CURRENT FRONTIERS AND PERSPECTIVES IN CELL BIOLOGY

Edited by **Stevo Najman**

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Cell Structures and Functions

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Tight Junctions

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1. Introduction

Epithelial cells cover the body (e.g. skin), cavities (e.g. stomach, uterus, bladder) and ducts (e.g. renal tubules, intestine) of multicellular organisms, and thus constitute the frontier between the individual and the external milieu. In areas that withstand strong mechanical or chemical stress (e.g. skin, esophagus, cornea, vagina) epithelia are stratified, whereas in the rest of the body, the epithelia independently of their morphology (e.g. columnar of the intestine, tubular of renal tubules, squamous of the lung), are organized in monolayers (Fig 1A). Epithelia protect the tissues that lie beneath, from microorganisms, toxins, trauma and water evaporation, and regulate the exchange of substances between the content of body cavities and ducts and the underlying tissues. Transport across epithelia occurs through the transcellular and paracellular pathways and requires the presence in epithelial cells of two basic features: a polarized plasma membrane and tight junctions (Fig. 1B).

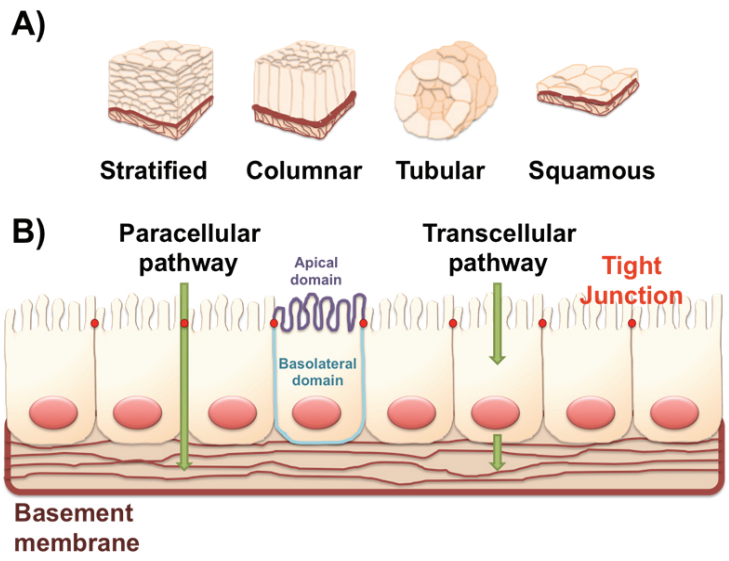

Fig. 1. Schematic representation of epithelia and transport pathways across a monolayer.

2. What are tight junctions?

Tight junctions are cell-cell adhesion structures present in epithelial cells at the limit between the apical plasma membrane that faces the exterior environment or the lumen of cavities and ducts, and the basolateral plasma membrane in contact with the internal milieu.

Tight junctions were first observed by transmission electron microscopy in thin sections of epithelial cells, as points of cell contact where the exterior membrane leaflets of the neighboring cells appeared to fuse, occluding in consequence the paracellular space (Fig 2A). When the interior of the membrane is observed by physically breaking apart a frozen biological sample with the freeze fracture technique, the tight junction is observed as a network of linear fibrils or chains of particles, termed TJ strands located bellow the apical microvilli (Fig 2B). By immunofluorescence, tight junction proteins display a cell border distribution that forms a chicken fence pattern when the sample is observed from above (Fig. 2C), while dots concentrated at the uppermost portion of the lateral membrane are seen when the tissue is viewed from the side (Fig. 2D)

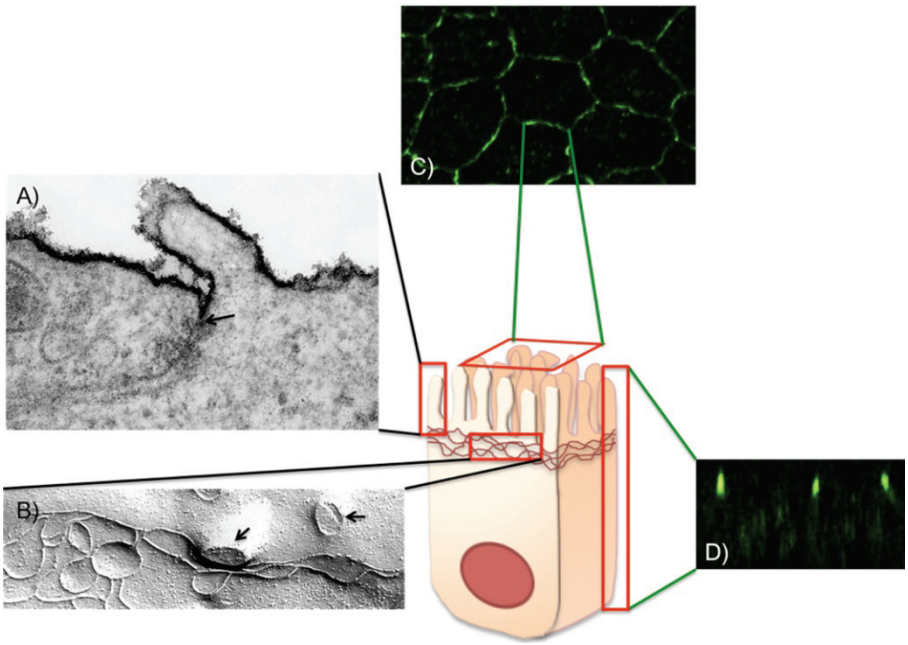

Fig. 2. Tight junction can be viewed by electron microscopy and immunofluorescence. A) By transmission **e**lectron microscopy, the tight junction appears in thin sections as points of cell-cell contact where the membranes of the adjacent cells appeared to fuse (arrow). Observe how the electrodense marker ruthenium red, added to the apical surface, does not stain the paracellular pathway bellow the tight junction. B) By freeze fracture, the tight junction appears as a network of strands bellow the apical microvilli (arrows). By immunofluorescence tight junction proteins give a chicken fence pattern when seen from above (C) while dots at the limit of the apical and the basolateral membrane are detected on a lateral view (D).

3. What are the canonical functions of tight junctions?

Tight junctions have two canonical functions that resemble those of a gate and a fence. The gate function refers to the capacity of tight junctions to regulate the passage of ions, molecules and water through the paracellular pathway. The gate function can be detected measuring the transepithelial electrical resistance (TER) of the tissue. The electric circuit in figure 3 shows how TER is the result of two resistances in parallel: the transcellular and the paracellular. The transcellular resistance is due to the resistance in series of the apical and the basolateral membranes. However since the electrical resistance across the plasma membrane is very high (2 GΩ) the current flows through the paracellular pathway regulated by the resistance offered by the tight junction. Therefore, the value of TER reflects the resistance of the tight junction. The gate function can also be evaluated by detecting the paracellular transit of molecules that are unable to cross through the transcellular pathway. Hence, these molecules must not be lipophilic, a target of carriers, pumps or co-transporters and subjected to a minimal degree of fluid phase endocytosis. When the observation is done by transmission electron microscopy, electrodense molecules like ruthenium red and lanthanum are chosen, whereas when the transit is evaluated across monolayers plated on transwell filters, molecules with fluorescent (e.g. FITC-dextran) or radioactive labels (^{3}H-mannitol) are used (Fig. 4).

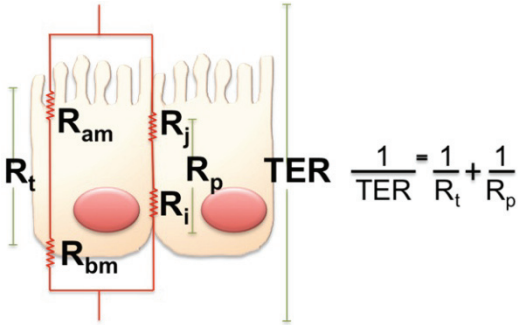

Fig. 3. Electrical circuit diagram and transepithelial electrical resistance equation of an epithelial monolayer. TER, transepithelial electrical resistance; R_t, transcellular resistance; R_{am}, apical membrane resistance; R_{bm}, basolateral membrane resistance; R_p, paracellular resistance; R_j, tight junction resistance; R_i, intercellular space resistance.

The fence function refers to the ability of tight junctions to restrict the movement of lipids and proteins within the membrane from the apical to the basolateral domains and vice versa. This function maintains the polarity of the plasma membrane and thus allows the vectorial transit of molecules across epithelia. The fence function of tight junctions is evaluated in monolayers cultured in transwell filters, by inserting a fluorescent lipid (e.g. fluorescent sphingomyelin) into the apical membrane and detecting if the fluorescent label reaches the basolateral membrane, or by chemically biotinylating the external domains of membrane proteins present at one of the cell surfaces (Fig. 4). In the latter, after generating a cell lysate, the polarized distribution of a particular membrane protein is detected by an immunoprecipitation with a specific antibody for the target protein, followed by a western blot with labeled (e.g. horse radish peroxidase or fluorescein) streptavidin, a molecule with an extraordinarily high affinity for biotin.

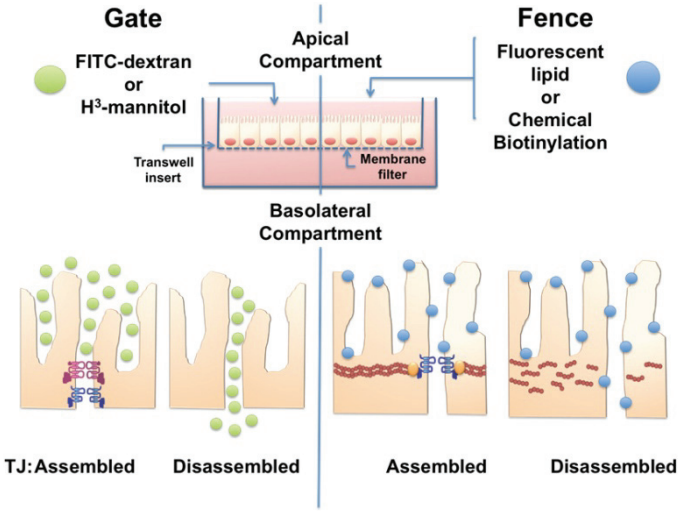

Fig. 4. The tight junction functions as a paracellular gate and a membrane fence. The gate function of the tight junction can be evaluated by measuring the passage of paracellular tracers from the apical to the basolateral compartment, whereas the fence function is determined by evaluating the free diffusion in the membrane plane of incomporated lipids and chemical probes.

4. Tight junctions are key players in the blood-brain, blood-retinal and blood-testis barriers and in the packing of myelin sheets

4.1 The blood-brain and blood-spinal cord barrier

The blood brain barrier separates circulating blood from the brain extracellular fluid and hence provides an optimal medium for neuronal function and protects the brain from fluctuations in ionic composition that occur after a meal or exercise that could perturb synaptic signaling. The blood brain barrier relies on the tight junctions present in brain capillaries. The latter differ from those in other organs for having a low rate of fluid-phase endocytosis, the absence of fenestrations and the presence of "tight" tight junctions. These characteristics restrict ion and fluid movement between the blood and the brain, and rely transendothelial traffic on specific ion transporters and channels. The signals that induce brain endothelial cells to express non-leaky tight junctions result from the specific interactions between capillary endothelial cells and the surrounding perivascular astrocytes and pericytes (Fig. 5A). The blood-spinal cord barrier is similar to the blood brain barrier and prevents the free passage of cells and blood substances to the spinal cord.

4.2 The blood-retinal barrier

The blood-retinal barrier confers protection or "immune privilege" to the ocular microenvironment. It is integrated by two separate anatomical sites: 1) the inner blood retinal barrier formed by the tight junctions present in the capillaries of the retina, and 2) the outer barrier integrated by tight junctions present between the retinal pigment epithelial cells (Fig 5C) that separate the neural retina from the choroidal vasculature.

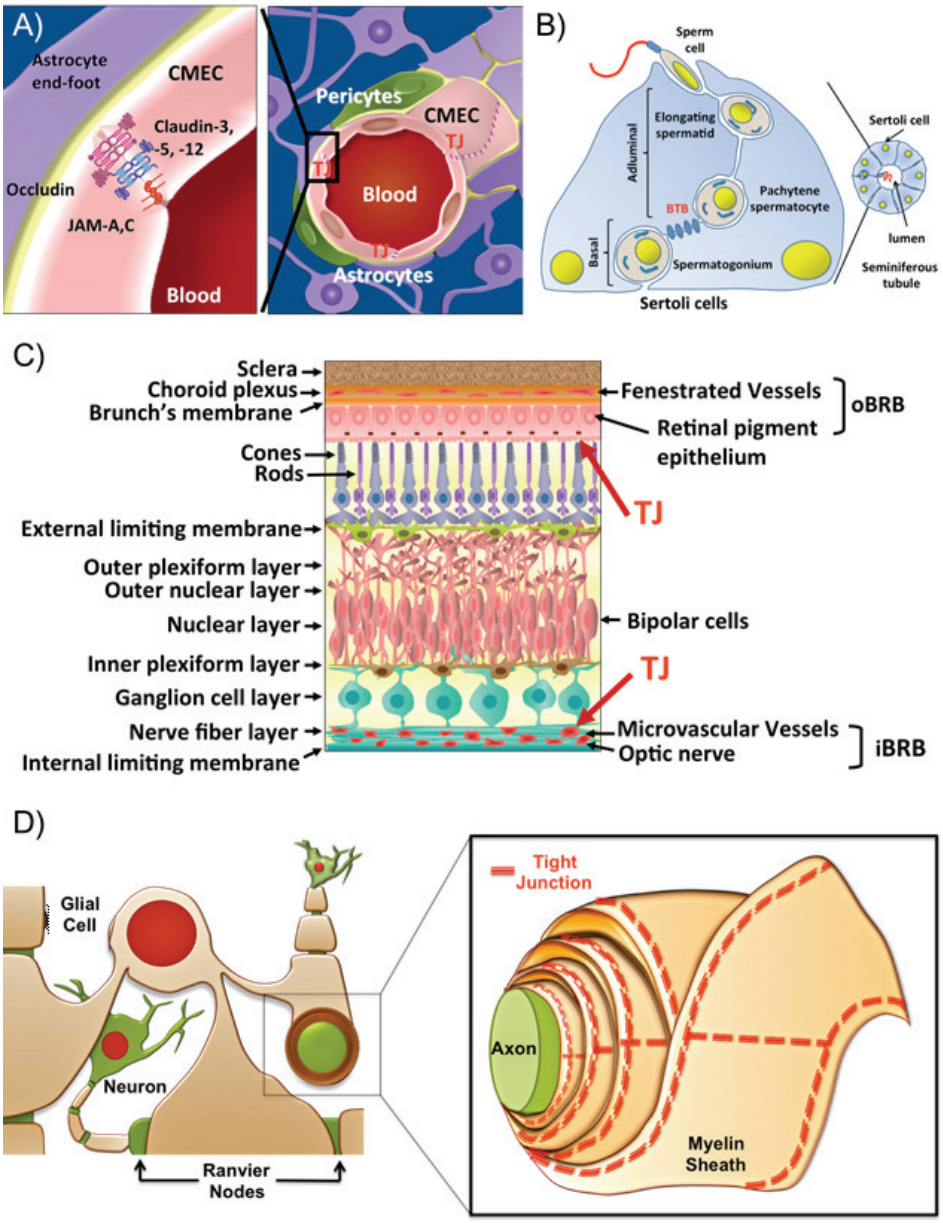

Fig. 5. Schematic representation of the blood-brain, blood-testis and blood-retinal barriers, and of the tight junctions present in myelinated axons. CMEC, cerebral microvascular endothelial cells; TJ, tight junction; oBRB, outer blood retinal barrier; iBRB, inner blood retinal barrier. A) Blood brain barrier B) Blood testis barrier C) Blood retinal barrier D) Glial cells tight junctions around myelin layers.

4.3 The blood-testis barrier

The tight junctions present between the epithelial cells of the testis known as Sertoli cells, form the blood testis barrier that divides the seminiferous epithelium into basal and adluminal compartments (Fig 5B). Germ cells at different stages of development move along the paracellular space of Sertoli cells in a basal to apical direction. Thus in the basal compartment the diploid spermatogonium and preleptotene spermatocytes are found whereas above the blood testis barrier, at the adluminal compartment, pachytene spermatocytes and round spermatids are present. Tight junctions of the blood testis barrier hence prevent the contact of systemic circulation with postmeiotic germ cells. This is important since spermatozoa and their surface antigens arise in puberty, long after self-tolerance is established in the fetus, and thus a compromise in the blood testis barrier could result in the generation of antibodies against the organism own sperm.

4.4 Tight junctions in myelinated axons

Myelinated axons are wrapped by continuous membrane layers derived from individual glial cells, the Schwann cells in the peripheral nervous system and the oligodendrocytes in the central nervous system (Fig 5D). Tight junctions mediate the adhesion among the successive layers of the myelin wrap. The compaction of these layers electrically insulates the axons and permits the saltatory conduction of action potentials that occurs by jumping from node to node, where axon insulation is interrupted.

5. Tight junctions have a complex molecular organization

Integral and peripheral proteins form tight junctions. The former are responsible for establishing cell-cell contact in the intercellular space, while the latter serve as a bridge between the integral proteins and the actin cytoskeleton. Peripheral proteins usually have multiple protein-protein binding domains that allow them to function as scaffolds for the attachment of a variety of signaling proteins (Fig. 6).

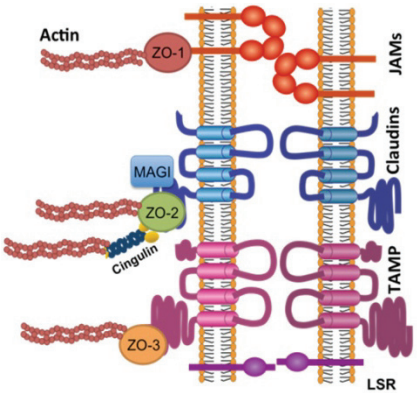

Fig. 6. Tight junctions are formed by a complex array of proteins. The integral proteins of the tight junction are single span like LSR and the family of JAMs, and tetraspan like the family of claudins and the TAMP proteins. Integral proteins establish cell-cell contact in the intercellular space. The peripheral proteins, act as a bridge between the integral proteins and the actin cytoskeleton

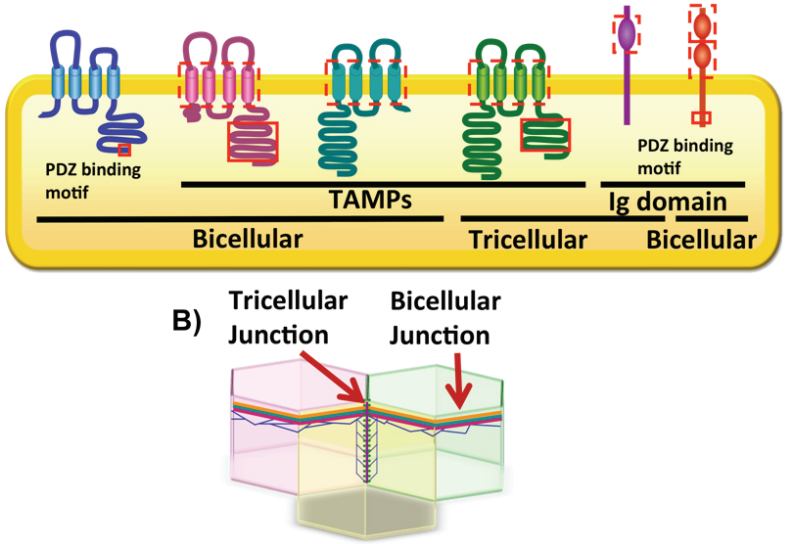

Fig. 7. Six different types of integral proteins constitute bicellular and tricellular tight junctions. A) Claudins and the TAMP proteins occludin, MarvelD3 and tricelullin are the tetraspan proteins of the tight junction. The latter three contain a marvel domain (boxed with discontinuous lines). Occludin and tricellulin share a highly homologous region in their carboxyl tail (boxed with continuous lines). LSR and JAM are single span proteins of the immunoglobulin family (Ig domains in boxes with discontinuous lines). B) Schematic drawing of the organization of bicellular and tricellular tight junctions. Tricellulin and LSR concentrate at the central sealing element of tricellular tight junctions, present at the corner where three cells meet, while the rest of the integral tight junction proteins are present in bicellular borders.

5.1 Integral tight junction proteins

The integral proteins of the tight junction establish cell-to-cell points of contacts or "kisses", where the outer leaflets of the membrane of apposing cells appear to fuse. There are two main classes of transmembrane proteins at the tight junction: the four and the single span proteins. The former include claudins, occludin, tricellulin and MarvelD3 and the latter are namely JAMs and LSR (Fig 7A). Occludin tricellulin and MarvelD3 are collectively called TAMPs (Tight junction associated MARVEL proteins) as they contain a conserved four transmembrane Marvel domain present in proteins concentrated in cholesterol rich microdomains and involved in membrane apposition and fusion events. Occludin and tricellulin carboxyl cytoplasmic tales are long and share a similar domain called ELL, whereas MarvelD3 has a short carboxyl segment and a long amino cytoplasmic domain similar to that in tricellulin. TAMPs have distinct but overlapping functions at the tight junction. JAMs and LSR belong to the immunoglobulin superfamily because they contain Ig domains which are regions of 55 to 75 amino acids separated by two cysteine residues that function as modules for protein-protein interaction. JAMs and LSR do not constitute tight junction strands and instead act as landmarks. Thus while LSR defines cell corners for

tricellular tight junction formation, JAMs are the first integral proteins to appear at the sites where bicellular tight junction will be assembled.

5.1.1 Claudins are integral proteins of the tight junction that regulate paracellular ionic selectivity

The word claudin derives from the Latin word "claudere" that means to close. Around 24 members comprise the claudin family in most vertebrates, although for example the puffer fish has 56 claudins. Since some claudin genes are closely linked, gene duplication is thought to have participated in the expansion of this family. In invertebrates like the fly and the worm *Caenorhabditis elegans*, claudin homologues have also been identified.

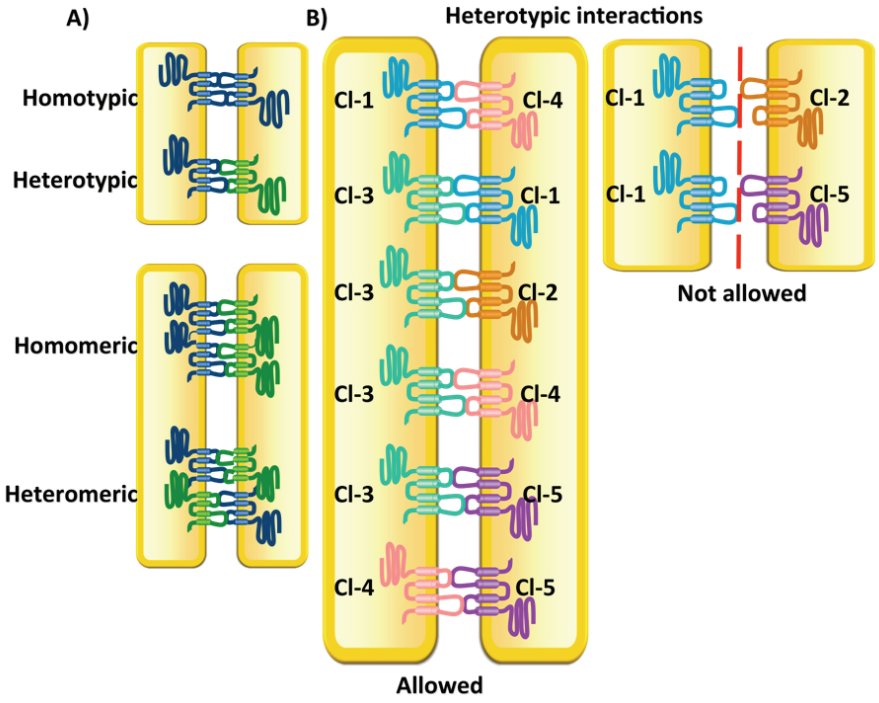

Fig. 8. Schematic representation of the interaction among claudins between neighboring cells. A) Between two cells, claudins establish homotypic and heterotypic interactions and dimerize in homomeric and heteromeric fashion. B) Drawing of the reported heterotypic interactions among claudins and of those proven not to occur.

Claudins like occludin are tetraspan proteins, yet they do not show any sequence similarity. Claudins size ranges from 20 to 34 kDa. The first extracellular loop of claudins is longer than the second one (around 52 Vs 16-33 amino acids) and contains a pair of cysteines that enhance stability by a disulfide bond, and charged amino acids that determine the ionic selectivity of the paracellular pathway. The second extracellular loop interacts with claudin molecules in the apposing cell membrane (trans-interaction) and participates in the side-to-

side oligomerization of claudins within the same membrane (cis-interaction). The cytoplasmic tails of claudins vary considerably among family members, constitute the site of posttranslational modifications that modulate junction tightness and stability, and end with two amino acids that form a PDZ binding motif that associates with adaptor proteins of the tight junction like ZOs and MUPP1.

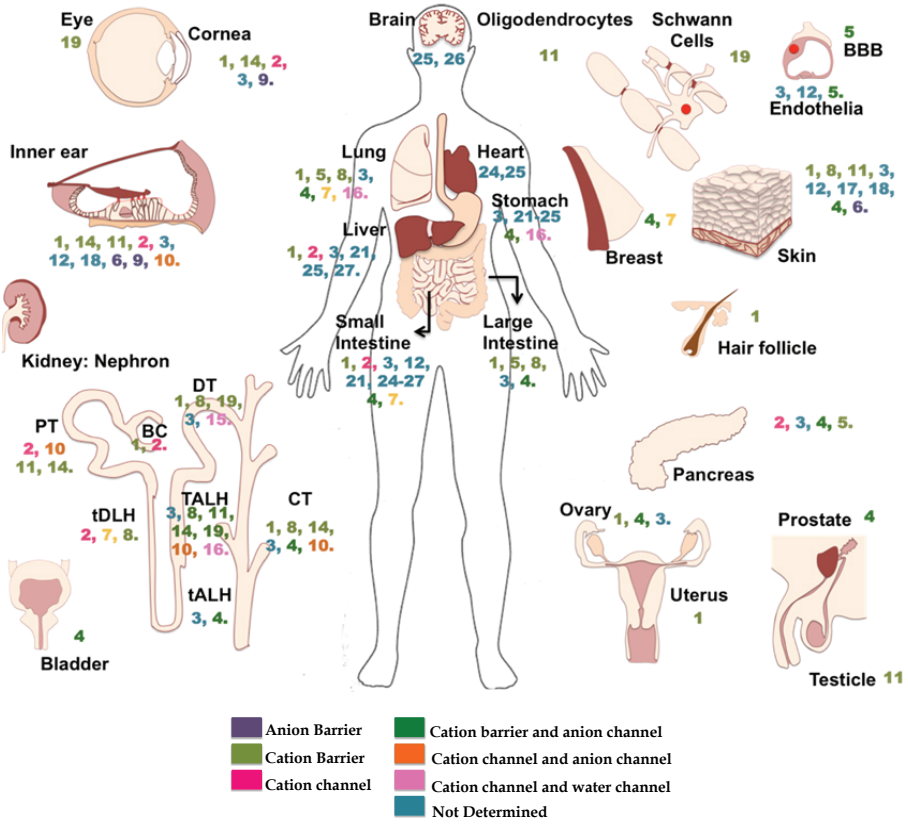

Fig. 9. Expression of different set of claudins in every tissue of the organism**.** BBB**,** blood-brain barrier; BC, Bowman capsule; PT, proximal tubule; tDLH, thin descending limb of Henle; tALH, thin ascending limb of Henle; TALH, thick ascending limb of Henle; DT, distal tubule; CT, collecting tubule.

Claudins are the building blocks of tight junction strands and upon transfection of claudins' cDNAs into cells that lack tight junctions like L-fibroblasts, well developed networks of filaments are formed, similar to *in situ* tight junctions. Claudins interact through homotypic and heterotypic associations and dimerize in homomeric and heteromeric manner (Fig. 8). Although all epithelial cells express claudins, each tissue exhibits a particular set of claudins, and some claudins are more ubiquitous than others (Fig. 9). For example claudins 1, 3 and 4 are present in a wide array of tissues, while claudin-5 is the dominant claudin in endothelia, and claudin-11 in ear stria vascularis basal cells, Sertoli cells and oligodendrocytes. The

expression of claudins is a dynamic process that responds to physiological and pathological conditions. For example, in the esophagus of subjects with reflux disease (Barrett's), a specialized columnar epithelium that expresses claudin-18 as the dominant claudin develops as replacement of the damaged squamous epithelia. Claudin-18 increases TER and reduces the paracellular permeability to H^+, making the columnar epithelium in comparison to the squamous epithelium, more resistant to acid. A somewhat similar case is found in the alveolar epithelium that after acute lung injury expresses claudin-4, a cation barrier forming claudin, as a mechanism to limit pulmonary edema. Another interesting example of a specific change in tight junction protein expression is observed in pouchitis, a remanifestation of the inflammatory bowel disease in the ileoanal pouch of patients that have undergone surgical treatment for severe therapy refractory ulcerative colitis. In these patients the expression in the pouch of claudin-1 decreases while claudin-2 increases. This change has a pathophysiological relevance as an elevated expression of the cation pore forming claudin-2 is observed in the colon of patients suffering from ulcerative colitis, Crohn's and celiac disease (Table 1).

Claudins might become major targets of drug development for electrolyte disorders since they regulate the ionic selectivity of the paracellular route due to their function as cation barriers (claudins 1, 4, 5, 8, 11, 14 and 19), cation channels (claudins 2, 10b, 15 and 16), anion barriers (claudins 6 and 9) and anion pores (claudins 4 and 10a) (Figure 9). It is noteworthy that claudin-18 forms paracellular water channels that mediate paracellular water transport in leaky epithelia and that the presence of certain claudins regulates the absorption of particular ions. In this respect it should be mentioned that claudin-16 was the first claudin identified as a paracellular channel, however recent data indicate that claudin-16 instead of being a channel for Mg^{2+}/Ca^{2+} as initially reported, forms a non-selective paracellular cation channel, whose absence results in a collapse of the transepithelial voltage, which is the driving force for Mg^{2+} and Ca^{2+} absorption in the thick ascending loop of Henle.

The intestinal tract and the nephron display specific expression of claudins in each of their respective segments. Thus in the proximal portion of these organs, namely duodenum and jejunum and the proximal tubule and thin descending limb of Henle's, a strong expression of paracellular channels like claudin-2 is observed, whereas in the more distal parts like the colon, distal tubule and collecting duct, more tightening claudins like 3, 4 and 8 are present (Figure 9).

The development of claudin knock out, knock down, transgenic, and mutated mice, with a particular phenotype, together with the identification of human and bovine hereditary diseases affecting the expression of claudins, has unveiled unique and non redundant role of certain claudins (Table 2). Hence it is observed that claudins 1 and 6 regulate the skin barrier in mice, whereas in humans, claudin-1 controls the permeability of liver biliary ducts. Claudin-5 is essential for the blood brain barrier, claudins 9 and 14 for the sensory epithelium of the cochlea, claudin-11 for the blood testis barrier and oligodendrocyte wrapping and claudins 2, 7, 16 and 19 for the renal reabsorption of ions. Claudin-15 promotes the proliferation of crypt cells of the small intestine and claudin-19 in mice is fundamental for Schwann cell wrapping, while in humans it is critical for the organization and development of the retina.

Tissue	Disease	TJ disruption	
		Upregulation	Downregulation
Brain	Alzheimer's disease and vascular dementia	Occ, Cl-2, -5 and -11	ND
	Cerebral aneurysm		Occ and ZO-1
	Chronic inflammatory pain	Cl-3 and -5	Occ
	Edema		Occ, Cl-5 and ZO-1
	Epilepsy		Cl-8 and ZO-1
	Massive intracraneal hemorrhage and congenital cataracts.		JAM-C mut
	Multiple sclerosis model (autoinmune encephalomyelitis)		JAM-A and ZO-1
	Multiple sclerosis	Cl-1 and -5 *	Occ, Cl-5 and ZO-1
	Rehumatoid arthritis (RA)		Occ
Ear	Age related nonsyndromic deafness (DFNA51)	ZO-2 genomic duplicarion	
	Nonsyndromic deafness (DFNB49)		Tricellulin mut
Esophagus	Barrett's esophagus	Cl-18	
	Reflux esophagitis	Occ, Cl-1, JAM-A and ZO-1	Cl-3
Eye	Diabetic retinopathy	Cl-1 **	Occ, Cl-5 and ZO-1 and -2
	Dry eye		Occ
	Gelatinous drop-like corneal dystrophy		Occ, Cl-1 and ZO-1
	Retina pigmentosa	ZO-1	
Intestine	Celiac disease	Cl-2, -3 and -4	Occ and ZO-1
	Collagenous colitis		Occ and Cl-4
	Crohn's disease	Cl-2	Occ, Cl-1, -5 and -8, JAM and ZO-1
	Non alcoholic fatty liver disease		ZO-1
	Obstructive jaundice	Cl-4	Occ, Cl-1 and -7
	Pouchitis	Cl-2	Cl-1
	Type I diabetes	Cl-2	Cl-1
	Ulcerative colitis	Cl-2	Occ, Cl-1, JAM and ZO-1
Liver	Intrahepatic cholestasis		7HG
	Necrotizing enterocolitis		Cl-3
	Primary biliary cirrhosis		7HG
	Primary sclerosing cholangitis		7HG
Lung	Acute lung inflammation		ZO-1, Cl-2, -4 and -5
	Acute lung injury	Cl-4	
	Asthma		Occ, Cl-1 and ZO-1
	Chronic alcoholic ingestion	Cl-5	Cl-1 and -7
	Sepsis		Occ, Cl-4 and -18
Spinal cord	Amyotrophic lateral sclerosis (ALS)		Occ and ZO-1
	ALS-mutant mice		Occ, Cl-5 and ZO-1
Vessels	Atherosclerosis		ZO-1

* In peripheral blood leukocytes (PBLs), predominantly in B and T lymphocytes and monocytes.

** In retinal pigment epithelium (oBRB).

Table 1. Pathologies related to tight junction dysfunction.

Claudin	KO, KD, TG and MT mice phenotype	Hereditary human/bovine diseases
1	KO: lethal, loss of skin barrier	H: 2 bp deletion → absence of Cl-1 → neonatal sclerosing cholangitis
2	KO: Defective reabsorption of NA^+,Cl^- and H_2O at proximal tubule	ND
3	NA	ND
4	NA	ND
5	KO: lethal, permeable BBB	ND
6	TG: permeable skin barrier	ND
7	KO: lethal, renal salt wasting and dehydratation	ND
8	NA	ND
9	MT: Deafness	ND
10a	NA	ND
10b	NA	ND
11	KO: Male sterility, hind limb weakness and deafness.	ND
12	NA	ND
14	KO: deafness	H: Single nucleotide deletion → loss of half of predicted protein → DFNB29 H: T254A/V85D → disrupts secondary structure in 2nd TMD → DFNB29
15	KO: Megaintestine	ND
16	KD: FHHNC, no accumulation of Cl-19 at TAL	H: FHHNC B: chronic interstitial nephritis (↑ blood urea nitrogen and creatinin, ↑ urinary proteins)
17	NA	ND
18	NA	ND
19	KO: disorganized Schwann cells TJs, abnormal animal behavior and peripheral neuropathy KD: FHHNC, no accumulation of Cl-16 in TAL	H: FHHNC and severe visual impairment. H: Mut G20D → disturbance of signal peptide sequence → perinuclear protein H: Mut Q57E (within W-GLW-C-C signature) → dimerization disruption H: Mut L90P → disrupts α-helix in 2nd TMD

Table 2. Genetic alterations of claudins that impact mice development and human or bovine health. B, bovine; BBB, blood-brain barrier; DFNB29, non syndromic deafness; FHHNC, familial hypomagnesemia with hypercalcuria and nephrocalcinosis; H, human; KD, knock-down; KO, knock-out; MT, mutation; NA, not available; ND, not determined; TAL, thick ascending limb of Henle; TG, transgenic; TMD, transmembrane domain.

5.1.2 Occludin and MarvelD3

From a group of monoclonal antibodies generated against a junctional fraction from chicken liver, three were selected for specifically recognizing an integral tight junction protein thereafter named occludin for the Latin word "occludere" that means to close up. Occludin has four membrane spanning domains and two loops with a high content of tyrosine and glycine residues exposed towards the extracellular space and flanked by a short amino and a large carboxyl terminal tail oriented towards the cytoplasm. The distal C-terminus of occludin forms a coiled-coil region essential for ZO-1 binding, while the amino tail associates to the E3 ubiquitin protein ligase Itch that regulates occludin degradation at the proteosome.

Transfection of occludin cDNA into L-fibroblasts induces the formation of only a small number of short strands, and is not only after the fibroblasts are co-transfected with claudin that well developed strands are formed, hence indicating that occludin is an accessory protein and not the main builder of tight junction strands.

The function of occludin has remained uncertain. On one hand the evidence indicates that occludin mediates adhesion at the tight junction since lowering the expression level of occludin, deleting the carboxyl tail of the protein, treating epithelia with peptides homologous to occludin extracellular loops and occludin endocytosis, disrupt the barrier function of tight junctions. In the other hand, the results with occludin knockout mice, reveal that the animals are viable with healthy epidermal, respiratory, renal and intestinal function but with small size, testicular atrophy, male infertility, gastritis, salivary gland dysfunction, thinning of compact bone and brain calcifications. These contrasting results indicate that although occludin is important for the establishment of cell-cell adhesion at the tight junction, other proteins can somehow replace its function. Thus it seems that the tretraspan proteins MarvelD3 and tricellulin, that respectively concentrate at bicellular (where two cells meet) and tricellular (where three cells meet) tight junctions, can partially compensate for occludin loss. In this respect for example it is observed that tricellulin is displaced to bicellular tight junctions upon occludin knockdown.

Recent evidence suggests a role for occludin in growth regulation. Thus occludin regulates the directional migration of epithelial cells by promoting the leading edge localization of the polarity proteins aPKC, Par3 and PATJ, and controls cell cycle progression by regulating centrosome separation and mitotic entry. This capacity is due to occludin localization at centrosomes during interphase and occludin phosphorylation at serine 490.

By SDS-PAGE occludin appears as a set of bands between 62 and 82 kDa. The lower bands are detergent soluble and correspond to the protein that distributes along the basolateral membrane, while the higher bands are insoluble, highly phosphorylated in serine residues and belong to the form of occludin that concentrates at tight junctions. The carboxyl terminal tail of occludin is a phosphorylation target of novel PKCδ and casein kinases 2 and Iε, and of protein phosphatases 2A and 1 that respectively dephosphorylate occludin in threonine and serine residues. Through this action both phosphatases negatively regulate the assembly of tight junctions. In contrast, in retinal pigment epithelial cells, endothelia and in the blood brain barrier, tyrosine phosphorylation of

occludin, induced by hepatocyte growth factor, ischemia and shear or oxidative stress, impairs the barrier integrity of tight junctions, and this process is accompanied by a concomitant increase in the activity of c-Src and FAK tyrosine kinases. Interestingly, the *in vitro* phosphorylation of occludin carboxyl terminal tail by c-Src, diminishes the capacity of occludin to interact with ZO proteins.

5.1.3 Tricellulin and LSR are proteins present in tricellular tight junctions

Freeze-fracture images show how at tricellular tight junctions, the most apical elements of the strands in bicellular tight junctions from both sides turn to and extend in the basal direction. Hence three pair of central sealing elements form a narrow tube in the extracellular space at the center of each tricellular contact. Short strands connect the bicellular tight junctions to the central sealing elements giving this structure an image that somehow resembles that of a fish skeleton placed upside down (Fig 7B).

Tricellulin and LSR concentrate in the central sealing elements of tricellular tight junctions. Tricellulin is a 65 kDa protein, structurally similar to occludin. Loss of the conserved carboxyl cytosolic domain of tricellulin is a cause of nonsyndromic deafness. Suppression of tricellulin expression compromises the barrier function of epithelial cells and overexpression of tricellulin increases the barrier towards ions and larger solutes. Tricellulin is excluded from bicellular tight junctions by occludin and is recruited to tricellular tight junctions by LSR, the receptor for triacylglyceride-rich proteins. The latter is a single span 585 amino acid protein with an extracellular Ig domain. LSR defines tricellular contacts in epithelial sheets, therefore LSR knockdown prevents the accumulation of tricellulin, while LSR accumulates at tricellular contacts even when tricellulin is knocked down.

5.1.4 JAMs are single span proteins important for tight junction assembly, cell migration, leukocyte transmigration, platelet activation and angiogenesis

JAM proteins constitute a family whose members exhibit two extracellular Ig domains, a single transmembrane region and a cytoplasmic tail with a canonical PDZ binding motif (Fig 7A). The family has two groups. One integrated by JAM-A, JAM-B and JAM-C with a short cytoplasmic tail of 45-50 residues and a type II PDZ binding motif, and another that includes CAR, ESAM, JAM4, CRTAM and BT-IgSF with cytoplasmic domains of 80-165 amino acids and type I PDZ domains. JAM-A is present in hematopoietic cells including monocytes, lymphocytes and red blood cells. Epithelial and endothelial cells exhibit JAM-A and CAR, whereas JAM-B, JAM-C and ESAM are expressed only in endothelial cells, and JAM4 and CRTAM only in epithelia. With the exception of JAM-B and CRTAM that localize along the lateral membrane and BT-IgSF that is present in neuron and glial cells, the rest of the proteins of the JAM family concentrate at the tight junction.

JAM proteins establish homophilic (e.g. endothelial JAM-A with platelet or endothelial JAM-A) and heterophilic interactions (Fig 10). The latter, with other members of the JAM family (e.g. endothelial JAM-B with endothelial or leukocyte JAM-C) and with other types of cell adhesion molecules such as integrins (e.g. endothelial JAM-A with integrin LFA-1 in leukocytes and integrin $\alpha v\beta 3$ in endothelial cells; endothelial JAM-B with integrin VLA4 in

leukocytes; and endothelial JAM-C with integrin MAC1 in leukocytes, and platelet JAM-C with integrins αxβ2 and MAC1 in leukocytes).

Crystal structural analysis of the extracellular regions of JAM-A reveals that the membrane distal V type Ig domain of two JAM proteins interconnect forming a U shaped cis homodimer, through a dimerization motif [R(V,I,L)E] also conserved in JAM-A JAM-B, JAM-C. These cis dimers are proposed to then interact between cells in trans, forming a zipper type seal (Fig. 10).

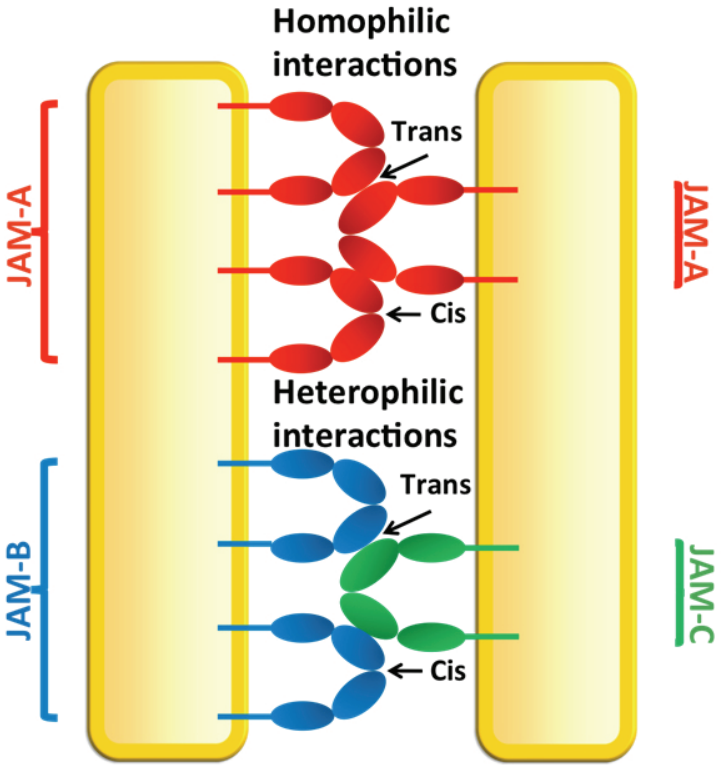

Fig. 10. Schematic representation of the homophilic and heterophilic interactions between JAM proteins. The scheme shows the homophilic interaction between JAM-A proteins and the heterophilic association between JAM-B and JAM-C. The cis interaction between the distal Ig domains of JAMs in the same cell forms a U shaped homodimer and the trans interaction between JAMs in neighboring cells forms a seal that resembles a zipper.

The function of JAMs is complex as they are important for tight junction assembly, cell migration, leukocyte transmigration, platelet activation, angiogenesis and virus binding.

Leukocytes bind JAMs present on the endothelial surface, adhere to the endothelia and then transmigrate interacting with JAMs proteins that form an adhesive "tunnel" at the paracellular pathway between endothelial cells.

JAMs are the first integral proteins to appear at tight junctions, where they tether other proteins to this location. Thus JAM-A transfected into fibroblasts promotes the localization of ZO-1, AF6, CASK and occludin to points of cell-cell contact, and in epithelial cells JAM association with Par3, tethers the complex Par3/Par6/aPKC to tight junctions. The correct location of this complex is crucial for the establishment of the apical-basal polarity of epithelial cells.

Decreasing or ablating the expression of JAMs has been another strategy recently employed to evaluate the roles of these proteins. Thus, for JAM-C, it was observed that most null mice die during postnatal development due to infections and that the surviving males are infertile and fail to produce mature sperm cells. This agrees with previous observations showing that in order for germ cells to move along Sertoli cells they need to establish various types of trans homo and heterodimers including that formed between JAM-C present on the spermatids and JAM-B present in Sertoli cells.

JAM-A knock out mice have a distinct phenotype characterized for an increased gastrointestinal permeability, colon neutrophil infiltration, increased colonic cell proliferation and a higher sensitivity for the development of experimental colitis when compared to wild type animals. JAM-A silencing in epithelial cell lines results in an impaired tight junction barrier function, an increased expression of the leaky claudins 10 and 15 and a concomitant decrease in claudin-1. Interestingly, the expression of JAM-A dominant negative mutants that are dimerization defective or lack the PDZ binding domain, reduces the rate of cell migration, which is important in endothelial cells for the promotion of angiogenesis and in epithelial sheets for the wound healing process. Apparently this is due to the fact that the down regulation of JAM-A reduces the level of active GTP-bound Rap1, which in turn reduces the stability of β1 integrin, which is necessary for cell migration. Similarly, it was observed that ESAM knock out mice have a retarded tumor growth that is associated to a diminished vascular density. This agrees with observations in ESAM null endothelial cells showing less migratory and angiogenic activity. These results hence indicate that ESAM is critical for blood vessel assembly.

5.2 Peripheral tight junction proteins

At the submembranous region of the tight junction more than 40 proteins have been identified. Some are signaling proteins that under certain circumstances concentrate at the tight junction. Such is the case of kinases, phosphatases, phospholipases, G proteins and transcription factors. In this section however we will only describe peripheral proteins essential for tight junction assembly and function. The latter can be classified based on the presence in their sequence of a domain known as PDZ. This domain establishes homotypic interactions with other PDZ domains and heterotypic associations with precise motifs (e.g. S/TXV or φXφ, where φ corresponds to a hydrophobic amino acid and X to any amino acid) present at the carboxyl terminal region of certain proteins. Some tight junction proteins like PAR6 contain a single PDZ domain, while others like PATJ and MUPP1 contain ten or more PDZ domains (Fig 11).

In addition to the PDZ domain, various peripheral proteins of the tight junction contain SH3 and guanylate kinase (GK) domains that serve as protein-protein binding domains with no

inherent catalytic activity. The presence of PDZ, SH3 and GK domains is the characteristic feature of proteins that belong to the MAGUK family. Among these proteins, ZO-1, ZO-2 and ZO-3, as well as Pals1, are present at the tight junction while other members of the MAGUK family localize at the adherens (e.g. Disc large) and synaptic (e.g. PSD95, SAP97 and Chapsyn-110) junctions. At the cytoplasmic region of the tight junction proteins named inverted MAGUKs or MAGIs are also present. These proteins contain six PDZ domains, a GK module and a WW domain instead of the SH3 region (Fig 11). Both the SH3 and WW domains establish molecular interactions with motifs with a precise proline consensus.

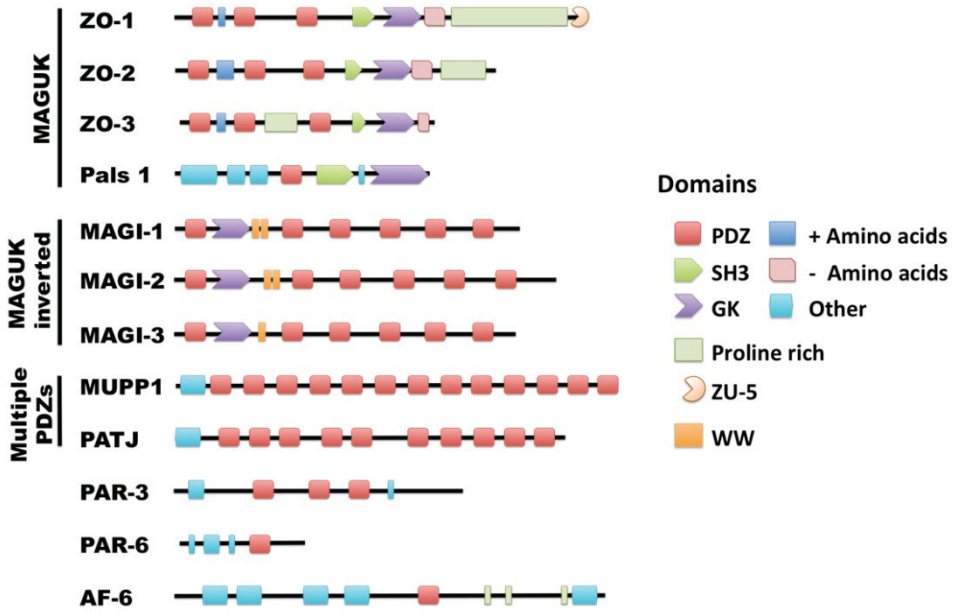

Fig. 11. Schematic representation of peripheral tight junction proteins with PDZ domains. Proteins that belong to the MAGUK protein family contain SH3 and GK domains in addition to PDZ modules. ZO proteins have 3 PDZ domains whereas Pals1 has a single one. MAGI proteins have an inverted organization since the GK domain is present before the majority of PDZ domains. MAGIs have WW domains instead of SH3. Multiple PDZ containing proteins include MUPP1 and PATJ that respectively have 13 and 10 PDZ domains.

In the peripheral tight junction proteins, the presence of multiple protein-protein binding domains like PDZ, SH3, WW and GK explains their function as molecular scaffolds for the formation of multiprotein complexes and for the linkage of the transmembrane proteins of the tight junction to the actin cytoskeleton.

5.2.1 ZOs are scaffolding proteins essential for claudin polymerization

The word ZO is an acronym of Zonula Occludens, the Latin name for tight junctions and number one in ZO-1 reveals that it was the first tight junction protein ever identified.

Although the genes of the MAGUK proteins MAGI and DLG are already present in unicellular protists, those of the ZO proteins are absent in unicellular organism. In the sponges, multicellular animals with no distinct embryonic cell layers and no true organs, the ancestral gene for ZO proteins named CARMA like is present and contains in addition to the PDZ, SH3 and GK domains a CARD region. The ZO gene first appears in the Placozoa, a flat multicellular animal that lacks tight junctions and instead exhibits adherens junctions. In *Hydra*, a Cnidaria with two embryonic cell layers, the endoderm and the ectoderm, and in the Bilateria *Drosophila* and *Caenorhabditis* that have the additional embryonic cell layer of the mesoderm, a ZO protein is present at the zonula adherens and no tight junction structure is distinguishable. In the Craniata an expansion of the gene gave rise to three ZO paralogues: ZO-1, ZO-2 and ZO-3 present at tight junctions. The ZO gene present in Placozoa, Cnidaria and Bilateria contains a ZU5 domain, similar to that present in Craniata ZO-1, hence suggesting that ZO-2 and ZO-3 arose as an expansion of ZO-1 that lost the ZU5 domain.

ZO proteins act as a bridge between the integral proteins of the tight junction and the actin cytoskeleton (Fig. 12). Thus through their first and third PDZ domains they respectively associate with claudins and JAMs, and by the SH3-GK region with occludin. ZOs interact with actin and actin binding proteins like 4.1, through a proline rich region that in ZO-1 and ZO-2 is located at the carboxyl segment, whereas in ZO-3 is present between PDZ-2 and PDZ-3. ZO-1 and ZO-2 also associate to myosin IIa.

ZO proteins have the capacity to form through their second PDZ domains homo and heterodimers. With respect to the latter, ZO-1/ZO-2 and ZO-1/ZO-3 but not ZO-2/ZO-3 interactions have been detected. ZO proteins also bind to other tight junction peripheral proteins like cingulin, and in the case of ZO-1 this association occurs through the GK domain.

In vertebrates, ZO proteins are not exclusively present in tight junctions. Thus in fibroblasts, and in epithelial cells, at the initial stages of assembly of the apical junctional complex, ZOs are detected at adherens junctions, where ZO-1 binds to afadin and α and γ catenins, ZO-2 to α-catenin and ZO-3 to afadin and p120 catenin. ZO proteins also interact with a wide variety of gap junction conexins.

ZO-1 and ZO-2 play a crucial role in the polymerization of claudins, as no tight junction strands are formed in ZO-1 knock out/ZO-2 knock down cells. They however appear to have a redundant role since the reintroduction of one protein or the other, provokes the reappearance of tight junction strands. Although claudins associate to the first PDZ domain of ZO-1 and ZO-2, a construct containing only the PDZ domains of ZO-1 (N-ZO-1) is incapable of inducing claudin polymerization, even when it is forcibly recruited to the plasma membrane by the introduction of a myristoylation sequence. Only after the addition of a homodimerizer, the N-ZO-1 segment is capable of producing the appearance of TJ strands throughout the lateral membrane. Instead, when a longer construct that includes the SH3-GK domain is used, claudins polymerize at the correct site, the limit between the apical and the lateral membrane. Taken together these results indicate that the dimerization of ZO-1 or ZO-2, mediated by PDZ-2, at the TJ region, determined by the SH3-GK domain, induces the polymerization of claudins.

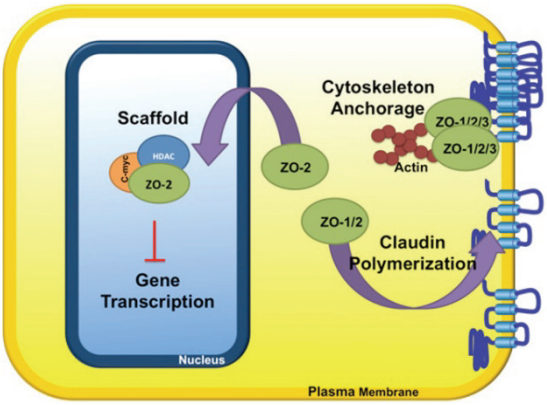

Fig. 12. ZOs form scaffolds that regulate claudin polymerization, integral proteins anchorage to the cytoskeleton and gene transcription. ZO-1 and ZO-2 dimerization and localization at the tight junction is required for claudin polymerization into strands. ZOs act as a bridge between the integral proteins of the tight junction and the actin cytoskeleton. At the nucleus ZO-2 associates to factors that regulate gene transcription.

During development ZO-1 and ZO-2 are essential as KO mice embryos are not viable. ZO-1 KO dies around embryonic stage 10.5 due to massive apoptosis at the neural tube, notochord and allantois and defective angiogenesis in the yolk sac. ZO-2 KO mice die shortly after implantation around E7.5 due to an arrest in early gastrulation. ZO-2 mice chimeras, obtained after injecting ZO-2 KO embryonic stem cells into wild type blastocyst, are viable, yet the males are infertile due to a defective blood testis barrier. Thus, indicating that ZO-2 is essential for the development of the extraembryonic tissue, and for the proper barrier function of tight junctions between Sertoli cells. In contrast, ZO-3 KO mice lack an obvious phenotype. This is interesting since in zebra fish ZO-3 KO embryos develop edema, loss of blood circulation, tail fin malformations and loss of the epidermal barrier.

In cultured epithelial monolayers, silencing of ZO-1 or ZO-2 retards tight junction formation and disorganizes the cortical ring of actin.

ZOs contain several putative nuclear localization (NLS) and exportation signals (NES). The four NES of ZO-2 are functional and the activity of NES-1 is regulated by PKCε phosphorylation. The presence of ZO-1 and ZO-2 at the nucleus is determined by the state of confluence of the culture. Thus, in sparse monolayers ZO-1 and ZO-2 concentrate at the tight junction and the nucleus, whereas in confluent cultures almost no nuclear staining of these proteins is detected. ZO-2 enters the nucleus at late G1 and departs at mitosis, thus explaining why in confluent quiescent cells the protein is absent from the nucleus. At the nucleus ZO-2 associates with nuclear lamina β1 and is distributed in speckles, rich in the essential splicing factor SC-35. ZO-1 and ZO-2 have the capacity to interact with transcription factors and to regulate gene transcription (Fig. 12). Thus ZO-2 associates with C/EBP, Jun and Fos transcription factors and inhibits the transcription of artificial promoters regulated by AP-1 sites, and through its association with c-myc negatively

regulates the transcription of cyclin D1. In accordance ZO-2 overexpression blocks cell cycle progression from G1 to the S phase of the cell cycle and inhibits cells proliferation. ZO-1 instead, through its SH3 domain, sequesters away from the nucleus, the Y box transcription factor ZONAB. Nuclear ZONAB stimulates cell proliferation by interacting with the cyclin D1 binding kinase CDK4, by promoting its nuclear accumulation and by inducing the transcription of CD1 and PCNA, a DNA replication and repair factor.

5.2.2 Cingulin and JACOP/paracingulin are tight junction proteins that regulate the activity of Rho GTPases

Cingulin is a 140 kDa protein exclusive of vertebrate tight junctions. Cingulin was identified with a monoclonal antibody raised against a preparation of chicken brush border myosin. As the immunofluorescence showed that the antigen localized as a belt below the apical surface it was named cingulin from the Latin word "cingere" meaning to encircle. Cingulin forms parallel homodimers, where each subunit consists of a large globular amino terminal head, a small globular carboxyl terminal tail and a coiled-coil rod domain homologous to the one present in conventional nonmuscle myosins (Fig 13). Cingulin is not an actin dependent motor protein but at least in vitro displays actin bundling activity. Through the head cingulin interacts with actin, myosin, ZO-1, ZO-2, ZO-3, afadin and the carboxyl terminal regions of JAM-A and occludin and through both rod and tail domains with non-muscle myosin II and ZO-3.

Disruption of cingulin gene does not prevent tight junction formation but alters the expression of other junctional proteins. Thus, in mouse embryoid bodies an increased expression of ZO-2, occludin and claudin-6 is observed concomitant with a decreased expression of ZO-1, whereas in MDCK cells, cingulin silencing increases protein levels of ZO-3 and claudin-2. The latter effect is mediated by an increment in RhoA activity. In this respect it is important to highlight that binding of cingulin at the tight junction to the guanine nucleotide exchange factor that activates RhoA named GEF-H1, results in downregulation of RhoA signaling and inhibition of cell proliferation.

Employing a monoclonal antibody against a chicken cytoplasmic antigen that localizes at the apical junctional complex, a protein similar to cingulin was identified and named JACOP for junction associated coiled-coil protein. Later, by searching EST sequences for their homologies to cingulin, a cDNA was identified and named paracingulin. JACOP and paracingulin are the same protein. JACOP/paracingulin is a 160 kDa protein with a domain organization similar to cingulin and 40% sequence identity. JACOP/paracingulin besides acting as a down regulator of RhoA through the recruitment of GEF-H1, promotes the activation of Rac1 by recruiting to junctions the GEF activator for Rac1 named Tiam1. In epithelial cells this is important since the acquisition of confluence is paralleled by a reduction of active Rho A levels and the activation of Rac1 and Cdc42, other members of the Rho family of small GTPases.

Unlike cingulin which is a tight junction specific protein, paracingulin localizes at both tight and adherens junctions. At the tight junction JACOP/paracingulin is recruited through ZO-1, whereas at the adherens junction it interacts with PLEKHA7, a protein that associates to the E-cadherin binding protein catenin p120.

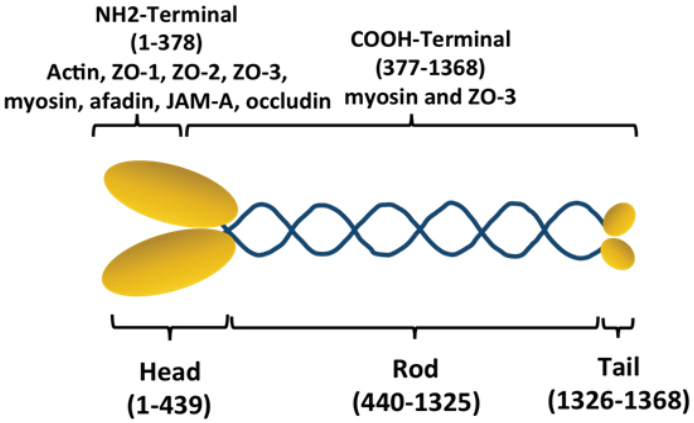

Fig. 13. Cingulin forms parallel homodimers. Each subunit of cingulin has a large globular amino terminal head, a small globular carboxyl terminal tail and a coiled-coil rod domain homologous to nonmuscle myosins.

6. Pathologies related to tight junction dysfunction

As table 1 shows, a wide array of pathologies affecting different organs are related to tight junction dysfunction. For example, the development of a leaky blood brain barrier is associated to various pathologies including stroke, cerebral aneurysms, cerebral edema, multiple sclerosis, epilepsy and Alzheimer disease. Frequently, the decreased expression of tight junction proteins precedes the onset of the clinical disease. Thus, brain edema surges as a consequence of impaired capillary endothelial cells; tight junction destruction is proposed to facilitate aneurism formation, and abnormal blood flow is thought to down regulate tight junction proteins, which leads to leakage of blood components into the central nervous system tissue and to the development of autoimmune diseases like multiple sclerosis and amyotrophic lateral sclerosis. Interestingly, the chronic ingestion of saturated fatty acids appears to generate blood brain barrier dysfunction enhancing the brain delivery of amyloid-β and exacerbating the amyloidogenic cascade that facilitates the development of Alzheimer disease. In other pathologies instead, a compromised blood brain barrier is a direct consequence of the disease. Such is the case of epilepsy, where seizures trigger the down regulation of tight junction proteins.

Disruption of the blood retinal barrier occurs in several blinding ocular diseases. For example in diabetic retinopathy breakdown of the inner blood retinal barrier is one of the initial alterations. Therefore strategies to revert the damage, have focused on the employment of drugs like the pseudo sugar derivative of cholesterol Sac-0601, the statin lovastatin, calcium dobesilate and rottlerin, that target VEGF, TNFα, MAPK and NF-κB, and PKCδ respectively, which are factors that promote tight junction protein breakdown in retinal endothelial cells.

In the intestine, the development of an impaired barrier function has two main consequences, leak flux diarrhea and the uptake of antigens that under normal conditions are prevented from entering the body, and that can aggravate or initiate inflammation as

well as autoimmune diseases. In pathological conditions of autoimmune origin like type I diabetes, ulcerative colitis, Crohn's and celiac disease, the transepithelial electrical resistance of the intestine is markedly reduced and an increase in the pore forming claudin-2 is generally observed.

Sometimes the failure of one organ leads to a major damage in another. For example, during acute liver failure, the generation of tumor necrosis factor α produces the disruption of the blood brain barrier and the consequent cerebral edema that leads to cerebral herniation and death, and non alcoholic liver failure and obstructive jaundice produce an increased intestinal permeability coupled to the loss of tight junction proteins.

Carcinoma	Claudins				TAMPs	JAMs	ZOs
	2	3	4	7			
Intestinal							
Prostate							
Renal							
Colorectal							
Endometrial							
Pleura							
Uterus							
Breast							
Gastric							
Head and neck							
Nasopharingeal							
Tongue							
Esophageal							
Ovarian							
Pancreatic							
Bladder							
Epidermoid							
Cervical							
Thyroid							
Lung							
Biliary Tract							
Liver							
Melanoma							
Meningioma							
Oral							
Testis							

Upregulated
Up/ Downregulated
Downregulated

Table 3. Regulation of tight junction proteins in cancer

Tight junctions are lost during cellular transformation. Therefore it came as a surprise that while the expression of TAMPs decreases in all types of cancers studied, that of other tight junction proteins like JAMs and ZOs is up or down regulated, depending on the type of cancer, and the stage of development of the disease. In the case of certain claudins like 2, 3, 4 and 7 the result is more surprising as they are generally overexpressed in cancerous tissue (Table 3). Downregulation of tight junction proteins in cancer is easy to understand, as the loss of cell adhesion promotes the transformation of polarized polygonal cells into mobile elongated cells with invasive potential. Instead the upregulation of tight junction proteins in cancer is more difficult to comprehend. Several explanations can be given. For example, the

aberrant expression might reflect cytosolic and nuclear accumulation of tight junction proteins, in a scenario where these molecules could serve as oncogenic factors. In this respect, it should be mentioned that claudin expression increases migration and motility, and promotes activation of metalloproteinases.

7. Pathogenic organism that affect the tight junction

Epithelia block the entry of microorganisms into the body, hence it is not surprising to find that a variety of pathogenic microorganisms have evolved mechanisms to control tight junctions in order to obtain a gateway of access to the underlying tissue. Some pathogens use tight junction proteins as their receptors for attachment and subsequent internalization. Such is the case of hepatitis C virus with occludin, claudins 1, 6 and 9; reovirus and feline calicivirus with JAM-A, and Coxsackie and adenovirus with CAR.

Viruses promote their spreading through the disruption of endothelial or epithelial barriers. Some viruses disrupt epithelial tight junctions by sequestering PDZ containing proteins. Such is the case for example of cancer causing human papillomaviruses types 16 and 18, whose E protein that contains a PDZ binding motif, targets MAGI-1 for degradation producing mislocalization of ZO-1 and loss of tight junction integrity. In a similar fashion the small envelope protein E of SARS coronavirus, the causative agent of severe respiratory infections, has a PDZ binding motif that targets PALS1, a tight junction associated protein member of the Crumbs-PALS1-PATJ complex, to the endoplasmic reticulum-Golgi region, disrupting in consequence tight junctions and the respiratory tract epithelial integrity.

HIV virus proteins Tat and gp120 open the blood brain barrier, the blood retinal barrier and the tight junctions of the intestine and female genital tract, allowing the microbial translocation to the brain, eye and body interstitium.

Some viral proteins like the spike forming VP8 protein of rotavirus, modulates the gate and fence function of tight junctions in epithelial cells.

Other pathogens destroy tight junctions and as a result open the paracellular pathway (Fig 14). For example, enteropathogenic *Escherichia coli* injects into the cytoplasm of host cells, the bacterial receptor Tir that binds to the bacterial adhesin intimin, and the pathogenic protein EspF. The latter that contains PDZ binding motifs and proline rich domains which act as actin nucleation centers, produces dissociation of occludin/claudin-1/ZO-1 complexes from the cell borders and recruits these proteins to actin rich pedestals formed beneath the bacteria, due to the phosphorylation of Tir and the consequent activation of the N-WASP-Arp2/3 pathway. Other bacteria like *Listeria monocytogenes* and *Shigella flexneri*, the respective causative agents of listeriosis and shigellosis, also recruit ZO-1 to the distal portion of actin filaments that like comet tails associate through, N-WASP or its *Listeria* homologue ActA, to the internalized bacteria.

Several bacterial toxins disassemble tight junction proteins. For example, lipopolysaccharide, the main component of the cell wall of Gram-negative bacteria, is an endotoxin that opens the blood brain barrier and induces neurological dysfunction. Other toxins disassemble tight junction proteins by altering the cortical ring of actin-myosin and its key regulators Rho and myosin light chain proteins.

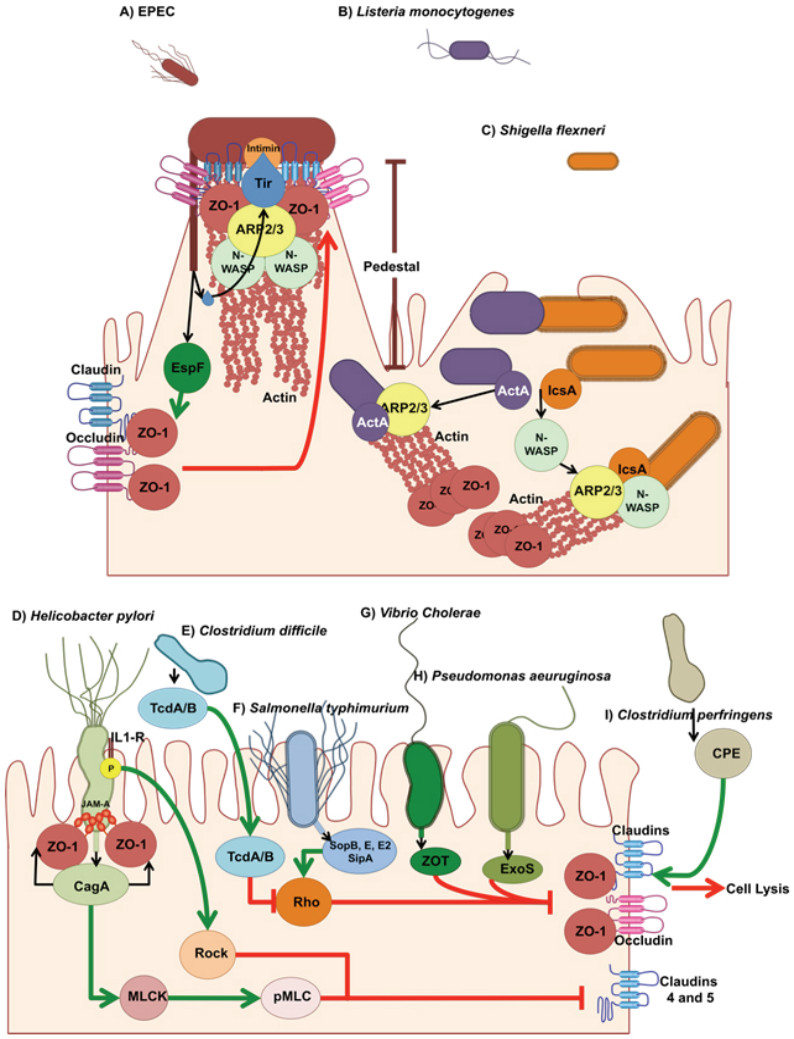

Fig. 14. Pathogens interaction with tight junction proteins A) EPEC produces dissociation of tight junction proteins from the cell borders and recruits these proteins to actin rich pedestals B) *Listeria monocytogenes* and C) *Shigella flexneri*, sequester ZO-1 to their actin comet tails, D) *Helicobacter pylori*, translocates CagA protein, disrupting claudins 4 and 5 expression. E) *Clostridium difficile*, secretes toxins TcdA and B leading to tight junction proteins dissociation. F) *Salmonella typhimurium*, inserts effector proteins that stimulate Rho, disrupting tight junction protein expression. H) *Vibrio cholera*, injects ZOT leading to ZO-1 and occludin disassembly from tight junction. H) *Pseudomonas aeruginosa* translocates toxin ExoS redistributing ZO-1 and occludin. I) *Clostridium perfringens* enterotoxin binds to the second extracellular loop of claudins 3, 4, 6, 7, 8 and 14, producing cell lysis. EPEC, Enteropathogenic *Escherichia coli*; MLCK, myosin light chain kinase; pMLC, phosphorylated myosin light chain; Rock, rho kinase; CPE, *C. perfringens* enterotoxin.

For example: 1) *Helicobacter pylori*, a causative agent of gastritis, gastric ulcers and cancer, translocates CagA protein to the host cell cytoplasm where it activates myosin light chain kinase producing the phosphorylation of myosin light chain and disrupting claudin 4 and 5 expression. In a manner independent of CagA and dependent of interleukin-1 receptor phosphorylation, *H. pylori* is able induce Rho kinase activation that disrupts claudin-4 expression. CagA also ectopically assembles ZO-1 and JAM-A at sites of bacterial attachment 2) In *Clostridium difficile*, the etiologic agent of pseudomembranous colitis, toxins TcdA and B inactivate Rho and hence disorganize the apical ring of actin and dissociate tight junction proteins from the lateral membrane. 3) *Salmonella typhimurium*, a major cause of gastroenteritis, secretes into the host cell the effector proteins SopB, E, E2 and SipA that stimulate Rho proteins and disrupt tight junction protein expression. 4) *Vibrio cholera*, the etiologic agent of cholera injects a protein named ZOT that induces PKCα activation and subsequent phosphorylation of myosin and ZO-1 that provokes the disengagement of ZO-1 from occludin and claudin at the tight junction. 5) *Pseudomonas aeruginosa*, a bacteria frequently present in cystic fibrosis and pneumonia patients, translocates toxin ExoS whose ADP-ribosylating domain disrupts the cytoskeleton and produces redistribution of ZO-1 and occludin.

Other bacterial toxins employ tight junction proteins as their receptors at the plasma membrane. Thus *Clostridium perfringens* enterotoxin binds to the second extracellular loop of claudins 3, 4, 6, 7, 8 and 14, and forms an insoluble complex that alters plasma membrane permeability, producing cell lysis. *Clostridium perfringens* enterotoxin might serve as strategy against cancer as it elicits cytolisis of breast, prostate, ovarian and pancreatic cancer cells that overexpress claudins 3 and 4.

Recommended reading

Claudins

(Furuse *et al.*, 1998), (Furuse *et al.*, 2002), (Nitta *et al.*, 2003), (Turksen and Troy, 2002), (Tatum *et al.*, 2010), (Nakano *et al.*, 2009), (Gow *et al.*, 1999), (Gow *et al.*, 2004), (Ben-Yosef *et al.*, 2003), (Tamura *et al.*, 2008), (Simon *et al.*, 1999), (Miyamoto *et al.*, 2005), (Hou *et al.*, 2009), (Angelow *et al.*, 2008)

Occludin

(Furuse *et al.*, 1993), (Traweger *et al.*, 2002), (Sakakibara *et al.*, 1997), (Saitou *et al.*, 2000), (Furuse *et al.*, 1998), (Du *et al.*, 2010), (Runkle *et al.*, 2011)

MarvelD3

(Raleigh *et al.*, 2010), (Steed *et al.*, 2009)

Tricellulin

(Ikenouchi *et al.*, 2005), (Ikenouchi *et al.*, 2008)

LSR

(Masuda *et al.*, 2011)

JAMs

(Hirabayashi and Hata, 2006), (Weber *et al.*, 2007), (Kostrewa *et al.*, 2001), (Laukoetter *et al.*, 2007), (Pellegrini *et al.*, 2011), (Ishida *et al.*, 2003)

ZO proteins

(de Mendoza *et al*., 2010), (Gonzalez-Mariscal *et al*., 2011), (Lopez-Bayghen *et al*., 2006), (Tapia *et al*., 2009), (Umeda *et al*., 2006), (Katsuno *et al*., 2008), (Xu *et al*., 2008) (Xu *et al*., 2009), (McCrea *et al*., 2009)

Cingulin and Paracingulin

(Guillemont and Citi, 2006), (Guillemot and Citi, 2006), (Guillemot *et al*., 2008), (Aijaz *et al*., 2005)

Tight junctions and disease

(Guttman and Finlay, 2009), (Singh *et al*., 2010), (Gonzalez-Mariscal *et al*., 2007)

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2

Tubulohelical Membrane Arrays, Annulate Lamellae and Nuclear Pores: Tripartite Membrane Architecture with the Participation of Nucleoporins

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1. Introduction

The interest in nucleoporins originates from their identification as constituents of nuclear pores. The latter are visible as prominent annuli in the electron microscope (Callan et al., 1949). Comprehensive studies carried out in the 1950s led to these general conclusions: i) all nuclear envelope (NE) have pores, and ii) irrespective of species or cell type, the pore complexes show similarities in shape and size. This very much supported the hypotheses suggesting that these pores might act as transport channels between the nucleus and the cytoplasm of eukaryotic cells. A breakthrough for these ideas was achieved by the combination of microinjection techniques with transmission electron microscopy (TEM). In 1962, Feldherr injected contrasting permeants into the cytoplasm of amebas and observed their path through pores using TEM. Together with the observation of giant polycystronic messengers in transit through nuclear pores (Stevens and Swift, 1966), these experiments determined the direction of further study in the context of nucleocytoplasmic transport.

The purpose-made transport machinery of nuclear pore complexes (NPCs), located at an interface as distinct as the NE, might be expected to be structurally unique. Surprisingly, however, the search for NPCs soon revealed quite similar annuli incorporated into reticulate cytoplasmic and nucleoplasmic membranes. For these alternative annuli-containing membranes Swift (1956) coined the term annulate lamellae (AL). In contrast to NPCs, there was no independent, strong incentive to elucidate the function of AL pore complexes (ALPCs); to date, all popular hypotheses on AL function are based on reconciling their role with the existence of nuclear pores. A critical review of the facts, however, makes clear that experimental proof for such suggestions is missing. Despite this problem and in the absence of a really comprehensive structural and biochemical comparison, equivalence of NPCs and ALPCs was nevertheless suggested (Kessel, 1981; Miller & Forbes, 2000).

Previously, the lack of knowledge on AL could be regarded as encouraging the search for their possible functions (Merisko, 1989). Richard Kessel, one of the authorities in AL

research, praised AL as the "last frontier in organelle research" (Kessel, 1992). These expectations were not met, however, and the research on AL is currently in decline. Ironically, Kessel´s publication in 1992 became the last comprehensive review on AL in an English journal, followed by the last review at all in the Russian Journal "Tsitologiia" by Morozova et al. (2005). Currently, the scarcity of publications on AL seems to have reached a point that leaves knowledge of their existence limited to a small community of experts.

Our review focuses on a third nucleoporin-containing membrane configuration, which, in conjunction with AL, has been overlooked in recent decades. Study of this enigmatic structure referred to as tubulohelical membrane array (TUHMA) (Reipert et al., 2009, 2010) might open new avenues in understanding AL in a functional context. In the following, NPCs and ALPCs are briefly summarized as annular nucleoporin configurations (Section 2). The TUHMAs are extensively introduced as novel, tubular nucleoporin configurations that differ significantly from the latter (Section 3). The formal separation into annular and tubular structures was chosen to differentiate TUHMAs as a novel player. Understanding them, however, will depend on studies of their dynamic interaction with AL, the NE and other organelles.

2. Annular assemblies of nucleoporins

NPCs and ALPCs manifest themselves in the TEM as ring-shaped pores incorporated into lipid bilayers, previously named as annuli (Feldherr et al., 1962). Their common overall morphology does not necessarily mean that they are equivalent. Studies of the fine structure by advanced EM methods, such as cryo EM and 3D-tomography, are still limited to NPCs. As a consequence, a comprehensive comparison of both structures at a fine structural level has not taken place. Also, the biochemical comparison of the nucleoporin composition of both structures has not been completed.

Here we give a short overview of both annular nucleoporin assemblies. At the same time we would like to refer to excellent overall reviews on NPCs by D'Angelo and Hetzer (2008), Lim et al. (2008), and Hoelz et al. (2011). In more detail, Wente and Rout (2010) addressed the current understanding of the transport mechanisms of the NPCs. The review by Liang and Hetzer (2011) highlights the relationship of NPCs and nucleoporins to gene regulation. Insights into the biogenesis of nuclear pores are provided by Antonin et al. (2008), and Fernandez-Martinez and Rout (2009).

2.1 The nuclear pore complex: A structure known for its major functions

NPCs are giant macromolecular ensembles which form transport channels through the nuclear membrane. They are incorporated into an annular, continuous, and strongly curved connection between the inner and outer nuclear membrane. The NPCs provide a permeable barrier between the nucleoplasm and the cytoplasm, across which only very small molecules (<30-40 KDa) can pass freely. For large soluble proteins, RNA and ribonucleo-particles the NPCs facilitate selective bi-directional transport. The macromolecules assigned for passage bear nuclear localization signals (NLSs) or nuclear export sequences. They are ushered through the nuclear pore channel with the help of transport factors, most of them summarized as karyopherins serving import (importins) and export (exportins). The driving

force for the transport is the hydrolysis of GTP by the GTPase Ran, which is maintained via a nucleocytoplasmic gradient between the two conformations, Ran-GTP and Ran-GDP (for details see: Wente & Rout, 2010). Since it turned out that nucleoporins also play critical roles in chromatin organization and gene regulation, NPCs are also under scrutiny for novel, related functions besides nucleocytoplasmic transport (Liang & Hetzer, 2011).

Despite morphological similarity and common functional aspects, there are differences in the NPCs of various species. Structural comparisons indicate that the vertebrate NPC is larger than its yeast counterpart and they also differ in molecular weight. The frequently quoted 125 MDa of a vertebrate NPC refers to estimates based on STEM analysis of NPCs of the frog *Xenopus laevis* (Reichelt et al., 1990). The weight of yeast NPCs was determined as roughly half of this weight. More recent calculations showed less dramatic differences of 60 MDa for vertebrate NPCs and 44 MDa for yeast NPCs (for review: Cronshaw et al., 2002).

Whether differences in molecular weight and size reflect modifications of the multifunctionality of pore complexes is not known. Variations in the NPC/NE organization as part of different concepts of eukaryotic cell division indicate such a possibility. Metazoa, for instance, undergo open mitosis characterized by the breakdown of the NE. The filamentous network of the nuclear lamina, otherwise tightly linked to NPCs, disassembles during this process. Yeast cells, in contrast, duplicate without NE breakdown. Interestingly, this major characteristic coincides with differences in the organization of the interface between the NPCs and the nucleoplasm of these cells. Yeast cells do not possess nuclear lamina at all (Adam, 2001). Moreover, different requirements for pore anchorage to the NE are indicated by identification of transmembrane nucleoporins in vertebrate and yeast pore complexes, which show no homology (Cronshaw et al., 2002). Here, we briefly introduce vertebrate NPCs, since they are closest in the context of finding TUHMAs in a mammalian cell line.

2.1.1 Structure

Cross-sectioned cell nuclei regularly display pore profiles in the TEM, which indicate the diameter of pore channels to be about 80 nm. If visualized *en face*, these pores appear enlarged in diameter by ring-like structures on their cytoplasmic and nucleoplasmic sides. Both the ring structures and aspects of the inner pore show a characteristic eight-fold symmetry that becomes prominent by TEM mapping based on Fourier analysis (Unwin & Milligan, 1982), and by analysis of frozen-hydrated pore complexes in the cryo-electron microscope. The "zooming in" on nuclear pores of amphibian oocytes by Unwin and Milligan highlighted details of a barrel-like pore architecture that were further elucidated in the following years by using advanced electron microscopic methods, such as high-resolution scanning electron microscopy (SEM), TEM analysis of frozen hydrated samples and electron tomography. Besides individual results of fine structural investigation, consensus has been reached over the major NPC architecture. Accordingly, the pore complex is built around a ring-like central framework that is inserted into the lipid double bilayer of the NE and anchored by trans-membrane proteins. The central framework itself is sandwiched between cytoplasmic and nucleoplasmic ring structures. Anchored to the cytoplasmic ring are filaments heading towards the cytoplasm, thereby giving the impression of individual motility. Filaments emanating from the nucleoplasmic ring,

however, are linked with a distal ring (Antonin et al., 2008). The resulting basket-like structures at the nucleoplasmic side were most clearly visible in association with a lamina network when studying amphibian pore complexes in the high-resolution SEM (Goldberg & Allen, 1992).

2.1.2 Biochemical composition

Historically, attempts to get information on the biochemical composition of NPCs were linked to efforts to isolate NPCs from mammalian NE. Based on NE isolation techniques Aaronson and Blobel (1975) prepared very distinct pore complex-lamina fractions (PCLFs) from rat liver NE, which were devoid of membranous components. The PCLF delivered detailed information on the composition of the nuclear lamina (Gerace & Blobel, 1980) and variations of lamins. Unfortunately, the tight association of the nuclear lamina with NPCs made proper separation of pores without protein losses impossible. A breakthrough in the biochemical characterization of the nuclear pore proteins was made by developing antibodies and subsequent screening of their labeling properties (Gerace et al., 1982; Davis & Blobel, 1986; Snow et al., 1987). While a number of nucleoporins could be identified this way, others remained inaccessible. Therefore, and in anticipation of equivalence between NPCs and ALPCs, AL generated *in vitro* were used as an alternative vertebrate nucleoporin source, which was known to be devoid of lamina (Miller & Forbes, 2000).

Currently, the number of proteins identified as constituents of the vertebrate NPCs includes about 30 nucleoporins (NUPs). For many of them immuno-EM, sometimes combined with differential extraction of membrane components and overlay assays, provided clues as to where they are located with respect to the pore architecture. If this is taken together with information on binding by overlays and immunoblotting it leads to a rather comprehensive view on how these nucleoporins are arranged with respect to each other (for review: Wente & Rout, 2010; Hoelz et al., 2011). Accordingly, the vertebrate NPCs are anchored to the NE by a transmembrane ring that connects to the core scaffold of the pore. This ring is built up of glycoprotein Gp210, pore membrane protein Pom121, and Ndc1. The connection between the nucleoplasm and the cytoplasm is achieved by a central tube which is anchored by linker proteins, Nup88 and Nup93. This central tube is lined and filled by a subset of NUPs containing phenylalanine-glycine (FG) repeat motives which are crucial for the translocation of cargo. The FG NUPs comprise centrally-located constituents (Nup98, Nup62, Nup54, Nup45, Nup48), as well as cytoplasmic (Nup358, Nup214, Nlp1) and nucleoplasmic constituents (Nup153, Tpr). The tube made from FG NUPs is confined by nucleoplasmic and cytoplasmic NUP-containing rings that form a core scaffold. The asymmetry in the composition of nucleoplasmic and cytoplasmic ring structures finds its continuity in filaments that extend towards the nucleoplasm and the cytoplasm. Filaments at the nucleoplasmic side are bound together by a distal ring composed of Nup153 (Panté et al., 1994), while cytoplasmic filaments provide the impression of being flexible.

Notably, whole complexes of NUPs are increasingly understood in a specific functional context. Interaction of FG NUPs of the central tube (Nup358, Nup214, Nup153, and Nup98) with mRNA export cargo, for instance, point to an essential role of these nucleoporins in mRNA export. It is thought that the Nup107-160 complex is involved in this process, too, since overexpression of specific fragments of individual components of the complex causes

marked defects in mRNA export. During mitosis, the Nup107-160 complex locates at kinetochores and spindle poles. The demonstration of its requirement for correct spindle assembly provides an interesting example for multifunctionality of complex nucleoporin ensembles.

2.1.3 Nuclear pore assembly

Metazoan cells undergo open mitosis, characterized by NE breakdown during prophase / metaphase and the reassembly of the NE during telophase. The NE breakdown is accompanied by disassembly of the NPCs. Consequently, NPCs have to reassemble as part of the formation of the nucleocytoplasmic interfaces of the daughter cells. Besides this mitosis-related process, NPCs also form while cells progress through interphase. As shown previously by Maul et al. (1972), this leads to a significant increase in NPC numbers in the interphase nuclei (doubling of pore number from 2000 to 4000 during S-phase in chemically synchronized HeLa cells).

Conditions of pore formation were previously mimicked by mixing precursor vesicle containing *Xenopus egg* extract with demembranated sperm chromatin (Lohka & Masui, 1983; Newport, 1987). As a result of an ATP- and GTP-driven process, vesicle fusion to a NE was observed which contained NPCs. Membrane fusion of the inner and outer membranes of the NE were seen as a possible starting point for pore formation. Currently, alternative scenarios are emerging (for review: Antonin et al., 2008; Webster et al., 2009), based on findings that transmembrane proteins of the NE get incorporated into ER instead of being sequestered in vesicles during mitosis (Daigle et al., 2001). Evidence was found that the contact between outgrowing ER tubules and chromatin initiates the assembly of the NE (Anderson & Hetzer, 2007). In consequence, processes related to the membrane curvature as the basis for membrane tubulation promoted interest in studies of pore biogenesis (Antonin et al., 2008). Moreover, chromatin was also identified as the site where prepores are formed after initial binding of several nucleoporins. How exactly sequestered tubular membranes and prepores find each other and are subsequently transformed into a flattened nuclear membrane is not yet clear.

For the assembly of NPCs into an intact NE, as happens during interphase of the cell cycle, several concepts have been suggested (for review: Fernandez-Martinez & Rout, 2009). The options are i) the *de novo* formation of pores in regions devoid of NPCs, ii) existing NPCs could duplicate, and iii) NPCs could assemble from cytoplasmic membranes or vesicular intermediates. Studies of pore assembly in cell free extracts and results of stable transfected HeLa cells expressing the transmembrane nucleoporin Pom121 support the idea of *de novo* biogenesis of NPCs (D'Ángelo et al., 2006). In the light of these data, it seems unlikely that cytoplasmic membranes in the form of AL play a role in NPC biogenesis. Therefore, one might wonder what functions other than serving the NPCs could be related to ALPCs.

2.2 The annulate lamellae pore complex: A structure on the search for a function

Up to the 1990s a tremendous amount of EM data was accumulated, either as short 'case reports' or in the form of systematic studies, which all provided evidence for the existence of AL in a wide variety of eukaryotic cell types and species (Kessel, 1989). Despite their

indicated omnipresence, AL were rarely encountered in TEM thin sections. This made systematic studies of the potentially new organelle difficult.

Later on, studies of AL profited from the development of antibodies against nucleoporins. Initial data indicating that ALPCs and NPCs share the central pore protein Nup62 (Dabauvalle et al., 1991; Cordes et al., 1995) were followed by more extensive immunohistochemical comparison of nuclear and cytoplasmic pores (Ewald et al., 1996). More recently, such comparative studies were complemented by observation of green fluorescent protein (GFP)-tagged nucleoporins expressed after transfection (Imreh & Hallberg, 2000; Daigle et al., 2001). Both immunohistochemistry and transfection techniques provided major technical improvements for systematic studies of AL in tissue culture cells. For the first time, the overall distribution of cytoplasmic (and also nucleoplasmic) nucleoporins became visible in the form of fluorescent spots. As demonstrated in Fig. 1, for PtK2 epithelial cells stimulated to generate cytoplasmic AL by vinblastine sulfate treatment, these spots could become large in size and strong in intensity.

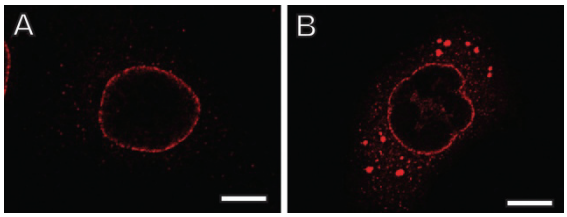

Fig. 1. Visualization of AL induced in PtK2 cells. A) Confocal section of an untreated cell displaying NPCs labeled with mAb 414. B) Treatment with vinblastine sulphate results in bright fluorescent spots in the cytoplasm reminiscent of AL. Bars, 10 μm.

Unfortunately, further technical advances were cut short by the conclusion that these spot-like labeling patterns represent AL. Whether such a correlation can be generalized or not is not yet clear, since correlative light and electron microscopy has so far spared AL as a field of research. Nevertheless, one can be confident that the fluorescently-labeled structures mostly represent AL, since it could be shown that antibodies applied at light microscopic level also labeled AL if used for immunogold labeling (Ewald et al., 1996). Accordingly, immunofluorescence microscopy was used for identification of cell lines which contain significant amounts of endogenous AL (Cordes et al., 1996). Despite this preparatory work, somatic cells were not much used in the past as models for research on AL.

Notably, isolation of AL for biochemical analysis and other studies poses a technical challenge. Generation of AL from amphibian oocytes has proven a useful strategy to overcome difficulties in isolation. In 1991, a method of generating AL cell-free from *Xenopus* egg extracts was published by Dabauvalle et al.. Further progress towards an assay was made by separation of membranous and other components that are not required for AL assembly *in vitro* by using ultracentrifugation (Meier et al., 1995). The resulting AL was separated for analysis of nucleoporins based on Western blotting. The cell-free AL formation assays were subsequently used in a couple of sophisticated experiments, which led to extension of the list of nucleoporins known to be located at both NPCs and ALPCs (Miller et al., 2000; Miller & Forbes, 2000).

A review of the publications on AL from previous years indicates that the incentive for elucidation of these structures seems to have changed. Initially, a systematic comparison of ALPCs and NPCs was seen as a way of possibly revealing any differences (Ewald et al., 1996; Cordes et al., 1996). As a result, a set of data was generated that concentrated on the similarities of both structures. To be precise, however, it should be stated that the biochemical comparison of ALPCs and NPCs is still incomplete, and that a systematic comparison of their fine structure is still at an early stage. Despite this, equivalence of NPCs and ALPCs was used as an argument in more recent publications (Miller & Forbes, 2000), and AL were referred to as NPC-containing membrane structures (Walther, et al., 2003).

2.2.1 AL structure and arrangement

AL are conspicuous in TEM thin sections, since they contain prominent annuli incorporated in reticular lipid membrane sheets (Fig. 2).

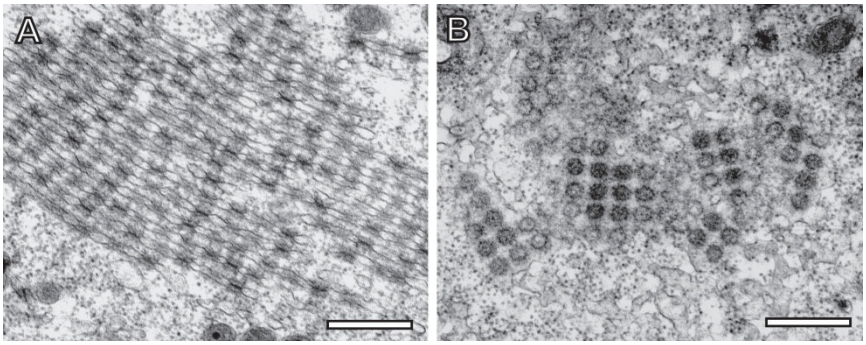

Fig. 2. AL in the cytoplasm of *Xenopus* oocytes visualized by TEM. A) Longitudinal epoxy resin section displaying an AL membrane stack endowed with numerous pore profiles. B) Transverse section displaying annuli in the *en face* view. Bars, 500 nm.

In cross sections, these annuli display a profile of about the same size as pore channels of nuclear pores. The *en face* view of the resin embedded or negatively stained annuli reveals ring structures around an inner pore channel, which are of eight-fold symmetry, quite similar to nuclear pores (Maul, 1970a; Franke et al., 1981). Taken together, conventional TEM allows the identification of AL by morphological criteria.

ALPCs are frequently densely packed thereby covering most of the membrane surface. This is particularly true for AL stacks of amphibian oocytes, as demonstrated in Fig. 3 representing a surface view on AL by scanning electron microscopy. Kessel (1989) described such an optimal use of membrane space by ALPCs as a tight hexagonal package. Notably, NPCs may sometimes reach similarly high densities by clustering of pores at the NE, as observed for nuclei of the *Xenopus* oocytes at developmental stage 2-3 (Kiseleva, unpublished results), in growing blastoderm nuclei in drosophila embryo (Gubanova & Kiseleva, 2008), and in hematopoietic cells under pathological conditions leading to cell death (Reipert et al., 1996).

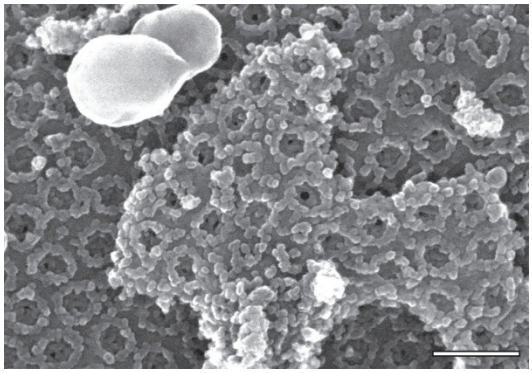

Fig. 3. Tightly distributed cytoplasmic pores of AL attached to the NE in *Xenopus* oocyte visualized by field emission in-lens scanning electron microscopy. Bar, 200 nm.

2.2.2 Occurrence and affecting factors

A review by Kessel (1989) meticulously summarized reports on the occurrences of AL in a variety of species and cell types, including observations related to pathological stages. Accordingly, AL were found in eukaryotes as different as mollusks, crustaceans, insects, amphibians, fish, reptiles, birds, plants and mammals. While many of the publications listed in this review referred to observations made in male and female germ cells, it also made very clear that the occurrence of AL is by no means restricted to them. AL was reported for somatic cells, including a variety of embryonic cells, epithelial cells, secretory cells, hormone secreting cells, muscle, and nerve cells. Kessel (1989) concluded that AL are present in all basic tissue cell types with the possible exception of some connective tissue cells.

Despite their wide-spread distribution, the likelihood of finding AL in random EM sections is rather low. This seems to be particularly true for somatic cells where the occurrence of AL might be vastly underestimated (Cordes et al., 1996). Only under conditions of metabolic activity, differentiation and rapid proliferation, does AL seem to be expressed more abundantly (Kessel 1989, 1992; Merisko, 1989). Some of these conditions are linked to diseases, including cancer, while others are part of normal cell physiological processes. Especially in germ cells and embryos, AL can be found in large amounts, organized in multiple, large stacks. Mature *Xenopus* oocytes for instance organize about 80% of all pore complexes in cytoplasmic stacks (Cordes et al., 1995). In consequence, systematic research on AL relies to a great extent on the use of oocytes and embryos.

Notably, the cellular distribution of AL can be affected by a diversity of external conditions imposed on cells and tissues. A comprehensive list of AL- affecting circumstances including drugs, culture conditions, and pathogens can be found in the review by Kessel (1992). Among the AL-affecting factors, tubulin-depolymerizing agents such as vinblastine sulfate have been proven to be effective inducers of AL in tissue culture cells (De Brabander & Borgers, 1975; Kessel & Katow, 1984; Ewald et al., 1996; Reipert et al., 2009). Given the

striking effectiveness of these agents, it was speculated that there is a possible relationship between formation of the AL network and the disassembly of microtubules (MTs) (De Brabander & Borgers, 1975; Merisko, 1989). A direct regulatory effect of tubulins on AL formation, however, has not yet been shown experimentally.

More recently, a direct metabolic link between importin β and AL formation in *Xenopus* egg extracts was reported (Walther et al., 2003) that, in the future, might be exploited for studying of AL in somatic tissue culture models. Based on a comprehensive set of experimental data, they suggested that RanGTP allows the incorporation of nucleoporins into membranes by releasing the inhibitory effect of importin β.

2.2.3 Biochemical composition

In contrast to NE, AL do not provide organelle compartmentalization. Instead, their membranes are surrounded by an exclusively cytoplasmic environment. The interface between cytoplasm and their laminar membrane sheets does not contain lamina built up from lamins, like the vertebrate nucleus (Chen & Merisko, 1988; Daigle et al., 2001). In agreement with this notion it was found that lamins are not required for assembly of AL in egg extracts. While antibodies against lamins can block formation of NPCs they do not hinder the assembly of AL stacks (Dabauvalle et al., 1991). Moreover, evidence was found that emerin, a transmembrane protein of the inner nuclear membrane, is not present in AL (Dabauvalle et al., 1999).

With the identification of nucleoporins as constituents of NPCs the question emerged whether they are also constituents of ALPCs. Labeling with the lectin wheat germ agglutinin (WGA) turned out to be positive for the O-linked N-acetylglucosamine (GlcNAc) containing moieties of glycoproteins in pore structures of both NPCs and ALPCs (Allen, 1990). More specifically, by using immunohistochemistry, immuno EM, and biochemical analysis of AL in *Xenopus* oocytes and egg extracts, the glycoprotein Nup62, a central constituent of nuclear pores, was identified as part of ALPCs (Dabauvalle et al., 1991; Cordes et al., 1995; Ewald et al., 1996; Daigle et al., 2001).

In 1996 Ewald et al. added further evidence of biochemical similarities; application of antibodies against nucleoporins located at both sides of the NPCs, namely cytoplasmic Nup180 and nucleoplasmic Nup153, demonstrated that these nucleoporins are also part of ALPCs. The nucleoporins Gp210 and Pom121, however, could not be detected as constituents of AL by this method. A molecular biological approach, based on expression of YFP-tagged Pom121 and Nup153 in tissue culture cells, however, also identified Pom121 as constituent of AL. Besides this, it confirmed previous data concerning the presence of Nup153 in AL (Imreh & Hallberg, 2000; Daigle et al., 2001). Furthermore, fluorescence recovery after photobleaching (FRAP) indicated that the turnover of Pom121 located in AL was rapid, while it was very slow in NPCs. Nup153, in contrast, seemed to be replaced continuously in both structures (Daigle et al., 2001).

Moreover, progress in biochemical analysis was achieved by studying AL formed under cell-free conditions from *Xenopus* egg extracts (Miller et al., 2000; Miller & Forbes, 2000). Aimed at identification of novel NPCs constituents by circumvention of the difficulties in

isolation of vertebrate NPCs, isolation of AL from egg extracts also led to the identification of a number of nucleoporins of *Xenopus* ALPCs. For this Miller et al. (2000) collected glycoproteins of the egg extract by using a WGA-Sepharose column, and biotin-tagged them prior to addition to the AL formation assay. As a result, Nup62, Nup98, and Nup214 were identified as constituents of the *in vitro* assembled AL. Furthermore, it was reasoned that Nup93, Nup205, and a newly identified Nup188 are present in *Xenopus* AL. Taken together this added to the evidence for similarities in the biochemical composition of ALPCs and NPCs. On the other hand, the nucleoporins identified so far represent just a fraction of those known to build up the vertebrate NPC.

2.2.4 Biogenesis

Concerning the biogenesis it is not yet clear how ALPCs are formed and from where they originate. For the latter, any suggestions are based on microscopic observations putting their cellular positioning in relation to other organelles. Accordingly, either a nuclear origin, or their formation in conjunction with rough endoplasmic reticulum (rER) were hypothesized (for review: Wischnitzer, 1970; Merisko, 1989; Kessel, 1989, 1992). Numerous reports proposed ideas in favour of one of these popular suggestions, because of their own observations of AL located proximal to the cell nucleus, or attached to rER. Also, an alternative suggestion was made based on much less frequent findings of AL linked to the Golgi complex, proposing a role of the Golgi complex in AL biogenesis (Maul, 1970b). That proposal was criticized for not being backed by EM data depicting the dynamic process related to AL formation (Kessel, 1989). In hindsight, however, all previous EM studies could only provide ideas on how biogenesis might occur. Since the observations in the EM sections occur almost randomly, there is the possibility that short but important steps in the dynamics of AL assembly were outnumbered by those observations that attracted regular attention. This seems to have happened with respect to the Golgi complex and AL. In consequence, studies of the putative relationship of both structures became sidelined.

Decades later, immunohistochemistry opened up the opportunity for a critical review of the linkage between AL and the Golgi complex. Initial double immunofluorescence labeling of AL and the Golgi complex of bovine epithelium cells, however, could not confirm any spatial relationship between both structures (Cordes et al., 1996). Based on a rather limited set of immunofluorescence data, Cordes et al. argued more generally against an intimate relationship between AL and the Golgi complexes.

An interesting aspect of AL biogenesis is its supposed coupling to the cell cycle indicated by the disassembly of AL during mitosis and their subsequent reappearance (Maul, 1970a; Erlandson & de Harven, 1971). Formation of AL as early as the telophase was confirmed later on by immunofluorescence microscopy (Cordes et al., 1996). In their morphometric studies of AL of synchronized HeLa cells Erlandson and de Harven (1971) discriminated between AL in direct continuity with rER and those showing no association with reticular membranes. From their data one could come to the conclusion that linkage with rER happens after AL is already formed, in preparation of cell division, at the S-phase of the cell cycle. Verifying the relationship of AL with respect to rER, the nucleus and other organelles during the cell cycle, therefore, could shed light on the sequence and nature of events in

which AL is involved. Such dynamics could possibly be coupled to microtubules (MTs). The latter seem to have been overlooked in systematic studies in the past despite reports of a possible association of MTs with AL (Kessel ,1992; Sutovsky et al., 1998). Perhaps, this neglect resulted from experience with MT-depolymerizing drugs, indicating that MTs are not required for AL formation itself.

3. Tubular assemblies of nucleoporins: The tubulohelical membrane array

The tubulohelical membrane array (TUHMA) was observed for the first time in the rat kangaroo kidney epithelial cell line PtK2 (Reipert et al., 2009). The initial observation was made by validating large numbers of cells for their structural preservation during rapid microwave-accelerated fixation with low concentration glutaraldehyde (Reipert et al., 2008). TUHMAs could also be observed in cryofixed and low-temperature processed samples (Reipert et al., 2009). Soon, however, it became clear that for identification of TUHMAs advanced EM preparation techniques are not required.

Initial studies indicated that TUHMAs are not very abundant within the asynchronously grown cell population of PtK2 cells. However, their single-organelle-like appearance only became obvious by immunofluorescence microscopy, using the monoclonal antibody (mAb) 414, as the most commonly used nucleoporin marker (Davis & Blobel, 1986). For successful labeling of the nucleoporins located at the tubular cores of TUHMAs, sufficient extraction of lipid material from the surrounding membranes was critical. The resulting tubular fluorescence patterns could be observed without interference from the positive cytoplasmic background, because PtK2 cells contain almost no endogenous AL (Fig. 1A), otherwise resulting in numerous fluorescent spots of various sizes. Because of both, the undisturbed view and the single-organelle-like appearance, the initial light microscopic studies could be effectively used to get hints of the extraordinary dynamics of TUHMAs.

Why did TUHMAs remain hidden to the discerning eye of electron microscopists for about half a century? We see an amalgamation of reasons: i) their transient nature, ii) their rare occurrence in thin sections, and iii) their structural hallmarks were overlooked in the two-dimensional view of TEM thin sections. In the following we provide an introduction to TUHMAs that should enable their identification in a diversity of cell types and species.

3.1 Structure

TUHMAs (Fig. 4) are lipid membrane arrays organized around tubular, proteinaceous electron-dense cores of 80 nm in diameter, confined by helix-like threads. Notably, this diameter is of about the same size as the inner pore channel of NPCs and ALPCs. The helical threads manifest themselves as zigzag-patterns resulting from their confinement within resin sections under conditions of almost parallel orientation with respect to the cutting plane. The zigzag-patterns are very clearly visible if the sections are of about the same thickness as the diameter of the core tubules of the TUHMAs. Such thin sections in the range of 80 to 100 nm are routinely produced with an ultramicrotome. It is important to note that the contrast patterns of TUHMAs cannot be explained by transverse alignment of annular pore complexes within AL membrane stacks (Hertig, 1968; Kessel, 1986).

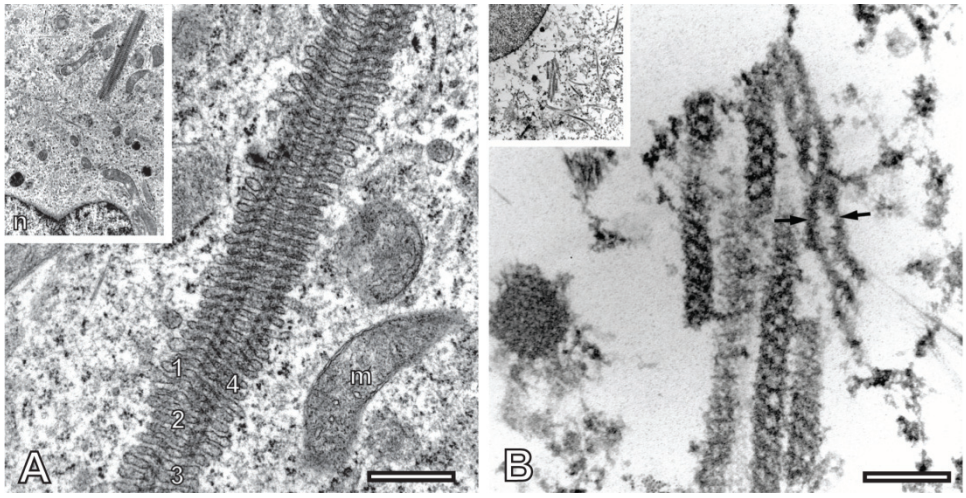

Fig. 4. TUHMA visualized by TEM. A) Aldehyde fixation followed by osmification. Tubules of uniform diameter of 80 nm, numbered 1–4, are incorporated in a regular stack of membranes and confined by helical threads resulting in black zigzag-patterns. Mitochondrion, m. B) Aldehyde fixation followed by extraction with Triton X-100 and osmification. The lipid membranes are dissolved, while the proteinaceous core tubules remain intact exposing their helix-like aspects. The core tubule on the right seems to detangle into separate threads (black arrows) (Reipert et al., 2010). Bars in A and B, 500 nm.

In agreement with our interpretation, we could follow the continuity of the helix-like threads of TUHMAs through stacks of tomographic sections (supplement in Reipert et al., 2009). Furthermore, we found that the helix-like threads lining core tubules withstand treatment of MW-fixed PtK2 cells with Triton-X100 (Reipert et al., 2010). Their helical nature was particularly apparent where disentanglement occurred (Fig. 4B). The latter indicates the possibility that the core tubule-confining threads might be double-helical in nature. However, a comprehensive analysis of the helix type is outstanding. Therefore, we use the more general terms here of helix-like or helical structures.

The core tubules provide the basis for an intermingled membrane scaffold of strongly curved lipid membranes. The overall lengths of the resulting array vary between 3-5 µm. The lipid membranes themselves are of particularly narrow curvature, and do not contain pore complexes. The nonlamellar character of these membranes was confirmed by electron tomography (see supplement in Reipert et al., 2009). Therefore, we conclude that the two major criteria characterizing AL structurally, i) ring-shaped annuli, and ii) lamellar membrane stacks (Merisko, 1989), are not applicable to TUHMAs.

3.2 Occurrence

Based on the structural definition given above, we reviewed previous publications on AL. As a result, we found a few structures resembling TUHMAs at different levels of visibility (Goldstein, 1971; Bhawan et al., 1978; Matsubara & Mair, 1979; Sun & White, 1979; Gracia-Navarro et al., 1980; Hill et al., 1984; Allen, 1988; Cheville, 1994; Alvaraz-Buylla et al., 1998).

With one exception, all authors interpreted their observations to be AL. Sun and White (1979), however, reported on a "peculiar configuration of agranular reticulum of braided channels" reminiscent of the nonlamellar membrane architecture of TUHMAs. Despite AL being the prime target of their studies, they provided an interpretation that avoided any confusion of both structures. From our point of view, their interpretation was furthered by fixation under hypotonic conditions, and very intense contrasting of lipid membranes. In consequence, the zigzag-patterns, hallmarks of TUHMAs, are less visible.

There are methods and circumstances whereby the visibility of the helix-like aspect can be enhanced. Intentional omission of membrane staining (Reipert et al., 2009), or pure contrasting of lipid membranes, both result in the almost exclusive display of helical patterns. In line with this argument, Denys Wheatley (1993, 1999) discovered helical structures in the murine fibroblast cell line 3T3, which closely resemble the proteinaceous aspects of TUHMAs. Wheatley clearly differentiated between findings described as "helical inclusions- or extrusions", and AL observed in close proximity to these structures. While this is very much in agreement with Reipert et al. (2009), it seems that the incorporation of these helical structures in complex lipid membrane arrays was overlooked by Wheatley. In consequence, he interpreted his findings as "helical pores", and postulated their extrusion from the nucleus into the cytoplasm. His attempt to substantiate this claim by immunolabeling of nucleoporins could possibly have failed because of insufficient permeabilization of the surrounding lipid membranes.

Notably, the references cited above refer to *in vivo* and *in vitro* studies in diverse somatic cells types and species. Accordingly, we would expect TUHMAs in species as different as amphibians (Gracia-Novarro et al., 1980); birds (Alvaraz-Buylla et al., 1998), and mammals (Bhawan et al., 1978; Matsubara & Mair, 1979; Sun & White, 1979; Cheville, 1994). With respect to the cell types, TUHMA-resembling structures were found in neuroblastoma cells (Goldstein, 1971), melanoma cells (Sun & White, 1979), striated muscle tissue (Matsubara & Mair, 1979), fibroblasts (Wheatley, 1993, 1999), Burkitt´s lymphoma cells (Allen, 1988), tissue of venereal sarcoma (Hill et al., 1984), hormone cells of the pituitary gland of amphibians (Gracia-Navarro et al., 1980), and neuronal precursor cells of the ventricular zone of the central nervous system of birds (Alvaraz-Buylla et al., 1998). While the majority of these examples refers to cancerous cells, the data by Gracia-Navarro et al. (1980) and Alvaraz-Buylla et al. (1997) were generated from tissues which were supposedly healthy and metabolically active by stimulated hormone secretion and neuronal differentiation, respectively.

3.3 Biochemistry

Studies of the biochemical composition of TUHMA are currently at an early stage. Isolation of TUHMAs from tissue culture has not yet been accomplished but efforts will be greatly facilitated by the availability of antibodies against nucleoporins that could serve as markers for TUHMAs in Western blotting. Notably, the mAb 414 recognizes not just one individual nucleoporin, but a whole set including the central pore protein Nup62, the nucleoplasmic Nup153, and the peripheral cytoplasmic proteins Nup214 and Nup358 (Davis & Blobel 1986; Cronshaw et al., 2002). After successful application of mAb 414 in immunohistochemistry, therefore, the question arises which of the nucleoporins stated above are indeed constituents of TUHMAs? By application of a human autoimmune serum well-characterized for

recognizing Nup62 (Wesierska-Gadek et al., 2008), we found evidence that this central constituent of porous annuli of both NPCs and ALPCs also occurs in TUHMAs. Identification of further constituents based on immunohistochemistry is currently limited by the availability of antibodies showing cross-reactivity with the marsupial species of our cell model, PtK2 cells.

Progress in studying TUHMAs can be expected by their isolation for proteomic and lipidomic analysis. Preliminary data (not shown) indicate that the cytoplasm of PtK2 cells indeed contains a nucleoporin-rich membrane fraction, clearly recognizable by Western blotting. If tested with antibodies against lamins this membrane fraction showed no indication for contamination with nuclear membranes. Therefore, and because of the almost complete absence of AL in PtK2 cells (Cordes et al., 1996; Reipert et al., 2009), the resulting signal should originate almost exclusively from TUHMAs. Following this argument, we hypothesize that all four nucleoporins recognized by mAb 414 are present in TUHMAs.

3.4 Association of TUHMAs with membrane-bound organelles

Besides information concerning the architecture of TUHMAs themselves, TEM provided insights into the association of this membrane array with other organelles. The initial data already contain surprisingly concise information on the interface between TUHMAs and adjacent organelles. Identification of nucleoporins as markers of TUHMAs greatly extended the possibilities for studying these entities in relation to other organelles, since it enables overall viewing, which incorporates a larger number of cells.

3.4.1 Association with the cell nucleus

Most remarkably, TUHMAs are able to take a polarized position with respect to the cell nucleus. Frequently, they can be found oriented either perpendicular or parallel to the nuclear membrane. Fig. 5 demonstrates these two preferential orientations of TUHMAs at light microscopic level with tubular fluorescence patterns directly attached to prophase nuclei. Correspondingly, TEM data showing TUHMAs in polarized positions with respect to the nucleus were published (Reipert et al., 2009). Besides this, polarized positioning of structures resembling TUHMAs has also been verified in previous publications (Goldstein, 1971; Matsubara & Mair, 1979; Hill et al., 1984; Wheatley, 1999).

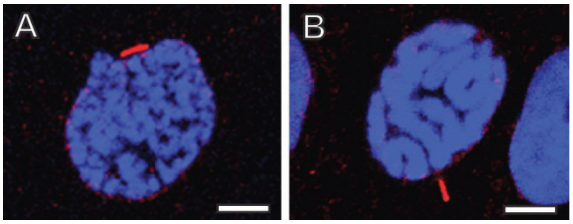

Fig. 5. Polarized positioning of TUHMAs with respect to the cell nucleus. Confocal section displaying tubular fluorescence patterns (red) labeled with mAb 414, in A) oriented parallel and in close proximity to an early prophase nucleus, and in B) perpendicular to a nucleus at a late stage of prophase. DNA counterstaining with Hoechst 33258. Bars, 5µm.

The positioning of TUHMAs close to cell nuclei in the light microscope indicates the possibility of a physical linkage. Such a linkage was confirmed by TEM serial sectioning, demonstrating that TUHMAs close enough to the nucleus are anchored via membranes to the nuclear membrane (Reipert et al., 2009). Importantly, such membrane bridges mediate the association with the nucleus regardless of the orientation of TUHMAs. Notably, these observations concerning TUHMAs differ significantly from the situation in chimpanzee oocytes. The latter displays arrays of AL with perfectly aligned ALPCs, which are linked exclusively perpendicular to the oocyte nucleus (Barton & Hertig, 1972). Both the variability in length of the nucleus-associated arrays, and the precise positioning of their ALPC columns in conjunction with NPCs, suggest the extrusion of AL from the oocyte nucleus. In contrast, TUHMAs of the somatic cell line PtK2 fused perpendicular to the nucleus show neither variation in their lengths, nor continuity of their core tubules with individual NPCs. Any TUHMAs proximal or fused to the nucleus in either direction seem to be "matured" with respect to their length, since no short fluorescent tubules could be found in this region. Therefore, we conclude that a scenario of extrusion of nucleoporins from the nucleus as part of TUHMA biogenesis does not appear to happen. Instead, we suggest that the assembly of TUHMAs occurs at some distance from the nucleus.

3.4.2 Association with ER and AL

EM revealed that TUHMAs, similar to AL, are in continuity with rER. Besides this apparent connection, TUHMAs are linked to a more extended membranous continuum. Small patches of AL were observed in association with TUHMAs; their ALPCs showed the characteristic eight-fold symmetry in the *en face* view (Reipert et al., 2009). Surprisingly, however, no immunofluorescent spots, reminiscent of AL, could be found next to TUHMAs that corresponded with the EM data. Since, otherwise, vinblastine-induced AL were clearly visible under the given preparation protocol, one may wonder what could have prevented identification of AL in its specific location next to TUHMAs. Independent of how this question may be answered in the future, the linkage of AL with TUHMAs is crucial for the understanding of both structures. In agreement with this notion, Bahwan et al. (1978), Matsubara & Mair (1979), Sun & White (1979), Gracia-Navarro et al. (1980), Hill et al. (1984), and Cheville (1994) all visualized AL in continuity with structures resembling TUHMAs. However, only Wheatley (1993) understood the necessity to differentiate between them. He made the point that there seems to be an "enigmatic relation" between helix-structures reminiscent of the core tubules of TUHMAs, and AL.

3.4.3 Association with the Golgi complex

Double labeling with mAb414 and antibodies against the Golgi matrix protein GM130 revealed that there is no fixed lateral constellation between TUHMAs and the Golgi complex within the asynchronously grown population of PtK2 cells. While most TUHMAs were located at a distance from the Golgi complex, a small fraction was linked to it. According to Reipert et al. (2010) the linkage between TUHMAs and the Golgi complex is mediated by intermediate membrane structures, named tubuloreticular structures (TRS) and AL proximal to the Golgi complex. Fig. 6 captured the rare event of getting all four structures visualized in a single TEM micrograph. If TUHMAs assemble as a result of membrane

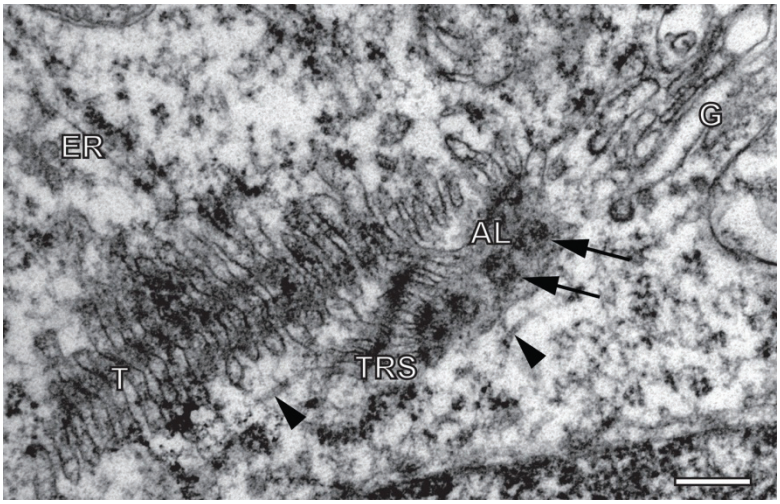

Fig. 6. The TUHMA/ TRS/ AL/ Golgi connection. A small patch of AL containing ALPCs (arrows) is located next to the Golgi complex (G). The connection between AL and a TUHMA is mediated by TRS. The TUHMA is also linked to rER. Note MTs (arrowheads) running in parallel to the axis of the TUHMA. Bars, 200 nm.

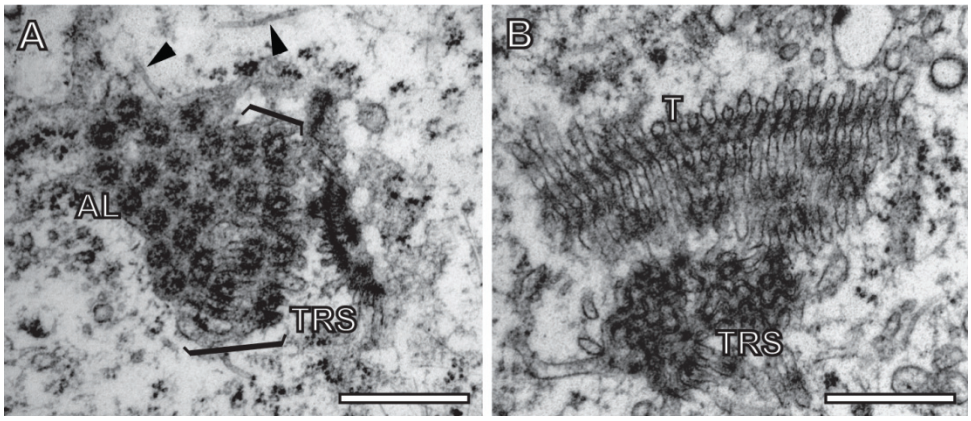

Fig. 7. TEM evidence for a possible transition of AL into TUHMAs. A) Patch of AL displaying structural intact ALPCs on its left side, and opened annuli in a transitional zone (in brackets), indicating membrane tubulation. The transition zone is continuous with TRS. Note also MTs (arrowheads). B) TUHMA linked to TRS containing darkly contrasted loops that resemble building elements of cubic membranes. Bars, 500 nm.

transformations proximal to the Golgi complex, as hypothesized previously, this should include transformation of annular nucleoporins into helical structures (Reipert et al., 2010). Fig. 7A demonstrates that AL indeed undergo a transformation. As indicated, they open up their nucleoporin-containing annuli, while at the same time the membrane sheet transforms into tubules continuously linked to TRS. The TRS themselves seem to undergo alterations

resulting in structures of a higher order. Consequently, they resemble in part a 'crystalline' lipid membrane formation, or so-called 3D-periodic, nonlamellar cubic membranes (Almsherqi et al., 2009). In conclusion, TRS, as a transitional structure linked to the Golgi complex and AL, should contain nucleoporins in an intermediate conformation before helical arrangement into TUHMAs.

3.5 Association with microtubules

On many occasions EM revealed the association of TUHMAs with MTs (Reipert et al., 2009). Therefore it seems reasonable to suggest a microtubular motor-driven dynamic being responsible for both the translocation and polarized alignment of TUHMAs with respect to the nucleus. As indicated by double labeling of TUHMAs and α-tubulin, variability in positioning of TUHMAs with respect to microtubule organizing centers (MTOCs), including those serving as basal bodies for primary cilia, is part of such a dynamic. To highlight the relationship between MTs and TUHMAs more specifically, a subset of tubulins was labeled with antibodies against detyrosinated tubulin (detyrTub), known to mark the axoneme of primary cilia and centrioles (Poole et al., 1997; Ou et al., 2003). As demonstrated in Fig. 8, it turned out that some TUHMAs showed partial co-localization with tubules built up from the longer-living form of tubulin (Infante et al., 2000). Others showed co-localization in combination with orthogonally-oriented tubules that were exclusively labeled by mAb against detyrTub. Even more striking, some axonemes of primary cilia, located in the upper section planes of the PtK2 cells, showed labeling for nucleoporin, besides labeling with Abs against detyrTub as their marker. The idea of a translocation of nucleoporins under participation of MTs was further supported by observation of single fluorescent dots of nucleoporin label in association with a small subset of primary cilia of serum starved PtK2 cells (Reipert et al., 2010). While, at first glance, the localization of nucleoporins at primary cilia and their axonemes may appear far-fetched, this could be an important aspect adding to the most recent research aiming to identify a "ciliary pore" similar to the NPC (Huang & Tsao, 2010). The current interest in such similarities was most recently inspired by evidence of the control of access of molecules to primary cilia via importin-β2 and the small GTPase Ran, and by identification of ciliary localization sequences (CLS), that, similar to nuclear localization sequences, mark the cargo for translocation (Dishinger et al., 2010).

3.6 Indications for a coupling of TUHMAs to the cell cycle

TUHMAs are apparently linked to the cycling cells, since they are almost absent in resting cell populations of so-called ´quiescent cells´ obtained by serum-starvation (Reipert et al., 2009). Accordingly, they show synchrony within individual colonies that could be followed for up to two cell divisions (results not shown). Moreover, immunofluorescence microscopy indicates that TUHMAs disappear during the course of mitosis, in a very similar way to AL (Cordes et al., 1996; Reipert et al., 2009). The latest signs for their existence can be observed in an advanced stage of prophase (Fig. 5). At this stage, TUHMAs are expected to be physically linked to the NE by membrane fusion. Their subsequent fade, therefore, very likely coincides with the disassembly of the NE and its pore complexes. In consequence, both AL and TUHMAs need to be re-assembled in the newly divided cells. While AL are already formed at late mitosis, TUHMAs would be expected to be seen much later. This can be concluded from the low number of cells containing TUHMAs that were observed in the

asynchronously grown cell population. Their presence in just ca. 5-10% of the cells would not fit with an early occurrence at the G1 phase of the cell cycle. This notion is very much in agreement with the hypothesis that TUHMA formation requires AL. If seen from the perspective of the ciliary cycle (Plotnikova et al., 2009), therefore, TUHMAs are not expected to play a role in the formation and outgrowth of primary cilia. However, they might be linked to processes of centriole duplication and ciliary resorbtion.

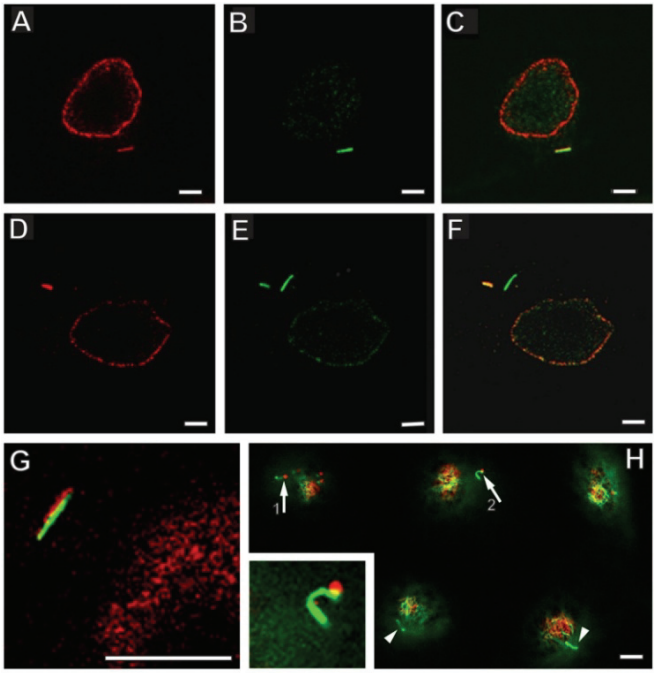

Fig. 8. Putative relation between TUHMAs and primary cilia. A) A TUHMA labeled with mAb 414 (red) and B) tubule-forming detyrTub (green) located in a confocal section close to the substratum of cell growth. C) overlay of A and B. D) TUHMA labeled with mAb414 (red) and E) detyrTub (green) forming two tubules positioned orthogonally to each other located in a confocal section close to the substratum of cell growth. F) Overlay of D and E indicating that just one of the detyrTub containing tubules co-localizes with the TUHMA in D. G) Co-localization of tubular fluorescence patterns labeled with mAb 414 and detyrTub in an upper confocal section perhaps harbouring the basal body. F) Two short cilia (arrows), numbered 1and 2, labeled with Ab against α-tubulin (green) are associated with red dots resulting from labeling with mAb 414. The insert displays cilium 2 in more detail. In contrast to cilia 1 and 2, two longer cilia in neighbouring cells (arrowheads) are free of labeling with mAb 414. (Reipert et al., 2010). Bars, 5 µm.

4. Conclusion

Taken together the initial data on TUHMAs indicate that nucleoporins are more versatile building elements than previously thought. Under as yet unknown conditions they are able

to organize in a non-annular, much more complex cytoplasmic arrangement. In the light of this finding we carried out a critical review of those structures already known to contain nucleoporins, namely NPCs and ALPCs. In the process it became apparent that the research on ALPCs lags far behind studies of the NPCs. Although this shortcoming makes a comprehensive comparison impossible, the current opinion tends to favor the equivalence of both structures. In consequence, AL are considered at best as subordinate structures serving the requirements of the NE.

Although a homeostasis involving NPCs and ALPCs was suggested, it has not so far been proven. On the contrary, recent morphometric analysis showed that the number of AL pores did not decrease to compensate for the growing number of nuclear pores during syncytial development of *Drosophila* embryos (Onischenko et al., 2004). Moreover it was found that during development of the *Drosophila* syncytium NPCs and ALPCs undergo a parallel regulation of their assembly/disassembly driven by the mitotic trigger Cdk1 and protein phosphatases (Onischenko et al., 2005). These studies are in line with previous observations of AL formation happening in parallel with the insertion of NPCs in the NE of pronuclei during fertilization of mammalian oocytes (Sotovsky et al., 1998). Taken together, these results neither indicate quantitative balancing between NPCs and ALPCs, nor accumulation of AL as a result of excessive nucleoporin synthesis. Proximity and sometimes even fusion of AL with the NE, however, point to the necessity of verifying their relationship to each other in future studies. A role of their connection in regulatory functions is indicated by observations that reported the accumulation of M-phase-promoting factor (Cdc2/Cyclin B2) in colocalization with AL in *Xenopus* oocytes (Beckhelling et al., 2003).

Alternative suggestions, which propose functions specific to ALPCs, have just recently emerged. Boulware and Marchant (2008) sought ALPC function in the context of differential regulation of cytoplasmic Ca^{2+} signaling. Their concept is based on the assumption that the numbers of ALPCs incorporated into reticulated cytoplasmic membranes affect the Ca^{2+} stores. As indicated by EM, however, function might also be related to the qualitative transition of AL into new structures in the form of TUHMAs. The supposed transformation of AL into TUHMAs manifests itself in the form of intermediates, so-called TRS. This finding might have some important implications:

1. Cytoplasmic annuli could possibly just be intermediates, which serve in the formation of structures of a higher order. Anticipating the energy needs for creation of highly ordered structures, a functional purpose appears to be reasonable. Peculiarities such as the combination of proteinaceous and lipid nanostructures, polarized orientation, single-organelle-like appearance, and a seemingly final destination of TUHMAs linked to the nuclear membrane, all indicate a yet unknown purpose.
2. The unique structure of TUHMAs is attracting the attention of biophysicists since the anisotropic, nanoperiodic design might serve in yet unknown concepts of cell communication and signaling (Reipert et al., 2010).
3. Since TUHMAs have not been visualized in germ cells so far, it appears reasonable to suggest that they exert functions exclusively in somatic cells. The indicated correspondence of TUHMAs with primary cilia found in PtK2 cells does not necessarily mean their occurrence is restricted, since primary cilia are omnipresent in a diversity of somatic cell types. Whether TUHMAs could serve particular functions in a subset of ciliated cells, as suggested for cells undergoing oriented cell division (Reipert et al.,

2010), awaits verification. Notably, observations of TUHMAs in a kidney epithelial cell line, namely PtK2, their occurrence in stratified muscle (Matsubara & Mair, 1979), as well as the finding of TUHMA aberrations in melanoma cells (Reipert et al., unpublished), all point to cells derived from tissues with well-defined cell division axes. Finding out how such division axes are maintained during morphogenesis still poses a challenge for current research. Since there is increasing evidence for the implication of defects in oriented cell division in polycystic kidney diseases and tumor genesis, this should be an incentive for investigation of possible nanophysical properties of TUHMAs that might be able to sense direction (Reipert et al., 2010).

4. Since both AL and TUHMA show a linkage to the cell cycle, studies of AL in this context have to be resumed and combined with the elucidation of TUHMA biogenesis. Particular attention has to be paid to a possible role of the Golgi complex that has been sidelined in research on AL in the past. Saraste et al. (2009) raised the interesting question whether an intermediate compartment (IC) expresses its own functional identity between the ER and the Golgi complex by carrying out or participating in some cellular functions. From our point of view, TUHMA biogenesis could be perceived as the dynamics of an IC between the Golgi complex and the rER that might result in a functional organelle.
5. Initial studies of TUHMAs indicate the importance of MTs for their existence and dynamics. TUHMAs disintegrate under the very same MT-destabilizing conditions under which AL are generated (Reipert et al., 2009). Both polarization and translocation of TUHMAs are processes that are most likely linked to MTs. Moreover, MTs could play an active role in the transformation of AL into TUHMAs by organizing the IC in conjunction with the Golgi complex. Temporal co-localization of TUHMAs with detyrosinated tubulin, as well as indications for the translocation of nucleoporins with respect to primary cilia (Reipert et al., 2010) could indicate the direction of future functional studies.
6. Initial evidence indicates that TUHMAs are formed away from the side of their final destination at the nuclear membrane. Consequently, they would have to undergo a three-step process: i) assembly next to the Golgi complex, ii) translocation towards the nucleus and oriented alignment with respect to the nuclear membrane, and iii) fusion of their membranes with the NE. Importantly, the latter does not include the fusion of core tubules with individual NPCs, which otherwise could easily be interpreted in the context of the nucleocytoplasmic transport. Instead, the fusion results in an extraordinary extension of the nuclear membrane lumen by the lumen of the membrane array. If the purpose of this extension is related to nucleocytoplasmic transport, a boost in the transport rate could possibly be achieved via a yet unknown luminal pathway that regulates NPC function.
7. Fluorescence microscopic observations interpreted as AL have to be handled with more care than previously, since intermediate structures, such as TRS, are likely to contribute to the fluorescent spots seen in the light microscope. Moreover, the immunofluorescence data have to be interpreted with caution because they depend on membrane permeabilization, and perhaps on the status of the nucleoporin organization itself. Notably, patches of AL, regularly seen next to TUHMAs in the electron microscope, were not detected by immunofluorescence microscopy, while AL induced by vinblastine sulfate treatment could be visualized using the very same protocol

(Reipert et al., 2009). Since nucleoporin markers do not seem to comprehensively mirror processes in the cytoplasm, correlative microscopy, comparing fluorescence patterns with EM fine structures is required in future studies.

The initial data on TUHMAs already indicate that these enigmatic structures do not have a life cycle independent of the other two nucleoporin-containing membrane configurations. Understanding of their possible function in the future, therefore, will require the elucidation of the tripartite nature of nucleoporin organization. Whether progress can be made in these studies will strongly depend on overcoming dogmas built up by previous research on AL, in particular concerning their relation to the Golgi complex and MTs.

Besides revealing their functions, the elucidation of TUHMAs will help to answer questions concerning the relationship between self-assembly of complex structures and gene expression. What governs the transformation of the proteinaceous pore assembles into helical structures? Is the tubulation of lamellar lipid membrane sheets driven by proteins known for their capability to curve membranes, such as reticulons and Yop1/DP1 (Voeltz & Prinz, 2007; Kiseleva et al., 2007), or do the nucleoporins themselves play a decisive role in membrane curvature? How might the cellular lipid composition affect the formation of nonlamellar membrane arrays and the nucleoporin architecture? Answers to these questions will expose "nanotechnologies" of the cell, which have previously gone undiscovered.

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3

Actin Folding, Structure and Function: Is It a Globular or an Intrinsically Disordered Protein?

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1. Introduction

Actin is the most abundant protein in eukaryotic cells. This protein is found in every living cell, but it is most plentiful in muscle cells, from which it was isolated more than 60 years ago (Straub, 1942). Since that time the continuous flow of investigations was started, involving more and more scientists. Even today, scientific interest in actin has not died down but is, rather, continuing to make noteworthy advances, as can be observed from the Scopus database (Figure 1). Initially, these investigations focused on muscle actin, with an emphasis on its ability to polymerize and to interact with the other main muscle protein, myosin, and several other regulatory proteins that control muscle relaxation and contraction. In addition, researchers examined the role of ATP hydrolysis as a source of energy and that of the bivalent cation Mg^{++}, an essential component of the polymerization-depolymerization process, that is replaced by Ca^{++} in vitro. In the early 1970s, it became evident that actin is an abundant protein, not only in muscle cells but also in every other type of eukaryotic cell. The main role of actin in non-muscle cells is the formation of the cytoskeleton, which functions to enable cell motility and inter-cell interaction. Further investigations showed that actin participates in many other vitally important cellular processes. Although the first report of actin in the nucleus appeared at virtually the same time as the discovery of the protein in the cytoplasm, the former observation was presumed to be an artifact. It was only recently that actin was proven to be an integral component that plays a key role in the nucleus, as it does in the cytoplasm. The actin role in nucleus begins with the nuclear scaffold formation and ends with the transcription process. Furthermore, it became evident that globular (G-) actin in nuclei participates in transcription and chromatin remodeling.

A large amount of data regarding actin has accumulated, with specific topics that include the structure of G-actin; the polymerization of G-actin to form fibrillar (F-) actin and the depolymerization of F-actin; the participation of the protein in muscle contraction; in the formation of the cytoskeleton in non-muscle cells; the localization of actin in different cell

compartments and its role there; and its interactions with numerous partners, of which more than 60 classes are currently known. These topics are discussed in multiple research works and a series of modern reviews (see, e.g., (dos Remedios et al., 2003; Winder & Ayscough, 2005; Reisler & Egelman, 2007; Schleicher & Jockusch, 2008; Vartiainen, 2008; Zheng et al., 2009; Skarp & Vartiainen, 2010; Wang et al., 2010; Dominguez & Holmes, 2011; Schoenenberger et al., 2011)). Therefore, in the present work, these questions will be discussed rather briefly, and the primary focus will be on the processes of actin folding and unfolding and on the validation of the hypothesis that G-actin belongs to the so-called "intrinsically disordered" (ID) proteins.

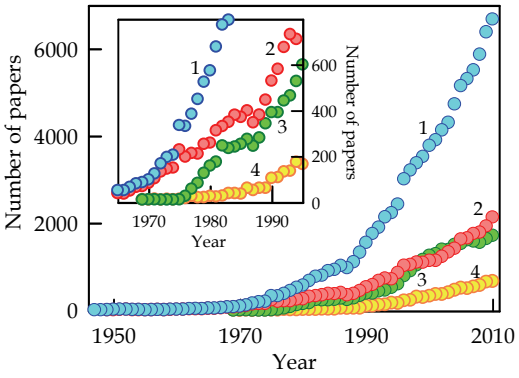

Fig. 1. The increase of researchers' interest in actin. The graph illustrates the increase in the total number of publications on actin (1, blue), with separate tracks showing the number of publications on muscle (2, red), cytoplasmic (3, green) and nuclear (4, yellow) actin from 1950 to the present.

2. Actin structure

Actin is a highly conserved protein of approximately 42 kDa, and its polypeptide chain consists of 375 amino acids (Oosawa, 1971). There are three isoforms of actin that are produced by different genes (alpha, beta and gamma), all of which are polymorphic proteins that are capable of polymerization. The actin isoforms differ by only a few amino acids, with most of the variation occurring toward the N-terminus (Herman, 1993). A distinctive feature of actin is its ability to polymerize. At low ionic strength *in vitro,* actin exists as a monomer (G-actin). In the presence of neutral salts, the protein polymerizes to form a single-stranded polymer (the so-called fibrous form of actin, or F-actin). F-actin forms the backbone of the thin filaments of muscle fibers and of the cytoskeleton. The tendency of actin to polymerize prevents the formation of its crystals, which is a requirement for 3D structure resolution via X-rays. However, actin loses its ability to polymerize after forming a complex with DNase I and/or other actin-binding proteins (ABPs). Actin was crystallized for the first time in complex with DNase I (Kabsch et al., 1990). This accomplishment was an important breakthrough in the study of actin structure. The actin monomer was revealed to be a relatively flat molecule of dimensions 55x55x35 Å. Actin folds into two major α/β-domains (Figure 2), as do other proteins of the structural superfamily to which the sugar kinases, hexokinases and Hsp70 proteins belong (Domingues end Holmes, 2011). Each large

domain consists of two subdomains, and traditionally, a four-subdomain nomenclature has been adopted (Kabsch et al., 1990). Subsequently, actin structures with certain other ABPs were determined. Nonetheless, it was unclear whether the structure of actin in complex with ABPs differs from that of free, native actin. This problem was solved when the structure of actin with a small molecule (ADP-actin with tetramethyl-rhodamine-5-maleimide, which prevents actin polymerization) was determined (Graceffa & Dominguez, 2003). To date, over 80 structures of actin in complexes with various ABPs have been reported (Dominguez & Holmes, 2011).

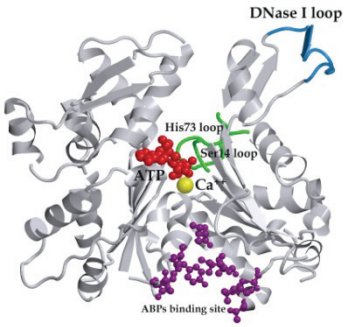

Fig. 2. The structure of the actin molecule. The figure was created on the basis of the PDB data (Dutta et al., 2009), the file 1ATN.ent (Kabsch et al., 1990), using the graphical software VMD (Hsin et al., 2002) and Raster 3D (Merritt & Bacon, 1997). ATP (red), Ca^{2+} (yellow), ATP/ADP sensing loops (green), the DNase I loop (blue) and ABPs binding sites are specially emphasized.

It appeared that irrespectively of modification, bound molecule or nucleotide (ATP or ADP) the conformation of the actin monomer was practically the same except though small but important differences. One of the important variable regions is the so called DNase I loop. This loop includes residues 39-51, which are located at the top of domain 2, and is referred to as the DNase I-binding loop because it is responsible for the formation of the actin complex with DNase I (Kabsch et al., 1990). At the same time this loop plays a critical role in the inter-subunit contacts in the F-actin filament. Any change in this loop, though it may not influence the structure of domain 2, leads to the loss of actin's ability to polymerize. Interestingly, this loop, which forms a β-strand in one crystal structure (Kabsch et al., 1990), was found to be disordered in several other crystal structures (McLaughlin et al., 1993) and to form an α-helix when ADP, rather than ATP, is bound to actin (Otterbein et al., 2001).

The differences between the ATP- and ADP-bound states are relatively minor and primarily involve two loops: the Ser14 β-hairpin loop, which is located in actin subdomain 1, and the sensor loop carrying the methylated His73 (Graceffa & Dominguez, 2003). The nucleotide-dependent conformational changes in these loops, though minor, are very important and can explain how interaction of actin-binding proteins (such as profilin and cofilin) with actin is regulated by the nucleotide bound to actin (Dominguez & Holmes, 2011).

Opposite the large cleft of the actin molecule, there is a smaller cleft. This region participates in the formation of inter-monomer contacts during actin polymerization, when the loop

containing residues 41-45 binds to residues 166-169 and 375 (Kabsch et al., 1990). There are two additional contacts between subdomains 3 and 4: residues 322-325 bind to residues 243-245, and the loop containing residues 286-289 binds to residues 202-204 (Holmes & Kabsch, 1991). This smaller cleft between domains 1 and 3 also appears to be the binding site of several major ABPs. This observation implies that actin bound to an ABP loses its ability to polymerize and can therefore be crystallized. The actin residues that participate in the formation of contacts with ABPs include Tyr143, Ala144, Gly146, Thr148, Gly168, Ile341, Ile345, Leu346, Leu349, Thr351 and Met355 (Figure 2) (Dominguez & Holmes, 2011). This cleft is sometimes referred to as hydrophobic, although not all of the residues mentioned above fit that description. It has been suggested that communication between the two clefts provides the structural basis by which nucleotide-dependent conformation changes modulate the binding affinities of ABPs (Dominguez & Holmes, 2011).

3. Folding of globular proteins

We have recently summarized the main principles of globular protein folding (Povarova et al., 2010). One of the fundamental laws of physics states that an isolated polypeptide chain must attain the conformation that corresponds to the free energy minimum, F:

$$F = H - TS, \tag{1}$$

where H is the enthalpy, the value of which is determined by all the interactions between the atoms of a polypeptide chain, as well as interactions with solvent molecules, and S is the entropy, a measure of the number of conformations, N, which determines the realization of a given protein state: $S = R \ln N$ (in this formula, R is the universal gas constant and T is the absolute temperature). In these terms, protein folding is the process of the attainment by a polypeptide chain of a thermodynamically stable state that corresponds to the global free energy minimum. It is important to remember, however, that alongside the single global minimum, the system can possess a series of local minima. The presence of such local free energy minima reflects the existence of intermediate states between the native (N) and unfolded (U) conformations. The state corresponding to a local minimum can also be a final state of the system if it is separated from other, deeper free energy minima by a high-energy barrier(s).

What is the nature of the state that corresponds to the free energy minimum for a given protein molecule? Will this state be native (biologically active)? To answer these questions, it is necessary to remember another fundamental law of nature – the biological law of evolutionary selection. An amino acid sequence must be composed in such a way that the protein becomes functionally active (native) after reaching the state corresponding to its free energy minimum. Thus, "nonsense" amino acid sequences that are nonfunctional in the state corresponding to the free energy minimum will be rejected by evolutionary selection. However, this theory implies that, if such so-called "nonsense" amino acid sequences are detected, then they likely serve some purpose, though this purpose may be unknown.

The unfolded state is entropically favorable because it represents a dynamic ensemble of a large number of conformations that originate from the rotational isomerization of the main chain. In contrast, any compact state imposes significant restrictions on the conformational freedom of the polypeptide chain and is therefore entropically unfavorable. The capacity of

a given polypeptide chain to attain a compact state is determined by its ability to form intramolecular contacts that compensate for the free energy increase that is caused by the decrease in the entropy component. The compactness of a structure formed by a polypeptide chain is determined by its amino acid composition and sequence. Therefore, depending on the peculiarities of their compositions and sequences, newly synthesized amino acid chains would adopt globular or partially or completely disordered structures. The structures formed by a polypeptide chain in water are significantly different from that of the Gaussian coil. This difference arises because water is a poor solvent for a polypeptide chain, not only due to the existence of numerous hydrophobic amino acid residues but also because water is a poor solvent for the protein backbone. In fact, despite the absence of hydrophobic residues, polar polypeptides (polyglutamine and glycine-serine block copolypeptides) prefer ensembles of collapsed structures in aqueous milieus (Crick et al., 2006; Tran et al., 2008). Furthermore, residual secondary structure is repeatedly found in unfolded globular proteins, even in concentrated solutions of strong denaturants, such as 8 M urea or 6 M guanidine hydrochloride (GdnHCl), which are much better solvents for polypeptide chains than water (Shortle & Ackerman, 2001; Shortle, 2002). Upon being unfolded, many globular proteins can refold into the same native structure, which means that all of the information necessary for a given polypeptide chain to fold into a unique tertiary structure is encoded in its amino acid sequence (Anfinsen et al., 1961). Furthermore, amino acid sequences must also bear information about to the pathways of their formation, because otherwise, the random optimization of a polypeptide chain of hundreds amino acids will take billion of years, as was shown by Levintal.

Another special feature of the polypeptide chain of each globular protein is that it proves the existence of a free energy barrier between the native and denatured states (Finkelstein & Ptitsyn, 2002). This circumstance is vital for the correct functioning of globular proteins because only the existence of a free energy barrier between the native and denatured states maintains the conformation of molecules of globular proteins in the native state. This precise circumstance provides the possibility of crystal formation by globular proteins in their native states.

In medias res, protein folding can be regarded as a realization of the second part of the genetic code because the amino acid sequence contains information on the functional 3D structure of the protein. The first part of the genetic code is predominantly solved, but the study of the problem of protein folding is far from completion.

The models of protein folding have changed several times with the progress of experimental studies. According to the current view, protein folding is achieved via various pathways that are determined by the protein's energy landscape. The energy landscape model not only elucidates the mechanisms of globular protein folding but also explains the nature of the so called intrinsically disordered proteins (see Section 5), describes the formation of their supramolecular complexes, and delineates the formation of potentially pathogenic oligomers, amorphous aggregates, amyloid, and amyloid-like fibrils.

4. Actin folding–unfolding

The first investigation of actin folding and unfolding was performed by Lehrer and Kerwar (Lehrer & Kerwar, 1972). In this work, it was shown that the release of calcium ion by EDTA or EGTA treatment leads to the transformation of G-actin into an inactivated form in which

the protein molecule loses its capability to polymerize. For many years, it was generally accepted that actin unfolds via a single intermediate state called inactivated actin (West et al., 1967; Lehrer & Kerwar, 1972; Contaxis et al., 1977; Tatunashvili & Privalov, 1984; Bertazzon et al., 1990; Le Bihan & Gicquaud, 1993; Turoverov et al., 1999a). The investigations of actin unfolding and refolding that we commenced in 1998-1999 led to an essential revision of the concept of inactivated actin (see section 4.1) and of our understanding of the transformations of actin induced by different concentrations of various agents (see section 4.2). In the conclusion of this section, data regarding actin folding *in vivo* will be presented (section 4.3).

4.1 Inactivated actin

The release of calcium ions by EDTA or EGTA treatment does not lead to actin unfolding but renders the protein inactive, i.e., a state in which the molecule loses its ability to polymerize (Lehrer & Kerwar, 1972; Turoverov et al., 1999a). Actin in this state was named inactivated actin (I). Inactivated actin may also be obtained by heat denaturation, exposure to moderate urea or GdnHCl concentrations, dialysis with 8 M urea or 6 M GdnHCl, or spontaneously during storage (Kuznetsova et al., 1988). On the basis of these data, inactivated actin (I) was considered to be an on-pathway intermediate between the native (N) and completely unfolded (U) states.

$$N \longrightarrow I \rightleftharpoons U, \tag{2}$$

All equilibrium experiments appeared to support this model. The spectrum of the intrinsic fluorescence of inactivated actin has its maximum at wavelength 340 nm, which is intermediate between that of the native (λmax = 325 nm) and completely unfolded protein (λmax = 350 nm). The fluorescence red shift can be caused by the polar environment formed by intrinsic residues of the protein (Turoverov et al., 1999a). It has been shown that the microenvironment of tryptophan residues is rather rigid (Turoverov et al., 1999b).

The secondary structure of inactivated actin is substantially distorted. Changes in the far UV CD spectrum caused by protein inactivation are consistent with the partial transformation of α-helices either into a disordered conformation or into the β-structure. The appearance of β-structural elements during denaturation has been described for a number of proteins and has been correlated with protein association or aggregation (Fink, 1998). It was shown that the far UV CD spectra of inactivated actin generated by different denaturing agents are practically identical. Moreover, protein inactivation was accompanied by a considerable increase of the fluorescence anisotropy value ($r = 0.09 \pm 0.01$, 0.17 ± 0.02 and 0.07 ± 0.01 for native, inactivated and unfolded actin, respectively), reflecting a considerable decrease in the internal mobility of the tryptophan residues in the inactivated actin (Kuznetsova et al., 1988).

A Perrin plot ($1/r$ versus T/η dependence, where T and η are temperature and viscosity, respectively) shows that inactivated actin is characterized by the independence of $1/r$ from T/η. This result is consistent with the assumption that inactivation is accompanied by the association of partially folded actin molecules in large particles. The rotational relaxation time of these particles is much greater than the Trp fluorescence lifetime. Interestingly, the Perrin plot measured for F-actin, which is known to be a long, rigid filament comprised of

numerous actin molecules, also has no visible dependence of $1/r$ on T/η. However, the intercepts of the Perrin plot on the Y axis ($1/r_o'$ value) for G- and F-actin practically coincide and exceed that for the inactivated protein (8.3±0.2 and 6.1±0.2, respectively). This fact indicates that the amplitude of high-frequency intramolecular mobility or the rotational relaxation time of inactivated actin, or both, are much lower than the corresponding values of native actin (Kuznetsova & Turoverov, 1983). Thus, it was shown that the interior of denatured (inactivated) actin has considerable mobility limitation.

The fact that the inactivated actin represents a specific aggregate and that its properties do not depend on the method of its generation was proven by gel-filtration and sedimentation experiments (Kuznetsova et al., 1999). The apparent molecular masses (M^{app}) of native and inactivated actins were determined using a gel-filtration column that was calibrated by a set of 20 native globular proteins with known molecular masses ranging from 5.78 to 660 kDa. The M^{app} for native and inactivated actin was determined as 40±2 and 710±20 kDa, respectively. For the determination of the real molecular mass (M^{true}), the Stokes radii (R_s) of native and inactivated actins were evaluated. These values for native and inactivated actins were determined to be 28±2 and 61±4 Å. Using these values and sedimentation coefficients, the M^{true} values for native and inactivated actin were determined to be 40.5 and 712.6 kDa, respectively (Kuznetsova et al., 1999). One can see that the value calculated for the native actin is consistent with the molecular mass estimated from its amino acid sequence (42.05 kDa). It can also be seen that, within the limits of experimental error, the values of the true molecular mass, calculated for native and inactivated actin from R_S, coincide with the apparent molecular mass values that were measured by gel-filtration.

It should be emphasized that this observation is very important because the use of a size-exclusion column calibrated with a set of native proteins with known M values allows one, in principle, to estimate the molecular mass for the protein of interest. This value should be considered to be an apparent one, as it will be correct only for native globular proteins. In contrast, the R_S value determined chromatographically reflects the real hydrodynamic dimensions of the given protein under the conditions studied, regardless of its conformational state (of course in the case if there is no interaction between the protein and column matrix). It is known that the sedimentation constant is a parameter that can be measured directly. Thus, two quantities that have been used for the calculation of M^{true} are independent of the model. The fact that M^{app} coincides with M^{true} is consistent with the conclusion that inactivated actin has hydrodynamic dimensions typical of the native globular protein with a molecular mass of ~700 kDa, which means that the protein in this form has overall native-like packing density. On the basis of the above experiments, it was concluded that inactivated actin represents an ordered aggregate: a supramolecular, monodisperse complex of 14-16 monomers of partially unfolded actin (Kuznetsova et al., 1999).

The interaction of inactivated actin with the hydrophobic fluorescent probe 1-anilinonaphthalene-8-sulfonic acid (ANS) is particularly interesting. High affinity to ANS is a well-known characteristic of many partially folded protein conformations (Fink, 1995; Ptitsyn, 1995). The formation of a complex between ANS and a protein molecule leads to a considerable increase in the dye fluorescence intensity. It has been established that ANS fluorescence is minimal in a solution with native and completely unfolded actin, whereas the inactivation of the protein is accompanied by a considerable increase in this parameter (Turoverov et al., 1999a).

In view of the finding that inactivated actin represents an ordered aggregate, the dependence of ANS interaction with actin on the concentrations of GdnHCl and urea was studied (Povarova et al., 2010). As can be expected, the intensity of ANS fluorescence weakly depends on the urea concentration, up to the concentration at which the supramolecular complexes are destroyed. However, the dependence of ANS fluorescence on the concentration of GdnHCl was found to follow a curve with maximum in a narrow range of small concentrations of the denaturant. Furthermore, the maximum of light scattering (or even precipitation at high protein concentration) was observed in the same range of GdnHCl concentrations. These findings indicate that inactivated actin forms large aggregates in this narrow range of GdnHCl concentrations and that the affinity of ANS molecules for these aggregates is very high. ANS incorporates into the hydrophobic pockets between the molecules that form the aggregates, resulting in the dramatic increase in its fluorescence intensity.

The protein aggregation was explained by interactions between the GdnHCl cations (GuH^+) and the side-chain C=O groups of the glutamic acid, glutamine, aspartic acid and asparagine residues of the actin molecule. The possibility of such interactions has been shown earlier (Anufrieva et al., 1994; Mason et al., 2004). In actin, the number of negatively charged groups from glutamic and aspartic acids (OD2 - 22 groups and OE2 - 28 groups) is greater than the number of positively charged groups from lysine (NZ – 18 groups), arginine (NH1 – 18 groups) and histidine (NE2 – 9 groups). Therefore, the actin molecule is negatively charged (pI 5.07) at a neutral pH. With an increase in the number of GuH+ ions bound to inactivated actin, the number of positively charged groups increases, and at a certain concentration of GdnHCl (0.2 – 0.3 M), the initially negatively charged molecules become neutral, which leads to their aggregation. Upon further increases in the GdnHCl concentration, the number of positively charged groups on the surfaces of the protein molecules will exceed the number of negatively charged groups. Therefore, the conditions will no longer be favorable for aggregation. This is the reason for the abrupt decrease in light-scattering intensity. The less-abrupt decrease in the intensity of ANS fluorescence in comparison with light scattering as the GdnHCl concentration increases can be explained by the higher affinity of the negatively charged ANS molecules for inactivated actin when it is positively charged, though aggregates are already destroyed (Povarova et al., 2010). Due to the complex process of actin denaturation and the dependence of the transition rates upon the GdnHCl concentration (Kuznetsova et al., 2002; Turoverov et al., 2002), the maximum of ANS fluorescence intensity shifts to a lower concentration of GdnHCl as the incubation time increases. Thus, after 24 h of incubation, the maxima of light scattering and of ANS fluorescence intensity are recorded at practically the same concentrations of GdnHCl as for initially inactivated actin.

Hydrophobic interactions apparently play a significant role in both the initial formation of inactivated actin and the formation of inactivated actin aggregates in the presence of low concentrations of GdnHCl. As mentioned above, due to the existence of hydrophobic pockets in inactivated actin, the ANS fluorescence intensity is 20 times greater in the presence of inactivated actin than in the presence of native actin. Inactivated actin already has hydrophobic clusters on its surface, but molecules of inactivated actin do not "stick together" because the negative charges on their surfaces prevent them from doing so. At low concentrations of GdnHCl, the aggregation of inactivated actin leads to a significant increase in the number of hydrophobic pockets and, consequently, to an increase in the

number of bound ANS molecules, that is detected by an increase in ANS fluorescence. Protein aggregation in the presence of low concentrations of GdnHCl is particularly pronounced for actin because large supramolecular complexes of inactivated actin (Kuznetsova et al., 2002; Turoverov et al., 2002) are involved in the aggregation.

Thus, the study of the characteristics of inactivated actin allowed us to make important conclusions regarding the properties of the molten globule state and of ANS fluorescence. It was shown that low concentrations of GdnHCl can cause the aggregation of proteins in a partially folded state and that the fluorescent dye ANS binds with these aggregates rather than with hydrophobic clusters on the surfaces of proteins in the molten globule state. This finding explains why an increase in ANS fluorescence intensity is often recorded during the process of protein denaturation by GdnHCl, but not by urea. Therefore, what was previously believed to be the molten globule state in the pathway of protein denaturation by GdnHCl in reality, for certain proteins, represents the aggregation of partially folded molecules (Povarova et al., 2010).

4.2 Intermediate states in the pathway of actin unfolding

To clarify the process of actin unfolding, the GdnHCl-induced changes in the intensity of intrinsic fluorescence, the parameter $A = I_{320}/I_{365}$, fluorescence anisotropy and CD were studied (Kuznetsova et al., 2002; Turoverov et al., 2002). The minima in the kinetic dependencies of fluorescence intensity in the range of 1.0 - 2.0 M GdnHCl indicate that the transition from the native to the inactivated state occurs via some essentially unfolded intermediate state of actin. Based on these experimental data, a kinetic scheme for actin unfolding was proposed:

$$\mathrm{N} \xrightarrow{k_1} \mathrm{U}^* \underset{k_2}{\overset{k_3}{\rightleftarrows}} \mathrm{I}, \tag{3}$$

where k_i are the rate constants of the corresponding processes and U* is an essentially unfolded kinetic intermediate, the fluorescence properties of which are similar to those of the completely unfolded state but the secondary structure is much more ordered. The GdnHCl dependencies of the rate constants, k_1, k_2 and k_3, illustrate the conditions in which the essentially unfolded intermediate state can be recorded. At 1.0 - 2.0 M GdnHCl, the value of the rate constant for the transition from native to essentially unfolded actin exceeds that for the following step of inactivated actin formation. This imbalance leads to the accumulation of essentially unfolded macromolecules early in the unfolding process, which in turn causes the minima in the time dependencies of tryptophan fluorescence intensity, parameter A, intrinsic fluorescence spectrum position, and tryptophan fluorescence anisotropy. To examine the properties of the newly identified kinetic intermediate U*, the predecessor of inactivated actin, and to elucidate the roles of inactivated actin and its kinetic predecessor in the processes of actin folding and unfolding, a parametric representation of the kinetic dependencies of the tryptophan fluorescence intensity changes recorded at two wavelengths was studied (Figure 4A). The use of a parametric relationship between two independent extensive characteristics of the system allowed us to determine whether protein unfolding is a two-state transition or if an intermediate state(s) is formed: if the parametric relationship between any two extensive characteristics is linear, then protein unfolding follows the model "all-or-none"; in contrast, a break in the line indicates the existence of an intermediate state (Kuznetsova et al., 2004).

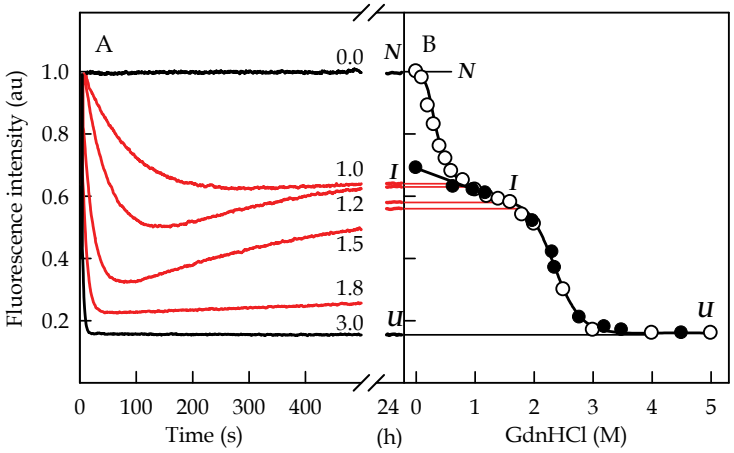

Fig. 3. Actin denaturation monitored by the change of intrinsic fluorescence intensity at 320 nm. A. The kinetics of actin denaturation induced by GdnHCl. The values on the curves indicate the concentration of GdnHCl (M). B. The fluorescence intensity of actin recorded after 24 h of incubation in the listed concentrations of GdnHCl. The open and closed symbols correspond to the unfolding and refolding experiments, respectively. In the refolding experiment, the appropriate concentrations of GdnHCl were obtained by the dilution of the actin solution in 5 M GdnHCl. λex = 297 nm, λem = 320 nm. N, I and U indicate the ranges of the predominant content of native, inactivated and unfolded actin, respectively, after 24 h of incubation of actin in solution with appropriate concentrations of GdnHCl. The values on the kinetic curves indicate the concentration of GdnHCl (M).

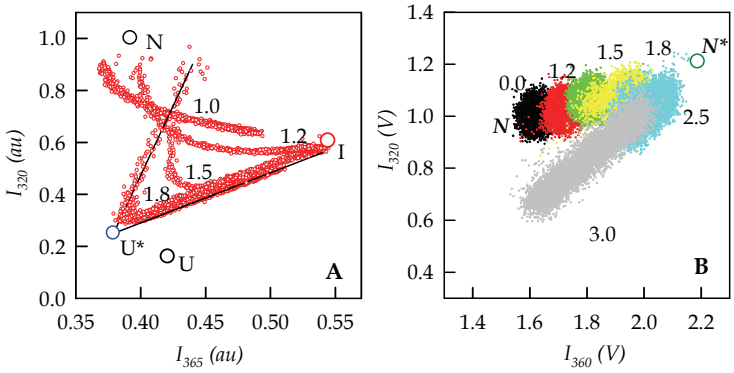

Fig. 4. Actin denaturation induced by GdnHCl. A. Parametric dependencies of fluorescence intensity at 320 and at 365 nm; the parameter is the time from the beginning of denaturation. The averaging time of signal is 0.6 s. The values on the curves indicate the concentration of GdnHCl (M). λex = 297 nm. B. Parametric dependencies of fluorescence intensity at 320 and at 360 nm; the parameter is the time from the beginning of denaturation. The averaging time of signal is 0.1 ms. The values on the curves indicate the concentration of GdnHCl (M). λex = 297 nm.

The fluorescence intensities recorded at the wavelengths 320 and 365 nm were used as independent extensive characteristics, and the time from the moment of protein solution mixture with a GdnHCl solution of the appropriate concentration was taken as a parameter. Figure 4A shows that these curves consist of two branches that are most pronounced for a concentration of 1.8 M. One branch corresponds to the N → U* transition and the other to the U* → I transition. This panel also shows that the fluorescence properties of the kinetic intermediate U* differ from those of completely unfolded actin (U) in 4-6 M GdnHCl. The kinetic intermediate has more blue fluorescence spectrum in comparison with the completely unfolded state of actin (Kuznetsova et al., 2002; Turoverov & Kuznetsova, 2003; Povarova et al., 2005; Povarova et al., 2007). Actin in this state, in contrast to the completely unfolded state, was shown to preserve its secondary structure. Furthermore, the parametric relationships between I_{320} and I_{365} were found to originate not from one point that corresponds to the native state of actin in the absence of GdnHCl but from distinct points (Figure 4A). This observation indicates that the change of the solution leads to rapid changes of the fluorescence properties of actin. The existence of one more intermediate states was proven in our experiments (Povarova et al., 2005) and in the work of Altschuler et al. (Altschuler et al., 2005) by stopped flow (Figure 4B). The total obtained data allowed us to propose a new kinetic pathway for actin unfolding and refolding induced by GdnHCl. In this scheme, the state N* precedes the transformation of native actin into the essentially unfolded state (U*). However, this state is far from a complete understanding and characterization. At the same time, the formation of an essentially unfolded state (U*) preceding the completely unfolded (U) or inactivated actin (I) has been proven. In the processes of folding and unfolding, the essentially unfolded state (U*) is an on-pathway intermediate, whereas inactivated actin (I) is an off-pathway associate, the appearance of which competes with the transition to the native state.

As was mentioned above, protein folding is associated with a decrease in entropy that leads to an increase of free energy transfer, which must be compensated by the formation of contacts. In this scenario, two competing processes are possible: formation of intramolecular contacts and formation of intermolecular contacts. In the first case, native protein appears, and in the second, amyloid fibrils, amorphous aggregates and associates such as inactivated actin appear. Investigation of the characteristics of such states and the factors that influence their appearance is not only of fundamental value but also important for medicine (in connection with the so called conformational diseases) and for biotechnology (in connection with the accumulation of insoluble aggregate forms of various proteins in inclusion bodies). The reversibility of the actin denaturation state in early works (Contaxis et al., 1977; Tatunashvili & Privalov, 1984; De La Cruz & Pollard, 1995) was not reproduced by us (Kuznetsova et al., 2002; Turoverov et al., 2002; Turoverov & Kuznetsova, 2003; Povarova et al., 2005). The Ca^{++} ion probably plays a crucial role in the irreversibility of actin denaturation: the native structure became energetically profitable only in the presence of the Ca^{++} ion, and otherwise, the structure is stabilized by intramolecular contacts, which leads to the formation of inactivated actin. Thus, the presence of chaperones is likely to be an essential requirement for actin folding.

The study of actin unfolding in different concentrations of GdnHCl allowed us to conclude that actin unfolding by 4 M GdnHCl also proceeds via the intermediate states N* and U*. The rate constants of the processes $N \longrightarrow N^*$, $N^* \longrightarrow U^*$ and $U^* \longrightarrow U$ increase with the GdnHCl concentration. The rate constants of these processes are very large in 4 M

GdnHCl, which is why it appears that actin unfolding by 4 M GdnHCl is a two-state transition, N ⟶ U. The Ca^{++} ion plays a stabilizing role in the structure of actin as ligands do for other proteins. Thus, Ca^{++} ions in actin can be regarded as triggers, the removal of which launches the cascade of conformational changes, i.e., the appearance of the kinetic intermediates N* and then U*. The dissociation of Ca^{++} in low concentrations of GdnHCl is a slow process (Kuznetsova et al., 2002). Actin molecules that transfer to the U* state are rapidly associated with the formation of inactivated actin (I) because the rate constant for the U* ⟶ I process is large in the absence of a denaturant. Due to this effect, the transition of native actin to the inactivated state occurs even spontaneously, in the absence of denaturant, during actin sample storage (Kuznetsova et al., 1988). However, the same process caused by EDTA is very rapid: the rate constant of the process N ⟶ N* is very large, and in the absence of GdnHCl, the rate constant of the process U* ⟶ I is also very large. This effect explains why it appear that the EDTA-induced unfolding of actin is a two-state process, N ⟶ I; in reality, however, it is a multi-state process in which the process N ⟶ N* ⟶ U* ⟶ I occurs. Experiments performed on stopped-flow device confirm the existence of the kinetic intermediate N*. In a temperature denaturation experiment, we also appear to record a two-state transition, N ⟶ I. However, in reality, as is the case in actin unfolding by EDTA, a multi-state process occurs: N ⟶ N* ⟶ U* ⟶ I, though in the case of temperature denaturation, the rate constants of the first transitions (N ⟶ N* ⟶ U*) are low, while the transition U* ⟶ I in the absence of denaturant is very fast.

Thus, the investigation of actin unfolding and refolding, as well as the data we obtained for certain other proteins, allowed us to conclude that, in the unfolding pathway, the order and number of the partially folded denatured states are independent of the denaturing agent. Overall, the obtained data allowed us to propose a new kinetic pathway for actin unfolding and refolding induced by GdnHCl:

$$\mathrm{N} \longrightarrow \mathrm{N^*} \longrightarrow \mathrm{U^*} \rightleftharpoons \mathrm{I_1\ldots} \underset{\text{[GdnHCl] > 3.0M}}{\overset{\text{[GdnHCl] < 1.8M}}{\rightleftharpoons}} \mathrm{I_n\ldots} \underset{\text{I=I}_{15}\text{ at [GdnHCl]=0M.}}{\overset{\text{[GdnHCl]=0.2M}}{\rightleftharpoons}} \mathrm{I} \rightleftharpoons \mathrm{I_{aggr.}} \qquad \mathrm{U^* \rightleftharpoons U} \tag{4}$$

In this scheme, the transition state N* precedes the transformation of native actin into the essentially unfolded state (U*). However, this state is far from representing a complete understanding and characterization of the underlying process. The formation of an essentially unfolded state (U*) preceding the completely unfolded (U) or inactivated actin (I) is proven. In the processes of folding and unfolding, the essentially unfolded state (U*) is an on-pathway intermediate, whereas inactivated actin (I) is an off-pathway associate, the appearance of which competes with the transition to the native state.

4.3 Actin folding *in vivo*

As was mentioned above, the conclusions regarding the reversible unfolding of actin that had been reported in earlier works (Kasai et al., 1965; Contaxis et al., 1977; Tatunashvili &

Privalov, 1984; De La Cruz & Pollard, 1995) were not reproduced (Kuznetsova et al., 2002; Turoverov et al., 2002; Turoverov & Kuznetsova, 2003; Povarova et al., 2005). Thus, the presence of chaperones is likely to be an essential requirement for actin folding.

Chaperones constitute a broad family of proteins with various molecular masses, structures and functions. Two classes of ATP-dependent chaperones, Hsp70 and the chaperonins, are known to play crucial roles in the folding of nascent, non-native polypeptides into their native, functional states inside eukaryotic cells. The Hsp70 chaperones, with the assistance of the co-chaperones of the DnaJ/Hsp40 family, interact with the small hydrophobic clusters of the newly synthesized polypeptide chain (Feldman & Frydman, 2000). These interactions are not specific because hydrophobic clusters are present in almost every partially folded polypeptide chain. The major role of Hsp70 is likely in preventing undesirable interactions that might result in the aggregation of the newly synthesized polypeptide chain with other molecules. For many proteins, interactions with Hsp70 are sufficient for correct folding. However, the folding of multidomain proteins requires the participation of other helpers. For example, the correct folding of actin relies on its interaction with prefoldin (PFD), which participates in the translocation of the partially folded actin to the CCT chaperonin. CCT consists of two stacked toroids, each of which contains eight three-domain proteins. The equatorial domains are responsible for the intertoroid interactions and for the interaction with ATP, whereas the apical domains mediate the interaction with the substrate and provide for the passage of the substrate to the central cavity. The folding of actin is a complex, multi-stage, ATP-dependent process controlled by CCT (Neirynck et al., 2006; Altschuler & Willison, 2008). The indispensable participation of PFD and CCT in actin folding is likely the reason that recombinant actin cannot be expressed in *E. coli* (Frankel et al., 1990) but can be expressed in yeast (Karlsson, 1988; Verkhusha et al., 2003). The refolding of EDTA-denatured actin in the presence of CCT *in vitro* was observed by Altschuler et al. (2005).

It is important to remember that, despite the crucial roles of chaperones in the folding of globular proteins *in vivo*, chaperones do not carry the structural information that is necessary for a newly synthesized polypeptide chain to fold into a unique, rigid and native globular structure. It is very likely that interactions with chaperones and other proteins are even more important for proteins the native states of which are partially or completely disordered. Such interactions would definitely protect these proteins from aggregation and proteolysis. Thus, the amino acid sequence of actin is such that it cannot fold into a compact state without chaperones. This phenomenon would be difficult to explain if, at the turn of the century, the so-called ID proteins had not been predicted.

5. Intrinsically disordered proteins

For a very long time, a protein's function was believed to depend on its prior folding into a unique three-dimensional structure. At the same time, over many decades, numerous proteins have been found to be either wholly disordered or to contain lengthy disordered segments, yet to carry out function. These proteins were typically considered to be outliers and were mostly ignored. However, at the turn of the century, it became clear that proteins without tightly folded 3D structures can also perform vital biological functions (Dunker et al., 2002; Daughdrill et al., 2005; Dyson & Wright, 2005; Dunker et al., 2008). The major structural characteristics of intrinsically disordered (ID) proteins include the inability to

form crystals, low circular dichroism signals in the near- and far-UV regions, a large hydrodynamic dimension, and high proteolytic sensitivity (Uversky, 2011). It has now been established that the inability of ID proteins to form rigid globular structures is linked to the peculiarities of their amino acid sequences. One of the reasons for native disorder to exist is encoded in the overall hydrophobicity and net charge of a given polypeptide chain. The smaller the content of hydrophobic residues and the higher the net charge of a polypeptide chain, the smaller the probability that this chain will fold into a compact globular state. The distinctiveness of the amino acid sequences of ID proteins formed the basis for the development of various computational tools for predicting such proteins (Ferron et al., 2006; Dosztanyi & Tompa, 2008). The application of disorder-predicting algorithms revealed that ID proteins are widely spread in nature. The length of the amino acid sequences that are unable to form ordered structures and the degree of disorderedness can vary significantly between ID proteins (Uversky, 2011). Because of this great variability, there is no strict boundary between globular and partially disordered proteins.

The atoms in the unstructured parts of a polypeptide chain possess a high degree of mobility, which is why they cannot be detected by X-ray analysis. The majority of globular proteins are enzymes and transporters that are naturally designed to have a strictly determined function. However, even in globular proteins, there is a some structural mobility. The most mobile atoms are the atoms in the active sites of enzymes, or the atoms in loops that might also be functional, participating in interactions with partners. Therefore, globular proteins also require a definite level of mobility for their functioning. Some polypeptide chains cannot fold into compact globular structures by themselves but can form compact structures while interacting with their partners if the free energy of the complex is lower than the free energies of the protein and its partner before their interaction. The potential for partially or completely disordered proteins to form complexes with their partners is the molecular basis of their functions in signaling, recognition and the regulation of different intercellular processes. Although many proteins are involved in such processes, special attention has been paid to the main regulatory proteins, which play key roles in the regulation of these complex processes. Many of these proteins, which are known as hubs and network concentrators and serve as "conductors" of these biological processes, were shown to be disordered (Dunker et al., 2005; Oldfield et al., 2008). Among such disordered hub proteins are α-synuclein, p53, HMG proteins, estrogen receptor α, and many others (Dunker et al., 2008; Uversky, 2008; Olovnikov et al., 2009). Proteome-wide analyses revealed that partially or completely ID proteins are more common in eukaryotes than in prokaryotes or archaea, likely due to the more complex regulation and signaling systems in higher organisms (Uversky, 2011). A striking example of this trend is p53, the function of which is inherent only in multicellular organisms. This protein monitors and coordinates practically all of the intercellular processes (Olovnikov et al., 2009), prioritizing the organism's needs over the interests of different cells: a damaged cell must either accelerate the repair processes or lose the possibility of division, and it may even die as a result of apoptosis (Olovnikov et al., 2009).

The ID proteins in such signalling net play a number of crucial roles in complex regulatory processes and are known as hub proteins or net concentrators. To avoid the risk of being digested and to escape the non-specific aggregation that potentially leads to the formation of oligomers, amorphous aggregates, and amyloid fibrils, disordered proteins should preferentially remain bound to their partners. The pathogenesis of conformational diseases

that are characterized by the formation of amyloids and amyloid-like fibrils is likely to be determined by the failures of the cellular regulatory systems rather than by the formation of proteinaceous deposits and/or by protofibril toxicity. It is evident that more or less actin meets the majority of the characteristics of ID proteins. First of all, like the ID proteins, actin cannot fold into a compact state without chaperones. Second, like many ID proteins, actin interacts with an enormous number of partners (Domingues end Holmes, 2011).

6. Actin localization and functions

Though it is now evident that actin can be found in any eukaryotic cell, it is most abundant in muscle cells, which consist almost entirely of actomyosin fibrils. Therefore, it is not surprising that the first purification of actin was performed in muscle cells. This historic experiment was conducted in Albert Szent-Gyorgy's laboratory by Bruno F. Straub, who studied muscular contraction (Straub, 1942). Straub showed that the purified protein was responsible for the activation of muscle contraction, for which reason he called it "actin". This work was a great breakthrough that determined the research guidelines for hundreds of scientific laboratories for decades. Today, muscle contraction has been studied in detail. Thousands of papers, reviews and books are devoted to this topic (see, e.g., (Oosawa, 1971)), and there is no use in repeating copy-book maxim again. We merely wish to emphasize that, the more muscle contraction was studied, the more it became evident that actin is not only an activator of muscle contraction but is also the main organizer and director of complex processes involving dozens of proteins other than actin and myosin, which have been given the general name of actin-binding proteins. Troponin and tropomyosin are notable among these proteins. In muscle, actin permanently exists in the form of F-actin. Once polymerized, it is integrated into the muscle and is not depolymerized unless the muscle is damaged. Accordingly, G-actin is required only for muscle generation in the course of muscle growth or repair.

Interestingly, the structure of F-actin was determined by X-ray analysis on the basis of the previously determined structure of the G-actin monomer (Holmes et al., 1990; Lorenz et al., 1993). F-actin was shown to form a single helix consisting of 13 molecules repeating in almost exactly six left-handed turns ((Holmes et al., 1990; Lorenz et al., 1993). Recently, this helix was directly visualized by electron cryomicroscopy (Fujii et al., 2010). At the same time electron microscopy of stained actin fibers showed F-actin to be made of two chains that twist gradually around each other to form a right-handed, two-chained long helix (Hanson & Lowy, 1963; Egelman, 1985; Dominguez & Holmes, 2011). Surprisingly, the image of F-actin as two-chained helix appeared to be so impressive that many researchers even today consider F-actin to be a two-chained helix. Nonetheless, this misconception is not inoffensive carelessness, as in this case, the model of the assembly and disassembly of actin filaments in principle differs from the generally accepted model.

In addition to its role in muscle cells, actin is an essential component of the cytoskeleton of all eukaryotic cells. This protein plays a crucial role in the generation and maintenance of cell morphology and polarity, in endocytosis and intracellular trafficking, and in contractility, motility and cell division. All of these functions are based on the highly ordered assembly and disassembly of actin filaments and the polymerization and depolymerization of F-actin. All of these processes are regulated by numerous ABPs that are, in turn, under the control of specific signaling pathways (dos Remedios et al., 2003;

Maciver, 2004). For these purposes, both F- and G-actin are required. The first crucial aspect of polymerization is nucleation, which refers to the formation of a nucleus of three associated monomers and constitutes the rate-limiting phase of polymerization. The main role in this phase is played by the complex of the Arp2 and Arp3 proteins, which is usually referred to as the Arp2/3 complex (Winder & Ayscough, 2005). The molecules of these ABPs have a similar tertiary structure to actin, such that, when the Arp2/3 complex binds to actin, it is regarded as a nucleus for polymerization. Then, in the processes of further filament growth, the Arp2/3 complex plays the role of a pointed end-capping protein that enhances the rapid growth of the filament from its barbed end. The Arp2/3 complex can also nucleate filament growth from the side of an existing filament. This ability is important for the dendritic branching that is found at the leading edges of motile cells (Pollard & Borisy, 2003). It has also been established that, *in vivo,* certain other proteins that take part in the regulation of filament growth participate in these processes (Paavilainen et al., 2004). The termination of filament growth is regulated by gelsolin and tensin. These proteins bind to the barbed end of the filament and block the addition of new monomers. Gelsolin also is known to participate in the severing of filaments (Burtnick et al., 2004), whereas tropomyosins (a highly conserved family of ABPs) are known to bind along the side of the actin filament to prevent its spontaneous depolymerization and even to protect it from severing by gelsolin. There are several other ABPs that participate in actin filament length determination. These proteins contain domains that allow them to interact with other proteins of the cell signaling networks. This interaction allows the remodeling of the actin cytoskeleton at appropriate times and places within the cell. An example of such an ABP is nebulin, which is an elongated protein with numerous low-affinity actin-binding sites (Winder & Ayscough, 2005).

When an actin filament is disassembled, F-actin must be depolymerized. The best-characterized proteins that drive depolymerization are the actin depolymerizing factor (ADF) and cofilin family members (Winder & Ayscough, 2005). After depolymerization, several highly conserved ABPs intervene in the process of actin turnover. These ABPs bind ADP-actin as it is released from the end of the filament (e.g., ADF/cofilin, twinfilin), facilitating the nucleotide exchange from ADP to ATP (e.g., profilin, CAP) and delivering the actin monomer to the barbed end of a filament to facilitate a new round of polymerization (e.g., profilin, twinfilin, verprolin/WIP, WASP). For rapid filament growth in cells, there must be a sufficiently large amount of ATP-actin ready to polymerize but preserved in the monomer form until an appropriate signal is given. For this purpose, there are special ABPs, the best studied of which are the thymosin family. A special signal triggers the activation of profilin, which leads to the release of thymosin from actin and results in the release of a large amount of ATP-actin that is ready to polymerize (Hertzog et al., 2004). Beyond these examples, F-actin interacts with many ABPs that do not influence its structure and dynamics. These ABPs include myosins that use actin as a track along which to move (Winder & Ayscough, 2005), cytoskeletal linkers (dystrophin, utrophin, vinculin) that interconnect different cytoskeletal elements and membrane anchors (annexins) that interact with both actin and the membrane.

Actin in the nucleus was discovered at practically the same time as in the cell (Lane, 1969), but this localization was taken to be an artifact. The focused study of nuclear actin began only recently. Currently, the presence of actin in the nucleus has been unequivocally demonstrated (Vartiainen, 2008). Actin has been shown to be an important regulator of

transcription (Miralles & Visa, 2006), transcription factor activity (Vartiainen et al., 2007), and chromatin remodeling (Zheng et al., 2009). At the same time, nuclear actin is less well studied than the cytoplasmic form. The existence of F-actin in the nucleus was controversial for a long time because it was not recognized by phalloidin fluorescence (Visegrady et al., 2005). However, all of the ABPs that interact with F-actin have been detected in the nucleus (Gonsior et al., 1999), and the actin monomer-sequestering drug Latrunculin has been reported to inhibit several nuclear actin-dependent functions, including the export of RNA and proteins (Hofmann et al., 2001), nuclear envelope assembly (Krauss et al., 2003), transcription (McDonald et al., 2006) and transcription-induced interchromosomal interaction (Nunez et al., 2008). These observations comprised indirect evidence that actin in the nucleus must be in a polymerized form. Several other studies have been published in which the authors try to prove the existence of actin filaments in the nucleus, but the most convincing is a recent microscopy study (McDonald et al., 2006). Approximately 20% of the total nuclear actin pool is in the polymeric state (McDonald et al., 2006). The failure of phalloidin to stain nuclear actin can be explained by its lower concentration relative to that in the cytoplasm (the nucleus contains approximately 1% as much actin as the cytoplasm), its decoration by ABPs (such as ADF/cofilin (McGough et al., 1997)) and possibly by a lower length (Vartiainen, 2008). At the same time, the dendritic actin branches have not been visualized in the nucleus, although Arp2/3 and other components that nucleate these filaments were found in the nucleus. The other unsolved problem is that of actin transport in and out of the nucleus. There is some evidence that actin can cross the nuclear pore complexes in a complex with profilin and exportin-6, although its import mechanism is still unclear (Vartiainen, 2008).

Interestingly, the "functional" form of actin differs in the muscle, the cytoplasm and the nucleus. In the muscle, once they are generated, filaments are not disassembled and new filaments appear only during muscle growth or reparation; therefore, the main functional form is F-actin. In non-muscle cytoplasm, although the cytoskeleton is composed of actin fibrils, it can be assembled and disassembled. Cell motility is also based on actin filament polymerization and depolymerization. Therefore, a sufficiently large amount of actin monomers must be stored in the cytoplasm to support the effective function of actin. In the nucleus, for the first time, actin monomers play a significant role by regulating SFR (serum response factor) activity. The actin monomer pool is involved in controlling the expression of many proteins that are themselves components of the actin cytoskeleton (Miralles & Visa, 2006).

7. Conclusion: Actin as a partially intrinsically disordered hub protein

Overall, actin has many characteristics that are typical of ID proteins. *In vitro,* its unfolding is irreversible, i.e., the information contained in its polypeptide chain is not enough for “regular” folding, or the intramolecular contacts that appear upon folding are not enough for the polypeptide to fold itself without chaperones and to maintain the folded native state without fastening it with Ca^{++} ions. Actin always exists in complexes: while folding, it successively interacts with the chaperone Hsp 70, then with PFD and finally with the chaperonin CCT, which provides for correct folding and Ca^{++} and ATP incorporation; fibrillar actin is formed by the self-association of G-actin molecules; in the cytoplasm or nucleus, actin is in complex with ABPs; and, in particular, the G-actin pool exists preserved in complex with profilin. Interestingly, inactivated actin is also a monodisperse complex

(not an amorphous aggregate) that, possibly, has some functional role. Actin not only cannot fold without chaperons but also cannot form a compact structure without its ligands, the Ca^{++} ion and ATP. Actin has several binding sites and can interact with an enormous number of partners. While interacting with numerous ABPs, actin acts as a hub protein, as is typical for ID proteins. Many of the ABPs themselves are ID proteins of the signaling system and interact with other hub proteins. Actin is ubiquitous. It is one of the main components of the system of muscle contraction, it forms the cytoskeleton, it is found in the cell nucleus in which, except for the motility and scaffold functions, actin acts as a regulatory protein that participates in the processes of transcription and chromatin remodeling. The analysis of the amino acid sequence of actin with the use of the PONDR® program (Obradovic et al., 2005; Uversky, 2011) reveals that actin contains segments of polypeptide chain that are prone to be disordered (Figure 5).

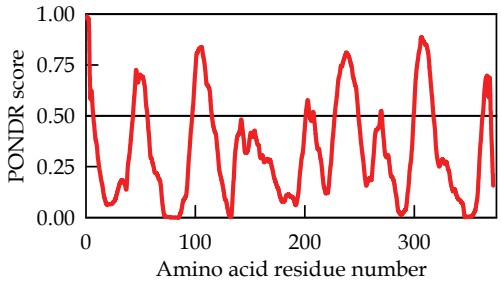

Fig. 5. PONDR score for actin. The PONDR® VLXT protein disorder predictor was used (Obradovic et al., 2005). The portions of the polypeptide chain for which the PONDR score > 0.5 are prone to form disordered fragments of polypeptide chains.

It appears that currently, the "actin" scientific community is not acquainted with, or may be acquainted with but not interested in, the most recent achievements in the field of protein structure and folding, while those researchers who study protein folding, ID protein folding in particular, do not seem to consider what an interesting object for their investigation actin can be. We will be pleased if this publication could help groups of researchers from diverse fields collaborate to join their efforts in the study of actin. Recently, V.N. Uversky published a work with the intriguing title "ID proteins from A to Z" (Uversky, 2011). We believe that this card file will not be full if, in the first cell (letter A), there is not a section entitled "Actin as an ID protein".

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4

Microtubules During the Cell Cycle of Higher Plant Cells

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1. Introduction

The microtubules control many cellular functions and play a key role in cell morphogenesis and development. They are dynamic heteropolymers composed of α- and β-tubulin and microtubule associated proteins (MAPs) that bind to microtubules and regulate their polymerization, organization and dynamic behavior. The structure of microtubules and tubulins are almost identical from yeast to human, yet the mechanisms that control the assembly, organization and behavior of microtubules, vary among different organisms. Thus, in animal cells the microtubule system is usually organized by the centrosome, the dominant microtubule organizing center (MTOC) that nucleates microtubules and controls spatial organization of the microtubules in the cell. Microtubule minus ends are anchored at MTOC, while the dynamic plus ends are oriented towards the cell periphery, forming aster-like array. Interphase cells typically assemble single "aster" with long microtubules often reaching the cell periphery. Upon the entry to mitosis, the spindle, composed of two asters with short microtubules, replaces interphase aster-like array. Unlike animal cells, plants do not have centrosome-like MTOC and assemble several distinct microtubule arrays, replacing each other during the cell cycle. The cortical microtubule system (CMT) is present during interphase (G1, S, and G2 phases) and plays a crucial role in the construction of the cell wall by guiding the deposition of new cell wall polymers. In many types of cells microtubules emanate from the nuclear surface towards the cell cortex, assembling another interphase array termed radial microtubule system (RMT). During cell division, microtubules rearrange into specialized arrays – the preprophase band (PPB), the spindle and the phragmoplast. The PPB and phragmoplast are unique to plants. The PPB (a circular array of microtubules) forms in G2 phase and disassembles prior to the nuclear envelope breakdown at the onset of prometaphase. The PPB defines the future division plane and the site of the cell plate formation during cytokinesis. The plant mitotic spindle provides equal distribution of chromosomes in mitosis, yet spindle assembly and organization differ from those in other eukaryotes, mainly due to the absence of the defined centrosome/MTOC in the spindle poles. The phragmoplast is a special microtubule array that substitutes the contractile ring of animal cells during cytokinesis. Phragmoplast directs the synthesis of a new cell wall that physically separates two daughter cells. Different microtubule arrays have distinctive features, use different tubulin isoforms, tubulin modifications and microtubule associated proteins in assembly of each array. In view on extensive studies of the molecular mechanisms underlying the cytoskeletal functions, this chapter will be focused both on specificity, and basic structural and functional aspects of organization of plant microtubule system.

2. Specific features of plant microtubules

2.1 Tubulin

Although tubulin α and β subunits have been highly conserved over the evolution of eukaryots, plant tubulin is immunologically and pharmacologically different from tubulin of animals and fungi. Thus, animal and plant tubulin have 79-81% sequence identity; the α subunit of plant tubulin have distinct peptide mapping pattern, its electrophoretic mobility is higher than of β subunit, what is in contrast with animal tubulin subunits (Fosket & Morejohn, 1992). Plant γ-tubulin shares 60% sequence identity with animal γ-tubulin, and, oppositely to animal cells, its electrophoretic mobility is higher than of α and β subunits (Liu et al., 1994). Nevertheless, microtubules comprising each plant array have an identical structure, and their functional diversity within the same cell is supported by multiple tubulin modifications. The heterogeneities of tubulin molecules are created on different levels by two mechanisms. On the genome level it is the activity of the multi-genes families, encoding plant α and β tubulins. Tubulin genes are not expressed uniformly during plant development and the expression of tubulin isotypes is tissue-specific. On the cytoplasmic level it is the posttranslational tubulin modifications. Tubulin is subjected to the detyrosination/tyrosination cycle, the removal of penultimate glutamate, polyglutamylation, polyglycylation, acetylation, phosphorylation and palmitoylation. Most modifications occur on microtubules rather than on unpolymerized tubulin, and notably contribute to the diversity of tubulins. Post-translational tubulin modifications create subpopulations of microtubules, involved in specific functions within particular cellular compartments, and play an essential role in the reorganization of the microtubule cytoskeleton during the life cycle of plant cells (Cai, 2010). Thus the development of specific plant cells and tissues is characterized by the expression of distinct tubulin genes and, consequently, by the use of distinct tubulin isotypes, which are post-translationally modified to control the microtubule properties and functions.

2.2 Resistance to microtubule depolymerizing compounds

Plant tubulin is pharmacologically different from tubulin of animals, fungi and slime molds. This is manifested by a low affinity to colchicine, an alkaloid produced by several plants of the *Liliacea* family. The drug is known as an anti-microtubule and anti-mitotic agent for both plant and vertebrate cells (Eigsti & Dustin, 1955). There are principal differences in the response of these organisms to colchicine. The minimal concentration required for mitotic block of animal and plant cells differs considerably. Thus, animal cells are about 100,000 more sensitive to the colchicine than plant cells. Colchicine is usually lethal to dividing animal cells even at the minimal concentrations necessary to block mitosis, yet plant cells can survive in a state of colchicine-induced mitotic arrest (C-mitosis) for several days and return to division after colchicine is removed (Eigsti & Dustin, 1955). It appears that plant microtubules as compared to vertebrate ones, are more resistant to colchicine treatment due to the low affinity of plant tubulin dimers to the drug, although they can be completely disrupted by extremely high concentrations of colchicine. Dinitroaniline herbicides (oryzalin, trifluralin), amiprophosmethyl (APM) and phenylcarbamates efficiently bind to plant tubulin and prevent microtubule polymerization. Plant tubulin has a high affinity to these herbicides, which, in turn, have no effect on animal tubulin; therefore these agents are broadly used in experimental studies of plant microtubule dynamic and function (Morejohn & Fosket, 1991).

2.3 Microtubule nucleation

Higher plant cells do not have a single dominant MTOC comparable to the centrosome of animal cells or spindle pole body of yeast. Instead, plant microtubules are initiated from dispersed nucleating sites - the γ-TuRCs (γ-tubulin ring complexes), which serve as the microtubule nucleation units. γ-TuRCs are composed of γ-tubulin small complexes (γ-TuSCs) and several other proteins. The conserved γ-TuSCs contain two molecules of γ-tubulin and one molecule each of Spc97 and Spc98 homologs, and are the minimal nucleation unit (Erhardt et al., 2002). Additional proteins in the larger ring complexes considerably enhance the nucleation activity, and some specific subunits are presumed to recruit the nucleation complexes to the particular cellular sites. γ-tubulin dependent nucleation of microtubules in plant cells occurs preferentially along the sidewalls of assembled microtubules, with new ones arising at discreet angles relative to assembled microtubules, and forming a Y-type or branched structure. A cytoplasmic γ-tubulin complex shuttles between the cytoplasm and the sides of microtubules and has nucleation activity only when bound to the microtubules.Thus microtubule nucleation in plant cells requires both existing microtubules and the presence of γ-tubulin (Murata et al., 2005).

2.4 Microtubule associated proteins (MAPs)

MAPs in plants regulate the assembly, repositioning and dismantling of all microtubule arrays throughout the cell cycle, and facilitate microtubule growth, dynamics, organization, and function (Sedbrook, 2004). Some MAPs are specific only to plants, while others are found in other organisms.

2.4.1 MOR1 (microtubule organization 1)

The *Arabidopsis* MICROTUBULE ORGANIZATION 1 MOR1/GEM1 is a homolog of XMAP215/TMP200 and belongs to a highly conserved MAP215/Dis1 group of MAPs found in all eukaryotes. MOR1 is essential for the organization of CMT array, proper organization and function of the PPB, the mitotic spindle, the phragmoplast formation and the progression of cell division. MOR1 and several other MAPs also contribute to microtubule-assembly dynamics (Kaloriti et al., 2007).

2.4.2 Plus end interacting proteins (+TIPs)

+TIPs are a diverse group of proteins characterized by their preferential localization to microtubule plus ends, where they regulate microtubule dynamic instability and mediate microtubule interactions with intracellular components such as the cell cortex, organelles, kinetochores, and other cytoskeletal elements (Bisgrove et al., 2004). Two important +TIPs in plants are END BINDING1 (EB1) and SPIRAL1 (SPR1). EB1 proteins are highly conserved in animals and fungi, yet SPR1 appears to be plant specific. Among known functions of EB1 proteins are the promotion of microtubule polymerization, stabilization, regulation of spindle positioning and chromosome segregation (Komaki et al., 2010). SPR1 and EB1 may act together to regulate directional cell expansion in response to the environmental stimulation. Another +TIP protein, CLASP, found in *Arabidopsis*, is involved in modulation of microtubule–cortex interactions and contributes to the PPB formation (Wasteneys & Ambrose, 2008).

2.4.3 Katanin

Katanin is a heterodimeric microtubule severing protein composed of an ATPase 60 kDa catalytic subunit with microtubule fragmenting activity and an 80 kDa regulatory subunit. The *Arabidopsis* katanin homolog exhibits a punctate localization pattern at the cell cortex and the perinuclear region. Overexpression of p60 katanin in *Arabidopsis* causes abnormal cortical microtubule bundling and fragmentation along their lengths. Plant katanin may function by severing microtubules at their minus ends, thus releasing the minus ends from the nucleating centers and allowing microtubules to be organized into bundles (Kaloriti et al., 2007).

2.4.4 MAP65 family

MAP65 family is a group of 60–65 kDa proteins that co-purify with microtubules. The *Arabidopsis* genome encodes nine MAP65 proteins of varying functions. Arabidopsis, tobacco, zinnia and carrot homologs bundle microtubules *in vitro* and, in some instances, *in vivo*. Some MAP65 members (AtMAP65-1, AtMAP65-3, NtMAP65-1a) belong to midzone MAPs because they localize at the antiparallel region of the developing phagmoplast and probably play essential role in cytokinesis by crossbridging microtubules that need to retain spatial organization in reorganizing microtubule arrays (Kaloriti et al., 2007). MAP65-4 regulates dynamic instability of microtubules by decreasing catastrophe and increasing rescue events. It is colocalized with mitotic microtubules, specifically with microtubules of the developing mitotic spindle during prophase and with the kinetochore fibers from prometaphase to the end of anaphase. Thus, MAP65-4 could mediate lateral interactions between spindle microtubules and participate in the formation and dynamics of microtubules within kinetochore fibers (Fashe et al., 2010).

2.4.5 Wave-dampened 2

WVD2 gene encodes a 23 kDa MAP that appears to regulate cell expansion through its association with and/or organization of cortical microtubules. WVD2 C-terminal domain is distantly related to the vertebrate microtubule-associated protein TPX2, involved in RanGTP-mediated spindle assembly around chromosomes.

2.4.6 AIR 9 (auxin induced root cultures 9)

AIR9 is a 187 kDa MAP, conserved in plants and found on microtubules of the cortical array and the PPB, but down regulated during mitosis. AIR9 reappears at the former PPB site when growing phragmoplast contacts the cortex, at the site of cell plate insertion and on the new cross walls, suggesting that AIR9 recognizes a component of the former PPB. Thus AIR9 may be involved in the maturation of those cell plates, which already contacted the established by the PPB cortical division site (Buschmann et al., 2006).

2.4.7 AtMAP70

MAP70 is a plant specific multi-gene family of proteins, sharing 70-80% identity and approximately 70 kDa molecular mass. AtMAP70-1 colocalizes with microtubules of all arrays, but is missing in microtubules of the midzone. AtMAP70-5 is smaller (58 kDa),

shares 47% sequence identity, and its possible functions are the regulation of microtubule dynamics and a role in anisotropic cell expansion and organ growth (Kaloriti et al., 2007).

2.5 Plant motors

Microtubule motor proteins play an essential role in the organization and function of microtubule arrays during cell division and cell growth in plants. They are responsible for the motility of macromolecular complexes and organelles, and the segregation of chromosomes during mitosis and meiosis.

2.5.1 Kinesins

61 genes encoding kinesins were identified in the genome of *Arabidopsis thaliana*, and most kinesins are evolutionarily divergent from their counterparts in animals and fungi (Lee & Liu, 2004). Kinesins are grouped into sub- families by phylogenetic analyses of their motor domains.

Plus end directed kinesins:

1. The N-terminal motor kinesin AtFRA1 (kinesin-4) belongs to the kinesin-4 or KIF4/chromokinesin subfamily. Animal chromokinesins typically play roles in chromosome condensation, chromatid motility and microtubule assembly, while AtFRA1 contributes to cellulose microfibril deposition in the cell wall. Thus the sequence similarity in the motor domain does not necessarily confer any functional relationship among kinesins from different organisms.
2. The internal motor kinesin - kinesin-13. Two *Arabidopsis* kinesins, AtKinesin-13A and AtKinesin-13B, belong to the kinesin-13 subfamily, previously known as MCAK or KIN I. AtKinesin-13A is specifically associated with plant Golgi stacks. Animal internal motor kinesins in this subfamily are not motors. Instead, they are microtubule depolymerases activated by microtubule end binding. Therefore, this again demonstrates that sequence homology in the motor domain alone does not guarantee functional similarity for kinesins from different organisms.
3. Kinesins in the BIMC (Kinesin-5) subfamily. Four *Arabidopsis* genes encode kinesins in the BIMC/Kinesin-5 subfamily. Besides the sequence conservation in the motor domain of these four kinesins and their animal and fungal counterparts, all kinesin-5 members contain a conserved phosphorylation site for the key cell-cycle kinase p34cdc2.

Minus end directed kinesins:

A number of C-terminal kinesins are minus end-directed motors. Among minus end-directed kinesins, however, some have the motor domain located at the N-terminus, while some others have it in the middle.

1. KATA/ATK1 and close relatives. These kinesins are closely related to the NCD (non-claret disjunction) kinesin-14 subfamily and are implicated in the organization of microtubules of the spindle and the phragmoplast.
2. The calmodulin-binding KCBP/ZWI kinesin. KCBP/ZWI is a single gene that encodes a unique calmodulin-binding kinesin, possibly involved in stabilization of microtubule ends.

3. The actin-binding KCH kinesins. Minus end-directed kinesins with a unique calponin-homology (CH) domain at the N terminus. They have only been reported in organisms of the kingdom Plantae. The presence of a CH domain in a kinesin is intriguing as it is typically found in actin-binding proteins like calponin and fimbrin. KCHs may serve as linkers between microtubules and actin microfilaments.
4. Other minus end-directed kinesins. There are nine other *Arabidopsis* kinesins in the category of minus end-directed motors. Four have the motor domain located at the N terminus, the feature that has never been reported for kinesins from animals and fungi.

Special group of kinesins involved in cytokinesis:

N-terminal kinesins are involved in cytokinesis and localize to the division site, yet in distinct manner.

1. AtNACK1/HIK kinesins and a MAP kinase cascade. AtNACK1/HIK is an activator of a mitogen-activated protein (MAP) kinase cascade and is essential for the completion of the cell plate formation. NACK1 is also required for cell plate expansion after initiation of the cytokinesis.
2. AtPAKRP1/AtKinesin-12A and similar kinesins. AtPAKRP1/AtKinesin-12A and two similar kinesins are specifically associated with the plus end of phragmoplast microtubules and are predicted as plus end-directed motors. They are implicated in the maintaining of orderly organization of microtubules within the phragmoplast.
3. Kinesins transporting Golgi-derived vesicles. Phragmoplast-associated kinesin, AtPAKRP2, is specifically associated with Golgi-derived vesicles in the phragmoplast. At least two other *Arabidopsis* kinesins are also exclusively associated with vesicles in the phragmoplast. Therefore, they are predicted to be examples of plus end-directed kinesin motors that deliver Golgi-derived vesicles during cytokinesis.

2.5.2 Dynein

Higher plants (angiosperms) lack cilia and flagella in their life cycle, and their non-motile sperm cells are conveyed to the egg by a growing pollen tube. These plants also lack the axonemal dynein, cytoplasmic dynein 2 and no full-length genes for cytoplasmic dynein 1 subunits, except for the light chain LC8, have been found in the sequenced genome of *Arabidopsis thaliana, Oryza sativa* and *Populus trichocarpa*. However, immunological and biochemical studies showed the presence of dynein heavy chain (DHC) related polypeptide in pollen tubes of *Nicotiana tabacum* (Moscatelli et al., 1998) and root cells of wheat *Triticum aestivum* L. (Shanina et al., 2009). Dynein is an ancient and evolutionary conservative multiprotein complex, found in diverse groups of organisms, from yeast to human, and DHC is the largest dynein subunit, containing ATP-binding/motor and microtubule-binding domain. To date, nothing is known about dynein in plants that have flagella apparatus at certain stages of their life cycle. For instance, the microtubules of the flagellar axonemes of water fern *Marsilea vestita* have the typical 9 + 2 arrangement with only inner dynein arms present (Hyams, 1985). Flagellated sperm cells also develop in ancient gymnosperms, Ginkgo and cycads, which are the only seed producing plants (spermatophytes) that have motile or free swimming sperm (Vaughn and Renzaglia, 2006) with abundant flagellar apparatus (100-50,000 flagella per cells) (Renzaglia and Garbary,

2001). Thus, higher plants have high molecular weight antigen that shares biochemical and immunological properties with DHC, yet its identity remains unclear.

2.6 Microtubule dynamics

The distinctive feature of plant microtubules is the unusual dynamic behavior, exemplified at least in some stages of microtubule life cycle. Cortical microtubules in plant cells exhibit the treadmilling (addition of tubulin subunits at plus ends and removal at minus ends), what is remarkably different from the behavior of microtubules in animal cells, when microtubules are attached to the centrosome with their minus ends, and plus ends exhibit the dynamic instability (slow growth – rescue, and rapid disassembly – catastrophe). Moreover, plant microtubules display a unique type of dynamics, a combination of dynamic instability and treadmilling behavior, termed "hybrid treadmilling", which involves dynamic instability behavior at the plus end, coupled with a slow depolymerization at the minus end. Polymerization-biased dynamic instability at one end and slow depolymerization at the other end results in sustained microtubule migration across the cell cortex (Shaw et al., 2003).

Another unique feature of higher plant microtubules is that unlike microtubules of most animal cells, they do not originate from a single or multiple MTOCs (Mineyuki, 2007; Murata et al., 2007). Plant microtubules change the arrangement with the progression of the cell cycle, assembling distinct types of microtubule arrays, which function at the appropriate stage of the cell cycle. The CMT system or/and RMT system is present during interphase, PPB, mitotic spindle and phragmoplast successively replace each other during mitosis (Fig. 1, 2).

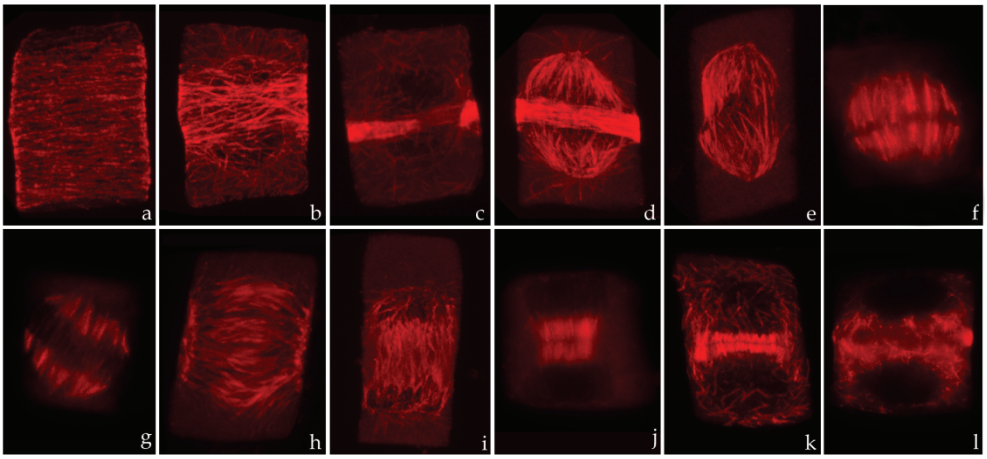

Fig. 1. Microtubules during the cell cycle of root meristem cells of *Triticum aestivum* L. (a) Cortical microtubule bundles in interphase cell. (b) The development of broad PPB during G2 phase. (c) Matured PPB in prophase cell. (d) PPB and prophase spindle. (e) Prometaphase spindle. (f) Metaphase spindle. (g) Anaphase spindle. (h) Initial stage of phragmoplast formation in anaphase. (i-k) Successive stages of phragmoplast formation and development during anaphase-telophase. (k-l) Radial microtubule network during transition to G1 phase.

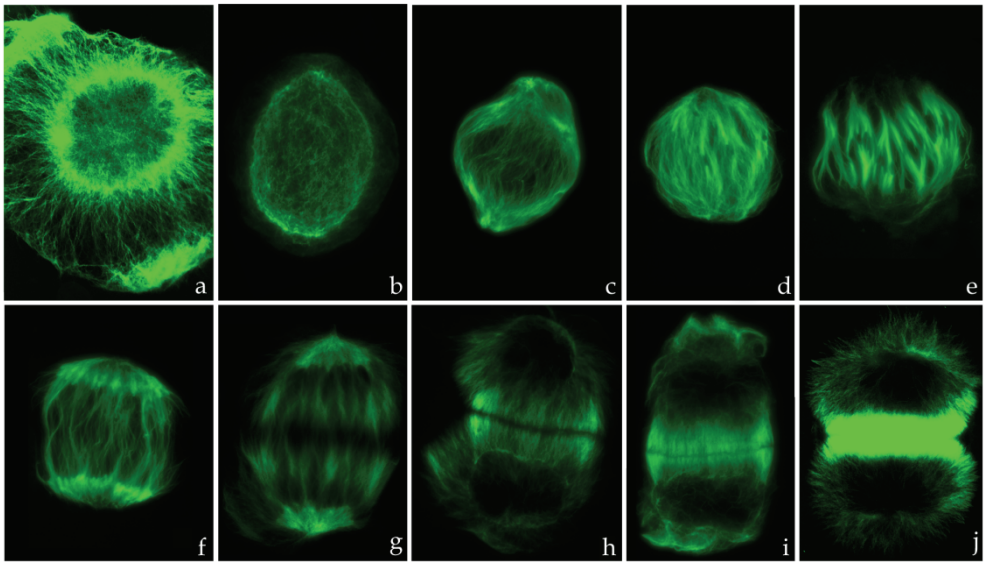

Fig. 2. Microtubules during the cell cycle of isolated endosperm cells of *Scadoxus katherinae* Bak. (a) Radial microtubule system in interphase cell. (b) Early stage of prophase spindle organization. (c) Prophase spindle. (d) Prometaphase spindle. (e) Metaphase spindle. (f, g) Spindle in mid and late anaphase. (h, i) Phragmoplast formation and development during telophase. (j) Radial microtubule arrays during transition to G1 phase.

3. Microtubule systems of interphase cells

3.1 Radial microtubule system (RMT)

RMT system is composed of microtubules radiating from the nuclear surface toward the cell periphery. During somatic division of flowering plants this microtubule array is observed for relatively short time, when the cell is completing the cytokinesis and before the establishment of the interphase CMT (Fig. 1k-l). RMT system is typical for some special cases of flowering plant cell division (Fig. 2a, j). In isolated endosperm cells of *Scadoxus*, RMT system is composed of elementary structural units – microtubule converging centers (MTCCs). The pointed tips of MTCCs are associated with the nuclear surface, and the divergent ends are oriented outwards and extend toward the plasma membrane. Cumulative evidence indicates that minus end of radial microtubules are associated with the nuclear surface, while plus ends are oriented toward the cell periphery. MTCCs could be the transitory structures, instrumental for orderly reorganization of RMT and other microtubule arrays in plant cells (Smirnova & Bajer, 1994, 1998). RMTs arrays emanating from the nuclear surface are also present in vacuolated cells, where they are termed endoplasmic microtubules (EMTs) (Dhonukshe et al., 2005). Thus nuclear surface may function as MTOC for RMTs, yet the microtubule nucleating factors have not been clearly identified. Chromatin affects microtubule nucleation through the nuclear membrane, as it was shown for reconstituted nuclei in *Xenopus* extracts (Heald et al., 1996, 1997). Targeting protein for Xklp2 (TPX2) is one of the proteins implicated in the nucleation of microtubules around chromosomes in a RanGTP-dependent manner. TPX2 orthologue was found in *Arabidopsis*, (Evrard et al., 2009) and experimental studies showed that it nucleates

perinuclear microtubules/RMTs, leading to the formation of a prophase spindle, and then may act as a spindle microtubule nucleator and stabilizer (Vos et al., 2008).

3.2 Cortical microtubule system (CMT)

Plant CMT network consists of a population of relatively short, overlapping microtubules that bundle into a higher order structures. In rapidly elongating cells, the CMT bundles typically circumference the cell and are arranged transverse to the elongation axis of the cell (Fig. 1a). Once the phase of cell elongation slows down, the transverse CMTs reorient into a predominantly oblique or longitudinal direction with respect to the cell's elongation axis (Wasteneys, 2004; Lucas & Shaw, 2008; Wasteneys & Ambrose, 2008).

Several mechanisms have been proposed in order to explain the assembly and dynamic organization of the cortical arrays:

1. Microtubules are initiated *de novo* or originate from the existing microtubules in the cortical cytoplasm. First, seed microtubules appear at the cortex of early interphase cells. Then γ-tubulin nucleates new microtubules along the lengths of existing microtubules, resulting in dispersed 'Y'-branched organizational centers (Murata et al., 2005). As a result, new cortical microtubules form on the pre-existing microtubules as branches with a defined angle (Hashimoto & Kato, 2006). Nascent microtubules detach form the nucleation sites and migrate to the cortex by "hybrid" treadmilling (Shaw et al., 2003).
2. Microtubules originate from the nuclear surface, then are severed and translocated to defined cortical positions, establishing CMT. At the M/G1 transition, microtubules are nucleated on the nuclear surface and extend to the cell cortex. γ-tubulin also first accumulates at the nuclear surface and then spreads to the cortex. Therefore, the radial/endoplasmic microtubules could be involved in distributing the γ-TuRCs from the nuclear surface to the plasma membrane. However, the majority of new microtubules are initiated *de novo* at the cell cortex and do not originate from the endoplasmic microtubules. New microtubule initiation sites have been detected only in associations with pre-existing microtubules and in cortical regions that have no detectable microtubules (Hashimoto & Kato, 2006; Wasteneys & Ambrose, 2008).
3. Lateral and axial sliding (translocation) of existing microtubules into new positions results in relocation, rearrangement and redistribution of microtubules in the cortex and/or from the perinuclear location toward the cortical cytoplasm. After microtubules are nucleated and released/severed from γ-TuRCs, both ends of the microtubules are free. The assembly dynamics of free microtubules may be marked by net polymerization at the plus-ends and net depolymerization at the minus-ends. The combined effect of these dynamic properties produces sustained treadmilling motility, when single microtubule translocates across the cell cortex. Thus, the migration of polymers to new positions is achieved by the balanced addition and removal of subunits at the microtubule ends.

3.2.1 Dynamic behavior of microtubules in the cortex

CMTs arise or arrive in random orientations throughout the cell's cortex, and this orientation is maintained in actively dividing meristem cells. In rapidly elongating cells, the discordant microtubule orientations do not persist for long, and microtubules of cortical arrays tend to acquire a common polarity by selective stabilization of concordant microtubules. Treadmilling leads to microtubule interactions, resulting in microtubules crossing over one another,

selectively disassembling, pausing or bundling. The latter depends on the angle of interaction between encountering microtubules. A steep angle of interaction (more than 40°) usually leads to the disassembly of the microtubules, while the shallow angle (less than 40°) results in alignment and lateral interaction of the encountering microtubules (Ehrhardt, 2008). MAPs, including +TIPs and those forming the cross-bridges between the lattices of adjacent microtubules (MAP65 family), may be involved in the organization of microtubule bundles. The combination of microtubule encounter activities ultimately determines the spatial organization of cortical microtubules in each cell types and explains the mechanism of self-organization of randomly nucleated microtubules into parallel arrays (Pastuglia & Buchez, 2007; Lucas & Shaw, 2008; Wasteneys & Ambrose, 2008).

3.2.2 Association of cortical microtubules with plasma membrane

After microtubules are initiated in the cortical cytoplasm, they usually detach from the original nucleation sites but remain tightly anchored to the cortex. The presence of cross-bridge structures between cortical microtubules and the plasma membrane points to the presumptive plasma-membrane-associated protein linkers anchoring microtubules to the cortex. Phospholipase is one of the molecules involved in attaching the CMTs to the plasma membrane (Kaloriti et al., 2007), yet cortical microtubule attachment could be also mediated by the +TIPs.

3.2.3 The functions of CMT array

Most plant cells have cellulose cell walls and CMT network beneath the plasma membrane. During interphase, CMTs are typically coaligned with the cellulose microfibrils, and, therefore, it has long been thought that the organization of the interphase CMTs regulates the axis of cell elongation by guiding the oriented deposition of cellulose microfibrils. The microfibrils, in turn, provide the constraints to restrict turgor-induced cell expansion to an axis perpendicular to the net orientation of the cellulose microfibrils. The functional association of microtubules with the cellulose synthase complex located on the plasma membrane (Paredez et al. 2006) strongly supports the concept that at least in some cell types, cortical microtubules can organize cellulose synthase complexes and guide their movement through the plasma membrane as they create the cell wall. The latest data confirmed that cortical microtubules not only guide the trajectories of cellulose synthase complexes in the plasma membrane, but also regulate the insertion and internalization of cellulose synthase complexes (Crowell et al., 2009). Moreover, cellulose synthase complexes also influence cortical microtubule array stability and organization (Paredez et al., 2008), indicating that while cortical array organization directs the trajectories of cellulose synthase complexes movement, the activity of cellulose synthase complexes affects cortical array organization as well. However, organized cortical microtubules are not always essential for maintaining or establishment of transversely oriented cellulose microfibrils in expanding cells, indicating that the relationship between CMT array organization, microfibril orientation and the axis of cell elongation is more complex. In this view, the template incorporation model and microfibril length regulation model are the alternative concepts of microtubules-microfibrils interplay (Wasteneys, 2004).

4. Preprophase band of microtubules (PPB)

Most differentiated and expanded cells have a system of parallel bundled microtubules oriented perpendicular to the longest axis of the cell and localized in the cortical cytoplasm

(CMTs). Prior to mitosis, this cortical system is replaced by the PPB, a unique plant array of cortical microtubules and actin filaments, encircling the nucleus and positioned just underneath the plasma membrane. PPB is formed in G2 phase and disassembled prior to prometaphase. The PPB at first occupies broad area of the cortex (about 2/3rd of the peripheral area), but then it gradually "matures", microtubules, forming the band, become more densely packed as the cell approaches the mitosis (Fig. 1b, c). PPB demarcates the cortical division site where the newly formed cell plate will be fused with the parental plasma membrane (Van Damme et al., 2007, Van Damme & Geelen, 2008; Müller et al., 2009).

4.1 The occurrence of PPB

The division events that involve a PPB is thought to be an adaptation for cells that are part of a complex multicellular architecture. Indeed, PPBs occur in meristems and meristemoids during vegetative growth, and absent in microsporogenesis, megasporogenesis, in the first assymetrical mitosis of the embryo, in embryo sac development (Mineyuki, 1999). In line with the idea that the PPB correlates with divisions in tissues, it was noticed that some suspension-cultured cells divide without the need for a PPB or frequently produce abnormal PPBs, but still manage to divide.

4.2 The origin of PPB

Few mechanisms have been proposed to explain the origin of the PPB: *de novo* assembly of PPB microtubules, recruitment of tubulin from the degraded cortical microtubules to microtubules of the PPB, rearrangement of existing cortical microtubules to the site of the PPB development. Current models implicate the distinctive dynamic behavior of cortical microtubules in PPB formation (Ehrhardt, 2008; Wasteneys & Ambrose, 2008; Müller et al., 2009). During interphase, microtubules are distributed throughout the cell cortex. The growing plus ends of dynamic microtubules that are bound to the cell cortex frequently run into other single microtubules and microtubule bundles. Steep-angle collisions promote microtubule shrinkage, whereas shallow-angle encounters facilitate coalignment into bundles. These interactions of microtubules may promote the self-organization of dynamic microtubules into a parallel arrangement. During PPB initiation at the transition from interphase to mitosis, the growth rate and the stochastic alternating frequencies between growing and shrinking phases increase in cortical microtubules, outside the PPB. Combined with the stabilizing activities in the developing PPB, these changes in microtubule dynamics may cause disassembly of cortical microtubules outside the PPB and accumulation of microtubules to form PPB. It has been assumed that at preprophase, cortical microtubules become restricted to the future plane of division via selective depolymerization of non-PPB microtubules and/or selective stabilization of microtubules in the PPB zone. However, the general hypothesis that bundling stabilizes the dynamic properties of the constituent microtubules was not confirmed, because no evidence that bundled microtubules are stabilized against depolymerization through changes to their dynamic properties was found.

4.3 The regulation of PPB formation

A variety of proteins is involved in PPB formation by differentially regulating nucleation, dynamics and stability of microtubules. Microtubule binding proteins like MOR1/GEM1 modulate microtubule dynamics and promote PPB formation. MAP65 members bundle

microtubules by forming cross bridges between overlapping microtubules and thus could potentially stabilize PPB microtubules via bundling. +TIP family member CLASP has been implicated in PPB organization and narrowing via modulation of microtubule dynamics in the PPB zone and/or by mediating microtubule-cortex interactions. *Arabidopsis* signaling proteins, TON1a, TON1b and TON2, are also crucial for the formation of PPB, because plants lacking any of these proteins do not form PPBs and, as a result, have misoriented cell divisions (Müller et al., 2009).

4.4 Functional properties of PPB

The position of the PPB in the cortical cytoplasm forecasts the division plane and the site where the cell plate inserts into the mother wall during cytokinesis. The PPB is dismantled prior to prometaphase, therefore the separation in time between the destruction of the PPB and the insertion of the cell plate led to the idea that the PPB leaves behind a landmark that will guide the expanding phragmoplast to the site where the PPB was positioned prior to its disassembly. After the PPB is disassembled, some type of "memory" of its location must remain throughout mitosis and cytokinesis. A number of negative and positive markers of the division site have been found in the cortex (Müller et al., 2009; Rasmussen et al., 2011).

Negative markers:

1. The actin-depleted zone (ADZ) of the cell cortex. Actin microfilaments are an integral part of the plant cytoskeleton. During interphase-mitosis transition, the actin network does not disappear throughout the peripheral space like the cortical microtubules. Upon breakdown of the PPB a region devoid of actin, termed the actin depleted zone (ADZ), is formed in the cortex. Because the position of the ADZ corresponds to that of the PPB, it too marks the division zone. It is important to note that this ''negative template'' remains throughout metaphase and anaphase, establishing the landmark at the plasma membrane. However, the role of ADZ as a negative marker of cortical division site remains ambiguous.
2. Cortical actin "twin peaks" – two bands of high actin density flanking the cortical division site. In this view, ADZ should be interpreted as a zone of low actin abundance rather than one with complete loss of the filaments.
3. The *Arabidopsis* kinesin-like protein KCA1 is another negative marker of the cortical division site, specifically the plasma membrane domain. Like cortical actin, it is locally depleted at the cortical division site during mitosis and cytokinesis, creating a KCA1-depleted zone (KDZ). KDZ coincides with the position of the disassembled PPB and established ADZ. Once established, the KDZ does no longer require an intact microtubule or actin cytoskeleton and persists throughout cytokinesis.
4. Endocytic vesicles form more frequently in the PPB zone than in other areas of the cell cortex, suggesting that endocytosis could be important for establishment of the division plane.

Positive markers:

TAN and RanGAP1 have been identified as positive markers of the division plane, continuously localizing there from preprophase through the completion of cytokinesis.

1. The TAN1 gene encodes a highly basic protein that binds to the microtubules of the PPB and localizes in the cortical division site during mitosis and cytokinesis. TAN is

implicated in guidance and linking of the phragmoplast to cortical division site throughout cytokineis.

2. Like TAN, RanGAP1 is recruited to the division plane, co-localizing with the PPB and remaining at the cortical division site throughout mitosis and cytokinesis. Ran-GAP1 is also localized to elsewhere in dividing cells including the nuclear envelope, spindle midzone, kinetochores and cell plate. It is not yet clear whether RanGAPs are required for PPB assembly/disassembly, or for phragmoplast guidance during cytokinesis.
3. A closely related *Arabidopsis* kinesins, POK1 and POK2 (phragmoplast orienting kinesins) are required for the correct localization of TAN and RanGAP1. POK1 and POK2 in combination are needed for localization of TAN to the PPB and cortical division site, suggesting that TAN becomes associated with the division plane as cargo of POK1 and POK2. By contrast, RanGAP1 does not require POK1 and/or POK2 for co-localization with PPBs, but does require these kinesins for its maintenance at the cortical division site after PPB disassembly.
4. The plasma membrane domain is defined by the localization of a plant specific protein TPLATE that has homology with coat proteins. During cytokinesis TPLATE accumulates in the cell plate and appears in the cortical division site just before cell plate fusion with plasma membrane. These finding suggests that TPLATE promotes vesicle trafficking to the cortical division site, where the cell plate contacts and fuses with plasma membrane.

Another essential PPB function is the proper positioning and orientation of the mitotic spindle. It is well known that cells lacking PPB may assemble multipolar prophase spindles, which are inevitably transformed into bipolar ones during prometaphase, after the nuclear envelope breakdown (Smirnova and Bajer, 1998). Alternatively, cells with PPB establish a bipolar cap-like organization of the prophase spindle perpendicular to the plane of division before nuclear envelope breakdown. It appeared that in vacuolated cells microtubules bridging the PPB and the prophase nucleus contribute to the bipolarity, orientation, and position of the prophase spindle by transmitting tensile forces that facilitate the organization of perinuclear microtubules from their initial random distribution, into two halves, oriented perpendicular to the PPB plane. This tension serves to co-align perinuclear microtubules in a direction similar to those of the bridge microtubules, thus providing a spatial cue for the orientation of the prophase spindle axis. Consequently, the PPB is an equatorial organizer of the prophase spindle (Ambrose & Cyr, 2008). However, non vacuolated cells have scarce or no microtubules bridging the PPB to the nucleus, therefore other mechanisms may control the spindle polarity and orientation.

Thus, the major PPB functions are the determination of the cortical division plane, promotion of the spindle morphogenesis and positional stability. PPB also controls premitotic migration and orientation of the nucleus in the cytoplasm of vacuolated cells, anchoring the premitotic nucleus in the central cytoplasm, and is a source of tubulin/microtubules pool for the assembling spindle.

5. Plant spindle

The mitotic spindles of higher plants do not have microtubule focusing structures at the spindle poles therefore it is usually barrel-shaped and lacks astral microtubules (Fig. 1f, 2d). The spindle consists of distinct kinetochore fibers (K-fibers), composed of kinetochore and non-kinetochore microtubules. The proximal parts of the K-fibers are attached to the

kinetochores, while convergent distal ends are oriented away from the chromosomes and terminate at the ill-defined polar areas. For instance, the mitotic spindle of the higher plant *Scadoxus* (formerly *Haemanthus*) is composed of multiple contiguous K-fibers, arranged as microtubule fir trees (MTFTs). Parallel central microtubules of MTFT, attached to the kinetochore, form a core of K-fiber (the trunk of the "fir tree") and non-kinetochore skew peripheral microtubules, represent the branches (Bajer and Molè-Bajer, 1982, 1986). All K-fibers/MTFTs within each half-spindle are aligned parallel to the spindle axis, and the structural integrity of the spindle is maintained by skew non-kinetochore microtubules, whereas the functional properties (support of chromosome movement) are provided by kinetochore microtubules. Each half-spindle may be composed entirely from K-fibers, conjoined together by non-kinetochore microtubules. The mitotic spindle is responsible for equal segregation of sister chromatids during cell division. This function is accomplished by the establishment of a plane of symmetry, which is materialized by the metaphase plate, orthogonal to the spindle axis and on which the chromosomes are positioned before separation.

5.1 The flexibility of plant spindle

Although conventional metaphase spindle is barrel-shaped and lacks astral microtubules, its shape may be altered during specialized types of division, for instance, during generative cell/nucleus division in pollen tube of different plants. In *Tradescantia virginiana* and *Convallaria majalis*, the spindle is comprised of an axial system of microtubule bundles, with kinetochores distributed along the length and depth of the tube. The same type of division in *Nicotiana tabacum* is characterized by oblique metaphase plate and the presence of distinct spindle with often pointed poles (Liu & Palevitz, 1991; Del Casino et al., 1999). During generative cell division in *Ornithogalum virens*, metaphase spindle has a conventional shape, encompassing diffuse poles (Banaś et al., 1996). Focused spindle poles are typical for divisions during microsporogenesis and cultured plant cells. Such loose organization of the spindle poles is crucial for spindle plasticity, required to counteract spatial constrains due to the presence of the rigid cell wall. Moreover, the focused spindle pole helps to maintain normal chromosome distribution and grouping, because if the spindle pole is not convergent, the division may lead to several developmental defects, such as the formation of micronuclei, multiple micro-spindles and phragmoplasts. Therefore, in most cells, anaphase chromosome movement occurs concomitantly with transformation of broad and ill-defined polar areas into convergent ones. It seems that dynamic changes of the spindle pole during metaphase-anaphase transition occur only in cells with broad metaphase spindle (Fig. 2 e-g), while tapered spindles retain their shape throughout division. Notably, reversed spindle pole transformations take place during prophase-metaphase transition, when pointed poles transform into diffuse ones (Fig. 1 d-f, 2 c-e). Chromosomes/kinetochores may play an important role in this process, because the changes in spindle pole configuration are triggered after the nuclear envelope breakdown at the onset of prometaphase.

5.2 The stages of spindle formation

The pathways of spindle assembly depend on the presence/absence of the PPB. In cells with PPB, the microtubules first assemble perinuclear network, which gradually transforms into two dense accumulations or polar caps, located on both sides of the nucleus and oriented perpendicular to the PPB axis. The polar caps are linked by microtubules thus forming the initial, yet already bipolar, prophase spindle (Fig. 1 d). Alternatively, the prophase spindle

may exhibit an especially variable pattern of organization in cells lacking PPB (division of isolated endosperm cells, cultured suspension cells), but during prometaphase, the spindle ultimately acquires bipolar configuration. This pathway of spindle assembly is not well understood, but the initial stage is marked by the formation of dense microtubule network around the nucleus in early prophase (Fig. 2 b). In mid-late prophase, microtubule network transforms into bipolar (Fig. 2 c), multipolar or apolar spindle-like configurations (Smirnova and Bajer, 1998). After nuclear envelope breakdown, which marks the onset of prometaphase, aberrations of the spindle structure are eliminated/corrected and final alignment of spindle axis takes place (Fig. 2 d, e). Although multipolar prophase spindles are common, multipolar metaphase spindles are extremely rare in all types of dividing higher plant cells. Thus, the formation of the bipolar spindle in prophase is not a prerequisite for bipolarity of the metaphase spindle.

5.3 MTOC-independent spindle assembly in higher plants

It is no longer doubted that the functional spindle can be assembled by MTOC-independent mechanism (Murata et al., 2007), yet the suggestion that higher plant spindles are formed by self-reorganization of microtubules in the absence of the centrosome/MTOC, was made before substantial molecular evidence came out (Smirnova and Bajer, 1992). Observations on microtubule reorganization in isolated dividing endosperm cells and cell fragments of *Scadoxus* revealed that microtubule system is comprised of elementary structural and functional units termed microtubule converging centers (MTCC) (Bajer & Molè-Bajer, 1986; Smirnova & Bajer, 1994, 1998). The remarkable cycle of MTCCs formation, exceptionally manifested in endosperm cells, is an expression of self-reorganization of microtubules, and the self-reorganization is also the major "driving force" for spindle assembly. Conceptual importance of this conclusion was that (1) it argued against the role of centrosome/MTOC in spindle organization and (2) invited speculations that MTCCs might have been developed in higher plants as a functional substitute of localized MTOC(s). In view of this concept, the consecutive stages of spindle organization were defined. Microtubules/MTCCs growth is initiated from the nuclear surface, with minus ends attached or directed toward the nucleus. Excessive formation of MTCCs in prophase would require disconnection or severing of assembled MTCCs from the nucleating sites. Free MTCCs coalesce around the nucleus making dense perinuclear cage. Closely located MTCCs interact according to the dynamic properties of their ends and action of microtubule-based motors (self-reorganize). Random translocation and reorientation of MTCCs result in appearance of microtubules arrays of mixed polarity, followed by sorting into bundles of uniform polarity. The metaphase spindle is composed of microtubules with minus ends located away from the nucleus/chromosomes (oppositely to interphase), therefore during spindle assembly minus ends must be extended away, cross-linked and converged into poles, while plus ends are captured and transiently stabilized by chromosomes/kinetochores. Thus, primary, yet not necessarily bipolar, spindle is formed in prophase. In prometaphase prophase spindle transforms into ultimate bipolar metaphase spindle due to the interference of the chromosomes/kinetochores in this process. Simultaneously, the focused appearance of the prophase spindle poles is usually disturbed. The splitting of the spindle pole into multiple sub-poles reflects the potential functional autonomy of each K-fiber. This is crucial in conditions of constrained cellular geometry that precludes the movement/rotation of the spindle within the cell and change of the cell shape. Consequently, higher plants facilitate spindle formation by assembling MTCCs as ready-to-use units, instead of single

microtubules. MTCCs form the spindle by self-reorganization therefore it is indeed "inside out" assembly of the spindle (Smirnova and Bajer, 1998).

The basic molecular mechanisms underlying MTOC-independent spindle assembly are now well understood (Wadsworth et al., 2010; Dunkan and Wakefiled, 2011). Briefly, in the initial phase of this process, chromosomes generate an environment enriched in RanGTP. Importins α and β direct traffic through nuclear pores during interphase and sequester several essential proteins. Proteins binding to importins in mitosis are the kinesin-like protein XCTK2, targeting protein for Xklp2 (TPX2) and nuclear protein of the mitotic apparatus (NuMA). These proteins have been proposed to be targets of RanGTP in the spindle assembly pathway. The importins-TPX2 complex is dissociated by RanGTP and once released TPX2 promotes microtubule nucleation in the vicinity of chromosomes. The size and shape of chromatin defines the shape and orientation of the initial spindle (Dinarina et al., 2009). Assembled microtubules are first randomly oriented; then, they coalesce and start to organize through the movement and sorting of randomly nucleated microtubules into bipolar structures. Two proteins Eg5 and Kid were suggested to be regulated by RanGTP and to participate in these events. Sorted microtubules within each of the two arrays become bunched together at their minus ends, thus giving the entire array the shape of a spindle. Microtubule minus ends may be focus into spindle poles, and this process involves two proteins regulated by RanGTP: NuMA and XCTK2. The TPX2 was confirmed to be a central regulator of spindle assembly in plant cells, because its absence or excess inhibits spindle formation. Plant TPX2 is predominantly nuclear during interphase and is actively exported before nuclear envelope breakdown to initiate prophase spindle assembly. Thus, plants have adapted nuclear–cytoplasmic shuttling of TPX2 to maintain proper spindle assembly without centrosomes (Vos et al., 2008). However, there is a basic difference in microtubule focusing and pole stabilizing activities between vertebrate and plant cells. The convergent pole of the prophase spindle is rearranged into unfocused one during prophase-metaphase, and inversely during metaphase-anaphase (part 4.1), indicating that the mechanisms of microtubule focusing and stabilization are switched on and off throughout mitosis. In conclusion, MTOC-independent spindle assembly appears to be exceptionally manifested in higher plants, yet our knowledge about the molecular mechanisms of this process is still fragmentary.

6. Phragmoplast

Cytokinesis in cells of flowering plants is achieved through the construction of a new cell wall from the inside-out. A cell plate forms between daughter nuclei at the end of mitosis and expands centrifugally to form a new cell wall positioned between new plasma membranes. It is initiated through the fusion of Golgi-derived vesicles, which contribute polysaccharides, proteins and membranous vesicles for the cell plate formation. These vesicles are guided to the cell plate by the microtubules of the phragmoplast, assembled in the interzonal area after chromosome's segregation in anaphase. Therefore cytokinesis in plants is considered as targeted secretion to the plane of division (Assaad, 2001; Jürgens, 2005; Müller et al., 2009). Phragmoplast is composed of two sets of interacting microtubules of opposite polarities, terminating in ribosome free, cell plate assembly matrix, actin filaments, ER and Golgi derived vesicles. It begins to form in the central part of the intersone already in anaphase, expanding laterally throughout telophase. Upon completion of cytokinesis it fuses with the parental cell wall at the site of the PPB location, where the cell plate attaches to the mother cell wall.

6.1 The phragmoplast assembly and development

The phragmoplast is assembled from microtubules of opposing polarity, interacting with their plus ends and pointing toward the equatorial region. These microtubules are aligned perpendicular to the future site of the cell plate localization. The phragmoplast is initiated in the midzone of the equatorial region in anaphase, between the trailing chromosome arms, and is shaped as cylindrical or barrel-like structure. Golgi-derived vesicles are carried from opposite sides, along microtubules toward the plus ends, where they fuse and first form vesicular-tubular membranous network, which is then transformed into the cell plate. During initial stages of formation, the cell plate is associated with the delivering vesicles microtubules of the phragmoplast. As the cell plate grows and expands centrifugally towards the parental cell wall, microtubules in the mid zone disassemble and in turn, assemble at the phragmoplast periphery, along the edge of the growing cell plate, resulting in displacement of microtubules toward the cell periphery. Consequently, the shape of the phragmoplast changes from cylindrical to ring-like, and at the end of cytokinesis two microtubule rings surround the edges of the newly formed cell plate. Microtubules of the phragmoplast provide the tracks for transport of vesicles to the site of the cell plate formation, yet the fusion of vesicles, development and maturation of the cell plate does not require microtubules. After the fusion of the cell plate with the parental cell wall, phragmoplast has an appearance of the microtubule ring, composed of the two sets of interacting antiparralel microtubules, and positioned at the site of the PPB localization. During specialized modes of cytokinesis, the phragmoplast is formed in cells dividing without a PPB, or even in syncytia between interphase nuclei, suggesting that the pathways of phragmoplast formations may vary among plant cells and tissues. For istance, during cellularization of endosperm, the multinucleate cytoplasm is reorganized into nuclear cytoplasmic domains defined by radial systems of microtubules, emanating from the nuclei. Adventitious phragmoplasts develop at the interfaces of microtubule systems emanating from adjacent nuclear cytoplasmic domains (Olsen et al., 1995), suggesting that microtubules of the spindle are not prerequisite for phragmoplast formation, and that phragmoplast-type of arrays may develop, where opposing sets of microtubules interact with their plus ends.

6.2 The origin of the phragmoplast

Phragmoplast is a ubiquitous plant structure, though the origin of phragmoplast microtubules and thereafter, the pathways of phragmoplast development and cell plate formation may differ among plant cells. For instance, in cells of apical meristem interzonal microtubules are abundant and give rise to the phragmoplast body (Staehelin & Hepler, 1996), while the central region of interzone in dividing endosperm cells is often nearly devoid of microtubules (Bajer and Molè-Bajer, 1982). Instead, numerous microtubules, initiated at the polar areas, appear between trailing chromosome arms in mid-anaphase, gradually extend toward the equator and give rise to a phragmoplast (Bajer and Molè-Bajer, 1986). These observations demonstrate that phragmoplast originates either predominantly or entirely from new microtubules, growing from the polar areas, and suggest that plant cells may have evolved multiple mechanisms of phragmoplast assembly and development.

6.2.1 Pathways of phragmoplast formation during “conventional” cytokinesis (i.e., in somatic plant tissues)

“Conventional” cytokinesis follows after mitosis (Fig. 1 h-k), and phragmoplast originates from the interpolar microtubules of the anaphase spindle between the sister nuclei.

Interpolar microtubules of each half-spindle interact in the midzone and establish anti-parallel arrays, giving rise to the cylindrical microtubule bundle. This bundle differentiates into two sets of microtubules of opposing polarity – the phragmoplast. Microtubules deliver Golgi-derived vesicles towards the plus ends of microtubules, where vesicles fuse and form continuous vesicular-tubular network, localized between opposing microtubule sets. The vesicular-tubular network first differentiates into tubular network and then into fenestrated plate-like structure. The plate expands laterally, forming numerous finger-like projections that fuse with the parental cell membrane. This is followed by the closing of the plate fenestrae and beginning of the cellulose synthesis. The formation of membranous network takes place in association with the phragmoplast microtubules, while cell wall construction within this network occurs after the disassembly of microtubules (Samuels et al., 1995).

6.2.2 Syncytial pathway of phragmoplast formation during "nonconventional" cytokinesis (i.e., during cellularization of nuclear endosperm)

"Nonconventional" cytokinesis takes place when mitosis is not immediately followed by cytokinesis, and the new cell walls are formed at a later stage between sister and non sister nuclei. The cell plate formation is preceded by synchronous appearance of microtubule sets of opposing polarity, followed by the assembly of numerous mini-phragmoplasts between non sister nuclei. Each mini-phragmoplast consists of two opposing sets of microtubules, which originate from the overlapping microtubule clusters. Golgi-derived vesicles are transported along these microtubules, fuse with each other and generate a network of wide membranous tubes. Subsequently, these wide tubes undergo a series of transformations that eventually give rise to the mature cell wall (Otegui and Staehelin, 2000a, 2000b). Thus interpolar microtubules of the spindle are not prerequisite for phragmoplast assembly. The spindle-independent phragmoplast formation also takes place during microsporogenesis, when microtubules of the radial systems, emanating from the nuclei, elongate and interact with plus ends, forming phragmoplast-type of arrays (Olsen et al., 1995).

Moreover, interpolar microtubules do not seem to be involved in the assembly of the phragmoplast even in certain types of dividing cells, because very few or no interpolar microtubules may be present in the interzone of endosperm cells during anaphase (Fig. 2 g). Therefore, these sets of spindle microtubules cannot contribute to the formation of the phragmoplast precursor (cylindrical microtubule bundle). Instead, numerous microtubules grow from the polar areas towards the cell periphery and the cell equator in anaphase-telophase, assembling aster-like microtubule configurations (Bajer and Mole-Bajer, 1986). The phragmoplast is assembled from the microtubules elongating from opposing polar areas and interacting in the mid zone (Fig. 2 h, i). The initial phragmoplast is a barrel-like or double dome structure, and at final stages of cytokinesis it is shaped as a circular, closed and narrow band.

6.3 Molecular components of the phragmoplast

Proteins associated with the phragmoplast are usually grouped according to the functions during cytokinesis. The phragmoplast plays an essential role in the targeted delivery of membrane vesicles to the plane of cell division, predicted by the PPB. Membrane associated functions are carried by classes of proteins involved in vesicles docking, fusion, budding and construction of the membrane. Cytoskeleton associated functions of the phragmoplast are predetermined by the PPB (part 4.3 and 4.4), and both structures share certain classes of

proteins, for instance, cross-linking and stabilizing of microtubules by MOR1/GEM 1 and MAP65 family members. A special class of cytokinetic kineisins (part 2.5.1) is directly involved in the organization and proper functions of the phragmoplast. Structural MAPs and kinesins regulate the dynamic stability of the microtubules in the phragmoplast body, maintain phragmoplast integrity and in this way provide tracks for targeted transport of vesicles and formation of the cell plate (Jürgens, 2005; Müller et al., 2009).

7. Concluding remarks

Extensive studies of plant microtubules for past decades led to the significant progress in understanding of the overall dynamics of plant microtubules, mechanisms underlying the nucleation, organization and function of cortical microtubule arrays, the specificity of plant cytokinesis and on the contrary, the unifying features with animal cytokinesis. The isolation, identification and characterization of vast family of microtubule associated proteins closed many gaps in our knowledge on the functional properties of plants microtubules and the regulation of their behavior. In spite of that, many questions remain unanswered, or may be simply overlooked. The goal of this chapter was to give an overview of basic and mainly structural aspects of plant microtubule cytoskeleton, which is, by all means, not complete due to space limitations, and draw attention to unsolved problems. Among the intriguing questions that remain are: the relationship between cortical microtubules and cellulose microfibrils orientation, the role of the radial microtubules and PPB in morphogenesis of the mitotic spindle, the molecular mechanisms of MTOC-independent plant spindle assembly as compared to other organisms, and even more, if one is enough curios and inquisitive to look inside the plant cell.

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Section 2

Genetic Material: Structure and Expression

5

Centromere Evolution: Digging into Mammalian Primary Constriction

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1. Introduction

Mammalian cell division requires even and complete distribution of chromosome complement in daughter cells. At this cell cycle stage, segregation fidelity is critical in order to prevent aneuploidy. Chromosomes attach by a proteinaceous bridge called kinetochore to the spindle apparatus (Rieder & Salmon, 1994). The chromosomal locus at which kinetochore is organized and chromatids align is represented by the centromere, historically and cytologically defined as *primary constriction*. Throughout the mammalian order, as well as more generally among all higher eukaryotes, a few distinctive features describe the centromeric chromatin, such as the incorporation of the centromeric protein CENP-A, a histone H3 variant (Sullivan, B. A. & Karpen, 2004; Sullivan, K. F. et al., 1994), and the CENP-B, an essential component of the mitotic chromosome scaffold (Masumoto et al., 1989).

Phylogenetic studies show that, in contrast to the high conservation of the chromosome segregation machinery, the primary sequence of centromeric DNA has undergone rapid evolution resulting strikingly different even among closely related mammalian species. These studies suggest that specialized chromatin structure is more critical in centromeric function rather than the presence of specific sequences (Torras-Llort et al., 2009), revealing a role of epigenetic mechanisms on centromeric identity and function (Karpen & Allshire, 1997).

In this chapter we review the variable DNA sequence that forms mammalian centromeres: the satellite DNA. The first pilot studies on mammal satellite DNA date back to 1960s and were accomplished on the mouse, guinea pig, and bovine genomes. Since then, plentiful analyses have been developed for the characterization of satellite DNA in several mammals, particularly in human. These studies enabled the formulation of different theories aiming to explain the rapid evolution of alphoid DNA in primates and clarify a possible role of these sequences in centromeric function. Therefore, this chapter provides an overview of the state of the art in the field of mammalian centromeric DNA organization and evolution.

2. Organization of centromeric satellite DNA in non-primate mammals

Mammalia is a class of air-breathing vertebrate animals, characterized by the possession of hair, three middle ear bones, a four-chambered heart, and mammary glands functional in

* Equally contributing

mothers with young. The class Mammalia is divided into two subclasses: the Theria, comprising the infraclasses Eutheria (the placentals) and Metatheria (the marsupials), and the Prototheria, comprising the order Monotremata of mammalian species that lay eggs (Prasad et al., 2008).

The centromeres of all Therian species examined consist of long arrays of head-to-tail tandemly repeated DNA families, the satellite DNA. One exception to this general rule is the sequence of newly formed centromeres (the centromere of horse chromosome 11 is an example), which is devoid of satellite DNA, thus demonstrating that centromeres can stably function over million years and many generations in the absence of satellite DNA (Wade et al., 2009). However, the acquisition and maintenance of satellite DNA is an obligated fate for all mammalian centromeres, since all mature centromeres possess satellite sequences. (Piras et al., 2010; Wade et al., 2009).

Centromeric satellite DNA sequences have been characterized in almost all mammalian orders. Here, we report exclusively Therian mammal centromeric satellite, because it has never been isolated from any species of the subclass Prototheria. Recently, an attempt to isolate centromeric satellite DNA of the Prototherian mammal platypus (order Monotremata) failed, suggesting that in this species centromeres are not enriched in satellite sequences (Alkan et al., 2010). Studies of other Prototherians such as the echidna might elucidate whether the lack of satellite DNA at centromeres is a platypus feature or it is common in the entire subclass.

The paragraph reports the state of the art about the sequence and organization of centromeric satellite DNA in mammalian species of the Eutherian orders Rodentia, Lagomorpha, Cetartiodactyla, Perissodactyla, Carnivora, Chiroptera, Cingulata, and Proboscidea and of the infraclass Metatheria, including the orders Diprotodontia and Didelphimorphia. The description of centromeric satellite DNA in Primates, to which the human species belongs, has a dedicate section since primate satellite sequences are the best characterized, both structurally and functionally, and plentiful information and studies have been developed in this field.

Despite its conserved function, the centromeric satellite is extraordinarily variable in the repeat unit length, sequence, organization, and relative quantity in respect to the total DNA even among closely related mammalian species. This singularity is known as the "centromere paradox" (Henikoff et al., 2001). In fact, the repeat unit length of the mammalian centromeric satellites ranges from 7 bp in the red-necked wallaby to 2.3 kb in the domestic cattle (**Table 1**). Exception to the high variability is the CENP-B box, a 17 bp sequence motif that is shared and conserved in all centromeric satellite families involved in the centromeric function (Masumoto et al., 1989). However, although this apparent lack of any rule among the satellite sequences, there is an evolutionary conserved pattern of sequence arrangement and organization (Sunkel & Coelho, 1995).

The study of centromeric satellite in different mammalian orders has contributed to the knowledge of centromeric satellite organization and evolution. Each mammalian order has its peculiarity, with some orders best revealing a particular aspect of the centromeric satellite organization. For examples in few primates the centromere is arranged in higher-order repeat (HOR) and in Cetartiodactyla satellite DNA is distributed among chromosomes according to the position of the centromere (acrocentric vs. (sub)metacentric), resulting in a similar centromeric satellite composition and organization among chromosomes that share the same centromere position.

Infraclass	Superorder	Order	Species	Common name	Karyotype	Satellite	Repeat unit size	Chromosomes	Localization	CENP-B box
Eutheria	Euarchontoglires	Rodentia	*Mus musculus*	house mouse	2n=20	minor	120 bp	autosomes, X	centromere	yes
						major	234 bp	autosomes, X	pericentromere	no
						MS3	150 bp	-	centromere	yes
						MS4	300 bp	-	centromere	yes
						Ymin	60-61 or 121 bp	Y	centromere	no
		Lagomorpha	*Oryctolagus cuniculus*	domestic rabbit	2n=44	Rsat I	375 bp	subgroup of autosomes	centromere	no
						Rsat II	585 bp	subgroup of autosomes	centromere	no
						Rsat IIE	585 bp	subgroup of autosomes	centromere	no
	Laurasiatheria	Cetartiodactyla	*Bos taurus*	domestic cattle	2n=60	I or 1.715	1400 bp	autosomes	centromere	-
						II or 1.722	700 bp	few autosomes	-	-
						III or 1.706	2350 bp	most autosomes	centromere	-
						IV or 1.709	3220 bp	few autosomes	pericentromere	-
			Ovis aries	domestic sheep	2n=54	I or 1.714	820 bp	autosomes	centromere	-
						II or 1.723	700 bp	autosomes, X	pericentromere	-
		Perissodactyla	*Equus caballus*	domestic horse	2n=64	major	221 bp	60 out of 64	centromere	-
						-	23 bp	58 out of 64	centromere	-
						-	80 bp	-	centromere	-
						-	80 bp	-	centromere	-
						ECAcons71	221 bp	24 out of 64	centromere	yes
						ECA3cons221	221 bp	60 out of 64	centromere	yes
						ECAcons70	419 bp	62 out of 64	centromere	yes
						ECAAcons450	450 bp	60 out of 64	centromere	yes
						ECA5cons451	451 bp	60 out of 64	centromere	yes
						ECAcons421+424	475 bp	60 out of 64	centromere	yes
		Carnivora	*Canis familiaris*	domestic dog	2n=78	CFA-SAT	737 bp	-	centromere	-
			Urocyon cinereoargenteus	grey fox	2n=66	UCI-SAT	880 bp	-	centromere	-
		Chiroptera	*Pipistrellus pipistrellus*	common pipistrelle	2n=44	-	418 bp	autosomes, X	pericentromere	yes
			Pipistrellus kuhli	Kuhl's pipistrelle	2n=42	*Pk*.SAT	1100 bp	-	-	yes
	Xenarthra	Cingulata	*Dasypus novemcinctus*	armadillo	2n=64	DNOcons173	173 bp	all chromosomes	centromere	yes
	Afrotheria	Proboscidea	*Loxodonta africana*	African elephant	2n=56	LAFcons842+936	1220 bp	all chromosomes	centromere	yes
Metatheria	Australidelphia	Diprotodontia	*Macropus robustus*	wallaroo	2n=16	major	-	all chromosomes	centromere	-
			Macropus rufus	red kangoroo	2n=20	#6 satellites	-	6 different subgroups	centromere	-
			Macropus rufogriseus	red-necked wallaby	2n=16	Mrb-sat1	342 bp	X, Y	centromere and pericentromere	no
						Mrb-sat23	178 bp	all chromosomes	centromere and pericentromere	yes
						Mrb-B29	7 bp	X, Y, 2p	centromere and pericentromere	no
	Ameridelphia	Didelphimorphia	*Monodelphis domestica*	short-tailed opossum	2n=18	MDOcons528	528 bp	8 out of 18	centromere	yes

Table 1. Satellite families at the centromeres of non-primate mammals. - not available data.

We start this excursus of mammalian centromeres with the description of centromeric satellites isolated from mammals other than primates of the superorder Euarchontoglires. This superorder includes the grandorder Euarchonta and the clade Glires. The grandorder Euarchonta ("true ancestors") comprises extant mammals belonging to the orders Primates, Scandentia and Dermoptera; the clade Glires includes the orders Rodentia and Lagomorpha. We illustrate the centromeric satellites of mammals of the orders Rodentia and Lagomorpha, since satellite DNA from mammals of the orders Scandentia and Dermoptera has never been characterized.

2.1 Rodentia

The satellite sequences of several Rodentia mammals have been investigated: *Mus musculus* (the house mouse), four *Acomys* species (the spiny mice) (Kunze et al., 1999), *Microtus chrotorrhinus* (the rock vole) (Modi, 1992), *Gerbillus nigeriae* (the Nigerian gerbil) (Volobouev et al., 1995), and a chromosome 2-specific centromeric DNA repeat from *Cricetulus griseus* (the Chinese hamster) (Fatyol et al., 1994). The *Microtus* and *Cricetulus* species show satellite sequences with the longest monomer size (>2.5 kb) reported until now among mammals. Here, we focus on the centromeric satellites of the house mouse, the most studied Rodentia species.

The centromere of all mouse telocentric chromosomes consists of two highly conserved, tandemly repeated sequences discovered by isopycnic centrifugation in CsCl gradient: the minor and major satellites (Pardue & Gall, 1970; Wong & Rattner, 1988). Minor satellite DNA represents 0.5–1% of the mouse genome and comprises an AT-rich 120 bp monomer that contains the CENP-B box motif (Yoda et al., 1992). The minor satellite array occupies 300–600 kb of the terminal region of all mouse telocentric chromosomes (the autosomes and the X chromosome) and coincides with the centric constriction and function (Kipling et al., 1991). It does not show evidence of higher-order organization. Major satellite DNA is a more abundant, AT-rich, 234 bp tandem repeat (Horz & Altenburger, 1981), organized into long arrays of 240–2000 kb (Vissel & Choo, 1989). It represents 5–10% of the total genomic DNA and shows pericentromeric localization, adjacent to the minor satellite. The major satellite monomer is internally repetitious and consists of eight 28-mer or 30-mer subrepeats, with a set of three related 9 bp ancestral sequence motifs (Horz & Altenburger, 1981). The minor satellite sequence is divided into two similar halves, which are further subdivided into quarter repeats. However, it does not have a primordial sequence similar to the 9 bp motif of the major satellite (**Fig. 1**).

The minor and major satellite sequences are highly conserved across the centromeres of all mouse telocentric chromosomes: the minor satellite monomers share a mean pairwise identity of 95% (Kalitsis et al., 2006); the major satellite monomers share a mean deviation from the consensus sequence of 3.9% (Vissel & Choo, 1989). This high degree of sequence conservation argues strongly for frequent recombinational exchanges between nonhomologous telocentric chromosomes driving sequence homogenization at mouse centromeres (Kalitsis et al., 2006; Vissel & Choo, 1989).

Besides these two AT-rich satellites, the major and minor satellites, two further GC-rich satellites have been identified at mouse centromeres: the Mouse Satellite 3 (MS3) and the

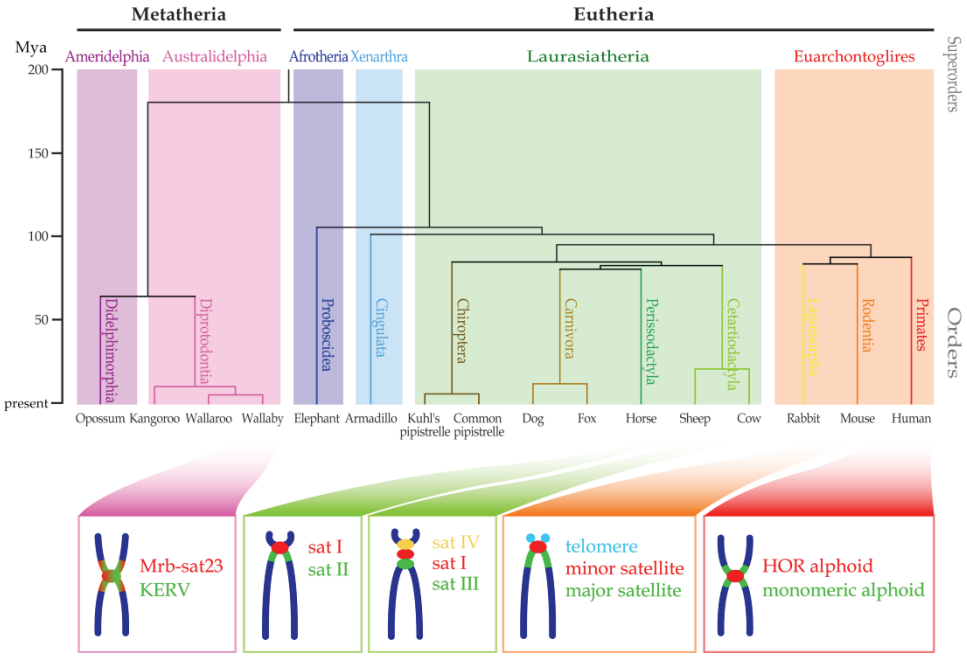

Fig. 1. Organization of centromeric satellites at mammalian centromeres. *top* Phylogenetic relationships among mammalian species discussed in the chapter with satellite DNA at their centromeres. *bottom* Spatial organization of satellite families at the centromere and pericentromere of *-from right to left-* human chromosomes, mouse telocentric chromosomes, cow autosomes, sheep autosomes, and wallaby autosomes. Chromosome arms are blue coloured. In the wallaby there is the co-existence of a satellite (Mrb-sat23) and a non-satellite element (KERV).

Mouse Satellite 4 (MS4). The MS3 monomer is 150 bp long and accounts for 2.2% of the total DNA; the MS4 monomer is 300 bp long and accounts for 1.6% of the total DNA. Both monomers contain the CENP-B box. Divergence between MS3 sequences is 0.7% due to single-nucleotide changes (Kuznetsova et al., 2005).

Mouse chromosome Y centromeric sequence has been recently investigated (Pertile et al., 2009), highlighting several features never described before at mouse centromeres. Mouse chromosome Y centromere has a chromosome-specific sequence and a unique multimeric HOR organization. It comprises a 90 kb array of an AT-rich and minor satellite-like tandem repeat (Ymin), with distant homology (76.8%) to mouse minor satellite. The Ymin satellite is closely associated with the kinetochore. It has a HOR sequence organization devoid of transposable elements, with a unit size of 2.3 (*Mus musculus molossinus*) or 1.6 (*Mus musculus domesticus*) kb, and a sequence identity among the repeated units of 99–100%. The HOR unit has a remarkably complex structure, consisting of an amalgam of highly diverged (<70% mean pairwise identity) monomers with a periodicity of 60–61 or 121 bp. The majority of the monomers form progressively larger and less diverged repeating HOR subunits, the largest comprising 2.4 copies of an 840 bp periodicity repeat (with 95% pairwise identity) that spans

the greater part of the HOR unit domain. The singularity of chromosome Y centromere argues for an intrachromosomal mode of sequence homogenization and an isolated evolution (Smith, 1976).

2.2 Lagomorpha

Ekes and collaborators described for the first time the satellite DNA families of a mammal belonging to the order Lagomorpha, the domestic rabbit, *Oryctolagus cuniculus* (Ekes et al., 2004). They found two major centromeric satellite DNA sequences, named Rsat I and Rsat II, which are not related to each other, and a divergent Rsat II-related subfamily, Rsat IIE. The Rsat I monomer has an average length of 375 bp, whereas repeat units Rsat II and Rsat IIE are ~585 bp long. These satellites do not provide a complete coverage of the rabbit complement, since seven autosome pairs and the sex chromosomes do not contain any of these satellites. Rsat I, Rsat II and Rsat IIE satellites are each distributed in variable amounts at the centromeres of a subgroup of rabbit chromosomes, with some chromosomes containing both Rsat I and Rsat II, or Rsat II and Rsat IIE. Part of Rsat I and Rsat II satellites shows a dimeric organization. However, further studies are required to isolate the rabbit sequences that constitute the centromeres devoid of the three known satellites, and to elucidate the higher-order repeat organization of rabbit satellites.

2.3 Cetartiodactyla

The Laurasiatheria is the evolutionarily closest superorder to the Euarchontoglires, and comprises the orders Cetartiodactyla, Perissodactyla, Carnivora, Chiroptera, Eulipotyphla, and Pholidota. The isolation of satellite DNA from a mammal of the last two orders has never been reported. Among mammals of the order Cetartiodactyla (cetaceans and even-toed ungulates), we describe the centromeric satellites isolated and characterized from two species of the family Bovidae: the domestic cattle (*Bos taurus*) of the subfamily Bovinae, and the domestic sheep (*Ovis aries*) of the subfamily Caprinae.

The cattle satellite I DNA (the 1.715 family) is a 1.4 kb tandem repeat that comprises 6-9% of the total genomic DNA (Kurnit et al., 1973). It constitutes the centromeric heterochromatin of all autosomes, but not of the sex chromosomes (Plucienniczak et al., 1982; Taparowsky & Gerbi, 1982) (**Fig. 1**). However, the primitive form of bovine X chromosome is acrocentric and has satellite I sequences at centromere (Chaves et al., 2005). Three additional bovine satellites, satellite II, III and IV, were localized at centromeric and pericentromeric regions of autosomes, respectively: satellite II is mostly localized at the autosomal centromeres; satellite III is present on most autosomes; satellite IV is present on less than half the autosomes (Kopecka et al., 1978; Kurnit et al., 1973). None of them is present on the sex chromosomes. The repeat unit of satellite III is 2,350 bp long and consists of two related and homogeneous 23-mer tandem subrepeats, the Pvu and the Sau motives, respectively (Pech et al., 1979). The three bovine satellites I, III and IV, or a subgroup of them, are organized on autosomes following always the same order: p-ter–sat IV–sat I–sat III–q (Chaves et al., 2003) (**Fig. 1**).

Two repetitive DNA families, satellite I (Buckland, 1983; Reisner & Bucholtz, 1983) and satellite II (Buckland, 1985) are the major components of the sheep centromeric and pericentromeric heterochromatin, respectively (Burkin et al., 1996; D'Aiuto et al., 1997) (**Fig.**

1). The sheep satellite I DNA (the 1.714 family) has a repeat unit of 820 bp and, as the bovine satellites, constitutes the centromeric heterochromatin of all autosomes, but not of the sex chromosomes (Buckland, 1983; Burkin et al., 1996; Chaves et al., 2000; Chaves et al., 2005). However, the amount and organization of satellite I DNA differ among the autosomes, with a lower amount at the centromeres of the biarmed chromosomes 1, 2, and 3, particularly of chromosome 1 (Burkin et al., 1996; D'Aiuto et al., 1997). The sheep satellite II DNA has a 700 bp monomer and constitutes the pericentromeric heterochromatin of all chromosomes with the exception of the Y chromosome (Burkin et al., 1996; D'Aiuto et al., 1997). Differently from sheep satellite I DNA, the satellite II family has a more variable chromosomal distribution: few acrocentric chromosomes are devoid and it is present at the centromeres of sheep metacentric and X chromosomes in large amounts (Burkin et al., 1996).

The bovine and ovine satellite I families show 70% sequence similarity and both consist of a degenerated 31 bp GC-rich tandem subrepeat (Novak, 1984; Reisner & Bucholtz, 1983). The presence of this 31 bp motif across the entire length of the satellite I repeat suggests that its present structure could have arisen from a tandemly amplification of an ancestral ~31 bp unit. The 31-mer motif sequence has been found also in other bovine satellites, like satellite III (Pluciennicza k et al., 1982; Taparowsky & Gerbi, 1982), and in the deer, muntjac, and pronghorn centromeric satellites (Bogenberger et al., 1985; Denome et al., 1994; Lee, C. & Lin, 1996), arguing that the amplification of the ~31 bp unit may have occurred in their common ancestral.

2.4 Perissodactyla

The order Perissodactyla (odd-toed ungulates) comprises the family Equidae, with eight living species all belonging to the genus *Equus*: two horses (*E. caballus* and *E. przewalskii*), two Asiatic donkeys (*E. kiang* and *E. hemionus*), one African donkey (*E. asinus*), and three zebras (*E. grevyi*, *E. Burchelli,* and *E. zebra*). Despite the *Equus* species can be crossbred and diverged recently, sharing a common ancestor about 2–3 million years ago, their karyotypes differ extensively and their satellite DNA has evolved rapidly (Wijers et al., 1993). Moreover, during the evolution of the genus *Equus*, centromere repositioning, the shift along the chromosome of the centromere without structural chromosome rearrangements, has occurred frequently (Carbone et al., 2006). It implies that several evolutionary new centromeres and ancestral now inactive centromeres are present in the *Equus* karyotypes.

Several satellite families have been identified in the horse genome with centromeric localization, suggesting a great diversity and variability in structure and organization of horse centromeric sequences. The horse major satellite accounts for 5–10% of the total genome and has a repeat unit of 221 bp. It is localized at the centromeric regions of 30 pairs of chromosomes and is missing at the centromere of chromosomes 2 and 11, both submetacentric. Repeat units share a sequence identity of 90–100% and have no internal repeat structure (Piras et al., 2010; Wijers et al., 1993).

In 1995, another horse satellite family with a repeat unit of 23 bp was isolated and localized at the centromeres of acrocentric but not metacentric horse chromosomes (Broad et al., 1995b). This pattern reminds the satellite distribution in Cetartiodactyla that occurs according to the position of the centromere along the chromosome.

Two further horse satellite families with a repeat unit of ~80 bp were identified, with a likely centromeric localization (Broad et al., 1995a). Alkan and colleagues extracted six distinct

satellite consensus sequences in the *E. caballus* genome of 221, 221, 419, 450, 451, and 475 bp, respectively. Their FISH hybridization patterns included the centromeres of all or a part of horse chromosomes except chromosome 11 (Alkan et al., 2010). All horse centromeres have either one or more than one satellite whereas horse chromosome 11 is the only one lacking any satellite (Alkan et al., 2010; Piras et al., 2010; Wade et al., 2009).

2.5 Carnivora

Few studies have been accomplished on centromeric satellites in species belonging to the order Carnivora. In 1988 and 1989, the major centromeric satellite of the domestic dog (*Canis familiaris*) and of the grey fox (*Urocyon cineroargenteus*), two species of the order Carnivora that diverged from a common ancestor 10–12 million years ago (Wayne et al., 1997), have been investigated in regard to their sequence and localization. Chromosomes of the domestic dog and grey fox are primarily acrocentric. The dog satellite monomer is 737 bp long and has a GC-content of 51%; the grey fox satellite monomer is 880 bp long and has a GC-content of 54% (Fanning, 1989). Recently dog fosmid clones containing satellite DNA were mapped to the centromere of a different subgroup of dog chromosomes. These heterogeneous patterns support the existence of a complex patchwork organization of satellites at dog centromeres, similar to horse centromeric sequence organization (Alkan et al., 2010).

2.6 Chiroptera

Bat genomes (order Chiroptera) are characterized by low DNA content, with a size approximately 50–87% the size of other eutherian genomes (Burton et al., 1989). Centromeric satellite DNA has been isolated from two bat species of the genus *Pipistrellus*, family Vespertilionidae, suborder Microchiroptera: the common pipistrelle (*Pipistrellus pipistrellus*) and the Kuhl's pipistrelle (*Pipistrellus kuhli*). Satellite DNA of the common bat represents approximately 3% of the whole genome and is organized in tandem repeats with a monomer size of 418 bp. The monomer units are highly similar, with a sequence identity of 95–100% and few base-substitutions randomly spread along the sequence. The common bat satellite has an AT-content of 62% and contains a putative CENP-B box motif. It is localized at the pericentromeric constitutive heterochromatin of all the autosomes and X chromosome, but it is absent from the Y chromosome (Barragan et al., 2003). *Pipistrellus kuhli* satellite represents approximately 5% of the total genomic DNA. The monomer unit is 1100 bp long and contains the CENP-B box as well as subrepeats, palindromes, and AT-rich tracts. The monomers group into two clusters (Fantaccione et al., 2005). Both pipistrelle satellites are absent in the genomes of other bat species analyzed, thus revealing that they might be species-specific.

2.7 Cingulata and Proboscidea

The study of centromeric satellite families in mammalian species belonging to the superorders Xenarthra and Afrotheria of the clade Eutheria has started very recently. In 2010, Alkan and colleagues included in their list of sequenced mammalian genomes to analyze in regard to centromeric satellite sequences, the armadillo (*Dasypus novemcinctus*), a species of the superorder Xenarthra, order Cingulata, and the African elephant (*Loxodonta*

africana), a species of the superorder Afrotheria, order Proboscidea. Using the RepeatNet algorithm, they extracted a 173 bp satellite consensus from the armadillo genome and a 1220 bp satellite consensus from the African elephant genome, and localized both satellites at the centromere of all chromosomes of the corresponding species (Alkan et al., 2010).

2.8 Diprotodontia and Didelphimorphia

The infraclass Metatheria comprises the marsupial mammals. Among the extant marsupials, species of the order Diprotodontia, family Macropodidae, and a species of the order Didelphimorphia were investigated in regard to their centromeric DNA organization.

The family Macropodidae (kangaroos, wallabies, and wallaroos), one of the largest in the infraclass Metatheria, exhibits an extraordinary karyotypic diversity and plasticity (Eldridge & Close, 1993; Hayman, 1990; Rofe, 1978). The Macropodidae centromere is a highly dynamic locus and has played a pivotal role in the karyotype evolution of this family, since all rearrangements, including centromere repositioning, fissions, fusions (Robertsonian translocations), pericentric inversions, and translocations, involved the centromeric loci as breakpoints (O'Neill et al., 2004; Rens et al., 2003).

Marsupial satellites are characterized by an uneven distribution among the centromeres of the different chromosomes. The centromeric satellite families isolated from each marsupial are also present in other marsupials analyzed, often in a different amount and with a different localization and distribution.

In 1981, Venolia and Peacock isolated a major satellite from the wallaroo (*Macropus robustus*) genome. It accounts for about 10% of the total DNA and localizes at the centromere of all chromosomes in different amounts and at the nucleolus organizer region (Venolia & Peacock, 1981). This satellite was localized in other *Macropus* species. In *M. rufus* and *M. rufogriseus* it is present mainly on the X chromosome at large, non-centromeric blocks, and in the region of the nucleolus organizer. In *M. rufogriseus* the satellite also occurs on the Y chromosome and in *M. rufus* at the centromere of four acrocentric autosomes.

Six different satellite DNA fractions have been isolated from the genome of the red kangoroo (*Macropus rufus*), each accounting for 1–3% of the total DNA. These satellites localize at the centromeres with each heterochromatic centromeric block differing in the amount and distribution of these satellites, as well as at interstitial regions and X chromosome telomeric heterochromatin (Elizur et al., 1982).

The red-necked wallaby (*Macropus rufogriseus*) karyotype has a distinctive feature: its chromosomes harbour an exceptional amount of centric and pericentric heterochromatin (Hayman & Martin, 1974), comprising almost 30% of the genome (Bulazel et al., 2006). They have unusually lengthened pericentromeric regions that are up to half the length of the chromosome, with the functional centromere restricted to a discrete point location within the larger region. In 2006, Bulazel and collaborators isolated three satellite families, named Mrb-sat1, Mrb-sat23, and Mrb-B29, from the red-necked wallaby genome. These satellites constitute the large centromeric and pericentromeric regions of the wallaby chromosomes and show a different chromosomal distribution. Mrb-sat23 constitutes the centromeric core as well as the large pericentric heterochromatic region of all chromosomes and is present in tandem arrays at all centromeres of most *Macropus* species (**Fig. 1**). In *M. rufogriseus*, Mrb-

sat23 experienced large-scale amplifications as it resides over the entire Y chromosome and is spread throughout the extensive X chromosome pericentromere. The presence of a CENP-B binding-competent domain on the Y of a marsupial suggests that ancestral mammalian sex chromosomes utilized CENP-B to differentiate centromere location and that the loss of CENP-B protein binding and CENP-B box DNA on the Y are derived when found within eutherian mammals. In this species the centromeric satellite constitution differs between the autosomes and the sex chromosomes: all autosomes have sequences of only Mrb-sat23, whereas the X and Y chromosomes harbour sequences of three satellites, Mrb-sat1, Mrb-sat23, and Mrb-B29, in different amounts (Bulazel et al., 2006).

Besides the satellite sequences, an active retroviral element, the Kangoroo Endogenous Retrovirus (KERV), is localized at the centromere and pericentromere in the genus *Macropus* (**Fig. 1**). It is considered thereof a major constituent of *Macropus* active and latent centromeres since it has undergone amplification at this locus (Ferreri et al., 2011; Ferreri et al., 2005; Ferreri et al., 2004; O'Neill et al., 1998). Particularly, in *M. rufogriseus* KERV is localized at the centromere of all autosomes, but it is absent or present in low copy number at the centromere of the sex chromosomes (Ferreri et al., 2011; Ferreri et al., 2004). Recently, Alkan and colleagues reported the centromeric satellite of the short-tailed opossum (*Monodelphis domestica*), a marsupial species of the order Didelphimorphia. They identified a 528 bp satellite, that is an LTR/ERV1 element, and localized it at the centromere of four homologous opossum chromosomes (Alkan et al., 2010). Such finding of a retroviral element at the centromeres of the short-tailed opossum, a marsupial belonging to a superorder different from the one of the Macropodidae marsupials, suggests that the use of a retroviral element as centromeric satellite might be ancestral in the infraclass Metatheria.

3. The centromeric alpha satellite in primates

The order Primates belongs to the subclass Theria, infraclass Eutheria (**Fig 1**). It includes an ancient group of which the size (the number of species) is still ambiguous. In fact, depending on whether some closely related groups are considered to be varieties of the same species or not, most taxonomic classifications refer to a range of 230–270 species.

The order Primates includes two suborders: Strepsirhini (including six families) and Haplorhini. Strepsirhini has a rhinarium, the tapetum lucidum, a bicornuate uterus, a toothcomb (with the exception of the Aye-aye), and a toilet-claw for grooming. On the other hand, the so-called “higher primates” compose the suborder Haplorhini, in turn divided in two main hyporders, the Anthropoidea, including the Platyrrhini (New World monkeys) and the Catarrhini (Old World monkeys and apes), and the Tarsiiformes (Goodman et al., 1989).

Primate predominant class of centromeric DNA is made up of long stretches of repeats consisting of 171 bp and AT-rich monomers, tandemly reiterated in a head-to-tail configuration (Vissel & Choo, 1987; Waye & Willard, 1987) commonly referred to as "alphoid satellite" DNA (Manuelidis, 1978). These sequences have been identified throughout the order Primates, including great apes, Old World and New World monkeys (Alves et al., 1994; Musich et al., 1980; Willard & Waye, 1987), with the exception of the suborder Strepsirhini (Lee, H. R. et al., 2011). Alpha satellite DNA is the most abundant repetitive DNA in all primate species studied, making up to 3–5% of each chromosome; it is pancentromeric in primate chromosomes and appears to be distinctive of the primate lineage (Vissel & Choo, 1987; Waye & Willard, 1987).

3.1 Great apes

At human centromeres alphoid DNA extends for ~250 kb up to ~5 Mb (Wevrick & Willard, 1989; Willard, 1990) and is known to exist in two forms designated (1) monomeric arrays and (2) higher-order repeats (HORs). In the monomeric arrays the 171 bp monomers lack further sequence structure and the head-to-tail configuration provides directionality to each satellite block. A higher-order repeat is rather composed of arrays in which a defined number of monomers has been homogenized as a unit which, in turn, is tandemly repeated many times to span several megabases, resulting in multiple copies of an alphoid multimer (Willard & Waye, 1987). At human centromeres monomeric alphoid satellites flank HORs (**Fig. 1**). Sequence analysis revealed that monomers within a higher-order repeat unit share as low as 72% average pairwise sequence identity however, adjacent individual higher-order repeated units are 98–100% identical (Rudd & Willard, 2004). Higher-order alpha satellite within an array is extremely homogeneous and appears to be uninterrupted by other (non-satellite) DNA sequences (Tyler-Smith & Brown, 1987; Warburton & Willard, 1990). In contrast, monomeric alpha satellite is more heterogeneous in sequence and is extensively interspersed with non-alpha-satellite sequences such as transposable elements (Guy et al., 2003; Kazakov et al., 2003; Schueler et al., 2001).

The human higher-order repeating structures can shape alphoid subfamilies. These subfamilies are classified according to whether they are specific for a single chromosome or shared by a small group of chromosomes (Choo et al., 1991). As a consequence, (i) some centromeres contain only the chromosome-specific subfamily, while others possess several distinctive alphoid subfamilies (Choo et al., 1991) organized in discrete and homogenized physical blocks (Schueler et al., 2001); (ii) multiple domains located on one chromosome may belong to the same or to different suprachromosomal families (Choo et al., 1991). Human alpha satellite sequences have been historically grouped into five suprachromosomal families (SFs) initially according to the higher-order repeat unit length, revealed by restriction site periodicity, and then founded on sequence-based phylogenetic analyses (Alexandrov, I. et al., 2001; Alexandrov, I. A. et al., 1993; Iurov Iu et al., 1988) (**Table 2**). Human SFs 1–3 likely derived from an ancestral sequence by interchromosomal exchange (Waye & Willard, 1986). The reconstruction of the ancestral monomer sequence revealed that it originated the actual two phylogenetic homology groups of monomers: J1-D2-W4-W5 belong to group A, J2-D1-W1-W2-W3 compose group B (Alexandrov, I. A. et al., 1993). Moreover, there are several subsets that appear to form two more homogenous families, which are characterized by an array of equally related monomers (SF 4) and an irregular alternation of two different types of monomers (SF 5) (Alexandrov, I. et al., 2001; Alexandrov, I. A. et al., 1993). SF 4 consensus monomer, M1, is closely related to D2 and W4 monomeric types of SF 2 and SF 3; also, consensus sequences R1 and R2 (SF 5) clearly belong to subset A and B, respectively. SF 4 is related both structurally and in sequence to the African green monkey alpha component, of which the consensus sequence is closely associated to the phylogenetic homology group A; besides, consensus sequences derived from groups A and B resulted to be nearly identical to R1 and R2. This shows that the amplification of the ancestral sequences that gave rise to the two homology groupings of alphoid monomers happened at the very beginning of primate evolution and that SF 5 consensus monomers may represent the ancestral form of primate A-B satellite. Among the primate lineages, the W1-W5 pentamers and D1-D2 dimers are present in gorilla, orangutan, chimpanzee, and human, while J1-J2 sequences are present in gorilla, chimpanzee, and human (Alexandrov, I. A. et al., 1993).

SF	Repetitive unit	Phylogenetic homology groups	Consensus monomers	Chromosomes
1	dimer	A-B	J1, J2	1,3,5,6,7,10,12,16,19
2	dimer	B-A	D1, D2	2,4,8,9,13,14,15,18,20,21,22
3	pentamer	B-B-B-A-A	W1,W2,W3,W4, W5	1,11,17,X
4	monomer	A	M1	5,7,13,14,15,19,21,22,Y
5	monomer	A-B	R1,R2	1,2,3,5,6,7,9,11,12,13,14,16,18,19,20,22

Table 2. Structure, periodicity, and localization of human suprachromosomal families. SF, suprachromosomal family; data from Alexandrov et al., 2001.

Among individuals, array length of a single satellite can be highly polymorphic. There is an extensive variation of centromere size between nonhomologous as well as homologous human chromosomes. For example, alpha-satellite array length of human chromosomes X and Y varies almost three times: in the case of chromosome X from 1380 to 3730 kb (Mahtani & Willard, 1990); in the case of chromosome Y from 285 to 1020 kb (Tyler-Smith & Brown, 1987).

Cross-hybridization studies of alphoid sequences among great apes show that the higher-order sequence organization of the chromosome X alpha-satellite subset has been conserved among closely related species (orthologous evolution) (Durfy & Willard, 1990). However, similar relationships were not found for the other subfamilies. In fact, experimental evidence suggests that the majority of human-derived chromosome-specific alphoid satellite DNA probes does not recognize orthologous chromosomes in great apes (Samonte et al., 1997).

3.2 Catarrhini

The first species in which primate centromeric sequence was identified is the African green monkey (AGM) (*Chlorocebus sabaeus*). The AGM is an Old World monkey, belonging to the family Cercopithecidae, genus *Chlorocebus*. In 1971, Maio and collegues identified the so-called component alpha DNA as the homogenous fraction composing the 20% of the total AGM genomic DNA, showing a behaviour similar to the mouse satellite DNA in renaturation kinetics experiments (Maio, 1971). Furthermore, two other Cercopithecidae species have been investigated in their centromeric main component: the Rhesus monkey (*Macaca mulatta*) and the baboon (genus *Papio*). Both their karyotypes show 20 pairs of autosomes plus the two sex chromosomes.

In the three species examined, the centromeric satellite DNA is the most abundant repetitive sequence, since it comprises up to 24% of the AGM and from 8 to 10% of the Rhesus monkey and baboon genomes (Musich et al., 1980). Besides, in the AGM genome, the tandemly repeated centromeric satellite monomeric unit is 172 bp long, while its length is doubled to 340 bp in the baboon and the nucleotide divergence among the satellite sequences of the two species is ~10% (Singer & Donehower, 1979) (**Fig. 2**). At Rhesus monkey centromeres, the underlying repetitive alphoid DNA is a 343 bp dimer (**Fig. 2**). The similarity between the two 171 and 172 bp component monomers is as much as 70%. Nevertheless, few high-identity regions have been detected, conserved not only between the two Rhesus monomers but also among all known Cercopithecidae monomers. Homologies between the macaque consensus alphoid sequence and the baboon, AGM and human alphoid sequences are >98%, 81%, and <70%, respectively (Musich et al., 1980).

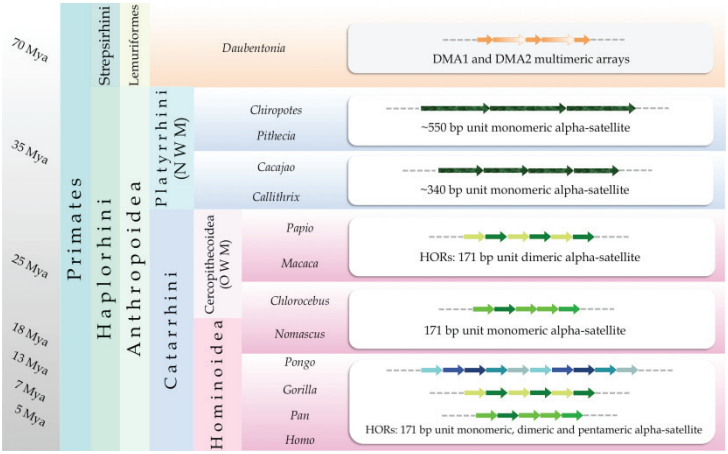

Fig. 2. Centromeric satellite organization in primates. *left* Simplified phylogenetic relationships among primate species. Estimated divergence times are reported in million of years. *right* Schematic organization of the alphoid arrays at primate centromeres. Arrows represent monomeric units. All Anthropoidea families have alphoid DNA at centromeres. For the genus Daubentonia (orange arrows) a model of the centromeric DMA1 and DMA2 satellites is proposed.

Among Catarrhini, a species belonging to the superfamily Hominoidea, family Hylobatidae, has been, lately, deeply characterized in its centromeric satellite: the white-cheecked gibbon (*Nomascus leucogenys*). In *N. leucogenys* alphoid monomers are 171 bp long and show four different hybridization patterns: telomeric, centromeric, telomeric-centromeric and Y-chromosome specific (**Fig. 2**). *N. leucogenys* is then the first primate species analysed in which alphoid DNA is detected at "ectopic" regions (telomeric and interstitial sites); though, the authors speculate that the different mapping pattern may be likely due to different sequence organizations rather than to site-specific sequence divergence. In fact, the gibbon karyotype is known to be extensively rearranged against the ancestral primate karyotype, leading to the hypothesis that alphoid sequences at telomeric level could represent the rest of the evolutionary fissions within centromeric breakpoints (Cellamare et al., 2009).

3.3 Platyrrhini

The group Platyrrhini includes five families: Callitrichidae, Cebidae, Aotidae, Pitheciidae, and Atelidae. Few centromeric sequences of several Platyrrhini species have been studied: three components of the family Pitheciidae (Alves et al., 1994) plus the common marmoset (Cellamare et al., 2009).

Centromeric sequence analysis on the black bearded saki (*Chiropotes satanas*) of the genus *Chiropotes* and the Rio Tapajós saki (*Pithecia irrorata*) of the genus *Pithecia* family Pitheciidae, have abundant amounts of satellite with monomeric unit of 539 and 559 bp, respectively. The difference in size between the two sequences is mainly due to 14 contiguous bases of which almost half consists in GA dinucleotides suggesting a strand slippage mechanism as a cause of the expansion.

The 539 bp alphoid satellite in *Chiropotes satanas* consists of four 170 bp subunits of which the third is incomplete. *C. satanas* alphoid DNA strongly hybridizes to *Pithecia*, while hybridization to the black-headed uakari (*Cacajao melanocephalus*) is much less intense, thus suggesting a satellite content loss in this species rather than a higher sequence divergence, since the divergence time between *Chiropotes* and *Cacajao* is thought to be about 5 Myr (millions years), while the divergence time between *Chiropotes/Cacajao* and *Pithecia* is 8 Myr (Schneider et al., 1993). Moreover, in *Cacajao melanocephalus* a substantial proportion of the satellite mass is composed by a 340 bp alphoid monomer, while the ~550 pb monomer constitutes a small subset. Nevertheless, it is likely that the ~550 bp monomer arose from an array of 340 bp repeats, leading to the conclusion that the ancestor of the *Pitheciini* harbored both structures (Alves et al., 1998) (**Fig. 2**).

Insights of the centromeric satellite among New World monkeys come from the genus *Callithrix*, more precisely from the common marmoset (*Callithrix jacchus*). Cellamare and collaborators characterized *C. jacchus* centromeric sequences by several cytogenetic and molecular approaches. Their analysis showed that, like for the above-mentioned New World monkeys, the alpha satellite monomer in this species is 340 bp long (**Fig. 2**). Thus, it is likely that two of these ancestral monomers fused and no further homogenization occurred between the two halves. The similarity between the first and second monomer is reported to be 40–50% and no results were obtained from sequence comparisons with great apes (human, chimpanzee, and gorilla), nor Old World monkeys (macaque) alphoid sequences (Cellamare et al., 2009).

3.4 Strepsirhini

Recently, the centromeric sequence of the aye-aye has been characterized (*Daubentonia madagascariensis*). Aye-aye centromeres are composed of two different, AT-rich, CENP-A-associated classes of repetitive DNA, termed DMA1 and DMA2, ~146 and ~268 bp long, respectively. DMA1 and DMA2 are often adjacent to one other at aye-aye centromeres and are completely unrelated to alphoid DNA in sequence composition though including a highly divergent CENP-B box. Moreover, their sequence analysis revealed significant homology values in the first 100 bp of both monomers, thus indicating that the two satellite classes share an evolutionary history (Lee, H. R. et al., 2011) (**Fig. 2**).

3.5 The evolution of alphoid DNA

Alphoid DNA in primates evolves rapidly (Mahtani & Willard, 1990; Wevrick & Willard, 1989); alpha-satellite DNA monomers evolve through a non-independent mechanism named *molecular drive* (Charlesworth et al., 1994; Smith, 1976; Stephan, 1986), a stochastic process in which mutations can accumulate, spread quickly through a repeat family, and fix in a population (Dover, 1982). In this evolutionary process mutations are homogenized throughout members of the satellite DNA family, and fixed within a species (Dover, 1982). Although Schindelhauer and Schwarz favour gene conversion as an explanation for both intrachromosomal and inter-homologue homogenization (Schindelhauer & Schwarz, 2002), only unequal crossover can explain the generation and maintenance of a multimeric higher-order repeat length, the extensive spread of sequence variants across megabases, and the rapid fall in sequence identity documented at the edge of the centromeric array.

Wu and Manuelidis proposed a two-step evolutionary process for the formation of tandem duplication arrays: after homology between two sequences is created, an unequal crossover might occur and thus result in dimer formation from divergent monomers; subsequently an amplification of the dimer into long tandem arrays might occur by subsequent unequal crossovers (Wu & Manuelidis, 1980). Eventually, a subset of monomers might be homogenized together to form HOR unit in which former monomers constitute subunits (Warburton & Willard, 1990; Willard & Waye, 1987). As the number of tandem repeats increases so will frequency of unequal crossovers between them. By this mechanism, variant nucleotides can be spread along tandem repeats at a rate much faster than, and independent of, the mutation rate (Choo, 1990). The two major types of phylogenetically distinct alpha satellite DNA existing in great apes are a consequence of the homogenization process: multimeric, higher-order repeats of ~171 bp units form centrally located, chromosome-specific alphoid domains (class A and B alphoid monomers), flanked by domains of more heterogeneous monomeric alpha-satellite from which they have evolved (class A alphoid monomers) lacking any further organization.

The final outcome of *molecular drive* is concerted evolution, exhibiting higher identity between HORs within a species than with the orthologous array in other species (Dover, 1982; Rudd et al., 2006; Willard & Waye, 1987), thus explaining the great diversity (24% divergence) seen between the human and chimpanzee alphoid regions on chromosomes 21 and 22. Given the neutral mutation rate of 0.13% per Myr and the estimated divergence of human and chimpanzee lineages 6-8 Mya (million years ago), those levels of divergence would have been absolutely unexpected. The extreme variability of HOR length among individuals may then be explained by the fact that unequal crossovers between higher-order repeat units will occur more frequently than between monomeric units because of the exceptionally high homology among HORs (Willard & Waye, 1987). Besides, these mechanisms appear to proceed in a localized, short-range fashion that leads to the formation of large domains of sequence identity, rather than among intra- or interchromosomal repeats (Dover, 2002), thus resulting in the chromosome-specific alphoid subdomains. As a consequence, adjacent monomers display a higher degree of sequence similarity (Durfy & Willard, 1989; Roizes, 2006; Schindelhauer & Schwarz, 2002; Willard & Waye, 1987) and monomers at array ends show lower identity due to the low efficiency of homogenization mechanisms at the edges of the satellite array (Schueler et al., 2005).

Unequal crossover events of alpha satellite arrays may represent both interchromosomal and intrachromosomal structural modifications. In the first case they will give rise to suprachromosomal families of higher-order alpha satellite (Alexandrov, I. A. et al., 1993; Waye & Willard, 1986), while in the latter they will result in chromosome-specific arrays of higher-order alpha satellite (Durfy & Willard, 1989; Schindelhauer & Schwarz, 2002; Schueler et al., 2001; Willard & Waye, 1987). In summary, the adjacent organization of higher-order and monomeric alpha satellite, as well as the fact that lower primates have only monomeric alpha satellite at their centromeres (Alves et al., 1994; Musich et al., 1980; Rosenberg et al., 1978), supports the hypothesis that higher-order alpha-satellite evolved from ancestral arrays of monomeric alpha-satellite and subsequently transposed to the centromeric regions of all great ape chromosomes (Alexandrov, I. et al., 2001; Kazakov et al., 2003; Schueler et al., 2001; Warburton et al., 1996). This is further confirmed by the age gradient revealed by L1 elements in alphoid regions. The theory is that after the insertion of

active LINEs that disrupts the centromeric periodicity, thus compromising the centromere function, an expansion of alphoid DNA occurs in order to compensate this unrest (Schueler et al., 2001; Shepelev et al., 2009). As a consequence, the analysis of LINEs can be used to deduce and calculate the age of different satellite blocks. These studies reveal that the most distal alpha-satellite domain is the oldest, with an age gradient advancing proximally through the satellite region.

Finally, it is clear that monomeric alpha satellite present within the pericentromeric regions of human chromosomes predates higher-order arrays of alpha satellite and thus may represent direct descendants of the ancestral primate centromere sequence. Thus, monomeric alphoid arrays are likely the remnants of the centromeres of our primate ancestors, once active and homogenous, that have been replaced by HOR sequences that are a much more efficient substrate for homogenization.

In the evolution of the order Primates, the 171 bp repeat unit seems to be the starting point. Two 171 bp monomers were firstly amplified together as a dimer, then in the Platyrrini lineage, the two monomers began to accumulate differences due to the decrease of homogenization mechanisms, thus forming the specific New World monkeys ~342 bp monomeric unit (variation of this unit generated the 550 bp in *Chiropotes* and *Cacajao*). In the Catharrini ancestor, instead, the ~171 bp dimer continued to be amplified by unequal crossover, thus forming the dimeric structure common to all the centromeres as reported in macaque and baboon. Moreover, in the superfamily Hominoidea the 171 bp monomer amplified and diverged in monomeric arrays in gibbon and in higher-order repeats in orangutan, gorilla, chimpanzee, and human.

4. Methods for centromeric sequence analysis: History and news

The study and analysis of repetitive elements such as centromeric satellite are particularly complicated due the low-variation and high-copy-number nature of these sequences. The development of specific techniques and the application of conventional assays have both contributed in satellite DNA analysis over the last decades.

Elective methods for the analysis of repetitive DNA sequences were the measurement of DNA renaturation kinetics (Britten et al., 1974), the isopycnic centrifugation in gradients of CsCl and $CsSO_4$ (Szybalski, 1968), and hybridization techniques. The application of these techniques to the analysis of mammalian genomes revealed the existence of several DNA fractions, which differed in their physical and chemical properties. In particular, they allowed the identification and separation of repeated DNA sequences taking advantage of the different copy number and density, respectively, from the bulk of the whole DNA. In fact, the term "satellite" derives from the satellite bands in the genomic DNA profile in CsCl density-gradient centrifugation assay. Southern blot analyses have been successfully applied to discover and examine the tandem-repetitive nature of satellite DNA, with cleavage sites for a number of different restriction endonucleases spaced at highly regular intervals. Further, the development of sequencing strategies has allowed its characterization at the nucleotide level. Most of these historical techniques are still widely used for centromeric satellite sequence analysis, in combination with the newly developed ones.

Further advances came from cytogenetics and molecular cytogenetics that provided some insights in the location and organization of satellite sequences. C-banding has been used to specifically stain the constitutive heterochromatin, including centromeric regions; the use of

satellite sequences as probes first in ISH (*in situ* hybridization) and then in FISH (Fluorescent *in situ* hybridization) experiments, has allowed the definition of their centromeric and pericentromeric localization and distribution among chromosomes. Thus, the application of such techniques clarified the role of satellite DNA as the main constituent of mammalian centromeric DNA. Besides the structurally characterization of centromeric heterochromatin, several functional studies of its protein constituents have been developed. The availability of the CREST antiserum, containing a mixture of antibodies against constitutive centromeric proteins, and of the anti-CENPA, anti-CENPB, anti-CENPC, and anti-CENPE specific antibodies, has allowed the localization of the centromeric functionality through immunofluorescence assays. The combination of satellite-FISH and immunofluorescence has definitely showed the co-localization of satellite DNA and centromeric functionality. The awareness of the binding between centromeric DNA and proteins has suggested the use of anti-CENP antibodies to isolate centromeric competent DNA from the bulk of the total DNA through chromatin immunoprecipitation (ChIP) assays. The subsequent FISH-localization, cloning, and sequencing of the isolated DNA allow the full characterization of centromeric competent DNA.

The recent development of high-throughput sequencing technologies has greatly increased the number of organisms with a sequenced genome. However, in genome sequencing projects of mammalian species, the assembly and characterization of centromeric regions cannot be directly achieved due to their repetitive and complex nature. In fact, in each human and other primate genome chromosome assembly there is no sequence in the existing gap between the p and q arms (Rudd & Willard, 2004). Nonetheless, whole-genome shotgun (WGS) sequence reads are not completely inapplicable for the characterization of centromeric regions and satellite DNA sequences. In fact, two computational methods were recently developed to isolate and characterize the centromeric repeats from WGS sequence data: HORdetect (Alkan et al., 2007) and RepeatNet (Alkan et al., 2010). HORdetect recovers alpha satellite sequences and predicts higher-order repeat structure in primate sequencing projects; RepeatNet allows the identification of higher-order repeat structures with no a priori information about the consensus, being thus applicable to any sequenced organism. This method has expanded the knowledge of mammalian satellite DNA and isolated for the first time centromeric satellites from species of the orders Cingulata, Proboscidea, and Didelphimorphia, previously reported in this chapter, although further efforts are needed to better characterize the isolated sequences.

5. Conclusion

The overall data collected so far allows the postulation of several considerations regarding centromeric satellite DNA in mammals. Several aspects are quite common among mammalian species, whereas others are shared among a subset of species, in some cases among evolutionarily distant ones, displaying an example of convergent evolution phenomena for satellite DNA. The main features described are summarized in **Table 1**.

Satellite DNA shows high variability across mammalian taxa in monomer size, nucleotide sequence, and quantity relative to the total genome. Although the satellite sequence is not evolutionarily conserved, there are recurrent elements among satellite sequences of closely related species. It is clear that satellite DNA follows a concerted evolution mechanism, so monomers are more similar to monomers of the same species than to monomers of other species. The amount of centromeric and pericentromeric satellite DNA is highly variable as well, and it is not related to the total genome size of the species. In fact, bats have a low C

value but do not show a small relative amount of satellite DNA, as it was thought before its evaluation: the percentage of satellite DNA relative to the total DNA, 3–5%, is very similar to the data of other mammals (**Table 1**).

Quite common features of satellite DNA are: i) the presence of internal direct and inverted subrepeats (Bogenberger et al., 1985; Lee, C. & Lin, 1996; Zhang & Horz, 1984); ii) the presence of the same satellite DNA families at the centromeres of autosomes and the X chromosome, but not at the Y chromosome centromere, like in mouse, sheep, and bat; iii) a greater divergence of the Y centromere sequence in comparison to the other centromeres, like in mouse and primates. Sheep, swine, and horse have a different satellite content in acrocentric and metacentric chromosomes. In two evolutionarily distant mammals, the marsupial *Macropus rufogriseus* and the rodent *Microtus chrotorrhinus*, giant sex chromosomes derived from a large block of heterochromatin at the centromeric and pericentromeric regions have been observed. Finally, evidence of a different rate at which autosomes and sex chromosomes accumulate and dissipate centromeric material has been found in cervid deer, muntjac, and in the genus *Macropus* (Bulazel et al., 2006; Li et al., 2005; Lin & Li, 2006), with the retention for longer periods of time of tandem arrays of ancestral satellites in the sex chromosomes that are not found in the autosomes.

The centromeric satellite is valuable as phylogenetic marker to establish the evolutionarily relationships among species, when they are not found or are ambiguous in the fossil record or other data (Saffery et al., 1999). The centromeric satellite was used as a phylogenetic marker in regard to two different aspects: its nucleotide sequence and its chromosome localization and distribution. The analysis of satellite DNA sequence, thanks to its high divergence rate and rapid evolution during speciation, was used to define the evolutionarily relationships among closely related species that diverged recently. An example is provided by the analysis performed on the spiny mice satellites (Kunze et al., 1999). On the other hand, the comparison of satellite DNA *in situ* hybridization patterns and the study of the nature and amplification of the satellite DNA families on the autosomes and the X chromosome allowed to infer phylogeny and increase the resolution of the evolutionary tree of the Artiodactyla (Chaves et al., 2000; Chaves et al., 2005; Modi et al., 1996). In the family Equidae, the phylogeny of four *Equus* chromosomes was reconstructed by centromere and satellite DNA localization (Alkan et al., 2010; Wade et al., 2009). Moreover, the analysis of satellite DNA sequence, organization, and chromosome distribution, in conjunction with karyotype analysis, is a valuable tool to measure species relationships while also elucidating important aspects of both genome and repetitive sequence evolution.

All the data collected up to date in mammals suggest that centromere satellite sequences are neither necessary nor sufficient for centromere function, and that repetitive DNA is more likely a consequence than a source of centromere function. Nevertheless, the pancentromeric presence of satellite DNA on all mammalian centromeres clearly indicates that a repetitive, ordered, and homogenous sequence is important for centromere maintenance during the evolution of the species.

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6

mRNA Biogenesis in the Nucleus and Its Export to the Cytoplasm

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1. Introduction

Fail-safe biogenesis of mRNA is crucial for translating genetic information into proteins in a high-fidelity manner. This process occurs in the nucleus and involves various mRNA processing steps. Recent findings indicate that these mRNA processing steps and the export of mRNA into the cytoplasm are linked in eukaryotic cells. This link is called 'coupling' and is thought to be indispensable for precise and efficient gene expression, because this coupling mechanism is conserved among many species. Through this coupling mechanism, only the properly-processed mRNAs are efficiently exported to the cytoplasm where protein synthesis occurs. Various mRNA binding proteins are identified to function in this system. Most of these proteins are recruited to the transcripts at an early stage of the mRNA lifecycle. Moreover, these proteins remain associated with the transcripts to a much later stage and function by prompting mRNA processing in the nucleus, coupling the mRNA processing steps and its export and surveillance of the improperly-processed mRNA in the cytoplasm. Here we review mRNA biogenesis in eukaryotic cells with an emphasis on the importance of this coupling mechanism for high-fidelity gene expression.

2. mRNA transcription by RNA polymerase II

The transcription of mRNA is the first step of translating genetic information into a protein. This genetic information encoded in the DNA is transcribed into mRNA by a huge molecular machine called RNA polymerase II (Pol II). Pol II is responsible for transcribing all the protein-coding genes.

2.1 Initiation

At the first stage of the transcription process, Pol II needs to be recruited to a gene promoter. Pol II cannot recognize the promoters of target genes alone. Instead, Pol II depends on a series of accessory factors known as general transcription factors (GTFs) (Orphanides et al., 1996; Roeder, 1996; Woychik and Hampsey, 2002). Pol II and the associated GTFs termed TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH assemble on the promoter to form the preinitiation complex (PIC). Mediator, a large multisubunit complex, regulates PIC

assembly, integrating various regulatory signals into the transcriptional activity (Conaway and Conaway, 2011). After the formation of PIC on the promoter, TFIIH, a complex harboring DNA helicases, melts the DNA to expose the template strand. Then RNA synthesis begins.

Once the first nucleotide bonds have been formed, Pol II is released from the promoter to facilitate downstream transcription. Most of the general transcription factors dissociate from the promoter, whereas mediator is likely to remain associated at the promoter to facilitate the next round of polymerase recruitment and reinitiation (Yudkovsky et al., 2000).

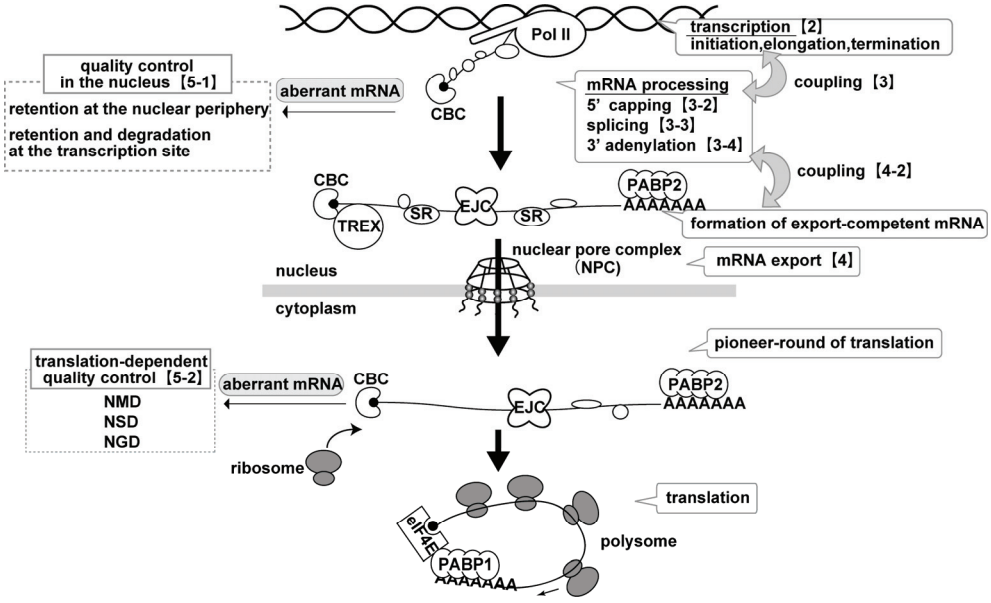

Fig. 1. mRNA lifecycle

RNA polymerase II complex (Pol II) transcribes all the protein-coding genes in the nucleus. Transcribed pre-mRNA undergoes the various processing steps. Immature mRNA is held in the nucleus and degraded. Only the properly-processed mRNA is exported from the nucleus to the cytoplasm. In the cytoplasm, there are other quality check systems that are associated with the pioneer-round of translation and degrade the aberrant mRNA. Hence, only the properly-processed mRNA managing to pass all of these exams can serve as the template for protein synthesis.

2.2 Promoter-proximal pausing

The recruitment of Pol II to the promoter is believed to be the rate-limiting step in gene expression (Saunders et al., 2006). However, recent genome-wide studies using drosophila and mammalian cells suggest that a number of developmental and inducible genes contain stalled Pol II in their promoter-proximal regions, that is 20–50 nt downstream from the transcription start site (Aida et al., 2006; Barboric and Peterlin, 2005; Bender et al., 1987;

Gilmour and Lis, 1986; Krumm et al., 1992; Saunders et al., 2006; Sims et al., 2004; Strobl and Eick, 1992). Moreover, activation of the stalled Pol II is thought to be responsible for the expression of these genes (Saunders et al., 2006). DRB sensitivity-inducing factor (DSIF) and the negative elongation factor (NELF) (Wu et al., 2003; Yamaguchi et al., 2002) are found to be associated with the pausing Pol II. TFIIS, TFIIF and the positive transcription-elongation factor-b (P-TEFb) assist in rescuing Pol II from this pause state (Adelman et al., 2005; Peterlin and Price, 2006). Pol II stalling is enriched at highly regulated genes that are essential for responses to stimuli or embryonic development (Muse et al., 2007; Zeitlinger et al., 2007). Pol II stalling at the promoter supports the quick expression of these genes (Lorincz and Schubeler, 2007; Saunders et al., 2006).

2.3 Elongation

A number of factors interact with Pol II during the transition into productive elongation, such as P-TEFb mentioned above. Once Pol II engages in productive elongation, the mature Pol II complex is remarkably stable and can transcribe hundreds of kilobases without dissociating from the DNA template (Singh and Padgett, 2009).

2.4 Termination

Transcriptional termination is crucial for the release of the transcripts from the transcription site. This process also facilitates Pol II release from the DNA template, promoting Pol II recycling for further rounds of transcription. Pol II release also contributes to proper gene regulation. Without such events, the correct function of neighboring genes may be influenced because of the "penetrating" Pol II from an upstream gene.

Currently, two models describing transcriptional termination of protein-coding genes are proposed. Pol II termination is known to be functionally coupled to an RNA maturation step called 3′ adenylation (see below). During the 3′ adenylation, the stalling of Pol II, the endonucleolytic cleavage of the nascent transcripts and the subsequent degradation of the downstream transcript occur. One of the models is called the 'allosteric model' which proposes that Pol II stalling during 3′ adenylation causes a conformational change in Pol II, which makes Pol II lose its processivity and leads to dissociation of Pol II from the template (Calvo and Manley, 2001; Greenblatt et al., 1993; Logan et al., 1987). The second model is the 'torpedo model'. The model proposes that a 5′ to 3′ RNA exonuclease involved in the clearance of downstream byproducts of the 3′ adenylation catches up to the elongating Pol II and causes it to terminate (Connelly and Manley, 1988).

3. Coupling transcription to mRNA processing steps

Once RNA synthesis starts, the transcribed pre-mRNA undergoes three major processing steps and these processing steps occur co-transcriptionally (Buratowski, 2009; Egloff and Murphy, 2008; Fuda et al., 2009; Koch et al., 2008; Kuehner et al., 2011). They are 5′ capping, splicing and 3′ adenylation. The proper completion of these processing steps is necessary for the production of export-competent mature mRNA molecules. Although the biochemical basis of each step is distinct, they are all coupled to transcription. A unique carboxy-terminal domain (CTD) on Rpb1, the largest subunit of Pol II, provides the platform for this

coupling system (Proudfoot et al., 2002). The data that 5′ capping, splicing and 3′ adenylation are all inhibited by the truncation of CTD without affecting transcription provides evidence for the important role of CTD in coupling transcription to these processing steps (McCracken et al., 1997).

3.1 Phosphorylation status of CTD during mRNA synthesis

CTD is a conserved repeat of the heptapeptide Tyr-Ser-Pro-Thr-Ser-Pro-Ser (Cramer et al., 2001). The number of repeats is 26 in yeast and 52 in humans. A unique feature of CTD is that its pattern of phosphorylation changes dynamically according to the transcriptional activity of Pol II (Buratowski, 2009; Egloff and Murphy, 2008; Fuda et al., 2009; Koch et al., 2008; Kuehner et al., 2011).

During the PIC assembly, Pol II with unphosphorylated CTD is recruited to the gene promoters. The mediator binds unphosphorylated Pol II. Then, within the PIC, TFIIH phosphorylates the CTD on Ser5, which leads to the release of mediator from Pol II. This allows Pol II to engage in processive transcription and depart from the promoter (Max et al., 2007). Even if Pol II harbors Ser5P CTD, it may still stall at the promoter-proximal region and synthesize short and abortive transcripts as described above. As Pol II manages to escape from pausing and transcribes further downstream, the level of Ser5P decreases; however, a low level of Ser5P is maintained throughout the elongation process. Subsequently, Ser2 is phosphorylated by Cdk9, a kinase subunit of the positive elongation factor P-TEFb as well as the elongation factor DSIF (also known as Spt4/Spt5), that leads to transcription into a gene body (Peterlin and Price, 2006). Along with transcriptional termination, Ser2P is dephosphorylated and this may help Pol II reinitiate transcription.

Each of these different modification patterns preferentially recruits a distinct set of processing factors at the right time during mRNA transcription and maturation. Furthermore, according to the X-ray study of yeast Pol II, CTD resides adjacent to the narrow tunnel for nascent transcripts to exit. This location appears to be appropriate for CTD-associating factors to work on pre-mRNAs (Fabrega et al., 2003) .

3.2 Coupling to 5’ capping

Capping is the first modification to the pre-mRNA. This is an m7GpppN structure added at the 5′ end of the nascent transcript. In the nucleus, this structure is recognized by the cap binding complex (CBC) (Izaurralde et al., 1994), which contains CBP20 and CBP80. The CBC-bound cap structure functions to protect the nascent transcript from attack by nucleases. It also has important roles both in the export of mature mRNAs from the nucleus and the quality control of mRNAs (Proudfoot et al., 2002). In the cytoplasm, the cap structure also serves as the binding site for the eukaryotic elongation factor eIF4E to recruit the ribosome.

Capping occurs after 20–30 nt of RNA is synthesized from the transcription start site and involves two enzymes, HCE (Human Capping Enzyme) and MT (RNA 7-methyltransferase) (Hirose and Manley, 2000). These capping enzymes bind specifically to the Ser5P CTD of Pol II (Fabrega et al., 2003). Ser5P CTD is most abundant in the promoter-proximal region and ensures the 5′capping of the pre-mRNA at the earliest stage of the transcription process.

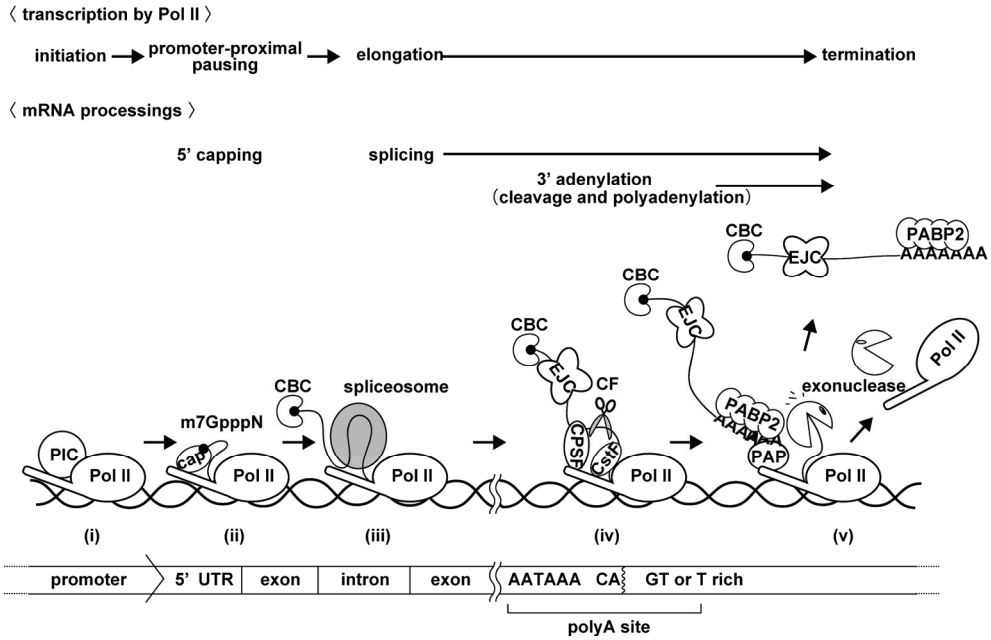

Fig. 2. Coupling transcription to mRNA processing steps

Here we present the overview of the coupling between Pol II transcription and mRNA processing steps. Transcription begins with the assembly of the transcriptional initiation complex (PIC) on the promoter (i). Soon after RNA synthesis begins, m7pppGN cap structure is added to the 5′ end of transcript (ii). Processive elongation stimulates the recruitment of spliceosome (iii). At the polyA site, pre-mRNA is cleaved and subsequently adenylated. 3′ adenylation provokes the transcriptional termination to make Pol II dissociate from DNA template (iv, v).

3.3 Coupling to splicing

Most mammalian genes contain introns. Since these sequences represent 'non-coding' parts of the gene, in order to make a functional protein, introns must be removed from the template by means of splicing. Along with the completion of splicing, the exon-exon junction complex (EJC) is deposited about 24 nt upstream of the exon–exon junctions (Le Hir and Andersen, 2008; Tange et al., 2004). EJC plays important roles in mRNA export from the nucleus and in cytoplasmic mRNA quality control (see below).

The pre-mRNA contains several *cis*-elements that are essential for the splicing reaction, such as 5′ splice site, the branch-point, the poly-pyrimidine tract and the 3′ splice site. These elements are recognized by the spliceosome, a large protein–RNA complex. The two sequential *trans*-esterification reactions carried out by the spliceosome lead to the ligation of the exon sequences and the excision of the introns. To accomplish the precise splicing, the proper recognition of these *cis*-elements by the spliceosome is crucial. To aid spliceosome deposition, there are other auxiliary sequences within the pre-mRNA. For example, a

sequence within an exon is called the 'exonic splicing enhancer' (ESE) (Chew et al., 1999; Wang et al., 2005; Wu et al., 2005). The serine/arginine-rich (SR) family of essential splicing factors play a particularly important role in splicing by binding to the ESE and recruiting the rest of the splicing machinery(Cartegni and Krainer, 2002; Cramer et al., 1999; Liu et al., 1998; Sun et al., 1993).

Transcripts derived from Pol II transcription are more efficiently spliced than those transcribed by T7 which lacks CTD (Das et al., 2006). Introducing mutations within the CTD greatly reduces the efficiency of splicing without affecting transcription as well as inhibition of CTD phosphorylation by kinase inhibitors (Bentley, 2005; Bird et al., 2004). Therefore, phosphorylated CTD seems to be crucial for the coupling of transcription and splicing. Several splicing factors such as U1 snRNP, a component of the spliceosome, and the SR protein factors directly associate with Pol II through the CTD (Das et al., 2007). Some SR proteins bind indirectly to the CTD of Pol II (Das et al., 2007; de Almeida and Carmo-Fonseca, 2008). These physical interactions seem to play a role in coordinating the transcriptional activity with the efficiency of splicing.

3.4 Coupling to 3' adenylation and transcription termination

Most of the protein-coding mRNAs undergo 3′ adenylation. At the 3′ end of the transcript, a polyadenosine tail (polyA tail) of approximately 200–300 nt is added (Danckwardt et al., 2008; Proudfoot et al., 2002). Pre-mRNA is cleaved prior to polyadenylation. The cleavage occurs at a CA dinucleotide defined by the sequence elements within the 3′ end region of the pre-mRNA. These elements consist of the AAUAAA consensus sequence and the U/GU-rich region (downstream sequence element or DSE) that is located 10–30 nt upstream and 30 nt downstream of the cleavage site, respectively. Multiple protein factors assemble onto these sequence elements to define the cleavage site. Cleavage and the polyadenylation specificity factor (CPSF) binds to the AAUAAA sequence, resulting in the pausing of Pol II transcription (Glover-Cutter et al., 2008). After the cleavage stimulation factor (CstF) binds to the U/GU-rich region, CPSF binds to CstF (Kazerouninia et al., ; Kuehner et al., 2011; Nag et al., 2007; Park et al., 2004) . Since the binding of CPSF to CstF is mutually exclusive with Pol II binding (Nag et al., 2007), that binding event induces the release of paused Pol II and the CPSF-mediated cleavage. The upstream cleavage product is polyadenylated, whereas the downstream cleavage product is degraded. CPSF is associated with the elongation complex by interaction with the Pol II body and CstF interacts with CTD. In yeast, Pcf11, a subunit of the cleavage/polyadenylation factor, binds preferentially to the Ser 2P CTD via its CID (CTD-interacting domain) (Licatalosi et al., 2002). Moreover, purified Pol II or CTD can stimulate 3' adenylation *in vitro* (Hirose and Manley, 1998). Thus, the efficiency of the 3′ adenylation is dramatically enhanced by coupling to transcription.

As mentioned above, transcriptional termination is tightly linked to 3′ adenylation (Logan et al., 1987; Whitelaw and Proudfoot, 1986). A functional polyA site (Zaret and Sherman, 1982) and several essential subunits of the cleavage factor including Pcf11 are necessary for Pol II termination(Birse et al., 1998; Gross and Moore, 2001; Minvielle-Sebastia et al., 1997). These data explain why CTD is required for optimal termination of transcription downstream of the polyA site (McCracken et al., 1997; McNeil et al., 1998). Thus, the elongation machinery stimulates both 3′ adenylation and transcriptional termination.

4. mRNA export

In eukaryotes, the nucleus is physically segregated from the cytoplasm by the nuclear envelope. Transcription and subsequent processing occur in the nucleus, whereas translation is a cytoplasmic event, therefore, processed mRNAs must be transported from the nucleus to the cytoplasm for translation.

Nucleocytoplasmic transport events occur through the nuclear pore complex (NPC) that penetrates the nuclear envelope (Kohler and Hurt, 2007; Mattaj and Englmeier, 1998). The NPC is imaged like a spouted basket, and allows the bidirectional transport of macromolecules such as proteins or RNA molecules between the nucleus and the cytoplasm. The transported cargoes, that are imported into or exported from the nucleus, are recognized by different transport receptors.

4.1 Tap-p15 mRNA export factor and its adaptors

The export receptor of mRNA is the Tap and p15 (Mex67 and Mtr2 in yeast) heterodimer complex (Gruter et al., 1998; Segref et al., 1997). The stable interaction with NPC is not accomplished only by Tap, but also requires heterodimerization with p15 (Guzik et al., 2001). In the absence of the Tap-p15 association, the mRNA is retained in the nucleus by the mRNA quality control system (Fasken and Corbett, 2009; Moraes, 2010).

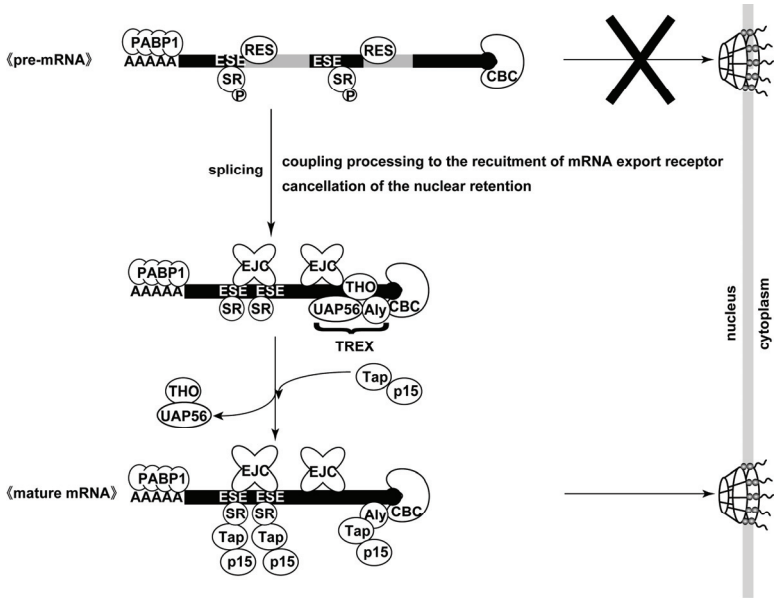

Fig. 3. Coupling splicing to export.
Because mRNA processing is coupled to its export, the proper processing facilitates mRNA export into the cytoplasm. Black bars represent exons and gray bars represent introns.
As splicing proceeds, the TREX complex is loaded onto mRNA and SR proteins on ESEs become hypophsophrylated. These factors function as adaptors for Tap/p15, mRNA export receptor. On the other hand, unspliced pre-mRNA is retained in the nucleus by the nulear retention activity of RES. Please see text for other nuclear retention mechanisms.

Numerous additional factors cooperate with the mRNA export receptor. The role of these factors is to establish a physical bridge between the mRNA and its export receptor Tap-p15. One of the important adaptors is the TREX (transcription-export) complex that consists of the Ref family protein Aly (Yra1 in yeast), UAP56 (Sub2 in yeast) and the THO complex (Masuda et al., 2005; Strasser et al., 2002). Because the direct interaction of mRNA and Mex67 appears to be weak, the TREX subunit Aly/Yra1 mediates interaction between mRNA and the Tap/Mex67 complex (Reed, 2003; Reed and Hurt, 2002).

Another class of mRNA export adaptors is the SR proteins. As mentioned above, the SR proteins have essential roles in splicing. Some of the SR proteins such as SF2/ASF and 9G8 function as mRNA export adaptors as well (Huang et al., 2003).

4.2 Coupling transcription and processing to nuclear export

Remarkably, these adaptors are attached to transcripts as pre-mRNA processing steps proceed.

The TREX complex is recruited onto mRNA during splicing (Masuda et al., 2005) and associates with the cap binding complex (CBC) through a direct interaction between Aly and CBC (Cheng et al., 2006; Nojima et al., 2007). Later the TREX complex is thought to be replaced by the Tap-p15 mRNA export receptor. Hence, the TREX complex integrates transcription or splicing with nuclear export. A study using electron microscopy revealed that the fully maturated mRNA is exported in the 5′ to 3′ direction (Visa et al., 1996); therefore, the association of the TREX complex at the cap site seems to guide the directional mRNA export.

SR proteins are recruited to the splicing machinery in a hyperphosphorylated form and become hypophosphorylated after splicing. The change in the phosphorylation state triggers the recruitment of TAP–p15 (Huang et al., 2003). The phosphorylation status of the SR proteins could act as a switch to signal the export competence of the spliced mRNP.

The importance of the coupling of mRNA export to transcription was initially suggested by the observation that spliced mRNAs exit the nucleus more efficiently than unspliced mRNAs or RNAs derived from cDNAs (Luo and Reed, 1999). Splicing-dependent recruitment and stabilization of these adaptors explains why mRNAs produced from intron containing genes are more efficiently exported than mRNAs derived from cDNAs.

Besides these factors described above, a complex called TREX-2 is proposed to functionally couple transcription and mRNA export. TREX-2 is composed of Sac3, Thp1, Sus1 and Cdc31 (Fischer et al., 2004; Rodriguez-Navarro et al., 2004). Sac3 was originally identified as an additional mRNA export adaptor in yeast (Fischer et al., 2002). Sac3 physically associates with the Mex67–Mtr2 export receptor. Sus1 in the TREX-2 complex interacts with SAGA, a large transcription initiation complex that catalyzes histone acetylation and deubiquitylation (Kohler et al., 2006; Shukla et al., 2006). TREX-2 and SAGA generate a physical contact with the NPC. TREX-2 therefore couples SAGA-dependent gene transcription to mRNA export both by facilitating the export receptor loading onto transcribed mRNAs and by bringing the transcription site closer to NPC.

4.3 The role of Dbp5-Gle1 and IP6 in the release of mRNA into the cytoplasm

To ensure the unidirectional transport of cargo, transport receptors need cues to determine which side of the NPC they are on. A shuttling of proteins between the nucleus and the cytosol requires a family of conserved nuclear transport receptors known as karyopherins (Chook and Blobel, 2001; Mosammaparast and Pemberton, 2004; Strom and Weis, 2001). Importinβ family proteins, the most studied karyopherins, directly or indirectly recognize cargo signals. The affinity for cargoes and the direction of nucleocytoplasmic transport by the importinβ family of proteins is determined by the asymmetric nucleus/cytoplasm distribution of RanGTP and RanGDP; the so-called 'Ran gradient' (Gorlich and Kutay, 1999; Pemberton et al., 1998). In the case of nuclear export, the interaction between karyopherin and the cargo is stabilized by the RanGTP concentrated in the nucleus, whereas in the cytoplasm, RanGDP, which is derived from RanGTP hydrolysis by RanGAP, causes the complex to disassemble and terminate the export step.

In contrast, bulk mRNA export does not depend on karyopherins or the Ran gradient. If the Tap-p15 heterodimer still interacts with mRNA at the NPC, mRNA could be returned to the nucleus. Dbp5-Gle1 and inositol hexakisphosphate (IP6) function to ensure the unidirectional export of mRNA into the cytoplasm.

In yeast, Dbp5 removes Mex67 from mRNA *in vivo* and displaces the RNA binding protein Nab2 *in vitro* (Lund and Guthrie, 2005; Tran et al., 2007). Consequently, Dbp5 remodels the profile of proteins binding to transcripts, thus preventing mRNA from returning to the nucleus. Dbp5 is a DEAD-box RNA helicases. DEAD-box RNA helicases are highly conserved among eukaryotes and contain a highly conserved core with ATP-binding and RNA-binding sites (Rocak and Linder, 2004). DEAD-box RNA helicases conjugate their ATPase activities to their helicase activities. The ATP-bound form of Dbp5, "closed form", sandwiches RNA, but the ADP-bound form, "open form", releases RNA (Ledoux and Guthrie, 2011). Moreover, only the ADP-bound Dbp5 can remove Nab2 from mRNA, whereas ATP-bound Dbp5 does not. Consequently, the alteration of ATP to ADP within Dbp5 may be crucial for its remodeling activity. Dbp5 exhibits quite low ATPase activity on its own. Gle1 and IP6 activate the ATPase activity of Dbp5 (Dossani et al., 2009; Weirich et al., 2006). Recently, the Dbp5-Gle1 and IP6 complex was shown to be structurally similar to the eIF4A-eIF4G complex which is essential for translation initiation. eIF4A is a DEAD-box family protein, and eIF4G activates eIF4A exactly as Gle activates Dbp5 (Montpetit et al., 2011). Dbp5 is concentrated at the cytoplasmic face of the NPC where it interacts with the nucleoporin Nup159 (NUP214/CAN in human) (Weirich et al., 2004). On the other hand, Dbp5 is relatively abundant and also localizes in the cytoplasm. Remarkably, Dbp5 shuttles between the nucleus and the cytoplasm, and the inhibition of Xpo1, an importinβ family protein, results in the accumulation of Dbp5 in the nucleus (Hodge et al., 1999). Moreover, it is reported that Dbp5 is recruited to mRNA at an early stage in the transcription process (Estruch and Cole, 2003). This raises the question of why Dbp5 removes Tap from mRNA only after transit through the NPC and not in the nucleus. The current model is shown in Fig.4. Gle1 is concentrated at the cytoplasmic face of the NPC via interaction with two cytoplasmic components of NPC, Nup42 (CG1 in human) and Nup159. As a result of this biased localization of Gle1, the ATPase of Dbp5 is activated only on the cytoplasmic side of the NPC. Then, Dbp5 should dissociate Mex67 from mRNA, and mRNA is unidirectionally exported to the cytoplasm.

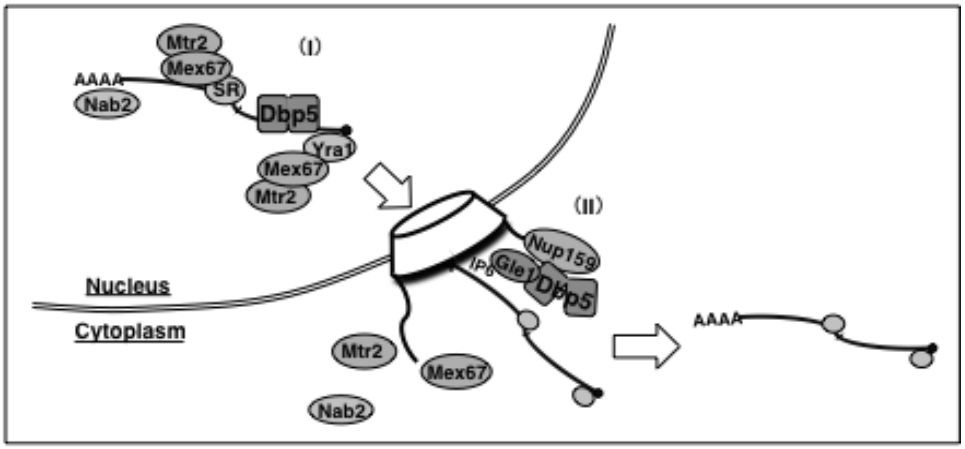

Fig. 4. The current model of mRNA export.

(I) Mex67-Mtr2 (Tap-p15 in human), mediated by Yra1 or SR proteins, exports mRNA to the NPC. (II) On the cytoplasmic side of the NPC, Dbp5, activated by Gle1 and IP6, removes RNA binding proteins including Mex67 from the mRNA, which is called 'remodeling'. Thereby, mRNA export to the cytoplasm is one-way traffic.

4.4 Non-canonical mRNA export pathway

The importinβ family protein Xpo1, also known as Crm1, does not have a major role in mRNA export. However, Xpo1 is shown to be involved in nuclear export of a subset of transcripts, such as mRNAs containing adenosine/uridine-rich elements (AREs) positioned in their 3′ untranslated regions (Barreau et al., 2005; Chen and Shyu, 1995).

5. mRNA quality control

When mRNA undergoes proper processing, it travels through the NPC to the cytoplasm for protein synthesis. However, if the mRNA is not properly processed, it should be held in the nucleus or be degraded to avoid the production of aberrant proteins that may disrupt cellular function. Currently, several mRNA quality control systems are found in the nucleus and the cytoplasm.

5.1 Nuclear retention of aberrant mRNA

Myosin-like proteins (Mlp1p and Mlp2p in yeast and Tpr in vertebrates) are filamentous proteins anchored at the NPC (Kohler and Hurt, 2007). Deletion of Mlp1 results in the leakage of unspliced transcripts into the cytoplasm (Galy et al., 2004). Mlp1 interacts with Nab2 (Fasken et al., 2008; Grant et al., 2008; Green et al., 2003; Vinciguerra et al., 2005), and the splice site-binding protein SF1 and the branch point-binding protein also interact with Mlp1 in an RNA-dependent manner (Galy et al., 2004). At the NPC, through sequestering unspliced transcripts or facilitating the export of mature mRNA, Mlps function as 'gatekeepers' to assure that only mature mRNAs are exported from the nucleus. In addition, pre-mRNA retention and

the splicing (RES) complex, which binds to the SF3b subcomplex in the spliceosome, are also implicated in pre-mRNA retention in the nucleus (Kaida et al., 2007). Some ESEs have been shown to possess activity to retain unspliced mRNAs, and the splicing reaction can reset the nuclear retention state caused by the ESEs (Taniguchi et al., 2007).

Several data suggest that the improperly-processed transcripts are submitted to the retention at the transcription site and subsequently undergo degradation. The nuclear exosome nuclease complex plays a crucial role in this mRNA quality control in the nucleus. (Abruzzi et al., 2006; Bousquet-Antonelli et al., 2000; Burkard and Butler, 2000; Custodio et al., 1999; Das et al., 2003; Dower et al., 2004; Dunn et al., 2005; Hilleren et al., 2001; Libri et al., 2002; Milligan et al., 2005; Torchet et al., 2002; Zenklusen et al., 2002).

The exosome is a ring-shaped multimolecular complex involved in degradation of various RNAs both in the nucleus and the cytoplasm (see below for the function of exosome in cytoplasmic mRNA quality control) (Belostotsky, 2009; Houseley et al., 2006; Lebreton and Seraphin, 2008; Lorentzen et al., 2008; Lorentzen and Conti, 2006; Lykke-Andersen et al., 2009; Schmid and Jensen, 2008; van Hoof and Parker, 1999; Vanacova and Stefl, 2007). In the nucleus, the exosome interacts with its nuclear-specific binding partner, Rrp6. When mRNA processing, such as splicing or 3′ adenylation, is perturbed, transcripts are retained within foci near or at the transcription site and are degraded in an Rrp6-dependent manner.

5.2 mRNA quality control in the cytoplasm

The mature mRNAs exported from the nucleus to the cytoplasm are now applied to translation. At the first translation round, so called the pioneer-round of translation, mRNA undergoes a quality check. At least three mechanisms are currently known (Houseley and Tollervey, 2008; Isken and Maquat, 2007; Shyu et al., 2008). They are nonsense-mediated mRNA decay (NMD) (Culbertson and Neeno-Eckwall, 2005; Isken and Maquat, 2008), non-stop decay (NSD) (Vasudevan et al., 2002) and no-go decay (NGD).

Some mutations can cause the emergence of a pre-mature termination codon (PTC) within the mRNA. Such nonsense mutations result in the production of C-terminally truncated proteins that may be harmful to the cell. After the splicing, EJC is deposited 20–24 nt upstream of the exon–exon junctions (Le Hir and Andersen, 2008; Tange et al., 2004).

Normal termination codons in mammalian transcripts are usually found within the last exon. Consequently, EJC should be displaced at a pioneer round of translation. However, if PTC is present more than 50-55 nt upstream of EJC, EJC remains on the transcripts and the translating ribosome meets EJC. This event triggers NMD to degrade PTC containing mRNA. Most PTC-containing mRNAs in mammals come from aberrant alternative splicing (McGlincy and Smith, 2008). Upf 1-3 proteins have a central role in facilitating the recognition of PTC (Muhlemann and Lykke-Andersen, 2010). PTC-containing mRNAs are degraded through two pathways. One is initiated through decapping at the 5′ end and/or deadenylation at the 3′ end. These reactions make it easy to degrade mRNA by exoribonucleases. In a second pathway, mRNA is first endonucleolytically cleaved by Smg6, and then degraded both in 5′ to 3′ and 3′ to 5′ direction by exonucleases (Muhlemann and Lykke-Andersen, 2010; Nicholson et al., 2010).

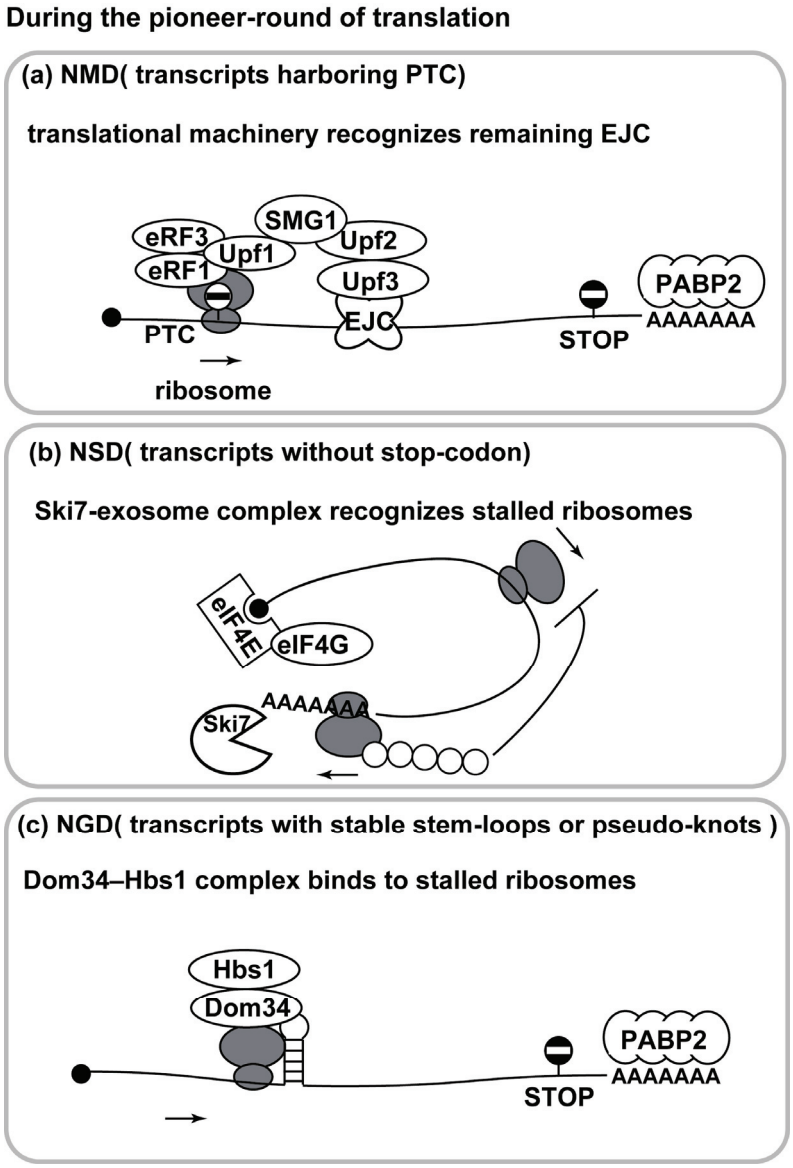

Fig. 5. mRNA quality control in the cytoplasm
When mRNA reaches the cytoplasm, it is applied to the first translation cycle; the so-called 'pioneer-round of translation'. During this translation, mRNA is submitted to the final quality check before the vigorous protein synthesis. Nonsense-mediated mRNA decay (NMD) for mRNAs harboring pre-mature termination codons (PTC) (a), non-stop decay (NSD) for mRNAs without functional termination codons (b) and no-go decay (NGD) for mRNAs with strong secondary structures are identified to participate in this translation-coupled quality control.

mRNAs that lack the termination codons are also targeted for the translation-dependent mRNA decay, NSD. When a ribosome translates through the polyA tail because of the lack of a termination codon and stalls at the 3'-end of the mRNA, Ski7, a component of the cytoplasmic exosome, interacts with the stalled ribosome and triggers the rapid degradation of the non-stop mRNA (Isken and Maquat, 2007).

Some mRNAs produced from aberrant alternative splicing contain strong secondary structures. Such structures prevent translation by the ribosome and cause the ribosome to stall. The ribosome is rescued by the NGD pathway. NGD clears the stalled ribosome and rapidly degrades the mRNA transcript (Doma and Parker, 2006). Dom34 and Hbs1 interact with the stalled ribosome and assist in the disassembly of the translational complex. They also trigger mRNA decay through endonucleolytic cleavage and subsequent exonucleolytic decay (Doma and Parker, 2007).

6. Concluding remarks

Various mRNA-processing proteins are recruited onto the transcripts at the early stage of the mRNA lifecycle. Besides their functions to facilitate pre-mRNA processing, they also serve as a 'dress-code' that indicates that the coded mRNA is properly processed and competent for export and translation. Pol II coordinates transcriptional activity for the recruitment of these factors to the transcripts. Hence, from its birth in the nucleus to its degradation in the cytoplasm, mRNA is kept under strict surveillance to ensure high-fidelity gene expression.

7. Acknowledgment

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7

Membrane Initiated Effects of 1α,25-Dihydroxyvitamin D_3 in Prostate Cancer Cells: Effects on AP1 and CREB Mediated Transcription

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1. Introduction

The biologically active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], is formed through a multistep process in the liver and the kidneys, initiated in the skin by solar UVB radiation. Vitamin D compounds are transported in the body by vitamin D binding protein (DBP) to either nuclear vitamin D receptors (nVDR) or putative membrane associated vitamin D receptors (mVDR) where it exerts its biological responses in target organs by nuclear- and membrane- initiated signaling pathways (Bouillon *et al.*, 1995; Holmén *et al.*, 2009). Finally, 1α,25$(OH)_2D_3$ becomes inactivated by 24-hydroxylase which transforms it into 1,24,25$(OH)_3D_3$, a substance with much lower affinity for VDR (Bouillon *et al.*, 1995).

In the nuclear initiated signaling pathway, occupancy of the nuclear vitamin D receptor (nVDR) by 1α,25$(OH)_2D_3$ leads to modulation of gene transcription of hormone-sensitive genes (Krishnan & Feldman, 2010). In conformity with several other receptors of the nuclear steroid/thyroid superfamily, nVDR forms heterodimers with retinoid X receptor (RXR) (Sutton *et al.*, 2003). Subsequent interaction with the vitamin D response element (VDRE) in the promoter sequence of target genes initiates induction or repression of transcription, hence generating a biological response (Haussler *et al.*, 2011).

Vitamin D exerts multiple actions in the organism including the well-known regulation of calcium and phosphate homeostasis (Holick, 2006), but it also possesses anti-proliferative, pro-differential and pro-apoptotic actions in cancer cells as well as increasing the effect of a number of established anti-cancer drugs (Trump *et al.,* 2010).

The association of vitamin D with human cancer is well described in adenocarcinoma of the prostate gland, i.e. prostate cancer (PCa). Clear correlation between vitamin D deficiency and risk factors for PCa, such as high age and darker pigmented skin, has been observed. Thus, the amount of vitamin D decline with age, and the elevated levels of melanin in African Americans partly inhibits sun initiated vitamin D synthesize (Hsing & Chokkaligam, 2006).

Furthermore, 1α,25$(OH)_2D_3$ has been proven to decrease the risk of PCa by controlling prostate cell proliferation (Holick, 2006).

In a previous paper, we have shown that 1α,25$(OH)_2D_3$ regulates prostate cell differentiation, apoptosis and proliferation via multiple pathways, which involves both nuclear and membrane receptors found in the JNK/SAPK (c-Jun N-terminal kinases/stress-activated protein kinase) pathway. (Hagberg *et al.*, 2008; Larsson *et al.*, 2008; Holmén *et al.*, 2009; Karlsson *et al.*, 2010).

The JNK/SAPK pathway may be induced by several different means, such as chemical and physical stress, UV-radiation and osmotic shock, as well as pro-inflammatory cytokines, and even G-coupled receptor signaling, (Matsukawa *et al.,* 2004). Among the cytokines that triggers the JNK/SAPK pathway, TNF-α is predominant, and it is also known to regulate cellular events associated with cancer cell phenotype, such as apoptosis, cell proliferation and differentiation. Still, it has been shown that cancer cells are resistant to apoptosis induced by TNF-α (Chopra *et al.,* 2004). This resistance seems to involve certain survival signals, one of which being the transcription factor, nuclear factor-kappa B (NF-κB). It is therefore thought that if NF-κB is inhibited, the cancer cells would become more sensitive to TNF-α induced apoptosis. Indeed, this seemed to be the case in TNF-α resistant leukemia cells that were treated with sulforaphane, a putative anti-cancer drug that showed a non-specific inhibition of the TNF-α induced NF-κB activation (Moon *et al.,* 2009). In addition, there were indications that this inhibition of NF-κB lead to prolonged JNK/SAPK activation in the leukemia cells. Other studies indicate that activation of JNK also seems to regulate and can be regulated by NF-κB (Nachmias *et al.,* 2004). A similar result to the sulforaphane inhibition of the TNF- α induced NF-κB has been reported for breast cancer cells treated with vitamin D. Thus, Michigan cancer foundation 7 (MCF-7) breast cancer cells, were found to become sensitized to apoptosis induced by TNF-α when treated with 1α,25$(OH)_2D_3$ (Golovko *et al.,* 2005).

Transcription factor NF-κB can be regulated in concert with another transcription factor, activator protein 1 (AP1). AP1 is a complex consisting of homodimers and heterodimers of the jun and fos families and the activity of AP1 seems to be regulated by differential expression of the jun and fos families. The c-Jun components of AP1 can be regulated by the phosphorylating activity of active JNK (Dedieu and Lefebvre, 2006). Thus, in the two prostate carcinoma cell lines PC-3 and LNCaP, overexpression of the early growth response protein EGR-1, selectively increased the activity of both NF-κB and AP1 and the activation of these transcription factors appeared to be essential for the induction of proliferation and anchorage independence (Parra *et al.,* 2011).

Another important mediator of cell proliferation, differentiation and apoptosis is the cyclic response element binding protein (CREB). CREB is part of the cAMP regulated pathway and is phosphorylated by protein kinase A (PKA). CREB does not have direct contact with the transcriptional machinery. Therefore it requires CREB binding protein (CBP) to achieve transcriptional activation. There are several steroid and thyroid hormones that act to bind CBP e.g. luteinizing hormone, glucagon and adrenaline which all exert influence over cAMP and PKA. There are no direct evidences that vitamin D receptors are coupled to a protein complex which includes adenylate cyclase. However, several studies have demonstrated that 1α,25$(OH)_2D_3$ evoke rapid increases in PKA activity, intracellular cAMP concentrations which have been found to be associated with G-protein-coupled signaling as well as regulation of Ca^{2+} transport through Ca^{2+}-channels (Massheimer *et al.*, 1999; Schwartz *et al.*, 2002; Dirks-Naylor & Lennon-Edwards, 2011).

Knowing how the cell cycle arrest and the anti-proliferative effects are induced on a molecular level is important when developing successful therapeutic tools against cancer. 1α,25$(OH)_2D_3$ stands out as a potential anti-cancer drug, even with its severe side effect of hypercalcemia, and treating LNCaP cells with 1α,25$(OH)_2D_3$ results in an accumulation of cells in the G1 phase, growth arrest, and to some extent apoptosis. In order to get a better understanding of how this kind of action of 1α,25$(OH)_2D_3$ is regulated we have in this study made the following investigations:

First, monitor the response on JNK/SAPK complex dependent activation of AP1 to 1α,25$(OH)_2D_3$ and TNF-α in LNCaP prostate cancer cells. Secondly, decide whether 1 α,25$(OH)_2D_3$ regulates TNF-α production and release by LNCaP cells and thus have an indirect effect via the TNF-α signaling pathway on cell growth, differentiation and apoptosis, and third, evaluate the PKA dependent activation of cyclic response element binding protein (CREB) in LNCaP cells treated with 1α,25$(OH)_2D_3$.

2. Materials and methods

2.1 Cell culture

LNCaP cells were cultured in Gibco RPMI 1640 media (Invitrogen, UK). The media contained FBS (10%), PEST (1%), L-glutamine (1%), HEPES and sodium pyruvate (1%). The cells were subcultured five days after the initial culture and seeded at a density of 20 000 cells/well onto a 96-well plate (Nunc, Thermo Fischer Scientific, US) or 50 000 cells/well at a 24 well plate (Nunc, Thermo Fisher Scientific). At the point of seeding to the plate, the growth media was substituted for Opti-MEM (Invitrogen, UK) with 5% FBS and 1% NEAA without phenol-red-free and without antibiotics to prepare for transfection. The cells were incubated at 37°C and 5% CO_2 for 48 hour prior 1α,25$(OH)_2D_3$ treatment.

2.2 Transient transfection and CREB reporter assay (cAMP/PKA)/AP1 reporter assay

SureFECT™ transfection reagent and Cignal™ CREB reporter/Cignal™ AP1 reporter kit was used according to the manufacturer's protocol (SA BioSciences, USA) to monitor cAMP/PKA pathway activity and the activity of AP1-regulated transduction pathways. The CREB reporter is a viral vector based on the Cytomegalo virus (CMV) that has been rendered replication incompetent and robbed of all virulence factors. It consist of inducible firefly luciferase that response to CREB and constitutively expressed *Renilla* constructs. The reporter is designed to monitor cAMP/PKA pathway activity and together with a Dual Glo™ Luciferase Assay System (Promega, USA), it provides an easy approach to study the activity of this pathway. The AP1 reporter contains a mixture of inducible AP1-responding firefly luciferase construct and a constitutively expressing Renilla luciferase construct. The luciferase construct codes for the firefly luciferase reporter gene which is under the control of a minimal cytomegalovirus (mCMV) promoter and tandem repeats of the TPA response element. The Renilla construct codes for the Renilla luciferase reporter gene, which acts under control of a CMV. It is used as a control for normalizing transfection efficiency and for monitoring cell viability.

Briefly, in both assays, the LNCaP cells were transfected using SureENTRY transfection reagent in a 96-well plate. The LNCaP cells were seeded at the time of transfection. The medium used for the transfection was Opti-MEM serum-free culture medium. Dilutions of

the AP1/CREB reporter and the positive and negative controls were prepared as well as dilutions for SureENTRY. The cells were then washed with PBS and trypsinised and then suspended in Opti-MEM serum-free cell culture medium. The cell pellet was the resuspended in Opti-MEM cell culture medium. A haemocytometer was used to determine cell density and 10 000 cells was then seeded into each well. The Cignal reporter, negative and positive control was added to the appropriate wells and SureENTRY was added to each well and incubated for 48 hours. The cells were then treated with $1\alpha,25(OH)_2D_3$ and luminescence measurements were made for selected interval.

2.3 $1\alpha,25(OH)_2D_3$ and G-protein coupled PKA/CREB-dependent gene expression in LNCaP cells

Following incubation, cells transfected with the CREB reporter, were treated with $1\alpha,25(OH)_2D_3$ in the concentrations 10^{-7} M with or without the G-protein inhibitor Guanosine 5′-[β-thio]diphosphate trilithium (GDP-β-S; 100 μM; Sigma-Aldrich). Ethanol (0,001%) was used as control.

2.4 Luminescence measurements

Luminescence levels were measured at four time points, 24, 48, 72 and 96h in a luminometer (FLUOstar Galaxy, BMG Labtech, Germany) using the Dual Glo™ Luciferase Assay System (Promega, US). At the first interval, Dual Glo Reagent™ was prepared by mixing Dual Glo Substrate™ with Dual Glo Buffer™ at a ratio of 1:1. Stop & Glo Reagent™ was prepared by mixing Stop & Glo Substrate™ with Stop & Glo Buffer™, at a ratio of 1:100 at each time interval, before measuring activity. At each measuring interval, 75 μl of Dual Glo™ was added to the examined wells to check inducible activity, according to manufacturer's recommendations and were incubated at room temperature for 12-15 minutes. Following the Firefly luciferase reading, 75 μl of Stop & Glo Reagent™ was added to check the non-inducible luciferase activity. As with Dual Glo Reagent™, the wells were incubated for 12-15 minutes. The principle is that Firefly luciferase (Dual Glo Reagent™) is inducible, while *Renilla* luciferase (Stop & Glo Reagent™) is not. This provides a reference point to compare and normalize obtained data.

2.5 Effects of $1\alpha,25(OH)_2D_3$ and TNF-α on AP1-dependent gene expression in LNCaP cells

The cells were treated with $1\alpha,25(OH)_2D_3$ (10^{-7} M) or TNF-α (10^{-9} M) with or without 20 μM SP600125 (JNK/SAPK inhibitor (0.001% of ethanol was used as a control)). Luminescence measurements were taken after 24, 48, 72 and 96h. The experiments were repeated in triplicates.

2.6 Effects of $1\alpha,25(OH)_2D_3$ on TNF-α production in LNCaP cells

Each well of a 24 well plate was seeded with 50 000 LNCaP cells. The cells were then treated with 10^{-7} M $1\alpha,25(OH)_2D_3$ and 20 μM of the JNK/SAPK inhibitor, SP600125. As a control, 0.001% of ethanol was used. TNF-α production in the cell culture media was measured post 72 and 96 hours treatment using a commercial TNF-α specific ELISAs according to the manufacturer's instructions (Promega, USA). Each experiment was repeated three times.

2.7 Statistics

Two-way ANOVAs were performed using GraphPad Prism to evaluate the data from the assays. For the AP1 reporter assay, triplicates were used for all four time points and all treatments except for the ethanol controls, in which case duplicates were used for all time points. For the TNF-α specific ELISA four replicates were used for all the samples and duplicates for the standards, control and blank wells. The P value threshold was set to 0.05.

3. Results and discussion

Previous studies on prostate cancer cells have reported that 1α,25$(OH)_2D_3$ regulates proliferation and cell survival through membrane initiated signaling pathways (Hagberg *et al.*, 2008; Larsson *et al.*, 2008). Similar observations have been made in vitamin D responsive tissues, where membrane initiated signaling have been linked to PKA, PKC and MAPK signaling pathways (Schwartz *et al.*, 2002; Dirks-Naylor and Lennon-Edwards, 2011). This study was aimed to further elucidate membrane initiated signaling by 1α,25$(OH)_2D_3$ in LNCaP prostate cancer cells by evaluating the effects of 1α,25$(OH)_2D_3$ on AP1 and CREB-dependent gene expression as well as testing the hypothesis that 1α,25$(OH)_2D_3$ might evoke membrane initiated effects through TNF-α and TNF-α initiated signaling pathways.

3.1 Effects of 1α,25$(OH)_2D_3$ on AP1-dependent gene expression

As shown in Figure 1, 1α,25$(OH)_2D_3$ increased AP1-dependent gene expression after treatment at 48 ($p<0.05$), 72 ($p<0.001$) and 96h ($p<0.0001$) compared to control treated LNCaP cells (0.001% ethanol). The effect was time-dependent and at 96h, a difference in AP1-dependent gene expression was observed between the cells treated with 1α,25$(OH)_2D_3$ and cells treated with 1α,25$(OH)_2D_3$ + SP600125 (JNK/SAPK inhibitor)($p<0.05$). Thus, 1α,25$(OH)_2D_3$ increase AP1-dependent gene expression through the JNK/SAPK signaling pathway in LNCaP prostate cancer cells.

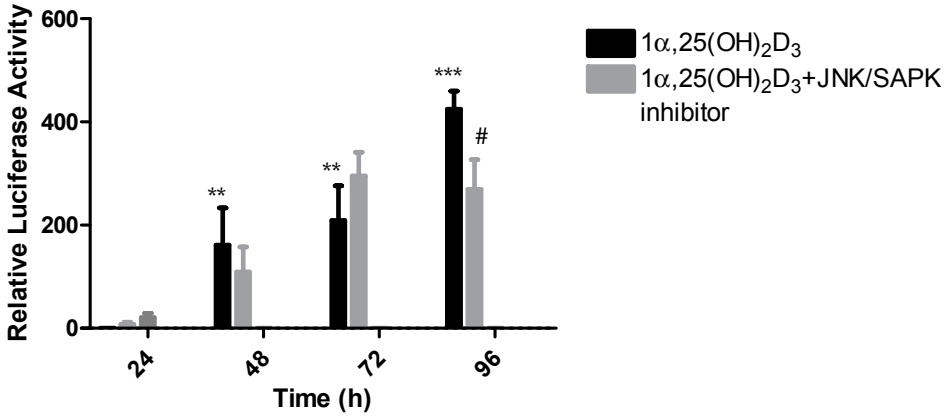

Fig. 1. Effects of 1 α,25$(OH)_2D_3$ (10^{-7} M) on AP1-dependent gene expression.

AP1-dependent gene expression was determined in LNCaP prostate cancer cells by a AP1 reporter assay (SA Biosciences) by measuring Firefly luciferase activity relative to the *Renilla* luciferase activity after treatment with 1 α,25(OH)$_2$D$_3$ (10^{-7} M) with or without SP600125 (20 μM), using ethanol as control. LNCaP cell were treated for 24, 48, 72 or 96h before measuring the AP1 activity. Data were normalised and are expressed as % of control. The level of significance was set to $p<0,05$. Data are presented as mean ± SEM

The data from this study supports previous observations in prostate cancer cells where 1α,25(OH)$_2$D$_3$ decrease cell proliferation (Larsson *et al.,* 2008) and where at least a part of the decreased proliferation has been connected an increase in the phosphorylation of JNK/SAPK and c-jun (Larsson *et al.*, 2008; Hagberg *et al.,* 2008; Karlsson *et al.,* 2010). Effects on MAPK signaling pathways by 1α,25(OH)$_2$D$_3$ is not limited to prostate cancer cells. In human myeloid leukemia HL-60 cells, Kim *et al.* (2007) 1α,25(OH)$_2$D$_3$ induced HL-60 cell differentiation through a pathway involved with PI3-K/PKC/ERK/JNK and in human osteosarcoma SaOS-2 cells (Wu *et al.,* 2007), 1α,25(OH)$_2$D$_3$ were reported to be involved in JNK/SAPK activation as well as ERK 1/2 MAPK signaling and that only sustained and not transient treatment with 1α,25(OH)$_2$D$_3$ induced AP1 activation.

3.2 Effects of TNF-α on AP1-dependent gene expression

TNF-α increased AP1-dependent gene expression after 72 ($p<0.01$) and 96h ($p<0.05$) compared to control treated LNCaP cells. The effect was not time-dependent and no differences in AP1-dependent gene expression was observed between the cells treated with TNF-α and cells treated with TNF-α + SP600125 (JNK/SAPK inhibitor) at any time-point. Thus, TNF-α increases AP1-dependent gene expression in LNCaP prostate cancer cells.

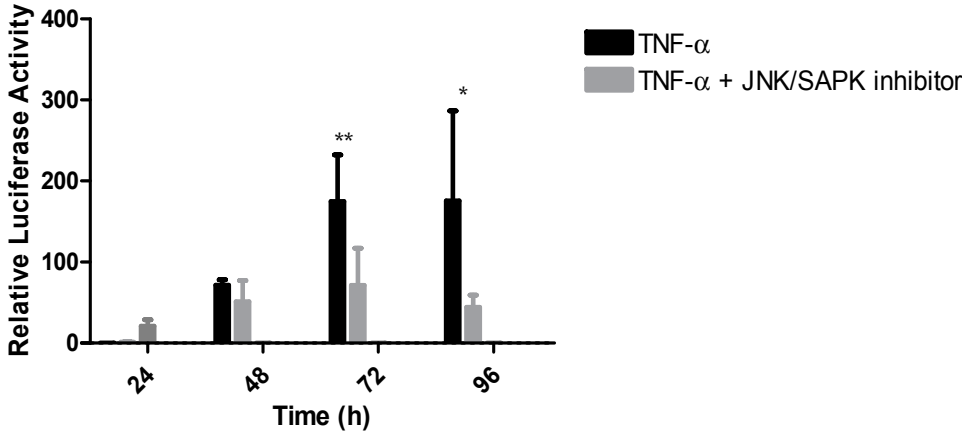

Fig. 2. Effects of TNF-α (1 nM) on AP1-dependent gene expression.

AP1-dependent gene expression was determined in LNCaP prostate cancer cells by a AP1 reporter assay (SA Biosciences) by measuring Firefly luciferase activity relative to the *Renilla* luciferase activity after treatment with TNF-α (1 nM) with or without SP600125 (20 μM), using ethanol as control. LNCaP cell were treated for 24, 48, 72 or 96h before measuring the

AP1 activity. Data were normalised and are expressed as % of control. The level of significance was set to $p<0,05$. Data are presented as mean ± SEM

The findings in the present study are in concert with reports from MIN6N8 pancreatic β-cells (Kim *et al.*, 2005) and MCF7 breast cancer cells (Yin *et al.* 2009), where the JNK/SAPK signaling pathway was reported to increase AP1 dependent gene expression. However, Yin *et al.* (2009) showed that the AP1 transactivation activity had its peak after 3 hours but was still significantly elevated after 24h. In the present study, the response in an increased AP1 activity came after 48 hours and persisted throughout the experiment. The difference in response reported in this study and by Yin *et al.* (2009) may be because of differences between breast cancer and prostate cancer cells but could also reflect that the concentration of TNF-α in experiments performed by Yin *et al.* (2009) could have been a limiting factor.

3.3 Effects of 1α,25(OH)$_2$D$_3$ on TNF-α production in LNCaP cells and TNF-α concentrations in culture media

The TNF-α specific ELISA showed that 1α,25(OH)$_2$D$_3$ did not affect the production of TNF-α in LNCaP cells and thus, does not have an effect on TNF-α signaling pathway by increased concentrations of the growth factor in the media. This suggest that TNF-α acts independently of 1α,25(OH)$_2$D$_3$ in activation of the JNK/SAPK signaling pathway. These results are consistent with the findings of Golovko *et al.* (2005), who reported that under physiological conditions, 1α,25(OH)$_2$D$_3$ does not affect the production of TNF-α, but that TNF-α mRNA expression was up-regulated by 1α,25(OH)$_2$D$_3$ as well as its analogue CB1093 in LNCaP and PC3 prostate cancer cells. Chopra *et al.* (2004) studied the role of TNF-α in regulation of growth and apoptosis in three different prostate cell lines: normal prostate epithelial cells, LNCaP cells and PC3 cells and could demonstrate that normal prostate epithelial cells and PC3 cells were resistant to growth arrest and apoptosis induced by TNF-α and LNCaP cells were highly sensitive to the growth factor. Thus, from the results in the present study as well as previous studies (Golovko *et al.*, 2005; Chopra *et al.*, 2004,) we conclude that 1α,25(OH)$_2$D$_3$ and TNF-α acts through independent pathways ending up in an up-regulation of AP1-dependent gene expression.

3.4 Effects of 1α,25(OH)$_2$D$_3$ on CREB-dependent gene expression

1α,25(OH)$_2$D$_3$ increased CREB-dependent gene expression compared to control treated LNCaP cells (Figure 3). The effect was time- and G protein-dependent where treatment with 10^{-7} M 1α,25(OH)$_2$D$_3$ increased CREB-dependent gene expression compared to cells treated with 10^{-7} M 1α,25(OH)$_2$D$_3$ + GDP-β-s (G protein inhibitor) at 24 ($p<0.05$), 48h ($p<0.0001$) but were decreased compared to the G-protein inhibited cells at 72h ($p<0.05$). Thus, 1α,25(OH)$_2$D$_3$ increases CREB-dependent gene expression through a G protein-dependent PKA/CREB signaling pathway in LNCaP prostate cancer cells.

PKA/CREB-dependent gene expression was determined in LNCaP prostate cancer cells by a CREB reporter assay (SA Biosciences) by measuring Firefly luciferase activity relative to the *Renilla* luciferase activity after treatment with 1α,25(OH)$_2$D$_3$ (10^{-7} M) with or without GDP-β-S (100 μM), using ethanol as control. LNCaP cell were treated for 24, 48, 72 or 96h before measuring the CREB activity. Data were normalised and are expressed as % of control. The level of significance was set to $p<0,05$. Data are presented as mean ± SEM

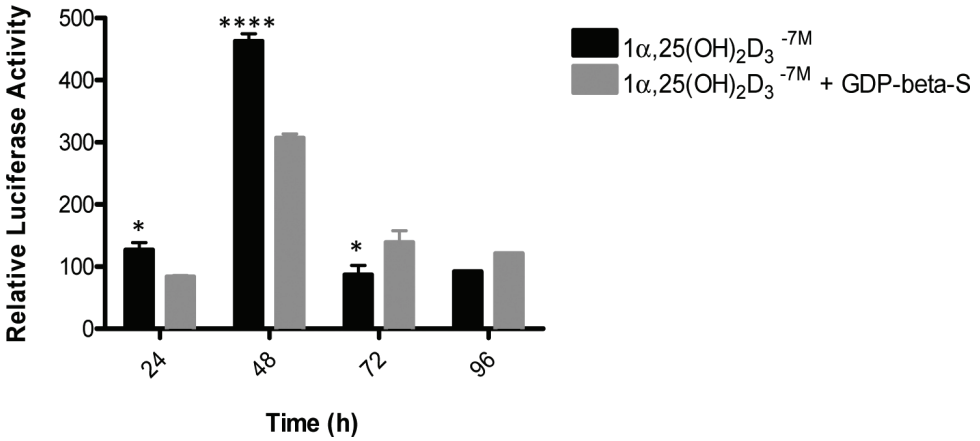

Fig. 3. Effects of $1\alpha,25(OH)_2D_3$ (10^{-7} M) on PKA/CREB-dependent gene expression.

The fact that $1\alpha,25(OH)_2D_3$ both activate JNK/SAPK and PKA/CREB-dependent gene expression indicate that $1\alpha,25(OH)_2D_3$ exert it effects through more than one pathway and mean that there might be more than one receptor that mediate the responses of this metabolite. Alternatively, the receptor could form different complexes that upon activation start unique signal cascades. An example of a similar observation is membrane initiated signaling in skeletal muscle, where six different signaling pathways have been described for $1\alpha,25(OH)_2D_3$ (Vasquez *et al.*, 1996; Capiati *et al.*, 2000; Dirks-Naylor and Lennon-Edwards, 2011). The point that there are two different vitamin D receptors (VDR, PDIA3) associated with the cell membrane (Holmen *et al.*, 2009; Karlsson *et al.*, 2010) and that PDIA3 has been suggested to form a trimer with at least three high affinity binding sites (Karlsson *et al.*, 2010) make us to postulate that depending on the docking site of $1\alpha,25(OH)_2D_3$ to the receptor, the resulting change in three dimensional structure of the hormone-receptor complex, starts a subsequent signaling cascade. The response will thus be dependent on both time and space where both short-term and long-term effect will be important in regulating prostate cell biology.

3.5 Conclusions

In conclusion, our findings support previous reports and suggest that $1\alpha,25(OH)_2D_3$ regulate prostate cell biology via multiple pathways and targeting of specific pathways for $1\alpha,25(OH)_2D_3$ might provide more effective therapies compared to the vitamin D therapies currently clinically tested and may serve as a complementary treatment in patients with androgen independent prostate cancer.

3.6 Future directives

The nature of membrane initiated signaling as a response to $1\alpha,25(OH)_2D_3$ is not yet clarified. It has been debated over the last two decades and currently there are two major candidates to be a membrane associated receptor for $1\alpha,25(OH)_2D_3$ (VDR and PDIA3). Our laboratory have for several years studied membrane initiated signaling by $1\alpha,25(OH)_2D_3$, *in silico* and *in vitro*, to elucidate signaling pathways and its key components with the goal to

clarify the biological role of the pathways in regulating prostate cancer. Focus on future work will be to create specific antagonists and agonists (the pharmacopore approach) to the putative membrane associated receptors to clarify their functions *in vitro* and hopefully get molecules that have a high specificity to single receptor binding site.

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8

Genetic Transformation and Analysis of Protein-Protein Interaction of Class B MADS-Box Genes from *Dendrobium moniliforme*

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1. Introduction

In angiosperm, flower formation has been initiated by transition of adult vegetative phase to reproductive phase under controlling of plant endogenous signals like hormone and circadian rhythm, and external factors such as photoperiod and temperature (Taiz and Zeiger, 2010). Floral organs of angiosperm are generally arranged in four whorls from outer part into inner part of a flower comprise; sepal-petal-stamen-carpel respectively. Analysis of molecular mechanism controlling flower development reveals that the formation of floral organs concerns functions of a group of transcription factors namely the ABC genes family. Coen and Meyerowitz (1991) have formulated the classical ABC model to explain function of these floral organ identity genes. Based on the classical ABC model, class A gene consists of *APETALA1* (*AP1*) and *APETALA2* (*AP2*) from *Arabidopsis*, act to specify sepal in whorl1. Combination of class A and class B genes, such as *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) in *Arabidopsis* and *DEFICIENS* (*DEF)* and *GLOBOSA* (*GLO)* in *Antirrhinum,* is necessary for petal and stamen development in whorls 2 and 3, respectively. A combination of class B and class C genes including *AGAMOUS* (*AG*) in *Arabidopsis* controls stamen development in whorl 3. The class C gene alone specifies carpel development in the forth floral whorl.

During the timeline of evolution, duplication event has once occurred in the ancestral B genes and gave rise of two gene lineages includes AP3/DEF and PI/GLO (Goto and Myerowitz, 1994; Jack et al., 1994 and Kramer et al., 1998). The second duplication has taken place in the *AP3* lineages causes separation of the B function genes into three sub-clades; eu*AP3* clade generally presents in higher eudicots, paleo *AP3* clade exists in lower eudicots, magnolid dicot, monocot and basal angiosperm (Kramer and Irish, 2000), and an additional sub-clade of paleo *AP3* named *TOMATO MADS BOX GENE6* (*TM6*) (Pnueli et al., 1991, Yu et al., 1999 and Hsu and Yang, 2002). Expression pattern and function of genes in the *TM6*-

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lineage are diverse than other class B gene lineages. In petunia, expression of *Petunia TM6* was detected in whorl 3 and 4 of flower and it plays a role like function of the C-class gene (Rijpkema et al., 2006). Recently, numerous of the class B genes have been widely proved for gene expression pattern and functions in several flowering plant species. In Orchidaceae, at least four *AP3/DEF*-like and one *PI/GLO*-like class B genes have been reported to contribute the developmental mechanism of perianth and reproductive organ development.

2. Molecular mechanism regulating orchids floral morphogenesis

Orchidaceae is one of the largest families in angiosperm containing more than 24,000 plant species (Fay and Chase, 2009). Flowers have been admired by people due to the great diversity of flower color and morphology. An orchid flower is usually comprised of 3 types of perianth arrange in 4 floral whorls include whorl 1 of three outer tepals (tepal is a technical term for sepal and petal that the floral morphology is similar), whorl 2 of inner tepals and whorl 3 and 4 of `gynostemium' or `column' which is a reproductive organ where the male and female are fused into a single unit. One tepal in the second floral whorl called `labellum' or 'lip' is typically large and colorful, which provides for trapping insect to help pollination. Another two inner petals are usually smaller than the lip and have morphologically similar structure of the three outer tepals.

Similarity between sepal and petal morphology is a dominant character in non-grass monocotyledonous plants such as lily, tulip, asparagus and orchids. van Tunen et al. (1993) has proposed a modified ABC model to explain the similarity of outer and inner tepals in tulip which is due to expanded expression of class B genes to the first floral whorl. The hypothesis has been confirmed by Kanno et al. (2003) that the identical of outer and inner tepals formation of tulip concerns regulatory mechanisms of two *DEF*- and *GLO*-like genes, in which all genes are expressed in both of the first and second floral whorls.

Formation of all type of perianth in orchid flower is regulated by the two lineages of class B-function genes: *AP3/DEF*-like and *PI/GLO*-like lineage associated with the E-function genes (Xu et. al., 2006). While only a *DEF*-like gene generally present in genome of dicotyledonous plants, at least four *DEF*-like genes have been detected in genome of *Phalaenopsis* orchids (Tsai et al., 2004). Therefore the chance to form heterodimeric interaction between *DEF*-like gene and *GLO*-like gene in orchid genome may help to generate different morphology of the petaloid organs.

Recently, several *DEF*- and *GLO*-like genes were isolated from orchid species. Given the *DEF*- and *GLO*-like genes phylogenic analysis, orchid *DEF*-like genes are the paleo *AP3* type. Mondrago´n-Palomino and Theißen (2008, 2009 and 2011) have formulated the 'orchid code' to explain orchid's perianth formation. In the orchid code, there are four clades of *DEF*-like and a clade of *GLO*-like lineage. Based on overall current research of *DEF*-like genes, clade 1 contains *PeMADS2*-like genes, including *PeMADS2* (*Phalaenopsis equestris*), *DcOAP3A* (*Dendrobium crumenatum*) and *OMADS5* (*Oncidium* Gower Ramsey). Clade 2 consists of *OMADS3*-like genes, including *OMADS3* (*O.* Gower Ramsey) and *PeMADS5* (*P. equestris*). Clade 3 contains *PeMADS3*-like genes, including *PeMADS3* (*P. equestris*), *DcOAP3B* (*D. crumenatum*) and *HrDEF* (*Habenaria radiata*). Clade 4 contains *PeMADS4*-like genes such as *PeMADS4* (*P. equestris*).

Results from phylogenic and gene expression analysis suggested that another gene duplication may be taken placed within the group of paleo *AP3*-like genes of orchid and

cause partition into two sister clades. Clades 1 and 2 are considered as a first sister clade in which gene expression is detected in both of outer and inner tepal formation. Clade 3 and 4 are another sister clade specifying only inner tepal development, but excluding outer tepal (Mondrago´n-Palomino and Theißen 2009, 2011).

In the *GLO*-like lineage, only one *GLO*-like gene was found in most of orchid genomes. However *H. radiata* has two *GLO*-like genes (Kim et al., 2007). Orchid *GLO*-like gene expression in all floral whorls of outer and inner tepals (Xu et al., 2006, Kim et al., 2007 and Sirisawat et al., 2010).

3. Isolation of class B MADS-box genes from genome of *Dendrobium moniliforme*

Dendrobium is a huge genus of orchid containing more than 1,200 species (Adams, 2011). Most of species are epiphytic and occasionally lithophytic. Flower of *Dendrobium* orchids usually has 3 outer tepals, 3 inner tepals in which one of them has developed to be labellum or lip that usually large, colorful to trap for pollination purpose. Female and male reproductive organ of *Dendrobium* orchid has fused as a single unit called column.

Dendrobium moniliforme or "SEKKOKU" in Japanese is a native orchid of Japan, and historical story about this orchid has been recorded since the Edo period. This species have a great variety of floral mutant phenotypes such as the double-petal mutant which lip has conversed to be normal petal, and the peloric mutant which flower has 3 outer tepals and 3 lips replaced of normal inner tepals (Figure 1). Therefore *D. moniliforme* serves as a good source for genetic analysis of floral organ identity. Perianth morphology of the double-petal mutant is similar to flower of *Apostasia* orchid, a genus of primitive orchid which has very simple gynostemium. Perianth forms in dimensional symmetry in which outer and inner tepals are similar in shape and color. This symmetrical flower is supposed to be a character of ancestral orchids (Mondrago´n-Palomino and Theißen 2008). Since lip development requires more heterodimeric complex between the *DEF*-and *GLO*-like genes than petal in *Phalaenopsis* (Tsai et al., 2004), lip is considered as an elevated perianth organ during evolution of orchid flower. Based on this hypothesis, the peloric mutant of *D. moniliforme* may be developed after the wild-type one.

Recently, at least 17 floral organ identity genes were isolated from several species of *Dendrobium* orchids comprising *D.* Madame Thong-In, *D. thyrsiflorum* (Reichb. f.), *D. crumenatum* and *D. moniliforme* (Yu and Goh, 2000, Skipper et al., 2006, Xu et al., 2006, Sirisawat et al., 2009, Sirisawat et al., 2010). Seven from 12 genes are members of class B MADS-box genes named *DcOAP3A, DMAP3A*, *DcOAP3B*, *DMAP3B*, *DMMADS4, DcOPI* and *DMPI* (Table 1).

In *D. moniliforme*, three paleo *AP3*-like and one *PI*-like genes were isolated and classified (Sirisawat et al., 2009 and 2010). Phylogenetic analysis using amino acid sequences showed that *DMAP3A* was a member of clade 1 *PeMADS2*-like genes and which was 96 and 88% identical to *DcOAP3A* and *PeMADS2,* respectively. *DMAP3B* was clustered in the clade 3 of *PeMADS3*-like genes and which was 96 and 88% identical to *DcOAP3A* and *PeMADS3* respectively. As a representative of *PeMADS4*-like genes in *Dendrobium* orchid, *DMMADS4* showed 87% identical to *PeMADS4* which belonged to clade 4 of *AP3/DEF*-like lineage of orchid (Figure 2).

Apart from *Habenaria radiata* which has two *GLO*-like genes, most of orchids have only one *GLO*-like gene in genome and the sequences are greatly similar. In particular, *DMPI* showed 96 and 93% identical to *PeMADS6* and *HrGLO2,* respectively (Figure 3). The great similarity of *GLO*-like sequences among *Orchidaceae* (more than 90%) indicated that functions of the group genes may also be highly conserve.

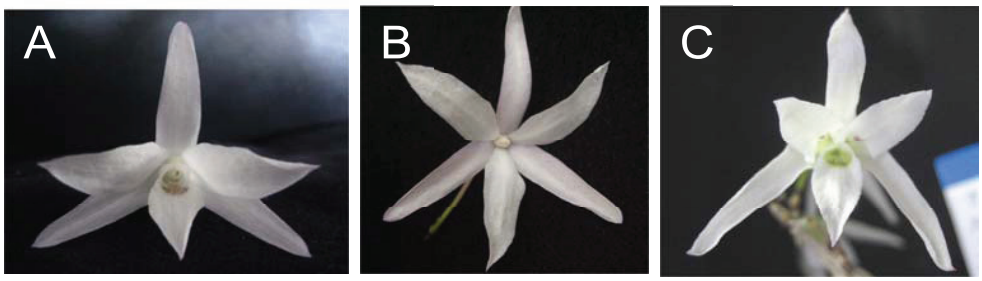

Fig. 1. Flower morphology of *Dendrobium moniliforme* wild-type (A), the double petal mutants (B) and the peloric mutant (C).

Class	Gene	Species	Sub Family	Accession No.	Reference
A	*DOMADS1*	*Dendrobium* Madame Thong-In	*AP1/AGL9*	AF198174	Yu and Goh, 2000
	DOMADS2	*D.* Madame Thong-In	*AP1/AGL9*	AF198175	Yu and Goh, 2000
	DOMADS3	*D.* Madame Thong-In	*AP1/AGL9*	AF198176	Yu and Goh, 2000
	*DthyrFL*1	*D. thyrsiflorum*	*AP1/FUL*	AY927236	Skipper et al., 2005
	*DthyrFL*2	*D. thyrsiflorum*	*AP1/FUL*	AY927237	Skipper et al., 2005
	*DthyrFL*3	*D. thyrsiflorum*	*AP1/FUL*	AY927238	Skipper et al., 2005
	DcOAP2	*D. crumenatum*	*AP2*	DQ119837	Xu et al., 2006
B	*DcOAP3A*	*D. crumenatum*	*AP3/DEF*	DQ119838	Xu et al., 2006
	DcOAP3B	*D. crumenatum*	*AP3/DEF*	DQ119839	Xu et al., 2006
	DMAP3A	*D. moniliforme*	*AP3/DEF*	EU056327	Sirisawat et al., 2010
	DMAP3B	*D. moniliforme*	*AP3/DEF*	EU056328	Sirisawat et al., 2010
	DMMADS4	*D. moniliforme*	*AP3/DEF*	GU132995	Sirisawat et al., 2009
	DcOPI	*D. crumenatum*	*PI/GLO*	DQ119840	Xu et al., 2006
	DMPI	*D. moniliforme*	*PI/GLO*	EU056326	Sirisawat et al., 2010
C	*DcOAG1*	*D. crumenatum*	*AG*	DQ119841	Xu et al., 2006
D	*DcOAG2*	*D. crumenatum*	*AG*	DQ119842	Xu et al., 2006
E	*DcOSEP1*	*D. crumenatum*	*SEP*	DQ119843	Xu et al., 2006

Table 1. List of floral organ identity genes isolated from genus *Dendrobium*

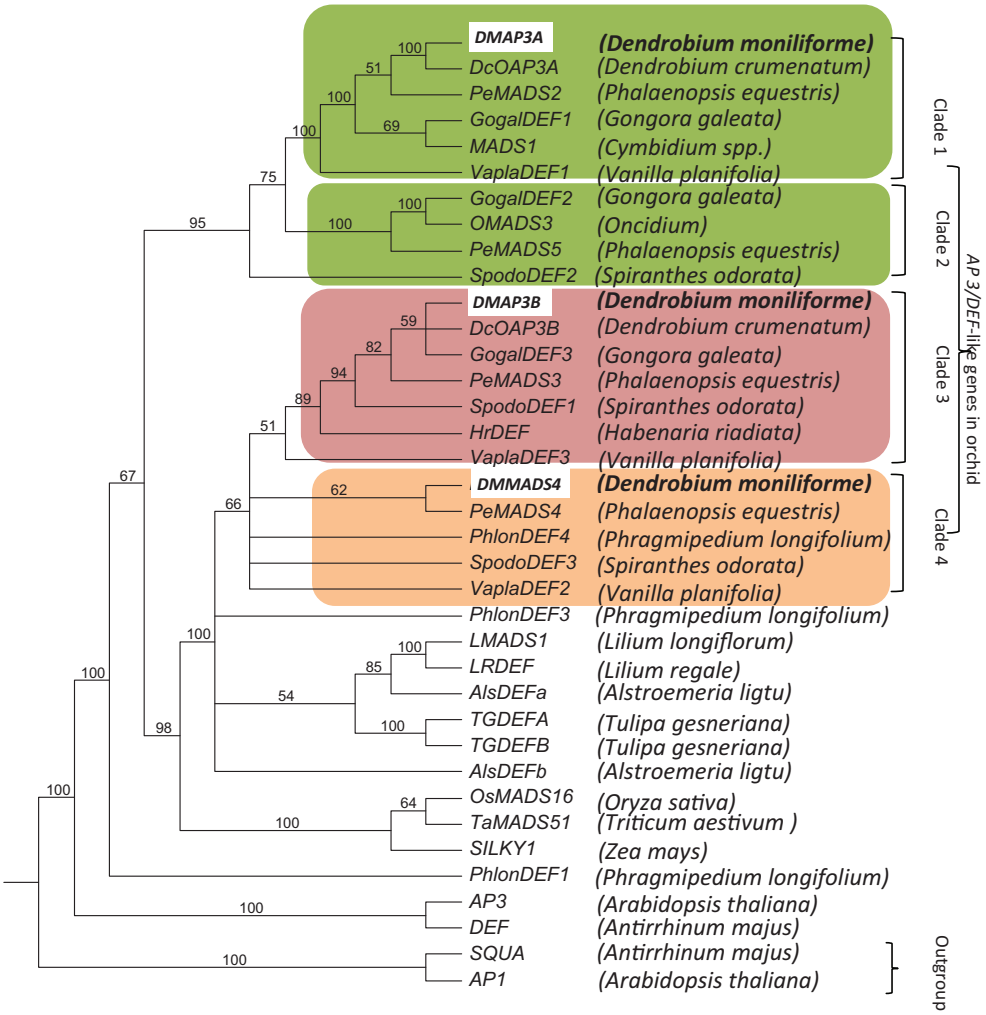

Fig. 2. The most parsimonious tree of *AP3/DEF*-like genes in plants based on an alignment of the full-length amino acid sequence using UPGMA method. Numbers are bootstrap values after 100 replicate runs. The four orchid *AP3/DEF* like clades were indicated by brackets. The B-class sequences isolated from *D. moniliforme* in this study were bolded. Two class A MADS-box genes were used as outgroup.

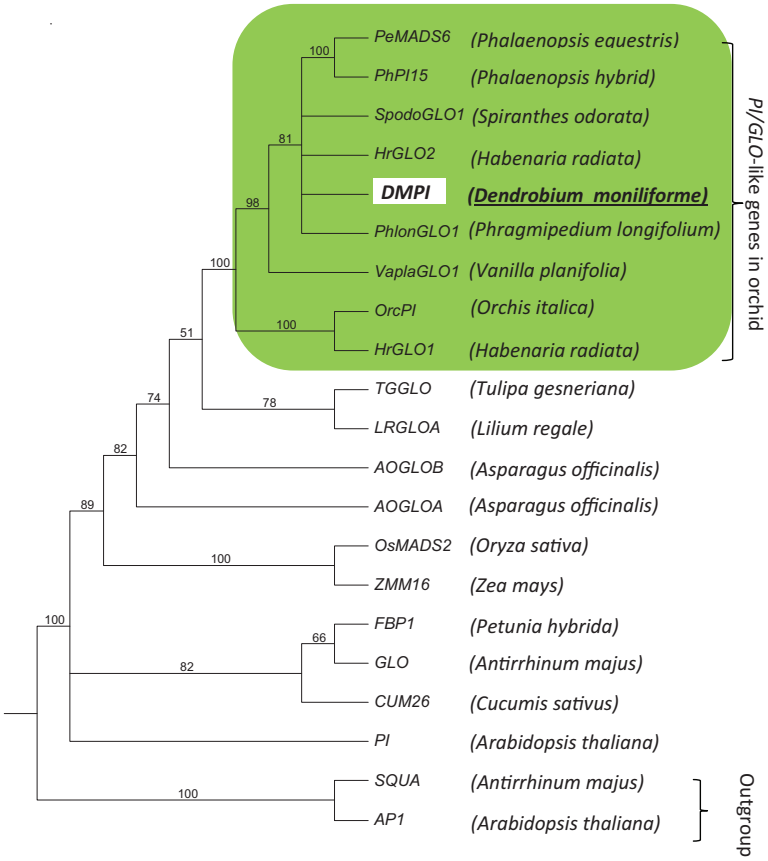

Fig. 3. The most parsimonious tree of *PI/GLO*-like genes in plants based on an alignment of the full-length amino acid sequence using UPGMA method. Numbers are bootstrap values after 100 replicate runs. The *PI/GLO*-like genes from orchid were indicated by brackets. The B-class sequences isolated from *D. moniliforme* in this study were boxed. Two class A MADS-box genes were used as outgroup.

4. Expression analysis of orchid class B-function genes by RT-PCR and Quantitative real-time PCR

Expression of *AP3/DEF*- and *PI/GLO*-like class B genes in angiosperm is usually detected in the second and third floral whorls of petal and stamen, respectively. Formation of double petals is a typical character in basal angiosperm and monocot plants. In non-grass monocots, expanded expression of the B function genes to the first floral whorl was found in tulip (Kanno et al., 2003), *Habenaria radiata* (Kim et al., 2007) , *D. crumenatum* (Xu et al., 2006) and *P. equestris* (Tsai et al., 2004) which contributes the petaloid-sepal formation in the first floral whorl in those plants.

Diverse expression pattern of *DEF*-like genes was found in orchid (Table 2). The combinational interaction between orchid *DEF*-and *GLO*-like genes is associated with distinct morphology of

all perianth organs such as outer tepals, two inner tepals and lip in orchid. In particular, expression of *GLO*-like genes is strongly detected in all floral whorls and highly conserved gene expression pattern is shown throughout *Orchidaceae* (Table 2). Similar to expression pattern of *GLO*-like genes, *DEF*-like genes in the clade 1 and 2 are strongly expressed in both of whorl 1 and whorl 2, whereas the signals of *DEF*-like genes of clade 3 and clade 4 are not detected in whorl 1 (Table 2). The results suggest that development of outer tepals in whorl1 of orchid is regulated by combinational interaction between *DEF*- and *GLO*-like genes of clade 1 and 2 (Mondrago´n-Palomino and Theißen, 2011).

Sub family	Gene name	Species	Sepal	Petal	Lip	Column	Ovary
AP3/DEF							
Clade 1	*DcOAP3A*[a]	*Dendrobium crumenatum*	+++	+++	+++	+++	ND
	DMAP3A[b]	*Dendrobium moniliforme*	+++	+++	+++	+++	++
	PeMADS2[c]	*Phalaenopsis equestris*	+++	+++	+	+	ND
	PhlonDEF2[d]	*Phragmipedium longifolium*	+++	+++	+	+++	++
	OMADS5[e]	*Oncidium* Gower Ramsey	+++	+++	++	ND	ND
	VaplaDEF1[d]	*Vanila planifolia*	++	++	+	+++	+
Clade 2	*OMADS3*[f]	*Oncidium* Gower Ramsey	+++	+++	+++	ND	ND
	PeMADS5[c]	*Phalaenopsis equestris*	++	+++	++	+	ND
	PhlonDEF1[d]	*Phragmipedium longifolium*	+++	+++	+	++	++
Clade 3	*DcOAP3B*[a]	*Dendrobium crumenatum*	-	+++	+++	+++	ND
	DMAP3B[b]	*Dendrobium moniliforme*	-	+++	+++	+++	-
	HrDEF[g]	*Habenaria radiata*	-	+++	+++	+	ND
	OMADS9[e]	*Oncidium* Gower Ramsey	-	+++	+++	ND	ND
	PeMADS3[c]	*Phalaenopsis equestris*	-	+++	+++	+	ND
	PhlonDEF3[d]	*Phragmipedium longifolium*	-	+++	+++	++	-
	VaplaDEF3[d]	*Vanila planifolia*	-	+++	+++	+++	-
Clade 4	*DMMADS4*[h]	*Dendrobium moniliforme*	-	+++	+++	+++	+++
	PeMADS4[c]	*Phalaenopsis equestris*	-	-	+++	+++	ND
	PhlonDEF4[d]	*Phragmipedium longifolium*	-	++	++	++	-
	VaplaDEF2[d]	*Vanila planifolia*	-	++	++	++	+
PI/GLO							
	DcOPI[a]	*Dendrobium crumenatum*	+++	+++	+++	+++	ND
	DMPI[b]	*Dendrobium moniliforme*	+++	+++	+++	+++	+++
	HrGLO1[g]	*Habenaria radiata*	+++	+++	+++	+++	ND
	HrGLO2[g]	*Habenaria radiata*	++	+++	+++	+	ND
	OMADS8[e]	*Oncidium* Gower Ramsey	+++	+++	+++	ND	ND
	PeMADS6[i]	*Phalaenopsis equestris*	+++	+++	+++	+++	+++
	PhlonGLO[d]	*Phragmipedium longifolium*	+++	+++	+++	+++	++
	VaplaGLO[d]	*Vanila planifolia*	++	++	++	++	++

The - sign indicates non of gene expression, + sign indicate level of gene expression, ND indicates data is not applicable.
[a]Data from Xu et al., 2006, [b]Data from Sirisawat et al., 2010, [c]Data from Tsai et al., 2004, [d]Data from Mondragón-Palomino and Theißen, 2011, [e]Data from Chang et al., 2010, [f]Data from Hsu and Yang et al., 2002, [g]Data from Kim et al., 2005, [h]Data from Sirisawat et al., 2009, [i] Data from Tsai et al., 2005.

Table 2. Summary of expression patterns of *AP3/DEF*-like and *PI/GLO*-like genes during the development of flower buds in different orchid tissues.

Strong expression of *PeMADS4*, a clade 4 *DEF*-like gene, in lip of orchid flowers indicates that this gene is a key gene to specify lip formation. However, the expression of other genes in the clade 4 such as *DMMADS4 (D. moniliforme)*, *PhlonDEF4 (Phragmipedium longifolium)* and *VaplaDEF2 (Vanilla planifolia*) is found in both whorls of petal and lip similar to

expression pattern of the clade 3 *DEF*-like genes (Table 2). This result suggests that the clade 3 and 4 *DEF*-like genes regulated both of petal and lip development. In particular, clade 3 and 4 *DEF*-like genes from *P. longifolium* and *V. planifolia* were strongly expressed in labellum rather than in petals (Mondrago´n-Palomino and Theißen, 2011). Additionally, expression of the four clades *DEF*-and *GLO*-like genes also associate with development of reproductive organs in the whorl 3 and 4 floral whorls.

Modulated signal of some *DEF*- and *GLO*-like gene expression was also found in immature ovary of orchid flower. Generally, ovary development in angiosperm is regulated by function of class D MADS-box genes (Lopez-Dee et al. 1999; Favaro et al. 2002), however molecular mechanism of class B MADS-genes related to ovary development is not well understood. In *Phalaenopsis*, expression of *PeMADS6*, a *GLO*-like gene, was strongly detected in immature ovary and the signal was decreased after pollination. Therefore expression of class B MADS-box genes in ovary is supposed to be regulated by pollination (Tsai et al., 2005).

5. Analysis of protein-protein interactions by the yeast 2-hybrid system

Yeast two-hybrid screening is a method to examine interaction between protein-protein, protein-DNA by detecting binding property of the protein-protein, protein-DNA in yeast cells. Heterodimer formations between AP3/DEF- and PI/GLO- like proteins are required for function of class B MADS-box genes in angiosperm. In *Arabidopsis* and *Antirrhinum*, AP3/DEF- like proteins needs to make heterodimer with the PI/GLO-like proteins to function accurately as transcriptional regulators (Schwarz-Sommer et al. 1992, Honma and Goto 2001, Immink et al., 2003).

Homodimeric formation of protein is supposed to be primarily characteristic of the class B floral homeotic proteins. In the gymnosperm *Gnetum gnemon,* GGM2 is able to bind DNA in a sequence-specific manner as a homodimer *(*Winter et al., 2002). In angiosperm, there are three sub-lineage of AP3/DEF-like proteins include paleo AP3, TM6 and euAP3 lineage. Some paleo AP3- and TM6-like proteins maintain homodimeric configuration, however most of protein in euAP3 lineage that generally present in dicotyledonous plants lost the ability of homodimeric formation and they need forming heterodimer with protein in the PI lineage (Winter et al., 2002, Hsu and Yang, 2002).

In non grass monocots, some class B function proteins are able to make homodimeric formation such as LMADS1 (*Lilium longiflorum*) (Tzeng and Yang, 2001) and TGGLO (*Tulipa gesneriana*) (Kanno et al., 2003). To confirm suspected interactions of DEF-and GLO-like proteins in *D. moniliforme*, we performed the Matchmaker Two-Hybrid assay (Clontech Co. Ltd.) according to the supplier's instruction. The entire coding sequence of *DMMADS4* gene was ligated to the pGBKT binding domain vector (pGBKT-DMMADS4) or the pGADT7 activation domain vector (pGADT7-DMMADS4). In the same way, the PCR fragments of *DMPI* were ligated to the pGBKT binding domain vector (pGBKT-DMPI) or the pGADT7 activation domain vector (pGADT7-DMPI). Several possible protein-protein interaction of the DMMADS4 and DMPI were screened by cotransformation to yeast using the lithium acetate method (Gietz et al., 1992). The transformants were selected on selection medium and then analyzed for the β- galactosidase activity. The results showed that the DMMADS4 was strongly interacted with the DMPI as heterodimer formation. Additionally, DMMADS4 is able to form homodimer weakly, whereas DMPI could not make the homodimeric interaction (Figure 4).

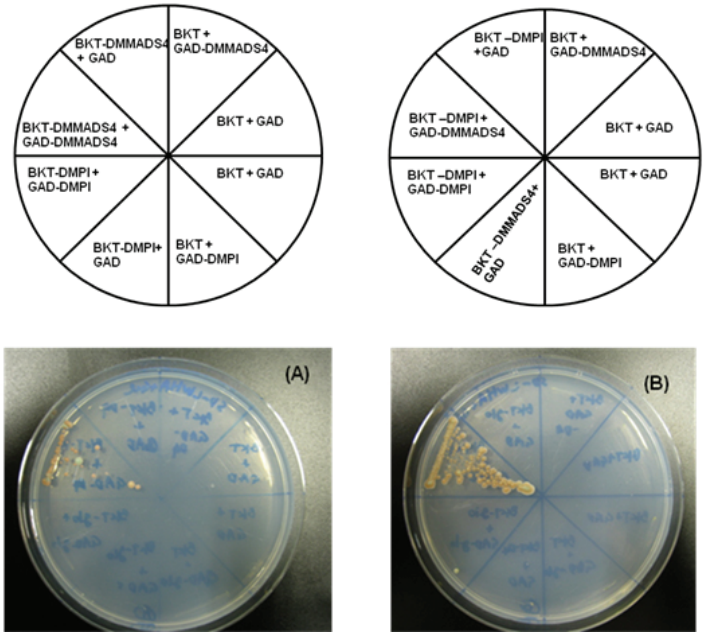

Fig. 4. Homodimeric interaction between pGBKT-DMMADS4 and pGADT-DMMADS4 (A) and heterodimeric interaction between pGBKT-DMMADS4 and pGADT-DMPI (B) (Sirisawat et al., 2009). Strong signal was detected when pGBKT-DMMADS4 and pGADT-DMPI form as heterodimer.

Sub family	Protein name	Species	Homodimer formation	Heterodimer formation with *PI/GLO*	Reference
AP3/DEF					
Clade 1	DcOAP3A	*Dendrobiun crumenatum*	-	+++ (DcOPI)	Xu et al., 2006
	PeMADS2	*Phalaenopsis equestris*	-	++ (PeMADS6)	Tsai et al., 2008
	OMADS5	*Oncidium* Gower Ramsey	++	- (OMADS8)	Chang et al., 2010
Clade 2	OMADS3	*Oncidium* Gower Ramsey	+	++ (OMADS8)	Chang et al., 2010
	PeMADS5	*Phalaenopsis equestris*	-	+ (PeMADS6)	Tsai et al., 2008
Clade 3	DcOAP3B	*Dendrobium crumenatum*	-	+++ (DcOPI)	Xu et al., 2006
	OMADS9	*Oncidium* Gower Ramsey	++	- (OMADS8)	Chang et al., 2010
	PeMADS3	*Phalaenopsis equestris*	-	+++ (PeMADS6)	Tsai et al., 2008
Clade 4	DMMADS4	*Dendrobium moniliforme*	+	+++ (DMPI)	Sirisawat et. al., 2009
	PeMADS4	*Phalaenopsis equestris*	++	+++ (PeMADS6)	Tsai et al., 2008
Sub family	**Protein name**	**Species**	**Homodimer formation**	**Heterodimer formation with *AP3/DEF***	**Reference**
PI/GLO					
	DcOPI	*Dendrobium crumenatum*	-	+++ (DMOAP3A)	Xu et al., 2006
	DMPI	*Dendrobium moniliforme*	-	+++ (DMMADS4)	Sirisawat et al., 2009
	OMADS8	*Oncidium* Gower Ramsey	-	++ (OMADS3)	Chang et al., 2010
	PeMADS6	*Phalaenopsis equestris*	+	+++ (PeMADS3)	Tsai et al., 2008

The – sign indicates no interaction between two proteins, + sign indicates level of protein-protein interaction

Table 3. Protein-protein interaction of class B MADS-box protein in *Orchidaceae*

Similar to other flowering plants, most of DEF- like proteins in orchid need to make heterodimer with the GLO-like protein to initiate flower organ development. Strong heterodimeric signal between the four clade DEF-and GLO-like proteins was detected in *P. equestris*, *D. crumenatum* and *D. moniliforme* (Table 3). In clade 4 DEF-like proteins, although PeMADS4 or DMMADS4 is able to make homodimer, a stronger signal was detected when the protein form heterodimer with the orchid GLO-like protein. Interestingly, the clade 1 and 3 DEF-like proteins of *O.* Grower Ramsey have also retained the ancestral characteristic of the B-MADS box protein since they are able to form as homodimer (Table 3). Several possibility of heterodimeric interaction between the DEF- and GLO-like protein may be help to improve the orchid flower diversity.

6. *Agrobacterium* mediated-transformation of class B MADS-box genes from *D. moniliforme* to *Arabidopsis*

Genetic transformation is a general molecular basis to learn functions of genes. In orchids, genetic transformation usually limit by problems of low transformation efficiency, extensive regeneration time due to long juvenile period of orchid and labor consumption to generate transgenic plants. As the results, most of orchid class B MADS-box genes were clarified their function by ectopically expressed in *Arabidopsis.* Additionally, rescue of *Arabidopsis* mutant phenotype is another way to know orchid gene function.

Ectopic expression of *AP3* in *Arabidopsis* causes conversion of carpel to stamen-like structures in whorl 4 (Jack et al., 1994). In orchids, most of *AP3/DEF*-like genes are paleo *AP3*-type in which the sequence is greatly different from *Arabidopsis AP3,* therefore over-expressing of several paleo *AP3*-like genes from orchids such as *OMADS3* (*Oncidium*), *DcOAP3A* (*D. crumenatum*), *DMAP3B* (*D. moniliforme*) in *Arabidopsis* do not affect the wild-type floral morphology (Hsu and Yang, 2002, Xu et al., 2006, Sirisawat et al., 2010). In contrast to *AP3/DEF*-like lineage, conserved function of the *PI/GLO*-like genes was found between *Arabidopsis* and orchids since ectopic expression of *PI/GLO*-like genes from several species of orchids, including *DcOPI*, *DMPI*, *PeMADS6* and *OMADS8,* causes partial transformation of sepal to petal-like organs in the first floral whorl (Figure 5A). This suggests that function of ancestral *PI/GLO*-like genes is slightly developing during the evolution of angiosperm.

In *Arabidopsis*, ectopically expressing *AP3/PI* caused transition of the whorl 1 sepal to be petal and the whorl 4 carpel to be stamen (Jack et al., 1994), therefore heterodimeric formation of *AP3* and *PI* play role to control petal and stamen development. In *D. moniliforme*, although the *DMMADS4* and *DMAP3B* were isolated from *D. moniliforme* and had the similar gene expression pattern, DNA sequence of *DMMADS4* was more related to *PeMADS4 (P. equestris*) than *DMAP3B*. Therefore, the *DMMADS4* is considered to be a member of the clade 4 *PeMADS4*-like genes rather than group of *DMAP3B* (Figure 2). Since functional analysis of orchid clade 4 *DEF*-like genes has not been confirmed in *Orchidaceae*, we over-expressed the *DMMADS4* in *Arabidopsis* and verify whether it can make heterodimeric interaction to regulate flower organ identity with the *DMPI*, a PI/GLO-like protein of *D. moniliforme*. F1 populations were generated by crossing the 35S::*DMMADS4* with the 35S::*DMPI*. The results showed that plants of 35S::*DMMADS4* have the same floral characteristics with wild-type *Arabidopsis*. Progenies derived from crossing population between 35S::*DMMADS4* and 35S::*DMPI* showed the phenotype resembling to *Arabidopsis* plants over-expressing *AP3/PI* (Krizek and Meyerowitz, 1996) in which the sepal in whorl 1converted into petal-like organs resulting in double petal formation (Figure 5B). Scanning electron microscope has been performed to verify differentiation between epidermal cell morphology between flower organ in whorl 1

and whorl 2 of the wild-type and transgenic plants. The results showed that the epidermal cells at the adaxial surface of the petaloid sepals in 35S::*DMMADS4*/35S::*DMPI* were similar to the epidermal cells at the base of petals (Figure 6). Thus heterodimer formation of *DMMADS4* and *DMPI* play roles in regulating the development of petals.

Unlike transgenic *Arabidopsis* plants ectopically expressing *AP3/PI* in which the number of stamens was increased due to the addition of a stamen in whorl 4, in 35S::*DMMADS4*/35S::*DMPI* plants the number of stamens in whorl 3 was equal to that in wild-type (6 stamens), and no carpel-to-stamen conversion was noted in whorl 4. However, the carpels and ovaries of the transgenic plants were poorly developed; leading to the production of short and rough siliques compared to wild type (Figure 7D). Additionally, the seeds within the siliques of the 35S::*DMMADS4*/35S::*DMPI* plants were tightly packed (Figure 7D), and the number of seeds per silique in the 35S::*DMMADS4*/35S::*DMPI* plants was reduced (data not shown), compared with wild-type plants. However, the seeds were fertile and had the same characteristics as the seeds of the wild-type plants.

Functional analysis of clade 3 and 4 *DEF*-like genes; *DMAP3B* and *DMMADS4* from *D. moniliforme* in *Arabidopsis* revealed that both genes are functional homology in order to control petal formation and regulation of ovary development (Figure 5B, 7A,7C, 7D). As the transgenic *Arabidopsis* obtains *DMAP3B/DMPI* or *DMMADS4/DMPI* showed indistinguishable phenotype of stamen and carpel compared to the wild-type plants, it is unclear how *DMAP3B/DMPI* and *DMMADS4/DMPI* are involved in stamen and carpel development. Because the male (stamen) and female (carpel) reproductive organs in orchids are fused together into a single unit named column, most of class-B genes are expressed in this organ. The functions of class-B proteins as they relate to column development should be clarified in further study.

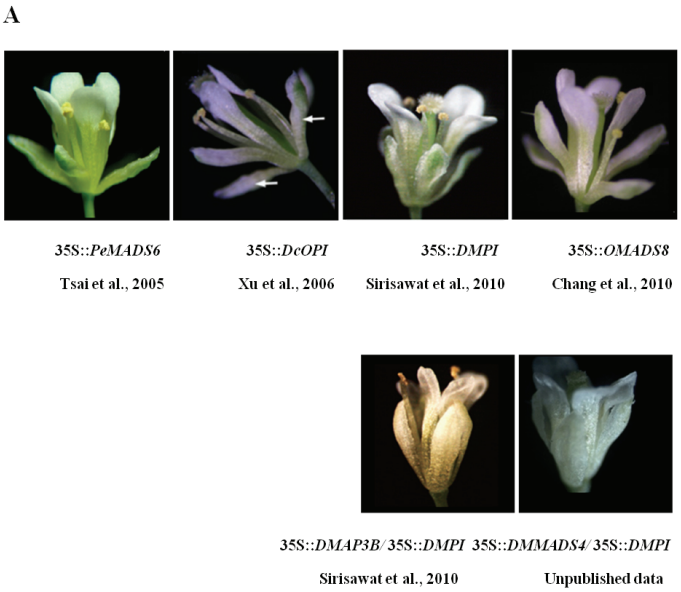

Fig. 5. Phenotypes of transgenic *Arabidopsis* overexpressing class B MADS-box genes from orchids

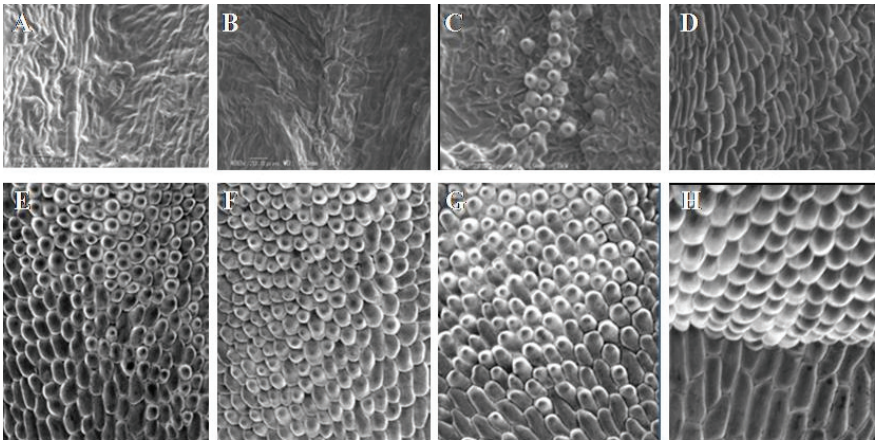

Fig. 6. Cell morphology of sepal and petal in wild-type, 35S::*DMMADS4*, 35S::*DMPI* and 35S::*DMMADS4*/35S::*DMPI*. Adaxial surface of sepal in 35S::*DMMADS4* (B) was similar to the wild-type sepal (A). Rounded cells were detected at the adaxial surface of petal in wild-type (E), 35S::*DMMADS4* (F), 35S::*DMPI* (G) and 35S::*DMMADS4*/35S::*DMPI* (H). The rounded cells were also presented at central region of the petaloid-sepal in 35S::*DMPI* plants (C). In 35S::*DMMADS4*/35S::*DMPI*, elongated cells of petal were observed over the entire area of the petaloid-sepal (D).

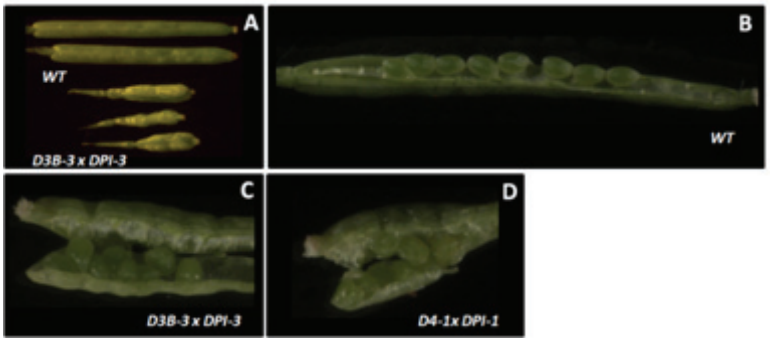

Fig. 7. Siliques of transgenic *Arabidopsis* overexpresing 35S::*DMAP3B*/35S:: *DMPI* (A, C), and 35S::*DMMADS4*/35S:: *DMPI* (D), and wild-type plants (B).

7. Conclusions

Three paleo *AP3*-like genes; *DMAP3A*, *DMAP3B* and *DMMADS4* and a *PI*-like genes; *DMPI* were isolated from *D. moniliforme* and clarified for gene expression pattern, protein-protein interaction and the gene functions. The results showed that all four genes have similar expression patterns to their homolog from other orchids and most of them need to make heterodimeric interaction to drive their transcriptional output. Functional analysis of a clade 4-paleo *AP3*-like genes; *DMMADS4* in *Arabidopsis* showed that it has functional homology with the clade 3 orchid paleo *AP3*-like genes (Xu et.al, 2006 and Sirisawat et.al.,2010) and also has highly conserved function to *Arabidopsis AP3* in controlling of petal formation.

Based on a systematic reviews drawing together with the results of several research studies on floral organ identity genes in orchids, Mondrago´n-Palomino and Theißen (2008-9, 2011) has defined 'orchid code' to explain molecular mechanism underlying orchid flower formation. In the 'orchid code', a duplication event is suggested to occur to an ancestral paleo *AP3*-like gene of orchid, this give rise of two sister clades in which the first sister clade consists of clade 1 and 2 paleo *AP3*-like genes and another sister clade includes clade 3 and 4 paleo *AP3*-like genes. Both sister clades have evolved under different rates of substitution (Mondrago´n-Palomino and Theißen, 2009). Genes in the clade 1 and 2 maintain some characters of ancestral *AP3*-like genes such as diverse expression pattern in all floral whorls, retaining its ability to form homodimeric of protein which most *AP3*-like genes of eudicot has lost the ability to form homodimers, they need to make heterodimeric interaction with *PI* (Hsu and Yang, 2002). Additionally, ectopic expression of *DcOAP3A*, a paleo *AP3*-like gene in clade 1 together with *DcOPI*, a *PI*-like gene, could not generate phenotypes to indicate the possible function of the heterodimeric interaction between these *AP3* and *PI*-like genes from orchid (Xu et al., 2006).

In contrast to the function of clade 1 and 2, the clade 3 and 4 paleo *AP3*-like have similar expression pattern to eudicot *AP3* in which the expression of genes was regularly found in whorl 2 and column, no signal was detected in the first floral whorl like that of the clade 1 and 3. Functional analysis suggests that clade 3 and 4 are functionally homolog in order to control petal development of *D. moniliforme* and show highly conserved function to *AP3*-like genes of dicotyledonous plants as *Arabidopsis,* suggesting that these two clade of paleo *AP3*-like genes are greatly elevated from the ancestral *AP3* throughout evolution while the clade 1 and 3 retain ancestral characterization of the B functional gene.

Additionally, ectopic expression of paleo *AP3*-like genes in clade 3 and 4 i.e. *DMAP3B* and *DMMADS4* with its potential partner *DMPI* caused suppression of ovary development in transgenic *Arabidopsis* obtained *DMAP3B/PI* or *DMMADS4/DMPI*, although it could not been found when the *AP3*-or *PI*-like gene was expressed singly. The results suggesting that heterodimeric interaction between the paleo *AP3*-like genes in clade 3 or 4 with a *PI*-like gene from *D. moniliforme* is not only required for petals development, but also they play some role during growth of other part of flower including ovary.

Ovary of orchid usually stay in the immature stage throughout anthesis, development of mature ovary is initiated after pollination (Nadeau et al., 1996). Since expression of some paleo *AP3*-like genes and *PI*-like gene were also detected in the immature ovary of orchids (Table 2), orchid B-function genes may involve mechanism related prolongation of the immature ovary. As the result, production of undersize silique in transgenic *Arabidopsis* obtained *DMAP3B/PI* or *DMMADS4/DMPI* may be due to functions of the heterodimeric interaction of those B-function genes in order to prolong immature stage of ovary growth.

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Section 3

Molecular and Cellular Regulatory Mechanisms

9

Exploring Secrets of Nuclear Actin Involvement in the Regulation of Gene Transcription and Genome Organization

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1. Introduction

Actin is one of the most abundant proteins in eukaryotic cells. It is a 43-kDa protein that was originally identified and purified from skeletal muscle. Once thought to be simply a component of muscle cells, actin has later been shown to be a highly conserved and ubiquitiously distributed protein in eukaryotic cells. It has been extensively studied as a cytoplasmic cytoskeletal protein that is involved in a wide range of cellular processes, including cell motility, growth and cytokinesis; endocytosis, exocytosis and secretion; signal transduction, synaptic transmission as well as intracellular trafficking (Ascough, 2004;Brakebusch and Fassler, 2003;Suetsugu and Takenawa, 2003). In the cytoplasm, actin exists in equilibrium between monomers (globular- or G-actin) and polymers (filamentous- or F-actin). The dynamics of actin, the coordinated assembly and disassembly of actin filaments in response to cellular and extracellular signaling, is critical for the diverse functions of actin and is tightly regulated by a plethora of actin-binding proteins (ABPs) in the cytoplasm (dos Remedios *et al.*, 2003). To date, over 70 distinct classes of ABPs have being identified and the inventory is still far from been completed (Pollard and Borisy, 2003).

While the cytoplasmic functions of actin are well established, the findings obtained from studies on nuclear actin have encountered consistent skepticism for many years. Presence of actin in the nucleus was considered to be cytoplasmic contamination from extraction or fixation procedures, or antibody cross-reactivity (Pederson and Aebi, 2002;Shumaker *et al.*, 2003). In addition, many known functions of actin in the cytoplasm are associated with the polymerization of actin into filaments, which can be detected by phalloidin staining. However, under normal conditions, nuclei cannot be stained by phalloidin. Nevertheless, in the past decade, there has been convincing data demonstrating that actin, actin-related proteins (Arps) as well as ABPs are not only present in the nucleus but also play important roles in diverse nuclear activities. Actin has been localized to specialized subnuclear compartments such as the nucleoli, splicing speckles and Cajal bodies (Fomproix and Percipalle, 2004;Gedge *et al.*, 2005;Saitoh *et al.*, 2004). In these subnuclear compartments, actin proves to be involved in almost all the processes associated with gene expression, from chromatin remodeling via transcription to ribonucleoprotein (RNP) assembly and maturation, as well as mRNA nuclear export (Blessing *et al.*, 2004;Chen and Shen, 2007;Olave *et al.*, 2002). Other nuclear processes in which actin is implicated, include

assembly of the nuclear structure (Krauss *et al.*, 2003;Krauss *et al.*, 2002;Olave *et al.*, 2002), genome organization, and regulation of transcription factor activity (Olave *et al.*, 2002;Vartiainen *et al.*, 2007).

In this chapter, the several aspects related to the nuclear actin presence and its importance in the regulation of gene expression will be reviewed.

2. Nuclear architecture and distribution of actin

The cell nucleus is a complex and multi-functional organelle, which displays a high degree of spatial organization and structural integrity. The most well characterized structural component of the cell nucleus is the nuclear lamina, mainly composed of A- and B-type laminas as well as lamina–associated proteins (Stewart *et al.*, 2007). The laminas are evolutionarily conserved nuclear-specific intermediate filaments that are essential for many nuclear functions, including the maintenance of nuclear shape, DNA replication, transcription, chromatin organization, cell cycle regulation and apoptosis (Andres and Gonzalez, 2009;Vlcek and Foisner, 2007;Wiesel *et al.*, 2008). Actin has been shown to interact with the c-terminus of A-type laminas (Sasseville and Langelier, 1998). A-type laminas are connected to the cytoskeleton by a linker of nucleoskeleton and cytoskeleton (LINC) complex found in the nuclear envelope. Connecting the A-type laminas to the cytoskeleton is necessary for nuclear migration and positioning within the cell as well as for transmitting mechanical signals from the cytoplasm to the nucleus (Starr, 2009;Tzur *et al.*, 2006;Worman and Gundersen, 2006).

Two important components of the LINC complex are Sun domain proteins and Nesprins. Located on the outer nuclear membrane, Nesprin 1 and 2 can interact with F-actin as well as Sun 1 and Sun 2 located on the inner nuclear membrane. Sun proteins, in turn, bind to lamina A (Crisp *et al.*, 2006;Ostlund *et al.*, 2009). Emerin, a lamina-associated protein, is also important for nuclear structure and has been shown to bind to actin. The interactions between actin, lamina and emerin indicate that an actin-containing structural network exists at the nuclear envelope and is involved in maintaining the nuclear structure and nuclear functions (Fairley *et al.*, 1999;Holaska and Wilson, 2007;Lattanzi *et al.*, 2003). The importance of actin in nuclear assembly was demonstrated using *Xenopus* egg extracts in which nuclear assembly is initiated after fluorescence-labelled actin is added. Moreover, the nuclear assembly gets blocked by Latrunculin A, which binds to G-actin and inhibits F-actin formation, suggesting that F-actin is required for nuclear assembly. In addition, the interaction between actin and protein 4.1 is implicated in this process (Krauss *et al.*, 2003;Krauss *et al.*, 2002).

Actin has also been associated with the nuclear matrix (Capco *et al.*, 1982;Okorokov *et al.*, 2002;Valkov *et al.*, 1989;Verheijen *et al.*, 1986). The nuclear matrix is a network of proteins throughout the inside of nucleus, which provides a structural framework for maintaining spatial order within nucleus and for proper nuclear functions , such as DNA replication and repair, gene transcription, RNA splicing and transport (Berezney, 2002;Berezney *et al.*, 1996;Hancock, 2000). It is tempting to speculate that nuclear actin acts as a component of intranuclear filament network (or nucleoskeleton) that is analogous to cytoskeleton. This was supported by a study showing a colocalization between actin and EAST (enhances adult sensory threshold), a structural protein of the nucleus. In a Drosophila model, EAST has been shown to be a ubiquitious nuclear protein forming a network throughout the

nucleus (Wasser and Chia, 2000). A number of studies have also confirmed that an actin-containing filament network exists in the nucleus. Studies of the *Xenopus* oocyte nuclei using electron microscopy have found that filaments containing actin and protein 4.1 form a network that attach to Cajal bodies and other subnuclear organelles (Kiseleva *et al.*, 2004). In this manner, the meshwork of actin-containing filaments might contribute to the nuclear compartmentalization.

3. Regulation of nuclear actin

3.1 The form of actin in the nucleus

Actin has been shown to be involved in diverse nuclear processes; but how and in what form actin takes part in these events remains to be elucidated. It has been suggested that nuclear actin coexists as a monomer (G-actin), short oligomer and polymer structure (Gieni and Hendzel, 2009;McDonald *et al.*, 2006). These different forms of nuclear actin are believed to be required for a variety of processes in the nucleus. There has been a great body of evidence in support of the presence of G-actin in the nucleus (Pederson and Aebi, 2002;Pederson and Aebi, 2005). Firstly, a number of G-actin binding proteins have been identified in the nucleus, including cofilin, profilin, β-thymosin, gelsolin and gelsolin-like protein (Huff *et al.*, 2004;Pendleton *et al.*, 2003;Percipalle, 2009;Prendergast and Ziff, 1991;Skare *et al.*, 2003). Secondly, using DNase I affinity chromatography, actin can be copurified with RNA polymerase I and II machinery (Fomproix and Percipalle, 2004;Kukalev *et al.*, 2005;Obrdlik *et al.*, 2008). DNase I binds to G-actin with very high affinity and F-actin with low affinity (Zechel, 1980). This suggesting that actin co-precipitated with RNA polymerase I and II is likely to be present in its monomeric or short oligomeric form. Thirdly, monoclonal antibodies directed against epitopes which are unique to monomeric or dimeric actin, display distinctive immunostaining of the nucleus (Jockusch *et al.*, 2006). Fourthly, the nuclear lamina proteins, such as lamina A (Sasseville and Langelier, 1998), emerin (Lattanzi *et al.*, 2003), and nesprin (Zhang *et al.*, 2002a) form complexes with actin. Biochemical evidence reveals that G-actin is present in these complexes.

It has been very challenging to document polymerization status of actin in the nucleus. Phalloidin staining is the most common method used for detecting actin filaments in the cytoplasm. Under physiological conditions, nuclear actin present in most of the cells cannot be detected by phalloidin staining, which specifically recognizes actin filaments of at least seven subunits in length. However, under certain cellular stress conditions, distinctive actin rods (also called bundles or paracrystals) can be induced in the nucleus in a variety of cell types. These conditions include dimethyl sulfoxide (DMSO) treatment (Sanger *et al.*, 1980a;Sanger *et al.*, 1980b), heat shock (Iida *et al.*, 1986;Welch and Suhan, 1985), Latrunculin B treatment and ATP deletion (Pendleton *et al.*, 2003) as well as viral infection (Charlton and Volkman, 1991;Feierbach *et al.*, 2006). Cellular stress-induced formation of actin filaments seems to be caused by an increased nuclear actin level because nuclear translocation and accumulation of actin are also observed at the same time. This is supported by the observation that actin filaments exist in the *Xenopus* oocytes, which have a very high concentration of actin (~2mg/ml) due to the lack of nuclear export receptor, exportin 6 (Bohnsack *et al.*, 2006;Clark and Rosenbaum, 1979;Roeder and Gard, 1994;Stuven *et al.*, 2003). In addition, some nuclear-actin dependent functions, such as nuclear export of RNA and proteins (Hofmann *et al.*, 2001), nuclear envelope assembly (Krauss *et al.*, 2003), transcription (McDonald *et al.*, 2006) and intranuclear movement of Herpes simplex virus-1 capsid

(Forest *et al.*, 2005) as well as movement of chromosome loci (Hu *et al.*, 2008) can be inhibited by Latrunculin B, a drug that binds G-actin with high affinity and prevents polymerization and thus F-actin formation (Spector *et al.*, 1989). These indirect evidence imply that some sort of polymerized actin exist in the nucleus to carry out corresponding nuclear functions. The presence of polymeric actin in the nucleus was also shown (McDonald *et al.*, 2006) in living cells using fluorescence recovery after photobleaching (FRAP) experiments. In that study, FRAP, which allows to analyze the dynamic properties of GFP-actin in the nucleus, shows that both a fast recovery and a slow recovery GFP-actin exist in the nucleus. Moreover, the latter type of actin is sensitive to actin mutants and Latrunculin B. Therefore, the slow species represents a polymeric form of actin with distinctive dynamics which is quite different from the actin dynamics observed in the cytoplasm. Interestingly, recent studies provided evidence that the nuclear polymeric actin is important for RNA polymerase I-mediated transcription and transcriptional activation of HoxB genes by RNA polymerase II (Ferrai *et al.*, 2009;Ye *et al.*, 2008).

3.2 Regulation of nuclear translocation of actin

Extracellular stress can induce nuclear translocation of actin. Sanger and colleagues demonstrated that a disappearance of stress fibers from the cytoplasm and a reversible translocation of cytoplasmic actin into the nucleus occur after treatment of PtK2 and WI-38 cells with 10% DMSO (Sanger *et al.*, 1980a;Sanger *et al.*, 1980b). Courgeon and colleagues showed that heat shock causes actin to accumulate in the nucleus of *Drosophila* cells (Courgeon *et al.*, 1993). In mast cells, entry of actin into the nucleus was induced by either treatment with Latrunculin B, or ATP depletion (Pendleton *et al.*, 2003). Most recently, nuclear translocation of actin was found in HL-60 cells and human peripheral blood monocytes when differentiated to macrophages by phorbol 12 myristate 13-acetate (PMA) (Xu *et al.*, 2010). These results suggest that actin is able to shuttle between the cytoplasm and the nucleus. To date, the molecular mechanism by which actin enters into the nucleus in response to cellular stress has not been established.

The nuclear envelope is a lipid bilayer that forms a barrier between the nuclear and cytoplasmic spaces. The traffic between nucleus and cytoplasm is mediated through nuclear pore complexes (NPCs) embedded in the nuclear envelope. NPCs allow passive diffusion of small molecules (such as ion and protein smaller than 40 kDa) but restrict the movement of larger molecules across the nuclear envelope. Macromolecules usually carry specific signals allowing them to access the nucleocytoplasmic transport machinery. Monomeric actin has a molecular weight of ~43 kDa, therefore it is unlikely to enter into nucleus by diffusion. Actin lacks a classical nuclear localization signal (NLS) and to date, no specific import receptor for actin has been identified. Therefore it most likely relies on an active carrier which guides it into the nucleus. Cofilin, an actin-binding protein, is suggested to be involved in the regulation of nuclear import of actin. Cofilin contains a NLS and it has been recognized as a component of intranuclear actin rods in response heat shock and DMSO treatment (Nishida *et al.*, 1987). A study by Pendleton *et al.* showed that stress-induced nuclear accumulation of actin was blocked by an anti-cofilin antibody, demonstrating that cofilin is required for actin import into the nucleus (Pendleton *et al.*, 2003).

For nuclear export, actin seems to use an active transport mechanism. The actin polypeptide has two well conserved nuclear export signals (NESs). In yeast, these two sequences were specifically recognized by chromosome region maintenance 1 (CRM1, also known as exportin 1), a general export receptor for cargos bearing leucine-rich export signals, and

actin can then be rapidly removed from nucleus. Transfection of cells with mutant actin lacking NESs or inhibition of CRM1 by leptomycin B results in nuclear accumulation of actin (Wada *et al.*, 1998). Exportin 6, a member of the importin β superfamily of transport receptor, is responsible for nuclear actin export in mammalian cells (Stuven *et al.*, 2003). Knockdown of exportin 6 by RNA interference also leads to nuclear accumulation of actin and the formation of actin rods. Interestingly, exportin 6 recognizes the actin:profilin complex rather than actin or profilin individually, suggesting a difference in the form of actin being presented to CRM1 and to exportin 6.

So far, the exact roles of nuclear accumulation of actin in response to external signals remain to be understood. Nuclear actin controls transcription of its target genes through several different ways: (1) Actin specifically binds to a 27-nt repeat element in the intron 4 of the endothelial nitric oxide synthase gene to regulate its expression (Ou *et al.*, 2005;Wang *et al.*, 2002); (2) Actin participates in chromatin remodeling for gene activation as a component of the chromatin remodeling complex (Rando *et al.*, 2002;Song *et al.*, 2007;Zhao *et al.*, 1998); (3) Actin plays a direct role in RNA transcription by being part of the pre-initiation complex with RNA polymerase II (Hofmann *et al.*, 2004). (4) Actin participates in transcriptional elongation as a component of RNP particles. Therefore, it is tempting to speculate that under stress, actin translocates into nuclei to function as a transcriptional modulator, playing an important role in the regulation of gene transcription along with stress-activated transcription factor. This hypothesis is supported by recent studies showing that nuclear accumulation of actin is involved in transcriptional activation of SLC11A1 gene during macrophage-like differentiation of HL-60 cells induced by PMA (Xu *et al.*, 2011;Xu *et al.*, 2010).

3.3 Regulation of actin polymerization

It is believed that the concentration of nuclear actin is sufficient for spontaneous polymerization. Therefore, in order to have dynamic equilibrium of the different forms of actin, an active process preventing polymerization is required.

Many of the regulators known to control cytoplasmic actin dynamics have also been shown to be present in the nucleus (Table 1). These regulators include Arps such as Arp 2/3; and ABPs such as cofilin, profilin and CapG; and signalling molecules (see section 3.4). In humans, Arp2/3 represents a stable complex of two Arps (Arp2 and Arp3) and five other subunits including p16, p20, p21, p34, p41 (Deeks and Hussey, 2005;Welch *et al.*, 1997). The Arp2/3 complex is capable of initiating *de novo* polymerization of actin and stimulating the formation of branched actin filaments when activated by members of Wiskott-Aldrich syndrome protein (WASP) family (Higgs and Pollard, 2001;Machesky and Insall, 1998;Pollard and Borisy, 2003;Volkmann *et al.*, 2001). The WASP family members share a common C-terminal verprolin-cofilin-acidic (VCA) region. Polymerization of actin is initiated by the interaction of the VCA region with both Arp2/3 complex and an actin monomer, forming the first subunit of *de novo* actin polymer (Dayel and Mullins, 2004;Kim *et al.*, 2000;Prehoda *et al.*, 2000;Rohatgi *et al.*, 1999). The potential role of Arp2/3 in the regulation of actin dynamics in the nucleus was suggested based on the viral infection studies, for example infection with baculovirus, results in accumulation of Arp2/3 complex in the nucleus, where it becomes activated by WASP-like virus protein p78/83. This event in turn results in Arp2/3-mediated actin polymerization that is essential for virus replication (Goley *et al.*, 2006). Furthermore, it has been demonstrated that N-WASP and Arp2/3 complex associate with RNA polymerase II and regulate the efficiency of gene transcription.

Induction of actin polymerization through the N-WASP-Arp2/3 complex pathway has been shown to be required for efficient transcription by RNA polymerase II (Wu *et al.*, 2006;Yoo *et al.*, 2007). Importance of the Arp2/3 complex –mediated actin polymerization in other nuclear actin-dependent processes remains to be fully elucidated.

Protein	Roles in the nucleus	References
Arp 2/3	De novo actin polymerization	Higgs and Pollard, 2001
	Formation of Branched actin filaments	Pollard et al., 2003
	Associated with transcription by pol II	Wu et al., 2006; Yoo et al., 2007
N-WASP	Activating ARP2/3-mediated actin polymerization	Higgs and Pollard, 2001; Volkmann et al. 2001
	Regulating transcription by pol II	Wu et al., 2006; Yoo et al., 2007
Gelsolin	Serving actin polymers	Ocampo et al., 2005
	Androgen receptor co-activator	Nishimura et al., 2003
Flightless I	Chromosome remodelling	Archer et al., 2005
Supervillin	Nuclear receptor-induced transcription	Ting et al., 2002
Filamin	Androgen receptor action	Ozanne et al., 2000
CapG	Unknown	De Corte et al., 2004
Profilin	Nuclear export of actin mediated by exportin 6	Stuven et al., 2003
	Possible involvement in pre-mRNA splicing	Skare et al., 2003
Thymosin β4	Sequestering actin and blocking actin polymerization	Hannappel et al., 2007; Huff et al., 2004
Cofilin	Nuclear import of actin	Pendleton et al., 2003
	Repressor of the glucocorticoid receptor	Ruegg et al., 2004
	A component of nuclear actin-rods	Nishida et al., 1987
Emerin	Nuclear architecture	Holaska et al., 2004
Myo1c/NM1	Transcription	Hofmann et al., 2006 ; Ye et al., 2008
	Chromatin remodeling	Percipalle et al., 2006
Tropomodulin	Unknown	Kong and Kedes, 2004
Protein 4.1	Nuclear assembly	Krauss et al., 2003
Actinin	Nuclear receptor activator (actinin alpha 4)	Khurana et al., 2011
	Regulation of DNase Y activity (actinin alpha 4)	Liu et al., 2004
Spectrin II α	Involved in DNA repair	Sridharan et al., 2003
Paxillin	Stimulating DNA synthesis and	Dong et al., 2009
	Promoting cell proliferation	
CAP2	Unkown	Peche et al., 2007
CABP14	Possible role in cell division	Aroian et al., 1997

Table 1. Proteins known to modulate cytoplasmic actin dynamics exist in nucleus

Actin filaments capping proteins bind the barbed (or fast growing end) of an actin filament and therefore block filament assembly or promote disassembly at that end. In the cytoplasm, members of the gelsolin family are characterized by the ability to cap, sever and bundle actin filaments in a Ca^{2+}-dependent manner in the cytoplasm (Archer *et al.*, 2005). Several members of gelsolin family has been detected in nucleus, including gelsolin (Nishimura *et al.*, 2003;Salazar *et al.*, 1999), CapG (De, V *et al.*, 2004;Onoda *et al.*, 1993), flightless (Lee *et al.*, 2004) and supervillin (Wulfkuhle *et al.*, 1999). In the nucleus, gelsolin has been found to be involved in chromosome decondensation by severing actin (Ocampo *et al.*, 2005). Flightless I has been found to bind to actin and Arp BAF53, a subunit of mammalian chromatin remodelling complex, and negatively regulates actin polymerization (Archer *et al.*, 2005). It is currently unclear whether other members regulate actin dynamics in the nucleus. Interestingly, many of them appear to function as transcriptional coactivators for nuclear hormone receptors (Gettemans *et al.*, 2005).

Many G-actin binding proteins are also present in the nucleus. Thymosin β4 is the most abundant polypeptide of the β-thymosin family in the cytoplasm and regulates F-actin polymerization by sequestering polymers (Huff *et al.*, 2004). In the nucleoplasm, thymosin β4 is present at a high level and suggested to sequester nuclear actin and block actin polymerization (Hannappel, 2007;Huff *et al.*, 2004). In addition, it has been shown to interact with ATP-dependent DNA helicase II to regulate specific gene expression (Bednarek *et al.*, 2008). Despite its small size (~4.9 kDa), Huff et al. showed that passive diffusion of thymosin β4 through the NPC can be ruled out (Huff *et al.*, 2004), and its nuclear localization has been reported to be regulated by the DNA mismatch repair enzyme human mutL homolog 1 (hMLH1) (Brieger *et al.*, 2007). Profilin is a small protein that binds specifically with G-actin. It enhances the nucleotide exchange on actin to convert ADP actin into ATP actin, which can readily be incorporated in to a growing filament. In the nucleus, formation of profilin-actin complex is required for nuclear export of actin through exportin 6 (Stuven *et al.*, 2003), to avoid excess actin polymerization in the nucleus. This was supported by Bohnsack and colleagues' work (Bohnsack *et al.*, 2006).

ADP/cofilins represent a family of small actin-regulatory proteins that bind to both actin monomers and filaments, and remove actin filaments by severing and depolymerising (Maciver and Hussey, 2002). Using fluorescence resonance energy transfer assay, they have been shown to bind to actin directly in the nucleus and at levels much higher than in the cytoplasm (Chhabra and dos Remedios, 2005). As mentioned in section 3.2, actin accumulates in the nucleus and forms intranuclear actin rods under a variety of cellular stress conditions. Cofilin has been recognized as a component of the actin rods (Gettemans *et al.*, 2005). The high level of cofilin present in the nucleoplasm and in the actin rods might explain the reason why actin filaments appear to be restricted in the nucleus since the cofilin/actin structures cannot be stained with phalloidin (Nishida *et al.*, 1987). The formation of nuclear actin rods is highly dynamic and is reversible when the cellular stress conditions are removed (Gieni and Hendzel, 2009), suggesting that cofilin might play a role in restricting the excess accumulation of polymeric actin, which otherwise could affect the polymeric actin-mediated nuclear process.

3.4 Signalling molecules regulating actin dynamics

The activities of ABPs are tightly controlled through various signalling pathways to ensure proper spatial and temporal regulation of actin dynamics in the cells. Several signalling molecules, including small GTPases, Ca^{2+} and phosphoinositides which display well-characterized effects on actin dynamics in the cytoplasm, are also found in the nucleus.

Small GTPases of the Rho family, such as Cdc42 and Rac1, have been found in the nucleus, (Williams, 2003). As discussed above, Arp2/3 is an important candidate for regulating nuclear actin polymerization and N-WASP, the most potent inducer of Arp2/3 –mediated actin nucleation remains to be the only member of the WASP family found in the nucleus (Suetsugu *et al.*, 2001;Zalevsky *et al.*, 2001). In the cytoplasm, N-WASP is activated by Cdc42, linking Rho family GTPase signalling with Arp2/3 –mediated actin polymerization (Rohatgi *et al.*, 1999). N-WASP is also activated by Rac1, and both Cdc42- and Rac1-mediated stimulation of N-WASP activity is further enhanced by the presence of phosphatidylinositol 4,5-bisphosphate (PIP2). The functional significance of the presence of Rho GTPases in the nucleus is not fully known. Some downstream effectors of Rho family GTPase, such as LIM

kinases (LIMK), has been shown to localize to the nucleus. LIMK can phosphorylate and inactivate cofilin, suggesting that Rho GTPase signalling pathway may play an important role in regulation of nuclear actin cytoskeleton. Rac1 was shown to shuttle in and out of the nucleus during the cell cycle and to accumulate in the nucleus in late G2 phase. In addition, GTP-bound Rac1 and a Rac1/Cdc42 GTPase activating- protein, MgcRacGAP, bind directly to phosphorylated transcription factors, STAT3 and STAT5, to mediate their translocation into the nucleus. Therefore, nuclear accumulation of Rac1 may also regulate actin polymerization influencing RNA polymerase II-mediated transcription.

Phosphoinositides (PIs) are major regulators of actin dynamics in the cytoplasm (Mao and Yin, 2007). PIs control actin polymerization by modulating the activity of regulatory proteins promoting actin assembly and inhibiting disassembly of actin filaments. For example, PIP2 activates nucleation of actin filaments induced by N-WASP-Arp2/3 complex and inhibits the actin-binding activity of cofilin (Hilpela *et al.*, 2004). PIP2 also binds and inhibits capping proteins, and seems to remove capping proteins from capped ends of actin filaments, which may help to stimulate actin assembly (Kim *et al.*, 2007). Based on the observations, one can speculate that PIs also modulate actin-binding activity of capping proteins in the nucleus. So far, the downstream targets of PI signalling remains poorly identified. Several studies have linked chromatin remodelling complexes with PIs. For example, PIP2 participates in the recruitment of mammalian chromatin remodelling complex, BRG1/BRM associated factor (BAF), to nuclear matrix-associated chromatin, upon activation of antigen receptor in T-lymphocytes (Zhao *et al.*, 1998). Further analysis has revealed that PIP2 can bind directly to BRG1, an ATPase subunit of the BAF complex, modulate the actin-binding activity of BRG1 (Rando *et al.*, 2002). Within the BAF complex, BRG1 is associated with β-actin and Arp BAF53 through two actin-binding domains. Interestingly, one of the acting-binding domains of BRG1 is required for PIP2 binding. Based on these findings, a model is designed in which interaction between PIP2 and BRG1 would essentially uncap β-actin or BAF53, thereby allowing them to interact with actin filaments in the nuclear matrix (Rando *et al.*, 2002).

In the cytoplasm, actin dynamics is also controlled by Ca^{2+} level. The activity of several ABPs, including members of gelsolin family, are regulated by Ca^{2+} influx (Archer *et al.*, 2005). For example, Ca^{2+} activates gelsolin to allow capping and severing of actin filaments. The importance of Ca^{2+}-regulated actin severing has been well-documented in platelet activation (Witke *et al.*, 1995). Gelsolin has six Ca^{2+} binding sites within domain S1-S6. When domains S5 and S6 are occupied by Ca^{2+} at submicromolar concentration gelsolin is activated to bind actin. However, for full activation of severing activity, higher Ca^{2+} concentrations are required most likely filling the sites on domains S1, S2 and S4 (Burtnick *et al.*, 2004;Choe *et al.*, 2002). It is clear that nuclear Ca^{2+} level is regulated which in turn regulates the activity of transcription factors, such as DREAM (Carrion *et al.*, 1999) and CREB (Chawla *et al.*, 1998). Likewise, it is possible that nuclear Ca^{2+} level could modulate the activity of actin-containing chromatin remodelling complex by controlling activity of certain nuclear ABPs.

4. Involvement of actin in chromatin remodelling

Eukaryotic DNA is tightly packaged into nucleosome repeats. Each nucleosome unit consist of a histone octamer core surrounded by a segment of 146 base pairs of double stranded

DNA. Histone octamer core is composed of two-molecule each of H2A, H2B, H3 and H4 proteins. This kind of packaging of genomic DNA in chromatin represents barriers that restrict access to a variety of DNA regulatory proteins involved in the processes of transcription, replication, DNA repair and recombination machinery. To overcome these barriers, eukaryotic cells possess a number of multiprotein complexes which can alter the chromatin structure and make DNA accessible. These complexes can be divided into two groups, histone-modifying enzymes and ATP-dependent chromatin remodelling complexes. The histone-modifying enzymes post-translationally modify the N-terminal tails of histone proteins through acetylation, phosphorylation, ubiquitination, ADP-ribosylation and methylation (Sterner and Berger, 2000;Wang *et al*., 2007). On the other hand, ATP-dependent chromatin remodelling complexes use the energy of ATP hydrolysis to disrupt the DNA-histone contact, move nucleosomes along DNA, and remove or exchange nucleosomes (Gangaraju and Bartholomew, 2007). Actin and Arps were first identified as integral components of the BAF complex, a mammalian SWI/SNF-like chromatin remodelling complex, that is involved in T-lymphocyte activation (Zhao *et al*., 1998). Since then, actin and Arps have been found to be present in a wide variety of chromatin remodelling and histone-modifying complexes (Figure 1C) in yeast, *Drosophila* and mammalian cells.

4.1 Actin–containing chromatin remodelling complex

The ATP-dependent chromatin remodelling complexes can be classified into at least four different families based on their central ATPases: SWI/SNF complex (or BAF complexes) with a SWI2/SNF2 ATPase; ISWI complex with an ISWI ATPase; Mi-2 (or CHD) complex containing a chromodomain-helicase-DNA binding protein ATPase; and INO80 complex with an INO80 ATPase (Farrants, 2008). Only the complexes of SWI/SNF and INO80 families have actin and Arps as subunits that are bound directly. Most members of SWI/SNF family contain actin and Arp4 homologues. In *Drosophilia*, the orthologous complexes BAP (Brahma associated proteins) and PBAP (polybromo-associated BAP) each contains actin and the Arp BAF55. In mammals, the orthologous complexes BAF, PBAF and p400 all contain actin and the Arp BAF53. The yeast SWI/SNF complex was the first to be discovered in *S. cerevisiae*. Many components of the SWI/SNF complex were initially identified in independent screens for genes that regulate mating-type switching (SWI genes) and sucrose non-fermenting (SNF genes) phenotype in yeasts (Abrams *et al*., 1986;Carlson *et al*., 1981;Nasmyth and Shore, 1987;Neigeborn and Carlson, 1984;Neigeborn and Carlson, 1987;Stern *et al*., 1984;Vignali *et al*., 2000). The yeast SWI/SNF complex contains 11 known subunits, of which the SWI2/SNF2 subunit possesses both chromatin remodelling and DNA-dependent ATPase activities. In yeast, the SWI/SNF complex and another orthologous complex RSC lack actin but were shown to contain two yeast specific Arps - Arp7 and Arp9 (Table 2). Interestingly, the yeast genome encodes both actin and Arp4 but they are replaced with novel Arps.

The INO80 family includes the yeast INO80 complex and its orthologues Pho-dINO80 (*Drosophila*) and INO80 (human); the yeast SWR1 complex and its orthologue SRCAP (human); and the yeast NuA4 complex and its orthologues TIP60 (*Drosophila*) and TRAAP/TIP60 (human) (Table 3). The yeast INO80 complex contains actin and Arp 4, Arp5 and Arp8, of which Arp5 and Arp8 and actin are conserved in *Drosophila* and mammals. Arp4 and actin are also components of yeast SWR1 and Nu4 complex. BAF53, a mammalian orthologue of Arp4, is present in the mammalian orthologous complex INO80, SRCAP, and TIP60 (Hargreaves and Crabtree, 2011).

Complex	SWI/SNF	RSC	BAP	PBAP	BAF	PBAF	nBAF	npBAF
Species	Yeast	Yeast	Drosophila	Drosophila	Human	Human	Mouse	Mouse
ATPase	Swi2/Snf2	Sth1	BRM	BRM	BRG1/hBRM	BRG1	BRG1	BRG1
Actin	No	No	β-actin	β-actin	β-actin	β-actin	β-actin	β-actin
ARP	ARP7, ARP9	ARP7, ARP9	BAP55 or BAP47	BAP55 or BAP47	BAF53a or BAF53b	BAF53a	BAF53b	BAF53a
Main subunits or			OSA	BAP170	BAF250a or	BAF200	BAF250a or	BAF250a
or					BAF250b		BAF250b or	BAF250b
							BAF200	BAF200
		Rsc1, Rsc2, Rsc4		Polybromo		BAF180		
and/or	Swi3	Rsc8	BAP155	BAP155	BAF155 and/or	BAF155 and/or	BAF155 and/or	BAF155
					BAF170	BAF170	BAF170	BAF170
	Swp73	Rsc6	BAP60	BAP60	BAF60a or BAF60b or BAF60c	BAF60a	BAF60a	BAF60a
BAF47/SNF5	Snf5	Sfh1	BAP45/SNR1	BAP45/SNR1	BAF47/hSNF5	BAF47/hSNF5	BAF47/SNF5	
			BAP111	BAP111	BAF57	BAF57	BAF57	BAF57
Unique Subunits	Swi1, Swp82 Taf14, Snf6 Snf11	Rsc3, 5, 7, 9, 10, 30 Htl1, Lbd7, Rtt102						

Table 2. Complexes of the SWI/SNF family

Complex	INO80	Pho-dINO80	INO80	SWR1	SRCAP	NuA4	TIP60	TRAAP/TIP60
Species	Yeast	Drosophila	Human	Yeast	Human	Yeast	Drosophila	Human
ATPase	Ino80	dIno80	hINO80	Swr1	SRCAP		Domino	p400
Actin	Act1	dActin	β-actin	Act1	β-actin	Act1	Act87E	β-actin
ARP	ARP4, ARP5 ARP8	dARP5, dARP8	BAF53a, ARP5 ARP8	ARP4, ARP6	BAF53a, ARP6	ARP4	BAP53	BAF53a
Main subunits	Rvb1, Rvb2	Reptin, Pontin	Tip49a, Tip49b	Rvb1, Rvb2	Tip49a, Tip49b		Reptin, Pontin	Tip49a, Tip49b
	Taf14			Yaf9	GAS41	Yaf9	dGAS41	GAS41
	Ies 2		hIes 2					
	Ies 6		hIes 6					
				Swc2	YL-1		dYL-1	YL-1
				Swc4	DMAP1	Swc4	dMAP1	DMAP1
				Bdf1			dBrd8	BRD8/TRCp120
				Swc6	Znf-HIT1			
						Tra1	dTra1	TRAAP
						Eaf3	dMRG15	MRG15
						Eaf6	dEaf6	FLJ11730
						Eaf7	dMRGBP	MRGBP
						Esa1	dTip60	Tip60
						Epl1	E(pc)	EPC1
						Yng2	dING3	ING3
Unique Subunits	Ies 1, Ies3, 4, 5 Nhp10	Pleiohomeotic	Amida, MCRS1, FLJ20309, UCH37 NFRKB, CCDC95	Swc3, 5, 7		Eaf1, 5		

Table 3. Complexes of the INO80 family

Actin was first identified in the mammalian BAF complex. Biochemical analysis indicated that actin is not only tightly bound to BRG1, the ATPase subunit of BAF, but also needed for the ATPase activity required for BAF association with chromatin (Zhao *et al*., 1998). To date, the molecular mechanisms that underlie the functions of actin and Arps in the chromatin remodelling remain largely unknown. Recently, a helicase-SANT-associated (HSA) domain was identified in the ATPase of several chromatin remodelling complexes. This domain is required for the binding of actin and Arps. Altering the HSA domain causes a loss of actin and Arps in these complexes and reduces ATPase activity, confirming the important role of actin and Arps in chromatin remodelling (Szerlong *et al*., 2008).

4.2 Actin recruits histone modifying enzymes

Actin has been identified as a component of pre-mRNA particles (pre-mRNPs) via binding to heterogeneous nuclear ribonucleoprotein particles (hnRNPs) in insects and mammals (Kukalev *et al*., 2005;Percipalle *et al*., 2003;Percipalle *et al*., 2002;Percipalle *et al*., 2001;Zhang *et al*., 2002b). In the dipteran insect *Chironomus tentans*, actin was found to bind directly to the nuclear protein HRP65-2 (HRP65 isoform 2). Disruption of this interaction by a competing peptide, which mimics the actin-binding motif of HRP65, inhibited RNA polymerase II-mediated transcription at the level of transcript elongation (Percipalle *et al*., 2003). The inhibitory effect of this peptide can be counteracted by a general inhibitor of histone deacetylases (trichostatin A), suggesting that actin-HRP65-2 interaction is involved in acetylation/deacetylation of histones. Indeed, HRP65-2 and actin were shown to form a complex with p2D10 *in vivo*. p2D10 is a histone H3 –specific acetyltransferase, a *C. tentans* ortholog of the largest subunit of the transcription factor TFIIIC (Sjolinder *et al*., 2005). Disruption of the interaction between HRP65-2 and actin releases p2D10 from RNA polymerase II-transcribing gene, coinciding with reduced H3 histone acetylation and inhibition of transcription, indicating that HRP65-actin interaction provides a molecular platform to recruit chromatin modifying factors to the transcribing genes allowing to maintain genes in an active state (Figure 1B) (Sjolinder *et al*., 2005).

Similarly, in human cells, the interaction between actin and hnRNP U, another component of pre-mRNPs, was also shown to be essential for RNA polymerase II-mediated transcription elongation. hnRNP U has been shown to bind to actin via a conserved actin-binding motif located at the C-terminus. Both actin and hnRNP U were shown to be associated with the phosphorylated C-terminal domain (CTD) of polymerase II and antibodies against either of these components are able to block transcription of class II genes (Kukalev *et al*., 2005). Furthermore, the actin - hnRNP U complex was shown to be required for the recruitment of histone acetyltransferase (HAT), PCAF, to actively transcribed genes, and they are all present at promoter and coding regions of constitutively expressed class II genes (Figure 1 B) (Obrdlik *et al*., 2008). It was previously shown that binding of hnRNP U to RNA polymerase II inhibited the phosphorylation of the CTD mediated by TFIIH, suggesting that actin-hnRNP U interaction might modify the inhibitory effect of hnRNP U on CTD phosphorylation, and that this modification is required for transcription elongation (Kim and Nikodem, 1999;Kukalev *et al*., 2005).

5. Involvement of actin in transcription machinery

Transcription is a process of synthesizing an RNA molecule from a sequence of DNA. The major steps of transcription include pre-initiation, initiation, elongation and termination.

Transcription is performed by an enzyme called RNA polymerase. Eukaryotic cells have three distinct classes of RNA polymerases characterized by the type of RNA they synthesize. RNA polymerase I is located in the nucleolus, a functionally highly specialized subnuclear compartment, and it is responsible for transcribing ribosomal RNA (rRNA). RNA polymerase II is located in nucleoplasm and responsible for synthesizing the precursors of messenger RNA (mRNA) and most small nuclear RNAs (snRNA) and microRNA (miRNA). RNA polymerase III is also located in nucleoplasm, and transcribes 5S rRNA, transfer RNA (tRNA), U6 snRNA and other small RNAs.

The first finding that demonstrated a role of nuclear actin in transcriptional process was documented by Smith et al. (Smith *et al.*, 1979). The authors found that actin co-purified with RNA polymerase II from the slime mold *Physarum polycephalum*. Following this original finding, the subsequent studies demonstrated that actin was present in transcriptionally active nuclear extracts from HeLa cells and calf thymus, and was able to initiate the transcription by RNA polymerase II *in vitro* (Egly *et al.*, 1984). Another study, published about the same time, showed that transcription of lampbrush chromosomes was inhibited when antibodies directed against actin or ABPs were microinjected into the nuclei of living oocytes of *Pleurodeles waltl*. This study provided first solid evidence for an association between actin and transcription (Scheer *et al.*, 1984). However, these two important findings were largely ignored and postulated as being artifacts of contamination. Although a number of key advances in the nuclear actin field occurred after 1984, skepticism of new data remained until two decades later when several studies finally provided convincing and non-contestable evidence for the involvement of actin in gene transcription (Grummt, 2006;Pederson and Aebi, 2002; Percipalle and Visa, 2006). However, many questions remain to be answered to fully understand the exact molecular mechanism of regulation of transcription by various forms of actin.

5.1 Role of actin and nuclear myosin 1 in gene transcription by RNA polymerase I

Actin has been shown to be present not only in mammalian nucleoplasm but also in nucleoli (Andersen *et al.*, 2005), suggesting a role for actin in transcription by RNA polymerase I. Indeed, co-immunoprecipitation and chromatin immunoprecipitation (ChIP) assays showed that actin is associated physically with RNA polymerase I and present on actively transcribing ribosomal genes at both the promoter and transcribed regions (Fomproix and Percipalle, 2004; Philimonenko *et al.*, 2004). Microinjection of anti-actin antibodies into the nuclei inhibited rRNA synthesis in living cells (Philimonenko *et al.*, 2004). Furthermore, *in vitro* transcription assays revealed that antibody against actin also inhibited rRNA synthesis in cell-free systems containing either naked rDNA or pre-assembled chromatin templates (Philimonenko *et al.*, 2004). Interestingly, anti-actin antibody did not affect the synthesis of initial trinucleotide (initiation phase) but inhibited the synthesis of run-off transcripts (elongation phase), indicating that actin is required for RNA polymerase I transcription in post-initiation steps.

Nuclear myosin 1 (NM1), a short-tailed myosin acting as an actin-dependent ATPase, has also been found in nucleoli (Fomproix and Percipalle, 2004), suggesting that actin and myosin might work together as actomyosin in transcription. NM1 and actin are present in a complex with RNA polymerase I (Fomproix and Percipalle, 2004; Philimonenko *et al.*, 2004). The same as actin, NM1 is present on the rDNA promoter and antibodies directly against

NM1 also inhibited RNA polymerase I transcription both in *in vivo* and *in vitro* transcription assays (Percipalle *et al.*, 2006;Philimonenko *et al.*, 2004). Previously, two contradictory findings were reported on this subject. One study showed that NM1 is not associated with the coding region (Philimonenko *et al.*, 2004); however another similar study using different anti-NM1 antibodies demonstrated that a fraction of nucleolar NM1 is associated with the coding region (Percipalle *et al.*, 2006). A possible reason for these discrepant findings could be that NM1 has different conformations during different steps of transcription, which could be recognized by different antibodies. Philimonenko and co-workers have shown that actin can directly interact with RNA polymerase I independent of whether or not it is engaged in transcription; however, NM1 binds to the transcription machinery via interaction with TIF-IA (Philimonenko *et al.*, 2004). TIF-IA is a RNA polymerase I-specific transcription initiation factor that mediates growth-dependent regulation of RNA polymerase I activity and rRNA transcription (Grummt, 2003). Based on these findings, one could speculate that actin and NM1 get close in proximity that they can interact with each other during the formation of transcription initiation complex and presumably activate RNA polymerase activity and rRNA synthesis. Ye and co-workers' studies provided further support to this hypothesis (Ye et al., 2008). In addition, NM1 was also found to be present on the coding region of 18S and 28S genes as a component of the chromatin remodelling complex B-WICH, which comprises the William syndrome transcription factor (WSTF) and SNF2h besides NM1, and is required for RNA polymerase I transcription activation and maintenance (Percipalle *et al.*, 2006). This suggests that MN1 is also implicated in the post-initiation phases of transcription. Recently, a study showed that knockdown of WSTF resulted in reduced recruitment of HATs at the rDNA which coincided with a lower level of histone acetylation.

5.2 Role of actin in transcription by RNA polymerase II

A number of studies have supported the role of actin in RNA polymerase II transcription machinery. Firstly, actin is co-purified with RNA polymerase II (Egly *et al.*, 1984;Hofmann *et al.*, 2004;Smith *et al.*, 1979), which maybe a general feature, as actin is also co-purified with RNA polymerase I (see 5.1) and III (see 5.3). Secondly, microinjection of anti-actin antibodies into the nuclei of *Xenopus* oocytes blocks chromosome condensation (Rungger *et al.*, 1979), and microinjecting antibodies directed against actin or Arps inhibit RNA polymerase II-mediated transcription (Hofmann *et al.*, 2004;Scheer *et al.*, 1984;Xu *et al.*, 2010). Thirdly, actin is a component of preinitiation complexes (Figure 1A), and the formations of preinitiation complexes are blocked by depletion of actin from nuclear extracts (Hofmann *et al.*, 2004). Fourthly, ChIP assays showed that actin can be recruited to the promoters of actively transcribed genes (Hofmann *et al.*, 2004;Xu *et al.*, 2010). For example, actin is absent from the promoters of the interferon-γ-inducible gene MHC2TA and interferon-α-inducible gene G1P3 before induction but it is associated with their promoters after gene induction. Recently, using ChIP-on chip assays, we have demonstrated that actin is recruited to a wide range of gene promoters during the PMA-induced macrophage-like differentiation of HL-60 cells. These data disprove the notion that actin might non-specifically interacts with the promoter regions. If this was the case, β-actin would have been found at the promoter of all genes even in the absence of induction. Even though significant progress has been made, the mechanisms of how actin is getting selectively recruited to the target genes still remains unknown.

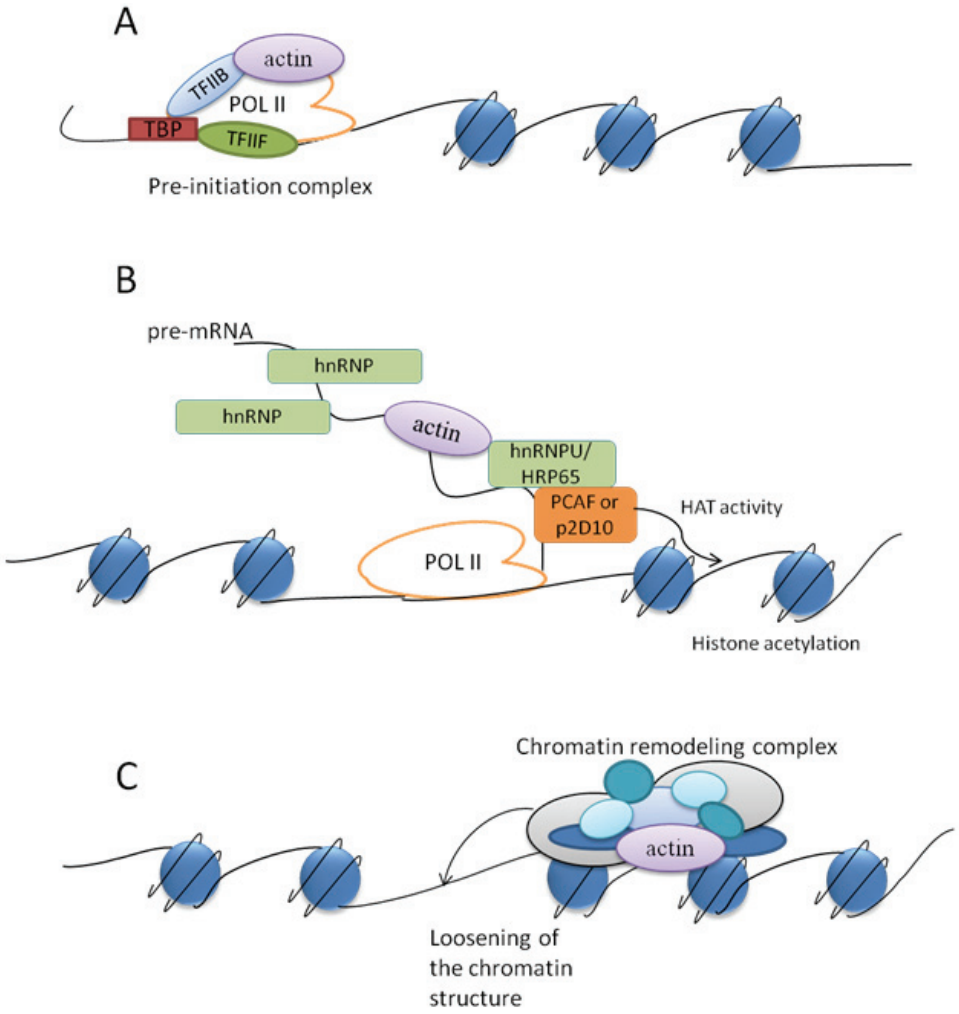

Fig. 1. Model for the function of actin in RNA polymerase II mediated transcription.

(A) Actin can interact with RNA polymerase II and is involved in the formation of preinitiation complex, and affect transcription directly. (B) Actin participates in the recruitment of histone modifying enzymes to protein-coding genes. Actin binds to hnRNP proteins and becomes incorporated into pre-mRNPs. Actin forms a complex with adaptor proteins, such as hnRNP U in mammals and HRP65 in *C. tentans*, and facilitates the recruitment of HATs, such as PCAF and p2D10 to the transcribing gene. HATs acetylate histones that maintain genes in active state. (C) Actin is implicated in chromatin remodelling as a component of ATP-dependent chromatin remodelling complexes. TBP, TATA binding protein; TFII B and TFII F, transcription factor II B and transcription factor IIF; Pol II, RNA polymerase II; hnRNP U, heterogeneous nuclear protein U; HAT, histone acetyltransferase.

5.3 Role of actin in RNA transcription by RNA polymerase III

Actin also plays an important role in RNA polymerase III transcription. Hu and colleagues have shown that actin can be co-purified with either tagged or endogenous RNA polymerase III, like with polymerase I and II (Hu *et al.*, 2003;Hu *et al.*, 2004). Moreover, β-actin was found to be associated with RNA polymerase III via direct protein-protein interactions with at least one of three RNA polymerase III subunits RPC3, RPABC2 and RPABC3. ChIP assays showed that β-actin is located at the promoter region of actively transcribed U6 gene, and can be dissociated from RNA polymerase III complex after inhibition of transcription by methane methylsulfonate (MMS), which resulted in the appearance of an inactivate RNA polymerase III. Notably, in an *in vitro* U6 transcription system, this inactive RNA polymerase III was not able to perform transcription, while adding exogenous β-actin reconstituted the transcriptional activity. These data indicate a crucial role for actin in RNA polymerase III-mediated transcription.

5.4 Regulation of subcellular localization and activity of transcription factor

It has been shown that nuclear actin is associated with the transcription activity of the serum response factor (SRF), a member of highly conserved MADX box family of transcription factors. SRF regulates the expression of immediate-early genes such as c-fos and actin, as well as muscle-specific genes, and these target genes are involved in cell growth, proliferation, differentiation and cytoskeletal organization (Miano, 2003). Myocardin and myocardin-related transcription factors (MRTFs) act as powerful cofactor of SRF in mammalian cells (Cen *et al.*, 2004;Parmacek, 2007). MRTF-A (also known as MAL and MKL1) is a G-actin binding protein and its subcellular localization and activity are regulated by the concentration of monomeric actin (Miralles *et al.*, 2003;Vartiainen *et al.*, 2007). In serum-starved NIH 3T3 cells, MRTF-A predominantly resides in the cytoplasm, where it interact with G-actin via its N-terminal RPEL domain (Guettler *et al.*, 2008;Miralles *et al.*, 2003). Activation of RhoA signalling, by stimulation with serum for example, results in increased actin polymerization and decreased G-actin level, respectively. Sensing depletion of the G-actin pool, MRTF-A dissociates from G-actin and rapidly accumulates in nucleus. In the nucleus, MRTF-As physically associate with SRF, facilitating the binding of SRF to CArG box to activate the transcription of target genes (Du *et al.*, 2004;Vartiainen *et al.*, 2007;Zhou and Herring, 2005). MRTF-A contains an unusually long bipartite NLS located within the RPEL domain. Pawlowski and colleagues demonstrated that importin α/β heterodimer competitively binds to the RPEL domain with G-actin via interaction with NLS. Importantly, this binding was shown to mediate the nuclear import of MATF-A (Pawlowski *et al.*, 2010). MRTF-A also binds G-actin in the nucleus and this association is required for export of MRTF-A from the nucleus (Guettler *et al.*, 2008;Vartiainen *et al.*, 2007). Furthermore, actin binding to MRTF-A in the nucleus inhibits the activity of the latter, and subsequently SRF-mediated transcription. Therefore, actin regulates SRF activity through modulating the sublocalization of MRTF-A and its activity within the nucleus (Vartiainen *et al.*, 2007). (Figure 2).

Striated muscle activator of Rho signalling (STARS), a muscle-specific ABP, is capable of stimulating the transcription activity of SRF through a mechanism involving RhoA activation and actin polymerization. MRTFs, including MRTF-A and MRTF-B, were shown to serve as a linker between STARS stimulation and SRF activity (Arai *et al.*, 2002). Studies have demonstrated that STARS can substitute for serum signalling and promote the nuclear traslocation of MRTF-A and MRTF-B, and subsequently activate the SRF-dependent transcription (Kuwahara *et al.*, 2005). The STARS protein contains a conserved actin-binding

domain within the 142 residues of C-terminus (Arai *et al.*, 2002). The C-terminal mutant of STARS, N233, which cannot bind actin, was unable to induce the nuclear translocation of MATF-A and MATF-B and to enhance the MRTF-mediated activation of SRF- dependent transcription. In contrast, the C-terminal 142 amino acids of STARS, which can bind actin , was shown to induce the nuclear accumulation of MRTFs and to synergistically enhance the MRTF-mediated transcription activation as efficiently as full-length STARS (Kuwahara *et al.*, 2005). In addition, stimulation of nuclear translocation of MRTFs by STARS can be inhibited by Latrunculin B. Similarly, inhibition of Rho A activity by treatment with C3 transferase or by the expression of dominant-negative Rho A prevents nuclear accumulation of MRTFs and subsequently MRTF-mediated SRF activation (Kuwahara *et al.*, 2005). Although Rho A activity is required in this process, it seems not to act as a downstream effector of STARS, since Rho A activity in STARS-transfected cells does not appear to be different from that observed in untransfected cells. However, it has been well documented that STARS requires Rho-actin signalling to evoke its stimulatory effects. As STARS binds to actin and induces actin polymerization (Arai *et al.*, 2002;Kuwahara *et al.*, 2005), it has been suggested that STARS stimulates the MRTF activity by inducing the dissociation of MRTF from actin and subsequently promoting its nuclear accumulation (Figure 2).

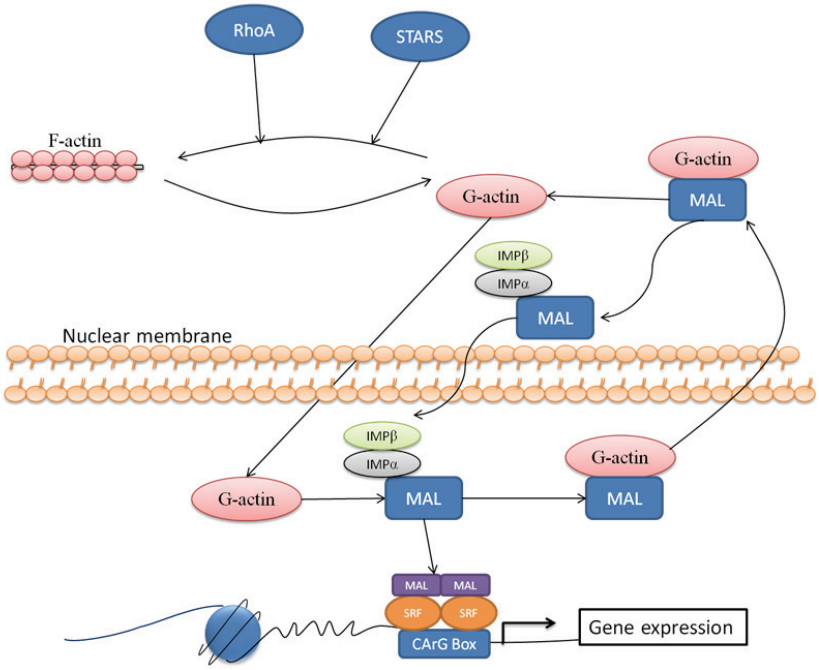

Fig. 2. Model for actin dynamics regulating SRF activity. Rho signalling (non-muscle cells) or STARS signalling (muscle cells) promote the assembly of F-actin from monomeric G-actin. Sensing depletion of G-actin, MAL dissociates from G-actin and is imported into nucleus through binding to heterodimer importin α/β. In the nucleus, MAL binds and activates SRF activity. G- actin also binds MAL in the nucleus and mediates the nuclear export of MAL. STARS, striated muscle activator of Rho signalling; MAL, also known as MRTF-A, myocardin-related transcription factor A; IMPα/β, importin α/β; SRF, serum response factor.

Interestingly, SRF activity is regulated by actin/MRTF interaction and in turn, SRF controls the transcription of many genes encoding actin isoforms and ABPs (Posern and Treisman, 2006;Sun *et al.*, 2006). SRF-regulated genes can encode structural components of actin microfilament (for example, actin), effectors of actin turnover (for example, cofilin 1) as well as regulator of actin dynamics (for example, filamin A)(Olson and Nordheim, 2010).

6. Roles of actin in genome organization

It is well known that the eukaryotic nucleus is highly organized into morphologically and functionally distinct subnuclear compartments, which spatially separate different physiological processes. These compartments include chromatin territories, proteinaceous nuclear bodies (e.g. nucleolus, Cajal body, speckle and PML body), compartmentalized multiprotein complexes such as transcription factories, and nuclear pore complexes that regulate nucleocytoplasmic transport of certain molecules. Within the interphase nuclei, chromosomes are non-randomly organized, but occupy discrete regions, known as chromosome territories (CTs). This was first suggested by Rabel in 1885, but until almost one hundred years later, Cremer and his group carried out experiments to indirectly demonstrate the existence of CTs (Cremer *et al.*, 1982). By using non-radioactive *in situ* hybridization, several groups unequivocally confirmed that the chromosomes are not distributed throughout the nucleus during the interphase, but confined to subnuclear domains (Borden and Manuelidis, 1988;Lichter *et al.*, 1988;Schardin *et al.*, 1985). Initial reports, based mainly on fluorescence *in situ* hybridization (FISH) demonstrated that these CTs are non-overlapping. However, Branco and co-workers (Branco and Pombo, 2006), using a high resolution cryo-FISH technique, revealed a significant intermingling of CTs in interphase, suggesting CTs interact more than previous thought. Interestingly, gene-rich CTs tend to occupy the interior of the nucleus, whereas many of the gene-poor chromosomes are associated with the nuclear periphery (Cremer *et al.*, 2001;Croft *et al.*, 1999). The gene-density-correlated radial organization of CTs was confirmed by analyses comprising all human chromosomes (Boyle *et al.*, 2001). This non-random radial distribution of CTs was also observed in rodents (Neusser *et al.*, 2007), cattle (Koehler *et al.*, 2009) and birds (Habermann *et al.*, 2001), suggesting that it has been evolutionary conserved. In addition, chromosome size-correlated radial arrangements have also been described (Sun *et al.*, 2000) and such organization seems to occur in flat nuclei (Bolzer *et al.*, 2005). However, the genome organization of eukaryotic cells is dynamic and chromosome arrangement can change in response to cellular signals.

An association of actin with various chromatin states has been shown by a number of studies (Milankov and De, 1993;Sauman and Berry, 1994). Bacterial actin was shown to be involved in plasmid and chromosome segregation (Becker *et al.*, 2006;Moller-Jensen *et al.*, 2007). In eukaryotic cell, chromosome segregation is generally driven by microtubules, however, in the oocyte of starfish, chromosome congression requires actin polymerization (Lenart *et al.*, 2005). Mehta and colleagues have recently demonstrated that CTs relocate in quiescent human fibroblasts (Mehta *et al.*, 2010). In the absence of serum stimulation, a number of chromosomes were observed to change position in the interphase nuclei; however, this kind of chromosome movement can be prevented by the inhibition of actin and myosin polymerization or knockdown of nuclear myosin 1β by RNA interference experiments. Inhibition of ATPase and/or GTPase also blocked the chromosome movement

upon serum withdrawal, suggesting that this kind of chromosome movement is an energy-dependent active process. In an earlier study, using a novel tool to visualize chromatin movement in living cells, Chuang and co-workers (Chuang *et al.*, 2006) reported long-range vectorial movements of chromatin exceeding 1μm. Furthermore, the authors found that repositioning of a chromatin locus was completely abolished in cells transfected with a nonpolymerizable actin mutant, whereas a mutant stabilizing filamentous actin accelerated locus redistribution. Transfection with a myosin mutant significantly delayed locus reposition. Dundr and colleagues (Dundr *et al.*, 2007) analyzed the dynamic association between Cajal bodies and U2 snRNA gene. Upon transcriptional activation, the chromosome region containing U2 snRNA genes moved toward the Cajal bodies which are relatively stably positioned. Inactivated U2 snRNA genes do not associate with the Cajal bodies. Similarly, this process was also found to be actin-dependent.

Actin and NM1 are also required for estrogen-induced interchromosomal interactions. Two estrogen-regulated loci, TFF1 and GREB1, located in different chromosomes colocalize after stimulation with 17β-estradiol (Hu *et al.*, 2008). This interaction was blocked by treatment of the cells with Latrunculin or jasplakinolide, which inhibit actin polymerization and depolymerisation, respectively. Depletion of actin or NM1 by siRNAs or nuclear microinjection of specific antibodies against NM1 also blocked the interaction. Furthermore, the inhibitory effect of anti-NM1 antibodies could be rescued by the expression of wild-type NM1, but not by the expression of NM1 mutant deficient in ATPase or actin-binding activity. The results demonstrate that the dynamics of nuclear actin affect the chromatin movement and gene positioning. How actin and NM1 cooperate to organize genome and facilitate the regulation of gene expression still needs to be studied in more detail.

7. Conclusion

The past decade has seen great advances in discovering the versatile functions of actin in the nucleus. Actin participates not only in the basal transcription mediated by all three RNA polymerases, as a component of RNA polymerase complex or pre-mRNP particles, but also in the transcriptional regulation as a component of chromosome remodelling complex. Actin also plays a role in movement, organization, and regulation of chromatin and activated genes in the nucleus. In addition, actin acts as a multifunctional brick in the nuclear architecture to help maintain nuclear shape, spatial order and nuclear functions. The challenge now is to understand the molecular mechanisms that underlie the many functions of actin in these nuclear processes.

In recent years, studies have shown that nuclear actin can exist as a polymeric form and that actin polymerization is implicated in transcription (Wu *et al.*, 2006;Ye *et al.*, 2008;Yoo *et al.*, 2007). However, the form of actin in various complexes associated with transcription seem to be monomeric and its functions in theses complexes do not appear to require polymerization/depolymerisation dynamics. There are two possible explanations for why this is so. First, actin polymerization may maintain a proper G-actin pool for transcription. Second, the polymeric state affects the movement of gene loci (Hu *et al.*, 2008), therefore, may affect the transcription. From this view point, it is important to address the dynamic behaviour of nuclear actin in order to understand how actin regulates transcription, for example, how actin polymerization is regulated inside the nucleus. What are the roles of

ABPs in the nucleus? It seems that many APBs have specific nuclear functions that are not related to the regulation of actin dynamics. Therefore, identifying novel ABPs is expected to be an important way to understand the regulation of actin polymerization. In addition, there is evidence to show the existence of a communication between cytoplasmic actin and nuclear actin pool (Vartiainen *et al*., 2007). But what are the signalling pathways that link cytoplasmic actin dynamics and nuclear actin behaviour? What are the mechanisms that controlling the nucleocytoplasmic shuttling of the actin? Although actin has functional NESs, it is always found to be exported in a complex with other cargo, for example with profilin and with MRTF-A.

Recently, studies have well documented that both nuclear actin and NM1 are implicated in the movement of CTs and chromosomal loci. The interactions of chromosomal loci with functional subnuclear domains, such as Cajal bodies, as well as interactions between distinct chromosomal loci are important for transcriptional regulation and genome-based nuclear processes. It is most likely that actin cooperates with NM1 as a motor to drive and direct the movement of chromosomal loci. Nevertheless, actin may also acts as a component of chromatin remodelling complex to relax the chromatin structure. New technologies need to be developed to investigate exact mechanisms of involvement of actin and NM1 in movement of chromosomal loci. In summary, there is still a long way to fully understanding the complexity of actin in the structural and functional networks of the nucleus.

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10

Signaling of Receptor Tyrosine Kinases in the Nucleus

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1. Introduction

Since the discovery of the first receptor tyrosine kinase (RTK) proteins in the late 1970s and early 1980s, many scientists have explored the functions of these important cell signaling molecules. The finding that these proteins are often deregulated or mutated in diseases such as cancers and diabetes, together with their potential as clinical therapeutic targets, has further highlighted the necessity for understanding the signaling functions of these important proteins. The mechanisms of RTK regulation and function have been recently reviewed by Lemmon & Schlessinger (2010) but in this review we instead focus on the results of several recent studies that show receptor tyrosine kinases can function from sub-cellular localisations, including in particular the nucleus, in addition to their classical plasma membrane location. Nuclear localisation of receptor tyrosine kinases has been demonstrated to be important for normal cell function but is also believed to contribute to the pathogenesis of several human diseases.

2. Classical signaling by receptor tyrosine kinases

The ability of a cell to receive signals from the outside, and deliver these inside so it can respond appropriately and in co-ordination with other cells, is required for the correct functioning of a multicellular organism as a whole. Cells communicate in two key ways – direct physical interaction or by way of communication molecules. These communication molecules, collectively called ligands, include those (eg steroid hormones, vitamins) that can pass directly through the lipid bilayer of the cell and interact with intracellular proteins and those such as protein hormones and peptide growth factors which cannot enter the cell directly. These latter ligands interact with plasma membrane-associated proteins called receptors to activate cascades of interactions between intracellular proteins that can result in a diverse range of responses and ultimately determine cell behaviour (Figure 1).

One large family of membrane receptors, the receptor tyrosine kinases (RTKs), is characterised by their intrinsic protein tyrosine kinase activity, an enzymatic function which catalyses the transfer of the γ phosphate of ATP to hydroxyl groups on tyrosine residues on target proteins (Hunter, 1998). Binding of the ligand stabilises dimers of the receptors to allow autophosphorylation *via* activation of the receptors' intrinsic tyrosine kinase activity that then initiates a network of sequentially acting components such as those of the

Ras/MAPK (mitogen-activated protein kinase) pathway, or single component systems, such as the STAT pathway. The combination of the activated signal transduction pathways constitute the mechanism by which this intracellular transfer of biochemical information is mediated and can determine the biological responses of cells to growth factors. Members of the RTK family play important roles in the control of most fundamental cellular processes including cell proliferation and differentiation, cell cycle, cell migration, cell metabolism and cell survival.

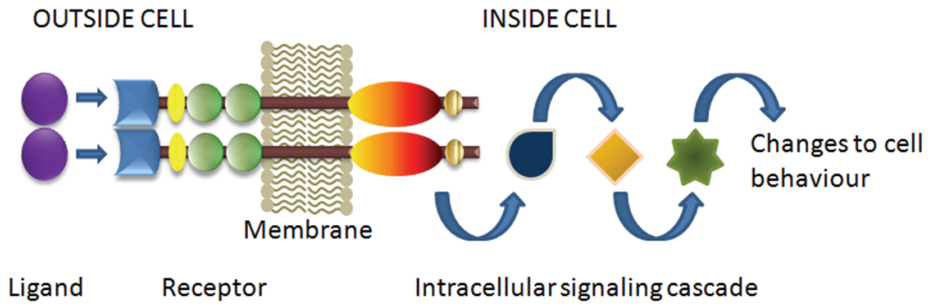

Fig. 1. Classical receptor tyrosine kinase signaling. Ligand binding stablilises dimers of the receptors within the plasma membrane. Autophosphorylation of one intracellular kinase domain by the other activates a signal transduction cascade into the cell so the cell can respond appropriately.

3. Protein structures of receptor tyrosine kinases

The general structure of RTK proteins is similar and all members of the RTK family have an intracellular kinase domain through which signaling is mediated by phosphorylation of tyrosine residues. In addition to the kinase domain, all RTKs have an extracellular domain, usually glycosylated, separated from the cytoplasmic part, containing the kinase domain, by a single hydrophobic transmembrane α helix. With the exception of the insulin (IR) and insulin-like growth factor (IGFR) receptor families, which are disulfide linked dimers of two polypeptide chains (α and β) that form a heterodimer (α2β2), RTKs are normally present as monomers in the cell membrane. Ligand binding induces receptor dimerisation resulting in autophosphorylation (the kinase domain of one RTK monomer cross-phosphorylates the other and *vice versa*). Receptor dimerisation is further stabilised by receptor:receptor interactions and the clustering of many receptors into lipid rich domains on the cell membrane (Pike, 2003). Further division of the 58 human RTKs into 20 different classes is based on similarities in primary structure, and the combinations of further functional domains in both extracellular and intracellular parts of the proteins (Figure 2).

4. Trafficking of receptor tyrosine kinases

Ligand activation of receptor tyrosine kinases present on the plasma membrane of cells promotes numerous downstream signal transduction pathways that result in cell responses including proliferation, migration and differentiation. Following ligand activation, virtually all receptor tyrosine kinases are rapidly endocytosed. This would allow the cell to

discriminate new signals from old ones but it has been suggested that, because trafficking is a complex and highly regulated process, it is likely that endocytosis provides more than just a mechanism for removal of receptor-ligand complexes from the cell surface. Endocytosed receptors can be either recycled back to the membrane after disengagement of the ligand, or targeted for lysosomal degradation. Most receptor tyrosine kinases are internalised *via* clathrin-coated pits which then shed the clathrin and deliver the internalised receptor-ligand complexes to early endosomes. Bifurcation of receptor trafficking occurs in the early endosomes, allowing either recycling back to the plasma membrane or degradation through lysosomes. In some cases continued signaling from the endosomes has also been demonstrated (Ceresa & Schmid, 2000; Di Fiore & De Camilli, 2001; Wang et al., 2004a).

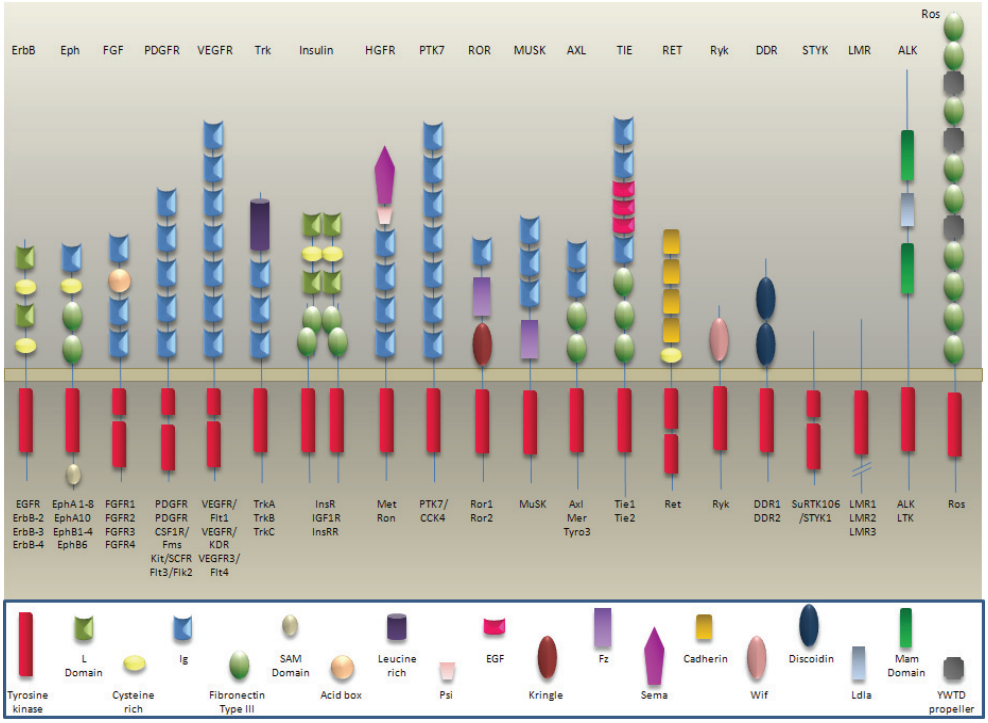

Fig. 2. Domain structures of 58 human receptor tyrosine kinases determines their sub-classification into 20 different families. The name of each family is shown above with the members listed below. A key indicates the various motifs common to individuals within that family.

Recent data also suggest that endocytosis controls sub-cellular localisation of activated receptors and their signaling complexes (Beguinot et al., 1984; Sorkin & Waters, 1993). For example, the prototypical receptor tyrosine kinase, the Epidermal Growth Factor Receptor (EGFR), has been found in caveoli, Golgi, endoplasmic reticulum, lysosome-like structures and nuclear envelopes (Carpentier et al., 1986; Lin et al., 2001). Given the continuity of the endomembrane system, linking endoplasmic reticulum, Golgi membranes, the plasma

membrane, vesicles of both the endosomal and lysosomal systems and even the nuclear membrane, it is probably not surprising that receptors would be found within the membranes of these structures.

It also appears that endocytosis and trafficking of vesicles is involved in localisation of receptor tyrosine kinases to the nucleus. Nuclear localisation of receptor tyrosine kinases has emerged as a highly significant occurrence in the last decade, with reports indicating that the EGFR (ErbB-1 and -2), FGFR1 and IGF-IR can all translocate to the nucleus as full-length receptors or protein fragments devoid of the extracellular domain. In some cases this has been found to be ligand-dependent, within as early as 2 minutes of ligand stimulation, although there are also cases in which nuclear translocation appears to be ligand-independent. Nuclear localisation of several receptor tyrosine kinases has been identified in cells of normal tissues, including EGFR in the nucleus of regenerating liver cells (Marti and Wells, 2000) and ErbB-4 in the nuclei of secretory epithelium in the lactating breast (Long et al., 2003; Tidcombe et al., 2003). For many receptor tyrosine kinases, also including EGFR and ErbB-4, nuclear localisation has been linked to diseases including cancer, diabetes and inflammation (Citri & Yarden, 2006; Lo & Hung, 2006; Massie & Mills, 2006; Bublil & Yarden, 2007; Wang & Hung, 2009; Wang et al., 2010). For example, the nuclear presence of EGFR is associated with high grade breast and ovarian cancers and is associated with the development of resistance to some radio-, chemo- and monoclonal antibody-therapies (Lo et al., 2005a; Xia et al., 2009).

5. Mechanisms of receptor tyrosine kinase translocation to the nucleus

It has been hypothesised that in order for a receptor tyrosine kinase to translocate to the nucleus it must somehow 'escape' from the lipid bilayer of the cell surface and/or the trafficking of the endomembrane system. Exactly how this happens is only just being explored experimentally, but Wells & Marti (2002) have proposed three potential 'escape' mechanisms using EGFR as a model receptor tyrosine kinase. In the first, a mutant EGFR protein, lacking the transmembrane domain, forms a dimer with a wild-type receptor on the cell surface. Binding of EGF causes internalisation of the mutant-wild-type dimer *via* a clathrin-coated pit into an early endosome. The mutant EGFR is disassociated from the wild-type protein in the endosome and released into the cytosol, and from there it is transported into the nucleus. In the second scenario, full-length wild-type EGFR is trafficked from the plasma membrane to the endoplasmic reticulum, where it interacts with an accessory protein that removes it from the membrane for translocation into the nucleus. In the third, EGFR is targeted by proteases at the plasma membrane and an intracellular fragment translocates to the nucleus again by interaction with nuclear transport proteins. Recently, Liao & Carpenter (2007) provided support for the second scenario by showing that EGFR in the endosome associates with an accessory protein Sec61β, a component of the Sec61 translocon and is then retrotranslocated from the ER to the cytoplasm and from there translocated to the nucleus by nuclear transport proteins.

6. Nuclear localisation sequences and importins

Transport of proteins into the nucleus through the nuclear-pore-complex can be facilitated by the dedicated nuclear transport receptors of the β-karyopherin family which includes the

importins (Gorlich and Kutay, 1999). Proteins translocated *via* importins contain nuclear localisation signals (NLS), a short stretch of amino acids that mediates the transport of proteins into the nucleus (Cokol et al., 2000). NLS motifs can be either monopartite, characterised by a cluster of basic residues preceded by a helix-breaking residue, or bipartite, where two clusters of basic residues are separated by 9–12 residues (Cokol et al., 2000). In the classical process of NLS-mediated nuclear translocation, an importin-α adaptor protein binds to a lysine-rich NLS in the cargo protein. An importin-β protein then binds to this importin-α/cargo complex through an NLS in the importin-α protein itself and guides the complex through the nuclear pore. Importin-β proteins are the key import mediators and can also bind non-classical NLS motifs, of which there are several types, to transport proteins without requiring importin-α interaction. In addition to basic NLSs, several other small epitopes have been identified that, when phosphorylated, can promote nuclear import (Nardozzi et al., 2010). These include the nuclear transport signal (NTS) of ERK1/2, which is a Ser-Pro-Ser (SPS) motif that, upon stimulation, is phosphorylated and functionally active as a binding site for the nuclear transport receptor importin-β7 (Chuderland et al., 2008).

7. Receptor tyrosine kinases reported to translocate to the nucleus

7.1 Epidermal Growth Factor Receptor (EGFR)/ErbB family

The Epidermal Growth Factor Receptor (EGFR) family of receptor tyrosine kinases, also known as ErbB (named after the viral oncogene v-erb-B2) or Human Epidermal growth factor Receptor (HER) receptors, contains four members: EGFR/ErbB-1/HER1, ErbB-2/HER2/Neu, ErbB-3/HER3 and ErbB-4/HER4. These receptors are expressed in various tissues of epithelial, mesenchymal and neuronal origin. Activation of ErbB receptors is controlled by the spatial and temporal expression of their 11 different ligands, all encoded by separate genes and all members of the EGF family of growth factors. These include EGF, epigen, transforming growth factor alpha (TGF-α), and amphiregulin, which bind EGFR; neuregulins (NRGs) 1,2,3,4, which bind ErbB-3 and/or ErbB-4, and betacellulin, heparin-binding EGF-like growth factor, and epiregulin, which bind EGFR and ErbB-4 (Riese & Stern, 1998). Ligand binding induces receptor dimerisation, and both homodimers and heterodimers with other ErbBs may be formed, and this then leads to the activation of a diverse range of downstream signaling pathways depending on the dimers and cross-activation of other ErbBs on the cell surface (Stern et al., 1986; Riese et al., 1995; Riese & Stern, 1998; Zaczek et al., 2005). Heterodimerisation is particularly important for signaling through ErbB-2, which lacks a conventional growth factor ligand, and ErbB-3, which has an inactive/impaired kinase domain.

Excessive EGFR, ErbB-2 and ErbB-3 signaling, as a result of receptor over-expression, mutations or autocrine stimulation, is a well known hallmark of a wide variety of solid tumours and leads to both increased cell proliferation and resistance to growth-inhibitory cytokines (Hynes & Lane, 2005). In contrast, ErbB-4 appears to be associated with growth suppression and improved patient prognosis in breast cancer (Jones, 2008; Muraoka-Cook et al., 2008). In addition, all four members of the ErbB family have a sub-membrane importin alpha-binding basic NLS that allows transport from the cytosol to the nucleus by the importin α/β complex. Consequently, ErbB proteins have been detected in the nucleus of both normal cells and cancer cells (Marti et al., 1991; Marti & Hug, 1995; Marti & Wells, 2000; Citri &Yarden, 2006; Lo & Hung, 2006; Massie & Mills, 2006; Bublil & Yarden, 2007; Wang &

Hung, 2009; Wang et al., 2010). In multiple cancer types, nuclear accumulation correlates with poor patient survival, tumor grade, and pathologic stage (Lo et al., 2005a; Psyrri et al., 2005; Junttila et al., 2005; Koumakpayi et al., 2006; Lo & Hung, 2006; Maatta et al., 2006; Hoshino et al., 2007; Xia et al., 2009; Hadzisejdic et al., 2010).

7.1.1 Epidermal Growth Factor Receptor (EGFR/ErbB-1/HER1)

Nuclear EGFR, and its ligands EGF and proTGF-α, were first observed in hepatocytes during liver regeneration (Raper et al., 1987; Marti et al., 1991; Marti & Hug, 1995; Marti & Wells, 2000; Grasl-Kraupp et al., 2002). Translocation of EGFR to the nucleus is also induced by DNA damage caused by irradiation (UV and ionizing) and cisplatin treatment but this appears to be ligand-independent (Dittmann et al., 2005; Xu et al., 2009). Full length EGFR is translocated into the nucleus through interactions with importin β-1, the nucleoporin protein Nup358 and proteins known to be involved in endocytotic internalisation of these proteins from the plasma membrane. Once in the nucleus, EGFR has three different roles depending on the initial signal, 1) as a direct regulator of gene transcription, 2) regulating cell proliferation and DNA replication *via* its kinase function, and 3) DNA repair and chemo- and radio-resistance through protein-protein interactions (Lin et al., 2001; Dittmann et al., 2005; Wang et al., 2006; Das et al., 2007; Kim et al., 2007; Wanner et al., 2008; Hsu & Hung, 2007). As a direct regulator of gene transcription, the C-terminal domain of EGFR directly interacts with the genome through binding and activating AT-rich sequences in the cyclin D1, nitric oxide synthetase (*iNOS*), *Aurora-A* and *B-myb* promoters (Liao and Carpenter, 2007; Lo, 2010). Nuclear EGFR interacts with STAT5 or STAT3 to transactivate the expression of the *Aurora-A* or *iNOS* genes respectively (Hung et al., 2008; Lo et al., 2005b). Nuclear EGFR can regulate cell proliferation and DNA replication by direct tyrosine phosphorylation of target proteins including chromatin bound proliferating cell nuclear antigen (PCNA) (Wang et al., 2006). EGFR kinase activity phosphorylates PCNA on tyrosine 211, stabilising the PCNA protein and stimulating DNA replication. In its third role, nuclear EGFR stimulates DNA repair by forming a direct protein-protein interaction with DNA-dependent protein kinase (DNA-PK) (Dittmann et al., 2005).

In addition to localisation to the plasma membrane and the nucleus, EGFR has also been found in the Golgi Apparatus, endoplasmic reticulum and the mitochondria (Carpentier et al., 1986; Lin et al., 2001; Boerner et al., 2004). EGFR was first reported in the mitochondria by Boerner et al., (2004) who found that in the presence of EGF, Src mediated the phosphorylation of EGFR residue Y845. EGFR phosphorylated at Y845 was found in the mitochondria and interacted with cytochrome c oxidase subunit II (CoxII) to possibly regulate cell survival. The method by which EGFR is translocated to the mitochondria is unknown, but was not related to endocytotsis of the EGFR protein and did not involve the function of Shc adaptor proteins (Yao et al., 2010). Furthermore, deletion studies showed that a putative mitochondrial-targeting signal between amino acids 646 and 660 was only partially responsible for migration (Boerner et al., 2004).

7.1.2 ErbB-2/HER2/Neu

Although ErbB-2 is catalytically active, it cannot bind the heregulin (HRG) ligand directly, but instead dimerises with either HRG-bound ErbB-3 or ErbB-4 to form a complex that is

capable of signaling through either ErbB-2 or ErbB-4 (ErbB-3 is catalytically inactive/impaired) (Carraway et al., 1994). Upon HRG stimulation, cell-membrane embedded ErbB-2 migrates from the cell surface *via* early endosomes and is then either targeted to lysosomes for degradation, or recycled back to the surface. By an as yet undefined mechanism, ErbB-2 can also be removed from the lipid bilayer to form a complex with both importin β1 and EEA1 (Giri et al., 2005). This complex then moves through the nuclear pore complex into the nucleus. Once in the nucleus, ErbB-2 can form a complex with β-actin and RNA polymerase-1, enhancing binding of RNA pol 1 to rDNA, and progressing the early and elongation steps of transcription to expedite rRNA synthesis and protein translation (Li et al., 2011). The nuclear function of ErbB-2 would appear to be unrelated to its normal signaling role transduced through PI3-K and MEK/ERK because inhibitors to these kinases (LY294002 and U0126, respectively) did not affect the levels of 45S pre-rRNA in these cells. In addition to this role in expediting overall rRNA synthesis and protein translation, nuclear ErbB-2 has also been shown to bind to the promoter of the cyclooxygenase enzyme (*COX-2*) and up-regulate its expression. *COX-2* catalyzes the conversion of lipids to inflammatory prostaglandin and contributes to increased anti-apoptotic, pro-angiogenic, and metastatic potential in cancer cells (Vadlamudi et al., 1999; Howe et al., 2001; Gupta & DuBois, 2001; Half et al., 2002; Subbaramaiah et al., 2002; Turini & DuBois, 2002). The promoters of *PRPK*, *MMP16* and *DDX10* have also been identified as direct targets of nuclear ErbB-2 (Wang et al., 2004b).

7.1.3 ErbB-3/HER3

The kinase domain of ErbB-3 has been described as either catalytically inactive or impaired. Despite this ErbB-3 forms dimers with other ErbB receptors, and can recruit novel proteins to activate diverse signaling pathways (Guy et al., 1994; Zaczek et al., 2005). Intact ErbB-3 was detected in nuclei of prostate cancer cells in metastatic specimens (Koumakpayi et al., 2006; Cheng et al., 2007). Nuclear localisation was then studied in a model of prostate cancer using the MDA-PC 2b cells and this demonstrated that both the tumour microenvironment and androgen status influenced nuclear localisation of ErbB-3 in these cells (Cheng et al., 2007). Metastasis of prostate cancer cells to the bone and depletion of androgens from subcutaneous tumours both increased the nuclear translocation of ErbB-3. This also correlated with a decrease in cell proliferation. Once the tumours resumed aggressive growth, ErbB-3 then relocalised from the nucleus to the membrane and cytoplasm of the prostate cancer cells. This suggests that nuclear ErbB-3 may be involved in the progression of prostate cancer in bone after androgen-ablation therapy. ErbB-3 has also been identified in the nucleus, and possibly within the nucleolus, of both normal and malignant human mammary epithelial cells (Offterdinger et al., 2002). The role of nuclear ErbB-3 in these cells has not been determined but yeast two-hybrid approaches have been used to identify several transcription factors that associate with ErbB-3 including p23/p198 (Yoo & Hamburger, 1999), early growth response-1 (Thaminy et al., 2003) and the zinc finger protein ZNF207 (Thaminy et al., 2003) suggesting a gene regulation function. Finally, alternative transcription initiation of the ErbB-3 gene in Schwann cells leads to the production of a nuclear targeted variant of ErbB-3 that binds to chromatin and regulates the transcriptional activity of the ezrin and *HMGB1* genes (Adilakshmi et al., 2011).

7.1.4 ErbB-4/HER4

ErbB-4 has multiple functions during embryogenesis (Gassmann et al., 1995) and expression has recently been shown to be essential during breast development and lactation. In the lactating breast, ErbB-4 localizes to the nuclei of secretory epithelium (Long et al., 2003; Tidcombe et al., 2003). A unique proteolytic cleavage mechanism leads to the nuclear translocation of an intracellular fragment of ErbB-4. Cell membrane expressed ErbB-4 is successively cleaved by TACE/ADAM17, to release the ectodomain, and then γ-secretase to release an 80 kDa soluble intracellular fragment (s80) (Ni et al., 2001). This active kinase fragment binds to YAP (Yes-associated protein) which facilitates its translocation to the nucleus (Komuro et al., 2003). ErbB-4 also has three potential polycationic NLSs in its carboxy-terminal part which may provide an alternative route for nuclear translocation (Williams et al., 2004). The ErbB-4 s80 fragment functions as a nuclear chaperone for the STAT5A, co-translocating this transcription factor and regulating the expression of target genes including β-casein by binding with STAT5 to the β-casein promoter (Long et al., 2003; Williams et al., 2004). ErbB-4 also contains a nuclear export signal (NES) recognised by exportin proteins allowing transport of the protein out of the nucleus as well.

7.2 Fibroblast growth factor receptor family

The fibroblast growth factor (FGF) family consists of 18 secreted polypeptidic growth factors that bind to four high-affinity receptors (FGFR1-4) and assist in the regulation of cell proliferation, survival, migration and differentiation during development and in adult tissue homeostasis (Wesche et al., 2011). FGFs also bind to low-affinity heparan sulfate proteoglycans (HSPGs) present on most cells, which assist in the formation of the FGF-FGFR complex and protect the ligands from degradation. Overactivity of FGFR signaling is associated with several developmental disorders and cancer (Wesche et al., 2011).

7.2.1 FGFR1 (Fibroblast growth factor receptor 1)

Nuclear localisation of full length FGFR1 has been reported in astrocytes, glioma cells, neurons, fibroblasts and retinal cells and has been shown to be important for neuronal differentiation in the central nervous system (Stachowiak et al., 2003a; Stachowiak et al., 2003b). Nuclear accumulation is induced by many different stimuli including activation of acetylcholine receptors, stimulation of angiotensin II receptors, activation of adenylate cyclase or protein kinase C. Biotinylation of cell surface proteins showed that nuclear FGFR1 was unlikely to have been derived from the cell surface (Stachowiak et al., 1997; Peng et al., 2002). Because nuclear FGFR1 is glycosylated the suggestion is that the protein is at least partially processed through the ER-Golgi but that it is not stable in the endomembrane system and is released into the cytosol (Myers et al., 2003). It is also not clear how FGFR1 is then translocated to the nucleus as it lacks a typical NLS. However, several members of the fibroblast growth factor (FGF) family, including FGF-1 and FGF-2, lack signal peptide sequences and are therefore found in trace amounts, if at all, outside of cells. Some of these, for example FGF-2, have nuclear localisation sequences and are highly concentrated in the cell nucleus and it is believed that these FGF ligands act as chaperones for the translocation of receptors like FGFR1 into the nucleus (Myers et al., 2003). Although FGFR1 in the nucleus has been demonstrated to have FGF-regulated kinase activity and is phosphorylated, there

appears to be limited co-localisation of FGF-2 and FGFR1 in the nucleus (Peng et al., 2002). Nuclear FGFR1 physically interacts with Ribosomal S6 Kinase isoform 1 (RSK1) and regulates its transcriptional activity (Hu et al., 2004). Target genes include *FGF-2*, *c-jun*, cyclin D1 and *MAP2*, genes that are involved in cell growth and differentiation (Reilly & Maher, 2001). FGFR1 has also been shown to be involved in the activation of the tyrosine hydroxylase promoter that is mediated through a cAMP responsive element (CRE) (Fang et al., 2005).

7.2.2 FGFR2

FGFR2 has been identified in the nuclei of quiescent Sertoli cells in the testes (Schmahl et al., 2004). In this study of FGF-9 knock-out mice, FGFR2 nuclear localisation was shown to correlate with male sex determination in the early gonads. The presence of FGFR2 in the nucleus coincides with the expression of the sex-determination gene *Sry* and the differentiation of progenitor cells in the gonads into Sertoli cells.

7.2.3 FGFR3

FGFR3 is a major negative regulator of linear bone growth and gain of function mutations cause the most common forms of dwarfism in humans as these are anti-proliferative (Colvin et al., 1996; Deng et al., 1996). Somatic mutations have been detected in several cancers where, by contrast, they are believed to drive proliferation and inhibit apoptosis (Trudel et al., 2004). Binding of FGF-1 to FGFR3 induces endocytosis *via* a dynamin/clathrin-mediated process to an endosomal compartment. Here the ectodomain is proteolytically cleaved possibly by an endosomal cathepsin although this has not yet been confirmed. The membrane anchored intracellular fragment is then cleaved in a second event by γ-secretase to generate a soluble intracellular domain that is released into the cytosol and can translocate to the nucleus. This requirement for endocytosis distinguishes FGFR3 proteolysis from that of most other RTKs.

7.3 VEGFR (Vascular endothelial growth factor receptor)

Cellular responses to the ligand vascular endothelial growth factor (VEGF) are activated through two structurally related receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR) and are critically important in the regulation of endothelial cell growth and function (Cross et al., 2003). Stimulation of endothelial cells with VEGF induced the translocation of VEGFR-2, eNOS and caveolin-1 into the nucleus (Feng et al., 1999). The consequences of nuclear localisation of these three proteins have yet to be clarified. Non-endothelial expression of VEGFR-2 has also been reported (Stewart et al., 2003). A recent study by Susarla et al., (2011) identified VEGFR-2 expression on normal thyroid follicular cells. The VEGFR-2 expressed by these cells was phosphorylated and, although there was some staining in the cytoplasm, the highest concentration of VEGFR-2 was seen in most nuclei. VEGFR-1 and VEGFR-3 immunoreactivity was also seen predominantly in the nucleus with VEGFR-1 also localised at points of cell to cell contact. The role that VEGF receptors play in the nucleus has not been determined but the intranuclear staining was not co-incidental with chromatin and it is therefore unlikely that VEGFR proteins act as transcription factors.

7.4 Insulin receptor

Insulin is secreted by pancreatic β-cells in response to an increase in circulating glucose level to trigger tissues to increase glucose uptake and suppress hepatic glucose release. This biological action of insulin is initiated by binding to the insulin receptor InsR (Youngren, 2007). The presence of InsR in the nucleus was first reported in 1987 by Podlecki et al., but more recently this was further characterised by Rodrigues et al., (2008) who demonstrated that the insulin receptor appears in the nucleus of hepatocytes within 2.5 min of stimulation with insulin. This translocation event was associated with selective hydrolysis of nuclear PIP2 and formation of InsP3-dependent Ca^{2+} signaling within the nucleus that regulates glucose metabolism, gene expression and cell growth (Poenie et al., 1985; Hardingham et al., 1997; Nathanson et al., 1999; Pusl et al., 2002; Rodrigues et al., 2007). Nelson et al., (2011) have identified two potential gene targets for InsR in the nucleus, the early growth response 1 (*egr-1*) gene that is involved in the mitogenic response, and the glucokinase (*Gck*) gene which encodes a key metabolic enzyme.

7.5 IGF-1R (Insulin-like growth factor 1 receptor)

The insulin-like growth factor 1 receptor (IGF-1R) plays crucial roles in development and is often over-expressed in cancer. Stimulation with insulin-like growth factor 1 (IGF-I) or 2 (IGF-II) promotes cell proliferation, anti-apoptosis, angiogenesis, differentiation and development. Over-expression of IGF-1R is common in cancer but the mechanisms underlying the role of IGF-1R are not fully understood. Recently, Sehat et al., (2010) showed that IGF-I promotes the modification of IGF-1R by small ubiquitin-like modifier protein-1 (SUMO-1) and this then mediates translocation of IGF-1R to the nucleus. Nuclear import was also enhanced by stimulation with IGF-II but only modestly by insulin, in keeping with the affinity of IGF-1R for these ligands. Full length IGF-1Rα and IGF-1Rβ chains which make up the multi-subunit IGF-1R are found in the nucleus (Aleksic et al., 2010). Although it has been reported that IGF-1R binds to chromatin and acts directly as a transcriptional enhancer, direct transcriptional effects of nuclear IGF-1R are yet to be identified.

SUMOylation is initiated by a SUMO activating enzyme, such as SAE1 or SAE2, followed by a transfer of the active SUMO to Ubc9, the only known SUMO-conjugating enzyme, which then catalyses the transfer of SUMO to the target protein (Wilkinson and Henley, 2010). Seventy-five percent of known SUMO targets are modified within the consensus motif ψKxD/E where ψ is a hydrophic amino acid and x is any residue (Xu et al., 2008). Four SUMO isoforms have been identified in mammalian cells and SUMO-1 is the most widely studied member. Modification by SUMO-1 can result in a variety of functional consequences ranging from transcriptional repression (Garcia-Dominguez & Reyes, 2009) to DNA repair, mainly through targeting of p53 and BRCA1 (Bartek & Hodny, 2010), protein stability (Cai & Robertson, 2010) and cytoplasmic-nuclear shuttling (Salinas et al., 2004; Miranda et al., 2010; Sehat et al., 2010). Currently, IGF-1R is the only receptor tyrosine kinase for which nuclear translocation may be regulated by SUMOylation.

7.6 Eph receptors

Eph receptors are the largest group of transmembrane receptor tyrosine kinases with 14 human members divided into 2 subclasses, EphA (EphA1–EphA8, EphA10) and EphB (EphB1–EphB4, EphB6) (Pitulescu & Adams, 2010). Eph receptors are activated by their

ligands the ephrins, proteins that are anchored to the plasma membrane of a neighbouring cell by either a glycosylphosphatidylinositol (GPI) anchor (type A) or a transmembrane amino acid sequence (type B). Eph-ephrin signaling plays important roles in neuronal and vascular development and many are over-expressed in various cancers (Flanagan & Vanderhaeghen, 1998; Adams & Klein, 2000; Stephenson et al., 2001; Lee et al., 2005; Pasquale, 2005; Chen et al., 2008).

To date only a single member of the Eph family, EphA4, has been reported in the nucleus (Kuroda et al., 2008). EphA4 is critically involved in development of neural tissue and more recently has been identified in hypertrophic chondrocytes and osteoblasts in the growth plate of developing mouse long bones (Kuroda et al., 2008). In the human osteoblastic cell line SaOS-2, EphA4 was found on the plasma membrane as expected, but also in the cytoplasm and in the nucleus. EphA4 accumulated in particular areas in the nucleus, but these were distinct from the nucleolus. It is not clear whether the EphA4 in the nucleus is full-length or a processed intracellular fragment and the role of EphA4 in the osteoblast nucleus has not been explored to date.

7.7 Ryk (Related to Receptor Tyrosine Kinase)

Ryk is a Wnt receptor that plays an important role in neurogenesis, neurite outgrowth, and axon guidance. Although a catalytically inactive receptor tyrosine kinase, Ryk is believed to signal *via* heterodimerisation with other receptor tyrosine kinases and has been shown to bind two members of the Eph receptor family, EphB2 and EphB3 (Halford et al., 2000). In neural progenitor cells, upon binding of Wnt3a, Ryk is cleaved at an intracellular site and the C-terminal cleavage product, Ryk ICD, translocates to the nucleus. Recently it was shown that Cdc37, a subunit of the molecular chaperone Hsp90 complex, binds to the Ryk ICD, promoting stabilization of the ICD fragment and providing the mechanism for nuclear translocation. Once in the nucleus, Ryk ICD regulates the expression of the key cell-fate determinants *Dlx2* (stimulated) and *Olig2* (inhibited) to promote GABAergic neuronal differentiation and inhibition of oligodendrocyte differentiation (Zhong et al., 2011).

7.8 Ror (RTK-like orphan receptor)

Ror1 and Ror2 receptor tyrosine kinases are involved in the development of mammalian central neurons (Paganioni & Ferreira, 2003; Paganioni & Ferreira, 2005). Although the ligand of Ror2 has been identified as Wnt-5A (Liu et al., 2008), Ror1 remains an orphan receptor protein tyrosine kinase without an identified interacting ligand. Tseng et al., (2010) used an in silico approach to predict receptor tyrosine kinases with likely nuclear localisation. Ror1 and Ror2 were identified in a panel that included receptors with known nuclear localisation including ErbB proteins, FGFR proteins and VEGFR proteins. The juxtamembrane domain of Ror1, responsible for nuclear localisation of this protein, was identified using deletion reporter constructs and the small GTPase Ran was identified as playing a key role in the nuclear transport. The function of Ror1 in the nucleus remains to be determined.

7.9 Trk (Tropomyocin Receptor Kinase)

Neurotrophins are a family of protein nerve growth factors that are critical for the development and functioning of the nervous system, regulating a wide range of biological

processes. The receptors for neurotrophins are the Trk receptors - TrkA (or NTRK1), TrkB (or NTRK2), and TrkC (or NTRK3). Binding of neurotrophins to Trk receptors promotes both neuronal cell survival and death by activating signal transduction cascades including Ras/MAPK (mitogen-activated protein kinase) pathway and the PI3K (phosphatidylinositol 3-kinase) pathway. TrkA accumulates in the nucleus and on the mitotic apparatus of the human glioma cell line U251 after binding the neurotrophin ligand, nerve growth factor (NGF) (Gong et al., 2007). Translocation of phosphorylated TrkA is *via* carrier vesicles which sort and concentrate the receptors. These vesicles then interact with the nuclear envelope but how the TrkA protein is then removed from the membrane to move into the nucleoplasm is unclear. Once in the nucleus of the U251 glioma cells, TrkA co-localises with α-tubulin at the mitotic spindle. Interestingly, it has been shown that NGF co-localises with γ-tubulin at the centrosomes or spindle poles. Zhang et al., (2005) suggest that NGF concentrated to the centrosome can recruit its receptor TrkA from the nucleoplasm, activate the tyrosine kinase activity of the receptor to phosphorylate the tubulin and promote the mitotic spindle assembly that modulates the mitosis of human glioma cells.

7.10 HGFR (Hepatocyte growth factor receptor)

The HGFR family includes three members, MET, RON and SEA, produced mainly by cells of epithelial origin, which bind hepatocyte and hepatocyte-like growth factors secreted by mesenchymal cells, to regulate cell growth, cell motility, and morphogenesis (Comoglio & Boccaccio, 1996). Members of the HGFR family are described as oncoproteins because over-expression and/or abnormal activity correlates with the poor prognosis of many cancers (Accornero et al., 2010).

7.10.1 MET

Hepatocyte growth factor (HGF) secreted by stromal cells is a mitogenic factor and binds to MET on hepatocytes to activate pathways involved in cell proliferation, differentiation, and related activities that aid tissue regeneration in the liver. Other cell targets of HGF include epithelium, endothelium, myoblasts, spinal motor neurons, and hematopoietic cells. MET over-expression and hyper-activation are reported to correlate with metastatic ability of the tumor cells of several different tissue origins. Gomes et al., (2008) used the SkHep1 liver cell line to show that stimulation of cells with HGF caused the rapid translocation of phosphorylated MET from the plasma membrane to the nucleus, with peak levels detected after only 4 min of HGF exposure. Translocation of MET to the nucleus was mediated by binding of Gab1, an adaptor protein that contains a NLS for importin-driven translocation. In the nucleus, MET was shown to initiate nuclear Ca^{2+} signaling that stimulates cell proliferation (Rodrigues et al., 2007).

7.10.2 RON (Recepteur d'origine nantais)

RON is a receptor tyrosine kinase whose expression is highly restricted to cells of epithelial origin (Wang et al., 2010). Its ligand is the HGF-like macrophage stimulating protein (MSP) which stabilises two monomers of RON as a homodimer on the cell membrane. RON has been shown to be aberrantly expressed or mutated in many cancers

including those from the bladder, breast, colon, lung, ovary, pancreas and prostate, particularly in aggressive tumours associated with poor patient survival (reviewed in Wang et al., 2010). Activated RON can promote c-Src activities that mediate cell-cycle progression, angiogenesis and survival of tumor cells (Danilkovitch-Miagkov et al., 2000; Feres et al., 2009). In bladder cancer cells, under conditions of serum starvation, RON has been shown to migrate from the cell membrane to the nucleus in a complex with EGFR with passage through the nuclear pore complex mediated by importins. In the nucleus, RON and EGFR co-operate in the transcriptional regulation of at least 134 different target genes known to participate in three stress-responsive networks: p53 (genes included *RBBP6*, *RB1*, *TP53BP2* and *JUN*), stress-activated protein kinase/c-jun N-terminal kinase (*JUN*, *MAPK8IP3*, *NFATC1* and *TRADD*) and phosphatidylinositol 3-kinase/Akt (*GHR*, *PPP2R3B* and *PRKCZ*) (Liu et al., 2010). Nuclear translocation of RON was therefore suggested to be a response to physiological stress. Furthermore, because MSP stimulation, homodimerisation and phosphorylation were not required for nuclear translocation, this is a ligand-independent response in these cells. A consensus sequence for binding nuclear RON was identified as GCA(G)GGGGCAGCG in genes that were both confirmed up-regulated (*FLJ46072*, *JUN*, *MLXIPL*, *NARG1* and *SSTR1*) and down-regulated (*RBBP6* and *POLRMT*) after serum starvation.

8. Conclusion

Although early reports of the presence of receptor tyrosine kinases in the nucleus of cells was met with scepticism, a significant collection of data now supports a role for many of these proteins in the nucleus of both normal and dysplastic cells. To date, 18 of the 58 human receptor tyrosine kinases have been found within nuclei and it is likely that more will be found. In general, the result of nuclear translocation of receptors is alterations to gene expression, but the full consequences of the presence of these proteins in the nucleus have yet to be determined. Only through further exploration can the complexity that nuclear localisation provides to receptor tyrosine kinase functions be determined.

9. References

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11

G Protein-Coupled Receptors-Induced Activation of Extracellular Signal-Regulated Protein Kinase (ERK) and Sodium-Proton Exchanger Type 1 (NHE1)

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1. Introduction

G-protein-coupled receptors (GPCRs) comprise a large family of cell-surface molecules, involved in signal transmission, accounting for >2% of the total genes encoded by the human genome. GPCRs have been linked to key physiological functions, including immune responses, cardiac- and smooth-muscle contraction and blood pressure regulation, neurotransmission, hormone and enzyme release from endocrine and exocrine glands. Thus, GPCRs contribute to embryogenesis, tissue remodelling and repair, inflammation, angiogenesis and normal cell growth. Their dysfunction contributes to multiple human diseases, and GPCRs represent the target of over 50% of all current therapeutic agents (Reviewed by Pierce et al., 2002). In addition, recent studies indicate that many GPCRs are overexpressed in various cancer types, and contribute to tumor cell growth when activated by circulating or locally produced ligands, suggesting a crucial role of GPCRs in cancer progression and metastasis. For example, many potent mitogens such as thrombin, lysophosphatidic acid (LPA), endothelin and prostaglandins stimulate cell proliferation by acting on their cognate GPCRs in various cell types. (Reviewed by Dorsam & Gutkind, 2007). The mechanisms that control cellular proliferation are important in normal physiology and disease states. Multiple mitogens that activate GPCRs stimulate the extracellular signal-regulated protein kinase (ERK) and lead to proliferation of mammalian cells. Another extensively studied mitogenic effector pathway in addition to ERK that ultimately leads to cell proliferation, is the ubiquitous plasma membrane sodium-proton exchanger type 1 (NHE1). NHE1 and ERK have both been implicated as key mediators of growth signals (Noel & Pouyssegur, 1995; Rozengurt, 1986; Kapus et al, 1994; Krump et al, 1997), therefore the regulatory relationships between NHE1 and ERK have been the subject of a number of studies over the last decade. Because both proteins can serve mitogenic functions, and because both are activated by similar stimuli, it has been hypothesized that

one may be a regulator for the other. Indeed, in some cell types ERK plays a clear role in either the short or long term activation of NHE1 (Aharonovitz & Granot, 1996; Bianchini et al., 1997; Wang et al., 1997; Sabri et al., 1998; Bouaboula et al., 1999; Gekle et al., 2001). However, several groups were unable to demonstrate any role of ERK in regulation of NHE1 in a number of cell types (Gillis et al., 2001; Kang et al., 1998; Pederson et al., 2002; Garnovskaya et al., 1998; Di Sario et al., 2003). In addition, a number of recent studies suggested that certain stimuli such as mechanical stretch, hypertrophy and inflammatory mediators require NHE1 to regulate ERK (Takewaki et al., 1995; Nemeth et al., 2002; Yamazaki et al., 1998; Javadov et al., 2006; Chen et al., 2007). At present, very little is known about GPCR-induced NHE1-dependent ERK regulation. One report suggests that NHE1 is not a regulator for LPA-induced ERK activation in C6 glioma cells (Cechin et al., 2005) and another paper demonstrates the lack of role of NHE1 in angiotensin II (Ang II)- and endothelin 1-induced ERK activation in cultured neonatal rat cardiomyocytes (Chen et al., 2007). At the same time, our group showed that NHE1 activation plays a necessary role in activation of ERK by AII AT_1 and serotonin 5-HT_{2A} receptors in vascular smooth muscle cells (VSMC) (Mukhin et al., 2004), and in bradykinin B_2 receptor-induced ERK activation in renal carcinoma A498 cells (Garnovskaya et al., 2008) thus suggesting that the critical role of NHE1 in GPCR-induced ERK activation is not restricted to one specific cell type and receptor. Studies on the involvement of NHE1 in ERK regulation may also have pathophysiological relevance. NHE1 is usually referred to as a "housekeeping" protein and is normally inactive, but it gets activated in response to multiple specific stimuli, and maintains homeostatic cell volume and pH through Na^+/H^+ transport. The role of NHE1 has been well established in the myocardial remodeling and heart failure process (reviewed by Karmazyn et al., 2008). NHE1 may play a key role in the maintenance of blood pressure because increased activity of NHE1 has been observed in cells and tissues from hypertensive animals and humans (Rosskopf, et al., 1993; Lucchesi et al., 1994). Northern blot analysis showed that cultured VSMC from Sprague-Dawley and Wistar-Kyoto rats express only the NHE1 isoform, and that steady-state mRNA levels are similar for normal and spontaneously hypertensive animals (Lucchesi et al., 1994; LaPointe et al., 1995). Because no mutations in the NHE1 DNA sequence have been found in hypertensive animals, this suggests that increased activity of the antiporter is caused by an alteration in the regulation of NHE1 (Lucchesi et al., 1994). In addition, NHE1-mediated intracellular alkalinization has been proposed to play role in cancer cells growth, and over-expression of NHE1 contributes to the transformed phenotype of multiple cancer cells (Cardone et al., 2005). The role of NHE1 in renal diseases is less known. Mice with a spontaneous point mutation that results in truncation between the 11^{th} and 12^{th} NHE1 trans-membrane domains and causes loss of NHE1 function (Cox et al., 1997) do not present visible renal phenotype, consistent with the concept that NHE1 "housekeeping" activity under normal conditions is not required. However, NHE1 activity was increased in cell lines derived from patients with diabetic nephropathy (Ng et al., 1994), suggesting that NHE1 activity may be important in the context of cellular stress. Further, it has been shown that genetic or pharmacological loss of NHE1 function causes renal tubule epithelial cell apoptosis and renal dysfunction in several models of kidney disease (ureteral obstruction, adriamycin-induced podocyte toxicity, and streptozotocin-induced diabetes), suggesting that NHE1 activity may be beneficial for chronic kidney disease (Schelling & Abu, 2008). Moreover both, ERK and NHE1, have been

proposed as key therapeutic targets for vascular illnesses, such as congestive heart failure (Kusumoto et al., 2001), myocardial infraction and reperfusion injury (Avkiran & Marber, 2002), ventricular fibrillation (Gazmuri et al., 2001), and ventricular hypertrophy (Chen et al., 2001). Therefore, studies devoted to the regulatory relationships between NHE1 and ERK have a potential clinical relevance.

The purpose of this review is to describe the relationship between NHE1 and ERK when both pathways are activated by GPCRs, with a particular emphasis on the situations when NHE1 is regulating ERK activity leading to cell proliferation.

2. Mitogen-activated protein kinases and Sodium-Hydrogen Exchanger-1 as mediators of growth signals

2.1 Mitogen-activated protein kinases

Mitogen-activated protein kinases (MAPKs) are a family of highly conserved proline-directed serine/threonine kinases that are activated by a large variety of extracellular stimuli and play integral roles in controlling many cellular processes, from the cell surface to the nucleus (Widmann et al, 1999). The MAPK family in mammals includes four distinctly regulated groups of MAPKs: extracellular signal-regulated kinase 1/2 (ERK), p38, c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPKs), and ERK5/Big MAPK (BMK1) (Chang & Karin, 2001; Bogoyevitch & Court, 2004; Johnson & Lapadat, 2002). MAPK cascades typically consist of three levels of protein kinases that are consecutively activated by phosphorylation events: MAPK kinase kinase (MAPKKK or MAP3K or MEKK) activates MAPK kinase (MAPKK (MKK or MEK) or MAP2K), which in turn activates MAPK (Figure 1). Even so, the different tiers are composed of many similar isoforms that can be activated by more than one MAPK, increasing the complexity and diversity of MAPK signaling. Regulation and function of different MAPKs as well as complexity of MAPK signaling have been recently discribed in several review articles (Pearson et al., 2001; Chang & Karin 2001; Shaul & Seger, 2007; Bodart; 2010). MAPKs are involved in transmitting signals from a wide variety of extracellular stimuli including those of growth factor receptors and GPCRs, as well as physical or mechanical stimuli. In fact, MAPKs are major components of signaling pathways regulating a large array of intracellular events, such as proliferation, differentiation, acute signaling in response to hormones, stress response, programmed cell death, and gene expression (Pearson et al., 2001; Chang & Karin 2001; Kim & Choi, 2010). ERK is one member of a family of kinases that participate in mitogenic signaling through complex phosphorylation cascades that convert cell surface signals into nuclear transcription programs. In the typical scenario, GTP-bound Ras, a small G protein, activates Raf-1 kinase. In an alternative scenario, protein kinase C (PKC) or other signaling molecules activate Raf-1. In either case, Raf-1 phosphorylates and activates mitogen and extracellular signal-regulated kinases kinase (MEK), which in turn phosphorylates and activates ERK (Cobb & Goldsmith, 1995). Activated ERK translocates to the nucleus, where it activates a number of transcription factors such as Elk-1.

Recently a number of scaffolding proteins that play important role in ERK regulation have been described. Examples of such proteins include the Kinase Suppressor of Ras (KSR), β-arrestins1/2, PEA15 (phosphoprotein enriched in astrocytes), paxillin, and Raf-1 (Kolch,

2005). Paxillin, a multi adaptor protein in focal adhesion assembly, serves as a connector between ERK and Focal Adhesion Kinase (FAK) signaling pathways binding Raf-1 and ERK in response to hepatocyte growth factor in epithelial cells (Ishibe et al., 2004).

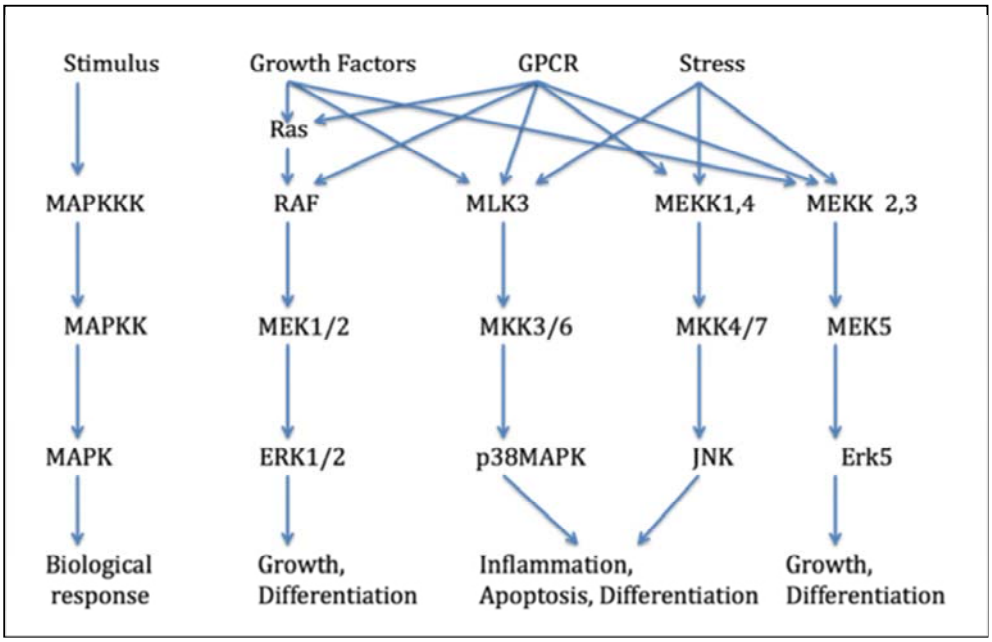

Fig. 1. Mitogen-Activated Protein Kinase Cascades.

2.1.1 Regulation of ERK by GPCRs

The ability of G-protein-coupled receptors (GPCRs) to generate signals that control cellular proliferation via activation of ERK pathways has been demonstrated in several studies (reviewed by Kranenburg & Moolenaar, 2001; and Luttrell, 2002). Although the mechanisms by which GPCRs control the activity of ERK vary between receptor and cell type, typically there are three categories of mechanisms: (1) signals that initiate classical G protein effectors, e.g., protein kinase A or protein kinase C causing the production of second messengers, (2) mechanisms that involve cross-talk between GPCRs and classical receptor tyrosine kinases, e.g., "transactivation" of epidermal growth factor (EGF) receptors, and (3) signals initiated by direct interaction between beta-arrestins and components of the MAP kinase cascade, e.g., beta-arrestin "scaffolds". Mitogenic pathways activated by different Gα families including activation of adenylyl cyclase/cAMP and phospholipase Cβ/protein kinase C second messenger pathways have been described in detail (reviewed by New & Wong, 2007). Angiotensin II promotes DNA synthesis and proliferation in many cell types by activating the G_q-coupled AT_1 receptor. AT_1 receptor activity in human adrenal cells induces Ras-dependent ERK activity, leading to increased levels of c-Fos and c-Jun transcription factors and to proliferation of the cells (Watanabe et al., 1996). Other mitogenic

GPCRs, including M_1 muscarinic and α_{1B}-adrenergic and purinergic receptors, induce ERK activity via the Ras-independent protein kinase C phosphorylation and activation of Raf-1 (Luttrell, 2002). G_s-coupled GPCRs utilize the adenylyl cyclase/ cAMP /Epac/Rap-1/B-Raf pathway to activate MAPK cascades and proliferation. In bone cells parathyroid hormone receptor promotes cAMP accumulation, which binds directly to the Rap-1 guanine nucleotide exchange factor Epac. Epac in turn activates Rap-1, a Ras family GTPase, which activates the kinase B-Raf, triggering ERK cascades (Fujita et al., 2002). Alternatively, PKA may directly activate Rap-1 (Luttrell, 2002).

However, activation of classical second messenger cascades cannot fully explain roles of GPCRs in stimulation of MAPK cascades. Additional signaling mechanisms including transactivation of the Receptor Tyrosine Kinases (RTKs) via the autocrine/paracrine release of epidermal growth factor (EGF)-like ligands at the cell surface and scaffolding of MAPK cascades, appear to contribute to GPCR-mediated MAPK activation. GPCR-mediated proliferation via the $G\alpha$ or $G\beta\gamma$ subunit transactivation of RTKs has been described in several cell types (Ohtsu et al., 2006; Schafer et al., 2004). Thus, ligands for the LPA, endothelin-1 and thrombin receptors all stimulate cell proliferation in Rat-1 fibroblasts by transactivation of the epidermal growth factor receptor (EGFR, an RTK). Such transactivation requires the activation of matrix metalloproteases (MMPs) to release EGF from its membrane bound form, which then stimulates the EGFR and downstream ERK pathways (Schafer et al., 2004). Studies from our group demonstrated that bradykinin B_2 receptor activates ERK via EGFR transactivation in kidney cells (Mukhin et al., 2003; Mukhin et al., 2006; Kramarenko et al., 2010). The similar MMP/EGFR/ERK pathway have been also demonstrated in kidney cancer cells stimulated by LPA and angiotensin II (Schafer et al., 2004). A significant advance in the understanding of how GPCRs activate MAPK cascades is the discovery that beta-arrestin, a protein well known for its roles in both receptor desensitization and internalization, serves as a scaffolding protein for the GPCR-stimulated the extracellular signal regulated kinase ERK cascade. For example, agonist stimulation of the proteinase-activated receptor-2 (PAR2) leads to the formation of a large complex, which includes the receptor and beta-arrestin, MAPKKK, Raf-1, and activated ERK. Similarly, activation of neurokinin-1 receptor with the substance P, results in the formation of a complex, which includes the receptor, and beta-arrestin, c-Src and ERK. (Reviewed by Pierce et al., 2001).

ERK activation occurring via EGF receptor transactivation or via pathways employing second messengers (PKA- or PKC-dependent pathways) typically leads to sustained ERK activity and nuclear translocation of the kinase, thus contributing to regulation of cell cycle progression (Kranenburg & Moolenaar, 2001; Luttrell, 2002). In contrast, beta-arrestin/endocytotic pathway usually results in the retention of ERK in the cytoplasm and transient ERK activity, which is probably not sufficient to stimulate cell proliferation (Luttrell, 2002).

The intracellular pathways that mediate GPCR -induced ERK activation and regulation of cellular proliferation were recently reviewed by New & Wong (New & Wong, 2007).

2.2 Sodium-Hydrogen Exchanger-1 (NHE1)

The Na^+/H^+ exchange system was described in 1977 by Aickin and Thomas (Aickin & Thomas, et al., 1977), and the first Na^+/H^+ exchanger (NHE) gene was cloned in 1989 (Sarget et al., 1989). To date nine mammalian isoforms (NHE 1-9) have been identified in the family of Na^+/H^+ exchangers (Kemp et al, 2008). In this review we will focus only on the

ubiquitously expressed, amiloride-sensitive integral plasma membrane protein NHE1 known as the "housekeeping enzyme", which is activated by various stimuli including growth factors, mitogens, and hyperosmolarity (Orlowski & Grinshtein, 2004; Wakabayashi et al., 1997). NHE1 is highly conserved across vertebrate species and is a major membrane transport mechanism, which plays an essential role in pH regulation, volume homeostasis, cell growth and differentiation (Bertrand et al., 1994). NHE1 is a phosphoglycoprotein of 815 amino acids that contains two functional domains: an NH2-terminal transmembrane ion translocation region with a proposed topology of 12 transmembrane domains, and a COOH-terminal cytoplasmic regulatory domain (Figure 2). The ion translocation domain catalyzes electroneutral exchange of extracellular sodium ion for intracellular hydrogen. Regulation of NHE1 activity in response to multiple stimuli including growth factors, hormones, and osmotic stress is mediated by a COOH-terminal cytoplasmic regulatory domain. The regulatory domain controls transport activity probably by altering affinity of a proton site in the transmembrane domain (Takahashi et al., 1999). This cytoplasmic domain includes a number of distinct subdomains modified either by phosphorylation or by the binding of regulatory proteins. The cytoplasmic domain contains high and low affinity Ca^{2+}/calmodulin-binding sites and several potential phosphorylation sites (Bertrand et al., 1994; Yan et al., 2001). Bertrand et al. first identified two calmodulin-binding sites on the

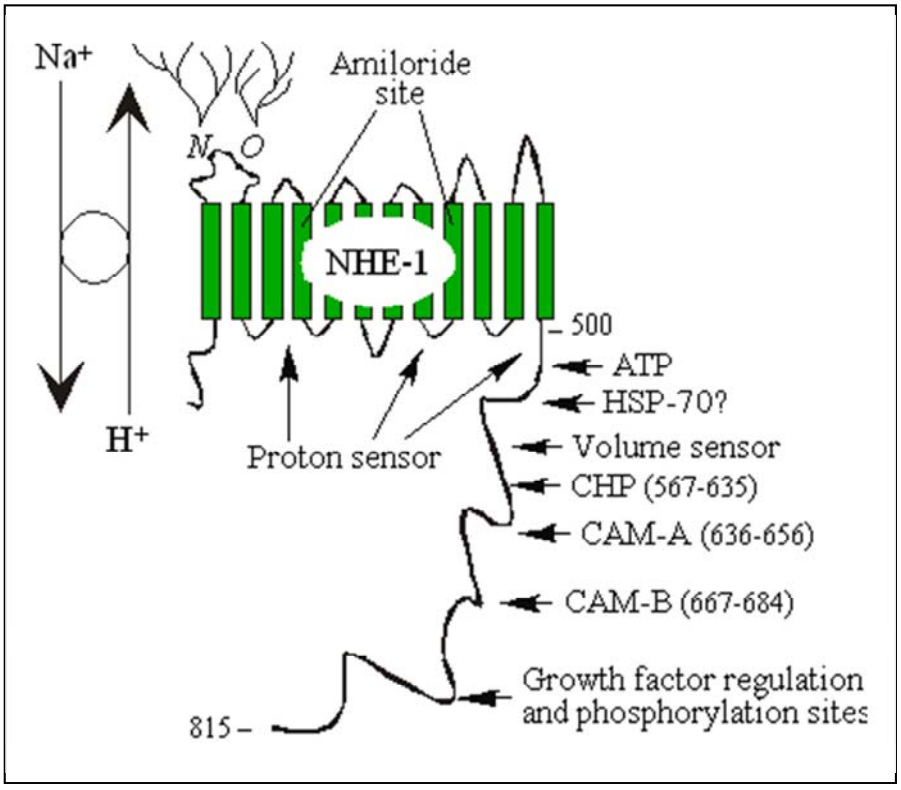

Fig. 2. Topographical model of NHE1.

cytoplasmic domain of NHE1 at amino acids 636-656 and 664-684, with high (Kd ~ 20 nM) and low (Kd~ 350 nM) affinities, respectively (Bertrand et al., 1994). In quiescent cells, the high-affinity calmodulin-binding domain may act as an autoinhibitory domain by interacting with the transmembrane domain, thus inhibiting ion translocation. Upon activation, NHE1 undergoes a conformational change that allows the Ca^{2+} -dependent binding of calmodulin (Wakabayashi et al., 1997). A phosphorylation domain at the distal COOH-terminus (amino acids 656-815 of human NHE1) contains a number of serine residues constitutively phosphorylated in quiescent cells that have increased phosphorylation levels in response to growth factors (Sarget et al., 1989). COOH-terminal serine residues on NHE1 molecule can be phosphorylated by the ERK-regulated kinase p90RSK (Tominaga et al., 1998) and by the Ste20-like Nck-interacting kinase (NIK) in response to growth factor receptors (Putney et al., 2002) and by Rho kinase 1 (ROCK1) in response to activation by GPCRs for thrombin and lysophosphatidic acid (Tominaga et al., 1998; Putney et al., 2002) and by integrin-induced cell adhesion (Tominaga & Barber, 1998). Because the cytoplasmic regulatory domain associates with multiple binding partners including the cytoskeleton-plasma membrane linker protein ezrin of the ezrin, moesin, radixin, (EMR) family (Denker et al., 2000), the calcineurin homolog protein CHP1 (Pang et al., 2001), calmodulin (CaM) (Yan et al., 2001), carbonic anhydrase II (Li et al., 2002), heat shock protein (Silva et al., 1995), and 14-3-3 protein (Lehoux et al., 2001), a novel function of NHE1 as a plasma membrane scaffold in the assembly of signaling complexes has been suggested (Baumgartner et al., 2004).

2.2.1 Regulation of NHE1 by GPCRs

While the activation of NHE1 and the kinetic alterations to the exchanger have been widely studied (Noel & Pouyssegur, 1995; Wakabayashi et al., 1997; Orlowski & Grinshtein, 1997) the signaling pathways that regulate NHE1 have not been fully elucidated. Because G protein-coupled receptors (GPCRs)-mediated regulation of sarcolemmal NHE activity is likely to play significant roles in modulating myocardial function in both physiological and pathophysiological conditions, most of the studies devoted to GPCR-induced NHE1 regulation were performed in cardiac myocytes (reviewed by Avkiran & Haworth, 2003). Sarcolemmal NHE activity is subject to exquisite regulation by a variety of extracellular stimuli, most of which act through GPCRs. Intriguingly, although the majority of the GPCR systems that have been studied to date have been shown to *stimulate* sarcolemmal NHE activity, there is also evidence that some may *inhibit* NHE activity or its stimulation through other pathways. A number of GPCRs, such as α_1-adrenergic receptors , angiotensin II AT_1 receptors, endothelin ET_A receptor, thrombin receptor, muscarinic receptors have been shown to increase sarcolemmal NHE activity through a change in the pH_i sensitivity of the exchanger. Interestingly, in contrast to the evidence that various G_q –coupled receptors (e.g. α_{1A}-ARs and angiotensin AT_1) mediate an increase in sarcolemmal NHE activity, GPCRs that signal through other G protein families (G_s and G_i) may attenuate NHE activity or its stimulation. Thus, β_1-AR stimulation *inhibits* sarcolemmal NHE activity, while adenosine A_1 and angiotensin AT_2 receptors attenuate stimulation of NHE1 by other ligands (Avkiran& Haworth, 2003). The mechanisms of GPCR-induced NHE1 activation are not fully understood. To date, several mechanisms of activation of NHE1 by G protein-coupled receptors have been proposed although not fully characterized : α_{1A}-adrenoceptor activates NHE1 through protein kinase C (Snabaitis et al., 2000; Avkiran & Haworth, 2003); lysophosphatidic acid stimulates NHE1 through RhoA and its effector ROCK (Tominaga et

al., 1998), and angiotensin II AT_1 receptor regulates NHE1 activity through RSK (Takahashi et al., 1999). In addition, Wallert et al provided evidence that the specific α_1-adrenergic agonist, phenylephrine and the lysophosphatidic acid (LPA) activate NHE1 in CCL39 cells, and demonstrated a direct involvement of ERK in the α_1-adrenergic activation of NHE1 and a significant role for both ERK and RhoA in LPA stimulation of NHE1 in CCL39 fibroblasts (Wallert et al., 2004). Our group reported that a fibroblast NHE1 can be rapidly stimulated through the transfected human serotonin 5-HT_{1A} receptor via pertussis toxin-sensitive G protein α-subunits $G_{i\alpha 2}$ and $G_{i\alpha 3}$, in CHO cells (Garnovskaya et al., 1997), by endogenously expressed G_q-coupled bradykinin B_2 receptor in kidney cells (Mukhin et al., 2001), and by endogenously expressed G_q-coupled angiotensin II AT_1 and serotonin 5-HT_{2A} receptors in vascular smooth muscle cells (Garnovskaya et al., 2003). While studying the signaling pathway of bradykinin B_2 receptor-induced NHE1 activation in mIMCD-3 kidney cells, we found a new mechanism for the GPCR-induced regulation of Na^+/H^+ exchange (Mukhin et al., 2001). This novel pathway involved activation of phospholipase C, elevation of intracellular Ca^{2+}, activation of the non receptor tyrosine kinase, Janus kinase 2 (Jak2), tyrosine phosphorylation of Ca^{2+}/calmodulin (CaM), and binding of CaM to NHE1. Bradykinin rapidly stimulated the assembly of a signal transduction complex that includes CaM, Jak2, and NHE1. We suggested that Janus kinase 2 is involved in the activation of NHE1 by increasing the tyrosine phosphorylation of calmodulin, which appears to be a direct substrate for phosphorylation by Janus kinase 2. Further the same pathway has been demonstrated for the bradykinin B_2 receptor-mediated activation of Na^+/H^+ exchange in KNRK and CHO cells (Lefler et al., 2003), and for the G_q-coupled angiotensin II AT_1 and serotonin 5-HT_{2A} receptors, which stimulated NHE1 activation in vascular smooth muscle cells (Garnovskaya et al., 2003), suggesting that this pathway represent a fundamental mechanism for the rapid regulation of NHE1 by G_q-coupled receptors in multiple cell types. Further we have shown that the G_i – coupled serotonin 5-HT_{1A} receptor also rapidly stimulates NHE1 through a pathway that involves 1) activation of Janus kinase 2 downstream of the 5-HT_{1A} receptor; 2) formation of a complex that includes NHE1, Jak2, and CaM; 3) tyrosine phosphorylation of CaM through Jak2; and 4) increased binding of CaM to the carboxyl terminus of NHE1 (Turner et al., 2007).

2.3 Relationships between NHE1 and ERK

2.3.1 MAPK regulates NHE1

Whereas it has been known for some time that mitogens typically activate both NHE1 and ERK in concert (Noel & Pouyssegur, 1995; Rozengurt, 1986; Kapus et al., 1994; Krump et al., 1997) the exact relationships between NHE1 and ERK have only recently been explored in any great detail. Recent studies have shown that multiple stimuli that rapidly activate ERK pathways also rapidly increase NHE activity in many cell types, particularly in fibroblasts. Those stimuli include, but are not limited to: growth factors that modulate tyrosine phosphorylation cycles, integrins, hyperosmotic stress or cell shrinkage, protein kinase C (PKC), tyrosine phosphorylation cascades and heterotrimeric G proteins (Clark & Limbird, 1991; Barber, 1991; Rozengurt, 1986; Lowe et al., 1990). Those similarities provide evidence to suggest that ERK may be a direct proximal component of an NHE regulatory pathway (Noel & Pouyssegur, 1995; Aharonovitz & Granot, 1996). There is a growing awareness that tyrosine phosphorylation cycles are critical in regulating NHE activities in a number of cell types (Donowitz et al., 1994; Yamaji et al., 1995; Good, 1995; Fukushima et al., 1996) as has also been shown for ERK (Blumer & Johnson, 1994). Other studies have demonstrated that

NHE and ERK activities are modulated by overlapping upstream enzymes, including phosphoinositide 3´-kinase (PI-3K), phospholipase C, and PKC (Levine et al., 1993; Kapus et al., 1994; Voyno-Yasenetskaya et al., 1994; Bertrand et al., 1994; Ma et al., 1994; Dhanasekaran et al., 1994; Inglese et al., 1995). In aggregate, those studies implicate G proteins, lipid-recognizing enzymes, tyrosine kinases, and NHEs as playing interrelated roles along with ERK in cell growth (Barber, 1991; Noel & Pouyssegur, 1995; Aharonovitz & Granot, 1996; Blumer & Johnson, 1994; Lin et al., 1996). Relevant to the hypothesis that ERK regulates NHE1, are studies showing that microinjection of activated Ras (Hagag et al., 1987) or transfection of the Ha-Ras oncogene (Doppler et al., 1987; Maly et al., 1989; Kaplan & Boron, 1994) stimulates NHE activity in fibroblasts. The classical effect of GTP-bound Ras is the activation of the ERK1 and ERK2 (Blumer & Johnson, 1994). This is thought to occur primarily through a linear signalling pathway that flows as follows: Ras-GTP → Raf-1 kinase → MEK (MAPK/ERK kinase) → ERK. Thus, because Ras functions upstream of both NHE and ERK activities, ERK has been proposed as a logical funnel for signals from extracellular stimuli to the effector NHE. The effect of NHE activation due to the sustained activation of ERK is most likely secondary to the activation of transcription cascades that upregulate the NHE message/protein or modulate expression of key regulators of NHE activity. However, several studies suggest that ERK might regulate NHE activity in the short term, as well. The possibility that ERK rapidly regulates NHE activity was tested in platelets by Aharonovitz and Granot (Aharonovitz & Granot, 1996) who showed that arginine vasopressin (AVP) and PMA rapidly activated NHE by a pathway which was sensitive to PD98059, a specific inhibitor of MEK1. Moreover, the signal initiated by AVP was sensitive to genistein, a broad-spectrum inhibitor of tyrosine kinases (Aharonovitz & Granot, 1996). Bianchini et al. (Bianchini et al., 1997) went further to characterize the role of ERK in regulating NHE when cells were stimulated by combinations of growth factors or serum. Specifically, they showed that expression of a dominant negative p44 ERK or of the MAPK phosphatase MKP-1, or treatment with the MEK1 inhibitor PD98059 reduced activation of NHE-1 by mixtures of growth factors by about 50%. Further, it has been shown that short-term activation of ERK leads to rapid stimulation of NHE1 in multiple cell types (erythrocytes, fibroblasts, MDCK-11 cells, rabbit skeletal muscle, and cultured rat neonatal and adult ventricular cardiomyocytes) when activated by diverse stimuli including growth factors, angiotensin II, and aldosterone (Wang et al., 1997; Sabri et al., 1998; Bouboula et al., 1999; Gekle et al., 2001; Wei et al., 2001; Moor et al., 2001; Snabaitis et al., 2002). At least in some cases, the short term-stimulation of NHE1 by ERK is mediated by phosphorylation of NHE1 either by ERK itself, or by p90RSK, an ERK-regulated kinase (Takahashi et al., 1999). Cuello et al. demonstrated that ERK- dependent 90kDa ribosomal S6 kinase (RSK) is the principal regulator of cardiac sarcolemmal NHE1 phosphorylation and NHE activity after α_1-adrenergic stimulation in adult myocardium (Cuello et al., 2007). Thus, there is clear evidence that ERK can increase the activity of NHE1 by increasing its expression and/or by stimulating the activity of existing NHE1 molecules.

2.3.2 MAPK and NHE1 do not regulate each other

On the other hand, several groups have been unable to show any role for ERK in activating NHE1 in multiple cell types, including Xenopus oocytes (Kang et al., 1998), Ehrlich Ascites cells (Pederson et al., 2002), CHO cells (Garnovskaya et al., 1998), or hepatic stellate cells (Di Sario et al., 2003). Moreover, there is one report in which ERK was shown to mediate inhibition of NHE1 activity in MTAL cells (Watts & Good, 2002). Our group tested the hypothesis that ERK could mediate rapid, short-term activation of NHE activity in

fibroblasts when both signals were initiated by a single G protein-coupled serotonin 5-HT_{1A} receptor (Garnovskaya et al., 1998). These studies revealed a number of similarities between the regulation of ERK and NHE. Activation of the two processes shared similar concentration–response and time-course characteristics. Receptor-activated NHE and ERK also shared an overlapping sensitivity to some pharmacological inhibitors of tyrosine kinases (staurosporine and genistein), PI-3K (wortmannin and LY294002), and PC-PLC (D609), and neither pathway was sensitive inhibition of PKC. However, definitive studies designed to block signaling molecules possessing well-defined roles in activating ERK through the 5-HT_{1A} receptor by transfecting cDNA constructs encoding inactive mutant PI-3K, Grb2, Sos, Ras, and Raf molecules were successful in attenuating ERK, but had essentially no effect upon NHE activation. Thus, our data do not support the hypothesis that ERK is a proximal short-term regulator of NHE in CHO cells when the signal is initiated by the $G_{i/o/z}$ protein-coupled 5-HT_{1A} receptor. Therefore, the ability of ERK to stimulate NHE1 activity has not been a universal finding.

2.3.3 NHE1 as a regulator of MAPK

Despite the increasing interest in potential roles for ERK in the activation of NHE1, much less is known regarding the role of NHE1 in regulating ERK. There have been several reports that suggest that NHE1 might play a role in regulating ERK activation (reviewed by Pedersen et al., 2007). Mitsuka et al. had shown that specific inhibitors of NHE1 could reduce neointimal proliferation in a rat model of carotid artery injury (Mitsuka et al., 1993). However, in C6 glioma cells although lysophosphatidic acid (LPA) - increased proliferation was sensitive to NHE1 inhibitors, LPA-induced ERK activation was unaffected (Cechin et al., 2005). Takewaki et al. presented some evidence that a potent antagonist of NHE1 could partially inhibit stretch-induced activation of ERK in the cultured cardiomyocytes (Takewaki et al., 1995). Later the same group reported that in cultured neonatal rat cardiomyocytes NHE1 inhibition blocked the stretch-induced activation of Raf-1 and ERK, while angiotensin II (Ang II)- and endothelin 1-induced ERK activation remained unaffected (Yamazaki et al., 1998). On the other hand, our work in vascular smooth muscle cells (VSMC) demonstrated that activation of ERK by AII and serotonin was strongly dependent of NHE1 activity, and the effect of NHE1 occurs at or above the level of Ras (Mukhin et al., 2004). In human colon cancer epithelial cells, NHE1 inhibition suppressed activation of ERK and NF-κB and led to decreased production of interleukin-8 in response to inflammatory signals (Nemeth et al., 2002). Recently it has been also demonstrated that NHE1 inhibition prevented ERK activation during phenylephrine-induced hypertrophy in neonatal rat cardiomyocytes (Javadov et al., 2006), and prevented glucose-induced ERK activation in a high glucose model of cardiomyocyte hypertrophy (Chen et al., 2007). In Ehrlich Lettre Ascites cells under osmotic cell shrinkage NHE1 regulates ERK acting at or above the level of MEK (Pederson et al., 2002). Therefore, NHE1-dependent regulation of ERK in most cases has been described in cells stimulated by mechanical stretch, osmotic shrinkage, hypertrophy and inflammatory mediators (Takewaki et al., 1995; Nemeth et al., 2002; Yamazaki et al., 1998; Javadov et al., 2006; Chen et al., 2007; Mitsuka et al., 1993; Pederson et al., 2007). Very little is known about GPCR-induced NHE1-dependent ERK regulation. One report suggests that NHE1 is not a regulator for LPA-induced ERK activation in C6 glioma cells (Cechin et al., 2005) and another paper demonstrates the lack of role of NHE1 in AII- and endothelin 1-induced ERK activation in cultured neonatal rat cardiomyocytes (Chen et al., 2007).

2.3.3.1 NHE1 regulates ERK activity in GPCR-activated VSMC

Angiotensin II (Ang II), a potent hypertrophic factor for vascular smooth muscle cells, mediates its effects via specific plasma membrane AT_1 receptors that belong to GPCR family. Ang II stimulates multiple signaling pathways (reviewed by Touyz & Schiffrin, 2000) including MAPKs, Src family kinases, phospholipase D, and Janus kinase (Jak2). Ang II also has been shown to stimulate NHE1 activity in VSMC (Berk et al., 1987) but does not appear to increase the steady state levels of NHE1 mRNA. There also are reports on relationship between Ang II-induced NHE1 and ERK activities in VSMC suggesting that activation of the AT_1 receptor first leads to activation of the MEK-ERK-p90RSK pathway, and that activated p90RSK in turn directly phosphorylates and activates NHE1 in VSMC (Takewaki et al., 1995). However, this suggestion was based mainly on *in vitro* experiments in which p90RSK immunoprecipitated from Ang II-stimulated VSMC was able to phosphorylate recombinant NHE1, and it is still not clear whether Ang II-induced phosphorylation of NHE1 takes place in VSMC *in vivo* and if this phosphorylation is physiologically significant. Our group has described a novel pathway of the regulation of NHE1 activity in VSMC by two mitogens, Ang II and serotonin (5-HT) that involves the activation of Jak2, tyrosine phosphorylation of Ca^{2+}/calmodulin, and binding of calmodulin (CaM) to NHE-1 (Garnovskaya et al., 2003). In the same study we were not able to support any role for ERK in Ang II-induced NHE1 activation in VSMC (Garnovskaya et al., 2003). Further, we specifically investigated the roles of NHE and ERK (as stimulated by either 5-HT or Ang II) in the activation of each other in VSMC (Mukhin et al., 2004), and we have found evidence to support a novel role for NHE in the activation of ERK in VSMC. This evidence includes 1) dual stimulation of NHE and ERK by Ang II and 5-HT, with the activation of NHE preceding that of ERK, 2) similar concentration-response relationships for the stimulation of NHE and the phosphorylation of ERK by 5-HT and Ang-II, 3) blockade of the activation of ERK induced by 5-HT and Ang II by chemical inhibition of NHE, 4) blockade of the activation of ERK induced by 5-HT and Ang II by removal of sodium from incubation buffers, and 5) phosphorylation of ERK during recovery from an imposed acid load, a maneuver that induces receptor-independent activation of NHE.

Moreover, in the case of receptor-induced activation of ERK, NHE appears to be located upstream of MEK and ERK, and downstream of Ang II and 5-HT-mediated transactivation of the EGF receptor. NHE intersects the classical pathway of activation of ERK at or above the level of Ras. Figure 3 depicts one possible scheme that can account for our findings. Because it has been described that G_q-coupled receptors such as Ang II AT_1 and serotonin $5\text{-}HT_{2A}$ receptors activate ERK in VSMC through transactivation and phosphorylation of the epidermal growth factor (EGF) receptor (Eguchi et al., 1999), we wanted to establish whether NHE regulates ERK activation upstream of the EGF receptor. It appeared that inhibition of NHE activity by depriving the exchanger of extracellular sodium, or by blockade with the specific inhibitors, amiloride analog, methylisobutylamiloride (MIA), prevents activation of ERK by two GPCR ligands, Ang II and 5-HT. Those same maneuvers have no effect on EGF-stimulated ERK, suggesting that there are some differences in the pathways used by Ang II and 5-HT to activate ERK when compared with that used by EGF. Interestingly, the close connection between NHE and ERK activation is further underscored by the observation that receptor-independent activation of NHE also results in ERK phosphorylation only when the exchanger is allowed to mediate recovery from an imposed

intracellular acid load. Thus, NHE activation is *necessary* for Ang II and 5-HT-induced activation of ERK, and is *sufficient* to activate ERK under conditions of an imposed acid load. In contrast, NHE activation is not *necessary* for EGF-mediated activation of ERK. The most likely explanation is that 5-HT or Ang II requires the parallel activation of NHE and the EGFR to activate ERK in VSMC. The two pathways intersect downstream of trans-phosphorylation of the EGFR, and upstream of ERK and MEK, most likely at or upstream of Ras (Figure 3). The precise mechanisms of NHE-dependent ERK activation by Ang II and 5-HT remain to be defined. One possibility is that NHE plays an accessory role in Ang II and 5-HT induced activation of ERK by facilitating cytoskeletal reorganization or by altering Na^+ or H^+ concentrations in cellular microdomains, thereby affecting enzyme activity or protein-protein interactions.

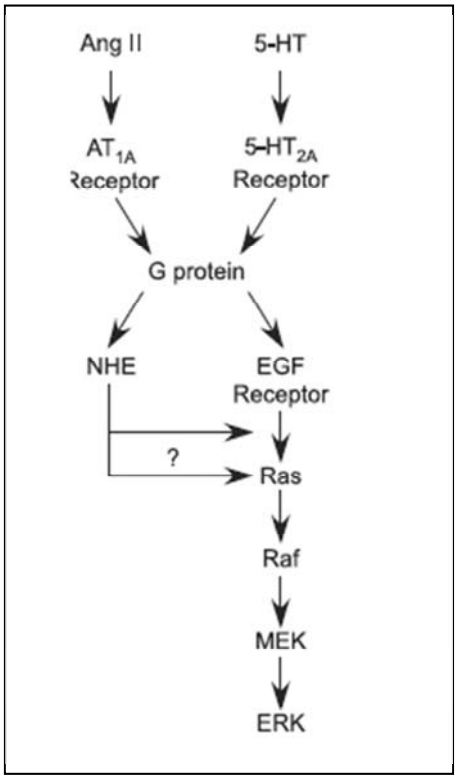

Fig. 3. Hypothetical scheme of NHE1-dependent ERK activation by GPCRs in VSMC.

The speculation regarding the cytoskeletal effects of NHE is particularly intriguing in light of work by Barber's group showing important functional links between NHE activity and the cytoskeleton (Denker et al., 2000). These findings have potential implications for the regulation of vascular tone, as well as for vascular pathobiology because Ang II and 5-HT are both potent vasoconstrictors, and Ang II has been shown to play major roles in various cardiovascular diseases including left ventricular hypertrophy and hypertension (Mitsuka et al., 1993).

2.3.3.2 NHE1 regulates ERK activity in Bradykinin-activated renal carcinoma cells

Because we have been able to detect the critical role of NHE in GPCR-mediated activation of ERK in cells of contractile phenotype, we thought that this relationship might be restricted to specific cell types and receptors. The cellular specificity of the relationship between NHE1 and ERK could be mediated by alternate accessory components of each signaling pathway, or by cell-specific compartmentalization of scaffolded signal transduction platforms (Luttrell & Lutrell, 2003). However, we have collected data that support a role of NHE1 in bradykinin B_2 receptor-induced ERK activation in renal carcinoma A498 cells, thus suggesting that the critical role of NHE1 in GPCR-induced ERK activation is not restricted to one specific cell type and receptor (Garnovskaya et al., 2008). In this study we investigated the endogenous intrarenal kinin hormone bradykinin (BK) that exerts its multiple pathophysiological functions via two known receptors, the bradykinin B_1 (BK B_1) and bradykinin B_2 (BK B_2) which belong to the superfamily of G protein-coupled receptors (GPCR) (Hess et al., 1992; Menke et al., 1994; Bagate et al., 2001). BK plays a significant role as a modulator of renal function such as electrolyte and water excretion (Mukai et al., 1996) as well as in renal cell growth and proliferation (El-Dahr et al., 1998; Jaffa et al., 1997). We have previously reported that BK activates NHE1 in a kidney cell line derived from the inner medullary collecting duct of mice (mIMCD-3 cells) (Mukhin et. al., 2001) via the similar pathway that Ang II and serotonin employ to activate NHE1 activity in VSMC, which involves the activation of Jak2, tyrosine phosphorylation of Ca^{2+}/calmodulin, and binding of calmodulin (CaM) to NHE-1 (Garnovskaya et al., 2003). We have also described that BK is a potent mitogenic factor for mIMCD-3 cells, and demonstrated that BK-induced cell proliferation was dependent on activation of epidermal growth factor receptor (EGFR) tyrosine kinase and subsequent activation of mitogen- and extracellular signal-regulated kinase kinase (MEK) and (Mukhin et al., 2003; Mukhin et al., 2006; Kramarenko et al., 2010). However, we were not able to establish the relationship between NHE1 and ERK in mIMCD-3 cells. Our data did not support either the hypothesis that ERK is a proximal short-term regulator of NHE or the hypothesis that NHE1 is necessary for the BK-induced ERK activation in normal kidney mIMCD-3 cells (Garnovskaya, unpublished data). Because there is evidence linking BK to the cancerogenic process (Bhoola et al., 2001; Chan et al., 2002), and because NHE1 has been proposed to play role in cancer cells growth (Cardone et al., 2005), we wanted to explore the possibility that BK exerts its mitogenic effects via activation of NHE in cancer cell lines. The expression of BK receptors has been demonstrated in clinical speciments of adenocarcinoma, squamous carcinoma, lymphoma, hepatoma and carcinoid, and in experimental mouse sarcoma 180 and colon adenocarcinoma 38 (Wu et al., 2002), in small cell and non-small cell carcinomas of the lung (Chee et al., 2008), and in oesophageal squamous cell carcinoma (Dlamini et al., 2005). The mitogenic effects of BK have been reported in primary cultured epithelial breast cancer cells and in MCF-7 breast cancer cell line, where BK stimulated cell proliferation through ERK activation (Greco et al., 2005; Greco et al., 2006). Because there were limited studies on the role of BK in renal cell carcinomas, we have chosen to use A498 cells, a transformed cell line derived from primary undifferentiated kidney carcinoma (Giard et al., 1973), which represents a widely used model for studying of renal carcinomas. Our results demonstrated that NHE1 is involved in BK-induced ERK activation and proliferation of A498 cells, and that BK B_2 receptor-induced ERK activation in A498 cells depends on NHE activity (Garnovskaya et

al., 2008), suggesting that the critical role of NHE1 in GPCR-induced ERK activation is not restricted to one specific cell type and receptor. Previously, NHE1-mediated intracellular alkalinization has been proposed to play role in cancer cells growth because it has been shown that increased pH_i of tumor cells is associated with increased *in vivo* tumor growth, DNA synthesis, and cell-cycle progression, suggesting that over-expression of NHE1 contributes to the transformed phenotype of multiple cancer cells (reviewed by Cardone et al., 2005). The cellular alkalinization of tumor cells induced by hyperactivation of NHE1 has been shown to be directly related to increased protein synthesis and tumor cell growth (Cardone et al., 2005; Harguindey et al., 2005). It has been suggested that the mechanism of NHE1-mediated tumor cell growth and metastasis does not depend of its ion-transporting activities but rather employs NHE1 as a scaffolding protein to directly regulate cytoskeletal dynamics (Cardone et al., 2005). Further it has been shown that NHE1 antisense gene suppresses cell growth, induces cell apoptosis, and partially reverses the malignant phenotypes of human gastric carcinoma cells (Liu et al., 2008). Similarly, silencing of NHE1 gene by siRNA interference and /or inhibition of NHE1 activity by amiloride analogs effectively blocked the invasiveness of human hepatocellular carcinoma cells (Yang et al., 2011). Thus, inhibition of NHE1 might result in an antiproliferative effect, and NHE1 may be a potential target for chemotherapeutics to treatment of renal carcinoma.

3. Conclusion

The elucidation and understanding of the relationship between NHE1 and ERK cascade has been one of the most active areas in biological research over the past few years. As discussed above, experimental studies have strongly implicated a role for NHE1 in the regulation of ERK activity, although the precise pathway, which leads from the activation of NHE1 to ERK regulation still has to be defined. One possibility is that GPCR-induced NHE1-dependent ERK activation depends on NHE1-mediated Na^+/H^+ exchange. In that sense, Grinstein et al have demonstrated uneven distribution of NHE1 molecules throughout the cell surface with the focal accumulation at or near terminal edges of fibroblasts and CHO cells, and the areas of increased NHE1 density closely corresponded to sites of accumulation of cytoskeletal proteins (Grinshtein et al., 1993). It is possible that NHE1 regulates ERK by altering Na^+ or H^+ concentrations in cellular microdomains, thereby affecting enzyme activity or protein-protein interactions. Another possibility is that NHE1 acts as a plasma membrane scaffold (Baumgartner et al., 2004) in the assembly of signaling complexes independent of its ion exchange activity bringing together GPCRs, and the members of ERK-activation cascade. Regardless of the mechanisms, the critical role of NHE1 as an upstream molecule in GPCR-induced ERK activation could have significant physiological and pathophysiological relevance.

Because ERK-dependent cell proliferation is thought to be a critical component in many pathologic conditions, and NHE is involved in a variety of complex physiological and pathological events that include regulation of intracellular pH, cell movement, heart disease, and cancer, improved understanding of the molecular mechanisms that regulate NHE and ERK may allow alternative approaches to the therapeutic manipulation of ERK and NHE activity to be developed.

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12

The Kinetochore and Mitosis: Focus on the Regulation and Correction Mechanisms of Chromosome-to-Microtubule Attachments

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1. Introduction

During mitosis, accurate segregation of the chromosomes duplicated at the S phase of the cell cycle relies on the successful of their proper attachment to microtubules of the mitotic spindle and their alignment at the metaphase plate before the onset of anaphase. Attachment errors or failure to align can lead to chromosome gains or losses, a condition known as chromosome instability (CIN) which is a common feature amongst many cancers.

The kinetochore, a multiprotein structure assembled on the centromeres, plays a key role in chromosome attachment and high fidelity of mitotic chromosome segregation between daughter cells.

The kinetochore structure provides the attachment site of microtubule polymers to chromosome. Each mitotic chromosome has a pair of kinetochores, positioned on opposite sides of the centromere, that allow for its bipolar attachment to the spindle. Besides this role in attachment, the kinetochore controls the metaphase-anaphase transition by inhibiting chromatid separation until all chromosomes are properly attached and aligned at the metaphase plate, thereby ensuring equitable sharing of chromosomes upon cell division.

During the last decade, considerable progress has been made in understanding the molecular composition of the kinetochore, shedding light on the higher order organization of the kinetochore and on it activity in mediating chromosome attachment to spindle microtubules and in regulating the fidelity of the metaphase to anaphase transition. We address these aspects, concentrating in particular on the role that the kinetochores have in sensing and resolving aberrant kinetochore-microtubule attachments and in preventing chromosome missegregation.

2. The events of Mitosis

In 1879, Walther Flemming was the first to describe in great detail cell division. The Fleming's great discovery was that during the cell division one longitudinal half of each

chromosome goes in each direction, so that each daughter nucleus is formed from a complete set of longitudinal halves. In 1882 he termed this process Mitosis (from the Greek mitos "thread-metamorphosis") (Baker, 1949; Paweletz, 2001).

2.1 Mitotic phases

Nowadays, it is known that Mitosis is a complex and highly regulated process, used by eukaryotes to generate two identical daughter cells from one original mother cell. This particular phase of the cell cycle, ensures that the cell faithfully segregates the sister chromatids, of the duplicated chromosomes, into the daughter cells, producing two cells that are identical to one another and to the original parent cell. Conventionally, mitosis is divided into five stages: Prophase, Prometaphase, Metaphase, Anaphase and Telophase (Fig. 1).

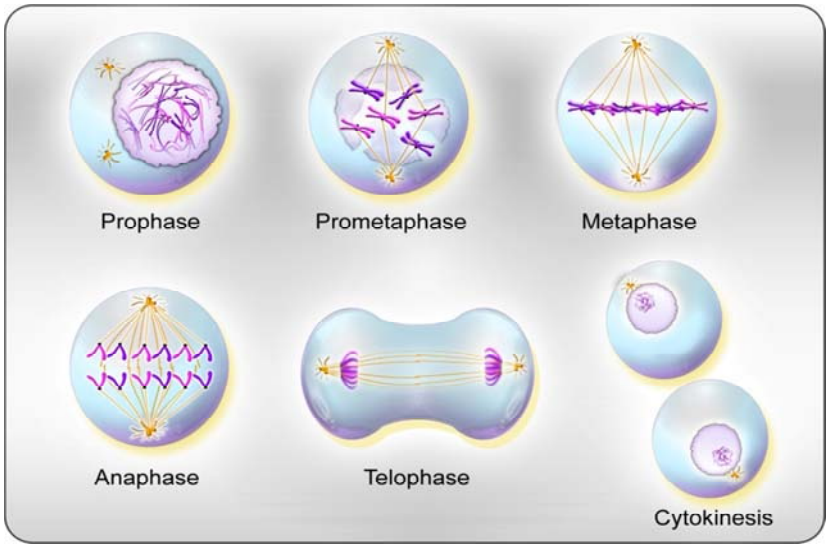

Fig. 1. Mitotic stages and Cytokinesis of animal cells. Prophase: chromatin condenses into chromosomes and centrosomes start to separate; Prometaphase: begins with nuclear envelope breakdown, chromosomes are captured by microtubules that are growing from opposite poles of the spindle; Metaphase: all chromosomes align at the equator of the cell; Anaphase: sister chromatids move to opposite poles; Telophase: sister chromatids reach the poles, decondense and nuclear membrane reforms around each identical daughter nuclei. Simultaneously, the division of the cytoplasm generates two independent daughter cells by a process called Cytokinesis.

Prophase, is marked by the appearance of condensed chromosomes that emerge as two identical filaments (sister chromatids, produced in the S phase of the cell cycle) and the centrosomes begin to separate to opposite sites initiating the mitotic spindle assembly. The next stage, Prometaphase, starts with the nuclear envelope breakdown allowing that microtubules, from the opposite centrosomes, occupy all the cytoplasmic space and fully

organize the mitotic spindle. These microtubules bind the chromosomes and help them to reach and align at the equatorial cell region; at this stage the cell has achieved the Metaphase. In Metaphase, chromosomes are aligned at the middle of the spindle forming the metaphase plate, awaiting the signal to the next stage, Anaphase. This transition, from Metaphase to Anaphase, is the most critical event of the cell cycle. This event occurs when the cohesion between sister chromatids is dissolved and the separated sisters migrate to opposite poles of the spindle. The last stage of mitosis is Telophase, during which the spindle disassembles, the chromosomes decondense and the nuclear envelop reappears. Simultaneously, the division of the cytoplasm generates two independent interphase daughter cells by a process called Cytokinesis. If these events do not occur properly and in the right sequence, the newly formed cells either die or carry on genetic damages that can lead ultimately to cancer.

2.2 Regulation of Mitosis

The timing and coordination of Mitosis progression relies mostly on mechanisms of protein Phosphorylation and Proteolysis (Nigg, 2001). While Phosphorylation is a reversible protein modification, and thus ideal for the control of reversible mitotic processes such as spindle assembly, Proteolysis, in contrast, by its chemical irreversibility, is more appropriate for controlling events that must not be reversed such as sister-chromatid separation (King et al., 1996; Morgan, 2007). The Mitosis-Promoting Factor (MPF), first called Maturation-Promoting Factor, was described as the "entity" whose activity controls entry into Mitosis (Masui & Markert, 1971). Now, it is well-established that the MPF is a heterodimer composed by one molecule of cyclin B and one of Cdc2 (cell division cycle). Later, Cdc2 became known as Cdk1 since it was established, at the Cold Spring Harbor Symposium on the Cell Cycle in 1991, that kinases that are associated with cyclins should be called "cyclin dependent kinases" or Cdks (Doree & Hunt, 2002). Cyclin B-Cdk1 (MPF) accumulates before entry into mitosis and its activation leads to phosphorylation of several substrates responsible for the morphological changes that occur in early stages of mitosis such as nuclear envelop breakdown, centrosome separation, spindle assembly, chromosome condensation, and endoplasmic reticulum and Golgi fragmentation. However, proteolysis-mediated disassembly of Cyclin-Cdk1 complexes is required for mitotic exit and cytokinesis (Nigg, 2001). Besides the direct role in regulating Cdk activity by controlling cyclin levels, proteolysis also drives cell cycle progression by directly triggering some key cell cycle events such as sister chromatids separation at metaphase-anaphase transition, thus providing directionality of the cell cycle (King et al., 1996). Therefore, these two mechanisms, phosphorylation and proteolysis, are interdependent since proteolysis events are controlled by phosphorylation and the mitotic kinases (M-Cdks) are inactivated by proteolytic destruction of cyclins (King et al., 1996; Morgan, 2007; Nigg, 2001).

The major events of mitosis are sister-chromatid separation and segregation. If these processes do not occur accurately the result would be the production of cells with extra or missing chromosomes, a state known as aneuploidy, which is a common characteristic of cancer cells (Holland & Cleveland, 2009; Schvartzman et al., 2010). To avoid the occurrence of aneuploidy, cells have developed a control system called mitotic checkpoint

or Spindle Assembly Checkpoint (SAC), which prevents the cell entry in anaphase until all chromosomes are correctly aligned, forming the metaphase plate, with proper attachment to the mitotic spindle (Rieder et al., 1995) and under a certain tension (Nicklas et al., 1995). When these conditions are satisfied, SAC is turned off. This checkpoint arrests cells in mitosis by blocking protein degradation. With its inactivation, the Anaphase Promoting Complex or Cyclosome (APC/C), an ubiquitin protein ligase whose activity depends on the activator protein Cdc20, targets Securin and Cyclin B for ubiquitylation and posterior proteolysis through the 26S proteasome. Destruction of Securin turns on Separase which cleaves the cohesion complex that holds sister chromatids together; destruction of Cyclin B leads to anaphase onset (Zachariae & Nasmyth, 1999).

2.3 The mitotic spindle

In order to congress and align at the center of the cell, and then segregate its sister chromatids to opposite poles, chromosomes use the mitotic spindle. The mitotic spindle is organized in a symmetric and fusiform structure composed of microtubules, polymers made of α- and β-tubulin heterodimers that being all oriented in the same way create a polar nature with β-tubulin exposed at one end (plus-end) and α-tubulin at the other end (minus-end) (Desai & Mitchison, 1997). Depending on the position of the microtubule plus-ends, spindle microtubules can be divided into three classes: astral-microtubules, interpolar-microtubules and kinetochore-microtubules; all contribute to the bipolarity of the mitotic spindle. Astral microtubules, emanate from the spindle poles and radiate out throughout the cytoplasm with the plus-ends interacting with the cell cortex. Interpolar microtubules, extend from the spindle poles to the spindle midzone where their plus-ends form an interdigitating system that connects the two spindle poles. Kinetochore microtubules, connect the spindle poles to chromosomes with the minus ends near the poles and the plus ends binding laterally or end-on, specifically to the kinetochores (an intricate protein complex raised on the centromeric DNA) (Hayden et al., 1990; Merdes & De Mey, 1990; Rieder & Alexander, 1990). These kinetochore-microtubules form a morphologically distinct bundle denominated K-fiber (kinetochore-fiber), made of up to 30 kinetochore-attached microtubules in higher eukaryotes, which is directly involved in chromosome congression and sister-chromatid segregation (Rieder & Salmon, 1998).

The ability of spindle microtubules to quickly assemble and disassemble (dynamic instability) provides them the necessary behavior to capture chromosomes. This statement becomes the basis of the first and favorite model for spindle assembly in systems with centrosomes, and is known as "search and capture" (Kirschner & Mitchison, 1986; Mitchison & Kirschner, 1984). This model postulates that, when nuclear envelop breaks down, chromosomes become accessible to microtubules radiated from centrosomes , which, through their dynamic nature, randomly explore the cytoplasm until capture a kinetochore, laterally or with the plus-end, forming an attachment that stabilizes the microtubule (Mitchison et al., 1986; Nicklas & Kubai, 1985). Although the plus-ends of microtubules can bind directly the kinetochore, the first contact usually occurs laterally. After binding one of the unattached sister kinetochores, the chromosome is rapidly transported along the side of the microtubule towards the spindle pole, in a mechanism

that is independent of microtubule depolymerization and involves the minus-end directed motor protein Dynein (Rieder & Alexander, 1990; Yang et al., 2007). Since the spindle pole has a high microtubule density, additional microtubules from the same pole will attach to the kinetochore, and the plus-ends of laterally associated microtubules shorten until reach the end-on binding, resulting in a stable K-fiber (Rieder, 2005). Since this model is based on "search and capture" of astral microtubules that radiate from centrosomes, it is not valid to cells lacking centrosomes, like higher plants and many animal oocytes. An alternative model, called "spindle self organization", proposes that microtubules are nucleated in the vicinity of chromatin, elongate and then, helped by motor proteins, are sorted into a bipolar array and focused at the poles (Walczak et al., 1998). Indeed, it was shown that cells with centrosomes, besides the "search and capture" mechanism, also use this chromosome-driven K-fiber formation for spindle assembly (Khodjakov et al., 2000; Maiato et al., 2004). In these "combined" system, the K-fibers nucleated from chromosomes are integrated at the spindle assisted by astral microtubules that search, capture and transport them toward the pole in a dynein-dependent manner (Khodjakov et al., 2003; Maiato et al., 2004). Several studies, in *Xenopus* extracts and in mammalian cells, show that the chromosome-driven microtubule formation relies in a Ran-GTP concentration gradient around mitotic chromosomes, which in turn induces a gradient of proteins that regulate microtubule nucleation/dynamics, by dissociating them from importin-β (Fuller, 2010; Kalab et al., 2002; Tulu et al., 2006). Furthermore, studies in mammalian somatic cells support a model in which the kinetochores are the key players in this chromosome-mediated spindle assembly (O'Connell et al., 2009). Another mechanism that contributes to spindle formation is the "search and transport", in which peripheral microtubules, similarly to K-fibers nucleated from chromosomes, are transported through aster microtubules to the poles, where they are incorporated into the spindle (O'Connell & Khodjakov, 2007; Tulu et al., 2003).

For a successful cell division, chromosomes must interact with spindle microtubules. Through their dynamic behavior, microtubules allow that chromosomes congress to the equatorial region of the spindle forming the metaphase plate and, are responsible for the segregation of sister chromatids to opposite poles. There are three major forces acting on chromosomes that drivethese movements: (1) The "Polar ejection force" or "Polar wind" that is generated by non-kinetochore microtubules and pushes chromosomes away from the spindle poles (anti-poleward movement) (Kapoor & Compton, 2002; Rieder et al., 1986); (2) "Microtubule flux", which consists in a flow of tubulin subunits from the spindle equator to the spindle poles as consequence of polymerization at the plus-ends, depolymerization at minus-ends, and translocation of the entire microtubule toward the spindle pole (Cassimeris, 2004; Mitchison, 1989); and (3) "Pacman" mechanism, in which the kinetochores catalyze the depolymerization of kinetochore-microtubule plus-ends resulting in chromosomal movement to the spindle poles by "chewing up" the microtubule track (Gorbsky et al., 1987; Mitchison et al., 1986).

3. The kinetochore structure

In 1894, Metzner was the first investigator to describe the "kinetic region", a specific chromatin area, located at the primary constriction on each side of the chromosome, that

leads the way of sister chromatids during the poleward motion (Metzner, 1894; Rieder, 2005; Schrader, 1944). Later, in 1934, Sharp coined these structures as "kinetochores", from the Greek 'kineto-' meaning 'move' and '-chore' meaning 'means for distribution' (Rieder, 2005; Schrader, 1936). In 1960, Bill Brinkley was the first to describe the mammalian kinetochore structure as a trilaminar proteinaceous disc structure that flanked the centromere: an electron-dense inner plate located on the surface of the centromeric heterochromatin, separated from an electron dense outer plate by a lighter middle layer (Brinkley & Stubblefield, 1966). However, using high-pressure frozen specimens, this electron-translucent middle layer is not visible, suggesting that it is an artifact produced during the classical EM fixation and/or dehydration procedures (McEwen et al., 1998). In 1967, Jokelainen shows the existence of a corona of electron opaque substance that covers the outer kinetochore layer, which after microtubule binding, becomes hard to detect by EM (Cassimeris et al., 1990; Jokelainen, 1967)(Fig. 2).

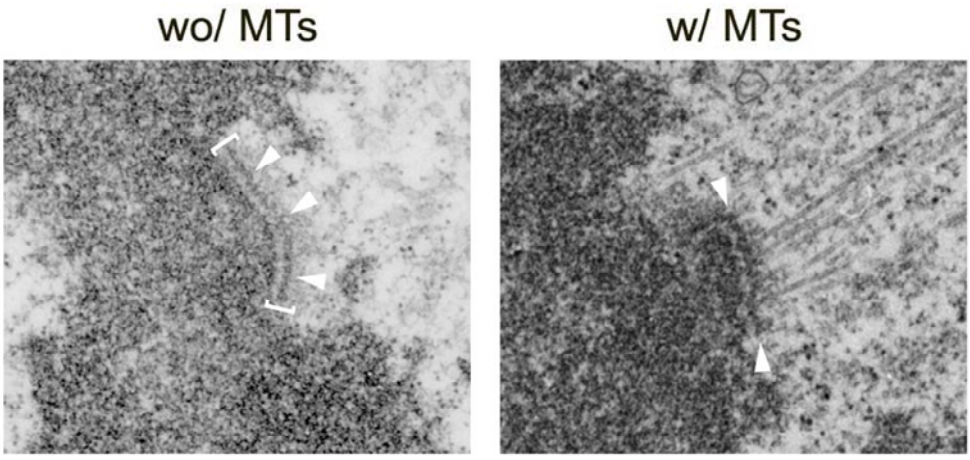

Fig. 2. Electron micrographs of kinetochores, from PTK1 cells, in absence and presence of microtubules. The trilaminar structure of kinetochore (brackets) is well defined without microtubules (wo/ MTs) and becomes distorted with microtubules (w/ MTs) embedded within the outer kinetochore plate (arrowheads). Courtesy of Dr. Helder Maiato (Maiato et al., 2006).

These mature kinetochores, with the trilaminar structure, occurs only in prometaphase after nuclear envelop breakdown. The kinetochore is built on the centromeric region of each sister chromatid by the assembly of multiprotein complexes (Fig. 3). In early G1 the typical layer conformation disappears giving rise to a condensed structure that unfolds in late G1 forming a linear, bead-like conformation that persists until S-phase, where it transforms into a loose fibrous bundle that duplicates at late S-phase. In late G2, pre-kinetochores refold into two separated and condensed structures. During prophase, these duplicated pre-kinetochores differentiate at the primary constriction of the sister chromatids originating the kinetochore layers at the time of nuclear envelop breakdown, completing the cycle (He & Brinkley, 1996).

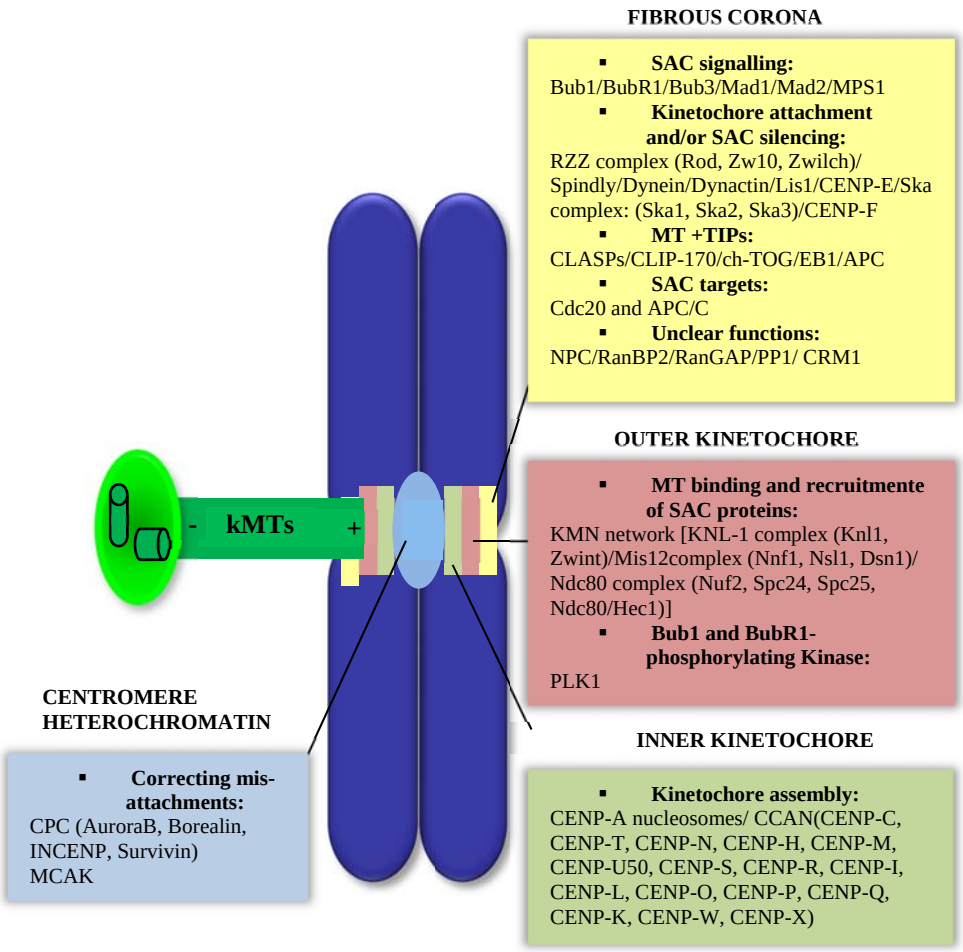

Fig. 3. Overview of protein complexes that build the kinetochore in animal cells. The kinetochore is built on the centromere as a trilaminar protein-rich structure: the inner kinetochore, the outer kinetochore and the fibrous corona. The inner and outer kinetochore layers are separated by the translucent interzone (not depicted). Proteins at centromere heterochromatin and at each kinetochore layer are indicated; the list is not exhaustive as it is continuously growing. Adapted from (Silva et al., 2011).

Kinetochores localize at the constriction region on each sister chromatid, assembling on the centromere, from the Greek 'centro-' (meaning 'central') and '-mere' (meaning 'part'), a specific chromatin region with distinct epigenetic marks (Cheeseman & Desai, 2008). In this particular region, the nucleosomal histone H3 is replaced by the variant CENP-A (CENtromere Protein A)(Vafa & Sullivan, 1997). Although it seems consensual that CENP-A is essential for specifying the site for kinetochore formation, the role of CENP-A in kinetochore assembly is still unclear (Bowers & Mellone, 2011). Some studied, using

overexpression/mistargeting of CENP-A show that CENP-A is not sufficient to originate functional kinetochores (Gascoigne et al., 2011; Van Hooser et al., 2001). Other studies, using ectopic targeting of HJURP (a CENP-A chromatin assembly factor) (Barnhart et al., 2011) and using *in vitro* kinetochores (Guse et al., 2011), demonstrate that CENP-A is indeed sufficient to form functional kinetochores. Besides CENP-A, several other proteins are present at vertebrate centromeres throughout the cell cycle. They were named CCAN for Constitutive Centromere Associated Network (Cheeseman & Desai, 2008). CCAN comprises at least 16 proteins, CENP-C, CENP-H, CENP-I, CENP-K-U, CENP-W, and CENP-X, grouped into several subcomplexes (Amano et al., 2009; Santaguida & Musacchio, 2009). It is known that whereas CENP-C, CENP-N and CENP-K associate specifically with CENP-A nucleosomes (Carroll et al., 2009; Guse et al., 2011), CENP-T/W complex are DNA-binding proteins that associate with histone H3 nucleosomes in the centromeric region (Guse et al., 2011; Hori et al., 2008). Although only few proteins are present at centromeres throughout the cell cycle, during mitosis this number increases substantially, indicating the existence of a complex assembly regulatory process. Gascoigne et al. have contributed with a piece of this puzzle, by demonstrating that phosphorylation of CENP-T by CDK can control kinetochore assembly in vertebrates. They saw that CENP-T becomes phosphorylated in G2, has a maximum at metaphase and drops until anaphase, and show that preventing CENP-T phosphorylation abolishes the recruitment of Ndc80 (Hec1 in mammals) (Gascoigne et al., 2011). Ndc80 is part of the Ndc80 complex that in turns is part of the so called KMN network, the microtubule-binding core of kinetochore, composed by the two-subunit Knl1 complex (containing Knl1/Blinkin and Zwint), the four-subunit complex Mis12 (containing Mis12, Dsn1, Nnf1 and Nsl1) and the four-subunit Ndc80 complex (containing Ndc80, Nuf2, Spc24 and Spc25). In the Ndc80 complex, Nuf2 and Ndc80 localize to the outer kinetochore region and bind directly to microtubules, and Spc24 and Spc25 localize at the inner kinetochore region and bind to the Mis12 and Knl1 complexes (Santaguida & Musacchio, 2009). A recent study, demonstrate that a conserved motif in the N-terminal region of Cenp-C binds directly to the Mis12 complex (Screpanti et al., 2011). In fact, several proteins from the CCAN, such as CENP-C, CENP-H, CENP-K, CENP-I, CENP-T/W, and CENP-X have been implicated in the recruitment of KMN proteins (Petrovic et al., 2010). These connections between the CCAN network and the KMN network link the inner to the outer kinetochore. KMN complex was considered as the essential core of the kinetochore. Besides the direct binding of centromeres to microtubules, it interacts, directly or indirectly, with proteins that are involved in crucial mitotic functions such as, regulation of the activity of spindle assembly checkpoint and kinetochore-microtubule interactions (Cheeseman & Desai, 2008; Przewloka & Glover, 2009). It allows, directly or indirectly, the recruitment/interaction of regulatory proteins such as Microtubule associated proteins (like CLASP, CLIP170, EB1, APC), motor proteins (like CENP-E, dynein/dynactin complex), protein involved in spindle assembly checkpoint (like Mad1, Mad2, Bub1, Bub3, BubR1, Bub1, RZZ complex, Mps1). It also interacts with the SKA (Spindle and Kinetochore Associated) complex, thought to be involved in stable end-on kinetochore-microtubule attachment; and with the CPC (Chromosome Passenger Complex- Aurora B, INCENP, Borealin and Survivin) complex, among others (Fig. 3). Indeed, depletion of any protein from the KMN network, in all eukaryotes, result in abnormal kinetochore structure or in a lack of kinetochore-microtubule attachment (Lampert & Westermann, 2011; Przewloka & Glover, 2009; Santaguida & Musacchio, 2009).

4. Kinetochore functions

The Kinetochores are dynamic structures with more than 100 proteins organized into networks of several complexes of probably intertwined functions during cell division (Cheeseman & Desai, 2008). They attach sister-chromatids to microtubules of the bipolar mitotic spindle and position the chromosome at the spindle equator; they inhibit anaphase onset until all chromosomes are properly attached and aligned at the metaphase plate; and provide correction mechanism of erroneous attachments. Next, we address the overall mechanisms and the molecular link between chromosome attachment to spindle microtubules, correction of attachment errors, and the spindle assembly checkpoint.

4.1 Kinetochores mediate bipolar attachment to the spindle

High-fidelity chromosome segregation at the onset of anaphase relies on the success of sister kinetochore bi-orientation on the mitotic spindle (Tanaka, 2010). Chromosome bi-orientation is a step-wise process that sequentially involves initial interaction of a kinetochore with the lateral surface of a microtubule; transport of the kinetochore to a spindle pole; conversion from the lateral to the end-on attachment; and attachment of the sister kinetochore to microtubules from the opposite spindle pole (Fig. 4).

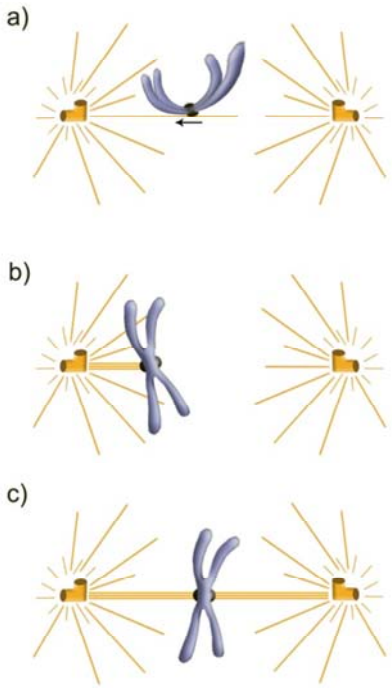

Fig. 4. Steps towards chromosome bi-orientation. Upon nuclear envelope breakdown, chromosome bi-orientation sequentially involves a) initial interaction of a kinetochore with the lateral surface of a microtubule and transport of the kinetochore to a spindle pole; b) conversion from the lateral to the end-on attachment; and c) attachment of the sister kinetochore to microtubules from the opposite spindle pole to achieve bipolar attachment.

As stated above, initial encounter of spindle pole microtubules with the kinetochore is mediated by the so-called "search and capture" process, guided by a Ran-GTP concentration gradient around the chromosome (Clarke & Zhang, 2008; Wollman et al., 2005). Additionally, and to avoid further delay in this initial encounter, kinetochore can nucleate microtubules which, by interacting with spindle-pole nucleated microtubules along their length, facilitate kinetochore loading onto centrosome-nucleated microtubules (Kitamura et al., 2010). The first step of the "search and capture" process is the interaction of the kinetochore with the lateral surface of a microtubule, called the lattice, followed by chromosome sliding along the microtubule lattice towards a spindle pole. The poleward kinetochore transport, powered by the minus end-directed dynein motor protein complex, brings chromosomes scattered throughout the cytoplasm to the mitotic spindle area (Sharp et al., 2000; Yang et al., 2007). Dynein binding to kinetochores requires the protein Spindly which in turn requires the RZZ complex [made of the proteins Rough-deal (ROD), Zeste-white (ZW10), and Zwilch] to localize to kinetochore (Chan et al., 2009; Griffis et al., 2007; Karess, 2005). The connection to the essential microtubule-binding core of the kinetochore is mediated by the protein Zwint-1 that links the RZZ complex to the KMN network (Wang et al., 2004).

During the association of the kinetochore with the microtubule lattice, shrinking of the microtubule plus-end leads to kinetochore tethering at the microtubule plus-end (end-on attachment) and to its further transport towards the spindle pole. End-on attachments are more robust than lateral attachments and are critical for bi-orientation and for the generation of load-bearing attachments (Joglekar et al., 2010). The mechanism of the conversion from a lateral into an end-on attachment remains unclear. Proteins thought to be required for this conversion include the *C. elegans* RZZ complex and Spindly/SPDL-1 (Gassmann et al., 2008), the vertebrate Ska1-3 (Gaitanos et al., 2009; Guimaraes & Deluca, 2009), and the *Saccharomyces cerevisiae* Ndc80 loop region (Tanaka, 2010).

The chromosome reaches the spindle pole with one kinetochore end-on attached to k-fiber microtubules from that pole and its sister kinetochore unattached, a state known as monotelic attachment. Aided by the back-to-back kinetochore geometry, the unattached kinetochore becomes attached when captured by microtubule searching from the opposite pole, thereby leading to chromosome bi-orientation (amphitelic attachment) (Fig. 4). Subsequently, k-fiber microtubule shrinking and elongation promote congression of the chromosomes towards the spindle equator in order to form the metaphase plate (Silva et al., 2011). As an additional mechanism that leads to bi-orientation, chromosomes can be transported towards the spindle equator by gliding alongside microtubules attached to other already bi-oriented chromosomes, driven by kinetochore-bound CENP-E, a plus end-directed microtubule motor of the kinesin-7 family (Kapoor et al., 2006).

4.2 Correcting aberrant kinetochore-microtubule attachments

Although the back-to-back orientation of sister kinetochores imposes a geometric constraint that favors chromosome bi-orientation, errors in kinetochore-microtubule attachments are frequent due to the stochastic nature of the search and capture mechanism. Such errors include monotelic (one kinetochore unattached while its sister attached to one spindle pole), syntelic (two sister kinetochores bound to microtubules from the same pole), and merotelic (one sister kinetochore bound to microtubules from both poles) attachments (Fig. 5) (Silva et al., 2011). Most of these errors occur at the beginning of prometaphase and, if left uncorrected, would lead to unequal chromosome segregation and aneuploidy (Kops et al., 2005).

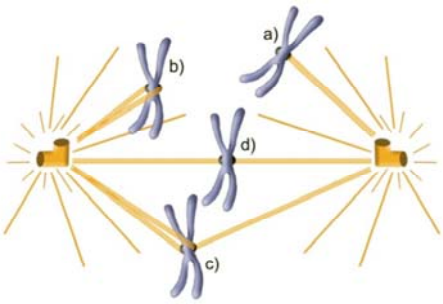

Fig. 5. Possible attachment errors during chromosome bi-orientation event. Errors include a) monotelic, with one kinetochore unattached while its sister attached to one spindle pole; b) syntelic, with two sister kinetochores bound to microtubules from the same pole; and c) merotelic, with one sister kinetochore bound to microtubules from both poles attachments. These errors are generally converted to d) amphitelic attachments, the only correct attachment configuration, by the error correction machinery.

Error correction is the result of biochemical changes induced by mechanical forces acting on the kinetochore. Bi-oriented kinetochores (amphitelic attachment) are under tension that results from the pulling forces of spindle microtubules in opposite directions. Cohesion between sister centromeres is necessary to generate this tension (Tanaka et al., 1999). Tension across the centromere stabilizes kinetochore-microtubule attachments, as evidenced by Bruce Nicklas in his classical micromanipulation experiments with insect spermatocytes (Nicklas & Koch, 1969; Nicklas & Ward, 1994). Tension artificially applied with a glass microneedle on grasshopper spermatocyte chromosomes stabilizes unipolar attachments by increasing the occupancy of microtubule attachment sites.

The first evidence of the translation of mechanical forces (tension) acting on the kinetochore into biochemical changes was provided by the identification of the Ipl-1 kinase in the budding yeast *Saccharomyces cerevisiae* (Biggins et al., 1999). In Ipl-1 defective yeast cells, kinetochores can interact with microtubules but sister-kinetochores often fail to bi-orient, suggesting that Ipl-1 promotes bi-orientation. Interestingly, in the same mutant, kinetochore-microtubule attachments are stabilized in the absence of tension, suggesting that Ipl-1 promotes bi-orientation by destabilizing tensionless attachments (Dewar et al., 2004; Tanaka et al., 2002). Defective Aurora B kinase (the mammalian functional homolog of Ipl-1) induces an increase in syntelic attachments, suggesting a similar work in mammalian cells (Hauf et al., 2003; Kallio et al., 2002). Aurora B localizes to the inner centromere, as the catalytic component of the chromosome passenger complex (CPC), together with the binding partners INCENP, Survivin, and Borealin (Ruchaud et al., 2007). While the molecular mechanism of error detection and correction is not fully understood, a current model proposes that Aurora B kinase promotes the turnover of erroneous kinetochore-microtubule attachments through phosphorylation of its substrates at the outer kinetochore, in a tension-dependent manner (Lampson & Cheeseman, 2011) . In this so-called spatial model, the distance between Aurora B and its outer kinetochore substrates is a critical determinant for their phosphorylation. When tension is low or absent, outer kinetochore substrates are phosphorylated due to their proximity to an Aurora B kinase activity gradient around the inner centromere (Wang et al., 2011), which destabilizes kinetochore-

microtubule attachments. For instance, in higher eukaryotes, Aurora B phosphorylation of the Ndc80 complex was reported to weaken its affinity to microtubules, while phosphorylation of the microtubule depolymerase kinesin MCAK activates its depolymerase activity (Cheeseman et al., 2006; Lan et al., 2004). Both changes promote destabilization of tensionless kinetochore-microtubule attachments, providing a further opportunity for chromosome to bi-orient. Bi-orientation locates outer kinetochore substrates away from the Aurora B activity gradient and close to the opposing and constitutively active phosphatase. In this way, dephosphorylation of Ndc80 and MCAK stabilizes amphitelic kinetochore-microtubule attachments. Therefore, tension provides the determinant for the error correction machinery to distinguish syntelic and merotelic from amphitelic attachments. Once all chromosomes become bi-oriented and aligned at the metaphase plate, Aurora B leaves the inner centromere and become concentrated on the spindle midzone. This presumably prevents turnover of kinetochore-microtubules attachments during anaphase, in which tension is reduced.

4.3 The spindle assembly checkpoint

Cells must be maintained in mitosis, by preventing securin and cyclin B degradation, until all chromosomes are properly bi-oriented and aligned at the metaphase plate. This is achieved by the evolutionary conserved mechanism called The "Spindle Assembly Checkpoint" (SAC) that prevents the E3 ubiquitin ligase APC/C (Anaphase Promoting Complex/Cyclosome) from targeting securin and cyclin B for degradation by the 26S proteasome, as long as unattached or improperly attached chromosomes are present (Silva et al., 2011).

Components of the SAC are conserved from yeast to human and include the core SAC proteins Mad1, Mad2, Bub1, Bub3, Mad3/BubR1, and the protein kinases Mps1 and Aurora B (Musacchio & Salmon, 2007). It is widely accepted that the SAC inhibits the APC/C through the Mad2 protein that sequesters Cdc20 an essential activator of the APC/C individually or in an inhibitory complex with BubR1 and Bub3, forming the "Mitotic Checkpoint Complex" (MCC) (Kallio et al., 2002; Sudakin et al., 2001). Unattached kinetochore is believed to provide the platform for the generation of this diffusible "wait anaphase" signal that inhibits mitosis. Indeed, checkpoint proteins dynamically associate with unattached kinetochores, reflecting the catalytic assembly and then release and diffusion of MCC. However this model is not universal as MCC formation does not require a kinetochore in yeast (Fraschini et al., 2001).

SAC becomes satisfied once all chromosomes are correctly bi-oriented at the metaphase plate. In order for cell to proceed to anaphase, the production of MCC must be halted and existing MCC must be disassembled. This is referred to as "SAC silencing", thought to be mediated by several mechanisms at least in mammalian cells (Fuller & Stukenberg, 2009; Vanoosthuyse & Hardwick, 2009). Kinetochore-mediated production of MCC is suggested to be stopped by Dynein-driven transport of SAC proteins from attached kinetochores towards spindle poles, along microtubules (Howell et al., 2001); and through dissociation of Mad2/Cdc20 complex due to competition binding of the protein $p31^{comet}$ to the dimerization interface of Mad2. Disassembly of existing MCC is promoted by $p31^{comet}$- and UbcH10-dependent Cdc20 autoubiquitination (Reddy et al., 2007; Stegmeier et al., 2007), and proteasomal degradation (Ma & Poon, 2011). The coordination between these mechanisms

during SAC inactivation and mitotic exit remains unclear. Once SAC is turned off, APC/C becomes activated and targets securin and cyclin B for degradation by the proteasome, thereby promoting sister-chromatid separation and exit from mitosis, respectively.

4.4 Relationship between microtubule attachment, attachment error correction, and SAC

The role of SAC in delaying anaphase onset, in the presence of unattached or improperly attached kinetochores, foresees the existence of a dynamic relationship between microtubule attachment, error correction machinery, and checkpoint signaling. This relationship could efficiently assure that attachment errors are detected; checkpoint signals are produced; and attachment errors are corrected only at the kinetochore platform, being all these activities in the right place at the right time.

A molecular link between microtubule attachment machinery and SAC activity has been suggested by the phenotype of yeasts with defective Ndc80 (Ndc80, Nuf2, Spc24, and Spc25) complex, a component of the KMN supercomplex (the core microtubule-binding interface of kinetochores). These mutants fail to attach chromosomes to the spindle and to activate SAC (Burke & Stukenberg, 2008; McCleland et al., 2003). In metazoans, the relationship between kinetochore-microtubule binding and SAC signaling is further suggested by the observation that KMN, through the Ndc80 complex, is required for kinetochore assembly of SAC proteins as well as for the generation of a SAC signal (Burke & Stukenberg, 2008). In addition, Blinkin (the human homologue of yeast kinetochore protein Spc105) directs Bub1 and BubR1 to kinetochores through interaction with their TPR domains (Bolanos-Garcia et al., 2011; Kiyomitsu et al., 2007). Preventing interaction between the Bubs and Blinkin, either by siRNA or by point mutation of the TPR domain of Bubs, abolishes the generation of SAC signals, suggesting that Blinkin has a role both in microtubule attachment and SAC signaling (Kiyomitsu et al., 2007). The SAC proteins Bub1, BubR1, Bub3, and Mps1 were themselves involved in the regulation of microtubule attachment, beside their role in SAC signaling (Logarinho & Bousbaa, 2008).

The highly conserved serine/threonine Aurora B kinase provides the main link between the error correction machinery and SAC. By destabilizing tensionless kinetochore-microtubule attachments, Aurora B creates unattached kinetochores that can be filled with checkpoint proteins to generate SAC signals (Burke & Stukenberg, 2008). This in turn allows time for error correction and bi-orientation. The direct involvement of Aurora B activity in SAC control is suggested by its requirement for SAC protein recruitment to unattached kinetochores (tensionless) artificially created in cells treated with the microtubule-depolymerizing drug nocodazole (Kallio et al., 2002). Moreover, recent studies strongly suggest that Aurora B directly contributes to SAC signaling independently of its error correction activity (Santaguida et al., 2011).

5. Conclusion

Accurate chromosome segregation during mitosis relies on the activities of the kinetochore. Here, we highlighted the event of mitosis and focused on the structure and functions of kinetochore in chromosome attachment, chromosome movement, error correction, and the generation of inhibitory signals that prevent anaphase in the presence of attachment errors.

The basic mechanisms of these kinetochore functions, their interplays, and regulatory pathways remain under investigation. Elucidating these mechanisms is crucial for future progress and is relevant to cancer aetiology and therapy. Indeed, failure in any of these kinetochore functions can lead to chromosome missegregation, with chromosome losses and gains, which may contributes to the aneuploidy phenotype that characterizes many cancers (Kops et al., 2005; Thompson et al., 2010).

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13

Molecular and Sub-Cellular Gametogenic Machinery of Stem and Germline Cells Across Metazoa

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1. Introduction

Metazoan life cycle and development include two main types of stem cells: the germline cells and the somatic stem cell lineages (Hogan, 2001; Rinkevich, 2009; Srouji & Extavour, 2011). In animals with asexual reproduction, the germ lineage is not segregated during embryogenesis, and the line of pluripotent stem cells is maintained continuously throughout the life of an individual or a colony, being predecessors of germ cells and a wide spectrum of somatic cells (Buss, 1987; Isaeva, 2011; Rinkevich, 2009; Sköld et al., 2009). Examples of such pluripotent gametogenic stem cells include sponge archaeocytes, cnidarian interstitial cells, planarian neoblasts, ascidian hemoblasts and stem cells of colonial rhizocephalans (reviews: Isaeva et al., 2008, 2009; Rinkevich et al., 2009; Sköld et al., 2009; Srouji & Extavour, 2011). In addition to the germline segregation by preformation, or mosaic developmental mode and epigenesis, or regulative mode (Extavour, 2008; Extavour & Akam, 2003; Gustafson & Wessel, 2010), somatic embryogenesis was also recognized (Buss, 1987; Blackstone & Jasker, 2003; Gustafson & Wessel, 2010; Rinckevich et al., 2009; Rosner et al., 2009). Earlier, somatic embryogenesis as natural cloning in animals was termed blastogenesis (Berrill, 1961; Ivanova-Kazas, 1996). In the life cycle of colonial animals, one generation of oozooid (an individual that has developed from an egg) alternates with numerous generations of blastozooids, with alternating morphogenetic processes: embryogenesis and blastogenesis (Ivanova-Kazas, 1996). Many animals, including placozoans, sponges, cnidarians, platyhelminths, nemerteans, entoproctans, ectoproctans, annelids, hemichordates and urochordates are capable of somatic embryogenesis (Buss 1987; Blackstone & Jasker, 2003; Gustafson & Wessel, 2010; Rosner et al., 2009). Among arthropods, many parasitic rhizocephalan crustaceans (Rhizocephala: Cirripedia: Crustacea) have asexual reproduction, somatic embryogenesis by budding without separation of blastozooids resulting in the emergence of colonial organization

(Høeg & Lützen, 1993, 1995; Høeg et al., 2005). We have found undifferentiated stem cells in stolons, buds and ovary rudiments of the colonial rhizocephalans *Polyascus polygenea* (Isaeva et al., 2001, 2004; Shukalyuk, 2002; Shukalyuk et al., 2005, 2007) and *Peltogasterella gracilis* (Isaeva et al., 2003; Shukalyuk et al., 2001, 2005). The rhizocephalan stem cells take part in the morphogenesis of the earliest buds, and later migrate to the developing ovary as primary germ cells. So, pluripotent gametogenic stem cells are a cellular source in the realization of reproductive strategy including both sexual and asexual reproduction in colonial rhizocephalan.

All stem cells are characterized by two common properties that extend across diverse taxa: first, the capacity for self-renewal, the ability to propagate without loss of stemness property; second, the ability to give rise to numerous progeny that are fated for further differentiation into specialized cells (Alié et al., 2011; Cox et al., 1998; Srouji & Extavour, 2011; Watanabe et al. 2009). Depending on the breadth of the potential range of cell differentiation, totipotent, pluripotent, multipotent, oligopotent, and unipotent stem cells are distinguished, but this terminology is not unified (see Isaeva, 2010; 2011).

Female germline cells can be qualified as unipotent, since they produce only one type of differentiated cells, and totipotent, taking into account their potential of developing into a whole organism. There is no doubt that differentiated and deeply specialized gametes are unipotent cells producing only oocytes or sperm under specific signaling control of their niche. However, the progenitors of germline cells are multipotent or even pluripotent, also capable of differentiating into somatic lineages *in vitro* or *in vivo,* causing various germline-based embryonic tumorogenesis.

Stem cells of invertebrates with asexual reproduction are capable of differentiation into both germline and all, most or many somatic cell types are traditionally referred to as totipotent, pluripotent or multipotent. We consider here these cells as gametogenic pluripotent stem cells. In asexually reproducing invertebrates no early segregation of the germ cell lineage is observed. The lineage of pluripotent, traditionally referred to as totipotent, stem cells ensures both sexual and asexual reproduction over the entire life span of an individual or a colony. These pluripotent stem cells can differentiate into gametes and somatic cells in adult organisms. We studied pluripotent gametogenic stem cells in asexually reproducing representatives of five animal types: archaeocytes in the sponge (Porifera) *Oscarella malakhovi* (Isaeva & Akhmadieva, 2011), interstitial cells in the colonial hydroids (Cnidaria) *Obelia longissima* and *Ectopleura crocea* (Isaeva et al., 2011), neoblasts in the planarian (Platyhelmintes) *Girardia tigrina* (Isaeva et al., 2005), stem cells in the colonial rhizocephalans (Arthropoda) *Peltogasterella gracilis* and *Polyascus polygenea* (Isaeva et al., 2003, 2004; Shukalyuk et al., 2005, 2007), hemoblasts, stem cells in the colonial ascidian (Chordata) *Botryllus tuberatus* (Akhmadieva et al., 2007), and also embryonic stem cells as a benchmark for pluripotency, using *in vitro* culture, electron microscopic, histological, histochemical and molecular methods.

Mammalian embryonic stem cells (ESCs) are considered as a standard cell culture model for studying pluripotency (Do & Schöler, 2009). In our studies, as well as in the present review, we compare our data on invertebrate pluripotent stem cells with the information on the molecular signature of pluripotent stem cells of various animals, including mouse ESCs (Isaeva et al., 2003; Shukalyuk, 2009; Shukalyuk et al., 2005, 2011; Shukalyuk & Stanford,

2008), taking into consideration that mammalian ESCs *in vitro* are in some sense artifacts of tissue culture (Shostak, 2006; Zwaka & Thomson, 2005).

In this review, we reveal some common principles in the sub-cellular and molecular machinery maintaining pluripotency and gametogenic potentiality. We hypothesize that evolutionary conserved molecular mechanisms underlie pluripotency, including gametogenic potentiality in germline, embryonic stem cells and other pluripotent stem cells of different metazoans.

2. The molecular and sub-cellular machinery of stem cell specifications

In all multicellular organisms, a stem cell system serves as a crucial cellular source during embryonic development building *de novo* the entire organism and during adulthood regenerating all types of cells and tissues of the individual. In colonial organisms with asexual reproduction in their life cycle, stem cells can be toti/pluripotent, producing not only all somatic types but germline as well. One of the critical features of the stem cell is self-renewal. Stem cells can divide indefinitely without losing their potent capacity and ability to differentiate. Typical stem cell morphology is characterized by a relatively organelle-free cytoplasm, large rounded nucleus and large prominent nucleolus (nucleoli) and diffuse euchromatin, presumably capable of genome-wide active transcription. They have also a significant proportion of inactive heterochromatin, which is silenced by histone methylation or siRNAs and appear as a compact electron-dense material at the ultrastructural level. Also, based on the chromatin organization state, stem cells commonly have the nuclear-to-cytoplasmic ratio shifted toward the nucleus of the cell. These morphological characteristics of stem cells are applicable for the germline cells as well. However, when germcells are specified they will stop actively proliferating until they reach the rudiment of the gonads. It is belived that germline cells will keep their toti-/pluripotent properties and self-renewal capacity while continuing to migrate and differentiate to gametes. Remarkable properties of germline cells, underlining their morpho-functional similarities and differences with other toti-/pluripotent cells, are our main focus in this section.

2.1 Germline cells

Germline cells across Metazoa are specialized cells which are usually specified in very early embryonic development, preserving their capacity to carry out important information about the entire organism, passing it down to the next generation. During preformation, maternally inherited factors of the egg cell, localized in specific areas of the cleaving zygote, leave a specific imprint in the blastomeres, which specialize into germline progenitors. It is believed that epigenesis gives an advantage for the multicellular organism in adaptation and species survival because it does not rely on the quality of a few blastomeres. Instead, during epiginesis, early blastomeres have equal developmental potential and have similar capacity contributing to the developing organism and germline specification (Fig. 1).

Germline cells can be indentified by their specific morpho-genetic signature. According to Extavour (Extavour, 2008; Extavour & Akam 2003), undifferentiated germ cells can be distinguished from somatic cells by several criteria, in addition to typical stem cell characteristics described above. The morphological features are mainly "default" characteristics of the undifferentiated state (Alié et al., 2011). An exception is the key

organelles of germline cells referred to as germ (germinal) granules or nuage. They are considered to be a germline hallmark across the animal kingdom (Brown& Swalla, 2007; Eddy et al., 1975; Ikenishi, 1998; Lim & Kai, 2007; Mahowald, 2001; Matova & Cooley, 2001). Evolutionary conserved germ-cell-specific gene expression marks germline cells distinguishing them from somatic cells in all studied metazoans (Ewen-Camden et al., 2010; Extavour, 2008; Leatherman, Jongens, 2003; Matova & Cooley, 2001; Seydoux & Braun, 2006; Sroji & Extavour, 2011).

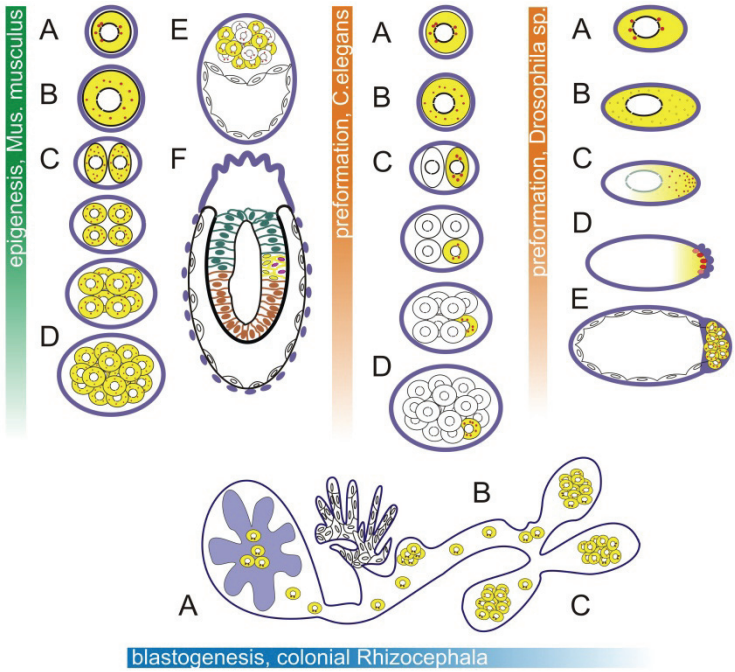

Fig. 1. Germline specification in Metazoa: epigenesis in mouse; preformation in *C. elegans* and *Drosophila* and blastogenesis in colonial Rhizocephala. Epigenesis and preformation: oocyte (A) with distinguished germ granules/germ plasm (red) by the nucleus; specific macromolecular complexes of cytoplasm (yellow), including proteins and transcripts of germline marker genes; mature egg or zygote (B), the germline granules presumably distribute evenly in the cell mass, in *Drosophila* the distinguished granules did not form yet; cleaving zygote (C—D), 2- to 16-cell blastomeres, maternal cytoplasm is distributing equally in mice and not equally in *C. elegans*, where only one blastomere inherited germ granules and the specific cytoplasmic factors, polarized cytoplasm in *Drosophila*, germ granules are located at the posterior pole; (E) in mouse, the group of pluripotent cells form the inner cell mass of the blastocyst, in *Drosophila*, germline cells specified by polarized germ granules; (F) germline specification occurs inside epiblast during mesoderm formation. Blastogenesis: in colonial Rhizocephala, gametogenic stem cells (SC, yellow) within stolon migrate to the rudiment of the gonad of the developing blastozooid (A), forming a germline lineage (blue colour, differentiating somatic tissues), totipotent SCs of the tubular-like stolon (B) forming buds (C).

2.1.1 Morphological evidence of "nuage"/germ granules/chromatoid bodies

"Germ plasm" (Keimplasma), Weismann's famous term, originally denoted the nuclear genetic material (Weismann, 1982, 1893) now is understood metaphorically as cytoplasmic compartment containing specific ultrastructural marker and a key organelle of metazoan germline cells (Amikura et al., 2001; Ikenishi, 1998; Lim & Kai, 2007; Matova & Cooley, 2001; Seydoux & Braun, 2006). Perinuclear germinal granules are almost universal specific organelles of germ cells. The ultrastructure of these organelles is similar, but they can be represented in cells of different organisms as either granules (bodies) or as a cloud (nuage) of fine-dispersed material. There are various terms for the specific electron-dense material: germ plasm granules, nuage, germ cell granules, polar, perinuclear, chromatoid, germinal, germ granules (bodies), dense bodies, etc. (Eddy, 1975; Flemr et al., 2010; Ikenishi, 1998; Isaeva, 2010, 2011; Mahowald, 2001; Seydoux & Braun, 2006; Lim & Kai, 2007). Despite the various terms for the germline granule in different model species, several common features are found (Fig. 2). It seems that the morphological appearance of the structure is linked to stage-specific function and macro-molecular composition, which will be discussed later in this review.

Nuage/germ granules/chromatoid bodies are morphologically and ultrastructurally identifiable and similar to each other. They are discrete, electron-dense organelles, composed of fibrillar and granular material, not bounded by a limiting membrane, often located in the perinuclear cytoplasm and usually associated with clusters of mitochondria. These bodies are found in germ cells in many stages of development, ranging from primordial germ cells in embryos to gametes in adult gonads. The observations suggest that germ granules/nuage/ chromatoid bodies represent different forms of the same material over time (Eddy, 1975; Ikenishi, 1998; Kloc et al., 2004; Mahowald, 2001; Parvinen, 2005). Germ granules have been called by a variety of names reflecting their different morphology at different developmental stages and in different organisms, for example, P granules in *Caenorhabditis elegans*, polar granules in primordial germ cells and nuage during later development in *Drosophila* and other insects, Balbiani body and germinal granules in *Xenopus*, chromatoid body in mammalian male germ cells (Eddy, 1975; Extavour, 2008; Kloc et al., 2004; Seydoux & Braun, 2006). Large complexes including other organelles as in *Xenopus* oocytes were called Balbiani body, mitochondrial cloud, intermitochondrial cement, yolk nucleus etc: the old nomenclature is "confusing and chaotic" (Kloc et al., 2004). The Balbiani body was also observed in oocytes of mouse (Pepling et al., 2007). Seydoux & Braun (2006) used the generic term "germ granules" to refer to all these structures considered as hubs for posttranscriptional regulation of gene expression. While the various names of these structures correspond to differences in morphology, composition, and animals in which they were first identified, it is believed that they are related entities (Eddy, 1975; Gustafson & Wessel, 2009; Parvinen, 2005). The exact relationship between all of these differently named structures has not been determined, but it is possible that they are all different morphological manifestations of the same germ line-specific body (Extavour & Akam, 2003). The chromatoid body of mammalian spermatocytes and spermatids is also suggested to be a mammalian counterpart of nuage on the basis of its structural features and protein composition (Parvinen, 2005; Pepling et al., 2007). Accumulating evidence indicates that the chromatoid body is involved in RNA storing and metabolism, being related to the RNA processing body (P-body: see below) of somatic cells (Kotaja et al., 2006; Nagamori et al., 2011). Here we will refer to these structures as germ plasm related bodies (GPRBs).

It was proposed that the determination of primordial germ cell fate in mammals is independent of germline-specific granules and occurs through an inductive process. Flemr and colleagues (2010) described the dynamics of the maternal stable untranslated transcripts (dormant maternal mRNAs) as components of P-bodies in mouse oocytes and reported that oocyte growth is accompanied by loss of P-bodies and a subcortical accumulation of several RNA-binding proteins, forming transient RNA-containing aggregates. The authors proposed that the cortex of growing oocytes contains a novel type of RNA granule related to P-bodies. Although early mouse oocytes contain granulo-fibrillar material reminiscent of germ cell granules in association with transiently appearing Balbiani bodies, later oocytes lack detectable germ granules (Flemr et al., 2010). Other authors argued that true P-bodies were not observed until the blastocyst stage of embryogenesis, providing evidence that mouse oocytes develop using molecular and developmental mechanisms widely conserved throughout the animal kingdom (Pepling, 2010; Pepling et al., 2007). Hubbard and Pera (2003) reasoned that basic germ-plasm machinery exists in mammalian germ cells as sub-microscopic complexes. In many organisms, GPRBs associate with nuclear pores (Eddy, 1975; Seydoux & Braun, 2006; Snee & Macdonald, 2004). The nuage is visible traversing the nuclear pores, so there is high probability that all or some of the nuage components originate in the nucleus or shuttle between the nucleus and nuage (Kloc et al., 2004). Continuity in electron-dense material between the nucleus and the chromatoid body through nuclear pore complexes has also been observed in male germ cells (Parvinen, 2005; Updike et al., 2011). Polysomes have been reported adjacent to nuage in *Drosophila* (Mahowald, 2001), and chromatoid bodies in rats (Parvinen, 2005). Perinuclear nuage clusters have remarkably dynamic composition, despite their relatively fixed positions around the nucleus (Snee & Macdonald, 2004). In the *Xenopus* oocyte and cleaving embryo, the germinal granules undergo constant transformation in size, number, and ultrastructure. Although the structure and behavior of germ line-specific structures show extraordinary variability, there are also striking similarities and common themes even among evolutionarily distant organisms (Kloc et al., 2004).

Thus, the presence of GPRBs with their specific organization and localization in the cell is an evolutionary conserved feature of metazoan germline cells. GPRBs have been found in more than 80 species of 8 animal types (Eddy, 1975). At least one new additional metazoan type can be added to Eddy's list – Porifera, because electron-dense bodies sometimes described as "nuclear extrusion" or "chromidia" were observed in oogonia and oocytes of several sponge species (see Harrison & De Vos, 1991; Isaeva & Akhmadieva, 2011). The germ granules, "the work horses" of germ cells, are thought to function as a specific cytoplasmic regulatory center, maintaining the genomic totipotency, preventing the expression of somatic differentiation genes, and protecting germline cells from somatic fate (Chuma et al 2006; Cinalli et al 2008; Extavour, 2008; Seydoux & Braun, 2006; Srouji & Extavour, 2011; Strome & Lehman, 2007), preventing somatic fate "by default" (Leatherman & Jongens, 2003).

2.1.2 Molecular signature

Germ granules and nuage contain products of marker germline genes, which are recognized as molecular signature of germline cells. GPRB's components include proteins, mRNAs, and noncoding RNAs. RNA-binding proteins in germinal granules are involved in mRNA localization, protection, and translation control. The molecular machinery and molecular

signature of germline cell specification includes a set of evolutionary conserved proteins such as Vasa, Piwi/Aubergine, Nanos, Tudor, Pumilio, Staufen and some others whose homologues have been identified in all metazoans studied (Extavour & Akam, 2003; Leatherman & Jongens, 2003; Kloc et al., 2004; Parvinen, 2005; Chuma et al., 2006; Pepling et al., 2007; Lim & Kai, 2007; Extavour, 2008; Gustafson & Wessel, 2010; Flemr et al., 2010; Srouji & Extavour, 2011). It was shown that some proteins of the germinal granules determine germ cell fate, and their genes are evolutionary conservative in all studied metazoans (Ikenishi, 1998; Matova & Cooley, 2001; Mochizuki et al., 2001; Seydoux & Braun, 2006; Srouji & Extavour, 2011). Every known nuage component has a role in one or more types of posttranscriptional control of gene expression; the presence of shared components reinforces the notion that nuage and polar granules are closely related structures (Snee & Macdonald, 2004). Genes related to *vasa* (*vas*) and other genes of the *DEAD* family (Raz, 2000; Shukalyuk et al., 2007) and to *piwi/argonaute* family (Funayama al., 2010) were found in a diverse range of eukaryotes from yeast to plants and animals; molecular and functional similarities of these genes were found across the kingdoms (Mochizuki et al., 2001; Watanabe et al. 2009). Products of the *vasa*- and *piwi*-related genes are the most widely used molecular germline markers for Metazoa (Extavour & Akam, 2003; Ewen-Camden et al., 2010; Gustafson & Wessel, 2010; Alié et al., 2011)

2.1.2.1 Vasa, DEAD box family

vasa protein of the *Drosophila* (or its homologues), germline-specific RNA helicase is a key determinant of the fate of germline cells found in GPRBs of germline cells across animal kingdom (Alié et al., 2011; Cinalli et al., 2008; Extavour & Akam, 2003; Ewen-Camden et al., 2010; Gustafson & Wessel, 2010; Shibata et al., 1999, 2010; Sroji & Extavour, 2011; Sunanaga et al., 2006). Products of *vasa*-related genes are necessary for the formation and maintenance of the structural organization of GPRBs and, presumably, for the maintenance of pluri/totipotency of cells. Vasa and Pl10 are members of the DEAD-box family of RNA helicases, proteins known to function in all eukaryotes, from yeast up to plants and animals, in wide aspects of RNA metabolism, including unwinding double-stranded RNAs and controlling their export, splicing, editing, stability, and degradation. They are involved in ribosome biogenesis, translation initiation, and mediating both RNA–RNA and RNA–protein interactions, promoting expression of other germline genes (Cinalli et al., 2008; Gustafson & Wessel, 2010).

The Vasa-like protein and a set of RNA-binding proteins, as well as other translational regulators are common and invariable components of GPRBs in many organisms. The presence of Vasa-like proteins in the germ plasm of different animals indicates the conservation of molecular mechanisms underlying the formation and maintenance of the germ plasm across Metazoa (Extavour, 2008; Ewen-Campen et al. 2010; Gustafson & Wessel, 2010; Juliano et al., 2010; Kloc et al., 2004).

2.1.2.2 Piwi, Piwi/Argonaute family

Piwi/Argonaute family members serve as epigenetic regulators of stem cells in many systems. Piwi/Ago proteins are an animal germline-specific subclass, highly conserved across eukaryotes, specifically expressed in germ cells and playing a key role in germ cell maintenance and self-renewal, transposon silencing, and RNA silencing. These proteins are

at the core of RNA-silencing machinery that uses small RNA molecules as guides to identify homologous sequences in RNA or DNA. The small RNAs regulate genes at the transcriptional or post transcriptional level affecting either chromatin structure or mRNA stability and mediating transcriptional gene silencing in germline maintenance (see Gustafson, Wessel, 2010; Peters, Meister, 2007; Thomson, Lin, 2009; Watanabe et al. 2009; Sroji & Extavour, 2011). Particularly, chromatoid bodies in male germ cells seem to operate as "intracellular nerve centers" of the microRNA pathway and function as subcellular concentration sites for components of the miRNA pathway, centralizing the miRNA posttranscriptional control system in the cytoplasm of haploid male germ cells (Kotaja et al., 2006; Nagamori et al., 2011). There are important interactions between Piwi and Vasa in the germline. PIWI-mediated microRNA pathways are evolutionarily conserved control mechanisms, found in bacteria, archaea and eukaryotes and are essential for stem cell division in both animal and plant kingdoms (see Ewen-Camden et al., 2010; Funayama et al., 2010; Watanabe et al., 2009).

2.1.2.3 Tudor-domain contained proteins

tudor (*tud*) gene products of *D. melanogaster* are key components of polar granules and nuage (see Anne, 2010; Arkov et al., 2006; Chuma et al 2006). Tudor motifs are found in many metazoan organisms and have been indentified to play a role in protein-protein interactions in which methylated protein substrates bind to these domains. Tudor protein interacts *in vitro* with Valois, which is a component of the methylosome in *Drosophila* (Mahowald, 2001; Anne, 2010). It also was shown to play a role in barrel-like folding, which creates the ability to bind and to recognize methylated histone H3-K4 and H4-K20 for a double Tudor-domain protein in human (Huang et al, 2006). The Tudor domain of the SMN (survival motor neuron) protein binds directly to spliceosomal SM proteins during spliceosome assembly (Selenko et al., 2001).

Thus, germline cells are relatively transcriptionally quiescent during most of embryonic development. Moreover, germ cells are typically mitotically quiescent from the time of their specification during embryogenesis, until the time that gametogenesis begins, usually during larval or adult life (Extavour, 2008). The transfer of most of the control of gene expression to the cytoplasm is an important evolutionary conservative acquisition ensuring plasticity for the germ cell genome (Seydoux & Braun, 2006).

2.2 Pluri/multipotent gametogenic stem cells of asexually reproducing animals

2.2.1 Germ granules/chromatoid bodies

In asexually reproducing invertebrates, stem cells capable of differentiating into germ and somatic cells can be identified by the presence of specific electron-dense cytoplasmic structures, morphologically similar or identical to germinal granules of germline cells (referring to GPRBs). In stem cells of asexually reproducing invertebrates, the germinal granules were revealed before our work in stem cells of cnidarians and flatworms. For example, the electron-dense bodies similar or identical to GPRBs of germline cells were found in interstitial cells of the hydra *Pelmatohydra robusta*. Bodies were associated with nuclear pores and mitochondria. The number and size of such dense bodies increased during early oogenesis and decreased during differentiation of somatic cells (cnidoblasts) from interstitial cells (Noda & Kanai, 1977). Studying many species across Metazoa, we also found significant similarity in the morphology of electron-dense bodies or granules (Fig. 2).

In planarians, electron-dense GPRBs were observed not only in germline cells but also in neoblasts. The chromatoid bodies in planarian neoblasts and germ cells are found near the nuclear envelope, in close proximity to mitochondria as well (Coward, 1974; Hori, 1982; Isaeva et al., 2005; Shibata et al., 1999). Nuage-like structures morphologically different from planarian chromatoid bodies were found in the flatworm *Macrostomum lignano* (Pfister et al., 2008). The chromatoid bodies in planarian neoblasts decrease in number and size during differentiation of somatic cells from neoblasts and disappear in completely differentiated cells, while in oogenic cells the chromatoid bodies were found during the entire life cycle (Hori, 1982; Shibata et al., 1999). These observations suggest that the chromatoid bodies are concerned with the cell totipotency maintenance (Shibata et al., 1999).

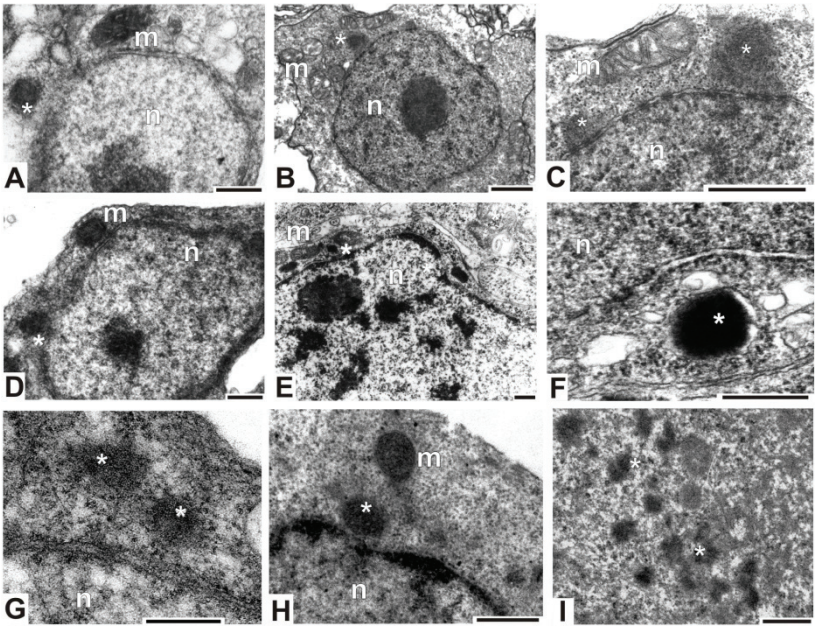

Fig. 2. Common morphofunctional feature of stem cells across Metazoa: transmition electron microscopic level of germ granules/nuage organization. Electron-dense granules (*) are usually localized near nucleus (n) pore and surrounded by mitochondira (m) in sponge *Oscarella malakhovi* (A, D and G), in planarian *Girardia tigrina* (E), in hydroids *Ectopleura crocea* (B and C) and *Obelia longissima* (F), as well as in mouse embryonic stem cells in culture (H, embryoid body, day 1) and in stem cells of inner cell mass of the mouse blastocyst (I). A, D and G, after Isaeva & Akhmadieva, 2011; E, after Isaeva et al., 2005; F, after Isaeva et al., 2011; H and I, Shukalyuk et al., unbubl. Scale bar is 0.1 μm.

The morpho-functional organization of pluripotent gametogenic stem and gonial cells in studied representatives of diverse metazoan phyla shares with germ and stem cells common properties as described above. Particularly, in the cytoplasm of archaeocytes in the sponge *Oscarella malakhovi* we have found germinal granules of a typical morphology located near the nuclear envelope and surrounded with polysomes (Isaeva & Akhmadieva, 2011). Electron-dense GPRBs were found earlier in the oogonia and oocytes of different sponges

but have not been previously described in the archaeocytes or any other cells of sponges. We revealed electron-dense GPRBs in interstitial cells of the colonial hydroids *Obelia longissima* and *Ectopleura crocea* (Isaeva et al., 2011), similar to "dense bodies" of interstitial and germ cells in *Pelmatohydra robusta* (Noda & Kanai, 1977) and cnidarian oocytes. The GPRBs surrounded by mitochondria and in contact with nuclear pores have been found near the nuclear envelope in neoblasts and gonial cells of the planarian *Girardia tigrina* (Isaeva et al., 2005). We revealed typical GPRBs in the cytoplasm of embryonic stem cells and stem cells of the colonial rhizocephalans, *Peltogasterella gracilis* and *Polyascus polygenea* (Shukalyuk et al., 2005, 2007, 2011). In the cytoplasm of some stem cells in the early buds of colonial ascidian *Botryllus tuberatus* we have found small electron-dense bodies (Akhmadieva et al., 2007), similar to disperse material of nuage, often found in vertebrates. Perinuclear electron-dense germinal granules often associate with the nuclear pore membrane and bear signs of mitochondrial origin, in particular, cristae of the inner mitochondrial membrane.

Pluripotent or multipotent gametogenic stem cells in all studied asexually reproducing animals belong to 5 animal types: Porifera, Cnidaria, Platyhelminthes, Arthropoda, and Chordata. They all feature the presence of the germinal granules similarly to germline cells. Evidently, the electron-dense germ granules are ultrastructural markers and key organelles both of metazoan germline and potentially gametogenic pluripotent stem cells of asexually reproducing invertebrates.

2.2.2 Molecular signature in pluripotent gametogenic stem cells

In asexually reproducing animals, both germ and pluripotent stem cells express evolutionary conserved germ cell markers such as products of genes related to *vasa/pl10, piwi/argonaute, nanos, tudor* as well as high activity of alkaline phosphatase (AP, Fig. 3) and telomerase (Extavour, 2008; Ewen-Camden et al., 2010; Funayama et al., 2010; Gustafson & Wessel, 2010; Isaeva, 2010, 2011; Mochizuki et al., 2001; Shukalyuk et al., 2005, 2007; Sroji, Extavour, 2011). Specifically, *vasa*-related gene expression is characteristic not only of germline cells, but also pluripotent gametogenic stem cells involved in their determination and maintenance. Vasa expression as well as a high activity of AP and telomerase became the classic selective markers of these stem cells (see Isaeva, 2011; Mochizuki et al., 2001; Rinkevich et al., 2009; Shibata et al., 1999; Shukalyuk et al., 2007; Sköld et al., 2009; Sroji & Extavour, 20110). In many of the invertebrates, such as cnidarians, acoels, planarians, annelids and colonial urochordates, expression of Piwi and Vasa are not restricted to the germline but are expressed in multipotent stem cells (Alié et al., 2011). Piwi is considered to be an omnipresent stemness flag for self-renewal and maintenance of germ line and stem cells in diverse multicellular organisms (Rosner et al., 2009). Several studies indicate a functional relationship between Vasa and both the small interfering RNA and micro-RNA processing pathways (Gustafson & Wessel, 2010). In sponges, cnidarians, flatworms, and colonial botryllid ascidians, germ cells derive from adult pluripotent stem cells (Agata et al., 2006; Extavour, 2008; Isaeva, 2010; 2011; Rinkevich et al., 2009; Sköld et al., 2009; Srouji & Extavour, 2011). Gametogenic potentiality was observed also for stem cells in colonial rhizocephalan crustaceans *P. polygenea* and *P. gracilis*; the stem cells migrated into the developing ovary becoming oogonial cells (Isaeva et al., 2004; Shukalyuk et al., 2005).

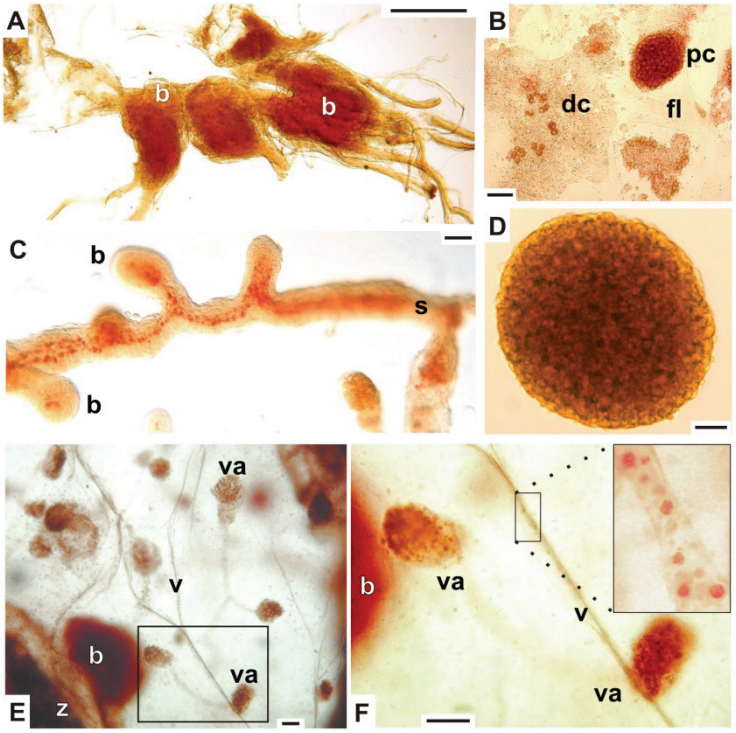

Fig. 3. Common histochemical feature of stem cells across Metazoa: selective expression of alkaline phosphatase (AP): A, buds (b) within a colony of *Peltogaster reticulatus* (Rhizocaphala); B, high AP-reaction in pluripotent *Mus musculus* ESCs and low expression in their differentiated colonies (dc) or fibroblasts (fl); C, AP-positive stem cells inside stolon (s) and buds (b) of *Peltogasterella gracilis* (Rhizocephala); D, AP reaction in neurosphere of *Mus musculus* ESCs in culture; E and F (selected E), AP-positive hemoblasts of *Botryllus tuberatus* inside zooid (z), bud (b), vessel (v) with AP-positive hemoblasts (enlarge in F), and vascular ampulla (va) during vascular budding. A, B & D, Shulalyuk, unpubl.; C, after Shukalyuk et al., 2005; D, after Akhmadieva et al., 2007. Scale bar is 20 μm (B, D—F) & 50 μm (A, C).

In the freshwater sponge Ephydatia fluviatilis (Porifera), expression of *piwi* orthologues was found in sponge archaeocytes and choanocytes, sponge pluripotent gametogenic stem cells (Funayama, 2008; Funayama et al., 2010). The expression of *nanos*-, *vasa*- and *PL10*-related genes (Mochizuki et al., 2000, 2001) was demonstrated for the adult interstitial and germline cells for the hydrozoan *Hydra magnipapillata* (**Cnidaria**); *piwi*-like expression was found in germline and stem cells of the jellyfish *Podocoryne carnea* (Seipel et al., 2004). *vasa*-like gene expresses in interstitial stem cells of *Hydractinia echinata* (Rebscher et al., 2008). Piwi/Ago, Pumilio, PCNA were revealed in the hydrozoan *Hydra magnipapillata* and the anthozoan *Nematostella vectensis*, whereas orthologues of Oct4 and Nanog were not found (Watanabe et al., 2009). In the acoel *Isodiametra pulchra* (**Acoelomorpha**) expression of *piwi* orthologue was shown in germ cells and neoblasts (De Mulder et al., 2009).

Planarian (**Plathyhelminthes**) neoblasts can differentiate into germ and somatic cells and express *vasa*, *piwi*, *nanos*, *pumilio, bruno, tudor* homologues (Agata et al., 2006; Pfister et al., 2008; Reddien et al., 2005; Rossi et al., 2007; Shibata et al., 1999, 2010; Solana et al., 2009). In *Dugesia japonica* two *vasa* homologues are expressed in the germ cells of the adult gonads. Only one of these homologues was expressed in neoblasts (Shibata et al. 1999). Flatworm *vasa* homologue of *Macrostomum lignano* was expressed in germ and stem cells (Pfister et al., 2008). Planarian homologues of *piwi* and *pumilio* genes were found specifically expressed in a neoblasts (Rossi et al., 2007) and the expression pattern of Piwi protein in planaria *Schmidtea mediterranea* coincides with the neoblasts (Reddien et al., 2005). The Tudor protein is a component of chromatoid bodies in germ cells and neoblasts in the planaria *Schmidtea polychroa* (Solana et al., 2009). Co-localization and co-expression of Piwi- and Tudor-related proteins also was detected in planarian neoblasts (see Shibata et al, 2010). In planarians, a high dose of irradiation significantly down-regulates neoblast's RNA metabolism, chromatin remodelling and transcription. However, a low dose of irradiation stimulates up-regulation of genes involved in signal transduction, cytoarchitecture organization, protein degradation, apoptosis, cell metabolism, intracellular trafficking and receptor/ligand activities (Rossi et al, 2007). Exposure to γ-irradiation demonstrates the presence of at least two irradiation-sensitive sub-populations of neoblasts in *Schmidtea mediterranea* (Eisenhoffer et al, 2008).

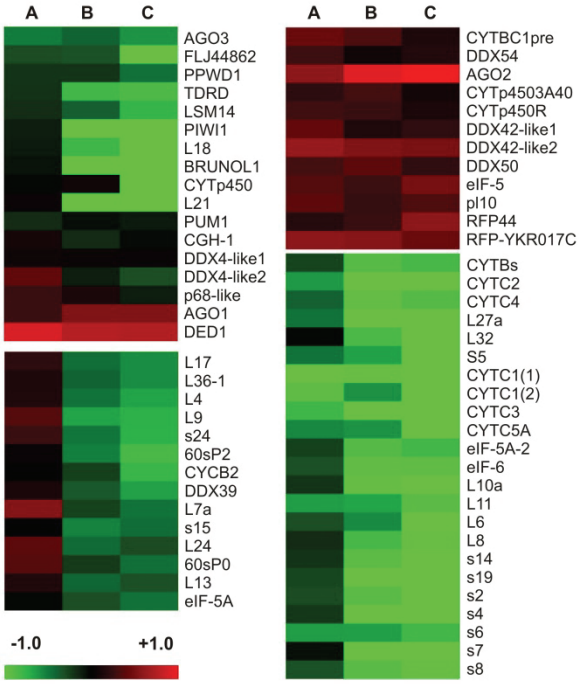

Fig. 4. Gene expression profile for neoblasts of planaria analyzed after irradiation at day 1 and day 7. Cluster analysis reveals set of genes which expression has changed slightly (right column) or dramatically (left column) from the level in wild type (A) to one day (B) or 7 days (C) post-irradiated animals. Heat map was generated using MultiExperiment Viewer v 4.3, emphasising gene expression in scale between -1.0 (bright green) and +1.0 (bright red).

A significant decrease of expression was observed in genes involved in translation, RNA processing, and chromatin transcription, synthesis and repair corresponding to the population of cells affected after 1 day or 7 days post-irradiation. However, the second neoblast population, presumably slower proliferating and more restricted, demonstrate significant down regulation of a specific genes only on day 7 post-irradiation, corresponding to the function of energy production, mitochondria maintenance, translation, metabolism and others. *piwi*- (smedwl-1) and *bruno*-like (brunol-1) genes were down-regulated in both days, marking a totipotent sub-population of neoblasts. Transcripts of mitochondrial carrier, cytochrome p450, ribosomal L21 and L18 proteins were specific to the 7-day irradiated neoblasts. We used publicly available MicroArray data (NCBI GEO #GSE11503; Eisenhoffer et al, 2008) to look at the expression profile of known components of germ plasm and nuage. In our analysis, we found it intriguing that as with Piwi1 and Brunol1 genes, expression of the tudor-like Tdrd gene was also down-regulated in both days post-irradiation (Fig. 4), as well as cytochrome C –family, L-family and S-family genes. However, high wild type expression of Ago2 up-regulated even more in both days post-irradiation, whereas Ddx42, Ddx50, and pl10 almost did not change in their expression (Fig. 4). In our opinion, mitochondrial cement might play an important role in gametogenic stem cell survival during specification by absorbing and utilizing some apoptotic factors that are released by mitochondria, such as cytochrome C, and typically found in the germline germ plasm functioning as stress relief granules. It is conceivable that Tudor, Piwi, and Bruno homologues form the core structure of the germ plasm and present all the time within the dynamic structure, playing an important role in self-renewal and stem cell maintenance.

Pluripotent stem cells of colonial parasitic rhizocephalan barnacles (Rhizocephala: Cirripedia: Crustacea: **Arthropoda**) are predecessors of somatic and germ cells, thereby ensuring the reproductive strategy with alternation of asexual and sexual reproduction. The earliest blastozooid primordia arise as epithelial buds of stolon-like structures filled with migrating stem cells; there is a cluster of undifferentiated stem cells within each bud; later stem cells migrate as germ cells into developing ovaries (Isaeva et al., 2001, 2003, 2004; Shulalyuk et al., 2005). Earlier we revealed the evolutionarily conserved sites of *vasa*- and *pl10*- related genes of the DEAD family, in DNA of the rhizocephalan crustaceans *Polyascus polygenea* and *Clistosaccus paguri* (Shukalyuk et al., 2007). Selective expression of RNA of the *vasa*- and *pl10*-related genes was observed in pluripotent stem cells, in oogenic and spermatogenic cells (Shukalyuk et al., 2007). We also found selectively high activity of AP histochemical marker in stem cells of *P. gracilis* along with expression of proliferating cell nuclear antigen (PCNA) in interna (Isaeva et al., 2003; Shukalyuk et al., 2005). Recently, we have shown for *P. gracilis* the presence of Piwi, Vasa and Nanog proteins in pluripotent stem cells, early blastozooids and early rudiments of the trophic system (Sukalyuk et al, 2011; Shukalyuk & Isaeva, unpubl. data).

Colonial ascidians (**Chordata**) can reproduce asexually, particularly, by palleal or vascular budding. In vascular budding of botryllid ascidians, pluripotent hemoblasts form buds generating a new individual. Hemoblasts are undifferentiated cells that can give rise to differentiated blood cells, somatic tissue cells of blastozooids during asexual reproduction, and evidently also to germline cells (see Rinkevich et al., 2009). *Vasa*-like gene expression was demonstrated in primary germline cells morphologically indistinguishable from hemoblasts in ascidian *Botryllus primigenus* (see Sunanaga et al., 2007). Brown and Swalla (2007) compared *vasa*-related gene expression in the solitary ascidian *Boltenia villosa* and the colonial ascidian *Botrylloides violaceus*. In *B. villosa*, mRNA of vasa-related gene was

expressed in germ cells whereas mRNA of *vasa*-related gene of the ascidian *B. violaceus* in mature colonies was expressed in germ cells, in some circulating in the blood cells, in differentiating buds and zooids. Gustafson & Wessel (2010) reported Vasa mRNA expression in germ lines along with hemoblast aggregates in *Botryllus primigenus* and *Polyandrocarpa misakiensis*. In the colonial *Botryllus schlosseri*, mRNA and the proteins of *vasa-*, *Pl10-*, *piwi-* and *Oct4-* orthologues are not expressed exclusively in germ cell lineages, but emerging *de novo* also in circulating hemoblasts, thus indicating somatic embryogenesis (Rosner et al., 2009). The results strongly suggest that germline hemoblasts are recruited from undifferentiated hemoblasts in budding tunicates (Rosner et al., 2009; Sunanaga et al., 2007). Data on marker gene expression in gametogenic stem cells of colonial ascidians and their interpretation are rather contradictory (see below).

Pluripotent stem cells in various invertebrates with asexual reproduction as well as cells of the germ lineage display the expression of conserved genes related to *vasa*, *piwi* and others which function in the specification and maintenance of both cell types across different metazoan phyla (Agata et al., 2006; Gustafson & Wessel, 2010; Juliano & Wessel, 2010; Rinkevich et al., 2009; Sköld et al., 2009; Srouji & Extavour, 2011; Wu et al., 2011). Besides the default characteristics of undifferentiated cells, these stem cells contain electron-dense perinuclear germ granules and express germline marker genes demonstrating that pluripotent stem cells display all of the morphological and functional features commonly used to identify germ cells.

2.3 Mammalian embryonic stem cells via germline connection

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of the developing embryo. During their adaptation for culture conditions these cells or some population of the originally extracted cells will gain some new properties and will become some sort of an artificial system. First, this adaptation includes the cell's ability to attach to the supporting surface: tissue culture treated plastic, extracellular matrix or feeder layer. Second, highly proliferating cells will be selected over time with self-renewing capacity. Third, cells that are selected within the culture will be responsive to the cell culture medium signaling, for example, from serum supplements and LIF (Leukemia Inhibitory Factor) for mouse ESCs or basic FGF (Fibroblasts Growth Factor) for human ESCs. However, it is commonly accepted that the ICM cells of the embryo are pluripotent cells equal in their properties to ESCs in culture, and can recapitulate normal embryonic development *in vitro* when placed under specific conditions.

2.3.1 Nuage/germ granules

The inner mass cells of mammalian embryos *in vivo* contain P-bodies (Pepling et al., 2007; Pepling, 2010), but we do not know any data in the literature on P-bodies, nuage or germ granules for embryonic stem cells cultured *in vitro*. As Clock and coauthors (2004) wrote, it will be interesting to see whether the embryonic stem cells in mice contain chromatoid body similar to that present in totipotent cells in planarians. We were the first to report morphological evidence for electron-dense germinal granules and more dispersed nuage material located near the nuclear membrane in the cytoplasm of mouse EMCs *in vitro* using confocal and light microscopy, with localization of mouse vasa homologue DDX4/MVH in perinuclear granules or nuage (Shukalyuk, 2009).

2.3.2 Molecular signature

In pluripotent mammalian ESCs, molecular signature and a core transcriptional regulatory network dedicated to establishment and preservation of pluripotency include a set of marker genes overlapping with gene signature of germline cells (Kim et al., 2008). The transcription factors Nanog, Oct4 and Sox2 are considered to be the core of the transcriptional network involved in pluripotency and commitment in human or mouse ESCs and have been recognized to be essential *in vivo* and *in vitro* for early development and coordinately regulating the epigenetic network supporting ES cell pluripotency (see Chambers et al., 2003; Do & Schöler, 2009; Jaenisch & Young, 2008; Kim et al., 2008; Rosner et al., 2009; Seydoux, Braun, 2006; Stice et al., 2006; Walker et al., 2007). Moreover, Oct4, Sox2 and Nanog can also indirectly regulate gene transcription by affecting chromatin structure, DNA methylation, microRNA and X chromosome inactivation, changes in local and higher order conformation of DNA, and RNA interference; so, Oct4, Sox2 and Nanog are involved in the cellular machinery, which has an important role in cell fate determination (Atkinson & Armstrong, 2008; Do & Schöler, 2009).

Nanog is a transcription factor, homeodomain protein found in mammalian pluripotent ES and developing germ cells, essential for mammalian embryogenesis. Nanog is thought to be a key factor underlying pluripotency in early development and ESCs, maintaining self-renewal of ESCs and developing germ cells. Nanog is considered a core element of the transcription network and regulatory circuits underlying pluripotency and reprogramming, a hallmark of pluripotent cells *in vivo* and *in vitro* (Do & Schöler, 2009; Stice et al., 2006; Jaenisch & Young, 2008; Kim et al., 2008; Watanabe et al. 2009). Both Nanog and Oct4 are not expressed in mammalian somatic stem cells and loss of Nanog is an early marker of differentiation (Do & Schöler, 2009).

Previously we reported (Shukalyuk & Stanford, 2008) as others have mentioned (Lacham-Kaplan, 2006) that some germline related genes are spontaneously expressed in mouse ESCs and reprogrammed mouse induced pluripotent stem cells (iPS) (Shukalyuk, 2009), even when cultured under the pluripotent and self-renewing condition maintained with LIF. We also found that the mouse homologues of Vasa (Ddx4), Stella, Dazl, Piwi (Miwi) and p68 (Ddx5) can be found in a surprisingly similar proportion of the cells in various ESC lines. We also observed reorganization of proteins and their accumulation in granules visible under confocal microscope after 72 hrs of initiating spontaneous differentiation by withdrawing LIF. We showed the co-localization of DDX4 protein with mitochondrial cytochrome C oxidase IV (COX IV) and single strand binding protein (SSPB1) in germ-like perinuclear granules of mouse ESCs. We also demonstrated that among others, Stau1 and Stau2, mRNA of *staufen*-related genes, were significantly enriched in ESC's RNA using immune-precipitation with anti-mDDX4 antibody (Shukalyuk, 2009).

Using a publicly available on-line micro array data set (NCBI GEO #GSE7506, Walker et al, 2007) we focused on the expression profile of germ-plasm related genes in mouse ESCs under pluripotent culture condition (LIF plus) and during spontaneous differentiation without LIF (LIF minus) or direct differentiation under retinoic acid treatment (RA plus). It is known that embryonic stem cells in culture are heterogeneous in their level of pluripotency marker expression.

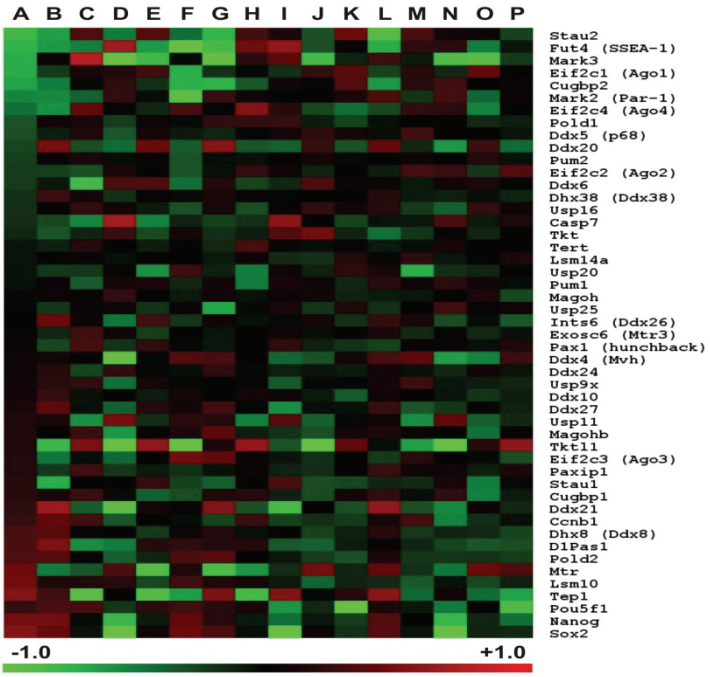

Fig. 5. Gene expression profile for OCT4-high (H), -medium (M) and -low (L) sub-populations of mouse ESCs differentiating over time under LIF- (5 days) or RA+ (2 days) conditions. Day0: A, LIF+(H); F, LIF+(M). Day1: B, LIF–(H); E, RA+(H); G, LIF-(M); J, RA+(M); L, LIF-(L); O, RA+(L). Day2: K, RA+(M), P, RA+(L). Day3: C, LIF-(M); H, LIF-(M), M, LIF-(L). Day5: D, LIF-(H); I, LIF-(M); N, LIF-(L). Heat map was generated using MultiExperiment Viewer v 4.3, emphasising the scale between -1.0 (bright green) and +1.0 (bright red).

In this particular data set, mouse ESCs were sorted based on their high, medium and low OCT4 (POU5F1) protein expression and each sub-population (high, medium and low) was differentiated under LIF-minus or RA-plus condition. As expected (Fig. 5), expression of Oct4, Sox2 and Nanog decreased overtime in each sub-population under both differentiating conditions. Expression of the Ddx4 gene, as compared to other DEAD-box contained genes (Ddx5, Ddx6, Ddx8, Ddx10, Ddx20, Ddx21, Ddx24, Ddx27), gradually increased, peaking on day 3, and dramatically decreased in each population by day 5 under LIFminus condition. An increase in Ddx4 expression was also observed for OCT4-low and OCT4-medium sub-populations of ESCs by day 2 under RAplus treatment (Fig. 5). Up-regulation of Ddx4 gene expression by day 3 during spontaneous differentiation is directly correlated with the same trend of Stau2, Eif2c4 (Ago4), Eif2c2 (Ago2), Eif2c1 (Ago1), Lsm14a and p68 (Ddx5) gene expression for all sub-populations but not for Stau1, Eif2c3 (Ago3) or Lsm10. Remarkably, significant down-regulation of germ-plasm members such as Ddx4, Stau2, Ago-family, p68 and Lsm14a oppositely correlates with Casp7 gene expression which is down-regulated at day 3 but significantly up-regulated by day 5 in all ESC populations (Fig. 5). A quick switch over time between levels of expression was indentified for mictotubule affinity-regulating kinase Mark3, as well as for telomerase associated Tep1 gene

and Tktl1, a known catalytic metal ion binding gene. These data are in line with existing *Drosophila* germ plasm composition studies revealing the presence, among transcriptional control regulators (Bruno, Nanos and Orb), of zinc ion binding and Ca^{2+} signaling element (Rangan et al., 2009). DDX4 is a DEAD-box family protein that, along with Oct4 and Nanog gene products, can express in the ICM, primordial germ cells (PGS) and ESCs (Chambers et al., 2003; Stice et al., 2006; Zwaka, Thomson, 2005). In undifferentiated mouse embryonic and induced pluripotent stem cells, we found expression of Ddx4/Vasa, Miwi/Piwi, Nanog and Oct4 (Shukalyuk, 2009). Futhermore, Oct4 expression appears to be crucial for the PGCs function and survival. Loss of Oct4 leads to the PGCs apoptosis in mammalians, instead promoting expected trophectodermal differentiation (Kehler et al., 2004).

Mammalian pluripoitent ESCs capable of differentiating into female and male germ cells *in vitro* are potentially gametogenic cells (Clark et al., 2004; Eguizabal et al., 2009; Mathews et al., 2009; Toyooka et al., 2003), along with invertebrate pluripotent stem cells. Because mammalian ESCs are capable of differentiating into germ cells, this suggests that these cells in mice contain all necessary components for the determination of germ cell fate and they are totipotent, despite their lack of differentiating towards extra-embryonic tissue. Observation of the common expression for protein and mRNA mammalian markers in both PGCs and ESCs has led to the hypothesis that embryonic stem cells are closely related, or even identical, to early germ cell precursors (Clark et al., 2004; Fox et al., 2007).

2.4 Evidence of *de novo* inducibility for germline markers

Generally, germline cells can be identified and retraced during development of an organism due to the availability of molecular markers. However, the molecular signature of pluripotent gametogenic stem cells becoming germline cells is not always and necessarily continuous during development and germline specification.

De novo epigenetical specification of hemoblasts into female germ cells was described in the colonial tunicates *Botryllus primigenus* and *Polyandrocarpa misakiensis* (Sunanaga et al., 2007; Rosner et al., 2009). In *B. primigenus*, *vasa* homologue expressing cells within the loose cell mass of the primary germline cells evidently arose from the *vasa*-negative cells at postembryonic stages. These results show that germ cell specification is inducible *de novo*. It has been suggested that germ cell formation in *B. primigenus* is a consequence of epigenetic induction during zooid differentiation. Similarly, in another budding ascidian, *P. misakiensis*, a *vasa* homolog was expressed strongly by loose cell aggregates and germ cells, indicating that germ cells arise *de novo* in developing zooids and suggesting that the *vasa* homologue plays a decisive role in switching the cell fate from coelomic stem cells to germ cells (Sunanaga et al., 2007). In *Botryllus schlosseri*, Vasa detected from the larva and the oozooid stages, repeatedly emerge *de novo* in the colony, independently of its sexual state (Rosner et al., 2009). The expression of *Pl10-*, *-piwi-* and *Oct4-* orthologues both in germline cells and also in circulating pluripotent stem cells, hemoblasts, in *B. schlosseri* in contrast to the observations in *B. primigenus* and *P. misakiensis* might reflect different modes of germ lineage sequestering between the species (Rosner et al., 2009). During development of the ascidian *Ciona intestinalis*, primary germ cells are localized to the tail of the tadpole and during metamorphosis migrate into the adult gonad rudiment. If the tail with primary germ cells is removed, adults still form mature germ cells, suggesting a compensatory mechanism that regulates ascidian germ line formation at a later ontogenetic stage (Takamura et al., 2002). In embryogenesis of the sea urchin *Strongylocentrotus purpuratus*, germ line determinants

accumulate in the small micromere lineage. Vasa protein is enriched in the 16-cell stage micromeres and subsequent small micromeres. Experimental removal of Vasa-positive cells induces Vasa expression *de novo* in adjacent blastomeres (Voronina et al., 2008).

2.5 Transient expression of germline marker genes during development and cell differentiation

Among cnidarians, in *Hydra magnipapillata, pl10* mRNA is expressed not only in undifferentiated cells (multipotent interstitial stem cells and germline cells) but also in differentiating somatic cells of the interstitial cell lineage. One of two *vasa*-related genes appears to be expressed in all kinds of undifferentiated cells: multipotent stem cells, germline cells and the ectodermal epithelial cells in the body column. However, none of the *vasa/PL10* genes were expressed in fully differentiated somatic cells in *Hydra* (Mochizuki et al., 2001). Analyses of the *piwi*-related gene during embryogenesis and medusa formation in the hydrozoan *Podocoryne carnea* have shown this gene expression in somatic stem cells as well as the germ line cells (Seipel et al., 2004). In sea anemone *Nematostella vectensis* (Cnidaria) members of the *vasa* and *nanos* families are expressed not only in presumptive germline cells but also in broad somatic domains during early embryogenesis and later are restricted to primary germ cells (Extavour et al., 2005).

During embryonic development of the planaria *Schmidtea polychroa,* Tudor-related protein is expressed in differentiating cells rather than neoblasts (Solana et al., 2009).

In the larvae of polychaete annelid *Platynereis dumerilii*, *piwi*-, *vasa*-, *PL10*- and *nanos*-related genes are expressed altogether at the mesodermal posterior growth zone in highly proliferative stem cells providing the somatic mesoderm and the germ line. *vasa*-like gene expression was revealed in the germ line as well as in multiple somatic tissues, including the mesodermal bands, brain, foregut, and posterior growth zone (Rebscher et al., 2007). During embryonic development of the oligochaete annelid *Tubifex tubifex,* transient *vasa* homologue expression was observed in cells in nongenital segments (Oyama & Shimizu, 2007). In polychaete *Capitella sp*. during embryonic, larval, and juveniles stages, *vasa* and *nanos* orthologues are coexpressed in somatic and germ line tissue. Both these genes reveals expression in multiple somatic tissues with largely overlapping but not identical expression patterns; following gastrulation, expression is observed in the presumptive brain, mesodermal bands, and developing foregut (Dill & Seaver, 2008).

In various sea urchin species, Vasa, Nanos, and Piwi are expressed in descendants of the small micromeres and subsequently become restricted to the coelomic pouches, from which the entire adult rudiment will form, suggesting that these conserved molecular factors are involved in the formation of multipotent progenitor cells that contribute to the generation of the entire adult body, including both somatic and germ cells (Juliano et al., 2010; Voronina et al., 2008). In addition, echinoderm species lacking small micromeres, such as sea stars, also have Vasa protein and/or transcripts enriched in the larval coelomic pouches, suggesting a conserved mechanism for the formation of multipotent progenitor cells in the coelomic pouch to produce an adult rudiment within the echinoderms (Juliano & Wessel, 2010; Wu et al., 2011). In the colonial ascidian *Botryllus schlosseri*, *Pl10*, *piwi* and *Oct4* orthologues are highly expressed in differentiating soma cells (Rosner et al., 2009). In the cephalochordate amphioxus *Branchiostoma floridae* (Chordata), Vasa and Nanos, in addition to the early localization of their maternal transcripts in the primary germ cells, are also expressed

zygotically in the tail bud, which is the posterior growth zone of highly proliferating somatic stem cells (Wu et al., 2011).

The data indicate a close relationship between presumptive germline cells and multipotent somatic stem cells during development (Wu et al., 2011) and suggest a common origin of germ cells and of somatic stem cells, which may constitute the ancestral mode of germ cell specification in Metazoa (Rebscher et al., 2007). A two-step model of germ cell specification was proposed as an ancestral mechanism involving co-specification of germ cells and stem cells: setting aside a population of undifferentiated pluripotent stem cells, which is excluded from somatic differentiation and has the potential to form both somatic and germ cells, from which the primary stem cells are segregated later (Rebscher et al., 2007).

2.6 Germline marker features beyond gametogenic stem cells

2.6.1 Processing bodies and cytoplasmic RNA granules in somatic cells

In eukaryotic somatic cells, mRNA metabolism is regulated by ribonucleoprotein (RNP) aggregates, RNP granules considered as possible equivalents of germ granules in germline cells. Post-transcriptional processes have a central role in the regulation of eukaryotic gene expression, and these processes are not only functionally linked, but are also physically connected by cytoplasmic granules (Eulalio et al., 2007). Cytoplasmic RNP granules function in determining mRNAs degradation, stabilization, intracellular localization, translational repression and RNA-mediated gene silencing. All RNA granules harbor translationally silenced mRNA. There are several classes of cytoplasmic granules in somatic cells named processing bodies (P-bodies, or P bodies), RNA or RNP granules, RNP particles, stress and neuronal granules (Anderson & Kedersha, 2006; Eulalio et al., 2007; Flemr et al., 2010; Lachke et al., 2011; Kiebler & Bassell, 2006; Kotaja et al., 2006; Seydoux, Brown, 2006).

In mammalian cells, P-bodies are the most common type of RNA granules and contain products of gene orthologues in germ cell granules (Flemr et al., 2010; Pepling, 2010). Processing bodies contain components of mRNA decay processes and microRNA-mediated silencing, serving as sites where mRNAs can be either stored or degraded (Kiebler & Bassell, 2006; Lachke et al., 2006). Argonaute proteins, and also miRNAs and miRNA-repressed mRNAs, were demonstrated to localize in P bodies in mammalian cells.

Unlike P bodies, stress and neuronal granules contain ribosomal subunits. Stress granules are dense aggregates accumulated in cells in response to environmental stress and regulated translational repression and mRNA recruitment to preserve cell integrity. Neuronal granules deliver mRNAs and inactive ribosomes to specific translation sites in dendrites (Anderson & Kedersha, 2006; Seydoux & Brown, 2006; Flemr et al., 2010). In mammalian neuronal cells, three classes of RNA granules were described: transport RNP particles, stress granules, and P bodies with potential functions in RNA localization, microRNA-mediated translational regulation, mRNA degradation, and localized translation of mRNAs involved in synapse formation or motility (Anderson & Kedersha, 2006; Kiebler et al., 2006). Electron dense perinuclear chromatoid body-like structures surrounding the nuclei of neurons were observed in the planaria *Dugesia japonica* (see Shibata et al., 2010).

Cytoplasmic RNP granules function in the posttranscriptional control of gene expression, but the extent of their involvement in developmental morphogenesis is unknown. Recently, a

Tudor domain-containing RNA binding protein (TDRD7) was identified as a component of a unique class of RNP granules with a conserved pattern of developmental expression in ocular lens fiber cells (Lachke et al., 2011). Furthermore, human TDRD7 mutations result in cataract formation via the misregulation of specific, developmentally critical lens transcripts. TDRD7 perturbation causes cataracts in chickens and mice. TDRD1, TDRD6, and TDRD7 have been associated with chromatoid bodies in mammalian male germ cells. Tdrd7 null mutant mice develop cataract and glaucoma; an arrest in spermatogenesis also was observed. Staining with the antibody of STAU1, a mammalian homologue of the *Drosophila* RNA-binding protein Staufen, revealed the presence of numerous STAU1-positive RNP particles in lens fiber cells co-localized to a high degree with TDRD7. The authors hypothesized that TDRD7 granules, either alone or through their interaction with STAU1- RNP granules and P bodies, might regulate the expression levels of specific lens transcripts (Lachke et al., 2011).

Several P body markers are highly concentrated in chromatoid bodies (Kotaja et al., 2006). These data suggest that the chromatoid bodies of male germ cells and P-bodies in somatic cells are functionally related, both acting as a site for mRNA decay and mRNA translational repression by the miRNA pathway (Anderson & Kedersha, 2006; Kotaja et al., 2006). P-bodies contain components of the RNA-dependent silencing machinery (Seydoux & Braun, 2006). P-body components are also present in two other classes of somatic RNP particles: stress granules and neuronal granules (Anderson & Kedersha, 2006). P-body components represent an ever-growing list of proteins involved in RNA metabolism, and the composition of P-bodies, stress granules in somatic cells and germ cell granules overlaps to some extent (Flemr et al., 2010). The data provide evidence of diversity of mammalian RNA granules. Although, they exhibit overlapping composition but different structures and functions (Flemr et al., 2010) sharing components and evolutionary conserved mechanism of post-transcriptional regulation with germ granules which function is distinguishably unique (Seydoux & Braun, 2006).

2.6.2 Germline marker gene expression in somatic stem pools and neurons

Among asexually reproducing animals, *vasa*- and *PL10*-related genes are expressed in somatic ectodermal epithelial cells (unipotent stem cells) in the hydrozoan *Hydra magnipapillata* (Mochizuki et al., 2001). In the colonial ascidians *Botryllus schlosseri*, *vasa*-related gene products are not exclusively expressed in germ lineages but also are strongly expressed in many embryonic and bud somatic cells. Expression of *vasa* and *piwi* orthologues were detected in somatic tissues and *Oct4*-related gene was also expressed in the somatic cells of the endostyle (Rosner et al., 2009).

Among animals without asexual reproduction, *piwi*, *vasa*, and *pl10* are expressed in somatic stem cells at the base of the tentacle bulb, giving rise to tentacle nematocytes in the hydromedusa (Cnidaria) *Clytia hemisphaerica* (Denker et al., 2008). Their expression, along with *bruno* orthologue, was found in germline cells in pluri/multipotent somatic stem cells in the tentacle root in *Pleurobrachia pileus* (Ctenophora), a species which reproduces only sexually (Alié et al., 2011). There is no experimental proof that ctenophore somatic stem cells are incapable of producing germ stem cells, but under normal conditions, this seems highly unlikely (Alié et al., 2011). There are also some other examples of canonic germline marker expression in somatic cells and tissues in bilaterian animals including vertebrates (see Alié et al., 2011; Gustafson & Wessels, 2010; Wu et al., 2011).

These data suggest two alternative hypotheses (see Alié et al., 2011). First, these genes are fundamentally associated with germinal potential, and when they are expressed in pluri/multipotent stem cells, they have the potential to generate germ cells. Second, these genes are components of an ancestral molecular toolkit of animal stem cells, whatever the fate of their progeny. Presumably, *piwi*, *vasa* and *pl10* belong to a gene network ancestrally acting in two contexts: germline and pluri/multipotent stem cells. Since the progeny of these multipotent stem cells includes both somatic and germinal derivatives, it remains unclear whether *vasa*, *piwi*, and *pl10* were ancestrally linked to stemness, or to germinal potential. Probably, total or partial restriction of these genes to the germline in some bilaterian groups (e.g., vertebrates and insects) is a derived, evolutionary secondary condition, and it is not appropriate to use these genes, including *vasa*, as germline markers (Alié et al., 2011). The fundamental reason why these genes are ancestrally linked to stemness, in addition to the germline, is probably the main function of the Piwi–piRNA pathway, i.e., genome protection through silencing. Genome protection is a crucial requirement not only for germ cells but also for somatic stem cells, and ancestral involvement of the same gene set in both the germline and somatic stem cells does not particularly imply their common origin in a genealogical sense, but the requirement of the same silencing pathway in two different contexts (Alié et al., 2011). Several conserved molecules are expressed in both germ cells and all types of stem cells (Sroji & Extavour, 2011).Tthe *piwi* gene family may represent the first class of genes with a common molecular mechanism shared by diverse stem cell types in diverse organisms (Cox et al., 1998).

There are some data on relationship of pluripotent stem cells and neuroblasts. Some proteins classically related to germ line development have been recently found to be involved in neuronal function and development. In the planaria *Schmidtea polychroa,* Tudor-related protein is expressed, beyond germline cells and neoblasts, in the central nervous system (Solana et al., 2009). *pumilio* and *bruno* planarian homologues are expressed similarly in neoblasts and in the central neural system, in perinuclear particles surrounding the nuclei of neurons (Salvetti et al., 2005; Guo et al., 2006). *nanos* and *pumilio* are involved in neuronal excitability, dendrite morphogenesis, and long-term memory in *D. melanogaster* (see Muraro et al., 2008; Solana et al., 2009). When mouse ESCs were cultured in serum- and feeder-deprived conditions colony-forming primitive neural stem cell populatiuons could be obtained (Stice et al., 2006). Tropepe and coauthors (2001) proposed neural fate specification from ESCs by a default choise.

All the data suggest that primordial germ cells can be segregated at almost any point during embryogenesis: before blastoderm formation; after embryonic rudiment formation but before germ layer separation; after germ layer separation but before gonadogenesis; or after gonadogenesis and continuously throughout adult life (Extavour & Akam, 2003).

2.7 Regulatory gene networks underlying gametogenic potential and pluripotency

The metazoan development program may be imagined as translation regulatory cascades. The regulatory transcriptional network to maintain stem cell function has been conserved during metazoan evolution (Watanabe et al., 2009). Genes *vasa/pl10*, *piwi/auberdine*, *nanos*, *tudor*, *pumilio*, and *staufen,* representing the core of the germline program, show striking evolutionary conservation (Alié et al., 2011; Chuma et al., 2006; Ewen-Camden et al., 2010; Extavour, 2008; Gustafson & Wessel, 2010; Leatherman & Jongens, 2003; Parvinen, 2005;

Sroji & Extavour, 2011). This gene network consists of gene modules whose interactions are highly stable and highly evolutionary conserved operating in similar ways both in different organisms, and in different places and/or times during the development of an animal organism. Interactions between *vasa* and other germ line genes have suggested a complex network of positive and negative regulation at multiple levels, including transcription, translation, and post-translational modification, epigenetic control of chromatin architecture mediated gene regulation crucial for the role in development (Cinalli et al., 2008; Ewen-Camden et al., 2010). Conserved germ cell-specific RNA networks repress transcriptional programs for somatic differentiation and promote germ cell maintenance (Cinalli et al., 2008). Maelstrom was identified as a nuage component that interacts with both mouse DDX4 and MIWI. It is required for spermatogenesis and also is involved in silencing transposable elements. Although still not definitive, the consistent association in multiple animals of Vasa and members of the RNAi pathway provides a strong argument that they have a functional relationship (Gustafson & Wessel, 2010).

We took adventage of the bioinformatic tool STRiNG (Snel at al, 2000) to construct and analyze germ plasm protein network. Using *Drosophila* known germ plasm components (Fig. 6A) and their homologues in mouse (Fig. 6B), we predicted interactions and indentified several pathways based on the protein functional domains, structure and sequence similarity (Fig. 6D). Similar to *Drosophila*, known germ plasm proteins (Ddx4, Tdrd1, Tdrd7, Tdrd9, Pum2, Nanos1, Nanos2, Nanos3 and others) formed a network (Fig. 6D) responsible for **germline differentiation** (module 3) upon RNA processing via sequence-specific RNA-binding, translation and mRNA-stabilisation (1, 4: Pum1, Nanos2, Pum2) as well as normal mRNA turnover and nonsense-mediated mRNA decay (15: Dcp1a, Dcp2, Edc3, Edc4). Some other important germline related functions are revealed: **transposable element repression** by piRNA machinery during spermatogenesis (5: Piwi-family, Mael, Tdrd6 and etc), **RNA-mediated gene silencing** (RNAi) by a RISC complex (2: Dicer1, Eif2-family/Ago2), **translational activation of mRNA** in the oocyte and early embryo (16, 17: Ddx3y, Ddx3x), and X-chromosome inactivation (8: Xiap). Several functions were related to **molecular metabolism** catalyzing the transsulfuration pathway from methionine to cysteine (20: Tdrd5, Cbs, Cth, Mthr-family), ribosomal protein complex (18: Mrps12, Mrps7, Mrpl11, Mrpl12, Rps20), proteasomal degradation and inhibition of the caspases (7, 8: Apaf1, Xiap, Casp7, Casp8ap2), mediation in activation of the stress-responsive elements upon DNA damage and also regulation of growth and apoptosis (9: Gadd-family, Casp7). A significant portion of the network is occupied by **cell cycle** pathways (10: Pcna, Cdk1) requiring the G2/M (mitosis) transition (7: Cyclin B1, Cdc20, Ccnb1, Ccnb2, Ccna1), DNA replication and polymerase function (11, 13: Fen1, Pold-family, Bub1b), progression from G1 to S phases (7: Lsm10, Lsm11) as well as the anaphase promoting complex/cyclosome regulation (12: Ube2c, Cdc-family, Mad-family). Presence of the cyclin B1 in the germ plasm of *Drosophila* was previously described by Dadly and Glover (1992). Other sub-sequential parts of the network are responsible for **tissue-specific regulation,** including alternative pre-RNA splicing (16: Ddx3y, Cugbp2, Usp9x, Ddx3x), endothelial cell motility and neurotrophic signalling for spinal and sensory neurons (19: Gpi1, Tkt-family), ventral **cell fate in the neural tube** and normal development of the vertebral column (14: Pax1, Pax9, Msx1, Shh), **survival of motor neurons** via spliceosomal Sm proteins function (6: Wdr77, Prmt5, Piwi-family, Lsm10, Lsm11) along with methylation of Piwi proteins required for interaction with Tudor-domain contained proteins.

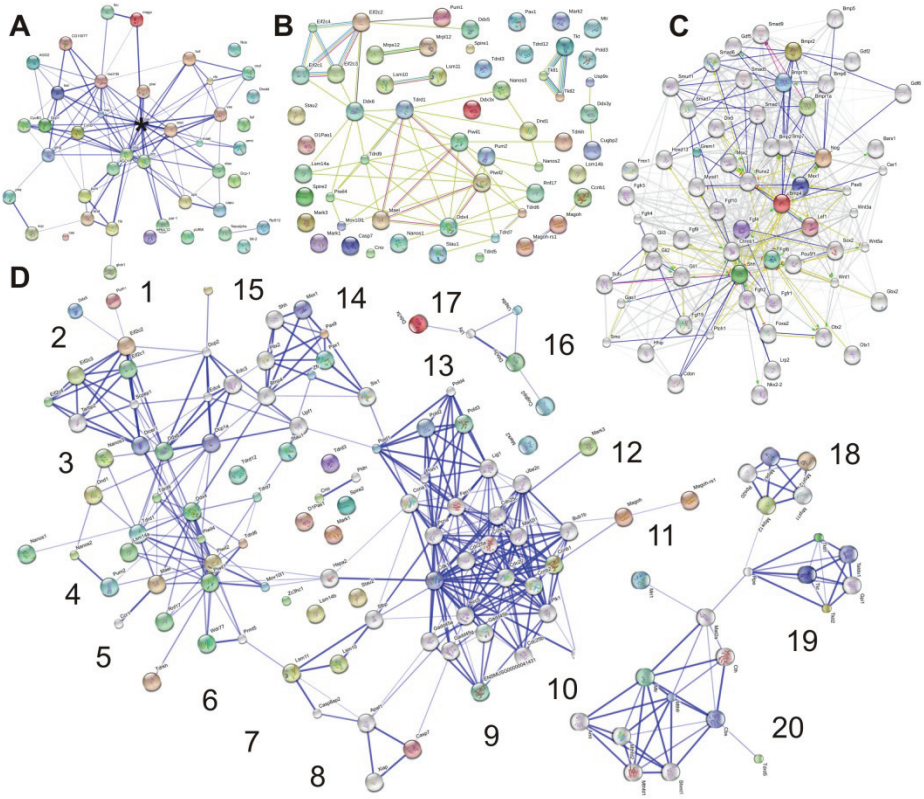

Fig. 6. Protein networks of *Drosophila melanogaster* germ-plasm (A, confidence mode), homologues (B, evidence mode) and expanded neighbouring interactions (D, confidence mode; 1—20 functional modules, see in the text) of *Mus musculus* germ-plasm; and interactions for *Mus musculus* BMP4 (C, action mode).

It was previously found that BMP-family proteins play an important role in primordial germline specification during normal mammalian development and are also required during germline differentiation of mouse and human ESCs *in vitro* (see Ying et al, 2001; Toyooka et al, 2003; Kee et al, 2006). The BMP4 protein, along with some nuage/germ granules components, is involved in a complicated network of transcriptional factors regulating early embryo development, stem cell fate and differentiation. Repression of the Shh gene, for example, prevents activation of the transcription for genes involved in ventral cell fate in the neural tube and also disrupts the polarizing signal for patterning of the anterior-posterior axis of the developing limb bud. It is not surprising that network control is looping from one gene to another (Wnt5a, Otx2, Shh, Bmp4, Runx2, Nog, and Fgf8) forming a "switch-off/switch-on" dynamic machinery in the cell cytoplasm (Fig. 6C). It is evident that the germ plasm or nuage complex is the sub-cellular localized organelle controling this "on and off" loop depending on the cell fate. Indeed, the sub-cellular organization and localization of the nuclear pore would serve as a major station processing specific mRNA. Such a station can sort and degrade specific mRNA upon cell fate and

differentiation via traditional nonsense-mediated mRNA decaying or by non-traditional Stau-based degeneration. Staufen is known to bind double-stranded RNA, along with other members of germ plasm (DEAD-box helicases), but Stau1 also binds tubulin cross-linking RNA macromolecular complexes (MMCs) to the cytoskeleton of the cell. Such organelle organization and function can quickly and reversibly mobilize MMCs to a specific compartment, preceding their post-transcriptional modification, and activate site-specific protein translation and localization. Furthermore, nuage can suppress or even put to silence transposable elements, preventing their mobilization via piRNA-based machinery. This can be done without significant structural changes in genome organization and without transcriptional de-activation of the crucial developmental loci and transcriptional factors that are involved in stem cell pluripotency (Pou5f1 and Sox2).

Besides the function in the germline, the germ plasm components control, or assist in the control, of tissue-specific pathways defining the pattern and fate in stem cell progenitors. They are also responsible for a wide range of gene transcription and translation regulation, as well as the cell cycle. It seems that the germ plasm acts as a switch between mesoderm and ectoderm fate. It stimulates endothelial cell motility, and possibly specifies or regulates the differentiation of cartilage and bone of notochord in chordates. Notochord induces neural plate formation and, by secreting SHH protein, signals differentiation of motoneurons in the neural tube.

The extensive molecular signatures and functional potential of germ cells and pluripotent stem cells suggest a shared evolutionary origin for these cell types and an ancestral pluripotency network including members of Vasa-like and Piwi-like class proteins, which are conserved components of both germ and stem cells across the metazoans (Alié et al., 2011; Ewen-Campen et al. 2010; Gustafson & Wessel, 2010; Sroji & Extavour, 2011). Based on the literature and our own data analysis, we support the idea that this regulatory gene network is not restricted to the germline cells but is expressed in stem cells that are capable of producing both somatic and germinal derivatives.

3. Conclusion

The data we have reviewed here suggest the existence of an evolutionary conserved basis of pluripotency and "stemness" of germ and gametogenic pluripotent stem cells. This mechanism is common for all studied metazoan representatives, from sponges to chordates, and operates at cellular, sub-cellular and molecular levels. In the studied asexually reproducing representatives of Porifera, Cnidaria, Platyhelminthes, Arthropoda and Chordata, stem cells serve as the predecessors of germ and somatic cells and are similar to cells of the germ lineage, displaying evolutionarily conserved features of the morphofunctional organization typical also of cells of the germ line (Ewen-Campen et al. 2010; Extavour, 2008; Funayama et al., 2010; Gustafson & Wessel, 2010; Isaeva et al., 2003, 2008, 2009; Rinkevich et al., 2009; Sköld et al., 2009; Shukalyuk et al., 2005, 2007, 2011; Sroji & Extavour, 2011). The reaction revealing the activity of alkaline phosphatase, earlier used for the identification of primary germ cells and embryonic stem cells in vertebrates, was successfully applied as a cytochemical marker of invertebrate stem cells (see Agata et al., 2006; Akhmadieva et al., 2007; Isaeva, 2011; Isaeva et al., 2003; Rinkevich et al., 2009; Shukalyuk et al., 2005; Sköld et al., 2009).

Since pluri/multipotent stem cells produce germline cells, they might be considered part of the germline (Mochizuki et al., 2001); such "primary" stem cells may be immortal

contributing to the germ line, in contrast to somatic tissues (Weismann, 1893; Sköld et al 2009). It is gametogenesis that gives us an "afterlife," propelling our genome into future generations (Seydoux & Braun, 2006). Pluripotent stem cells of animals with asexual reproduction are predecessors of primary germ cells. Pluripotent gametogenic stem cells and germline cells share many morphological features and rely on the activity of related genes; their evolutionary and ontogenetic relationship has been proposed (Extavour, 2008; Extavour & Akam, 2003; Sköld et al., 2009; Strouji & Extavour, 2011).

Adult pluripotent stem cell systems are not restricted to primitive animals and probably evolved as components of asexual reproduction (Agata et al., 2006). The data on the asexual reproduction in some arthropods and chordates contradicts the dogma that asexual reproduction is common exclusively among the lower animals (Isaeva, 2010, 2011).

The term "somatic embryogenesis" (Buss, 1987; Blackstone & Jasker, 2003) suggests that stem cells, which ensure the asexual reproduction, are recognized as somatic ones; pluripotent stem cells in animals with asexual reproduction are often referred as somatic (Blackstone & Jasker, 2003; Extavour & Akam, 2003; Extavour, 2008; Rinkevich, 2009; Sköld et al., 2009; Funayama et al., 2010). However, pluriponent gametogenic stem cells of asexually reproducing invertebrates, like primary germ cells, do not belong to any germ layer, differentiated tissue, and population of specialized somatic cells or their somatic stem cells (Isaeva, 2010, 2011). Such pluripotent stem cells are dispersed in the organism, do not display contact inhibition of cell reproduction and movement and are similar to primary germ cells in their ability to perform amoeboid movements and large-scale migrations within the organism, directed to the localities of asexual reproduction and regeneration or to the gonads, respectively (Isaeva et al., 2008, 2009; Rinkevich et al., 2009; Sköld et al., 2009). We believe that the evolutionarily and ontogenetically related cells of early embryos, pluripotent gametogenic stem cells and germline cells belonging to cell populations capable of realizing the entire developmental program, including gametogenesis (and, potentially, subsequent embryogenesis) are not identical to somatic cells.

Pluripotent cells in invertebrates with asexual reproduction are similar in their potential and their molecular signature to mammalian embryonic stem cells, although the latter are artificial cell systems cultured *in vitro*. Thus, published and original data indicate the existence of evolutionary conserved, sub-cellular and molecular bases of toti/pluripotency and "immortality" and similarity of studied morphofunctional features and molecular signature of pluripotent stem cells in metazoans with asexual reproduction from sponges and cnidarians to chordates, germline and embryonic stem cells (Fig. 7).

Recent data indicate the broad and partially overlapping spectrum of gene expression in ECSs, germ, and pluri/multipotent potent stem cells, in particular, the possible inducibility of germline cells *de novo* without continuous expression of molecular markers of the germ line. The data also show a transient molecular signature typical of the germline in broad somatic domains during embryogenesis and the expression of germline marker genes in somatic stem pools. Embryonic stem, germ and pluripotent stem cells of various metazoans share the expression *piwi, vasa*-related and other germline marker genes. It is possible a functional diversification of paralogues of *vasa*, *piwi* and other marker "germline" genes fulfilling different functions in germ and other stem cells. In the animal kingdom, *vasa*-like genes are present in numbers from one to four (Shibata et al., 1999; Rebscher et al., 2007; Extavour et al., 2005; Pfister et al., 2008). In mammals, four Argonaute subfamily members have been shown to be involved in the miRNA pathway (Parvinen, 2005; Kotaja et al., 2006).

Although, the canonic, classical germline molecular markers remain reliable for germ cell identification within developing individual across Metazoa, more studies need to be done in order to understand molecular and cellular events underlying pluri/totipotency, stem cell self-renewal and self-preservation during germline specification.

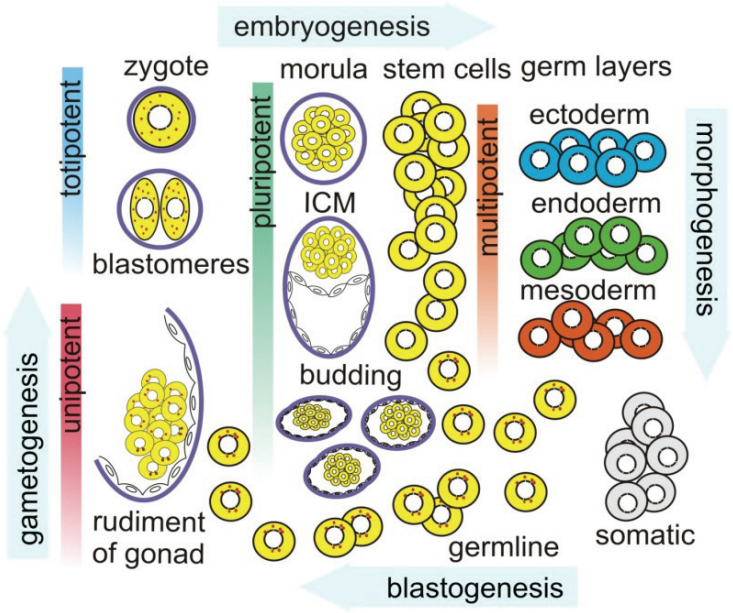

Fig. 7. Germ-plasm material on the sub-cellular level in animals with sexual or both sexual and asexual reproduction in their life span. In animals with epimorphosis, early blastomeres after first few divisions are believed to be fully totipotent along with zygote and capable of producing an entire organism. Macromolecular complexes (MMC, yellow) distributed in cytoplasm and germ-plasm granules around nucleus (in red) are present in zygote and early blastomers marking their totipotent ability. Through the embryonic development, the visual manifestations of the granules are disappeared in the pluripotent cells of morula, inner cell mass of blastocyst or embryonic stem cells in culture. However, specific MMCs are distributed in cytoplasm (yellow) marking their potency. Morphogenesis followed after specification of the three germ layers (ectoderm, endoderm and mesoderm) segregates true somatic cells lacking their potency (grey color) and the germline. The germline lineage expresses the specific MMCs and germ markers *de novo* during specification. The germ-plasm activates in granules (red) near nucleus pores. Germline specification occurs from the mesoderm (in mammalians) as well as spontaneously in culture of embryonic stem cells, and from the pool of the stem cells in asexually reproducing organisms. Presumably, re-activation of the germ-plasm in germline forms a unique sub-cellular niche for regulating and manipulating cellular pluripotency. Finally, germline cells with their specific molecular signature migrate and populate the rudiment of the developing gonad. A new signalling niche will significantly restrict germline abilities (to unipotency) during further gametogenesis. Eventually, cells will undergo dramatic morphological and molecular changes (oogenesis and spermatogenesis) ensuring protection and passing of undamaged information to the next generation.

Germ granules, chromatoid bodies, nuage, P-bodies and so on are the physical embodiment of overlapping but not identical gene networks. The patterns arose ancestrally as a gametogenic/stem program. Germ granules may be as diverse as P-granules of somatic cells, with functions ranging from RNA localization and decay to translational activation and repression (Anderson & Kedersha, 2006; Seydoux & Braun, 2006). It is also possible, that in some cases, this machinery exists in mammals as submicroscopic complexes that are rich in RNAs and RNA-binding proteins (Hubbard & Pera, 2003). The Oskar protein nucleates the formation of polar granules *de novo*, from cytoplasmic pools of the components shared with nuage. In this model, nuage could be an organelle that concentrates and thus potentiates the activity factors normally present in all cells, but that must be especially active in germline cells because of their intensive reliance on post-transcriptional controls of gene expression (Snee & Macdonald, 2004). Basic germ-plasm machinery may exist as discrete granules or bodies, large complexes as Balbiani bodies, dispersed nuage, small particles (for example, P-bodies) or submicroscopic RNP aggregates. It seems to us that similar networks in mammalians overlapped with the macromolecular frame of the germinal granules emerging earlier in germline cell evolution. Given that the factors that are associated with germ granules in non-mammalian species are also expressed in mammalian germ cells, we speculate that all multicellular animals share basic germ-plasm machinery, a nuage-like sub-cellular frame, which operates in a similar manner across Metazoa and might recruit other tissue-specific networks within.

Protection against apoptosis is very important for embryonic, germ and pluripotent stem cells as well as for long living neural precursors and neurons. Differentiation of toti-/ pluripotent cells irrevocably drags their descendants into programmed death. Undifferentiated cells have only two choices: stay undifferentiated and immortal or start to differentiate and die. Breaking this rule leads cells to cancerogenesis. The germline cells and neuronal precursors evolutionarily obtained unique machinery for circumventing this rule, which allows them to continue their differentiation but keep their immortality over the life-span of the individual. However, cell death does occur during neurogenesis, matching the number of neurons to the number of target cells. It is also known that in zebrafish, loss of *piwi*-related gene function results in a progressive loss of germ cells due to apoptosis during larval development (Houwing et al., 2007). A mutation of mouse *vasa* homolog gene (*Mvh/Ddx4*) leads to restricted expression. In homozygotes, premeiotic germ cells cease differentiation and undergo apoptotic death (Tanaka et al., 2000). Studies of apoptosis have revealed the key role of the apoptosis induction factor of mitochondrial origin and apoptogenic functions of cytochrome C in this important, evolutionary conserved process (Green & Reed, 1998; Martinou, 1999). Contact with mitochondria is a typical property of germinal granules in diverse multicellular animals. The germinal granules in the cytoplasm of germline cells of *Drosophila* contain ribosomal RNAs of mitochondrial origin (Amikura et al., 2001). It is not coincidence that several cytochrome C oxidase and other mitochondrial products were found within the germ plasm. Moreover, we propose mitochondrial participation in biogenesis of germ granules (Isaeva et al., 2005; Isaeva, 2011). VASA protein homologue has been found both in germ granules and in the mitochondrial matrices in germ cells of *Xenopus* embryo (see Watanabe et al., 2009). Similarly, the protein encoded by the *tudor*-related gene, is present not only in polar granules, but also inside the mitochondria of early embryos of *Drosophila* (see Ding & Lipshitz, 1993). We also postulate the presence of molecular defence against apopthosis localized in germinal granules and related

cytoplasmic structures in germline, embryonic, pluripotent stem and neural cells ensuring self-preservation against cell aging and death.

The hypothesis has been advanced that the germline originally evolved from primary stem cells (Sköld et al., 2009). Extavour (2008) also proposed that germ cells have their evolutionary origins in a pluripotent stem cell population. Taking a different position, Zwaka & Thomson (2005) hypothesized that embryonic stem and embryonic germ cells represent a family of related pluripotent cell lines, whose common properties reflect a common origin from germ cells. Rebscher et al. (2007) proposed a model in which *vasa*, *piwi*, and *pl10* ancestrally carried out a first step in germline determination by specifying a multipotent population of stem cells within which PGCs are sorted out later. An ancient association of "germline genes" with stemness (Watanabe et al. 2009) and "an ancestral gene fingerprint of stemness" (Alié et al. 2011) were proposed. The genes *vasa* and *piwi* are the most extensively studied of the genes, known as germline markers, which appear to be involved in the ancestral molecular signature of stemness and expressed in pluri-/ multipotent stem cells across animal phyla. According to the hypothesis put forward by Alié et al. (2011), *piwi* and *vasa*-related genes belong to a gene network ancestrally associated with stemness. These genes determine gametogenic potential, but the main function of these genes is genome silencing. Agata et al. (2006) proposed that the pluripotent stem cell system supporting both asexual and sexual reproduction in many adult animals represents one type of origin of stem cell systems; the other system developed by separating multiple functions of primitive pluripotent stem cells into specialized cell lineages.

We suppose mainly common and partially overlapping molecular signatures in pluri-/ multipotent stem cells of a wide range of animals from sponges to chordates. But we also suspect a wide spatial and temporal ontogenetic context as a continuum of toti-, pluri-, multipotent state within stem cells. Such a continuum ranges from pluripotent gametogenic stem cells to germline cells and up to multipotent somatic stem cells lacking gametogenic potential. All animals possess pluripotent gametogenic stem cells in different ontogenetic periods: as a transient state in early cleavage until segregation of germ line (preformation), as a longer state during embryogenesis (epigenesis), or as a continuous state during the entire life of asexually reproducing organisms. Pluripotent stem cells have the capacity to move away from pluripotency towards a special, restricted stem cell identity as germ cells (Sroji & Extavour, 2011) or to restricted identities as somatic multipotent stem cells, oligopotent stem cells and so on. A core regulatory gene network of pluripotent gametogenic stem cells, germline cells and multipotent stem cells evidently overlap to a large extent including also some specific distinctions and fluctuations of key gene expression. Genes of the molecular machinery of stem cells appeared to be interconnected in related pathways that are involved in post-transcriptional regulation and epigenetic modification acting in a coordinated manner, as part of a complex network of signal cascades that are known to regulate the balance between cell death and survival (Rossi et al., 2007). Since all animals have a common ancestor in single cell organisms it is possible to identify common principles in the regulatory mechanisms for the transcriptional and epigenetic machinery but at present there is no clear picture to what extent the regulatory transcriptional network to maintain stem cell function has been conserved during metazoan evolution (Watanabe et al. 2009), in germline cells or beyond the germ line (Gustafson & Wessel, 2010).

Taking into consideration our own data and the supporting literature, we see that there is enough evidence to suggest the existence of an ancient molecular basis of toti-, pluri-, or multipotency of germ and stem cells, common for all studied representatives of multicellular animals. However, the detailed molecular mechanisms and overlapping regulatory networks in different stem cell systems appeared to be more complex that was viewed before. Interconnecting regulatory networks of stem cells and germ cells still remain unclear in their capacity to decide cell fate transferring the control from the nuclear transcriptional networks to the cytoplasmic post-transcriptional machinery. Further comparative studies of stem cells in a wide variety of metazoans may provide significant and crucial data for our understanding of the common, evolutionary conservative basis of stemness, pluripotency and potential "immortality" of germ and stem cells across Metazoa.

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14

Drosophila: A Model System That Allows *in vivo* Manipulation and Study of Epithelial Cell Polarity

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1. Introduction

Epithelia are specialized tissues that emerged early in evolution to subdivide the body into distinct parts and to form barriers by lining both the outside (skin) and the inside cavities and lumen of bodies. Functions of epithelial cells include secretion, absorption, protection, transcellular transport, sensation detection and selective permeability. To achieve these different tasks, several types of epithelial tissues emerged during evolution, all of them having one feature in common that is indispensable for their function: the cells are attached to each other in order to form a layer that works as a barrier. Epithelial cells thus show a ordered morphology, they are polarized. Disruption of polarity is an important feature of epithelial cancers that accounts for more than 90% of fatal malignancies in adults, rendering the understanding of the fundamental processes needed for polarity an ongoing subject of high interest.

Epithelial cells are highly polarized and the cells are oriented so that their external, so-called apical, surfaces face the outside or a central lumen and the internal, or basolateral, surfaces are in contact with other cells and the basement membrane. These characteristics were discovered using histological and thus descriptive analyses. With the advent of molecular genetics, biochemistry and molecular cell biology our knowledge about polarization processes increased dramatically, allowing for a better understanding of the mechanisms used by epithelial cells to fulfill their specialized tasks and to act as barriers. Thus, the polarization is not only reflected in the morphology of the cells, but also in the positioning of their organelles and in the apico-basal (AB) localization of polarity protein complexes at the plasma membrane. The plasma membrane can be divided into an apical, a junctional and a basolateral domain, each domain comprising its own set of polarity proteins that are widely conserved in eukaryotes. This AB polarity is required for formation of functional epithelial tissues. The asymmetrical deployment of proteins is mediated through subcellular trafficking and the polarized localization of transcripts. Genetic studies have revealed that the polarity protein complexes function in a sequential but interdependent manner to regulate the establishment and maintenance of cellular polarity.

The proteins involved in epithelial cell polarization are largely conserved between species, as mentioned above, even between vertebrates and invertebrates. Much of our hitherto

knowledge stems from studies performed in model organisms and until now it is impossible to culture whole epithelia for a long time. Existing *in vitro* cell culture systems give important insights into epithelial cell function, but mechanisms following biological input from the living and developing organism are obviously missed in these systems. Therefore, the fruit fly, *Drosophila,* provides an excellent model system for studying columnar epithelial cells (Fig. 1), allowing their static and especially dynamic analysis in the context of a whole living organism. Furthermore, state of the art microscopy imaging techniques can be easily coupled to the extensive genetic tools available in the *Drosophila* system, allowing for *in vivo* analysis and the concomitant dissection of pathways needed for epithelial cell polarity.

In this chapter we would like to introduce *Drosophila* as a model system to study epithelial cell function, establishment and maintenance. First, we will show the similarities and differences between invertebrate and vertebrate columnar epithelial cells. Second, we will depict the current knowledge of polarity protein complex regulation, highlighting the achievements derived from work in *Drosophila*. Finally, we will provide examples that nicely show how easy *in vivo* studies on epithelial cells can be performed using *Drosophila*.

This chapter will not only give a background on epithelial cell polarity regulation, but it will highlight the importance of whole organismal studies for the understanding of epithelial tissues. Furthermore it will reveal the value of a model system like the fruit fly when deciphering mechanisms underlying biological processes.

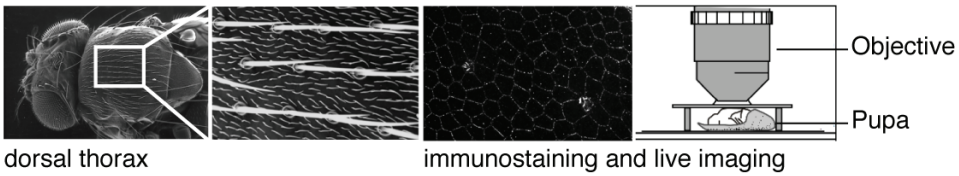

Fig. 1. Dorsal thorax of *Drosophila melanogaster*.

The dorsal thorax serves as a model system for epithelial maintenance and can easily be analyzed by immunostaining or non-invasive live imaging, using for example the dorsal thorax of the immobile, developing pupa.

2. Establishment of epithelial cell polarization in metazoans

Epithelial cells have an adhesive belt that encircles the cells apically, the zonula adherens (ZA). It assembles from the aggregation of spot adherens junctions. Basally, integrin-based focal contacts connect the epithelial cells to the basement membrane. Vertebrate epithelial cells develop a tight junction (TJ) apical to the ZA, which impedes intercellular diffusion and forms a region of close membrane contacts. In *Drosophila*, the functional equivalent to TJs is the septate junction (SJ) that lies basal to the ZA. A domain with similar protein composition as found in TJs is located apically of the ZA in the subapical region (SAR). Only a single junction, the *C. elegans* apical junction (CeAJ), has been identified in *C. elegans*, which resembles the ZA of *Drosophila* and vertebrates (Knust & Bossinger, 2002; Tepass et al., 2001) (Fig. 2). Thus, the overall structure of epithelial cells is highly conserved between species. The polarity protein complex located close to the ZA, the junctional Par complex, has been shown to play an essential role in the establishment of epithelial polarity.

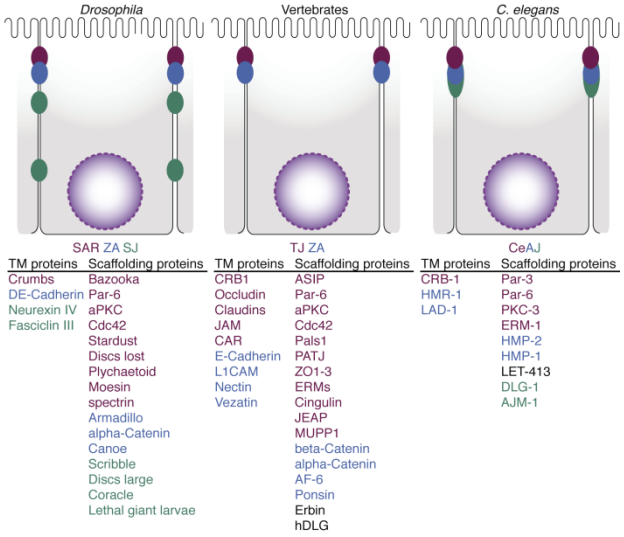

Fig. 2. (adapted from Knust & Bossinger, 2002). Epithelial cell characteristics and typical polarity protein composition for *Drosophila*, vertebrate and *C. elegans* epithelial cells.

The transmembrane (TM) and scaffolding proteins are color-coded depending on their localization. SAR: subapical region; ZA: zonula adherens; TJ: tight junction; CeAJ: *C. elegans* apical junction.

2.1 The junctional Par complex

About 20 years ago, Kemphues and colleagues identified six Par (partitioning defective) proteins, Par-1 to Par-6, and an atypical protein kinase C (aPKC, known as PKC-3 in *C. elegans* and homolog to human PKCζ) in a screen for partition defective cell division in the one cell stage embryo in *C. elegans*, resulting all in loss of the anterior-posterior axis of the embryo when mutated (Cheng et al., 1995; Kemphues et al., 1988; Kirby et al., 1990). Five of the Par proteins - all but Par-2 - as well as aPKC are highly conserved throughout the animal kingdom and are needed for cell polarization. Par-1, Par-4 (also known as LKB1) and aPKC are kinases, Par-3 and Par-6 are PDZ (PSD95, DlgA, ZO-1)-domain containing proteins and Par-5 is a 14-3-3 protein. Par-3, Par-6 and aPKC form a complex localized at the anterior cell cortex of the one cell stage *C. elegans* embryo, while Par-1 and Par-2 remain at the posterior cortex and Par-4 and Par-5 localize uniformly at the cortex. The polarized localization of these proteins is triggered upon fertilization by sperm entry, which enriches the RacGAP CYK-4 (Cytokinesis defect-4) at the posterior pole to give a spatial cue for polarity. CYK-4 functions as a GTPase activating protein (GAP) for small GTPases like Rho, Cdc42 or Rac. Thus its localized activity leads to a gradient of acto-myosin via the inactivation of small GTPases, which distributes the Par proteins in a polarized manner (Jenkins et al., 2006) (Fig. 3). One of the essential functions of the Par complex comprised of Par-3, Par-6 and aPKC, is to set-up epithelial polarity in metazoans in response to the formation of initial cell-cell contact or discrete membrane domains, whereas the basolateral Par complex (Par-1) functions to promote the expansion of the lateral membrane. In general, Par proteins have been found to regulate cell polarization in many different contexts in diverse animals: in epithelia, in directed cell migration, in polarized

cells like neurons or in self-renewing cells; suggesting that they form part of an ancient and fundamental mechanism of cell polarization.

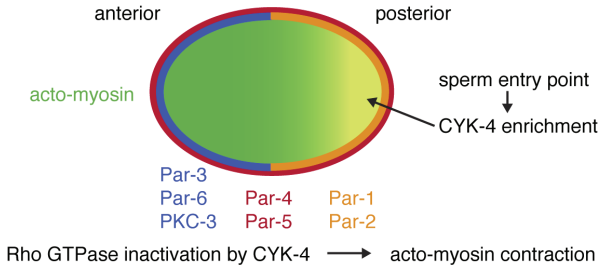

Fig. 3. Par protein distribution in the one cell stage *C. elegans* embryo.

Sperm entry marks the posterior pole and leads to an enrichment in CYK-4 protein, which functions as a GAP for Cdc42, Rho and Rac, thus leading to a gradient of acto-myosin and to the spatial restriction of the different Par complexes.

2.2 The apical Crumbs complex

The apical region of polarized epithelial cells harbors Crumbs (Crb), a transmembrane protein with 30 EGF-like repeats in the extracellular domain that binds with its intracellular domain to the MAGUK (membrane-associated guanylate kinase) protein Pals1 (protein associated with Lin7; also known as Stardust in *Drosophila*). Crb also recruits PATJ (Pals1-associated tight junction protein) into the most-apical complex (Tepass & Knust, 1993). Pals1 and PATJ are cytoplasmic scaffolding proteins with several protein-protein interaction domains including L27 domains, SH3 (Src homology 3) domains, guanylate kinase (GUK) domains and PDZ domain. Overexpression or siRNA-mediated down-regulation of any of the three components in mammalian epithelial cells leads to defects in AB polarity formation suggesting the requirement of the complex as a whole for the proper development of cell polarity (Roh et al., 2003; Shin et al., 2005; Straight et al., 2004).

2.3 Basolateral domain: Lethal giant larvae, Discs large and Scribble

The basolaterally localized proteins Lethal giant larvae (Lgl), Discs large (Dlg) and Scribble (Scrib) cooperatively regulate cell polarity, junction formation and cell growth in epithelial cells. All three genes were identified as tumor suppressors in *Drosophila*. Lgl has at least four WD40 repeats, forms homo-oligomers and associates with the cytoskeleton by binding to non-muscle myosin II. Dlg contains three PDZ domains, a SH3 domain and a GUK domain. Scrib is a LAP (leucine-rich repeats and PDZ domain) protein, containing 16 leucine-rich-repeats (LRRs) and 4 PDZ domains (Yamanaka & Ohno, 2008).

2.4 Adherens junctions proteins

The adherens junctions harbor the E-Cadherin/β-Catenin/α-Catenin (E-Cad/β-Cat/α-Cat) complex that is indispensable for establishing and maintaining cell-cell adhesion. Cadherins are a large family of transmembrane glycoproteins that form homophilic, calcium-dependent interactions with neighboring cells (Gumbiner et al., 1988). E-Cadherin is the predominant epithelial isoform of cadherin. Its extracellular domain is composed of five

ectodomain modules (EC1-EC5), with the most membrane-distal module (EC1) mediating binding with the E-Cad on the adjacent cell. Calcium ions bind between the EC domains to promote a rod-like conformation required for trans-interactions (Fig. 4). The cytoplasmic tail binds to β-Cat (known as Armadillo, Arm in *Drosophila*) in order to link E-Cad to α-Cat in metazoans (Pacquelet & Rørth, 2005). Arm is furthermore linked to the Par complex in *Drosophila* by binding to Bazooka (Baz), the *Drosophila* Par-3 homolog (Capaldo & Macara, 2007; Oda et al., 1994).

Stable epithelial adhesions require the F-actin network (Cavey et al., 2008) and the E-Cad/β-Cat/α-Cat complex is linked to actin via α-Cat, vinculin and α-actinin. It is a matter of debate whether the α-Cat-actin interaction indeed stabilizes the AJ complex since it has been shown in mammalian cells that α-Cat binds only in its homodimeric state to actin, whereas its binding to β-Cat is restricted to the monomeric form. Thus, the interaction of the E-Cad/β-Cat/α-Cat complex with the actin cytoskeleton is dynamic by way of association and dissociation of α-Cat with the complex and with actin filaments. Homodimeric α-Cat directly regulates actin-filament organization by suppressing Arp2/3 mediated actin polymerization, most likely by competing with the Arp2/3 complex for binding to actin filaments (Drees et al., 2005; Yamada et al., 2005). The observations made by Drees, Yamada and colleagues thus suggests a 'dynamic stabilization' of the AJ complex through actin-α-Cat interactions. In the light of a developing epithelial tissue, where a modulation of the cell-cell contacts needs to take place in order to allow for cell rearrangements and growth without compromising epithelial integrity and barrier properties (Classen et al., 2005), such a dynamic interaction between the stabilizing actin cytoskeleton and the AJ complex seems to be indispensable. Thus, this suggests the existence of adjustable mechanisms stabilizing the adherens junctions proteins at the junctions allowing for plasticity of the epithelial tissue; mechanisms that needed to be further elucidated *in vivo* to completely understand epithelial development and maintenance and associated E-Cad transport and turnover.

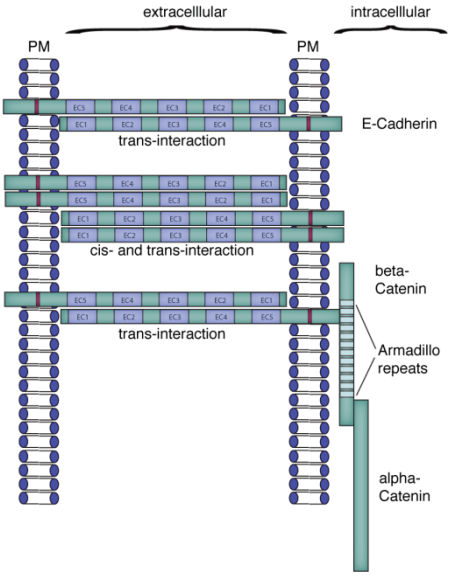

Fig. 4. E-Cadherin/β-Catenin/α-Catenin interaction.

β-Catenin binds to the cytoplasmic tail of E-Cadherin. α-Catenin binds to the N-terminal region of β-Catenin. E-Cadherin molecules can interact with each other in the extracellular space in cis and in trans, both interactions need Ca^{2+}. PM: plasma membrane.

3. Establishment of epithelial cell polarization in *Drosophila*

In *Drosophila*, the syncytial blastoderm gives rise to the first epithelium during cellularization. Membrane invaginations form, the so-called furrow canals, which already display discrete domains apical and lateral to the nuclei (Foe & Alberts, 1983; Mavrakis et al., 2009; Schejter & Wieschaus, 1993). Baz localizes below aPKC and Par-6 as the epithelium forms and its positioning is independent of aPKC and Par-6 but dependent on cytoskeletal cues given by the apical scaffold and dynein-mediated basal-to-apical transport as well as by cues that still need to be elucidated. Baz recruits Par-6 and aPKC, and subsequently Baz and Par-6 recruit Crb and PATJ respectively. aPKC in turn stabilizes apical Crb. Par-6 positioning is dependent on aPKC and on activated Cdc42 (Harris & Peifer, 2005). A transient basal adherens junction made up of ZA proteins E-Cad, Arm, α-Cat and PATJ forms and its assembly is coupled to correct Baz positioning (Hunter & Wieschaus, 2000). Once cellularization is complete, the lateral dispersed spot-like adherens junctions coalesce apically to form the belt-like ZA. In the SAR, Crb binds to Stardust (Sdt, Pals1 homolog of *Drosophila*) via its C-terminus and recruits PATJ into the complex. Crb also provides a link to the apical membrane cytoskeleton, which might reinforce the ZA, by binding to *Drosophila* Moesin (membrane-organizing extension spike protein) via its FERM (4.1, Ezrin, Radixin, Moesin) binding site and to spectrin (Médina et al., 2002). Spectrin is a tetrameric actin crosslinking protein. The spectrin cytoskeleton composition differs in the apical and basolateral domain of epithelial cells, thus giving spatial cues. The basolateral localization of the proteins Lgl, Dlg and Scrib depends on the presence of each of the three proteins and a failure of localization leads to the expansion of the apical Crb complex into more lateral domains. Consequently, the ZA does not form correctly, leading to multilayering of cells. Dlg and Scrib localize to the basolateral domain and the septate junctions just below the ZA, whereas Lgl co-localizes only partially with them below the septate junctions. Lgl is also found in the cytoplasm (Bilder & Perrimon, 2000; Bilder et al., 2000; Woods & Bryant, 1991).

Thus, epithelial polarity establishment has been extensively studied in *Drosophila*, which is due to the fact that the syncytial blastoderm-to-epithelium transition can be easily studied in wild-type and mutant conditions. Freshly laid eggs are collected from the flies and subsequently staged to observe cellularization (defects) at different timepoints.

4. Establishment of epithelial cell polarization in vertebrates

The TJs of vertebrate epithelial cells separate apical and basolateral membrane domains and harbor the transmembrane proteins occludin, claudin family members and junctional adhesion molecules (JAMs) (Fig. 2). All TJ proteins interact directly with cytoplasmic, PDZ-domain containing proteins like ZO-1, ZO-2, ZO-3 (Zonula occludens 1-3), Par-3 and Pals1. These cytoplasmic proteins recruit other cytoskeletal (F-actin) or signaling molecules (Mertens et al., 2005).

Many results explaining epithelial polarity establishment in vertebrates rely on a cell-based system: In MDCK (Madin-Darby canine kidney) cells, ZO-1 binds to the C-terminus of claudins and JAM via two of its PDZ domains and Par-3 directly associates with the C-terminus of JAM. Subsequently, and similar to what has been shown in *Drosophila*, Par-6, aPKC and Cdc42 are recruited to Par-3 and form a complex that is needed for the development of normal tight junctions (Afonso & Henrique, 2006; Chen & Macara, 2005; Joberty et al., 2000). Claudins play a central role in polarity establishment, since they also interact with the Crb complex: PATJ, whose expression depends on Pals1, interacts with claudin and ZO-3 via two of its PDZ domains and with Pals1 via its N-terminus. Pals1 interacts with its PDZ domain with the cytoplasmic tail of Crb, which reinforces the association of Crb with the Par complex (Lemmers et al., 2003; Straight et al., 2004).

5. Regulation of cell polarity in the epithelium

5.1 Regulation of the junctional Par complex

As described above, Par-3/Baz recruits Par-6 and aPKC in vertebrates and *Drosophila* and this has been confirmed by ectopic expression of Par-3, which leads to ectopic Par-6 and aPKC recruitment in mammalian cells (Joberty et al., 2000), indicating a general mechanism for this recruitment. Par-6 also plays an important role in Par complex localization and activation since it interacts with Cdc42 via its PDZ and semi-CRIB domain, with Par-3/Baz via its PDZ domain and with aPKC through its PB1 (Phox and Bem1) domain. Concomitant binding to aPKC and Cdc42 causes a conformational change in Cdc42-GTP, leading to aPKC activation (Hutterer et al., 2004; Joberty et al., 2000). Activated aPKC in turn is needed for correct Par-3 localization in mammalian cells and has been shown to spatially regulate the basolateral protein complex in *Drosophila*. aPKC phosphorylates Par-3 which in turn dissociates from the Par complex, possibly allowing the regulative interaction of aPKC with other proteins and thus leading to correct cell-cell contact formation and epithelial polarization (Joberty et al., 2000; Nagai-Tamai et al., 2002). At the basolateral side, Par-6 localization is excluded by Lgl in *Drosophila*, however the exclusion of the Par complex in mammalian cells has been shown to be regulated by Par-1 kinase activity: Par-1 phosphorylates Par-3, which leads to the binding of Par-5 (14-3-3) to Par-3 and thus inhibits the formation of the Par complex by blocking Par-3 oligomerization and binding to aPKC (Benton & St Johnston, 2003). Par-1 activation is regulated by Par-4 through phosphorylation of the activation loop of the Par-1 kinase domain (Suzuki & Ohno, 2006) and it is inactivated by phosphorylation by aPKC, which causes binding to Par-5 (14-3-3) and inhibition of plasma membrane binding in the apical domain (Hurov et al., 2004) (Fig. 5).

Proteins that have so far not been linked to epithelial polarity can of course also affect the functionality of the junctional Par complex, and in the upcoming years new regulators will for sure be identified. One example for such a regulation is protein phosphatase-2A (PP2A), a heterotrimeric serine/threonine phosphatase with broad substrate specificity and diverse cellular functions like cell growth and regulation of the cytoskeleton. PP2A inhibits aPKC function and dephosphorylates TJ components thereby triggering junction disassembly in mammalian cells (Nunbhakdi-Craig et al., 2002) (Fig. 5).

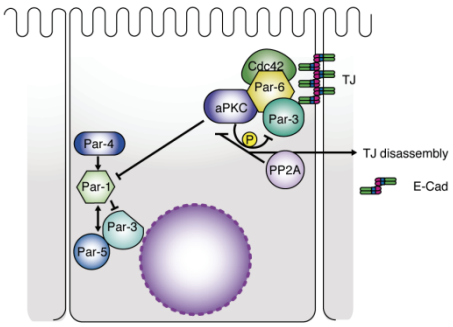

Fig. 5. Regulation of the junctional Par complex in mammalian cells.

aPKC inhibits the function of Par-1, which inactivates Par-3 in the basolateral domain. Par-4 activates Par-1. Cdc42 binds to Par-6 and thereby activates the Par complex. aPKC phosphorylates Par-3, which then dissociates from the Par complex. PP2A blocks aPKC function and leads thus to TJ disassembly. TJ: tight junction; PP2A: protein phosphatase-2A.

5.2 Regulation of the junctional E-Cad/β-Cat/α-Cat complex

The junctional E-Cadherin/β-Catenin/α-Catenin complex is needed for cell-cell adhesion in epithelial cells and its localization and maintenance must thus be strictly regulated. In mammalian cells, disassembly of the apical junctional complex is driven by reorganization of apical F-actin involving cofilin-1-dependent depolymerization and Arp2/3-assisted repolymerization as well as myosin II mediated contraction (Ivanov et al., 2004). Actin organization, myosin II phosphorylation and therefore localization and regulation of gene transcription and E-Cad localization is affected by Rho, which counteracts Cdc42 and Rac activity and thus inhibits AJ formation (Sturge et al., 2006). Cdc42, Rac and Rho are indispensable for epithelial polarity regulation at the junctional domain. Cdc42 promotes Par complex formation in vertebrates and *Drosophila* as depicted above and it promotes TJ development by activation of Rac in mammalian cells. Rac is also activated at the junctional domain through the GEF Tiam1 (T-cell lymphoma invasion and metastasis-1), which also binds to Par-3, providing a link between Rac and the junctional polarity protein complex. This activation is needed to counteract Rho activity, since Rho favors TJ disassembly through Rho kinase (ROCK) mediated myosin II phosphorylation (van Leeuwen et al., 1999). In *Drosophila*, Cdc42/Par-6/aPKC furthermore regulate E-Cad endocytosis, by recruiting and interacting with the actin and dynamin machinery needed for vesicle scission (Georgiou et al., 2008, Harris & Tepass, 2008, Leibfried et al., 2008) (Fig. 6).

Myosin II is a motor that converts chemical energy of ATP into mechanical forces, mediating the contractility of the actin cytoskeleton. It is activated by phosphorylation of its light chain through ROCK or MLCK (myosin light chain kinase). Rho activity is therefore down-regulated at the AJs in polarized epithelial cells via the interaction of the GAP p190RhoGAP and the catenin p120-Catenin. p190RhoGAP translocation to the AJs is mediated by Rac activity, a process taking also place in *Drosophila* epithelial cells, thus suggesting a general and not organism-specific down-regulation of Rho at the adherens junctions (Magie et al., 2002; Wildenberg et al., 2006). In mammalian cells, Rac has been shown to recruit actin to sites of cell-cell contacts, where it leads to the internalization of the E-Cad/Cat complex

(Akhtar & Hotchin, 2001). Therefore, Rac seems to have a dual role in E-Cad/Cat complex maintenance by recruiting p190RhoGAP and at the same time leading to E-Cad/Cat internalization at the junctions.

Echinoid (Ed) is a component of *Drosophila* AJs that stabilizes the adhesion complex through cooperation with E-Cad and by linking the AJs to actin filaments. The C-terminal PDZ domain of Ed furthermore binds to Baz, which leads to a strong linkage between the Par complex and the AJs, since E-Cad is also bound to Baz via its interaction with Arm (Laplante & Nilson, 2006; Wei et al., 2005). Moreover, actin filaments are organized by a pathway that is regulated by Bitesize, a synaptogamin-like protein that binds to Moesin and PIP2 at the apical domain, leading to stabilization of E-Cad at the AJs (Pilot et al., 2006). These results show that the regulation of the adhesion complex depends strongly on small GTPases and the underlying acto-myosin network as well as on protein-protein interactions between adhesion and junctional polarity proteins. Thus they form part of an ancient and fundamental mechanism of cell polarization.

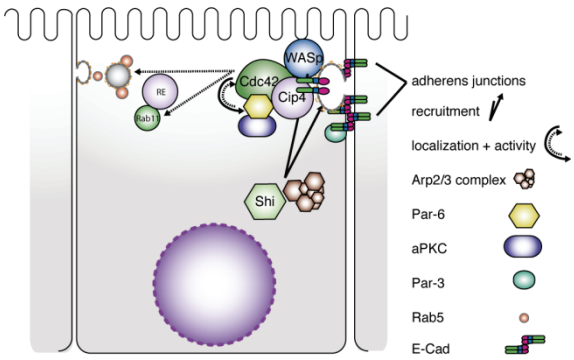

Fig. 6. Regulation of E-Cad endocytosis at the AJs in *Drosophila* epithelial cells.

Cdc42 most likely recruits together with Par-6 and aPKC the actin regulators Cip4 and WASp as well as dynamin, which is needed for endocytic vesicle scission, therefore allowing for correct endocytosis of junctional material.

5.3 Regulation of the apical Crumbs complex

The correct localization of the Crb complex depends on the Crb/Pals1/PATJ proteins themselves, on motor proteins, the Par complex and on regulation of the cytoskeleton. Most of our current knowledge is based on studies conducted on *Drosophila* epithelial cells: The apical localization of the Crb complex is highly dependent on its individual components. Crb localization depends on PATJ and on the transport of Crb protein and transcript by a cytoplasmic dynein complex. PATJ localization is in turn partially dependent on apical Crb localization, possibly resulting in a positive feedback loop between Crb and PATJ targeting (Horne-Badovinac & Bilder, 2008; Li et al., 2008; Michel et al., 2005; Tanentzapf et al., 2000). This positive regulation might be antagonized during later stages of epithelial development by the FERM protein Yurt in order to prevent an expansion of the apical domain. Yurt is localized at the basolateral domain, also in mammalian epithelial cells, but Crb recruits it to the apical membrane late during epithelial development where it counteracts Crb activity (Laprise et al.,

2006). Crb also recruits Pals1 to the apical domain and the same dynein complex used for Crb apical targeting can transport Pals1 transcript. The junctional Par complex regulates the Crb complex by phosphorylation. aPKC can interact with Crb in presence of PATJ and Pals1. As a consequence, Crb gets phosphorylated which is indispensable for correct membrane targeting of Crb and PATJ and for Crb activity (Sotillos et al., 2004). PATJ localization and ZA formation depend furthermore on Par-6 activity (Hutterer et al., 2004).

The apical and basolateral complex regulate each other and loss of the basolateral complex leads to the expansion of the Crb complex and loss of polarity, which can be rescued by the concomitant loss of the Crb complex (Bilder et al., 2003; Tanentzapf & Tepass, 2003). The discrete membrane domains are interdependent not only during epithelial establishment but also during its maintenance. For example loss of adherens junctions proteins E-Cad and Arm leads to loss of Crb and apical polarity via the disruption of the lateral spectrin and actin cytoskeleton (Tanentzapf et al., 2000), therefore resulting in a complete loss of polarity.

5.4 Regulation of the basolateral Lgl complex

Scrib stabilizes the AJ complex in the junctional domain, but Lgl localization is accurately restricted to the basolateral domain by aPKC. Lgl, aPKC and Par-6 can interact, leading to the phosphorylation of Lgl by aPKC at three conserved serine residues in mammalian cells and *Drosophila* (Betschinger et al., 2003). Phosphorylation inactivates Lgl on the apical side and inhibits its binding to the plasma membrane in both mammalian and *Drosophila* epithelial cells by changing the conformation of the protein (Betschinger et al., 2005; Plant et al., 2003). Lgl and aPKC seem to mutually regulate each other, since loss of Lgl (leading to an overproliferation phenotype) can be suppressed by concomitant loss of aPKC in *Drosophila* (Rolls et al., 2003) (Fig. 7). In mammalian cells, Lgl phosphorylation by aPKC is furthermore facilitated by the concomitant interaction of Lgl with P32 (Bialucha et al., 2007). Thus, the interplay between junctional and basolateral proteins maintains distinct membrane domains in polarized epithelial cells.

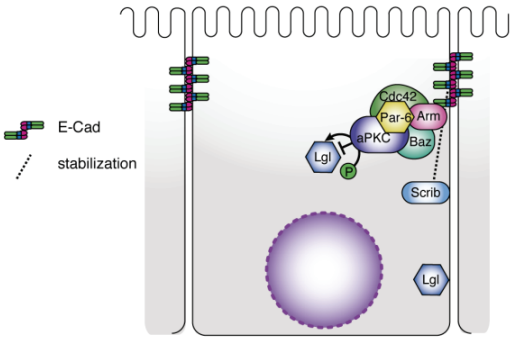

Fig. 7. Regulation of basolateral Lgl and stabilization of E-Cad by Scrib in *Drosophila* epithelial cells.

aPKC phosphorylates Lgl, which impedes the binding of Lgl to the cortex in the junctional and apical domain. Scrib leads to the stabilization of E-Cad. Arm: Armadillo; Baz: Bazooka; Lgl: Lethal giant larvae; Scrib: Scribble.

5.5 Regulation of epithelial polarity via TGF-β and EGF signaling

TGF-β and EGF signaling pathways are two out of several important signaling pathways needed for the correct development of an organism. They have an influence on epithelial polarity and can lead for example to epithelial to mesenchymal transition (EMT). Therefore, the inhibition of both pathways is important for epithelial polarity maintenance.

Transforming growth factor beta (TGF-β; Decapentaplegic, Dpp in *Drosophila*) controls proliferation and cellular differentiation in most cells and TGF-β signaling can lead to loss of cell polarity: in mammalian cells, the TGF-β downstream effector Rho controls EMT by changing the actin cytoskeleton (Ozdamar et al., 2005), whereas the downstream transcription factors of the Snail family concomitantly lead to down-regulation of claudins, occludin, E-Cad and Crb (Xu et al., 2009). Therefore, to maintain epithelial polarity, Par-6 interacts with the TGF-β receptor, which phosphorylates Par-6 so that it can recruit the E3 ubiquitin ligase Smurf1. Smurf1 leads to degradation of Rho and thus to a block of EMT. Snail protein activation is blocked by Smad6 and Smad7, thereby preventing down-regulation of TJ and apical proteins.

Epidermal growth factor (EGF) signaling also plays an important role in the regulation of cell growth, proliferation and differentiation and it can modulate mammalian TJ formation, which allows for concerted dissociation and re-establishment of cell-cell adhesion essential for morphogenesis. Two different pathways achieve this modulation. EGF signaling activates Src (sarcoma) family kinases that phosphorylate TJ proteins, including ZO-1, ZO-2, occludin, E-Cad and Par-3, leading to positive and negative regulation of TJ formation in mammalian cells (Chen et al., 2002; Shen et al., 2008). The down-regulation of E-Cad by Src promotes EMT because it alters E-Cad trafficking by redirecting E-Cad from a recycling pathway to a lysosomal pathway (Shen et al., 2008). In both mammalian and *Drosophila* cells, EGF signaling also induces the MAPK (mitogen-activated protein kinase) pathway which leads to E-Cad and claudin expression and subsequent translocation of junctional proteins from the cytoplasm to cell-cell contacts (O'Keefe et al., 2007; Wang et al., 2006). E-Cad and EGF receptor interact at cell-cell contacts to negatively regulate the MAPK pathway in mammalian cells, suggesting a general negative feedback loop to regulate adhesion and junctional integrity (Qian et al., 2004). These results suggest that EGF signaling needs to be tightly controlled to promote either EMT or the stabilization of junctions and they emphasize the need of a strict regulation of signaling pathways in the cell in order to maintain epithelial integrity.

5.6 Regulation of epithelial polarity via phosphatidylinositol signaling

Recent studies integrate phosphatidylinositol-phosphate signaling to polarization in both mammalian and *Drosophila* epithelial cells. Phosphatidylinositol-phosphate signaling was mainly known to regulate cell size by interacting with the insulin receptor, but Martin-Belmonte, von Stein and colleagues propose that it also enhances polarity establishment.

PDZ domains can bind to phosphatidylinositol lipid membranes and *Drosophila* Baz binds to both, phosphatidylinositol lipid membranes and to the phosphatase PTEN (phosphatase and tensin homolog), thereby possibly recruiting this protein to the apical domain (von Stein et al., 2005). PTEN converts PIP3 (Phosphatidylinositol-(3,4,5)-triphosphate) to PIP2 (Phosphatidylinositol-(4,5)-bisphosphate) and this leads to Cdc42 recruitment via Annexin-2 and subsequently to aPKC recruitment to the apical domain in mammalian cells (Martin-Belmonte et al., 2007). PIP3 is produced by activation of PI3K (phosphatidylinositol-3

kinase) at the adherens junctions, as shown in *Drosophila* epithelial cells, which locally activates Cdc42 and recruits more E-Cad to the junctions (von Stein et al., 2005). This suggests a general model where the Par complex recruits PTEN by binding to phosphatidylinositol lipid membranes, and where PTEN converts PI3K-produced PIP3 to PIP2. This might mediate the establishment of epithelial polarity by the recruitment of Cdc42, aPKC and E-Cad (Fig. 8).

PIP2 and PIP3 regulate furthermore the actin cytoskeleton: PIP2 binds to actin-associated proteins that link the actin cytoskeleton to the plasma membrane or to proteins that are involved in the initiation of *de novo* actin polymerization, and PIP3 activates WASp family proteins (like WASp, WAVE and WASH proteins) and the Arp2/3 complex via interaction with Rho GTPases (Fig. 8). These results point toward a spatio-temporal fine-regulation of Cdc42 recruitment during polarization and a coeval regulation of cell polarity establishment and cytoskeleton controlled by the balance between PIP2 and PIP3, leading to an enhanced effect for both Cdc42-mediated pathways.

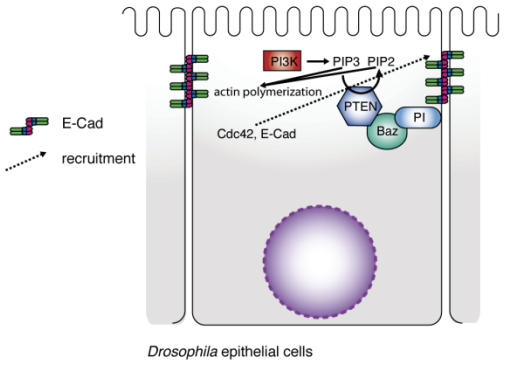

Fig. 8. Regulation of epithelial polarity via phosphatidylinositol signaling.

Baz can bind to PI and to PTEN. PTEN mediates PIP3 to PIP2 conversion, which leads to the recruitment of Cdc42 and E-Cad. PIP3, which is generated by PI3K activity in the apical domain, and PIP2 lead to actin polymerization at the junctions. PI: phosphatidylinositol; PIP3: Phosphatidylinositol-(3,4,5)-triphosphate; PIP2: Phosphatidylinositol-(4,5)-bisphosphate; PI3K: phosphatidylinositol-3 kinase; Baz: Bazooka.

5.7 Regulation of epithelial polarity via the acto-myosin cytoskeleton

Cell polarity also requires fine regulation of the cytoskeleton. Actin is needed for the furrow-canal formation during cellularization in *Drosophila* and AJ proteins are linked to the actin cytoskeleton. Furthermore, spectrin has a crucial role in anchoring Crb at the apical domain (Tanentzapf et al., 2000). In general, the regulation of the actin cytoskeleton is dependent on the activity of Rac and Cdc42, which both lead to actin nucleation upon their activation. Downstream of Rac and Cdc42 are WASp family proteins, which are activated through binding to the Cdc42 and Rac-binding domain (CRIB). WASp protein activity leads to actin nucleation by activation of the Arp2/3 complex. In mammalian cells, the binding of Cdc42-GTP and PIP2 to WASp synergistically enhances the activation of the protein (Parsons et al., 2005) and WASp upregulates the GEF activity of the Cdc42-

specific GEF intersectin (Malacombe et al., 2006), suggesting a positive feedback loop between Cdc42 and WASp activity. Cdc42 is also directly linked to epithelial polarity via the junctional Par complex (see 5.2). Downstream of Cdc42 is furthermore PAK1, which promotes microtubule formation, thus localizing actin nucleation and microtubule formation to the same confined space (Parsons et al., 2005), possibly needed for correct plasma membrane identity in polarized cells. Cell polarity is furthermore maintained by the AMP-activated protein kinase (AMPK), which alters the acto-myosin cytoskeleton in response to energetic stress situations. High AMP levels lead to a conformational change of the protein and Par-4 phosphorylates AMPK to activate it as indicated by biochemical data (Hawley et al., 2003). In *Drosophila*, Dystroglycan, which is localized at the basal domain, where it interacts with the extra-cellular matrix protein Perlecan, transduces a signal from the cellular energy sensor AMPK to myosin II, thus activating myosin II and regulating AB polarity (Mirouse et al., 2009). To conclude, regulation of both the actin cytoskeleton and myosin II by GTPases and polarity proteins seems to be indispensable for maintaining AB polarity.

6. *In vivo* studies on epithelial cells using *Drosophila*

As depicted above, most of our hitherto knowledge of epithelial polarity stems from genetic and biochemical studies performed in cell culture or on model systems. Though extensive, these two approaches cannot reveal all mechanisms underlying the process of polarization. Cell polarity is not only needed for the establishment and maintenance of the single cell, but also for the correct development of the whole multicellular organism. Some events, like spatio-temporal protein or organelle localization can only be captured when analyzing a whole, developing, epithelium. Whole tissue analysis can be easily performed using *Drosophila*: epithelia can be imaged *in vivo* by confocal microscopy (see Fig. 1) while the animal develops. This imaging techniques and the vast genetic tools available (e.g. gene knock-down or over-expression, expression of fluorescent tagged proteins in the living fly) have allowed for the dissection of new mechanisms regulating epithelial polarity.

6.1 Junction-cytoskeleton interaction dissected *in vivo*

The interaction of α-Catenin with E-Cad and the cortical actin control both stability and remodeling of adhesion. How this occurs is, as mentioned in section 2.4., not elucidated. Live imaging of *Drosophila* embryos expressing fluorescent E-Cad and actin (F-actin) revealed that E-Cad is not evenly distributed around the adhesion belt in epithelial cells as previously expected. Stable microdomains intersperse with mobile domains. Laser nano-ablation of actin and FRAP (Fluorescence Recovery After Photobleach) and photo-conversion experiments for E-Cad show that the stability and mobility of these microdomains depend on two actin populations, a stable network and one that rapidly turns over (Cavey et al., 2008).

Further *in vivo* studies using FRAP and nano-ablation show that the myosin-II forces needed for actin remodeling at the junctions is not as previously assumed based on polarized activity of junctional myosin-II, but by the polarized flow of medial actomyosin pulses towards a specific junctional domain (Rauzi et al., 2010).

6.2 Planar cell polarity mechanisms dissected *in vivo*

The morphogenesis and function of an epithelial tissue relies on the precise arrangement of its constituent cells. Tissue patterning and organization during development depends on the establishment of concentration gradients of signaling molecules along the tissue that furthermore can lead to the formation of polarized structures in the plane of the epithelium (like hairs). This type of polarization of a field of cells is referred to as planar cell polarity (PCP), where the spatial information that organizes planar polarity is transmitted locally from one cell to the next (for review see Seifert & Mlodzik, 2007). Thus, epithelial tissues not only show an apico-basal polarity, but also a positional oriented appearance in the plane in order to generate polarized structures and to orient themselves in a directed fashion. An evolutionary conserved set of genes control establishment of planar polarity in flies and vertebrates, the core Frizzled/PCP (Fz/PCP) factors Flamingo (Fmi), Strabismus (Stbm), Dishevelled (Dsh), Diego (Dgo), Fat, Dachsous (Ds) and Prickle (Pk) (Jenny et al., 2005). The Fz/PCP factors polarize a field of cells along a specific axis. Local differences in Fz activity between neighboring cells provide directional information required for planar polarity, in other words a gradient of the morphogen is needed for correct PCP. As a result, wing hairs point away from the site of highest Fz activity in *Drosophila* (Adler et al., 1997).

Drosophila wing epithelial cells are irregularly arranged throughout most of development, but they become hexagonally packed shortly before hair formation. PCP proteins promote hexagonal packing in the *Drosophila* wing by polarizing membrane trafficking (Classen et al., 2005). Planar polarity arises during growth due to a cell flow that is triggered by tension arising from the wing hinge contraction. *In vivo* imaging of *Drosophila* wing epithelial cells expressing fluorescent polarity proteins Stbm and E-Cad led to these observations, that would be very difficult to dissect *in situ* or *ex vivo* (Aigouy et al., 2010).

6.3 Asymmetric cell division of epithelial cells dissected *in vivo*

Planar cell polarity proteins, amongst others, are also needed to align the mitotic spindle correctly in order to allow for oriented mitosis to occur. Live imaging of the spindle in the *Drosophila* dorsal thorax using fluorescent microtubule components have revealed the mechanism that keeps the spindle in the correct plane and correct apico-basal tilt. This positioning controls the correct asymmetric cell division needed in the mechanosensory organ precursor cell, which resides in the dorsal thorax. Mutations in either Fz/Dsh or the NuMa homolog Mud as well as mutations in Ric-8, a guanine nucleotide-exchange factor for heterotrimeric G proteins, result in the mis-orientation of the spindle during division and the subsequent mal-formation of the sensory organ (David et al., 2005, Ségalen et al., 2010). The alignment of the spindle during mitosis can only be captured using *in vivo* studies and the underlying mechanism would have stayed unknown without the use of *Drosophila* for *in vivo* imaging and the vast genetic techniques available for this model system.

7. Concluding remarks

Epithelial cell function relies in both vertebrates and invertebrates on the tight regulation of the underlying polarity protein machinery, which is highly conserved (Leibfried, 2009). This regulation has been analyzed in epithelial cells in cell culture, but an analysis in a whole living organism, integrating all cellular and environmental cues that an epithelium is exposed to, remains an interesting task, comprising the truth about epithelial function and

the regulatory networks needed for it. The use of *Drosophila* as a model system allows us to better study epithelial establishment, maintenance and plasticity in the context of a whole organism. Furthermore, green-fluorescent-protein (GFP) and its derivates give us the opportunity to analyze epithelial cell function in a spatio-temporal manner by live imaging. Thus, *Drosophila* will also in the future help to better understand epithelial establishment, maintenance and plasticity thanks to today's microscopy imaging and manipulation techniques, the extensive genetic tools available and the feasibility to study the epithelium in the context of the whole organism.

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Development and Cell Polarity of the *C. elegans* Intestine

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1. Introduction

1.1 The nematode *C. elegans* as a model organism

Much of our knowledge on development of multicellular organisms and the underlying cellular and molecular processes is derived from the studies of model organisms, like *C. elegans*, *Drosophila*, *Xenopus*, zebrafish and mouse. These model organisms were selected based on their amenability to experimental studies.

In 1963, Sydney Brenner realized that "Part of the success of molecular genetics was due to the use of extremely simple organisms which could be handled in large numbers: bacteria and bacterial viruses." He further argued "…..that the future of molecular biology lies in the extension of research to other fields of biology, notably development and the nervous system". Thus, he proposed to the Medical Research Council: "we want a multicellular organism which has a short life cycle, can be easily cultivated, and is small enough to be handled in large numbers, like a micro-organism. It should have relatively few cells, so that exhaustive studies of lineage and patterns can be made, and should be amenable to genetic analysis.

We think we have a good candidate in the form of a small nematode worm, Caenorhabditis….." (cited after: Wood, 1988).

C. elegans genetics started in October 1967 with Sydney Brenner's first mutant hunt, which produced two mutants showing a "dumpy" and a "variable abnormal" phenotype (Brenner, 2009). In 1974, the article entitled "The genetics of *Caenorhabditis elegans*" (Brenner, 1974) reported a study of 300 EMS-induced mutants and a map of about 100 genes on six linkage groups, which provided an excellent starting point for future *C. elegans* research.

Since that time many key steps towards the total description of *C. elegans* have been undertaken:

- complete description of cellular development (cell lineage, Fig.2) from egg to adult (Sulston and Horvitz, 1977; Sulston et al., 1983)
- complete description of the nervous system: all branches and connections determined (White et al., 1986)

- first use of green fluorescent protein as a marker for gene expression in a multicellular organism (Chalfie et al., 1994; Hunt-Newbury et al., 2007)
- first draft genome sequence of a multicellular organism completed (The_C_elegans_Sequencing_Consortium, 1998)
- basic mechanism of double-stranded(ds) RNA-mediated interference worked out (Fire et al., 1998)
- nearly all predicted genes tested for function by RNAi (Fraser et al., 2000; Gönczy et al., 2000; Kamath et al., 2003)
- comprehensive databases on *WormBase* (http://www.wormbase.org) (Harris et al., 2010), *Wormatlas* (http://www.wormatlas.org) and *WormBook* (http://www.wormbook.org).

In the 1990s, the popularity of *C. elegans* climbed sharply, as indicated by the increase in the number of research publications per year. Thirteen and 744 research articles were published in 1974 and 2009, respectively (Han, 2010). Over the past decade, research on the nematode *C. elegans* was granted three Nobel prizes for groundbreaking discoveries such as programmed cell death (apoptosis), dsRNA-mediated interference and the use of the green fluorescent protein. The Nobel prize for Physiology or Medicine went to H. Robert Horvitz, John Sulston and Sydney Brenner in 2002 (Brenner, 2003; Horvitz, 2003; Sulston, 2003) and to Andrew Fire and Craig Mello in 2006 (Fire, 2007; Mello, 2007). The Nobel prize for Chemistry went to Martin Chalfie (with Osamu Shimamura and Roger Tsien) in 2008 (Chalfie, 2009; Tsien, 2009).

Caenorhabditis elegans is a small, free-living nematode (Blaxter, 2011) that survives by feeding primarily on bacteria. In the laboratory *C. elegans* normally grows at temperatures between 12 °C and 26 °C on agar plates, which are seeded with *E. coli* bacteria as a food source (Fig.1A). The animals can also be grown in liquid culture for biochemical analyses. Starved worm cultures retain their viability for months and strains can be frozen and stored at -80 °C or lower (http://www.cbs.umn.edu/CGC/). Such frozen stocks are stable for > 40 years. *C. elegans* is an important model system for biological research in many fields including genomics, cell biology, neuroscience and aging (http://www.wormbook.org). Among its many advantages for study are its short life cycle (Fig.1B), compact genome (100 x 10^6 base pairs, Fig.1C), invariance in cell number and anatomy, ease of propagation and small size. The simplicity and invariance permit complete and exhaustive descriptions. There are two *C. elegans* sexes: a self-fertilizing hermaphrodite (Fig.1A) and a male. The adult body plan is anatomically simple with about 1031 and 959 somatic cells in hermaphrodites and males, respectively. The *C. elegans* hermaphrodite produces a large number of progeny per adult (> 200) and is amenable to genetic crosses. *C. elegans* can be examined at the cellular level *in vivo* by Nomarski differential interference contrast microscopy, because it is transparent throughout its life cycle. The life cycle is temperature dependent and by a temperature shift from 16 °C to 25 °C the time needed for development can be accelarated about 100% (Fig.1B).

Since 1974, when Sydney Brenner published his pioneering genetic screen (Brenner, 1974), researchers have developed increasingly powerful methods for identifying genes and genetic pathways in *C. elegans* (Jorgensen and Mango, 2002). The long history of *C. elegans* as a genetic model organism means that there are a large number of mutants available. The *C. elegans* Genetics Center (CGC) houses the community collection of *C. elegans* mutant strains and related nematode strains (http://www.cbs.umn.edu/CGC/). Due to the efforts of the *C. elegans* Gene Knockout Consortium (http://www.celeganskoconsortium.omrf.org/) in

the United States and Canada and the National BioResource Project in Japan, deletion alleles have been obtained for about 5,500 out of 20,000 predicted genes (Mitani, 2009; Moerman and Barstead, 2008).

Working with existing mutants can be advantageous for several reasons: **First**, temperature-sensitive conditional alleles allow the analysis of otherwise lethal mutations. They may also provide a way of analyzing gene function during a specific developmental process. **Second**, genetic mutants avoid inconsistencies sometimes observed in RNAi phenotypes that may arise from variability in the bacterial expression of dsRNA or from the amount of bacteria ingested by the worm strain used. **Third**, genetic alleles may encode partially functional proteins or gain-of-function gene products, thus providing additional information about the structure-function features of the gene product.

To further analyze the function of a gene product, it is often helpful to have a complete loss-of-function allele. If such a mutant is not available, there are three knock-out consortiums (see above) that are generating large collections of deletion alleles for the *C. elegans* community. If a knock-out of your gene-of-interest does not exist, one can request a new screen through the websites. With new approaches to generate targeted deletion mutants and to control gene expression the arsenal of methods to investigate gene functions in *C. elegans* is growing (Boulin and Bessereau, 2007; Calixto et al., 2010b; Frokjaer-Jensen et al., 2010; Robert and Bessereau, 2007).

Obtaining strains containing heritable null mutations in every gene (see above) is complementary to RNAi, a so-called reverse genetics approach (Baylis and Vazquez-Manrique, 2011). RNAi in *C. elegans* (Fig.3) was first described in the 1990s (Guo and Kemphues, 1996) and quickly became an important laboratory tool for investigating gene function. RNAi is easily achieved in the worm and the availability of the genome sequence (The_C_elegans_Sequencing_Consortium, 1998) helped to make RNAi the reverse genetics tool of choice, particularly for genome-wide studies of developmental processes (Fraser et al., 2000; Gönczy et al., 2000; Kamath et al., 2003; Sönnichsen et al., 2005). The effectiveness of RNAi in *C. elegans* is even maintained during spaceflight (Etheridge et al., 2011). RNAi seems to be an evolutionary conserved cellular response to dsRNA, and the mechanism is thought to originate from an ancient endogenous defense mechanism against viral and other heterologous dsRNAs (Lu et al., 2005; Schott et al., 2005; Wilkins et al., 2005). In mammalian cells, introduction of dsRNAs longer than 30 bp activates antiviral pathways, leading to nonspecific inhibition of translation and cytotoxic responses.

To inactivate gene expression in early *C. elegans* embryos and to analyze the resulting phenotype, worms can e.g. be fed bacteria expressing dsRNA corresponding to the gene of interest (Fig.3). Because the adult hermaphrodite continuously produces oocytes, pre-existing mRNA is eliminated with each egg that is laid. Embryos born early after the initiation of RNAi are only mildly depleted of the gene product whereas embryos born later are usually highly depleted. The time required for efficient depletion varies among target genes, but generally 24 - 30 hours after the initiation of feeding, mRNA levels are reduced significantly, protein levels are almost undetectable and phenotypes are visible.

A problem often arises when looking for phenotypes by RNAi in later embryogenesis. If the gene product of interest is involved in a developmental process prior to the one to be observed or in multiple cell types, making specificity of the phenotype unclear. Worm

strains that are sensitive to RNAi only in a particular tissue have now been generated (Calixto et al., 2010a; McGhee et al., 2009; Pilipiuk et al., 2009; Qadota et al., 2007). One strategy relies on a genetic background that is resistant to RNAi due to a mutation in an essential RNA processing protein, e.g. RDE-1 (Fig.3) and complementation in the tissue of interest by tissue-specific promoter induction of wild-type protein. Tissue-specific RNAi largely circumvents the problems mentioned but does rely on having promoters that turn on early enough in the tissue to have sufficient depletion by the developmental stage of interest. Nevertheless, RNAi has a few intrinsic limitations. First, RNAi efficiency is sensitive to the experimental conditions, and the result can be variable. Second, residual gene expression persists to an extent that is difficult to predict for a given gene. Third, some tissues are partially resistant to RNAi (Zhuang and Hunter, 2011).

In summary, the discovery of RNAi has led to a much greater reliance on the reverse genetics approach but with the advent of next-generation DNA sequencing technologies and the ensuing ease of whole-genome sequencing are reviving the use of classical genetics to investigate *C. elegans* development (Bowerman, 2011; Hobert, 2010).

1.2 Introduction to epithelial tissues

Epithelia are polarized tissues (Fig.4A) that outline the cavities (e.g. the digestive tract) and surfaces (e.g. the epidermis, Fig.4B-C) of the body (de Santa Barbara et al., 2003; Fuchs, 2007; Noah et al., 2011). They are specialized for secretion, absorption, protection or sensory functions. Polarization of epithelial cells is manifested by distinct apical and basolateral membrane domains, which are separated by cell junctions that form belt-like structures around the apex of the cells (Fig.4A; Knust and Bossinger, 2002; Nelson, 2003; Nelson, 2009; Weisz and Rodriguez-Boulan, 2009). Epithelial cell junctions serve the adhesion, communication, vectorial transport, and morphogenetic properties of epithelia. Two of the most important features for the functions of epithelia are to create a diffusion barrier between two biological compartments and to build a cell adhesion system between their cells. Cell-cell adhesion is regulated by cell-specific mechanical and biochemical constraints. For instance, fibroblasts and neuronal cells are involved in more labile and plastic interactions, whereas endothelial and epithelial cells require a strong adhesion.

During the process of epithelial polarization the organization and maintenance of the boundary between apical and basolateral membranes must be regulated. In vertebrate epithelia, this fence function is established by a specific intercellular junction, the tight junction (TJ; Anderson and Van Itallie, 2009; Ebnet, 2008; Eckert and Fleming, 2008; Tsukita et al., 2001). TJs are the most apical cell junction in vertebrate epithelia and lie adjacent to the more basally localized zonula adherens (ZA; Harris and Tepass, 2010; Wang and Margolis, 2007). TJs provide a fence to lateral diffusion of membrane proteins and a barrier to the diffusion of molecules in between the individual epithelial cells. In invertebrates, TJs have not been found thus far. However, a region just apical to the ZA in *Drosophila* epithelia harbors a probably larger protein complex, called the subapical region (SAR; Bulgakova and Knust, 2009). It has been suggested that one of the functions of this protein complex is the fence function of vertebrate TJs (Müller, 2000; Wodarz et al., 2000). In many invertebrate epithelia the paracellular transport through the epithelium is controlled by a unique invertebrate structure, the septate junction (SJ; Müller and Bossinger, 2003). In the nematode *C. elegans* SJ (Lints and Hall, 2009) have thus far only been found in the spermatheca

epithelium (Pilipiuk et al., 2009), raising the interesting question as to how embryonic epithelia in these animals maintain a diffusion barrier. Claudins with four transmembrane domains are major cell adhesion molecules working at TJs in vertebrates. In *C. elegans* four claudin-related proteins (CLC-1 to -4) exist and two of them, CLC-1 and CLC-2, seem to be involved in the pharynx and epidermis barrier, respectively (Asano et al., 2003).

2. Development and differentiation of the *C. elegans* embryonic intestine

The *C. elegans* digestive tract is one of the most complex portions of the nematode anatomy and is composed of a large variety of tissues and cell types (Altun and Hall, 2009c; Bird and Bird, 1991; Kormish et al., 2010; White, 1988). It forms a separate epithelial tube running inside the cylindrical body wall, separated from it by the pseudocoelomic body cavity, and placed parallel to the gonad. The *C. elegans* digestive tract is divided into the foregut (stomodeum; buccal cavity and the pharynx; Altun and Hall, 2009d; Mango, 2007), the midgut (intestine; Altun and Hall, 2009b; McGhee, 2007), and the hindgut (proctodeum; rectum and anus in hermaphrodites and cloaca in males; Altun and Hall, 2009a) and contains a total of 127 cells (Schnabel et al., 1997; Sulston et al., 1983). In comparison to human digestive tracts, it lacks both an intestine-sheathing innervated muscle layer and a renewable/regenerating stem cell population. In *C. elegans*, ingested *E. coli* bacteria flow through the digestive tract by the muscular pumping and peristalsis of the pharynx at the anterior end, and the waste material is discarded through the opening of the anus at the posterior end by the action of the enteric muscles. Developmentally, the intestine (midgut) is endodermal in origin, deriving clonally from the E-lineage whereas the foregut and hindgut have a mixed lineage from ectodermal and mesodermal origins (Fig.2).

The *C. elegans* intestine is a large organ (~ 1/3 of the somatic tissue) that carries out multiple functions executed by distinct organs in higher eukaryotes (McGhee, 2007): digestion of food, absorption of processed nutrients, synthesis and storage of macromolecules, nurturing of oocytes by producing yolk, and initiation of an innate immune response to pathogens (Kimble and Sharrock, 1983; Schulenburg et al., 2004). Remarkably, despite a large increase in tissue volume during larval and adult development (Fig.1B), the intestine continues to grow without further cell or nuclei divisions. Intestinal cells become binucleate and polyploid during post-embryonic development. By the adult stage, the intestine is composed of only 20 (Fig.5A-E) cells with a total of 30-34 nuclei, which have increased their ploidy to 32C (Hedgecock and White, 1985; Sulston and Horvitz, 1977). Age-related changes in the intestine include the loss of critical nuclei, the degradation of intestinal microvilli, and changes in the size, shape, and cytoplasmic contents of the intestine (McGee et al., 2011).

The intestinal epithelium consists of 20 cells that are mostly positioned as bilaterally symmetric pairs to form a long tube around a lumen. Each of these cell pairs forms an intestinal ring (II-IX int rings). The anteriormost intestinal ring (int ring I) is an exception and is comprised of four cells (Fig.5E Leung et al., 1999; Sulston et al., 1983). The intestine is composed of large cells, with distinct apical, lateral and basal membrane domains. Each intestinal cell forms part of the intestinal lumen at its apical pole (Fig.5E'-E'') and contains a basal lamina at its basal pole (Kramer, 2005), whose constituents are either made by the intestine itself (laminin α and β nidogen/entactin) or by the muscle and somatic gonad (type IV collagen). Many microvilli extend into the lumen from the apical surface, forming a brush border. The microvilli are anchored into a strong cytoskeletal network of intermediate

filaments at their base, called the terminal web. The core of each microvillus has a bundle of actin filaments that connects to this web (Bossinger et al., 2004; Carberry et al., 2009; Hüsken et al., 2008; MacQueen et al., 2005). Each intestinal cell is sealed laterally to its neighbors by large apical adherens junctions and connects to the neighboring intestinal cells via gap junctions on the lateral sides (Altun et al., 2009; Bossinger and Schierenberg, 1992; Cox and Hardin, 2004; Hardin and Lockwood, 2004; Labouesse, 2006; Michaux et al., 2001).

The molecular and cellular events that lead to the formation of the intestinal epithelial tube have been described and reviewed in great detail elsewhere. In brief, these events include the correct specification and asymmetric division of the intestinal founder cell EMS (Bossinger and Schierenberg, 1996; Goldstein, 1992; Han, 1997; Kormish et al., 2010; Schierenberg, 1987; see Fig.2 for further details), the ingression of the intestinal precusor cell Ea and Ep during gastrulation (Fig.5B-C; Chisholm, 2006; Putzke and Rothman, 2003; Rohrschneider and Nance, 2009; Sawyer et al., 2009; Schierenberg, 2005; Schierenberg, 2006), the cytoplasmic polarization of intestinal primordial cells (Fig.5D; Achilleos et al., 2010; Bossinger et al., 2001; Leung et al., 1999; Totong et al., 2007), the formation of apical adherens junction and the generation of the future lumen within the primordium (Fig.5E'-E''; Leung et al., 1999), the intercalation of specific sets of cells (Hoffmann et al., 2010; Leung et al., 1999), the invariant 'twist' in the anterior of the intestinal primordium (Hermann et al., 2000), and finally the differentiation of the late embryonic, larval and adult intestine that has been proposed to be under the control of the GATA-factor ELT-2 (McGhee et al., 2009; McGhee et al., 2007; Pauli et al., 2006).

3. Apicobasal polarity complexes in the *C. elegans* intestine

From genetic studies on *Drosophila* ectoderm and mammalian culture cells, it appears that at least four spatially restricted membrane associated protein-scaffolds are required for regulating the maturation of the ZA in epithelial cells: the PAR-3–PAR-6–aPKC (PPC) complex, the Crumbs–Stardust–Patj complex, the Scribble–Dlg–Lgl complex, and the Yurt–Coracle group (Betschinger et al., 2003; Bilder et al., 2003; Harris and Peifer, 2005; Harris and Peifer, 2007; Krahn et al., 2010a; Krahn et al., 2010b; Laprise et al., 2009; Plant et al., 2003; Tanentzapf and Tepass, 2003; Yamanaka et al., 2003).

In the *C. elegans* embryo, a single electron-dense structure, the "*C. elegans apical junction*" (CeAJ, McMahon et al., 2001), is a prerequisite for correct epithelial cell functions (Cox and Hardin, 2004; Labouesse, 2006; Lynch and Hardin, 2009; Michaux et al., 2001; Müller and Bossinger, 2003). The CeAJ is a belt-like junctional structure that encircles the apex of polarized epithelial cells and resembles the ZA in other systems. By immunohistochemistry, the apicolateral membrane domain can be subdivided into four subdomains (Fig.6): the PPC together with the *Drosophila* Crumbs homolog CRB-1 and the multi PDZ-domain containing protein MAGI-1 (Achilleos et al., 2010; Aono et al., 2004; Bossinger et al., 2001; Stetak and Hajnal, 2011; Totong et al., 2007), the catenin–cadherin complex (CCC; Costa et al., 1998; Grana et al., 2010; Kwiatkowski et al., 2010), the DLG-1–AJM-1 complex (DAC; Bossinger et al., 2001; Firestein and Rongo, 2001; Köppen et al., 2001; Lockwood et al., 2008; McMahon et al., 2001) and the LET-413 protein (Bossinger et al., 2004; Legouis et al., 2000; Legouis et al., 2003; Lockwood et al., 2008; Pilipiuk et al., 2009; Segbert et al., 2004).

Epithelial polarization of the *C. elegans* intestine can be subdivided into three processes, **first** the appearance of junctional complexes, i.e. the CCC and DAC (Köppen et al., 2001;

Kwiatkowski et al., 2010; Lockwood et al., 2008) at the future apical pole (Achilleos et al., 2010), **second** the assembly of a junctional belt around the apex of epithelial cells (Totong et al., 2007), **third** and **fourth** the maintenance of epithelial cell polarity (Bossinger et al., 2004; Legouis et al., 2000) and cell-cell adhesion (Segbert et al., 2004; van Fürden et al., 2004).

4. Targeting of junctional complexes

At the end of the *C. elegans* proliferation phase, when the intestinal primordium consists of 16, so-called E-cells (E^{16}, Fig.5D), foci of the CCC and DAC accumulate at the apical surface (Fig.7A-C) under the control of *par-3* and *let-413* gene functions, respectively (Fig.1C-E; Achilleos et al., 2010; Legouis et al., 2000). In very elegant experiments, a targeted protein degradation strategy was used to remove both maternal and zygotic PAR-3 (*par-3M/Z*) from *C. elegans* embryos before epithelial polarization starts (Achilleos et al., 2010; Totong et al., 2007).

While localization of the CCC is mainly PAR-3 regulated, the DAC is under control of PAR-3 and LET-413. Interestingly, apical but not basolateral localization of LET-413 in intestinal primordial cells seems to be PAR-3 dependent too (Achilleos et al., 2010), suggesting that PAR-3 presumably acts via LET-413 to promote apical targeting of the DAC (Fig.7I). Consistent with this idea in *let-413(RNAi)* embryos the DAC reaches its apical position less efficiently (compare Figs.1E and 1F; Köppen et al., 2001; Legouis et al., 2000; McMahon et al., 2001; Segbert et al., 2004), a phenotype reminescent of embryos depleted for maternal and zygotic PAR-3 (Achilleos et al., 2010).

Using RNAi to deplete PAR-3 and LET-413 in developing larvae of *C. elegans*, Aono et al. (2004) and Pilipiuk et al. (2009) only discovered a requirement for these proteins in spermathecal development but not in other epithelia. Spermathecal precursor cells are born during larval development and differentiate into an epithelial tube for the storage of sperm. In PAR-3 and LET-413–depleted worms, the distribution of the DAC and apical microfilaments are severely affected in spermathecal cells, suggesting that the primary defect is in the organization of the apical domain.

How PAR-3 and LET-413 become localized apically in intestinal primordial and spermathecal cells is not known. In *Drosophila* membrane targeting of Bazooka/PAR-3 is mediated by direct binding to phosphoinositide lipids (Krahn et al., 2010b). Recent deletion and point mutation analyses of three LAP proteins, using *C. elegans* LET-413, human Erbin and human Scribble demonstrate that their LRR domain is crucial for membrane targeting (Legouis et al., 2003). Importantly, functional studies of LET-413 in *C. elegans* show that the LRR domain but not the PDZ domain is necessary for LET-413 to function during embryogenesis (Legouis et al., 2003).

5. Assembly of the junctional belt

During the early morphogenesis phase of *C. elegans*, the assembly of junctional complexes into an adhesive belt encircling the apex of epithelial cells (Figs.5E'',7F) depends on LET-413, DLG-1 and PAR-6 gene functions (black arrows in Fig.7I). In mid-morphogenesis of *let-413(RNAi)* embryos, long stretches of normal DAC localization form at the subapical cortex of epithelial cells, which are separated intermittently by gaps completely lacking DAC (Legouis et al., 2000). In contrast, the AJM-1 pattern in DLG-1 depleted embryos is

characterized by small aggregates separated by large regions in which AJM-1 is almost completely missing (Bossinger et al., 2001; McMahon et al., 2001). In *let-414;dlg-1(RNAi)* embryos AJM-1 localization is nearly completely abolished (Köppen et al., 2001).

The N-terminal leucine-rich repeats of LET-413, which mediate basolateral localization, show good similarity with the Ras-interacting protein SUR-8 (Legouis et al., 2003). Among the small GTPase families, the Rab proteins are well known for their role in vesicle trafficking (Jordens et al., 2005) and it has been postulated that many charcteristics of LET-413 qualify this protein for acting as a docking platform in a trafficking pathway, which is controlled by small GTPases and ensures assembly of the CeAJ (Legouis et al., 2000).

For several reasons, and consistent with data from cell culture (see above), we do not favor the F-actin network as a major player in early steps of CeAJ biogenesis. First, *C. elegans* mutants defective in components of the CCC show severe defects in actin filament bundling without interfering with the formation of an adhesive junctional belt (Costa et al., 1998). Second, depletion of ERM-1, the only Ezrin-Radixin-Moesin homolog in *C. elegans*, almost completely abolishes establishment of the F-actin network in the apical cortex. Nevertheless, the CeAJ continuously forms around the apex of intestinal cells (van Fürden et al., 2004). Third, both described phenotypes are quite different from *let-413/dlg-1* induced defects, in which clustering of CeAJ proteins becomes the predominant phenotype (Bossinger et al., 2001).

There are nine α-tubulins (TBA-1-9) and six β-tubulins (TBB-1-6) in the *C. elegans* genome. Microtubules (MTs) are oriented circumferentially in dorsal and ventral epidermal cells, but are less well-organized in lateral seam cells (Costa et al., 1998). During organogenesis of the *C. elegans* intestine, MTs are concentrated near the apical cortex, where they appear to emerge in a fountain-like array and extend along the lateral surfaces of the cells. Numerous MTs are in the vicinity of the centrosomes, suggesting that there might be a MT organizing center at the apical cortex (Leung et al., 1999). By contrast, in many other epithelial cells most MTs are noncentrosomal and align along the apicobasal polarity axis. They create asymmetry by orienting their minus- and plus-ends towards the apical and basal membrane domains, respectively (Bacallao et al., 1989; Bre et al., 1990).

The polarized MT cytoskeleton in the *C. elegans* embryonic intestine is ideally suited to transport vesicles from the basally located Golgi toward the apical surface (Leung et al., 1999). During *Drosophila* cellularization, strong MT nucleation from apical centrosomes is likely necessary for the assembly of lateral MTs that promote the apical transport of lipids/proteins to form cell membranes and the initial apical positioning of AJs (Harris and Peifer, 2005; Lecuit and Wieschaus, 2000; Papoulas et al., 2005). In the *C. elegans* intestine, centrosomal MTs might also help direct the symmetric positioning of the CeAJ around the subapical domain. MT motors have been previously implicated in AJ assembly. For example, dynein interacts with β-catenin and may tether MTs to AJs assembling between cultured epithelial cells (Ligon et al., 2001). Kinesin transports AJs proteins to nascent AJs in cell culture (Chen et al., 2003; Mary et al., 2002) and MKLP-1/ZEN-4 is required for apical targeting of AJM-1 in the *C. elegans* pharynx epithelium (Portereiko et al., 2004). During early epithelial development in *Drosophila* positioning of Bazooka/PAR-3 relies on cytoskeletal cues, including an apical scaffold and dynein-mediated basal-to-apical transport (Harris and Peifer, 2005).

The similarity of *let-413* and *dlg-1* phenotypes and the fact that many CeAJ proteins show comparable phenotypes after depletion of LET-413 and DLG-1 is remarkable. These observations suggest that both proteins might somehow control the release of vesicles from MTs, either by providing a docking platform as discussed for LET-413 (see above) or by directly interacting with motor proteins. In *Drosophila* neuroblasts, Discs large, kinesin Khc-73, and astral MTs induce cortical polarization of Pins/Gαi. Khc-73 localizes to astral MT plus ends, and Dlg/Khc-73 and Dlg/Pins coimmunoprecipitate, suggesting that MTs induce Pins/Gαi cortical polarity through Dlg/Khc-73 interactions (Siegrist and Doe, 2005). In *C. elegans*, the clustering of CeAJ proteins after interfering with *let-413* and *dlg-1* gene functions would then indicate a jam in vesicular trafficking.

6. Maintenance of epithelial cell polarity

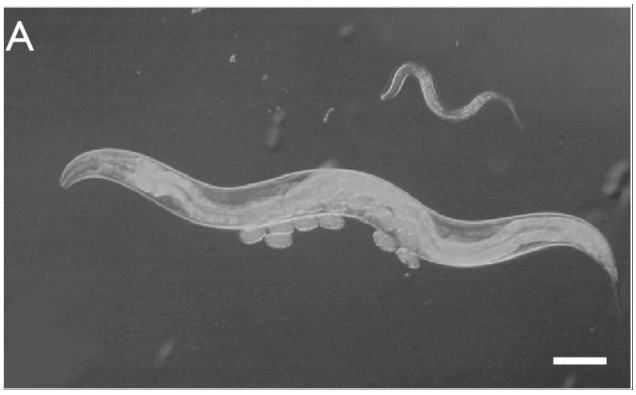
During late morphogenesis of *let-413* mutant or RNAi embryos, apical membrane markers in the epidermis as well as in the intestine progressively spread into the lateral membrane, suggesting that LET-413 acts to maintain polarity (Bossinger et al., 2004; Köppen et al., 2001; McMahon et al., 2001).

Surprisingly, worms treated with *let-413(RNAi)* during larval and adult life are sterile and exhibit spermathecal defects but otherwise develop normally, suggesting that depletion of LET-413 level does not restrict the function of major epithelia, like the pharynx, the intestine, or the hypodermis (Pilipiuk et al., 2009). How this function is maintained during post-embryonic development in *C. elegans* remains puzzling and might depend upon so far unidentified proteins that either completely replace LET-413 function or act redundantly.

7. Maintenance of cell-cell adhesion

During *C. elegans* morphogenesis, only double-knockdowns, e.g. HMR-1/E-cadherin + SAX-7/L1CAM (Hoffmann et al., in preparation), HMP-1/α-catenin + DLG-1 (Segbert et al., 2004), or HMR-1/cadherin + ERM-1 (van Fürden et al., 2004) give rise to intestinal cell-cell adhesion defects. HMR-1/E-cadherin and SAX-7/L1CAM also function redundantly in blastomere compaction and non-muscle myosin accumulation during *C. elegans* gastrulation (Grana et al., 2010). Interestingly, early embryonic and epithelial cells lacking PAR-6 can separate from one another inappropriately (Nance, 2003; Totong et al., 2007). Hence, PAR-6 seems to function reiteratively to control cell-cell adhesion in the *C. elegans* embryo. While *par-6* gene function clearly interferes with the correct localization of the CCC and DAC in intestinal primordial cells (Totong et al., 2007) this relationship still has to be demonstrated for early embryogenesis. The enhancement of hypodermal defects through functional loss of the DAC in mutations of *vab-9* (encoding a claudin homolog orthologous to human brain cell membrane protein 1; Simske et al., 2003) is another example of functional redundancy concerning cell-cell adhesion in the *C. elegans* embryo.

In summary, these genetic data suggest that cell-cell adhesion in the intestine is regulated by at least two redundant systems, which both act at the level of cell adhesion molecules, linker proteins and cytoskeletal organizers.



B

Developmental time (h) and length (µm) of *C. elegans* larva (L1-L4) and adult at different temperatures (°C)

STAGE	*25°C*	*20°C*	*16°C*
egg laid	0 h / 55 µm	0 h / 55 µm	0 h / 55 µm
egg hatches (L1)	8-9 h / 250 µm	10-12 h / 250 µm	16-18 h / 250 µm
first molt (L2)	18 h / 380 µm	26 h / 370 µm	36.5 h / 360 µm
second molt (L3)	25.5 h / 510 µm	34.5 h / 480 µm	48 h / 490 µm
third molt (L4)	31 h / 620 µm	43.5 h / 640 µm	60 h / 650 µm
fourth molt (young adult)	39 h / 940 µm	56 h / 850 µm	75 h / 900 µm
egg-laying begins (adult)	~ 47 h / 1110 µm	~ 65 h / 1060 µm	~90 h / 1150 µm
egg-laying ends (old adult)	~ 88 h	~ 96 h	~ 180 h

based on Byerly et al., 1976

C

***C. elegans* genome**

Base pairs:	100,267,233
Coding Sequences: (11,068,632 base pairs)	25244 (100%) (20470 from protein-coding genes)
Confirmed:	12052 (47.7%)
Partially confirmed:	11172 (44.3%)
Predicted:	2020 (8.0%)

(based on http://wiki.wormbase.org/index.php/WS227)

Fig. 1. *Caenorhabditis elegans* development and genome

(A) Shows a DIC micrograph of a *C. elegans* larva (top) an adult hermaphrodite (middle) and embryos (bottom) maintained on agar plates with *E. coli* as food source (scale bar: 100 µm). **(B)** The table summarizes the developmental time (in hours) of *C. elegans* at different temperatures (°C), starting with the eggs released from the mother's uterus (0 h), completing embryogenesis (8-18 h), passing through four larval stages (L1-L4) and finally reaching adulthood (47-90 h). The length of the egg, larva and adult at each stage is given in micrometers (µm). **(C)** provides a short summary of the *C. elegans* genome (The_C_elegans_Sequencing_Consortium, 1998) that contains 100,267,633 base pairs and is estimated to have 25244 coding sequences (CDS) from which 47.7% have been confirmed (every base of every exon has transcription evidence). 44.3% CDS are partially confirmed (some, but not all exon bases are covered) and 8.0% CDS show no transcriptional evidence at all. Recent meta-analysis of results from four orthology prediction programs has yielded a set of 7633 *C. elegans* genes ("OrthoList") having human orthologs (Shaye and Greenwald, 2011).

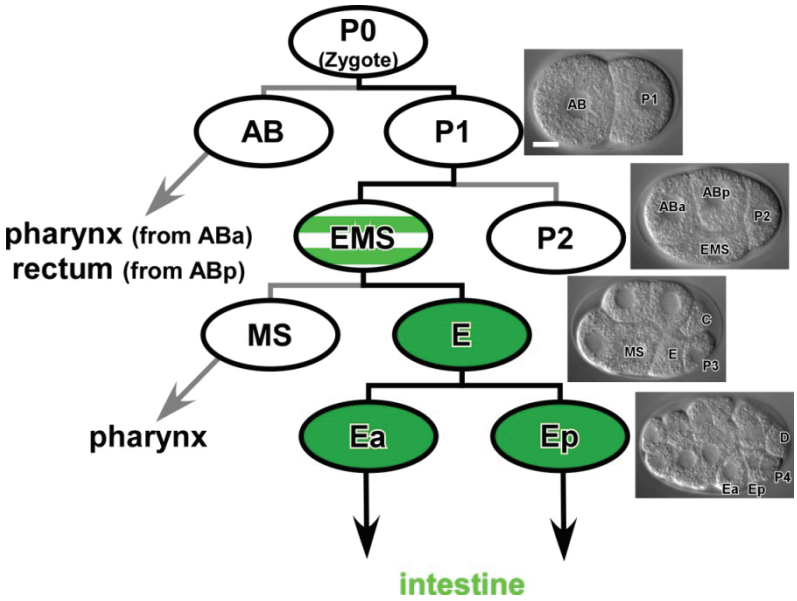


Fig. 2. Early cell lineage of *C. elegans*

The *C. elegans* one-cell embryo, also called zgote or P0, is a widely studied model of cell polarity (summarized in, Cowan and Hyman, 2004b; Gönczy, 2008; Nance and Zallen, 2011). The unfertilized oocyte has no developmentally significant polarity. Polarity is established shortly after fertilization in response to a signal contributed by the sperm (Cowan and Hyman, 2004a). This signal leads to the establishment of two distinct cortical domains defining the anterior-posterior axis of the embryo. The one-cell embryo divides asymmetrically according to the axis such that one cell inherits the anterior cortical domain and the other cell inherits the posterior domain. The division is also physically asymmetric: the volume of the posterior P1-cell is approximately half that of the anterior AB-cell (see DIC micrograph). The resulting cells are already functionally distinct. The anterior AB-cell

proceeds along a differentiation pathway producing ectoderm (hypodermis, pharynx, and neurons). The posterior P1-cell re-establishes anterior-posterior polarity and again divides asymmetrically (into P2 and EMS; see DIC micrograph) in a stem cell-like mode of division. These stem cell-like divisions establish the founder cells for the somatic lineages of the worm (AB, MS, E, C and D; see DIC micrographs) and maintaining a single stem cell (P4; see DIC micrographs) for the germline, which finally produces sperms and oocytes in the adult hermaphrodite.

The complete *C. elegans* digestive tract consists of three "organs" derived from four distinct embryonic cell lineages (Sulston et al., 1983): pharynx (57 cells from ABa; 38 cells from MS), intestine (20 cells from E; green), and rectum (11 cells from ABp; Sewell et al., 2003). Only the intestine is a pure clone of 20 E-cells; the three other lineages produce cells both inside and outside of the digestive tract. The intestine is one of the few cell lineages in the *C. elegans* embryo where a plausible sequence of direct molecular interactions can be proposed throughout the life cycle (Kormish et al., 2010; McGhee, 2007), beginning with maternally-derived factors in the cytoplasm of the early embryo (e.g. SKN-1 and SYS-1/POP-1), progressing through a small number of zygotic transcription factors (e.g. END-1/3 and ELT-2), and ending with the transcription of e.g. vitellogenin genes in the adult intestine. ELT-2 has been proposed to participate directly in the regulation of most intestinal genes expressed from the E^2 cell stage (Ea and Ep, see DIC micrograph) and later (McGhee et al., 2009; McGhee et al., 2007). The molecular mechanisms that lead to the asymmetric division of the EMS blastomere (green striated) into a larger MS- and a smaller E blastomere (see DIC micrograph) and the correct specification of their cell fates, central to the formation of the pharynx and intestine has been describe in great detail elsewhere (Maduro, 2010; Mango, 2007; Sugioka et al., 2011). **Orientation** (DIC micrographs): anterior, left, dorsal top; scale bar: 10 µm.

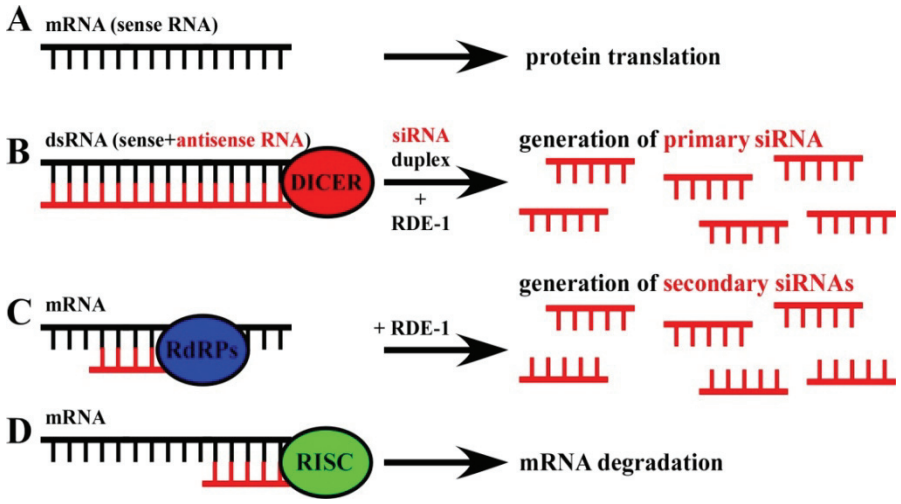


Fig. 3. RNA-mediated interference (RNAi) in *C. elegans*

Over the last decades, RNAi has been found not only be effective in *C. elegans* but also in other organisms and cell culture. The cartoon depicts a very simplified scheme of the exogenous RNAi-mechanism in *C. elegans* (for detailed reviews see: Ahringer, 2006; Fischer, 2010; Maine, 2008) that leads to targeted destabilization of endogenous, homologous mRNA molecules by double stranded RNA (dsRNA; Fire et al., 1998). **(A)** In a cell, RNA is used as a "messenger" (mRNA) to carry genetic information from the nucleus into the cytoplasm, where it is translated into proteins. **(B)** In *C. elegans,* exogenous dsRNA can be either applied by injection, "feeding" or "soaking" (Maeda et al., 2001; Mello et al., 1991; Timmons and Fire, 1998). dsRNA is then cut into ~22 nt primary siRNAs by a protein complex containing the RNAse III enzyme Dicer (DCR-1) and the dsRNA binding protein RDE-4 (Ketting et al., 2001; Tabara et al., 2002). The Argonaute protein RDE-1 (Tabara et al., 1999) binds siRNAs and seems only required for their stability (Parrish and Fire, 2001). Finally, RDE-1 slicer activity removes the passenger strand from the guide strand in the siRNA duplex (Steiner et al., 2009), which is necessary to allow guide-strand accessibility to the mRNA target. **(C)** RNAi in *C. elegans* includes an amplification step (Alder et al., 2003; Fire et al., 1998). The mRNA that is targeted by siRNAs serves as a template for the generation of secondary siRNAs mediated by RNA-dependent RNA polymerases (RdRPs). Secondary siRNAs are always antisense and have 5′ triphosphates instead of the 5′ monophosphate characteristic of Dicer cleavage. Secondary siRNAs are made by unprimed RNA synthesis by RdRPs, which are recruited to the target mRNA bound to the primary siRNA in complex with RDE-1 (Pak and Fire, 2007; Sijen et al., 2007). *In vitro* studies suggest that secondary siRNA generation is Dicer-independent (Aoki et al., 2007). **(D)** siRNAs present in the cell are associated with an effector complex called the RISC (RNA-induced silencing complex). In *C. elegans* multiple such complexes exist (Caudy et al., 2003; Chan et al., 2008; Gu et al., 2007), which finally drive mRNA destabilization.

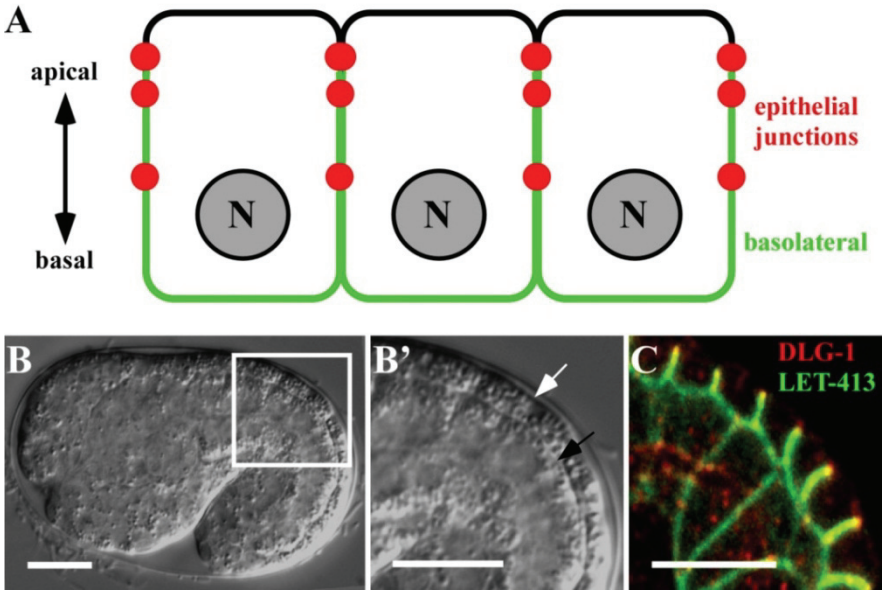


Fig. 4. Epithelial cell polarity and junctions

(A) Epithelial cells in general show a pronounced apicobasal polarity that becomes manifested by the establishment of apical (black) and basolateral (green) membrane domains that differ in the compositions of proteins and lipids. A hallmark of epithelial differentiation is the assembly of junctional complexes (red) along the lateral membrane domain, which fulfill different functions during epithelial development. **(B-B')** Shows a DIC micrograph of a *C. elegans* embryo during the elongation phase (B), focusing on two epithelia (B'), the epidermis (white arrow) and the intestine (black arrow). **(C)** Depicts an immunofluorescence micrograph of an embryo in B' stained against junctional protein DLG-1 (red) and basolateral protein LET-413 (green). See text for further details. **Orientation** (B-C'): anterior, left, dorsal top (A-E''); scale bar: 10 µm.

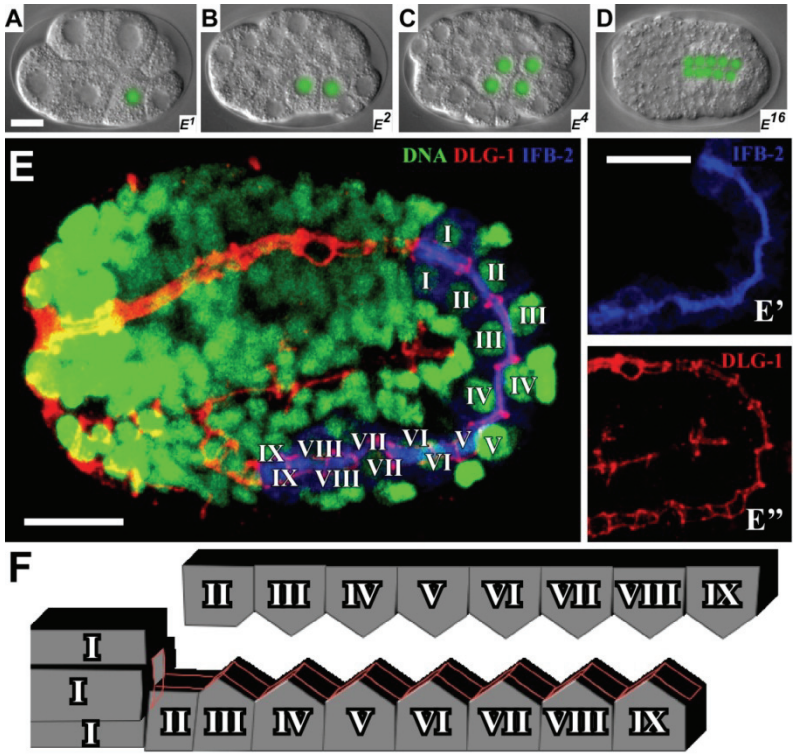


Fig. 5. Development and differentiation of the *C. elegans* embryonic intestine

The *C. elegans* intestine, the whole endoderm of the animal, consists of only 20 cells, which derive from a single somatic founder cell, the so-called E-cell (Deppe et al., 1978; Leung et al., 1999; Sulston et al., 1983). **(A-D)** Shows a series of DIC micrographs with E-cell nuclei colored in green. The E-cell is born at the 8-cell stage (A) and with the beginning of gastrulation (24-cell stage), 2 E-cells (E^2) ingress into the embryo (B) where they further undergo cell divisions (C, 4 E-cells, E^4). The ingression of Ea and Ep cells depends on correct cell fate specification and polarization of the machinery that orchestrates cell shape changes and cell migration (Lee and Goldstein, 2003; Sawyer et al., 2011). Among these, PAR-3 and

PAR-6 proteins regulate apical accumulation of myosin heavy chain, and a Wnt-Frizzled signaling pathway modulates contraction of the actomyosin network that drives apical constriction and finally leads to correct ingression of endodermal precursor cells (Cabello et al., 2010; Grana et al., 2010; Lee et al., 2006). Gastrulation in *C. elegans* later continues with the internalization of other cells including mesoderm and germline progenitors (Chisholm and Hardin, 2005; Nance et al., 2005). During early morphogenesis, the intestinal precursor cells (E^{16}) start to polarize (D, 16 E-cells, E^{16}, only 10 E-cells in focal plane) and finally an intestinal tube of 20 E-cells forms during ongoing morphogenesis of *C. elegans*. **(E-E'')** Shows micrographs of a mid-morphogenesis stage (similar to D) stained against DNA (E, green, YoYo), the intestinal-specific intermediate filament protein IFB-2 localized in the apical cortex (E', blue, mabMH33), and the junctional protein DLG-1 (E'', red, anti-DLG-1 antibodies). **(F)** The cartoon depicts the organization of the intestinal epithelial tube in nine units (I-IX), which are connected by the CeAJ (red). **Orientation** (A-E''): anterior, left, dorsal top (A-E''); scale bar: 10 µm.

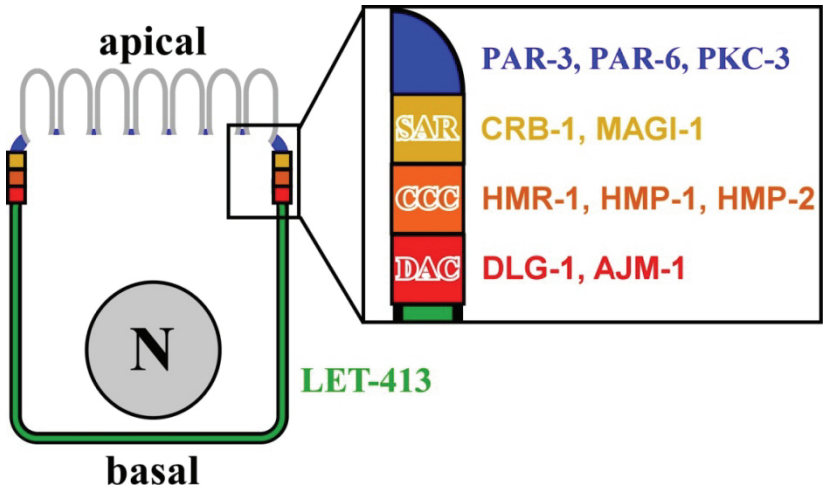


Fig. 6. Apical junctional complexes in the *C. elegans* intestine

Epithelia of the *C. elegans* embryo contain a single electron-dense apical junction (about 250 nm; Carberry et al., 2009; Müller and Bossinger, 2003), also referred to as "*C. elegans* apical junction" (CeAJ; McMahon et al., 2001) that has been subdivided into distinct parts by immunohistochemistry. In the basal part of the CeAJ, the DLG-1–AJM-1 complex (DAC; Köppen et al., 2001; Lockwood et al., 2008) is organized, while more apically the catenin–cadherin complex (CCC; Costa et al., 1998; Kwiatkowski et al., 2010), consisting of the proteins HMR-1 (E-cadherin), HMP-1 (α-catenin) and HMP-2 (β-catenin) can be found. The subapical region harbours the proteins MAGI-1 and probably CRB-1 (Bossinger et al., 2001; Stetak and Hajnal, 2011). By immunofluorescence analysis all these proteins show a typical, "junctional" staining pattern (e.g. DLG-1, Fig.5E'') that reflects the correct formation of the CeAJ within the embryonic intestine. Most apically, the PAR-3–PAR-6–PKC-3 complex (PPC; Achilleos et al., 2010; Leung et al., 1999; Totong et al., 2007) is localized, showing a more "cortical" staining pattern, comparable to that of intermediate filament proteins (e.g. IFB-2, Fig.5E').

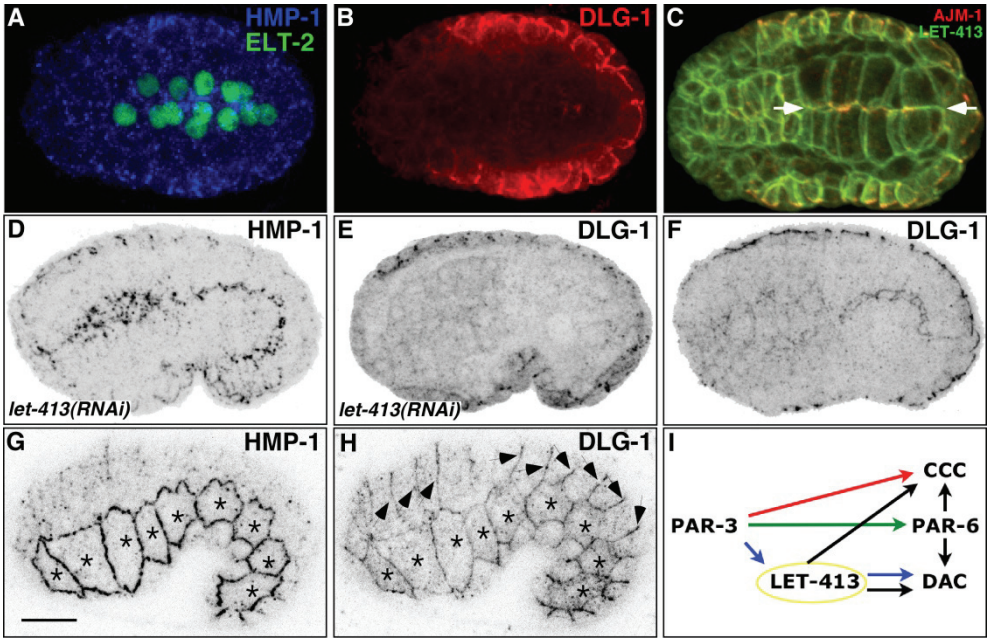


Fig. 7. Establishment of cell polarity and assembly of junctional complexes during development of the *C. elegans* intestine

(A-C) Early morphogenesis stages showing immunofluorescences (IF) of the catenin–cadherin complex (CCC, blue in A, anti-HMP-1/α-catenin IF), the intestine-specific GATA-factor ELT-2 (green in A, anti-GFP IF; McGhee et al., 2009; McGhee et al., 2007), the DLG-1–AJM-1 complex (DAC, red in B and C, anti-DLG-1/Discs large IF and anti-AJM-1 IF), and the LET-413/SCRIB protein (green in C, anti-CFP). **(D-E)** Mid morphogenesis stages after RNAi (Fire et al., 1998) against *let-413* gene function displaying anti-HMP-1 and anti-DLG-1 IFs. **(F)** During early morphogenesis stage, the *C. elegans* apical junction (CeAJ) forms around the apex of intestinal primordial cells (anti-DLG-1 IF). **(G-H)** IF analysis shows that the CCC (G) but not the DAC (H) moves away from the CeAJ (arrows in H) prior to the onset of cell fusion in the dorsal hypodermis (Oren-Suissa and Podbilewicz, 2007; Oren-Suissa and Podbilewicz, 2010). In contrast, both complexes clearly localize at the CeAJ in lateral seam cells (asterisks in G,H). **(I)** Schematic drawing of key players involved in epithelial polarization (colored arrows), formation of the junctional belt around the apex (black arrows) and maintenance of cell polarity (yellow circle). PAR-3 is a PDZ domain-containing protein orthologous to mammalian atypical PKC isotype-specific interacting protein (ASIP) and *Drosophila* Bazooka. PAR-6 contains PB1, CRIB and PDZ domains and is also conserved in *Drosophila* and mammals. LET-413 belongs to the LAP (LRR (for leucine-rich repeats) and PDZ (for PSD-95/Discs-large/ZO-1)) protein family and contains one PDZ domain and 16 LRR (Bilder et al., 2000; Legouis et al., 2000; Legouis et al., 2003). The DLG-1–AJM-1 complex (DAC; Köppen et al., 2001; Lockwood et al., 2008) is composed of DLG-1/Discs large (a MAGUK with three PDZ, one SH3, and one GUK domain) and AJM-1 (apical junction molecule) a coiled-coil protein. See text for further explanations. **Orientation**

(A-H): anterior (left), dorsal (top); scale bar: 10 μm. A-B, C and G-H: photo courtesy of Tobias Wiesenfahrt, Jennifer Pilipiuk and Eva Horzowski, respectively.

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16

Intercellular Communication

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1. Introduction

Intercellular communication (transfert of information) is an essential issue for continuity of life in multicellular organisms. Several types of communication systems coordinate body functions to maintain homeostasis (Guyton & Hall, 2000). Until now, it has been accepted that two major organ systems control all physiologic processes within the human body: The **endocrine system** and the **nervous system** (Greenspan & Gardner, 2004). Beside them, a third organ system, **immune system,** is a super-system which provide recognition and destroy of foreign cells by specific coordination between their cells again within the body. The contact and communication between immun cells are used for the distinction between self and non-self. In recent years, considerable data supported the existence of dynamic interactions between these super-systems. For example, neuro-endocrine, neuro-immune, psycho-neuro-immuno-endocrinological cross communications have been identified (Downing & Miyan, 2000; Sternberg, 1997; Weihe et al., 1991). In addition, within organ systems, **autocrine**, **paracrine**, **juxtacrine**, **neurocrine, lumencrine (exocrine)** and finally **intracrine** communications have been defined (di Sant'Agnese, 1992; Greenspan & Gardner, 2004; Guyton & Hall, 2000; Hansson & Abrahamsson, 2001; Krantic et al., 2004; Miller, 2003; Patel et al., 1993; Re & Bryan, 1984; Re, 1989; Ruan & Lai, 2004; Sporn & Todaro, 1980; Sporn & Roberts, 1992; Zimmerman et al., 1993) (Figure 1). Intercellular communication in the organism is realized by specific molecules, except neural transmission exerted by action potentials (Despopoulos & Silbernagl, 2003; Faller & Schuenke, 2004; Guyton & Hall, 2000) and except information transfer by biophoton emission which has been reported very recently (Albrecht-Buehler, 1992; Cohen & Popp, 1997; Fels, 2009; Jaffe, 2005; Musumeci et al., 1999; Niggli, 1992). This review especially addresses chemical communication systems in the human body by simplified examples.

2. Modes of communication

This chapter will attempt to summarize the modes of communications in this order below; **autocrine** (including intracrine), **paracrine** (including juxtacrine, gap junctional, via Tunneling Nano-Tube like structures), **endocrine**, **neurocrine** (including neuro-endocrine) and **lumencrine** communications.

2.1 Autocrine communication

Autocrine communication (derived from *auto*: self and *krinein*: to secrete, Greek) is an activity of a hormone or growth factors (GFs) that binds to and affects the same cell that

secreted it. These substances directly stimulate (or inhibit) the cell via their surface receptors. Autocrine secretion was described first by Sporn and Todaro in 1980. It explains self-regulation of cells. This concept is now not only important to explain malignant transformation, but is also mainstay of embryogenesis and morphogenesis. Autocrine regulation provides selective growth advantages during the earliest stages of embryogenesis before the development of a functioning circulatory system and endocrine function (Dockray, 1979; Sporn & Todaro 1980).

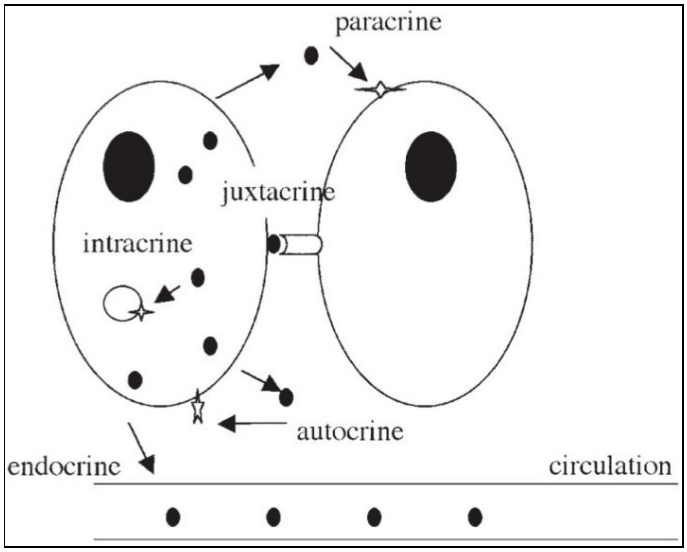

Fig. 1. Several intercellular modes of communication (courtesy of Mao-De Lai, corresponding author of the article Ruan & Lai, 2004)

Autocrine secretion is an important phenomenon in the regulation of the behavior of many normal cells such as macrophages, lymphocytes, fibroblasts and vascular smooth muscle cells. This regulation can be positive or negative manner. For example, oncogenes such as EGF, TGF-α and PDGF enhance autocrine pathways to increase cell replication during carcinogenesis. Same peptides also have an important autocrine role in tissue repair and wound healing in normal physiology. On the other hand TGF-β is a significant negative autocrine regulator in the adenoma-carcinoma sequence of human colon carcinogenesis (Sporn & Roberts, 1992). But, TGF-β is bifuntional like many other peptide growth factors and its stimulatory or inhibitory effects depends on many factors including cell type specificity, cell growth condition, and some other factors (Sporn & Roberts, 1988; Ruan & Lai 2004). In fact, cells are different than simple mechanical devices, they take new information from its environment and depending on conditions they give an appropriate response (Sporn & Roberts, 1992). For example, a specific autocrine cytokine such as TGF-β or interleukin-6 can act either positive or negative signal for growth in a given cell (Sporn & Roberts, 1988; Akira et al., 1990). Autocrine communication can be considered as a primitive mechanism of humoral regulation than endocrine secretion (Sporn & Todaro, 1980).

2.1.1 Internal autocrine (intracrine) communication

Another type of autocrine regulation is realized inside the cell. Internal autocrine or intracrine mode of action which is described first Re RN et al in 1984, indicates that some peptide hormones and growth factors (intracrines) bind and act in the cellular interior either after internalization by target cells or retention in their cells of synthesis (Re & Bryan, 1984; Re, 1989). Some endogenous cytokines such as interleukin-3 or PDGF can be modified and retained within the cell to ensure internal cellular action and they have high degree of intracellular biologic activity (Bejcek et al, 1989; Dunbar et al, 1989; Keating & Williams 1988). As shown in Figure 1, a chemical mediator (peptide growth factor or hormone) interacts with its specific receptor within the cell, bypassing the need of secretion outside, to exert functional activity. An intracrine system, in contrast to endocrine system, requires minimal amounts of biologically active hormones to exert their maximum hormonal effects. For this reason, the intracrine system plays an important role especially in the development of hormone-dependent neoplasms such as breast, prostate malignancies. As another example, locally produced bioactive androgens and/or estrogens exert their action in the cells where synthesis occurs without release in the extracellular space including circulation (Sasano et al., 2008). Labrie and colleagues described the formation of active androgens (such as DHT) from the inactive adrenal precursors in the some tissues or cells in adenocarcinoma of the prostate where biosynthesis takes place without release into the extracellular space as "intracrine activity" (Labrie et al., 1995, 2003, Sasano et al., 2008). On the other hand, estrogen-dependent breast carcinoma in which aromatase converts circulating androgens to estrogens (from androstenedione to estrone and from testosterone to estradiol, respectively) should also be considered as "intracrine tissue". One of the most studied example of intracrine function is about local renin-angiotensin system (RAS). As it is known, the proteolytic enzyme renin (an aspartyl protease) which cleaves angiotensinogen, is secreted mostly from juxtaglomerular cells in the renal afferent arteriole. Reduced renal arteriolar blood pressure and then the activation of local β2-adrenoreceptor stimulates the secretion of renin. The major source of plasma angiotensinogen is the liver, but it is also formed for local use in the heart and the brain. Cleavage of angiotensinogen by renin yields angiotensin I which has no biological activity. Further, angiotensin II is produced from angiotensin I by endothelial angiotensin-converting enzyme (ACE) and angiotensin III is produced from angiotensin II by aminopeptidase. Angiotensin II is a potent constrictor of vascular smooth muscle, and this action is mediated by the AT_1 receptor. Angiotensin II and III act on zona glomerulosa cells in the adrenal cortex and promote secretion of aldosterone. Today we know that renin is not simply a circulating enzyme but is a hormone and it is also an intracrine. Renin and angiotensin are also active within cells. A complete intracrine RAS exists in some cells (Re & Bryan, 1984). For example, an adrenal intracellular RAS has been reported (Peters et al., 1999). Prorenin and renin can bind to specific cellular receptors. Prorenin, and to a lesser extent, renin, can be internalized by cells where angiotensin II is produced (Re, 2003a). Internalized, activated prorenin causes both hypertension and cardiac injury. Nuclear angiotensin receptors were also reported (Re, 1999). There is a renin transcript in some cells (adrenal, brain) lacking the sequence encoding the secretory signal piece (renin exon 1A) (Clausmeyer et al., 2000; Peters et al.,

1999). Renin exon 1A generates angiotensin in mitochondria which have angiotensin receptors and stimulates aldosterone secretion in the adrenal cortex. This aldosterone secretion is inhibited by the angiotensin receptor blocker losartan (Peters et al., 1999). Renin exon 1A is upregulated by nephrectomy. Adrenal mitochondrial renin granules increase following nephrectomy (Peters et al, 1999). Release of angiotensin II in the intracellular space upregulates a series of genes including PDGF which stimulated proliferation (Re, 2003b). It has been reported that renin exon 1A upregulated in the ventricles of rats after myocardial infarction. Intracrine RAS may have a reasonable role in the processes like left ventricular hypertrophy, cardiac fibrosis and some forms of arrhytmia (Re, 2003b). So, the existence of intracrine RAS can be clinically important. Another important area related to intracrinology is the angiogenesis. Many intracrines (angiogenin, FGF-2, angiotensin) are angiogenic either directly or through the stimulation of vascular endothelial growth factor (VEGF) (Li & Keller, 2000; Re, 1999). Angiogenin is an RNase and needs nucleolar translocation to stimulate angiogenesis. VEGF also is an intracrine. Lee and colleagues, demonstrated that VEGFR1 expression was abundant in breast cancer cells (Lee et al., 2007). It was predominantly expressed internally in MDA-MB-231 and MCF-7 breast cancer cells and VEGFR1 antibody had no effects on the survival of these cells. Learning this intracrine concept has a practical significance because the usage of therapeutic antibodies against GFs have serious limitations in that an internal autocrine loop can not be accessible to antibody therapy (Sporn & Roberts, 1992).

2.2 Paracrine communication

Paracrine communication (derived from *para*: from beside by, Greek) is an activity of an agent (hormone or growth factor) that binds to and affects neighboring cells. The agent is directly released into the intercellular space and may involve many nearby cells that have receptors for this agent (Öberg, 1998; Raybould et al., 2003). One nice example of paracrine communication is the interaction between vascular endothelial cells and pericytes. The control of proliferation and migration of vascular endothelial cells can be mediated by neighboring cells; pericytes in a capillary (Antonelli-Orlidge et al, 1989), or smooth-muscle cells (Dennis & Rifkin, 1991) in an artery. A capillary endothelial cell synthesize latent TGF-β and a pericyte is required for activation of this latent molecule. This is a cooperative interaction via paracrine way. Loss of paracrine activation by pericytes may contribute diabetic proliferative vascular retinopathy (Antonelli-Orlidge et al, 1989). During recent years, interactions between endothelial cells and mural cells (pericytes and vascular smooth muscle cells) have gained increasing attention in physiological and pathological conditions including tumor angiogenesis, diabetic retinopathy, hereditary telangiectasia, lymphedema and hereditary stroke and dementia syndrome (Armulik et al, 2005). Some signaling pathways such as angiopoietin-Tie2, PDGF-B/PDGFR-β are described between endothelial cells and pericytes. Nitric oxide (NO) is also a paracrine agent; endothelial cells produce NO (and citrulline) from arginine as a substrate by endothelial nitric oxide synthase, and it diffuses into smooth muscle where induces relaxation and dilatation of blood vessels (Schechter & Gladwin, 2003). As another example, paracrine interactions between immune cells and fibroblasts are required for the normal repair of injured tissue (Sporn & Roberts, 1992). On the other hand, it has been demonstrated recently the multicellular autocrine and paracrine cross

talk in the inflammatory tumor microenvironment; for example RAGE (receptor for advanced glycation end products) engagement in cancer cell surface with its ligands (AGEs, S100/calgranulins, amyloid A, amyloid-β and DNA-binding protein HMGB1) which are expressed and secreted by many cell types within the tumor microenvironment including fibroblasts, leukocytes and vascular cells, produces activation of multiple intracellular signalling mechanisms involved in several inflammation-associated clinical entities, such as cancer, diabetes, renal and heart failures and neurodegenerative diseases (Rojas et al, 2010). Intercellular bidirectional paracrine communication is essential also either in spermatogenesis or development of an egg competent to undergo fertilization and embryogenesis (Matzuk et al, 2002).

2.2.1 Juxtacrine communication

Another kind of paracrine communication between signaling and target cells is juxtacrine interactions. **Juxtacrine** mode of action (derived from *juxta*: nearby, Latin) is a direct and intimate contact between two cells such as macrophage-T lymphocyte, spermatogonia-Sertoli cell or endothelial cell (EC) and the leukocyte (Krantic et al., 2004; Patel et al., 1993; Zimmerman et al., 1993). This signaling form provides a mechanism for strict spatial control of activation of one cell by another and juxtacrine signaling is likely to be common in physiologic events that require tight regulation (Zimmerman et al., 1993). The term "juxtacrine" was coined by Anklesaria and Massagué and colleagues in 1990 (Anklesaria et al, 1990; Massagué, 1990). In juxtacrine systems the signaling factor acts while associated with the surface of signaling cells, rather than acting in the fluid phase. In the example of spermatogonia-Sertoli cell, spermatogonia produce somatostatin and Sertoli cells express sst2 receptors. Activation of sst2 receptor by somatostatin binding leads to a diminished expression of stem cell factor (SCF) expression by Sertoli cells. This inhibition of SCF is associated with a decrease in spermatogonia proliferation (Krantic et al, 2004). Immunologic synapse which involve multiple adhesion and regulatory molecules between antigen-presenting cell (APC) and T-cell can also be considered juxtacrine communication (Bromley et al. 2001; Biggs et al, 2011). Juxtacrine secretion provides a unique mechanism for preventing an undesirable diffuse action of a given cytokine on innocent bystander cells (Sporn and Roberts, 1992). For example tumor necrosis factor (TNF) is a cytokine that can act by a juxtacrine mechanism has been implicated as a critical mediator of cachexia, septic shock, rheumatoid artritis, autoimmune states, induction of HIV expression and the killing of tumor cells. Transmembrane form of TNF is highly active and cell-to-cell contact, without secretion into the intercellular space, is sufficient for TNF to kill a target tumor cell (Perez et al., 1990). Disruption of juxtacrine signaling may lead to pathologic outcomes, oxidant-injured endothelial cells is one example and this disruption may be a fundamental process in adult respiratory distress syndrome, shock and similar tissue injuries (Zimmerman et al., 1993).

2.2.2 Gap Junctional Intercellular Communication (GJIC)

GJIC is different than the other modes of communication where a ligand and its receptor interaction exists by diffuse (autocrine, paracrine, endocrine) or non-diffuse (juxtacrine) mechanism. This type of communication between adjacent cells is mediated via

intercellular channels that cluster in specialized regions of the plasma membrane to form gap junctions (Robertson 1963, Revel and Karnovsky 1967, Wei et al 2004, as cited in Meşe et al, 2006). Gap junctional channels link the cytoplasm of two cells, and provide the exchange of ions (K^+, Ca^{2+}), second messengers (cAMP, cGMP, IP3) and small metabolites like glucose (Kanno & Loewenstein, 1964; Lawrence et al., 1978, as cited in Meşe et al., 2006) . Valiunas et al (2005), recently showed that transfer of small interfering RNAs between neighboring cells trough gap junctions. GJIC is essential for many pysiological events such as cell synchronization, differentiation, cell growth, and metabolic coordination of avascular organs including epidermis and lens (Vinken et al., 2006; White and Paul, 1999). GJIC forms a close electrical and metabolic unit (syncytium). It is present in the epithelium, many smooth muscles, the myocardium, and the glia of the central nervous system (Despopoulos & Silbernagl, 2003). Electric coupling permits the transfer of excitation (*electrical synapses*); many examples can be given for this wave of excitation in the body such as atrium and ventricles of the heart, stomach, intestine, biliary tract, uterus and ureter. Gap junctions are formed by two unrelated protein families, the pannexins and connexins (Meşe et al, 2006). Connexins have four transmembrane domains and six connexins oligomerize to form hemichannels called "connexons". One connexon docks with another connexon on the adjacent cell, thereby forming a common channel which substances with molecular masses of up to around 1 kDa can pass (Despopoulos & Silbernagl, 2003). This organization requires the membranes of two adjacent cells leaving a 2-4 nm gap (Bruzzone et al., 1996; White & Paul 1999). Gap junction channels are selective permeable. There are at least 21 connexin isoforms and connexons can be formed either from a single type of connexin or from more than one type, leading to the formation of either homomeric or heteromeric hemichannels, respectively (Meşe et al, 2006) and this characteristic can explain selective permeability. For example connexin32 homomeric hemichannels were permeable to both cAMP and cGMP whereas connexin26/connexin32 hemichannels were permeable mainly to cGMP. The "Contact inhibition" process can be mediated in some cells by gap junctions (Trosko, 2007).

2.2.3 Intercellular communication via Tunneling Nano-Tube (TNT) like structures

Very recently, a novel mechanism for intercellular communication was discovered by which nanotubular structures, consisting of thin membrane bridges, mediate membrane continuity between mammalian cells (Rustom et al., 2004). These channels, referred to as tunneling nanotubes (TNT), were shown to actively traffic cytosolic content from cell to cell within the interior of their filaments (Rustom et al., 2004). TNTs were first described in cultured rat pheochromocytoma PC12 cells. Calcium ions, MHC class I proteins, prions, viral and bacterial pathogens, small organelles of the endosomal/lysosomal system and mitochondria are among identified TNT cargos until now (Eugenin et al., 2009; Gerdes, 2009; Gerdes et al., 2007; Gurke et al., 2008; Koyanagi et al., 2005). Intercellular exchange via TNT based cell-communication was reported in cells which have high motility and plasticity like progenitor cells, immune cells and tumor cells. The exchange of endosome-related organelles and other cellular components over long distances and the coordination of signaling between the connected cells are realized by this way (Rustom et al. 2004, Gerdes et al. 2007; Gerdes & Carvalho, 2008). Domhan et al (2011) reported also

intercellular exchange by TNT, between human renal proximal epithelial cells; this may play an important role in renal physiology.

2.3 Endocrine communication

Endocrine system (derived from *endon*: inside, *krinein*: to secrete, Greek) is a radio-like communication system. It consists of endocrine glands and specialized groups of cells within organs of multicellular organism. The endocrine glands sends its hormonal messages like a radio broadcast to essentially all cells of human body by secretion into the circulation of blood. Hormones are chemical messengers of endocrine communication. They are transported through the bloodstream and cells which have a receiver (a *receptor*) take this message (Greenspan & Gardner, 2004). Hormones can be proteins (eg growth hormone, FSH, LH), peptides or peptide derivatives (eg ACTH), amino acid derivatives (eg catecholamines, thyroid hormones). Steroid hormones and vitamin D are derived from cholesterol. Retinoids are derived from carotenoids and eicosanoids are derived from fatty acids. Some hormones (eg insulin, growth hormone, prolactin, catecholamines) bind cell surface receptors, others (steroids, thyroid hormones) bind to intracellular receptors that act in the nucleus. Hormone binding alters receptor conformation and this alteration transmits the binding information into postreceptor events that influence cellular function (Greenspan & Gardner, 2004). Hormones serve as messenger substances that are mainly utilized for *slower, long-term transmission of signals*; they are carried by the blood to *target structures great distances away* (Despopoulos & Silbernagl, 2003). Endocrine system is essentially responsible for control and integration of multicellular organism. The principal functions of endocrine hormones at the target level, are to control and regulate enzyme activity, transport processes, growth, secretion of other hormones, exert negative or positive feedback control and coordinate cells of same type. Endocrinology is a great and expanding discipline of science (Table 1).

2.4 Neurocrine communication

Nervous communication is point-to-point through nerves and *electrical in nature* and *fast*. By this aspect, communication by nervous system is similar to sending messages by conventional telephone so it is a cable phone-like system. In neurocrine communication, neuronal cells release their products directly into the synaptic space; they act on another cell type (Öberg, 1998). A synapse is the site where the axon of a neuron communicates with effectors or other neurons (Despopoulos & Silbernagl, 2003). According to the termination of an axon, the synapse may be axo-dendritic, axo-axonic or axo-somatic (Faller and Schuenke, 2004). Chemical synapses utilize (neuro)transmitters for the transmission of information. The arrival of action potential to the synapse in the axon triggers the release of transmitter from the presynaptic terminals. The transmitter then diffuses across the narrow intercellular gap (synaptic cleft) which is approximately 10-50 nm, and it binds postsynaptic receptors in the membrane of a neuron or a glandular or muscle cell (Despopoulos & Silbernagl, 2003; Faller & Schuenke, 2004). Transmitters are released by exocytosis of synaptic cytosolic storage vesicles. Depending on the type of transmitter and receptor involved, the effect on the postsynaptic membrane may be excitatory or inhibitory. Neuroscience, like endocrinology is also another essential interdisciplinary science (Table 1).

2.4.1 Neuro-endocrine communication

In this system, neurocrine and endocrine communications exist together. Neuroendocrinology is a studying science the interactions between nervous and endocrine systems (Greenspan & Gardner, 2004). There are two major mechanism of neural regulation of endocrine function; the first is *neurosecretion* which refers to neurons that secrete hormones into the circulation. For example, hypothalamic neurons synthesize and secrete hormones (*releasing or release inhibiting hormones*) into blood vessels (a kind of portal venous system) that communicate with the anterior pituitary. Posterior pituitary hormones (oxytocin and ADH) are transported from hypothalamic neurons to the ends of the axons in the neurohypophysis where enter the systemic circulation directly (Despopoulos & Silbernagl, 2003). The second is the *direct autonomic innervation* of endocrine tissues (such as adrenal medulla, pancreatic islets and gut) which couples central nervous system signals to hormone release. Enterochromaffin cells (EC) in the gastrointestinal tract have close contact with nerve elements of both afferent and efferent type adjacent to the basal lamina of the mucosa and true synapses have been identified (Ahlman & Dahlström, 1983). One of the interesting example of neuro-endocrine communication is the stimulation of gastric secretion in the cephalic phase (Guyton & Hall, 2000). In this phase of gastric secretion, when we see or smell a nice food while we are hungry, gastric secretion increases even before food enters the stomach (Pavlov's sham-feeding assay). Neurogenic signals come by vagus to stomach. In this point the first chemical messengers are acetylcholine and gastrin-releasing peptide (GRP). It has been demonstrated that G cells have muscarinic receptors and muscarine-like action, particularly in the M3 receptor-mediated route, plays a significant role in acetylcholine-mediated gastrin secretion (Matsuno et al., 1997). And we know also that cephalic phase of acid secretion is augmented predominantly by acetylcholine and gastrin while histamine is of major importance during the gastric phase (Schusdziarra, 1993). Martinez and colleagues demonstrated similarly that atropine and gastrin antibody decrease basal acid secretion while gastrin antibody only did not block the rise in acid during sham feeding (Martinez et al., 2002). Gastrin stimulated acid secretion is through releasing histamine from ECL (enterochromaffine-like) cells (Waldum et al., 2002). Cholecystokinin 2 (gastrin) receptors in the stomach are only in the ECL cells (Waldum et al., 2002). Then, ECL cells release histamine and stimulate oxinthic cells via paracrine way (by binding H_2 receptors) to produce HCl. As a control of gastric acid secretion, somatostatin (SS14) secreted by D cells inhibits both G cell and the parietal cell.

2.5 Lumencrine (exocrine) communication

Another type of communication is lumencrine (exocrine) secretion in the open cell types like pancreas and prostate. Like the other modes of regulations, lumencrine mechanism can also play an important regulatory role both during growth and differentiation of the prostate as well as in the secretory process of the mature gland (di Sant'Agnese, 1992). Calcitonin, GRP (bombesin) and somatostatin have been reported in semen that they may be directly secreted into the ejaculate (Arver & Sjoberg, 1982; Bucht et al, 1986; Gnessi et al, 1989; Sasaki & Yoshinaga, 1989; Sjoberg et al, 1980; cited in di Sant'Agnese, 1992). It has also been shown that a decrease in seminal ionized calcium correlates with a decrease in motility of sperm

(Prien et al., 1990). On the other hand, lumencrine secretion of pancreatic enzymes, water and ions play a major role in the duodenal phase of the digestion (Raybould et al., 2003) (Figure 2).

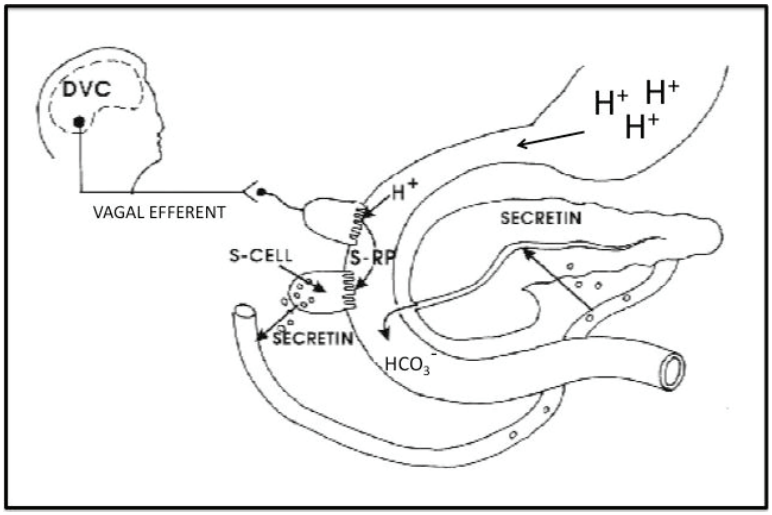

Fig. 2. Lumencrine, endocrine and neuro-endocrine communications during the regulation of pH in duodenum (modified from Li et al., and cited in Konturek et al, 2003). DVC: Dorsal vagal center, S-RP: Secretin releasing peptide.

2.6 Hypotheses about intercellular communication

Information theory was developed by Shannon, approximately more than 60 years ago (Shannon, 1948), to determine *quantitative* aspects of information exchange between a biologic source and a biologic receiver (Mayer and Baldi, 1991). According to this theory, peptidergic cell-to-cell communication between neurocrine, endocrine and growth factor-mediated messages require different encoding and decoding strategies. On the other hand *qualitative* component of the exchanged message is concerned with semantic information such as human language. The word "information" means "knowledge of order" in common language (Vincent, 1994). The laws of linguistics and semantics are valid not only at the organismic level, but also at the cellular and molecular level (Vincent, 1994). Today, we know that bacteria communicate by quorum sensing molecules (Miller & Bassler, 2001). Microbian language contains two component system which consists of a signal (input) and a response (output) (Pechère, 2007). Sensors receive the signal, effectors make the response. Cells use a molecule-based language called *cellese* which has the counterparts of sound- and visual-based human language (*humanese*) (Ji, 1999). What is transmitted is the meaning of the message (significance) which can be memorized by the cell, providing a possible following use (Vincent, 1994). It was suggested that cytokines can be viewed as symbols in an intracellular language (Sporn & Roberts, 1988). The participation of the extracellular matrix in the language of intercellular communication is a way that multicellular organisms can use past experience to determine the response to

cytokines and interactions with matrix enable cytokines to elicit adaptive responses (Nathan & Sporn, 1991). Unique phenotype of cells based their carbohydrate determinants on their cell surfaces is another area of interest (glycobiology) in intercellular communication (Sporn & Roberts, 1992). Many peptide growth factors and cytokines are described and they are multifunctional ((Sporn & Roberts, 1988). It is apparent that they form part of complex cellular signaling language, in which the individual peptides are the equivalent of characters of an alphabet or code (Sporn & Roberts, 1988). Five years ago, we proposed an intercellular network model (message-adjusted network) in the physiology of gastro-entero-pancreatic (GEP) endocrine system, based on up-to-date information from medical publications (Aykan, 2007). In this network; **message** is an input which can affect the physiologic equilibrium, **mission** is an output to improve the disequilibrium, **aim** is always maintenance of homeostasis. Messages are picked up by biologic sensors or detectors. If we orientate to a transmission of a unique, physiologic, simple message we can design its proper network (Figure 3). In this model, different cells use different chemical messengers via different modes of regulations to transmit the same message. These **message-adjusted intercellular networks** may be most important (or unique) determinants in the formation of proper, environmentally adaptive multi-cellular organizations in the biology and it should be tested in the laboratory.

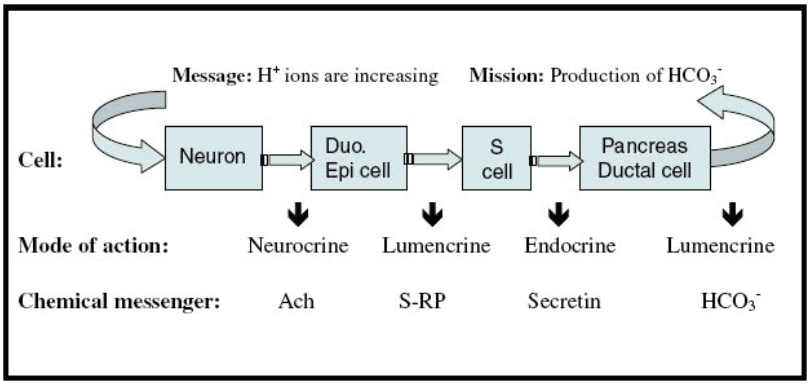

Fig. 3. A message-adjusted network model in the regulation of pH at the duodenum. Unbuffered hydrogen ions coming from stomach stimulate secretin release from S cells in the duodenum. Major effect of secretin is the secretion of bicarbonate ions from pancreatic ductal epithelium. Vagal stimulation results also small stimulation of pancreatic bicarbonate secretion.

Human body has more than 200 different cells (Alberts et al., 1994). The crucial question in the field of communication physiology is that "how they communicate each one with another?" Today, we know the words that are chemical messengers (bioactive peptides like hormones and neuromediators, cytokines, growth factors, some ions and other molecules), but we don't know languages specific to cell populations (tissue specific languages!). Although there is an expanding knowledge in molecular biology, some scientific disciplines which should be related to modes of communication are not developped yet in the literature (Table 1).

Intercellular Communication	Scientific Discipline
Intracrine	Intracrinology
Autocrine	?
Paracrine	?
Juxtacrine	?
Endocrine	Endocrinology
Neurocrine	Neuroscience
Neuro-endocrine	Neuroendocrinology
Lumencrine	?

Table 1. Modes of intercellular communication and related scientific disciplines.

On the other hand, we suggest that pheromones can play a role in lumencrine interindividual unconscious communication (Mayer & Baldi, 1991; Brennan & Keverne, 2004; Knecht et al., 2003) but we don't know if there is a wireless-like intercellular communication. This type of communication, if there is, should be between mobile cells, such as circulating cells, spermatozoa or mature oocyte and it should be bi-directional to provide cell to cell crosstalk. One potential example can be the induction of hypertrophy of draining lymph nodes by mast-cell derived tumour necrosis factor during infection (Mc Lachlan et al., 2003). The mode of this action of mast cells is defined as a remote control mechanism (Buckland, 2003). A second candidate for wireless communication can be again in the immune system; substance P and its receptors have been detected in granulocytes, monocytes and lymphocytes (Ferone et al, 2001). A third and most interesting example may be in the spermatozoa-egg communication; recent studies indicate that olfactory receptors might be a role in the chemotaxis of spermatozoa (Sliwa, 2003; Spehr et al, 2004; Fukuda et al, 2004; Eisenbach & Tur-Kaspa, 1999).

3. Conclusion

After human genome project (HU-GO) and protein organisations (HU-PO), it is time to resolve all parts of intercellular communication. Clarifying intercellular communication systems is as important as intracellular signal mechanisms. Finally, we believe that intercellular communication in our world becomes by specific molecules; these molecules are the words of the cell language.

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Section 4

Cellular Basis of Disease and Therapy

17

Adult Stem Cells in Tissue Homeostasis and Disease

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1. Introduction

Stem cells (SCs) are a rare population of cells characterized by the ability to self-renew in order to preserve the SC pool and to differentiate in different lineage to produce progeny needed for the physiological functions of tissues and organs. SC can be classified as embryonic SC (ESC) and adult or somatic SC (ASC): ESC have been isolated from the inner cell mass of the blastocyst and are pluripotent cells, that is cells able to differentiate into all the cell types required to form an entire organism (Smith, 2001); ASC are tissue-resident SC that, based on their differentiation potency, can be classified as multipotent, oligopotent or even unipotent. It is still controversial whether every mammalian tissue and organ possesses an ASC, but many tissue-specific ASC have been successfully identified and isolated e.g., hematopoietic SCs (HSCs), mammary SCs, muscle SCs (satellite cells), intestinal SCs, and mesenchymal SCs. All these tissues need to constantly replace damaged or dead cells throughout the life of the animal. This process of continual cell replacement critical for the maintenance of adult tissues, is called tissue homeostasis, and is maintained through the presence of ASC (Fig. 1). The homeostatic replacement of cells varies substantially among different tissues. The epithelium of the intestine is one of the most rapidly self-renewing tissue in adult mammals and it completely self-renews in around 5 days (van der Flier & Clevers, 2009). By contrast, interfollicular epidermis takes 4 weeks to renew (Blanpain & Fuchs, 2009), whereas the lung epithelium can take as long as 6 months to be replaced (Rawlins & Hogan, 2006). Moreover, apart from the maintenance of tissue homeostasis, ASC are devoted to the regeneration and repair of highly specialized tissues. Regeneration refers to the proliferation of cells to replace lost structures, such as the growth of an amputated limb in amphibians. In mammals, whole organs and complex tissues rarely regenerate after injury, but tissues with high proliferative capacity, such as the hematopoietic system and the epithelia of the skin and gastrointestinal tract, renew themselves continuously and can regenerate after injury, as long as the SC of these tissues are not destroyed (Fig. 1). Repair most often consist of a combination of regeneration and scar formation by the deposition of collagen which relative contribution depends on the ability of the tissue to regenerate and the extent of the injury. For instance, in superficial injury of the skin, wound can heal through the regeneration of the surface epithelium. However, scar formation is the predominant healing process that occurs when the extracellular matrix framework is damaged by severe injury (Fig. 1). This last mechanism results in restoration of tissue continuity but with or without function (Gurtner et al., 2008).

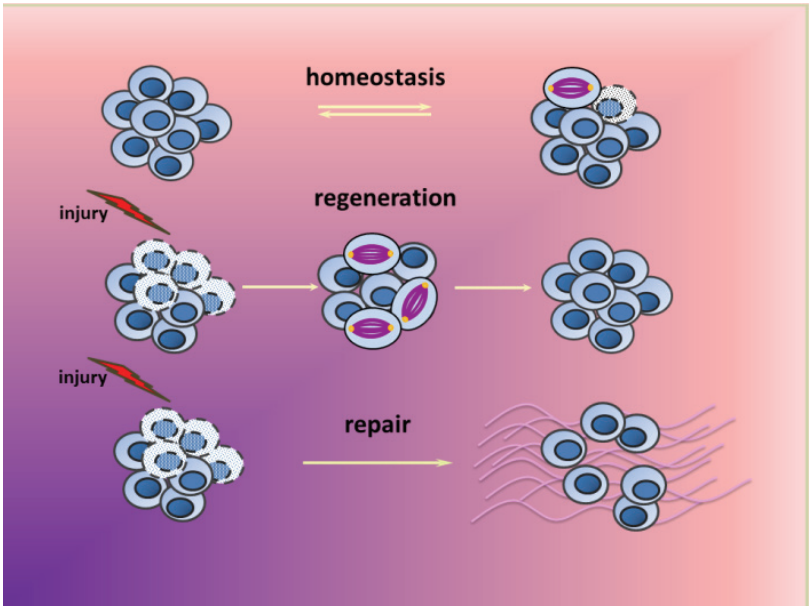

Fig. 1. Normal homeostasis and healing responses. In normal homeostasis a balance between proliferation and cell death maintains the tissue structure and function. Healing after acute injury can occur by regeneration, that restores normal tissue structure, or repair with deposition of collagen fibers and scar formation.

2. SCs and their niches

Self-renewal and differentiation of ASC are supported by two types of cell division known as symmetric and asymmetric (Morrison & Kimble, 2006). With symmetric division both the daughter cells acquire similar fates, while the asymmetric division, a fundamental and nearly universal mechanism for the generation of cellular diversity and pattern, gives rise to daughter cells with dissimilar fates. Divergent fates in daughter cells may be recognized by various characteristics: (i) morphological, such as cell size and shape; (ii) molecular, such as the segregation of proteins into only one daughter cell; or (iii) behavioural, such as the subsequent descendant types produced by either of the daughter cells. One mechanism for fate determination of daughter cells following symmetric and asymmetric cell divisions is the partitioning of fate-determining molecules during mitosis of the mother cell (Tajbakhsh et al., 2009). The idea that specific molecules can be partitioned unequally to daughter cells and behave as fate determinants had been hypothesized over a century earlier, following observations of cell divisions in simple organisms. When an intrinsic mechanism is used, cells establish an axis of polarity, orient the mitotic spindle along this axis and localize cell fate determinants to one side of the cell. During cytokinesis, determinants are then segregated into one of the two daughter cells where they direct cell fate (Betschinger & Knoblich, 2004). However, this hypothesis was only experimentally validated a little under two decades ago, with the identification of the first asymmetrically segregated cell fate determinant – Numb (Rhyu et al., 1994).

Alternatively, the SC depends on the contact with the surrounding microenvironment (the SC "niche") for maintaining the potential to self-renew (Li & Xie, 2005). By orienting its mitotic spindle perpendicularly to the niche surface, the SC will placed the two daughters in distinct cellular environments either inside or outside the SC niche, leading to asymmetric fate choice. However, when SC divides parallel to the niche it may also generate two identical SC in order to increase SC number or to compensate for occasional SC loss (Yamashita et al, 2010). The concept of the "niche" was proposed first by Schofield (Schofield, 1978) who hypothesized that proliferative, hematopoietic cells derived from the spleen displayed decreased proliferative potential when compared to HSC obtained from the bone marrow because they were no longer in association with a complement of cells, the "niche", which supports long term SC activity. This concept subsequently has proven relevant to many different SC systems, and the definition of the niche has been expanded further to include functional regulation of SC by both cellular and acellular (extracellular matrix) component of the niche. Thus the niche comprise all the microenvironment surrounding SCs, which provides diverse external cues to instruct SCs activities, preserve their proliferative potential and block maturation (Jones & Wagers, 2008).

3. Signaling pathways regulating SC function

Despite morphological and functional differences among different ASC, common signaling pathways appear to control SC self-renewal, activation, and differentiation, including Notch and Wingless-type (Wnt).

3.1 Notch signaling pathway

The Notch signaling pathway was discovered in flies more than 90 years ago (Morgan, 1917), and it is among the most well-conserved signaling pathways in animals. It arose with the evolution of multicellular organisms and the concomitant need for juxtacrine cell-to-cell communication to coordinate development. In mammals, four Notch transmembrane receptors (Notch1-4) have been described. Notch ligands are also transmembrane proteins comprising two different subtypes (Delta, Jagged), each containing several members (Jagged1-2, Delta-like1, 3, and 4) (Kopan & Ilagan, 2009). In Notch signaling, a 'signal-sending cell' presents the Notch ligand to the 'signal-receiving cell', which expresses the Notch receptor. Triggering of Notch receptor by ligand binding promotes two proteolytic cleavage events at the Notch receptor (Fig. 2) (Kopan & Ilagan, 2009). The first cleavage is catalyzed by the ADAM-family of metalloproteases, whereas the second cleavage is mediated by γ-secretase, an enzyme complex that contains presenilin, nicastrin, PEN2 and APH1. The second cleavage releases the Notch intracellular domain (NICD), which is free to translocate to the nucleus where it engages CSL, converting it from a transcriptional repressor to an activator and activates transcription of genes containing CSL binding sites (Kopan & Ilagan, 2009). In the absence of a Notch signal, CSL represses transcription of Notch target genes by interacting with the basal transcription machinery and recruiting ubiquitous corepressor proteins to form multiprotein transcriptional repressor complexes (Lai, 2002). In the presence of a Notch signal, NICD binding to CSL displaces corepressors from CSL. The best characterized Notch target genes belong to the hairy enhancer of split (Hes) complex and consist of the b-HLH transcription factors Hes (1-7) and Hey (1-3) (Bray & Bernard, 2010).

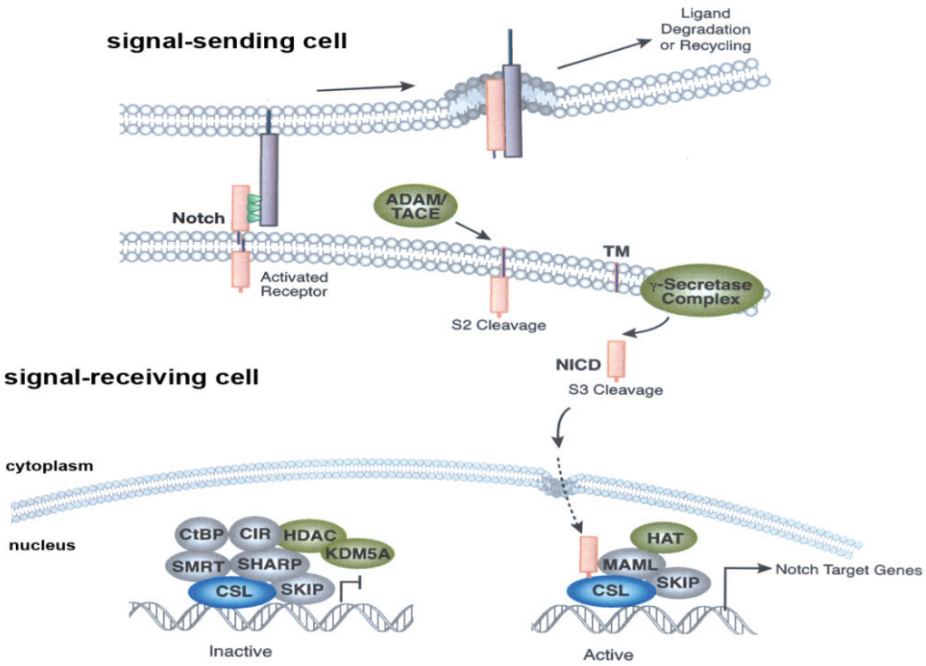

Fig. 2. Model of Notch signaling pathway. See the text for detail.

3.2 Wnt signaling pathway

The Wnt signaling pathway is a highly conserved developmental pathway, and orchestrates development and morphogenesis in many different tissues. Wnt proteins are secreted proteins, that bind to receptors of the Frizzled family (FZD) (Wodarz & Nusse, 1998), of which 10 members were found, and several coreceptors such as lipoprotein receptor-related protein (LRP)-5/6, (Pinson et al., 2000) Ryk, or Ror2 (Logan & Nusse, 2004). Wnt signals can be transduced to the canonical, or Wnt/β-catenin, pathway and to the noncanonical, or β-catenin independent, pathway.

3.2.1 Canonical Wnt signaling pathway

The canonical Wnt pathway involves the multifunctional protein β-catenin (MacDonald et al., 2009). In the absence of Wnt, β-catenin is targeted to a multimeric destruction complex with adenomatous polyposis coli (APC) and Axin and is phosphorylated by casein kinase 1α, followed by phosphorylation by glycogen synthase kinase (GSK)3β (Fig.34) (Ikeda et al., 1998). This phosphorylation targets β-catenin for ubiquitination and degradation by the proteasome. The binding of Wnt ligands to the FZD receptors results in the disassembly of the destruction complex and the stabilization of β-catenin. This process also involves the protein dishevelled (DVL). Cytoplasmic β-catenin accumulates and is eventually imported into the nucleus, where it serves as a transcriptional coactivator of transcription factors of the TCF/LEF family (Arce et al., 2006). TCF/LEF target genes are then involved in regulating cell proliferation, SC maintenance, or differentiation.

3.2.2 Noncanonical Wnt signaling pathway

Different noncanonical Wnt signals are transduced through FZD receptors and coreceptors. Depending on the major intracellular mediators used, those are called the Wnt/JNK (Veeman et al., 2003) or Wnt/calcium pathway (Fig. 3). The core element of the Wnt/JNK pathway (or planar cell polarity –PCP- pathway) includes the activation of small GTPases of the rho family, such as rac, cdc42, and rhoA. The GTPases can activate more downstream mediators like JNK or rho kinase (ROK). In this branch, Dvl is also recruited by a FZD receptor and promotes the asymmetrical localization of the PCP core proteins within the cell (Montcouquiol, et al. 2006). The asymmetrical subcellular localization of these elements in an epithelial sheet directs cytoskeletal reorganization. The same mechanism is used in mesenchymal cells to direct cell movement and migration during gastrulation (convergent and extension movements) (Roszko, et al., 2009).

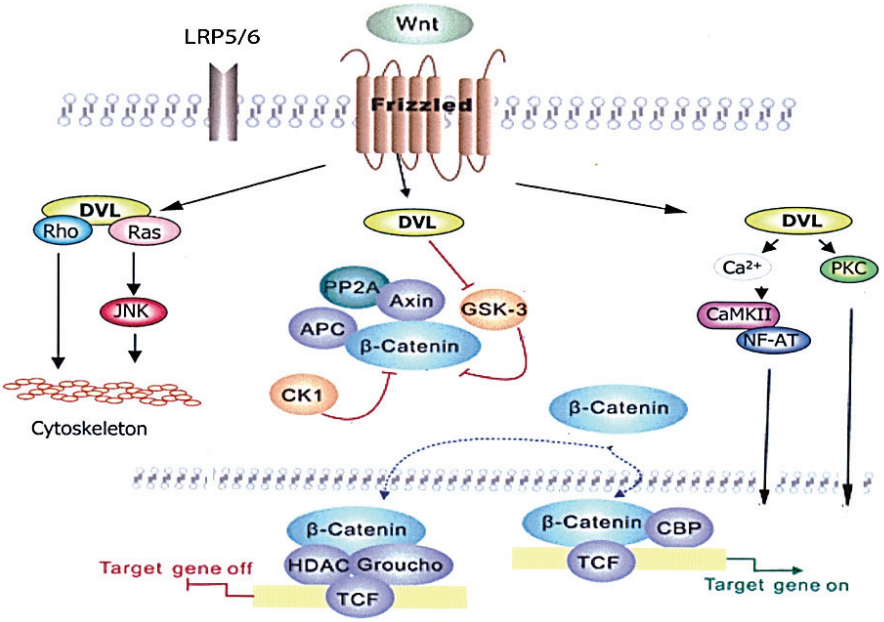

Fig. 3. Model of canonical and noncanonical Wnt signaling pathway. See the text for detail.

The existence of the Wnt/calcium pathway was hypothesized because injection of RNA coding for certain Wnts or FZD into early zebrafish embryos triggered intracellular calcium release (Slusarski et al., 1997) and loss of Wnt-11 or Wnt-5A function resulted in reduced intracellular calcium signaling (Eisenberg & Eisenberg, 1999; Westfall et al., 2003). This finding was subsequently expanded by the observation that the Wnt-induced release of intracellular calcium is sufficient to activate different intracellular calcium-sensitive enzymes such as protein kinase C, PKC (Sheldahl et al., 1999), calcium–calmodulin-dependent kinase II, CamKII (Kuhl et al., 2000) and the calcium-sensitive phosphatase calcineurin (Saneyoshi et al., 2002). Through calcineurin the Wnt/calcium pathway connects to NFAT (nuclear factor of activated T cells) transcription factor and gene expression.

Presently, a series of recent findings clearly indicate that different Wnt signaling pathways are simultaneously active within the same cell type, supporting the idea that Wnt pathways are highly connected to form a Wnt signaling network. This network seems to be activated by either one or more ligands acting on a certain cell type (Kestler & Kuhl, 2008).

3.3 Wnt signaling inhibitors

Secreted frizzled-related proteins (SFRP1, 2, 3, 4, 5), WIF1, DKK1, -2, -3, and -4 are secreted-type Wnt signaling inhibitors. WIFs and SFRPs can directly bind to Wnt proteins in the extracellular space, thereby affecting receptor occupancy and, ultimately, the cellular response (Bovolenta et al., 2008). DKK1 is among the best-characterized inhibitors of the canonical Wnt pathway. DKK1 itself is a target gene of Wnt/β-catenin signaling, thereby establishing a negative-feedback loop (Niida et al., 2004). There are two possible mechanisms by which DKK1 inhibits β-catenin signaling. One possible mechanism is that DKK1 prevents the formation of Wnt–FZD–LRP6 complexes on the cell surface by binding to LRP6 (Seto et al., 2006). Another possibility, which is related to the internalization of LRP6, is that DKK1 binds to another class of receptor, Kremen (Krm). In this model, the binding of DKK1 to LRP6 and Krm results in the formation of a ternary structure and induces rapid endocytosis and the removal of LRP6 from the plasma membrane, and thereby attenuates β-catenin signaling (Mao et al., 2002).

4. Hematopoietic SCs

In adult mammals, HSCs form a rare population of multipotent SCs that reside primarily in the bone marrow (BM). They have the capability to both self-renew and constantly give rise to lineage-specific progenitor cells and effector blood cells that perform the physiological functions of the hematopoietic system. Blood cells can be classified into various cell types, from the myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells), and lymphoid lineages (T-cells, B-cells, NK-cells) (Liu et al., 2010).

HSCs are functionally defined by their capacity to reconstitute the hematopoietic system of immunodeficient animals such as NOD/SCID mice or contribute to functional reconstitution in human transplant settings. HSCs can be identified and isolated by a combination of presence and absence of cell surface markers. The most commonly used combination is characterized by the positive expression of the tyrosine kinase receptor c-Kit (CD117) and the membrane glycoprotein Sca-1 (Okada et al., 1992), together with the lack of markers of terminal differentiation (Ter119, Gr-1, Mac-1, B220, CD4 and CD8), collectively known as Lineage markers. The resulting c-Kit+ Sca-1+ Lin- population, is commonly referred to as KSL cells. More recently, an alternative method was described, using a signature of SLAM (Signaling lymphocyte activation molecule) family of cell surface molecules, CD150+ CD244- CD48- (Kiel et al, 2005). This is the first family of receptors whose combinatorial expression precisely distinguishes HSCs from hematopoietic progenitor cells (HPC).

The BM microenvironment –also called niche- plays an important role in the regulation of self-renewal and differentiation of HSCs. It is composed of different types of cells and structures surrounding the bone, which regulates the fate of hematopoietic cells through

direct or indirect means, facilitating a stable generation of all the blood cells needed in a steady state situation. But the niche also adapts in times of hematopoietic stress. A failure to maintain a strict regulation of the hematopoietic cells can lead to a variety of malignancies such as leukemia, the most common form of cancer in humans (Renstrom et al., 2010).

4.1 Notch pathway as a regulator of HSC behavior

All Notch receptors and ligands are expressed on HSCs (Singh et al., 2000) and it is now well established that Notch signaling is essential for the production of HSCs during embryogenesis. However, its role in subsequent stages of mammalian HSC development is still controversial (Liu et al, 2010; Radtke et al., 2010).

In adult hematopoiesis, activation of Notch signaling has been reported to promote HSCs self-renewal, proliferation and differentiation *in vitro* and *in vivo*, and in both mice and humans. Constitutive expression of NICD by HSCs, leading to the constitutive activation of the Notch pathway, enhances proliferation and consequently delays hematopoiesis. Conversely, it inhibits differentiation in response to various cytokines, mostly under myeloid promoting conditions (Carlesso et al, 1999). Several reports show that HSCs stimulated with soluble or membrane-bound Notch ligand Delta 1 (Karanu et al, 2001) or Jagged1 (Karanu et al. 2000) increase in expansion potential *in vitro* and in reconstitution capacity *in vivo*. Although these gain-of-function studies show an important role for Notch in expanding the HSC pool, they do not prove that Notch is essential for post-natal hematopoiesis. The controversy arises from several loss-of-function studies in mice that did not fully support the previous conclusions. In particular, inactivation of Notch receptors (Notch1, Notch2), ligands (Jagged1) or downstream effectors (CSL/RBPJ, Mastermind-like1) does not impair HSC function (Cerdan & Bhatia, 2010). Additional studies failed to identify a protective role for Notch when HSCs were exposed to oxidative stress. Taken together, these results show that Notch signaling is not a major regulator of adult HSC maintenance *in vivo*. Downstream of HSCs, Notch signaling plays a critical role in cell fate decision of a variety of oligopotent progenitor cells in the hematopoietic system, such as in T-cell development. Inactivation of Notch signaling in HPCs results in early blockade of T-cell lymphopoiesis, due to a failure in commitment to the T-cell lineage. Transgenic mice with a conditional deletion of Notch1 do not develop T-cells but develop ectopic B-cells in the thymus, while immunodeficient mice expressing a constitutively active form of Notch1 develop ectopic T-cells in the bone marrow (BM) but no B-cell (Tanigaki & Honjo, 2007). Additionally, Notch1 signaling is necessary at various stages of T-cell development, such as progression through thymocyte maturation, regulation of T-cell Receptor β (TCR-β) gene rearrangement, regulation of lineage decisions between αβ and γδ lineages (Tanigaki & Honjo, 2007).

4.2 Role of Notch in T-cell leukemia

The pathological role for a deregulated Notch signaling was first described in a rare human T-cell acute lymphoblastic leukaemia/lymphoma (T-ALL), in which a t(7;9) chromosomal translocation results in the generation of a constitutively active, but truncated form of the Notch1 receptor named TAN1 (Translocation Associated Notch homolog) (Ellisen et al., 1991). Evidence that constitutively active Notch1 is responsible for disease development was provided by murine BM reconstitution experiments. Irradiated mice transplanted with BM

progenitors expressing activated forms of Notch1 developed clonal hematopoietic tumors characterized as T-ALL. Experiments performed using other truncated Notch isoforms, including Notch2 and Notch3, showed similar results. However, mice having a defect in T-cell development failed to produce tumors. These results reveal that Notch1 has a special oncotropism for T-cell progenitors (Radtke et al., 2010). These findings became extremely relevant when a study of a large number of T-ALL patients revealed in more than 50% of them the presence of at least one gain-of-function mutation in the Notch1 receptor, emphasizing the oncogenic role of Notch (Weng et al., 2004). Notch1 mutations found in T-ALL affect critical domains responsible for preventing the spontaneous activation of the receptor in the absence of ligand or for terminating Notch1 signaling in the nucleus.

Studies of the genes and pathways controlled by Notch in T-ALL identified Notch1 as a central regulator, promoting leukemia cell growth by multiple direct and indirect mechanisms (Fig. 4) (Paganin & Ferrando, 2011). Analysis of Notch1 expression in T-ALL showed that it acts as a direct transcriptional activator of multiple genes. Notch1 also promotes the expression of the MYC oncogene, which in turn further enhances its direct effect on anabolic genes and facilitates cell growth. Indeed, many of the anabolic genes directly controlled by Notch1 are also direct targets of MYC, creating a feed-forward-loop transcriptional network that promotes leukemic cell growth (Palomero et al., 2006). Additionally, Notch1 facilitates the activation of the PI3K-AKT-mTOR signaling pathway, a critical regulator of cell growth and metabolism, via transcriptional downregulation of the PTEN tumor suppressor gene by Hes1, a transcriptional repressor directly downstream of Notch1 signaling (Palomero et al., 2007). The mTOR signaling was suppressed in T-ALL cells upon inhibition of Notch signaling, illustrating the importance of this indirect mechanism of regulation. The transcriptional program activated by oncogenic Notch1 also has a direct effect on cell cycle progression, promoting of G1/S cell cycle progression in T-ALL. This effect is mediated in part by transcriptional upregulation of CCND3, CDK4 and CDK6. Moreover, Notch1 induces the transcription of the S phase kinase-associated protein 2 (SKP2), which mediate the proteasomal degradation of CDKN1B (p27/Kip1) and CDKN1A (p21/Cip1), promoting premature entry of the cells into S phase (Sarmento et al, 2005). Notch1 can also modulate the survival of T-ALL cells by interacting with NF-κB, upregulating its activity by increasing expression of IκB kinase and upregulating both the expression and the nuclear localization of NF-κB. Inhibition of NF-κB in T-ALL can efficiently restrict tumor growth both *in vitro* and *in vivo* (Vilimas et al., 2007).

In addition, Notch1 modulates the NFAT cascade through the activation of calcineurin, which is a calcium-activated phosphatase that is important for the activation and translocation of NFAT factors to the nucleus. Calcineurin inhibition resulted in T-ALL cell death, as well as tumor regression and prolonged survival of leukemic mice (Medyouf et al., 2007). Finally, Notch1 regulates the activity of p53, lowering its expression through repression of the ARF-mdm2-p53 surveillance network. Attenuation of Notch signaling led to increase p53 expression and to tumor regression by inducing apoptosis (Beverly et al., 2005). A strong body of evidence supports a central role of Notch1 in promoting cell metabolism, growth and proliferation, as well as in enhancing the activity of signaling pathways that reinforce these functions and also promote cell survival. These results suggest that blocking Notch1 signaling may reduce the self-renewal capacity of T-ALL cells and/or selectively affect the leukemia initiating cell population.

Only few Notch mutations have been reported in myelogenous leukemias, but it is unclear whether Notch aberrant expression is responsible for the disease.

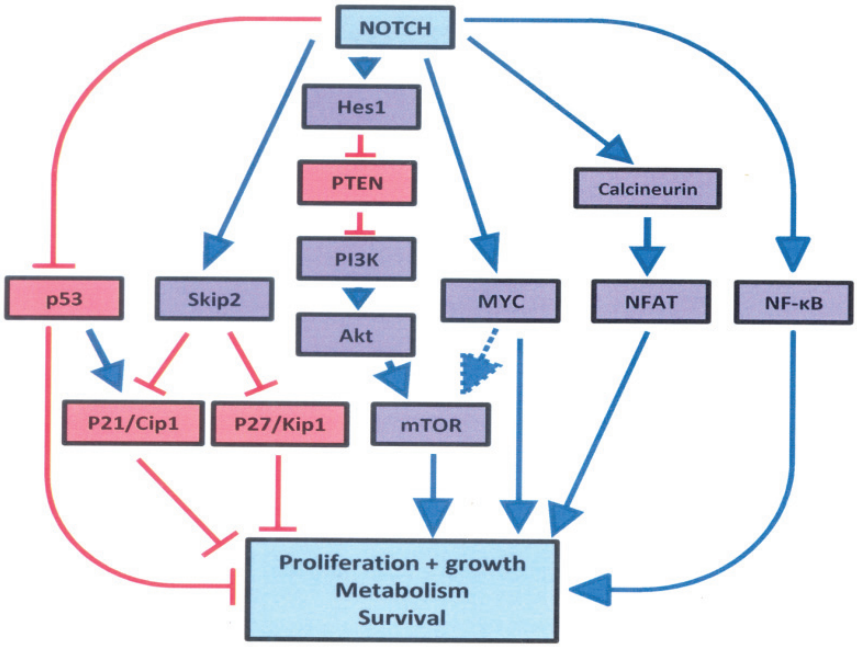

Fig. 4. Genes and pathways controlled by Notch in T-ALL

4.3 Wnt pathway and HSC

In hematopoiesis, Wnt pathway activity is required in the BM niche to regulate HSC proliferation and preserve self-renewal capacity (Malhotra & Kincade, 2009). Even though the role of canonical signaling on the regulation of adult hematopoiesis has been studied in great detail, controversy remains, possibly explained by differences in strength and duration of Wnt signaling or redundancy with other pathways. A role for Wnt signaling in hematopoiesis is supported by observations that Wnt ligands enhance proliferation of HSCs ex vivo (Van Den Berg et al, 1998) and that Wnt antagonists inhibit HSC proliferation and reconstitution. In particular, only short-term repopulation was reported using HSCs from normal mice cultured with Wnt3A (Reya et al., 2003; Willert et al., 2003). Subsequent studies reported that noncanonical Wnt5a inhibited canonical Wnt3a-mediated signaling to promote the maintenance of quiescent, functionally transplantable HSCs. In addition constitutively active nuclear β-catenin signaling reduces HSC quiescence and blocks HSC differentiation (Kirstetter et al., 2006). On the other hand, osteoblast-specific expression of Dkk1 results in increased HSC cycling and reduced regenerative capacity (Fleming et al, 2008). These findings suggest that Wnt pathway activation in the niche limits HSC proliferation and preserves self-renewal. These observations suggest that fine-tuning of Wnt/β-catenin activity in the microenvironment is crucial for maintaining SC quiescence.

The canonical Wnt pathway has also been shown to be necessary for appropriate HSC development (Zhao et al., 2008). In this model, Ctnnb1-/- bone marrow cells are deficient in long-term HSC maintenance and compete poorly against wild-type cells. However, experiments in adult HSC revealed that Ctnnb1 is dispensable for HSC maintenance in fully developed HSC (Koch et al., 2008). This indicates differential requirements for self-renewal pathways in development versus maintenance of HSC.

In the context of development, genetic studies have demonstrated the requirement for canonical signaling in the formation of mesoderm (Kelly et al., 2004; Liu et al., 1999). Recent advances have provided insights into the uniqueness of the biological functions of canonical and noncanonical pathways. It has been found that non-canonical and canonical Wnts affected different target populations and stages of hematopoietic development (Vijayaragavan et al., 2009). Consistent with its previously defined role in human adult cells (Van Den Berg et al., 1998), canonical signaling increased proliferation of blood committed progenitors when administered during the proper window of time during EB development. However, a short pulse of non-canonical signaling was necessary and sufficient to control exit of hESCs from the pluripotent state and subsequent entry into the mesendoderm/mesoderm lineages (Vijayaragavan et al., 2009). Taken together, these findings provide the first evidence of a unique role for non-canonical signaling in early specification of hematopoiesis from hESCs, whereas canonical signaling affects the proliferation of cells already fated to blood. These studies provide a valuable model system for examining the possibility of chronological activation and interaction between non-canonical and canonical signaling in the cellular progression from mesoderm to blood. The controversial function of canonical signaling on the reconstituting capacity of adult HSCs, combined with these present findings in hESCs, underscores the importance of fine tuning the strength and duration of Wnt signaling towards therapeutically exploiting the balance between self-renewal and lineage commitment of HSCs.

However, there are conflicting reports on the requirement for Wnt/β-catenin signaling in basal hematopoiesis: conditional disruption of β-catenin in adult HSCs does not affect their ability to self-renew and reconstitute hematopoietic lineages (Huang et al, 2009). In addition, although overexpression of stabilized β-catenin increases immunophenotypic HSCs, this is associated with a loss of repopulating activity and hematopoietic failure *in vivo* (Kirstetter et al., 2006), findings that appear incompatible with a positive role for β-catenin in hematopoiesis. A general conclusion from these apparently conflicting reports is that the role of Wnt signaling in hematopoiesis is complex and context dependent (Staal & Sen,. 2008). However, although the β-catenin loss-of-function studies suggest that canonical Wnt signaling is not essential for basal hematopoiesis in adults, they do not rule out a possible role for the Wnt/β-catenin pathway under nonbasal conditions and are still compatible with gain-of-function experiments in which the pathway is activated.

4.4 Wnt signaling and malignant HSC

Stem cell quiescence is closely associated with protection from myelotoxic insults (Cheshier et al, 1999). Similar to the role of tissue SCs in normal tissues, several cancers are also propagated by small populations of quiescent cancer stem cells (CSCs) that are resistant to both conventional chemotherapy and targeted therapies, and are retained and contribute to relapse following discontinuation of therapy (Dick, 2008).

When Ctnnb1 was deleted contemporaneously with activation of BCR-ABL using retroviral infection and transformation of HSC, chronic myeloid leukemia stem cell (CML-LSC) failed to engraft in secondary recipient mice (Hu Y et al., 2009). These experiments clearly indicate a pivotal role of Wnt signaling in CML-LSC development. More recently, Ctnnb1 has been investigated in the maintenance of already engrafted CML-LSC. In this clinically relevant setting, pharmacologic or genetic inactivation of Ctnnb1 after onset of the myeloproliferative disease acted synergistically with imatinib, reduced LSC numbers, and improved survival in a BM transplant model (Abrahamsson et al., 2009). Thus, despite its dispensability for adult HSC, CML-LSCs seem to retain dependency on canonical Ctnnb1 to maintain self-renewal capacity. In human disease, Ctnnb1 activation via the canonical Wnt pathway has been shown to occur in CML-blast crisis LSCs. Aberrant splicing of GSK3 appears to contribute to this hyperactivation in blast crisis samples (Abrahamsson et al., 2009). Thus, there is growing evidence that canonical Wnt signaling is an attractive target pathway in the treatment of CML-LSC. Moreover, cell extrinsic inhibition of Wnt signaling through ectopic DKK1 expression impairs leukemia cell proliferation in vitro (Zhu et al., 2009).

5. Intestinal SCs

Homeostasis of the intestinal epithelium is maintained by an intestinal SC (ISC) compartment that resides at the bottom of the crypt, safely far from the shear stresses and potentially toxic agents. These ISC are at the top of a cellular hierarchy and are crucial for the renewal of the differentiated progeny within the intestinal layer (Medema & Vermeulen, 2011). Indeed, as they migrate out of their niche, they cease to proliferate and initiate differentiation into the different cell lineages of the mature villi: absorptive enterocytes, mucin-secreting-goblet cells, peptide hormone-secreting neuroendocrine cells, and microbicide-secreting Paneth cells. Until relatively recently, ISCs were a rather elusive entity at the bottom of the intestinal crypt, and the discovery of ISC markers has only partly detailed the organization of the intestinal crypt and villi. Briefly, the marker LGR5 identifies crypt base columnar cells (CBCC) located in between the Paneth cells at the crypt bottom (Barker et al., 2007), whereas the markers BMI1 and TERT identify the +4 position in the crypt, just above the Paneth cells (Montgomery et al., 2011; Sangiorgi & Capecchi, 2008). Knock-in constructs that allow expression of GFP and Cre from the Lgr5 locus show that LGR5 expression is confined to CBCCs, and that these cells give rise to the variety of epithelial cells present in crypts, proving that CBCCs function as ISCs as well (Barker et al., 2007, Sato et al., 2009). The existence of these different types of ISC remains a matter of debate and notably, remains to be determined whether and how BMI1+ +4 cells ISCs and LGR5+ ISCs relate to each other. Interestingly, recent data indicate that TERT-expressing ISCs can generate LGR5+ISCs (Montgomery et al., 2011) suggesting that these different ISC types may act in a hierarchical fashion. Regardless of this dispute about ISC identity, there is a consensus that ISCs reside in a niche that provides the cells with essentials signals such as Wnt, Notch and Hedgehog. Under normal circumstances, the Paneth cell signals dictate the size of the SC pool to maintain the total number of SCs within the niche constant. SCs may divide asymmetrically, so that one SC remains within the niche, resulting in self-renewal, whilst the other daughter cell gives rise to progenitor cells that can migrate up the crypt and become more differentiated as they reach the top. Alternatively, two recent studies (Lopez-Garcia et al., 2010; Snippert et al., 2010) support that SCs may divide symmetrically either

forming two daughter SCs (leading to expansion) or two daughter non-stem progenitor cells (leading to extinction). Several pathways play a role in maintaining and regulating stem ISCs, including Wnt and Notch.

5.1 Notch signaling in intestinal epithelium

In the intestine, Notch activity determines lineage decisions between enterocyte and secretory cell differentiation. Several components of the Notch pathway are expressed in adult intestinal crypt cells, suggesting a role for Notch signaling in gene expression programs in immature proliferating compartment cells (Sander & Powell, 2004; Schroder & Gossler, 2002). The first evidence that Notch signaling plays a role in cell-type specification in the intestine was reported in Hes1 knockout mice (Jensen et al., 2000). The deletion of the Hes1 gene resulted in the generation of excessive numbers of goblet cells, enteroendocrine cells, and Paneth cells. Subsequently, it was shown that Math1 (mouse atonal homolog1), one of the genes repressed by Hes1, is required for the differentiation into the three secretory lineages, because the intestinal epithelium of Math1-mutant mice is populated only by absorptive cells (Yang et al., 2001). These data suggest that the choice between the absorptive or secretory fate might be the first decision made by each progenitor cells, and that Hes1 and Math1 activated by Notch signal play opposite roles in this decision making. Recently, using the villin promoter to drive the expression of a constitutively active form of mouse Notch1 receptor, it was noticed an expansion of proliferating intestinal progenitor cells (Fre et al., 2005). Moreover, Notch activation inhibited the differentiation of secretory cells in the mouse intestine, as there was a complete depletion of goblet cells, a marked reduction in enteroendocrine cells, and a low expression of early marker for Paneth cells. These results clearly suggest that Notch signaling is required for maintaining crypt cells in a proliferative state, at least in part, through its negative regulation of Math1. Conversely, conditional removal of the Notch pathway transcription factor CSL/RBP-J increases the proportion of goblet cells in the murine intestine, and a similar phenotype was observed using a γ-secretase inhibitor (van Es et al., 2005). These results suggest that Notch pathway is not only a gatekeeper for proliferating crypt progenitor cells, but is also involved in controlling the balance between secretory and absorptive cell types. Data suggest that the ISC microenvironment delivers Notch-activating signals to maintain stemness, which is consistent with the observation that Paneth cells express Notch ligands (Sato et al., 2011). In particular, recent papers identified Dll1 and Dll4 as the physiologically relevant Notch1 and Notch2 ligands within the small intestine of the mouse. These ligands cooperate and exhibit a partial functional redundancy to maintain the crypt progenitor compartment (Pellegrinet et al., 2011). However, Notch seems to have dual functions in the crypt, as it acts together with Wnt to affect significantly crypt homeostasis (Fre et al., 2005; van Es et al., 2005).

5.2 Canonical Wnt signaling in intestinal epithelium

The Wnt pathway proteins regulate cellular fate along the crypt-villus axis in normal gut epithelium and have been implicated in ISC self-renewal. The nuclear accumulation of β-catenin is preferentially observed in cells located at the base of crypts and decreases as cells move toward the top of the crypts (van der Wetering et al., 2002). Wnt target genes EphB2

and EphB3 control crypt cellular segregation (Batlle et al., 2002), Sox9 regulates Paneth cell differentiation (Mori-Akiyama et al., 2007), and Lgr5 (Barker et al., 2007). TCF4 null mice died shortly after birth and showed an embryonic epithelium made entirely of differentiated cells without proliferative compartments in the crypts (Korinek et al., 1998) suggesting that TCF4 maintains the proliferation of SCs in the murine small intestine. Notably, deletion of the Wnt/TCF4 target gene c-Myc led to a loss of intestinal crypts in a murine model (Muncan et al., 2006). The importance of the Wnt signaling pathway in maintaining the architecture and homeostasis of the adult intestinal epithelium was also shown in a murine model through adenoviral expression of Dkk1. This induced Wnt inhibition in fully adult mice, resulted in inhibition of proliferation in the small intestine and colon, with progressive loss of crypts, villi and glandular structure (Kuhnert et al., 2004). By contrast, when the Wnt pathway is overactivated by mutations in APC or β-catenin, many of the epithelial cells enter into the proliferative state and display a failure of the differentiation programs (Andreu et al., 2005; Sansom et al., 2004). According with these data, recent papers demonstrated that injection of R-spondin1 (R-Spo1), a potent activator of the Wnt signaling pathways, induced rapid onset of crypt cell proliferation displaying epithelial hyperplasia in the intestine of normal mice through β-catenin stabilization and subsequent transcriptional activation of target genes such as murine Axin2, Ascl2, and Lgr5 (Kim et al., 2005; Takashima et al., 2011). The effects of R-Spo1 administration determine protection against radiation-induced colitis by stimulating proliferation of intestinal SCs and protect them against a damage after allogeneic bone-marrow transplantation, suppressing inflammatory cytokine cascades and donor T cell activation (Takashima et al., 2011). These, *in vivo,* data suggest that Wnt signaling is directly linked to the promotion of cellular proliferation and, more specifically, the regulation of progression through cell cycle. In this regard, previous papers pointed to the downregulation of $p21^{cip1waf1}$, a cyclin-dependent kinase inhibitor (CKI), as an important mechanism that might mediate Wnt-dependent growth promotion. A microarray analysis showed that $p21^{cip1waf1}$ was one of the genes whose expression was increased by inhibition of Wnt signaling in human colorectal cancer-derived LS174T cells (van der Wetering et al., 2002). Furthermore, the TCF4 target gene c-Myc has been shown to play a central role in Wnt-mediated repression of $p21^{cip1waf1}$ expression at the transcriptional level through its direct binding to the $p21^{cip1waf1}$ gene promoter (van der Wetering et al., 2002). These data suggest that the repression of $p21^{cip1waf1}$ by c-Myc might be the intracellular mechanism by which Wnt signaling regulates the G1/S transition and cell cycle progression. This signaling cascade has been shown to be functional *in vivo*, because abnormal features of proliferation/differentiation in the adult murine intestine, which occur with the single deletion of APC, are mostly rescued when c-Myc gene is simultaneously deleted (Sansom et al., 2007). Furthermore, this restoration of the morphologically normal phenotype in double mutant mice for APC and c-Myc is accompanied by restoration of p21 expression within the crypts, suggesting the involvement of p21 in the Wnt-c-Myc pathway-mediated growth control of progenitor cells. Indeed, raises the possibility that p21 is an intracellular molecular switch between proliferation and differentiation. Moreover, it has been shown that conditional expression of $p21^{cip1waf1}$ alone allow cells to differentiate (van der Wetering et al., 2002) suggesting that the cell fate choice between proliferation and differentiation is regulated by modulation of the expression of $p21^{cip1waf1}$ via the direct induction of c-Myc by Wnt signaling.

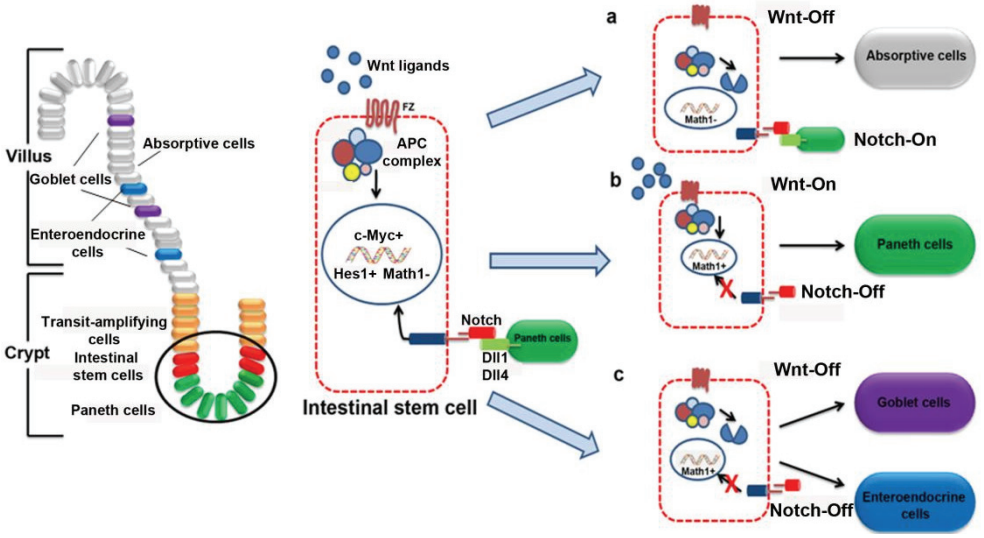

Fig. 5. The role for Notch and Wnt pathways in intestinal epithelial proliferation and differentiation. The ISC can give rise to four lineages of terminally differentiated cells: a is absorptive cells, b and c (Paneth, goblet and enteroendocrine cells) have secretory phenotypes. See the text for detail.

In general, the data strongly support a model in which Notch directs proliferation when Wnt signal activity is high, and directs enterocyte differentiation when Wnt activity levels drop towards the top of the crypt. The multipotent progenitors require both Wnt and Notch signals to be activated for fulfilling continuous proliferation without differentiation. Once some cells in this Wnt and Notch-activated population escape from the Notch signal, they stop proliferating and acquire the Math1 function. These cells raise the terminally differentiation in secretory cells in areas where the Wnt signal is not active (Pinto et al., 2003), whereas they differentiate in Paneth cells if they remain at the bottom of the crypt where Wnt ligands are abundant. By contrast, if cells in this Wnt and Notch-active population lose the Wnt signal, for example, because of their positional changes along the vertical axis, they differentiate as absorptive cells (Fig. 5).

5.3 SCs and the origin of intestinal cancer

Despite stringent homeostatic maintenance in the intestine, the high number of patients with colorectal cancer (CRC) indicates that these regulatory mechanisms often fall short in protecting against malignant transformation. Both environmental and genetic risk factors have been defined for CRC, and deregulation of morphogenetic pathways plays a key part in cancer development. Notably, the vast majority of sporadic CRC cases carry Wnt pathway mutations, highlighting the importance of this pathway in CRC. The hit that induces transition from normal to polypoid tissue is accompanied by several changes in crypt appearance and behavior, cells show a more immature phenotype and a higher proliferative index which results in expansion of the pre-malignant clone. Although

mutation of APC or β-catenin is an early event in the transformation of colonic epithelial cells, studies have revealed that colon carcinomas do not contain nuclear β-catenin homogeneously (Fodde & Brabletz, 2007). This so-called β-catenin paradox indicate that Wnt signaling has a preponderant role only for a subset of tumour cells, cancer SCs (CSCs), which are endowed with tumorigenic capacity (Vermeulen et al., 2008). Indeed, the past decade has seen a shift in the way tumours are perceived, and the now widely accepted model is that tumours contain a small population of self-renewing CSCs, as well as a large compartment of more differentiated tumour cells (Vermeulen et al., 2008). Cellular hierarchy within CRC is maintained, at least in part, by microenvironmental factors regulating stemness and differentiation. In agreement, tumour cells located next to myofibroblast-rich regions, have a much higher incidence of nuclear-localized β-catenin, suggesting for microenvironment-modulated Wnt signaling (Fodde & Brabletz, 2007). A recent paper point to hepatocyte growth factor (HGF) as the myofibroblast-derived signal that, at least in part, orchestrates this intimate relationship and enhances Wnt activity in more differentiated tumour cells, thereby reinstalling CSCs features (dedifferentiation) (Vermeulen et al., 2010). Indeed, using a TCF/LEF reporter that directs the expression of enhanced green fluorescent protein, authors provided evidence that Wnt signaling activity is a marker for colon CSCs and is regulated by the microenvironment. Moreover, they show that differentiated cancer cells can be reprogrammed to express CSC markers and regain their tumorigenic capacity when stimulated with myofibroblast-derived factors (Vermeulen et al., 2010). Although, these data clearly ascertain a role for the Wnt pathway in CRC stemness, Notch inhibition with an antibody against the Notch ligand Dll4 results in human colon CSCs differentiation, reduction of CRC growth in a xenotransplantation model and chemosensitization (Hoey et al., 2009).

6. Identification of Renal SCs

The mammalian kidney shares with the majority of organs the ability to repopulate and at least partially repair structures that have sustained some degree of injury. Indeed, tubular integrity can be rescued after acute damage, and even severe glomerular disorders sometimes may undergo regression and remission, suggesting that glomerular injury is also reparable (Imai & Iwatani, 2007; Remuzzi, et al., 2006). However, the existence of renal SC (RSC) has been a matter of long debate. Recently, converging data definitively demonstrated the existence of a population of stem/progenitor cells in the parietal epithelium of the Bowman's capsule of adult human kidney (Sagrinati, et al., 2006) (Fig.6). These SC coexpress both CD24, a surface molecule that has been used to identify different types of human SC, and CD133, a marker of several types of adult tissue SC, lack lineage-specific markers, express transcription factors that are characteristic of multipotent SC, and exhibit self-renewal, high clonogenic efficiency and multidifferentiation potential. When injected intravenously in SCID mice that had acute kidney injury, RSC regenerated tubular structures from different portions of the nephron and also reduced the morphological and functional kidney damage (Sagrinati, et al., 2006).

In addition, it was demonstrated that RSC are arranged in a precise sequence within Bowman's capsule of adult human kidneys (Ronconi, et al., 2009) (Fig. 6).

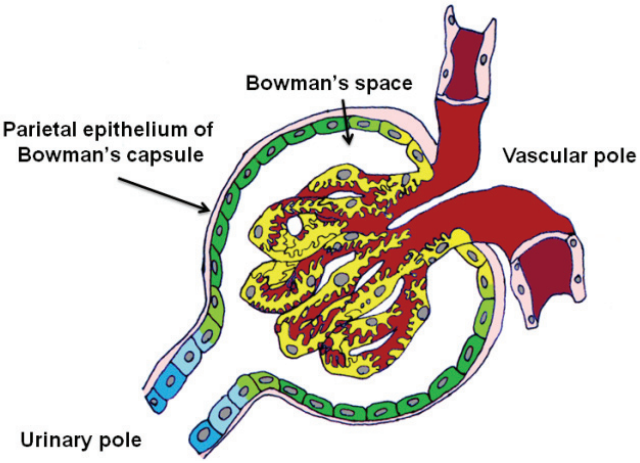

Fig. 6. Localization of RPC in the glomerulus. RPC (green) are localized in the Bowman's capsule epithelium. A transitional cell population (podocyte progenitors, green/yellow) displays features of either RPC or podocyte (yellow) and localize between the urinary pole and the vascular stalk. Cells that express only podocyte markers and the phenotypic features of differentiated podocytes (yellow) localize at the vascular stalk of the glomerulus.

These findings obtained in human kidneys were confirmed in a parallel study performed in murine kidney by Appel (Appel, et al., 2009), who also demonstrated the existence of transitional cells with morphological and immunohistochemical features of both parietal epithelial cells and podocyte in proximity of the glomerular vascular stalk and that podocytes are recruited from parietal epithelial cells, which proliferate and differentiate from the urinary to the vascular stalk, then generating novel podocytes (Fig. 6). This occurs as the kidney grows, during childhood and adolescence, and may also take place following an injury which allows a slow, regulated generation of novel podocytes, such as uninephrectomy. Recently, a rare subpopulation of CD133+CD24+ cells has also been describe in renal tubules (Lindgren, et al., 2011). These cells are able to proliferate and differentiate after tubular injury. Accordingly, tubular epithelium regenerating on acute tubular necrosis displayed long stretches of CD133+CD24+ cells, further substantiating that the cells that are repairing tubular epithelium may simply represent the result of proliferation and differentiation of CD133+CD24+ tubular progenitors.

6.1 Involvement of RSC in glomerular disorders and cancer

It has been widely recognized that a disruption in the strictly regulated balance of SC self-renewal and differentiation not only impairs regenerative mechanisms but can even generate disorders. In the glomerulus, the response to podocyte injury may cause aberrant epithelial cell proliferation, hypercellular lesions formation and Bowman's space obliteration, as seen in collapsing glomerulopathy and in crescentic glomerulonephritis (Albaqumi & Barisoni, 2008; Thorner, et al., 2008). Until now, theories explaining the origin of aberrant epithelial cells in collapsing glomerulopathy and crescentic glomerulonephritis have been controversial. One possibility is that these cells are exclusively of parietal epithelial origin (Thorner et al., 2008), while another is that some dedifferentiated

podocytes acquire markers of parietal epithelial cells (Moeller et al., 2004). It was recently demonstrated that the majority of cells present in the hyperplastic lesions in collapsing glomerulopathy or crescentic glomerulonephritis exhibits the RSC markers CD133 and CD24, with or without coexpression of podocyte markers (Smeets et al., 2009). Therefore, it is suggested that the glomerular hyperplastic lesions are generated by RSC of Bowman's capsule at different stages of their differentiation towards mature podocytes. Support for this hypothesis came from lineage tracing experiments performed in transgenic mice with genetically labeled parietal epithelial cells in a model of inflammatory crescentic glomerulonephritis, and of collapsing glomerulopathy (Smeets et al., 2009).

Finally, a close relationship between the transcriptome of CD133+ tubular progenitors and the one derived by papillary renal cell carcinomas was demonstrated (Lindgren et al. 2011). Moreover, a strong CD133 expression was observed in the papillary renal cell carcinomas analysed. Thus, these observations raise the provocative hypothesis that papillary renal cell carcinomas may directly derive from $CD133^{+}CD24^{+}$ renal tubular progenitors, whereas clear renal cell carcinomas may derive from other more differentiated proximal tubular cells.

6.2 Signaling pathway regulating the RSC niche

The molecular mechanisms regulating the proliferation of RSC, as well as the cell fate determination in the podocyte lineage are unknown. We recently demonstrate the role of the Notch signaling pathway in both these processes (Lasagni et al., 2010). Notch activation triggers the expansion of renal progenitors by promoting their entry into the S-phase of the cell cycle and mitotic division. Moreover, Notch downregulation is required for differentiation toward the podocyte lineage. However, Notch downregulation was neither sufficient nor necessary for the acquisition of a podocyte phenotype, but an impaired downregulation of the Notch pathway led to podocyte death. Indeed, renal progenitor differentiation into podocytes was associated with cell cycle checkpoint activation and G_2/M arrest, reflecting an intrinsic barrier to replication of mature podocytes. Persistent activation of the Notch pathway induced podocytes to cross the G_2/M checkpoint, resulting in cytoskeleton disruption and cell death (Lasagni et al., 2010). Notch expression was virtually absent in the glomeruli of healthy adult kidneys, while a strong upregulation was observed in renal progenitors and podocytes in patients affected by glomerular disorders. Accordingly, inhibition of the Notch pathway in mouse models of focal segmental glomerulosclerosis ameliorated proteinuria and reduced podocyte loss during the initial phases of glomerular injury, while inducing reduction of progenitor proliferation during the regenerative phases of glomerular injury with worsening of proteinuria and glomerulosclerosis. Taken altogether, these results suggest that the severity of glomerular disorders depends on the Notch-regulated balance between podocyte death and regeneration provided by renal progenitors (Lasagni et al., 2010).

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Claudins in Normal and Lung Cancer State

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1. Introduction

The epithelial cells form a physical barrier that serves to separate two different environments, and interact with neighboring cells through various kinds of cell-cell communication systems. Among the systems of cell-cell communications have been described 3 types of intercellular junctions: gap-junctions, adherents-junctions and tight-junctions.

The tight junctions are critical for the sealing of cellular sheets and controlling paracellular ion flux. Tight junctions are composed primarily of 3 components: The IgG-like family of junctional adhesion molecules (JAMs), occluding and claudin families. Claudins are the main constituents of tight junctions. The claudin family proteins is composed of approximately 24 transmembrane proteins, all of which are closely related, most of them are well characterized at the level of gene and protein. The claudins are present in variety of normal tissues, hyperplastic conditions, but have also been found in benign neoplams and cancers that exhibit epithelial differentiation. Loss of claudins expression has been reported in various malignant diseases. The differential expression of several members of the family of the claudins in various cancers has been used to confirm the histological identity of certain types of cancer.

The permeability barrier in the terminal airspaces of the lung is due in large part to tight junctions between alveolar epithelial cells, which regulate the flow of molecules between apical and basolateral extracellular compartments. Disruption of the paracellular alveolar permeability barrier is a significant pathological consequence of acute lung injury. Little is known about the expression and localization of claudins in normal bronchial epithelium and lung cancer. So that is in our interest to describe the expression of claudins in normal and lung cancer, also describe the cellular and molecular mechanisms.

2. Tight junctions

The cellular polarity is critical for a variety of cellular functions, such as directed migration, asymmetric cell division and the vectorial transport of molecules. Polarity is studied in

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epithelial cells where apical and basolateral surface domains with different lipid and protein compositions can be distinguished (Steed, et al., 2010). In vertebrate epithelia, the two membrane domains are separated by tight junctions (TJ), who act as an intramembrane diffusion barrier and also as a paracellular seal that prevents diffusion of molecules across the epithelial cell layer. TJs are structures appearing as discrete sites of fusion between the outer plasma membrane of adjacent cells. The TJ regulates the diffusion of solutes with size and charge selectivity and that it is functionally different in physiologically diverse epithelial cell types. To understand the molecular mechanism controlling TJ structure and function, it is important to determine their molecular composition and organization (Anderson & Cereijido 2001; Steed, et al., 2010).

2.1 Tight junction molecular structure

The molecular components of the TJ have been separated into 3 groups: 1) The integral transmembrane proteins, 2) The peripheral or cytoplasmic and 3) TJ-associated/regulatory proteins. 1) The integral transmembrane proteins are essential for correct assembly of the structure: occludin, claudins and junctional, immunoglobulin superfamily membrane proteins with two extracellular Ig-like domains, including JAM-A, JAM4, coxsackie adenovirus receptor (CAR), and endothelial cell-selective adhesion molecule (ESAM). The integral transmembrane proteins are the critical for correct assembly of the TJ structure and controlling TJ functions via homotypic and heterotypic interactions. 2) The peripheral or cytoplasmic or plaque anchoring proteins: the membrane-associated guanylate kinase (MAGUK) family proteins ZO 1, ZO 2, and ZO 3 bind to the C-terminal cytoplasmic domain of claudins, occludin, tricellulin, and JAM-A. In addition, MAGI-1, MAGI-3, MUPP1, PATJ and ASIP/PAR3 are known to be PDZ domain-containing proteins that directly bind to claudins or other TJ-associated membrane proteins. The plaque anchoring proteins act as a scaffold to bind the raft of TJ molecules together and provide the link to the actin cytoskeleton and the signaling mechanism of the cell. 3) TJ-associated/regulatory proteins — α-catenin, cingulin, paracingulin, etc., (for review see: Blasig, et al., 2006; Furuse 2010; Hamazaki, et al., 2002; Itoh, et al., 1999, Tsukita & Furuse 2000a, 2000b).

2.2 Paracellular transport

Separation of functional compartments is necessary for higher organisms. The structures that separate such compartments, epithelia and endothelia, consist of cell layers with diverse properties according to the organism's actual demands. While such structures prevent uncontrolled diffusion and convection of substances, they also provide selective transport processes via secretion (exocrine and endocrine glands), absorption (intestine), or reabsorption (kidney). Such transport processes are realized via the transcellular pathway involving resorption across the apical membrane, transfer through the cytoplasm, and extrusion at the basolateral membrane. In general, transcellular transport is an energy-dependent process, but it allows the organism to reabsorb substances that are indispensable, even against an existing electrochemical gradient. Moreover, since this pathway is controlled at several steps, it allows fine-tuning according to actual demands. On the other hand, paracellular transport occurs through the intercellular space of

adjacent cells. This transport is passive and dependent on an electrochemical gradient. This form of transport allows bulk reabsorption with a minimum of energy expenditure. The key structure of the intercellular space, and thus the major determinant of paracellular transport, is the tight junction (TJ). The ion conductance of tight junctions varies from tissue to tissue and can be experimentally manipulated by expressing or removing specific pores. The specificity of these pores is determined by the claudin composition and, more precisely, by the properties of their extracytoplasmic loops, such as electrostatic interaction sites. The molecular mechanisms that underlie size-selective paracellular diffusion are unclear. However, several studies reported a functional dissociation between transepithelial electrical resistance and size-selective paracellular diffusion upon specific modifications of either junctional components or signaling pathways that affect permeability. Thus, the molecular bases of ion-selective and size-selective permeation seem to be distinct (see Figure1) (Amasheh, et al., 2009; Steed, et al., 2010; Tsukita &Furuse 2000a, 2000b; Will, et al., 2008).

3. Claudins

The tight junctions consist of several components: integral membrane proteins, cytoplasmic proteins and cytoskeletal proteins (Brennan, et al., 2010). To date, a number of integral membrane proteins are associated with TJ, occludin, adhesion molecules, claudin family, etc., (Tsukita & Furuse 2000a, 2000b, Dhawan, et al., 2005; Furuse, 2010). Gradually has been shown that the molecular architecture of these complex is more numerous, the TJ is made up of at least 40 different components (Figure 1) (for review see: Schneeberger & Lynch, 2004; http://www.genome.jp/kegg/pathway/hsa/hsa04530.html). Among the elements that form part of integral membrane proteins, the claudin family (Clds; present active infinitive of *claudeō*, means close), has attracted the attention because of its relatively recent identification, the family includes 24 members in mammals (Furuse 2010, Brennan, et al., 2010), although Tsukita group recently has reported three new genes that code for Cldns 25, 26 and 27 (Mineta, et al., 2011). The Cldns were identified in 1998 by Dr. Tsukita in membrane fractions from chicken liver (enriched with TJ) through sucrose gradients. Among the protein components two bands of 23 kDa were obtained, with similar in size but not identical. Analysis of the amino acid sequence showed that these proteins were structurally related (30% identical at the amino acid sequence), calling claudin 1 and 2, respectively (Furuse et al., 1998). However, earlier reports this had already been described genes with similar sequences (Briehl & Miesfeld, 1991, Katahira, et al., 1997). This information allowed proposes the existence of a large family of proteins. Currently there are over 558 articles that involve claudins studies (updated to April 20, 2011), describing various aspects of molecular, cellular, regulation, operation, including its expression/co-expression, localization in tissues and organs, and their potential involvement in diseases.

Before the discovery of the claudins, it is believed that the tight junctions were composed mainly of occludin. Even thought that occludin and claudin were members of the same family, but the report of the genetic sequence of the Cldns confirmed that these were different from those occludins proteins, and showed no similarity between them (Furuse, et al., 1998a). To date is accepted that the central part proteins responsible for the paracellular barrier are the claudins (Angelow, et al., 2008, Tsukita & Furuse, 2000).

Claudins are found in the tight junction at the interface of the basolateral and apical membranes of polarized epithelial and endothelial cells, and also at paranodes in compact myelin. Transfected claudins are capable of forming tight junction 'strands' or 'fibrils', the freeze–fracture descriptions of a branching and anastomosing network of rows of intramembranous particles characteristic of tight junctions. Claudins are also found in the basolateral membranes, possibly as precursors to the fibrils (Peter & Goodenough 2004).

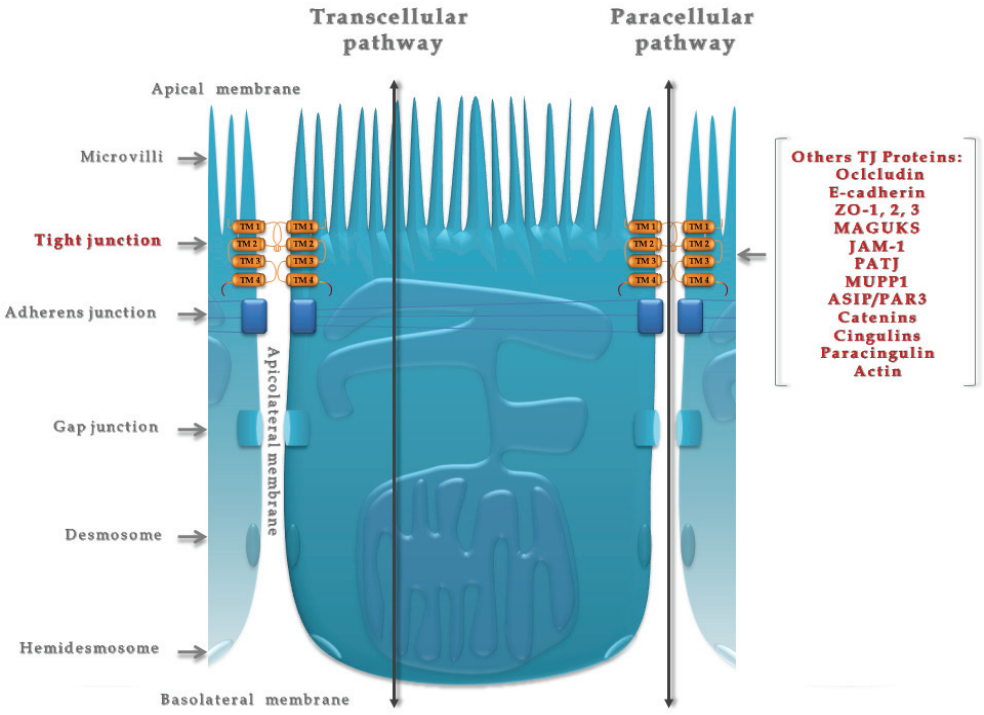

Fig. 1. Schematic diagram of the molecular organization of epithelial.

Cell-cell interactions are mediated by intercellular junctional complexes: gap junctions, adherent junctions, desmosome and thigh junctions, each of which have different had asymmetric distribution in epithelial cells, TJ are located at the apical-basal border and they contribute to maintain cell polarity, regulate the solute and fluid exchange between basolateral and apical domains, and also regulated paracellular permeability. TJ membrane proteins are linked to the cytoskeleton (F-actin) via a complex network of adaptor proteins.

3.1 Claudin evolution

The mechanism by which the family of claudins evolves is unknown; however, the data suggest that this family expanded by gene duplication early in the evolution of chordates

(for review sees: Lal-Nag & Morin 2009, Loh, et al., 2004, Kollmar, et al., 2001). When the septate junctions (the corresponding structure of invertebrates), were replaced by tight junctions. In the same way as other groups of genes were extended, the claudins diversified into the body of vertebrates from the chordates, leading to new structures: the skull, pairs of sense organs and appendages (Kollmar, et al., 2001). The search for claudins in the Genebank of *Drosophyla melanogaster* and *Caenorhabditis elegans* showed no similarity to genes previously reported (Venter & Adams, 2001; Kolmar, et al., 2001). However, in *D. melanogaster*, was recently found three genes, which encode for three different proteins that are required in the paracellular transport: Megatrachea (Mega) Sinuous (Sinu) and Kune Kune. Mega, a transmembrane protein homologous to claudins, and show that it acts in septate junctions, this protein has transepithelial barrier function similar to the claudins, and is necessary for normal tracheal cell morphogenesis but not for apico-basal polarity or epithelial integrity (Behr, et al., 2003). The gene sinuous encodes a protein that is molecularly and functionally similar to vertebrate claudins. Sinuous share several characteristic with vertebrate claudins as has all of the amino acids absolutely conserved across vertebrate claudins and has as much sequence similarity to canonical vertebrate claudins as do some of the more divergent vertebrate claudins. Also has functional similarity because it localizes to and is required for the function of paracellular barrier junctions (Wu, et al., 2004). Kune Kune, this protein localizes to septate junctions and is required for junction organization and paracellular barrier function, but not for apical-basal polarity (Nelson, et al., 2010). In *C. elegans* genome database identified four claudin-related, ~20-kDa integral membrane proteins (CLC-1 to -4), which showed sequence similarity to the vertebrate claudins. The expression and distribution of CLC-1 was mainly expressed in the epithelial cells in the pharyngeal region of digestive tubes and colocalized at their intercellular junctions. In CLC-1-deficient worms, the barrier function of the pharyngeal portion of the digestive tubes appeared to be severely in experiments performed with RNA interference. CLC-2 was expressed in seam cells in the hypodermis, and it also appeared to be involved in the hypodermis barrier (Asano, et al., 2003). In addition VAB-9 is a predicted four-pass integral membrane protein that has greatest similarity to BCMP1 (brain cell membrane protein 1, a member of the PMP22/EMP/Claudin family of cell junction proteins) and localizes to the adherents junction domain of *C. elegans* apical junctions. In this nematode *C. elegans* protein VAB-9 regulates adhesion and epidermal morphology (Simske, et al., 2003). In *Danio rerio* (Zebra fish) have been located at least 15 genes for Cldns, some of which have their orthologous in human (Kollmar, et al., 2001), and among non-vertebrate *Halocynthia roretzi* (Sea pineapple) as also found a gene that encodes to claudins (Kollmar, et al., 2001). The presence of these genes suggests that the origin of the claudins may be quite ancient and that a claudin ancestor pre-dates the establishment of the chordates (Kollmar, et al., 2001).

3.2 Claudin structure

The claudins belong to the peripheral myelin protein (PMP22)/ epithelial membrane protein (EMP)/ epithelial membrane protein or membrane protein (MP20)/claudin superfamily of four transmembrane-spanning domains. The 24 mammalian members are 20 to 34 kDa in size (Lal-Nag & Morin, 2009, Peter & Goodenough 2004), and recently others members of the claudin family 25, 26 and 27 were reported (Mineta, et al., 2011). The proteins are

predicted, on the basis of hydropathy plots, to have four transmembranal helices (Morita, et al., 1999; Lal-Nag & Morin, 2009), with their NH_2-and COOH-terminal tails extending into the cytoplasm (Lal-Nag & Morin, 2009). Sequence analysis of Cldns has led to classification into two groups: classic claudins (1–10, 14, 15, 17, 19), and non-classic claudins (11–13, 16, 18, 20–24) (Table 2), according to their degree of sequence similarity to conserved structural features at ECL1 for classic claudins (Krause, et al., 2008). The typical claudin protein contains a small intracellular cytoplasmic NH_2-terminal sequence of approximately 4 to 5 residues followed by a huge extracellular loop (EL1) of approximately 60 residues, a short 20-residue intracellular loop, another extracellular loop (EL2) of about 24 residues, and a COOH-terminal cytoplasmic. The size of the COOH-terminal tail is more variable in length; it is typically between 21 and 63 residues. The amino acid sequences of the first and fourth transmembrane domains are highly conserved among Cldns, and the second and third are more diverse. The first loop contains several charged amino acids and, as such, is thought to influence paracellular charge selectivity, and two highly conserved cysteine residues are hypothesized to increase protein stability by the formation of an intramolecular disulfide bond. It is assumed that the first extracellular loop is critical for determining the paracellular tightness and the selective paracellular ion permeability (see Table 2). It has been suggested that the second extracellular loop, can form dimers with Cldns on opposing cell membranes through hydrophobic interactions between conserved aromatic residues and that the second extracellular loop may cause narrowing of the paracellular cleft (Lal-Nag & Morin 2009; Krause, et., al 2008, 2009).

The region that shows the most sequence and size heterogeneity among the claudin proteins is the COOH-terminal tail. It contains a PDZ-domain-binding motif that allows claudins to interact directly with cytoplasmic scaffolding proteins, such as the TJ-associated proteins MUPP1, PATJ, ZO-1, ZO-2 and ZO-3, and MAGUKs (see figure 1 and 2). Furthermore, the COOH-terminal tail upstream of the PDZ-binding motif is required to target the protein to the TJ complex, and also functions as a determinant of protein stability and function. The COOH-terminal tail is the target of various post-translational modifications, such as serine/threonine and tyrosine phosphorylation and palmitoylation, which can significantly alter claudin localization and function. Most cell types express multiple claudins, and the homotypic and heterotypic interactions of claudins from neighboring cells allow strand pairing and account for the TJ properties, although it appears that heterotypic head-to-head interactions between claudins belonging to two different membranes are limited to certain combinations of claudins, and stoichiometry have yet to be determined (Lal-Nag & Morin, 2009; Peter, & Goodenough 2004).

The extracellular domains of claudins in one cell are thought to interact with those in an opposing cell to form a new class of ion channel (see Figure 1). These channels confer ion selectivity to the paracellular pathway between luminal and basolateral extracellular compartments. The permeability properties of the paracellular pathway have the biophysical characteristics of conventional ion channels, including ion selectivity, anomalous mole fraction effects, pH dependence and a diameter of ~6Å. Exchanging the first extracellular loop between claudin-2 and claudin-4 changes the Na^+ and Cl^- selectivity of the paracellular pathway in cultured epithelial cells (Peter & Goodenough 2004; Ben-Yosef, et al., 2003).

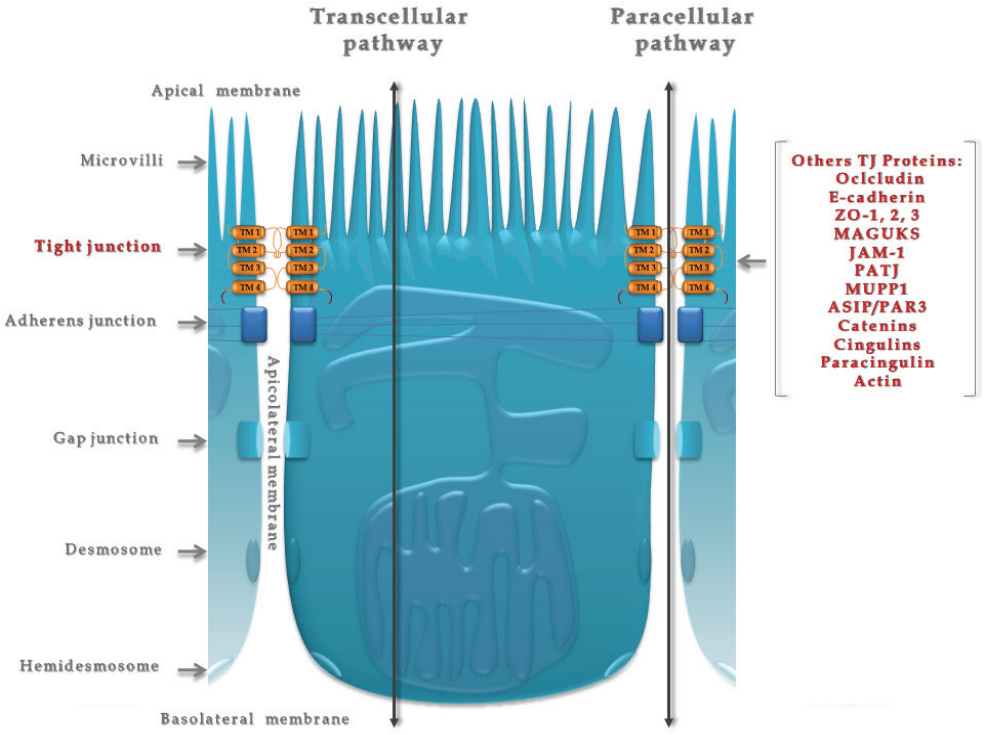

Fig. 2. Schematic representation of structure and molecular interactions of typical claudins. Claudins are proteins with four transmembrane-spanning domains (TM 1, TM 2, TM 3, and TM 4), two extracellular loops (EL1 and EL2) related with paracellular ion selectivity and oligomerization respectively. The NH_2 and COOH terminal are localized in the cytosol, intracellular loops are posttranscriptional modify. COOH terminal contains PDZ-binding domain that is important for signal transduction.

4. Epithelial cells

Epithelia have three basic functions in multicellular organisms, first, cover the outer surface of the body and the cavities and formed a physical barrier that separates two environments. This physical barrier provides protection against mechanical damage, the entry of microorganisms and water losses. Epithelia are also involved in secretion and absorption process. These three functions of epithelia are given primarily by the type of cellular arrangements that characterizes them. Epithelial cells are polarized and form sheets of cells attached to each other through complex mechanisms. Some of these mechanisms of cell-cell interactions will be discussed in this chapter. Thus in an epithelial cell it can distinguish two basic functional components: a) the basal domain and b) the apical domain (Feing & Muthuswamy, 2009). The basal domain participated in cell-

extracelular matrix (ECM) interactions; in particular, it is in contact with a structure formed by type IV collagen, laminin and proteoglycans called basal lamina (Gumbiner, 1996). The basal lamina allows epithelial cells to be attached to the underlying connective tissue. In specialized epithelial tissue basal domain is also involved in endocrine secretion. The apical domain is located in the opposite direction of the basal. Depending on its functions basal domain is involved in exocrine secretion and absorption. Alternatively, another fundamental property of epithelial tissue is the close cohesion between the cells, which allows the formation adherents selectively permeable layers that are at once very strong mechanical barriers. Cell-cell interactions are mediated by intercellular junctional complexes that consist of gap junctions (GJ), adherent junctions (AJ), desmosomes (Ds) and thigh junctions (TJ), each of which have different functions and properties (Itoh & Bissell et al., 2003; Feing & Muthuswamy, 2009). These junctional complexes had asymmetric distribution in epithelial cells, TJ are located at the apical-basal border and they contribute to maintain cell polarity, regulate the solute and fluid exchange between basolateral and apical domains, and also regulated paracellular permeability (Itoh & Bissell et al., 2003; Feing & Muthuswamy, 2009). TJ are widely explained in section 3 of the present chapter. On the other hand, AJ are basal to TJ and they are considered the primary determinants of cell-cell adhesion. AJ are ubiquitously represented by cadherins, transmembranal Ca^{2+}-dependent receptors, which form complex with catenins, cytoplasmic plaque proteins, and actin cytoskeleton. Cell adhesion regulates the organization of cell patters and architecture of tissues (Gumbiner, 1996). In epithelia, it can be found different cell shapes (flat, cylindrical or cubic) in at least three basic forms of cell arrangement, a) simple epithelia (single sheet of cells), b) stratified (multiple cell sheets) and c) pseudostratified (single sheets of cells with several sizes and shapes that give the appearance of true stratified epithelia).

4.1 Lung epithelial cells

The adult human lung is lined by specialized types of airway epithelia organized in tree-like form with three anatomical and functional units: a) trachea and bronchi (tracheobronchial), b) bronchioles and acinar ducts (bronchiolar), and c) peripheral saccular-alveolar structures (alveoli) (Maeda, et al., 2007). Tracheabronchial and bronchiolar units form conducts that provide inhaled gases to alveoli unit, there; epithelial alveolar cells and capillaries exchange oxygen and carbon dioxide required for respiration (Maeda, et al., 2007). Each of these airway units is composed of distinct types of epithelial cells that are important for maintaining normal lung function, in Table 1 is shown a summary of the main types of intrapulmonary epithelial cells with their respective function.

Trachea and bronchi are characterized by pseudostratified epithelia, whereas bronchioles, acinar ducts and alveoli are mainly characterized by simple cubic epithelia. Epithelial cells throughout of airway show major functions such as protection (Ciliated cells), progenitor cells (Basal and Clara cells), exocrine secretion (Goblet, Clara and Alveolar type II cells), endocrine secretion (Neuroendocrine pulmonary cells), and gas exchange (Alveolar type I cells) (Table 1) (Herzog, et al., 2008; Linnoila, 2006; Maeda, et al., 2007; Reynolds, et al., 2007; Rock, et al., 20010; Rogers, 2007).

Airway unit	Epithelia type	Epithelial cell types	Cell function	Biomarkers
TRACHEOBRONCHIAL	Pseudo-stratified	Goblet	Mucus secretion	Mucin 5AC (MUC5AC)
		Basal	Attachment with the basement membrane; Progenitor cells	Transcription factor p63, cytokeratins 5 and 14
		Clara	Mucus secretion; Mucocilliary clearance; Detoxify xenobiotics and oxidant gasses; Progenitor of ciliated cells; Regulation of immune system	Uteroglobin, Surfactant apoproteins A, B and D
		Ciliated	Mucocilliary clearance	Calpastatin, ezrin
BRONCHIOLAR	Simple columnar-cuboidal	Clara	See above	See above
		Ciliated	See above	See above
		Pulmonary neuroendo-crine cells	Endocrine and paracrine secretion in lung development; Oxygen sensors	Gastrin-releasing peptide, bombesin, calcitonin gene-related peptide, synaptophysin
ALVEOLI	Simple columnar	Alveolar type I cells (squamous cells)	Mediate gas exchange	T1-α, aquaporin-5 (AQP-5)
		Alveolar type II cells	Synthesis, secretion and recycle the lipid and protein component of surfactant Innate immunity	Surfactant apoprotein C and ATP-binding cassette A3 (ABCA3)

Table 1. Characteristics of intrapulmonary epithelia of human. Sources: Herzog, et al., 2008; Linnoila, 2006; Maeda, et al., 2007; Reynolds, et al., 2007; Rock, et al., 2010; Rogers, 2007.

The complex patterns of intrapulmonary epithelial cells, organization, numbers and types of cells, are regulated by several humoral signals and cell-cell interactions. It is know that

several physiopathological conditions could modify the lung epithelial cell pattern such as infection, cytokines, inflammatory mediators, pollutants and injury that are associated with common airway diseases, including chronic obstructive pulmonary disease, asthma, cystic fibrosis and cancer (Ballaz & Mulshine, 2003; Maeda, et al., 2007; Rock, et al., 2010).

4.2 Lung cancer

Lung cancer is one of the most important epithelial neoplasias in the world with high incidence and mortality (Jemal, et al., 2011). Currently lung cancer is the most commonly diagnosed cancer, as well as, the leading cause of cancer death in males in worldwide. Among females, it represents the fourth most commonly diagnosed cancer and the second leading cause of cancer death. Even more, lung cancer is the leading cause of cancer-related deaths around the world, accounting for more deaths than those caused by three of the most diagnosed cancers combined (prostate, breast and colorectal cancers). In 2008 estimated lung cancer related deaths in worldwide were 1, 378, 400 whereas estimated related cancer deaths by prostate, breast and colorectal cancers were 1, 004, 900 (Jemal, et al., 2011). Moreover, whereas the five-year survivor over time was improved in prostate, breast and colorectal carcinomas in last 15 years, at 99%, the five-year survivor rate of lung cancer was relatively unchanged at 15% (Borczuck, et al., 2009; Schwartz, et al., 2007). The high mortality of lung cancer could be explained in part by histological heterogeneity and late detection (Borczuck, et al., 2009; Schwartz, et al., 2007). On the other hand, smoking is the most important cause of lung cancer, 80-90% of lung cancer cases are associated with smoking but only 15%of the smokers developed lung cancer and 10% of lung cancers occur in never-smokers (Borczuck, et al., 2009; Schwartz, et al., 2007). Other lung carcinogens are asbestos, arsenic, radon, polycyclic aromatic hydrocarbons and air pollution (Jemal, et al., 2011). The World Health Organization (WHO) reported that the cancer is a leading cause of death worldwide and accounted for 7.6 million deaths (around 13% of all deaths) in 2008. The main types of cancer are: lung (1.4 million deaths), stomach (740 000 deaths), liver (700 000 deaths), colorectal (610 000 deaths) and breast (460 000 deaths). More than 70% of all cancer deaths occurred in low- and middle-income countries. Deaths from cancer worldwide are projected to continue to rise to over 11 million in 2030 (http://www.who.int/mediacentre/factsheets/fs297/en/index.html).

Lung cancer is divided into two histological types, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC is the most common lung cancer; it represents between 80-85% of cases and consists in a heterogeneous group of cancers that can divide into three major subtypes: squamous cell carcinoma (SCC), adenocarcinoma (AC) and large-cell carcinoma. This histological heterogeneity is only the reflection of lung cancer biology complexity and it has very important implications in initiation, treatment and prognosis.

Multi-step models of carcinogenesis, genetic and genomic approaches are developed to understand lung carcinogenesis (Borczuck, et al., 2009; Schwartz, et al., 2007; Wistuba, et al., 2002). In this way, an emergent field of lung carcinogenesis is open, the role that play the loss of polarity and dysregulation cell-cell adhesion molecules in initiation and invasion process of cancer cells.

CLDN	Aa	M W	Cl	Transport	Organism	Detection	Disease
1	211	22,744	C	PT	H, C, R, M, D, Cw	Protein RTi_PCR	Up: LC-S Down: LC-A
2	230	24,549	C	PT: Na+, K+, water	H, C, R, M, D,Cw, G	RTi_PCR	
3	220	23,319	C	PB: mono, divalent ions;	H, C, R, M		
4	209	22,077	C	PB: Na^+ PT: Cl^-	H, C, R, M	RTi_PCR	Up: P-MC
5	218	23,147	C	PB	H, C, R, M	RTi_PCR	Up: LC-A Down: LC-S
6	220	23,292	C		H, C, R, M		
7	211	22,390	C	PB: Na^+ PT: Cl^-	H, C, R, M	RTi_PCR	
8	225	24,845	C	PBdivalent cations	H, C, R, M	RTi_PCR	
9	217	22,848	C	PT: Na^+, K^+	H, R, M	RTi_PCR	
10	a: 226 b: 228	24,251 24,488	NC NC		H, C, R, M H, C, R, M		
11	207	21,993	NC		H, C, R, M		
12	244	27,110	NC		H, C, R, M	RTi_PCR	
13					M		
14	239	25,699	C	PB: K^+	H, C, R, M	IE	
15	228	24,356	NC		H, C, R, M		
16	305	33,836	NC	PT: Na^+, cations	H, C, R, M	RTi_PCR	
17	224	24,603	C		H, C, R, M		
18	a: 261 b: 261	27,856 27,720	NC NC		H, C, R, M H, C, R, M	RTi_PCR	
19	a: 224 b: 211	23,229 22,076	C C	PT: Na^+ cations	H, C, R, M H, C, R, M		
20	219	23,515	C		H, C, R, M		
21	229	25,393	NC		H, C, M		
22	220	25,509	NC		H, C, R, M		
23	292	31,915	NC		H, C, R, M		
24	205	22,802	NC		H, C, R, M		
25	276				M		
26	223				M		
27	320				M		

Table 2. Molecular characteristics of claudins. Gene; Aminoacids (Az); Molecular Weight (MW); Classification: Classical (C) and Non-Classical (NC); Paracellular Transport (PT) and Paracellular Barrier (PB); Organism: Human (H), Chimpanzee (C) Rat (R), Mouse (M), Dog (D), Cow (Cw), Chicken (G); Lung expression: Real-time PCR (RT_PCR); Disease type: Adenocarcinoma (LC-A), Lung Cancer (LC-S), Pleura (metastatic adenocarcinoma) (P-MC). Source: Amasheh, et al., 2009; Angelow, et al., 2006; Hewitt, et al., 2006; Hou, et al., 2006; 2007, 2008, 2009; Krause, et al., 2008; Milatz, et al., 2010; Mineta, et al., 2011; Singh, et al., 2010; Wen, et al., 2004; http://www.genecards.org/.

4.3 Claudins and lung cancer

The tight junctions exist in lung epithelium, but knowledge of their development, normal, disease and cancer phases, but exact function and distribution in the developing and adult human lung is incomplete. Epithelial cells often express multiple claudin types, and they show a variable expression profile in different epithelia. Similarly, expression of different claudins varies between different types of epithelial, endothelial and mesothelial tumors Kaarteenaho, et al., 2010). The expression of the different claudins during ontogenesis of human lung might vary since they have distinct expression profiles in normal human lung (Kaarteenaho, et al., 2010). Disruption of the paracellular alveolar permeability barrier is a significant pathologic consequence of acute lung injury. The permeability barrier in terminal airspaces of the lung is due in large part to tight junctions between alveolar epithelial cells, which regulate the flow of molecules between extracellular apical and basolateral compartments (Boitano, et al., 2004). In humans, very little is known about the expression and localization of claudins in normal bronchial epithelium and also in lung cancer. The expression of different claudins was studied in freshly excised human airways using immunfluorescence staining and confocal microscopy bronchi and bronchioles expressed claudins 1, 3, 4, 5, and 7, but not claudins 2, 6, 7, 9, 11, 15, and 16 (Coyne, et al., 2003). Claudins 1, 3, 4, 5, and 7 are expressed in developing human lung from week 12 to week 40 with distinct locations and in divergent quantities. The expression of claudin 1 was restricted to the bronchial epithelium, whereas claudin 3, 4 and 7 were positive also in alveolar epithelium as well as in the bronchial epithelium. All claudins studied are linked to the development of airways, whereas claudin 3, 4, 5 and 7, but not claudin 1, are involved in the development of acinus and the differentiation of alveolar epithelial cells (Kaarteenaho, et al., 2010). In human lung tumors by using cDNA microarray and in large cell carcinomas relatively low levels of claudin-4 and 7 expressions were found as compared with other types of lung cancer, such as adenocarcinoma, squamous cell carcinoma and small cell lung cancer (Garber, et al., 2001). Claudin 1 expression was stronger in squamous cell carcinomas than in adenocarcinomas, whereas claudin 4 and claudin 5 expression was stronger in adenocarcinomas (Jung, et al., 2009). 10 Hydroxycamptothecin (HCPT) elicits strong anti-cancer effects and is less toxic making it widely used in recent clinical trials, HCPT-loaded nanoparticles reduced the expression of cell-cell junction protein claudins, E-cadherin, and ZO-1, and transmission electron microcopy demonstrated a disrupted tight junction ultrastructure (Zhang, et al., 2011). Keratinocyte growth factor (KGF) augments barrier function in primary rat alveolar epithelial cells grown in culture, specifically whether KGF alters tight junction function via claudin expression. KGF significantly increased alveolar epithelial barrier functions in culture as assessed by transepithelial electrical resistance (TER) and paracellular permeability (LaFemina, et al., 2010). Alveolar epithelial cells cultured for 5 days formed high-resistance barriers, which correlated with increased claudin-18 localization to the plasma membrane (Kolval, et al., 2010). Bronchial BEAS-2B cells and SK-LU1 cells respond to tobacco smoke by changing their claudin mRNA synthesis and resulting tight junction permeability changes may thus contribute to tobacco induced carcinogenesis both during initiation and progression (Merikallio, et al., 2011). Zeb1 and twist regulate expression of genes which take part in epitheliomesenchymal transition (EMT). Carcinomas metastatic to the lung showed a significantly higher expression of these transcriptional factors than primary lung tumors, indicating their probable importance in the metastatic process. Zeb1 and twist were inversely associated with several claudins, indicating a role in their down-regulation (Merikalio, et al., 2011).

Despite many questions, recent insights into the molecular structure of tight junctions and claudins are beginning to explain their important physiological differences and contribution to paracellular transport and their importance in several disease and neoplasias, as well as, in healthy tissues.

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Biology of Cilia and Ciliopathies

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1. Introduction

Cilia and flagella are microtubule-based appendages extending from the basal body of most eukaryotic cells, and are classified as either motile or primary. Motile cilia or flagella can be found on many cells such as *Chlamydomonas*, sperm, and respiratory tract epithelial cells. This type of cilia is responsible for movement of the cell itself or generation of fluid flow. In contrast, primary cilia are non-motile organelles that are critically involved in visual, olfactory and auditory signal transduction and play key roles in the regulation of gene expression, development, and behavior. This chapter reviews the current understanding of the various mechanisms involved in cilia and flagellar assembly and maintenance. Consistent with the nearly ubiquitous cellular distribution, cilia have been implicated in numerous human diseases collectively known as ciliopathies. This chapter also discusses several major ciliopathies including primary ciliary dyskinesia, hydrocephalus, polycystic kidney disease, Bardet-Biedl syndrome, and cancer.

2. Mechanics of ciliogenesis

2.1 Basic biology of cilia

Cilia and flagella are long, slender structures protruding from the body of ciliated cells and are composed of a microtubule-based core known as the axoneme. The main structural element is an array of nine doublet microtubule pairs. The a-subfiber of these pairs exists as a fully enclosed filament and is fused to the incomplete b-subfiber containing fewer tubulin subunits. The individual pairs of subfibers are linked together by nexin proteins, forming an enclosed cylinder around a central pair of singlet subfibers; this layout is known as the "9+2" arrangement. The axoneme itself originates from the basal body, a modified form of the centriole consisting of nine triplet microtubules which anchors the cilia to the plasma membrane. The area between the triplet microtubules of the basal body and doublet pairs of the axoneme is referred to as the transition zone. Proteinaceous extensions from this area called transition fibers serve to mark the enclosure of the flagellar compartment and create a barrier from the cytoplasm. The outer and inner dyneins, located on the a-subfiber of the axoneme, are motor proteins that produce the bending and sliding of the microtubules by exerting force on the b-subfiber via ATP hydrolysis. Defects in the inner and outer motor complexes can yield paralyzed or uncoordinated sliding of the axoneme, resulting in inefficient movement. The outer doublet ring and the central pair of microtubules are connected by structures known as radial spokes [1].

Cilia can be distinguished into two types: primary (nonmotile) and motile cilia. While they have the same basic structure, the biological functions of these two types can be very different. Primary cilia typically do not contain the central pair of microtubules (having a "9+0" structure), and also lack other accessory proteins important for generating the ciliary waveform stroke. This form of cilia is considered a sensory antenna for the cell due to a highly specialized membrane protein profile and ability to extend in the luminal space of various tissues. Historically, motile cilia/flagella are known to be important for locomotion in single-celled organisms. In humans, however, motile cilia are important for various physiological processes, ranging from mucous clearing in the trachea to aiding in establishing proper left-right symmetry in developing organisms [2]. Because of their structural similarities, the terms cilia and flagella will be used interchangeably.

2.2 Intraflagellar transport at a glance

Intraflagellar transport (IFT) is a term used to describe the bi-directional movement of non-membrane protein particles that move along the microtubule doublet core (or axoneme), between the space of the ciliary membrane and the axoneme. IFT was originally discovered by Kozminksi and colleagues in 1993, using digital interference contrast (DIC) microscopy to visualize the continuous movement of "bulges" beneath the membrane of a *Chlamydomonas reinhardtii* mutant with paralyzed-cilia [3]. Anterograde movement, towards the ciliary tip or plus end of microtubules, is powered by heterotrimeric kinesin-2, and retrograde movement is driven by cytoplasmic dynein1b [4-14]. A multi-meric protein complex known as the IFT particle attaches to the motor complex and is itself comprised of two large protein sub-complexes [5]. The axoneme is undergoing constant turnover at its tip, meaning tubulin and other accessory proteins must be constantly replenished at the distal tip [15]. The well-conserved IFT motors and particles are tasked with assembling and maintaining the whole cilia structure by serving as adaptors for the transport of axonemal precursors and the recycling of turnover products [1]. A secondary, though equally important, function of IFT is to ferry in ciliary membrane proteins through a secondary adapter complex known as the BBSome [16, 17].

2.2.1 IFT Motors

2.2.1.1 Anterograde motor

First isolated in sea-urchin, the anterograde IFT motor is a heterotrimeric kinesin-2, comprised of three individual subunits [18]. Homologs are found in a variety of ciliated organisms, including *Tetrahymena, Caenorhabditis elegans*, and humans. In *Chlamydomonas*, FLA10 and FLA8 comprise the motor portion of the complex and together they interact with FLA3, a kinesin-associated protein [19]. First evidence for the role of the kinesin-2 in anterograde movement came from the characterization of a temperature sensitive mutant in FLA10. While incubated at the permissive temperature (22°C), the biflagellated green algae possess two, full-length flagella. However, following a shift to the non-permissive temperature (32°C), FLA10 subunit denatures and levels of IFT proteins significantly reduce within the first hour [6]. Cessation of IFT results in the dismantling of the axoneme and the entire ciliary structure is retracted back into the cell body because of the normal turnover [5]. In addition, an isolated null mutant for the FLA10 subunit produces no flagella [20]. Taken together, these studies demonstrate the importance of kinesin-2 to ciliogenesis.

These observations, however, are not entirely consistent among all ciliated organisms. Mutations in the kinesin-2 motor subunits of different species do not result in a cilia-less cell phenotype because of a secondary, homodimeric kinesin known as OSM-3 in *C. elegans* and KIF17 in *Homo sapiens*[13]. Studies investigating the function of OSM-3 conclude that the canonical kinesin-2 motor and OSM-3 work in a concerted effort to build sensory cilia in *C. elegans* [13]. Single mutants in KLP-11 (FLA8) and KAP-1 (FLA3) in *C. elegans* appear to form intact sensory cilia due to the redundancy of OSM-3 function in ciliogenesis (Signor et al, 1999). However, perturbations of OSM-3 results in loss of the ciliary distal segment comprised of singlet microtubule extensions beyond the doublet axoneme core. In these mutants, the heterotrimeric anterograde motor still allows formation of the middle segment.

It could be possible the transferring of the IFT particle from the canonical kinesin-II to OSM-3 may insure proper, sequential construction of the cilia. However, it has been well documented that OSM-3 speed actually increases in disrupted kinesin-II mutants [21], suggesting that kinesin-II may in fact be negatively regulating OSM-3. If so, kinesin-II would ultimately be involved in determining the re-supply rate of axonemal precursors to the flagella compartment. Defects in retrograde IFT clearly demonstrate the negative impact that excess precursors and turnover products have on proper ciliary function. Therefore, accumulation of axonemal components, due to a faster influx of proteins by OSM-3, could also unbalance the natural turnover vs. assembly in favor of creating longer cilia, which is a phenotype that has been observed in kinesin-II mutants. Recently, a null mutant for a relatively new kinesin, KLP-6 in *C. elegans* males, demonstrated a slower procession of OSM-3/KAP-1-associated IFT particle within the ciliary compartment [22]. Although it was observed moving independently of the canonical IFT particle/motor complex, KLP-6 function may have a positive influence on ciliary length. This conclusion is supported by a reduction of *klp-11/klp-6* double mutant cilia compared to the single *klp-11* mutant; *klp-11* mutant has comparatively longer cilia than wild-type.

2.2.1.2 Retrograde motor

IFT-dynein, cytoplasmic dynein 2 (previously known as dynein 1b), powers the retrograde movement of IFT [12]. To date, four proteins are confirmed members of the dynein 2 complex: heavy chain DHC1b, light chain LC8, light intermediate chain D1bLIC, and an intermediate chain FAP133 [7-9, 14, 23-25]. *C. elegans* null mutants defective in dynein components undergo normal anterograde movement but accumulate large amounts of IFT proteins and turnover products within the ciliary compartment [26]. Retrograde-defective cilia are severely truncated and contain protein aggregates that appear as noticeable large, electron-dense clots. These results suggest IFT dynein is responsible for the retrograde movement of the IFT; this result has been seen in *Chlamyomonas*, where defects in IFT dynein lead to protein accumulations in the flagella compartment [27]. Anterograde movement remains active in these mutants; however, the characteristic bulbous cilia are present as a result of axonemal turnover outpacing the dysfunctional retrograde IFT. It has become fairly evident that IFT particles do not passively diffuse out of the flagella compartment and turnover products must be actively removed by dynein 2 in order to allow unhindered trafficking of the IFT trains.

The current model for retrograde activation is fragmented at best. IFT-dynein is carried into the compartment in an inactivated form as part of the IFT cargo. Once it reaches the tip, a

poorly understood remodeling occurs, initiating the dynein-powered return of the IFT train back to the cell body [27, 28]. During retrograde movement, the kinesin is inactivated, but it is unknown whether kinesin-2 is removed by the IFT particle as part of the turnover cargo or simply diffused out. IFT-dynein has been historically shown to associate with complex A in *Chlamydomonas*, primarily due to IFT-A temperature sensitive mutants exhibiting similar phenotypes as retrograde mutants [29, 30]. During remodeling at the distal tip, IFT-A likely facilitates the activation of dynein-2 in order to initiate retrograde movement, although a detailed mechanistic overview is lacking [28].

The newest addition to the retrograde movement model suggests OSM-3 and kinesin-II may directly transport IFT dynein, independently of the IFT particle in *C. elegans* [11]. The conclusion is derived from IFT-dynein undergoing normal IFT transportation speeds despite the uncoupling of IFT complex A/kinesin-2, and complex B/OSM-3. Another new concept suggests IFT172 may in fact mediate the interaction between inactivated dynein and the IFT particle during anterograde movement [28]. A new study in *C. elegans* has revealed the presence of a new retrograde dynein motor, specific to outer labial quadrant neurons, which are able to form full functional cilia in canonical IFT dynein mutants [11].

2.2.1.3 Regulation of the motors

IFT motor regulation continues to be of high interest in the cilia field due to the motors' important functions. Defects in FLA3, the kinesin associated protein, lead to mislocalized kinesin-II and subsequently produce a bald or flagella-less phenotype in *Chlamydomonas [31]*. Isolation of DYF-11 null mutant, a homolog of human microtubule-interacting protein (MIP)-T3 and IFT54 in *C. elegans*, reveals that this protein may serve as an anchoring protein for the priming/loading of the entire IFT motor/particle complex onto the transition zone of cilia [32]. KIF17, OSM-3 homolog of *C. elegans*, in human primary cilia was discovered to be under the regulation of a RAN gradient between the cell body and flagellar compartment. This mechanism operates in similar fashion to the RAN gradient active in regulating the trafficking of proteins across the nuclear pore complex [33]. A ciliary localization signal (CLS) at the tail end of KIF17 was shown to contribute to the interaction with another accessory protein known as importin-β2, a nuclear import protein; this interaction was inhibited by RAN-GTP. Similar CLS signals may exist for additional proteins in other model organisms due to the conserved nature of IFT. Recent study into the regulation of IFT-dynein flagellar entry has suggested that *Chlamydomonas* IFT172, a peripheral protein of IFT-B subcomplex, may be directly involved in the transport of IFT-dynein into the flagellar compartment [28].

2.3 IFT particle

By comparing the flagellar proteome of a *fla10^{ts}* mutant after incubation at the non-permissive temperature to a wild-type proteome, Cole and colleagues biochemically observed the depletion of certain proteins from the flagellar compartment [5]. After further analysis, members of the IFT particle were discovered. Results from this study demonstrated that the IFT particle was actually comprised of two sub-complexes, IFT-A and IFT-B, which to date consist of 6 and 12 polypeptides, respectively [19]. A recent study shed new light on the organization of the IFT-B subcomplex, demonstrating it can be separated further into two tetrameric subdomains: IFT25/27/74/81/72 and IFT52/46/88/70 [34-36].

IFT52 serves as the interface between the IFT74/81 and IFT52/46/88/70 [36]; IFT74/81 functions as the intermediate complex between IFT25/27 and IFT52/46/88/70. Currently IFT-A complex is understood to be composed of IFT144/121/140/121/139/43 [30], however its structural organization remains unclear.

A majority of the IFT members are enriched in WD40 and tetracopeptide repeats (TPR), multi-protein binding domains that possibly form a circularized beta-propeller structure and alpha helical solenoid, respectively, to behave as scaffolding elements [19]. WAA is another binding motif present in these proteins, although it is poorly understood. Most of the IFT proteins contribute to the overall integrity of their respective complexes, evident by the subsequent instability and depletion of complex-mates following disruption of certain IFT proteins. Depending on which complex is disrupted, flagella morphology is typically affected in one of two ways: structurally sound but severely truncated cilia (IFT-B mutants) or short bulbous flagella (IFT-A) [1, 13, 27]. The resulting phenotype reveals the different nature of the two complexes; short flagella convey IFT-B's importance in anterograde movement and protein buildup in the flagella compartment suggest IFT-A is involved in retrograde IFT. Nonetheless, the many parts of IFT machinery must work in a concerted effort to strike an efficient balance between retrograde and anterograde transportation dynamics.

Defects in other IFT-B proteins typically lead to a bald phenotype, making any biochemical analysis a challenge to determine individual function. A null mutant of *ift88*, the first IFT protein to be implicated in disease [37], displays a bald phenotype in *Chlamydomonas*. The absence of IFT54/MIP-T3 causes the entire IFT motor/particle complex, with the exception of OSM-3, to mislocalize at the ciliary base in *C. elegans* [32]. In IFT46 mutants, IFT-B complex still assembles the complex B core proteins but stability is severely affected, evident by the presence of structural sound yet short flagella in *Chlamydomonas* [38]. A suppressor IFT46 mutant sufficiently stabilizes the IFT complex to produced full length flagella. However, upon closer analysis, the axoneme lack outer dynein arms [38]. Thus, IFT46 serves as an adaptor protein for the specific transport of ODA16, a component of the outer dynein arms [39]. It is unclear whether IFT46 functions as a structural protein, since it appears not to be an essential contributor to complex B structural integrity (Richey and Qin, unpublished). It could possess a secondary function as a molecular indicator or chaperone for the IFT-B complex assembly, since the IFT-B can still assemble on a sucrose density gradient (Richey and Qin, unpublished). In addition to the known IFT core proteins, there are a few peripheral proteins associated with complex B: IFT57, IFT20, IFT172, and IFT80 [34]. IFT20 is a particularly interesting protein, because it is the only IFT protein that can localize in the Golgi apparatus, the central hub for the sorting and packaging of macromolecules for secretion. The current model suggests IFT20 is involved in directing vesicles transporting ciliary-specific proteins near the basal body, and participating in the trafficking of membrane proteins into the flagellar compartment [40]. IFT57 can target to the transition fibers of the axoneme, and serves as an anchoring protein for IFT20 to IFT-B in zebrafish [41]. IFT172 is also an interesting protein, since it readily dissociates from the IFT particle and has been shown to be important for retrograde movement. Using temperature sensitive mutant *fla11*^{ts} (IFT172), Pederson et al. 2006 concluded that IFT172 directly interacts with CrEB1, a protein exclusively located at the flagella tip, and accumulates IFT-B but not IFT-A nor IFT dynein proteins in the flagella [42, 43]. Recently, evidence of IFT172 involvement in the flagellar entry of IFT-dynein was detected in *Chlamydomonas*. Upon

incubation at the non-permissive temperature, IFT-dynein is depleted from the flagella compartment of the temperature sensitive IFT172 mutant while the rest of IFT particle remains at wild-type levels [28].

The function of individual IFT-A sub-complex proteins are even more enigmatic. Much like IFT-B proteins, disruption or depletion of a single IFT-A protein leads to the instability of the complex and subsequent depletion from the cell body [28]. Mouse IFT122 was shown to regulate members of the sonic hedgehog pathway in a number of ways by uniquely affecting the localization of certain proteins differently than IFT-A and IFT dynein mutants [44]. In *Drosophila*, an IFT140 mutant does not have detectable levels of ciliary TRPV ion channels; while the mRNA levels were unchanged, IFT140 may instead be important for the post-translational stability of the ion channels [45]. The more predominant understanding of the IFT-A function comes from its importance to retrograde movement. At the permissive temperature, electron-dense bulges are present within the cilia of temperature-sensitive *Chlamydomonas* mutants in IFT139 (*fla17*) and IFT144 (*fla15*). Following a shift to the non-permissive temperature leads to the complete breakdown of retrograde IFT and retraction of the axoneme, resulting in lolli-pop shaped bulbs filled with IFT-B proteins [29, 30]. This phenotype is also observed in IFT dynein mutants, thought to arise from the possible hindrance of retrograde IFT activation, and ultimately leading to the buildup of turnover products and IFT particles within the flagellar compartment [43]. In *C. elegans*, IFT-A directly interacts with kinesin-II while IFT-B is transported by OSM-3. IFT-A and IFT-B are linked together by the BBSome, a secondary adaptor complex important for ciliary membrane biogenesis [21, 46].

2.4 BBSome role in membrane biogenesis

Originally discovered during genetic disease screens, the BBSome protein complex functions as an adaptor complex for the IFT particle and facilitates the transport of ciliary membrane proteins. Interaction assays using BBS4 led to the discovery of the seven conserved proteins, BBS1/2/4/5/7/8/9, that comprise the BBSome complex [47]. In the same study, BBS5 was found to interact with phosphoinositides, phospholipids important for recruitment of trafficking proteins to the plasma membrane, implicating a role for the BBSome in vesicle trafficking. In addition, the BBS1 was shown to interact with Rabin8, a guanine nucleotide exchange factor (GEF) for Rab8, two proteins important for ciliary protein trafficking [16]. BBS1 direct association with Rabin8 stimulates the protein's GEF-activity to promote Rab8 activation. Rab8 and Rabin8 contribution to ciliogenesis will be discussed below. Arl6 (BBS3), although not important for BBSome assembly, is important for the recruitment of BBSome to primary cilia and purified liposomes [48]. In *Chlamydomonas*, perturbation of BBSome proteins does not lead to any morphological defects; however, cells are unable to undergo phototaxis, suggesting a role in signal transduction. The ciliary membrane in BBS1, BBS4, and BBS7 mutants accumulate several proteins which are thought to hinder Ca2+ signaling pathways involved in the phototaxic response [17]. In *C. elegans*, the BBSome serves as a linking bridge between IFT-A/kinesin-II and IFT-B/OSM-3; disruption of the complex results in the uncoupling of the IFT machinery and leads to cilia morphology defects in some cases [21, 46]. Protein models predict the BBSome functions as a vesicle coat, much like clathrin and COPI/II coat, directing post trans-Golgi network (TGN) vesicles to the ciliary compartment and accompanying them as a mediator between the IFT machinery [48]. The BBSome also sporadically "falls off" the IFT train, possibly in the event

of cargo unloading [17]. However, only a few ciliary membrane proteins have been confirmed to be BBSome-dependent for proper localization, most notably Somatostatin receptor 3 (SSTR30) and some G-coupled receptors. Thus, it is unclear whether the BBSome-dependent ciliary transport is a general mechanism or a protein-specific system [17].

2.5 Role of GTPases in ciliogenesis

Research in small GTPases involved in ciliogenesis is a growing branch of the field and the results have been quite interesting. ADP-ribosylating factor–like (ARL) 13, BBS3 and the BBSome are involved in the targeting and entry of flagellar membrane proteins into the compartment [16]. ARL-13 and ARL-3 are small G-proteins antagonistically operating to maintain the stability of IFT particles during middle segment transport in *C. elegans* (IFT A and B) [49, 50]. ARL-13 may also have roles involved in maintaining axonemal integrity since null mutant animals have a variety of gross cilia abnormalities [49-51]. It has been suggested that ARL13 may in fact regulate the coupling of IFT-A and IFT-B, while ARL3 regulates IFT-B interaction with OSM-3; together they regulate the integrity of the IFT machinery in *C. elegans* [50]. Rab8 is recruited to the transition zone by Rabin8 (Rab8GEF), following stimulation from BBS1, a core member of the BBSome; this ultimately results in the fusion of post-Golgi vesicles shuttling ciliary membrane proteins near the basal body [52, 53]. Dominant negative and constitutive active constructs demonstrate the impact that the nucleotide state of Rab8 has on its entry into the ciliary compartment and its role in ciliogenesis [47]. Arf4 and Rab11 form a complex with Arf GTPase activating protein ASAP1 and FIP3 to package and transport rhodopsin from the trans-golgi-network to photoreceptor cilia [54]. This interaction between rhodopsin and Arf4 is dependent on a VxPx motif, a ciliary localization signal also found in other ciliary membrane proteins [16]. Recently, the VxPx motif has been shown to be essential for the trafficking of polycistin-1 protein, and to be involved in the recruitment of Rab8, thereby promoting fusion of ciliary membrane protein-containing vesicles [55]. Another small GTPase, Rab23, was found to be responsible for the turnover of sonic hedgehog signaling protein, Smoothened, from the ciliary compartment [56]. As mentioned in IFT motor regulation section, a RAN-GTP ciliary/cytoplasmic gradient regulates the entry of kinesin motor KIF17 in the primary cilia of cultured cells [33].

Two mysterious members of the IFT-B complex, IFT27/RABL4 and IFT22/RABL5, are the only small GTPases known to directly interact with the IFT particle. IFT25 is a phosphoprotein of unknown function , though it is known to interact with the small GTPase-like IFT27 [57]. Recent work on IFT27 confirmed its GTP binding and GTPase activity along with solving the crystal structure of the sub-complex IFT25/27. However, the exact function of IFT25/27 remains unknown [58]. IFT22 has been the more controversial of the two, since in recent studies with *C. elegans* and *Trypanosome* IFT22 homologs produced conflicting results. In *C. elegans*, a putative constitutive active form (GTP-locked) of the IFTA-2 (IFT22 homolog) can enter the ciliary compartment while dominant negative (GDP-locked) diffusely localizes throughout the neuronal cell body and is notably excluded from the ciliary compartment [59]. IFTA-2 null mutants exhibited extended lifespans, reminiscent of insulin IGF-1-like signaling pathway defects, and a failure to enter dauer formation, a type of survival mode. The null IFTA-2 (IFT22) mutant had intact sensory cilia, effectively suggesting IFTA-2 is not essential to ciliogenesis. However, RNAi knockdown experiments of *Trypanosome* RABL5 lead to the buildup of IFT particles in the flagella compartment and

subsequent shortening of the flagella [60]. This phenotype is similar to mutants with defective retrograde IFT, suggesting RABL5 is important for ciliogenesis.

2.6 Gating the ciliary compartment

As mentioned above, the transition fibers mark the entrance of the flagella compartment by tethering the plasma membrane to the base of the flagella. The ciliary proteome contains various proteins not found at such concentrated levels in cytoplasm, implying an inherent selectivity to the transition zone barrier [61]. Although the complete regulatory pathway remains poorly understood, various studies have begun to demonstrate the complexity of the flagella gating mechanism. Recent biochemical characterization of *cep290* mutant in *Chlamydomonas* revealed that the protein CEP290 functions as an intricate member of the transition zone barrier proteins [62]. CEP290 is part of the MKS/MKSR/NPHP proteins (Meckel-Gruber syndrome/related and nephronophthisis), shown to localize at the base of the flagella. Together these proteins form the transition zone and function as the ciliary selective barrier, evident by the accumulation of non-ciliary proteins in the cilia of various TZ mutants [63, 64]. Although IFT anterograde movement was normal and retrograde slightly slower, the *cep290* flagella accumulated IFT-B proteins and BBS4, yet had a reduction of IFT-A, some membrane proteins and axonemal precursors. This phenotype suggests CEP290 plays a role in the mechanical selectivity of the transition zone; it could be possible that IFT-B binding/priming at the transition zone requires CEP290, which could explain the mostly unhindered movement of IFT and the buildup of IFT-B and not IFT-A.

Additional selectivity mechanisms have become more apparent, such as the requirement of ciliary transport signal (CTS) for access to the flagella compartment [16]. The VxPx motif, a CTS, is important for the targeting and entry of ciliary membrane proteins polycistin-1 and rhodopsin. In contrast, a recent study discovered a mechanism for molecular retention, whereby passive diffusion into the ciliary membrane is inhibited by a transferable retention signal [65]. Podoclayxin was shown to contain a four- amino acid PDZ binding motif that facilitated its interaction with NA+/H+ exchanger 3 regulatory factor NHERF1, a protein attached to the apical actin cytoskeleton [66]. The conserved four- amino acid sequence in the PDZ binding motif was shown to be sufficient to prevent passive diffusion into the cilary membrane domain [65]. Although the ciliary entry is passive, the ciliary membrane protein retention appears to require the protein to be firmly attached to the axoneme. Thus, a ciliary retention signal is likely to be necessary for membrane protein accumulation in the ciliary compartment. Additionally, much like the gating system for the nuclear pore complex, a RAN-GTP has been found to exist between the cilia and cytosol that is important for import of KIF17, via its Ran-GTP dependent association with importin-β2 [33].

2.7 Microtubule post translational modifications

The microtubule component of the axoneme undergoes various post-translational modifications (PTMs) that play a vital role in promoting the mechanical movement of the organelle. The dramatic impact of microtubule PTMs has been well characterized, but recent studies have begun to deepen our understanding of how PTMs affect ciliary assembly and maintenance. For an extensive review on the impact of PTMs on ciliogenesis and cell motility, please see a recent review by Wloga and Gaertig [67, 68].

2.7.1 Acetylation of tubulin

N-Acetylation is the only PTM that occurs within the microtubule core, at the highly conserved K40 residue on α-tubulin [69-71]. Mammalian microtubules undergo acetylation of lysine residues on multiple sites located on both α- and β- tubulin [72]. Recently MEC-17, a previously uncharacterized protein now known as αTAT1 (α-tubulin acetyltransferase), may be the sole enzyme responsible for the acetylation of K40 in mammalian cilia [73]. Knockdown of αTAT1 does not produce any severe morphological defects nor does it affect microtubule polymerization. However, as a BBSome-associated protein, it was suspected to be involved in cilia assembly. The depletion of αTAT1 leads to a delayed assembly of primary cilia; taken together with recent information, K40 acetylation may be involved in the dynamics of axonemal assembly and disassembly [74]. Recent studies demonstrate that acetylation of α-tubulin may target these subunits for degradation, since they are preferentially selected to be ubiquitinated over β-tubulin during disassembly [75]. A newly discovered BBSome subunit, BBIP10, functions as a positive regulator of microtubule stability, as a reduction of cytoplasmic microtubules and increase in free tubulin is seen in BBIP10-depleted cells. In addition, overexpression of BBIP10, microtubule acetylation was dramatically increased. The function of BBIP10 on microtubule stability appears to be either dependent or independent of the BBSome [76]

2.7.2 Glutamylation and glycylation

Glutamylation has been observed on microtubules in general, while glycylation has been found to be restricted to the ciliary microtubules of flagellated cell types [77]. These side chains are synthesized in distinct steps of initiation and elongation, typically carried out by two types of enzymes known as tyroslytubulin ligase-like proteins (TTLLs) [78, 79]. Recent studies have begun to investigate the function of these types of PTMs for ciliogenesis, most notably by the impact of polyglutamylation on inner dynein dynamics [80, 81].

3. Ciliopathies

Ciliated cells can be found in various tissues throughout the human body. These include the eye, the trachea, the kidney, the reproductive tract, the intestines, the heart, and many others. In each of these tissues, the cilia perform a significant role in allowing proper function of the tissues. Since ciliated cells are in most important organ tissues, malfunctioning cilia contribute substantially to human disease. Diseases caused or related to faulty cilia are called ciliopathies. The list of ciliopathies is just as diverse as the variety of tissues in which cilia are found. These diseases support the fact that cilia, once thought to be unimportant cellular appendages, are essential for sustaining health in the human body. There are too many ciliopathies to mention, and the list continues to grow. Some major ciliopathies include Primary Ciliary Dyskinesia (PCD), Hydrocephalus, Polycystic Kidney Disease (PKD), Bardet-Biedl Syndrome (BBS), and even cancer [82].

3.1 Primary Ciliary Dyskinesia (PCD)

The relationship between Primary Ciliary Dyskinesia (PCD) and ciliary defects was first discovered in the 1970's [83], making PCD the first human disorder found to be linked to cilia function [84]. PCD is a multi-symptomatic ciliopathy that is present in all major ethnic

groups and occurs 1 in every 20,000 live births, although this is likely an underestimation due to a failure to properly diagnose the disorder [85, 86]. PCD was first called "immotile cilia syndrome," but was renamed because it was later found that the cilia were not always immotile, but often had abnormal motility [87].

PCD is characterized by many symptoms that are expressed to various degrees [84]. It affects mainly the respiratory system beginning at birth or within the first month of life. Early signs typically involve a persistent cough and chronic nasal congestion. Other symptoms often include other respiratory problems such as sinusitis and bronchiectasis [87]. The respiratory symptoms of PCD are caused by the lack of uniform ciliary movement to transport particles, or mucous in or out of the organs or the cells themselves. There are about 200 motile cilia in the respiratory tract of a healthy individual. Beating coordinately, these cilia function to remove mucous and debris from the airway in a process called mucocilliary clearance [88]. When the cilia malfunction, there is buildup of mucous and debris in the tract, which leads to respiratory difficulties.

However, this disease is not only associated with the respiratory system. It also has an impact in development, fertility, and aural health. Fifty percent of patients with PCD have total situs inversus, with organs developing on the opposite side of the body. This is thought to be due to the importance of cilia in producing correct direction of nodal flow during embryo development. This was seen in a 2002 study which showed that when direction of nodal flow was artificially reversed to right instead of left, mice developed organs that were a mirror image to normal orientation [89]. There are three known genes involved in left-right axis (LRA) determination, including lrd (left-right dynein), hfh-4 (hepatocyte nuclear factor/forkhead homologue 4), and kif3B (kinesin member 3B) [90-92]. PCD also affects fertility. Males with PCD are typically infertile, and females have a higher rate of ectopic pregnancies. Significant hearing impairment is seen in about fifty percent of children with PCD [93]. This is likely due to the condition known as chronic secretory otitis, which is found almost universally in PCD patients [94]. This is a condition that causes collection of fluid in the middle ear and can cause serious hearing loss and pain. Studies have been done to show that people with chronic secretory otitis have a slower ciliary beat frequency, resulting in failure to move fluid out of the ear canal, leading to infection [95].

PCD is a genetically heterogeneous disease typically caused by autosomal recessive mutations in ciliary protein genes. The most common mutations occur in DNAH5 (dynein heavy chain) and DNAI1 (intermediate chain dynein) genes. Studies show that the disease is caused by abnormalities in the axonemal structure of the cilia. In most patients, the outer dynein arms are missing, of which fifty percent of these patients have mutations in DNAH5 and DNAI1 [96-98]. However, it is thought that lacking the inner dynein arms, central pair of microtubules, or radial spokes can also lead to PCD. Seventy to eighty percent of patients have outer or inner dynein arm defects, while only five to ten percent are missing radial spokes. PCD can also be caused by a disorientation of cilia and transposed microtubules [85]. Transposed microtubules occur when a cilium lacks the central microtubule pair and a peripheral doublet with dynein arms transposes to the center of the axoneme. This microtubule defect, coined "central microtubule agenesis," causes a circular rotation of the cilia rather than the normal back and forth motion [99]. In addition to genetic causation, PCD can also be a result of acquired defects caused by epithelial damage by chronic infection or irritant exposure [100].

Beat frequency, beat patterns, and protein localization are all parameters measured in diagnosis of the disease. Nasal brush biopsies are most commonly used to collect a patient's cells, but bronchoscopic brush biopsies can also be used [101]. Typically, a diagnosis is made by examining fixed cells under a transmission electron microscope to identify ciliary structural defects in addition to measuring ciliary beat frequency in live cells by video microscopy. However, in the case of central microtubule agenesis, it is important to also look at ciliary beat pattern, since the cilia typically maintain a normal beat frequency, with aberrant movement. These patients also tend to have normally functioning nodal cilia, which allow normal situs, accounting for the fact that not all PCD patients have situs inversus [99]. This may pose a problem for diagnosis if doctors are using organ orientation as a diagnostic factor. Protein localization is a relatively new and uncommon means of diagnosis. In respiratory cells, certain ciliary proteins have been shown to mislocalize in the case of PCD. These include DNAH5 and DNAI1, which under normal circumstances, colocalize throughout the axoneme. In patients with PCD caused by a mutation in the DNAI1 gene, the protein fails to localize to the distal tip, leading to abnormal motility. In patients with a mutation in DNAH5, the protein is entirely absent from the axoneme, causing complete paralysis. This is useful for diagnosis since immunofluorescence can show if this mislocalization is present [102]. Genetic testing can also be done, but is not very reliable since the disease is very genetically heterogeneous, with the discovery of at least ten related genes from various loci on multiple chromosomes [103]. False positive diagnoses can occur during or after a respiratory infection or inflammation, since these conditions show impaired ciliary function. Therefore diagnosis is only accurate at least four to six weeks post-infection [101].

Screening for the disease is also very important to help rule PCD out for patients with similar symptoms. Among the screening techniques, measuring levels of nasal nitric oxide (nNO) is relatively new but fairly promising. Very low levels of nNO are exhaled from patients with PCD. Therefore, if the patient has high or normal levels, they likely do not have PCD. Levels are not diagnostic of PCD, however, since low levels can also be found in accordance with other diseases such as cystic fibrosis, chronic sinusitis, and others. One downfall of this screening technique is that it is ineffective for children under five years old, since younger children will not be able to blow into the apparatus [104]. Saccharin testing is also used for PCD screening. This is a measurement of the time it takes for a patient to be able to taste saccharin, which is related to the function of the cilia in the taste bud cells. However, this is not a very useful test since it is unreliable in children under twelve. Radioaerosol mucociliary clearance testing, which measures how well mucous is removed from the respiratory tract, is useful in infants for screening, but again is not diagnostic of PCD [101].

Current treatment for PCD is mainly focused on treating and preventing the symptoms of the disease. Studies are being done to test the efficacy of antibiotics, airway clearance, and anti-inflammatory treatments [101]. Nebulized DNase has also shown promise in a 1999 case study with a PCD patient. The patient's symptoms were not relieved from treatment with antibiotic and bronchodilator treatments alone. However, when DNase was used in addition to these treatments, there was a significant improvement in symptoms overnight. This showed the therapeutic potential of DNase, but more studies need to be done to further test the efficacy [105]. Gene therapy is also being considered. A 2010 pilot study showed that a lentiviral vector can incorporate ciliary protein into the axoneme to restore ciliary

function [96]. Further studies need to be done to make these treatments more mainstream. In addition to treatments, preventative measures such as avoiding respiratory irritations and exercising, can be very helpful. If treatments and preventative measures are unsuccessful, it is often necessary to undergo surgery.

PCD research has taught us a lot about cilia. It has shown the importance of cilia in development, in respiratory function, in aural health, and in fertility. It has also taught us a lot about the structure of cilia and the function of various ciliary proteins. In fact, a 2010 study revealed 208 potential ciliary genes based on PCD research [106]. PCD will continue to give us new insights on ciliary function and will continue to be an important area of ciliopathy research.

3.2 Hydrocephalus

Hydrocephalus is a disease in which cerebral spinal fluid (CSF) accumulates in the brain. CSF is mostly produced by the choroid plexuses of the lateral, third, and fourth ventricles [107]. To maintain equilibrium it is important for the excess fluid to drain into the subarachnoid space where it is resorbed into the venous system [108]. When this process malfunctions, the fluid builds up, causing swelling in the brain that leads to many complications. The disease dates back to the time of Hippocrates when he described a "liquefaction of the brain" that showed symptoms such as headache, vomiting, and visual impairment [109]. Other symptoms can include high intracranial pressure, in addition to impairments in gait, cognition, alertness, and continence [110]. Although this disease can have various causes, it has been shown that there is a higher prevalence of hydrocephalus in patients that are known to have ciliary defects, and the disease can also be linked to genes known to impact cilia function or structure [111]. Hydrocephalus is often seen in conjunction with other ciliopathies such as PKD as seen in the Tg737 mouse [112] and more rarely in PCD [113].

Although hydrocephalus has a rich history in research, the disease as a ciliopathy is a relatively new area of study, and much is yet to be learned about its link with cilia. In animal models, 43 mutations from 9 different genes are known to be related to hydrocephalus. In contrast, there is only one gene that has been identified in humans [114]. X-linked hydrocephalus is caused by a mutation in the neural cell adhesion molecule L1 (L1CAM) gene [115]. This form of the disease was first discovered in 1949 [116] and occurs about 1 in every 30,000 male births [117]. No research has been done up to this point to determine if there is a link between L1CAM and cilia.

Many mouse models, however, have allowed identification of multiple ciliary genes that, when mutated, can lead to hydrocephalus. Accumulation of CSF can be caused by malfunctions in CSF production, CSF flow, or CSF absorption. Tg737 mice are missing intraflagellar transport protein IFT88/Polaris. This protein is important for ion transport and the mutation shows an overproduction of CSF, leading to the development of hydrocephalus [107]. This suggests the importance of cilia in signaling and regulation of CSF levels. Mice with this mutation also have abnormal beating of the motile cilia of the ependymal cells, disrupting the CSF flow. However, this is not causal in this case since development of the disease occurs before the motile cilia form [107]. In contrast, CSF flow disruption seems to be the cause of hydrocephalus in Mdnah5 (axonemal dynein heavy chain) and Hydin mutants. These mutants cause structural defects in the axoneme of the

cilia, leading to impaired motility [111, 118-120]. In these two mutants, the cilia are unable to create sufficient flow to remove CSF from the ventricles. Other hydrocephalus mutants are thought to be linked to important development pathways that may disrupt the CSF equilibrium. Inactivation of Pten and β-catenin leads to hydrocephalus [121]. These proteins are key players in Wnt signaling pathway and important for proper midbrain development, in which the function of primary cilia could be involved [122]. Polycystin-1, a ciliary membrane protein, is also shown to be important in development and regulating fluid in the brain [112]. Ptch1 and parkin-qk1 mutations also lead to hydrocephalus [123] most likely due to a disruption in the Hedgehog pathway, which also involves the primary cilia [124]. All these findings show the importance of cilia in signaling, development, and movement of fluid, and their roles in maintaining a healthy CSF balance in the brain.

An early method of diagnosis required ventricular puncture to test for dilation and occlusive lesions. This procedure was risky, so it was eventually replaced by computed tomography (CT) and magnetic resonance imaging (MRI) [109]. Prenatal diagnosis can be done for x-linked recessive hydrocephalus by doing serial ultrasound scans to test for abnormal growth of the baby's head [125]. Since there is only one known human hydrocephalus gene, and it is for the rare x-linked type, genetic testing is not yet useful for diagnosis.

The oldest treatment known for hydrocephalus was to tightly bandage the baby's deformed swollen head to decrease the size and swelling. This method was abandoned since it increased the intracranial pressure. In the 18th and 19th centuries, special diets were recommended and dehydration was induced with laxatives, diuretics, potassium iodide, etc. In 1957 acetazolamide was first used in practice and is still used to reduce production of CSF. Other treatments were abandoned such as isosorbide and irradiation of the choroid plexus [109]. Vasoconstrictors such as dihydroergotamine have shown promise in allowing better arterial pulsation and reducing ventricular dilation [126]. Various surgical treatments have been used including external CSF drainage, serial lumbar puncturing, and implantation of an internal shunt, which is a catheter that allows drainage of CSF out of the ventricles. These surgical procedures brought about complications such as infections, improper placement of shunts, and hydraulic mismanagement due to body positioning. These complications necessitated further research and improvements. One of the most pivotal improvements is the development and modification of shunts with adjustable, autoregulating, antisiphon, and gravitational valves. There are currently at least 127 designs of valves and more than 20 shunting procedures that have been suggested, with ventriculoperitoneal shunts being the most commonly utilized. Valved shunts are now standard treatment for hydrocephalus, being the choice treatment for about 80% of cases [109, 127]. In elderly patients shunt implantation is very risky. Repeated lumbar puncturing in patients with communicating hydrocephalus can potentially prevent the need for shunt surgery in these patients [110]. The newest addition to shunt technology is antibiotic-impregnated shunts that help prevent post-surgical infections [128].

The relationship between cilia and hydrocephalus and the genes that are involved are poorly understood in humans. Advancements in this knowledge may eventually lead to better, less invasive forms of treatment and a better understanding of how cilia function in the brain.

3.3 Polycystic Kidney Disease (PKD)

There are two types of PKD, autosomal dominant polycystic kidney disease (ADPKD) and autosomal recessive polycystic kidney disease (ARPKD). ADPKD is the most common of all the potentially lethal autosomal dominant diseases, with an incidence of 1 in 1000 [129]. Symptoms typically do not present themselves until between the ages of 35-50 years. These symptoms include acute abdominal and lower back pain, hypertension, palpable kidneys, recurrent urinary tract infections, shortness of breath, early satiety, hydrocephalus, kidney stones and cysts in the kidney, liver, thyroid, subarachnoid space, and seminal vesicles [130]. ADPKD most often ends in end stage renal disease (ESRD) between 55 and 75 years old [82]. ARPKD, on the other hand presents itself immediately. Although delayed presentation is possible, ARPKD can often be seen in utero, and leads to ESRD in the neonatal stage and infants often die due to respiratory complications. Also unlike ADPKD, there is usually no cyst formation in other organs in the case of ARPKD [130]. It is also much more rare, with an incidence of 1 in 20000, and has a very high infant mortality rate [131].

Although other factors may be involved, strong correlations have been made between PKD and cilia function. Primary cilia are now known to act as mechanosensors that regulate Ca^{2+} influx. Fluid flow in the kidney causes the primary cilia to bend, which allows the Ca^{2+} channels to open, allowing increased intracellular Ca^{2+}. This process is disrupted in some forms of PKD. However, primary cilia have also been implicated in pathways such as Hedgehog, Wnt, cAMP, and Planar Cell Polarity. Disruptions of these pathways can lead to abnormal polarity, differentiation, and proliferation, which can lead to cyst formation [130]. Therefore the role of primary cilia in cystogenesis is not only important, but is very multidimensional.

In human, ADPKD is caused by a mutation in PKD1 (85 % of the time) or PKD2 (15% of the time). There are more than 500 mutations known in PKD1 and 120 in PKD2 [132, 133]. These genes code for proteins polycystin-1 and polycystin-2, which both localize to renal cilia [134]. These proteins prove to be important in renal tube development and cell differentiation in the kidney. They are involved in the calcium signal transduction cascade that regulates proliferation and differentiation [135, 136]. ARPKD is caused by a mutation in PKHD1, which encodes fibrocystin, a receptor-like protein associated with the membrane and colocalizes with polycystin-2 in primary cilia [82, 137]. It also plays a role in collecting duct and biliary cell differentiation [138]. Studies in other species have made other connections between cilia and PKD. In addition to hydrocephalus, Tg737 mice have cysts in kidney and pancreas, hepatic fibrosis, and polydactyly. Mice with this mutation have elevated polycystin-2 levels and have cilia that are much shorter than normal [135, 139]. These factors result in cyst formation. A mutation in Kif3A (a subunit of kinesin-II in kidney epithelium) causes increased canonical Wnt activity. Deletion of this gene leads to absence of cilia, and after the cilia are lost, cystogenesis occurs [140]. Seahorse is another mutation that shows a disruption in Wnt signaling in zebrafish and may function downstream from cilia [141]. Out of the 11 genes identified in zebrafish that relate to PKD, 6 were found to be ciliary genes [142]. These mutants and more, show the importance of cilia and pathways in regulating renal cells.

Although CT and MRI techniques can be used, diagnosis by ultrasound and positive family history are the choice methods to test for PKD [143]. ARPKD can even be detected in utero by ultrasound which reveals large kidneys that take up most of the fetal abdominal cavity

and the lack of urine in the bladder [130]. Nuclear magnetic resonance (NMR) spectroscopy also allows discrimination between PKD and other kidney diseases with an accuracy of over 80%, by creating a fingerprint of urinary protein biomarkers with key PKD features [143]. Since most PKD patients have mutations in known PKD genes, genetic testing may be plausible. However, since there are hundreds of possible mutations, the only commonly used genetics testing is direct sequencing to screen for the disease [144].

There is currently no effective treatment that is widely used for PKD. Transplantation and dialysis are often the only options. Apart from that, pain control, antibiotics for urinary tract infections, increase in fluid intake, and refraining from smoking and caffeine, are ways of lessening the symptoms [130]. However, many potential drugs for treating cystogenesis are under investigation. Many drugs have shown to slow cyst growth in animal models. Rapamycin helps regulate cell proliferation by inhibiting the mTOR pathway, which is often overexpressed in PKD patients. This drug increases apoptosis and shedding of cystic cells, which decreases kidney size and restored kidney function [145]. Roscovitine, currently used in cancer treatment because it inhibits cell cycle, show positive effects in PKD models [146]. Lisinopril, an inhibitor of angiotensin converting enzyme (ACE) also alters proliferative and apoptotic pathways, reducing cyst development [147]. Patients with PKD show high levels of circulating vasopressin. Tolvaptin is a vasopressin-2 receptor antagonist and shows reduction in kidney and cyst volumes [148]. Ocreatide, an analogue of the hormone somatostatin, inhibits cAMP production. In doing so, it inhibits secretion and reduces liver cysts and kidney volume. However, it does not seem to improve renal function [149, 150]. Epidermal growth factor receptor (EGFR) tyrosine kinase inhibition using EKI-785 also showed promise in PKD animal studies [151]. Anti-inflammatory drug, colchicine, is a microtubule inhibitor that has been shown to delay formation of cysts and is a candidate for prolonged clinical use [152].

PKD mutants are giving us a better understanding of the importance of cilia in many regulatory pathways and sensory functions. The disease further emphasizes how complex these seemingly simple cellular organelles are, and how necessary they are in maintaining systemic health and prevention of cystic growth.

3.4 Bardet-Biedl Syndrome (BBS)

Many syndromes are related to cilia, Bardet-Biedl syndrome being the most well-known. Bardet-Biedl Syndrome (BBS) is a multi-symptomatic disorder with symptoms including obesity, retinitis pigmentosa, genital hypoplasia, polydactyly, and mental retardation [153]. Typically at age 8, night blindness occurs which can eventually lead to complete blindness between ages 15 and 20. Kidney cysts are also common, making end-stage renal failure the most common cause of premature death in BBS patients [154]. Mid-facial deformities are often seen in humans with BBS and also in mice mutants for BBS4 and BBS6 [131]. Although this is a very multi-symptomatic disorder, obesity is what it is most known for. Ninety-eight percent of BBS patients become obese with a body mass index greater than 30% [155]. Diabetes occurs secondary to obesity, and patients often show lower locomotor activity than normal [156, 157].

Phototransduction proteins and others necessary for vision are produced in the inner segment of photoreceptor cells. To maintain the outer segments, the phototransduction

proteins have to be transported to the outer segments of the photoreceptors through the connecting cilia, the only bridge between outer and inner segments. BBS proteins form a BBSome and are believed to be responsible for recruiting membrane vesicles to the cilia [16, 47]. BBS proteins are also involved in important pathways such as Wnt and Hedgehog pathways which are very important for proper development and function [153]. Hedgehog and Wnt signaling are anti-adipogenic, preventing obesity [158-160]. It is now believed that obesity in BBS patients is linked to the impairment of ciliated cells in the hypothalamus to sense satiety, inducing hyperphagia [161]. BBS proteins may be important in transport of leptin in and out of the cells, causing loss of leptin signaling ability when cilia are lost. Moreover, melanin concentrating hormone receptor-1 (Mchr1) is involved in regulation of feeding behavior, and fails to localize to cilia in BBS mutants [161, 162]. These are just a few of the possible factors that may be involved in causing obesity in BBS patients.

BBS is an autosomal recessive disease showing pleiotropy, with multiple traits being affected. Development of the disorder often requires more than three mutations in at least 2 BBS genes [163]. Apart from the causative mutations, other BBS mutations often serve as disease modifiers. So far there are 12 known BBS genes, BBS1-12, which code for proteins important in trafficking cargo to the basal body and along the cilia. Other ciliary proteins have also been linked to BBS [154]. Among these are Kif3A and Tg737 mutants, which show hyperphagic activity and obesity, in addition to elevated plasma levels of glucose, insulin, and leptin [164].

Diagnosis of BBS typically requires the presence of at least four primary symptoms or three primary in addition to two secondary symptoms. Primary symptoms include cone-rod dystrophy, polydactyly, obesity, learning disability, genital defects, and renal anomalies. Secondary symptoms include speech impairment, brachydactyly (short digits), syndachtyly (fused digits), developmental delay, polyuria (excessive urination), ploydypsia (excessive thirst), ataxia (lack of muscle coordination), diabetes, heart and liver problems, olfactory deficits, and defects in pain and temperature sensation [154].

Since BBS is syndromic and affects multiple systems, treatment can be multi-faceted. Gene therapy has shown promise in treating vision impairments. Mice, in which Bbs4 has been deleted, show an inability of rhodopsin to localize to rod cilia and cone opsins to localize to cone cilia. This failure to localize leads to photoreceptor apoptosis and the deterioration of electroretinogram (ERG) a- and b-waves, causing serious vision impairment. Recently, adeno-associated viral (AAV) vectors have shown to be successful in incorporating Bbs4 into these mutants and restoring the localization of rhodopsin. In doing so, photoreceptor death was prevented, function of the retina was restored, and mice showed recovery of visual behavioral responses [165]. Surgical techniques are used for other symptoms of BBS. For instance renal transplantation is used in the case of cystic kidneys. Obesity is important to treat, since it has been shown to cause a fifty percent increase in mortality rates most likely caused by complications from secondary diseases. These include diabetes, cardiovascular diseases, cerebrovascular diseases, digestive disorders, gall bladder cancer, breast cancer, endometrium cancer, prostate cancer, etc [166, 167]. Bariatric surgeries such as gastric bypass and gastric band operations greatly improve weight loss in patients with obesity. These operations also show a dramatic improvement in reducing the risk of secondary diseases that are known to be associated with obesity. Because of the reduction in the incidence of these diseases, the mortality rate decreases substantially [168]. In

addition to surgical procedures, drug treatments have also been under review for the treatment of obesity. There are only two drugs currently accepted by the U.S. Food and Drug Administration for long term obesity treatment. This is due to the high prevalence of severe side effects correlated with the use of weight loss drugs. These side effects include heart attack, gastrointestinal distress, liver damage, anxiety, memory problems, suicide, and are often habit-forming. Because of these serious issues, it is important to be skeptical in determining which drugs are safe to be used to treat obesity [169]. These are only a few of the current treatments being used for BBS-related symptoms, and many others are yet to be discovered.

Again, BBS adds further support to the idea that cilia are important in many areas throughout the body, and they are very important in maintaining the overall health of the individual.

3.5 Cancer

Relating cancer to cilia is one of the newest areas of ciliary research in the field today. The cilia assembly-disassembly cycle is closely linked with the cell cycle. Cilia assemble upon exit of mitosis to the stationary phase, and resorb when the cell exits the S-phase and enters mitosis [170]. When the ciliogenesis is disrupted, it may have adverse effects on cell cycle and lead to cancer. This may be due to centrosomal amplification and genetic instability [171]. Proteins necessary for ciliogenesis colocalize to the centrosome, also supporting the link between cilia and cell cycle [172]. Cilia have also been found to play roles in important pathways such as Sonic Hedgehog, signal transduction pathways, and ligand-induced signaling. These pathways have recently been shown to relate to cancer. New insights are being made to connect ciliary dysfunction to carcinogenesis [172].

Primary cilia appear to have dual opposing functions in development of different types of cancer, so that some refer to them as being an On/Off switch, regulating tumorigenesis [173, 174]. One of the most important pathways related to ciliogenesis and cancer is the Hedgehog (Hh) pathway. Hh is normally suppressed by Ptch1 which prevents the trafficking of Smo in primary cilia. When Smo fails to localize to cilia, it prevents Hh signaling. Under cancerous conditions, however, Smo is able to localize to the cilia and Hh signaling is overexpressed leading to oncogenesis [175]. SmoM2 leads to brain tumors only if primary cilia are present. However, Gli2ΔN induces brain tumors only in the absence of cilia, since absence of cilia causes a disruption in Gli3, a repressor of Gli2ΔN [174, 176]. It appears that primary cilia are required both for the suppression and expression of oncogenisis. Primary cilia suppress oncogenic mutations that act downstream of cilia, but allow expression of oncogenic mutations upstream of cilia [173, 174, 177]. Therefore, cilia can play opposite roles depending on the causal mutation of the cancer development [173, 174].

Disruptions in Hh, Wnt, and PDGF pathways all have been linked to tumor formation. Cilia-associated genes such as Gli1, RPGRIP1, and DNAH9 are often mutated in breast cancer [177]. Nek8 is also localized to cilia and upregulated in breast cancer [178, 179]. Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer deaths in the United States [180]. In this case, excessive Hh signaling is caused by a mutation in a Kras gene[181], which codes for a protein known to be important in ciliogenesis. Therefore affected cells lack primary cilia, and are unable to regulate proliferation [182].

German pathologist, Rudolf Virchow, first defined cancer in the mid-1800s when he realized that Leukemia was caused by a rapid duplication of healthy cells that had mutated and in response, multiplied. Before the advent of modern-day technology, cancer was not diagnosed or treated until the tumors became visible and palpable, in which case they were surgically removed. Since then, however, an emphasis has been placed on early detection so that doctors can treat the cancer before it turns into large fatal tumors. Most doctors recommend certain regular screening for some types of cancer. Among these are mammograms for breast cancer, colonoscopies for colon cancer, Pap smears for cervical cancer, and prostate exams for prostate cancer. Other diagnostic tests include blood tests, ultrasounds, computed tomography (CT), X-ray, magnetic resonance imaging (MRI), and fine-needle biopsies. These techniques are used to detect early signs of cancer, allowing an earlier treatment and an attempt to prevent fatality [183].

Cancer treatment is one of the most prioritized areas of research today. Many treatments are currently being used and even more are being tested. These treatments include chemotherapy, radiation therapy, surgery, and gene therapy. Perhaps the implications in the relationship with cilia and cell cycle will help lead to the development of new treatments.

Primary ciliary dyskinesia, hydrocephalus, polycystic kidney disease, Bardet-Biedl syndrome, and cancer are only a few of the known ciliopathies, and more are still being discovered. These diseases are not only important to study for diagnostic and treatment purposes, but also give us a clearer understanding about cilia and their role in most critical bodily functions. Further ciliopathy studies will continue to shed new light on these important cellular structures.

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The Roles of ESCRT Proteins in Healthy Cells and in Disease

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1. Introduction

Endocytosis is a process that occurs in all eukaryotes and is an essential mechanism for internalizing membrane proteins and controlling intracellular trafficking (Bishop, 2003). Membrane proteins such as active epidermal growth factor receptors (EGFRs) are endocytosed via clathrin-dependent or independent-pathways and are typically first delivered to the early endosome (Bishop, 1997; Tarrago-Trani & Storrie, 2007) (Figure 1). The early endosomes (or sorting endosomes) have a crucial role in sorting the endocytosed cargo to three alternative destinations: (i) recycling the cargo back to the plasma membrane (receptor sequestration), (ii) transferring the cargo to the *trans* Golgi network (TGN), (iii) transporting the cargo into intraluminal vesicles (ILVs) of maturing endosomes known as multivesicular bodies (MVBs) (reviewed by Gruenberg & Stenmark, 2004; Russel et al., 2006; Piper & Katzmann, 2007). The ultimate consequence of such sorting is the exposure of the ILVs and their contents to lysosomal hydrolases after fusion of the MVB with lysosomes (receptor down-regulation) (reviewed by Sorkin & von Zastrow, 2009; Wegner et al., 2011). MVBs also play an important role in the traffic of lysosomal enzymes from the TGN, and in the secretion of exosomes from cells (Lakkaraju & Rodriguez-Boulan, 2008; Simons & Raposo, 2009, Thery et al., 2009). MVBs functions extend beyond cargo sorting - they also serve as MHC class II compartments for antigen presentation, T-cell secretory granules and melanosomes in specialised cell types (Raiborg et al., 2003).

Efficient sorting at the early endosome and the MVB compartments typically requires mono- or polyubiquitination of cell surface receptors. The molecular machinery that recognises the ubiquitinated cargo at the early endosome and mediates its sorting into MVBs is a set of interacting protein complexes, the endosomal complexes required for transport (ESCRTs'). The ESCRTs' were first identified in yeast and were initially referred to as class E Vps (vacuolar protein sorting) proteins (Raymond et al., 1992). Characterisation of the 18 class E

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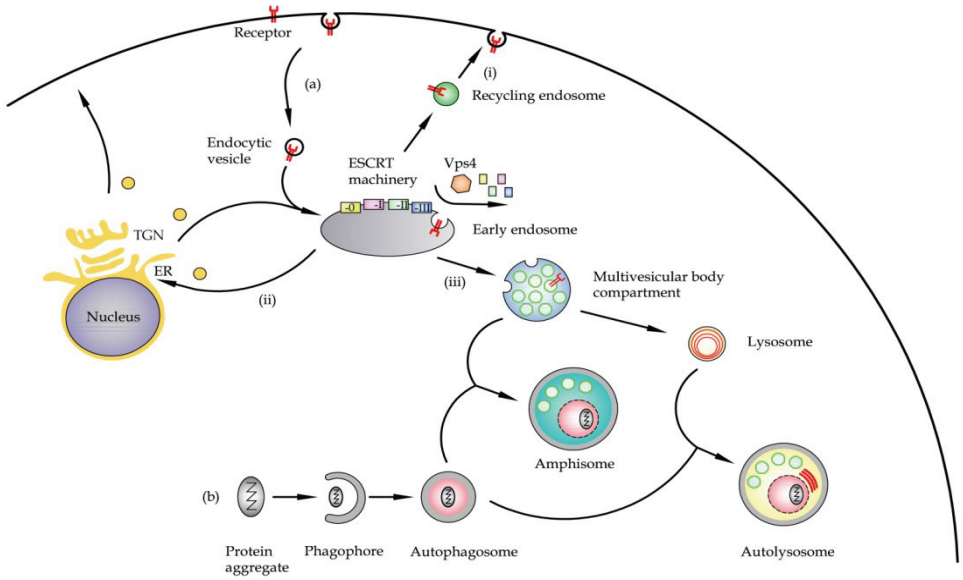

Fig 1. Interrelationships between the endocytic and autophagic pathways.
(a) Receptor-mediated endocytosis involves internalization of plasma membrane cargo into the cell. Endocytosed cargo is first delivered to the early endosome, which also receives cargo from the TGN. From here on, selected cargo can be delivered to three alternative destinations: (i) can be recycled back to the plasma membrane (receptor sequestration), or (ii) sorted into the TGN or (iii) incorporated into ILVs of MVBs. The cargo within the MVB compartment is subsequently transported to the lysosome where the constituents are broken down by lysosomal hydrolases (receptor down-regulation). The MVB biogenesis and the sorting of ubiquitinated cargo is controlled by four ESCRTs', -0, -I, -II -III, and the action of a AAA+ type ATPase Vps4. (b) In contrast to endocytosis, autophagy digests intracellular material by encapsulating damaged organelles or protein aggregates by a phagophore. The resulting autophagosome can fuse directly with the lysosome forming an autolysosome or indirectly via the MVB compartment forming a hybrid organelle, termed an amphisome.

genes revealed that ten of these encode subunits of the ESCRT system, whereas the others encode ESCRT-related proteins or upstream or downstream interactors (reviewed by Slagsvold et al., 2006) (Table 1).

Extensive genetic, biochemical and structural studies using yeast, Drosophila and mammalian model systems have revealed the molecular roles of the ESCRTs'. The ESCRT system consists of four different complexes termed ESCRT-0, -I, -II and –III, and a number of associated proteins such as Vps4 (Babst et al., 2002a, 2002b; Katzmann et al., 2001) (Figure 2). Ubiquitinated endosomal cargo targeted for lysosomal degradation is initially recognised by ESCRT-0. ESCRT-I, -II and -III which are subsequently recruited to the endosomal membrane by protein-protein interactions between the four complexes (reviewed by Roxrud et al., 2010). The ubiquitinated cargo is further concentrated on the

endosomal membrane by the action of ESCRT-I and –II, furthermore invaginations that form from the endosomal membrane to become ILVs depend upon ESCRT-III and Vps4 - facilitated membrane abscission (Elia et al., 2011; Babst et al., 2011). The endocytosed contents in the ILVs are ultimately terminated via lysosomal degradation (Figure 1). Following protein sorting into MVBs, the ATPase Vps4 catalyzes the release of the ESCRT machinery from the limiting membrane of the MVB compartment into the cytosol for further rounds of cargo sorting.

The ESCRTs' also have alternative cellular roles beyond lysosomal trafficking. A subset of ESCRTs' have a well-established function in eukaryotic cell abscission (cytokinesis) (Spitzer et al., 2006; Carlton & Martin-Serrano, 2007; Morita et al., 2007), viral budding (Morita & Sundquist, 2004; Fujii et al., 2007) and autophagy (Filimonenko et al., 2007; Lee et al., 2007). Given their importance in fundamental cellular processes, it is not surprising that ESCRT dysfunction is associated with numerous diseases, including neurodegenerative disorders, cancer and infectious diseases. The dynamics and regulation of the ESCRT machinery have been extensively reviewed (Hurley & Emr, 2006; Saksena et al., 2007; Williams & Urbe, 2007; Raiborg & Stanmark, 2009; Hanson et al., 2009; Carlton & Martin-Serrano, 2009; Hurley, 2010; Roxrud et al., 2010; Henne et al., 2011) and will only be mentioned briefly here. This review focuses on understanding the role of the ESCRTs' in disease using model systems, to better understand the mechanisms behind their role in pathogenesis.

2. Evolutionary conservation of ESCRTs'

Comparative genomic and phylogenetic analysis has revealed in great detail the conservation of the molecular machineries involved in cargo sorting and membrane trafficking. The phylogenetic data has shown that most ESCRT genes emerged early during the evolution of eukaryotes (Slater & Bishop, 2006, Field et al., 2007; Leung et al., 2008, Field & Dacks, 2009). However the ESCRT-III complex and Vps4 have been identified in Archaea, suggesting an even earlier, ancestral function for these components (Lindas et al., 2008; Ghazi-Tabatabai et al., 2009; reviewed by Makarova et al., 2010; Samson et al., 2008, 2011). It has even been suggested a similar mechanism may contribute to bacterial outer membrane vesicle production (Kulp & Kuehn, 2011). All of the other ESCRT complexes with the exception of ESCRT-0, are present across all of the eukaryotic lineages. ESCRT-0 appears to be specific to the opisthokonts (metazoa and fungi) and is absent from *Dictyostelium discoideum*, a member of their sister lineage the Amoebozoa, as well as from plants (Winter & Hauser, 2006; Leung et al., 2008; Field & Dacks, 2009). However *D. discoideum* contains instead a minimal, possibly ancestral ESCRT-0 in which DdTom1 interacts with ubiquitin, clathrin and the ESCRT-1 protein Tsg101 (Blanc et al., 2009). MVBs were also recently identified in the basal amoebozoan *Breviata anathema*, strengthening the conclusion that the ESCRTs' are a common feature of this supergroup (Herman et al., 2011). In mammals and plants several *VpsE* genes such as *Vps37*, *Vps4*, *Vps32, Mvb12* and *Bro1* have undergone gene duplications. The domain structure of VpsE proteins, especially the domains involved in protein-protein and protein-lipid interactions is well conserved across yeast, metazoa and plants (reviewed by Michelet et al., 2010) (Table 1). Collectively, these data suggests that the fundamental structure and the role of the ESCRTs' is well conserved among many eukaryotic organisms.

ESCRT complex and activity	Yeast Protein names	Metazoan Protein names	Domains/Motifs[1]	Biological function
ESCRT-0 *Clusters ubiquitinated cargo*	Vps27	Hrs	VHS, FYVE, UIM (yeast) DUIM (metazoan), PTAP, GAT, coiled-coil core, clathrin binding	Binds PtdIns3P, ubiquitinated cargo, ESCRT-I and clathrin
	Hse1	STAM1, 2	VHS, UIM, SH3, GAT, coiled-coil core, clathrin binding	Binds ubiquitinated cargo and DUB enzymes
ESCRT-I *Membrane deformation and budding*	Vps23	Tsg101	UEV, Pro-rich linker, stalk, headpiece	Cargo and ESCRT-0 (Vps27) interaction, contains the viral PTAP motif
	Vps28	Vps28	Headpiece, C-terminal	Binds ESCRT-II (Vps36)
	Vps37	Vps37A, B, C, D	Basic helix, head piece	Binds membranes
	Mvb12	MVB12A, B	Stalk, ubiquitin binding domain	Stabilizes ESCRT-I subunits, binds ubiquitin
ESCRT-II *Membrane deformation and budding*	Vps22	EAP30, Snf8	Coiled-coil, WH	Binds membranes
	Vps25	EAP20	PPXY, WH	Binds ESCRT-III (Vps20), cargo
	Vps36	EAP45	GLUE, NZF1, 2 (yeast), WH	Binds membranes, ubiquitin and ESCRT-I (Vps28)
ESCRT-III *Membrane scission*	Vps20	CHMP6	Charged, coiled-coil, MIM	Initiates membrane scission
	Vps32/* (Snf7)	CHMP4A, B, C	Charged, coiled-coil, MIM	Membrane scission, binds Bro1 domains
	Vps24	CHMP3	Charged, coiled-coil, MIM	Completes membrane scission
	Vps2/(Did4)	CHMP2A, B	Charged, coiled-coil, MIM	Recruits Vps4; initiates ESCRT disassembly
Vps4 *Disassembly of ESCRTs'*	Vps4	Vps4A,B/(SKD1, 2)	AAA+ ATPase, MIT	ESCRT disassembly and recycling
	Vta1	VTA1/LIP5	MIT, VSL	Positively regulates of Vps4
Other	Vps31/(Bro1)	ALIX/AIP1	Bro1, Proline-rich domain	ESCRT-III interaction by recruiting Snf7; Doa4 recruitment, interacts with apoptosis regulators, contains viral YPXP domains
	Vps60/(Mos10)	CHMP5	Charged, coiled-coil	ESCRT-III like protein, binds Vta1
	Vps46/(Did2)	CHMP1A, B	Charged, coiled-coil	ESCRT-III like protein, recruits Vps4
	Ist1	IST1	MIM1, MIM2	The tandem ESCRT III domains bind SNF7B and the DUB UBPY (USP8)
	Doa4	UBPY/USP8	Rhod, UBP	Removes ubiquitin

[1]**Domain acronyms:** Bro1, Bro1 domain-containing protein 1; CHMP, charged multivesicular body protein; DID, DOA4-independent degradation protein; DUB, deubiquitylating enzyme; DUIM, double-sided ubiquitin-interacting motif; ESCRT, endosomal sorting complex required for transport; GAT, GLUE, GRAM-like ubiquitin-binding in EAP45; Hrs, hepatocyte growth factor-regulated Tyr kinase substrate; Ist1, increased sodium tolerance protein 1; MIM, MIT-interacting motif; MIT, microtubule-interacting and transport; MVB, multivesicular body; NZF, Np14-type zinc finger; SH3, SRC homology 3; UBPY, ubiquitin isopeptidase Y; UEV, ubiquitin E2 variant; UIM, ubiquitin-interacting motif; VHS, Vps27, Hrs and STAM; Vps, vacuolar protein sorting; VSL and VTA1; WH2, winged helix 2. *Alternative names are provided in brackets.

Table 1. Components of the ESCRT machinery.
(Table is modified from Hurley & Hanson, 2010, see cited paper for further details on domain/motif structure)

3. Structure and function of ESCRTs' in normal cells

3.1 Composition of the ESCRT complexes

In order to understand the role of the ESCRTs' in disease, a brief overview of the composition of each complex is provided (Figure 2). ESCRT-0, -I and –II are stable heterotetrameric complexes, while ESCRT-III is formed by polymers formed by four core protein subunits.

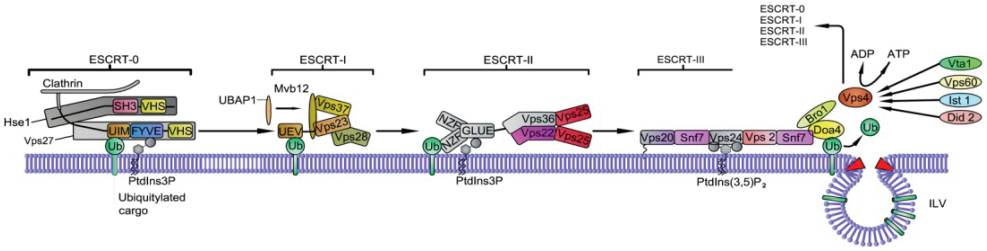

Fig. 2. Composition and molecular interactions of the ESCRTs'.

Interactions between the four ESCRTs' are indicated, as are interactions with ubiquitylated cargo, accessory molecules such as phosphatidylinositol 3-phosphate (PtdIns(3)P), deubiquitylating enzymes (DUBs), Bro1 and the ATPase Vps4. Yeast protein names have been used but the figure above is a composite of data obtained from studies of several model systems containing the ESCRTs'.

3.1.1 ESCRT-0

The ESCRT-0 complex has an early role in MVB biogenesis and in the sorting of ubiquitinated proteins into the MVB pathway. ESCRT-0 binds and clusters ubiquitinated cargo destined for delivery into MVBs, and recruits clathrin and deubiquitinating enzymes (Wollert et al., 2010). The ESCRT-0 complex consists of two subunits, Hrs (Vps27 in yeast) and STAM1/2 (Hse1 in yeast). Hrs contains a FYVE zinc finger domain which binds PtdIns(3)P providing membrane recruitment and endosomal specificity for the ESCRT-0 complex (Mao et al., 2000). Hrs and STAM1/2 bind ubiquitin via their UIM and VHS ubiquitin domains respectively, which are essential for efficient sorting of ubiquitinated proteins (Bishop et al., 2002; Mizuno et al., 2003; Bache et al., 2006). Hrs binds directly to the tumour susceptibility gene–101 product (Tsg101) recruiting ESCRT-I to the endosomal membranes (Bishop et al., 2002) (Figure 2).

3.1.2 ESCRT-I

The ESCRT-I complex along with ESCRT-II is required for further concentrating ubiquitinated cargo on the endosomal membrane and initiating the first stages of membrane invagination (Wollert et al., 2010). Mammalian ESCRT-I is composed of four subunits Tsg101 (Vps23 in yeast), Vps28, Vps37 (four isoforms, A-D) and Mvb12 (two isoforms A/B); the yeast ESCRT-I contains single copies of the four subunits (Chu et al., 2006; Curtiss et al., 2007; Kostelansky et al., 2007; Oestreich et al., 2007) (Figure 2). A novel ESCRT-I component was recently identified in mammalian cells, termed UBAP1. UBAP1 contains a region

conserved in Mvb12 and binds Bro1 proteins involved in cytokinesis (Stefani et al., 2011). The ESCRT-I structure is organised as a headpiece core with flexibly connected modules that mediate interactions with other partners such as ESCRT-0, ubiquitin, Alix (Bro1 in yeast) and ESCRT-II. The Tsg101 subunit can also directly bind Vps20, an ESCRT-III component, surpassing both ESCRT-I and –II (Katzmann et al., 2003; Bilodeau et al., 2003; Pornillos et al., 2003).

3.1.3 ESCRT-II

The ESCRT-II complex is recruited to the endosomal membrane by the interaction between the ESCRT-I subunit Vps28 and the ESCRT-II subunit Vps36 (Saksena et al., 2009) (Figure 2). The ESCRT-II complex is a heterotetramer with one copy of Vps22 and Vps36 and two copies of Vps25 (Hierro et al., 2004; Im & Hurley, 2008; Teis et al., 2010). Mammalian Vps36 binds PtdIns(3)P and ubiquitin via the GLUE domain and is important for efficient cargo sorting (Teo et al., 2006). The yeast Vps36 contains a GLUE domain with two NZF insertions. NZF1 binds to ESCRT-I (Gill et al., 2007) and NZF2 binds to ubiquitinated cargo (Alam et al., 2004). The C-terminal domain of Vps25 provides a direct link to ESCRT-III by binding to CHMP6 (Vps20).

3.1.4 ESCRT-III

The ESCRT-III complex plays an important role in membrane scission and is responsible for pinching off the neck of the invagination, forming an ILV (Wollert et al, 2009, Wollert & Hurley, 2010) (Figure 2). Mammalian ESCRT-III consists of multiple subunits, CHMP2 (two isoforms A/B,) (in yeast Vps2), CHMP3 (in yeast Vps24), CHMP4 (four isoforms A-D) (in yeast Snf7), and CHMP6 (in yeast Vps20) (Babst et al, 2002a; Bajorek et al., 2009b). The other ESCRT-III subunits CHMP1 (two isoforms A/B), (in yeast Did2), CHMP5 (in yeast Vps60) and Ist1 are not strictly essential for function and appear to assemble with the rest of the ESCRT-III subunits at a later stage. Did2 and Vps60 recruit and activate Vps4, while Ist1 inhibits Vps4 activity (Nickerson et al., 2006; Dimaano et al., 2008). Vps4 is an AAA-ATPase, which has an important role in catalysing and energizing the dissociation of the ESCRT machinery form the endosomal membrane back to the cytosol, for further rounds of cargo sorting. The ESCRT-III complex does not bind ubiquitin, however it recruits Alix, which plays a key role in the endosomal recruitment of Doa4, a deubiquitinating enzyme (Babst et al., 1997; 1998; Scott et al., 2005; Muziol et al., 2006; Shim et al., 2007; Yu et al., 2008; Teis et al., 2008; Lata et al., 2008; Ghazi-Tabatabai et al., 2009).

3.2 Biological roles of the ESCRTs'

3.2.1 Cytokinesis

In eukaryotes, cytokinesis consists of at least three key steps: (i) assembly of the central spindle, (ii) formation of the cleavage furrow, (iii) and membrane abscission at the midbody (Yang et al., 2008; reviewed by Saksena & Emr, 2009). The membrane scission and the creation of the membrane curvature required in cytokinesis is topologically similar to the curvature needed during MVB sorting and viral budding. Studies have shown that components of ESCRTs' are required for membrane abscission, the final step of cytokinesis. For instance, ESCRT-III is specifically recruited to the midbody to mediate membrane fission

and Vps4 is important in the release of ESCRT-III in cytokinesis (Spitzer et al., 2006; Obita et al., 2007; Carlton & Martin-Serrano, 2007). Furthermore, depletion of either Ist1 and Did2 (ESCRT-III and Vps4 human homologues) leads to an arrest in cytokinesis (Agromayor et al., 2009; Bajorek et al., 2009a). Additionally, the ESCRT-I subunit Tsg101 and the ESCRT-III associated protein Alix were found to competitively associate with Cep55 (a multimeric cell division protein essential for late stage cell division) to facilitate recruitment of ESCRT-III and Vps4 for abscission of the two daughter cells (Carlton & Martin-Serrano, 2007; Morita et al., 2007). The role of ESCRT-II in cytokinesis is unclear, although studies conducted by Langelier et al., 2006 indicate that Vps22 of ESCRT-II is located on the centrosomes and is involved in the maturation of these organelles. The mechanisms behind ESCRT mediated scission and their role in microtubule disassembly have been recently reviewed in detail by Henne et al., 2011 and Roxrud et al., 2010 and will not be further discussed in this review.

3.2.2 Autophagy

In the mammalian system there are two pathways that intersect with the lysosome, the MVB pathway as described in the introduction and the autophagy pathway. To date, three autophagy pathways have been described in higher eukaryotes: microautophagy (MA), chaperone-mediated autophagy (CMA) and macroautophagy (Mizushima et al., 2008; Cuervo, 2010). Microautophagy was originally described in yeast, but is not yet well characterised in other eukaryotes (Marzella et al., 1981). In this pathway, the lysosome invaginates and internalizes cytosolic components, which are subsequently degraded in the lumen of the lysosome. Chaperone-mediated autophagy is a more selective autophagy that does not involve vesicle formation but rather a direct translocation of a specific set of proteins across the lysosomal membrane. The cytosolic chaperone hsc70, a major component of the CMA pathway recognises the pentapeptide 'KFERQ' sequence in proteins destined for lysosomal degradation (Sahu et al., 2011). The lysosome-associated protein type 2A (LAMP2A) binds and translocates the KFERQ proteins to the lysosome, through a yet-unclear-mechanism (Orenstein & Cuervo, 2010; reviewed by Shpilka & Elazar, 2011). A recent study has identified a new macroautophagy-like degradation pathway that is distinct from CMA and occurs in lysosomes (Orenstein & Cuervo, 2010). Endosomal microautophagy was shown by Sahu et al., 2011 to occur during MVB formation and requires both ESCRT-I and –III, as well as hsc70 for delivery of KFERQ proteins from the cytosol into MVBs. This study provided fresh insights into the mechanisms of autophagy in mammalian model systems and also extended the role of ESCRTs' to degradation of cytosolic compartments. The role of the ESCRTs' is best characterized in macroautophagy and this will be the focus here.

Macroautophagy (henceforth simply referred to as autophagy) is a bulk degradation pathway responsible for the removal of damaged organelles and for clearance of protein aggregates (reviewed by Mehrpour et al., 2010). The fundamental molecular mechanisms of the autophagy pathway have been extensively studied in yeast, using genetic screening to identify autophagy genes (*atg)* (Klionsky et al., 2003). Subsequent inactivation of *atg* orthologues in higher eukaryotes has shown that the autophagic machinery is highly conserved. The autophagic pathway involves multiple steps: (i) sequestration of cytoplasmic constituents by a double membrane phagophore, resulting in the formation of an autophagosome and (ii) direct fusion of autophagosomes with the lysosome, where the

cytoplasmic material is degraded in the resulting autolysosome or alternatively (iii) fusion of the autophagosome with the MVB compartment, forming a hybrid component termed an amphisome, which then fuses with the lysosome (Lawrence & Brown, 1992; Berg et al., 1998; Liou et al., 1997) (Figure 1).

Many age-related neurodegenerative disorders are characterised by an accumulation of ubiquitin-positive aggregates in affected brain regions. Autophagy is necessary for the clearance of these proteins, as aggregates essentially become toxic for postmitotic cells like neurons (reviewed by Eskelinen & Saftig, 2009). Defects in the autophagic pathway are associated with neurodegenerative diseases such as Alzheimer's, Huntington's and Parkinson's diseases. For instance, in Alzheimer's disease (AD) neuronal autophagy is activated in the early stages, however autophagic degradation becomes impaired as the disease progresses (Boland et al., 2008). Similarly in Huntington's disease (HD), active autophagy helps in the clearance of toxic polyglutamine-containing proteins (Ravikumar et al., 2004). In Parkinson's disease (PD) mutant α-synuclein blocks its own degradation via the chaperone-mediated autophagy pathway resulting in a gain-of-function neurotoxicity (Cuervo et al., 2004).

Studies conducted using slime moulds, nematodes, flies and mammals as model systems to study neurodegenerative disease have revealed that the ESCRT machinery plays a role in autophagy. Genetic disruption of ESCRT-I, -II and -III in mammalian and *Drosophila* cells leads to an increase in autophagosomes and toxic protein aggregates increase the severity of HD (Lee et al., 2009). Similarly, in rodent cortical neurons, loss of the CHMP2B subunit leads to an accumulation of autophagosomes (Lee et al,. 2007). Autophagosome and amphisome accumulation was also observed in HeLa cells when Tsg101 and CHMP3/Vps24 were knocked down or CHMP2B was disrupted (Lee et al., 2007). Consistent with the above data, downregulation of Vps4 in HeLa cells resulted in autophagosome accumulation, impaired degradation of autophagy substrates and impaired delivery of endosomal constituents to autophagosomes (Nara et al., 2002). The observed increase in autophagosomes suggests that there is either an enhanced initiation of autophagy in the cell or a decreased autophagic flux. The ESCRT machinery is therefore predicted to be involved in one or more key stages of the autophagic pathway. The possibilities include: (i) ESCRTs' are involved in signalling pathways that induce autophagy, (ii) ESCRTs' are required for phagophore closure or (iii) ESCRTs' are involved in the fusion of autophagosomes with the lysosome and/or the fusion of the autophagosomes with the MVB (reviewed by Rusten & Stenmark, 2009).

To date, little is known about the underlying mechanisms allowing the ESCRTs' to mediate fusion of autophagosomes with the MVB compartment and lysosomes. It has been shown that tethering of lysosomes to endosomes and autophagosomes is mediated by Rab7 (Bucci et al., 2000, Gutierrez et al., 2004; Jager et al., 2004) and the HOPS complex, which brings the membranes in close proximity (Wurmser et al., 2000; Seals et al., 2000; reviewed by Metcalf & Isaacs, 2010). ESCRT proteins interact directly with the HOPS complex which binds Rab7, as determined by a recent study which revealed that mutant CHMP2B (an ESCRT-III subunit) leads to impaired recruitment of Rab7 (Urwin et al., 2010). This suggests that functional ESCRTs' are required either for recruiting the vesicular fusion machinery to the MVB compartment or for delivery of the fusion machinery to lysosomes or autophagosomes. A number of other proteins are also implicated in autophagosome fusion

with endosomes/lysosomes including UVRAG, Rubicon and LAMP-2. It is not yet known whether the ESCRT machinery has an effect on these proteins and processes.

3.2.3 Downregulation of receptor-mediated signaling

Receptor tyrosine kinases (RTKs) are growth factor receptors that play a important regulatory roles in controlling cell growth, proliferation, differentiation, survival and metabolism in several tissues and organs (Hunter, 2000; Pawson et al., 2001). Dysfunction of RTKs or mutations in key components of their downstream signaling pathways results in a variety of diseases, such as cancer, diabetes, immune deficiencies and cardiovascular disorders (Blume-Jensen & Hunter, 2001). EGFR is one of the best studied RTKs, and its uncontrolled signaling is associated with the development of a number of human cancers, including mammary carcinomas, squamous carcinomas and glioblastomas (Hunter, 2000; Pawson et al., 2001). The multivesicular body pathway silences RTK signaling via lysosome sequestration and degradation and thus plays an important role in modulating the amplitude and kinetics of amide signaling pathways from activated receptors (Saksena et al., 2007; Hurley & Emr, 2006; Williams & Urbe, 2007). Defects in ESCRT-mediated sorting of these receptors to lysosomal degradation pathways can thus lead to sustained receptor signaling either because of prolonged residence and activity in the endosomal membrane or as a result of increased recycling of the receptors to the plasma membrane.

Drosophila studies have shown that EGFR degradation is impaired and signalling is prolonged by dysfunctional ESCRT-0 (Hrs) (Lloyd et al., 2002), ESCRT-I (Tsg101) (Vaccari & Bilder, 2005) or ESCRT-II (Vps25) (Thompson et al., 2005). In mammals, depleting Tsg101 causes sustained EGFR signaling (Bache et al., 2006), whereas depletion of CHMP3 (ESCRT-III) (Bache et al., 2006) or Eap30 (ESCRT-II) (Malerod et al., 2007) causes delayed EGFR degradation but not sustained signaling (Table 2). Sustained signaling observed in ESCRT-0, -I and -II *Drosophila* mutants and after ESCRT-I depletion in mammals may result from increases in the residence time of receptors in the endosomal membrane and their recycling back to the plasma membrane. Mutations in ESCRT-III subunits do not cause sustained signaling (Bache et al., 2006), possibly because ESCRT–III recruitment occurs after signal termination. This may also explain why ESCRT-III subunits so far have not been implicated in cancer.

The Notch signaling pathway is highly conserved from *Drosophila* to humans and plays a central role in the normal development of many tissues and cell types. It controls various effects on differentiation, survival, and/or proliferation that are highly dependent on signal strength and cellular context. Dysfunction of the Notch signaling pathway leads to many human diseases such as lung and skin cancer (Radtke & Raj, 2003; Allenspach et al., 2002). Studies in *Drosophila* have shown that Notch signaling is terminated via lysosomal degradation suggesting a role for the ESCRT machinery in the regulation of Notch. In *Drosophila,* depletion of Hrs or mutation of Tsg101 or Vps25 leads to an accumulation of the cell-surface receptors Notch, Delta, Thickveins and EGFR (Thompson et al., 2005; Vaccari & Bilder, 2005; Moberg el al., 2005). Notch accumulation stimulates cell proliferation in the eye disc (Chao et al., 2004, Tsai & Sun, 2004) and results in overgrowth phenotypes in surrounding wild-type cells via the JAK/STAT pathway. Furthermore, inactivation of Tsg101 or Vps5 in *Drosophila* results in loss of epithelial cell polarity, which is associated with malignant transformation, suggesting that ESCRT components have a role in

organizing the actin and/or microtubule cytoskeleton (Thompson et al., 2005; Vaccari & Bilder, 2005; Moberg el al., 2005; Saksana & Emr, 2009). In summary, there is growing evidence that implicates functional ESCRTs' in suppressing malignant transformation and preventing cancer.

4. The roles of ESCRTs' in disease

4.1 Neurodegenerative diseases

The most direct evidence that ESCRT dysfunction causes neurodegenerative disease comes from the identification of autosomal dominant *CHMP2B* mutations found to cause a rare form of frontotemporal dementia (FTD3) (Skibinski et al., 2005) and amyotrophic lateral sclerosis (ALS) (Parkinson et al., 2006). FTD is the second most common form of early-onset dementia after Alzheimer's disease (Ratnavalli et al., 2002; Harvey et al., 2003) and is characterised by the presence of either tau neurofibrillary tangles or ubiquitin deposits. FTD with the presence of tau or ubiquitin pathology is termed FTLD-U (frontotemporal lobar degeneration with ubiquitin-immunoreactive inclusions) (Neary et al., 2005). Both FTLD-U and ALS are characterised by abnormal accumulation of ubiquitin-positive protein deposits (including TDP-43) that contain p62, tau and α-synuclein-negative neuronal cytoplasmic inclusions (Arai et al., 2006; Neumann et al., 2006). The adapter protein p62 is commonly found in protein inclusions associated with neurodegenerative disease (Talbot & Ansorge, 2006), it binds polyubiquitin (Vadlamudi et al., 1996) and interacts with the autophagic associated protein Atg8/LC3 (Bjorkoy et al., 2005; Pankiv et al., 2007). Collectively, these data implicate p62 as a link between protein accumulation and aggregation with autophagy-mediated clearance (reviewed by Saksena & Emr, 2009). Similarly, ESCRT-depleted cells and cells overexpressing CHMP2 in flies, mice and humans, showed impaired autophagic degradation leading to an accumulation of autophagosomes and protein aggregates containing p62, thereby contributing to the pathogenesis of FTD3. A recent study has shown that deletion of the ESCRT proteins Tsg101 and Vps24 resulted in accumulation of TDP-43, suggesting that impaired MVB function could have a role in TDP-43 aggregate formation in FTLD-U and ALS (Filimonenko et al., 2007). Furthermore, Vps24 was found to be essential in the clearance of expanded polyglutamine aggregates associated with Huntington's disease (Table 2) (Filimonenko et al., 2007). Collectively, these data suggest that efficient autophagic degradation requires functional ESCRTs' and dysfunction of this machinery is associated with neurodegenerative phenotypes and disorders.

Several indirect links also implicate the ESCRTs' in various neurodegenerative disorders, and several ESCRT-interacting proteins are products of genes that are associated with inherited forms of neurodegeneration (reviewed by Stuffers et al., 2009a). For instance, in mice, a null mutation in Mahoganin, an E3 ubiquitin ligase that ubiquitinates Tsg101, causes spongiform neurodegeneration, a recessively transmitted prion-like disease (Kim, et al., 2007; Jiao et al., 2009). Two putative ESCRT-III interacting proteins, spartin and spastin are mutated in spastic paraplegia, an inherited neurodegenerative disease that paralyzes the lower limbs (Reid et al., 2005). The exact mechanism of CHMP4 contribution to this disease remains unclear and requires further investigation. Finally, Niemann-Pick disease type C is an inherited neurodegenerative disorder characterized by a disruption of lipid trafficking and is caused by a mutation in either of the two genes, *npc1* and *npc2* (reviewed by Eskelinen & Saftig, 2009). A dominant-negative mutant of Vps4 was found to cause an accumulation of ubiquitinated

NPC1 (Ohsaki et al., 2006). Together, these data indicate that dysregulation of ESCRT pathways may contribute to a broad spectrum of degenerative diseases.

4.2 Cancer

The first hint that ESCRTs' play a role in cancer came from the identification of *Tsg101* and *Vps37A* as tumour suppressor genes on the basis that they map to chromosomal regions deleted or mutated in cancer (Li & Cohen, 1996; Xu et al., 2003). Genomic deletions and splice variants of *Tsg101* were found in sporadic forms of breast cancer (Li et al., 1997) and other malignancies such as myeloid leukaemia and prostate cancer (Table 2) (Sun et al., 1997; Lin et al., 1998). In addition, Vps37A expression in hepatocellular carcinomas was found to be dramatically reduced or undetected suggesting that Vps37A may be a potential tumour suppressor (Xu et al., 2003). Similar results were observed with CHMP1A, as overexpression of this protein inhibited cell growth and tumour formation in human pancreatic tumor cells (Li et al., 2009).

Mutations that prevent c-Cbl-mediated ubiquitination of EGFRs and thereby inhibit ESCRT-mediated receptor down-regulation are associated with a number of cancers, particularly acute myeloid leukemia. For example, a mutant EGFR lacking only the direct c-Cbl-binding site transduces stronger mitogenic signals when compared to the wild-type receptor (Waterman et al., 2002; Saksena & Emr, 2009). The c-Met RTK (also known as HGFR) regulates invasive growth and is critical for normal development and wound repair. Its overexpression causes uncontrolled proliferation and growth and consequently is associated with a variety of human cancers (Haddad et al., 2001). In part c-Cbl-mediated ubiquitination controls cellular c-Met levels and therefore ubiquitination and functional ESCRTs' are needed to avoid c-Met-related malignant transformation (Peschard et al., 2001).

Collectively, the foregoing studies indicate that the ESCRTs' have a negative regulatory role in growth receptor signaling, however several independent studies have shown that ESCRTs' also have a positive role in growth factor signaling. For instance, Tsg101 was recently found to be overexpressed, rather then reduced in breast, thyroid, ovarian and colon cancer (Ma et al., 2008). Furthermore, depletion of Tsg101 prevented tumorigenicity in several cancer lines (Zhu et al., 2004). To further support ESCRTs' positive role in oncogenic signaling, the ESCRT-0 component Hrs was found to be essential for cell proliferation and tumorigenesis in both HeLa and mouse fibroblast cells (Toyoshima et al., 2007).

A positive regulatory role in growth factor signalling for the ESCRTs' has also been observed in *Drosophila melanogaster* (Vaccari et al., 2005; Thompson et al., 2005; Moberg et al., 2005; Vaccari et al., 2009; Herz et al., 2006; Rodahl et al., 2009). For example, *Tsg101* is essential for normal cell growth and cell survival in the fruit fly and clonal loss of this gene in epithelial cells causes hyperplasia of surrounding tissue despite the mutant cells dying via apoptosis (Moberg et al., 2005; reviewed by Stuffers et al., 2009a). Loss of Vps25 causes a similar effect, whereas loss of Hrs is without effect (Vaccari & Bilder, 2005; Thompson et al., 2005). It is important to note that the proapoptotic signaling pathways Hippo, JNK and Hid are activated in the Vps25 *Drosophila* mutants. Expression of the caspase inhibitor p35 in the Vps25 mutant cells restores cell growth and even results in overgrowth, suggesting that mutations in both the ESCRT pathway and the apoptotic pathway are required for overgrowth. Blocking apoptosis by expressing *Ark* (an essential component of the apoptotic

pathway) or *Diap1* (*Drosophila* inhibitor of apoptosis protein 1), again results in overgrowth of the Vps25 mutant tissue. Collectively, these results suggest that the ESCRTs' in *Drosophila* do not act as conventional tumor suppressors.

Overall, the ESCRTs' have been implicated in both positive and negative roles in growth factor receptor signaling and cancer, suggesting that the exact role of the ESCRTs' in tumourigenesis may be cell-type and context-dependent. Alternatively, ESCRT-mediated actions in controlling cell proliferation may reflect diverse endosomal sorting roles on a broad range of molecular targets with many different roles in cellular homeostasis (reviewed by Lobert & Stenmark, 2011). Further research needs to be conducted using different model systems to better understand the complex roles of the ESCRTs' in signaling and cell proliferation. More specifically, future studies need to address whether ESCRTs' act as genuine tumour suppressors in mammals, since at this stage this is still unclear.

4.3 Infectious diseases

4.3.1 Microbial infections

The endocytic and autophagic pathways play an important role in innate immunity. Multiple studies have now shown that these host cell pathways can be manipulated by viruses and microorganisms in order to facilitate infection (von Schwedler et al., 2003; Vieira et al., 2004; Philips et al., 2008; Morita & Sundquist, 2004; Martin-Serrano & Marsh, 2007; McCullough et al., 2008). ESCRTs' play an important role in degenerative endosomal trafficking, so it is not surprising that they are involved in killing many microorganisms. For example, functional ESCRTs' have been shown to restrict mycobacterial growth and infection (Philips et al., 2008). Mycobacteria may invade macrophages and are able to survive and replicate intracellularly due to their ability to prevent fusion of bacteria-containing phagosomes with lysosomes. In both the *Drosophila* model system, and in mammalian macrophages, mutation of ESCRTs' renders cells susceptible to mycobacterial infections. Similarly, overexpression of Vps4 in the host cell results in deficient differentiation and virulence of the intracellular protozoal pathogen *Leishmania major (*Table 2) (Vieira et al., 2004; Philips et al., 2008). Furthermore, autophagosome accumulation was also observed, and both functional endosomal and autophagic pathways are required for optimal *L. major* virulence and infection (Besteiro et al., 2006). The mechanisms by which ESCRTs' mediate resistance to microbial infection have not been defined. It is possible that ESCRTs' are required for the delivery of the pathogen to the lysosome, more specifically having a role in phagosome maturation and fusion between the phagosome and lysosome. Like the involvement of the ESCRTs' in the autophagic pathway these results suggest that the ESCRTs' affect multiple cellular trafficking events. The finding that ESCRT components restrict the growth of intracellular microbial pathogens means that they can now be considered as therapeutic targets for treatment of these infections which cause millions of deaths every year.

In the case of eukaryotic pathogens, the ESCRTs' of the pathogen may also play important roles in virulence. *Candida albicans* causes opportunistic fungal infections and its ESCRT proteins have multiple roles in pathogenesis. The fungal ESCRT components are suggested to contribute to diverse fungal functions including cell signaling, nutrient acquisition and possibly cell wall architecture (Cornet et al., 2005; Wolf et al., 2010). However the role of ESCRTs' in candidiasis is not yet fully understood.

Complex	Component	Dysfunction/disease	Pathogenesis	Model systems
Cancer				
ESCRT-0	Hrs (Vps27)	Tumourigenesis and metastatic potential	Hrs depletion is associated with the upregulation of E-cadherin and reduced β-catenin signalling[1]	Human cancer cells, MEF, mice
ESCRT-0 associated	Hrs (Vps27)	Benign brain tumours (e.g. Schwannomas, meningiomas, ependymomas)	Interaction with neurofibromatosis 2 tumour suppressor protein schwannomin/merlin, regulating STAT signalling[13, 14]	Human cancer cells, rat cells
ESCRT-I	Vps37A	Hepatocellular ca. (HCC) and metastasis	Growth inhibitory protein, suppressing proliferation, transformation and invasion; strongly reduced levels in HCC[2]	Human tissue and cancer cells
	Tsg101 (Vps23)	Ovarian cancer	Up regulation of Tsg101: suppression of p21 expression and posttranslational regulation through MAPK signalling[3, 4]	Human tissue and cancer cells
	Tsg101 (Vps23)	Mammary cancer	Overexpression of Tsg101: increased signalling through MAPK[5]	Human tissue, transgenic mice
	Tsg101 (Vps23)	Papillary thyroid cancer, gastrointestinal stromal tumours	Overexpression of Tsg101 (consequences not known)[6, 7]	Human tissue
ESCRT-I/II	Erupted Tsg101/Vps25	Neoplastic transformation (ovary and imaginal discs), over-proliferation of adjacent WT cells	Enhanced Notch and growth factor signalling in mutant cells[8, 9, 10]	*Drosophila*
ESCRT-III	CHMP3 (Vps24)	Prostate cancer	CHMP3 induces neuroendocrine cell differentiation[11]	Human cells
	CHMP3 (Vps24)	Non-small cell lung cancer	CHMP3 has a functional role in neuroendocrine cell differentiation[12]	Human cancer cells
ESCRT-III associated	CHMP1A	Ductal pancreatic cancer	Tumour suppressor, regulating tumour growth potentially through p53 signalling pathway[15]	Human cells, mice
Neurodegenerative diseases				
ESCRT-I/III	Tsg101 (Vps23) / CHMP3 (Vps24) / CHMP2B	Neurodegeneration (FTLD-U, ALS, Huntington's disease (HD))	Reduced autophagic degradation, accumulation of Ub-protein aggregates containing TDP-43; reduced clearance of Huntington-positive inclusions[18]	Human cells, mouse cells
ESCRT-I associated	Tsg101 (Vps23)	Spongiform neurodegeneration (hallmark of prion disease)	E3 ubiquitin-protein ligase Mahogunin ubiquitinates Tsg101; depletion of Mahogunin disrupts endosomal trafficking[21]	Human cells, rat tissue
ESCRT-I associated	Tsg101 (Vps23)	Charcot-Marie-Tooth disease (CMT1C)	Interaction with SIMPLE; SIMPLE plays a role in the lysosomal sorting of plasma membrane proteins[22]	
ESCRT-III	CHMP2B (Vps2)	FTLD-U and ALS	Disruption of endosomal trafficking, protein accumulation[16, 17]	Human cells
	CHMP4B (Snf7-2) / CHMP2B	Neurodegeneration (FTLD-U, ALS)	Accumulation of autophagosomes; failure of mutant CHMP2B to dissociate properly leading to dysfunctional ESCRT-III on late endosomes[20]	*Drosophila*, mice
ESCRT-III associated	CHMP1B	Hereditary spastic	Interaction with spastin; spastin	Monkey cells

Table 2. ESCRT-associated diseases in various model systems
(Modified from Stuffers et al., 2009a)

References: [1]Toyoshima et al., 2007; [2]Xu et al., 2003; [3]Young et al., 2007; [4]Young et al., 2007; [5]Oh et al., 2007; [6]Liu et al., 2002; [7]Koon et al., 2004; [8]Moberg et al., 2005; [9]Vaccari & Bilder et al., 2005; [10]Thompson et al., 2005; [11]Wilson et al., 2001; [12]Walker et al., 2006; [13]Gutmann et al., 2001; [14]Scoles et al., 2002; [15]Li et al., 2008; [16]Parkinson et al., 2006; [17] Skibinski et al., 2005; [18]Filimonenko et al., 2007; [19]Rusten et al., 2007; [20]Lee et al., 2007; [21] Kim et al., 2007; [22] Shirk et al., 2005; [23]Reid et al., 2005; [24]Vieira et al., 2004; [25]Cornet et al., 2005; [26]Wolf et al., 2011; [27]Babst et al., 1998; [28]Spitzer et al., 2006; [29]Besteiro et al., 2006; [30]Shiels et al., 2007.

4.3.2 Viral infections

The beneficial role of ESCRTs' in protecting against intracellular bacteria is reversed in viral infections. Many membrane-enveloped viruses hijack the ESCRT machinery to bud out of host cells. Retroviruses (HIV-1), filoviruses (Ebola virus), rhabdoviruses and arenaviruses encode short sequence motifs termed L-domains (late domains) within their structural (Gag) polyproteins that are essential for the release of assembled viruses from the host cells (reviewed by Carlton & Martin-Serrano, 2009; Stuffers et al., 2009b). The P(S/T)AP motif found on the HIV-1 Gag protein for example binds directly to the UEV domain of Tsg101 of ESCRT-I. Even though HIV-1 budding is normally ESCRT-I dependent, if Tsg101 is unavailable, the virus alternatively binds to Alix via the YPxL domain and buds (Stark et al., 2003). Both ESCRT-I and Alix can independently recruit ESCRT-III, which together with Vps4 are required for efficient virus budding. Recent studies have shown that ESCRT-III and Vps4 can be recruited independently of either Tsg101 or Alix by the herpes simplex virus type-1 (Pawliczek & Crump et al., 2009) and the hepatitis C virus (Corless et al., 2010). ESCRT-II was found not to be essential for HIV- budding (Langelier et al., 2006), however ESCRT-II was discovered recently to be essential for release of the avian sarcoma virus (Pincetic et al., 2008). Other viruses such as the rabies virus can indirectly recruit the ESCRTs' by using the PPxY motif to specifically recruit WW-domain-containing E3 ubiquitin ligases of the Nedd4 family (Kikonyogo et al., 2001). Disruption of ESCRT function by RNA interference or dominant-negative Vps4 arrests viral release at the plasma membrane (Garrus et al., 2001; Martin-Serrano & Neil, 2011; Demirov et al., 2002; Strack et al., 2003; reviewed by Carlton & Martin-Serrano, 2009). Collectively, this data confirms that different enveloped viruses require specific proteins for budding and that the ESCRT machinery regulates viral release from the plasma membrane.

5. Conclusions

The ESCRT machinery is ubiquitous in eukaryotes and has been highly conserved in evolution due to its vital functions including endocytosis, cytokinesis and autophagy. Our understanding of the ESCRTs'' roles in endocytosis, receptor downregulation, membrane deformation and scission has made great progress over the past few years and the study of various model systems has contributed significantly to this. We know that the ESCRTs', in particular ESCRT-III and Vps4 have an intrinsic budding and scission activity that is focused on the neck of the ILVs and that they are important regulators of cytokinesis (Spitzer et al., 2006; Obita et al., 2007; Carlton & Martin-Serrano, 2007). Model systems have implicated the ESCRTs' in autophagic fusion events and in endosome-lysosome degradation. Impaired function of these pathways causes various neurodegenerative disorders, cancers and is implicated in microbial infections. Genetic disruption of ESCRT-I, -II and –III in mammalian and *Drosophila* systems has been shown to result in an accumulation of autophagosomes and toxic aggregates which accelerates neurodegeneration (Lee et al., 2007). Mutations in the ESCRT-III subunit CHMP2B, have been shown to cause FTD3 (Skibinski et al., 2005) and ALS (Parkinson et al., 2006). Furthermore, the ESCRTs' and their associated proteins are also indirectly implicated in causing spongiform neurodegeneration (Kim, et al., 2007; Jiao et al., 2009), spastic paraplegia (Reid et al., 2005) and Niemann-Pick type C neurodegeneration (Ohsaki et al., 2006). Sustained receptor signaling is a key event in carcinogenesis, and Tsg101 (Li et al., 1997, Sun et al., 1997; Lin et

al., 1998), Vps37A (Xu et al., 2003) and CHMP1A (Li et al., 2009) have been identified as potential tumor suppressors. However several other subsequent studies found Tsg101 to play a role in cell cycle control, a conclusion that is in contradiction to the tumor suppressor properties of Tsg101 (Zhu et al., 2004). In *Drosophila* ESCRT-I and -II were found to behave as tumor suppressors (Li & Cohen, 1996; Xu et al., 2003; Li et al., 2008). Tissues expressing mutant ESCRT-I or -II were found to form tumors that are largely attributable to the cell non-automous stimulation of proliferation caused by excessive cytokine production by the mutant cells. This is triggered by overactive Notch signaling from endosomes, signifying that the ESCRT machinery is crucial for silencing Notch signaling and thereby for tumor suppression in flies. It has not yet been clarified whether this is the case in mammals. The ESCRTs' were found to have a beneficial role in innate immunity by restricting microbial growth and infection (von Schwedler et al., 2003; Vieira et al., 2004; Philips et al., 2008; Morita & Sundquist, 2004; Martin-Serrano & Marsh, 2007; McCullough et al., 2008). The ESCRTs' however, are turned against the host in viral infections. Several viruses, such as HIV-1 use the ESCRT components to bud out cells and cause infection (reviewed by Carlton & Martin-Serrano, 2009; Stuffers et al., 2009b). Further dissection of the roles of the ESCRTs' in these events will shed light on the basic mechanism of vesicular traffic and provide new insights into disease pathogenesis and preventative and therapeutic strategies.

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21

Autologous Grafts of Mesenchymal Stem Cells – Between Dream and Reality

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1. Introduction

During the last decade, the characterization of Adult Stem Cells (ASC) incited extraordinary infatuation for the development of autologous cellular therapy. The number of directed cellular differentiation essays of hematopoietic and mesenchymal stem cells demonstrated, against the classical rules of embryology, unsuspected capacities to generate *ex vivo* practically all cellular types. Nevertheless, the difficulties of revealing these spectacular capacities during clinical applications of tissue reparation suggest a random evolution of cell cultures. The major influence on this reality may play fragmentary knowledge of transductional mechanisms controlling the cellular fate and above all, the quality of isolated cells. This last condition seems to represent the one of essential technical barriers. In fact, the process of cellular isolation principally residing in the employment of cellular adherence and/or magnetic field able to retain cells marked by tagged antibodies. Unfortunately, the proteins recognized by antibodies are not expressed by sole stem cells but also by committed progenitors. In fact, the bone marrow precursors represent very heterogenic population of mononuclear cells whose affiliation seemed to be recently questioned. For instance, the antibody against CD34 protein is employed for isolation of Hematopoietic Stem Cells (HSC) while the absence of CD45 (universal hematopoietic cell marker) is recognized as sufficient to qualify Mesenchymal Stem Cells (MSC). Consequently, if previous observations revealed promising potential of bone marrow stem cells to be used for development of cellular therapy, their utilization must be preceded by detailed studies of their biology with particular focus on specific markers and transductional pathways permitting a high purity of isolation and control of differentiation protocols. The proposed chapter is based on our recent work indicating the heterogeneity of mesenchymal stem cells isolated from rabbit bone marrow which, placed in the context of recent studies, allows to propose a novel hierarchic organization of bone marrow cells. As *ex vivo* differentiation of stem cells would be dependent on transductional mechanisms we also propose to discuss how pharmacological modulation of activity of molecular target implicated in calcium homeostasis may influence cellular differentiation.

2. Heterogeneity of MSC

2.1 Introduction

Besides tissues having properties of self-renewal such bone marrow, the liver represents in Man the sole internal organ endowed with a spectacular capacity of regeneration illustrated already by the ancient myth of Prometheus. Interestingly, this process intervenes only after physical damage of the hepatic parenchyma which, destabilizing the entirety of the extracellular matrix, highlights the crucial role of epigenetic modulation on the proliferation and cellular differentiation (Michalopoulos & DeFrances, 1997; for review). Even if this natural phenomenon does not seem to be reproduced in internal organs, the recent isolation of adult multipotent, dormant within the various organs and tissues, stem cells seemed open the way towards a Regenerating Cellular Therapy. Indeed, the bibliographical data indicate a great plasticity of stem cells and in particular those taking from bone marrow like hematopoietic stem cells (HSC) and mesenchymal stem cells (MSC). Certain reports, already conclusive in the rat and the mouse, indicate a possibility of directed in situ and in vitro differentiation of stem cells and open exciting therapeutic prospects for tissue and various organs repair, without exposing the host to the failure of an allogeneic transplant rejection. Thus, contrary to embryonic stem cells (ESC) whose clinical application is still not unanimously accepted, ASC initially appeared as an ideal solution to prepare various autologous graft. Nevertheless, this dream about the imminent clinical application of ASC to cure a number of diseases as diabetes, cystic fibrosis, myocardial infarction and many others physiopathological states appeared more difficult to accomplish than initially expected. With the perspective of recent dynamic works it is conceivable to think that this is just a problem of a better knowledge of their diversity as well on the fundamental level as from the point of view of their therapeutic use.

This observed *ex vivo* pluripotency of HSC and MSC was a cause of noted infatuation. Surprisingly, HSC known to date for their capacity of renewal of blood morphotical elements, were also able differentiated toward skeletal muscle (Ferrari, 1998), cardiac (Orlic et al., 2001), nervous (Mezey et al., 2000), liver (Lagasse et al., 2000) or epithelial cells (Krause et al., 2001). Despite the notable example of post infarct myocardium wall repair, these works did not open the way to the routine clinical application (Agbulut et al., 2004). Troublesome, these results appeared as not reproducible and the parabiosis experiments between exposed to radiation and green fluorescent protein (GFP)-transgenic mousses did not demonstrate this supposed regenerating power of HSC (Wagers et al., 2002).

The *ex vivo* experiments carried out with MSC reporting relatively similar observations. For a long time, MSC were considered as having potential of differentiation limited to mesenchymal family cells as osteoblasts, chondrocytes, adipocytes or muscle precursors (Ashton et al., 1980). More recently, MSC revealed *in vitro* abilities to generate cells distinguished also by ecto- and endodermal features (Reyes et al., 2002; Woodbury et al., 2000; Sato et al., 2005). However, this pluripotentiality was objected by certain unsettled findings. At first, Hardeman et al. (1986) showed formation of cellular hybrids like myofibroblast which was forming by a fusion of fibroblasts with myoblastes able to conserve muscular character (Hardeman et al., 1986). Recently, the fusion of neuronal stem cells with ESC yielded cells expressing both characters (Ying et al., 2002). Since labeling technique with DNA coding enzymes or fluorescent proteins, these observations question

the reality of observed differentiation *in situ*. On the other hand, the possibility of phenotype modification of gene expression according to culture conditions could contribute to observed *in vitro* differentiation (Discher et al., 2009). It is also plausible that these divergent observations reflect a greate heterogeneity of MSC characterized by a certain ability of multipotency revealed during *ex vivo* manipulation where large majority of them represent committed progenitor cells rather than really pluripotent stem cells. Then, the random results of directed differentiation may be explained by imperfect approach of cell isolation. In fact, majority of protocols used is based rather on adhesion capacity of MSC than on specificity of a membrane marker not yet identified.

At the first time, on the basis of morphological differences, the heterogeneity of MSC was brought up by Colter et al. (2000) which proposed three types of MSC. Two first, named RS-1 and RS-2, characterized by a little size and absence or presence of granulations, were considered as self-renewal cells. The third type, distinguished by apparently bigger size, seemed corresponded to already partially differentiated (CSMm) cells (Colter et al., 2000). Thus, the authors hypothesized that RS-1 and RS-2 cells were progenitors of CSMm but since more quiescent state, RS-1 population appeared as precursor of RS-2 that differentiated to CSMm cells. In the proposed schema, RS-2 population would have had the capacity to maintain equilibrium of CSMm production by the ability to reprogramming towards the ground RS-1 state (Colter et al., 2000). In reality, the ulterior antigenic study of these three cellular populations that matching these morphological differences revealed yet more important cellular heterogeneity than initially supposed (Colter et al., 2001).

2.2 Evidence of rabbit MSC heterogeneity

The above data indicated the necessity to explore this proposed heterogeneity of MSC on the molecular level with particular insight into differences between clonal colonies which seems to be essential in elaboration of final approach of directed differentiation. Thus, we carried out the study having for objective the molecular characterization of colonies proliferating from individual $CD45^-$ mononuclear cells isolated from bone marrow of rabbit. This model was chosen for relative facility to obtain a biological material. This advantage being unfortunately associated with limited knowledge of rabbit genome, we have employed the Differential-Display Reverse Transcription-Polymerase Chain Reaction (DDRT-PCR) technique to analyze expressed respective mRNAs (Sturtevant, 2000). This approach resides in use of several non-specific primers able to hybridize with certain extracted mRNA during low temperature reaction (for more details see original paper of Sturtevant, 2000). In this way, the comparison of obtained patterns of mRNA in analyzed colonies showed differences in genes expression. The colonies were cultured separately after isolation of each clone proliferated from one cell on the surface delimited by cylinder (Figure 1A). After the harvest, the mRNA extract of each colony was analyzed with DDRT-PCR approach. In the figure 1B, we present the DDRT-PCR patterns of amplicons obtained after analyze of 14 colonies with couple 1 of DDRT-PCR primers purchased from Seegen (Seoul, Korea). Thus, these patterns, despite a certain similitude, vary by five differentially expressed mRNAs marked by the arrows. These genes correspond to proteins implicated in different cellular functions as follows: 1 – TBC1D7- cellular growth and proliferation; 2 – Filamine – cell migration; 3 – Cystatine 10 – chondrogenesis; 4- LUC7-like – inhibition of myogenic differentiation; 5 – MTHFR – inhibition of intracellular methylation. Their expression seem to be convergent with expression of OCT-4 gene (Figure 1C) considering as a marker of non differentiated cellular state (Tondreau et al., 2005).

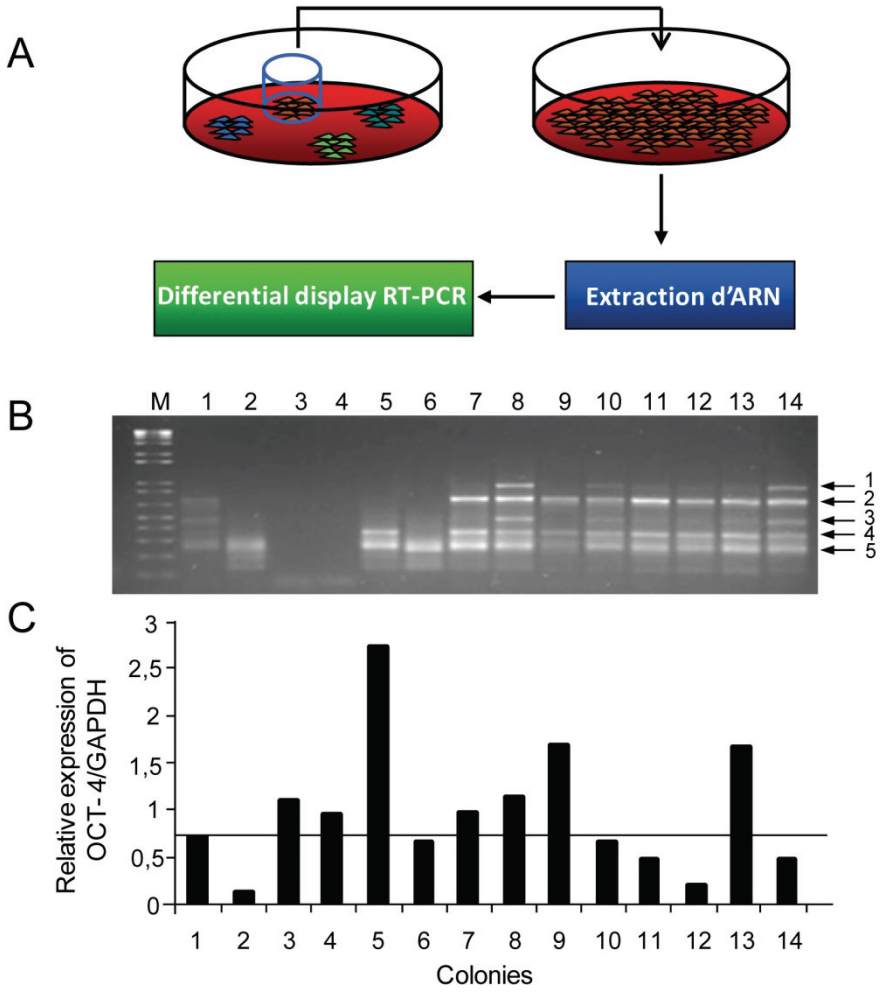

Fig. 1. **Contribution to the hypothesis of MSC cells heterogeneity.** A. Schematic representation of MSC clonal colonies development. After delimitation of one cell in the cylinder space and its proliferation, young colony is displaced in Petri dish where continues to proliferate. Just before confluence, cells are harvested for extract of RNAs. B. Differential Display Reverse Transcription-Polymerase Chain Reaction (DDRT-PCR) gel containing the amplicons obtained from 14 colonies extracts. The arrows indicate sequenced bands corresponding to TBC1D7, Filamine, Cystatine 10, LUC7-like, MTHFR gens. See the text for more details. C. Histograms representing the expression of OCT-4 gen. The line visualizes the mean level of relative OCT-4 expression.

It was interesting to observe, that in the medium LIF-free (Leukemia Induced Factor is employed in view to preserve non differentiated state during cell proliferation) the colonies were able spontaneously differentiated to muscle precursor cells with unequal capacities (Figure 2).

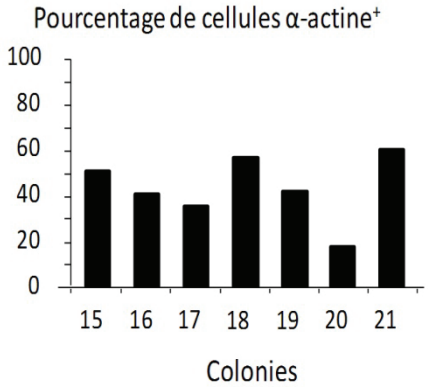

Fig. 2. **Relative spontaneous expression of α-actin gene in studied colonies.** The presence of actin was revealed by indirect immunocytochemistry where the presence of specific antibody was detected with horseradish peroxydase system.

All these results clearly indicate that mononuclear CD45- cells, still considered as MSC, form a heterogenic population characterized by different non differentiated and committed state. Our results did not determine the definitive number of cellular clones but suggest that currently practiced isolation of MSC may conducted toward random results of directed differentiation.

2.3 Toward a new hierarchy of bone marrow stem cells

Our conclusion seems to be strongly supported by the similar results obtained by microarray analysis of human MSC (Mareddy et al., 2009; Tormin et al., 2009). In addition, common distribution of certain membranes markers as CD44, CD73, CD90 or CD105 indicates that use of relative antibodies cannot be considered as discriminative tool for cell isolation. Thus, our results raised the question concerning hierarchy of organisation of bone marrow stem cells and place the observations previously published by groups of Verfaillie and Ratajczak at the special place. These key studies made mention of the very special cells named multipotent adult progenitor cells (MAPC) and very small embryonic-like cells (VSEL) respectively (Jiang et al., 2002; Kucia et al., 2006). Even if these results cannot be reproduced by other laboratories, MAPC possessing similar morphology to MSC are able generate mature cells characterizing by ecto-, meso- and endodermal features. This pluripotentiality, attesting their immature character, allows thinking that MAPC may be direct precursor of MSC as well as HSC (Jiang et al., 2002). Conceivably, this hypothesis may explain the random results of directed *ex vivo* MSC differentiation.

In contrast, VSEL cells are a very small, morphologically similar to embryo cells which being probably attracted by the chemical gradient of SDF-1, colonize bone marrow during embryogenesis (Kucia et al., 2006). Amazingly, the grafts of VSEL in irradiated mice indicated that their weak number seems to be responsible for acceleration of senescence process which suggesting their participation in internal organs and tissues regeneration (Kucia et al., 2008). Convergently, the increased number of VSEL, expressing myogenic Nkx2.5 protein, detected in general circulation in patients suffering from cardiac ischemia

suggests the possibility of their implication in cardiovascular repair (Wojakowski et al., 2009). In the case of definitive clinical confirmation, this observation may open extremely promising horizons of cellular therapy. Nevertheless, identification of these new cellular populations not responds to the question concerning the origin of MSC.

In this context, our results support hypothesis that MSC cannot be considered as pluripotent stem cells having the potential to generate all cells naturally deriving from tree embryonic layers. In the Figure 3, we propose to attribute this role to MAPC and VSEL cells. There are two possibilities, either we observe a coexistence of three cellular populations or existence an ontogenetic hierarchy. In the first situation, each cellular type possesses the variable potency of differentiation: i) VSEL that of committed precursor, ii) MAPC would be pluripotent and iii) MCS just mesodermal. In the second situation, VSEL would be direct precursor of MAPC generating MSC among other. In this way, the apparition of all cellular populations in bone marrow would reflect an ontogenic hierarchy formed during embryogenesis where initial number of VSEL cells determines a capacity of hypothetic organ repair during adult life. It appears that however the reality may be, the strategy of preparation of graft from MSC should be revised taking into account the recent clinical trials lacking therapeutic effects as published recently (Menasche, 2011). In fact, the clinical use of MSC is actually recognize for their immunomodulatory effects known in diminution of graft reject or graft versus host disease (Ringden et al., 2006; Ucceli et al., 2007; Le Blanc et al., 2008). In this way, two novel axes of fundamental research seem to be profiled: **i)** definite establishment of hierarchy of bone marrow cells in regard of MAPC and VSEL cells, **ii)** exploration of intracellular pathways in view to determine cellular fate during directed ex vivo differentiation.

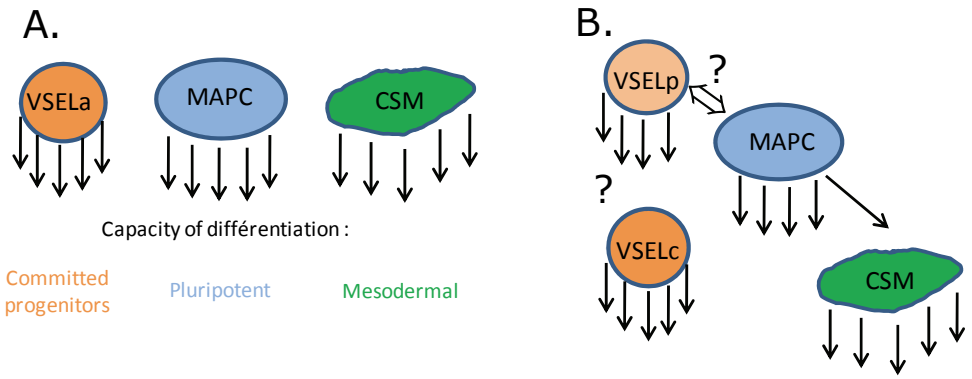

Fig. 3. **Schematic representation of bone marrow stem cells hierarchy.** A. Are we in the presence of three types of CSA which evolved separately according to the physiological regulation? B) Can one envisage a hierarchic ontogenic organization where VSEL cells would be pluripotent stem cells generating all bone marrow cells?

3. Calcium signaling and fate of MSC

3.1 Introduction

The random results of *ex vivo* differentiation of MSC during preparation of autologous grafts indicate that promising potential may be revealed by the microenvironment of cellular

culture. This hypothesis seems to open a new area for proceedings of directed differentiation which may be based on modulation of activity of molecular targets of MSC. Given the dependence of phenotypical gene expression, self renewing, migration or proteolytical enzyme secretion on increase of cytosolic calcium concentration $[Ca^{2+}]_c$, pharmacological modulation of calcium signalling would represent a key to control cellular fate. The various membrane channels and ionic transporters having potential to modulate $[Ca^{2+}]_c$ appears then as appropriate molecular targets. Recent reports indicate that MSC express several ionic membrane channels generating sodium (Na), calcium (Ca) and potassium (K) inward and outward currents characterized by molecular biology and patch-clamp approaches (Li et al., 2005; Li et al., 2006; Deng et al., 2006; Kawano et al., 2003; Kawano et al., 2002; Heubach et al., 2004). Nevertheless, the capacity of these channels to modulate $[Ca^{2+}]_c$ in MSC was not yet evaluated. Some data indicate that in human MSC, the $[Ca^{2+}]_c$ may change upon cyclic oscillatory variations via a mechanism implicating inositol trisphosphate receptors (IP_3Rs), store operating channels (SOCs), L-type voltage dependent calcium channels (L-VDCaCs) as well as Na^+-Ca^{2+} exchangers (NCX) (Kawano et al., 2003; Kawano et al., 2002). We have recently shown that MSC express also several genes coding the proteins of transient receptor potential cation channel (TRPC1/2/4/6) family (Torossian et al., 2010) possessing a major role in cell proliferation as already documented in cancers (El Boustany et al., 2008). Over it, dependence of immature cell proliferation or myoblasts fusion (Lory et al., 2006) on activity of voltage dependent T-type calcium channel reinforces idea that pharmacological modulation of calcium signalling could reveal potential to improve efficiency of protocols employed in directed differentiation of adult stem cells.

In the present study, using functional and molecular biology approaches, we pursued two major objectives: *i)* evaluation of efficiency of membrane voltage dependent ionic channels (VDCaC, VDNaC, VDKC) and transporters (Na/K-dependent ATPase and NCX) to modulate calcium homeostasis on the basis of kinetics of $[Ca^{2+}]_c$ variations occasioned by selective activators and blockers, *ii)* demonstration that inactivation of chosen targets such T- or L- type VDCaC and TRPC1 reduced cellular proliferation and that high concentration of nifedipine activated neuroglial differentiation.

3.2 Efficiency of molecular targets to modulate calcium homeostasis

Figure N°4 illustrates that equilibrium state in single MSC is disturbed by modifications of the extracellular medium or by the presence of selective pharmacological agents which changing Ca^{2+}, Na^+ or K^+ gradients induce the $[Ca^{2+}]_c$ variations with different kinetics. The gathered histograms representing the areas under curves (AUC) were obtained from individual profiles whose averages are expressed in the Figures 4 and 5. The highest calcium mobilization was observed in the presence of depolarizing solution of KCl as well as 2-diazo-4,6-dinitrophenol (DDNP) or bepridil, well known respective blockers of BK_{Ca} channels and NCX. Even if each product activated this increase by different mechanism, the obtained AUCs were very similar and corresponded to 5.5, 5.25 and 5.1 µM/L (Fig. 4). The depolarizing solution of KCl imposing membrane potential to value inferior to -30 mV activates low threshold VDCaCs as "L"- and/or "N"-type channels. Similar effect obtained with DDNP (Fig. 5D) revealed a high capacity of the BK_{Ca} channel inactivation to membrane depolarization subsequent to cytosolic K^+ accumulation. The action of bepridil eliciting the reverse mode of NCX action is responsible for calcium influx which considered its proximity

with endoplasmic reticulum, induces intracellular calcium mobilization (Niggli et al., 1991). In the same way, figure 4 shows also that the action of Na/K-dependent ATPase having the capacity to modify sodium gradient induced cytosolic calcium increase by recruitment of NCX (Hilgemann et al., 1992). In contrast, the effect of other depolarizers as $CaCl_2$ solution, tetraethylammonium (TEA) or veratridine (Fig 5B, 4C and Fig 6) appeared as less efficient.

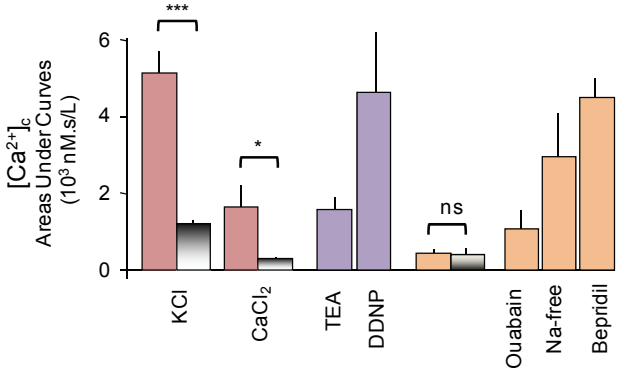

Fig. 4. **Mean values of area under curves (AUC) of cytosolic calcium mobilization in rabbit mesenchymal (MSC) stem cells.** KCl (25 mM) in the absence (n= 10) or in the presence of 10 µM nifedipine (▭ ; n= 15), $CaCl_2$ (10 mM) in the absence (n= 14) or in the presence of 10 µM nifedipine (▭ ; n= 17), TEA (3 mM; n= 14), DDNP (10 µM; n= 6), veratridine alone (100 µM; n= 29) or in the presence of 1 µM TTX (▭ ; n= 22), ouabain (10 µM; n= 6), Na-free (n= 11) and bepridil (100 µM; n= 17) were injected in the vicinity of MSC (***, $p<0.001$; **, $p<0.01$; *, $p<0.1$). The mean values (±SEM) of areas under curves were numerically integrated from individual microfluorimetric (Indo-1) recordings by trapeze method using Excel programme. Each recording represents the same number of points acquired every 250 ms by PC-assisted system developed by Notocord Systems (Paris, France).

The type of VDCaCs was determined with nifedipine, a dihydropyridine derived L- type channel blocker and by RT-PCR experiments. The significantly reduced, but never totally abolished stimulatory effect of KCl and $CaCl_2$ solutions on calcium mobilization in the presence of the blocker indicating the involvement of both nifedipine-sensitive and insensitive VDCaCs. RT-PCR convergent experiments, carried out with Cav1.2, Cav2.2 and Cav3.3 specific primers, confirmed expression of L-, N- and T-type of VDCaCs in MSC (Fig. 4, boxes). L-type channels (Cav1.2 subunits) were already reported in human and rat MSC (Li et al., 2005; Li et al., 2006). In contrast, the existence of T channels in MSC are a matter of debate since contradictory reports concluding to the absence of Cav3.1 and Cav3.2 subunits (Li et al., 2005; Heubach et al., 2004) or to the presence of Cav3.2 whose functionality was however not determined (Kawano et al. 2002). The importance of expression of T channel in MSC is illustrated by observations in ESC where the sustained increase in $[Ca^{2+}]_c$ is responsible for cell proliferation (Lory et al., 2006) or fusion of differentiated myoblasts (Bijlenga et al., 2000). The expression of N-type VDCaCs in MSC is not surprising because its functionality in differentiating cells evolves through an expression pattern (Arnhold et al., 2000). Thus, during neuronal differentiation of ESC, transitory high expression of N-type

channel in initially apolar phenotype matched with cellular migration whereas its reappearance in differentiated neuron coincided, similarly to mature cells (Yokoyama et al., 2005), with synaptogenesis and modification of the exocytose level (Jones et al., 1997). As MSC are known for their secretory and migratory activities, similar functionality may be expected. Consequently, calcium fluxes in MSC can be modified by opening of three types of VDCaCs which filling up the different cytosolic microdomains with calcium can separately control gene expression, cellular proliferation and migration or exocytosis (Lory et al., 2006; Yokoyama et al., 2005; Yang et al., 2006; Yoo et al., 2007).

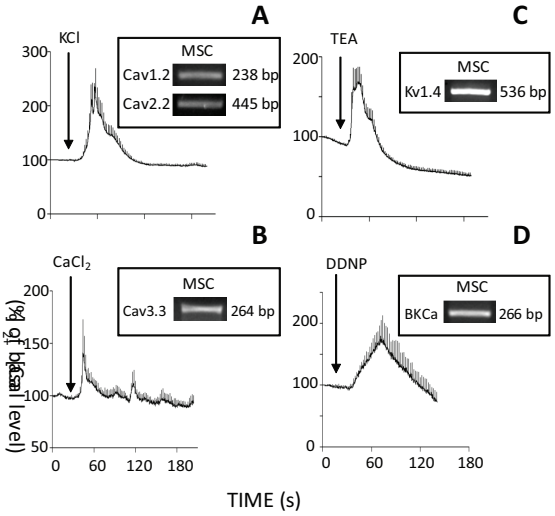

Fig. 5. **Effects of modulation of calcium and potassium channels on $[Ca^{2+}]_c$ in rabbit mesenchymal stem cells (MSC).** The arrows indicate the pressure-ejected administration of depolarizing solution KCl (25 mM) (A), $CaCl_2$ (10 mM) (B) and voltage or calcium dependent potassium channels blockers as TEA (3 mM) (C) and DDNP (10 μM) (D) in the vicinity of the cells. The right-placed boxes represent RT-PCR obtained amplicons of mRNA coding L, N, T-type voltage-dependent calcium channels (Cav1.2, Cav2.2 and Cav3.3 subunits), voltage dependent (Kv1.4 subunit) and calcium dependent (BK_{Ca}) potassium channels. The curves represent a mean from 10 (A), 14 (B), 14 (C) and 6 (D) individual cell recordings. The spontaneous level of $[Ca^{2+}]_c$ (100% basal level) was calculated for each experiment as the mean concentration during 30 s preceding the administration of ionic solutions or potassium channel blockers.

Interestingly, the activation of T-type VDCaC *in vivo* appears to be directly dependent on a potassium gradient demonstrating the crucial role of K^+ channels in the evolution of stem cell fate. This astute mechanism is based on cooperation between three types of ionic channels. Briefly, VDKC or/and CaDKC provoke transitory membrane hyperpolarization conducting to depolarizing potassium influx through delayed-rectifier potassium channel responsible for T-type VDCaC activation and [Ca2+]c increase. Such membrane hyperpolarization, detected in rat MSC (Deng et al., 2007) during progress of cell cycle from G(1) to S phase, seems to be dependet on the balance of expression between KCa3.1 and

delayed-rectifier (Kv1.2/Kv2.1) subunits which since their down-regulation with the specific RNAi appeared crucial for cell proliferation. Thus, the cooperation between IK_{Ca} and KDR channels that generate hyperpolarizing efflux and subsequently delaying influx of K^+ may vary membrane polarity near the threshold value of T-type VDCaC activation.

Amazingly, unlike the mechanism described above, we found pharmacological way to obtain *ex vivo* a similar effect on [Ca2+]c increase in MSC. Using functional and RT-PCR experiments, we observed that the blockage of Kv1.4 and BK_{Ca} channels (fig.4C, D) by TEA (51%; 32 of 63 cells) and DDNP (46%; 39 of 84 cells) induced $[Ca^{2+}]_c$-increase after VDCaC activation due to intracellular membrane depolarization triggered by cytosolic K^+ accumulation. Further studies are needed to show whether such blockage of BK_{Ca} channel would stabilize cell proliferation and immaturity.

The pharmacological activation of VDNaC represents another way to augment [Ca2+]c. Similarly to excitable cells like neurons or cardiocytes, the opening of VDCaCs in MSC results also from progressive membrane depolarization initiated by low threshold T-type VDCaC and/or VDNaC. In our experiments, veratridine (non-selective opener of VDNaCs) (Yang et al., 2006) started Na-induced depolarization which reaching activation threshold of VDCaCs was responsible for increase of $[Ca^{2+}]_c$ (84% given 54 of 64 cells). Not significant reduction of this effect by TTX, a VDNaCs blocker (t=0.38; 79% given 55 of 69 cells) (Fig. 5A) and identification of mRNA encoding Nav1.9 subunit (Fig 5A) indicated the expression of TTX-resistant VDNaCs in MSC. Noticeably, the type of VDNaC expression in MSC appears to be controversial. Using identical primer as Deng et al. (2006), we were unable to confirm their observation on expression of Nav1.1 subunit in rabbit MSC but we found relative transcript in extracts from rabbit nervous system which suggests non-expression of this subunit in our cultures. Divergent findings on the expression of VDNaC may also be noted in human MSC. While Heubach et al. (2004) failed to identify both TTX-resistant and TTX-sensitive channels, Li et al. (2005) detected a functional TTX-sensitive inward current. These discrepancies may result from the different experimental protocols used. In our study, mononuclear cells were separated with CD45 antibody instead of their capacity to adhesion already reported (Li et al., 2005). Moreover, our mRNA samples were obtained at the final stage of the first passage contrary to the 4^{th} or even the 8^{th} as previously described (Li et al., 2005; Deng et al., 2006). As expression of sodium channel unit *in vivo* changes throughout cellular maturation (Benn et al., 2001), these observed *in vitro* differences reveal modulation of gene expression by microenvironment. Nevertheless, the weak kinetics of calcium mobilization induced by veratridine seems indicate that VDNaCs did not appear as interesting target to modulate a fate of MSC.

On the contrary, NCX having capacity to exchange cytosolic/extracellular Ca^{2+} for Na^+ in normal or reverse mode (Niggli et al., 1991) within chemical gradient of both ionic populations, appears as powerful $[Ca^{2+}]_c$ enhancer in MSC. As shown in Fig.6, Na-free medium (38% given 17 of 45 cells), bepridil (44% given 31 of 70 cells) or ouabain (62% given 23 of 37 cells) led to transient increases in $[Ca^{2+}]_c$. The RT-PCR-detected expression of genes coding NCX and Na^+/K^+-ATPases (Figs. 6B, C) matched our functional observations. Similarly to other cellular models (Hilgemann et al., 1992), cytosolic overloading with sodium after ouabain-induced inactivation of Na^+/K^+-ATPase triggered a $[Ca^{2+}]_c$ increase resulting from exchange of sodium for calcium during reverse mode action of NCX (Niggli et al., 1991). In human MSC, the NCX seems to take part in the induction of calcium

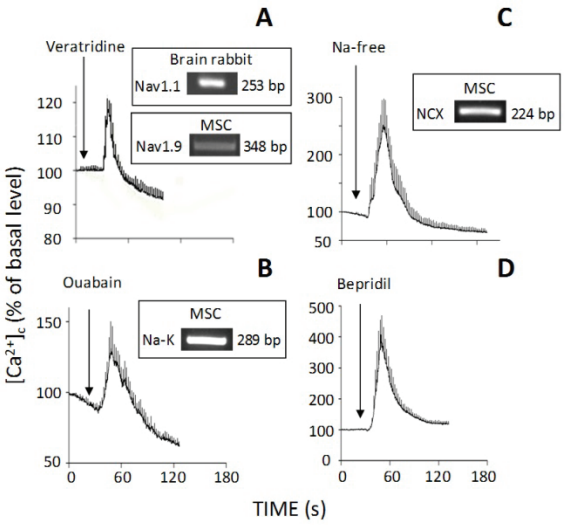

Fig. 6. **Effects of modulation of voltage dependent sodium channels, ATPase Na^+/K^+ dependent and sodium-calcium exchanger activities on $[Ca^{2+}]_c$ in rabbit mesenchymal stem cells (MSC).** The arrows indicate the pressure-ejected administration of Veratridine (100 µM) (A), Ouabain (10 µM) (B), Na^+-free medium (C) and Bepridil (100 µM) in the vicinity of MSC. The right-placed boxes represent the RT-PCR obtained amplicons of mRNA coding voltage-dependant sodium channel (Nav1.9 subunit) in MSC, Nav1.1 being detected only in brain rabbit extract, ATPase Na-K dependend (B) and Na^+-Ca^{2+} exchanger (NCX) (C). The curves represent a mean from 22 (A), 6 (B), 11 (C) and 17 (D) individual cell recordings. The spontaneous level of $[Ca^{2+}]_c$ (100% basal level) was calculated for each experiment as the mean concentration during 30 s preceding the administration of ionic solutions or potassium channel blockers.

oscillations (Kawano et al., 2003). In the present work, its activation induced a transient increase in $[Ca^{2+}]_c$ followed by a slow basal calcium level recovery. It is like during early stage of cardiomyocyte differentiation of mouse ESC, where without modifying transient calcium variations, NCX enhanced the basal level of $[Ca^{2+}]_c$ (Fu et al., 2006). This may indicate the crucial role of NCX in the stabilization of higher basal $[Ca^{2+}]_c$ in immature cells where its activity may be improved by direct intracellular phosphorylation or by increase of Na-gradient during opening of VDNaC or Na^+/K^+-ATPase inhibition.

3.3 Effects of VDCaCs inactivation on MSC cell culture

Taken account of highest capacity to modify calcium homeostasis, VDCaCs was chosen as more appropriate target to evaluate pharmacological modulation of MSC fate. Another choice is related to TRPC1 protein which being largely expressed by rabbit MSC (Torossian et al., 2010) is known as one of essential factors managing calcium distribution during cancer cell proliferation (El Boustany et al., 2008; El Hiani et al., 2009).

Then, the blockage of L- and T-type VDCaC pointed their implication in the control of cellular proliferation and differentiation. Mibefradil and nifedipine induced a dose-

dependent decrease in cell numbers corresponding to 25 and 15 % of cells respectively at 10 μM concentrations attaining very significant inhibition (65 and 50 %; $p<0.005$) when treated with 30 μM doses (Fig. 7). Similarly, inducing 45% inhibition of MSC proliferation, the specific siRNA demonstrated a major role of TRPC1 protein in this process.

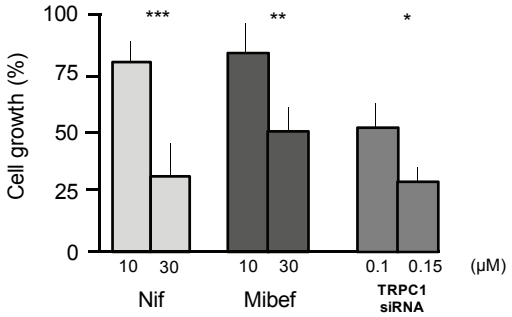

Fig. 7. **Relative mesenchymal stem cells (MSC) proliferation in the presence of calcium channel blockers.** MSC were cultured during 48 hours in the medium containing nifedipine, mibefradil (10 and 30 μM, both) or siRNA of TRPC1 (0.1 or 0.15 μM). The results represent the means (±SEM) from four independent experiments expressed as a percentage of proliferating cells. Each culture contained initially 20000 MSC and after 24h incubation period in expansion medium, the blockers at respective concentrations were administered in the plates. After 48h period of incubation the cells were fixed in acetic alcohol, stained with crystal Violet and extracted with acetic acid after drying. The optical density of extractions was evaluated using spectrophotometric measurement at 570 nm and compared to the standard range to obtain the number of cells. The relative effect of the drugs on cell proliferation was evaluated in comparison to non-treated cells (***, $p<0.001$; **, $p<0.01$; *, $p<0.1$).

Noticeably, the presence of a higher concentration of nifedipine (100 μM) induced apparition of two types attached irregularly shaped cells. The first type, representing about 85%, was characterized by expression of Glial Fibrillary Acidic Protein (GFAP) (Fig. 8B, D, F) whereas the second remained GFAP negative. This result may be particularly relevant in comparison to the control LIF-free culture where cells showed varied morphology and ability to spontaneously differentiate into myogenic precursor cells since relative to α-smooth muscle actin staining (Fig. 8A)

For the first time, we show that blockade of L-type channels in MSC may generate neural precursor cells already shown for their GFAP staining (Imura et al., 2003). We observed two kinds of $GFAP^+$ cells corresponding to a low number of neural-like cells accompanied the large majority of staining cells displaying astrocyte-like morphology. The absence of GFAP staining in the LIF-free expansion medium and the disappearance of myogenic character after treatment with nifedipine, fully support the idea that pharmacological modulation of calcium homeostasis would reinforce strategy for directed differentiation of stem cells. These observations suggest that the reduction of higher and persistent $[Ca^{2+}]_c$ appears like a turning point between proliferation and differentiation where favouring proliferation, the persistent calcium level avoids differentiation.

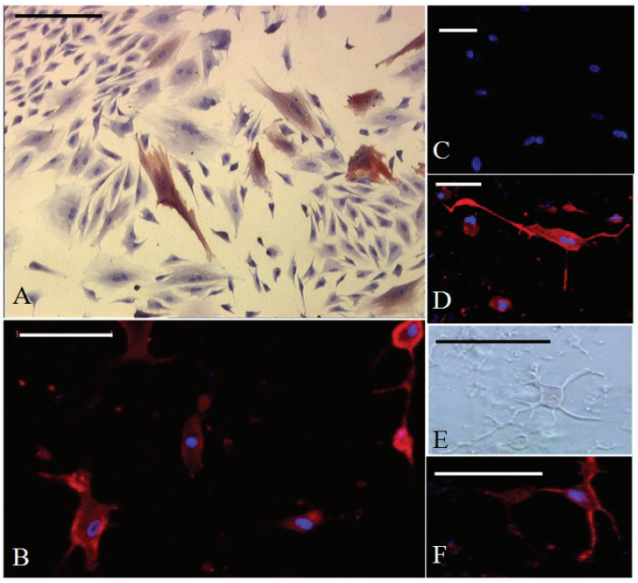

Fig. 8. **Spontaneous myogenic and nifedipine induced GFAP⁺ cells derived from mesenchymal stem cells (MSC).** (A) MSC stained with α-smooth muscle actin cultured in the LIF-free expansion medium. (B) Expression of GFAP in MSC cultured in the LIF-free expansion medium supplemented with 100 µM nifedipine. (C) Absence of GFAP expression in MSC cultured in the LIF-free expansion medium in the absence of nifedipine and counter staining with nuclear dye Hoechst. (D) Neural-like GFAP⁺ **cells**. (E,F) Morphological aspects of astrocyte-like cultured MSC in the LIF-free expansion medium supplemented with 100 µM nifedipine (E, viable cell; F, GFAP⁺ fixed cell). Scale bars, 100 µm.

The results obtained during prolonged exposition of MSC on both anticalcics seem to corroborate this hypothesis. As shown in the Fig. 9, the 10 µM doses were able introduce morphological modifications indicating the initiation of differentiation process. Cells growing in the presence of mibefradil (Fig. 9B) seem to display a more elongated and spindle shape while nifedipine favoured formation of cell extensions (Fig. 8C). Theirs action coincided with apparition of numerous vacuoles apparently more large and swollen in the presence of nifedipine (Fig. 8B, C). Since negative staining with oil red O, hematoxylin-eosine, toluidine blue or periodic acid-Schiff (data not shown) these vacuoles did not contain lipids, glycoproteins nor mucopolysaccharides. Such formation, attributable to an intensification of autophagy process (Mizushima & Levy, 2010), was transiently observed during erythrocyte or lymphocyte differentiation (Kundu et al., 2008; Mortensen et al., 2010) and appeared crucial to adipogenesis (Baerga et al., 2009). According to information recently reported in human U-251 glioblastoma cells (Johnson et al., 2006) or maturating foetal hepatocytes (Matsunga et al., 2008), the mechanism of this process may be explain by not well understood dependency of initial stage of differentiation upon Ca-dependent PI3-kinase activity. Interestingly, the siRNA-inactivation of TRPC1 expression did not modify cell morphology suggesting that unlike T and L type channels this protein is not implicated in MSC differentiation.

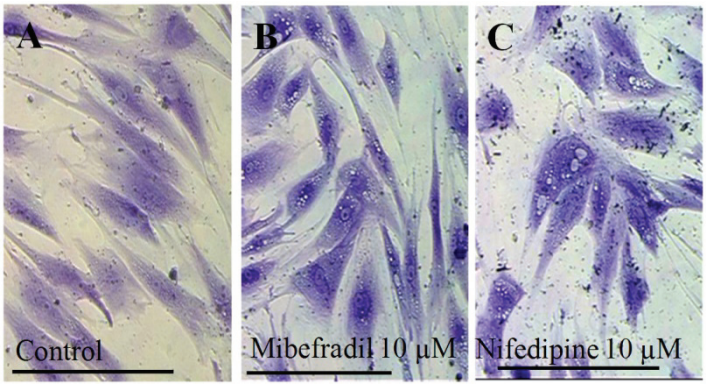

Fig. 9. **Morphological modifications of mesenchymal stem cells (MSC) induced by calcium channel blockers.** Culture of MSC in the LIF-free expansion medium in the absence (A) and in the presence of mibefradil, "T-type" (B) or nifedipine, "L-type" calcium channel blockers (C). Both blockers were employed at 10 µM concentration. Scale bar, 100 µm.

3.4 Conclusion

Taken together, our data demonstrated that pharmacological modulation of ionic carriers' activity, as particularly T- and L-type VDCaC or TRPC1 protein, may reinforce the strategies employed *ex vivo* for directed differentiation of stem cells. Further studies should demonstrate whether pharmacologically induced modulation of $[Ca^{2+}]_c$ in stem cells would maintain their immaturity or begin their differentiation.

4. General discussion and perspectives

The heterogeneity of MSC and their various commitments, as discussed above, perfectly explain why clinical application appears restraint their supposed pluripotentiality on immunological and mesenchymal capacity. In fact, not expressing II class MHC molecule, MSC are therefore not antigen-presenting cells and would be ignored by the host's immune system (Tse et al., 2003; Krampera et al., 2003). By their constitutive secretory activity (Caplan, 2009), MSC have capacity to create microenvironment favourable to combat graft-versus-host-disease (Koc et al., 2000) as well as attenuate inflammatory bowel symptoms in Crohn disease grafted patients (Caplan, 2009). Their aptitude for differentiate into osteoblasts was exploited in clinical trial for the treatment of *osteogenesis imprfecta* patients (Horowitz et al., 1999; 2002). One of very interesting work representing the regeneration of surgically amputated meniscus in goat by knee injection of MSC with hyaluronan delivery vehicle, provides perspective in the treatment of arthritis (Murphy et al., 2003). In contrast, use of MSC in view of cardiac post infracted reparation which seems to provide therapeutic improvement appeared to be not exerted by cardiomyocyte differentiation (Caplan, 2009). Convergently, the results of clinical trials realized with autologous MSC, HSC and mononuclear bone marrow cells (MNC) in about 1600 (Menasche, 2011) patients suffering from acute myocardial infarction, refractory angina or chronic heart failure did not give expected benefits indicating that heterogenic MNC of bone marrow, while remaining immunologically neutral, appear to be not therapeutically reliable to repair other than hard

tissues like bone or cartilage. Nevertheless, the existence of great variability in the functionality of MSC retrieved from patients indicating that pluripotent differentiation would be ascribed to more immature cells which are able generating MSC. Our study shows clearly that MSC should be considered as heterogeneous and composed by lineage-committed cells that may be multipotent but certainly non pluripotent cells. In addition, parallelism between decrease in MSC number with age (Lennon et al., 1996) and acceleration of the senescence process in mouse grafted with a low number of VSEL cells (Kucia et al., 2008) strongly suggests that this role may be ascribed to VSEL cells which would represented this pluripotent cellular population. In this way, the unequal number of VSEL cells in isolated samples may explain the random results of *ex vivo* differentiation. It is therefore conceivable that specific isolation of this cell population represents the first problem to resolve. In fact, the number of data suggest that bone marrow may be considered as reserve of pluripotent cells but this property cannot be attributed to MSC.

Our study of calcium signaling raises a second problem of directed differentiation representing by epigenetic reprogramming of gene expression which in an unpredictable manner would change the cellular fate. This conclusion is supported by divergence concerning the expression of VDCaCs and VDNaC in MSC. This inconvenience could be avoided in the cultures composed of a homogenous population of stem cells able reproducing stable microenvironment. Microenvironmental stability appears then as one of the more important conditions allowing prediction of cellular evolution and an objective comparison of the effects occasioned by experimentally introduced modifications. Our results indicated that pharmacological modulation of calcium homeostasis may influence cellular behavior seem open the perspectives for research of experimental protocols having potential to control the cell proliferation and differentiation.

Taken together, it can be concluded that in view to realize a dream about autologous regenerative grafts it would be necessary to direct the basic research toward two major objectives: i) to find the strategy to facilely isolate pluripotent stem cells from the bone marrow and ii) to perfect protocols allowing control the evolution of cellular cultures.

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Section 5

New Methods in Cell Biology

22

Salivary Glands: A Powerful Experimental System to Study Cell Biology in Live Animals by Intravital Microscopy

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1. Introduction

Mammalian cell biology has been studied primarily by using *in vitro* models. Among them, cell cultures are the most extensively used since they make possible to study in great detail the molecular machineries regulating the biological process of interest. Indeed, cell cultures offer several advantages such as, being amenable to both pharmacological and genetic manipulations, reproducibility, and relatively low costs. However, their major limitation is that the architecture and physiology of cells *in vitro* may differ considerably from the *in vivo* environment. This reflects the fact that cells in a living organism i) have a three-dimensional architecture, ii) interact with other cell populations, iii) are surrounded by an extracellular matrix with a specific and unique composition, and iv) receive a number of cues from the vasculature and from the nervous system that are essential for maintaining their functions and differentiation state (Cukierman et al., 2001; Ghajar and Bissell, 2008; Xu et al., 2009). In the last two decades, cell biology has greatly benefited from major technological advances in light microscopy that have enabled imaging virtually any cellular process at different levels of resolution. The development of genetically-encoded fluorescently tagged proteins (Chalfie et al., 1994) has triggered the development of novel technologies such as FRAP, FLIM, FRET, BRET, photo-activation, photo-switching and photo-conversion (Diaspro, 2002; Lippincott-Schwartz, 2011a, b), and the realization of more sophisticated microscopes, which have significantly improved the limits of light microscopy in terms of both temporal (spinning disk, resonant scanners) and spatial (PALM, STORM, STED) resolution (Lippincott-Schwartz, 2011a, b). However, the application of these very powerful technologies has been primarily restricted to *in vitro* systems. One of the major breakthroughs in light microscopy is the realization of instruments based on non-linear emissions (Denk et al., 1990; Mertz, 2004; Zipfel et al., 2003b), which has opened the door to the development of intravital microscopy (IVM). IVM encompasses a series of light microscopy-based techniques aimed at studying several physiological processes in live animals (Amornphimoltham et al., 2011; Fukumura et al., 2010; Weigert et al., 2010). In particular, two-photon microscopy (TPM) has been instrumental in developing fields such as neuroscience, immunology and tumor biology For example,, TPM has made possible imaging the behavior of single neuronal populations in the brain of live animals leading to fundamental discoveries in neuronal plasticity and neurotransmission, thus

increasing our understanding of pathological conditions such as the Alzheimer's disease or ischemia-induced damages (Serrano-Pozo et al., 2011; Svoboda and Yasuda, 2006; Zhang and Murphy, 2007). In immunology, TPM has been instrumental in analyzing the interactions among the cells of the immune system during the immune response and has provided valuable information on host-pathogen interactions (Cahalan and Parker, 2008; Germain et al., 2005; Miller et al., 2002; Textor et al., 2011). Finally, the ability to image tumors *in situ* during cell growth and invasion, and to monitor the tumor microenvironment has provided with formidable tools to unravel several key mechanisms regulating tumor progression, thus leading to the design and test of novel therapeutic approaches (Andresen et al., 2009; Fukumura et al., 2010; Fukumura and Jain, 2008; Orth et al., 2011). The first attempt to image submicron structures in a live animal has been in the brain, where long term imaging of dendritic spines has been accomplished (Pan and Gan, 2008), whereas the first attempts to image the internalization of fluorescently labeled molecules into highly dynamics sub-cellular structures, such as the endosomes, has been performed in the kidney of live rats and mice (Dunn et al., 2002; Dunn et al., 2003; Sandoval et al., 2004; Sandoval and Molitoris, 2008). However, the motion artifacts due the heartbeat and the respiration of the animal have precluded a detailed analysis of the dynamics of these events. Recently, we have developed an experimental system that has enabled us to follow the dynamics of endosomes and secretory granules in the salivary glands (SGs) of live rodents by using IVM (Masedunskas et al., 2011; Masedunskas and Weigert, 2008; Sramkova et al., 2009). In this chapter, we will review some of the most recent applications of IVM, aimed at studying various aspects of cell biology in live rodents, and will highlight the fact that the salivary glands (SGs) represent a perfect model organ for these studies since they offer unique advantages: first, they can be easily externalized and positioned to completely eliminate the motion artifacts due to the respiration and the heartbeat (Masedunskas and Weigert, 2008), and second they can be easily manipulated both pharmacologically and genetically providing thus with the opportunity to dissect and unravel molecular machineries (Masedunskas and Weigert, 2008, Sramkova et al., 2009). Our goal is to persuade the readers that this approach has a wide range of applicability in different areas of the biomedical field and has the potential to address several fundamental biological questions.

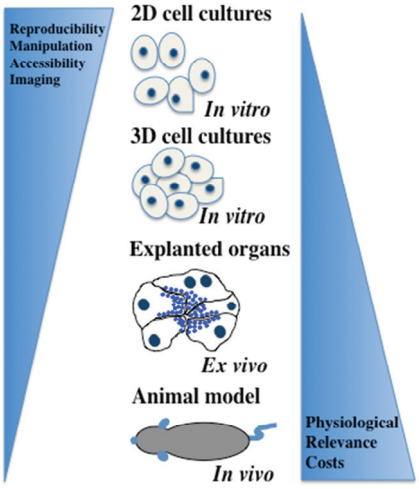

Fig. 1. Comparison among the various experimental systems utilized to study cell biology in mammalian system

Increased complexity in the architecture of the experimental model systems utilized to study cell biology: cell cultures grown on bi-dimensional surfaces (i.e. plastic or glass), cell cultures grown in three dimensions (i.e. purified components of the extracellular matrix), explanted organs, and live animals.

2. Basic principles of intravital microscopy

Biologists have been always fascinated by the possibility to observe biological process in live organisms. One of the major challenges in IVM is to expose the tissue of interest taking care of minimizing damages and maintaining its functionality during the observation period. To this aim, appropriate surgical techniques have been developed since the early days of IVM. The first intravital studies were performed in the early 30's, although they were limited to the examination of the vasculature and its cellular components by using bright field illumination (Beck and Berg, 1931). Advances in optical methods and particularly the development of fluorescence light microscopy, have increased the level of resolution, thus extending the number of biological processes that can be observed *in vivo* (Amornphimoltham et al., 2011; Weigert et al., 2010). Fluorescence light microscopy is based on the generation of contrast by the excitation of the energy levels of molecules (referred as fluorophores) that are either naturally present in the tissue of interest or are administered exogenously. The excitation is achieved by illuminating the specimen with a light source such as a mercury lamp or a laser. The emission can be either directly proportional to the excitation (linear) or exhibit a more complex dependence (non-linear). In the last two decades, microscopes based on e various non-linear processes have been developed, making possible to perform deep tissue imaging (Denk et al., 1990; Mertz, 2004; Zipfel et al., 2003b). Below, we will briefly describe and compare some of the linear (confocal microscopy, CM) and non-linear (multi-photon and harmonic generation) techniques that are commonly used to perform IVM.

2.1 Confocal microscopy

In CM, the excitation of the fluorophore is achieved by using single photons with wavelengths ranging from ultraviolet (UV) to visible light (Fig.2). In order to gather the signal coming from the focal plane and to avoid off-focus emissions that reduce the spatial resolution, the emitted light is forced to pass through a pinhole. This allows to modify the thickness of the sampled area providing an easy way to balance resolution and signal intensity. Confocal microscopes are widespread tools and have been extensively used for IVM (Guan et al., 2009; Masedunskas et al., 2011). However, CM has some limitations. First, UV and visible light are scattered by biological specimen, thus limiting the imaging to the first 50-60 μm below the surface of the specimen and making CM the optimal choice for cell cultures and optically transparent tissues. Second, long term illumination with UV and visible light may lead to photobleaching and phototoxicity, limiting the use of CM to short term imaging as documented by several reports of radiation-induced cellular damage or impairment in tissue development (Dela Cruz et al., 2010). However, when tissues are homogeneous and biological processes are not dependent on the depth, CM can be successfully used providing a better spatial resolution than other techniques (Masedunskas et al., 2011).

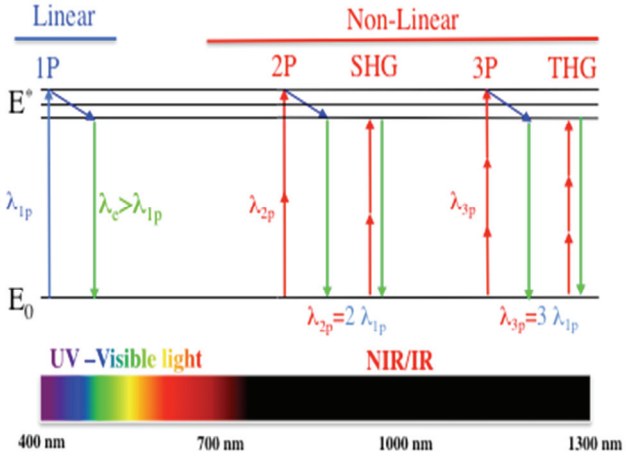

Fig. 2. Comparison between linear and non-linear modalities of fluorescence light microscopy.

Single photon excitation: the energy gap between the ground (E_0) and the excited (E*) state in the fluorophore is filled by a single photon in the UV or visible range. Following some energy dissipation, a single photon is emitted at a higher wavelength (red-shift). Two- and three-photon excitation: the same energy gap is filled by two or three photons respectively, which have half or a third of the energy required for single photon excitation (NIR or IR light). Second and third harmonic generation (SHG and THG): two or three photons interact with the fluorophore and recombine generating a photon with half or a third of the wavelength of the incident ones.

2.2 Multiphoton microscopy (MPM)

Multiphoton emission is based on the fact that a fluorophore can be excited by the almost simultaneous absorption (within femto or atto seconds) of two or three photons that have a half or a third of the energy required to fill the energy gap in the fluorophore (Fig. 2). This requires the use of infrared (IR) light which has a lower intrinsic scattering in biological specimen when compared to UV or visible light. The non-linear nature of multi-photon excitation and the low probability for a multiphoton transition to occur, require that a high number of photons are focused in a restricted volume (1 fl–1 μm^3). This is achieved with pulsed lasers, such as the tunable titanium:sapphire laser, which generates high power beams (in the order of 2-4 W), that are focused in the focal point with high numerical aperture lenses (McMullen and Zipfel, 2010). This implies that all the emitted light generated from the focal point can be utilized to generate the image without the need for a pinhole. This simplifies the geometry of multiphoton microscopes, which require detectors with high sensitivity placed as close as possible to the specimen. Another implication of the fact that photons are absorbed in a confined volume is that photobleaching and phototoxicity are reduced, extending the duration of the experimental observations without any tissue damage, and enabling the realization of long term longitudinal studies that are fundamental in fields such as tumor biology. In terms of depth, MPM enables to extend the range of observation when compared with CM. For example, by using high numerical

aperture objectives, subcellular structures can be resolved up to a depth of 100-150 μm. Lowering the level of resolution and using lenses with longer working distances cellular structures can be routinely resolved at a depth of 300-500 μm. Furthermore, some tissues, such as the brain, exhibit lower light scattering enabling imaging up to a 1 mm depth. Recently, alternative approaches based on the use of either longer excitation wavelengths through the use of optical parametric oscillators (OPO) or regenerative amplifiers, have extended the limits of imaging depth (Andresen et al., 2009; Theer et al., 2003). Two final advantages of multiphoton excitation are: first, the fact that several endogenous molecules can be easily excited providing a contrast that provides numerous information on tissue and cell architecture (Campagnola and Loew, 2003; Dela Cruz et al., 2010; Weigert et al., 2010; Zipfel et al., 2003a), and second, that due to their broad multiphoton absorption spectra, multiple fluorophores can be excited simultaneously using a single excitation wavelength. This avoids the use of multiple lasers, thus reducing further the risk of photodamage.

2.3 Second and third harmonic generation (SHG and THG)

SHG and THG do not involve energy absorption since the incident photons are scattered and recombined into a single photon in a process without energy loss (Campagnola and Loew, 2003; Schenke-Layland et al., 2008; Zoumi et al., 2002). Molecules that generate second harmonic signals such as, collagen, microtubules, and muscle myosin are usually assembled in highly ordered and repeated structures with non-centrosymmetric symmetries, whereas third harmonic signals are typically generated at the interface between optically heterogeneous biological materials (Campagnola and Loew, 2003; Debarre et al., 2006; Gualda et al., 2008). SHG has been extensively utilized to study the properties of the extracellular matrix under both physiological and pathological conditions, and shows also an incredible potential for diagnostic purposes. THG has been used to image lipid bodies in small organisms, to study early embryogenesis dynamics in zebrafish, and to study the process of demyelination in models for neurodegenerative disorders. SHG and THG have the advantage of being nontoxic since no energy is absorbed by the specimen during imaging (Fig. 2), and they can be combined with MPM providing with the opportunity to perform multimodal imaging (Campagnola and Loew, 2003; Chen et al., 2009; Debarre et al., 2006; Farrar et al., 2011; Radosevich et al., 2008).

3. The salivary glands as a versatile model to perform intravital microscopy and to manipulate cellular pathways

3.1 Architecture and physiology of the salivary glands

Salivary glands (SGs) are major exocrine glands responsible for the production and secretion of saliva into the oral cavity (Gorr et al., 2005; Melvin et al., 2005). In mammals there are two kinds of SGs: the major and the minor glands. The major SGs include: parotid glands, which secrete primarily enzymes involved in digestion (e.g. amylase), submandibular glands, which secrete enzymes required to defend the oral cavity from pathogens (e.g. peroxidases, kallikrein), and sublingual glands, which secrete molecules required to protect the oral cavity (e.g. mucins). Saliva is a mixture of water, proteins, and electrolytes that is primarily released from the acini, the main secretory units of the SGs, into the acinar canaliculi and from there discharged into the ductal system (Fig. 3). Acini are formed by polarized acinar cells, with the apical plasma membrane (APM) facing the lumen of the acinar canaliculi, and

the basolateral membrane facing the basement membrane and the stroma. The secretion of water and proteins is under the control of G protein-coupled receptors: muscarinic stimulation is the primary signal regulating water secretion, whereas protein secretion is regulated by either the beta-adrenergic (submandibular and parotid) or the muscarinic receptors (sublingual) (Gorr et al., 2005; Melvin et al., 2005). The ductal system is also formed by polarized cells and its main function is to modify the electrolyte composition of the primary saliva and convey it into the oral cavity. In rodents, a subpopulation of the ductal cells, the granular convoluted tubules, secrete large amount of growth factors (such as EGF and NGF) that are stored in large secretory granules (Peter et al., 1995).

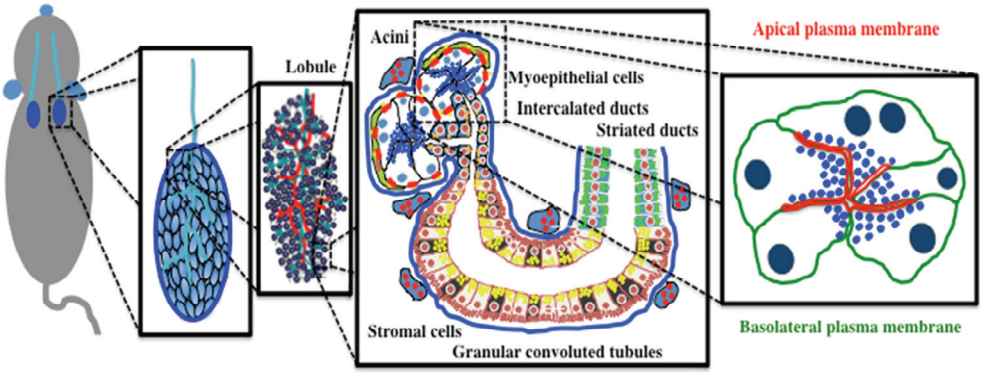

Fig. 3. Diagram of rodent submandibular salivary glands.

The SGs are formed by interconnected lobules, which contain both acini and ductal structures. Acini are formed by polarized epithelial cells, which contain secretory granules that fuse with the apical plasma membrane and release their content in the acinar canaliculi. The canaliculi merge in larger ducts, the intercalated ducts, than enlarge forming the granular convoluted tubules and later the striated ducts. The striated ducts merge with the major excretory duct

3.2 Delivery of molecules, drugs and gene transduction in the salivary glands

SGs are ideal organs to perform IVM for various reasons. First, in rodents the glands are located in the neck area, where the motion artifacts due to the heartbeat and the respiration are significantly reduced (Masedunskas and Weigert, 2008). Second, the SGs can be exposed with relatively minor surgical procedures, which do not involve the exposure of the body cavity, which may effect the overall health status of the animal. Finally, the epithelium of the SGs can be easily accessed from the oral cavity by introducing fine polyethylene tubings into the major excretory ducts (called Wharton's duct in the submandibular glands and Stensen's duct in the parotid glands) that can be utilized to selectively deliver various molecules into the ductal system (Masedunskas et al., 2011; Masedunskas and Weigert, 2008; Sramkova et al., 2009) (Fig. 4A). We have shown that fluorescent dyes can be delivered into the ductal system through injection or by gravity diffusion, and utilized to study endocytosis, exocytosis or various aspects of water secretion (Fig. 4B) (Masedunskas and Weigert, 2008; Sramkova et al., 2009, Masedunskas et al., 2011). The same route has been utilized to selectively deliver drugs to the SGs. This approach offers two advantages: 1) to specifically

target the SGs avoiding the side effects due to systemic injections, and second, to precisely control the doses of the drugs administered (Masedunskas and Weigert, 2008).

SGs have been widely used as a target organ for the viral-mediated expression and gene delivery of various transgenes both in live animals and in humans (Baum et al., 2010; Cotrim and Baum, 2008). Indeed, these organs have the potential to be utilized for gene therapy to correct various diseases including Sjogren's syndrome and protein deficiencies (Baum et al., 2004; Voutetakis et al., 2004). Notably, for viral-mediated gene therapy in humans, the SGs offer several advantages with respect to other organs: i) the encapsulation of the SG tissue prevents the dissemination of the virus in the rest of the body (Baum et al., 2004; Voutetakis et al., 2004), ii) in case of potential health issues the SGs can be removed since they are not essential for life, iii) the differentiation of the cells provides a relatively stable cell populations for non-integrating vectors, and iv) duacrine (both exocrine and endocrine) protein secretion allows to direct the expressed molecules into either the saliva or the blood stream (Baum et al., 2004). Numerous studies have shown successful gene transfer into both rat and mouse submandibular glands using viral-based approaches, which offer the advantage of a more robust expression of the transgenes (Andresen et al., 2009; Baum and Tran, 2006; Delporte et al., 1996; Honigman et al., 2001; Mastrangeli et al., 1994; Morita et al., 2011; Palaniyandi et al., 2011; Perez et al., 2011; Samuni et al., 2008; Wang et al., 2000; Zheng et al., 2009). However, non viral-mediated approaches have also been utilized, although limited to a small percentage of the cells in the parenchyma (Goldfine et al., 1997; Honigman et al., 2001; Niedzinski et al., 2003a; Niedzinski et al., 2003b; Passineau et al., 2010; Sramkova et al., 2009). Furthermore, the majority of the studies on rodent SGs were focused on submandibular glands and only few studies were performed in parotid glands. Recent studies demonstrated efficient gene transfer into rat parotid glands, as shown by the effective delivery of human erythropoietin and human parathyroid hormone (Adriaansen et al., 2010; Kagami et al., 1998; Mastrangeli et al., 1994; Zheng et al., 2009). The rationale behind developing strategies to deliver transgene into parotid glands is their use in humans as main target for clinical applications (Zheng et al., 2011).

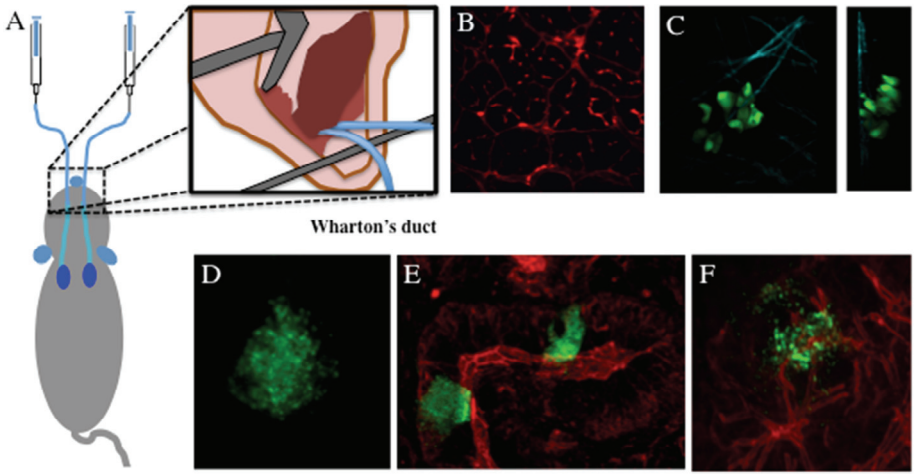

Fig. 4. Non viral-mediated gene transfer in the submandibular SGs of live rats.

A. Fine polyethylene cannulae are introduced in the oral cavity in the Wharton's duct of live rats. B– 10 kDa Texas Red-dextran is injected in the SGs and the ductal system is highlighted. C-F. Plasmid DNAs encoding for the fluorescent protein pVenus (B), GFP-ribonuclases (D), Aquaporin 5-GFP (E), and GFP-Clathrin (F) were injected in the submandibular glands. After 16 hrs, the glands were exposed and imaged by two-photon IVM (C and D) or excised, fixed, labeled with rhodamine-phalloidin, and imaged by CM (E and F). C. Cluster of pVenus expressing-cells (green) localized below the surface of the glands as shown by SHG, which reveals the collagen fibers (cyan). Excitation 930 nm. D. GFP-ribonuclease is localized in intracellular vesicles. Excitation 930 nm. E,F. GFP-Acquaporin is expressed in large ducts (E) and GFP-clathrin in acini (F) as revealed by labeling for the actin cytoskeleton (red).

We have utilized plasmid DNA in live rats and shown that the transgenes under the appropriate conditions can be targeted to specific subpopulations of the SGs (Sramkova et al., 2009). The main advantage in using naked DNA vs. viral-based vectors is the possibility to screen very rapidly for multiple genes without dealing with the time-consuming steps of designing, cloning and preparing the viral particles. We have injected plasmid DNA encoding for various fluorescent proteins into the Wharton's duct of rat submandibular glands, and after 16 hours we have observed their expression in the SGs epithelium (Fig 4C-F). Specifically, we have found that when plasmid DNA is injected alone, the reporter molecule is expressed in approximately 0.05% of the cells of the parenchyma, which we have identified as intercalated ducts. The addition of empty replication-defective adeno-viral (rAd5) particles increases the level of transduction up to 0.5-2% of the cells and notably, the fluorescent reporter is expressed primarily in the large striated and granular ducts and to a lesser extent in the acinar cells (Fig. 4C, 4E and 5) . In both instances, the expression of the reporter molecule is transient and lasts for 72 hrs, a window of time sufficient to be utilized for IVM. Furthermore, since our goal is to transduce genes primarily into acinar structures, we sought to find a more specific way to target these cells. We reasoned that plasmid DNA might be internalized by the acinar cells via the endocytic pathways and for this reason we stimulated compensatory endocytosis by activating the beta-adrenergic receptors during plasmid DNA injection (see below). Notably, under these conditions, the reporter molecule is expressed in 1% of the cells of the parenchyma and primarily in the acinar cells (Fig. 4D, 4F and 5) (Sramkova et al., 2009). It is important to emphasize, that although the efficiency of gene expression is low, the absolute number of cells that can be imaged by IVM is still very high. This implies that viral-based approaches may still have to be utilized whenever a different readout, such as a biochemical assay, needs to be used.

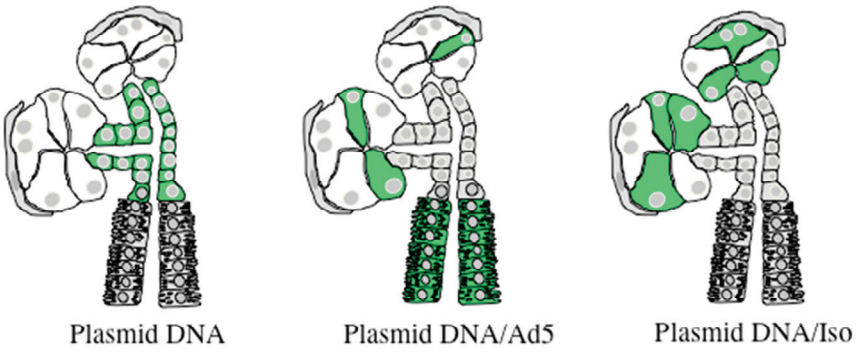

Fig. 5. Naked DNA is targeted to different cell populations of the salivary glands.

When naked DNA is injected in the absence of any other manipulation the transgene is expressed in the intercalated ducts. When naked DNA is pre-mixed with empty adenoviral particles, the transgene is expressed primarily in the large ducts but also in some acinar cells. When naked DNA is injected and compensatory endocytosis is elicited by stimulation of the beta-adrenergic receptor (sub-cutaneous injections of isoproterenol) the transgene is expressed in acinar cells.

This approach can be utilized to express any fluorescently tagged protein, enabling the expansion of the repertoire of compartments that can be visualized in a short period of time (Fig. 4C-F). Moreover, this strategy has provided us with a powerful tool to interfere with specific molecular machineries by introducing molecules acting as positive or negative regulators of the specific processes of interest. Finally, this approach can be used to genetically modify the target tissue by silencing certain genes. Small interfering RNA (siRNA) has been successfully delivered in live rats. siRNA targeting the cystic fibrosis transmembrane conductance regulator (CFTR) were injected intraductallly into rat submandibular glands resulting in the effective silencing of CFTR (Ishibashi et al., 2008; Ishibashi et al., 2006). Notably, in order to complement and confirm the results with the siRNA, a specific inhibitor of CFTR ($CFTR_{inh-}$ 172) and suramin, a non-specific P2 receptor antagonist, were also injected, further highlighting the power of this approach. Although the efficiency in siRNA delivery is low, novel approached has been introduced to overcome this issue. For example, silencing of GAPDH in rat parotid glands was performed in combination with microbubble-enhanced sonoporation, improving the efficiency of siRNA transfer by 10-50% (Sakai et al., 2009).

4. Imaging membrane trafficking and the actin cytoskeleton in salivary glands by intravital microscopy

Membrane traffic is an important field in cell biology that studies the processes and the machineries involved in the transport of various molecules among different compartments within the cell. Transport steps are mediated by membranous containers, termed "transport intermediates", which are very heterogeneous in size, shape, contents and modality of transport. Their biogenesis, trafficking, delivery to the target compartments, and dynamic behavior are dictated by the architecture of the cells and by the organization of the cytoskeletal elements (e.g. microtubules and microfilaments). Most of the data on the dynamics of the transports steps have been derived from cell culture models. As, previously discussed, the architecture of the cells in a living organism differs considerably from the architecture of cells in culture. Although IVM offers a very powerful opportunity to study membrane trafficking in physiological conditions, the challenges in controlling the motion artifacts have discouraged several investigators from pursuing this approach and only few labs have invested in high resolution imaging of live animals. For example, submicron structures were imaged dynamically in the brain of live mice, where structural changes in the architecture of dendritic spines were observed under conditions such as epileptic seizures (Mizrahi et al., 2004; Pan and Gan, 2008; Svoboda and Yasuda, 2006). In kidney, various subcellular processes were analyzed, such as endocytosis of selected molecules (Dunn et al., 2002; Dunn et al., 2003; Molitoris and Sandoval, 2006; Sandoval et al., 2004; Sandoval and Molitoris, 2008), exocytosis of renin (Toma et al., 2006), and mitochondrial function (Hall et al., 2009). Recently, mitochondrial dynamics and lipid bodies have been analyzed in live animals (Debarre et al., 2006; Roberts et al., 2008; Zhong et al., 2008).

Another area where imaging membrane trafficking in vivo has provided novel information is tumor biology. Very recently, nuclear dynamics and mitotis were observed using in murine xenograft model of human cancer and compared to cells in culture (Orth et al., 2011) with profound implications for drug development and cancer therapy (Amornphimoltham et al., 2011). Furthermore, using QD conjugated either to an anti-HER2 antibody or to EGF-conjugated nanotubes the delivery and the uptake of these molecules by tumor cells was analyzed (Bhirde et al., 2009; Tada et al., 2007).

Here, we review our work using the SGs, which represent a robust model to study several aspects of membrane trafficking, particularly because the motion artifacts can be easily reduced using various strategies described in detail elsewhere (Masedunskas et al. 2011b). Although the SGs are exocrine glands, which represent a perfect model system to study exocytosis, they are also a powerful model to study endocytic processes, such as receptor-mediated endocytosis, which occur at the basolateral plasma membrane of the epithelium, compensatory endocytosis that is triggered upon exocytosis at APM, fluid phase endocytosis in stromal cells, polarized trafficking of plasma membrane proteins in the epithelium, and mitochondrial dynamics.

4.1 Endocytosis

The endosomal system is utilized as a transport route, to shuffle proteins, lipids and membranes to and from the cell surface, and towards other sub-cellular organelles. Endocytosis occurs in every cell and is involved in several processes such as nutrient uptake, cell adhesion, migration, cytokinesis, polarity and signaling (Maxfield and McGraw, 2004; Mellman, 1996). Endocytosis and recycling mediate the removal and retrieval of membrane components from the cell surface and these tightly coupled processes are highly regulated. Notably, endocytic pathways are very diversified in terms of molecular machinery, as shown by the fact that multiple endocytic routes have been described (Conner and Schmid, 2003; Doherty and McMahon, 2009; Grant and Donaldson, 2009; Mayor and Pagano, 2007). Much of our understanding of the endosomal system is derived from studies performed in cell cultures and few studies have been performed in live organisms such as rodents. The first attempt to image endocytosis in vivo was realized in the kidney of live rats and mice, where fluorescently labeled dextrans of different molecular weight were injected systemically (Dunn et al., 2002). Similar studies were performed imaging the receptor-mediated endocytosis of the antibiotic gentamicin and the internalization of the folate-receptor (Dunn et al., 2002; Sandoval et al., 2004). Although the diffusion of the injected probes along the tubular system in the kidney was imaged, their internalization could be followed only for short period of times due to the high levels of the motion artifacts. In this respect, the SGs provide with a more controlled experimental system. Indeed, we injected fluorescently-labeled molecules in the tail artery and tracked the dynamics of the endosomal compartments for over 60 minutes (Masedunskas and Weigert, 2008) (Fig. 6A). To further distinguish among the different endocytic sub-compartments, Texas red-dextran (TXR-D) was injected systemically and allowed to accumulate into the lysosomes (Fig. 6B). After 24 hours, Alexa-488 dextran (488-D) was injected and imaged in time-lapse mode. 488-D was first internalized into small vesicles, and then delivered to early endosomes that grew over time due to homotypic fusion events. Later, 488-D was delivered to late endosomes and lysosomes and the process was imaged providing novel insight on the dynamics of the endo-lysosomal system (Fig. 6B) (Masedunskas and Weigert, 2008). In a separate study

lysosomal fusion events were captured at a higher resolution, almost comparable to that achieved in cell culture (Weigert et al., 2010).

Interestingly, Cytochalasin D and Latrunculin A, two actin-disrupting agents significantly reduced the uptake of fluorescent dextran in SGs, suggesting for a role of actin during internalization (Fig. 6C and 6D) (Masedunskas and Weigert, 2008). The requirement for actin during endocytosis has been demonstrated in yeast but has been controversial in mammalian cells (Galletta et al., 2010). The conflicting results could be due to differences in the organization of the actin cytoskeleton or to a different organization of the endocytic pathways. Additional work is required to address these fundamental questions and particularly in defining the endocytic routes *in vivo* and their reciprocal relationship.

Another important issue is the fact that different cell populations within the same organ exhibit different rate of internalization. For example we found that in the SGs, fibroblasts and dendritic cells internalized molecules at a much faster rates than the acinar or the ductal cells in the parenchyma (Fig. 6E). This may reflect the presence of barriers such as the basement membrane and the tight junctions that controls the delivery of molecules from the blood stream.

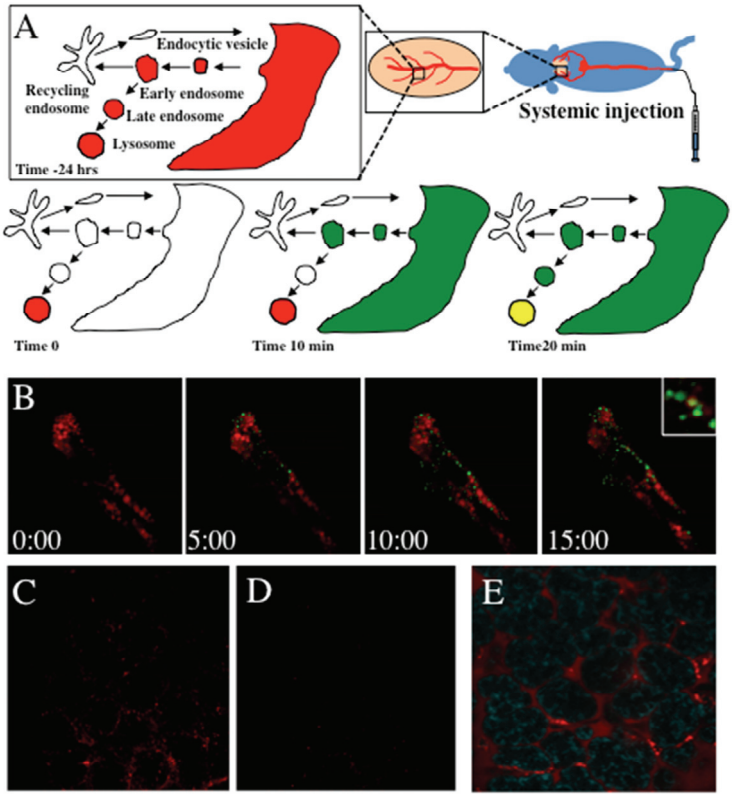

Fig. 6. Endocytosis of systemically-injected fluorescently-labeled probes in the salivary glands of live rats

A. Diagram of the experimental design to study endocytosis in SGs. The fluorescent probes are injected into the tail artery, reach the SGs through the circulation, and diffuse out of the vasculature from the fenestrated capillaries. B. TXR-D (red) is accumulated into the lysosomes after 24 hrs from the injection (time 0:00). Alexa 488-dextran is first internalized into early endosomes (time 5:00 and 10:00) and later reach the lysosome (time 15:00 and inset). C,D. TXR-D was injected and the SGs were imaged after 20 min. C. Control glands D. SGs treated with latrunculin A. E. Lower magnification of the SGs after the injection of TXR-D. The probe is accumulated in the stroma, internalized in stromal cells, and was excluded from the acini that are revealed by two-photon-stimulated intrinsic emission (Masedunskas and Weigert, 2008; Weigert et al., 2010).

Endocytosis from the APM of the SGs was also analyzed by either injection or slow gravity-mediated infusion of small molecular weight dextrans though the Wharton's duct. Under resting conditions most of the probes underwent a low but detectable level of endocytosis in both the ducts and the acinar cells. However, upon stimulation of protein but not water secretion, the probes were primarily internalized into the acini, most likely by the activation of a process known as compensatory endocytosis (Masedunskas et al., 2011; Sramkova et al., 2009). Interestingly, this process did not involve any of the currently characterized endocytic processes and studies are undergoing to further elucidate this it s machinery. Finally, in order to study receptor-mediated endocytosis at the basolateral plasma membrane we have transfected in the acinar cells of live rats, transferrin receptor, as a model for constitutive endocytosis and beta2-adrenergic receptor, as a model for agonist-induced endocytosis. The ectopically expressed receptors are properly targeted to the acinar cells and at the plasma membrane as predicted (Fig. 7C and 7D).

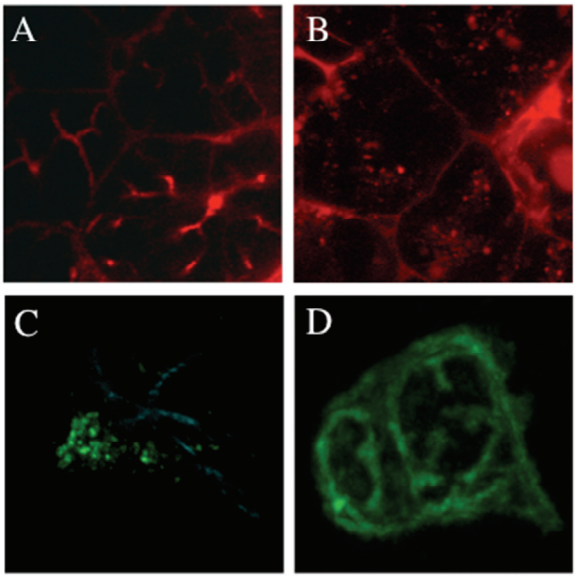

Fig. 7. Endocytosis from the apical and the basolateral plasma membrane of the salivary glands

A,B. 10 kDa TXR-D was injected into the Wharton's duct to fill the acinar canaliculi. After 20 minutes, minimal internalization of the probe was observed (A). Five minutes after the SC injection of isoproterenol smaller endocytic vesicles formed from the APM (B). C,D. GFP-Transferrin receptor (C) and YFP-beta2-adrenergic receptor were transfected in the acinar cells of live rats and imaged by using IVM. Maximal projections of Z-stacks are shown. Transferrin receptor is primarily localized in intracellular vesicles, whereas beta2-adrenergic receptor is primarily localized at the basolateral plasma membrane.

4.2 Exocytosis

SGs are a well-established model for exocrine secretion. Proteins destined to secretion are synthesized in the endoplasmic reticulum and transported through the Golgi apparatus to the trans-Golgi network (TGN) where they are packed in large vesicles, secretory granules (SCGs), which are released into the cytoplasm, and transported to the cell periphery. Here, upon stimulation of the appropriate G protein-coupled receptor (GPCR), the SCGs fuse with the APM, releasing their content into the lumen of the canaliculi. Although, exocytosis in SGs has been extensively studied in ex-vivo models (Castle et al., 2002; Castle, 1998; Gorr et al., 2005), very little is known about the molecular mechanisms regulating this process. Several studies have reported contradicting findings about the stimuli triggering exocytosis, the modalities of fusion, and the requirement for the actin cytoskeleton in this process (Eitzen, 2003; Nashida et al., 2004; Segawa et al., 1998; Segawa and Riva, 1996; Segawa et al., 1991; Sokac and Bement, 2006; Warner et al., 2008). This variability probably reflects the different experimental conditions utilized to isolate and culture *ex-vivo* the acinar cells. To overcome this issue, we have utilized IVM and studied the dynamics of the SCGs in live animals. To this aim, we used a series of transgenic mouse models expressing selected fluorescently labeled molecules, combined with the ability to transduce genes, and selectively deliver molecules and pharmacological agents, as described above and elsewhere (Masedunskas et al., 2011b). This approach has provided novel and valuable information on the structure and the physiology of the acinar cells (Masedunskas et al., 2011). We estimated that in resting conditions, the major SGs contain approximately 2500-3000 granules per acinus, most of them accumulated in the sub apical area of the PM. Our analysis on the effect of various agonists of GPCRs has revealed three major differences between in vivo and ex-vivo models: 1) the stimulation of the beta-adrenergic but not the muscarinic receptors, enhances the mobility of the secretory granules promoting their docking and subsequent fusion at the APM; 2) muscarinic receptors do not play any synergistic role with the adrenergic receptor during exocytosis; and 3) the maximal rate of fusion of the secretory granules in live animal (10-15 granules/cell/min) is 3-4 times faster than previously reported for *ex-vivo* systems. Furthermore, by using another mouse model, which expresses the Tomato fluorescent protein fused with a di-palmitoylated peptide (m-Tomato), a well-established marker for the plasma membrane, we discovered that the secretory granules after fusing with the plasma membrane completely collapse within 30-40 seconds (Masedunskas et al., 2011). This result underscores another major difference between *in vivo* and *ex-vivo* models, in which compound exocytosis (i.e. the sequential fusion of strings of SCGs), has been described as the primary modality of fusion (Warner et al., 2008). Notably, we also observed that the granules in close proximity of the APM recruit a series of cytosolic proteins including actin, suggesting a role for the cytoskeleton during granule exocytosis. To address this issue we have transduced the salivary glands of live rats with the small peptide Lifeact fused with GFP, a novel tool to label dynamically F-actin (Riedl et al., 2008). We determined that F-actin filaments are polymerized onto the surface of the granules only after

fusion has occurred, and persisted until their complete collapse. The impairment of the dynamics of the actin cytoskeleton, using pharmacological agents such as cytochalasin D (cyto D) or latrunculin A (lat A), did not affect the fusion of the secretory granules with the APM, but it blocked substantially their collapse leading to the accumulation of fused granules which often expanded in size. Finally, we found that myosin IIa and IIb, two actin-based motor proteins are recruited on the fused secretory granules and that their motor activity is required to drive the gradual collapse of the granules. These results suggest that the acto-myosin complex provides a contractile scaffold around the secretory granules that facilitates the completion of the fusion at the APM and preventing an aberrant influx of membrane inside the cell (Masedunskas et al., 2011). This novel approach provided new insights into the molecular mechanisms of exocytosis in SGs, captured the exocytosis process dynamically and established important tools to study this process. This approach can be extended to study exocytosis in other exocrine glands such as pancreas, lacrimal glands, and mammary glands

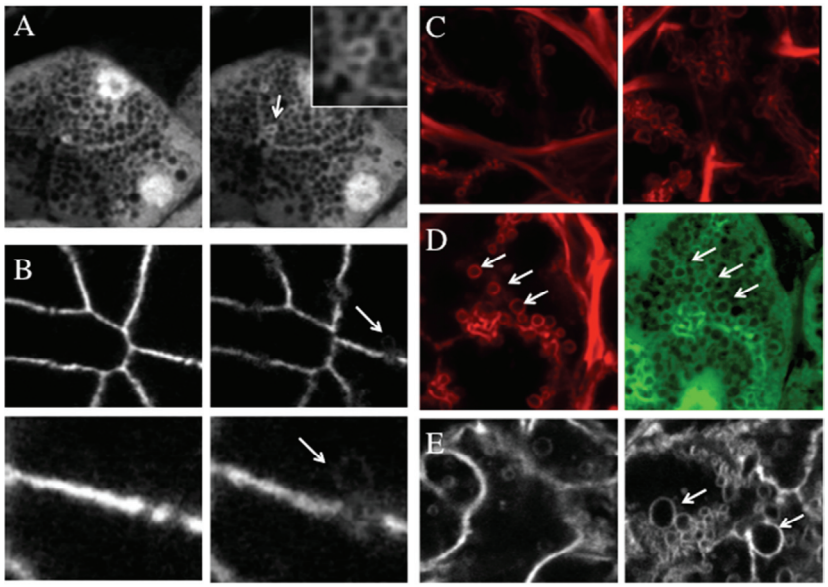

Fig. 8. Regulated exocytosis in the salivary glands of live rodents.

A. SGs of mice expressing cytoplasmic GFP. GFP is excluded from the large SCGs that appear as dark vesicles in the cytoplasm. Upon stimulation with isoproterenol, SCGs fuse with the APM. Fusing SCGs are characterized by an accumulation of GFP on the limiting membranes of the SCGs (arrow), as previously described (Masedunskas et al., 2011). B. SGs in the m-Tomato mice. The m-Tomato probe labels all the cellular membranes. Upon stimulation with isoproterenol, the m-Tomato diffuses into the membrane of the SCGs (arrows). C. The SGs of a live rat were labeled with rhodamine-phalloidin to reveal the actin cytoskeleton. The left panel shows the enrichment of actin at the APM in control conditions. The right panel shows the recruitment of actin around the SCGs upon stimulation with isoproterenol. D. A GFP mouse was stimulated with isoproterenol and labeled with rhodamine-phalloidin. Arrows point to the SCGs at the plasma membrane that are enriched in GFP and actin. E. The effect on exocytosis of actin-disrupting agents. The SGs of a live m-Tomato mouse were treated with

latrunculin A and stimulated with isoproterenol (right panel). SCGs fail to collapse at the plasma membrane and increase in size forming large vacuolar structures.

4.3 Actin cytoskeleton

The actin cytoskeleton plays a fundamental role in many cellular events. We have recently shown a role for actin in endocytosis and a novel role for actin and the actin motor protein myosin II in exocytosis in the SGs of live animals (Masedunskas et al., 2011; Masedunskas and Weigert, 2008). Notably, by using IVM we revealed a novel function for the actomyosin complex that was not completely appreciate in cell cultures. Specifically, we found that actin serves: 1) as a barrier preventing the unwanted homotypic fusion between the SCGs, 2) as a scaffold to prevent the hydrostatic pressure generated by fluid secretion to disrupt the exocytic events, and 3) as a platform to generate a contractile scaffold that facilitate the collapse of the SCGs with the apical plasma membrane (Masedunskas et al., 2011). We took an advantage of the methodology reviewed here to pharmacologically disrupt the actin cytoskeleton and fluorescently tag proteins for labeling the cytoskeleton *in vivo*. Specifically, we have used GFP-lifeact as a tool to follow the dynamics of F-actin (Riedl et al., 2008). This tool is more effective and less toxic than GFP-actin (Fig. 9A and 9C). However, one of the drawbacks of our transfection system is that the expression of the protein of interest is limited to one or two cells per acinus, limiting the possibility to study the behavior of the actin cytoskeleton in groups of cell. Recently, a transgenic mouse expressing GFP-life act has been generated (Riedl et al., 2010). These mice are superior to the GFP-actin transgenic mice in terms of viability, level of protein expression and cellular toxicity and represent a formidable tool to study several aspect of the involvement of actin in various cellular processes. These mice are nicely complemented by other transgenic mice expressing the GFP-tagged versions of the actin motor proteins myosin IIa and IIb (Bao et al., 2007).

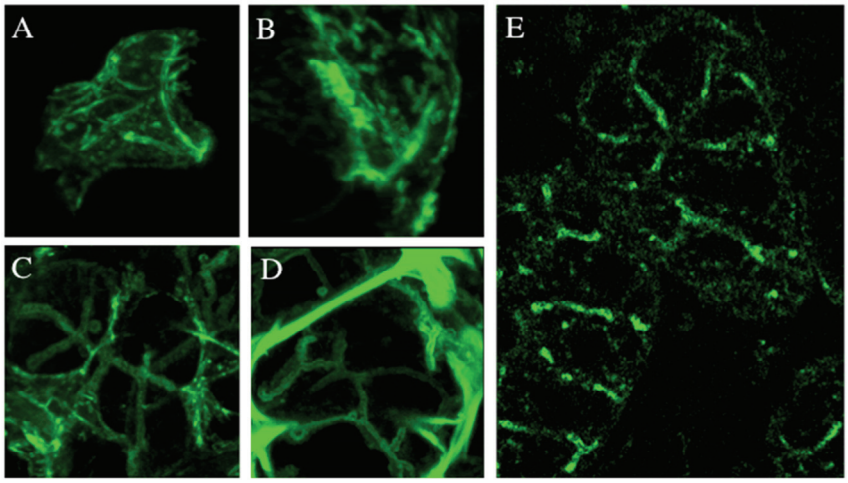

Fig. 9. Imaging the actin cytoskeleton in the salivary glands of live animals.

A, B. GFP-actin or GFP-lifeact were transfected in the SGs of live rats. Both molecules were expressed in acinar cells and exhibited filamentous cortical localization. However, the precise localization of the two probes with respect to the apical or the basolateral pole

cannot be assessed in a single cell. C, D. Transgenic mice expressing GFP-actin (C) or GFP-lifeact (D). The acinar structure in the salivary glands show the typical enrichment at the apical plasma membrane. E. Transgenic mouse expressing GFP-myosin IIb. In the acini of the SGs, GFP-myosin IIb is localized at the apical plasma membrane as previously described for the endogenous myosins (Masedunskas et al., 2011).

5. Conclusions

We have provided several examples from our recent work that the combination of IVM as imaging technique, and the SGs as a model organ is a very versatile tool that can be successfully used to address several biological questions under physiological conditions. The possibility to either express or down regulate proteins in an acute fashion, combined with the plethora of available transgenic and knockdown mouse models, offer a unique set of opportunities to study processes in live animals at a molecular level. Furthermore, the ability to image at a subcellular level in a live animal has opened the possibility to study several aspects of cell biology in a dynamic fashion. We have utilized this approach to study membrane trafficking and the dynamics of the actin cytoskeleton, which represent only a fraction of the fields that can be studied *in vivo*. We envision that soon IVM will be extended to study other processes, such as cell cycle, signal transduction, mitochondrial dynamics and metabolisms to name a few.

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23

Evaluation of Mitochondrial DNA Dynamics Using Fluorescence Correlation Analysis

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1. Introduction

Mitochondria are the sites of oxidative phosphorylation and generate ATP when electron is transferred from respiratory substrates to oxygen by a series of redox reaction in which respiratory enzymes pump protons across the mitochondrial inner membrane from the matrix space[1]. In isolated mitochondria as well as in intact cells, respiration frequently produces reactive oxygen species (ROS). Especially, ROS increased if respiration is perturbed, e.g., ischemia-reperfusion injury [2]. ROS can attack almost all biomolecules unspecifically. In case of DNA, ROS causes single- and double –strand breaks, and base damage[3]. Nevertheless mitochondria metabolize them only partially. Due to the defense system, mitochondrial DNA (mtDNA, ~17 kbp) that mitochondria contain independently of nucleus is particularly vulnerable because it is partially associated with the inner mitochondrial membrane as shown in Fig.(**1A**) [4]. Moreover, mtDNA repair system is weaker than that of nucleus[5]. Since mtDNA codes a part of respiratory enzymes[6], the damages would be harmful to mitochondrial function. On the other hand, because mtDNA forms a complex with proteins and is not naked, a concept that mtDNA have a resistance against ROS is also favored[7]. Therefore, one may need to reconsider mtDNA damage using a newly developed methodology which is able to detect symptoms failed to be found in the previous studies. Among symptoms, the changes in mtDNA dynamics are noticed.

2. mtDNA nucleoid

As shown in Fig.(**1B**), mtDNA molecules are usually clustered within mitochondria as protein-DNA complexes called nucleoid[8]. Cells contain tens to hundreds of nucleoid dependent on the species, growth conditions, differentiation, developmental stage and so on. Each nucleoid contains several mtDNA copies. Among nucleoid proteins reported previously, main proteins were (i) transcription factor A of mitochondria, TFAM[9], (ii) mitochondrial single-stranded DNA binding protein, mtSSB[10], (iii) mtDNA helicase, Twinkle[11], and (iv) mtDNA polymerase, POLG[8]. These proteins would participate in the maintenance of mtDNA. When a cell divides during cell cycle, daughter cells need to receive mtDNA. However, since mtDNA damage probably affects the interaction with nucleoid proteins, transmission of damaged mtDNA may differ from that of intact mtDNA as discussed in heteroplasmy[15]. Indeed, in contrast to neutral polymorphisms, severe mtDNA mutations responsible for diseases in a heteroplasmic state, almost never returned

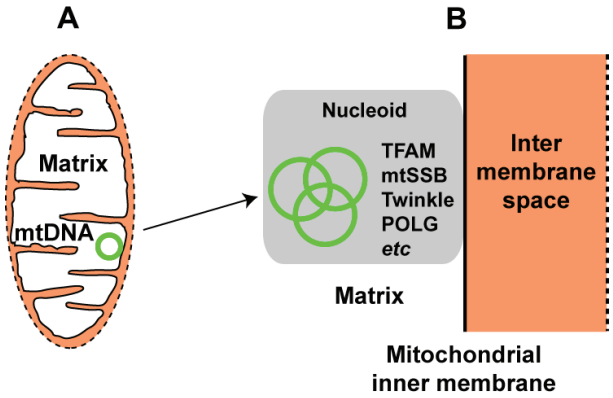

Fig. 1. Structural organization of mitochondrial DNA, mtDNA. (A) Mitochondria have own genetic materials within the matrix. (B) mtDNA nucleoid structure. Several mtDNA molecules within a nucleoid associate to mitochondrial inner membrane through the complex formed with nucleoid proteins such as TFAM, mtSSB, Twinkle, POLG and so on (see text).

to homoplasmy[16]. Large deletions (~5 kb) of mtDNA were very rarely transmitted to the offspring[17]. It was reported that deleterious heteroplasmic mtDNA mutation occurred in early stage of development of primary oocytes from a woman carrying a mtDNA mutation responsible for MELAS, mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes[18].

Moreover, mitochondria itself are dynamic organelles in post mitotic state as well [12]. When mitochondria move along cytoskeletal tracks, each mitochondrion encounters and undergoes fusion[13]. Consequently, mitochondrial networks spread within an entire cell in some cases. On the other hand, each mitochondrion yields two or more shorter mitochondria when fission occurs[14]. Therefore, in addition to mtDNA damage, mutations in nuclear-encoded genes that play an important role in mitochondria dynamics would also cause changes in mtDNA dynamics. For example, heterozygous mutations in optic atrophy gene 1 product, OPA1, caused autosomal dominant optic atrophy, the most common heritable form of optic neuropathy[19]. Mutations in gene encoding Twinkle or POLG cause the autosomal dominant progressive external opthalmoplegia[11, 20]. Although the organization of nucleoid proteins in fixed cells was revealed by immunocytochemistry, effects of the organization on mtDNA dynamics remained fully unclear. Methods by which mtDNA dynamics can be evaluated would permit to diagnose biopsy samples from patients suspected of having these diseases. Moreover, when we search candidates for compounds that modulate mtDNA dynamics by high throughput screening, image correlation method is one of the useful methods.

3. Direct measurement of mtDNA dynamics

Time-lapse fluorescence microscopy was successfully used to study mtDNA dynamics. When nucleoids were observed in cells that expressed GFP-Twinkle, the average displacement velocity of GFP-Twinkle spots was 0.01 μm/s [21]. However, in this method,

because only a few mtDNAs selected within a cell were analyzed, it is probably hard to determine mtDNA dynamics in a whole cell. In order to analyze mtDNA dynamics in wide area of cytoplasm, image correlation spectroscopy (ICS) are proper because fluorescent particles, namely mtDNAs, are not selected. ICS is an imaging analog of fluorescence correlation spectroscopy (FCS)[22]. Therefore, prior to ICS, principles of FCS are described. In FCS, fluorescent molecules entering a tiny detection area generated by confocal optics emit photons and those exiting the area due to Brownian motion cease to emit them[23, 24]. Based on the fluctuations of fluorescence intensity, the motion of fluorescent molecules is evaluated as diffusion constant. As shown in Fig.(**2**), the fluctuation signal is dependent on molecular weight and the number of molecules.

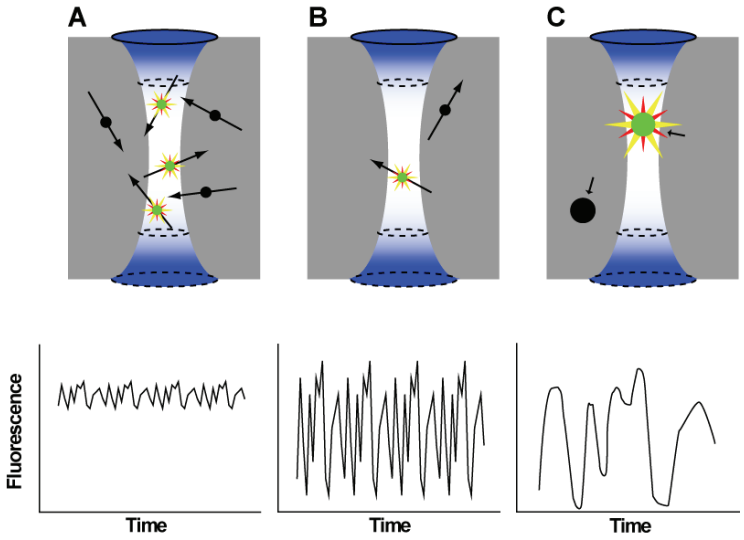

Fig. 2. Effect of molecule number and molecular weight on fluorescence fluctuation. (A) When fluorescent molecules enter and exit tiny detection area (confocal volume element) due to Brownian motion, fluorescence intensity fluctuates. (B) When the fluorescent molecules decrease, the relative fluctuation of fluorescence intensity against the average value increases. (C) When the fluorescent molecules become larger, the fluctuation become slower.

Since fluorescence intensity fluctuates with only a few fluorescent molecules diffusing in and out of the volume element, the intensity at time t, $I(t)$, changes into $I(t+\tau)$, τ seconds later. The normalized autocorrelation function commonly used is calculated from the random fluctuation of fluorescence intensity:

$$G(\tau) = \frac{\langle I(t) I(t+\tau) \rangle}{\langle I(t) \rangle^2} \tag{1}$$

To evaluate the experimentally obtained autocorrelation function, the following analytical expression has been derived [25]:

$$G(\tau) = 1 + \frac{1}{N}\left(1 + \frac{\tau}{\tau_d}\right)^{-1}\left(1 + \frac{s^2\tau}{\tau_d}\right)^{-\frac{1}{2}} \quad (2)$$

In fact, the equation indicates simple diffusion properties, and then represents the time-dependent correlation function for translational diffusion based on fluorescence fluctuation due to Brownian motion of three dimensions, where N is the average number of molecules in the volume element. τ_d is the diffusion time that the molecules take to traverse the detection area in the radial direction. s is the ratio of the axial half-axis to the lateral half-width of the detection area and it can be previously obtained with an authentic material such as rhodamine 6G. When Eq. (2) is fitted to the experimentally obtained autocorrelation function, τ_d and N can be obtained. Although Eq. (2) represents a one-component model for the autocorrelation function, depending on the application, practically, a two-component [26-28] or multicomponent model [29, 30], or even analytical expression for the cross-correlation function [31, 32] is also adopted.

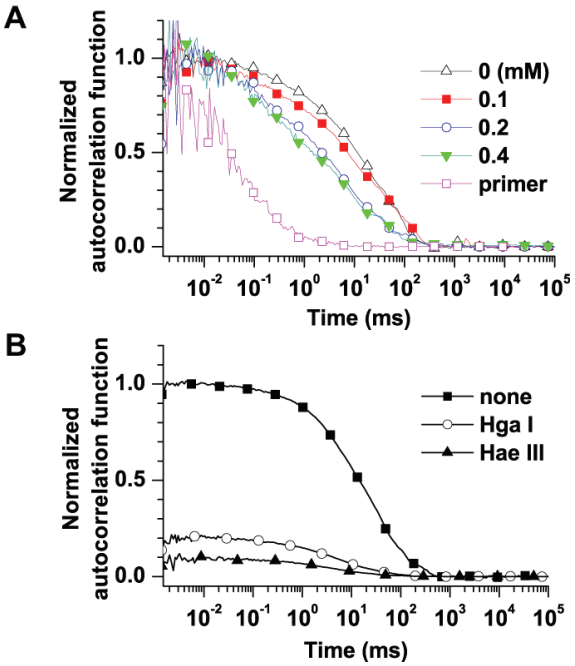

Fig. 3. Fluorescence correlation analysis of mtDNA damage *in vitro*. (A) Changes in normalized autocorrelation functions of long PCR products (~17 kbp) for mtDNA isolated from the cells exposed to H_2O_2 (0 ~ 0.4 mM). For comparison, normalized autocorrelation function of primer is also shown. A decrease in fraction of slow-moving components (long PCR products) shifted normalized autocorrelation function to the left hand side. (B) Effect of restriction digestion on the normalized autocorrelation function of long PCR products for mtDNA. An increase in fluorescent molecules due to the fragmentation resulted in the decrease in amplitude of autocorrelation function.

In order to estimate the vulnerability of mtDNA to oxidative stress, using FCS, the complete mtDNA genome isolated from the cells exposed to H_2O_2 was amplified by long PCR and the product (~17 kbp) was fluorescently labeled with an intercalating dye, YOYO-1 [34]. As shown in Fig.(**3A**), normalized autocorrelation function (normalized ($G(\tau)$-1)) of long PCR for mtDNA product was shifted to the left with the increment of H_2O_2 concentration. When the data were analyzed by a 2-component model, a decrease in the slow component due to mtDNA damage was revealed. In further study, we quantified size distribution of restriction fragments in long PCR product for mtDNA with Hga I and Hae III [30] (Fig.(**3B**)), which indicated changes in molecular number due to fragmentation. Using a multi-component model which was considered as a fragment length-weighted correlation function, we calculated the correlation amplitude expected theoretically and compared it to that measured by FCS (refer [30]). Since these were coincident well, the amplitude measured by FCS would be a very useful index for primary screening for alterations in the entire mitochondrial genome using restriction enzymes that have several polymorphic restriction sites.

4. Image correlation spectroscopy

In ICS which is an imaging analog of FCS, the raw data for image correlation analyses is an image series which is recorded as a function of space and time. The images are usually obtained from a confocal laser scanning microscope (LSM), two-photon LSM or evanescent wave imaging[35]. A generalized spatiotemporal correlation function is defined as:

$$r(\xi,\eta,\tau)=\frac{\left\langle \delta I(x,y,t)\,\delta I(x+\xi,y+\eta,t+\tau)\right\rangle}{\left\langle I(x,y,t)\right\rangle^{2}} \tag{3}$$

where a fluctuation in fluorescence, $\delta I(x, y, t)$, is given by:

$$\delta I(x,y,t)=I(x,y,t)-\left\langle I(x,y,t)\right\rangle \tag{4}$$

where $I(x,y,t)$ is the intensity at pixel (x, y) in the image recorded at time t, and $\langle I(x,y,t)\rangle$ is the average intensity of that image at time t. Every image acquired on a LSM is a convolution of point spread function (PSF) for the microscope with the point-source emission from the fluorophores due to diffraction [36]. This convolution causes the signal from a point-emitter to be spread over a number of pixels. Correlation of fluctuations arising from fluorescent particles within the microscope PSF also confers some critical limitations on ICS approaches. In ICS introduced here, the spatial correlation function is firstly computed and then number of particle is obtained. Next, using the value, when the temporal correlation function is fitted to an analytical model derived from diffusion theory, diffusion coefficient is calculated.

With spatial ICS, a spatial autocorrelation function is calculated from the intensities recorded in the pixels of individual images (Fig.(**4A**)) As shown in the colored surface of Fig.(**4B**), the spatial autocorrelation function of the image is given by Eq. 3 when $\tau = 0$:

$$r(\xi,\eta,0)=\frac{\left\langle \delta I(x,y,t)\,\delta I(x+\xi,y+\eta,t)\right\rangle}{\left\langle I(x,y,t)\right\rangle^{2}} \tag{5}$$

where the angular brackets denote spatial averaging over the image, and ξ and η are spatial lag variables corresponding to pixel shifts of the image relative to itself in the x and y directions. The correlation function is then fitted to a 2D Gaussian using a nonlinear least squares algorithm (the grey mesh in Fig.(**4B**)):

$$r(\xi,\eta,0) = g(0,0,0)\cdot\exp\left[-\frac{\xi^2+\eta^2}{\omega_0^2}\right] + g_\infty \tag{6}$$

where $g(0,0,0)$ is the zero-lags amplitude, and g_∞ is the long-spatial lag offset to account for an incomplete decay of the correlation function. Fitted parameters are $g(0,0,0)$ and g_∞. In Eq.(6), a Gaussian function is used because the laser beam acts as the spatial correlator and has a Gaussian intensity profile. The zero-lags amplitude of the correlation function is inversely proportional to the number of independent fluorescent particles per beam area. The beam radius of the microscope PSF (ω_0) can be determined using methods such as imaging of fluorescent microspheres with diameter less than diffraction limit [36]. Because the size of ω_0 is wavelength-dependent, the PSF should be measured at excitation wavelength same as the ICS experiment.

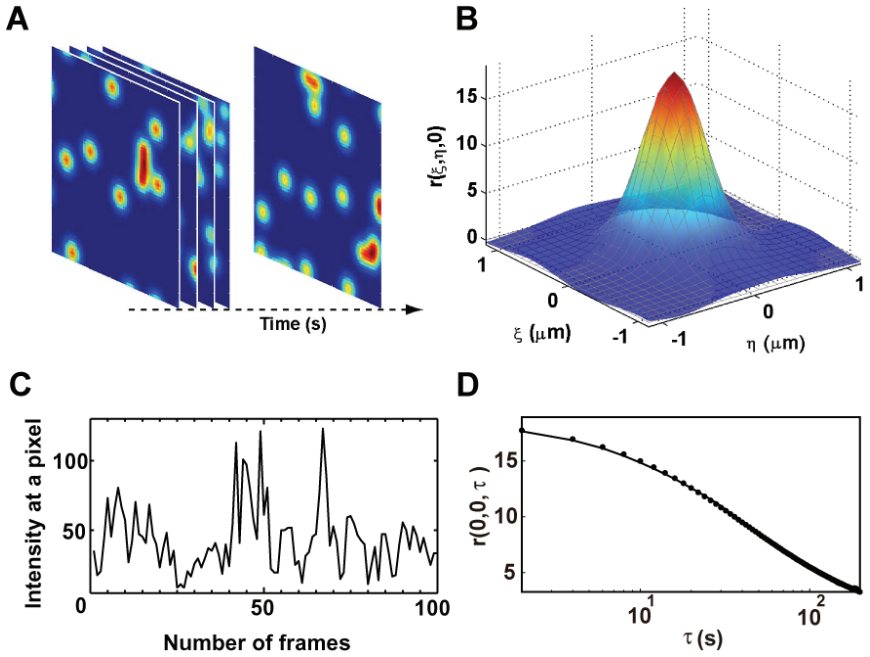

Fig. 4. Image correlation analysis of computer-generated simulations in the case of τ_d = 0.001 μm²/s, 0.1 particles/μm² and ω_0 = 0.4 μm. (**A**) Temporal image series with 5 μm × 5 μm / 2 s. (**B**) The raw correlation function is denoted by the colored surface, and the fitted 2D Gaussian function is denoted by the grey mesh. (**C**) Intensity at a pixel fluctuates with number of frames separated 2 s interval. (**D**) Temporal image correlation function derived from temporal image series (**A**).

Next, as shown in Figs.(**4C** and **D**), temporal autocorrelation function of an image series as a function of time lag τ is obtained from Eq. 3 when ξ and $\eta = 0$:

$$r(0,0,\tau) = \frac{\langle \delta I(x,y,t)\delta I(x,y,t+t)\rangle}{\langle I(x,y,t)\rangle^2} \tag{7}$$

where the angular brackets denote spatial and temporal averaging. Experimentally, τ values are determined by the time between subsequent images in the image series. Depending on the microscope system used, sampling time of image acquisition is usually between 0.03 and 10 s. Here, because it can be assumed that mtDNA in cytoplasm of cells attached strongly on a culture dish behaves as 2D diffusion, the correlation function $r(0,0,\tau)$ was fitted to a simple one component model which was diffusing freely:

$$r(0,0,\tau) = \frac{g(0,0,0)}{\left(1+\frac{\tau}{\tau_d}\right)} + g_\infty \tag{8}$$

where $g(0,0,0)$ is the zero-lags amplitude dependent on number of fluorescent particles, and g_∞.is the long-time offset. For confocal excitation, the characteristic diffusion time, τ_d is related to the diffusion coefficient, D by:

$$D = \frac{\langle \omega_0 \rangle^2}{4\tau_d} \tag{9}$$

where ω_0 is e^{-2} radius of the focused beam of the microscope.

5. mtDNA dynamics

Prior to analysis of mtDNA dynamics in living cells, mtDNA localization was determined by cross-correlation analysis of dual-labeled images with specific dyes for mtDNA (PicoGreen [37], PG) and mitochondria (MitoTracker Deep Red, MT) as shown in Fig.(**5A**). The cross-correlation function was calculated by shifting the red image over a distance ξ in the x-direction with respect the green image with $-4 \le \xi \le 4\mu m$ [38]. For each value of ξ, Pearson's correlation coefficient $r_p(\xi)$ was calculated according to:

$$r_p(\xi) = \frac{\sum_{(x,y)} \left[I_g(x,y) - \langle I_g \rangle\right]\left[I_r(x+\xi,y) - \langle I_r \rangle\right]}{\sqrt{\sum_{(x,y)} \left[I_g(x,y) - \langle I_g \rangle\right]^2} \sqrt{\sum_{(x,y)} \left[I_r(x,y) - \langle I_r \rangle\right]^2}} \tag{10}$$

where $I_g(x,y)$ and $I_r(x,y)$ are the intensity of green and red channel at pixel (x,y), and $\langle I_g \rangle$ and $\langle I_r \rangle$ are average intensity, respectively. As shown in Fig.(**5B**), the cross-correlation function was obtained by plotting $r_p(\xi)$ against ξ. In principle, a cross-correlation function can be determined for shifts in any direction of the x, y, z-space, but shifts in the x,y-plane are

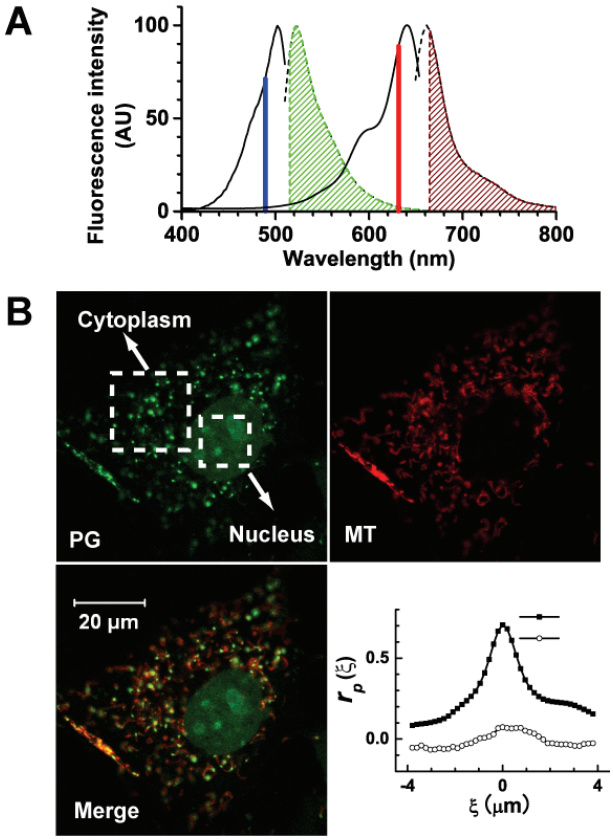

Fig. 5. Localization of mtDNA within mitochondria. (**A**) fluorescence spectra of suspension of cells dual-stained with PicoGreen (ex. 488 nm) for mtDNA and MitoTracker Deep Red (ex. 633 nm) for mitochondria. A solid line shows each excitation spectrum. Using LSM, the hatched area of emission spectra (broken line) was observed. (**B**) Confocal images of mtDNA (PG) and mitochondria (MT) are merged. In right panel, cross-correlation analysis of a dual-labeled image is shown in cytoplasmic (filled squares) and nuclear area (open circles).

preferred because of the limited *z*-resolution of LSM. For simplicity, cross-correlation function for shifts in the *x*-direction was analyzed here. As shown in Fig.(**5B**), in contrast to nucleus, PG signals in cytoplasm was partially localized within mitochondria, as confirmed by $r_p(0)$. Therefore, PG signal would show mtDNA.

As shown in Fig.(**6**), temporal autocorrelation function of mtDNA in living cells could be fitted well using Eq.8. Average of diffusion coefficient of mtDNA was 9.4 x 10^{-3} μm^2/s (25 cells), was stable among 0.2~2 s of sampling time, and was comparable to that of mitochondria (8.2 x 10^{-3} μm^2/s) although graphical data are not shown. Therefore, using sequential frames acquired by a LSM, ICS allowed evaluating both dynamics simultaneously. Diffusion coefficient of mtDNA in single living cells was two digits smaller than that *in vitro* [34], and was comparable to that of mitochondria. This suggests that mtDNA would be bound to an internal structure of mitochondria matrix.

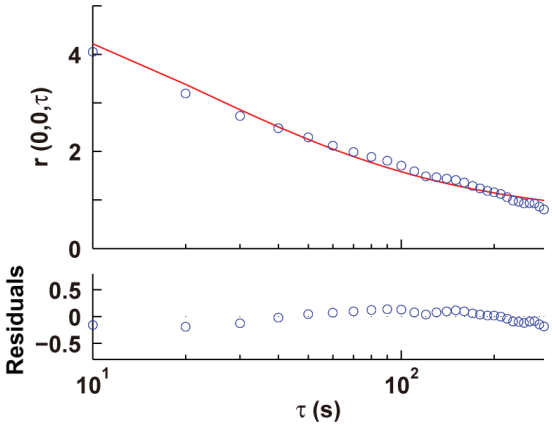

Fig. 6. Typical dynamics of mtDNA analyzed by ICS. In upper panel, temporal image correlation function of raw data (open circles) and fitted analytical model (solid line) are shown. Lower panel is the residuals.

6. Concluding remarks

Because diffusion coefficient depends on molecular weight and/or molecular interaction, diffusion coefficient of mtDNA would allow detecting large deletion of ~5 kb and abnormal transmission. In contrast to recent methods studying mitochondrial genetics such as its RNA expression (e.g., [39]), the present technique is useful for quantifying mtDNA dynamics in single living cells.

7. Acknowledgments

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Regeneration and Recycling of Supports for Biological Macromolecules Purification

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1. Introduction

Great evolution and improvement in biological molecules purification have been achieved in the last 20 years, giving advantages in both product quality and yield, and speed of purification methods. Purity levels, once poorly suitable for large scale production, as well as requiring long and very expensive procedures, are today achievable using simple procedures. For example, till few decades ago, the only way to obtain ultrapure nucleic acids was the ultracentrifugation on cesium chloride gradient; today, the same -if not higher- purity is easily achieved using solid-phase anion-exchange separations. The purification of recombinant proteins has also been dramatically improved by using more precise affinity techniques.

However, despite their routine use, these procedures are often quite expensive, making it very convenient the possibility of using the same purification devices several times, instead of wasting them after one use only.

The possibility of recycling purification matrices has to be considered desiderable and convenient not only in the laboratory research field, where most purifications are anyway performed on small-medium scale, but also in large scale production. Several attempts to reuse purification systems have been made in the last years, showing how many critical points have to be considered.

In fact, most of the previous procedures tested, especially on DNA purification columns, failed to fully decontaminate them (Chang *et al.*,1999; Fogel and McNally, 2000; Kim *et al.*, 2000), resulting in a substantial carry-over contamination, because of the remaining of substantial amounts of material into the matrix after elution (Esser *et al.*, 2005), so that the main challenge in every regeneration procedure is the complete removal of any detectable trace of the previously purified molecules, to avoid the presence of contaminating molecules in downstream applications. Moreover, the use of very sensitive analysis systems (like PCR) reduced dramatically the threshold of acceptable contamination levels. Only in recent years reliable decontamination methods have been proposed, as it will be discussed in the chapter.

Regeneration procedures of columns used in nucleic acids purification are based on nucleic acids hydrolysis, and take advantage of DNA and RNA chemical properties, so that different protocols may be needed for DNA and RNA efficient removal, depending on the decontamination procedure used. To this aim, the proper knowledge of the basis of binding

and elution (including the incomplete release of sample molecules), chemical properties and tolerance of both binding matrices and biomolecules to different reagents is needed, to ensure the proper management and improvement of decontamination procedures without impairing the matrix performances.

1.1 DNA columns

DNA purification strategies using columns take advantage of the chemical nature of the molecule, an highly negatively charged polyanion. Silica, glassfiber and silica-based anion exchange supports are among the most used DNA purification systems, ensuring rapid procedures with good yields and quality without any organic extraction. Such columns are commercially available for the purification of either small molecules (PCR fragments, plasmids, etc.) and genomic DNA, suitable for most applications. Anion-exchange matrices are usually based on polysaccharidic or mineral (silica) derivatized supports, where the active chemical group on resin surface is usually the DEAE (DiEthyl-AminoEthyl). The density of DEAE groups, much higher in silica based matrices (as found in Qiagen resins, Fig. 1A), seems to strongly affect not only the column capacity, but also the binding properties and the selective release of different molecules, each in specific conditions.

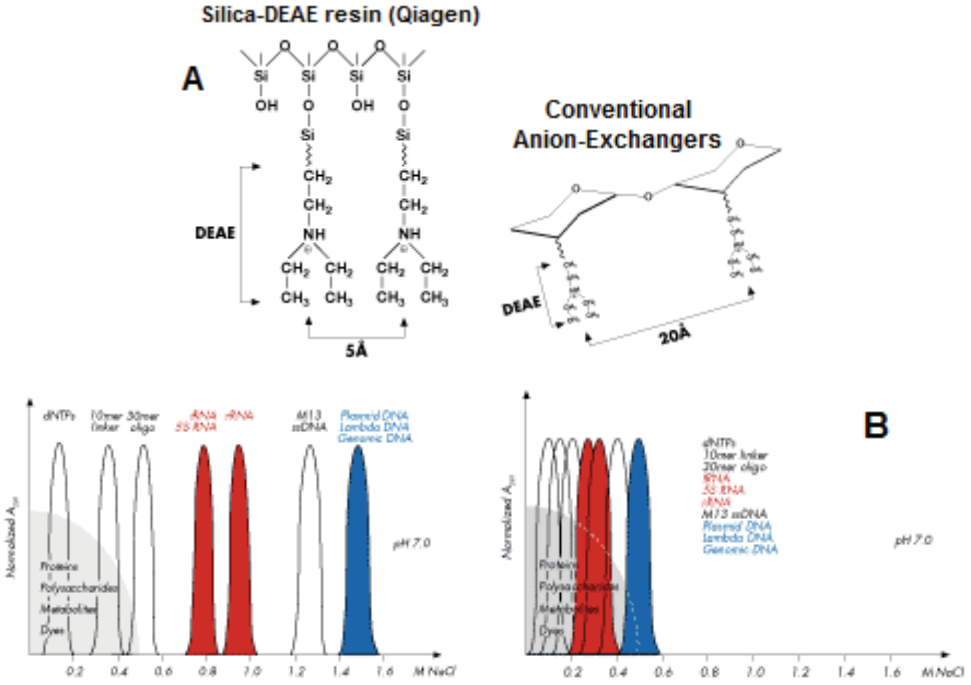

Fig. 1. Structure of anion-exchange DEAE resins (A) and elution profiles (B) (from Qiagen, Purification Technologies)

Such devices have a very broad range of separation, and allow the highly selective binding of nucleic acids, due to the negatively charged phosphate backbone, to the positively charged DEAE groups. Different column suppliers propose various purification conditions, using

either changes of both pH and ionic strength or constant pH (usually at neutral values) and different saline concentration to achieve binding (sometimes in the presence of some amount of ethanol or isopropanol) and elution. These parameters are also tuned to sequentially purify DNA and RNA, or nucleic acid molecules of different sizes (Fig. 1B)

The DNA purification by means of silica gel, proposed first in batch and later in column, has been the most largely used method to recover DNA from both dissolved gel bands and cell lysates, giving high quality DNA without time consuming procedures.

Silica and glassfiber matrices bind DNA because of its negative charge. However, the interactions between silica and nucleic acids occur in different ways depending on chemical conditions.

Binding properties of silica surface depend on its hydratation status (silica-gel) and on the pH value.

In particular, acidic or neutral pH combined with high ionic strength (Fig. 2) allows the silica surface to be positively charged and the DNA to tightly bind silica particles. Binding occurs in the presence of high concentrations of chaotropic salts (usually guanidinium hydrochloride or thiocyanate, sometimes sodium perchlorate). These chemicals alter the hydratation status of macromolecules and facilitate silica-nucleic acid interactions. Under these conditions, DNA remains selectively bound to the matrix, while other molecules (RNA, proteins, polysaccharides and other biological molecules) flow through. Such interaction is still strong under low ionic strength in the presence of high alcohol concentrations, so that this condition is used to perform one or more washes, allowing any trace of salts or soluble contaminants to flush out. Finally, DNA is recovered by elution in pure water or very low salt buffer (frequently Tris-HCl 10 mM, pH 7.5-8.5). In most columns, the maximum DNA release occurs above pH 8.

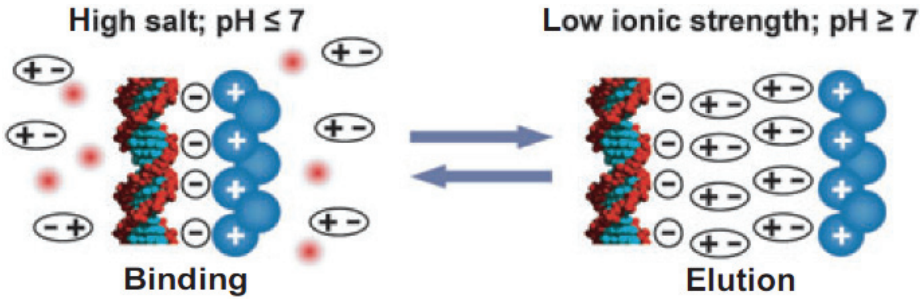

Fig. 2. Interactions between nucleic acids and silica in conditions employed for binding and elution (from Esser *et al.*, 2006)

1.2 RNA columns

Devices for fast total RNA purification are based on the same principles described above. In these columns, a guanidinium salt is always used to protect RNA from degradation, besides promoting its binding to the resin.

Columns can be employed which allow the purification of DNA or RNA, or both (silica-DEAE resins in particular), simply using different buffers. Some silica-based columns, like silica gel itself, show an enhanced RNA binding capacity when the chaotropic, high salt

binding buffer is supplemented with ethanol and/or 2-propanol; in these conditions, DNA binds the silica with low efficiency. Due to the different supports and/or binding and elution conditions, molecules shorter than 200 nucleotides usually fail to bind or are lost during washes, although recently columns allowing the recovery of the whole RNA population, including molecules few tens of nucleotides long, became commercially available.

Affinity columns are largely used to purify the poly-A^+ fraction of eukaryotic mRNA. Such purifications can be performed either in batch or in columns, although the mechanism is the same. The resin employed consists of polymeric, hydrophilic beads (often agarose) whose surface is coated of covalently linked oligo-dT. The interaction with target RNAs occur because of the complementarity between the mRNA poly-A tail and the resin-linked oligo-dT, leaving other molecules unbound. The following elution allows the recovery of the poly-A^+ fraction (Fig. 3).

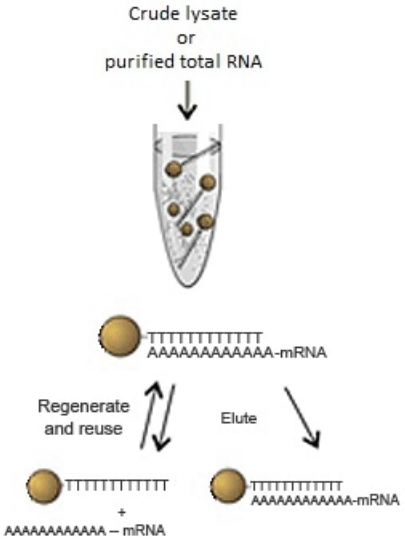

Fig. 3. Purification of the eukaryotic poly-A^+ mRNA fraction. (modified from Invitrogen)

All the systems described above are largely used in most molecular biology laboratories and in many biotechnological companies for both research applications and large scale productions, as they offer the possibility to get large quantities of high quality products using short and simple procedures.

2. Limits

The major disadvantage of the purification systems and devices described above is the cost, as they can only be used once because of the substantial amount of DNA which remains into the matrix after elution.

In fact, the nucleic acids recovery has been estimated to be not more than 90-95% of the input. The remaining part is lost during purification (because of binding failure or leakage

during washes) or remains inside the matrix. The incomplete elution may have various explanations:

1. some molecules are not released from the resin;
2. a small volume of eluent is always retained by the resin, leaving free molecules inside;
3. some molecules might be included into unsolubilized protein particles;
4. some molecules might be associated with cellular fragments, especially of bacterial origin.

Whatever the cause, after the elution (even if sequential rounds are performed) the resin contains relatively large amounts of sample (Fig. 4), making it impossible to reuse the same matrix for further purifications, especially of different samples, because of the high risk of cross-contamination and reduction in the column binding capacity due to trapped particles (especially when a crude lysate had passed trough the column).

All these conditions require particular attention for any attempt of recycling purification columns, so that only methods whose reliability has been proven should be employed.

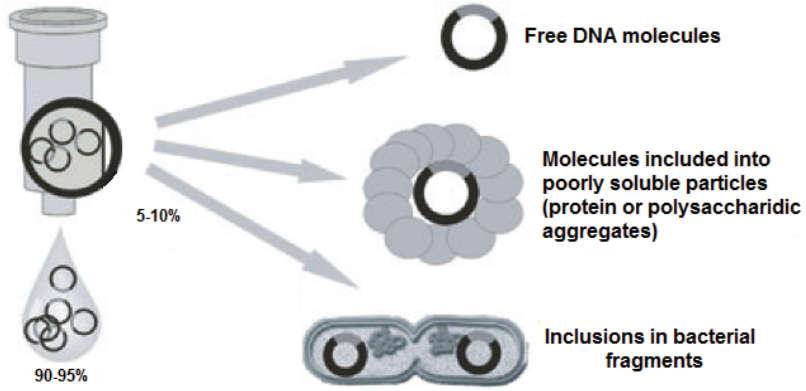

Fig. 4. Residual DNA into the column after elution (modified from Esser *et al.*, 2006).

3. Regeneration

Whereas working with a large number of DNA samples (or large volumes) could represent a problem because of the columns or, in general, purification matrices cost, the possibility of recycling them becomes attractive. The main challenge in every regeneration procedure is the complete elimination of any detectable DNA trace.

In the last years several attempts have been made to set fast and safe procedures ensuring a true complete decontamination without impairing the resin binding properties.

Thus, chemical procedures are needed that could ensure the complete hydrolysis or functional/chemical inactivation of nucleic acids. Moreover, the treatment should be able to remove, at least partially, particles (mainly composed of proteins) trapped into the matrices.

The methods proposed earlier did not avoid carry-over contamination (Chang *et al.*,1999; Fogel and McNally, 2000; Kim *et al.*, 2000).

The first reliable method has been proposed (Esser *et al.*, 2005) and became commercially available as a kit (patented in USA in 2009).

It is based on the hydrolysis (single and double strand breaks) of nucleic acids in the presence of ferric salts in a reducing, buffered acidic environment. Other biomolecules, including lipids and proteins are damaged by the treatment, too. This procedure is claimed to be effective within minutes or hours, ensuring the complete decontamination (assessed by PCR assay) of columns used to purify plasmids or PCR products. Moreover, the composition of the two active solutions included in the kit is claimed to be safe and not hazardous. In fact, the first solution presumably consist of ferric chloride, citric acid, ascorbic acid (as reducing agent), detergents and phosphate buffer at mild acidic pH.

The ability of iron salts (and other transition elements salts, like those of copper, zinc, cobalt, etc.) to damage DNA and RNA is well known since a long time, so that Fe^{2+}-mediated DNA hydrolysis has been used in earlier studies on chromatin structure (Hertzberg and Dervan, 1982) and even today for the footprinting of DNA-protein complexes (Swapan and Tullius, 2008).

Several reactions have been hypothesized to occur, and the so called Fenton's reaction is the most widely recognized and used up today in several fields where fast and efficient oxydative degradation of organic compounds is required.

Fenton's reaction (see below) consists in the iron(II) salt-dependent decomposition of hydrogen peroxide (hypothesized to occur *via* an oxoiron(IV) intermediate), which generates the highly reactive hydroxyl radical. When a reducing agent is added, it leads to a cycle which greatly enhance the damage to biomolecules.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

This reaction is believed to occur together with the Haber-Weiss reaction, which triggers the following cycle:

$$H_2O_2 + OH^{\bullet} \rightarrow H_2O + O_2^{-} + H^{+}$$

$$H_2O_2 + O_2^{-} \rightarrow O_2 + OH^{-} + OH^{\bullet}$$

Although the exact sequence of these reactions and the identity of the reactive species involved in the various conditions (i.e. presence or absence of chelating agents and/or reducing agents) is still controversal (for a discussion, see Barbusiński, 2009), it's widely accepted that the formation of the hydroxyl radical is determinant for the subsequent DNA damage.

Besides the reactions reported above, the presence of chelated Fe(III) instead of Fe(II), together with reducing agents, can lead to strong DNA damage resulting in multiple strand breaks. For example, it has been reported that Fe(III)-nitrilotriacetate (NTA) in the presence of either H_2O_2 (able to act both as reductant and oxidizing agent (Buettner and Jurkiewica, 1996), ascorbate (which in certain conditions can act as pro-oxidizing) or cysteine, produced DNA single and double strand breaks as a function of reductant concentration, *via* a mechanism involving the reduction of Fe(III) to Fe(II) and the formation of H_2O_2. The latter, in turn, enters in the Fenton/Haber-Weiss reactions, where the presence of a reducing agent supports the "iron redox cycle". In all these cases, H_2O_2 seems to be a common intermediate (in fact, catalase activity is able to block these events, leading to the reduction of DNA damage), while the $OH^{\bullet}$ hydroxyl radical is the reactive species which attacks DNA (Toyokuni and Sagripanti, 1992). Moreover, the auto-oxidation of ascorbate in the presence of Fe(II) ions, chelating agents and

phosphate buffer, with the concurrent formation of hydroxyl radicals ($OH^{\cdot}$) has been reported (Prabhu and Krishnamurthy, 1993). Figure 5 shows the chemical reaction resulting in DNA (or RNA) multiple breaks, although other reactions may occur simultaneously, which result in direct bases damage caused by the $OH^{\cdot}$ radical.

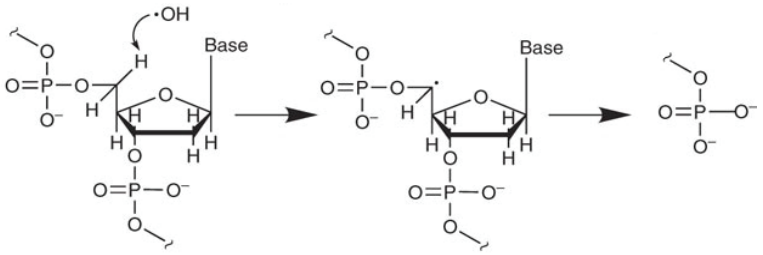

Fig. 5. DNA break by exposure to hydroxyl radicals (modified from Swapan and Tullius, 2008)

Thus, this method makes use of relatively non-toxic and environmental friendly chemicals, allowing the virtually complete removal of small nucleic acid molecules from purification columns. A disadvantage is that the solutions are guaranteed for 18 months only, because reagents undergo chemical modifications making them ineffective. The supplier of the recycling kit claims that resins can undergo about 20 regeneration cycles. Unfortunately, no data are available on the possibility of efficiently decontaminate devices used for genomic DNA purification, whose features are expected to make it more resistant to chemical treatmets, thus leading to the possibility of DNA carry-over.

The first report of a simple and efficient home-made decontamination method has been published in 2008. It achieves the nucleic acids removal by acidic hydrolysis, but its major limits were the need of very long incubation times (Siddappa *et al.*, 2007), and the secure efficacy on low molecular weight nucleic acids only.

The principle used in that method was the DNA and RNA degradation by a treatment with strong acids. In particular, nucleic acids are known to be susceptible to acid catalyzed hydrolysis, which involves the cleavage of the *N*-glycosidic bond of purine nucleosides. As shown in Fig. 6, the reaction results in the formation of an AP-site (apurinic or abasic sites), which causes DNA or RNA break. In fact, the hydrolysis of the *N*-glycosidic bond unmasks the latent aldehyde functionality at the C1′ position, rendering the 3′-phosphate group susceptible to β-elimination (1), which results in strand break. Moreovever, such products are highly sensitive to further alkaline hydrolysis (Fig. 6), so that depurinate molecules can easily undergo fragmentation following exposure to bases (2). These are, for example, reactions used for the controlled, partial DNA hydrolysis in molecular biology protocols, where the short exposure to relatively low concentrations of HCl lead to DNA fragmentation.

However, when the acid concentration is high and/or the exposure time is extended, the depurination extent is so high that, after alkaline treatment, very short DNA fragments or even nucleotides are obtained.

The column regeneration method proposed by Siddappa *et al.* (2007) consisted in a 24 hours incubation of used columns in a HCl solution, followed by several washes. Data reported showed that no detectable nucleic acids were still present in the column, whose binding capacity were claimed to be mantained.

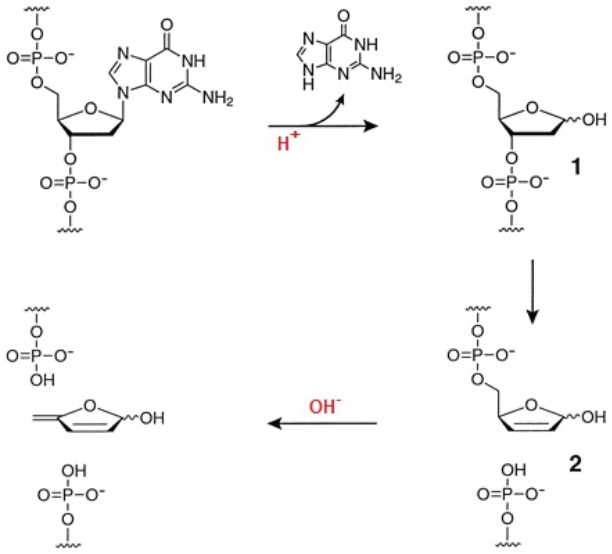

Fig. 6. DNA strand break resulting from H^+ catalyzed depurination and subsequent β-elimination at the AP site. Hydrolysis results in release of the purinic nucleotide and formation of an AP site (1). The α- and β-hemiacetals are in equilibrium with the open chain aldehyde, which is susceptible to β-elimination that results in cleavage of the adjacent 3′ phosphoester (2). This product in turn undergoes cleavage of the 5′ phosphoester under alkaline conditions. (modified from Sheppard *et al.,* 2000).

The lack of evidences about the efficacy of the method even on columns contaminated by genomic DNA and the time-expensiveness of the procedure prompted further tests to improve the procedure.

In fact, if silica columns are used to purify small molecules, contaminating DNA can be virtually completely eliminated by commercial kits (Esser *et al.*, 2005) or using the procedure reported in Siddappa *et al.* (2007), as they make any trace of the previous sample undetectable. However, the efficacy of both methods in eliminating genomic DNA remains uncertain.

The fastest and most effective home-made procedure available up today for the decontamination of silica-based columns consist in an improvement of that described above, as it's also based on DNA depurination and hydrolysis, and addresses the main limits of previously proposed protocols.

Silica-bound DNA could be expected to be efficiently depurinated and removed by treatments with strong acids even after short exposures. However, after such a regeneration procedure small amounts of amplifiable DNA are actually still detectable.

Such failure might be hypothesized to be due to an incomplete permeation of the acidic solution into the silica matrix, where the nucleic acid might be still bound to silica or trapped because of its high molecular weight (Esser *et al.*, 2005). Moreover, any molecules included into aggregates might be somewhat resistant to chemical treatments. All these conditions might allow variable amounts of DNA to escape the depurinating agent, resulting in residual amplifiable traces, making it necessary a very long incubation in HCl solutions.

These limitations have been overcome by the procedure described by Tagliavia *et al.* (2009) and reported below. It can be completed in about 45' (instead of more than 24 hours), and allows not only to regenerate silica columns contaminated by DNA of any size, but also to save time.

The method consists in sequential alkaline and acidic treatments which denature and depurinate, respectively, any DNA still present into the column (depurination rate in denaturated DNA is higher than in native DNA (Lindahl and Nyberg, 1972). A further alkaline treatment hydrolyzes long depurinated DNA molecules reducing them into very small fragments (Siddappa *et al.*, 2007). These chemical treatments are performed in the presence of a non-ionic detergent at low concentration, which seems to enhance their action. In fact, given the structure of the column resins, the detergent is supposed to allow a more even permeation of the solutions employed in the treatment, as it modifies their surface tension. Moreover the tensioactive (which is important to be non-ionic to reduce any dependence of its action on pH and ionic strength), along with the initial alkaline treatment, helps dissolving aggregates, making trapped molecules more exposed to NaOH and HCl.

The efficacy of the method has been demonstrated both by assays using radiolabeled DNA and by PCR, using columns contaminated by large amounts of either genomic DNA or short PCR products.

The protocol steps are briefly reported in box 1.

Box 1. DNA silica column regeneration protocol

1. Load the silica columns with a 1 N NaOH/0.1% Triton X-100™ solution
2. Incubate 5 minutes at room temperature
3. Spin for 30 seconds
4. Load the silica columns with a 1 N HCl/0.1% Triton X-100™ solution
5. Let a little amount of the solution flow through by gravity
6. If dropping tends to empty the column, put it into a tube containing the same solution
7. Incubate at room temperature for 30 minutes
8. Spin for 30 seconds
9. Load the silica columns with a 1 N NaOH/0.1% Triton X-100™ solution
10. Incubate at room temperature for 5 minutes
11. Spin for 30 seconds
12. Load the column with sterile ddH_2O
13. Spin 30 seconds

Note: it's essential to treat not only the resin, but any surface even potentially contaminated by DNA.

The use of the regeneration systems described above is safer on silica-based columns, but not on those supports consisting of polysaccharidic compounds, as they might be hydrolysed or their structure impaired by chemicals employed. Alternative methods, some of which based on radical-driven nucleic acids degradation different from that described above, are under investigation.

Regeneration methods, besides their first application in reusing purification supports, might become of wider use, even for the pre-treatment of new columns before first use. There are many commercially available kits that rely on DNA binding columns to extract and purify DNA from tissues or cultured cells, and a recent paper (Erlwein et al., 2011) reported that, in independent tests, some DNA purification columns from different kits were contaminated with DNA of diverse provenance, including human and murine DNA. Although further investigations are needed, the need of a preliminar columns decontamination step should be considered, at least for particular experiments or analyses have to be carried out.

3.1 RNA columns

Total RNA purification is carried out using columns working exactly like those employed in DNA purification. As discussed earlier, different conditions used for binding and/or elution allow the selective recovery of RNA only.

The same problems described for DNA columns occur in RNA columns, too. However, RNA is well known to be very sensitive to a variety of conditions and chemicals, but treatments are needed that ensure not only the complete degradation of any residual RNA, but also the maintainance of the columns RNase-free state.

The commercial system based on the earlier discussed iron-mediated degradation is effective, but a home-made, simple and inexpensive method is available (Nicosia *et al.*, 2010).

In fact, the methods described in two previous reports (Siddappa *et al.*, 2008; Tagliavia *et al.*, 2009) are time expensive or include steps not required for RNA hydrolysis, so that a faster and more efficient protocol has been set up. It is based on the RNA high sensitivity to alkali, omitting acidic treatments. Indeed, the exposure of RNA to high pH is able to completely hydrolyse RNA, since it is directly cleavable by the OH^- due to the presence of the 2'-OH group in the molecule (Fig. 7).

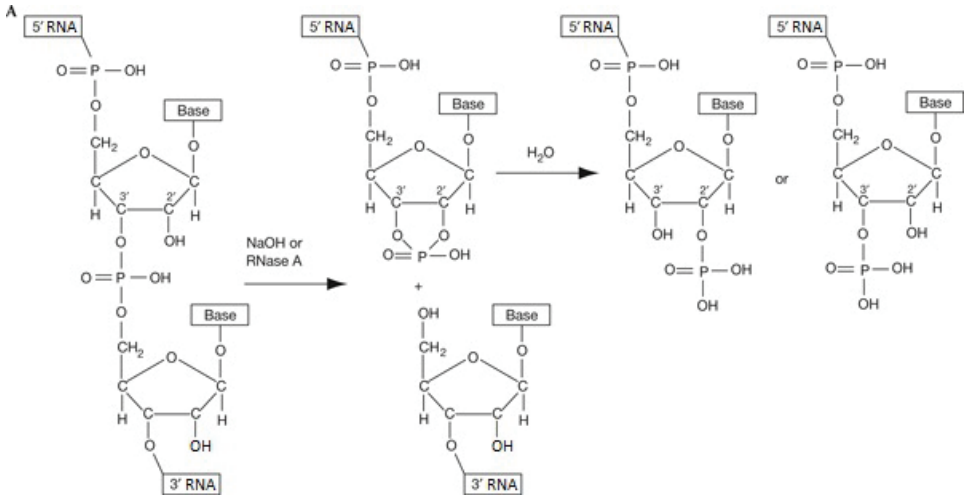

Fig. 7. Alkali catalyzed RNA hydrolysis. The 2'-OH group, present in RNA only, makes it OH^- sensitive. Besides a 5'-OH end, a cyclic 2',3'-P intermediate is released, which in turn produces a 3'-P or 2'-P end (modified from Vengrova and Dalgaard, 2005).

Thus, a strong base like NaOH is employed in the presence of low concentrations of a non-ionic surfactant, whose role has been earlier discussed. Treatments are performed using prewarmed solutions, so as to allow the reduction of both alkali concentration and exposure time. Indeed, it should be remembered that silica does not tolerate high alkali concentrations, as it forms silicates, resulting in matrix destruction and loss of binding properties. This is the reason why time of exposure to NaOH, its concentration and the temperature, as described in Nicosia *et al.* (2010), are crucial for the successful decontamination without impairing the columns integrity and efficieny, making it possible to reuse them several times.

The regeneration protocol is briefly reported below.

Box 2. RNA silica column regeneration protocol

1. Fill the silica column with a prewarmed (75°C) solution containing 0.2 N NaOH and 0.1% (v/v) Triton X-100™
2. Incubate 5 minutes at room temperature
3. Spin for 1 minute
4. Repeat step 1, incubate for 10'
5. Spin for 1 minute
6. Add a volume of a 50 mM sodium acetate/acetic acid buffer (pH 4) solution
7. Incubate at room temperature for 1 minute
8. Spin for 1 minute
9. Load the column with sterile ddH_2O
10. Spin for 1 minute

A different strategy is used to purify the poly-A^+ fraction of eukaryotic mRNAs, aiming to exclude the most abundant RNA classes like rRNAs, where oligo-dT covalently linked on the surface of polysaccharidic beads or similar solid supports are employed, as described earlier.

Many suppliers indicate, in instruction of such kits, that oligo-dT supports may be reused, and provide regeneration protocols always based on RNA hydrolysis by NaOH treatments, which will destroy any RNA traces, leaving unmodified the DNA component (oligo-dT).

4. Protein purification resins

4.1 IMAC

The use of immobilized-metal affinity chromatography (IMAC) for protein purification was firstly described and showed by Porath *et al.* (1975). Initially developed for purification of native proteins with an intrinsic affinity to metal ions, IMAC shows numerous application fields spanning from chromatographic purification of metallo and phosphorylated proteins, antibodies and recombinant His-tagged proteins. IMAC is also used in proteomics approaches where fractions of the cellular protein pool are enriched and analyzed differentially (phosphoproteome and metalloproteome).

IMAC is a chromatography method that can simply be scaled up linearly from milliliter to liter volumes (Block *et al.*, 2008; Hochuli *et al.*, 1988; Kaslow and Shiloach, 1994; Schäfer *et al.*, 2000) and Ni-NTA Superflow columns are in use for biopharmaceutical production processes.

It is based on the known affinity of transition metal ions such as Zn^{2+}, Cu^{2+}, Ni^{2+}, and Co^{2+} to certain amino acid in aqueous solutions (Hearon, 1948). Amino acids as histidine, cysteine, tryptophan, tyrosine, or phenylalanine, working as electron donors on the surface of proteins, are able to reversibly bind transition metal ions that have been immobilized by a chelating group covalently bound to a solid support. Histidine represents the preferential choice in protein purification using IMAC since it binds selectively immobilized metal ions even in presence of free metal ions excess (Hutchens and Yip, 1990b); additionally, copper and nickel ions have the greatest affinity for histidine.

Great improvement in development of IMAC chromatographic procedures was achieved by the introduction of DNA engineering techniques allowing the construction of fusion proteins in which specific affinity tags as 6xHis tag are added to the N-terminal or C-terminal protein sequence; the use of these strategies simplifies purification of the recombinant fusion proteins (Hochuli *et al.*, 1988). Moreover the identification or invention of chelating agent able to be both covalently bound to a support and interact with transitional metal ions contributed to the definition of IMAC for high-quality protein purification.

The chelating group that has been first used for IMAC proteins purification is iminodiacetic acid (IDA) (Porath *et al.*, 1975). IDA was charged with metal ions such as Zn^{2+}, Cu^{2+}, or Ni^{2+}, and then used to purify a variety of different proteins and peptides (Sulkowski, 1985)

The tridentate IDA group binds to three sites within the coordination sphere of divalent metal ions such as copper, nickel, zinc, and cobalt (Fig. 8). When copper ions (coordination number of 4) are bound to IDA, only one site remains available for interaction with proteins (Hochuli *et al.*, 1987). For nickel ions (coordination number of 6) bound to IDA, three valencies are available for imidazole ring interaction while it is unclear whether the third is sterically able to participate in the interaction binding to proteins. Thus Cu^{2+}-IDA complexes are stable on the column but have lower capacity for protein binding. Conversely, Ni^{2+}-IDA complexes bind proteins more avidly, but Ni^{2+}-protein complexes are more likely to dissociate from the solid support.

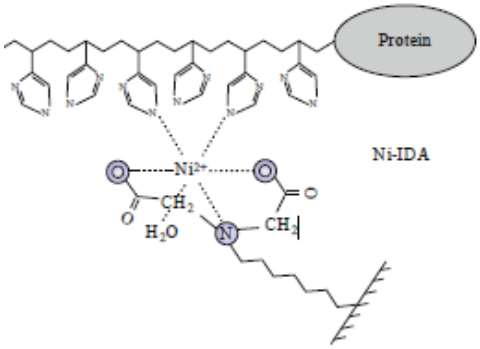

Fig. 8. Model of the interaction between residues in the His tag and the metal ion in tridentate (IDA) IMAC ligand.

The development of a new metal-chelating adsorbent, nitrilotriacetic acid (NTA), has provided a convenient and inexpensive tool for purification of proteins containing histidine residues (Hochuli *et al.*, 1987). The NTA chelating agent coordinates Ni^{2+} with four valencies

(tetradentate, coordination number 4) leaving two valencies available for binding to electron donor groups (i.e., histidine) on the surface of proteins (Fig. 9).

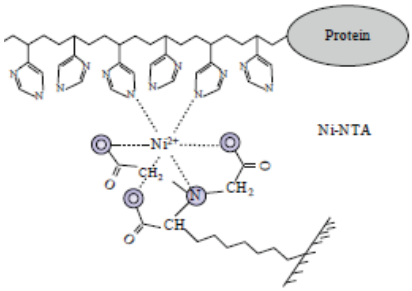

Fig. 9. Model of the interaction between residues in the His tag and the metal ion in tetradentate (NTA) IMAC ligand.

The coordination number plays an important role regarding to the quality of the purified protein fraction but not in protein yield. IDA has only 3 metal-chelating sites and cannot tightly bind metal ions, a relative weak binding leads to ion leaching after loading with strongly chelating proteins or during washing steps. This results in impure products, and metal-ion contamination of isolated proteins; meanwhile protein recovery is usually similar between the two chelating agent. Thus the advantage of NTA over IDA is that the divalent ion is bound by four rather than three of its coordination sites. This minimizes leaching of the metal from the solid support and allows for more stringent purification conditions (Hochuli, 1989).

The NTA also binds Cu^{2+} ions with high affinity, but this occupies all of the coordination sites, rendering the resulting complex ineffective for IMAC. Another tetradentate ligand is a chelating agent commercially known as Talon resin, consisting in carboxymethyl aspartate (CM-Asp), available as cobalt-charged (Chaga *et al.*, 1999).

The lowest metal leaching is obtained using *N*,*N*,*N*'-tris(carboxymethyl)ethylenediamine (TED), a pentadentate ligand (Fig.10). Because TED coordinates ions extremely tightly, such chelators represent a valid alternative expecially if low metal ion contamination is needed; nevertheless only one coordination site is avalaible for His tag binding and protein recovery is substantially lower than IDA or NTA

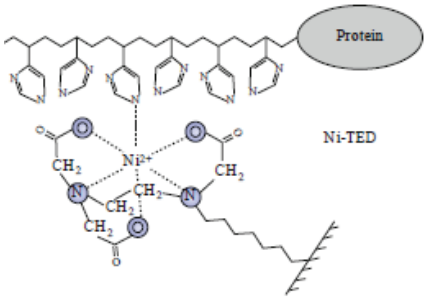

Fig. 10. Model of the interaction between residues in the His tag and the metal ion in pentadentate (TED) IMAC ligand.

The choice of the metal ion immobilized on the IMAC ligand depends on the application.

Whereas trivalent cations such as Al^{3+}, Ga^{3+}, and Fe^{3+}(Andersson and Porath, 1986; Muszynska *et al.*, 1986; Posewitz and Tempst, 1999) or tetravalent Zr^{4+} usually immobilized to IDA (Zhou *et al.*, 2006) are preferred for phosphoproteins and phosphopeptides capturing, divalent Cu^{2+}, Ni^{2+}, Zn^{2+}, and Co^{2+} ions are preferentially used for purification of His-tagged proteins. Combinations of a tetradentate ligand that ensure strong immobilization, and a metal ion that leaves two coordination sites available free for imidazole interaction (Ni^{2+} and Co^{2+}) allow similar recovery yield and eluted proteins quality. Immobilized copper or nickel ions bind native proteins with a K_d of $1x10^{-5}$ M and $1.7x10^{-4}$ M, respectively (Hutchens and Yip, 1990a). The K_d value is reduced for protein produced, using recombinant DNA technology, as chimeric constructs with an epitope containing six or more histidine residues. Addition of six histidines to the protein results only in 0.84 kDa protein mass excess whereas other fusion protein systems utilize much larger affinity groups that must be often removed to allow normal protein function (e.g., glutathione-*S*-transferase, protein A, Maltose Binding Protein). Furthermore the lack of His-tag immunogenic activity allows injection into animals for antibody production without tag removal. Addition of a His-tag results in an enhanced affinity for Ni^{2+}-NTA complex binding due to K_d value of 10^{-13} M at pH 8.0 even in the presence of detergent, ethanol, 2 M KCl (Hoffmann and Roeder, 1991), 6 M guanidine hydrochloride (Hochuli *et al.*, 1988), or 8 M urea (Stüber *et al.*, 1990) allowing protein purification under both native and denaturing conditions, as well as both oxidizing and reducing conditions providing a stringent environment avoiding host strain proteins co-purification (Jungbauer *et al.*, 2004). Nevertheless proteins intrinsically expressing chelating amino acids, such as histidine on their surface, are able to interact with an IMAC support and, although usually with lower affinity than a His-tagged protein, co-purify. In *E. coli*, proteins observed to copurify with His-tagged target proteins, especially in native conditions, can be classified into four groups (Bolanos-Garcia and Davies, 2006):

1. proteins with natural metal-binding motifs,
2. proteins displaying histidine clusters or stretches on their surfaces,
3. proteins interacting directly or not with heterologously expressed His-tagged proteins,
4. proteins showing affinity to IMAC support such agarose or sepharose based supports.

Furthermore, some copurifying proteins seem to have a binding preference for Co^{2+} over Ni^{2+} (or other ions) and others vice versa. Several options have been developed in order to reduce the contaminating amount of copurified quote or avoiding their adsorption to the matrix, including additional purification steps, adjusting the His-tagged protein to resin ratio, to using an engineered host strain that does not express certain proteins, using an alternative support, tag cleavage followed by reverse chromatography and reduction of non specific binding by including imidazole in the lysis and washing buffer.

Since there is an higher potential of binding background contaminants under native conditions than under denaturing conditions, low concentrations of imidazole in lysis and wash buffers (10–20 mM) could be used. The imidazole ring is part of histidine structure and it's responsible for Ni-NTA interaction (Fig. 11).

At low imidazole concentrations, non specific binding is prevented, while 6xHis-tagged proteins, because of the K_d value derived, still bind strongly to the Ni-NTA matrix allowing greater purity in fewer steps.

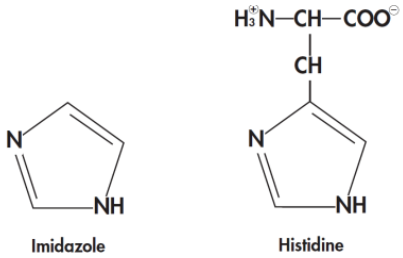

Fig. 11. Chemical structures of histidine and imidazole.

Binding of tagged proteins to Ni-NTA resin is not conformation-dependent and is relatively not affected, within a certain concentration range, by most detergents and denaturants, so Triton X-100 and Tween 20 (up to 2%), or high salt concentrations (up to 2 M NaCl) can be used, resulting in nonspecific binding reduction without affecting specific interaction.

As previously described, purification of tagged proteins under native conditions is often associated with copurification of coupled proteins such as enzyme subunits and binding proteins present in the expressing host (Le Grice and Grueninger-Leitch, 1990; Flachmann and Kühlbrandt 1996). Purification in denaturing condition is performed in presence of strong chaotropic agents such as 6 M GuHCl or 8 M Urea. Under these conditions the 6xHis tag on the protein surface is fully exposed so that binding to the Ni-NTA matrix will improve, and the efficiency of the purification procedure will be maximized by reducing the potential of non specific binding. The histidine tail binds to the Ni^{2+}-NTA resin via the imidazole ring of the histidine residues. At pH ≥7.0, the imidazole side chain is deprotonated, leading to a net negative charge interacting with Ni^{2+}-NTA; at pH 5.97 (corresponding to imidazole pK_a), 50% of the histidines are protonated; finally, within pH values ≤4.5, almost all of the histidines are protonated and unable to interact with Ni^{2+}-NTA. Thus, there are, generally, three different methods for His-tagged proteins recovery after washing steps based on chemical and cinetical counterpart features that can be used for both native or denaturing purifications.

A"competition derived approach"based on Ni^{2+}-NTA affinity for imidazole, working as competitor, increasing imidazole concentrations results in protein displacement from the support at constant pH. Under these conditions the 6xHis-tagged protein can no longer bind to the nickel ions and will dissociate from the Ni-NTA resin.

An alternative procedure uses buffers of decreasing pH to elute the histidine tail ensuring efficient recovery from Ni^{2+}-NTA (Hochuli *et al.*, 1988). Disadvantages are that the pH must be maintained accurately at all temperatures and that some proteins may not be able to withstand the extreme pH change required for protein elution.

An optional method is based on the stripping ability of certain reagents such as EDTA or EGTA in chelation of nickel ions and their removal from the NTA groups. This results in the 6xHis-tagged protein elution as a protein–metal complex. NTA resins, so stripped, appear white in color because they have lost their nickel ions and must be recharged if additional purification steps have to been performed.

Whereas all elution methods (imidazole, pH, and EDTA) are equally effective, imidazole is recommended under native conditions, when the protein would be damaged by a pH reduction or when the presence of metal ions in the eluate needs to be avoided

4.2 Cleaning and regeneration of Ni-NTA resins

The suitability of IMAC for industrial production purposes has been largely demonstrated and it can be expected that IMAC-based procedures will acquire increasing application because of its robustness and relatively low requirements for individual optimization. In contrast to these facilities it's noteworthy the production of a large amount of discarted materials consisting in metal-chelating groups, IMAC supports such as agarose and sepharose ones and, above all, considerable metal transition amounts to be disposed.

In order to reduce the environmental impact of such wastes, several IMAC commercially manufacturers have introduced and developed protocols allowing to reuse the same resin after regeneration and equilibration step cycles. Regeneration methods, enabling the flush out of any contaminating materials from previously purified samples, can be divided into 2 different classes:

1. CIP (cleaning-in-place) protocols;
2. Stripping and recharging.

A simple and effective cleaning procedure for Ni-NTA resins used to purify proteins from different samples is represented by the incubation of such resins with a non-flammable, bacteriostat 0.5M NaOH solution for 30 min in 15 column volumes (Schäfer *et al.*, 2000) allowing denaturation and desorption of unspecifically resin-attached proteins.

Box 3. Ni-NTA agarose regeneration protocol

1. Wash the column with 2 volumes of Regeneration Buffer (6 M GuHCl, 0.2 M acetic acid).
2. Wash the column with 5 volumes of H_2O.
3. Wash the column with 3 volumes of 2% SDS.
4. Wash the column with 1 volume of 25% EtOH.
5. Wash the column with 1 volume of 50% EtOH.
6. Wash the column with 1 volume of 75% EtOH.
7. Wash the column with 5 volumes of 100% EtOH.
8. Wash the column with 1 volume of 75% EtOH.
9. Wash the column with 1 volume of 50% EtOH.
10. Wash the column with 1 volume of 25% EtOH.
11. Wash the column with 1 volume of H_2O.
12. Wash the column with 5 volumes of 100 mM EDTA, pH 8.0.
13. Wash the column with H_2O.
14. Recharge the column with 2 volumes of 100 mM $NiSO_4$.
15. Wash the column with 2 volumes of H_2O.
16. Wash the column with 2 volumes of Regeneration Buffer.
17. Equilibrate with 2 volumes of a suitable buffer

Resins stored for long terms in up to 1 M NaOH do not show any significant effect on metal-leaching rates corresponding to 1 ppm under any conditions without compromising its performance.

For repeated reuse of a Ni-NTA column, the CIP procedures had to be followed by a reequilibration step. Furthermore for long-term storage, resin may be kept in 30% (v/v) ethanol to inhibit microbial growth. No significant changes of metal-ion leaching were observed during five CIP runs, moreover the binding capacities for 6xHis-tagged protein of Ni-NTA resins remained unchanged from run 1 to run 5 (Schäfer *et al.*, 2000).

Due to the high chelating strength and the resulting low metal-leaching rate of all Ni-NTA IMAC resins, stripping is not required even after repeated reuse or long-term storage. However, reduction in binding capacity or resin damages for example, by repeated purification of samples containing chelating agents, could happens. In this cases Ni-NTA may be stripped and recharged with nickel or a different metal ion using combination of chelating steps (EDTA treatments) ensuring a Ni^{2+} free medium, followed by nickel salts incubation. Metal chloride and sulfate salts, (e.g. 0.1 M $NiSO_4$) are commonly used. Here we report (box 3)a stripping and recharging protocol based on Qiagen instruction for relative Ni-NTA agarose resins

4.3 IMAC for industrial-scale protein production and Ni^{2+} environmental impact

IMAC for production of proteins in industrial scale, has not been used until quite recently due to worries regarding allergenic effects of nickel leaching from an IMAC matrix. During protein purification 1ml or resins is usually used for each 30-40 mg recombinant proteins. Several data describing nickel leaching from resins show that nickel concentrations in the peak elution fractions is below 1 ppm under all conditions, including denaturant or native conditions. More specifically even after several purification steps followed by CIP, the level of nickel contamination in the peak elution fractions is comprised between 0.3 and 0.6 ppm for native and denaturing conditions, respectively (Schäfer *et al.*, 2005). The discarded cations are released as liquid or dry waste into the environment where it's just present under many forms.

Nickel, occurs naturally in the earth's crust, in various forms such as nickel sulphides and oxides, its sources arise from earth's molten core where it is trapped and unusable to volcanic eruptions, soils, ocean floors, and ocean water (Stimola, 2007).

Such divalent cation is used not only in metallurgic industries to make stainless steel but also in other application fields such as in coinage in various forms of 'costume' or 'fashion' jewellery. The different forms of nickel include elemental nickel (Ni), nickel oxide (NiO), nickel chloride ($NiCl_2$), nickel sulphate ($NiSO_4$), nickel carbonate ($NiCO_3$), nickel monosulfide (NiS), and nickel subsulfide (Ni_3S_2) (ATSDR, 2005).

Human exposure to nickel is associated with drinking water, food, or smoking tobacco containing nickel or direct contact with nickel-containing products, such as jewelry, stainless steel and coins. The average concentration of nickel in different categories of soil span from 4 to 80 ppm, but this number has increased significantly (up to 9,000 ppm) around nickel producing industries (ATSDR, 2005). Skin contact is the usual source of contamination from the ground unless for children who are more likely to ingest soil particles. Foods such as tea, coffee, chocolate, cabbage, spinach and potatoes contain high levels of nickel, making these foods a major source of exposure. The average amount of nickel introduced is 70 micrograms of nickel per day.

This rapid analysis suggests nickel concentrations typically observed in protein preparations obtained from tetradentate IMAC resins are low and content in expected daily doses of protein used such as biopharmaceutical will be far below the typical daily intake of nickel.

4.4 Amylose affinity chromatography

The expression and purification of recombinant proteins compared to native ones represent an efficient system to product any protein. As previously described for IMAC tag, recombinant DNA techniques allow the construction of fusion proteins in which specific affinity tags are added to the protein sequence of interest, facilitating the recombinant fusion proteins purification by the use of affinity chromatography methods.

Maltose-binding protein (MBP) is one of the older and more popular fusion partners used for recombinant proteins production in bacterial cells; it's coded by the *malE* gene of *Escherichia coli* as part of maltose/maltodextrin system (Nikaido, 1994). MBP, despite the molecular weight (42.5 kDa) is considered one of the best choises to solve problems related to heterologous protein expression since it acts as protein production and solubilisation enhancer by mechanisms far to be completely understood (Randall *et al.*, 1998; Nomine *et al.*, 2001; Sachdev and Chirgwin, 1998). Several commercial plasmid DNA vectors have been constructed allowing expression of a cloned protein or peptide by fusing it to MBP (Guan *et al.*, 1988; Bedouelle and Duplay, 1988; Maina *et al.*, 1988). The isolation and purification of recombinant proteins MBP fused can be performed using an easy affinity column procedure amylose based resins dependeding on MBP affinity for maltose packaged in the amylose resins (K_d value of MBP for maltose is 3.5 µM) (Kellerman and Ferenci, 1982). A crude cell extract, in absence of detergent or chaotropic agents, is prepared and passed over a column containing an agarose resin derivatized with amylose, a polysaccharide consisting of maltose subunits.

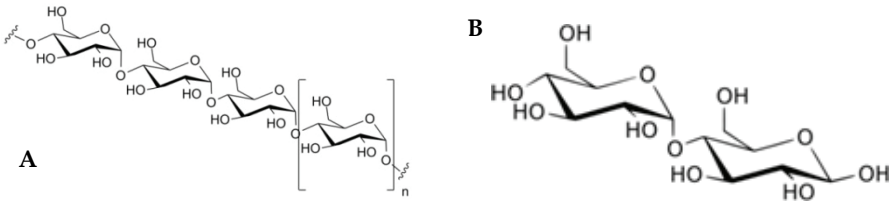

Fig. 12. Chemical structures of amylose (A) and maltose (B). Glucose monomers (2 units in maltose, several hundreds in amylose) are joined with an α(1→4) bond.

Such resin can be purchased from commercial suppliers in it's original form (amylose based) or in an maltoheptaose version similar to amylose one, but with lower molecular weight glucose polymers resulting in a theorical larger number of potential binding sites. Three amylose affinity chromatography matrices are manufactured by New England BioLabs (Cattoli and Sarti, 2002):

1. Amylose magnetic beads;
2. Amylose agarose resin;
3. High flow support matrix.

Amylose magnetic beads have a binding capacity up to 10 µg/mg (supplied as a 10 mg/ml suspension). Amylose agarose has a binding capacity of 3 mg/mL for MBP and 6 mg/ml for an MBP-β-galactosidase protein. The typical flow velocity of the amylose resin is 1 ml/min in a 2.5 cm x 10 cm column, and the matrix can withstand small manifold vacuums (universally known as "piglet"). The amylose matrix can suffer from flow restrictions. So that total protein loading should be ≤2.5 mg/ml. Amylose high flow has a binding capacity of approximately 7 mg/ml for an MBP-paramyosin protein. The exact chemical nature of the

matrix is not described but has a pressure limit of 0.5 MPa (75 psi), a maximum flow velocity of 300 cm/h, and recommended velocities are below 60 cm/h being 10–25 ml/min (for Ø1.6-cm and Ø2.5-cm columns respectively).

Alternatively, home-made amylose-agarose resin can be prepared following procedures described by Lee *et al.* (1990). Pratically, sepharose beads are washed with water and then incubated with 1M sodium carbonate pH 11 allowing to react in presence of vinyl sulfonic acid. Activated resin is derivatized by mixing, in 1 M sodium carbonate pH 11 environment, with an amylose solution. The resulting matrix can be freshly used or in 20% ethanol stored.

In contrast with an IMAC conformation-independent binding of tagged proteins to Ni-NTA resin, MBP's affinity to amylose and maltose depends on hydrogen bonds patterns derived from the three-dimensional structure of the protein; agents interfering with hydrogen bonds or the protein structure interfere with binding as well. For these reasons protein purification of tagged proteins can be performed under native conditions only, (Tris-HCl, MOPS, HEPES, and phosphate, buffers at pH values between 6.5 and 8.5) in presence or absence of optional additives as 1 mM sodium azide, 10 mM β-mercaptoethanol or 1 mM DTT. Such reducing agents can be added to mantein reduced cysteins avoiding non specific disulphide bridges formation resulting in tedious aggregations. Moreover higher ionic strength does not adversely affect MBP binding to amylose, so that 1M NaCl can be used to reduce non specific protein binding to resin.

Despite MBP's affinity of some fusions to amylose is dramatically reduced in presence of nonionic detergents (0.2% Triton X–100 or 0.25% Tween 20) resulting in<5% binding, other fusions are unaffected. Binding is efficient in the presence of 5% ethanol or acetonitrile, as well as in 10% glycerol. 0.1% SDS completely eliminates binding.

Furthermore low levels of residual detergents, especially from regeneration solutions, (see below) can still remain; removal of detergent and mixed micelles can be achieved using dilute methanol-containing solutions

After several washing steps, protein elution and recovery is performed in a "competition derived approach" based on MBP affinity for maltose. Maltose working as competitor at 10 mM concentration, results in protein displacement from amylose at constant pH value.

Because the presence of substantial amounts of amylases in the crude extracts interferes with binding, by cutting the fusion off the column or by releasing maltose that elutes the fusion from the column, the amylose resin "half-life" depends on incubation time with trace amounts of contaminant. Manufacturers instructions and recommendations explain (e.g. NEB): "Under normal conditions defined as 15 ml of amylose agarose matrix processing, 1 liter of LB media supplemented with 0.2% glucose (producing 40 mg MBP fusion protein); a matrix binding capacity reduction of 1–3% after each purification step is reported. It is stated that such a column may be used up to 5 times before a decrease in yield is detectable (5–15% lost binding capacity), and up to 10 times to achieve an evident reduction (10–30% lost binding capacity)".

Column reuse and regeneration can be performed according to New England BioLabs following sequence of washes in water, saline buffer (20 mM Tris-HCl, 200 mM NaCl and 1 mM EDTA), and 0.1% SDS, or by a very short treatment using 0.1 N NaOH followed by a neutralization step.

Alternatively Pattenden *et al.* (2008) proposed a regeneration procedure based on sequential amylose resin treatments with two different regeneration solutions:

1. Regeneration 1: 50 mM HEPES, 4 M Urea, 0.5% w/v SDS pH 7.4.
2. Regeneration 2: 50 mM HEPES, 150 mM $(NH_4)_2SO_4$, 2 mM EDTA, 2 mM EGTA pH 7.4.

Regenerated resin can be stored in 20% ethanol at 4 °C

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