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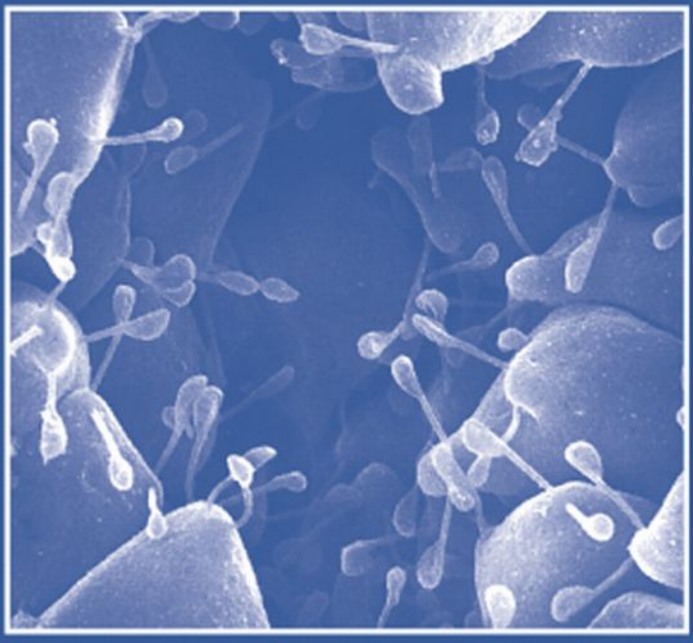

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CHAPTER ONE

Physiological Roles of Ribosomal Protein S6: One of Its Kind

Oded Meyuhas*

Contents

Abstract

The phosphorylation of ribosomal protein S6 (rpS6), which occurs in response to a wide variety of stimuli on five evolutionarily conserved serine residues, has attracted much attention since its discovery more than three decades ago. However, despite a large body of information on the respective kinases and the signal transduction pathways, the role of this phosphorylation remained obscure. It is only recent that targeting the genes encoding rpS6, the phosphorylatable serine residues or the respective kinases that the unique role of rpS6 and its posttranslational modification have started to be elucidated. This review focuses primarily on the critical role of rpS6 for mouse development, the

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pathways that transduce various signals into rpS6 phosphorylation, and the physiological functions of this modification. The mechanism(s) underlying the diverse effects of rpS6 phosphorylation on cellular and organismal physiology has yet to be determined. However, a model emerging from the currently available data suggests that rpS6 phosphorylation operates, at least partly, by counteracting positive signals simultaneously induced by rpS6 kinase, and thus might be involved in fine-tuning of the cellular response to these signals.

Key Words: Ribosomal protein S6, S6 kinase, RSK, mTOR, Protein synthesis, TOP mRNAs, Cell size, Cell proliferation, Glucose homeostasis.

1. Introduction

The higher eukaryotic ribosomes are composed of two subunits that are designated as 40S (small) and 60S (large) subunits. The mammalian 40S subunit is composed of a single RNA molecule, 18S ribosomal (r) RNA, and 33 proteins, whereas the 60S subunit has three RNA molecules: 5S, 5.8S, and 28S rRNAs, and 46 proteins (Wool *et al.*, 1996). Of all ribosomal proteins, it is ribosomal protein S6 (rpS6) that has attracted much attention, since it is the first, and was for many years the only one, that has been shown to undergo inducible phosphorylation.

The ribosome biogenesis takes place in the nucleolus and starts with the synthesis of 5S and 45S pre-rRNA by distinct RNA polymerases and requires the import of ribosomal proteins from the cytoplasm. A complex pathway that involves both endo- and exonucleolytic digestions enables the release of mature rRNAs from the pre-rRNA. Concomitantly, rRNAs are extensively modified and bound by the ribosomal proteins before the assembled pre-40S and pre-60S subunits are exported separately to the cytoplasm (Fromont-Racine *et al.*, 2003; Zemp and Kutay, 2007). High-resolution cytological analysis has recently disclosed the fate of rpS6 from its biosynthesis site in the cytoplasm to the pre-40S subunit. Thus, rpS6 enters the nucleus of HeLa cells, reaches, via Cajal bodies, the nucleolus, where it is assembled with other proteins and rRNA into pre-40S subunit. The latter is then released to the nucleoplasm prior to its export through the nuclear pores to the cytoplasm (Cisterna *et al.*, 2006). Interestingly, the nuclear import, as well as the nucleolar localization of human rpS6 and yeast rpS6A, rely on motifs, whose number, nature, and position are evolutionary conserved (Lipsius *et al.*, 2005; Schmidt *et al.*, 1995).

The phosphorylation of rpS6 has attracted much attention in numerous labs since its discovery in 1974 (Gressner and Wool, 1974b). However, it is only recently that the role of rpS6 and its posttranslational modification has started being disclosed by genetic targeting of the *rpS6* gene and of the

respective kinases. Hence, this review includes a brief account on the evolutionary conservation of rpS6, as well as the enzymes that conduct, and the cues that affect its phosphorylation, and a comprehensive discussion on the critical role of rpS6 for mouse development, pathways that transduce various signals into rpS6 phosphorylation, and the physiological role of this modification.

2. General Background

2.1. Evolutionary conservation of rpS6

rpS6 is an evolutionary conserved protein that spans 236–253 residues in species as remote as yeast, plants, invertebrates, and vertebrates (Fig. 1.1), yet no homology with any ribosomal protein in *E. coli* or archebacteria has

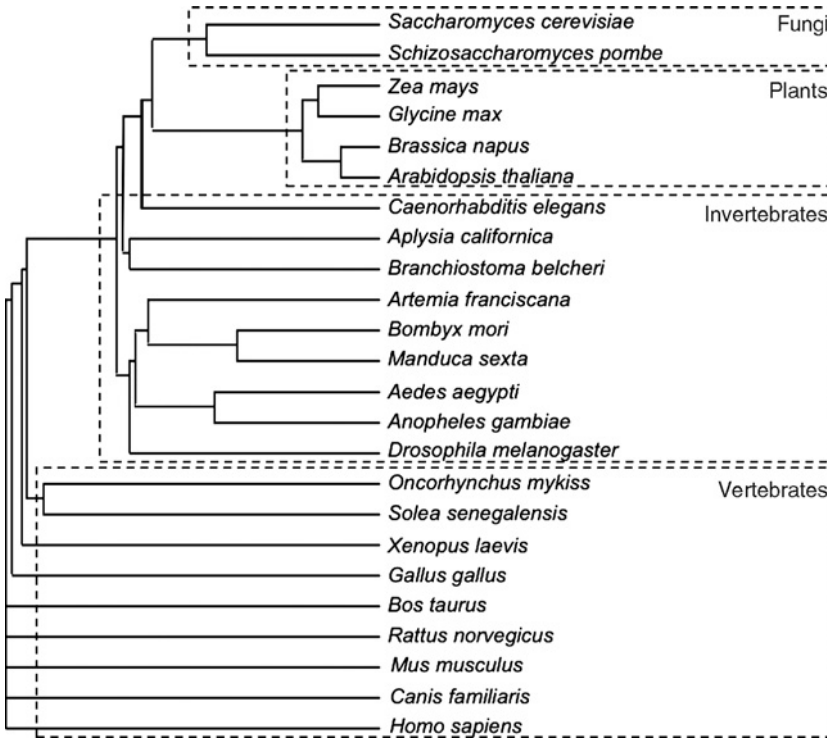

Figure 1.1 The phylogenetic tree of sequences of rpS6 orthologs in different eukaryotic species. Alignments and the tree were generated using the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw/index.html).

been detected (Wool *et al.*, 1996). Interestingly, rpS6 with C-terminal extensions, ranging in length from 81 to 190 amino acids and enriched for lysine and alanine, is widespread among the Culicomorpha (an infraorder of Nematocera that includes mosquitoes and black flies) (Fallon and Li, 2007). The C-terminal extensions on rpS6 from the mosquitoes *Aedes aegypti* and *Aedes albopictus* are 42–49% homologous with histone H1 proteins from other multicellular organisms (Hernandez *et al.*, 2003).

2.2. rpS6 is an indispensable ribosomal protein

The role of rpS6 was first addressed by conditional knockout of the respective gene in adult mouse liver (Volarevic *et al.*, 2000). Hepatocytes that lacked *rpS6* gene failed to synthesize the 40S ribosomal subunit and consequently to proliferate following partial hepatectomy. This failure to progress through the cell cycle correlated with a block in expression of cyclin E gene. Nonetheless, the expression of *rpS6* gene was not required for liver growth when starved mice were refed. Moreover, the relative engagement of liver ribosomes in translation, as exemplified by their polysomal association, was indistinguishable between rpS6-containing and -lacking hypertrophying livers (Volarevic *et al.*, 2000).

The critical role of rpS6 is not confined to the regenerating liver, as thymus-specific knockout of *rpS6* gene, but not conditional deletion of one allele, had devastating effect on the gland development (Sulic *et al.*, 2005). rpS6 heterozygosity ($rpS6^{wt/del}$), however, had a remarkable effect on the number of mature T cells in peripheral lymphoid organs (spleen and lymph nodes). The deficiency of one *rpS6* allele led to a proportional diminution in the abundance of rpS6 and ribosome contents in purified $rpS6^{wt/del}$ T cells, yet with no effect on their total protein content or their ability to undergo normal stimulated cell growth (Sulic *et al.*, 2005). Likewise, 30–50% reduction in rpS6 content of HeLa cells by siRNA only mildly affected global protein synthesis (Montgomery *et al.*, 2006). Nevertheless, while wild-type T cells progressed *in vitro* through several divisions upon mitogenic stimulation, their $rpS6^{wt/del}$ counterparts failed to proliferate, as a result of a block at the G1/S checkpoint of the cell cycle, and partially due to increased apoptosis. Interestingly, deletion of both *p53* alleles almost completely resumed the proliferative capacity of stimulated $rpS6^{wt/del}$ T cells. These observations strongly support the notion that impaired ribosome biogenesis, associated with rpS6 deficiency, activates a p53-dependent checkpoint to eliminate defective T cells (Sulic *et al.*, 2005).

$rpS6^{wt/del}$ embryos died during gastrulation at day 8.5. However, already at day 6.5, their cells failed to show dephosphorylation and activation of Cdk1 and to enter mitosis. Moreover, the embryonal death was preceded by induced apoptosis. The fact that *p53* gene knockout enabled $rpS6^{wt/del}$ embryo to develop past gastrulation stage, suggests that rpS6 heterozygosity

triggers a p53-mediated checkpoint during gastrulation. Interestingly, ribosome biogenesis is defective in *rpS6*$^{wt/del}$/*P53*$^{-/-}$ embryo, as well as in the corresponding mouse embryo fibroblasts (MEFs). However, while neither cell cycle progression nor cell growth is impaired in *rpS6*$^{wt/del}$/*P53*$^{-/-}$ MEFs, compromised cell proliferation was observed in the liver from *rpS6*$^{wt/del}$/*P53*$^{-/-}$ embryo. This decreased in hepatic proliferation might be explained by the relative deficiency of cyclins D1 and D3, observed in this organ (Panic *et al.*, 2006).

Lesions in *Drosophila rpS6* gene expression, due to insertion of P element upstream of the transcription initiation site, had a mixed response: hyperplasia of lymphglands on the one hand and growth inhibition of most larval organs on the other hand (Stewart and Denell, 1993; Watson *et al.*, 1992).

The critical role of rpS6 is underscored by the fact that it is the only ribosomal protein, for which it has been shown, so far, that heterozygosity leads to early embryonal lethality (Panic *et al.*, 2006). Thus, *rpL24*$^{-/-}$ mice die before E9.5, yet the heterozygotes are viable, even though exhibiting dysmorphic feature and reduced somatic growth (Oliver *et al.*, 2004). Similarly, *rpS19* null mutation is lethal prior to implantation, whereas rpS19$^{+/-}$ mice have normal growth and organ development (Matsson *et al.*, 2004). Unlike rpS6, rpL29 seems to be a dispensable ribosomal protein, as mice with disruption of both *rpL29* alleles suffer of global growth deficiency, yet they are viable (Kirn-Safran *et al.*, 2007). Similarly, it has recently been shown that efficient depletion (2% residual activity) of rpL13a in human monocytic cells by short hairpin RNA had no significant effect on global protein synthesis, translational fidelity, or cell proliferation. These results suggest, therefore, that rpL13a is dispensable for canonical ribosome function (Chaudhuri *et al.*, 2007).

3. Phosphorylation of rpS6

3.1. Stimuli inducing rpS6 phosphorylation

A pioneer study conducted by David Kabat has shown that a 33-kDa protein, termed F protein, which resided in the small ribosomal subunit undergoes phosphorylation in rabbit reticulocytes (Kabat, 1970). Later, it has been identified as rpS6, and that it is the only ribosomal protein that undergoes phosphorylation during rat liver regeneration (Gressner and Wool, 1974b). A flood of subsequent reports has demonstrated that rpS6 is subject to phosphorylation in response to numerous physiological, pathological, and pharmacological stimuli (see Table 1.1). Notably, this modification can be detected in both the cytosol and the nucleus (Pende *et al.*, 2004).

Table 1.1 rpS6 is phosphorylated by multiple

Treatment	Organism/cell	Phosphorylation	References
(A) Mitogenic stimulation			
(a) Liver regeneration	Rat	⇑	Gressner and Wool (1974b)
(b) Growth factors and cytokines			
(1) Serum, IGF	Chick embryo fibroblasts	⇑	Haselbacher *et al.*, (1979)
(2) EGF	Mouse Swiss 3T3 cells	⇑	Thomas *et al.*, (1982)
(3) NGF	Rat PC12 cells	⇑	Halegoua and Patrick (1980)
(4) PDGF	Mouse Swiss 3T3 cells	⇑	Nishimura and Deuel (1983)
(5) Interleukin 2	Mouse T lymphocytes	⇑	Evans and Farrar (1987)
(B) Hormones			
(a) Insulin	Mouse 3T3-L1 cells	⇑	Smith *et al.*, (1979)
(b) Glucagon	Rat liver	⇑	Gressner and Wool (1976)
(c) Progesterone	*Xenopus* oocyte	⇑	Nielsen *et al.*, (1982)
(d) Estrogen	Rooster hepatocytes	⇑	Cochrane and Deeley (1984)
(e) PTTH	Tobacco hornworm	⇑	Song and Gilbert (1997)
(f) Juvenile hormone	Flesh-fly	⇑	Itoh *et al.*, (1987)
(C) Nutrients			
(a) Amino acids	Human HEK293 cells	⇑	Tang *et al.*, (2001)
(b) Leucine	Rat L6 myoblasts	⇑	Kimball *et al.*, (1999)
(c) Glucose	Mouse MIN6 β-cells	⇑	Gleason *et al.*, (2007)
(D) Lipid compounds			
(a) Diacyl glycerol	Mouse T lymphocytes	⇑	Evans and Farrar (1987)
(b) Prostaglandin $F_{2\alpha}$	Mouse Swiss 3T3 cells	⇑	Thomas *et al.*, (1982)
(E) Viral infection			
(a) Vaccinia virus	Human HeLa cells	⇑	Kaerlein and Horak (1976)

Table 1.1 (*continued*)

Treatment	Organism/cell	Phosphorylation	References
(b) Pseudorabies	Hamster fibroblasts	⇑	Kennedy *et al.*, (1981)
(c) Polyoma virus	Hamster fibroblasts	⇑	Kennedy and Leader (1981)
(d) Simian virus	Hamster fibroblasts	⇑	Kennedy and Leader (1981)
(e) Avian sarcoma virus	Chick embryo fibroblasts	⇑	Decker (1981)
(f) AMLV	Mouse NIH 3T3 fibroblasts	⇑	Maller *et al.*, (1985)
(g) Alphavirus	Human HEK293	⇓	Montgomery *et al.*, (2006)
(F) Stresses			
(a) Hypoxia	Human HEK293 cells	⇓	Arsham *et al.*, (2003)
	Maize root tips	⇓	Williams *et al.*, (2003)
(b) Heat shock	*Drosophila*	⇓	Glover (1982)
	Human HeLa cells	⇓	Kennedy *et al.*, (1984)
	Tomato cell suspension	⇓	Scharf and Nover (1982)
(c) Hyperosmolarity	Mouse myeloma	⇓	Kruppa and Clemens (1984)
(G) Pharmacological agents			
(a) Translation inhibitors			
(1) Cycloheximide	Rat liver	⇑	Gressner and Wool (1974a)
(2) Puromycin	Rat liver	⇑	Gressner and Wool (1974a)
(b) Transcription inhibitors			
(1) d-Galactosamine	Rat liver	⇑	Gressner and Greiling (1977)
(2) DRB	Human HeLa cells	⇑	Duncan and McConkey (1984)
(c) Energy depletion			
(1) 2-Deoxyglucose	Human HEK293	⇓	Inoki *et al.*, (2003)

(*continued*)

Table 1.1 (*continued*)

Treatment	Organism/cell	Phosphorylation	References
(2) 5-Thioglucose	Mouse embryo fibroblasts	⇓	Hahn-Windgassen *et al.*, (2005)
(d) Phorbol esters	Rat hepatoma cells	⇑	Trevillyan *et al.*, (1984)

Notes: ⇑, increase; ⇓, decrease; AMLV, Abelson murine leukemia virus; DRB, dichlororibofuranozyl benzimidazole; EGF, epidermal growth factor; IGF, insulin-like growth factor; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PTTH, prothoracicotropic hormone.

3.1.1. Evolutionary conservation of rpS6 phosphorylation sites

The phosphorylation sites in rpS6 in mammals and *Xenopus laevis* have been mapped to five clustered residues: S^{235}, S^{236}, S^{240}, S^{244}, and S^{247} (Bandi *et al.*, 1993; Krieg *et al.*, 1988; Wettenhall *et al.*, 1992), whose location at the carboxy terminus of higher eukaryotes is evolutionarily conserved (Table 1.2). It has been proposed that phosphorylation progresses in an ordered fashion, with Ser236 as the primary phosphorylation site (Flotow and Thomas, 1992; Wettenhall *et al.*, 1992). A similar organization of phosphorylation sites, relative to the carboxy terminus was described for *Drosophila melanogaster* rpS6 (Radimerski *et al.*, 2000 and Table 1.2).

Maize (*Zea mays*) rpS6 appears to be encoded by two genes and the resulting proteins are identical except for two amino acid substitutions. The relative location of the five phosphorylation sites is comparable with that of vertebrate rpS6, yet this set of sites is not confined to serines, as it include also a threonine residue (Williams *et al.*, 2003 and Table 1.2).

The first report on the phosphorylation of rpS6 (S10 according to an older nomenclature) in *Saccharomyces cerevisiae* lagged behind that of its mammalian counterpart (Hebert *et al.*, 1977). Yeast rpS6 is phosphorylated after transfer of a stationary culture to fresh nutrient medium, as well as at an early stage of germination, and as in other eukaryotes, the protein is dephosphorylated during heat shock (Jakubowicz, 1985; Szyszka and Gasior, 1984). However, yeast rpS6, unlike higher eukaryotes, bears only two phosphorylatable serine residues (Ser232 and Ser233) that correspond to Ser235 and Ser236 in the mammalian protein.

3.1.2. S6 kinase (S6K1 and S6K2)

Characterization of an S6 kinase at a molecular level was first achieved in *Xenopus* oocytes wherein the dominant form of S6 kinase detected after mitogenic stimulation had been purified as a 90-kDa polypeptide (Erikson and Maller, 1985), later termed as p90 ribosomal protein S6 kinase (RSK, also known as $p90^{RSK}$). Purification of the avian and mammalian major

Table 1.2 Sequences of the carboxy-terminus of rpS6 from different eukaryotes

Organisms	Phosphorylated serine residues	NCBI accession number
Yeast		
Saccharomyces cerevisiae (baker yeast)	AEKAEIRKRRA**SS**LKA^{236}	NP_015235
Schizosaccharomyces pombe (fission yeast)	QKREVVKARRASSLKK^{239}	NP_592833
Plants		
Zea mays (corn)	KRR**S**KL**S**-AAAKA**S**AA**TS**A^{251}	AAG02240
Arabidopsis thaliana (thale cress)	KKR*S*RL*S*SAAAKP*S*V-*T*-A^{250}	AF034217
Brassica napus (rape)	KKR*S*RL*S*SAAAKP*S*V-*T*-A^{249}	AAP46142
Glycine max (soybean)	KRR*S*KL*S*SAAKAAV^{247}	AAS47511
Invertebrates		
Caenorhabditis elegans	RRRSSASHHSESEVKKTSKK^{247}	Q9NEN6
Aplysia californica	RKRSNSRSKGDSIRESTSKK^{247}	AAG60623
Artemia franciscana	RRRSASVRSAGH^{242}	ABS18359
Branchiostoma belcheri (Japanese lancelet)	KRR*S*SARES*S*LRESK*S*KA^{244}	ABK32076
Aedes aegypti (yellow fever mosquito)[a]	RRR*S*RL*S*SMRDSRSSVGEER^{243}	AF154067
Anopheles gambiae (malaria mosquito)[b]	RRH*S*RL*S*SIRDSRSSLTSEK^{244}	BN000162
Bombyx mori (domestic silkworm)	RRR*S*A*S*MRD*S*KS*S*SQ*S*APQK^{253}	NP_001037566
Manduca sexta (tobacco hornworm)	RRR*S*A*S*MRD*S*KS*S*NQ*S*APQK^{253}	AAB06459
Drosophila melanogaster (fruit fly)	RRR**S**A**S**IRE**S**KS**S**VS**S**DKK^{248}	AAB05982
Vertebrates		
Oncorhynchus mykiss (rainbow trout)	RRRL*S*SLRA*S*TSK*S*ES*S*QK^{249}	AAD01429

(*continued*)

Table 1.2 (*continued*)

Organisms	Phosphorylated serine residues	NCBI accession number
Solea senegalensis (sole)	RRRL*S*S*L*RA*S*TSK*S*ES*S*QK^{249}	BAF45894
Xenopus laevis (African clawed frog)	RRRL**S**S*L*RA**S**TSK**S**ES**S**QK^{249}	AAC38014
Gallus gallus (chicken)	RRRL*S*S*L*RA*S*TSK*S*ES*S*QK^{250}	NP_990556
Rattus norvegicus (rat)	RRRL**S**S*L*RA**S**TSK**S**ES**S**QK^{249}	NP_058856
Mus musculus (mouse)	RRRL**S**S*L*RA**S**TSK**S**ES**S**QK^{249}	NP_033122
Canis familiaris (dog)	RRRL*S*S*L*RA*S*TSK*S*ESSQK^{249}	XP_531949.1
Bos taurus (cattle)	RRRL*S*S*L*RA*S*TSK*S*ES*S*QK^{249}	NP_001015548
Homo sapiens (human)	RRRL*S*S*L*RA*S*TSK*S*ES*S*QK^{249}	NP_001001

Notes: Bold letters, identified phosphorylation sites; italic letters, putative phosphorylation sites based on complete sequence conservation among closely related species. Alignments in metazoans and plants assume conservation of a stretch of three basic residues preceding the first phosphorylated serine.

[a,b] These proteins contain a C-terminal extension and span a total of 306 and 346 amino acids, respectively.

rpS6 kinases recovered 65–70 kDa polypeptides (Blenis *et al.*, 1987; Jenö *et al.*, 1988) that are currently referred to as S6K.

Mammalian cells contain two forms of S6K, S6K1, and S6K2 (also known as S6Kα and S6Kβ, respectively), which are encoded by two different genes and share a very high level of overall sequence homology. S6K1 has cytosolic and nuclear isoforms (p70 S6K1 and p85 S6K1, respectively), whereas both S6K2 isoforms (p54 S6K2 and p56 S6K2) are primarily nuclear (Martin *et al.*, 2001 and references therein) and partly associated with the centrosome (Rossi *et al.*, 2007). Analysis of rpS6 phosphorylation in mouse cells, deficient in either S6K1 or S6K2 suggests that both are required for full S6 phosphorylation, with the predominance of S6K2 (Pende *et al.*, 2004). Notably, the phosphorylation of the evolutionary conserved sites of *Drosophila* rpS6 (Table 1.2) is carried out by dS6K that is encoded by a single gene (Watson *et al.*, 1996).

Two putative S6K homologs, originally named atpk1/ATPK6 and atpk2/ATPK19, sharing 87% sequence homology were identified in *Arabidopsis* (Mizoguchi *et al.*, 1995; Zhang *et al.*, 1994). They were later referred to as atS6K1 and atS6K2, respectively (Turck *et al.*, 1998), of which

S6K2 was suggested to be an ortholog of the mammalian S6Ks, because this kinase, and not atS6K2, was able to phosphorylate the rpS6 (Zhang *et al.*, 1994).

Phosphorylation of yeast rpS6 is known for three decades (Hebert *et al.*, 1977), yet the identity of the respective kinase is still obscure. It should be noted, however, that Loewith and colleagues (Urban *et al.*, 2007) have recently suggested that Sch9 kinase might be the long sought for yeast S6K. This notion has been supported by several lines of circumstantial evidence: (a) Sch9, but not catalytically inactive Sch9, efficiently phosphorylated rpS6 *in vitro*; (b) a mutant rpS6, which contains two amino acid substitutions (S232A and S233A) previously shown to abolish phosphorylation of rpS6 *in vivo* (Kruse *et al.*, 1985), was not phosphorylated by Sch9; and (c) Sch9 seems to undergo phosphorylation by the yeast orthologs of the mammalian kinases that are known to phosphorylate S6K.

3.1.3. 90-kDa ribosomal protein S6 kinase (*RSK1–RSK4*)

RSKs are central mediators of extracellular signal-regulated kinase (ERK; for further details on this pathway, see Section 3.2.1.2) in regulation of cellular division, survival, and differentiation via phosphorylation of numerous intracellular proteins (Dümmler *et al.*, 2005 and references therein). Four RSK genes (*RSK1–RSK4*) have been identified in mammals, and RSK orthologs have been described in *Drosophila melanogaster* and *C. elegans*, but not in yeast and plants (Hauge and Frödin, 2006).

The discovery that S6K is the predominant rpS6 kinase in somatic cells (Ballou *et al.*, 1991; Chung *et al.*, 1992) has led to a widely accepted belief that RSK, despite its name, is physiologically irrelevant for rpS6 phosphorylation. However, recent observations have challenged this dogma. Thus, phosphorylation of rpS6 at Ser235 and Ser236 can still be detected, albeit at a much lower level, in cells lacking both S6K1 and S6K2. This phosphorylation is abolished by treatment by either U0126 [a mitogen-activated protein (MAP) and ERK kinases (MEK) inhibitor] or PD184352 (an ERK inhibitor), indicating the involvement of a MEK/ERK-dependent kinase (Pende *et al.*, 2004). Likewise, a recent study with HEK293E cells has shown that Ser235 and Ser236 remained partly phosphorylated in cells treated with rapamycin, that completely inhibits mammalian target of rapamycin (mTOR) and thereby its downstream target S6K, indicating the presence of an mTOR-independent pathway leading to rpS6 phosphorylation at these sites. Moreover, it has been shown that this phosphorylation is carried out, both *in vitro* and *in vivo*, by RSK, which phosphorylates rpS6 exclusively at Ser235 and Ser236 in a response to serum, growth factors, tumor-promoting phorbol esters, and oncogenic Ras (Roux *et al.*, 2007).

The consensus recognition sequences of S6K and RSK are similar, RxRxxS and R/KxRxxS, respectively, where x represents any amino acid and the carboxy terminal S is the phosphorylated serine residue

(Flotow and Thomas, 1992; Hauge and Frödin, 2006). Notably, however, the sequence context of serine 236 in rpS6 is the only one, among the phosphorylatable serine residues, that conforms to the consensus recognition sequence of these enzymes.

3.1.4. rpS6 dephosphorylation

The steady-state level of rpS6 phosphorylation is the product of a dynamic equilibrium between the activities of the respective kinases (S6Ks and RSKs) and the opposing phosphatases. Nonetheless, the fluctuations in rpS6 phosphorylations have been attributed, in nearly all the relevant reports, to parallel changes in the kinase(s) activity. In a few cases, however, the phosphorylation status of rpS6 has been primarily ascribed to the activity of a phosphatase rather than a kinase. Thus, Rous sarcoma virus-transformed chick embryo fibroblasts show attenuated dephosphorylation of rpS6 during mitosis and a parallel decrease in the activity of the protein phosphatase type 1 (PP1). This observation suggests that it is the PP1 activity that might control rpS6 phosphorylation under these circumstances (Belandia *et al.*, 1994). Conversely, rpS6 phosphorylation is not detectable in murine erythroleukemia or other hematopoietic cells and this constitutive dephosphorylation state appears to be due to the action of a phosphatase that is likely to act directly on rpS6 (Barth-Baus *et al.*, 2002). Notably, unlike the relatively detailed picture that has been generated regarding rpS6 phosphorylation and the respective kinases, the involving phosphatase(s) has yet to be identified. Clearly, unequivocal establishment of the physiologically relevant phosphatase will require the demonstration that its knockdown and/or conditional knockout renders rpS6 constitutively phosphorylated in cultured cells or a specific organ.

3.1.5. Other phosphorylated ribosomal proteins

Following many years, during which rpS6 "starred" as the only ribosomal protein that is phosphorylated in response to changes in the physiological milieu, it has recently been demonstrated that also rpL13a undergoes inducible phosphorylation. Thus, rpL13a is completely released from the ribosome upon 24-h interferon-γ treatment of monocytic cells. This release is associated with phosphorylation of the entire cellular pool of rpL13a that consequently assembles into a complex that operates as a translational repressor of ceruloplasmin mRNA (Mazumder *et al.*, 2003). Interestingly, rpL13a is the first, and so far the only, case of ribosomal protein that can leave completely the organelle and assemble in a different functional multicomponent complex.

Finally, the large ribosomal subunit in eukaryotic cells contains three acidic ribosomal proteins: P0, P1, and P2, whose constitutive phosphorylation is required for their assembly into the 60S subunit (Wool *et al.*, 1996 and references therein).

3.2. Signaling to rpS6 phosphorylation

Following the initial wave of descriptive reports on rpS6 phosphorylation, much effort has been invested in an attempt to establish the pathways that transduce various signals into activation or inhibition of the respective kinases. It should be noted, however, that at least for one stimulus, heat shock, there is a discrepancy between the apparent decrease in rpS6 phosphorylation (Glover, 1982; Kennedy *et al.*, 1984) and the lack (Vries *et al.*, 1997), or even opposite, effect on S6K1 activity (Jurivich *et al.*, 1991; Lin *et al.*, 1997). Hence, pathways discussed below are only those that have been documented to exert a parallel effect on both rpS6 phosphorylation and the activity of the respective kinase.

3.2.1. Growth factors

3.2.1.1. PI3K/Akt/TSC/Rheb/TORC1/S6K pathway Signaling to S6 phosphorylation by growth factors starts by activation of the respective receptor tyrosine kinase (RTK, Fig. 1.2). This, in turn, leads to activation of class I phosphatidylinositol 3-kinase (PI3K), either through direct binding to the

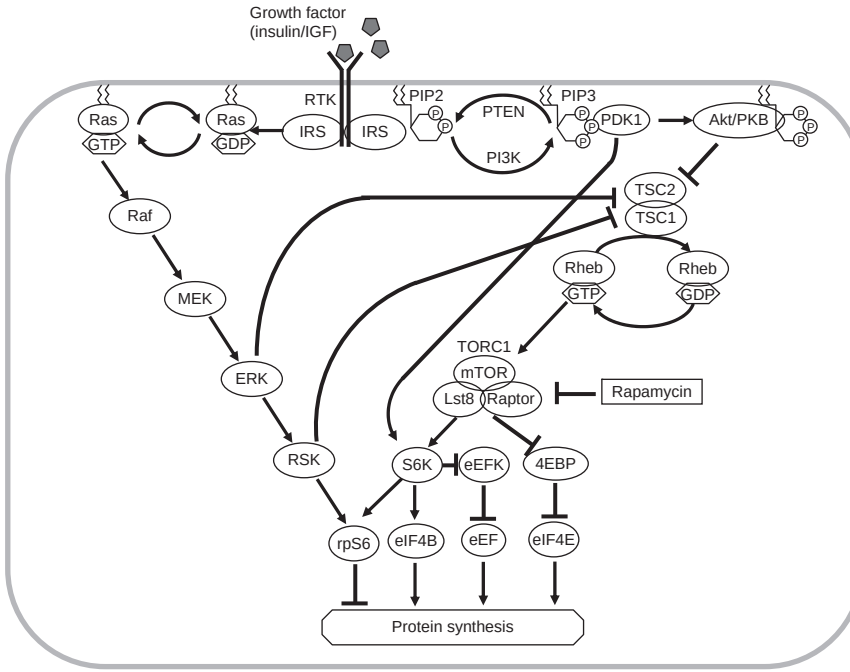

Figure 1.2 **Pathways transducing signals emanating from growth factors to rpS6 phosphorylation. Growth factors are represented by insulin of insulin-like growth factor (IGF). Arrows represent activation and bars inhibition. See text for details.**

phosphorylated receptor or through tyrosine phosphorylation of scaffolding adaptors, such as insulin receptor substrate (IRS), which then binds and activates PI3K (Cantrell, 2001). PI3K converts the lipid phosphatidylinositol-4,5-P2 (PIP2) into phosphatidylinositol-3,4,5-P3 (PIP3), in a reaction that can be reversed by the PIP3 phosphatase PTEN (phosphatase and *tensin* homolog deleted from chromosome 10) (Leslie and Downes, 2002). PIP3 recruits both 3-phosphoinositide-dependent kinase 1 (PDK1) and Akt [also known as protein kinase B (PKB)] to the plasma membrane (Brazil and Hemmings, 2001; Lawlor and Alessi, 2001), and PDK1 phosphorylates and activates Akt at T308 (Belham *et al.*, 1999). Activated Akt phosphorylates at multiple sites tuberous sclerosis complex 2 (TSC2), within the TSC1–TSC2 tumor suppressor dimmer (Inoki *et al.*, 2002; Manning *et al.*, 2002; Potter *et al.*, 2002). This phosphorylation blocks the ability of TSC2 to act as a GTPase-activating protein (GAP) for Rheb (Ras-homolog enriched in brain), thereby allowing Rheb–GTP to accumulate and to operate as an activator of the rapamycin-sensitive TOR complex 1 (TORC1) (Avruch *et al.*, 2006). The latter is consisting of target of rapamycin (TOR), Raptor (regulatory-associated protein of TOR), and LST8 (also known as GβL) (Bhaskar and Hay, 2007; Yang and Guan, 2007). Since it is TORC1 that conveys signals to S6Ks and rpS6, it will be mentioned in the remainder of this review, rather than mTOR, when transduction of signals is discussed. Akt can also activate TORC1 independently of TSC1–TSC2 by phosphorylating PRAS40, thereby relieving the PRAS40-mediated inhibition of TORC1 (Sancak *et al.*, 2007; Vander Haar *et al.*, 2007). Finally, it has been suggested that Akt can activate TORC1 not only by direct phosphorylation of TSC2, but also by elevating cellular energy (Hahn-Windgassen *et al.*, 2005). According to this model, Akt maintains a high ATP level that causes a decrease in the AMP/ATP ratio and thereby inhibits AMP-dependent kinase (AMPK)-mediated phosphorylation and activation of TSC2 (for further details on AMPK and TORC1, see Section 3.2.3).

Active TORC1 phosphorylates two translational regulators, S6Ks and eukaryotic initiation factor 4E (eIF-4E)-binding protein (4E-BP1, 2, and 3) (Hay and Sonenberg, 2004). Activation of S6Ks requires also phosphorylation by PDK1 in a reaction that does not need binding of PDK1 to PIP3 (Alessi *et al.*, 1998). Finally, activated S6K phosphorylates rpS6, as well as many other substrates (Ruvinsky and Meyuhas, 2006).

3.2.1.2. Ras/Raf/MEK/ERK/RSK pathway Activation of the second family of rpS6 kinases, the RSKs involves a distinct signaling pathway, even though it might share the same initial event, namely the ligand binding, with the PI3K/Akt/TORC1 pathway. Since RSK seemed to be a minor rpS6 kinase, the pathway leading to its activation is only very briefly described here. Thus, binding of insulin, as well as many other growth factors, to their receptors induces the activation of the small GTPase Ras

and consequently the recruitment of Raf to the membrane for subsequent activation by phosphorylation. Raf activates MAP kinase kinases 1 and 2 (MEK1/2), which in turn phosphorylates and activates ERK1 and ERK2. Activated ERKs phosphorylate and activate a vast array of substrates localized in all cellular compartments such as the RSK family [see Fig. 1.2 and refer to the following reviews for a more comprehensive picture (McCubrey *et al.*, 2007; McKay and Morrison, 2007)].

Interestingly, TSC2 is repressed by the Ras/MAPK pathway in addition to its downregulation by the PI3K/Akt pathway, as evidenced by the observation that activated Erk1/2 directly phosphorylates TSC2 at sites that differ from the Akt target sites, thereby causing functional inactivation of the TSC1–TSC2 complex (Ma *et al.*, 2005). Moreover, the MAPK-activated RSK1 also phosphorylates TSC2 at a unique site. This RSK1-mediated phosphorylation inhibits the TSC1–TSC2 complex and thereby increases TORC1 signaling toward S6K1 (Roux *et al.*, 2004).

3.2.2. Amino acid availability

Amino acid starvation, unlike serum starvation, fails to downregulate PI3K or PKB (Hara *et al.*, 1998; Wang *et al.*, 1998), yet it results in a rapid dephosphorylation of S6K1 and rpS6. Furthermore, readdition of amino acids restores the phosphorylation of these targets in a TORC1-dependent (rapamycin-sensitive) fashion (Tang *et al.*, 2001 and references therein). The requirement of the TSC1–TSC2 complex for amino acid-induced rpS6 phosphorylation was first suggested by showing that overexpression of TSC1 and TSC2 blocks amino acid-induced activation of S6K (Tee *et al.*, 2002). However, studies using TSC2-deficient mouse cells have yielded conflicting results, proposing that amino acids activate TORC1 in both a TSC1–TSC2-dependent (Gao *et al.*, 2002) and TSC1–TSC2-independent (Nobukuni *et al.*, 2005; Smith *et al.*, 2005) manner (Fig. 1.3).

Several lines of evidence suggest that Rheb mediates the effect of amino acids on TORC1 activity: (a) overexpression of Rheb in mammalian (Garami *et al.*, 2003) and *Drosophila* (Saucedo *et al.*, 2003) cells allows TORC1 signaling in the absence of amino acids; (b) knockdown of Rheb with siRNA greatly decreased TORC1 activation by amino acids (Nobukuni *et al.*, 2005); and (c) GTP-bound Rheb dissociated from mTOR upon depletion of amino acids. It should be noted, however, that others failed to detect amino acid-regulated binding of Rheb to mTOR (Smith *et al.*, 2005). Moreover, it is not yet clear how Rheb–mTOR interaction is controlled, as the GTP charging of Rheb is independent of amino acids (Long *et al.*, 2005; Smith *et al.*, 2005; Zhang *et al.*, 2003a).

Another mechanism whereby amino acids could affect TORC1 activity is via hVPS34 (vacuolar protein sorting 34). This class III PI3K (converts phosphatidylinositol to phosphatidylinositol-3-phosphate) has been shown to transduce the signal of amino acid sufficiency to TORC1 independently

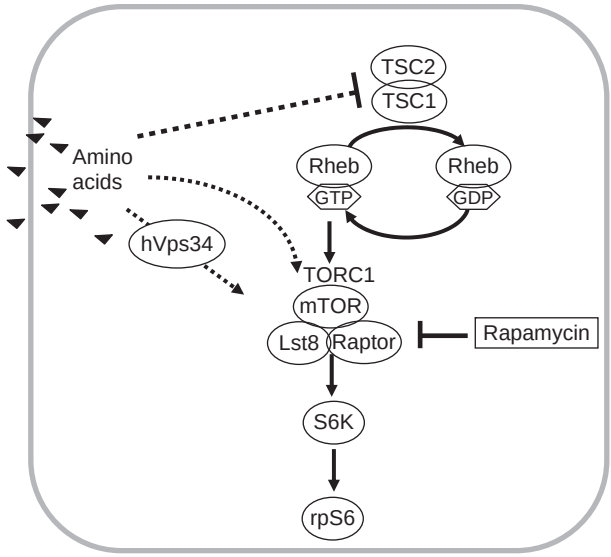

Figure 1.3 Amino acid signaling to rpS6 phosphorylation. Arrows represent activation, bars inhibition, and dotted lines putative links. See text for details.

of the TSC1–TSC2/Rheb axis (Byfield *et al.*, 2005; Nobukuni *et al.*, 2005). Nevertheless, the mechanism by which hVPS34 regulates mTOR is unknown.

A third mechanism for controlling TORC1 activity by amino acids suggests a direct effect on TORC1 integrity. Thus, withdrawal of amino acids in mammalian cells alters the binding of raptor to mTOR (Kim *et al.*, 2002). Clearly, the mechanism(s) by which amino acids sufficiency is transduced to TORC1 activation and thereby to rpS6 phosphorylation requires further studies.

3.2.3. Energy balance

Glucose starvation of mammalian cells or treating them with glycolytic [e.g., 2-deoxyglucose (2-DG)] or mitochondrial (e.g., valinomycin, antimycin A, oligomycin) inhibitors, depletes cellular energy and causes a concomitant decrease in TORC1 activity (Dennis *et al.*, 2001; Inoki *et al.*, 2003; Kim *et al.*, 2002). The prevailing model (Fig. 1.4) to explain how energy levels couple to the regulation of mTOR is via activation of AMP-activated protein kinase (AMPK) (Fig. 1.4). AMPK acts as a sensor of cellular energy status and is activated by increases in the cellular AMP: ATP ratio caused by metabolic stresses that either interfere with ATP production (e.g., deprivation for glucose or oxygen) or that accelerate ATP consumption (e.g., muscle contraction). Activation in response to

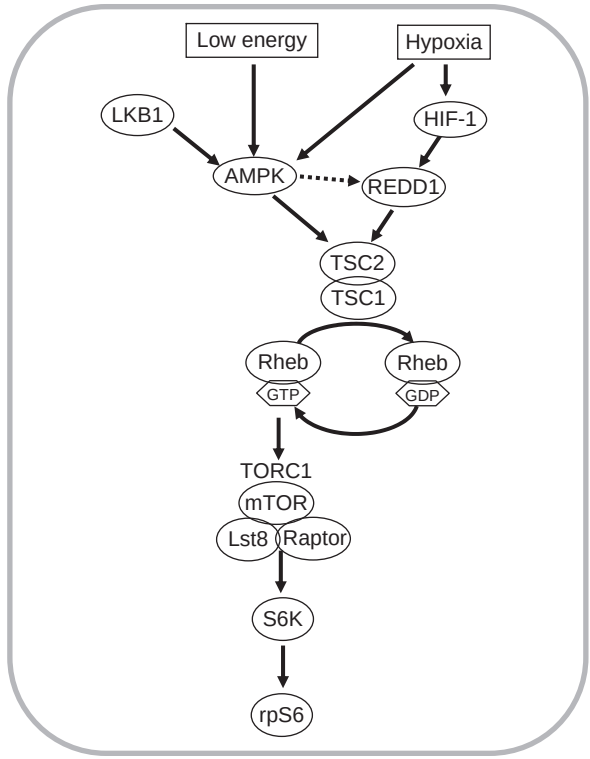

Figure 1.4 Pathways transducing energy balance and hypoxic signals to rpS6 phosphorylation. Arrows represent activation, bars inhibition, and dotted lines putative links. See text for details.

increases in AMP involves phosphorylation by an upstream kinase, the tumor suppressor LKB1 (Towler and Hardie, 2007), as AMPK activation in response to low energy conditions is blocked in LKB1 null cells (Corradetti *et al.*, 2004). Furthermore, LKB1 mutant cells exhibit hyperactive TORC1 signaling (Corradetti *et al.*, 2004; Shaw *et al.*, 2004). Activation of AMPK by 5-aminoimidazole-4-carboxyamide (AICAR), an AMP analog, inhibits TORC1-dependent phosphorylation of S6K1 (Bolster *et al.*, 2002). Likewise, expression of an activated form of AMPK decreases S6K1 phosphorylation, whereas a dominant-negative form of AMPK increases S6K1 phosphorylation (Kimura *et al.*, 2003).

AMPK phosphorylates several targets to enhance catabolism and suppresses anabolism in response to low energy, and exerts this effect by directly phosphorylating and activating TSC2, and thereby downregulates TORC1 (Inoki *et al.*, 2003). Thus, the phosphorylation of S6K1 is more resistant to glucose deprivation in $TSC2^{-/-}$ cells or cells whose mutant TSC2 cannot be

phosphorylated by AMPK (Inoki *et al.*, 2003). It appears, therefore, that energy depletion is sensed by AMPK and relayed to TORC1 through the TSC1–TSC2 complex (Fig. 1.4). However, it seems that cells can convey energy stress signals to TSC2 also through upregulation of the mRNA encoding REDD1 (Regulated in Development and Damage Responses 1, also known as RTP801). REDD1, like AMPK, activates TSC2 to inhibit Rheb, and this in turn leads to inhibition of TORC1 signaling to S6K1 (Sofer *et al.*, 2005). Yet, the fact that TORC1 is refractory to energy starvation in REDD1$^{-/-}$ cells, despite normal activation of AMPK and AMPK-dependent activation of TSC2, suggest that the effect of REDD1 on TSC2 is predominant over that of AMPK. This notion is further supported by the observation that overexpression of REDD1 can suppress TORC1 activity even in the presence of dominant-negative AMPK (Sofer *et al.*, 2005). Taken together, these results imply that REDD1 may act in parallel with, or downstream of AMPK toward TSC2. It should be noted, however, that conflicting results have shown that acute treatment of TSC2$^{-/-}$ with 2-DG leads to inactivation of S6K1 (Smith *et al.*, 2005), suggesting that signals from energy starvation might be transduced into suppression of TORC1 also in a TSC1–TSC2 complex-independent fashion.

3.2.4. Oxygen supply

Hypoxia has a prominent inhibitory effect on TORC1 activity, which is mediated in part by REDD1 (Fig. 1.4), as REDD1$^{-/-}$ mouse cells are defective in hypoxia-mediated inhibition of S6K activation (Brugarolas *et al.*, 2004). This effect, however, relies on an intact TSC1–TSC2 complex, as S6K phosphorylation is refractory to hypoxic treatment of TSC1$^{-/-}$ or TSC2$^{-/-}$ mouse cells (Brugarolas *et al.*, 2004). Transcriptional activation of *REDD1* gene under conditions of hypoxia (Shoshani *et al.*, 2002) is mediated by HIF-1, the master regulators of oxygen homeostasis (Brahimi-Horn *et al.*, 2007). Hypoxia can also inhibit TORC1 independently of REDD1 via the induction of energy stress, possibly due to reduced oxidative phosphorylation. AMPK is upregulated under these conditions, thereby activates TSC2 and inhibits TORC1 (Liu *et al.*, 2006). It should be noted, however, that prolonged exposure to low oxygen leads to a reduced mTORC1 activity independently of TSC2 by an unknown mechanism (Liu *et al.*, 2006).

3.2.5. Osmolarity

An increase in the concentration of solutes outside the cell relative to that inside is termed as hyperosmotic stress. Such a stress causes water to diffuse out of the cell, resulting in cell shrinkage, which can lead to DNA and protein damage, cell cycle arrest, and ultimately cell death (Burg *et al.*, 2007). Hyperosmotic stress that is induced by treating cells with either sorbitol (Kruppa and Clemens, 1984) or high salt concentration (Naegele and Morley, 2004) elicits reversible dephosphorylation of rpS6

in mammals. A closer look at the sorbitol effect has suggested the involvement of a phosphatase, as calyculin A, a phosphatase inhibitor, was able to prevent sorbitol-induced suppression of S6K (Parrott and Templeton, 1999).

Hyperosmotic-dependent S6K inhibition has been shown also in mannitol-treated tobacco leaves. Downregulation of S6K activity appears to have a protective effect against sustained osmotic stress, as *Arabidopsis* plants expressing high levels of S6K were hypersensitive to mannitol treatment (Mahfouz *et al.*, 2006; Williams *et al.*, 2003).

4. Physiological Roles of rpS6 Phosphorylation

4.1. Global protein synthesis

Numerous early studies showing temporal correlation between rpS6 phosphorylation and initiation of protein synthesis following mitogenic or nutritional stimuli (Kruppa and Clemens, 1984) set the ground for the claim that rpS6 phosphorylation is involved in regulation of protein synthesis. This model has been further supported by UV cross-linking experiments that have localized rpS6 to the interface between the two ribosomal subunits and demonstrated its interaction with tRNA, initiation factors, and mRNA (reviewed in Nygard and Nilsson, 1990). However, attempts to establish a causal relationship between rpS6 phosphorylation and efficiency of protein synthesis have yielded conflicting results. Thus, analyses of the distribution of rpS6 have shown that polysomes have a higher percentage of the phosphorylated rpS6 than subpolysomal fractions, suggesting that the 40S subunit with the highest proportion of phosphorylated rpS6 has a selective advantage in mobilization into polysomes (Duncan and McConkey, 1982; Thomas *et al.*, 1982). To the contrary, later studies have provided evidence that increased rpS6 phosphorylation by itself is not sufficient to enhance the mobilization of 40S subunits into protein synthesis (Kruppa and Clemens, 1984; Montine and Henshaw, 1990; Tas and Martini, 1987). An early attempt to directly address this issue by genetic manipulation has disclosed that substitution of the two phosphorylatable serine residues in yeast rpS6 to alanine had no detectable effect on yeast growth, under a wide variety of nutritional conditions (Johnson and Warner, 1987). This observation implies that rpS6 phosphorylation, at least in yeast, has no obvious role in protein synthesis or other cellular functions. However, this conclusion could not be extrapolated to higher eukaryotes that have five, rather than two phosphorylatable serine residues.

Recently, the role of rpS6 phosphorylation in regulation of protein synthesis in mammalian cells has been explored using a knockin mouse

(rpS6$^{P-/-}$), in which all phosphorylatable serine residues in rpS6 were substituted by alanines (Ruvinsky *et al.*, 2005). The rates of global protein synthesis (incorporation of radio-labeled amino acids) and of the accumulation of steady state levels of protein were significantly increased in MEFs derived from knockin mice, relative to those measured with wild-type MEFs (Ruvinsky *et al.*, 2005). It appears therefore, that protein synthesis, at least in this cell type, is downregulated by rpS6 phosphorylation. Although slightly faster elongation rate was determined in rpS6$^{P-/-}$ MEFs, the augmentation in overall protein synthesis in these cells is mainly attributable to enhanced translation initiation by an as yet unknown mechanism. A lack of stimulatory effect of rpS6 phosphorylation on global protein synthesis has also been shown in mouse liver. Thus, monitoring the relative proportion of ribosomes engaged in translation (associated with polysomes) has demonstrated a similar proportion in the liver of both rpS6$^{P-/-}$ and wild-type mice. Furthermore, this similarity was apparent even in regenerating liver, in which rpS6 undergoes extensive phosphorylation only in the wild-type (Ruvinsky *et al.*, 2005). Clearly, these results indicate that rpS6 phosphorylation is dispensable for efficient polysomal recruitment of liver ribosomes.

In assessing the physiological significance of rpS6 phosphorylation to global protein synthesis, one should take into account that in addition to rpS6, activated S6K phosphorylates two other proteins that might affect the rate of protein synthesis, elongation factor 2 (EF2) kinase (Wang *et al.*, 2001), and initiation factor eIF4B (Raught *et al.*, 2004). The phosphorylation of the former leads to its inactivation and thus to dephosphorylation and derepression of EF2 (Wang *et al.*, 2001). Likewise, S6K1-dependent phosphorylation of eIF4B promotes its recruitment to the eIF3:40S ribosomal subunit preinitiation complex (Holz *et al.*, 2005). It has been speculated that this brings eIF4B into close proximity with eIF4A, which can then fully exert its helicase activity for efficient translation of mRNAs with a long structured 5′ untranslated region. Taken together, S6K seems to operate two opposing mechanisms: upregulation of global protein synthesis through phosphorylation of EF2 kinase and/or eIF4B, on the one hand, and its downregulation through rpS6 phosphorylation (Fig. 1.2). This, seemingly futile effect of rpS6 phosphorylation might turn out to be a mechanism for fine-tuning the activation of protein synthesis following mitogenic or nutritional signals, in order to ensure balanced protein synthesis, and thus to restrain energy wastage.

4.2. Translational control of TOP mRNAs: The rise and fall of a myth

The synthesis of many proteins, associated with the translational apparatus of higher eukaryotes (ribosomal proteins, elongation factors, and poly(A)-binding protein, etc.), is selectively regulated by mitogenic and nutritional

signals, at the translational level (Hornstein *et al.*, 2001). The corresponding mRNAs are characterized by the presence of a 5′ Terminal OligoPyrimidine tract (5′TOP) and therefore are referred to as TOP mRNAs. This structural motif comprises the core of the translational *cis*-regulatory element of these mRNAs (Meyuhas, 2000). The translational control of TOP mRNAs is manifested by their selective recruitment into polysomes when resting cells are induced to grow (increase in their mass) or to proliferate, or when amino acid starved cells are refed (Stolovich *et al.*, 2002; Tang *et al.*, 2001). Activation of S6K and consequently rpS6 phosphorylation closely correlate, under some physiological circumstances, with translational upregulation of TOP mRNAs (reviewed in Meyuhas, 2000). Furthermore, inhibition of rpS6 phosphorylation with rapamycin, a specific inhibitor of mTOR, was shown to repress the translational activation of TOP mRNAs upon mitogenic stimulation of at least some cell lines (reviewed in Hornstein *et al.*, 2001). These correlative data has led to the hypothesis that rpS6 phosphorylation increases the affinity of ribosomes for TOP mRNAs and thus facilitates the initiation of this class of mRNAs (Jefferies *et al.*, 1994).

The proposed model has been widely accepted and is still frequently referred to in the scientific literature. However, subsequent biochemical and genetic studies with cultured cells have disproved the causal relationship between rpS6 phosphorylation and translation efficiency of TOP mRNAs: (a) Hyperphosphorylation of rpS6 by overexpression of RSK2 or inhibition of rpS6 phosphatase by calyculin A failed to relieve the translational repression of TOP mRNAs in amino acid-starved cells (Tang *et al.*, 2001). (b) The translation of TOP mRNAs is constitutively repressed in dividing lymphoblastoids, even though their S6K1 is active and rpS6 is phosphorylated (Stolovich *et al.*, 2005). (c) TOP mRNAs are translationally activated by serum refeeding or amino acid replenishment in $S6K^{-/-}$ ES cells (Stolovich *et al.*, 2002; Tang *et al.*, 2001) or in mouse erythroleukemia cells (Barth-Baus *et al.*, 2002), even though their rpS6 is constitutively unphosphorylated or dephosphorylated, respectively. (d) Mistargeting of upstream signals by overexpression of a kinase-dead S6K1 mutant, completely abolished any S6K activity and rpS6 phosphorylation without a concomitant inhibitory effect on translation of TOP mRNAs (Tang *et al.*, 2001). (e) LiCl can alleviate the translational repression of TOP mRNAs in quiescent HEK293 and P1798 cells, even though S6K remains inactive and rpS6 is unphosphorylated in these cells (Stolovich *et al.*, 2005). (f) Rapamycin completely blocks S6K activity and rpS6 phosphorylation, yet it exerts only a minor or no repressive effect on the translational activation of TOP mRNAs in several cell lines (Stolovich *et al.*, 2002 and references therein). (g) TOP mRNAs are unloaded from polysomes upon hormone-induced *Xenopus* oocyte maturation (Hyman and Wormington, 1988) and during gastrulation (Pierandrei-Amaldi *et al.*, 1982), yet rpS6 becomes increasingly phosphorylated during these developmental

stages (Nielsen *et al.*, 1982; Schwab *et al.*, 1999). Consistently, it has been shown that the translation of TOP mRNAs is normally regulated in hepatocytes from mice lacking both S6K1 and S6K2 ($S6K1^{-/-}/S6K2^{-/-}$) (Pende *et al.*, 2004). However, rpS6 in these mice was still phosphorylated at Ser235 and/or Ser236, most probably by RSK, and therefore, it could be argued that this residual phosphorylation is essential and/or sufficient for efficient translation of TOP mRNAs. Nevertheless, polysomal analyses of TOP mRNAs in regenerating liver and serum stimulated MEFs from mice lacking any phosphorylatable serine residue ($rpS6^{P-/-}$), and from wild-type mice, showed a similar translational activation of these mRNAs (Ruvinsky *et al.*, 2005). Collectively, all these observations indicate that TOP mRNAs are translationally controlled in an S6K- and rpS6 phosphorylation-independent fashion.

4.3. rpS6 phosphorylation as an effector of TORC1 in determining cell size

Previous reports have demonstrated that the TORC1 pathway is an integral cell growth regulator (reviewed in Lee *et al.*, 2007). Thus, treatment of mammalian cells by rapamycin decreases their size. This mTOR-dependent regulation of the cell size involves its downstream targets, S6K1 and 4E-BP (Fingar *et al.*, 2002; Ohanna *et al.*, 2005). Indeed, S6K has been implicated as an important positive regulator of cell and body size. Thus, most dS6K null *Drosophila* exhibit embryonic lethality, with the few surviving adults having a severely reduced body size, due to a decrease in cell size rather than a decrease in cell number (Montagne *et al.*, 1999). $S6K1^{-/-}$ mice are significantly smaller at birth, due to a proportional decrease in the size of all organs (Shima *et al.*, 1998). A smaller cell size in these mice was reported for pancreatic β-cells (Pende *et al.*, 2000) and myoblasts (Ohanna *et al.*, 2005). In contrast, the birth weight of $S6K2^{-/-}$ mice, as well as the size of their myoblasts, are similar to those of wild-type mice (Ohanna *et al.*, 2005; Pende *et al.*, 2000). In accordance with the phenotypes of each of these mutant mice, the embryonic and postnatal growth, as well as the size of myoblasts of the double knockout mice, $S6K1^{-/-}/S6K2^{-/-}$, are similar to those of $S6K1^{-/-}$ mice (Ohanna *et al.*, 2005; Pende *et al.*, 2004). The fact that mammalian cell size is predominantly determined by S6K1 and not S6K2 posed a question regarding the effector(s) of S6K1 involved in this mode of regulation.

Of the known multiple substrates of S6K1, it is rpS6 phosphorylation that has been shown to be directly involved in the control of cell size. Thus, a wide variety of cell types derived from $rpS6^{P-/-}$ mice are significantly smaller than their wild-type counterparts. These include pancreatic β-cells, interleukin 7-dependent cells derived from fetal livers, MEFs (Ruvinsky *et al.*, 2005), muscle myotubes, and hepatocytes (I. Ruvinsky, M. Katz,

Y. Bolkier, and O. Meyuhas, unpublished results). It appears, however, that the small cell phenotype is not ubiquitous, as acinar cells in the pancreas display a similar size regardless of the absence of S6K1 (Pende *et al.*, 2000) or phosphorylatable serine residues in rpS6 (Ruvinsky *et al.*, 2005).

Several studies have demonstrated that cell cycle progression and cell growth are separable and are therefore distinct processes, at least in some mammalian cells (reviewed in Conlon *et al.*, 2004; Fingar and Blenis, 2004). Thus, overexpression of S6K1 or initiation factor 4E (eIF-4E) resulted in increased cell size, due to augmented cell growth and not from delayed cell cycle progression (Fingar *et al.*, 2002). Likewise, overexpression of c-Myc in serum-starved B cells or aphidicolin treatment of Schwann cells accelerated their growth independent of cell division (Conlon *et al.*, 2001; Schumacher *et al.*, 1999). Conversely, the decreased cell size observed upon overexpression of 4E-BP1 resulted from decreased cell growth and not from accelerated cell cycle progression (Fingar *et al.*, 2002).

The apparent small size of $rpS6^{P-/-}$ MEFs is accompanied by accelerated division (Ruvinsky *et al.*, 2005), yet several lines of evidence lend support to the notion that the small cell size phenotype reflects impaired growth, rather than being a by-product of enhanced cell division: (a) $rpS6^{P-/-}$ MEFs remained smaller then their wild-type counterparts, even when progression through the cell cycle was arrested by aphidicolin, an inhibitor of DNA polymerase-α. (b) The size of immortalized $rpS6^{P-/-}$ MEFs is increased to the extent that it equalize with that of $rpS6^{P+/+}$ MEFs. Nevertheless, this increase was not accompanied by lengthening of the doubling time, as would be expected if the size was inversely proportional to the division rate (Ruvinsky *et al.*, 2005). (c) $rpS6^{P+/-}$ primary MEFs are still smaller than and $rpS6^{P+/+}$ MEF, even though they have a similar doubling time (M. Katz and O. Meyuhas, unpublished results).

Interestingly, rapamycin treatment decreased the size of $rpS6^{P+/+}$ MEFs, whereas the size of $rpS6^{P-/-}$ MEFs remained unchanged (Ruvinsky *et al.*, 2005). The rapamycin resistance displayed by the latter is reminiscent of that exhibited by $S6K1^{-/-}$ myoblasts (Ohanna *et al.*, 2005), implying that cells already displaying a small size phenotype, due to deficiency of S6K1 or of phosphorylatable serine residues in rpS6, are not further affected by rapamycin. Furthermore, it seems that rpS6 phosphorylation is a critical effector of mTOR in regulation of cell growth and that its absence is equivalent to inhibition of mTOR. Notably, the small size of $S6K1^{-/-}$ myoblasts is apparent, even though their rpS6 is still phosphorylated, most probably by S6K2 (Ohanna *et al.*, 2005). It is likely, therefore, that this small cell size phenotype reflects a reduced activity of yet another S6K1-specific effector(s), which is involved in this mode of regulation, such as SKAR (Richardson *et al.*, 2004). Alternatively, if S6K2 is inactive during muscle differentiation in early embryo, then it is possible that S6K1 deficiency is indeed equivalent to the lack of phosphorylatable serine in rpS6. If the latter is the case, then it

should be assumed that once the growth of a specific cell lineage is blocked by a temporary deficiency of rpS6 phosphorylation, as a result of S6K1 deficiency, the small size is maintained thereafter, regardless of the later phosphorylation of rpS6 by a different kinase (S6K2, for example).

4.4. Cell proliferation

The puzzling observations of a similar birth weight of $rpS6^{P-/-}$ and $rpS6^{P+/+}$ mice, despite a smaller size of $rpS6^{P-/-}$ embryonic cells (MEFs and interleukin 7-dependent cells), have been reconciled by the findings that $rpS6^{P-/-}$ newborns contain a higher DNA content, which reflects a higher cell number (Ruvinsky *et al.*, 2005). It is conceivable, therefore, that a faster proliferation compensates for the smaller size of embryonic $rpS6^{P-/-}$ cells. Indeed, this possibility has been further supported by the apparent shorter population doubling time of $rpS6^{P-/-}$ MEFs, as well as the faster protein and nucleic acids accumulation in this cells. This accelerated cell division reflects primarily a shortening of the G1 phase in $rpS6^{P-/-}$ MEFs (Ruvinsky *et al.*, 2005). Notably, the deficiency of both S6K1 and S6K2, unlike the mutation in all phosphorylatable serine residues in $rpS6^{P-/-}$, had no effect on the doubling time of MEFs or primary myoblasts (Ohanna *et al.*, 2005; Pende *et al.*, 2004). This difference might reflect the fact that rpS6 in $S6K1^{-/-}/S6K2^{-/-}$ is still phosphorylated at Ser235/236.

4.5. Glucose homeostasis

It has previously been shown that insulin secretion closely correlates with the size of β-cells (Giordano *et al.*, 1993; Pende *et al.*, 2000). Mice deficient for S6K1 exhibited impaired glucose homeostasis, due to insufficient insulin secretion in response to glucose load. The reason for this defect was proposed to be the small size of β-cells in $S6K1^{-/-}$ mice (Pende *et al.*, 2000). This phenotype is recapitulated in $rpS6^{P-/-}$ mice, which show a twofold reduction in both circulating levels and pancreatic content of insulin, in addition to a higher and prolonged hyperglycemic response after glucose challenge, relative to wild-type mice (Ruvinsky *et al.*, 2005).

Interestingly, the apparent glucose intolerance in $rpS6^{P-/-}$ and $S6K1^{-/-}$ mice is reminiscent of impaired glucose tolerance observed in offspring of rats that were undernourished during pregnancy, or in adult human beings after prenatal exposure to famine (Ravelli *et al.*, 1998 and references therein). Moreover, low protein diet during pregnancy leads to reduction of pancreatic cell proliferation, islet size, islet vascularization, and insulin content in the fetal rat (Dahri *et al.*, 1991). Possibly, malnutrition during pregnancy leads to insufficient signals through mTOR, an integrator of nutritional signals (Dann *et al.*, 2007; Proud, 2007), which in turn leads to

reduced activation of S6K1 and hypophosphorylation of rpS6 during a critical stage of pancreatic development and consequently to impaired pancreatic function in the adult organism. It should be pointed out, however, that the effect of perinatal famine on the size of β-cells, a hallmark of rpS6$^{P-/-}$ and S6K1$^{-/-}$ mice, is currently unknown.

Notably, the similarity in the phenotypic manifestations associated with glucose homeostasis in both rpS6$^{P-/-}$ knockin and S6K1$^{-/-}$ knockout mice (Pende *et al.*, 2000; Ruvinsky *et al.*, 2005), strongly suggests that it is the failure to phosphorylate rpS6 that can account for the common defects in both types of mutants. Nevertheless, verifying this hypothesis should await the availability of data on the phosphorylation status of rpS6 in S6K1$^{-/-}$ β-cell. It should be stressed, however, that S6K1$^{-/-}$ mice, unlike rpS6$^{P-/-}$ mice, also display an *in utero* developmental defect manifested in smaller birth size (Shima *et al.*, 1998) and the disruption of both S6K1 and S6K2 leads to decreased viability due to perinatal lethality (Pende *et al.*, 2004). Clearly, these phenotypes attest to the involvement of S6K targets other than rpS6, in normal *in utero* development.

4.6. rpS6 phosphorylation as a diagnostic marker

The usage of phospho-rpS6(Ser235/236) as a biomarker for activation of the PI3K/TORC1/S6K pathway in tissue samples from tumor biopsies (Robb *et al.*, 2006, 2007) or transplants (Lepin *et al.*, 2006) has recently been proposed. However, these sites can also be phosphorylated by RSK (Roux *et al.*, 2007), and therefore, their phosphorylation cannot be used as an indication for therapeutic strategy involving blockage of PI3K/TORC1/S6K signaling. Indeed, it has recently been shown that phosphorylation of Ser235/236 might be upregulated in tumors with activation of the Ras/Raf/ERK pathway, rather than activation of the PI3K/TORC1/S6K pathway (Chow *et al.*, 2006; Ma *et al.*, 2007). Hence, future differential diagnosis of the activated pathway may depend on the use of specific biomarkers such as phospho-rpS6(Ser240/244) for the PI3K/mTOR/S6K pathway and phospho-ERK or phospho-RSK for the Ras/Raf/MEK pathway.

5. Concluding Remarks and Future Perspectives

The preceding sections have shown that rpS6 is an indispensable ribosomal protein that undergoes phosphorylation in response to a wide variety of stimuli, and that this modification plays a critical regulatory role in multiple cellular and organismal processes. However, despite extensive

experimental work, many questions remain unresolved, including the following major ones:

a. What is so unique about rpS6 that heterozygotic embryos ($rpS6^{wt/del}$) exhibit lethal haploinsufficiency (Panic *et al.*, 2006), whereas all other examined ribosomal proteins exhibit a modest to sever phenotype only after knocking out both alleles (Kirn-Safran *et al.*, 2007; Matsson *et al.*, 2004; Oliver *et al.*, 2004)? One plausible explanation assumes that rpS6 plays a key role during assembly of the 40S ribosomal subunit, and therefore heterozygosity leads to a reduction by half of the number of ribosomes. This might have a devastating consequence at a critical developmental stage when maximal ribosome dosage is required. Contrarily, ribosome assembly and function are not strictly dependent on any of the other examined ribosomal proteins. Clearly, verification of this model needs further information regarding the order at which individual ribosomal proteins are assembled into the mammalian 40S ribosomal subunit.

b. What is the physiological meaning of rpS6 phosphorylation in response to each of the many stimuli that up- or downregulates it? A closer look at the multiple stimuli detailed in Table 1.1 poses a puzzling question regarding the role of the up- or downregulated rpS6 phosphorylation in physiological consequences exerted by these stimuli. Thus, growth factor and nutrients that support cell proliferation induce rpS6 phosphorylation, whereas, signals, like hypoxia, energy depletion, heat shock, and hyperosmolarity, that are expected to block cell proliferation, lead to rpS6 dephosphorylation. However, the study with $rpS6^{P-/-}$ cells has clearly shown that rpS6 phosphorylation is inversely, rather than directly correlates with cell proliferation (Ruvinsky *et al.*, 2005). A likely explanation assumes that rpS6 phosphorylation is required for restraining cell proliferation, most probably by diminishing the rate of protein synthesis (see Section 4.1). Thus, when cells are induced to proliferate they operate, in addition to the machinery that initiates and sustains cell cycle progression, a ''brake'' system that involves rpS6 phosphorylation, in order to limit the rate of proliferation. Accordingly, blocking cell cycle progression by stress signals will lead to release of the ''brake'' and to rpS6 dephosphorylation.

c. What is the mechanism(s) underlying the role of rpS6 phosphorylation in regulating processes as diverse as global protein synthesis, cell proliferation, cell growth, and glucose homeostasis. Several testable models can be proposed to account for these unique physiological functions of rpS6 phosphorylation: (i) rpS6 might be one of the many bifunctional ribosomal proteins, that can carry out extraribosomal tasks often unrelated to the mechanics of protein synthesis (Wool, 1996). Thus, rpS6 can be detected in association with the chromatin of primary hepatocytes and undergoes hormone-dependent phosphorylation within the nucleus

(Franco and Rosenfeld, 1990). This suggests that rpS6 might be involved in a nuclear function, as has been shown for *Drosophila* rpS3, which is tightly associated with the nuclear matrix where it is engaged in DNA repair (Wilson *et al.*, 1994). (ii) The phosphorylation of rpS6 within, or outside, the ribosome might affect the translation efficiency of specific mRNAs as precedented by rpL13a and rpL26 (Mazumder *et al.*, 2003; Takagi *et al.*, 2005). An unbiased search for mRNAs whose abundance (through transcriptional regulation) or translatability is affected by rpS6 phosphorylation, should be greatly aided by genome-wide transcriptional and translational profiling of total and polysomal RNAs, in combination with proteomics analyses. (iii) Phosphorylated rpS6 does not affect protein synthesis, but instead interacts with cellular protein(s), which consequently becomes active or inactive, and thus affects the cell physiology. Thus, the extraribosomal association of L11, L5, and L23 with HDM2, which prevents the ubiquitination and degradation of P53 and thereby induces p53-dependent cell cycle arrest (Dai and Lu, 2004; Dai *et al.*, 2004; Zhang *et al.*, 2003b), and the ability of phosphorylated rpL13a to operate outside the ribosome (Mazumder *et al.*, 2003) exemplify this possibility. Furthermore, several extraribosomal proteins have been reported to be coimmunoprecipitated with rpS6, suggesting an *in vivo* interaction, either directly or indirectly with these proteins. The latter include heat-shock protein 90 (Kim *et al.*, 2006), alphavirus nonstructural protein (Montgomery *et al.*, 2006), and death-associated protein kinase (DAPK) (Schumacher *et al.*, 2006). Clearly, any future attempt to establish the mechanistic base for the complex physiological roles of rpS6 phosphorylation will also require the verification of its potential extraribosomal function.

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REFERENCES

Alessi, D., Kozlowski, M., Weng, Q., Morrice, N., and Avruch, J. (1998). 3-Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p70 S6 kinase *in vivo* and *in vitro*. *Curr. Biol.* **8,** 69–81.

Arsham, A. M., Howell, J. J., and Simon, M. C. (2003). A novel hypoxia-inducible factor-independent hypoxic response regulating mammalian target of rapamycin and its targets. *J. Biol. Chem.* **278,** 29655–29660.

Avruch, J., Hara, K., Lin, Y., Liu, M., Long, X., Ortiz-Vega, S., and Yonezawa, K. (2006). Insulin and amino-acid regulation of mTOR signaling and kinase activity through the Rheb GTPase. *Oncogene* **25,** 6361–6372.

Ballou, L., Luther, H., and Thomas, G. (1991). MAP2 kinase and 70K S6 kinase lie on distinct signalling pathways. *Nature* **349,** 348–350.

Bandi, H. R., Ferrari, S., Kreig, J., Meyer, H. E., and Thomas, G. (1993). Identification of 40S ribosomal protein S6 phosphorylation sites in Swiss mouse 3T3 fibroblasts stimulated with serum. *J. Biol. Chem.* **268,** 4530–4533.

Barth-Baus, D., Stratton, C. A., Parrott, L., Myerson, H., Meyuhas, O., Templeton, D. J., Landreth, G. E., and Hensold, J. O. (2002). S6 phosphorylation-independent pathways regulate translation of 5′-terminal oligopyrimidine tract containing mRNAs in differentiating hematopoietic cells. *Nucleic Acids Res.* **30,** 1919–1928.

Belandia, B., Brautigan, D., and Martín-Pérez, J. (1994). Attenuation of ribosomal protein S6 phosphatase activity in chicken embryo fibroblasts transformed by Rous sarcoma virus. *Mol. Cell. Biol.* **14,** 200–206.

Belham, C., Wu, S., and Avruch, J. (1999). Intracellular signalling: PDK1—a kinase at the hub of things. *Curr. Biol.* **9,** R93–R96.

Bhaskar, P. T., and Hay, N. (2007). The two TORCs and Akt. *Dev. Cell* **12,** 487–502.

Blenis, J., Kuo, C. J., and Erikson, R. L. (1987). Identification of a ribosomal protein S6 kinase regulated by transformation and growth-promoting stimuli. *J. Biol. Chem.* **262,** 14373–14376.

Bolster, D. R., Crozier, S. J., Kimball, S. R., and Jefferson, L. S. (2002). AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling. *J. Biol. Chem.* **277,** 23977–23980.

Brahimi-Horn, M. C., Chiche, J., and Pouyssegur, J. (2007). Hypoxia signalling controls metabolic demand. *Curr. Opin. Cell Biol.* **19,** 223–229.

Brazil, D. P., and Hemmings, B. A. (2001). Ten years of protein kinase B signalling: A hard Akt to follow. *Trends Biochem. Sci.* **26,** 657–664.

Brugarolas, J., Lei, K., Hurley, R. L., Manning, B. D., Reiling, J. H., Hafen, E., Witters, L. A., Ellisen, L. W., and Kaelin, W. G., Jr. (2004). Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. *Genes Dev.* **18,** 2893–2904.

Burg, M. B., Ferraris, J. D., and Dmitrieva, N. I. (2007). Cellular response to hyperosmotic stresses. *Physiol. Rev.* **87,** 1441–1474.

Byfield, M. P., Murray, J. T., and Backer, J. M. (2005). hVps34 is a nutrient-regulated lipid kinase required for activation of p70 S6 kinase. *J. Biol. Chem.* **280,** 33076–33082.

Cantrell, D. A. (2001). Phosphoinositide 3-kinase signalling pathways. *J. Cell Sci.* **114,** 1439–1445.

Chaudhuri, S., Vyas, K., Kapasi, P., Komar, A. A., Dinman, J. D., Barik, S., and Mazumder, B. (2007). Human ribosomal protein L13a is dispensable for canonical ribosome function but indispensable for efficient rRNA methylation. *RNA* **13,** 2224–2237.

Chow, S., Minden, M. D., and Hedley, D. W. (2006). Constitutive phosphorylation of the S6 ribosomal protein via mTOR and ERK signaling in the peripheral blasts of acute leukemia patients. *Exp. Hematol.* **34,** 1183–1191.

Chung, J., Kuo, C. J., Crabtree, G. R., and Blenis, J. (1992). Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 kinases. *Cell* **69,** 1227–1236.

Cisterna, B., Necchi, D., Prosperi, E., and Biggiogera, M. (2006). Small ribosomal subunits associate with nuclear myosin and actin in transit to the nuclear pores. *FASEB J.* **20,** 1901–1903.

Cochrane, A. W., and Deeley, R. G. (1984). Estrogen-dependent modification of ribosomal proteins. Effects of estrogen withdrawal on the distribution of constitutive and hormonally regulated mRNAs. *J. Biol. Chem.* **259,** 15408–15413.

Conlon, I. J., Dunn, G. A., Mudge, A. W., and Raff, M. C. (2001). Extracellular control of cell size. *Nat. Cell Biol.* **3,** 918–921.

Conlon, I., Lloyd, A., and Raff, M. (2004). Coordination of cell growth and cell-cycle progression in proliferating mammalian cells. *In* "Cell Growth: Control of Cell Size" (M. N. Hall, M. Raff, and G. Thomas, Eds.), pp. 85–99. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Corradetti, M. N., Inoki, K., Bardeesy, N., DePinho, R. A., and Guan, K. L. (2004). Regulation of the TSC pathway by LKB1: Evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome. *Genes Dev.* **18,** 1533–1538.

Dahri, S., Snoeck, A., Reusens-Billen, B., Remacle, C., and Hoet, J. J. (1991). Islet function in offspring of mothers on low-protein diet during gestation. *Diabetes* **40,** 115–120.

Dai, M. S., and Lu, H. (2004). Inhibition of MDM2-mediated p53 ubiquitination and degradation by ribosomal protein L5. *J. Biol. Chem.* **279,** 44475–44482.

Dai, M. S., Zeng, S. X., Jin, Y., Sun, X. X., David, L., and Lu, H. (2004). Ribosomal protein L23 activates p53 by inhibiting MDM2 function in response to ribosomal perturbation but not to translation inhibition. *Mol. Cell. Biol.* **24,** 7654–7668.

Dann, S. G., Selvaraj, A., and Thomas, G. (2007). mTOR Complex1-S6K1 signaling: At the crossroads of obesity, diabetes and cancer. *Trends Mol. Med.* **13,** 252–259.

Decker, S. (1981). Phosphorylation of ribosomal protein S6 in avian sarcoma virus-transformed chicken embryo fibroblasts. *Proc. Natl. Acad. Sci. USA* **78,** 4112–4115.

Dennis, P. B., Jaeschke, A., Saitoh, M., Fowler, B., Kozma, S. C., and Thomas, G. (2001). Mammalian TOR: A homeostatic ATP sensor. *Science* **294,** 1102–1105.

Duncan, R., and McConkey, E. H. (1982). Preferential utilization of phosphorylated 40-S ribosomal subunits during initiation complex formation. *Eur. J. Biochem.* **123,** 535–538.

Duncan, R., and McConkey, E. H. (1984). S6 phosphorylation accompanies recruitment of ribosomes and mRNA into polysomes in response to dichlororibofuranosyl benzimidazole. *Exp. Cell Res.* **152,** 520–527.

Dümmler, B. A., Hauge, C., Silber, J., Yntema, H. G., Kruse, L. S., Kofoed, B., Hemmings, B. A., Alessi, D. R., and Frödin, M. (2005). Functional characterization of human RSK4, a new 90-kDa ribosomal S6 kinase, reveals constitutive activation in most cell types. *J. Biol. Chem.* **280,** 13304–13314.

Erikson, E., and Maller, J. (1985). A protein kinase from Xenopus eggs specific for ribosomal protein S6. *Proc. Natl. Acad. Sci. USA* **82,** 742–746.

Evans, S. W., and Farrar, W. L. (1987). Interleukin 2 and diacylglycerol stimulate phosphorylation of 40 S ribosomal S6 protein. Correlation with increased protein synthesis and S6 kinase activation. *J. Biol. Chem.* **262,** 4624–4630.

Fallon, A. M., and Li, L. (2007). The C-terminal extension that characterizes mosquito (Diptera: Culicidae) ribosomal protein S6 is widespread among the Culicomorpha. *J. Med. Entomol.* **44,** 608–616.

Fingar, D. C., and Blenis, J. (2004). Target of rapamycin (TOR): An integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. *Oncogene* **23,** 3151–3171.

Fingar, D. C., Salama, S., Tsou, C., Harlow, E., and Blenis, J. (2002). Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. *Genes Dev.* **16,** 1472–1487.

Flotow, H., and Thomas, G. (1992). Substrate recognition determinants of the mitogen-activated 70K S6 kinase from rat liver. *J. Biol. Chem.* **267,** 3074–3078.

Franco, R., and Rosenfeld, M. (1990). Hormonally inducible phosphorylation of a nuclear pool of ribosomal protein S6. *J. Biol. Chem.* **265,** 4321–4325.
Fromont-Racine, M., Senger, B., Saveanu, C., and Fasiolo, F. (2003). Ribosome assembly in eukaryotes. *Genes Cells* **313,** 17–42.
Gao, X., Zhang, H., Arrazola, P., Hino, O., Kobayashi, T., Yeung, R. S., Ru, B., and Pan, D. (2002). Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling. *Nat. Cell Biol.* **4,** 699–704.
Garami, A., Zwartkruis, F. J., Nobukuni, T., Joaquin, M., Roccio, M., Stocker, H., Kozma, S. C., Hafen, E., Bos, J. L., and Thomas, G. (2003). Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. *Mol. Cell* **11,** 1457–1466.
Giordano, E., Cirulli, V., Bosco, D., Rouiller, D., Halban, P., and Meda, P. (1993). B-cell size influences glucose-stimulated insulin secretion. *Am. J. Physiol.* **265,** C358–C364.
Gleason, C. E., Lu, D., Witters, L. A., Newgard, C. B., and Birnbaum, M. J. (2007). The role of AMPK and mTOR in nutrient sensing in pancreatic beta-cells. *J. Biol. Chem.* **282,** 10341–10351.
Glover, C. V. (1982). Heat shock induces rapid dephosphorylation of a ribosomal protein in Drosophila. *Proc. Natl. Acad. Sci. USA* **79,** 1781–1785.
Gressner, A. M., and Greiling, H. (1977). The phosphorylation of liver ribosomal protein S6 during the development of acute hepatic cell injury induced by D-galactosamine. *FEBS Lett.* **74,** 77–81.
Gressner, A. M., and Wool, I. (1974a). The stimulation of the phosphorylation of ribosomal protein S6 by cycloheximide and puromycin. *Biochem. Biophys. Res. Commun.* **60,** 1482–1490.
Gressner, A. M., and Wool, I. G. (1974b). The phosphorylation of liver ribosomal proteins *in vivo*. Evidence that only a single small subunit protein (S6) is phosphorylated. *J. Biol. Chem.* **249,** 6917–6925.
Gressner, A. M., and Wool, I. G. (1976). Influence of glucagon and cyclic adenosine 3′:5′-Monophosphate on the phosphorylation of rat liver ribosomal protein S6. *J. Biol. Chem.* **251,** 1500–1504.
Hahn-Windgassen, A., Nogueira, V., Chen, C. C., Skeen, J. E., Sonenberg, N., and Hay, N. (2005). Akt activates the mammalian target of rapamycin by regulating cellular ATP level and AMPK activity. *J. Biol. Chem.* **280,** 32081–32089.
Halegoua, S., and Patrick, J. (1980). Nerve growth factor mediates phosphorylation of specific proteins. *Cell* **22,** 571–581.
Hara, K., Yonezawa, K., Weng, Q.-P., Kozlowski, M. T., Belham, C., and Avruch, J. (1998). Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. *J. Biol. Chem.* **273,** 14484–14494.
Haselbacher, G. K., Humbel, R. E., and Thomas, G. (1979). Insulin-like growth factor: Insulin or serum increase phosphorylation of ribosomal protein S6 during transition of stationary chick embryo fibroblasts into early G1 phase of the cell cycle. *FEBS Lett.* **100,** 185–190.
Hauge, C., and Frödin, M. (2006). RSK and MSK in MAP kinase signalling. *J. Cell Sci.* **119,** 3021–3023.
Hay, N., and Sonenberg, N. (2004). Upstream and downstream of mTOR. *Genes Dev.* **18,** 1926–1945.
Hebert, J., Pierre, M., and Loeb, J. E. (1977). Phosphorylation *in vitro* and *in vivo* of ribosomal proteins from Saccharomyces cerevisiae. *Eur. J. Biochem.* **72,** 167–174.
Hernandez, V. P., Higgins, L., Schwientek, M. S., and Fallon, A. M. (2003). The histone-like C-terminal extension in ribosomal protein S6 in Aedes and Anopheles mosquitoes is encoded within the distal portion of exon 3. *Insect Biochem. Mol. Biol.* **33,** 901–910.

Holz, M. K., Ballif, B. A., Gygi, S. P., and Blenis, J. (2005). mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. *Cell* **123,** 569–580.

Hornstein, E., Tang, H., and Meyuhas, O. (2001). Mitogenic and nutritional signals are transduced into translational efficiency of TOP mRNAs. *Cold Spring Harb. Symp. Quant. Biol.* **66,** 477–484.

Hyman, L. E., and Wormington, W. M. (1988). Translational inactivation of ribosomal protein mRNAs during *Xenopus* oocyte maturation. *Genes Dev.* **2,** 598–605.

Inoki, K., Li, L., Zhu, J., Wu, J., and Guan, K. L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat. Cell Biol.* **4,** 648–657.

Inoki, K., Zhu, T., and Guan, K. L. (2003). TSC2 mediates cellular energy response to control cell growth and survival. *Cell* **115,** 577–590.

Itoh, K., Ueno, K., and Natori, S. (1987). Counteraction by 20-hydroxyecdysone of the effect of juvenile hormone on phosphorylation of ribosomal protein S6. *FEBS Lett.* **213,** 85–88.

Jakubowicz, T. (1985). Phosphorylation-dephosphorylation changes in yeast ribosomal proteins S2 and S6 during growth under normal and hyperthermal conditions. *Acta Biochim. Pol.* **32,** 7–12.

Jefferies, H. B. J., Reinhard, C., Kozma, S. C., and Thomas, G. (1994). Rapamycin selectively represses translation of the 'polypyrimidine tract' mRNA family. *Proc. Natl. Acad. Sci. USA* **91,** 4441–4445.

Jenö, P., Ballou, L., Novak-Hofer, I., and Thomas, G. (1988). Identification and characterization of a mitogenic-activated S6 kinase. *Proc. Natl. Acad. Sci. USA* **85,** 406–410.

Johnson, S., and Warner, J. (1987). Phosphorylation of the *Saccharomyces cerevisiae* equivalent of ribosomal protein S6 has no detectable effect on growth. *Mol. Cell. Biol.* **7,** 1338–1345.

Jurivich, D. A., Chung, J., and Blenis, J. (1991). Heat shock induces two distinct S6 protein kinase activities in quiescent mammalian fibroblasts. *J. Cell. Physiol.* **148,** 252–259.

Kabat, D. (1970). Phosphorylation of ribosomal proteins in rabbit reticulocytes. Characterization and regulatory aspects. *Biochemistry* **9,** 4160–4175.

Kaerlein, M., and Horak, I. (1976). Phosphorylation of ribosomal proteins in HeLa cells infected with vaccinia virus. *Nature* **259,** 150–151.

Kennedy, I. M., and Leader, D. P. (1981). Increased phosphorylation of ribosomal protein S6 in hamster fibroblasts transformed by polyoma virus and simian virus 40. *Biochem. J.* **198,** 235–237.

Kennedy, I. M., Stevely, W. S., and Leader, D. P. (1981). Phosphorylation of ribosomal proteins in hamster fibroblasts infected with pseudorabies virus or herpes simplex virus. *J. Virol.* **39,** 359–366.

Kennedy, I. M., Burdon, R. H., and Leader, D. P. (1984). Heat shock causes diverse changes in the phosphorylation of the ribosomal proteins of mammalian cells. *FEBS Lett.* **169,** 267–273.

Kim, D. H., Sarbassov, D. D., Ali, S. M., King, J. E., Latek, R. R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D. M. (2002). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* **110,** 163–175.

Kim, T. S., Jang, C. Y., Kim, H. D., Lee, J. Y., Ahn, B. Y., and Kim, J. (2006). Interaction of Hsp90 with ribosomal proteins protects from ubiquitination and proteasome-dependent degradation. *Mol. Biol. Cell* **17,** 824–833.

Kimball, S. R., Shantz, L. M., Horetsky, R. L., and Jefferson, L. S. (1999). Leucine regulates translation of specific mRNAs in L6 myoblasts through mTOR-mediated changes in availability of eIF4E and phosphorylation of ribosomal protein S6. *J. Biol. Chem.* **274,** 11647–11652.

Kimura, N., Tokunaga, C., Dalal, S., Richardson, C., Yoshino, K., Hara, K., Kemp, B. E., Witters, L. A., Mimura, O., and Yonezawa, K. (2003). A possible linkage between AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signalling pathway. *Genes Cells* **8,** 65–79.

Kirn-Safran, C. B., Oristian, D. S., Focht, R. J., Parker, S. G., Vivian, J. L., and Carson, D. D. (2007). Global growth deficiencies in mice lacking the ribosomal protein HIP/RPL29. *Dev. Dyn.* **236,** 447–460.

Krieg, J., Hofsteenge, J., and Thomas, G. (1988). Identification of the 40 S ribosomal protein S6 phosphorylation sites induced by cycloheximide. *J. Biol. Chem.* **263,** 11473–11477.

Kruppa, J., and Clemens, M. J. (1984). Differential kinetics of changes in the state of phosphorylation of ribosomal protein S6 and in the rate of protein synthesis in MPC 11 cells during tonicity shifts. *EMBO J.* **3,** 95–100.

Kruse, C., Johnson, S., and Warner, J. (1985). Phosphorylation of the yeast equivalent of ribosomal protein S6 is not essential for growth. *Proc. Natl. Acad. Sci. USA* **82,** 7515–7519.

Lawlor, M. A., and Alessi, D. R. (2001). PKB/Akt: A key mediator of cell proliferation, survival and insulin responses? *J. Cell Sci.* **114,** 2903–2910.

Lee, C. H., Inoki, K., and Guan, K. L. (2007). mTOR pathway as a target in tissue hypertrophy. *Annu. Rev. Pharmacol. Toxicol.* **47,** 443–467.

Lepin, E. J., Zhang, Q., Zhang, X., Jindra, P. T., Hong, L. S., Ayele, P., Peralta, M. V., Gjertson, D. W., Kobashigawa, J. A., Wallace, W. D., Fishbein, M. C., and Reed, E. F. (2006). Phosphorylated S6 ribosomal protein: A novel biomarker of antibody-mediated rejection in heart allografts. *Am. J. Transplant.* **6,** 1560–1571.

Leslie, N. R., and Downes, C. P. (2002). PTEN: The down side of PI 3-kinase signalling. *Cell. Signal.* **14,** 285–295.

Lin, R. Z., Hu, Z. W., Chin, J. H., and Hoffman, B. B. (1997). Heat shock activates c-Src tyrosine kinases and phosphatidylinositol 3-kinase in NIH3T3 fibroblasts. *J. Biol. Chem.* **272,** 31196–31202.

Lipsius, E., Walter, K., Leicher, T., Phlippen, W., Bisotti, M. A., and Kruppa, J. (2005). Evolutionary conservation of nuclear and nucleolar targeting sequences in yeast ribosomal protein S6A. *Biochem. Biophys. Res. Commun.* **333,** 1353–1360.

Liu, L., Cash, T. P., Jones, R. G., Keith, B., Thompson, C. B., and Simon, M. C. (2006). Hypoxia-induced energy stress regulates mRNA translation and cell growth. *Mol. Cell* **21,** 521–531.

Long, X., Ortiz-Vega, S., Lin, Y., and Avruch, J. (2005). Rheb binding to mammalian target of rapamycin (mTOR) is regulated by amino acid sufficiency. *J. Biol. Chem.* **280,** 23433–23436.

Ma, L., Chen, Z., Erdjument-Bromage, H., Tempst, P., and Pandolfi, P. P. (2005). Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. *Cell* **121,** 179–193.

Ma, L., Teruya-Feldstein, J., Bonner, P., Bernardi, R., Franz, D. N., Witte, D., Cordon-Cardo, C., and Pandolfi, P. P. (2007). Identification of S664 TSC2 phosphorylation as a marker for extracellular signal-regulated kinase mediated mTOR activation in tuberous sclerosis and human cancer. *Cancer Res.* **67,** 7106–7112.

Mahfouz, M. M., Kim, S., Delauney, A. J., and Verma, D. P. (2006). Arabidopsis TARGET OF RAPAMYCIN interacts with RAPTOR, which regulates the activity of S6 kinase in response to osmotic stress signals. *Plant Cell* **18,** 477–490.

Maller, J. L., Foulkes, J. G., Erikson, E., and Baltimore, D. (1985). Phosphorylation of ribosomal protein S6 on serine after microinjection of the Abelson murine leukemia virus tyrosine-specific protein kinase into Xenopus oocytes. *Proc. Natl. Acad. Sci. USA* **82,** 272–276.

Manning, B. D., Tee, A. R., Logsdon, M. N., Blenis, J., and Cantley, L. C. (2002). Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide-kinase/akt pathway. *Mol. Cell* **10,** 151–162.

Martin, K. A., Schalm, S. S., Romanelli, A., Keon, K. L., and Blenis, J. (2001). Ribosomal S6 kinase 2 inhibition by a potent C-terminal repressor domain is relieved by mitogen-activated protein-extracellular signal-regulated kinase kinase-regulated phosphorylation. *J. Biol. Chem.* **276,** 7892–7898.

Matsson, H., Davey, E. J., Draptchinskaia, N., Hamaguchi, I., Ooka, A., Leveen, P., Forsberg, E., Karlsson, S., and Dahl, N. (2004). Targeted disruption of the ribosomal protein S19 gene is lethal prior to implantation. *Mol. Cell. Biol.* **24,** 4032–4037.

Mazumder, B., Sampath, P., Seshadri, V., Maitra, R. K., DiCorleto, P. E., and Fox, P. L. (2003). Regulated release of L13a from the 60S ribosomal subunit as a mechanism of transcript-specific translational control. *Cell* **115,** 187–198.

McCubrey, J. A., Steelman, L. S., Chappell, W. H., Abrams, S. L., Wong, E. W., Chang, F., Lehmann, B., Terrian, D. M., Milella, M., Tafuri, A., Stivala, F., Libra, M., *et al.* (2007). Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochim. Biophys. Acta* **1773,** 1263–1284.

McKay, M. M., and Morrison, D. K. (2007). Integrating signals from RTKs to ERK/MAPK. *Oncogene* **26,** 3113–3121.

Meyuhas, O. (2000). Synthesis of the translational apparatus is regulated at the translational level. *Eur. J. Biochem.* **267,** 6321–6330.

Mizoguchi, T., Hayashida, N., Yamaguchi-Shinozaki, K., Kamada, H., and Shinozaki, K. (1995). Two genes that encode ribosomal-protein S6 kinase homologs are induced by cold or salinity stress in Arabidopsis thaliana. *FEBS Lett.* **358,** 199–204.

Montagne, J., Stewart, M. J., Stocker, H., Hafen, E., Kozma, S. C., and Thomas, G. (1999). *Drosophila* S6 kinase: A regulator of cell size. *Science* **285,** 2126–2129.

Montgomery, S. A., Berglund, P., Beard, C. W., and Johnston, R. E. (2006). Ribosomal protein S6 associates with alphavirus nonstructural protein 2 and mediates expression from alphavirus messages. *J. Virol.* **80,** 7729–7739.

Montine, K. S., and Henshaw, E. C. (1990). TPA stimulates S6 phosphorylation but not protein synthesis in Ehrlich cells. *Biochem. Biophys. Res. Commun.* **166,** 1340–1345.

Naegele, S., and Morley, S. J. (2004). Molecular cross-talk between MEK1/2 and mTOR signaling during recovery of 293 cells from hypertonic stress. *J. Biol. Chem.* **279,** 46023–46034.

Nielsen, P. J., Thomas, G., and Maller, J. L. (1982). Increased phosphorylation of ribosomal protein S6 during meiotic maturation of Xenopus oocytes. *Proc. Natl. Acad. Sci. USA* **79,** 2937–2941.

Nishimura, J., and Deuel, T. F. (1983). Platelet-derived growth factor stimulates the phosphorylation of ribosomal protein S6. *FEBS Lett.* **156,** 130–134.

Nobukuni, T., Joaquin, M., Roccio, M., Dann, S. G., Kim, S. Y., Gulati, P., Byfield, M. P., Backer, J. M., Natt, F., Bos, J. L., Zwartkruis, F. J., and Thomas, G. (2005). Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. *Proc. Natl. Acad. Sci. USA* **102,** 14238–14243.

Nygard, O., and Nilsson, L. (1990). Translational dynamics. Interactions between the translational factors, tRNA and ribosomes during eukaryotic protein synthesis. *Eur. J. Biochem.* **191,** 1–17.

Ohanna, M., Sobering, A. K., Lapointe, T., Lorenzo, L., Praud, C., Petroulakis, E., Sonenberg, N., Kelly, P. A., Sotiropoulos, A., and Pende, M. (2005). Atrophy of S6K1$^{-/-}$ skeletal muscle cells reveals distinct mTOR effectors for cell cycle and size control. *Nat. Cell Biol.* **7,** 286–294.

Oliver, E. R., Saunders, T. L., Tarle, S. A., and Glaser, T. (2004). Ribosomal protein L24 defect in Belly spot and tail (Bst), a mouse Minute. *Development* **131,** 3907–3920.

Panic, L., Tamarut, S., Sticker-Jantscheff, M., Barkic, M., Solter, D., Uzelac, M., Grabusic, K., and Volarevic, S. (2006). Ribosomal protein S6 gene haploinsufficiency is associated with activation of a p53-dependent checkpoint during gastrulation. *Mol. Cell. Biol.* **26,** 8880–8891.

Parrott, L. A., and Templeton, D. J. (1999). Osmotic stress inhibits p70/85 S6 kinase through activation of a protein phosphatase. *J. Biol. Chem.* **274,** 24731–24736.

Pende, M., Kozma, S. C., Jaquet, M., Oorschot, V., Burcelin, R., Le Marchand-Brustel, Y., Klumperman, J., Thorens, B., and Thomas, G. (2000). Hypoinsulinaemia, glucose intolerance and diminished beta-cell size in S6K1-deficient mice. *Nature* **408,** 994–997.

Pende, M., Um, S. H., Mieulet, V., Sticker, M., Goss, V. L., Mestan, J., Mueller, M., Fumagalli, S., Kozma, S. C., and Thomas, G. (2004). S6K1$^{-/-}$/S6K2$^{-/-}$ mice exhibit perinatal lethality and rapamycin-sensitive 5′-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. *Mol. Cell Biol.* **24,** 3112–31124.

Pierandrei-Amaldi, P., Campioni, N., Beccari, E., Bozzoni, I., and Amaldi, F. (1982). Expression of ribosomal protein genes in *Xenopus laevis* development. *Cell* **30,** 163–171.

Potter, C. J., Pedraza, L. G., and Xu, T. (2002). Akt regulates growth by directly phosphorylating Tsc2. *Nat. Cell Biol.* **4,** 658–665.

Proud, C. G. (2007). Amino acids and mTOR signalling in anabolic function. *Biochem. Soc. Trans.* **35,** 1187–1190.

Radimerski, T., Mini, T., Schneider, U., Wettenhall, R. E., Thomas, G., and Jeno, P. (2000). Identification of insulin-induced sites of ribosomal protein S6 phosphorylation in Drosophila melanogaster. *Biochemistry* **39,** 5766–5774.

Raught, B., Peiretti, F., Gingras, A. C., Livingstone, M., Shahbazian, D., Mayeur, G. L., Polakiewicz, R. D., Sonenberg, N., and Hershey, J. W. (2004). Phosphorylation of eukaryotic translation initiation factor 4B Ser422 is modulated by S6 kinases. *EMBO J.* **23,** 1761–1769.

Ravelli, A. C., van der Meulen, J. H., Michels, R. P., Osmond, C., Barker, D. J., Hales, C. N., and Bleker, O. P. (1998). Glucose tolerance in adults after prenatal exposure to famine. *Lancet* **351,** 173–177.

Richardson, C. J., Broenstrup, M., Fingar, D. C., Julich, K., Ballif, B. A., Gygi, S., and Blenis, J. (2004). SKAR is a specific target of S6 kinase 1 in cell growth control. *Curr. Biol.* **14,** 1540–1549.

Robb, V. A., Astrinidis, A., and Henske, E. P. (2006). Frequent [corrected] hyperphosphorylation of ribosomal protein S6 [corrected] in lymphangioleiomyomatosis-associated angiomyolipomas. *Mod. Pathol.* **19,** 839–846.

Robb, V. A., Karbowniczek, M., Klein-Szanto, A. J., and Henske, E. P. (2007). Activation of the mTOR signaling pathway in renal clear cell carcinoma. *J. Urol.* **177,** 346–352.

Rossi, R., Pester, J. M., McDowell, M., Soza, S., Montecucco, A., and Lee-Fruman, K. K. (2007). Identification of S6K2 as a centrosome-located kinase. *FEBS Lett.* **581,** 4058–4064.

Roux, P. P., Ballif, B. A., Anjum, R., Gygi, S. P., and Blenis, J. (2004). Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. *Proc. Natl. Acad. Sci. USA* **101,** 13489–13494.

Roux, P. P., Shahbazian, D., Vu, H., Holz, M. K., Cohen, M. S., Taunton, J., Sonenberg, N., and Blenis, J. (2007). RAS/ERK signaling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates cap-dependent translation. *J. Biol. Chem.* **282,** 14056–14064.

Ruvinsky, I., and Meyuhas, O. (2006). Ribosomal protein S6 phosphorylation: From protein synthesis to cell size. *Trends Biochem. Sci.* **31,** 342–348.

Ruvinsky, I., Sharon, N., Lerer, T., Cohen, H., Stolovich-Rain, M., Nir, T., Dor, Y., Zisman, P., and Meyuhas, O. (2005). Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. *Genes Dev.* **19,** 2199–2211.

Sancak, Y., Thoreen, C. C., Peterson, T. R., Lindquist, R. A., Kang, S. A., Spooner, E., Carr, S. A., and Sabatini, D. M. (2007). PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. *Mol. Cell* **25,** 903–915.

Saucedo, L. J., Gao, X., Chiarelli, D. A., Li, L., Pan, D., and Edgar, B. A. (2003). Rheb promotes cell growth as a component of the insulin/TOR signalling network. *Nat. Cell Biol.* **5,** 566–571.

Scharf, K. D., and Nover, L. (1982). Heat-shock-induced alterations of ribosomal protein phosphorylation in plant cell cultures. *Cell* **30,** 427–437.

Schmidt, C., Lipsius, E., and Kruppa, J. (1995). Nuclear and nucleolar targeting of human ribosomal protein S6. *Mol. Biol. Cell* **6,** 1875–1885.

Schumacher, M., Staege, M. S., Pajic, A., Polack, A., Weidle, U. H., Bornkamm, G. W., Eick, D., and Kohlhuber, F. (1999). Control of cell growth by c-Myc in the absence of cell division. *Curr. Biol.* **9,** 1255–1258.

Schumacher, A. M., Velentza, A. V., Watterson, D. M., and Dresios, J. (2006). Death-associated protein kinase phosphorylates mammalian ribosomal protein S6 and reduces protein synthesis. *Biochemistry* **45,** 13614–13621.

Schwab, M., Kim, S., Terada, N., Edfjäll, C., Kozma, S., Thomas, G., and Maller, J. (1999). p70(S6K) controls selective mRNA translation during oocyte maturation and early embryogenesis in *Xenopus laevis*. *Mol. Cell. Biol.* **19,** 2485–2494.

Shaw, R. J., Bardeesy, N., Manning, B. D., Lopez, L., Kosmatka, M., DePinho, R. A., and Cantley, L. C. (2004). The LKB1 tumor suppressor negatively regulates mTOR signaling. *Cancer Cell* **6,** 91–99.

Shima, H., Pende, M., Chen, Y., Fumagalli, S., Thomas, G., and Kozma, S. C. (1998). Disruption of the $p70^{s6k}/p85^{s6k}$ gene reveals a small mouse phenotype and a new functional S6 kinase. *EMBO J.* **17,** 6649–6659.

Shoshani, T., Faerman, A., Mett, I., Zelin, E., Tenne, T., Gorodin, S., Moshel, Y., Elbaz, S., Budanov, A., Chajut, A., Kalinski, H., Kamer, I., *et al.* (2002). Identification of a novel hypoxia-inducible factor 1-responsive gene, RTP801, involved in apoptosis. *Mol. Cell. Biol.* **22,** 2283–2293.

Smith, C. J., Wejksnora, P. J., Warner, J. R., Rubin, C. S., and Rosen, O. M. (1979). Insulin-stimulated protein phosphorylation in 3T3-L1 preadipocytes. *Proc. Natl. Acad. Sci. USA* **76,** 2725–2729.

Smith, E. M., Finn, S. G., Tee, A. R., Browne, G. J., and Proud, C. G. (2005). The tuberous sclerosis protein TSC2 is not required for the regulation of the mammalian target of rapamycin by amino acids and certain cellular stresses. *J. Biol. Chem.* **280,** 18717–18727.

Sofer, A., Lei, K., Johannessen, C. M., and Ellisen, L. W. (2005). Regulation of mTOR and cell growth in response to energy stress by REDD1. *Mol. Cell. Biol.* **25,** 5834–5845.

Song, Q., and Gilbert, L. I. (1997). Molecular cloning, developmental expression, and phosphorylation of ribosomal protein S6 in the endocrine gland responsible for insect molting. *J. Biol. Chem.* **272,** 4429–4435.

Stewart, M. J., and Denell, R. (1993). Mutations in *Drosophila* gene encoding ribosomal protein S6 cause tissue overgrowth. *Mol. Cell Biol.* **13,** 2524–2535.

Stolovich, M., Tang, H., Hornstein, E., Levy, G., Cohen, R., Bae, S. S., Birnbaum, M. J., and Meyuhas, O. (2002). Transduction of growth or mitogenic signals into translational activation of TOP mRNAs is fully reliant on the PI3-kinase-mediated pathway, but requires neither S6K1 nor rpS6 phosphorylation. *Mol. Cell Biol.* **22,** 8101–8113.

Stolovich, M., Lerer, T., Bolkier, Y., Cohen, H., and Meyuhas, O. (2005). Lithium can relieve translational repression of TOP mRNAs elicited by various blocks along the cell

cycle in a glycogen synthase kinase-3- and S6-kinase-independent manner. *J. Biol. Chem.* **280,** 5336–5342.

Sulic, S., Panic, L., Barkic, M., Mercep, M., Uzelac, M., and Volarevic, S. (2005). Inactivation of S6 ribosomal protein gene in T lymphocytes activates a p53-dependent checkpoint response. *Genes Dev.* **19,** 3070–3082.

Szyszka, R., and Gasior, E. (1984). Phosphorylation of ribosomal proteins during differentiation of Saccharomyces cerevisiae. *Acta Biochim. Pol.* **31,** 375–382.

Takagi, M., Absalon, M. J., McLure, K. G., and Kastan, M. B. (2005). Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. *Cell* **123,** 49–63.

Tang, H., Hornstein, E., Stolovich, M., Levy, G., Livingstone, M., Templeton, D. J., Avruch, J., and Meyuhas, O. (2001). Amino acid-induced translation of TOP mRNAs is fully dependent on PI3-kinase-mediated signaling, is partially inhibited by rapamycin, and is independent of S6K1 and rpS6 phosphorylation. *Mol. Cell. Biol.* **21,** 8671–8683.

Tas, P. W., and Martini, O. H. (1987). Are highly phosphorylated 40-S subunits preferentially utilized during protein synthesis in a cell-free system from HeLa cells? *Eur. J. Biochem.* **163,** 561–567.

Tee, A. R., Fingar, D. C., Manning, B. D., Kwiatkowski, D. J., Cantley, L. C., and Blenis, J. (2002). Tuberous sclerosis complex-1 and -2 gene products function together to inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling. *Proc. Natl. Acad. Sci. USA* **99,** 13571–13576.

Thomas, G., Martin-Pérez, J., Siegmann, M., and Otto, A. M. (1982). The effect of serum, EGF, $PGF_{2\alpha}$ and insulin on S6 phosphorylation and the initiation of protein and DNA synthesis. *Cell* **30,** 235–242.

Towler, M. C., and Hardie, D. G. (2007). AMP-activated protein kinase in metabolic control and insulin signaling. *Circ. Res.* **100,** 328–341.

Trevillyan, J. M., Kulkarni, R. K., and Byus, C. V. (1984). Tumor-promoting phorbol esters stimulate the phosphorylation of ribosomal protein S6 in quiescent Reuber H35 hepatoma cells. *J. Biol. Chem.* **259,** 897–902.

Turck, F., Kozma, S. C., Thomas, G., and Nagy, F. (1998). A heat-sensitive Arabidopsis thaliana kinase substitutes for human p70s6k function *in vivo*. *Mol. Cell. Biol.* **18,** 2038–2044.

Urban, J., Soulard, A., Huber, A., Lippman, S., Mukhopadhyay, D., Deloche, O., Wanke, V., Anrather, D., Ammerer, G., Riezman, H., Broach, J. R., De Virgilio, C., *et al.* (2007). Sch9 is a major target of TORC1 in Saccharomyces cerevisiae. *Mol. Cell* **26,** 663–674.

Vander Haar, E., Lee, S. I., Bandhakavi, S., Griffin, T. J., and Kim, D. H. (2007). Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. *Nat. Cell Biol.* **9,** 316–323.

Volarevic, S., Stewart, M. J., Ledermann, B., Zilberman, F., Terracciano, L., Montini, E., Grompe, M., Kozma, S. C., and Thomas, G. (2000). Proliferation, but not growth, blocked by conditional deletion of 40S ribosomal protein S6. *Science* **288,** 2045–2047.

Vries, R. G., Flynn, A., Patel, J. C., Wang, X., Denton, R. M., and Proud, C. G. (1997). Free Full Text Heat shock increases the association of binding protein-1 with initiation factor 4E. *J. Biol. Chem.* **272,** 32779–32784.

Wang, X., Campbell, L. E., Miller, C. M., and Proud, C. G. (1998). Amino acid availability regulates p70 S6 kinase and multiple translation factors. *Biochem. J.* **334,** 261–267.

Wang, X., Li, W., Williams, M., Terada, N., Alessi, D. R., and Proud, C. G. (2001). Regulation of elongation factor 2 kinase by $p90^{RSK1}$ and p70 S6 kinase. *EMBO J.* **20,** 4370–4379.

Watson, K. L., Konrad, K. D., Woods, D. E., and Bryant, P. J. (1992). *Drosophila* homolog of the human S6 ribosomal protein is required for tumor suppression in the hematopoietic system. *Proc. Natl. Acad. Sci. USA* **89,** 11302–11306.

Watson, K. L., Chou, M. M., Blenis, J., Gelbart, W. M., and Erikson, R. L. (1996). A Drosophila gene structurally and functionally homologous to the mammalian 70-kDa s6 kinase gene. *Proc. Natl. Acad. Sci. USA* **93,** 13694–13698.

Wettenhall, R. E., Erikson, E., and Maller, J. L. (1992). Ordered multisite phosphorylation of Xenopus ribosomal protein S6 by S6 kinase II. *J. Biol. Chem.* **267,** 9021–9027.

Williams, A. J., Werner-Fraczek, J., Chang, I. F., and Bailey-Serres, J. (2003). Regulated phosphorylation of 40S ribosomal protein S6 in root tips of maize. *Plant Physiol.* **132,** 2086–2097.

Wilson, D. M., 3rd, Deutsch, W. A., and Kelley, M. R. (1994). Drosophila ribosomal protein S3 contains an activity that cleaves DNA at apurinic/apyrimidinic sites. *J. Biol. Chem.* **269,** 25359–25364.

Wool, I. G. (1996). Extra ribosomal functions of ribosomal proteins. *Trends Biochem. Sci.* **21,** 164–165.

Wool, I. G., Chan, Y.-L., and Glück, A. (1996). Mammalian ribosome: The structure and the evolution of the proteins. *In* "Translational Control" (J. W. B. Hershey, M. B. Mathews, and N. Sonenberg, Eds.). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Yang, Q., and Guan, K. L. (2007). Expanding mTOR signaling. *Cell Res.* **17,** 666–681.

Zemp, I., and Kutay, U. (2007). Nuclear export and cytoplasmic maturation of ribosomal subunits. *FEBS Lett.* **581,** 2783–2793.

Zhang, S. H., Lawton, M. A., Hunter, T., and Lamb, C. J. (1994). atpk1, a novel ribosomal protein kinase gene from Arabidopsis. I. Isolation, characterization, and expression. *J. Biol. Chem.* **269,** 17586–17592.

Zhang, Y., Gao, X., Saucedo, L. J., Ru, B., Edgar, B. A., and Pan, D. (2003a). Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. *Nat. Cell Biol.* **5,** 578–581.

Zhang, Y., Wolf, G. W., Bhat, K., Jin, A., Allio, T., Burkhart, W. A., and Xiong, Y. (2003b). Ribosomal protein L11 negatively regulates oncoprotein MDM2 and mediates a p53-dependent ribosomal-stress checkpoint pathway. *Mol. Cell. Biol.* **23,** 8902–8912.

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CHAPTER TWO

Molecular Domains in Epithelial Salt Cell$_{NaCl}$ of Crustacean Salt Gland (Artemia)

Frank P. Conte*

Contents

Abstract

The salt secretory cell has two distinct patterns of plasma membrane development. First, the basolateral surface forms a tubular labyrinth. It contains the subunit alpha-2 of the Na^+-K^+-ATPase bound together with a beta subunit for structural attachment within the lipid bilayer. Second, the apical plasma membranes form a multiple array of extending tufts. These tufts contain the subunit alpha-1 of the Na^+-K^+-ATPase bound together with a beta subunit for structural integrity within the lipid bilayer. The presence of an active transporter for chloride remains as an open question. It has been taken as preliminary evidence from brine shrimp cystic fibrosis toxicity that a cystic fibrosis transmembrane conductance regulator chloride channel could be present in the apical region. The presence of cytoskeletal elements being involved in the

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construction of a hypo-osmoregulatory apparatus is supported by the homeobox gene products derived from APH-1 m RNA found in the salt gland.

Key Words: Artemia, Salt secretory cell, Chloride cell, Crustacean salt gland, Glandular epithelium.

1. Introduction

The ultrastructural features and functional activity of the surface epithelium forming the crustacean larval salt gland in naupliar stage-1 of the brine shrimp (Artemia) was reviewed earlier by Conte (1984). That data formed the physiological foundation which established that the larval epithelium performed the task of salt secretion. Since that date, a great deal more information on the cytoarchitectural components involved in ion transport has been revealed (Clegg and Trotman, 2002). The purpose of the present chapter is to integrate the current biochemical, genetic, transcriptional, and translational data with the past mechanisms that were proposed to be or are taking place within the glandular epithelium. These mechanisms provide for the movement of sodium (Na^+) and chloride (Cl^-) ions from the hemolymph to the extracellular environment. It is hoped that this review will also offer a refined view on the ultrastructural and spatial arrangements of the molecular domains that form the hypo-osmoregulatory apparatus in the developing epithelial cells of stage-1 naupliar larval salt gland.

2. Epithelium

Epithelium lines both the inner and outer surfaces of multicellular organisms and forms the border between the external and internal environments. It is a tissue composed of cells tightly bound to each other. There are specializations of the plasma membrane that play an important role in maintaining the integrity of the tissue. The functions of epithelium are many and varied but have been conveniently divided into two major categories: metabolic and protective. Metabolic functions of epithelium include metabolite exchanges; for example, the passing of electrolytes between the outer and inner environments as described in active ion transport. Another example is the passing of glandular secretions from the specialized epithelial cells of the gland to the outer environment. Spatial polarity is another architectural feature of epithelial cells that plays an important role in its function. Polarized cells display different morphological areas of the surface plasma membrane in which apical, lateral, and basal

entities can be shown to occur. These structural modifications are unlike other tissues, where structural polarity is not found. At the present time, the crustacean larval epithelium can be classified as a simple metabolic epithelium composed of a single layer of homogenous cells demonstrating a unique spatial polarity related to its functional role of ion transport (Hootman and Conte, 1975).

2.1. Chloride cell or salt cell$_{NaCl}$

The pioneering work of Copeland (1967) on the ultrastructure of the salt secretory cell of the adult brine shrimp metepipodite established the fact that the cell was laden with large numbers of mitochondria surrounded by various configurations of plasma membranes. These extremely close associations with flattened mitochondria led Copeland to postulate the existence of a ''mitochondrial pump system'' as the possible site of active ion transport but there was little physiological evidence to support this hypothesis. Probably what was most important was his interpretation that the use of $AgNO_3$ and its reduction to free silver aided in the location of excess chloride as postulated by Koch (1938) and Wigglesworth (1938) for aquatic invertebrates. Therefore, these cells were similar in function to the ''chloride'' cells postulated earlier in fish gill. Since that time, ion-transporting cells were termed chloride cells despite the fact that there was no physiological or biochemical evidence supporting active transport of chloride ions. Therefore, for the ion-secretory cell to be called a chloride cell is ''a half-truth of a misnomer.'' I propose the use of the term ''salt cell'' with subscript to indicate what ions that can be shown to be actively transported by an identifiable carrier molecule (including channel-type molecules).

2.2. Toxins to ionic regulation (cf. Conte, 1984)

2.2.1. Sodium–potassium transporter

It was found that ouabain, a specific inhibitor of Na^+-K^+-activated ATPase, inhibited naupliar osmotic and ionic regulation of the hemolymph in the stage-1 nauplius. Isolation and purification of the enzyme from the developing embryo, nauplius—stage-1, and the isolated salt gland took place subsequently in our laboratory (Conte *et al.*, 1977; Lowy and Conte, 1985a,b; Peterson *et al.*, 1978a,b). Our findings of two isoforms of the Na^+-K^+-ATPase enzyme was the first for this enzyme and was substantiated by the continuation and the improvement of the purification protocol by Peterson *et al.* (1982a,b). Therefore, the finding of two forms of the alpha and beta subunit gave us opportunity to look for spatial arrangements of the subunits in the plasma membrane of these polar cells. If one found either alpha-1 or alpha-2 subunit restricted to either the apical or basal–lateral domains, it would provide a breakthrough in understanding of vectorial ion and solute transport in many types of polar

epithelial tissues. As will be described below, this avenue of investigation became the major focus of Hokin and coworkers.

2.2.2. Chloride transporter/carrier

It was found that acetazolamide, a specific inhibitor of chloride–bicarbonate exchange, also inhibited naupliar osmotic and ionic regulation of the hemolymph in the stage-1 nauplius. At this time, our laboratory pursued the isolation and purification of carbonic anhydrase (CA) which had been postulated to catalyze the exchange reaction (Maetz, 1971). After an exhaustive search, the naupliar tissue was found to have very little CA (Conte, 1984). Therefore, it was felt that CA was not responsible for movement of chloride ion needed to maintain osmotic and ionic balance. The major puzzle at this time was the *trans*-epithelial potential found across both the intact and isolated salt gland epithelium indicating active transport for both sodium and chloride.

2.2.3. Cystic fibrosis toxins/osmoregulation

Shortly after it was found that the amount of naupliar CA could not account for the active transport of chloride (Conte, 1984), cystic fibrosis (CF) toxins became of interest. This was due to the reports that blood serum or saliva taken from CF patients contained factors that would arrest the movement of ciliated epithelia cells, such as those found in oyster gills (Bowman *et al.*, 1969, 1970). Therefore, it seemed possible that CF fluids would interact with naupliar brine shrimp and in some way alter their behavior. Similar to our earlier pharmaceutical techniques of mixing enzyme inhibitors with various salines, we mixed CF patient saliva and control (non-CF patient) saliva into different concentrations of salines. The results showed that some unknown CF-factor not found in normal saliva was altering naupliar behavior. In many ways, the CF-factor was similar to ouabain and acetozolamide toxicity in that it showed both salinity/dose dependency. How to interpret the way that the saliva was affecting nauplii became a problem due to complexities of shrimp behavior. In normal conditions, when nauplii emerge from the cyst stage, they show swimming motility, phototaxis which can be both positive and negative, and osmotic and ionic regulation. When early emerged nauplii (24-h old) are placed in a CF-saliva saline mix, they become nonmotile within a short period of time (4 h), but remain positively phototactic by staying at the surface near the light source. However, they begin to show a loss of buoyancy (6 h) and gradually sink to the bottom. We devised a technique called ''the submarine assay'' (Fig. 2.1). In preliminary tests, we used a white porcelain chemical spot plate to observe shrimp behavior. Later we devised the test tube assay. In a large test tube (15 × 180 mm), we covered the entire tube with aluminum foil. At the top, we opened a small slit to allow light to shine at the top for positive phototactic attraction and to allow observation as to motility or lack

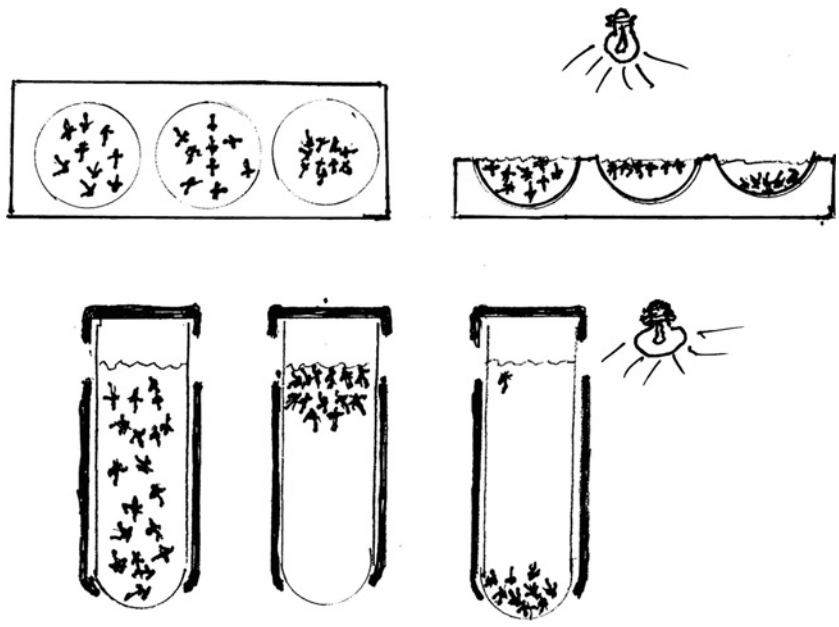

Figure 2.1 Submarine assay using naupliar stage-1 brine shrimp for detection of cystic fibrosis (CF). See text for details.

of it. Near the bottom of the tube, on the opposite darkened side, a small slit was made to detect the nonmotile and sunken shrimp which we considered to have lost osmoregulatory ability. The goal of the "submarine assay" was to distinguish not only the presence of CF but to determine whether the parents were homozygous or heterozygous. The "submarine assay" proved to be successful in the hands of numerous medical technicians using nauplii as described above but the results of the genetic evaluation showed the assay was a failure. However, the "submarine assay" was neither better nor cheaper than the sweat test and thus the novel technique was dropped and forgotten.

As I will elaborate later, it appeared possible that the CF-toxicity, observed in all the CF assaying laboratories, was the result of an unknown CF-saliva-carrying inhibitor that blocks osmoregulation through some type of interference with active chloride transport. At that time (1979), there was no evidence for the existence of any type of active chloride molecular complex or channel. Later, Kerem *et al.* (1989) published a paper on the genetic analysis of CF which showed the identity of CF gene as being responsible for encoding the cystic fibrosis transmembrane conductance regulator (CFTR). The function of CFTR is now well established (Dawson and Smith, 1997). As yet there has been no experimental evidence

identifying the cause of CF-toxicity in nauplii nor has the presence or absence of CFTR been confirmed in the naupliar stage-1 brine shrimp.

2.3. Spatial arrangements of polar epithelium

The research pursued in late 1980s and early 1990s was to unravel the unique structural configurations of the plasma membrane in the epithelial cells. It depended upon the use of distinct molecular probes. As stated earlier, the finding of subunits for the sodium–potassium ion-transport enzyme gave us an important tool in that endeavor.

2.3.1. Sodium–potassium ion-transporter subunits

In 1982, Hokin's group under the guidance of Peterson and Hokin, focused on four aspects dealing with the two isoforms of the Na^+-K^+-ATPase enzyme:

1. The new and improved purification protocols (Churchill, 1984a,b; Churchill *et al.*, 1984; Peterson *et al.*, 1982a,b) provided for clarity as to molecular weight of both subunits and to the holoenzyme.
2. Fisher *et al.* (1984, 1986) established the sites of Na^+-K^+-ATPase mRNA synthesis. They harvested 16 h whole nauplii and extracted RNA from isolated membrane-bound and free polysomes. The polysomal RNA (mRNA) was translated, the proteins isolated by immunoprecipitation and resolved on SDS-polyacrylamide gels. The results showed two distinct sites of synthesis occurring for alpha versus beta subunits. Membrane mRNA provide two distinct alpha subunits, whereas the nonmembrane (free) mRNA translated into a single beta subunit. Subsequent investigation on the temporal pattern of the mRNAs showed that messages were present in the cysts and early gastrula stage but not translated or synthesized. Emergence of naupliar stage brought about large increases in mRNA and synthesis of alpha-1 and alpha-2 subunits. However, regulation of mRNA and the rate of protein synthesis of the two alpha units are quite different suggesting that other cytoskeletal mechanisms must be involved in the formation and insertion of the ion-transport enzyme.
3. Baxter-Lowe *et al.* (1989) provided the first information on genetic construction of the brine shrimp Na^+-K^+-ATPase by focusing on the molecular cloning of the alpha subunit. In this study, 18-h brine shrimp were used to provide total RNA. The polyadenylated RNA was reverse transcribed to form a cDNA library. Details of the library and its use of expression vectors and sequencing were described by Baxter-Lowe *et al.* (1989). The results showed that the brine shrimp strain (*A. franciscana*) alpha subunit contains 996 amino acids with a molecular mass of 111 kDa. This sequence contains five potential glycosylation sites.

Since the alpha subunit of the brine shrimp has nearly 70% conformity with reported sequences of other species, the conserved regions may be useful for the identification of structural components needed in the formation and insertion of the enzyme into the plasma membrane. Bhattacharyya *et al.* (1990) continued this type of exploration by focusing on the molecular cloning of the beta subunit. Since the holoenzyme consisted of equimolar amounts of the catalytic alpha subunit and the structural (?) glycosylated beta unit, it was worth pursuing screening of cDNA libraries for the beta subunit. Details of the library and its use of expression vectors is describe in Bhattacharyya *et al.* (1990). The results showed the beta subunit of the same strain of brine shrimp contains a polypeptide of 315 amino acids with a molecular mass of 36 kDa, which is in agreement with other molecular weights of beta subunit of other species. This sequence contains only three potential glycosylation sites. The functional significance of the beta subunit or the existence of more than one isoform was not elaborated.

4. Sun *et al.* (1991) using the avidin–biotin–peroxidase complex immunostaining technique revealed the location of the alpha subunits in tissue cells in the various stages of brine shrimp development. Unfortunately, both active and inactive isoforms of the alpha subunit will show positive staining. The alpha subunit was not detected in the gastrula stage or early emergent stage (0 and 12 h). In late emergence (16 h) and naupliar-1 stage (24 h), as seen in the photographs (Fig. 2.2A–C), there was positive staining found to be located in epidermal cells lining the mandibular (brain) region and the mesenchymal cells of the prospective region of the antennal gland. The epithelial cells of the salt gland showed positive staining. In naupliar-2 stage (36 h) through stage-3 (48 h), the positive staining increased in the salt gland epithelium, epidermal cells, mesenchymal cells of the labrum region, and midgut mucosal cells. When immunostaining technique was used to locate the beta subunit presence in embryonic tissue, the findings matched the alpha-subunit pattern. Therefore, the developmental pattern of both alpha and beta subunits matched the pattern found by earlier investigations using intact enzyme. In addition, these data support the hypothesis that the switch from the passive, glycerol-mediated transport of water to sodium-mediated transport processes occur somewhere in the pre-naupliar stage (Conte *et al.* 1977). Unfortunately, this investigation did not use isolated salt glands (Lowy and Conte, 1985a,b) which contains only homogenous epithelial cells and would have given a greater degree of clarity in regard to the position of the enzyme in the spatial domains of the plasma membrane. Is the alpha subunit located on the basal–lateral domains? Does the beta subunit have a different location, such as apical domain? D.Y. Sun *et al.* (1992) took a different perspective in the problem of localization of the Na^+-K^+-ATPase transport complex. They used *in situ* hybridization

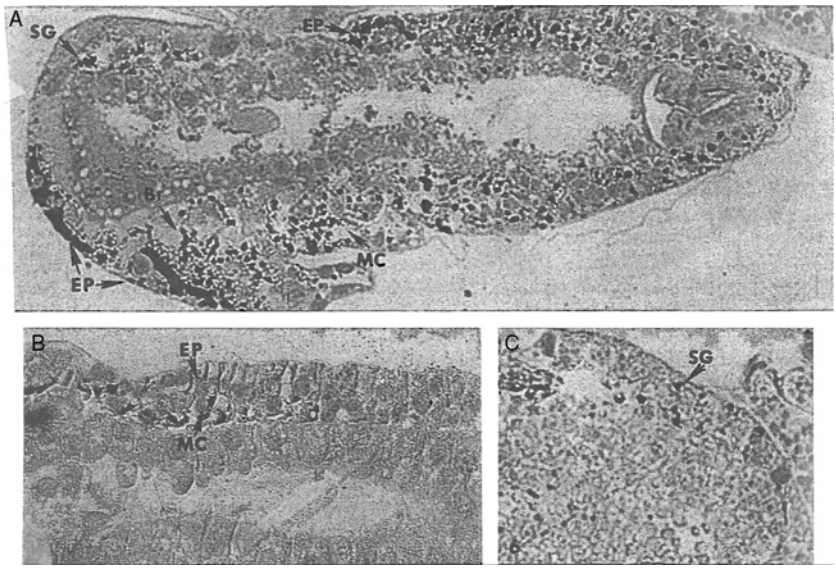

Figure 2.2 *In situ* **localization of alpha subunit mRNA by immunocytochemical technique. Viewed by light microscopy (dark stain). Salt gland (SG), epidermal cells (EP), mesenchyme cells (MC). Taken from Sun *et al.* (1991).**

histochemistry which utilized oligonucleotide probes to localize the mRNAs encoding the alpha-1 and alpha-2 subunits and beta subunit. Details of the preparation of probes and specificity of probes together with the *in situ* hybridization techniques are found in Sun *et al.* (1992). The results using alpha-1 mRNA (5.1 kb) which could not hybridize with alpha-2 probe using mRNA (3.5 kb) was thought to be the main subunit found in later developmental stages in which the antennal gland and leg metepipodites are formed and become the main osmoregulatory tissues in the adult. The alpha-2 mRNA was absent in early embryos (0–6 h) but the beta mRNA was low in abundance. After emergence (16 h) and into the naupliar-1 stage (24 h), the increase in both alpha-2 and beta mRNA was phenomenal. The autoradiographic localization in microscopic sections showed the major sites were in the epithelial layer of the salt gland, presumptive gut mucosa, and epidermal cells (Fig. 2.3). Later naupliar stage-3 (36 h) showed similar pattern but a decline of intensity of grain count. The author's interpretation of the data was to suggest that in the early emergent stages, the epidermal cells perform an initial role in osmoregulation and later after emergence, the salt gland takes over the osmotic and ionic balance together with the epidermal cells. This poses the question as to "how do these two tissues coordinate the functional duties?" It is equally clear that alpha-2 subunit

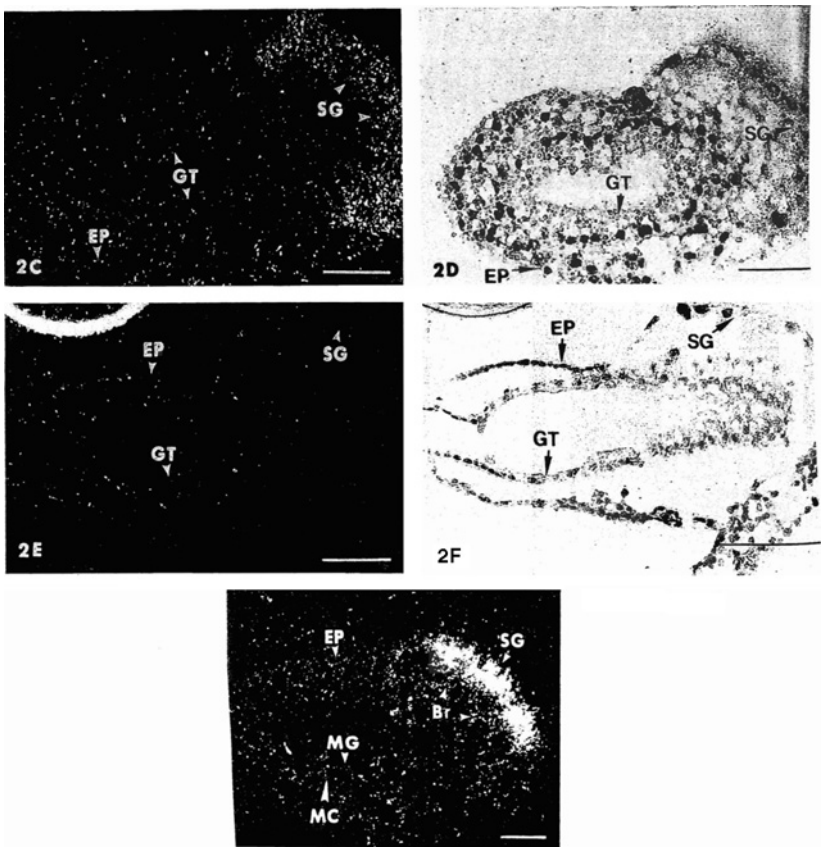

Figure 2.3 *In situ* localization of alpha-2 subunit mRNA by immunochemical and autoradiographic techniques. Light field microscopy (dark grains) and dark field microscopy (light grains). Salt gland (SG), epidermal cells (EP), gut mucosa (GT), midgut (MG), and the brain region (BR). Taken from Sun *et al.* (1992).

together with an appropriate beta subunit are joined at the baso-lateral region in formation of the active transport system. It still remains unclear as to whether alpha-1 subunit and other beta subunits are present. This latter problem was resolved by Escalante *et al.* (1994, 1995) using *in situ* hybridization analyses of Na^+-K^+-ATPase subunits during larval development. In the earlier paper, Escalante's group showed that the alpha-1 and alpha-2 subunits had different temporal patterns of mRNA expression coming from two different cDNA clones. One cDNA clone (pArATNa136) yielded a probe that specifically hybridized to a 4.5-kb mRNA and was termed to be an alpha-1 subunit probe. The alpha-2 probe hybridized to a 3.6-kb mRNA. The expression of the two alpha

probes on naupliar-1 stage embryo is shown in (Fig. 2.4). The main sites of expression were the salt gland, the analog of the antennal gland and the midgut. The pattern of expression is that both alpha-1 and alpha-2 together with the beta subunits are all expressed in the salt gland at all stages of development (24–62 h), Thus, salt cell$_{NaCl}$ differentiation involves the synthesis of both alpha subunits and the beta subunit. If the alpha-2 is located in the basolateral region, does this finding infer that the alpha-1 is located in the apical region, possibly in alignment with a chloride channel. Again, I must emphasize the use of isolated salt glands in this type of investigation would aid in answering the polarity problem.

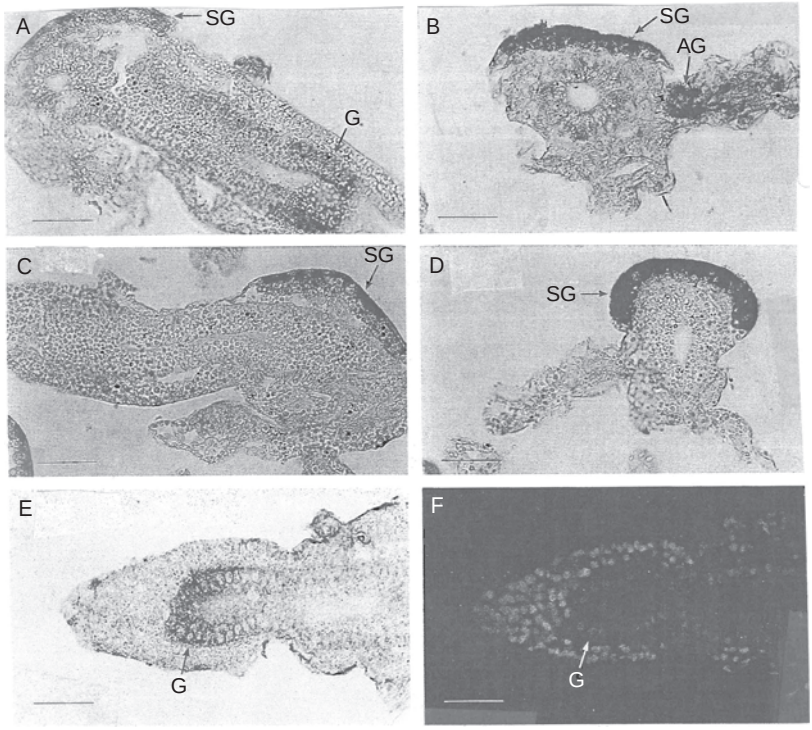

Figure 2.4 *In situ* localization of alpha-1 subunit mRNA as seen in (A, B, E, F) and alpha-2 subunit mRNA as seen in (C, D) by immunocytochemical technique as detailed by Escalante *et al.* (1995). Sections were viewed by light or ultraviolet microscopy (dark stain). Salt gland (SG), antennal gland (AG), and midgut (G).

2.3.2. Chloride transmembrane channel

Unlike the abundance of information on proteins involved in sodium ion transport, there is a paucity of information on any kind of macromolecule responsible for chloride movement across any type of secretory epithelium in larval brine shrimp. Toxins have played major role in elucidating the function of anion permeation in vertebrate tissue. The discovery that in the human disease of CF, the loss of a chloride channel referred to as the cystic fibrosis transmembrane conductance regulator (CFTR) is responsible for the disease. Sheppard and Welsh (1999) reviewed the structure and function of the CFTR chloride channel. CFTR is located in the apical membrane of epithelia. The chloride channel requires phosphorylation via ATP to open. The phosphorylation is accomplished by a cytosolic cAMP-dependent protein kinase. The CFTR is composed of five domains: two membrane-spanning domains (MSDs), two nucleotide-binding domains (NBDs), and a regulatory (R) domain. The MSDs for the channel pore complex, phosphorylation of the R domain determines channel activity, and ATP hydrolysis by the NBDs control channel gating. The CFTR is a unique member of the family of ATP binding cassette (ABC) ATP binding cassette transporters. The CFTR has been isolated from the amphibian, *Xenopus laevis* by PCR amplification. The mRNA of 4455 bp sequence encoded a polypeptide of 1485 amino acids which had an overall homology of 77% for the amino acids to those found in human CFTR. Unfortunately, while searching CFTR literature, I found no citation which identified a CFTR isolated from invertebrates. Genetic probes designed to detect CFTR in brine shrimp would certainly prove useful in establishing the existence or nonexistence of a chloride channel.

3. Polar Domains

3.1. Cytoskeleton associated with polar domains

The cytoskeleton is an intracellular network of fibers that traverse the cytosol linking various macromolecular complexes which have been or are being inserted into lipid bilayer of the plasma membrane. Research on the multifunctional protein CFTR found in vertebrate epithelial tissue has given a great deal of new insights in the regulation of membrane traffic and spatial polarization. The summations by Jilling and Kirk (1997) and Van der Wouden *et al.* (2003) reveal the fact that many ion-transporter molecules and other ion channels are surrounded by actin filaments, actin-binding proteins, microtubules, all associated with *trans*-Golgi network. These structural complexes are vital for the secretory epithelial cell in maintaining vectorial ion movement, that is, from intracellular to extracellular

environments. Invertebrate epithelial tissues, especially the epithelial lining of the larval salt gland, have not been investigated for this aspect of regulation of spatial distribution of polar domains. If CFTR is detected in naupliar brine shrimp, the cytoskeleton interaction with chloride channels would yield fruitful information on this aspect of the problem.

3.2. Genetic regulation of spatial distribution of polar domains

An important class of developmental regulators is transcription factors that control cell- or tissue-specific gene expression (Verrifzer and Van der Vliet, 1993). Many of these factors belong to evolutionary conserved protein families. One of these families is the homeodomain family in which belong the POU domain proteins. In 1999, Chavez *et al.* (1999) screened the brine shrimp (*A. franciscana*) genome for the presence of POU genes. Using POU domain sequences from established POU genes, probes were made by PCR technique and used as a hybridization probe of a cDNA library from stage-3 nauplius. The results gave a fragment identical to a sequence of POU domain and termed APH-1 for Artemia POU homeogene and belongs to the class III POU homeoproteins. Interestingly, only two other type Class III POU proteins have been found in arthropods, Cf1-a in the fruit fly (*Drosophila*) by Johnson and Hirsh (1990) and the POU-M1 in the silkworm (*Bombyx mori*) by Fukuta *et al.* (1993). The latter is thought to control transcription of the silk protein genes. The developmental and spatial expression of APH-1 gene was determined by RT-PCR assays at each larval stage for APH-1 mRNA and its tissue location for spatial distribution by whole-mount *in situ* hybridization. The pattern of APH-1 mRNA was that low levels of mRNA were found just before emergence and increased dramatically during naupliar stage-1 through stage-4 and were undetectable in adulthood. The probes used to locate APH-1 mRNA were labeled with digoxigenin for microscopic visualization (Fig. 2.5 is taken from Chavez *et al.*, 1999). A strong signal was observed in both the developing salt gland of pre-naupliar stage and osmoregulating salt gland of stage-2 nauplius. It has been suggested by these investigators that the APH-1 gene plays an important role in the formation of the osmoregulatory apparatus in the epithelial salt cell $_{NaCl}$.

3.3. Death of larval salt gland

Prior to the onset of establishing an adult osmoregulatory apparatus within the leg segments, the larval salt gland is destroyed. The mechanism of cell death (apoptosis) by which the ion-secretory epithelium is destroyed is entirely unknown. Recently, studies on the molecular mechanisms of apoptosis induced by a variety of physical forces have been summarized

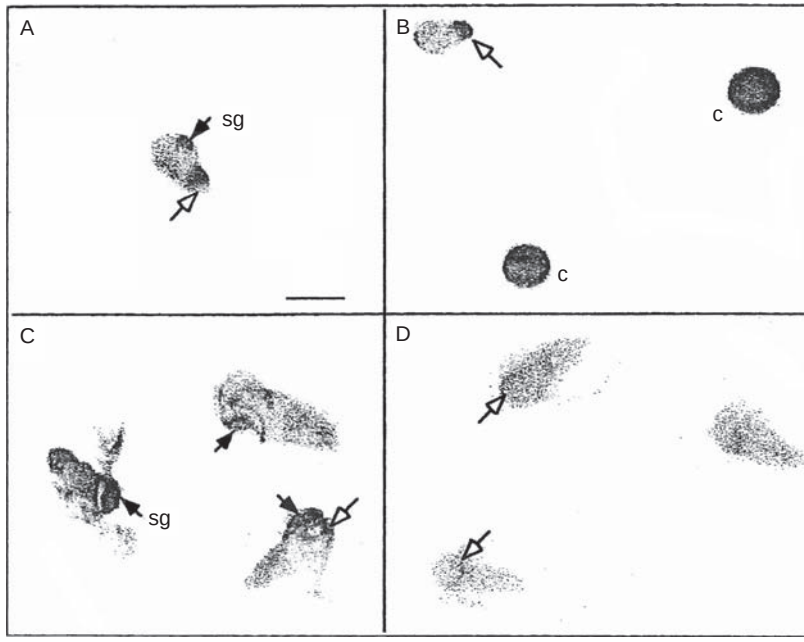

Figure 2.5 Pattern of APH-1 POU domain in pre-nauplius (A, B) and nauplius stage-1 (C, D). Positive APH-1 mRNA probe (A, C) while control antisense probe was negative (B, D). Taken from Chavez *et al.* (1999).

by Hsieh and Nguyen (2005). It has been shown that physical forces act through receptor-like molecules found in the cytoskeleton. These molecules in turn activate a limited number of protein kinase pathways which amplify the signal and activate caspases that promote apoptosis. Interestingly, Steve Hand and his colleagues who have been studying cellular stasis in the dormant brine shrimp embryo for a long period, began to investigate how apoptosis is avoided in the dormant stages. Evaluation of hydrated diapaused embryo extracts (nauplii and shells are discarded) revealed that caspase-9 and caspase-3 were present (Menze and Hand, 2007). However, the caspase enzymes were not activated by cytochrome *c* (other proapoptotic factors of mitochondrial origin were not evaluated). But an array of nucleotides, such as ADP, ATP, and GTP caused an inhibition of the caspases activity. It was concluded that multiple mechanisms may be in place to suppress apoptosis under limited nucleotide energy pools. Since naupliar extracts were not investigated, it would be interesting to compare caspases activity in isolated naupliar stage-1 larval salt glands to later naupliar stages as it metamorphs into the adult form (without the presence of salt gland). These observations would certainly give insight into the programmed death of the larval salt gland.

3.4. Model of larval salt gland: Polar domains of ion transporter (Na,K) and channel (Cl) in salt cell$_{NaCl}$

The mature salt secretory cell, when functional, has two distinct allomorphic patterns of plasma membrane growth. First, the basolateral plasma membranes form a tubular labyrinth. It is derived from invagination of basolateral surfaces and this infolding is closely associated with mitochondria. Second, the apical surfaces form numerous irregularly shaped tufts which are not associated with mitochondria and lie beneath the cuticular shell. The apical surface membrane is separated from the basolateral membrane by extensive septate junctions. Additionally, the entire epithelial complex is devoid of being structural supported by a basal lamina. The large nucleus with prominent nucleoli I is located in the central area of the elongated cell (Conte, 1984).

3.4.1. Molecular domains of the apical membrane tufts

The presence of ion transporters in the apical region for the outward sequestration of intracellular sodium is supported by the evidence of finding the isoform subunit alpha-1 of the Na^+-K^+-ATPase and beta isoform for structural attachment. In addition, the site location of mRNA for the subunits of the Na^+-K^+-ATPase supports this view. However, the presence of an ion transporter for chloride being in the apical region is only hypothesized since there is no data to substantiate the presence of CFTR. Taking the preliminary evidence that CF-toxicity of naupliar brine shrimp points in the direction of a CFTR chloride channel being present, then its location would be in the apical membrane. Figure 2.6 shows how these polar domains might appear in the apical tufts. The involvement of cytoskeletal elements being involved in the formation of the completely functional sodium chloride osmoregulatory system is supported by the homeobox gene products derived from APH-1 mRNA found in the salt gland.

3.4.2. Molecular domains of the basolateral membrane labyrinth

The presence of ion transporters in the basolateral labyrinth for the inward movement of intracellular sodium is supported by the evidence of finding the isoform subunit alpha-2 of the Na^+-K^+-ATPase and beta isoform for structural attachment. In addition, the site location of mRNA for the subunits of the Na^+-K^+-ATPase is supportive. Inward movement of chloride is open to question since CFTR is only found in apical regions of epithelial cells. However, Covi and Hand (2007) have recently postulated that V-ATPases perform both the role of maintaining acidification of the intracellular compartment and possibly in maintaining energization of the plasma membrane in the absence of membrane Na^+-K^+-ATPases

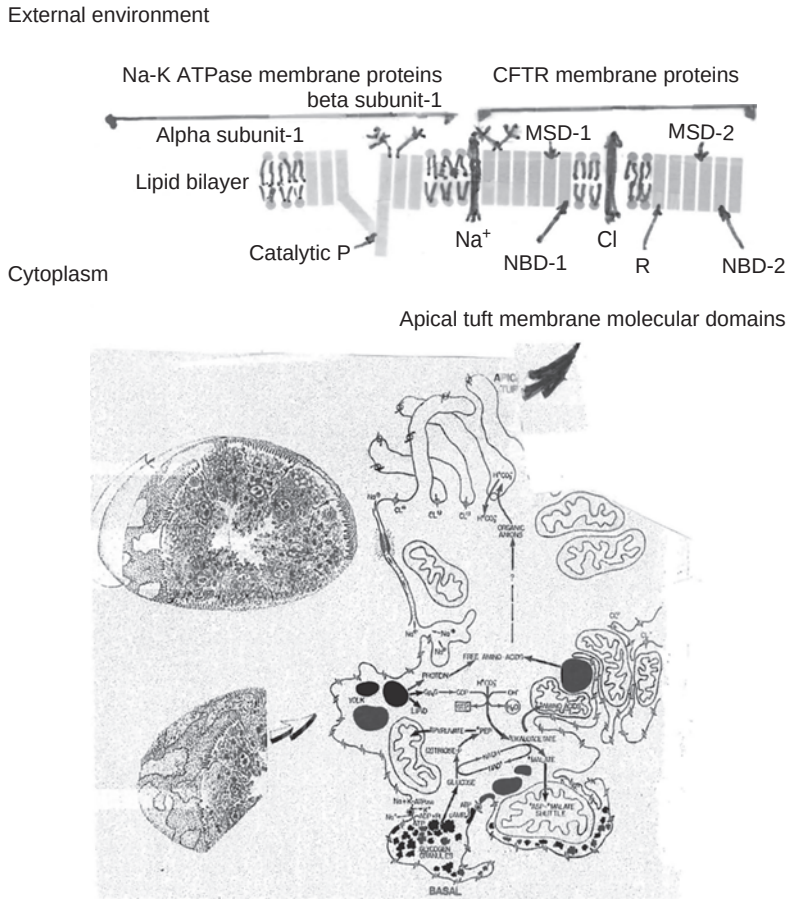

Figure 2.6 Drawing of larval salt gland with cytoarchitecture of established metabolic complexes. Enlargement of apical tufts in the insert showing existing molecular domains for ion transport.

(Covi and Hand, 2005; Covi *et al.*, 2005). Since these V-ATPases are associated with the intracellular nucleotide pool, they may be called upon to respond at later naupliar stages to changing levels of high energy demands brought about by increasing levels of ion pumping by Na^+-K^+-ATPase or CFTR. Could these metabolic interactions be the mitochondrial—pump system that was postulated many years ago by Copeland? Figure 2.7 shows how these polar domains might appear in the basolateral labyrinth. Like the suggestion of cytoskeletal elements being involved in this aspect of forming the glandular osmoregulatory system, the homeobox gene products of APH-1mRNA are needed.

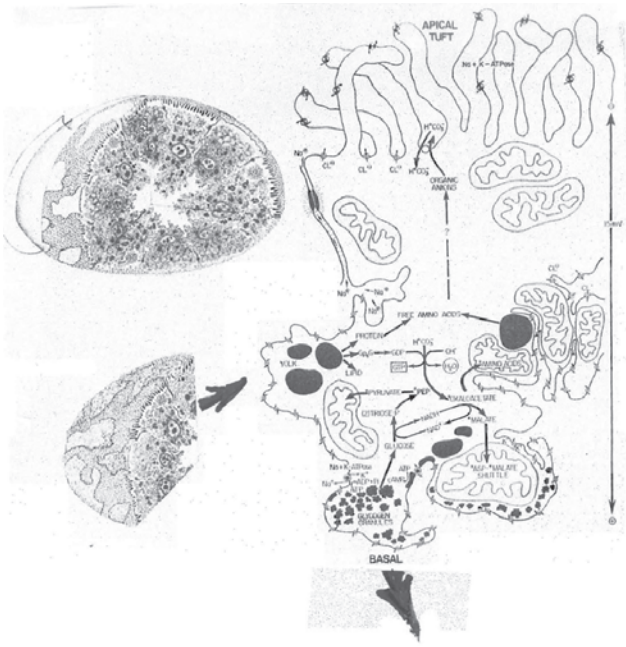

Figure 2.7 Drawing of larval salt gland with cytoarchitecture of established metabolic complexes. Enlargement of basolateral tufts in the insert showing existing molecular domains for ion transport.

4. Concluding Remarks

The crustacean (Artemia) salt gland has proven to be a very useful model in our understanding of biogenesis in epithelial ion transport. The spatial localization of the two isoforms of the sodium transporting enzyme has been a major achievement. Alpha-2 subunit is shown to be located on the basolateral surfaces, while alpha-1 subunit is at the apical surface. It

remains to be seen if presence of the APH-1 m RNA in the salt gland indicates that additional proteins are involved in building the cellular osmoregulatory apparatus. Thus, the finding of homeobox genes in this transient structure may portend a more important role of other cytoskeletal proteins in the evolution of salt secretory cells.

REFERENCES

Baxter-Lowe, L. A., Guo, J. Z., Bergstrom, E. E., and Hokin, L. E. (1989). Molecular cloning of the Na, K-ATPase alpha subunit in developing brine shrimp and sequence comparison with higher organisms. *FEBS Lett.* **257,** 181–187.

Bhattacharyya, K. K., Bergstrom, E. E., and Hokin, L. E. (1990). Molecular cloning of the beta subunit of the Na, K-ATPase in the brine shrimp, Artemia. *FEBS Lett.* **269,** 233–238.

Bowman, B. H., Lockhart, L. H., and McComb, M. L. (1969). Oyster ciliary inhibition by cystic fibrosis factor. *Science* **164,** 325–326.

Bowman, B. H., McComb, M. L., and Lockhart, L. H. (1970). Cystic fibrosis: Characterization of the inhibitor to ciliary action in oyster gills. *Science* **167,** 871–873.

Chavez, M., Landry, C., Loret, S., Muller, M., Figueroa, J., Peers, B., Rentier-DeIrue, F., Rousseau, G. G., Krauskopf, M., and Martial, J. A. (1999). APH-1, a POU homeobox gene expressed in the salt gland of the crustacean *Artemia franciscana*. *Mech. Dev.* **87,** 207–212.

Churchill, L. (1984a). Differences in phosphorylation of the two large subunits of brine shrimp Na, K-ATPase. *J. Exp. Zool.* **231,** 335–341.

Churchill, L. (1984b). A reversal in relative mobility of the two large subunits of brine shrimp (Na^+-K^+)-adenosinetriphosphatase. *J. Exp. Zool.* **231,** 351–354.

Churchill, L., Hall, C. C., Peterson, G. L., Ruoho, A. E., and Hokin, L. E. (1984). Photoaffinity labeling of the Quabain binding site in Na, K-ATPase in developing brine shrimp. *J. Exp. Zool.* **231,** 343–350.

Clegg, J. S., and Trotman, C. N. A. (2002). Physiological and biochemical aspects of Artemia ecology. *In* "Artemia: Basic and Applied Biology" (T. J. Abatzopoulos, J. A. Beardmore, J. S. Clegg, and P. Sorgeloos, Eds.), pp. 129–170. Kluwar Academic Publishers, Dordrecht, The Netherlands.

Conte, F. P. (1984). Structure and function of the crustacean larval salt gland. *Int. Rev. Cytol.* **91,** 45–106.

Conte, F. P., Droukas, P. C., and Ewing, R. D. (1977). Development of sodium regulation and *de novo* synthesis of Na^+-K^+-activated ATPase in larval brine shrimp. *J. Exp. Zool.* **202,** 339–362.

Copeland, D. E. (1967). A study of salt secreting cells in the brine shrimp (Artemia salina). *Protoplasma* **63,** 363–384.

Covi, J. A., and Hand, S. C. (2005). V-ATPase expression during development of Artemia franciscana embryos: Potential role for proton gradients in anoxia signaling. *J. Exp. Biol.* **208,** 2783–2798.

Covi, J. A., Hand, S. C., and Treleaven, W. D. (2005). V-ATPase inhibition prevents recovery from anoxia in Artemia franciscana embryos: Quiescence signaling through dissipation of proton gradients. *J. Exp. Biol.* **208,** 2799–2808.

Covi, J. A., and Hand, S. C. (2007). Energizing an invertebrate embryo: Balfilomycin-dependent respiration and the metabolic cost of proton pumping by the V-ATPase. *Physiol. and Biochem. Zool.* **80,** 422–432.

Dawson, D. C., and Smith, S. S. (1997). Commentary cystic fibrosis transmembrane conductance regulator. *J. Gen. Physiol.* **110,** 337–339.

Escalante, R., Garcia-Saez, A., Ortega, M. A., and Sastre, L. (1994). Gene expression after resumption of development of *Artemia franciscana* cryptobiotic embryos. *Biochem. Cell Biol.* **72,** 78–83.

Escalante, R., Garcia-Saez, A., and Sastre, L. (1995). *In situ* hybridization analyses of Na, K-ATPase alpha subunits expression during early larval development of *Artemia franciscana*. *J. Histochem. Cytochem.* **43,** 391–399.

Fisher, J. A., Baxter-Lowe, L. A., and Hokin, L. E. (1984). Site of synthesis of the alpha and beta subunits of the Na, K-ATPase in brine shrimp nauplii. *J. Biol. Chem.* **259,** 14217–14221.

Fisher, J. A., Baxter-Lowe, L. A., and Hokin, L. E. (1986). Regulation of Na, K-ATPase biosynthesis in developing Artemia salina. *J. Biol. Chem.* **261,** 515–519.

Fukuta, M., Matsuno, K., Hui, C., Nagata, T., Takiya, S., Xu, P., Ueno, K., and Suzuki, Y. (1993). Molecular cloning of POU domain-containing factor involved in the regulation of the Bombyx serecin-1 gene. *J. Biol. Chem.* **268,** 9471–9475.

Hootman, S. R., and Conte, F. P. (1975). Functional morphology of the larval salt gland in Artemia salina nauplii. *J. Morphol.* **145,** 371–386.

Hsieh, M. H., and Nguyen, H. T. (2005). Molecular mechanism of apoptosis induced by mechanical forces. *Int. Rev. Cytol.* **245,** 45–89.

Jilling, T., and Kirk, K. L. (1997). The biogenesis, traffic and function of the cystic fibrosis transmembrane conductance regulator. *Int. Rev. Cytol.* **172,** 193–241.

Johnson, W. A., and Hirsh, J. (1990). Binding of a *Drosophila* POU domain protein to a sequence element regulating gene expression in specific dopaminergic neurons. *Nature* **343,** 467–470.

Kerem, B., Rommens, J. M., Buchanan, J. A., Markiewicz, D., Cox, T. K., Chakravarti, A., Buchwald, M., and Tsui, L. C. (1989). Identification of the cystic fibrosis gene: Genetic analysis. *Science (Washington, DC)* **245,** 1073–1080.

Koch, H. J. (1938). The absorption of chloride ions by the anal papillae of Diptera larvae. *J. Exp. Biol.* **15,** 152–160.

Lowy, R. J., and Conte, F. P. (1985a). Isolation and functional characteristics of the larval brine shrimp salt gland (Artemia salina). *Am. J. Physiol.* **17,** R702–R708.

Lowy, R. J., and Conte, F. P. (1985b). Morphology of isolated crustacean larval salt gland. *Am. J. Physiol.* **17,** R709–R716.

Maetz, J. (1971). Fish gills: Mechanisms of salt transfer in freshwater and seawater. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **B 262,** 209–249.

Menze, M. A., and Hand, S. C. (2007). Caspase activity during cell stasis: Avoidance of apoptosis in an invertebrate extremeophile, *Artemia franciscana*. *AJP Regul. Integr. Comp. Physiol.* **292,** R1–R9.

Peterson, G., Ewing, R. D., and Conte, F. P. (1978a). Membrane differentiation and *de novo* synthesis of the (Na^+-K^+)-activated adenosine triphosphatase during development of Artemia salina nauplii. *Dev. Biol.* **67,** 90–98.

Peterson, G., Ewing, R. D., Hootman, S. R., and Conte, F. P. (1978b). Large scale partial purification and molecular and kinetic properties of the (Na^+-K^+)-activated adenosine triphos-phatase from Artemia salina nauplii. *J. Biol. Chem.* **253,** 4762–4770.

Peterson, G., Churchill, L., Fisher, J. A., and Hokin, L. E. (1982a). Structure and biosynthesis of (Na,K)-ATPase in developing brine shrimp nauplii. *Ann. N. Y. Acad. Sci.* **402,** 185–206.

Peterson, G., Churchill, L., Fisher, J. A., and Hokin, L. E. (1982b). Structural and biosynthetic studies on the two molecular forms of the (Na,K)-activated adenosine triphosphatase large subunit in Artemia salina nauplii. *J. Exp. Zool.* **221,** 295–308.

Sheppard, D. N., and Welsh, M. J. (1999). Structure and function of the CFTR chloride channel. *Physiol. Rev.* **79**(Suppl 1), S23–S45.

Sun, D. Y., Guo, J. Z., Hartmann, H. A., Uno, H., and Hokin, L. E. (1991). Na, K-ATPase expression in the developing brine shrimp Artemia. Immunochemical localization of the alpha- and beta-subunits. *J. Histochem. Cytochem.* **39,** 1455–1460.

Sun, D. Y., Guo, J. Z., Hartmann, H. A., Uno, H., and Hokin, L. E. (1992). Differential expression of the alpha-2 and beta messenger RNAs of Na, K-ATPase in developing brine shrimp as measured by *in situ* hybridization. *J. Histochem. Cytochem.* **40,** 555–562.

Van der Wouden, J. M., Maier, O., van Ijzendoorn, S. C. D., and Hoekstra, D. (2003). Membrane dynamics and the regulation of epithelial polarity. *Int. Rev. Cytol.* **226,** 127–164.

Verrifzer, C. P., and Van der Vliet, P. C. (1993). POU domain transcription factors. *Biochim. Biophys. Acta* **1173,** 1–21.

Wigglesworth, V. B. (1938). The regulation of osmotic pressure and chloride concentration in the hemolymph of mosquito larvae. *J. Exp. Biol.* **15,** 235–247.

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CHAPTER THREE

Natriuretic Peptides in Vascular Physiology and Pathology

Geoffrey E. Woodard* *and* Juan A. Rosado†

Contents

Abstract

Four major natriuretic peptides have been isolated: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), and *Dendroaspis*-type natriuretic peptide (DNP). Natriuretic peptides play an important role in the regulation of cardiovascular homeostasis maintaining blood pressure and extracellular fluid volume. The classical endocrine effects of natriuretic peptides to modulate fluid and electrolyte balance and vascular smooth muscle tone are complemented by autocrine and paracrine actions that include regulation of coronary blood flow and, therefore, myocardial perfusion; modulation of proliferative responses during myocardial and vascular remodeling; and cytoprotective anti-ischemic effects. The actions of natriuretic peptides are mediated by the specific binding of these peptides to three cell surface receptors: type A natriuretic peptide receptor (NPR-A), type B natriuretic peptide receptor (NPR-B), and type C natriuretic peptide receptor (NPR-C). NPR-A and

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NPR-B are guanylyl cyclase receptors that increase intracellular cGMP concentration and activate cGMP-dependent protein kinases. NPR-C has been presented as a clearance receptor and its activation also results in inhibition of adenylyl cyclase activity. The wide range of effects of natriuretic peptides might be the base for the development of new therapeutic strategies of great benefit in patients with cardiovascular problems including coronary artery disease or heart failure. This review summarizes current literature concerning natriuretic peptides, their receptors and their effects on fluid/electrolyte balance, and vascular and cardiac physiology and pathology, including primary hypertension and myocardial infarction. In addition, we will attempt to provide an update on important issues regarding natriuretic peptides in congestive heart failure.

Key Words: ANP_{1-28}, ATP, Renal glomeruli, ATPγS, Hypertensive rats, Wistar Kyoto (WKY) rat.

Abbreviations

AAPH	2,2′-Azobis (2-amidinopropane) dihydrochloride
ANP	atrial natriuretic peptide
BNP	brain natriuretic peptide
CNP	C-type natriuretic peptide
CHF	congestive heart failure
DNP	*Dendroaspis*-type natriuretic peptide
NPR	natriuretic peptide receptor
SHR	spontaneously hypertensive rats
SOCE	store-operated calcium entry
TNFα	tumor necrosis factor α

1. Introduction

Natriuretic peptides are a family of cardiac- and vascular-derived hormones that play a relevant role in cardiovascular homeostasis mainly through the regulation of blood volume and pressure. In the second half of the 1950s, electron microscopy revealed the presence of secretory granules in cardiac atrial cells. Later, in the 1980s, de Bold *et al.* reported that injection of atrial myocardial extracts into rats was able to induce natriuresis and consequently a decrease in blood pressure (de Bold *et al.*, 1981), suggesting the existence of a hormone that was identified in 1983 as the atrial natriuretic peptide

(ANP; Kangawa and Matsuo, 1984). Although the endocrine function of the heart has long been recognized, the physiological activities of natriuretic peptides go beyond their classical endocrine effects regulating blood volume and pressure. Natriuretic peptides also exert autocrine and paracrine effects within circulation in normal and pathological conditions, regulate renin secretion, progesterone release, endothelin secretion, and vasopressin secretion (Anand-Srivastava, 2005).

The mammalian family of natriuretic peptides consists of ANP, brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), and the recently identified *Dendroaspis*-type natriuretic peptide (DNP). BNP was isolated in 1988 from porcine brain (Sudoh *et al.*, 1988), although the major source of circulating BNP are cardiac ventricles (Saito *et al.*, 1989), and showed a similar biological activity to ANP. Two years latter, another structurally related peptide from porcine brain, CNP, was isolated and identified (Sudoh *et al.*, 1990). DNP was isolated in the first half of the 1990s from the venom of the green mamba (*Dendroaspis angusticeps*) (Schweitz *et al.*, 1992) and its physiological role was suggested by the observation of DNP immunoreactivity in human plasma (Schirger *et al.*, 1999). These peptides are encoded by different genes but the mature active forms of all the natriuretic peptides possess a characteristic 17-amino acid ring structure, which contains several invariant amino acids (Fig. 3.1). Three natriuretic peptides, ANP, BNP, and CNP, have been shown to regulate cardiovascular homeostasis by the occupation of three membrane receptors; two are guanylyl cyclase-coupled receptors, known as natriuretic peptide receptor type A (NPR-A) and natriuretic peptide receptor type B (NPR-B), while natriuretic peptide receptor type C (NPR-C) lacks enzymatic activity (Potthast and Potter, 2005; Potter *et al.*, 2006). NPR-A shows high affinity and is activated by ANP and BNP (Nakao *et al.*, 1992b), NPR-B is activated by CNP (Koller *et al.*, 1991) and, finally, the NPR-C binds all natriuretic peptides (Anand-Srivastava, 2005).

2. Natriuretic Peptides and Their Receptors

2.1. Natriuretic peptides

2.1.1. Atrial natriuretic peptide

ANP is a 28-amino acid peptide released in physiological state by the cardiac atria in response to wall stretch resulting from blood pressure and volume loading, as well as to physical exercise (Lang *et al.*, 1985; Vuolteenaho *et al.*, 1985). The human ANP gene encodes a preprohormone that contains 151 amino acids (Oikawa *et al.*, 1984), which is posttranslationally modified to obtain a 126-amino acid proANP that is stored in secretory granules in the atrial myocytes (Vuolteenaho *et al.*, 1985). During the secretory process,

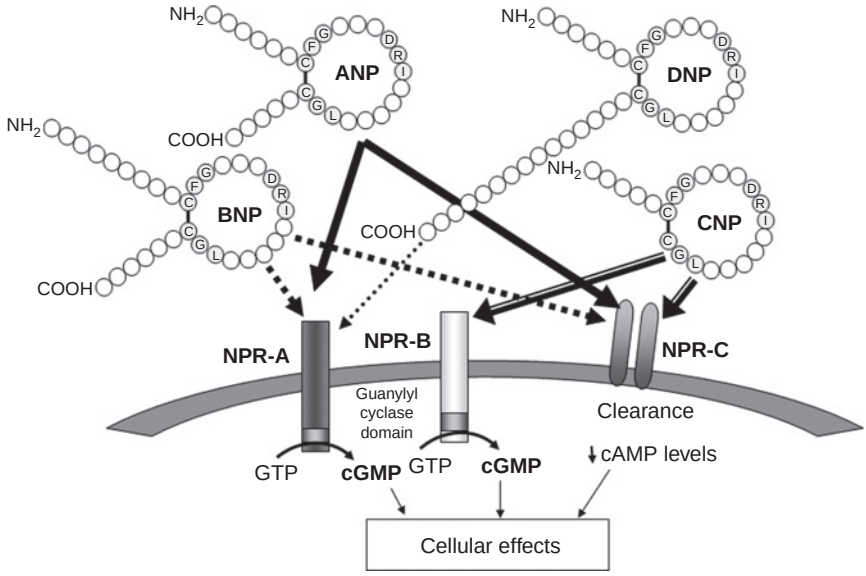

Figure 3.1 Natriuretic peptide signaling and peptide selectivity. Mature active forms of natriuretic peptides show a common 17-amino acid ring formed by disulfide bond formation between two cysteine residues. Variable C-terminal and N-terminal tails are present in ANP, BNP, and DNP, while CNP lacks the C-terminal tail. NPR-A and NPR-B are activated by ANP, BNP, CNP, and DNP, as indicated, and occupation of these receptors results in the stimulation of cGMP generation leading to physiological responses. The third type of natriuretic peptide receptor, NPR-C is involved in peptide internalization and clearance, and is also coupled to adenylyl cyclase through a Gi protein. ANP, BNP, and CNP have been reported to interact with NPR-C and inhibit adenylyl cyclase activity, which results in a decrease in intracellular cAMP concentration.

proANP is cleaved by the transmembrane cardiac serine protease called corin to the biologically active 28-amino acid mature C-terminal ANP ($ANP_{99–126}$) (Yan *et al.*, 2000). Alternative processing of proANP by a protease in the kidney generates a 32-residue peptide named urodilatin, which is important in regulating sodium and water excretion.

The expression of ANP is low in the ventricles in the physiological state and that it is increased in a number of cardiovascular disorders, such as left ventricular hypertrophy and upon volume and pressure overload (D'Souza *et al.*, 2004; Ruskoaho, 1992). Hormones such as endothelin (Stasch *et al.*, 1989), angiotensin (Soualmia *et al.*, 1997), and arginine–vasopressin (Lachance *et al.*, 1986) stimulate ANP release (Thibault *et al.*, 1999), as well as water immersion (Ogihara *et al.*, 1986) and head down posture (Hollister *et al.*, 1986). Plasma levels of ANP in normal patients are ~10 fmol/ml and are elevated 10- to 30-fold in patients with congestive heart failure (CHF)

(Cody *et al.*, 1986; Mukoyama *et al.*, 1991; Potter *et al.*, 2006). ANP circulates in the blood reducing vascular tone and promoting diuresis/natriuresis to lower blood volume and pressure (Ahluwalia *et al.*, 2004).

2.1.2. Brain natriuretic peptide

BNP was initially purified from porcine brain extracts (Sudoh *et al.*, 1988). BNP is a polypeptide that contains 32 amino acids and is mainly secreted by cardiac atria and ventricles (Weber and Hamm, 2006), although, in contrast to ANP, that is predominantly secreted in the atria, the cardiac ventricle is the major site of synthesis and secretion of BNP (D'Souza *et al.*, 2004; Ogawa *et al.*, 1991). As for ANP, the human BNP gene encodes a 132-amino acids preprohormone that is posttranslationally modified to the 108-amino acids prohormone ($BNP_{1–108}$). The proBNP is subsequently cleaved resulting in an inactive 76-residue amino-terminal fragment and a 32-amino acid C-terminal fragment, which is the mature biologically active peptide (Sudoh *et al.*, 1989).

In the cardiac atria, BNP is stored with ANP in granules; however, BNP is not stored in granules in the ventricles. Instead, ventricular BNP production is transcriptionally regulated by cardiac wall stretch (Potter *et al.*, 2005).

BNP is expressed in response to venous volume and/or blood pressure. BNP expression has been found to be constant during chronic cardiac overload, although its levels are increased in hypertrophied heart. Healthy individuals have plasma BNP concentrations of ~1 fmol/ml (3.5 pg/ml) or approximately one-tenth that of ANP (Potter, 2005). In contrast, plasma BNP concentrations of patients with CHF are elevated between 200- and 300-fold. The enormous range of plasma BNP concentrations between healthy subjects and patients with heart failure and the rapid induction of BNP expression in response to acute overload has been widely used as an indicative of elevated myocardial loading and cardiac stress (Mukoyama *et al.*, 1991; Tokola *et al.*, 2001). Other stimuli that induce BNP secretion are hypoxia, ischemia, and states of nephrosis and cirrhosis that are associated to an increase in central blood volume (D'Souza and Baxter, 2003; Toth *et al.*, 1994; Wong and Blendis, 1994).

2.1.3. C-type natriuretic peptide

CNP is the third member of the natriuretic peptide family identified. CNP is mainly expressed in the nervous system and vascular endothelial cells (Ogawa *et al.*, 1991; Woodard *et al.*, 2002a). Small amounts of CNP have been found in the cardiac tissue or circulating in blood (Yandle, 1994); however, in the nervous system the most abundant form of natriuretic peptide is CNP (Yandle, 1994). In addition, expression of CNP has also been found in the vascular endothelium, where it might exert its autocrine/paracrine roles in vascular tone and muscle cell growth (Komatsu *et al.*, 1992; Woodard *et al.*, 2002a); in fact, CNP has been shown to be more

potent than ANP inducing smooth muscle relaxation but shows a weaker effect on natriuresis and diuresis (Sudoh *et al.*, 1990). The cardiovascular effects of CNP are more likely mediated by local effects on blood vessel or by central actions on vasopressin and adrenocorticotropine release (Fowkes and McArdle, 2000; Komatsu *et al.*, 1992).

CNP is expressed and stored as a preprohormone that consists of 103 amino acids, which is then converted to two fragments of 22- and 53-amino acids through a multistep process involving a ''pro-protein convertase'' named furin (Wu *et al.*, 2003). The 22-amino acid fragment is the mature and more active CNP form (Ogawa *et al.*, 1992).

In contrast to ANP and BNP, which contains 5- and 6-amino acid residues in the C-terminal tail, respectively, CNP completely lacks this tail (Fig. 3.1). CNP gene expression is induced in response to a number of physiological and pathological vasoactive mediators including interleukin 1β, vascular endothelial growth factor (VEGF), transforming growth factor (TGF), tumor necrosis factor α (TNFα) (Suga *et al.*, 1992, 1993; Woodard *et al.*, 2002a), as well as by shear stress (Chun *et al.*, 1997), and is suppressed by insulin (Igaki *et al.*, 1996).

2.1.4. *Dendroaspis*-type natriuretic peptide

DNP is a recently isolated 38-amino acid peptide that shares structural and functional properties with the other members of the natriuretic peptide family. DNP immunoreactivity has been reported in human plasma, although purification of DNP from human plasma has not yet been achieved (Schirger *et al.*, 1999). The source of DNP has not been clearly identified although DNP-like immunoreactivity has been reported in rat aorta, carotid artery, and renal vasculature and tubules. DNP-like immunoreactivity was found to be regulated by endothelin-1, angiotensin II, and sodium nitroprusside but not by TGF-β (Woodard *et al.*, 2002b).

DNP binds to the ANP and BNP NPR-A (see Section 2.2), inducing vasorelaxation (Best *et al.*, 2002). In addition, DNP has been reported to elicit a marked inhibitory effect on DNA synthesis in culture rat aortic vascular smooth muscle cells (Woodard *et al.*, 2002b).

2.1.5. Other natriuretic peptides

In addition to ANP, BNP, CNP, and DNP, other peptides have been reported to exert a similar effect in mammalians. Urodilatin is a peptide obtained by alternative cleavage of the C-terminal 32 amino acids of proANP in the distal tubules of the kidney, where it exerts its natriuretic effect (Schulz-Knappe *et al.*, 1988). This peptide is synthesized by the same gene that synthesizes ANP, but in the kidney, in contrast to all other tissues that have been investigated, the ANP prohormone is processed differently, resulting in the formation of urodilatin rather than ANP (Levin *et al.*, 1998; Nakao *et al.*, 1992a; Schulz-Knappe *et al.*, 1988).

The intestinal epithelium expresses two peptides, guanylin and uroguanylin, which are involved in water absorption in the gut (Beltowski, 2001). Guanylin and uroguanylin participate in the prevention of hypernatremia and hypervolemia after salty meals. Ingestion of a salty meal induces secretion of both peptides into the intestinal lumen, where they inhibit Na^+ absorption and induce Cl^-, HCO_3^-, and water secretion. In addition, these hormones have been shown to stimulate renal electrolyte excretion by inducing natriuresis, kaliuresis, and diuresis (Fonteles *et al.*, 1998; Forte *et al.*, 2000).

A peptide with similarity to natriuretic peptides was identified by two separate groups in bone and muscle cells; for this reason, this peptide is currently referred to as osteocrin/Musclin (Nishizawa *et al.*, 2004; Thomas *et al.*, 2003). This peptide binds with high affinity to NPR-C, but not to NPR-A or NPR-B, in a manner that is competitive with ANP; therefore, in cells expressing NPR-A and NPR-C osteocrin/Musclin increases ANP-dependent cGMP generation, presumably by blocking NPR-C mediated ANP clearance. Although the physiological role of osteocrin/Musclin is not completely understood, its expression in human skeletal tissue, particularly in osteoblasts in developing human neonatal bone and at sites of bone remodeling, such as in iliac crest bone biopsies from adults, suggests a role for this protein in bone formation (Bord *et al.*, 2005).

2.2. Natriuretic peptide receptors

Natriuretic peptides exert their physiological effects by the occupation of three membrane receptors. Two subtypes are membrane-associated guanylyl cyclase-coupled receptors, known as NPR-A and NPR-B, which mediate most of the physiological actions of natriuretic peptides. Both receptors are particulate guanylyl cyclases, different from the soluble guanylyl cyclase, heterodimeric heme-containing cytosolic enzyme of monomers with molecular masses of 70 and 80 kDa activated by nitrovasodilators, nitric oxide, and free radicals. The NPR-C lacks guanylyl cyclase activity and among other functions acts as a clearance receptor.

2.2.1. Natriuretic peptide receptors-A and -B

NPR-A and NPR-B are single-transmembrane receptors with a similar basic structure. NPR-A is an ~120-kDa glycoprotein that contains a variable extracellular natriuretic peptide-binding region, a conserved intracellular kinase homology domain (KHD), which has been reported to be essential for the regulation of NPR-A activity and receptor sensitivity, and a guanylyl cyclase domain with enzyme activity (Hamad *et al.*, 2003). NPR-A shows high affinity and is activated by ANP, BNP, and DNP (Best *et al.*, 2002; Nakao *et al.*, 1992b; Schweitz *et al.*, 1992), but BNP is approximately tenfold less potent than ANP. In contrast, CNP does not increase guanylyl

cyclase activity of NPR-A. NPR-B is activated only by CNP and neither ANP nor BNP increase guanylate cyclase activity in NPR-B expressing cells (Ogawa *et al.*, 1992; Yandle, 1994). The study of the regulation of guanylyl cyclase activity of NPR-A revealed that the enzymatic activity of purified NPR-A cannot be stimulated solely by $ANP_{1–28}$ (Kuno *et al.*, 1986), suggesting that accessory mechanisms likely involving ATP, as well as a number of cations, including Mg^{2+}, regulate the activation of guanylyl cyclase in NPR-A (Chang *et al.*, 1991; Kurose *et al.*, 1987). ATP has been reported to regulate NPR-A activity through the interaction with the receptor KHD (Joubert *et al.*, 2005) or the involvement of an ATP-regulated module, which represses the catalytic activity of NPR-A (Duda and Sharma, 2005).

Occupation of these receptors by natriuretic peptides induce cellular responses through the elevation of intracellular cGMP levels, which has been demonstrated in all tissues and cells that express NPR-A and NPR-B upon stimulation with natriuretic peptides. The increase in intracellular cGMP concentration following the occupation of NPR-A or NPR-B leads to the activation of a cGMP-dependent protein kinase (PKG) that transfers a phosphate from ATP to a serine of threonine residue in a target protein, which, in turn, mediate a specific physiological function. cGMP-mediated signaling is terminated by cGMP phosphodiesterases, which modulate the intracellular concentrations of cGMP and the duration and magnitude of the responses (D'Souza *et al.*, 2004).

2.2.2. Natriuretic peptide receptor-C

NPR-C is a transmembrane receptor with an extracellular domain of ~440 amino acids that shares ~30% homology with NPR-A and NPR-B, a transmembrane domain, and a 37-amino acid cytosolic domain (Porter *et al.*, 1990). Two different cDNAs encoding NPR-C have been identified (Murthy *et al.*, 1998). Consistent with this two vascular NPR-C of ~67 and 77 kDa have been reported (Kato *et al.*, 1991), suggesting the existence of two subtypes of NPR-C (Savoie *et al.*, 1995; Trachte *et al.*, 1995).

NPR-C is the most abundant NPR in most tissues. NPR-C is widely distributed in several cells and tissues including vascular smooth muscle cells, renal glomeruli and collecting ducts, adrenals, platelets, lungs, cerebral cortex, and cardiac purkinje fibers (Anand-Srivastava, 2005; Porter *et al.*, 1990). NPR-C binds ANP, BNP, and CNP with high affinity, although its affinity is higher for ANP and CNP than for BNP (Bennett *et al.*, 1991).

NPR-C has been involved in peptide clearance. It has been reported that NPR-C removes ANP and BNP from the circulation (Maack *et al.*, 1993). In addition, it has also been suggested that NPR-C may be involved in other biological effects of natriuretic peptides through second messengers different from cGMP, such as the adenylyl cyclase/cAMP pathway (Murthy *et al.*, 2000). The adenylyl cyclase/cAMP system is one of the best

characterized signal transduction mechanisms and mediates the physiological actions of a variety of hormones. This pathway is composed of three elements: a receptor; a cyclase with enzymatic activity; and a stimulatory or inhibitory guanine nucleotide regulatory protein, the G proteins, which act as transducers and in the presence of guanine nucleotides communicates the signal from the membrane hormone receptor to the cyclase, resulting in an increase (if a Gs is involved) or decrease (when the transducer is a Gi) in the generation of cAMP. Natriuretic peptides have been reported to inhibit cAMP synthesis in a number of tissues, an effect that is dependent on the presence of guanine nucleotides, suggesting the involvement of a G protein in the NPR-adenylyl cyclase transduction system (Anand-Srivastava, 2005). The functional coupling between NPR-C and adenylyl cyclase was confirmed by the use of the ring deleted analog of ANP des[Glu^{18}, Ser^{19}, Gln^{20}, Leu^{21},Gly^{22}]ANP_{4-23}-NH_2 (C-ANP_{4-23}) that binds specifically to NPR-C. C-ANP_{4-23} has been reported to inhibit cAMP generation in a number of tissues including aorta, adrenal cortical membranes, vascular smooth muscle cells (Anand-Srivastava *et al.*, 1990), and renal glomeruli (Woodard *et al.*, 2004a, 2005). We have recently provided evidence that the 77 kDa protein is involved in peptide internalization and clearance, whereas the 67 kDa NPR-C-like protein inhibits adenylate cyclase activity; therefore, both NPR-C-like proteins are involved in the regulation of cardiovascular physiology (Woodard *et al.*, 2002c).

3. Functions of Natriuretic Peptides

3.1. Vascular effects of natriuretic peptides

In addition to the effects of natriuretic peptides on nonvascular cells, involving actions on renal tubular and mesangial cells, inhibition of renin, vasopressin or aldosterone release, which are important for their diuretic and natriuretic actions, a number of relevant systemic actions ascribed to natriuretic peptides are mediated to the reduction in total peripheral resistance. This effect might be achieved by direct effect on the vessel wall, such as the effect of natriuretic peptides inducing dilation of the renal afferent arterioles, which induces an increase in glomerular filtration and subsequently diuresis. In addition, the vascular effects of natriuretic peptides might be mediated by regulation of the sympathetic tone and catecholamine release from peripheral sympathetic neurons.

The direct vasorelaxant actions of natriuretic peptides are mediated by the activation of PKG. This enzyme regulates the opening of ion channels in vascular smooth muscle cells inducing hyperpolarization and, therefore, vascular relaxation (Dora *et al.*, 2001). In addition, cGMP decreases cytosolic free Ca^{2+} concentration by a mechanism that is likely mediated by

PKG (Rosado *et al.*, 2001). The effects of cGMP on intracellular Ca^{2+} homeostasis include a reduction in Ca^{2+} release from intracellular stores, reduced Ca^{2+} entry, and an increased Ca^{2+} removal from the cytosol either by promoting Ca^{2+} sequestration into the internal stores or increasing Ca^{2+} extrusion through the plasma membrane (Geiger *et al.*, 1992). Finally, natriuretic peptides might induce vasorelaxation through the activation of cGMP-regulated phosphodiesterases (D'Souza *et al.*, 2004). Natriuretic peptides increase the cellular levels of cGMP that activates phosphodiesterase-II, which is expressed in heart, platelets, liver, and adrenals. Activation of phosphodiesterase-II results in the decrease of intracellular cAMP concentration and thus impairs cAMP-mediated responses (Whalin *et al.*, 1991).

The vascular smooth muscle can regulate itself in the absence of endothelium and can provide a braking mechanism to the cellular proliferative effects common to disease and injury to the lumen of the arterial wall. Recent studies have shown that CNP is expressed in mammalian vascular smooth muscle independent of endothelial cells (Kelsall *et al.*, 2006; Mendonca *et al.*, 2006; Woodard *et al.*, 2002a). This expression could be modulated by cytokines expressed by immune cells and platelets as demonstrated in vascular smooth muscle cells treated with PDGF (Mendonca *et al.*, 2006), basic fibroblast growth factor (bFGF) (Mendonca *et al.*, 2006; Woodard *et al.*, 2002a), and TGF-β (Mendonca *et al.*, 2006; Woodard *et al.*, 2002a). Indeed, serum addition (10% fetal bovine serum) after 48 h serum starvation of human aortic vascular smooth muscle cells shows a greater than 60-fold induction of CNP-transcript levels after only 8 h (Mendonca *et al.*, 2006). Interestingly, human aortic endothelial cells show a much lower response in CNP-transcript level to serum addition, reaching a level of only threefold control value at 3 h and declining toward time-matched control values at 8 and 24 h after serum addition (Mendonca *et al.*, 2006). This positive effect of serum treatment on CNP mRNA was significantly reduced or abolished in the presence of genistein, a tyrosine kinase inhibitor, as tyrosine kinase is a known downstream effector of most serum-derived growth factors such as bFGF, PDGF, and VEGF. Likewise, the most profound effect of serum stimulation of CNP seemed to work through protein kinase C activation (PKC; Mendonca *et al.*, 2006).

Gene therapy using an adenoviral construct of CNP in vascular smooth muscle cells has also been shown to have a profound reduction in vascular smooth muscle cell proliferation and restenosis following balloon angioplasty (Pelisek *et al.*, 2006). This is not surprising considering very high levels of NPR-C have been found in neointimal vascular smooth muscle cells up to 9 months after percutaneous coronary intervention (Naruko *et al.*, 2005).

A recent study has reported that the bioactive lipid sphingosine-1-phosphate (S1P) to have a strong but acute inhibitory effect on NPR-B activity, as measured by cGMP, in A10 vascular smooth muscle cells

(Abbey-Hosch *et al.*, 2004). A different study suggested that the CNP-induced inhibitory effect on smooth muscle cell intimal proliferation might be somehow mediated by the regulation of the α_1-adrenoreceptor and IP_3 receptor (Xiaohong *et al.*, 2000).

3.2. Natriuretic peptides in cardiac physiology and pathology

ANP and BNP have been reported to show either negative or no effects on cardiac contractility when administered exogenously and even in the same species the findings are conflicting. ANP has been shown to have no effect in isolated rat cardiomyocytes or in isolated rat, feline or canine heart (Burnett *et al.*, 1987; Hirose *et al.*, 1998; MacDonell and Diamond, 1997; Yanagisawa *et al.*, 1987); however, ANP exerts a negative inotropic effect on avian cardiomyocytes or rat ventricular trabeculae (Stone *et al.*, 1990; Vaxelaire *et al.*, 1989). The inotropic effects of CNP are not less inconsistent and contradictory, and both positive and negative effects have been reported (D'Souza *et al.*, 2004). The reason of these conflicting results is still not clear but evidences suggest a possible concentration-dependent effect of natriuretic peptides on cardiac contractility. According to this hypothesis, low concentrations of natriuretic peptides might have positive inotropic effects while high concentrations of natriuretic peptides, sufficient to get micromolar concentrations of cGMP might induce negative inotropic effects due to inhibition of intracellular Ca^{2+} mobilization, especially Ca^{2+} entry through voltage-dependent channels and therefore by interfering with the cardiac contractile mechanism (Massion and Balligand, 2003). In any case, the clinical benefits of natriuretic peptides due to their negative inotropic effects have been considered insignificant compared to the cardiovascular effects observed due to their diuretic and natriuretic actions. For instance, nesiritide, a recombinant form of human BNP, may offer tolerability and clinical advantages over currently used vasodilators, inodilators, and inotropes; specially, nesiritide does not exert proarrhythmic effects. Nesiritide is effective and well tolerated in patients receiving concomitant β-blocker therapy or with renal insufficiency, which suggests nesiritide as a suitable first option for the treatment of patients with acutely decompensated CHF (Keating and Goa, 2003).

All the natriuretic peptides investigated have been shown to induce vasorelaxant effects in the coronary circulation, based on the effect of blockade of the guanylyl cyclase-coupled receptors NPR-A and NPR-B with the antagonist HS-142-1 (Supaporn *et al.*, 1996). Most studies have been focused on the administration of ANP, which exerts a concentration-dependent vasodilator action in canine and feline coronary arteries *in vivo* (Chu and Cobb, 1987; Yanagisawa *et al.*, 1987). The vasodilator actions of ANP are especially relevant in the epicardial coronary arteries (Adachi *et al.*, 1989). Similar effects have been reported for BNP in humans; although little effect of BNP on the

coronary resistance vessels have been demonstrated (Okumura *et al.*, 1995). The effects of ANP are endothelium independent and resistant to inhibitors of the soluble guanylyl cyclase in both canine and feline coronary arteries (Matsumoto *et al.*, 1999; Yanagisawa *et al.*, 1987). Although there are evidences supporting that ANP stimulates nitric oxide synthase in human renal proximal tubular cells (McLay *et al.*, 1995), its relevance in coronary vasodilatation remains unclear.

CNP has also been shown to induce vasorelaxant effects in porcine and human coronary arteries (Wei *et al.*, 1994; Wiley and Davenport, 2001). Although the mechanism mediating the coronary vasorelaxant actions of CNP are not completely understood, recent studies have suggested a role for nitric oxide synthase and soluble guanylyl cyclases (Brunner and Wolkart, 2001a,b). In addition, CNP has been presented as an endothelium-derived hyperpolarizing factor via activation of NPR-C and the opening of a G-protein-gated inwardly rectifying K^+ channel (Chauhan *et al.*, 2003). Likewise, vascular smooth muscle hyperpolarization elicited by CNP/EDHF depends on inwardly rectifying K^+ (KIR) channels and Na^+/K^+-ATPase (blocked by Ba^{2+} and ouabain, respectively; Chauhan *et al.*, 2003). The hyperpolarizing effects of CNP provide new insight for the development of therapeutic strategies to alter perfusion of isolated vascular beds (Ahluwalia *et al.*, 2004). Recent studies have reported that the actions of CNP on vascular tone are not limited to its hyperpolarizing effects and includes other vascular actions that mimics some of the anti-atherogenic actions of nitric oxide and prostaglandin I_2, thus CNP is an important regulatory factor for coronary blood flow and provide a cardioprotective effect preventing myocardial injury during ischemia/reperfusion (Chauhan *et al.*, 2003; Hobbs *et al.*, 2004).

Myocardial ischemia is one of the main causes of death in developing societies. Acute occlusion of an epicardial coronary artery following atherosclerotic plaque rupture is a primary cause of myocardial ischemia, which leads to myocyte loss and the development of a wave of necrosis and infarction of the ischemic tissue (D'Souza *et al.*, 2004). The consequences of myocardial infarction depend on the duration of the ischemia, and, therefore, the extent of the ischemic territory. The regulation of the cardiovascular function by the neurohormonal response to myocardial infarction has recently been widely investigated and special attention should be taken to the effect of natriuretic peptides. BNP concentration increases rapidly in the early phase of acute myocardial infarction. The plasmatic concentration of BNP increases within hours of the onset of acute myocardial infarction to reach a concentration that might be more than 100 times the physiological concentration (Morita *et al.*, 1993). Experimental studies have demonstrated that after left ventricle ischemia, BNP concentration in the ventricle increased about twofold in the first 12 h and fivefold at day 1 compared to the physiological levels. The concentration of BNP in

the coronary effluent was found to be dependent on the duration of the ischemia (D'Souza and Baxter, 2003). In addition, BNP expression was demonstrated in the ischemic and non-ischemic areas of the left ventricle as well as in the right ventricle (Hama *et al.*, 1995). ANP mRNA and concentration has also been shown to increase immediately after myocardial infraction (Galipeau *et al.*, 1988; Kleber *et al.*, 1992). These findings provide evidence that BNP, and probably ANP, are sensitive markers of acute myocardial infarction than ANP.

Rapid BNP release in response to experimental coronary artery occlusion has also been demonstrated in rat hearts (Toth *et al.*, 1994). The same authors reported that ANP and BNP secretion was stimulated by hypoxic perfusion, independently of myocardial damage, which suggests that natriuretic peptide release is associated to hypoxia and, subsequently, to a decrease in the cellular energy state due to impairment of the oxidative metabolism (Toth *et al.*, 1994).

Rapid release of natriuretic peptides following infarction has been suggested to provide a protective action on the ischemic myocardium, as well as other mediators of vascular, myocardial, and neuronal origin, including bradykinin, adenosine, and endogenous opioids (D'Souza *et al.*, 2004). Experiments carried out in rat hearts perfused with BNP prior and during coronary artery occlusion have demonstrated that BNP significantly reduces infarct size in a concentration-dependent manner (D'Souza *et al.*, 2003a). The protective effects of BNP are associated to an increase in myocardial cGMP concentration and are impaired by K_{ATP} channel blockers and inhibition of nitric oxide synthase and soluble guanylyl cyclase, suggesting that activation of nitric oxide-dependent soluble guanylyl cyclase is somehow involved in the cardioprotective effects of BNP over infarction size (D'Souza *et al.*, 2003b).

3.3. Antiproliferative effects of natriuretic peptides

Proliferation of vascular smooth muscle cells is an important response of arteries to several vascular injuries, and is involved in a number of cardiovascular pathologies such as atherosclerosis, restenosis after angioplasty, hypertensive left ventricular hypertrophy, postinfarction myocardial remodeling, cardiac failure, or ventricular dilatation. Cytokines and growth factors, released by the injured vascular wall and activated platelets, stimulate proliferation of the vascular smooth muscle cells (Clowes *et al.*, 1983). Natriuretic peptides have been reported to inhibit cell proliferation in several cell types, such as vascular smooth muscle cells through the generation of cGMP. In support of this hypothesis, nitric oxide, a cGMP-elevating agent, elicits antiproliferative effects in vascular smooth muscle cells (Garg and Hassid, 1989). ANP and BNP have been reported to modulate cell

growth and proliferation in smooth muscle cells and cardiac myocytes and fibroblasts, preventing the secretion of extracellular matrix (Abell *et al.*, 1989; Horio *et al.*, 2000; Tsuruda *et al.*, 2002). In vascular smooth muscle cells, CNP, which is commonly referred as an endothelium-derived relaxing factor (Brown *et al.*, 1997), has been presented as the most potent inhibitor of growth and proliferation, an effect mediated by the occupation of the guanylate cyclase-coupled NPR-B (Porter *et al.*, 1992). Consistent with this, CNP has been shown to inhibit arterial intimal thickening *in vivo*, most likely through the inhibition of vascular smooth muscle proliferation induced by vascular injury (Brown *et al.*, 1997). The presence of transcripts of ANP, and predominantly CNP, as well as mRNAs coding for all three NPRs in cultured rat vascular smooth muscle cells has been reported (Woodard *et al.*, 2002a). The production and secretion of CNP in vascular smooth muscle cells are stimulated by TGF-β while bFGF plays an inhibitory role, suggesting that ANP, and mainly CNP, are coexpressed with the NPRs in rat vascular smooth muscle cells, providing evidence for a vascular natriuretic peptide autocrine system of physiological relevance in these cells (Woodard *et al.*, 2002a). Recent studies have reported that small peptide fragments containing 12 amino acids irrespective of the region of the cytoplasmic domain of NPR-C inhibit proliferation induced by vasoactive peptides, such as angiotensin II, endothelin-1, and arginine vasopressin, through $G_{i\alpha}$ protein and MAP kinase/phosphatidylinositol 3-kinase/AKT pathways, which demonstrate the antiproliferative effects of NPR-C (Hashim *et al.*, 2006).

3.4. Natriuretic peptides and hypertension

Primary hypertension is a genetically determined disorder affecting the homeostatic systems that normally regulate blood pressure that is modulated by superimposed environmental factors. These control systems include endocrine, renal, vascular, cardiogenic, and neurogenic mechanisms that interact in order to achieve blood pressure homeostasis. Among those control systems, the interaction between blood pressure and the kidney is especially relevant and kidney might both play an essential role in the pathogenesis of hypertension and equally be a prime target of damage caused directly or indirectly by hypertension. Kidney is extremely sensitive to changes in blood pressure and responds in several ways to maintain circulatory homeostasis (Raine, 1994). In addition, several renal function abnormalities, such as increased renal vasoconstriction, increased renin and aldosterone secretion, or altered activity of monovalent ion transporters play an important role in the genesis of human hypertension (Orlov *et al.*, 1999; Van Hooft *et al.*, 1991). Among the potential pathogenic factors involved in hypertension, ANP is an obvious candidate due to its relevant effects of natriuresis, diuresis, and hypertension itself. In spontaneously hypertensive

rats (SHR), where hypertension is a polygenic trait and both autosomal and sex-linked genes have been suggested to influence blood pressure (Hilbert *et al.*, 1991), an exaggerated natriuretic and diuretic response to exogenous ANP has been reported compared to normotensive strains (Pollock and Adrendshorst, 1990). Consistent with this, renal glomerular NPR-A shows a higher affinity for $ANP_{1–28}$ and lower maximal binding capacity in SHR compared to age-matched normotensive rats at all ages investigated (Woodard *et al.*, 2004b). In addition, $ANP_{1–28}$ induced a higher cGMP production rate in renal glomeruli from SHR than in age-matched normotensive Wistar Kyoto rats, both in young (3-week-old rats before the development of hypertension in SHR) and in adult rats (Woodard *et al.*, 2004b). The abnormality in NPR-A-mediated signaling is unlikely mediated by hypertension itself since adult (12-week old) DOCA-salt hypertensive rats, which showed the same or even higher blood pressure as age-matched SHR, failed to show a significant difference either in basal or $ANP_{1–28}$-stimulated cGMP production compared with their control Wistar Kyoto rats (Woodard *et al.*, 2004b). In addition, we have reported that expression of NPR-A and NPR-C in isolated glomerular membrane homogenates in normotensive and DOCA-treated hypertensive rats is similar (Woodard *et al.*, 2006). Therefore, the high rate of cGMP production in SHR occurs before the development of hypertension, indicating its primary role in the pathogenesis of hypertension.

Abnormalities in NPRs in other structures have been reported in hypertensive subjects. It is well known that several brain structures play an important regulatory role in blood pressure via peripheral or central neurogenic and neurohormonal mechanisms. Several studies have reported an increase in ANP content in the hypothalamus and the olfactory bulb after stimulation with several hormones, including catecholamines, or ethanol, which is probably involved in the modulation of the increases in blood pressure (Bastos *et al.*, 2001; Guillaume *et al.*, 1997). In addition, the number of ANP-binding sites has been shown to be reduced in all the areas of the brain investigated in SHR compared to normotensive rats (Brown and Czarnecki, 1991; Saavedra *et al.*, 1986; Tang *et al.*, 1993; Woodard *et al.*, 2003), together with a higher rate of cGMP production in NPR-A from olfactory bulb and hypothalamus in SHR (Woodard *et al.*, 2003).

3.5. Natriuretic peptides and oxidative stress

Oxidative stress is the result of an imbalance between the production of oxidants and the ability of the antioxidant systems to neutralize the radicals and to prevent or repair the damage. Among the mechanisms involved in the enhanced endogenous production of ROS are specially relevant the mitochondria, xanthine oxidase, superoxide-dismutase, the activation of arachidonic acid metabolism, the metabolism of phosphoinositides, and the

activity of NADH/NADPH oxidase (Iuliano *et al.*, 1997; Li *et al.*, 2002; Seno *et al.*, 2001; Tsutsui, 2004). Oxidative stress is associated with the accumulation of reactive species, such as reactive oxygen species (ROS), reactive nitrogen species, reactive nitrogen oxygen species, as well as unbound metal ions (Davis *et al.*, 2001). ROS include oxygen radicals such as superoxide radical, hydroxyl radical, as well as non-radical derivatives of oxygen including hydrogen peroxide (H_2O_2). Reactive nitrogen species include nitric oxide radical, and reactive nitrogen oxygen species include the highly reactive oxidant species peroxynitrite, which is a derivative of the reaction between nitric oxide and the superoxide radical. These reactive species are highly oxidizing and potently damaging to redox-sensitive intracellular proteins and DNA (Aschner *et al.*, 2007). In addition, they are also associated to peroxidation of membranes and to premature aging.

ROS, such as H_2O_2, have also been presented as intracellular messengers required for the activation of a large number of signal transduction mechanisms, especially those mediated by tyrosine kinases (Salmeen *et al.*, 2003; Yada *et al.*, 2003). In this context, a large number of physiological agonists have been reported to stimulate H_2O_2 production in several cell types (Frank *et al.*, 2000; Kimura *et al.*, 1995; Redondo *et al.*, 2005; Rosado *et al.*, 2004; Seno *et al.*, 2001). ROS induce the activation of a number of tyrosine kinase proteins, such as proteins of the MAPK pathway, proteins of the Src family, and focal adhesion proteins (Abe *et al.*, 1997; Ben Mahdi *et al.*, 2000; Finkel, 1998). The latter are involved in the reorganization of the actin cytoskeleton (Rosado *et al.*, 1998), another major target of ROS that has been shown to contribute significantly to the inherent sensitivity of cells to oxidative stress (Haarer and Amberg, 2004; Rosado *et al.*, 2002). Consistent with this, H_2O_2 (10 μM) has been shown to induce a temporal reorganization of the actin cytoskeleton in platelets, similar to that induced by agonists on actin microfilaments and microtubules (Bouaziz *et al.*, 2007; Redondo *et al.*, 2006). This actin remodeling consists of an initial net depolymerization followed by a net increase in the actin filament content (Redondo *et al.*, 2004a). Our observations are consistent with the involvement of ROS in the activation of the *de novo* conformational coupling between the type II IP_3 receptor in the endoplasmic reticulum and hTRPC1 channels in the plasma membrane, which has been proposed to mediate store-operated Ca^{2+} entry (SOCE) in human platelets (Ben-Amor *et al.*, 2006; Lopez *et al.*, 2006; Rosado and Sage, 2000, 2002; Rosado *et al.*, 2005; Woodard *et al.*, 2007), where cortical actin filaments prevent constitutive SOCE activation blocking the approach of portions of the ER to the PM (Rosado and Sage, 2001; Rosado *et al.*, 2000). The correlation between Ca^{2+} release and entry and actin reorganization induced by H_2O_2 suggests that low concentrations of H_2O_2 might act as physiological molecules in human platelets, as previously reported (Rosado *et al.*, 2004; Seno *et al.*, 2001). However, ROS have also been found to exerts a biphasic concentration-dependent

effects on intracellular Ca^{2+} homeostasis, so that at high concentrations H_2O_2 induces Ca^{2+} release from mitochondrial and non-mitochondrial Ca^{2+} stores most likely through the inhibition of the sarcoendoplasmic reticulum Ca^{2+} ATPase, the sensitization of IP_3 receptors and the inhibition of Ca^{2+} extrusion mechanisms, which leads to an abnormally elevated basal cytosolic Ca^{2+} concentration (Redondo *et al.*, 2004b; Suzuki and Ford, 1992).

Although free radicals are known to play a physiological role in optimal cell function, excessive oxidative stress has been involved in a variety of cardiovascular disorders. It has been reported that increased production of ROS has been associated with the development of hypertension, an effect that might be associated to elevated methylglyoxal level, which, in turn, might induce generation of ROS (Chang and Wu, 2006). A number of studies support the role of oxidative stress in the pathogenesis of hypertension. In hypertensive animal models such as SHR and DOCA-salt induced-hypertension, supplementation with the antioxidants vitamin C or vitamin E reduced blood pressure and vascular remodeling (Chen *et al.*, 2001). Consistent with the role of oxidative stress in hypertension, overexpression of superoxide dismutase, which neutralizes superoxide ions, and catalase, which decomposes H_2O_2, reduces hypertension and increases NO availability in different models of experimental hypertension (Chu *et al.*, 2003). In addition, tempol (a superoxide dismutase mimetic) decreases blood pressure; increases NO bioavailability; and improves endothelium-dependent relaxation, kidney damage, and glomerular filtration in SHR and DOCA-salt hypertensive rats (Chen *et al.*, 2001; Kawada *et al.*, 2002; Kojsova *et al.*, 2006).

Oxidative stress also contributes importantly to the pathophysiology of inflammation in the cardiovascular system. ROS play a key role in regulation of matrix metalloproteinases and the development of apoptotic events in smooth muscle cells (Yuan *et al.,* 2007). Recent evidences suggest that ROS and reactive nitrogen species are associated with abdominal aortic aneurysm formation in several experimental models and humans (McCormick *et al.*, 2007). Furthermore, it is well known that oxidative stress is involved in cardiac hypertrophy and in the pathogenesis of cardiomyopathies, ischemic heart disease, and CHF. ROS have been reported to be able to induce cellular damage and acceleration of cell death through apoptosis and necrosis, which is especially relevant in advanced heart failure (Suematsu *et al.*, 2003). Moreover, ROS can impair cellular energetics through actions on mitochondrial enzymes in the heart, a tissue that is remarkably sensitive to oxygen deprivation (Solaini and Harris, 2005). A large body of evidence suggests a role for increased ROS production in the processes underlying left ventricular hypertrophy, left ventricular remodeling, and heart failure. In isolated cardiomyocytes, hypertrophy induced by a number of agonists, including catecholamines, angiotensin II, endothelin-1, or TNFα has

been shown to involve enhanced ROS generation (Hirotani *et al.*, 2002; Nakamura *et al.*, 1998; Pimentel *et al.*, 2001). In addition, enhancement of superoxide by inhibition of superoxide dismutase results in cardiomyocyte hypertrophy and apoptosis, which further supports a role for oxidative stress in the pathogenesis of myocardial remodeling and failure (Siwik *et al.*, 1999). Enhanced ROS production has also been shown to be involved in left ventricular remodeling resulting from experimental myocardial infarction or chronic CHF, which can be prevented by ROS scavengers such as by long-term administration of dimethylthiourea, a hydroxyl radical scavenger (Kinugawa *et al.*, 2000), or by treatment with the antioxidant probucol (Sia *et al.*, 2002), which exert multiple beneficial morphological effects including better left ventricular function, reduced neurohumoral activation, and preservation of renal function. In addition to cardiomyocyte hypertrophy, enhanced production of ROS has been shown to induce reexpression of fetal gene programs (Hare, 2001; Kono *et al.*, 2006).

It has been reported that oxidative stress is increased in most of the body regions, including the myocardium, in patients with heart failure (Mallat *et al.*, 1998). There is also a good correlation between oxidative stress, myocardial dysfunction, and the progression and severity of heart failure in CHF patients, (Hornig *et al.*, 1998; Mallat *et al.*, 1998; McMurray *et al.*, 1993). Oxidative stress has been shown to be increased in ischemic and non-ischemic CHF, a process where neutrophils may play an important role. Short- but mainly long-term administration of vitamin C reduces oxidative stress, decreases neutrophil superoxide anion generating capacity, and increases flow-mediated dilation (Ellis *et al.*, 2000). Among the different sources of ROS, the NADPH oxidases, which are specifically activated by several stimuli such as angiotensin II, cytokines, and mechanical forces, have been suggested to be especially important in modulating redox-sensitive signaling pathways that might underlie the development of cardiomyocyte hypertrophy and ventricular remodeling (Cave *et al.*, 2005).

In bovine carotid artery endothelial cells, treatment with 0.5 and 1 mmol/l H_2O_2 resulted in nine- and tenfold increases of CNP concentration in the media. Further RT-PCR analysis reported that CNP mRNA expression in these cells was rapidly enhanced within 1 h with 1 mmol/l H_2O_2, reaching a peak at 3 h to show a tenfold increase. These findings suggest that H_2O_2 augments endothelial secretion of the endothelium-derived relaxing peptide CNP, which might be an endothelial response under oxidative stress to compensate the impaired nitric oxide-dependent vasorelaxation in hypertension and atherosclerosis (Chun *et al.*, 2000).

Recent studies have reported that infusion of human ANP (carperitide), in addition to its known effects improving hemodynamics in patients with heart failure, shows antioxidant actions, reducing the level of superoxide in cardiomyocytes induced by addition of H_2O_2 (Shono *et al.*, 2007). Due to the expression of NPR-A throughout the body, it is expected that

carperitide exerts its effects in many organs, such as the kidneys, blood vessels, and heart. However, treatment of isolated cardiomyocytes with exogenous ANP at low concentration (10^{-8} mol/l or 10^{-9} mol/l; comparable with the therapeutic dose of carperitide), rapidly and strongly suppressed the expression of thioredoxin, a multifunctional protein that contains a redox-active dithio/disulfide in the active site and exerts cytoprotection against oxidative stress (Hare, 2001), which suggests that infused carperitide shows a direct antioxidant action on the failing heart *in vivo* (Shono *et al.*, 2007). The antioxidant efficiency of ANP has been reported in neonatal rat cardiomyocytes, where the hypertrophic response of the heart to stimuli such as angiotensin II, consisting of increase in cardiomyocyte size, β-myosin heavy chain and *c*-fos expression, superoxide generation, and gp91phox expression, was significantly reduced by ANP. These effects have been shown to be mimicked by the superoxide dismutase mimetic, tempol. ANP, as well as tempol, also significantly inhibited endothelin-1-induced cardiomyocyte hypertrophy and superoxide anion generation. Altogether, these findings demonstrate that the antihypertrophic actions of ANP are accompanied by a reduction in the cellular levels of superoxide (Laskowski *et al.*, 2006), suggesting that an antioxidant action contributes to the well known antihypertrophic effects of ANP.

The protective effects of ANP against oxidant-induced injury have also been reported in rat aortic smooth muscle cells. In these cells, treatment with the water-soluble 2,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH), a free radical generating system, induces a significant increase in cytosolic free Ca^{2+} concentration and phospholipase D (PLD) activity as well as a decrease in intracellular pH due to the activity of the Ca^{2+}/H^{+} exchanger, that operates the efflux of Ca^{2+} coupled to H^{+} influx. Pretreatment of rat aortic smooth muscle cells with pharmacological ANP concentrations have been reported to attenuate the effects of AAPH, although no effects were observed with physiological ANP concentrations, suggesting a possible role of the natriuretic peptide as protective effector against early events of the oxidative stress (De Vito *et al.*, 2003). In these cells, lysophosphatidic acid has been reported to induce cell growth and ROS production. Both effects were impaired by physiological concentrations of ANP, without having any significant effect on lysophosphatidic acid receptors expression. The effect of lysophosphatidic acid on cell growth and ROS production has been suggested to be mediated by activation of the lipid kinase, phosphatidylinositol 3-kinase, and its substrate Akt, since ANP-induced inhibition of lysophosphatidic acid-activated responses was mimicked by cell treatment with wortmannin, an inhibitor of phosphatidylinositol 3-kinase, and physiological concentrations of ANP reduced the activation of Akt by lysophosphatidic acid. These findings also point out that the pathway phosphatidylinositol 3-kinase/Akt might be a target for ANP-induced regulation of cell proliferation (Baldini *et al.*, 2005a).

In Hep G2 cells and human monocytes or macrophages, ANP has been shown to regulate ROS production through either the recruitment of a signal pathway involving an increase in p38 MAPK phosphorylation, downregulation of mRNA NHE-1 expression, and regulation of phospholipase D activity or the involvement of diacylglycerol and phosphatidic acid (Baldini *et al.*, 2003, 2005b), pointing to ANP as a modulator of ROS production.

4. Further Aspects of Natriuretic Peptides in Cardiovascular Medicine

CHF, a condition in which the heart cannot pump enough blood to the organs, leading to tissue congestion and affecting the ability of the kidney to dispose of sodium and water and increasing edema, affects a large number of patients. Only in the United States, nearly 5 million people suffer from this pathology. Among the cardiac risk factors involved in the development of CHF smoking, high cholesterol, high blood pressure, diabetes, and obesity are especially relevant. Current medications for the treatment of CHF include diuretics, inotropes, vasodilators, and β blockers, which are designed to overcome the alterations on single components of the diverse pathways contributing to CHF. For instance, diuretics help the kidneys to eliminate excess fluid, thereby reducing blood volume and heart workload, vasodilators facilitate blood flow through the peripheral arteries and β blockers slow the heart rate and reduce blood pressure by blocking the effects of catecholamines. Despite the treatment of CHF with multiple drugs, almost all CHF patients experience one or several episodes of acute CHF that require hospitalization. A major factor leading to the less than optimal treatment of CHF is its focus on the symptoms of the pathology rather than addressing the underlying cause. In most CHF cases, the inability of the heart to pump blood efficiently is the result of other medical conditions, such as high blood pressure or reduced kidney function.

It has been reported that patients with heart disease show attenuated natriuretic peptide response, which might be attributed to the recently described downregulation in the density of NPR-A in heart and coronary artery of patients with ischemic heart disease (Singh *et al.*, 2006).

As previously mentioned, BNP has several physiological effects, such as a vasodilatation, or the increase in the excretion of sodium (natriuresis) and fluid (diuresis). When the heart is unable to pump blood efficiently, BNP is produced and its actions work in concert on the vessels, heart, and kidney to decrease the fluid load on the heart, allowing the heart to function better and improving cardiac performance (Fig. 3.2). Recent studies have reported evidence supporting that adjunctive therapy with nesiritide (human recombinant BNP; hBNP) may be beneficial for patients with advanced heart

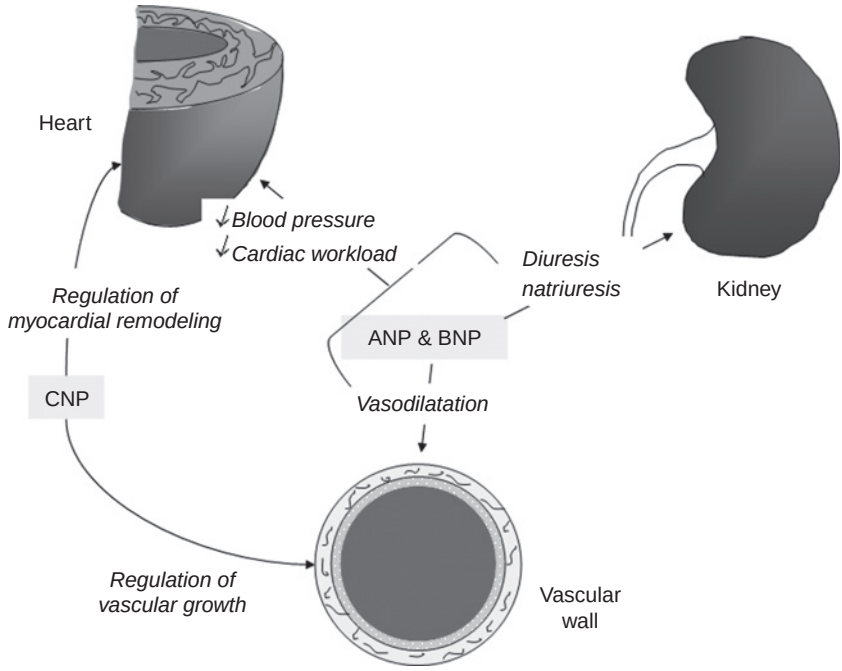

Figure 3.2 **Proposed roles of natriuretic peptides in congestive heart failure. In response to cardiac failure endogenous or exogenous administered ANP and BNP enhanced natriuresis and diuresis and induce local vasodilatation. CNP reduces the development of cardiac remodeling and attenuates vascular smooth muscle cell proliferation. The net result of these actions is a decrease in blood pressure and circulatory volume, thus reducing cardiac workload.**

failure and renal insufficiency (Yancy and Singh, 2006). In patients hospitalized with decompensated CHF, nesiritide improves hemodynamic function and their clinical status (Colucci *et al.*, 2000). The major hemodynamic effects of hBNP reported includes decrease in cardiac preload and systemic vascular resistance, improvement of cardiac output without increasing heart rate, and a reduced plasma norepinephrine and aldosterone levels; therefore, hBNP has been presented as a pharmacologically active peptide with potential in the therapy for decompensated heart failure (Abraham *et al.*, 1998), and compared with dobutamine, a pharmacological tool commonly used as a means of treating decompensated CHF, nesiritide is a safer short-term treatment for patients with decompensated CHF (Burger *et al.*, 2002).

Recombinant human ANP (carperitide) has also been shown to be useful for management of acutely decompensated CHF. Carperitide has been approved for the clinical management of acute decompensated CHF in Japan since 1995 (Chen and Burnett, 2006). Investigation of the cardiovascular effects of carperitide reported that this compound has a negligible

effect on coronary perfusion pressure or heart rate, but attenuated the contractile force in isolated guinea pig hearts. Intravenous infusion of carperitide decreased arterial blood pressure and total peripheral resistance in the anesthetized and conscious dogs. In addition, intravenous carperitide reduced pulmonary capillary wedge pressure, pulmonary pressure, and right atrial pressure in dogs with CHF induced by coronary artery occlusion and saline loading, suggesting that carperitide can improve the hemodynamic alterations in animals with acute experimental heart failure (Fig. 3.2) (Hidaka *et al.*, 1993).

As recently found, an increase in the plasma levels of CNP of myocardial origin and of its precursor was observed in CHF, suggesting that CNP is produced in the heart during cardiac failure where it may elicit important compensatory physiological effects on ventricular remodeling (Fig. 3.2) (Del Ry *et al.*, 2006a; Kalra *et al.*, 2003). Continuous administration of CNP in rats with experimental myocardial infarction has shown that CNP improved left ventricular dysfunction and reduced the development of cardiac remodeling due to its potent antifibrotic and antihypertrophic actions (Soeki *et al.*, 2005). CNP may represent an important new local autocrine and endocrine antiremodeling mediator in CHF (Del Ry *et al.*, 2006b).

CNP has also been shown to attenuate the development of restenosis, a significant clinical problem limiting the long-term therapeutic success of balloon dilation or stent implantation. Recent studies investigating the therapeutic effects of periadventitial liposome-mediated CNP gene transfer *in vivo* reported a significant long-term reduction of neointimal formation without compromising endothelial repair. The therapeutic effect of CNP gene transfer was better than single CNP administration. The advantages of CNP based therapy during angioplasty are its physiological origin and the simultaneous inhibition of vascular smooth muscle cell proliferation while promoting re-endothelialization (Fig. 3.2) (Pelisek *et al.*, 2006). Therefore, the inclusion of CNP in drug-eluting stents might be used to reduce the risk of restenosis, providing a considerable improvement to cardiovascular treatments so that future adverse coronary events can be averted.

5. Concluding Remarks

In addition to its mechanical function, the heart is an endocrine organ that upon cardiac stretch and overload releases natriuretic peptides, which, in turn, exert a variety of beneficial actions to the cardiovascular system, including vasodilation, natriuresis, and diuresis. Cardiac-derived natriuretic peptides, mostly BNP, have emerged as useful biomarkers for the diagnosis and prognosis of patients showing heart failure. In fact, the inactive amino-terminal fragment of the BNP prohormone (NT-proBNP), which is more

stable than mature BNP, has been presented as a tool for the diagnosis of cardiac dysfunction and elevated NT-proBNP plasma concentrations have been reported to be predictive of poor prognosis in a variety of cardiovascular diseases (Costello-Boerrigter and Burnett, 2005). In addition to cardiac-secreted natriuretic peptides, CNP, synthesized and released by vascular endothelial cells shows strong antiproliferative effects, inhibiting arterial intimal thickening through the inhibition of vascular smooth muscle proliferation induced by vascular injury. A number of studies have provided evidence supporting that adjunctive therapy with nesiritide (human recombinant BNP) or carperitide (human recombinant ANP) may be beneficial for patients with advanced heart failure and renal insufficiency due to their effects modulating fluid and electrolyte balance, vascular smooth muscle tone, and the regulation of coronary blood flow, myocardial perfusion and proliferative responses during myocardial and vascular remodeling; although further clinical studies are necessary to evaluate risks and benefits, safety, and comparative efficacy of the use of natriuretic peptides with other treatments.

REFERENCES

Abbey-Hosch, S. E., Cody, A. N., and Potter, L. R. (2004). Sphingosine-1-phosphate inhibits C-type natriuretic peptide activation of guanylyl cyclase B (GC-B/NPR-B). *Hypertension* **43,** 1103–1109.

Abe, J., Takahashi, M., Ishida, M., Lee, J. D., and Berk, B. C. (1997). c-Src is required for oxidative stress-mediated activation of big mitogen-activated protein kinase 1. *J. Biol. Chem.* **272,** 20389–20394.

Abell, T. J., Richards, A. M., Ikram, H., Espiner, E. A., and Yanker, T. (1989). Atrial natriuretic factor inhibits proliferation of vascular smooth muscle cells stimulated by platelet-derived growth factor. *Biochem. Biophys. Res. Commun.* **160,** 1392–1396.

Abraham, W. T., Lowes, B. D., Ferguson, D. A., Odom, J., Kim, J. K., Robertson, A. D., Bristow, M. R., and Schrier, R. W. (1998). Systemic hemodynamic, neurohormonal, and renal effects of a steady-state infusion of human brain natriuretic peptide in patients with hemodynamically decompensated heart failure. *J. Card. Fail.* **4,** 37–44.

Adachi, H., Tomoike, H., Nishijima, H., Egashira, S., and Nakamura, M. (1989). Sustained dilatation of large coronary artery by alpha-human atrial natriuretic peptide in conscious dogs: A comparison with nitroglycerin. *Eur. J. Pharmacol.* **161,** 189–196.

Ahluwalia, A., MacAllister, R. J., and Hobbs, A. J. (2004). Vascular actions of natriuretic peptides. Cyclic GMP-dependent and -independent mechanisms. *Basic Res. Cardiol.* **99,** 83–89.

Anand-Srivastava, M. B. (2005). Natriuretic peptide receptor-C signaling and regulation. *Peptides* **26,** 1044–1059.

Anand-Srivastava, M. B., Sairam, M. R., and Cantin, M. (1990). Ring-deleted analogs of atrial natriuretic factor inhibit adenylate cyclase/cAMP system. Possible coupling of clearance atrial natriuretic factor receptors to adenylate cyclase/cAMP signal transduction system. *J. Biol. Chem.* **265,** 8566–8572.

Aschner, M., Syversen, T., Souza, D. O., Rocha, J. B., and Farina, M. (2007). Involvement of glutamate and reactive oxygen species in methylmercury neurotoxicity. *Braz. J. Med. Biol. Res.* **40,** 285–291.

Baldini, P. M., De Vito, P., Martino, A., Fraziano, M., Grimaldi, C., Luly, P., Zalfa, F., and Colizzi, V. (2003). Differential sensitivity of human monocytes and macrophages to ANP: A role of intracellular pH on reactive oxygen species production through the phospholipase involvement. *J. Leukoc. Biol.* **73,** 502–510.

Baldini, P. M., De Vito, P., D'aquilio, F., Vismara, D., Zalfa, F., Bagni, C., Fiaccavento, R., and Di Nardo, P. (2005a). Role of atrial natriuretic peptide in the suppression of lysophosphatydic acid-induced rat aortic smooth muscle (RASM) cell growth. *Mol. Cell. Biochem.* **272,** 19–28.

Baldini, P. M., De Vito, P., Vismara, D., Bagni, C., Zalfa, F., Minieri, M., and Di Nardo, P. (2005b). Atrial natriuretic peptide effects on intracellular pH changes and ROS production in HEPG2 cells: Role of p38 MAPK and phospholipase D. *Cell. Physiol. Biochem.* **15,** 77–88.

Bastos, R., Favaretto, A. L., Gutkowska, J., McCann, S. M., and Antunes-Rodrigues, J. (2001). Alpha-adrenergic agonists inhibit the dipsogenic effect of angiotensin II by their stimulation of atrial natriuretic peptide release. *Brain Res.* **895,** 80–88.

Beltowski, J. (2001). Guanylin and related peptides. *J. Physiol. Pharmacol.* **52,** 351–375.

Ben-Amor, N., Redondo, P. C., Bartegi, A., Pariente, J. A., Salido, G. M., and Rosado, J. A. (2006). A role for 5,6-epoxyeicosatrienoic acid in calcium entry by *de novo* conformational coupling in human platelets. *J. Physiol.* **570,** 309–323.

Ben Mahdi, M. H., Andrieu, V., and Pasquier, C. (2000). Focal adhesion kinase regulation by oxidative stress in different cell types. *IUBMB Life* **50,** 291–299.

Bennett, B. D., Bennett, G. L., Vitangcol, R. V., Jewett, J. R., Burnier, J., Henzel, W., and Lowe, D. G. (1991). Extracellular domain-IgG fusion proteins for three human natriuretic peptide receptors. Hormone pharmacology and application to solid phase screening of synthetic peptide antisera. *J. Biol. Chem.* **266,** 23060–23067.

Best, P. J., Burnett, J. C., Wilson, S. H., Holmes, D. R., Jr., and Lerman, A. (2002). *Dendroaspis* natriuretic peptide relaxes isolated human arteries and veins. *Cardiovasc. Res.* **55,** 375–384.

Bord, S., Ireland, D. C., Moffatt, P., Thomas, G. P., and Compston, J. E. (2005). Characterization of osteocrin expression in human bone. *J. Histochem. Cytochem.* **53,** 1181–1187.

Bouaziz, A., Ben Amor, N., Woodard, G. E., Zibidi, H., Lopez, J. J., Bartegi, A., Salido, G. M., and Rosado, J. A. (2007). Tyrosine phosphorylation/dephosphorylation balance is involved in thrombin-evoked microtubular reorganisation in human platelets. *Thromb. Haemost.* **98**(2), 375–384, Doi: 10.1160/TH07-01-0061.

Brown, J., and Czarnecki, A. (1991). Receptor subtypes for natriuretic peptides in the brains of hypertensive rats. *Am. J. Physiol. Renal Physiol.* **260,** 441–447.

Brown, J., Chen, Q., and Hong, G. (1997). An autocrine system for C-type natriuretic peptide within rat carotid neointimal during arterial repair. *Am. J. Physiol. Heart Circ. Physiol.* **272,** 2919–2931.

Brunner, F., and Wolkart, G. (2001a). Endothelial NO/cGMP system contributes to natriuretic peptide-mediated coronary and peripheral vasodilation. *Microvasc. Res.* **61,** 102–110.

Brunner, F., and Wolkart, G. (2001b). Relaxant effect of C-type natriuretic peptide involves endothelium and nitric oxide-cGMP system in rat coronary microvasculature. *Cardiovasc. Res.* **51,** 577–584.

Burger, A. J., Horton, D. P., LeJemtel, T., Ghali, J. K., Torre, G., Dennish, G., Koren, M., Dinerman, J., Silver, M., Cheng, M. L., and Elkayam, U. (2002). Effect of nesiritide (B-type natriuretic peptide) and dobutamine on ventricular arrhythmias in the treatment of patients with acutely decompensated congestive heart failure: The PRECEDENT study. *Am. Heart J.* **144,** 1102–1108.

Burnett, J. C., Jr., Rubanyi, G. M., Edwards, B. S., Schwab, T. R., Zimmerman, R. S., and Vanhoutte, P. M. (1987). Atrial natriuretic peptide decreases cardiac output independent of coronary vasoconstriction. *Proc. Natl. Acad. Sci. USA* **186,** 313–318.

Cave, A., Grieve, D., Johar, S., Zhang, M., and Shah, A. M. (2005). NADPH oxidase-derived reactive oxygen species in cardiac pathophysiology. *Philos. Trans. R. Soc. B* **360,** 2327–2334.

Chang, T., and Wu, L. (2006). Methylglyoxal, oxidative stress, and hypertension. *Can. J. Physiol. Pharmacol.* **84,** 1229–1238.

Chang, C. H., Jiang, B., and Douglas, J. G. (1991). Calcium reveals different mechanisms of guanylate cyclase activation by atrial natriuretic factor and ATP in rat lung membranes. *Biochim. Biophys. Acta* **1093,** 42–46.

Chauhan, S. D., Nilsson, H., Ahluwalia, A., and Hobbs, A. J. (2003). Release of C-type natriuretic peptide accounts for the biological activity of endothelium-derived hyperpolarizing factor. *Proc. Natl. Acad. Sci. USA* **100,** 1426–1431.

Chen, H. H., and Burnett, J. C., Jr. (2006). Clinical application of the natriuretic peptides in heart failure. *Eur. Heart J.* **8,** 18–25.

Chen, X., Touyz, R. M., Rark, J. B., and Schiffrin, E. L. (2001). Antioxidant effects of vitamins C and E are associated with altered activation of vascular NADPH oxidase and superoxide dismutase in stroke-prone SHR. *Hypertension* **38,** 606–611.

Chu, A., and Cobb, F. R. (1987). Effects of atrial natriuretic peptide on proximal epicardial coronary arteries and coronary blood flow in conscious dogs. *Circ. Res.* **61,** 485–491.

Chu, Y., Iida, S., Lund, D. D., Weiss, R. M., Dibona, G. F., Watanabe, Y., Faraci, F. M., and Heistad, D. D. (2003). Gene transfer of extracellular superoxide dismutase reduces arterial pressure in spontaneously hypertensive rats: Role of heparin-binding domain. *Circ. Res.* **92,** 461–468.

Chun, T. H., Itoh, H., Ogawa, Y., Tamura, N., Takaya, K., Igaki, T., Yamashita, J., Doi, K., Inoue, M., Masatsugu, K., Korenaga, R., Ando, J., *et al.* (1997). Shear stress augments expression of C-type natriuretic peptide and adrenomedullin. *Hypertension* **29,** 1296–1302.

Chun, T. H., Itoh, H., Saito, T., Yamahara, K., Doi, K., Mori, Y., Ogawa, Y., Yamashita, J., Tanaka, T., Inoue, M., Masatsugu, K., Sawada, N., *et al.* (2000). Oxidative stress augments secretion of endothelium-derived relaxing peptides, C-type natriuretic peptide and adrenomedullin. *J. Hypertens.* **18,** 575–580.

Clowes, A. W., Reidy, M. A., and Clowes, M. M. (1983). Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. *Lab. Invest.* **49,** 327–333.

Cody, R. J., Atlas, S. A., Laragh, J. H., Kubo, S. H., Covit, A. B., Ryman, K. S., Shaknovich, A., Pondolfino, K., Clark, M., Camargo, M. J., Scarborough, R. M., and Lewicki, J. A. (1986). Atrial natriuretic factor in normal subjects and heart failure patients. Plasma levels and renal, hormonal, and hemodynamic responses to peptide infusion. *J. Clin. Invest.* **78,** 1362–1374.

Colucci, W. S., Elkayam, U., Horton, D. P., Abraham, W. T., Bourge, R. C., Johnson, A. D., Wagoner, L. E., Givertz, M. M., Liang, C. S., Neibaur, M., Haught, W. H., and LeJemtel, T. H. (2000). Intravenous nesiritide, a natriuretic peptide, in the treatment of decompensated congestive heart failure. Nesiritide Study Group. *N. Engl. J. Med.* **343,** 246–253.

Costello-Boerrigter, L. C., and Burnett, J. C. (2005). The prognostic value of N-terminal proB-type natriuretic peptide. *Nat. Clin. Pract. Cardiovasc. Med.* **2,** 194–201.

Davis, K. L., Martin, E., Turko, I. V., and Murad, F. (2001). Novel effects of nitric oxide. *Annu. Rev. Pharmacol. Toxicol.* **41,** 203–236.

de Bold, A. J., Borenstein, H. B., Veress, A. T., and Sonnenberg, H. (1981). A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. *Life Sci.* **28,** 89–94.

De Vito, P., Di Nardo, P., Palmery, M., Peluso, I., Luly, P., and Baldini, P. M. (2003). Oxidant-induced pHi/Ca^{2+} changes in rat aortic smooth muscle cells. The role of atrial natriuretic peptide. *Mol. Cell. Biochem.* **252,** 353–362.

Del Ry, S., Maltinti, M., Piacenti, M., Passino, C., Emdin, M., and Giannessi, D. (2006a). Cardiac production of C-type natriuretic peptide in heart failure. *J. Cardiovasc. Med.* **7,** 397–399.

Del Ry, S., Passino, C., Emdin, M., and Giannessi, D. (2006b). C-type natriuretic peptide and heart failure. *Pharmacol. Res.* **54,** 326–333.

Dora, K. A., Garland, C. J., Kwan, H. Y., and Yao, X. (2001). Endothelial cell protein kinase G inhibits release of EDHF through a PKG-sensitive cation channel. *Am. J. Physiol. Heart Circ. Physiol.* **280,** 1272–1277.

D'Souza, S. P., and Baxter, G. F. (2003). B-type natriuretic peptide: A good omen in myocardial ischaemia? *Heart* **89,** 707–709.

D'Souza, S. P., Yellon, D. M., Martin, C., Schulz, R., Heusch, G., Onody, A., Ferdinandy, P., and Baxter, G. F. (2003a). B-type natriuretic peptide limits infarct size in rat isolated hearts via KATP channel opening. *Am. J. Physiol. Heart Circ. Physiol.* **284,** 1592–1600.

D'Souza, S. P., Yellon, D. M., and Baxter, G. F. (2003b). Cardioprotective action of B-type natriuretic peptide is NP-dependent. *Br. J. Pharmacol.* **138,** 21P.

D'Souza, S. P., Davis, M., and Baxter, G. F. (2004). Autocrine and paracrine actions of natriuretic peptides in the heart. *Pharmacol. Ther.* **101,** 113–129.

Duda, T., and Sharma, R. K. (2005). Two membrane juxtaposed signaling modules in ANF-RGC are interlocked. *Biochem. Biophys. Res. Commun.* **332,** 149–156.

Ellis, G. R., Anderson, R. A., Lang, D., Blackman, D. J., Morris, R. H., Morris-Thurgood, J., McDowell, I. F., Jackson, S. K., Lewis, M. J., and Frenneaux, M. P. (2000). Neutrophil superoxide anion generating capacity, endothelial function and oxidative stress in chronic heart failure: Effects of short- and longterm vitamin C therapy. *J. Am. Coll. Cardiol.* **36,** 1474–1482.

Finkel, T. (1998). Oxygen radicals and signaling. *Curr. Opin. Cell. Biol.* **10,** 248–253.

Fonteles, M. C., Greenberg, R. N., Monteiro, H. S., Currie, M. G., and Forte, L. R. (1998). Natriuretic and kaliuretic activities of guanylin and uroguanylin in the isolated perfused rat kidney. *Am. J. Physiol.* **275,** F191–F197.

Forte, L. R., London, R. M., Freeman, R. H., and Krause, W. J. (2000). Guanylin peptides: Renal actions mediated by cyclic GMP. *Am. J. Physiol. Renal Physiol.* **278,** F180–F191.

Fowkes, R. C., and McArdle, C. A. (2000). C-type natriuretic peptide: An important neuroendocrine regulator? *Trends Endocrinol. Metab.* **11,** 333–338.

Frank, G. D., Motley, E. D., Inagami, T., and Eguchi, S. (2000). PYK2/CAKbeta represents a redox-sensitive tyrosine kinase in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **270,** 761–765.

Galipeau, J., Nemer, M., and Drouin, J. (1988). Ventricular activation of the atrial natriuretic factor gene in acute myocardial infarction. *N. Engl. J. Med.* **319,** 654–655.

Garg, U. C., and Hassid, A. (1989). Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J. Clin. Invest.* **83,** 1774–1777.

Geiger, J., Nolte, C., Butt, E., Sage, S. O., and Walter, U. (1992). Role of cGMP and cGMP-dependent protein kinase in nitrovasodilator inhibition of agonist-evoked calcium elevation in human platelets. *Proc. Natl. Acad. Sci. USA* **89,** 1031–1035.

Guillaume, P., Gutkowska, J., and Gianoulakis, C. (1997). Alterations in brain levels of atrial and C-type natriuretic peptides after chronic moderate ethanol consumption in spontaneously hypertensive rats. *Eur. J. Pharmacol.* **319,** 215–224.

Haarer, B. K., and Amberg, D. C. (2004). Old yellow enzyme protects the actin cytoskeleton from oxidative stress. *Mol. Biol. Cell.* **15,** 4522–4531.

Hama, N., Itoh, H., Shirakami, G., Nakagawa, O., Suga, S., Ogawa, Y., Masuda, I., Nakanishi, K., Yoshimasa, T., and Hashimoto, Y. (1995). Rapid ventricular induction

of brain natriuretic peptide gene expression in experimental acute myocardial infarction. *Circulation* **92,** 1558–1564.

Hamad, A. M., Clayton, A., Islam, B., and Knox, A. J. (2003). Guanylyl cyclases, nitric oxide, natriuretic peptides, and airway smooth muscle function. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **285,** 973–983.

Hare, J. M. (2001). Oxidative stress and apoptosis in heart failure progression. *Circ. Res.* **89,** 198–200.

Hashim, S., Li, Y., and Anand-Srivastava, M. B. (2006). Small cytoplasmic domain peptides of natriuretic peptide receptor-C attenuate cell proliferation through Gi{alpha} protein/MAP kinase/PI3-kinase/AKT pathways. *Am. J. Physiol. Heart Circ. Physiol.* **291,** 3144–3153.

Hidaka, T., Aisaka, K., Inomata, N., Furuya, M., Inoue, T., Omata, M., Yoshida, M., Miyazaki, T., Ohno, T., and Ishihara, T. (1993). Effect of carperitide (alpha-human atrial natriuretic polypeptide) on the cardiovascular system in experimental animals. *Nippon Yakurigaku Zasshi* **101,** 309–325.

Hilbert, P., Lindpaintner, K., Beckmann, J. S., Serikawa, T., Soubrier, F., Dubay, C., Cartwright, P., De Gouyon, B., Julier, C., Takahasi, S., Vincent, M., Ganten, D., *et al.* (1991). Chromosomal mapping of two genetic loci associated with blood-pressure regulation in hereditary hypertensive rats. *Nature* **353,** 521–529.

Hirose, M., Furukawa, Y., Kurogouchi, F., Nakajima, K., Miyashita, Y., and Chiba, S. (1998). C-type natriuretic peptide increases myocardial contractility and sinus rate mediated by guanylyl cyclase-linked natriuretic peptide receptors in isolated, blood-perfused dog heart preparations. *J. Pharmacol. Exp. Ther.* **286,** 70–76.

Hirotani, S., Otsu, K., Nishida, K., Higuchi, Y., Morita, T., Nakayama, H., Yamaguchi, O., Mano, T., Matsumura, Y., Ueno, H., Tada, M., and Hori, M. (2002). Involvement of nuclear factor-kB and apoptosis signal-regulating kinase 1 in G-protein-coupled receptor agonist-induced cardiomyocyte hypertrophy. *Circulation* **105,** 509–515.

Hobbs, A., Foster, P., Prescott, C., Scotland, R., and Ahluwalia, A. (2004). Natriuretic peptide receptor-C regulates coronary blood flow and prevents myocardial ischemia/reperfusion injury; novel cardioprotective role for endothelium-derived C-type natriuretic peptide. *Circulation* **110,** 1231–1235.

Hollister, A. S., Tanaka, I., Imada, T., Onrot, J., Biaggioni, I., Robertson, D., and Inagami, T. (1986). Sodium loading and posture modulate human atrial natriuretic factor plasma levels. *Hypertension* **8,** II106–II111.

Horio, T., Nishikimi, T., Yoshihara, F., Matsuo, H., Takishita, S., and Kanagawa, K. (2000). Inhibitory regulation of hypertrophy by endogenous atrial natriuretic peptide in cultured cardiac myocytes. *Hypertension* **35,** 19–24.

Hornig, B., Arakawa, N., Kholer, C., and Drexler, H. (1998). Vitamin C improves endothelial function of conduit arteries in patients with chronic heart failure. *Circulation* **97,** 363–368.

Igaki, T., Itoh, H., Suga, S., Komatsu, Y., Ogawa, Y., Doi, K., Yoshimasa, T., and Nakao, K. (1996). Insulin suppresses endothelial secretion of C-type natriuretic peptide, a novel endothelium-derived relaxing peptide. *Diabetes* **45,** S62–S64.

Iuliano, L., Colavita, A. R., Leo, R., Practico, D., and Violi, F. (1997). Oxygen free radicals and platelet activation. *Free Radic. Biol. Med.* **22,** 999–1006.

Joubert, S., Jossart, C., McNicoll, N., and De Lean, A. (2005). Atrial natriuretic peptide-dependent photolabeling of a regulatory ATP-binding site on the natriuretic peptide receptor-A. *FEBS J.* **272,** 5572–5583.

Kalra, P. R., Clague, J. R., Bolger, A. P., Anker, S. D., Poole-Wilson, P. A., Struthers, A. D., and Coats, A. J. (2003). Myocardial production of C-type natriuretic peptide in chronic heart failure. *Circulation* **107,** 571–573.

Kangawa, K., and Matsuo, H. (1984). Purification and complete amino acid sequence of alpha-human atrial natriuretic polypeptide (alpha-hANP). *Biochem. Biophys. Res. Commun.* **118,** 131–139.

Kato, J., Lanier-Smith, K. L., and Currie, M. G. (1991). Cyclic GMP down-regulates atrial natriuretic peptide receptors on cultured vascular endothelial cells. *J. Biol. Chem.* **266,** 14681–14685.

Kawada, N., Imai, E., Karber, A., Welch, W. J., and Wilcox, C. S. (2002). A mouse model of angiotensin II slow pressor response: Role of oxidative stress. *J. Am. Soc. Nephrol.* **13,** 2860–2868.

Keating, G. M., and Goa, K. L. (2003). Nesiritide: A review of its use in acute decompensated heart failure. *Drugs* **63,** 47–70.

Kelsall, C. J., Chester, A. H., Sarathchandra, P., and Singer, D. R. (2006). Expression and localization of C-type natriuretic peptide in human vascular smooth muscle cells. *Vascul. Pharmacol.* **45,** 368–373.

Kimura, T., Okajima, F., Sho, K., Kobayashi, I., and Kondo, Y. (1995). Thyrotropin-induced hydrogen peroxide production in FRTL-5 thyroid cells is mediated not by adenosine 3′,5′-monophosphate, but by Ca^{2+} signaling followed by phospholipase-A2 activation and potentiated by an adenosine derivative. *Endocrinology* **136,** 116–123.

Kinugawa, S., Tsutsui, H., Hayashidani, S., Ide, T., Suematsu, N., Satoh, S., Utsumi, H., and Takeshita, A. (2000). Treatment with dimethylthiourea prevents left ventricular remodelling and failure after experimental myocardial infarction in mice: Role of oxidative stress. *Circ. Res.* **87,** 392–398.

Kleber, F. X., Nussberger, J., Niemoller, L., and Doering, W. (1992). Mechanisms involved in cardiac enlargement and congestive heart failure development after acute myocardial infarction. *Cardiology* **81,** 213–220.

Kojsova, S., Jendekova, L., Zicha, J., Kunes, J., Andriantsitohaina, R., and Pechanova, O. (2006). The effect of different antioxidants on nitric oxide production in hypertensive rats. *Physiol. Res.* **55,** 3–16.

Koller, K. J., Lowe, D. G., Bennett, G. L., Minamino, N., Kangawa, H., Matsuo, H., and Goeddel, D. V. (1991). Selective activation of the B natriuretic peptide receptor by C-type natriuretic peptide (CNP). *Science* **252,** 120–123.

Komatsu, Y., Nakao, K., Itoh, H., Suga, S., Ogawa, Y., and Imura, H. (1992). Vascular natriuretic peptide. *Lancet* **340,** 622.

Kono, Y., Nakamura, K., Kimura, H., Nishii, N., Watanabe, A., Banba, K., Miura, A., Nagase, S., Sakuragi, S., Kusano, K. F., Matsubara, H., and Ohe, T. (2006). Elevated levels of oxidative DNA damage in serum and myocardium of patients with heart failure. *Circ. J.* **70,** 1001–1005.

Kuno, T., Andresen, J. W., Kamisaki, Y., Waldman, S. A., Chang, L. Y., Saheki, S., Leitman, D. C., Nakane, M., and Murad, F. (1986). Co-purification of an atrial natriuretic factor receptor and particulate guanylate cyclase from rat lung. *J. Biol. Chem.* **261,** 5817–5823.

Kurose, H., Inagami, T., and Ui, M. (1987). Participation of adenosine 5′-triphosphate in the activation of membrane-bound guanylate cyclase by the atrial natriuretic factor. *FEBS Lett.* **219,** 375–379.

Lachance, D., Garcia, R., Gutkowska, J., Cantin, M., and Thibault, G. (1986). Mechanisms of release of atrial natriuretic factor. I. Effect of several agonists and steroids on its release by atrial minces. *Biochem. Biophys. Res. Commun.* **135,** 1090–1098.

Lang, R. E., Tholken, H., Ganten, D., Luft, F. C., Ruskoaho, H., and Unger, T. (1985). Atrial natriuretic factor-a circulating hormone stimulated by volume loading. *Nature* **314,** 264–266.

Laskowski, A., Woodman, O. L., Cao, A. H., Drummond, G. R., Marshall, T., Kaye, D. M., and Ritchie, R. H. (2006). Antioxidant actions contribute to the

antihypertrophic effects of atrial natriuretic peptide in neonatal rat cardiomyocytes. *Cardiovasc. Res.* **72,** 112–123.

Levin, E. R., Gardner, D. G., and Samson, W. K. (1998). Natriuretic peptides. *N. Engl. J. Med.* **339,** 321–328.

Li, J. M., Gall, N. P., Grieve, D. J., Chen, M., and Shah, A. M. (2002). Activation of NADPH oxidase during progression of cardiac hypertrophy to failure. *Hypertension* **40,** 477–484.

Lopez, J. J., Salido, G. M., Pariente, J. A., and Rosado, J. A. (2006). Interaction of STIM1 with endogenously expressed human canonical TRP1 upon depletion of intracellular Ca^{2+} stores. *J. Biol. Chem.* **281,** 28254–28264.

Maack, T., Okolicany, J., Koh, G. Y., and Price, D. A. (1993). Functional properties of atrial natriuretic factor receptors. *Semin. Nephrol.* **13,** 50–60.

MacDonell, K. L., and Diamond, J. (1997). Cyclic GMP-dependent protein kinase activation in the absence of negative inotropic effects in the rat ventricle. *Br. J. Pharmacol.* **122,** 1425–1435.

Mallat, Z., Philip, I., Lebret, M., Chatel, D., Maclouf, J., and Tedgui, A. (1998). Elevated levels of 8-iso-prostaglandin F2 in pericardial fluid of patients with heart failure: A potential role for *in vivo* oxidant stress in ventricular dilatation and progression to heart failure. *Circulation* **97,** 1536–1539.

Massion, P. B., and Balligand, J.-L. (2003). Modulation of cardiac contraction, relaxation and rate by the endothelial nitric oxide synthase (eNOS): Lessons from genetically-modified mice. *J. Physiol.* **546,** 63–75.

Matsumoto, T., Wada, A., Tsutamoto, T., Omura, T., Yokohama, H., Ohnishi, M., Nakae, I., Takahashi, M., and Konoshita, M. (1999). Vasorelaxing effects of atrial and brain natriuretic peptides on coronary circulation in heart failure. *Am. J. Physiol. Heart Circ. Physiol.* **276,** 1935–1942.

McCormick, M. L., Gavrila, D., and Weintraub, N. L. (2007). Role of oxidative stress in the pathogenesis of abdominal aortic aneurysms. *Arterioscler. Thromb. Vasc. Biol.* **27,** 461–469.

McLay, J. S., Chatterjee, P. K., Mistry, S. K., Weerakody, R. P., Jardine, A. G., McLay, N. G., and Hawksworth, G. M. (1995). Atrial natriuretic factor and angiotensin II stimulate nitric oxide release from human proximal tubular cells. *Clin. Sci. (Lond.)* **89,** 527–531.

McMurray, J., Chopra, M., Abdullah, I., Smith, W. E., and Dargie, H. J. (1993). Evidence of oxidative stress in chronic heart failure in humans. *Eur. Heart J.* **14,** 1493–1498.

Mendonca, M. C., Doi, S. Q., Glerum, S., and Sellitti, D. F. (2006). Increase of C-type natriuretic peptide expression by serum and platelet-derived growth factor-BB in human aortic smooth muscle cells is dependent on protein kinase C activation. *Endocrinology* **147,** 4169–4178.

Morita, E., Yasue, H., Yoshimura, M., Ogawa, H., Jougasaki, M., Matsumura, T., Mukoyama, M., and Nakao, K. (1993). Increased plasma levels of brain natriuretic peptide in patients with acute myocardial infarction. *Circulation* **88,** 82–91.

Mukoyama, M., Nakao, K., Hosoda, K., Suga, S., Saito, Y., Ogawa, Y., Shirakami, G., Jougasaki, M., Obata, K., Yasue, H., Kambayashi, Y., Inouye, K., *et al.* (1991). Brain natriuretic peptide as a novel cardiac hormone in humans. Evidence for an exquisite dual natriuretic peptide system, atrial natriuretic peptide and brain natriuretic peptide. *J. Clin. Invest.* **87,** 1402–1412.

Murthy, K. S., Teng, B.-Q., Jin, J. G., and Makhlouf, G. M. (1998). G-protein-dependent activation of smooth muscle Enos via natriuretic peptide clearance receptor. *Am. J. Physiol. Cell. Physiol.* **275,** C1409–C1416.

Murthy, K. S., Teng, B. Q., Zhou, H., Jin, J. G., Grider, J. R., and Makhlouf, G. M. (2000). G(i-1)/G(i-2)-dependent signaling by single-transmembrane natriuretic peptide clearance receptor. *Am. J. Physiol. Gastrointest. Liver Physiol.* **278,** 974–980.

Nakamura, K., Fushimi, K., Kouchi, H., Mihara, K., Miyazaki, M., Ohe, T., and Namba, M. (1998). Inhibitory effects of antioxidants on neonatal rat cardiac myocyte

hypertrophy induced by tumor necrosis factor-α and angiotensin II. *Circulation* **98,** 794–799.

Nakao, K., Ogawa, Y., Suga, S., and Imura, H. (1992a). Molecular biology and biochemistry of the natriuretic peptide system. I. Natriuretic peptides. *J. Hypertens.* **10,** 907–912.

Nakao, N., Ogawa, S., Suga, S., and Imura, H. (1992b). Molecular biology and biochemistry of the natriuretic peptide system. II: Natriuretic peptide receptors. *J. Hypertens.* **10,** 1111–1115.

Naruko, T., Itoh, A., Haze, K., Ehara, S., Fukushima, H., Sugama, Y., Shirai, N., Ikura, Y., Ohsawa, M., and Ueda, M. (2005). C-type natriuretic peptide and natriuretic peptide receptors are expressed by smooth muscle cells in the neointima after percutaneous coronary intervention. *Atherosclerosis* **181,** 241–250.

Nishizawa, H., Matsuda, M., Yamada, Y., Kawai, K., Suzuki, E., Makishima, M., Kitamura, T., and Shimomura, I. (2004). Musclin, a novel skeletal muscle-derived secretory factor. *J. Biol. Chem.* **279,** 19391–19395.

Ogawa, Y., Nakao, K., Mukoyama, M., Hosoda, K., Shirakami, G., Arai, H., Saito, Y., Suga, S., Jougasaki, M., and Imura, H. (1991). Natriuretic peptides as cardiac hormones in normotensive and spontaneously hypertensive rats. The ventricle is the major site of synthesis and secretion of brain natriuretic peptide. *Circ. Res.* **69,** 491–500.

Ogawa, Y., Nakao, K., Nakagawa, O., Komatsu, Y., Hosoda, K., Suga, S., Arai, H., Nagata, K., Yoshida, N., and Imura, H. (1992). Human C-type natriuretic peptide. Characterization of the gene and peptide. *Hypertension* **19,** 809–813.

Ogihara, T., Shima, J., Hara, H., Tabuchi, Y., Hashizume, K., Nagano, M., Katahira, K., Kangawa, K., Matsuo, H., and Kumahara, Y. (1986). Significant increase in plasma immunoreactive atrial natriuretic polypeptide concentration during head-out water immersion. *Life Sci.* **38,** 2413–2418.

Oikawa, S., Imai, M., Ueno, A., Tanaka, S., Noguchi, T., Nakazato, H., Kangawa, K., Fukuda, A., and Matsuo, H. (1984). Cloning and sequence analysis of cDNA encoding a precursor for human atrial natriuretic polypeptide. *Nature* **309,** 724–726.

Okumura, K., Yasue, H., Fujii, H., Kugiyama, K., Matsuyama, K., Yoshimura, M., Jougasaki, M., Kikuta, K., Kato, H., and Tanaka, H. (1995). Effects of brain (B-type) natriuretic peptide on coronary artery diameter and coronary hemodynamic variables in humans: Comparison with effects of systemic hemodynamic variables. *J. Am. Coll. Cardiol.* **25,** 342–348.

Orlov, S. N., Adragna, N. C., Adarichev, V. A., and Hamet, P. (1999). Genetic and biochemical determinants of abnormal monovalent ion transport in primary hypertension. *Am. J. Physiol. Cell Physiol.* **276,** 511–536.

Pelisek, J., Fuchs, A. T., Kuehnl, A., Tian, W., Kuhlmann, M. T., Rolland, P. H., Mekkaoui, C., Gaedtke, L., and Nikol, S. (2006). C-type natriuretic peptide for reduction of restenosis: Gene transfer is superior over single peptide administration. *J. Gene Med.* **8,** 835–844.

Pimentel, D. R., Amin, J. K., Xiao, L., Miller, T., Viereck, J., Oliver-Krasinski, J., Baliga, R., Wang, J., Siwik, D. A., Singh, K., Pagano, P., Colucci, W. S., *et al.* (2001). Reactive oxygen species mediate amplitude-dependent hypertrophic and apoptotic responses to mechanical stretch in cardiac myocytes. *Circ. Res.* **89,** 453–460.

Pollock, D. M., and Adrendshorst, W. J. (1990). Exaggerated natriuretic response to atrial natriuretic factor in rats developing spontaneous hypertension. *Hypertension* **16,** 72–79.

Porter, J. G., Arfsten, A., Fuller, F., Miller, J. A., Gregory, L. C., and Lewicki, J. A. (1990). Isolation and function expression of the human atrial natriuretic peptide clearance receptor cDNA. *Biochem. Biophys. Res. Commun.* **171,** 796–803.

Porter, J. G., Catalano, R., McEnroe, G., Lewicki, J. A., and Protter, A. A. (1992). C-type natriuretic peptide inhibits growth factor-dependent DNA synthesis in smooth muscle cells. *Am. J. Physiol. Cell Physiol.* **263,** 1001–1006.

Potter, L. R. (2005). Domain analysis of human transmembrane guanylylcyclase receptors: Implications for regulation. *Front. Biosci.* **10,** 1205–1220.

Potter, L. R., Abbey-Hosch, S., and Dickey, D. M. (2006). Natriuretic peptides, their receptors, and cyclic guanosine monophosphate-dependent signaling functions. *Endocr. Rev.* **27,** 47–72.

Potthast, R., and Potter, L. R. (2005). Phosphorylation-dependent regulation of the guanylyl cyclase-linked natriuretic peptide receptors. *Peptides* **26,** 1001–1008.

Raine, A. E. G. (1994). Hypertension and the kidney. *Br. Med. Bull.* **50,** 322–342.

Redondo, P. C., Salido, G. M., Pariente, J. A., and Rosado, J. A. (2004a). Dual effect of hydrogen peroxide on store-mediated calcium entry in human platelets. *Biochem. Pharmacol.* **67,** 1065–1076.

Redondo, P. C., Salido, G. M., Rosado, J. A., and Pariente, J. A. (2004b). Effect of hydrogen peroxide on Ca^{2+} mobilisation in human platelets through sulphydryl oxidation dependent and independent mechanisms. *Biochem. Pharmacol.* **67,** 491–502.

Redondo, P. C., Jardin, I., Hernandez-Cruz, J. M., Pariente, J. A., Salido, G. M., and Rosado, J. A. (2005). Hydrogen peroxide and peroxynitrite enhance Ca^{2+} mobilization and aggregation in platelets from type 2 diabetic patients. *Biochem. Biophys. Res. Commun.* **333,** 794–802.

Redondo, P. C., Harper, M. T., Rosado, J. A., and Sage, S. O. (2006). A role for cofilin in the activation of store-operated calcium entry by *de novo* conformational coupling in human platelets. *Blood* **107,** 973–979.

Rosado, J. A., and Sage, S. O. (2000). Coupling between inositol 1,4,5-trisphosphate receptors and human transient receptor potential channel 1 when intracellular Ca^{2+} stores are depleted. *Biochem. J.* **350,** 631–635.

Rosado, J. A., and Sage, S. O. (2001). Activation of store-mediated calcium entry by secretion-like coupling between the inositol 1,4,5-trisphosphate receptor type II and human transient receptor potential (hTrp1) channels in human platelets. *Biochem. J.* **356,** 191–198.

Rosado, J. A., and Sage, S. O. (2002). The ERK cascade, a new pathway involved in the activation of store-mediated calcium entry in human platelets. *Trends Cardiovasc. Med.* **12,** 229–234.

Rosado, J. A., Salido, G. M., Jensen, R. T., and Garcia, L. J. (1998). Are tyrosine phosphorylation of p125(FAK) and paxillin or the small GTP binding protein, rho, needed for CCK-stimulated pancreatic amylase secretion? *Biochim. Biophys. Acta* **1404,** 412–426.

Rosado, J. A., Jenner, S., and Sage, S. O. (2000). A role for the actin cytoskeleton in the initiation and maintenance of store-mediated calcium entry in human platelets. Evidence for conformational coupling. *J. Biol. Chem.* **275,** 7527–7533.

Rosado, J. A., Porras, T., Conde, M., and Sage, S. O. (2001). Cyclic nucleotides modulate store-mediated calcium entry through the activation of protein tyrosine phosphorylation and altered actin polymerization in human platelets. *J. Biol. Chem.* **276,** 15666–15675.

Rosado, J. A., Gonzalez, A., Salido, G. M., and Pariente, J. A. (2002). Effects of reactive oxygen species on actin filament polymerisation and amylase secretion in mouse pancreatic acinar cells. *Cell Signal.* **14,** 547–556.

Rosado, J. A., Redondo, P. C., Salido, G. M., Gomez-Arteta, E., Sage, S. O., and Pariente, J. A. (2004). Hydrogen peroxide generation induces $pp60^{src}$ activation in human platelets: Evidence for the involvement of this pathway in store-mediated calcium entry. *J. Biol. Chem.* **279,** 1665–1675.

Rosado, J. A., Redondo, P. C., Sage, S. O., Pariente, J. A., and Salido, G. M. (2005). Store-operated Ca^{2+} entry: Vesicle fusion or reversible trafficking and *de novo* conformational coupling? *J. Cell. Physiol.* **205,** 262–269.

Ruskoaho, H. (1992). Atrial natriuretic peptide: Synthesis, release, and metabolism. *Pharmacol. Rev.* **44,** 479–602.

Saavedra, J. M., Correa, F. M., Plunkett, L. M., Israel, A., Kurihara, M., and Shigematsu, K. (1986). Binding of angiotensin and atrial natriuretic peptide in brain of hypertensive rats. *Nature* **320,** 758–760.

Saito, Y., Nakao, K., Itoh, H., Yamada, T., Mukoyama, M., Arai, H., Hosada, K., Shirakami, G., Suga, S., and Minamino, N. (1989). Brain natriuretic peptide is a novel cardiac hormone. *Biochem. Biophys. Res. Commun.* **158,** 360–368.

Salmeen, A., Andersen, J. N., Myers, M. P., Meng, T. C., Hinks, J. A., Ronks, N. K., and Barford, D. (2003). Redox regulation of protein tyrosine phosphatase 1B involves a sulphenyl-amide intermediate. *Nature* **423,** 769–773.

Savoie, P., de Champlain, J., and Anand-Srivastava, M. B. (1995). Brain natriuretic peptide and C-type natriuretic peptide inhibit adenylyl cyclase activity: Interaction with Anf-R2 receptors. *FEBS Lett.* **370,** 6–10.

Schirger, J. A., Heublein, D. M., Chen, H. H., Lisy, O., Jougasaki, M., Wennberg, P. W., and Burnett, J. C., Jr. (1999). Presence of *Dendroaspis* natriuretic peptide-like immunoreactivity in human plasma and its increase during human heart failure. *Mayo Clin. Proc.* **74,** 126–130.

Schulz-Knappe, P., Forssmann, K., Herbst, F., Hock, D., Pipkorn, R., and Forssmann, W. G. (1988). Isolation and structural analysis of "urodilatin," a new peptide of the cardiodilatin-(ANP)-family, extracted from human urine. *Klin. Wochenschr.* **66,** 752–759.

Schweitz, H., Vigne, P., Moinier, D., Frelin, C., and Lazdunski, M. (1992). A new member of the natriuretic peptide family is present in the venom of the green mamba (*Dendroaspis angusticeps*). *J. Biol. Chem.* **267,** 13928–13932.

Seno, T., Inoue, N., Gao, D., Okuda, M., Sumi, Y., Matsui, K., Yamada, S., Hirata, K., Kawashima, S., Tawa, R., Imajoh-Ohmi, S., Sakurai, H., *et al.* (2001). Involvement of NADH/NADPH oxidase in human platelet ROS production. *Thromb. Res.* **103,** 399–409.

Shono, M., Yoshimura, M., Nakayama, M., Yamamuro, M., Abe, K., Suzuki, S., Mizuno, Y., Sugiyama, S., Saito, Y., Nakao, K., Yasue, H., and Ogawa, H. (2007). Predominant effect of A-type natriuretic peptide on reduction of oxidative stress during the treatment of patients with heart failure. *Circ. J.* **71,** 1040–1046.

Sia, Y. T., Lapointe, N., Parker, T. G., Tsoporis, J. N., Deschepper, C. F., Calderone, A., Pourdjabbar, A., Jasmin, J. F., Sarrazin, J. F., Liu, P., Adam, A., Butany, J., *et al.* (2002). Beneficial effects of long-term use of the antioxidant probucol in heart failure in the rat. *Circulation* **105,** 2549–2555.

Singh, G., Kuc, R. E., Maguire, J. J., Fidock, M., and Davenport, A. P. (2006). Novel snake venom ligand dendroaspis natriuretic peptide is selective for natriuretic peptide receptor-A in human heart: Downregulation of natriuretic peptide receptor-A in heart failure. *Circ. Res.* **99,** 183–190.

Siwik, D. A., Tzortzis, J. D., Pimental, D. R., Chang, D. L., Pagano, P. J., Singh, K., Sawyer, D. B., and Colucci, W. S. (1999). Inhibition of copper-zinc superoxide dismutase induces cell growth, hypertrophic phenotype, and apoptosis in neonatal rat cardiac myocytes *in vitro*. *Circ. Res.* **85,** 147–153.

Soeki, T., Kishimoto, I., Okumura, H., Tokudome, T., Horio, T., Mori, K., and Kangawa, K. (2005). C-type natriuretic peptide, a novel antifibrotic and antihypertrophic agent, prevents cardiac remodeling after myocardial infarction. *J. Am. Coll. Cardiol.* **45,** 608–616.

Solaini, G., and Harris, D. A. (2005). Biochemical dysfunction in heart mitochondria exposed to ischaemia and reperfusion. *Biochem. J.* **390,** 377–394.

Soualmia, H., Barthelemy, C., Masson, F., Maistre, G., Eurin, J., and Carayon, A. (1997). Angiotensin II-induced phosphoinositide production and atrial natriuretic peptide release in rat atrial tissue. *J. Cardiovasc. Pharmacol.* **29,** 605–611.

Stasch, J. P., Hirth-Dietrich, C., Kazda, S., and Neuser, D. (1989). Endothelin stimulates release of atrial natriuretic peptides *in vitro* and *in vivo*. *Life Sci.* **45,** 869–875.

Stone, J. A., Backx, P. H., and ter Keurs, H. E. (1990). The effect of atrial natriuretic factor on force development in rat cardiac trabeculae. *Can. J. Physiol. Pharmacol.* **68,** 1247–1254.

Sudoh, T., Kangawa, K., Minamino, N., and Matsuo, H. (1988). A new natriuretic peptide in porcine brain. *Nature* **332,** 78–81.

Sudoh, T., Maekawa, K., Kojima, M., Minamino, N., Kangawa, K., and Matsuo, H. (1989). Cloning and sequence analysis of cDNA encoding a precursor for human brain natriuretic peptide. *Biochem. Biophys. Res. Commun.* **159,** 1427–1434.

Sudoh, T., Minamino, N., Kangawa, K., and Matsuo, H. (1990). C-type natriuretic peptide (CNP): A new member of natriuretic peptide family identified in porcine brain. *Biochem. Biophys. Res. Commun.* **168,** 863–870.

Suematsu, N., Tsutsui, H., Wen, J., Kang, D., Ikeuchi, M., Ide, T., Hayashidani, S., Shiomi, T., Kubota, T., Hamasaki, N., and Takeshita, A. (2003). Oxidative stress mediates tumor necrosis factor-alpha-induced mitochondrial DNA damage and dysfunction in cardiac myocytes. *Circulation* **107,** 1418–1423.

Suga, S., Nakao, K., Itoh, H., Komatsu, Y., Ogawa, Y., Hama, N., and Imura, H. (1992). Endothelial production of C-type natriuretic peptide and its marked augmentation by transforming growth factor-β. Possible existence of "vascular natriuretic peptide system." *J. Clin. Invest.* **90,** 1145–1149.

Suga, S., Itoh, H., Komatsu, Y., Ogawa, Y., Hama, N., Yoshimasa, T., and Nakao, K. (1993). Cytokine-induced C-type natriuretic peptide (CNP) secretion from vascular endothelial cells-evidence for CNP as a novel autocrine/paracrine regulator from endothelial cells. *Endocrinology* **133,** 3038–3041.

Supaporn, T., Wennberg, P. W., Wei, C. M., Kinoshita, M., Matsuda, Y., and Burnett, J. C., Jr. (1996). Role of the endogenous natriuretic peptide system in the control of basal coronary vascular tone in dogs. *Clin. Sci. (Lond.)* **90,** 357–362.

Suzuki, Y. J., and Ford, G. D. (1992). Superoxide stimulates IP_3-induced Ca^{2+} release from vascular smooth muscle sarcoplasmic reticulum. *Am. J. Physiol.* **262,** H114–H116.

Tang, W., Paulding, W. R., and Sumners, C. (1993). ANP receptors in neurons and astrocytes from spontaneously hypertensive rat brain. *Am. J. Physiol. Cell Physiol.* **265,** 106–112.

Thibault, G., Amiri, F., and Garcia, R. (1999). Regulation of natriuretic peptide secretion by the heart. *Annu. Rev. Physiol.* **61,** 193–217.

Thomas, G., Moffatt, P., Salois, P., Gaumond, M. H., Gingras, R., Godin, E., Miao, D., Goltzman, D., and Lanctot, C. (2003). Osteocrin, a novel bone-specific secreted protein that modulates the osteoblast phenotype. *J. Biol. Chem.* **278,** 50563–50571.

Tokola, H., Hautala, N., Marttila, M., Magga, J., Pikkarainen, S., Kerkela, R., Vuolteenaho, O., and Ruskoaho, H. (2001). Mechanical load-induced alterations in B-type natriuretic peptide gene expression. *Can. J. Physiol. Pharmacol.* **79,** 646–653.

Toth, M., Vuorinen, K. H., Vuoltheenaho, O., Hassinen, I. E., Uusimaa, P. A., Leppaluoto, J., and Ruskoaho, H. (1994). Hypoxia stimulates release of ANP and BNP from perfused rat ventricular myocardium. *Am. J. Physiol. Heart Circ. Physiol.* **266,** 1572–1580.

Trachte, G. J., Kanwal, S., Elmquist, B. J., and Ziegel, R. J. (1995). C-type natriuretic peptide neuromodulation via clearance receptors. *Am. J. Physiol. Cell Physiol.* **268,** 978–984.

Tsuruda, T., Boerrigter, G., Huntley, B. K., Noser, J. A., Cataliotti, A., Costello-Boerrigter, L. C., Chen, H. H., and Burnett, J. C., Jr. (2002). Brain natriuretic peptide is produced in cardiac fibroblasts and induces matrix metalloproteinases. *Circ. Res.* **91,** 1127–1134.

Tsutsui, H. (2004). Novel pathophysiological insight and treatment strategies for heart failure. *Circ. J.* **68,** 1095–1103.

Van Hooft, I. M. S., Grobbee, D. E., Derkx, F. H. M., de Leeuw, P. W., Schalekamp, M. A. D. H., and Hofman, A. H. (1991). Renal haemodynamics and the renin-angiotensin-aldosterone system in normotensive subjects with hypertensive and normotensive parents. *N. Engl. Med.* **324,** 1305–1311.

Vaxelaire, J. F., Laurent, S., Lacolley, P., Briand, V., Schmitt, H., and Michel, J. B. (1989). Atrial natriuretic peptide decreases contractility of cultured chick ventricular cells. *Life Sci.* **45,** 41–48.

Vuolteenaho, O., Arjamaa, O., and Ling, N. (1985). Atrial natriuretic polypeptides (ANP): Rat atria store high molecular weight precursor but secrete processed peptides of 25–35 amino acids. *Biochem. Biophys. Res. Commun.* **129,** 82–88.

Weber, M., and Hamm, C. (2006). Role of B-type natriuretic peptide (BNP) and NT-proBNP in clinical routine. *Heart* **92,** 843–849.

Wei, C. M., Hu, S., Miller, V. M., and Burnett, J. C., Jr. (1994). Vascular actions of C-type natriuretic peptide in isolated porcine coronary arteries and coronary vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **205,** 765–771.

Whalin, M. E., Scammell, J. G., Strada, S. J., and Thompson, W. J. (1991). Phosphodiesterase II, the cGMP-activatable cyclic nucleotide phosphodiesterase, regulates cyclic AMP metabolism in PC12 cells. *Mol. Pharmacol.* **39,** 711–717.

Wiley, K. E., and Davenport, A. P. (2001). Physiological antagonism of endothelin-1 in human conductance and resistance coronary artery. *Br. J. Pharmacol.* **133,** 568–574.

Wong, F., and Blendis, L. (1994). Pathophysiology of sodium retention and ascites formation in cirrhosis: Role of atrial natriuretic factor. *Semin. Liver Dis.* **14,** 59–70.

Woodard, G. E., Rosado, J. A., and Brown, J. (2002a). Expression and control of C-type natriuretic peptide in rat vascular smooth muscle cells. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **282,** 156–165.

Woodard, G. E., Rosado, J. A., and Brown, J. (2002b). Dendroaspis natriuretic peptide-like immunoreactivity and its regulation in rat aortic vascular smooth muscle. *Peptides* **23,** 23–29.

Woodard, G. E., Zhao, J., Rosado, J. A., and Brown, J. (2002c). A-type natriuretic peptide receptor in the spontaneously hypertensive rat kidney. *Peptides* **23,** 1637–1647.

Woodard, G. E., Zhao, J., Rosado, J. A., and Brown, J. (2003). Differences between natriuretic peptide receptors in the olfactory bulb and hypothalamus from spontaneously hypertensive and normotensive rat brain. *Neurosci. Res.* **47,** 421–429.

Woodard, G. E., Li, X., and Rosado, J. A. (2004a). Water deprivation enhances the inhibitory effect of natriuretic peptides on cAMP synthesis in rat renal glomeruli. *Am. J. Physiol. Renal Physiol.* **287,** 418–426.

Woodard, G. E., Zhao, J., Rosado, J. A., and Brown, J. (2004b). Patterning of renal cGMP production by the natriuretic peptide receptor type A and blood pressure in spontaneously hypertensive rats. *Regul. Pept.* **119,** 45–51.

Woodard, G. E., Li, X., and Rosado, J. A. (2005). Characteristics of the renal C-type natriuretic peptide receptor in hypertrophied and developing rat kidney. *J. Mol. Endocrinol.* **35,** 519–530.

Woodard, G. E., Li, X., and Rosado, J. A. (2006). Renal atrial natriuretic peptide receptors binding properties and function are resistant to DOCA-salt-induced hypertension in rats. *Regul. Pept.* **137,** 114–120.

Woodard, G. E., Sage, S. O., and Rosado, J. A. (2007). Transient receptor potential channels and intracellular signaling. *Int. Rev. Cytol.* **256,** 35–67.

Wu, C., Wu, F., Pan, J., Morser, J., and Wu, Q. (2003). Furin-mediated processing of pro-C-type natriuretic peptide. *J. Biol. Chem.* **278,** 25847–25852.

Xiaohong, W., Jun, Y., Lijia, T., Jingyi, S., Chaoshu, T., and Naikui, L. (2000). C-type natriuretic peptide inhibits upregulation of alpha1-adrenoceptor and inositol 1,4,5-trisphosphate receptor in rat vascular smooth muscle after vascular endothelial injury. *Chin. Med. Sci. J.* **15,** 73–78.

Yada, T., Shimokawa, H., Hiramatsu, O., Kajita, T., Shigeto, F., Goto, M., Ogasawara, Y., and Kajiya, F. (2003). Hydrogen peroxide, an endogenous endothelium-derived hyperpolarizing factor, plays an important role in coronary autoregulation *in vivo*. *Circulation* **107,** 1040–1045.

Yan, W., Wu, F., Morser, J., and Wu, Q. (2000). Corin, a transmembrane cardiac serine protease, acts as a pro-atrial natriuretic peptide-converting enzyme. *Proc. Natl. Acad. Sci. USA* **97,** 8525–8529.

Yanagisawa, A., Osborne, J. A., Stahl, G. L., and Lefer, A. M. (1987). Coronary vascular actions of synthetic atrial natriuretic factor in isolated vascular preparations. *J. Cardiovasc. Pharmacol.* **10,** 320–326.

Yancy, C. W., and Singh, A. (2006). Potential applications of outpatient nesiritide infusions in patients with advanced heart failure and concomitant renal insufficiency (from the Follow-Up Serial Infusions of Nesiritide trial). *Am. J. Cardiol.* **98,** 226–229.

Yandle, T. G. (1994). Biochemistry of natriuretic peptides. *J. Intern. Med.* **235,** 561–576.

Yuan, Q., Jiang, D. J., Chen, Q. Q., Wang, S., Xin, H. Y., Deng, H. W., and Li, Y. J. (2007). Role of asymmetric dimethylarginine in homocysteine-induced apoptosis of vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **356,** 880–885.

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CHAPTER FOUR

New Insights into the Cell Biology of Insect Axonemes

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Contents

Abstract

Insects do not possess ciliated epithelia, and cilia/flagella are present in the sperm tail and—as modified cilia—in mechano- and chemosensory neurons. The core cytoskeletal component of these organelles, the axoneme, is a microtubule-based structure that has been conserved throughout evolution. However, in insects the sperm axoneme exhibits distinctive structural features; moreover, several insect groups are characterized by an unusual sperm axoneme variability. Besides the abundance of morphological data on insect sperm flagella, most of the available molecular information on the insect axoneme comes from genetic studies on *Drosophila* spermatogenesis, and only recently other insect species have been proposed as useful models. Here, we review the current knowledge on the cell biology of insect axoneme, including contributions from both *Drosophila* and other model insects.

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1. Introduction

Several reasons underlie the growing interest for insect biology. First, insects constitute the largest living animal group: They have colonized almost all possible ecological niches, attaining specific and efficient adaptations to a variety of life styles, and several species are currently studied as useful indicators for altered environmental conditions. Second, research on insect biology has important implications in agriculture and biomedicine. Third, some insects can be easily grown as laboratory strains, and the availability for some species of a sound genetic background and of the possibility to produce and manipulate mutants, has allowed to overcome many of the evident difficulties that insects pose for traditional biochemical researches. As a matter of fact, studies on *Drosophila melanogaster*, currently one of the most established model organisms, have provided fundamental contributions to our present knowledge of animal cell biology. Finally, the whole genome sequence has been either established or is currently being assembled for as many as 50 insect species.

In the last decade, much attention has been paid to the study of ciliary assembly, function, and organization. This has been mainly due to the discovery that cilia and flagella, beside being motile organelles, are involved in sensory perception and cell signaling processes; as such, they play a crucial role in the development of living organisms and—when altered—are responsible for several important genetic diseases (Bisgrove and Yost, 2006; Davis *et al.*, 2006; Marshall and Nonaka, 2006; Satir and Christensen, 2007; Scholey and Anderson, 2006). The basic organization of the cilium has been conserved during evolution. This organelle is based on a membrane-enveloped core cytoskeletal structure—the axoneme—which is commonly formed by nine microtubular doublets arranged around two central singlet microtubules, a disposition that is usually referred to as the "9+2" pattern. The outer doublets are connected to each other by series of projections—the nexin links and the outer (ODAs) and inner (IDAs) dynein arms—and to the central microtubules by the so-called radial spokes. Such an organization is found in motile cilia and flagella of most eukaryotic phyla; on the other hand, a 9+0 pattern, that is, with no central pair, is a common feature of sensory and primary cilia. The possible evolutionary origin of this complex and peculiar microtubular array has been the subject of a long-standing debate (see e.g., Cavalier-Smith, 1982; Li and Wu, 2005; Mitchell, 2004; Satir *et al.*, 2007).

Among insects, cilia/flagella are present in sperm cells, in sensory cells as modified cilia, and in epidermal secretory units as transient cilia; the absence of any ciliated epithelium is a peculiar feature that, along with moulting, groups insects and nematods in Ecdysozoa. The abundance of morphological data available on insect sperm flagella has shown that they generally share the basic 9+2 axonemal organization, but also that some peculiar features have been added during the evolution of pterygotans, and that a variety of unusual axoneme patterns has been achieved by several insect orders (Dallai *et al.*, 2006; Jamieson *et al.*, 1999).

While the detailed knowledge of the ultrastructure of insect sperm axonemes has provided significant phenotypic features that proved to be useful for the study of phylogenetic relationships among different insect orders, the molecular data available on insect axonemes have essentially derived, over the last 30 years, from observations carried out on *Drosophila* mutants, and only recently other species have been proposed as suitable experimental models.

Here, we review the current information on the cell biology of the insect axoneme, with the aim to report about the contribution that studies on insect models can provide toward the general comprehension of axoneme biology.

2. Structural Organization of the Axoneme in Insect Cilia and Flagella

2.1. Flagellar axoneme: From 9+2 to various axoneme models

In insect sperm flagella, the 9+2 axoneme pattern is present only in the basal wingless Collembola, and, as a derived condition, in a few pterygotan orders (Dallai *et al.*, 2006). Most insects are in fact characterized by a peculiar synapomorphic feature, that is, a crown of nine microtubules—the accessory tubules—which encompass the central 9+2 axoneme, so that the common model is the so-called 9+9+2 pattern (Fig. 4.1A–C). During spermiogenesis, accessory tubules originate from each microtubular doublet as a short projection, which extends from a definite point of the B-subtubule in a direction opposite to dynein arms, progressively grows and finally detaches from the doublet giving rise to an independent complete microtubule (Cameron, 1965; Dallai and Afzelius, 1993); later, an electron dense material is accumulated in the space between two adjacent accessory tubules.

A considerable diversity has been described in the structure of the accessory tubules, which is genetically determined (Raff *et al.*, 1997; see Section 3.1.1); most commonly, they consists of 16 protofilaments, but

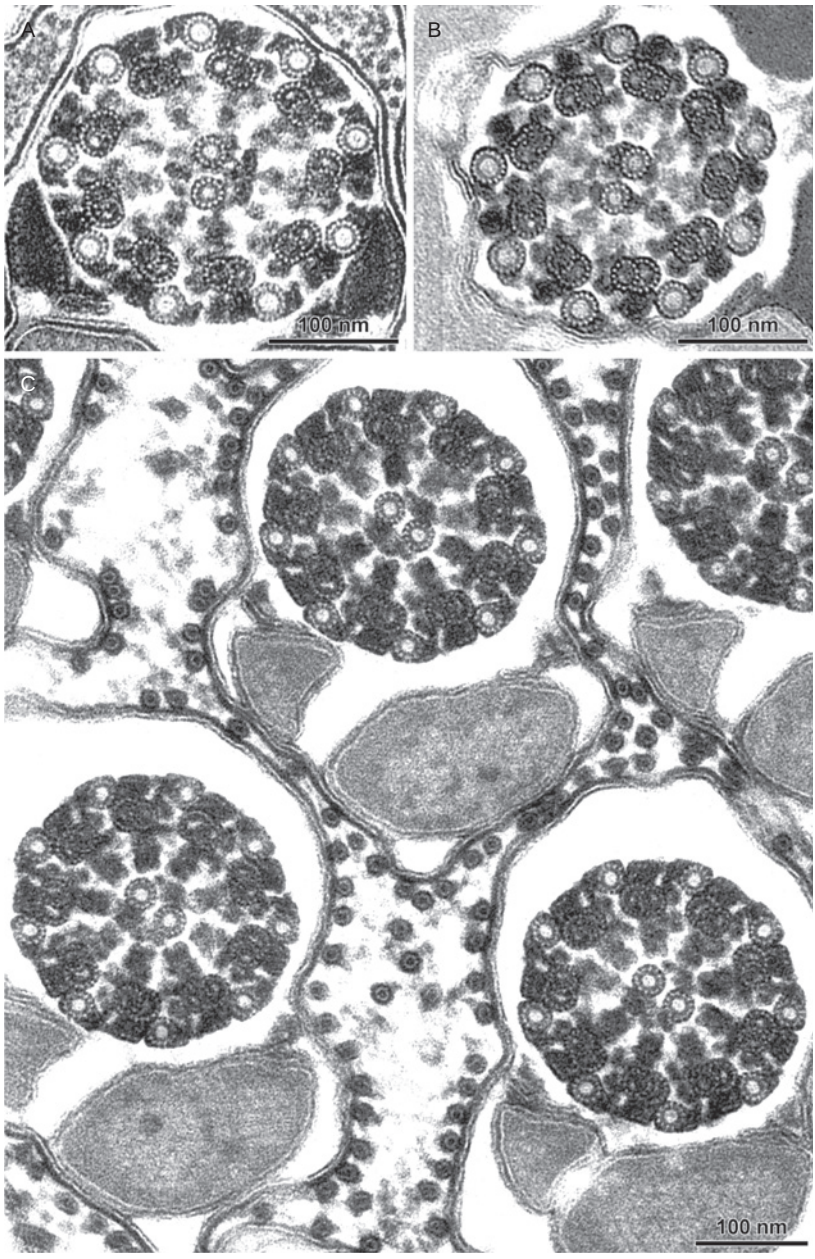

Figure 4.1 (A and B) Cross sections of insect sperm tails showing the most common 9+9+2 axoneme pattern found among insects: The accessory tubules are formed by 16 protofilaments. (A) The coleopteran *Ptilophorus dufouri* (from Jamieson *et al.*, 1999) and (B) the basal nematoceran Diptera *Macrorhyncus ancae* (from Dallai *et al.*, 1995). (C) Cross section through the spermatozoon of the brachyceran Diptera *Ramphomyia* sp.: The accessory tubules are provided with 13 protofilaments (from Dallai *et al.*, 1993).

different protofilament numbers can be found in some groups, varying from 13 up to the exceptional number of 40 protofilaments recently described in the atypical spermatozoa of the neuropteran *Perlamantispa perla* (Dallai *et al.*, 2006).

Although the 9+9+2 axoneme pattern is the common insect model, several taxa express a modified sperm tail structure, which can vary from the "simple" absence of a given axoneme component, to very odd axoneme organizations. Interestingly, in different insect evolutionary lineages, the first affected structure is most often the central pair, which may be either modified or absent; this first event is then followed by—or is concomitant with—either the loss of dynein arms, and/or alterations in the number and disposition of microtubular doublets. Thus, it seems that during insect evolution, alterations of the central complex would have led to an increased "evolutionary instability" of the sperm axoneme. Such a morphological variability of the insect sperm tail has been recently reviewed by Jamieson *et al.* (1999) and by Dallai *et al.* (2006).

Examples of this trend can be found in the wingless Protura and primitive paleopteran order Ephemeroptera, in which the lack of the central pair is concomitant with the loss of the ODA (Dallai *et al.*, 1992), or in the immotile spermatozoa of the trichopteran Annulipalpia, in which the central pair is either absent or consists of three or seven microtubules arranged in a regular array, and both dynein arms are absent (Fig. 4.2A). Supernumerary outer microtubules also characterize the sperm tail of these insect groups. Protura contain a number of axonemal doublets varying from 12 to 16 in the different genera examined. Similarly, the sperm axoneme of the trichopteran *Wormaldia copiosa* consists of 13 doublets, devoid of dynein arms and surrounding a central vesicle (Fig. 4.2A), while in the trichopteran family Hydropsychidae, which also lacks the central complex, supernumerary doublets are assembled *ex novo* at different levels along the axoneme with an assembly process that does not involve the basal body (Dallai *et al.*, 1995; Friedländer and Morse, 1982) and are localized in finger-like appendages of the sperm cell (Fig. 4.2B).

The greatest sperm variability has been described in the dipteran suborder Nematocera. Here, the tendency toward an increased number of doublets is maximally expressed in the two closely related families: Sciaridae and Cecidomyiidae, which are characterized by highly aberrant axonemes. In the former family, *Sciara* and *Rhynchosciara* possess a sperm axoneme that is devoid of the central apparatus and consists of a spiral composed, respectively, by 60–90 and 350 doublets, each one associated with its own accessory tubule and still endowed with dynein arms. An interesting series of progressive variations of the axoneme patterns has been described within Cecidomyiidae. In the members of the most primitive subfamily Porricondylinae, the central apparatus is either formed by one, two, or three microtubules, or is absent. Then in all the species belonging to the subfamily Lestreminae, the axoneme lacks the central apparatus and comprises an

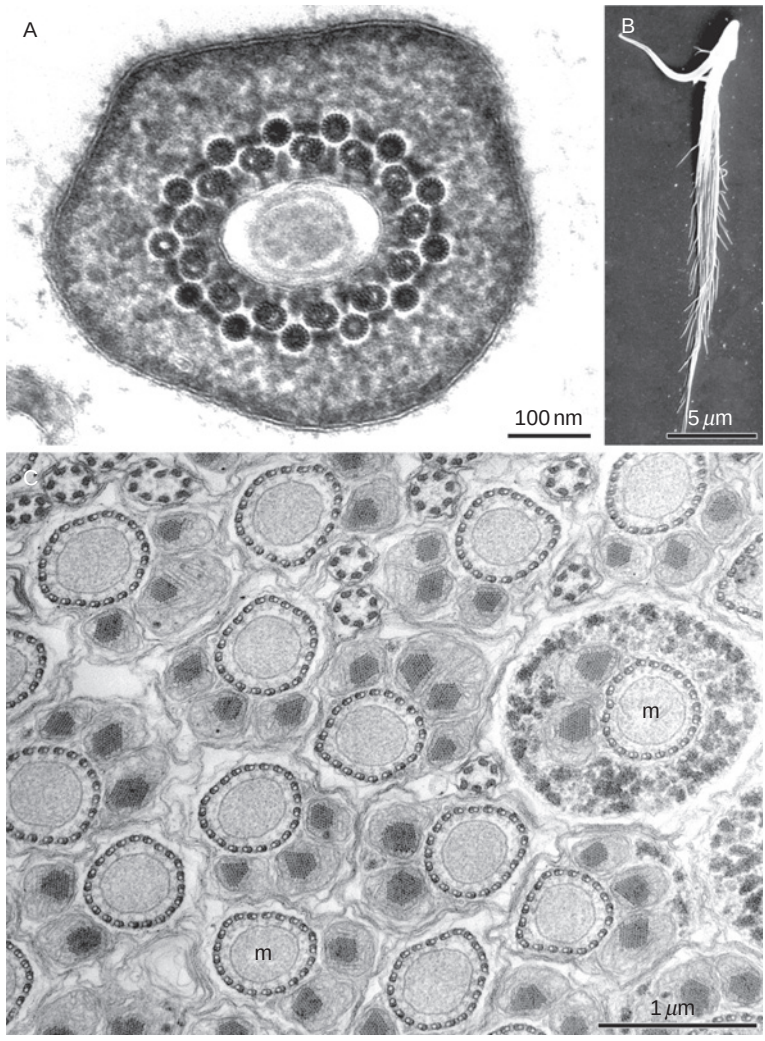

Figure 4.2 (A) Cross section through the *Wormaldia copiosa* spermatozoon. The axoneme is provided with 13 microtubular doublets and lacks the central complex (from Dallai *et al.*, 1995). (B) SEM micrograph of the caddisfly *Hydropsyche pellucidula* spermatozoon; note the numerous appendages that are present along the sperm (from Dallai *et al.*, 1995). (C) Cross section through the spermatozoa of the lestremiid Diptera *Anaretella cincta*. The flagellar axoneme consists of 20 microtubular doublets surrounding an axial mitochondrion (m) (from Dallai *et al.*, 1996).

increasing number of microtubular doublets that are arranged around a central axial mitochondrion [e.g., *Heterogenella* (Fig. 4.3C) and *Anaretella* (Fig. 4.2C)], or are disposed in two antiparallel rows, containing up to 150 microtubules. Both subfamilies still retain their dynein arms. The evolution

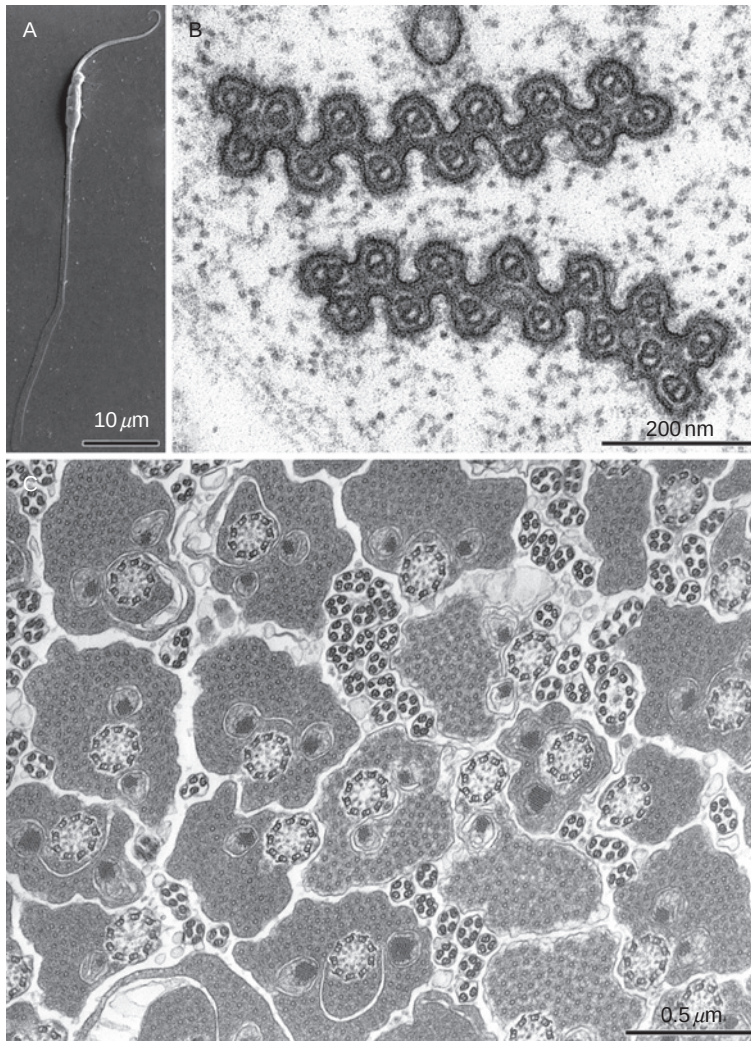

Figure 4.3 (A) SEM micrograph of the gall midge dipteran *Allocontarinia sorghicola* spermatozoon; note the flattened flagellar axoneme (from Dallai *et al.*, 1993). (B) Cross section through the flattened sperm flagellar axoneme of the gall midge dipteran *Myricomyia mediterranea*. The single microtubular doublets devoid of arms are hosted in pockets of the plasma membrane (from Dallai *et al.*, 1993). (C) Cross section through *Heterogenella* sp. spermatozoa. The axoneme is provided with 9 microtubular doublets and lacks the central complex (from Dallai *et al.*, 1996).

of the more advanced subfamily Cecidomyiinae is characterized first by the appearance of highly aberrant axonemes consisting of a huge number of doublets (up to 2500 in *Asphondylia ruebsaameni*) arranged in a single or in a double parallel spiral and provided with the ODAs only (Lupetti *et al.*, 1998;

Mencarelli *et al.*, 2001) (Fig. 4.8); successively, along the evolution of Cecidomyiinae, both dynein arms disappear and the immotile flagellum becomes a flattened structure, with the doublets hosted in small outpockets of the plasma membrane (Fig. 4.3A and B).

Quite often the central pair is replaced by other axial structural elements. In an axoneme cross section, these can appear as a ring of dense material—as in Ephemeroptera, in the nematoceran Diptera *Bibio* and in some gall midges, for example, *Bryomyia* and *Xylopriona*—or as a compact rod, as in Culicidae (Dallai *et al.*, 2006, 2008).

The oddness of some insect sperm models concerns not only the axoneme structure, but also the number of axonemes. The mature spermatozoon of thrips (Thysanoptera) has a bizarre flagellar axoneme consisting of 27 microtubule elements, which derive from three amalgamated 9+0 axonemes (Paccagnini *et al.*, 2007). A multiflagellate spermatozoon was also described by Baccetti and Dallai (1978) in the isopteran *Mastotermes darwiniensis*, with each flagellum consisting of a 9+0 axoneme.

How the variety of unconventional sperm axoneme models that has been realized during insect evolution might have occurred and maintained their viability is a puzzling and still unsolved question. In particular, the frequency of modifications affecting the central apparatus raises several questions, given the important regulatory role that this structure plays in 9+2 axoneme motility (Kamiya, 2002). Further contributions from both genetic and molecular analyses as well as new studies on insect reproductive biology and phylogeny are necessary to answer this question.

2.2. Ciliary axoneme

2.2.1. Primary cilium

The occurrence of primary cilia as a widespread feature of most vertebrate cells, and their crucial role as organelles able to transduce a variety of both mechanical and chemical extracellular stimuli has been firmly established over the past years by many studies (Davis *et al.*, 2006; Marshall and Nonaka, 2006; Pan and Snell, 2007; Quarmby and Parker, 2005). But still, information on primary cilia in insects is extremely limited. The modified chemo- and mechanosensory cilia are considered as derived from primary cilia of the sensory neurons, and they show the 9+0 axoneme model that is typical of the primary cilium. Besides these structures, primary cilia have been till now reported only in the primary spermatocytes of some insect species belonging to different orders, that is, the heterometabolic locust (Daub and Hauser, 1988) and a few holometabolic insects, the lepidopterans *Bombyx mori* (Friedländer and Wahrman, 1970; Holm and Rasmussen, 1980), *Euproctis chrysorrhea* (Leclercq-Smekens, 1978), *Spodoptera littoralis* (Godula, 1985), and *Calpodes ethlius* (Lai-Fook, 1982), the trichopteran *Potamophylax rotundipennis* (Wolf and Klein, 1995), and the dipteran *Dermatobia hominis*

(Quagio-Grassiotto and de Lello, 1996). In these species, the spermatocyte shows four short cilia, located at the cell pole and directed toward the cyst lumen; the apex of each cilium is swollen (Fig. 4.4A and B). According to Friedländer and Wahrman (1970), the occurrence of four cilia marks the primary spermatocyte in the early meiotic profase, while the two secondary spermatocytes are provided each with a pair of cilia. In *P. rotundipennis*, the cilium originates from a basal body possessing the typical triplets only in its

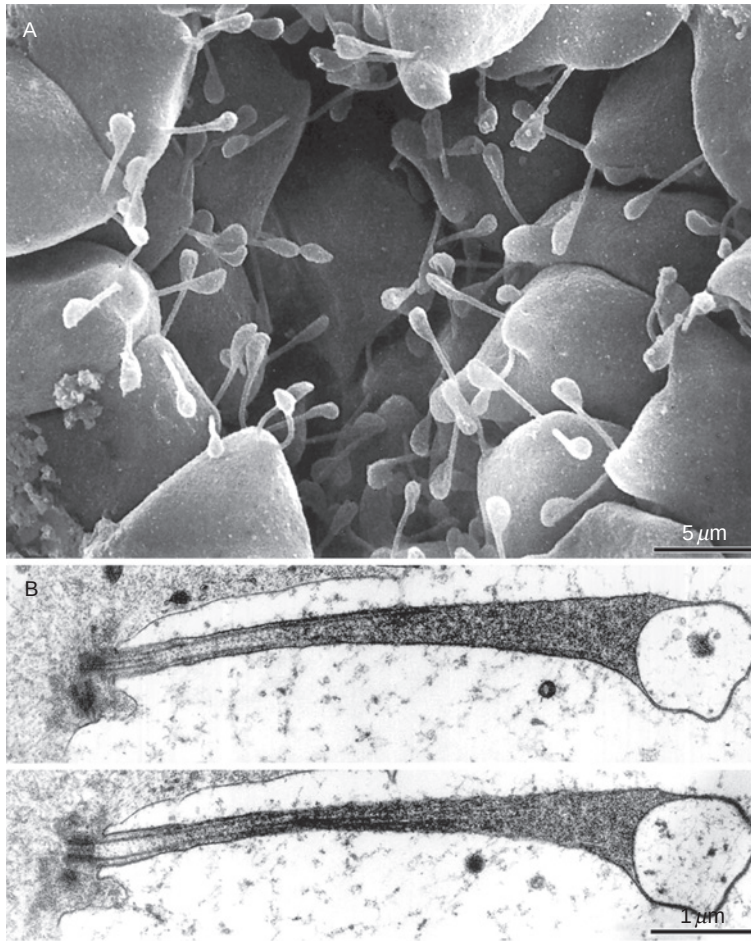

Figure 4.4 (A) SEM micrograph showing the primary cilia of the primary spermatocytes of the moth *Spodoptera littoralis*; note the swollen cilium tip (from Godula, 1985). (B) TEM micrographs of two consecutive longitudinal sections of primary cilia from the primary spermatocytes of the caddisfly *Potamophylax rotundipennis*; the cilium lacks the central microtubules and shows a swollen tip (from Wolf and Klein, 1995).

most distal region, close to the transition zone, and ends in a swollen apical region containing electron transparent material and only a few dense granules (Fig. 4.4B). The primary cilia are supposedly reabsorbed at the end of the meiotic process and, during spermiogenesis, spermatids assemble the sperm flagellum *ex novo*.

A peculiar ciliary structure that is typical of insect species and that might be related to the primary cilium is the ephemeral cilium expressed during epidermal gland morphogenesis (Fig. 4.5). These glands—defined as glands of the 3rd type—possess a quite complex ontogenetic process, which has

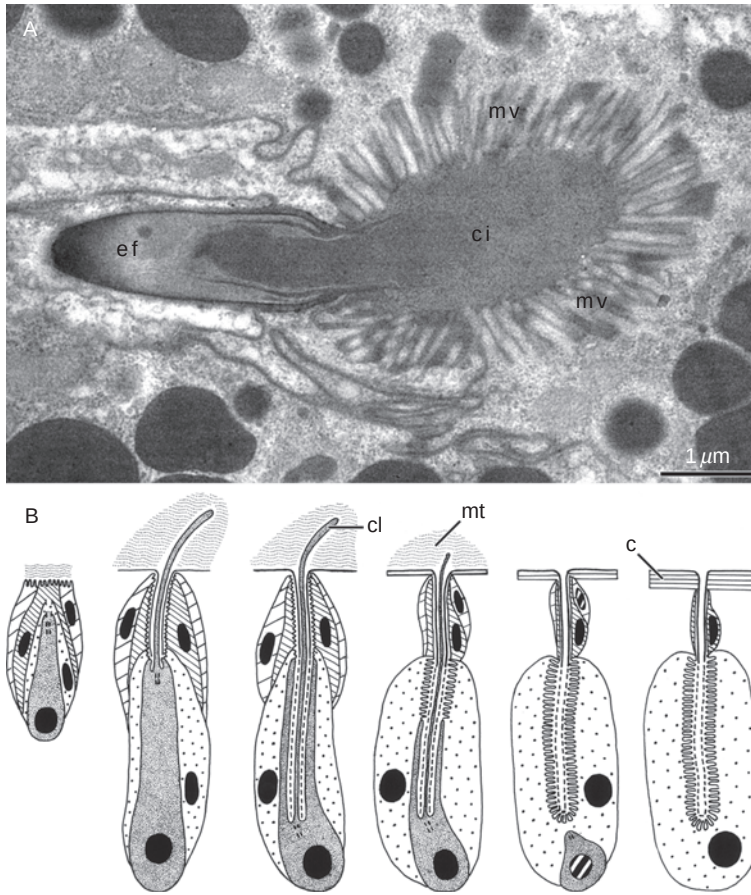

Figure 4.5 (A) Cross section through the extracellular cistern (ci) where the secretion is stored. Note the microvilli (mv) bordering the cistern lumen and the efferent duct (ef) (from Dallai and Burroni, 1981). (B) Schematic drawing of the ontogenesis of the epidermal secretory cells of 3rd type. Explanation in the text: cl, ephemeral cilium; mt, moulting fluid; c, cuticle (from Quennedey, 1998).

been well described by Sreng and Quennedey (1976). Gland formation involves four cells, enwrapping one another (Fig. 4.5B). The innermost cell of each glandular unit assembles a ciliary process, and progressively invaginates to form a sort of muff around the basal part of the cilium, whereas the three other cells form the efferent duct; the invaginated part of the ciliated cell will contribute to form the secretory canal. Successively, the cilium is reabsorbed and the cells undergo a degeneration process, except one cell located beneath the external cuticle, that will become the duct-forming cell, and an inner cell, that will increase in size and form the secretory cell (Fig. 4.5A and B). In apterygotans, which continue to moult even when they have reached the adult stage, the ciliary cells are still present in the adult, and the cilium is partly reabsorbed at each moult (Bitsch, 1981; Bitsch and Palévody, 1976). As it is noted below, the development of the 3rd type gland is somehow reminiscent of the development of sensory organs, which, in its simplest cases, also implicates a tetrad of cells.

2.2.2. Modified cilium of insect sensory cells

In insects, the transmission of mechanical and chemical stimuli is mediated by a series of specialized Type I sensory organs which—though showing a series of structural adaptations—all share a common feature: The sensory cell is always a ciliated bipolar neuron and the ciliated dendrite is the site of transduction. Type II sensory cells are instead single, nonciliated multidendritic neurons.

Type I organs can be further subdivided into chordotonal organs and external sensory organs, which include bristle (either olfactory or mechanosensory) and campaniform sensilla (for a detailed description of the structural organization of these organs, see Hallberg and Hansson, 1999; Keil, 1992, 1997; Yack, 2004). The bipolar neuron is always surrounded by specialized supporting cells, which form a large subcuticular cavity around the dendrite, the receptorlymph space (Fig. 4.6A and B). The neuron and the supporting cells originate from the same stem cell through a series of asymmetric divisions; this morphogenetic process is schematized in (Fig. 4.6D). The first division produces two secondary precursors: One cell will further divide into the so-called tormogen and trichogen cells, which will be implicated in the formation of the bristle shaft and the socket; the other cell will first divide into a small cell and another precursor cell, which will finally divide to produce the sensory neuron and the thecogen cell, implicated in the secretion of an electron dense sheath around the outgrowing dendrite. All Type I sensilla arise from variations on this lineage (Bardin *et al.*, 2004; Kernan, 2007). The ciliary structure—the so-called connecting cilium—subdivides the sensory cell into an inner and an outer segment. Within the cilium, the axoneme always exhibits a 9+0 organization (Fig. 4.7A) and a typical basal body; a ciliary rootlet extends into the inner segment of the cell.

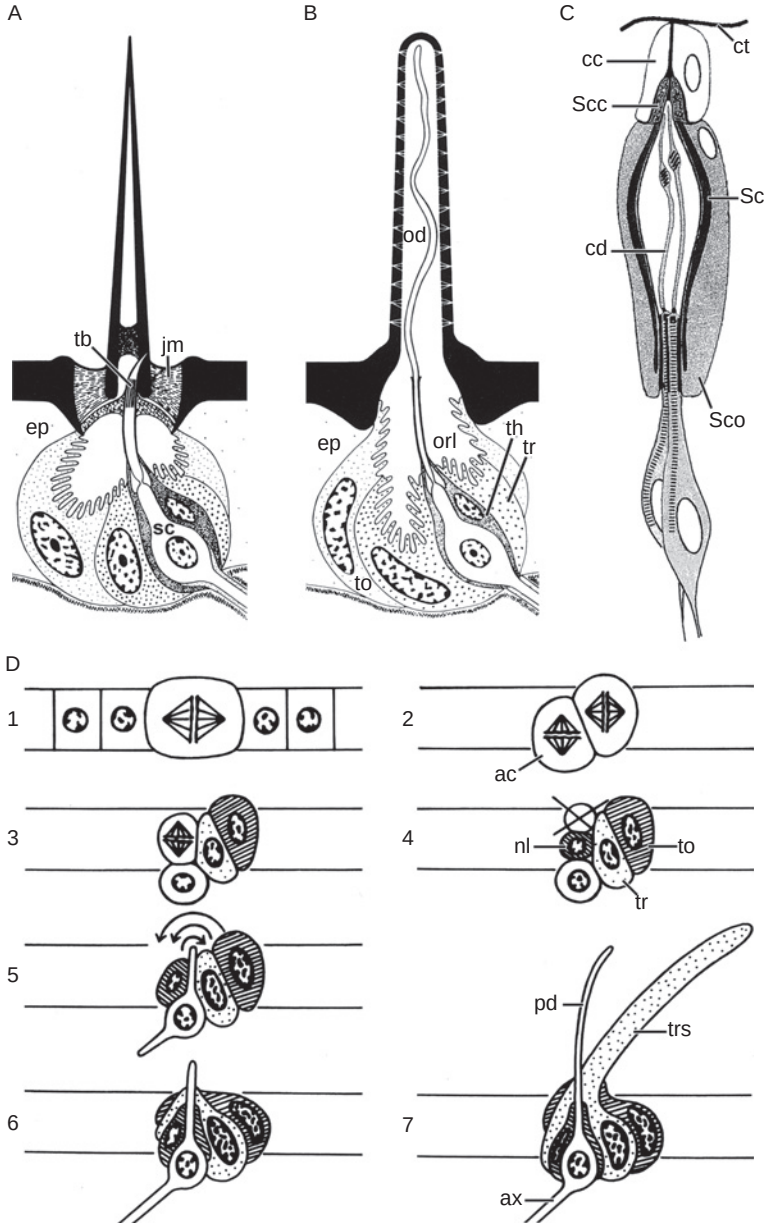

Figure 4.6 (A–C) Schematic drawings of sensilla: (A) mechanoreceptor, (B) olfactory sensillum, (C) chordotonal organ. In (A), the apical end of the dendrite forms the "tubular body" (tb); in (B), the ciliary dendrite prolongs into the lumen of the seta. to, tormogen cell; pd, primary dendrite; tr, trichogen cell; ax, axon; nl=th, neurilemma=thecogen cell; ep, epidermal cell; tb, tubular body; jm, joint membrane; sc, sensory cell; orl, receptorlimph cavity; od, outer dendritic segment. Note the perforated cuticular wall in the olfactory sensillum. (C) In the chordotonal organ, a scolopidium provided with the scolopale cap

In mechanosensory bristles (Fig. 4.6A) and in campaniform sensilla, the ciliary outer segment contacts the base of the bristle or of the cuticular dome, respectively. The cilium is highly modified; a very short connecting cilium with a 9+0 axoneme pattern soon expands into the distal part of the outer segment, which contains many tightly packed singlet microtubules—the tubular body—embedded in an electron dense matrix. The effective stimulation is thought to occur when a local deformation of the cuticle compresses the ciliary membrane between an extracellular dentritic cap and the tubular bundle. In olfactory sensilla, instead, a long cilium extends within a porous bristle shaft (Figs. 4.6B and 4.7B–D); depending on the type of sensillum, the outer segment may or may not divide into several terminal dendritic branches, each containing singlet microtubules extending from the tubular body.

Chordotonal (or scolopidial) organs are tension receptors attached under the cuticle, with no visible external sensillum (see Yack, 2004). In each sensory unit—the scolopale—a fluid-filled capsule, formed by the scolopale cell, encloses the long cilium of 1–3 sensory neurons (Fig. 4.6C). The cilium retains the 9+0 axoneme structure for most of its length; approximately in the distal third of the axoneme, doublets bulge out to form a dilation that encloses an electron dense inclusion with a tubular or paracrystalline substructure, the function and composition of which are unknown. The ciliary tip is covered by an extracellular tube-shaped dendritic cap, required for the transmission of stimuli, which is secreted by the scolopale cell.

Interestingly, while in external sensory organs the 9+0 axoneme is devoid of both inner and outer arms, both dynein arms are instead present on the doublets of the modified cilium in the chordotonal organs (Fig. 4.7A) (Boo and Richards, 1975; Corbière-Tichané, 1971), suggesting the occurrence of a distinct functional mechanism for the cilium in the two types of organs (see below).

3. Molecular Composition of the Insect Axoneme

In the last decade, comparative genomic analyses and proteomic studies have provided significant information on the molecular composition of cilia and flagella, evidencing that these organelles are far more complex than previously thought (Avidor-Reiss *et al.*, 2004; Broadhead *et al.*, 2006; Inglis *et al.*, 2006; Li *et al.*, 2004a; Ostrowski *et al.*, 2002; Pazour *et al.*, 2005;

(Scc) embedding the ciliary dendrite (cd), is present. ct, cuticle; cc, cap cell; Scc, scolopale cap; Sc, scolopale; cd, ciliary dendrite; Sco, scolopale cell. (D) Schematic drawing of the origin of a sensillum complex. Explanation in the text. to, tormogen cell; nl, neurilemma = thecogen cell; ac, accessory cell; pd, primary dendrite; trs, tricogen cell sprout; ax, axon (A, B, D from Keil and Steinbrecht, 1984).

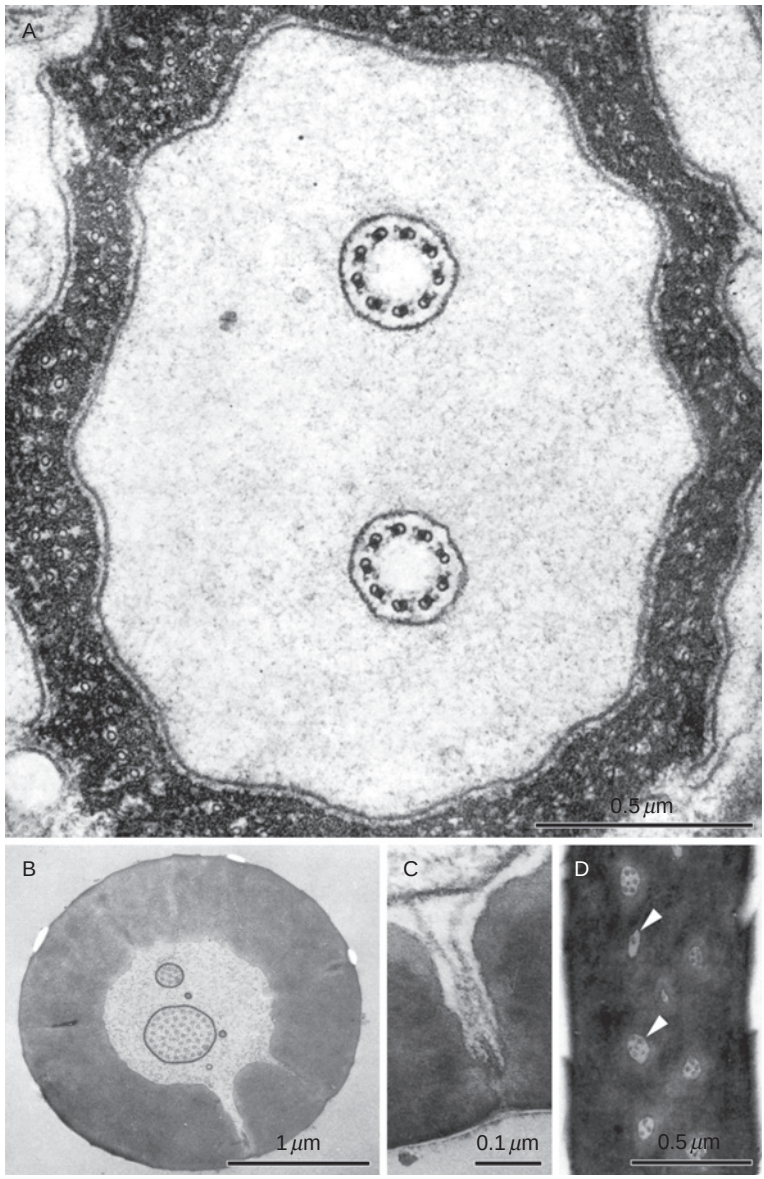

Figure 4.7 (A) Cross section through the antennal scolopidium of the beetle *Speophyes lucidulus*. Note the two outer dendritic ciliated segments, with a 9+0 axoneme; the axonemal doublets are provided with dynein arms (from Corbière-Tichané, 1971). (B–D) Cross section through the distal region of an olfactorium sensillum to show the distal ends of two outer dendritic segments and the slender pore tubule in the cuticle (arrowheads), through which the odor molecules reach the dendrite membrane (from Keil and Steinbrecht, 1984).

Smith *et al.*, 2005). Most recently, the proteomic analysis of whole sperm cells from *D. melanogaster* identified 381 proteins, most of which have been found to have orthologs in *Anopheles gambiae* (Dorus *et al.*, 2006). Both searching of the genome for homologues of known axoneme proteins (Goldstein and Gunawardena, 2000) and the analysis of the sperm proteome (Dorus *et al.*, 2006) have identified in *Drosophila* several known axoneme components, including α and β tubulins, tektins, radial spoke proteins, and dynein subunits. Among the unknown proteins identified in the fly sperm proteome, several appear to be evolutionarily conserved, since they possess orthologs in cilia from other model organisms, ranging from protists to mammals (Inglis *et al.*, 2006); however, a certain degree of organism-specific variation in the protein complement is expected, reflecting the known structural differences among flagella of evolutionarily divergent organisms. Comparing different *Drosophila* sp., the sperm proteome, as a whole, appears to be evolving quite conservatively (Dorus *et al.*, 2006); this is in contrast with the rapid protein divergence rate that characterizes other parts of the *Drosophila* reproductive apparatus, for example, male accessory glands (Mueller *et al.*, 2005; Swanson *et al.*, 2001), and suggests the occurrence of strong evolutionary constraints on sperm structure and function.

Besides these contributions, most part of the current molecular information on the insect axoneme derives from the genetic analysis of spermatogenesis in *D. melanogaster*, which allowed to explore extensively the relationship between tubulin structure and axoneme assembly and function. Recently, some unconventional insect sperm flagella have also been proposed as interesting models relevant for other aspects of axoneme biology, namely, the analysis of tubulin posttranslational modifications (PTMs) and the molecular architecture of the ODA.

3.1. Structural features of axonemal tubulins

Notwithstanding the high level of sequence conservation exhibited among widely distant organisms, α and β tubulins—the main components of microtubules—are characterized by a substantial degree of structural heterogeneity. Tubulin diversity can arise at the gene level, through the expression of multiple isotypes encoded by a multigene family, and can be further elaborated by an extensive array of reversible PTMs (Kierszenbaum, 2002; Ludueña, 1998; Westermann and Weber, 2003). While the conserved sequence features are thought to reflect structural constraints imposed by the basic microtubule function, the expression of different tubulin isotypes and of different sets of tubulin modifications has been related to the specificity of microtubule structure and function in a given cellular context, thus reflecting structural protein adaptation for specialized microtubule arrays.

Most part of tubulin variability, either due to primary structure differences or PTMs, concerns its highly acidic, highly heterogeneous carboxy-terminal region, which is located on the outer surface of the microtubule and is therefore well positioned to modify the properties of the polymer and to mediate interactions with other proteins. In fact, this domain is not required for assembly of microtubules per se and has been implicated in binding to microtubule-associated proteins (Sackett, 1995). The carboxy-terminus has been conserved in corresponding tubulin isoforms in different species, and is therefore considered an isotype-defining domain determining the functional microtubule specificity (Sullivan, 1988). Comparative sequence analyses have also identified the internal variable sequence comprised between amino acids 33 and 57 as a second isotype-defining region (see Sullivan, 1988).

We summarize first the valuable information that studies on *Drosophila* sperm axonemes have contributed to the identification of the tubulin primary structural features that are essential for the proper assembly of the axoneme; then, we analyze the available data on the expression of tubulin PTMs in either conventional or aberrant insect sperm axonemes.

3.1.1. Primary structural requirements for axonemal tubulin

Most of the present knowledge on the tubulin structural features required for the assembly of a 9+2 axoneme comes from the ultrastructural analysis that Raff and colleagues performed on the spermatogenesis of transgenic flies carrying different lesions in the testis-specific β tubulin molecule. These studies have revealed that axoneme function is strongly dependent on the intrinsic properties of the constituent β-tubulin isotype, and that a high degree of stringency has occurred during eukaryotic evolution in the structure–function relationship between β-tubulin sequence and axoneme morphogenesis.

D. melanogaster possesses four genes coding for α tubulin and four genes for β tubulin; however, all the microtubule-mediated processes occurring during spermatogenesis are mediated by a single tubulin heterodimer consisting of the testis-specific $\beta 2$ isotype paired with the predominant α isotype (α84B), that is constitutively expressed also in other tissues (Raff, 1994). $\beta 2$ tubulin is first synthesized in mature primary spermatocytes and is then required for all subsequent sets of microtubules, including meiotic spindle, cytoplasmic microtubules responsible for nuclear shaping and mitochondrial derivative elongation, and axonemal microtubules, with the only exception of the basal body, which is completed in earlier stages of spermatogenesis and is therefore constituted by a different β isotype, $\beta 1$ tubulin.

Spermatogenesis is strictly dependent on the expression of $\beta 2$: when this was hampered by mutations altering $\beta 2$ stability, spermatogenesis failed (Fackenthal *et al.*, 1993, 1995). Moreover, gene replacement experiments have shown that the divergent $\beta 3$ isotype (Hoyle and Raff, 1990) and even the highly similar (95% identity) $\beta 1$ isotype (Raff *et al.*, 2000), which are not

normally expressed in the male germ line, cannot functionally replace β2. β3 can support only a subset of the multiple functions normally performed by β2, that is, the assembly of microtubules involved in mitochondrial derivative elongation, but neither meiosis, nor nuclear shaping and axoneme assembly, indicating that the different spermatogenic microtubule arrays impose different structural requirements on β tubulin (Hoyle and Raff, 1990). Although sharing with β2 a higher degree of sequence conservation, β1 is also unable to assemble a correct axoneme, and allows the assembly only of 9+0 axonemes, which distally lose their integrity (Raff *et al.*, 2000). This phenotype is coherent with the role that β1 normally plays in the assembly of the short 9+0 axonemes of sensory cilia (Dettman *et al.*, 2001).

These data indicated that the information for the construction of a motile 9+2 axoneme resides in some primary structural features of the tissue-specific β2-tubulin isotype, and that small sequence differences between isotypes, like those existing between β1 and β2, can mediate large differences in microtubule assembly capacity. In this respect, it is interesting to note that β1 and β3 affect the axoneme structure in different aspects and to a different level.

A deeper insight into the structural relationship between the axoneme and its constituent β2 tubulin was provided by the analysis of a series of stable, partially functional tubulin variants, each altered in a different region of the molecule; this mutational analysis showed that the two isotype-defining variable regions, that is, the internal region between residues 33 and 57, and the last carboxyterminal 15 amino acids, are indeed critical for β2 function in axoneme assembly.

In the $B2t^6$ mutant, which carries an amino acid substitution at position 56, the axoneme is normally assembled but, as far as its maturation proceeds, an electron dense luminal filament appears in the A subfibers of doublet microtubules, that normally occurs only in the singlet microtubules, that is, in the central pair and in the accessory tubules (Fackenthal *et al.*, 1995). Thus, this phenotype reflects some β2 sequence requirements for the specification of doublet morphology. Interestingly, an almost reciprocal phenotype was observed when the predominant α84B subunit, normally expressed during spermatogenesis, was replaced by the α85E subunit, a minor isotype present in a restricted set of somatic cells (Hutchens *et al.*, 1997); only the singlet microtubules of the axoneme were affected, and both the central pair and accessory tubules were characterized by the absence of the internal luminal filament. So, different subsets of axoneme microtubules have separable requirements for α- and β-tubulin structures.

The crucial role of the β2 carboxyterminus was initially suggested by the analysis of transgenic flies expressing β2ΔC, a truncated β2 molecule lacking the terminal 15 amino acids (Fackenthal *et al.*, 1993, 1995). This tubulin variant is unable to support the assembly of the proper axoneme architecture, and only unorganized clusters of microtubules with presumptive axonemal

identity, that is, microtubules with shared walls and microtubules with electron dense filling, occur in the spermatid cytoplasm. Spokes and any other microtubule connecting structures are also absent. Thus, the $\beta 2$ carboxyterminus is essential for the proper organization of microtubules into a functional higher-order 9+2 structure with all its associated nontubulin components.

Although the $\beta 2\Delta C$ and $B2t^6$ variants affect different aspects of axoneme morphogenesis—specification of microtubule identity and organization of microtubules in a motile axoneme—they do not complement each other for proper axonemal morphology (Fackenthal *et al.*, 1995). This finding suggested that the two isotype-defining regions are both required for $\beta 2$ function, and it was proposed that they interact to form a 3D structure necessary for proper axoneme assembly (Fackenthal *et al.*, 1995).

If the C-terminus is essential for the correct organization of doublets and singlets into the axoneme, then the replacement of this domain in the $\beta 3$ molecule with that of $\beta 2$ should confer on the former isotype new functional capabilities. Starting from this hypothesis, Hoyle *et al.* (1995) constructed a gene encoding for the chimeric protein $\beta 3\beta 2C$, with a $\beta 3/\beta 2$ fusion at the position corresponding to amino acid 344 of the $\beta 2$ molecule. Although still not equivalent to $\beta 2$, this chimeric protein indeed possesses additional functions. In particular, even if an overall complete 9+2 axoneme is still not formed, some suprastructural features are nevertheless present, namely, doublets exhibit architecturally complete linkers and spokes, and morphologically normal accessory tubules are also assembled. The occurrence of these elements even on incomplete arrays of doublets provided evidence that their assembly and the addition to their specific-binding sites is a process occurring independently at each doublet, and strictly dependent on sequence information located in the $\beta 2$ carboxyterminus. The involvement of this domain in the regular positioning of nontubulin components along the axoneme was then confirmed by the analysis of transgenic flies coexpressing $\beta 2\Delta C$ along with the endogenous $\beta 2$ (Hoyle *et al.*, 2001); incorporation of $\beta 2\Delta C$ disrupts the longitudinal repeat of radial spokes and central pair components. The $\beta 2$ carboxyterminus is therefore crucial in conferring to the doublet microtubules the capacity to associate with other axoneme structural components.

Comparative sequence analyses on β tubulins from different organisms and tissues identified a sequence motif that is specifically present in the C-terminus of axonemal β tubulins (Raff *et al.*, 1997). This motif consists of the consensus sequence $EGEF(E/D)_3$ and has been evolutionarily conserved throughout different taxa, but is not present in β tubulins from organisms that lack motile cilia and flagella (Ludueña, 1998). An extended version of this consensus sequence—$EGEF(E/D)_{2-5}G/A$—was successively proposed by Duan and Gorovsky (2002). Some indications about the role

that this sequence plays in axoneme morphogenesis came from the analysis of a series of chimeras constructed from β1 and β2 tubulin (Nielsen *et al.*, 2001). As reported above, β1-mediated axonemes have a 9+0 architecture. It was found not only that the replacement of the whole β1 carboxyterminal 15 amino acid sequence with the corresponding β2 sequence allows the assembly of 9+2 axonemes, but also that even the substitution of just the two amino acids that are required to introduce the axoneme motif into the β1 C-terminus, rescued the assembly of the central pair complex (Nielsen *et al.*, 2001). However, axonemes assembled by these chimeras did not maintain their full-length integrity, and additional changes introducing β2-specific residues in the internal variable region (amino acids 55–57), did not significantly improve axoneme integrity.

This set of data indicated that the short axoneme motif in the β2 carboxyterminus carries information for the assembly of the central microtubules and is presumably required to mediate critical protein interactions. A truncated β2-tubulin variant missing the seven amino acids carboxyterminal to the core axoneme motif is still able to support the assembly of an apparently normal axoneme (Popodi *et al.*, 2005). Thus, only the proximal eight residues of the carboxyterminus—which still contain sites for PTMs (see below)—appear to be essential for the interaction properties of this protein domain. However, these data indicated also that other features of the β2 molecule are necessary to form an axoneme stable over its whole length; this is suggested also by the phenotype of the $B2t^7$ mutant, which carries an amino acid substitution at position 144 and expresses axonemes devoid of one or both the central microtubules, which fray apart within a few microns from the basal body (Fuller *et al.*, 1988). This is likely to be also the reason why the β2 carboxyterminus did not rescue the capability to assemble the central pair complex when inserted in the context of the more divergent β3 molecule (see above).

Besides the sequence features required to build a motile 9+2 axoneme—which are broadly conserved—the testis-specific β-tubulin isotype should also contain some aspects responsible for species-specific structural features, which are conserved over a more restricted phylogenetic range; the latters include, for example, structural features that are characteristic of a given insect order. This was suggested by the analysis of transgenic flies in which β2 was replaced by the *Heliotis virescens* ortholog, Hvβt (Raff *et al.*, 1997). The moth β tubulin is unable to support any spermatogenetic microtubule array when expressed as the sole β-tubulin isotype in the postmitotic male germ line, but supports the assembly of partial axonemes when coexpressed with β2. Interestingly, when Hvβt comprises more than 6% of the total β-tubulin pool, it imposes on the accessory tubules the 16-protofilament structure that is typical of moths, while *Drosophila* accessory tubules are normally formed by 13 protofilaments. The finding that only the accessory tubules are affected, and not doublets or central pair singlets, is likely to

reflect their peculiar morphogenetic process. So, the testis-specific β-tubulin sequence appears to contain also structural information for axoneme species-specific features.

Overall, these studies revealed that the testis-specific β2 tubulin exerts a considerable control over different levels of microtubule organization within the axoneme, and that several features of this organelle—including microtubule identity, the assembly of the central pair, the regular positioning of binding sites for spokes and linkers, up to the general organization of the axoneme—are specified by definite structural aspects of this tubulin isotype. This means that use in axoneme assembly has strictly constrained β2-tubulin evolution, and, indeed, β tubulins used in motile axonemes fall among the most conserved β-tubulin sequences. Nielsen and Raff (2002) suggested the occurrence of intramolecular cooperative interactions and the assembly of the central pair as the main features that have contributed to this stringent structure/function relationship. The general occurrence of a 9+2 array in motile axonemes would thus reflect a positive selection for molecular mechanisms—mediated by the central apparatus—that are able to provide a fine tuning of the beating and hence a more efficient motility. As a matter of fact, several motile flagellar models are known that exhibit unconventional axoneme geometries—most of them among insects—but they are all characterized by a poor motility.

Interestingly, the mutation rate of testis-specific β tubulin seems not to be the same when different insect groups are considered over comparable times of evolutionary separation, as it emerged from the analysis of testis-specific β-tubulin sequences from two lepidopteran and two dipteran species (Nielsen *et al.*, 2006). In the same study, an almost complete stasis in β2 evolution was shown in the genera *Drosophila* and *Hirtodrosophila*, which was related to the presence of special constraints, unique to this clade and possibly related to requirements imposed on the β2 sequence by the assembly of the very long sperm axoneme (up to 5.8 cm long in *D. bifurca*) that characterizes these insects. Surprisingly, residues evolving more rapidly in lepidopterans with respect to dipterans are those involved in longitudinal contacts with α tubulin—both inter- and intradimers—and in lateral contacts between protofilaments, while the most slowly evolving residues are those of the carboxyterminus, which do not play an actual structural role in microtubule assembly. Such a difference in β2 evolution rate between dipterans and lepidopterans has been suggested to be related to the absence of a testis-specific α-tubulin isotype in *Drosophila* and to its presence in *Bombyx* (Kawasaki *et al.*, 2003; Nielsen *et al.*, 2006); use of both α and β testis-specific isotypes might have released testis tubulin evolution, allowing a mutation rate greater than when the α subunit of the dimer has multiple function, as occurs in *Drosophila*, which uses the same major α isotype for both somatic and axonemal functions.

The above reported study is based on the analysis of quite a limited number of species. The two dipterans used for this mutational rate analysis,

D. melanogaster and *A. gambiae*, belong respectively to Brachycera and Nematocera, the two dipteran suborders. We note here that, while brachycerans are characterized by an absolute conservation of the 9+9+2 pattern, a trend toward increasingly aberrant axoneme architectures, up to some of the most amazing ever described, occurred in nematocerans (see Dallai *et al.*, 2006; Jamieson *et al.*, 1999). Having a basal phylogenetic position within this dipteran group, *Anopheles* still maintains the circumferential organization of the nine doublets and the related accessory tubules, though it has already lost the central pair complex. Therefore, it would be interesting to obtain sequence data on testis-specific β tubulin from higher nematoceran species that possess highly modified axonemes.

3.1.2. Posttranslationally modified tubulin isoforms

A conserved axoneme feature is the presence of a high tubulin polymorphism, which is essentially contributed by a series of PTMs (Verhey and Gaertig, 2007; Westermann and Weber, 2003). Such a molecular complexity of axonemal tubulin is likely to be correlated with the great number of protein interactions occurring in the 9+2 structure and to the requisite organization of specific anchoring sites on the microtubular lattice.

With the exception of acetylation of Lys^{40} on α tubulin, all axoneme PTMs occur in the tubulin carboxyterminal domain. These include the tyrosination/detyrosination cycle and the removal of the terminal glutamyl–tyrosine dipeptide on the α tubulin, and two polymodifications that are common to both α and β tubulins, that is, polyglutamylation and polyglycylation, which consist in the addition, respectively, of several glutamate (up to 20) or glycine (up to 34) residues onto the lateral chain of specific glutamic residues in the tubulin C-terminal domain, thus forming polyglutamate or polyglycine side chains. Polymodifications provide major contributions to axonemal tubulin heterogeneity, due to variations in the length and structure of the added side chain (Bré *et al.*, 1998; Redeker *et al.*, 1991, 1998; Vinh *et al.*, 1999; Wolff *et al.*, 1994).

All tubulin PTMs have been detected in axonemes from phylogenetically distant species, from protists to mammals, and in insects as well (Bobinnec *et al.*, 1999; Bré *et al.*, 1996; Bressac *et al.*, 1995; Mancini *et al.*, 2005; Mencarelli *et al.*, 2000a, 2004, 2005; Taddei *et al.*, 2000). Such a widespread distribution is suggestive of a relevant role for tubulin PTMs during axoneme morphogenesis and function, but the specific contribution provided by each modification has not yet been definitely established.

PTMs are unlikely to affect microtubule stability directly; rather, they seem to biochemically distinguish different microtubule sites and to adapt them for specific functions. Glutamylation and glycylation could allow to modify to a different extent the structure and the charge properties of the C-terminus—where their modification sites partially overlap with the axoneme motif (see Popodi *et al.*, 2005 and references therein)—and to

differentially modulate its capability to interact with other axonemal components. The structural variability of the added side chains could provide a differentiated scaffold for multiple protein interactions. Evidence supporting this hypothesis has been accumulating during the last years. The involvement of glutamylation in regulating the interaction between microtubules and microtubule-associated or motor proteins has been demonstrated *in vitro* (Bonnet *et al.*, 2001; Boucher *et al.*, 1994; Larcher *et al.*, 1996; Okada and Hirokawa, 2000); both polyglycine and polyglutamate side chains have been implicated in the interaction of flagellar dynein with the B-tubule during its mechanochemical cycle (Bré *et al.*, 1996; Gagnon *et al.*, 1996; Million *et al.*, 1999).

Further observations correlate with a role of polymodifications in specifying microtubule-binding properties. First, in metazoans, glycylation is mostly restricted to axonemal microtubules, and in protists, only monoglycylation occurs on cytoplasmic microtubules, while the longer polyglycine side chains are restricted to the hyperstable axonemal array (Bré *et al.*, 1998). Second, axoneme morphogenesis is characterized by a developmentally regulated expression of glycylation—monoglycylated isoforms occur in newly assembled microtubules while longer polyglycine side chains mark the end of sperm maturation (Iftode *et al.*, 2000; Iomini *et al.*, 1998; Kann *et al.*, 1998). Third, in axonemes from protists, echinoderms and mammals, polymodifications have been found to be differentially distributed among the different kinds of axonemal microtubules (Johnson, 1998; Multigner *et al.*, 1996), among different doublets (Fouquet *et al.*, 1996; Kann *et al.*, 1998; Prigent *et al.*, 1996) and also along the axoneme (Bré *et al.*, 1996; Huitorel *et al.*, 2002; Lechtreck and Geimer, 2000).

Recently, the analysis of *Tetrahymena* mutant strains carrying alterations of the glycylation sites has shown that a certain threshold level of microtubule glycylation is required for both cell viability and correct axoneme morphogenesis (Redeker *et al.*, 2005; Thazhath *et al.*, 2002, 2004; Xia *et al.*, 2000). Glycylation deficiencies were shown to result in the failure to initiate the construction of the central pair, and in the capability to assemble only short-size 9+0 immotile cilia; furthermore, the overall structural integrity of the axoneme is compromised. On the basis of these results, it was proposed a function for tubulin glycylation during axoneme morphogenesis; possibly, shorter or even monoglycylated side chains could be required during the early stages of assembly, while longer chains could be involved in distinct functions occurring later during axoneme maturation, for example, motility. PTMs could specifically recruit proteins implicated in central pair assembly. The above described mutant phenotypes, however, could be due to a defect of both glycylation and glutamylation, since it has been shown that the two polymodifications are coordinated (Redeker *et al.*, 2005); in fact, mutations in the glycylation sites of either α or β tubulin modify the level of glutamylation along with glycylation on the

mutated tubulin molecule, and also affect the levels of both polymodifications on the nonmutated subunit of the tubulin dimer.

The axonemal defects of *Tetrahymena* glycylation mutants resemble some of the phenotypes described in *Drosophila* mutants with an altered β-tubulin isotype. Up to now, tubulin PTMs have been studied only in the sperm flagella of a limited number of insect species, mainly by the use of isoform-specific monoclonal antibodies. Bressac *et al.* (1995) and Bré *et al.* (1996) analyzed the expression of glycylated isoforms during *Drosophila* spermatogenesis, and found that glycylation is developmentally regulated, occurring at the end of spermatogenesis and marking the end of sperm maturation. The presence of glutamylated α and β tubulin in *Drosophila* testis was reported by Bobinnec *et al.* (1999).

A more detailed analysis of tubulin isoform content and distribution was carried out by Mencarelli *et al.* (2000a) in the 9+9+2 sperm axoneme of the hymenopteran *Apis mellifera*. In this species, an enhanced stability of accessory versus axonemal microtubules allowed the separation and comparative analysis of the two classes of microtubules. The results of this study evidenced a specific compartmentalization of tubulin isoforms within the honeybee sperm axoneme. Tubulin heterogeneity is overall much higher in axoneme than in accessory tubules, with significant differences occurring between the two classes of microtubules. First, the tubulin tyrosination extent of accessory tubules is far lower than that of axonemal microtubules, whereas the reverse is observed for detyrosination. This finding confirms at the molecular level the origin of accessory tubules as outgrowths from the B-tubules of outer doublets, which contain a much higher amount of detyrosinated tubulin than the A-tubules (Johnson, 1998; Multigner *et al.*, 1996). This result was confirmed by Taddei *et al.* (2000), who reported a preferential occurrence of tyrosinated α tubulin on axonemal doublets in the sperm tail of *Bacillus rossius*. Similarly, accessory tubules resemble the B-tubules also by containing glycylated variants which—in other organisms—have been reported to be specifically located in the B-subfiber (Multigner *et al.*, 1996). Second, tubulin polyglutamylation occurs differentially, and a higher amount of β-tubulin molecules are modified in axonemal than in accessory microtubules. Third, the most acidic variants, likely to be those with longer polyglutamate side chains, are restricted to axonemal microtubules.

Thus, the structural and functional organization of the honeybee sperm tail is based on a finely tuned differentiation of the two microtubular assemblies, which is at least in part created by the intrinsic properties of tubulin in each system. The different stability of axoneme and accessory microtubules is, however, unlikely to be generated by their tubulin content, but rather is more probably contributed by different and specific sets of associated proteins. Hence, the differential distribution of PTMs may be related to specific structural requirements imposed by the different interactions that the two microtubular systems need to establish during axonemal

morphogenesis. In this view, the higher level of heterogeneity exhibited by axoneme microtubules might be due to the more complex protein interactions they are involved in and to the more specialized functions they have to perform in axonemal motility.

No information is currently available about the mechanisms that generate such microtubule differentiation during insect spermatogenesis. The knowledge on the enzymes responsible for microtubule PTMs is still limited (Verhey and Gaertig, 2007), and the question of how axoneme microtubules can be differentially modified still remains a central issue.

In a successive series of experiments, Mencarelli *et al.* (2004, 2005) analyzed the content of tubulin PTMs in a group of unconventional axonemes. The models selected for this study included (i) three species—the ephemeropteran *Ecdyonurus* and the dipterans *Bibio* and *Anopheles*—characterized by axonemes devoid of the central pair microtubules, which are substituted by central axial elements of unknown nature and (ii) two species—the cecidomids *Monarthropalpus* and *Asphondylia*—the sperm axonemes of which have lost not only the central pair/radial spokes complex, but also the IDAs and even the ninefold symmetry of the conventional axoneme (Fig. 4.8) (Lupetti *et al.*, 1998; Mencarelli *et al.*, 2000b, 2001). Hence, these axonemes are aberrant both in their assembly mechanism and in their motility (see below), and all recall *Tetrahymena* glycylation site mutants for the absence of the central pair microtubules. The authors found that such axonemal alterations are concomitant with a strong reduction in tubulin molecular heterogeneity, which results from extremely low levels of glycylation and glutamylation on both α and β tubulins (only *Asphondylia* β tubulin—which also is not significantly glycylated—still retains a certain level of glutamylation), or even from the lack of both polymodifications, as occurs in *Bibio*. This common feature is unlikely to be due to evolutionary relationships, given the unrelated positions of the analyzed species in the insect phylogenetic tree. In fact, *Anopheles* belongs to a dipteran superfamily (Culicoidea) different from that of the other dipterans (Bibionidae); moreover, dipterans have strongly diverged from the more primitive order Ephemeroptera, to which *Ecdyonurus* belongs (Jamieson *et al.*, 1999). So, in these insects, a correlation appears to exist between alterations in axonemal pattern—namely the absence of the central pair—and a reduced expression of the two polymodifications.

The nature of the molecular defect/s concomitantly underlying the unusual axoneme architecture and PTMs deficiencies has not yet been established. The finding of a contemporary reduction of both polymodifications on both tubulin subunits fits well with the above reported observations by Redeker *et al.* (2005), on the existence of a coordinate expression of glycylation and glutamylation.

It is remarkable that, while removal of β-tubulin glycylation is lethal in *Tetrahymena*, and cilia and flagella of other organisms have always been

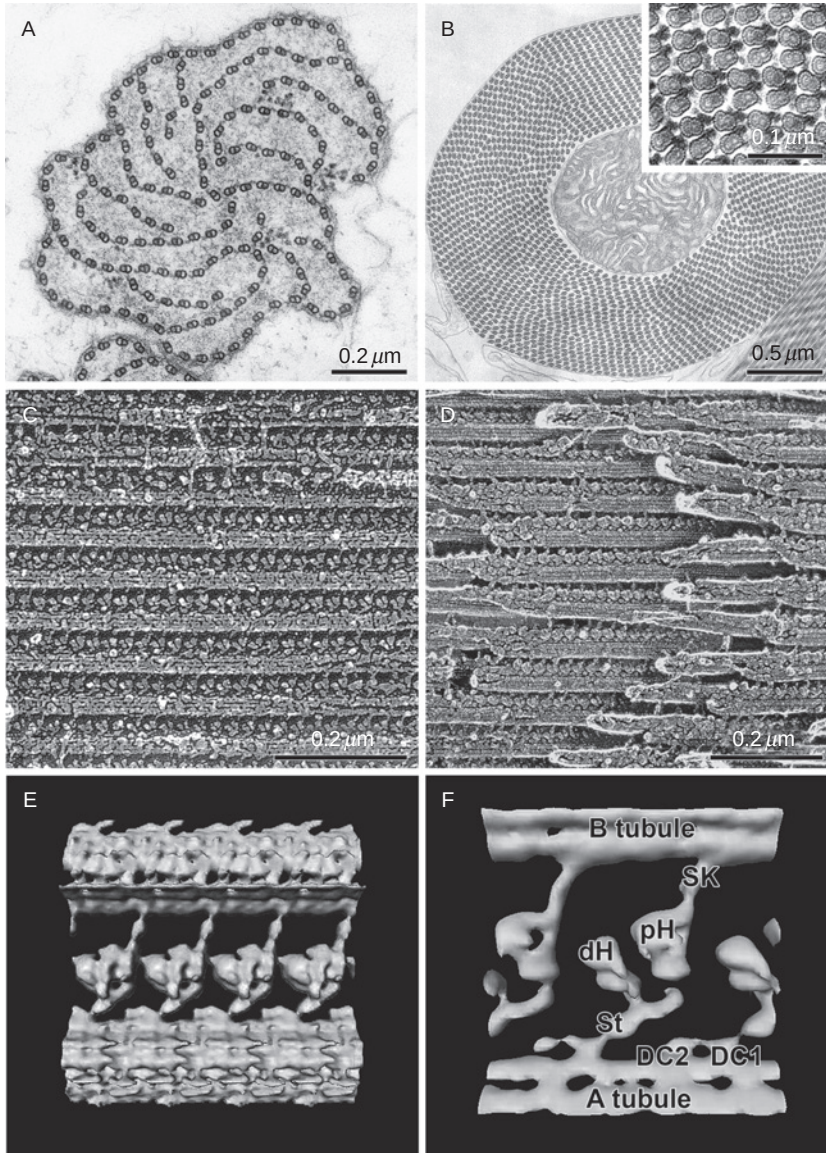

Figure 4.8 (A). Transverse section through the sperm tail of *Monarthropalpus flavus*. In this species of gall midge fly, the axonemes are made of cartwheel oriented laminae of microtubular doublets connected by outer dynein arms only (original unpublished). (B) Transverse section through mature spermatozoa of the gall midge *Asphondylia ruebsaameni*. In this species, outer dynein arms are visible connecting microtubular doublets that are arranged in double parallel spirals (see inset in this figure) around an axial stack of mitochondria (original unpublished). (C) Metal replica of demembranated, rapidly frozen, cryofractured sperm axonemes of *Monarthropalpus flavus*. The axoneme was fractured parallel to the longitudinal axis of the sperm thus showing many

reported to be enriched at least in one polymodification, in these insects neither sperm viability nor its capability to beat are affected. This raises the question of how this condition has been allowed and maintained during the evolution of these species. With respect to protists, insects could have been less affected by alterations in tubulin PTMs since they express a testis-specific β-tubulin isotype, while in *Tetrahymena* only one α and one β-tubulin isotype (see Xia *et al.*, 2000) are responsible for the assembly of all the multiple microtubular arrays of the cell, so that mutations in the tubulin molecule and the ensuing PTMs are expected to interfere with multiple vital functions. Alternatively, this discrepancy might be related to the different process underlying axoneme assembly in insect sperm flagella and in *Tetrahymena* cilia. In *Tetrahymena*, as in most organisms, axoneme morphogenesis depends on intraflagellar transport (IFT); polymodifications have been proposed to affect IFT (Pathak *et al.*, 2007; Redeker *et al.*, 2005). During insect spermatogenesis, on the contrary, axoneme assembly occurs in the spermatid cytoplasm, and does not require IFT (Han *et al.*, 2003; Sarpal *et al.*, 2003; Witman, 2003). Therefore, if polymodifications are indeed required for the interactions between IFT motors and microtubules, then the peculiar way that insects use to build their sperm axoneme might allow them to more easily tolerate a decrease in the polymodifications level. This hypothesis is supported by the observation that, while axonemes depending on IFT are characterized by an early onset of monoglycylation and by a delayed polyglycine side chain lengthening (Iftode *et al.*, 2000; Kann *et al.*, 1998), in *Drosophila* spermatogenesis both mono- and polyglycylations occur only in mature spermatozoa (Bré *et al.*, 1996).

A detailed analysis of the distribution of glutamylated tubulin isoforms was carried out in *Asphondylia* spermatozoa (Mencarelli *et al.*, 2004). This highly aberrant axoneme does not originate from a conventional basal body, but it is formed by a very peculiar assembly process, in which doublets originate in the postnuclear region, from aggregates of dense fibrous material and membranous cisternae (Mencarelli *et al.*, 2000b). In the mature sperm tail, the highest level of glutamylation is localized in the very

dynein outer arms intercalated among parallel rows of microtubules (original unpublished). (D) Same preparation as in (C) but from mature spermatozoa of *Asphondylia ruebsaameni*. Also in this case, the fracture plane was parallel to the longitudinal axis of the sperm tail and many outer dynein arms are visible in rows intercalated among parallel microtubules (original unpublished). (E) 3D surface rendering model from a fourier-filtered double tilt tomographic map of *Monarthropalpus* spermatozoa prepared like that shown in (C) (original unpublished). (F) 3D surface rendering of two neighboring outer arm dyneins. The model was obtained by single particle analysis performed on a tomogram from *Monarthopalpus* sperm axoneme like that shown in (C). The ring presumably corresponding to the distal head heavy chain is visible after this strategy of image analysis. SK, stalk; dH, distal head; pH, proximal head; St, stem; DC1 and DC2, docking complex (original unpublished).

proximal region of axonemal doublets, close to the nucleus. Such a high level of glutamylation is commonly observed in basal bodies of cilia and flagella of a variety of species (Lechtreck and Geimer, 2000; Péchart *et al.*, 1999), and tubulin glutamylation has been shown to be crucial for microtubule stabilization in centrioles (Bobinnec *et al.*, 1998). So, tubulin glutamylation could be required for the stabilization of the nascent shared-wall microtubules per se, independently from the supramolecular architecture of the axoneme.

Along the *Asphondylia* sperm axoneme, glutamylated epitopes exhibit a uniform distribution, rather than the differential distribution in a proximo-distal gradient that characterizes all the till now analyzed 9+2 axonemes (Bré *et al.*, 1996; Fouquet *et al.*, 1996; Huitorel *et al.*, 2002; Kann *et al.*, 1998, 2003; Lechtreck and Geimer, 2000; Mencarelli *et al.*, 2000a; Prigent *et al.*, 1996). The gradient distribution of tubulin isoforms has been related to the differential distribution of the various IDA isoforms along the 9+2 axoneme—more specifically, to the need of providing a scaffold of differentiated, specific-binding sites along the axoneme. Thus, it is notable that in *Asphondylia*, the absence of a gradient distribution of tubulin glutamylated isoforms is concomitant with the lack of IDAs (Mencarelli *et al.*, 2001). The inhibition exerted on the motility of reactivated sperm flagella by an anti-glutamylated tubulin antibody (Mencarelli *et al.*, 2001) indicates, however, that this PTM is still involved in the peculiar motility of this aberrant axoneme, which is based on the activity of the ODA only.

The above reported data on insect axonemes emphasize the view that polymodifications are involved in the establishment and/or stabilization of the 9+2 architecture. Thus, not only precise sequence features but also PTMs of the tubulin carboxyterminus are strictly required for axoneme construction, and, as a part of a tightly constrained developmental system, tubulin PTMs are likely to be also subjected to evolutionary constraints and to coevolutionary adaptive changes.

3.2. Dynein

Axonemal dyneins are minus end-directed molecular motors connecting microtubular doublets in motile axonemes of cilia and flagella. Most ultrastructural and molecular information about axonemal dyneins has been obtained in studies of the biflagellate green alga *Chlamydomonas reinhardtii*, the ciliate *Tetrahymena pyriformis* and sea urchin spermatozoa, while only limited work has been performed in insects. All the dynein isoforms that have been identified biochemically are large multisubunit proteins with a mass of 0.6–2 million daltons, composed of 1–3 heavy chains (MW $\sim$ 530 kDa) as well as of a number of intermediate (MW 60–135 kDa) and light (MW $<$ 25 kDa) chains (Mitchell, 1994; Porter, 1996). The heavy chains correspond to morphologically identifiable heads, each containing a molecular motor domain with the

hydrolytic site for ATP; they bind to microtubules in an ATP-dependent manner and allow dynein arms to force neighboring doublets to slide against each other (Summers and Gibbons, 1971; Warner *et al.*, 1989).

Several dynein gene products and at least five biochemically defined forms of dynein can be found in a given type of flagellum (Hook and Vallee, 2006). The spatially and temporally coordinated activity of different dynein isoforms arranged on doublets in two longitudinal rows of ODAs and IDAs is usually necessary for ciliary and flagellar motility (Asai and Wilkes, 2004; Kamiya, 2002; Porter and Sale, 2000).

After the complete sequencing of *Drosophila* euchromatic genome (Adams *et al.*, 2000; Rubin *et al.*, 2000), 12 dynein heavy chain genes could be identified; among them most appeared to be axonemal isoforms and 3 appeared to be new genes (Goldstein and Gunawardena, 2000).

Correlating dynein genes with specific dynein structures in the axoneme and understanding the functional morphology of axonemal dyneins is a long, challenging, but very important process. The first details about the molecular morphology of isolated dynein complexes were obtained by quick freeze-deep etch (QF-DE) microscopy of molecules adsorbed onto mica (Goodenough and Heuser, 1982, 1984; Sale *et al.*, 1985) and later by negative stain and image analyses on purified minimal motor domains (Burgess *et al.*, 2003, 2004). These studies showed that each heavy chain forms a ring-shaped head domain, from which two other domains emerge: A slender stalk, which is able to bind to the B-tubule in an ATP-dependent manner and an elongated tail domain, which binds to the A-tubule. The ODA has three heads (motor domains) in protozoans but only two are retained in metazoans. The IDAs are two- and one-headed and are arrayed in a repeating pattern along the microtubule (Goodenough and Heuser, 1984). Information on dynein arm organization *in situ* has come initially from the analysis of metal replicas of quick-frozen deep-etched axonemes of *Chlamydomonas* flagella (Goodenough and Heuser, 1982, 1984).

Successively Burgess *et al.* (1991) introduced the use of computer-aided image analyses of metal replicas and studying 9+2 axonemes of domestic fowl produced the first tentative 3D models for ODAs, showing the positioning of the head domain, the B-link (stalk), and the stem (tail) in both the rigor and relaxed mechanochemical states of the complex.

The first studies on the molecular structure of outer arm dyneins in insect spermatozoa were performed on sperm axonemes of asphondyliid and cecidomid gall midges (Lupetti *et al.*, 1998; Mencarelli *et al.*, 2001). Spermatozoa of these insects are motile but their axonemes show a very peculiar ultrastructure characterized by the presence of many microtubular doublets arranged in spirals and connected by ODAs only (Fig. 4.8A and B) (Dallai, 1988; Dallai and Mazzini, 1980). IDAs, central pair complex, radial spokes, and nexin links: All the structures involved in the regulation of flagellar beating in normal 9+2 axonemes are missing, while ODAs are

clearly functional in these axonemes and able to support microtubule sliding in an ATP-dependent fashion (Mencarelli *et al.*, 2001). Among the many hundred existing species of gall midges, the molecular composition of axonemal dynein was analyzed comparatively in *A. ruebsaameni* (supertribe Asphondyliidi) and *Monarthropalpus flavus* (supertribe Cecidomyiidi) (Lupetti *et al.*, 1998; Mencarelli *et al.*, 2001). In the first species, dynein possesses the typical metazoan composition comprising two different heavy chains, while in the genome of *Monarthropalpus* only one functional gene codifying for an axonemal dynein heavy chain could be identified, and a closely-related additional sequence was proposed to be a pseudogene (Lupetti *et al.*, 1998; Mencarelli *et al.*, 2001). Interestingly, all the outer arm dynein molecules analyzed so far comprise different heavy chains that are thought to play different functional roles. It was therefore speculated that both heavy chain genes were originally expressed, as it still occurs in the heterodimeric outer arm of *Asphondylia*, but that an incorrect excision of an intron from one gene led to its loss of function in *Monarthropalpus*, thus giving rise to the unusual homodimeric ODA of this species (Lupetti *et al.*, 1998; Mencarelli *et al.*, 2001). These data indicate the occurrence in the cecidomid family of a progressive evolutionary trend toward simplification of the dynein heavy chain. The early loss of all the regulatory structures for the dynein activity occurred in the sperm axoneme of these insects would have allowed accumulation of mutations leading to a progressive loss of dynein heavy chain gene function in cecidomids. The ultrastructural analysis performed by QF-DE on *in situ* ODAs confirmed the presence of two heads in both species but with some morphological differences (Fig. 4.8C and D). An additional globular domain was identified in *Asphondylia* in close vicinity of the ODAs connection with A-tubule. A single B-link was identified projecting obliquely from the proximal head. Treatment of demembranated sperm cells with ATP and vanadate induced conformational changes in the dynein outer arms that were interpreted as the result of rotation of the dynein head with respect to what observed in ATP depleted axonemes (in rigor condition) (Lupetti *et al.*, 1998; Mencarelli *et al.*, 2001).

Electron tomography was applied to study metal replicas obtained by QF-DE from rigor sperm axonemes of *M. flavus* (Lupetti *et al.*, 2005). The several microtubular laminae composing these axonemes proved to be sufficiently planar to allow the visualization of many doublet microtubules with their dynein complexes within the same fracture face. This structural feature allowed to recover a significant number of equivalent objects and to improve the signal to noise ratio of the dynein reconstruction by applying advanced averaging filtration protocols (Fig. 4.8E). The 3D model obtained by this strategy showed the following interesting structural features: First, the two head domains of each ODA are almost parallel to each other and are obliquely oriented with respect to the longitudinal axis of microtubules. The two heads are, therefore, positioned at different distances from the

surface of the A-tubule. Second, each head domain consists of a series of globular structures that are positioned on the same plane. Third, a stalk domain originates as a conical region from the proximal head and ends with a small globular domain that contacts the B-tubule. Fourth, the tail region presents two distinct points of anchorage to the surface of the A-tubule. Finally, and most importantly, contrary to what has been observed in isolated dynein molecules adsorbed to flat surfaces, the stalk and the stem domains are not in the same plane as the head. This implies that the force generated during the power stroke would only act along the longitudinal axis of microtubules if a substantial rearrangement occurred in the stalk-head orientation (Lupetti *et al.*, 2005).

After this study, Nicastro *et al.* (2005, 2006) presented a comprehensive analysis of the molecular architecture of ODA and IDA complexes *in situ* in *Chlamydomonas* cilia and sea urchin spermatozoa performed by cryo electron tomography. The densities corresponding to heavy chain rings were identified and interdynein linkers that may provide wiring to coordinate motor action were shown between neighboring ODAs.

Further and even better resolved details have been obtained by cryoelectron microscopy and single particle average of outer arm dynein complexes in *Chlamydomonas* flagella (Ishikawa *et al.*, 2007). The comparative analysis of flagella from wild-type cells and ODA11 mutant (which lacks alpha-heavy chain) allowed to localize this domain in wild-type ODAs; the links with A-tubule were also resolved; two alternative models for the spatial distribution of gamma heavy chain have been proposed together with some hypotheses about the mechanism for the generation of power stroke (Ishikawa *et al.*, 2007).

We are currently performing double tilt axis tomography on metal replicas from cecidomid sperm axonemes followed by single particle analysis. The preliminary data we have obtained so far allowed to resolve additional and important structural details like the heavy chain rings in the proximal head of rigor ODAs (Fig. 4.8F).

Molecular data obtained on axonemal dynein from *Chlamydomonas* indicate that heavy chains are tightly associated with one or more light chains that may directly regulate motor function (King and Patel-King, 1995); moreover, the base of the outer arm dynein particle consists of an additional subcomplex formed by two closely-related intermediate chains that contain WD repeats. At least one of these two chains is involved in the ATP-independent binding of the arm to the A-tubule. Additionally, several light chains—some of which are shared between different dynein classes and are essential for dynein assembly—are found at the base of the outer arm dynein (Bowman *et al.*, 1999). Among these, Roadblock (robl) is a 97-amino acid polypeptide identified in *Drosophila* that is 70% similar to the 105-amino acid *Chlamydomonas* outer arm dynein-associated protein LC7. Both robl and LC7 have homology to several other genes from fruit fly, nematodes, and

mammals (Bowman *et al.*, 1999). Evidence has been obtained that Roadblock/LC7 family members may modulate specific dynein functions; LC7a, for example, has been shown in *Chlamydomonas* to stabilize both the outer arms and the inner arm I1, while both LC7a and LC7b are involved in multiple intradynein interactions within both dyneins (DiBella *et al.*, 2004).

As to the molecular evolution of insect dyneins, it is worth to mention that evidences have been obtained about the recent evolutionary origin in *D. melanogaster* of *Sdic*, a chimeric gene coding for an axonemal dynein intermediate chain originated by events involving the genes coding for an annexin and for an intermediate chain of cytoplasmic dynein, through a process of duplication and gene fusion accompanied by the recruitment of new promoter elements and the formation of a new exon encoding the amino-terminus of the polypeptide chain (Nurminsky *et al.*, 1998; Ranz *et al.*, 2003); *Sdic* has only recently been created—it is not found in closely related species that diverged from *D. melanogaster* within the last 1–3 million years—and is apparently still in the process of evolving. From what we have discussed so far it appears that, though maintaining a quite conserved molecular organization, dynein subunits seem to evolve at a different rate with respect to structural components like tubulins. Additional evidences from molecular genetic studies are needed to corroborate and further discuss the implications for this hypothesis.

3.3. Other axonemal components

Other proteins have been implicated in different aspects of *Drosophila* sperm tail motility or integrity, but their precise localization and function has not yet been established. A group of proteins specifically expressed in the male-germ line has been identified as components of the sperm tail that have been evolutionarily conserved among *Drosophila* sp. (Schäfer *et al.*, 1993). These proteins are encoded by the *Mst(3)CGP* gene family, and are characterized by the presence of multiple copies of the CysGlyPro (CGP) motif. It has been proposed that in *Drosophila*, these proteins are constituents of the so-called satellites (accessory tubules and the associated intertubular material), and that they might stiffen the axoneme or provide it with elastic properties, but their precise localization has not yet been determined.

Homologues of some mammalian sperm tail components have also been identified in *Drosophila* by amino acid sequence comparison; these include the highly insoluble Shippo1 protein, characterized by the occurrence of several Pro-Gly-Pro repeats (De Carvalho *et al.*, 2002), and the members of the Spef family of proteins (Chan *et al.*, 2005). While in mammals, these proteins appear to be mainly associated with the structural elements surrounding the axoneme, that is, the outer dense fibers and the fibrous sheath, their localization in fruit fly sperm tail is unknown.

4. Assembly of the Insect Axoneme

The mechanisms of ciliogenesis have been investigated both in sensory cilia and motile flagella from several model organisms obtaining evidences that cilia formation begins when the basal body, a centriole-related structure, serves as a template for the assembly of the axoneme. It has also been demonstrated that the formation of cilia and flagella can be achieved by two different mechanisms. In most motile and sensory cilia, the process starts with docking of basal body to the plasma membrane, the axoneme is then assembled beneath the plasma membrane, and the cilium grows progressively, projecting out from the cell body surrounded by its own plasma membrane. Characteristic of such mechanism of ciliogenesis is that axoneme and plasma membrane are assembled concurrently as a separate compartment from the cell body; for this reason, the process has been described as "compartmentalized ciliogenesis" (Avidor-Reiss *et al.*, 2004). Another mechanism, alternative to compartmentalization and indicated as cytosolic biogenesis, has been demonstrated in the sperm cells of *Drosophila* (Tokuyasu *et al.*, 1975) and in the flagellated stage of malaric plasmodia (Sinden *et al.*, 1976). In these cells, the complete axoneme is first assembled inside the cytosol and then it is either extruded or matures into a flagellum. During compartmentalized ciliogenesis, the assembly process—which starts from the basal body and takes place at the growing distal tip of the axoneme—is performed by the motility process called IFT. Flagellar precursors produced in the cell soma are bound to protein complexes (IFT particles) comprising at least 18 polypeptides, and are then transported to the flagellar tip by kinesin II. When ciliary assembly is complete, a dynamic trafficking is maintained at the tip of the axoneme: Flagellar components are continuously added and released, then bound to retrograde IFT complexes and carried back by cytoplasmic dynein 1b to the cell body for recycling (Cole, 2003; Qin *et al.*, 2004; Rosenbaum and Witman, 2002). The whole process occurs in the space between the flagellar membrane and outer doublets (Kozminski *et al.*, 1995), and is required for both the assembly and maintenance of the eukaryotic cilium.

In insects, compartmentalized cilia are found only in chemo- and mechanosensory neurons. These cells are characterized by the expression of a specific group of proteins that are thought to be involved in cilia mophogenesis and axoneme assembly, but are not found during spermatogenesis (Avidor-Reiss *et al.*, 2004). These include IFT proteins, Bardet–Biedl syndrome (BBS)-related polypeptides, C2 domain-containing proteins, small G proteins, and a novel family of proteins (OSEGs: outer segments) containing structural features—WD- and TPR-like repeats—which are involved in reversible protein–protein interactions (Avidor-Reiss *et al.*, 2004). OSEGs

are thought to function as ciliary transport proteins and to be required for the assembly of the outer segment in mechano- and chemosensory neurons, as is suggested by the phenotype of *oseg1* and *oseg2* mutants, which possess normal basal body and connecting cilium but show defects in the structure of the tubular body and the outer segment (Avidor-Reiss *et al.*, 2004). These results also suggest that the assembly of the outer segment is a process independent of the assembly of the connecting cilium.

The assembly of the axoneme is strictly dependent on IFT in sensory cilia, but not in spermatids. Mutants in the protein NOMPB, the *Drosophila* homologue of *Chlamydomonas* IFT88, fail to form sensory cilia in chordotonal organs and sensory sensilla; on the contrary, they produce motile sperm (Han *et al.*, 2003). A similar phenotype was observed in *Drosophila* mutants with defects in the kinesin-associated protein DKAP, which plays an essential role in kinesin II function; however, the flagellar assembly in spermatids is not affected (Sarpal *et al.*, 2003).

Spermatogenesis has been well characterized at the ultrastructural level in *Drosophila* (Fuller, 1993). Centrioles do not replicate during meiosis, so that each spermatid possesses a single centriole, but, starting from the primary spermatocyte, they undergo a series of ultrastructural modifications that will finally lead to a substantial increase in length (Gonzales *et al.*, 1998). How conserved this pathway is among insects is not known. Differently from what occurs in compartmentalized ciliogenesis, the centriole/basal body migrate and strictly associate with the nuclear membrane of the spermatid. A pericentrin-like protein (D-PLP)—which is not strictly required for mitosis—has been reported to be instead essential in maintaining the structural integrity of this large centriole, which often partially fragment during spermatogenesis in *d-plp* mutants (Martinez-Campos *et al.*, 2004). How a centriole is reconfigured as a ciliogenic basal body is essentially unknown. An early role in this process has been proposed for the protein product of the *unc* (*uncoordinated*) gene (Baker *et al.*, 2004). Mutations in this protein were initially identified as affecting the function of ciliated mechanosensory neurons (Eberl *et al.*, 2000; Kernan *et al.*, 1994), and later found to result also in male sterility (Baker *et al.*, 2004); thus, *unc* mutations identify a general feature common to both compartmentalized and cytosolic ciliogenesis in *Drosophila*. In these mutants, sensory cilia are either absent or truncated, with the distal basal body also disrupted, and the spermatids show nuclei detached from basal bodies and disorganized flagellar axonemes. In the elongating spermatid, the UNC protein has been localized at a position corresponding to the so-called centriole adjunct, a mass of electron dense material that surrounds the basal body; at this site, γ-tubulin is also present (Wilson *et al.*, 1997). Consistently with the presence of the UNC protein in the early spermatocyte, in *unc* mutants the centriolar structure is already defective in mature spermatocytes, prior to meiosis, though the cellular consequences of *unc* mutations are not seen until spermatid elongation. The specific function

of this protein is unknown, but it has been proposed to be an early marker for the conversion of the centriole to a basal body.

The close apposition of the basal body and the nuclear membrane has been shown to implicate also cytoplasmic dynein and the Grip75 and Grip128 proteins.

A male-sterile null mutant for the 14 kD light chain (tctex-1) subunit of cytoplasmic dynein is characterized by a disrupted linkage between the nucleus and the flagellar basal body (Li *et al.*, 2004b). In wild-type early spermatids, cytoplasmic dynein is concentrated to form a hemispherical nuclear cap that lies juxtaposed to the associated basal body, resembling the arrangement of a second nuclear membrane that assembles limited to the nuclear side adjacent to basal body; the dynein cap is missing in the mutant. It has been proposed that this nuclear-associated dynein cap interacts with the cytoplasmic microtubules surrounding the centriole prior to its anchoring to the nucleus, thus facilitating its attachment and the morphogenesis of the basal body. The lack of sperm motility occurring in this null mutant is probably due to a concomitant impairment of axonemal dyneins, given that this light chain in *Drosophila* is a component of both cytoplasmic and axonemal dyneins.

Grip75 and Grip128 proteins are components of the γ-tubulin ring complex, an essential template for microtubule nucleation *in vivo* (Gunawardane *et al.*, 2000). In wild-type spermatids, γ-tubulin is localized at the junction between the nucleus and elongating flagellum (Wilson *et al.*, 1997); in *Grip75* and *Grip128* mutants this association is lost—the axoneme is correctly assembled, but its basal body is no longer associated with the nucleus (Vogt *et al.*, 2006).

In *Trypanosoma*, the assembly of central pair and of outer doublet microtubules has been shown to pose different requirements for microtubule nucleation (McKean *et al.*, 2003). While formation of the outer doublets is a γ-tubulin independent process that requires only the preexisting template of the basal body triplets, the *de novo* nucleation of the central pair microtubules is absolutely dependent on γ-tubulin, possibly located in a central structure at the basal body transition zone (McKean *et al.*, 2003). In *Drosophila*, γ-tubulin has been localized in the electron dense material forming the so-called centriole adjunct (Wilson *et al.*, 1997), but no information is available on its possible occurrence in the core of flagellar basal bodies. Also, mutations that disrupt centrosomin function in *Drosophila* affect not only the meiotic spindle organization and cytokinesis, but also axoneme morphogenesis (Li *et al.*, 1998). While the outer doublets, the associated radial spokes, and the accessory tubules are not affected, the central pair is frequently missing. This suggests that centrosomin is associated with the basal body, and may affect its internal organization, possibly by recruiting other proteins, including γ-tubulin.

Once the central pair is assembled, specific factors appear to be required to maintain its structural integrity, as is suggested by the phenotype of *dfmr*

mutants. In fact—besides affecting neuronal synaptogenesis—mutations in the fruit fly homologue of the mammalian *fragile X mental retardation 1* gene (*dfmr*, also known as *dfxr*) cause highly specific defects in late spermatids, that is, the central pair is gradually lost, due to a lack of stability, while the outer doublets show no discernable defects (Zhang *et al.*, 2004). The cause of this specific loss is unknown. The protein product of the *dfmr* gene is a widely expressed RNA-binding translational regulator. A proteomic approach has identified a limited number of proteins whose expression is affected in the spermatogenesis of *dfmr* mutants; interestingly, among these are the chaperone proteins: Hsp60B, Hsp68, and the Hsp90-related protein TRAP1, which have been previously implicated in microtubule stability and in *Drosophila* spermatogenesis (Timakov and Zhang, 2001; Yue *et al.*, 1999).

Occasionally, the central pair of microtubules is reported missing also in the *Drosophila whirligig* mutants, along with the accessory tubules; the product of this gene is unknown, but it has been supposed to interact with microtubules (Green *et al.*, 1990). Mutant phenotypes are characterized by the frequent appearance of triplets instead of doublets in the axoneme, suggesting that the *wrl* gene product might be involved in the transition from the basal body (triplets) to the axoneme organization (doublets plus accessory tubules).

The whole body of data we have herein summarized indicates the central pair microtubules as the most frequently affected components of *Drosophila* sperm axoneme, and suggests that their assembly is a critical step for axoneme stability, which is regulated independently from the assembly of the outer doublets and implicates the coordination of several protein interactions and processes. It is interesting to underline how this evidence parallels what is suggested by the phylogenetic analysis of sperm ultrastructure in insects (see Section 2), which indicates the occurrence of a defect in the central apparatus as the first one of the series of successive events leading in several insect lineages to a disorganization of the axoneme and to unorthodox axoneme patterns.

A very limited information is available on the assembly mechanism of insect unconventional axoneme models, and this derives only from ultrastructural studies. A giant basal body has been described in *Sciara* (Phillips, 1967); it consists of several dozens of short singlet microtubules and forms from a 9-membered centriole during spermatogonial divisions; this giant centriolar structure is usually associated with a region of dense fibrous material. An apparently similar electron dense material has been found associated with the 18-doublets basal body of the cecidomid *Mycodiplosis* (Dallai and Mazzini, 1980) and in *A. ruebsaameni* (Mencarelli *et al.*, 2000b). In the latter species, no discernible basal body structure occurs in spermatids, but rather the hundreds of axoneme doublets are assembled from laminae of singlet microtubules which are embedded in a felt of dense fibrous material associated with membranous cisternae in the postnuclear region. How these

microtubular structures are formed during the previous stages of spermatogenesis—whether they originate from a modified centriole, as in *Sciara*, or whether they form *de novo*—is not known. Recent data have provided evidence of a dual mode of centriole/basal body assembly, either templated or *de novo*, in diverse cell types including spermatogenic cells (Beisson and Wright, 2003; Dawe *et al.*, 2007). In *Asphondylia*, the fibrous material associated with membranes in the spermatid postnuclear region appears to act as a potent nucleation center, which is morphologically similar to the noncentrosomal microtubule nucleating centers described in other cell systems (Mogensen and Tucker, 1987).

The morphogenesis of thrips (Thysanoptera) sperm flagella has been recently shown to be characterized by a striking basal body instability (Paccagnini *et al.*, 2007). In these species, the early phases of spermiogenesis lead to a spermatid endowed with three centrioles, each one of them forming a distinct 9+0 axoneme; later, the three axonemes disorganize and amalgamate, implicating a substantial rearrangement of either the basal body and/or the connecting region between the basal body and the axoneme.

In both protists and vertebrates, the sequential assembly of doublets and triplets in the basal body requires specific members of the tubulin superfamily, δ and ε tubulins (Marshall and Rosenbaum, 2003; McKean *et al.*, 2001). The *Chlamydomonas* mutants, *bld2–1* and *uni3*, characterized respectively by reduced levels of ε tubulin or by the absence of δ tubulin, form centrioles containing singlets or doublets instead of triplets. The exact role of these tubulin isoforms is not known, but it is probable that they play a stabilizing role in the centriole. Neither δ nor ε tubulins are expressed in *Drosophila*; the absence of these specific tubulin isoforms is, however, circumvented in the fruitfly spermatid, which assembles its flagellum from a triplet-containing basal body. We do not know whether the absence of δ and ε tubulins is a general feature of insects, and whether this absence may result, or not, in a somehow less stable basal body structure.

5. Axoneme Function

5.1. Flagellar motility

Current knowledge on the mechanisms of insect sperm motility is limited, being hampered by the small amount of material available and by the difficulties intrinsic to *in vivo* studies. Information on this aspect of insect sperm biology is, however, of relevance, not only for possible contributions to the control of insect reproduction, but also for the elucidation of the evolutionary processes underlying the wide diversity of insect sperm structure. It appears in fact reasonable that selective pressures acting on sperm function—either through a sperm competition process and/or through the

interaction with specific fertilization environments—have been important in the evolutionary divergence of insect sperm morphology.

How does the peculiar organization of the insect 9+9+2 sperm axoneme affect its function? How and to which extent is motility altered in the variety of aberrant axoneme models occurring among insect spermatozoa? And how could these modifications of the basic axoneme organization and function—some of which absolutely astounding—have been accepted during insect evolution and divergence? These are just some of the main still unsolved questions we have to face in the analysis of insect sperm axoneme function.

In the conventional insect axoneme model, the central 9+2 microtubule array is not only encircled by the nine accessory tubules and their associated intertubular material, but is also associated, along almost its entire length, with mitochondrial derivatives and accessory bodies. The available biochemical information on these structures is very limited, but all of them appear to be highly insoluble components (Baccetti *et al.*, 1977; Mencarelli *et al.*, 2000a, and our unpublished results). Thus, it appears conceivable that they might act to strengthen the insect axoneme against mechanical damage and/or as elastic elements that passively transmit and amplify the mechanical power generated by microtubule sliding. As a matter of fact, while the 9+2 flagellum of the insect *Ctenocephalus canis* forms an almost planar wave, the sperm 9+9+2 flagellar axonemes of the few species till now analyzed all exhibit a complex, 3D helicoidal beating pattern, consisting of a major wave superimposed to a minor wave (Dallai *et al.*, 2006). A double-wave pattern is maintained also in the backward motility exhibited by the spermatozoa of some species, which are able to reverse the direction of wave propagation (Baccetti *et al.*, 1989; Curtis and Benner, 1991). It has been proposed that the minor wave is actively generated by doublet sliding, while the major wave would result from mechanical restrictions imposed by the passive components of the flagellum. In this regard, it is interesting to note that the so-called 9+9+"1" sperm flagellum of the mosquito *Aedes notoscriptus*—which possesses accessory tubules but is devoid of the central regulatory complex—still maintains a double-wave motility (Swan, 1981).

Such a structural reinforcement for the function of insect sperm axoneme might have been required by the peculiarities of insect reproductive organs; in fact, spermatozoa commonly occur in a bent, rolled up configuration both in the male and female narrow reproductive ducts and storage organs. Other structural components have been implicated in the mechanical stabilization of the very long sperm tail of *Drosophila* sp. characterized by giant spermatozoa. First, the small family of Dhmst101 proteins, which is specifically expressed during the spermatogenesis of *D. hydei*, is likely to possess elastic properties due to the molecular structure and the occurrence of a high percentage of disulfide cross-links; these components have been localized to the outer sheath (Neesen *et al.*, 1999). Second, *Drosophila* sp.

with very long spermatozoa possess an external membrane specialization, known as "zipper line," which has been proposed to act as a reinforcing structure during sperm bending and torsion within seminal vesicles and female spermatheca (Dallai *et al.*, 2008).

In the study of insect sperm motility, it is important to bear in mind that the observations carried out *in vitro* may not reproduce the conditions in which sperm function *in vivo*, not only for possible differences in parameters like pH, osmolarity, and molecular composition of the medium, but also for the peculiar hydrodynamic conditions that result both from the interaction of the spermatozoa with the surrounding fluid and from the structure of male and female reproductive apparatus. This consideration could explain the absence of a progressive motility observed *in vitro* in the 9+9+2 sperm flagella of some species, though they are able to beat *in vitro* and are fully motile both within the male and female storage organs (*Drosophila* sp., Bressac *et al.*, 1991; *Megaselia scalaris*, Curtis and Benner, 1991). Sperm of the rove beatle *Drusilla canaliculata* are similarly characterized by weak motility and no progressive motion when observed *in vitro*, but exhibit vigorous beating when constrained within the narrow spermathecal duct, and it has been proposed that spatial restriction might contribute to enhanced motility within the female (Werner *et al.*, 2007).

Alternatively, the ability to perform a robust progressive movement could not be strictly required in some insect groups, for example, dipterans, in which female processes are likely to contribute to the sperm transfer within the spermatheca, and active sperm motility seems to play only a minor role (Arthur *et al.*, 1998; Linley and Simmons, 1981; Simmons *et al.*, 1999).

The latter condition might have been—at least in some instances—one of the factors that made acceptable some of the oddest axoneme organizations during the evolution of insect sperm. This is well exemplified by the two cecidomid species: *M. flavus* and *A. ruebsaameni*, whose sperm flagella constitute the only till now described axoneme models endowed with the ODAs only (Lupetti *et al.*, 1998; Mencarelli *et al.*, 2001). While mutant strains of the protist *Chlamydomonas* lacking the IDAs are immotile (Huang *et al.*, 1979), the sperm flagella of these insect species appear to be motile within both the male deferent ducts and the female spermatheca, where they are stored rolled up and are involved into a continuous train of bends. When released *in vitro* upon dissection, however, they become immediately immotile except from those that are maintained in a bent configuration by some mechanical constraints (e.g., by having some tracts attached to the glass, or still entrapped into the reproductive ducts), which keep undergoing a helicoidal beating (Lupetti *et al.*, 1998; Mencarelli *et al.*, 2001). In these species, the female reproductive apparatus is characterized by the presence of a very short spermathecal duct. This anatomical feature might have contributed to render these odd axonemes functional, since it would not require the sperm to move any long distance, but simply allows them to

contact the egg as soon as it descends through the oviduct and faces the opening of the spermatheca. Thus, the evolution of this peculiar model of axoneme organization and function is likely to have occurred concomitantly to the development of a peculiar configuration of the female reproductive apparatus.

Interestingly, the analysis of these aberrant cecidomid axonemes have provided also some clues on the mechanisms that regulate dynein activity. In the 9+2 axoneme, dynein activity is controlled by the transmission of chemical signals from the central pair/radial spoke system through the dynein regulatory complex associated to the IDAs, so that flagella from mutant organisms lacking any of these components are immotile (Sakato and King, 2004). However, other observations have suggested the existence of a second mechanism of dynein activation, based on external mechanical stimuli (Hayashibe *et al.*, 1997; Morita and Shingyoji, 2004). Paralyzed mutant flagella lacking most of the IDAs are actually able to display temporary beating when stimulated by an external force, while mutants lacking the ODAs are not able to propagate preformed bends and remain immotile. It has been therefore suggested that the axoneme contains two motor systems, subjected to different mechanisms of activation, one mainly based on chemical signals—the IDAs—and the other based on the mechanical state of the axoneme—mainly based on the ODAs. In *Asphondylia* and *Monarthropalpus*, only the latter motor system seems to be present, since flagellar motility depends on the presence of an external force that imposes a bend onto the axoneme and activates ODAs bringing some adjacent doublets closer one to each other. A similar mechanism of dynein activation seems to be required also by the highly aberrant axoneme of thrips spermatozoa (see Section 4) (Paccagnini *et al.*, 2007).

Interestingly, the redundant doublet number and the loss of both IDAs and the central apparatus that characterize *Asphondylia* and *Monarthropalpus* sperm flagella are concomitant with a progressive simplification of the axoneme molecular composition, since both the dynein heavy chain complement (Lupetti *et al.*, 1998; Mencarelli *et al.*, 2001) and the molecular heterogeneity and differential localization of posttranslationally modified tubulin isoforms (Mencarelli *et al.*, 2004, 2005) appear to be reduced. An earlier loss of IDAs and/or of the central apparatus, occurred in insect taxa basal to cecidomids (Dallai *et al.*, 2006), and would have been followed by a successive simplification of the motility process as well as of the underlying molecular components.

5.2. Role of the axoneme in sensory cilia

Sensory function depends on the integrity of the modified cilium expressed in sensory neurons, as has been indicated by the analysis of a series of *Drosophila* mutants exhibiting alterations in hearing, touch insensitivity or

severe proprioception defects, and uncoordination. Several genes and their related products have been implicated in different steps of the process of sensory perception (Caldwell and Eberl, 2002; Kernan, 2007), including both membrane proteins—ion channels and proteins required for the attachment of the ciliary tip to the extracellular dentritic cap—and proteins involved in the correct assembly and function of the axoneme. Among the latters are the above mentioned proteins UNC (Baker *et al.*, 2004) and D-PLP (Martinez-Campos *et al.*, 2004). Defective sensory function has been demonstrated also in mutants with an affected IFT, either anterograde, like in the *nompB*, *Klp64D*, and *KAP* mutants (Han *et al.*, 2003; Sarpal *et al.*, 2003), or retrograde, like in *oseg1* and *btv* mutants (Eberl *et al.*, 2000). Mutants defective in components of the anterograde transport lack sensory cilia and mechanosensory responses; those defective in the Oseg1 protein (the homologue of the IFT122 subunit of the *Chlamydomonas* retrograde complex A) have reduced outer segments (Avidor-Reiss *et al.*, 2004). IFT is presumably required not only for axoneme assembly, but also to convey membrane proteins and receptors to their correct localization in the ciliary membrane, as has been demonstrated in other model systems (Qin *et al.*, 2005).

While these data clearly indicate that the integrity of the ciliary apparatus is required for sensory function, they do not provide any information about the mechanisms that integrate the activity of membrane proteins with matrix and cytoskeletal elements of the cilium to transduce and transmit stimuli. Is the axoneme required only as a framework for the transport machinery to correctly build the transducing components of the sensory cilium or it may play a more active role in stimulus transmission?

The only piece of information that is available concerns chordotonal cilia. It has, in fact, been demonstrated that in the femoral chordotonal organ of the grasshopper *Melanoplus bivittatus*, a pronounced bend at the base of the sensory cilia occurs after stimulation, thus suggesting that these sensory cilia are able of active movement (Moran *et al.*, 1977). This observation is coherent with the presence of dynein arms in axonemes of the chordotonal organs. Given that the chordotonal cilium is immobilized at both its base and tip, an active stroke would induce an axoneme coiling and thus a shortening of the cilium; the resulting increase in tension would activate other components of the sensory apparatus, thus transmitting and amplifying the mechanical stimulus. A speculative model for the chordotonal organ function has been proposed, which implicates such an active role for the ciliary axoneme (Todi *et al.*, 2004). It has also to be noted here that, while mutations in genes like *unc* and *nompB* affect both external and chordotonal organs, other mutations (*btv* and *tilB*) specifically affect only chordotonal organs and, moreover, are characterized by male sterility due to defects in the sperm axoneme (Eberl *et al.*, 2000); interestingly, *tilB* sperm axonemes are devoid of both dynein arms and often split.

6. Perspectives

While *Drosophila* has been proved to be a valuable model for the study of several aspects of spermatogenesis and for the analysis of the structure/function relationship between the axoneme and its components, the peculiar unconventional sperm axonemes of some insects have been recently proposed as models useful to approach other aspects of axoneme biology. Interestingly, some of the ultrastructural features found in mutant axonemes of transgenic flies are reminiscent of the unconventional axoneme patterns exhibited by some insect species, which can thus be considered as a sort of "natural" mutants. Studies on these unconventional models might be helpful to clarify not only aspects of insect evolution and reproduction, but also more general questions concerning axoneme structure and function.

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REFERENCES

Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A., Galle, R. F., George, R. A., Lewis, S. E., *et al.* (2000). The genome sequence of *Drosophila melanogaster*. *Science* **287,** 2185–2195.

Arthur, B. I., Jr, Hauschteck-Jungen, E., Nöthiger, R., and Ward, P. I. (1998). A female nervous system is necessary for normal sperm storage in *Drosophila melanogaster*: A masculinized nervous system is as good as none. *Proc. R. Soc. Lond. B* **265,** 1749–1753.

Asai, D. J., and Wilkes, D. E. (2004). The dynein heavy chain family. *J. Eukaryot. Microbiol.* **51,** 23–29.

Avidor-Reiss, T., Maer, A. M., Koundakjian, E., Polyanovsky, A., Keil, T., Subramanian, S., and Zuker, C. S. (2004). Decoding cilia function: Defining specialized genes required for compartmentalized cilia biogenesis. *Cell* **117,** 527–539.

Baccetti, B., and Dallai, R. (1978). The spermatozoon of Arthropoda. XXX. The multiflagellate spermatozoon in the termite *Mastotermes darwiniensis*. *J. Cell Biol.* **76,** 569–576.

Baccetti, B., Dallai, R., Pallini, V., Rosati, F., and Afzelius, B. A. (1977). Protein of insect sperm mitochondrial crystals. Crystallomitin. *J. Cell Biol.* **73,** 594–600.

Baccetti, B., Gibbons, B. H., and Gibbons, B. R. (1989). Bidirectional swimming in spermatozoa of tephritid flies. *J. Submicrosc. Cytol. Pathol.* **21,** 619–625.

Baker, J. D., Adhikarakunnathu, S., and Kernan, M. J. (2004). Mechanosensory-defective, male-sterile *unc* mutants identify a novel basal body protein required for cliogenesis in *Drosophila*. *Development* **131,** 3411–3422.

Bardin, A. J., Le Borgne, R., and Schweisghuth, F. (2004). Asymmetric localization and function of cell-fate determinants: A fly's view. *Curr. Opin. Neurobiol.* **14,** 6–14.

Beisson, J., and Wright, M. (2003). Basal body/centriole assembly and continuity. *Curr. Opin. Cell Biol.* **15,** 96–104.

Bisgrove, B. W., and Yost, H. J. (2006). The roles of cilia in developmental disorders and disease. *Development* **133,** 4131–4143.

Bitsch, J. (1981). Ultrastructural modifications of the glandular epithelium of the *receptaculum seminis* in *Thermobia domestica* (Insecta: Thysanura) during the moulting period. *Cell Tissue Res.* **220,** 99–113.

Bitsch, J., and Palévody, C. (1976). Ultrastructure des glandes vésiculaires des Machilidae (Insecta, Thysanura) pendant l'intermue; présence de cellules ciliaires associées aux cellules glandulaires. *Arch. Zool. Exp. Gén.* **117,** 141–168.

Bobinnec, Y., Khodjakov, A., Mir, L. M., Rieder, C. L., Eddé, B., and Bornens, M. (1998). Centriole disassembly *in vivo* and its effect on centrosome structure and function in vertebrate cells. *J. Cell Biol.* **143,** 1575–1589.

Bobinnec, Y., Marcaillou, C., and Debec, A. (1999). Microtubule polyglutamylation in *Drosophila melanogaster* brain and testis. *Eur. J. Cell Biol.* **78,** 671–674.

Bonnet, C., Boucher, D., Lazereg, S., Pedrotti, B., Islam, K., Denoulet, P., and Larcher, J. C. (2001). Differential binding regulation of microtubule-associated proteins MAP1A, MAP1B, and MAP2 by tubulin polyglutamylation. *J. Biol. Chem.* **276,** 12839–12848.

Boo, K. S., and Richards, A. G. (1975). Fine structure of scolopidia in Johnston's organ of female *Aedes aegypti* compared with that of the male. *J. Insect Physiol.* **21,** 1129–1139.

Boucher, D., Larcher, J.-C., Gros, F., and Denoulet, P. (1994). Polyglutamylation of tubulin as a progressive regulator of *in vitro* interactions between the microtubule-associated protein tau and tubulin. *Biochemistry* **33,** 12471–12477.

Bowman, A. B., Patel-King, R. S., Benashski, S. E., McCaffery, J. M., Goldstein, L. S., and King, S. M. (1999). Drosophila roadblock and Chlamydomonas LC7: A conserved family of dynein-associated proteins involved in axonal transport, flagellar motility, and mitosis. *J. Cell Biol.* **146,** 165–180.

Bré, M.-H., Redeker, V., Quibell, M., Darmanaden-Delorme, J., Bressac, C., Johnson, T., Adoutte, A., and Levilliers, N. (1996). Axonemal tubulin polyglycylation probed with two monoclonal antibodies: Widespread evolutionary distribution, appearance during spermatozoan maturation and possible function in motility. *J. Cell Sci.* **109,** 727–738.

Bré, M.-H., Redeker, V., Vinh, J., Rossier, J., and Levilliers, N. (1998). Tubulin polyglycylation: Differential posttranslational modification of dynamic cytoplasmic and stable axonemal microtubules in *Paramecium*. *Mol. Biol. Cell* **9,** 2655–2665.

Bressac, C., Joly, D., Davaux, J., Serres, C., Feneux, D., and Lachaise, D. (1991). Comparative kinetics of short and long sperm in sperm dimorphic *Drosophila* species. *Cell Motil. Cytoskeleton* **19,** 269–274.

Bressac, C., Bré, M.-H., Darmanaden-Delorme, J., Laurent, M., Levilliers, N., and Fleury, A. (1995). A massive new posttranslationaly modification occurs on axonemal tubulin at the final step of spermatogenesis in Drosophila. *Eur. J. Cell Biol.* **67,** 346–355.

Broadhead, R., Dawe, H. R., Farr, H., Griffiths, S., Hart, S. R., Portman, N., Shaw, M. K., Ginger, M. L., Gaskell, S. J., McKean, P. G., and Gull, K. (2006). Flagellar motility is required for the viability of the bloodstream trypanosome. *Nature* **440,** 224–227.

Burgess, S. A., Dover, S. D., and Woolley, D. M. (1991). Architecture of the outer arm dynein ATPase in an avian sperm flagellum, with further evidence for the B-link. *J. Cell Sci.* **98,** 17–26.

Burgess, S. A., Walker, M. L., Sakakibara, H., Knight, P. J., and Oiwa, K. (2003). Dynein structure and power stroke. *Nature* **421,** 715–718.

Burgess, S. A., Walker, M. L., Sakakibara, H., Oiwa, K., and Knight, P. J. (2004). The structure of dynein-C by negative stain electron microscopy. *J. Struct. Biol.* **146,** 205–216.

Caldwell, J. C., and Eberl, D. F. (2002). Towards a molecular understanding of *Drosophila* hearing. *J. Neurobiol.* **53,** 172–189.

Cameron, M. J. (1965). Some details of ultrastructure in the development of flagellar fibers of the *Tenebrio* sperm. *Can. J. Zool.* **43,** 1005–1010.

Cavalier-Smith, T. (1982). The evolutionary origin and phylogeny of eukaryote flagella. *Symp. Soc. Exp. Biol.* **35,** 465–493.

Chan, S. W., Fowler, K. J., Choo, K. H. A., and Kalitsis, P. (2005). Spef1, a conserved testis protein found in mouse sperm flagella. *Gene* **353,** 189–199.

Cole, D. G. (2003). The intraflagellar transport machinery of *Chlamydomonas reinhardtii*. *Traffic* **4,** 435–442.

Corbière-Tichané, G. (1971). Ultrastructure de l'équipement sensoriel de la mandibule chez la larve de *Speophyes lucidulus* Delar. (Coléoptère Cavernicole de la souis-famille des Bathysciinae). *Z. Zellforsch* **112,** 129–138.

Curtis, S. K., and Benner, D. B. (1991). Movement of spermatozoa of *Megaselia scalaris* (Diptera: Brachycera: Cyclorrhapha: Phoridae) in artificial and natural fluids. *J. Morphol.* **210,** 85–99.

Dallai, R. (1988). The spermatozoon of Asphondyliidi (Diptera, Cecidomyiidae). *J. Ultrastruct. Mol. Struct. Res.* **101,** 98–107.

Dallai, R., and Afzelius, B. A. (1993). Development of the accessory tubules of insect sperm flagella. *J. Submicrosc. Cytol. Pathol.* **25,** 499–504.

Dallai, R., and Burroni, D. (1981). La struttura delle ghiandole tegumentali delle zampe anteriori dei Proturi (Insecta). *Redia* **64,** 165–172.

Dallai, R., and Mazzini, M. (1980). Characteristics of the motile spermatozoa in 5 species of gall-midges (Diptera: Cecidomyiidae). *Int. J. Insect Morphol. Embryol.* **9,** 383–393.

Dallai, R., Xuè, L., and Yin, W.-Y. (1992). Flagellate spermatozoa of Protura (Insecta, Apterygota) are motile. *Int. J. Insect Morphol. Embryol.* **21,** 137–148.

Dallai, R., Bellon, P. L., Lanzavecchia, S., and Afzelius, B. A. (1993). The dipteran sperm tail: Ultrastructural characteristics and phylogenetic considerations. *Zoologica Scripta* **22,** 193–202.

Dallai, R., Lupetti, P., and Afzelius, B. A. (1995). Sperm structure of trichoptera. III. Hydropsychidae, polycetropodidae and philopotamidae (Annulipalpia). *Int. J. Insect Morphol. Embryol.* **24,** 171–183.

Dallai, R., Lupetti, P., Afzelius, B. A., and Mamaev, B. M. (1996). The sperm structure of the gall-midge *Anaretella* and *Lestremia* (Insecta, Diptera, Cecidomyiidae). *Tissue Cell* **28,** 331–338.

Dallai, R., Lupetti, P., and Mencarelli, C. (2006). Unusual axonemes of hexapod spermatozoa. *Int. Rev. Cytol.* **254,** 45–99.

Dallai, R., Lombardo, B. M., Mercati, D., Vanin, S., and Lupetti, P. (2007a). Sperm structure of Limoniidae and their phylogenetic relationship with Tipulidae (*Diptera, Nematocera*). *Arthropod. Struct. Dev.* **37,** 81–92.

Dallai, R., Mercati, D., and Giusti, F. (2008). Structural organization of the "zipper line" in *Drosophila* species with giant spermatozoa. *J. Struct. Biol.* **161,** 43–54.

Daub, A. M., and Hauser, M. (1988). Primary cilia in locust spermatocytes, formation, fate and possible function. *Z. Naturforsch.* **43,** 455–462.

Davis, E. E., Brueckner, M., and Katsanis, N. (2006). The emerging complexity of the vertebrate cilium: New functional roles for an ancient organelle. *Dev. Cell* **11,** 9–19.

Dawe, H. R., Farr, H., and Gull, K. (2007). Centriole/basal body morphogenesis and migration during ciliogenesis in animal cells. *J. Cell Sci.* **120,** 7–15.

De Carvalho, C. E., Tanaka, H., Iguchi, N., Ventelä, S., Nojima, H., and Mishimune, Y. (2002). Molecular cloning and characterization of a complementary DNA encoding sperm tail protein SHIPPO 1. *Biol. Reprod.* **66,** 785–795.

Dettman, R. W., Turner, F. R., Hoyle, H. D., and Raff, E. C. (2001). Embryonic expression of the divergent Drosophila beta3-tubulin isoform I required for larval behavior. *Genetics* **158,** 253–263.

DiBella, L. M., Sakato, M., Patel-King, R. S., Pazour, G. J., and King, S. M. (2004). The LC7 light chains of *Chlamydomonas* flagellar dyneins interact with components required for both motor assembly and regulation. *Mol. Biol. Cell* **15,** 4633–4646.

Dorus, S., Busby, S. A., Gerike, U., Shabanowitz, J., Hunt, D. F., and Karr, T. L. (2006). Genomic and functional evolution of the *Drosophila melanogaster* sperm proteome. *Nat. Genet.* **38,** 1440–1445.

Duan, J., and Gorovsky, M. A. (2002). Both carboxyterminal tails of α- and β-tubulin are essential, but either one will suffice. *Curr. Biol.* **12,** 313–316.

Eberl, D. F., Hardy, R. W., and Kernan, M. J. (2000). Genetically similar transduction mechanisms for touch and hearing in *Drosophila*. *J. Neurosci.* **20,** 5981–5988.

Fackenthal, J. D., Turner, F. R., and Raff, E. C. (1993). Tissue-specific microtubule functions in *Drosophila* spermatogenesis require the β-tubulin isotype-specific carboxy-terminus. *Dev. Biol.* **158,** 213–227.

Fackenthal, J. D., Hutchens, J. A., Turner, F. R., and Raff, E. C. (1995). Structural analysis of mutations in the Drosophila β2-tubulin isoform reveals regions in the β-tubulin molecule required for general and for tissue specific microtubule functions. *Genetics* **139,** 267–286.

Fouquet, J.-P., Prigent, Y., and Kann, M. L. (1996). Comparative immunogold analysis of tubulin isoforms in the mouse sperm flagellum: Unique distribution of glutamylated tubulin. *Mol. Reprod. Dev.* **43,** 358–365.

Friedländer, M., and Morse, J. C. (1982). The aberrant spermatozoa of Hydropsychidae caddis flies (Trichoptera). *J. Ultrastruct. Res.* **78,** 84–94.

Friedländer, M., and Wahrman, J. (1970). The spindle as a basal body distributor. *J. Cell Sci.* **7,** 65–89.

Fuller, M. T. (1993). Spermatogenesis. *In* "The Development of Drosophila melanogaster" (M. Bate and A. Martinez Arias, Eds.), Vol. 2, pp. 71–147. Cold Spring Harbor Laboratory Press, New York.

Fuller, M. T., Caulthon, J. H., Hutchens, J. A., Kaufman, T. C., and Raff, E. C. (1988). Mutations that encode partially functional β2 tubulin subunits have different effects on structurally different microtubule arrays. *J. Cell Biol.* **107,** 141–152.

Gagnon, C., White, D., Cosson, J., Huitorel, P., Eddé, B., Desbruyères, E., Paturle-Lafanechère, L., Multigner, L., Job, D., and Cibert, C. (1996). The polyglutamylated lateral chain of alpha tubulin plays a key role in flagellar motility. *J. Cell Sci.* **109,** 1545–1553.

Godula, J. (1985). Quadriflagellar primary spermatocytes in the cotton leafworm, *Spodoptera littoralis* (Boisd.) (Noctuidae, Lepidoptera). *Cell Tissue Res.* **242,** 681–683.

Goldstein, L. S. B., and Gunawardena, S. (2000). Flying through the Drosophila cytoskeletal genome. *J. Cell Biol.* **150,** F63–F68.

Gonzales, C., Tavosanis, G., and Mollinari, C. (1998). Centrosomes and microtubule organization during *Drosophila* development. *J. Cell Sci.* **111,** 2697–2706.

Goodenough, U. W., and Heuser, J. E. (1982). Structure of the outer dynein arm. *J. Cell Biol.* **95,** 798–815.

Goodenough, U. W., and Heuser, J. E. (1984). Structural comparison of purified dynein proteins with in situ dynein arms. *J. Mol. Biol.* **180,** 1083–1118.

Green, L. L., Wolf, N., McDonald, K. L., and Fuller, M. T. (1990). Two types of genetic interaction implicate the whirligig gene of *Drosophila melanogaster* in microtubule organization in the flagellar axoneme. *Genetics* **126,** 961–073.

Gunawardane, R. N., Martin, O. C., Cao, K., Zhang, L., Dej, K., Iawamatsu, A., and Zheng, Y. (2000). Characterization and reconstitution of Drosophila gamma-tubulin ring complex subunits. *J. Cell Biol.* **151,** 1513–1524.

Hallberg, E., and Hansson, B. S. (1999). Arthropod sensilla: Morphology and phylogenetic considerations. *Microsc. Res. Tech.* **47,** 428–439.

Han, Y.-G., Kwok, B. H., and Kernan, M. J. (2003). Intraflagellar transport is required in *Drosophila* to differentiate sensory cilia but not sperm. *Curr. Biol.* **13,** 1679–1686.

Hayashibe, K., Shingyoji, C., and Kamiya, R. (1997). Induction of temporary beating in paralyzed flagella of *Chlamydomonas* mutants by application of external force. *Cell Motil. Cytoskeleton* **37,** 232–239.

Holm, P. B., and Rasmussen, S. W. (1980). Chromosome pairing, recombination nodules and chiasma formation in diploid *Bombyx* males. *Carlsberg Res. Commun.* **45,** 483–548.

Hook, P., and Vallee, R. B. (2006). The dynein family at a glance. *J. Cell Sci.* **119,** 4369–4371.

Hoyle, H. D., and Raff, E. C. (1990). Two *Drosophila* beta tubulin isoforms are not functionally equivalent. *J. Cell Biol.* **11,** 1009–1026.

Hoyle, H. D., Hutchens, J. A., Turner, F. R., and Raff, E. C. (1995). Regulation of β-tubulin β_{III} function and expression in *Drosophila* spermatogenesis. *Dev. Genet.* **16,** 148–170.

Hoyle, H. D., Turner, F. R., Brunick, L., and Raff, E. C. (2001). Tubulin sorting during dimerization *in vivo*. *Mol. Biol. Cell* **12,** 2185–2194.

Huang, B., Piperno, G., and Luck, D. J. (1979). Paralyzed flagella mutants of *Chlamydomonas reinhardtii*. Defective for axonemal doublet microtubule arms. *J. Biol. Chem.* **254,** 3091–3099.

Huitorel, P., White, D., Fouquet, J.-P., Kann, M.-L., Cosson, J., and Gagnon, C. (2002). Differential distribution of glutamylated tubulin isoforms along the sea urchin sperm axoneme. *Mol. Reprod. Dev.* **62,** 139–148.

Hutchens, J. A., Hoyle, H. D., Turner, F., and Raff, E. C. (1997). Structurally similar Drosophila α-tubulins are functionally distinct *in vivo*. *Mol. Biol. Cell* **8,** 481–500.

Iftode, F., Clérot, J.-C., Levilliers, N., and Bré, M.-H. (2000). Tubulin polyglycylation: A morphogenetic marker in ciliates. *Biol. Cell* **92,** 615–628.

Inglis, P. N., Boroevich, K. A., and Leroux, M. R. (2006). Piecing together a ciliome. *Trends Genet.* **22,** 491–500.

Iomini, C., Bré, M.-H., Levilliers, N., and Justine, J.-L. (1998). Tubulin polyglycylation in Platyhelminthes: Diversity among stable microtubule networks and very late occurrence during spermiogenesis. *Cell Motil. Cytoskeleton* **39,** 318–330.

Ishikawa, T., Sakakibara, H., and Oiwa, K. (2007). The architecture of outer dynein arms in situ. *J. Mol. Biol.* **368,** 1249–1258.

Jamieson, B. G. M., Dallai, R., and Afzelius, B. A. (1999). "Insects: Their Spermatozoa and Phylogeny." Scientific Publishers, Enfield, New Hampshire, USA.

Johnson, K. A. (1998). The axonemal microtubules of the *Chlamydomonas* flagellum differ in tubulin isoform content. *J. Cell Sci.* **111,** 313–320.

Kamiya, R. (2002). Functional diversity of axonemal dyneins as studied in Chlamydomonas mutants. *Int. Rev. Cytol.* **219,** 115–155.

Kann, M.-L., Prigent, Y., Levilliers, N., Bré, M.-H., and Fouquet, J.-P. (1998). Expression of glycylated tubulin during the differentiation of spermatozoa in mammals. *Cell Motil. Cytoskeleton* **41,** 341–352.

Kann, M.-L., Soues, S., Levilliers, N., and Fouquet, J.-P. (2003). Glutamylated tubulin: Diversity of expression and distribution of isoforms. *Cell Motil. Cytoskeleton* **55,** 14–25.

Kawasaki, H., Sugaya, K., Quan, G.-X., Nohata, J., and Mita, K. (2003). Analysis of α- and β-tubulin genes of *Bombyx mori* using an EST database. *Insect Biochem. Mol. Biol.* **33,** 131–137.

Keil, T. A. (1992). Fine structure of a developing insect olfactory organ: Morphogenesis of the silkmoth antenna. *Microsc. Res. Tech.* **22,** 351–371.
Keil, T. A. (1997). Functional morphology of insect mechanoreceptors. *Microsc. Res. Tech.* **39,** 506–531.
Keil, T. A., and Steinbrecht, R. A. (1984). Mechanosensory and olfactory sensilla of insects. *In* "insect ultrastructure," (R. C. King, and H. Akai, Eds.), vol. 2. Plenum Press, New York, and London.
Kernan, M. J. (2007). Mechanotransduction and auditory transduction in *Drosophila*. *Eur. J. Physiol.* **454,** 703–720.
Kernan, M. J., Cowan, D., and Zuker, C. (1994). Genetic dissection of mechanosensory transduction: Mechanoreception-defective mutations of Drosophila. *Neuron* **12,** 1195–1206.
Kierszenbaum, A. L. (2002). Sperm axoneme: A tale of tubulin posttranslational diversity. *Mol. Reprod. Dev.* **62,** 1–3.
King, S. M., and Patel-King, R. S. (1995). Identification of a Ca^{++}-binding light chain within Chlamydomonas outer arm dynein. *J. Cell Sci.* **108,** 3757–3764.
Kozminski, K. G., Beech, P. L., and Rosenbaum, J. L. (1995). The *Chlamydomonas* kinesin-like protein FLA10 is involved in motility associated with the flagellar membrane. *J. Cell Biol.* **131,** 1517–1527.
Lai-Fook, J. (1982). Structural comparison between eupyrene and apyrene spermiogenesis in *Calpodes ethlius* (Hesperiidae, Lepidoptera). *Can. J. Zool.* **60,** 1216–1230.
Larcher, J.-C., Boucher, D., Lazereg, S., Gros, F., and Denoulet, P. (1996). Interaction of kinesin motor domains with α- and β-tubulin subunits at a tau-independent binding site. Regulation by polyglutamylation. *J. Biol. Chem.* **271,** 22117–22124.
Lechtreck, K.-F., and Geimer, S. (2000). Distribution of polyglutamylated tubulin in the flagellar apparatus of green flagellates. *Cell Motil. Cytoskeleton* **47,** 219–235.
Leclercq-Smekens, M. (1978). Reproduction d'*Euproctis chrysorrhea* L. (Lépidoptère Lymantriidae). Étude de l'organogenèse des testicules et de la spermatogenèse. *Bull. Classe Sci. Acad. Royale Belgique* **64,** 304–317.
Li, J. Y., and Wu, C. F. (2005). New symbiotic hypothesis on the origin of eukaryotic flagella. *Naturwissenschaften* **92,** 305–309.
Li, K., Xu, E. Y., Cecil, J. K., Turner, F. R., Megraw, T. L., and Kaufman, T. C. (1998). Drosophila centrosomin protein is required for male meiosis and assembly of the flagellar axoneme. *J. Cell Biol.* **141,** 455–467.
Li, J. B., Gerdes, J. M., Haycraft, C. J., Fan, Y., Teslovich, T. M., May-Simera, H., Li, H., Blacque, O. E., Li, L., Leitch, C. C., Lewis, R. A., Green, J. S., *et al.* (2004a). Comparative genomics identifies a flagellar and basal body proteome that includes the BBS5 human disease gene. *Cell* **117,** 541–552.
Li, M., Serr, M., Newman, E. A., and Hays, T. S. (2004b). The Drosophila tctex-1 light chain is dispensable for essential cytoplasmic dynein functions but is required during spermatid differentiation. *Mol. Biol. Cell* **15,** 3005–3014.
Linley, J. R., and Simmons, K. R. (1981). Sperm motility and spermatechal filling in lower diptera. *Int. J. Inver. Rep.* **4,** 137–146.
Ludueña, R. F. (1998). Multiple forms of tubulin: Different gene products and covalent modifications. *Int. Rev Cytol.* **178,** 207–275.
Lupetti, P., Mencarelli, C., Rosetto, M., Heuser, J. E., and Dallai, R. (1998). Structural and molecular characterization of dynein in a gall-midge insect having motile sperm with only the outer arm. *Cell Motil. Cytoskeleton* **39,** 303–317.
Lupetti, P., Lanzavecchia, S., Mercati, D., Cantele, F., Dallai, R., and Mencarelli, C. (2005). Three-dimensional reconstruction of axonemal outer dynein arms in situ by electron tomography. *Cell Motil. Cytoskeleton* **62,** 69–83.

Mancini, K., Báo, S. N., Fernandes, A. P., and Dolder, H. (2005). Immunocytochemical localization of tubulins in spermatids and spermatozoa of *Euptoieta hegesia* (Lepidoptera: Nymphalidae). *Tissue Cell* **37,** 81–89.

Marshall, W. F., and Nonaka, S. (2006). Cilia: Tuning in to the cell's antenna. *Curr. Biol.* **16,** R604–R606.

Marshall, W. F., and Rosenbaum, J. L. (2003). Tubulin superfamily: Giving birth to triplets. *Curr. Biol.* **13,** R55–R56.

Martinez-Campos, M., Basto, R., Baker, J., Kernan, M., and Raff, J. (2004). The *Drosophila* pericentrin-like protein is essential for cilia/flagella function, but appears to be dispensable for mitosis. *J. Cell Biol.* **165,** 673–683.

McKean, P. G., Vaughan, S., and Gull, K. (2001). The extended tubulin superfamily. *J. Cell Sci.* **114,** 2723–2733.

McKean, P. G., Baines, A., Vaughan, S., and Gull, K. (2003). Gamma-tubulin functions in the nucleation of a discrete subset of microtubules in the eukaryotic flagellum. *Curr. Biol.* **13,** 598–602.

Mencarelli, C., Bré, M.-H., Levilliers, N., and Dallai, R. (2000a). Accessory tubules and axonemal microtubules of *Apis mellifera* sperm flagellum differ in their tubulin isoform content. *Cell Motil. Cytoskeleton* **47,** 1–12.

Mencarelli, C., Lupetti, P., Rosetto, M., and Dallai, R. (2000b). Morphogenesis of the giant sperm axoneme in *Asphondylia ruebsaameni* Kertesz (Diptera, Cecidomyiidae). *Tissue Cell* **32,** 188–197.

Mencarelli, C., Lupetti, P., Rosetto, M., Mercati, D., Heuser, J., and Dallai, R. (2001). Molecular structure of dynein and motility of a giant sperm axoneme provided with only the outer dynein arm. *Cell Motil. Cytoskeleton* **50,** 129–146.

Mencarelli, C., Caroti, D., Bré, M.-H., Levilliers, N., Mercati, D., Robbins, L. G., and Dallai, R. (2004). Glutamylated and glycylated tubulin isoforms in the aberrant sperm axoneme of the gall-midge fly, *Asphondylia ruebsaameni*. *Cell Motil. Cytoskeleton* **58,** 160–174.

Mencarelli, C., Caroti, D., Bré, M.-H., Levilliers, N., and Dallai, R. (2005). Tubulin glycylation and glutamylation deficiencies in unconventional insect axonemes. *Cell Motil. Cytoskeleton* **61,** 226–236.

Million, K., Larcher, J., Laoukili, J., Bourguignon, D., Marano, F., and Tournier, F. (1999). Polyglutamylation and polyglycylation of alpha- and beta-tubulins during in vitro ciliated cell differentiation of human respiratory epithelial cells. *J. Cell Sci.* **112,** 4357–4366.

Mitchell, D. R. (1994). Cell and molecular biology of flagellar dyneins. *Int. Rev. Cytol.* **155,** 141–180.

Mitchell, D. R. (2004). Speculations on the evolution of 9+2 organelles and the role of central pair microtubules. *Cell Motil. Cytoskeleton* **96,** 691–696.

Mogensen, M. M., and Tucker, J. B. (1987). Evidence for microtubule nucleation at plasma membrane-associated sites in *Drosophila*. *J. Cell Sci.* **88,** 95–107.

Moran, D., Varela, F., and Rowley, J. (1977). Evidence for active role of cilia in sensory transduction. *Proc. Natl. Acad. Sci. USA* **74,** 793–797.

Morita, Y., and Shingyoji, C. (2004). Effects of imposed bending on microtubule sliding in sperm flagella. *Curr. Biol.* **14,** 2113–2118.

Mueller, J. L., Ravi Ram, K., McGraw, L. A., Bloch Qazi, M. C., Saggia, E. D., Clark, A. G., Aquadro, C. F., and Wolfner, M. F. (2005). Cross-species comparison of Drosophila male accessory gland protein genes. *Genetics* **171,** 131–143.

Multigner, L., Pignot-Paintrand, I., Saoudi, Y., Job, D., Plessmann, U., Rüdiger, M., and Weber, K. (1996). The A and B tubules of the outer doublets of sea urchin sperm axonemes are composed of different tubulin variants. *Biochemistry* **35,** 10862–10871.

Neesen, J., Heinlein, U. A., Heinz Glätzer, K., and Bünemann, H. (1999). Proteins with tandemly arranged repeats of a highly charged 16-amino-acid motif encoded by the

Dhmst101 gene family are structural components of the outer sheath of the extremely elongated sperm tails of *Drosophila hydei*. *Dev. Growth Differ.* **41,** 93–99.

Nicastro, D., McIntosh, J. R., and Baumeister, W. (2005). 3D structure of eukaryotic flagella in a quiescent state revealed by cryo-electron tomography. *Proc. Natl. Acad. Sci. USA* **102,** 15889–15894.

Nicastro, D., Schwartz, C., Pierson, J., Gaudette, R., Porter, M. E., and McIntosh, J. R. (2006). The molecular architecture of axonemes revealed by cryoelectron tomography. *Science* **313,** 944–948.

Nielsen, M. G., and Raff, E. C. (2002). The best of the world or the best possible world? Developmental constraint in the evolution of beta-tubulin and the sperm tail axoneme. *Evol. Dev.* **4,** 303–315.

Nielsen, M. G., Turner, F. R., Hutchens, J. A., and Raff, E. C. (2001). Axoneme-specific beta-tubulin specialization: A conserved C-terminal motif specifies the central pair. *Curr. Biol.* **11,** 529–533.

Nielsen, M. G., Caserta, J. M., Kidd, S. J., and Phillips, C. M. (2006). Functional constraint underlies 60 million year stasis of Dipteran testis-specific β-tubulin. *Evol. Dev.* **8,** 23–29.

Nurmirnsky, C. M. D. I., Nurminskaya, M. V., De Aguiar, D., Harti, D. L., *et al.* (1998). Selective sweep of a newly evolved sperm-specific gene in Drosophila. *Nature* **396,** 572–575.

Okada, Y., and Hirokawa, N. (2000). Mechanism of single-headed processivity: Diffusional anchoring between the K-loop of kinesin and the C-terminus of tubulin. *Proc. Natl. Acad. Sci. USA* **97,** 640–645.

Ostrowski, L. E., Blackburn, K., Radde, K. M., Moyer, M. B., Schlatzer, D. M., Moseley, A., and Boucher, R. C. (2002). A proteomic analysis of human cilia: Identification of novel components. *Mol. Cell Proteomics* **1,** 451–465.

Paccagnini, E., Mencarelli, C., Mercati, D., Afzelius, B. A., and Dallai, R. (2007). Ultrastructural analysis of the aberrant axoneme morphogenesis in Thrips (Thysanoptera, Insecta). *Cell Motil. Cytoskeleton* **64,** 645–661.

Pan, J., and Snell, W. (2007). The primary cilium: Keeper of the key to cell division. *Cell* **29,** 1255–1257.

Pathak, N., Obara, T., Mangos, S., Liu, Y., and Drummond, I. A. (2007). The zebrafish fleer gene encodes an essential regulator of cilia tubulin polyglutamylation. *Mol. Biol. Cell* **18,** 4353–4364.

Pazour, G. J., Agrin, N., Leszyk, J., and Witman, G. B. (2005). Proteomic analysis of a eukaryotic cilium. *J. Cell Biol.* **170,** 103–113.

Péchart, I., Kann, M.-L., Levilliers, N., Bré, M.-H., and Fouquet, J.-P. (1999). Composition and organization of tubulin isoforms reveals a variety of axonemal models. *Biol. Cell* **91,** 685–697.

Phillips, D. M. (1967). Giant centriole formation of *Sciara coprofila* sperm. *J. Cell Biol.* **30,** 499–517.

Plessmann, U., and Weber, K. (1997). Mammalian sperm tubulin: An exceptionally large number of variants based on several posttranslational modifications. *J. Protein Chem.* **16,** 385–390.

Popodi, E. M., Hoyle, H. D., Turner, F. R., and Raff, E. C. (2005). The proximal region of the β-tubulin C-terminal tail is sufficient for axoneme assembly. *Cell Motil. Cytoskeleton* **62,** 48–64.

Porter, M. E. (1996). Axonemal dyneins: Assembly, organization, and regulation. *Curr. Opin. Cell Biol.* **8,** 10–17.

Porter, M. E., and Sale, W. S. (2000). The 9+2 axoneme anchors multiple inner arm dyneins and a network of kinases and phosphatases that control motility. *J. Cell Biol.* **151,** 37–42.

Prigent, Y., Kann, M. L., Lach-Gar, H., Pechart, I., and Fouquet, J. P. (1996). Glutamylated tubulin as a marker of microtubule heterogeneity in the human sperm flagellum. *Mol. Reprod. Dev.* **2,** 573–581.

Qin, H., Diener, D. R., Geimer, S., Cole, D. G., and Rosenbaum, J. L. (2004). Intraflagellar transport (IFT) cargo: IFT transports flagellar precursors to the tip and turnover products to the cell body. *J. Cell Biol.* **164,** 255–266.

Qin, H., Burnette, D. T., Bae, Y. K., Forscher, P., Barr, M. M., and Rosenbaum, J. L. (2005). Intraflagellar transport is required for the vectorial movement of TRPV channels in the ciliary membrane. *Curr Biol.* **15,** 1695–1699.

Quagio-Grassiotto, I., and de Lello, E. (1996). Centriole behaviour during meiosis of male germ cells of *Dermatobia hominis* (Diptera: Cuterebridae). *Cytobios* **85,** 73–80.

Quarmby, L. M., and Parker, J. D. (2005). Cilia and the cell cycle? *J. Cell Biol.* **6,** 707–710.

Quennedey, A. (1998). Insect Epidermal Gland Cells: Ultrastructure and Morphogenesis. *In* "Microscopic Anatomy of Invertebrates", vol. 11A (Insecta), (F. W. Harrison and M. Locke, Eds.). Wiley-Liss, NY.

Raff, E. C. (1994). The role of multiple tubulin isoforms in cellular microtubule function. *In* "Microtubules" (J. S. Hyams and C. W. Lloyd, Eds.), pp. 85–109. John Wiley and Sons, New York.

Raff, E. C., Fackenthal, J. D., Hutchens, J. A., Hoyle, H. D., and Turner, F. R. (1997). Microtubule architecture specified by a beta-tubulin isoform. *Science* **275,** 70–73.

Raff, E. C., Hutchens, J. A., Hoyle, H. D., Nielsen, M. G., and Turner, F. R. (2000). Conserved axoneme symmetry altered by a component beta-tubulin. *Curr. Biol.* **10,** 1391–1394.

Ranz, J. M., Ponce, A. R., Hartl, D. L., and Nurminsky, D. (2003). Origin and evolution of a new gene expressed in the Drosophila sperm axoneme. *Genetica* **118,** 233–244.

Redeker, V., Le Caer, J.-P., Rossier, J., and Promé, J.-C. (1991). Structure of the polyglutamyl side chain posttranslationally added to the α-tubulin. *J. Biol. Chem.* **266,** 23461–23466.

Redeker, V., Rossier, J., and Frankfurter, A. (1998). Posttranslational modifications of the C-terminus of α-tubulin in adult rat brain: α4 is glutamylated at two residues. *Biochemistry* **37,** 14838–14844.

Redeker, V., Levilliers, N., Vinolo, E., Rossier, J., Jaillard, D., Burnette, D., Gertig, J., and Bré, M.-H. (2005). Mutations of tubulin glycylation sites reveal a crosstalk between the C-termini of α- and β-tubulin and affect the ciliary matrix in *Tetrahymena*. *J. Biol. Chem.* **280,** 596–606.

Rubin, G. M., Hong, L., Brokstein, P., Evans-Holm, M., Frise, E., Stapleton, M., and Harvey, D. A. (2000). *A Drosophila* complementary DNA resource. *Science* **287,** 2222–2224.

Rosenbaum, J. L., and Witman, G. B. (2002). Intraflagellar transport. *Nat. Mol. Cell Biol.* **3,** 813–825.

Sackett, D. L. (1995). Structure and function in the tubulin dimer and the role of the acidic carboxyl terminus. *Subcell. Biochem.* **24,** 255–302.

Sakato, M., and King, S. M. (2004). Design and regulation of the AAA^+ microtubule motor dynein. *J. Struct. Biol.* **146,** 58–71.

Sale, W. S., Goodenough, U. W., and Heuser, J. E. (1985). The substructure of isolated and *in situ* outer dynein arms of sea urchin sperm flagella. *J. Cell Biol.* **101,** 1400–1412.

Sarpal, R., Todi, S. V., Sivan-Loukianova, E., Shirolikar, S., Subramanian, N., Raff, E. C., Erickson, J. W., Ray, K., and Eberl, D. F. (2003). *Drosophila* KAP interacts with the kinesin II motor subunit KLP64D to assemble chordotonal sensory cilia, but not sperm tails. *Curr. Biol.* **13,** 1697–1696.

Satir, P., and Christensen, S. T. (2007). Overview of structure and function of mammalian cilia. *Annu. Rev. Physiol.* **69,** 377–400.

Satir, P., Guerra, C., and Bell, A. J. (2007). Evolution and persistence of the cilium. *Cell Motil. Cytoskeleton* **64,** 906–913.

Schäfer, M., Börsch, D., Hülster, A., and Schäfer, U. (1993). Expression of a gene duplication encoding conserved sperm tail proteins is translationally regulated in Drosophila melanogaster. *Mol. Cell Biol.* **13,** 1708–1718.

Scholey, J. M., and Anderson, K. V. (2006). Intraflagellar transport and cilium-based signaling. *Cell* **5,** 439–442.

Sinden, R. E., Canning, E. U., and Spain, B. (1976). Gametogenesis and fertilization in Plasmodium yoelii nigeriensis: A transmission electron microscope study. *Proc. R. Soc. Lon. B Biol. Sci.* **193,** 55–76.

Simmons, K. R., Parker, G. A., and Stockley, P. (1999). Sperm displacement in the yellow dung fly, *Scatophaga stercoraria*: An investigation of male and female processes. *Am. Nat.* **153,** 302–314.

Smith, J. C., Northey, J. G., Garg, J., Pearlman, R. E., and Siu, K. W. (2005). Robust method of proteome analysis by MS/MS using an entire translated genome: Demonstration on the ciliome of *Tetrahymena termophila*. *J. Proteome Res.* **4,** 909–919.

Sreng, L., and Quennedey, A. (1976). Role of a temporary ciliary structure in the morphogenesis of insect glands. An electron microscope study of the tergal glands of male *Blattella germanica* L. (Dictyoptera, Blattellidae). *J. Ultrastruct. Res.* **56,** 78–95.

Sullivan, K. F. (1988). Structure and utilization of tubulin isotypes. *Annu. Rev. Cell Biol.* **4,** 687–716.

Summers, K. E., and Gibbons, I. R. (1971). Adenosine triphosphate-induced sliding of tubules in trypsin-treated flagella of sea-urchin sperm. *Proc. Natl. Acad. Sci. USA* **101,** 12865–12869.

Swan, M. A. (1981). The generation and propagation of double waves in mosquito (*Aedes notoscriptus*) sperm-tails. *Gamete Res.* **4,** 241–250.

Swanson, W. J., Clark, A. G., Waldrip-Dail, H. M., Wolfner, M. F., and Aquadro, C. F. (2001). Evolutionary EST analysis identifies rapidly evolving male reproductive proteins in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **98,** 7375–7379.

Taddei, A. R., Gambellini, G., Fausto, A. M., Baldacci, A., and Mazzini, M. (2000). Immunolocalization of different tubulin epitopes in the spermatozoon of *Bacillus rossius* (Insecta, Phasmatodea). *J. Submicrosc. Cytol. Pathol.* **32,** 735–643.

Thazhath, R., Liu, C., and Gaertig, J. (2002). Polyglycylation domain of β-tubulin maintains axonemal architecture and affects cytokinesis in *Tetrahymena*. *Nat. Cell Biol.* **4,** 256–259.

Thazhath, R., Jerka-Dziadoszm, M., Duan, J., Wloga, D., Gorovsky, M. A., Frankel, J., and Gaertig, J. (2004). Cell context-specific effects of the beta-tubulin glycylation domain on assembly and size of microtubular organelles. *Mol. Biol. Cell* **15,** 4136–4147.

Timakov, B., and Zhang, Y. Q. (2001). The *hsp60B* gene of *Drosophila melanogaster* is essential for spermatid individualization process. *Cell Stress Chaperones* **6,** 71–77.

Todi, S. V., Sharma, Y., and Eberl, D. F. (2004). Anatomical and molecular design of the *Drosophila* antenna as a flagellar auditory organ. *Microsc. Res. Tech.* **63,** 388–399.

Tokuyasu, K. T. (1975). Dynamics of spermiogenesis in *Drosophila melanogaster*. VI. Significance of "onion" nebenkerrn formation. *J. Ultrastruct. Res.* **53,** 93–112.

Verhey, K. J., and Gaertig, J. (2007). The tubulin code. *Cell Cycle* **6,** 2152–2160.

Vinh, J., Langridge, J. I., Bré, M.-H., Levilliers, N., Redeker, V., Loyaux, D., and Rossie, J. (1999). Structural characterization by tandem mass spectrometry of the posttranslational polyglycylation of tubulin. *Biochemistry* **38,** 3133–3139.

Vogt, N., Koch, I., Schwarz, H., Schnorrer, F., and Nüsslein-Volhard, C. (2006). The γTuRC components Grip75 and Grip128 have an essential microtubule-anchoring function in the *Drosophila* germline. *Development* **133,** 3963–3972.

Warner, F. D., Satir, P., and Gibbons, L. R. (1989). Section 2 of "Cell Movement," Vol. 1, pp. 111–188. Alan R Liss, New York.

Werner, M., Gack, C., Speck, T., and Peschke, K. (2007). Queue up, please! Spermathecal filling in the rove beetle *Drusilla canaliculata* (Coleoptera, Staphylinidae). *Naturwissenschaften* **94,** 837–841.

Westermann, S., and Weber, K. (2003). Post-translational modifications regulate microtubule function. *Nat. Rev. Mol. Cell Biol.* **4,** 938–947.

Wilson, P. G., Zheng, Y., Oakley, C. E., Borisy, G. G., and Fuller, M. T. (1997). Differential expression of two gamma-tubulin isoforms during gametogenesis and development in Drosophila. *Dev. Biol.* **184,** 207–221.

Witman, G. B. (2003). Cell Motility: Deaf *Drosophila* keep the beat. *Curr. Biol.* **13,** R796–R798.

Wolf, K. W., and Klein, C. (1995). Development of the sperm head in the caddisfly *Potamophylax rotundipennis* (Insecta, Trichoptera). *Zoomorphology* **115,** 109–115.

Wolff, A., Houdayer, M., Chillet, D., de Néchaud, B., and Denoulet, P. (1994). Structure of the polyglutamyl chain of tubulin: Occurrence of alpha and gamma linkages between glutamyl units revealed by monoreactive polyclonal antibodies. *Biol. Cell* **81,** 11–16.

Xia, L., Hai, B., Gao, Y., Burnette, D., Thazhath, R., Duan, J., Bré, M.-H., Levilliers, N., Gorovsky, M. A., and Gaertig, J. (2000). Polyglycylation of tubulin is essential and affects cell motility and division in *Tetrahymena thermophila*. *J. Cell Biol.* **149,** 1097–1106.

Yack, J. E. (2004). The structure and function of auditory chordotonal organs in insects. *Microsc. Res. Tech.* **63,** 315–337.

Yue, L., Karr, T. L., Nathan, D. F., Swift, H., Srinivasan, S., and Lindquist, S. (1999). Genetic analysis of viable Hsp90 alleles reveals a critical role in *Drosophila* spermatogenesis. *Genetics* **151,** 1065–1079.

Zhang, Y. Q., Matthies, H. J. G., Mancuso, J., Andrews, H. K., Woodruff, E., Friedman, D., and Broadie, K. (2004). The *Drosophila* fragile X-related gene regulates axoneme differentiation during spermatogenesis. *Dev. Biol.* **270,** 290–307.

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CHAPTER FIVE

New Insights into the Mechanism of Precursor Protein Insertion into the Mitochondrial Membranes

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Contents

Abstract

Mitochondria are surrounded by a double membrane system that forms four intra-organelle compartments: the outer membrane, inner membrane, intermembrane space, and matrix. Each of the two membranes contains a unique set of proteins defining specific functions of that membrane. The vast majority of mitochondrial proteins including those of the mitochondrial membranes are nuclear encoded and synthesized as precursor proteins in the cytosol. Subsequently, they are targeted to the mitochondria and become sorted to the correct submitochondrial destination. A small portion of the mitochondrial inner membrane proteins is encoded by the mitochondrial genome. These proteins are synthesized on mitochondrial ribosomes and are inserted by dedicated machinery into the inner membrane. This chapter summarizes our current knowledge of the signals that target mitochondrial membrane proteins to their correct intracellular location, and describes the mechanisms by which mitochondrial

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translocation machineries recognize precursor proteins and mediate their insertion into mitochondrial membranes.

Key Words: Membrane insertion, Mitochondria, Oxa1, Protein import, TIM complex, TOB/SAM complex, TOM complex.

1. Introduction

Inner and outer mitochondrial membranes differ considerably in their functions, protein to lipid ratio, lipid composition, and structural characteristics. The mitochondrial outer membrane is a subcellular compartment that mediates numerous interactions between the mitochondrial metabolic and genetic systems and the rest of the eukaryotic cell. This membrane harbors a diverse set of proteins such as components of the preprotein translocation machinery, enzymes, translocators of soluble metabolites, pore structures, regulators of programmed cell death, and proteins regulating the morphology of the organelle. The inner membrane is the place where the oxidative phosphorylation takes place and thus this membrane is the center of energy production for the whole eukaryotic cell. In addition, mitochondrial inner membrane proteins mediate various metabolic processes and are pivotal for the dynamic morphology of the organelle.

The outer membrane has a high content of lipids and the lipid composition is similar to that of the endoplasmic reticulum (ER). Currently ~40 different proteins are estimated to reside in the mitochondrial outer membrane of fungi (Schmitt *et al.*, 2006; Zahedi *et al.*, 2006). In contrast, proteins make up ~80% of the mass of the inner membrane and just 20% are lipids. This makes the inner membrane one of the protein-richest membranes of the eukaryotic cell. Among the 1000–1500 estimated proteins in mammalian mitochondria, several hundreds are part of the inner membrane (McDonald *et al.*, 2006). The lipid composition of the inner membrane is unique: It is characterized by significant amounts of *cardiolipin* which is only found in small amounts in the outer membrane and not present in other membranes of the cell. Many protein complexes of the inner membrane contain cardiolipin in their quaternary structure (Mileykovskaya *et al.*, 2005). For some of these complexes, cardiolipin functions as a structural stabilizer. Furthermore, cardiolipin might contribute directly to the catalytic activity of some membrane-embedded enzymes. Although cardiolipin and other lipids are much more than just structural grease between the membrane proteins, our knowledge on the molecular functions of specific lipids is strongly limited, mainly due to lack of the required methodology to study these functions.

The outer membrane appears to be quite homogeneous in its microscopic structure. In contrast, the inner membrane can be subdivided into

two regions: the inner boundary membrane which is adjacent to the outer membrane, and the cristae membrane forming the invaginations of the inner membrane that protrude into the interior of the organelle. Recent studies suggest that both areas of the inner membrane differ in their protein composition and hence might exhibit different functions: Whereas the protein translocases and presumably also other transporters might be predominantly found in the inner boundary membrane, the cristae appear to resemble areas that are specialized on the electron transport processes of respiration (Vogel *et al.*, 2006; Wurm and Jakobs, 2006).

2. The Protein Import Machinery of Mitochondria

Mitochondria are extremely complex organelles. Even in simple organisms like baker's yeast, ~800–1000 different proteins need to be targeted to mitochondria (Mootha *et al.*, 2003; Sickmann *et al.*, 2003). These proteins are recognized on the mitochondrial surface by receptors and imported into the different subcompartments of mitochondria: the outer membrane, the intermembrane space, the inner membrane, and the matrix. These translocation reactions are facilitated by a small number of membrane-embedded translocases: the translocase of the outer mitochondrial membrane (the TOM complex) and two translocases of the inner mitochondrial membrane (the TIM22 and the TIM23 complexes). Moreover, a fourth protein complex, the Oxa1 translocase is involved in the insertion of proteins into the inner membrane. These four translocases operate in a sequential but concerted manner using the membrane potential across the inner membrane and/or the hydrolysis of ATP to drive protein translocation. The translocation machinery was primarily studied in baker's yeast and in the following mainly the yeast system will be described. However, recent studies in mammalian and plant cells suggest that the translocases and their mode of function are largely conserved among eukaryotes (Millar *et al.*, 2006).

2.1. Translocation across and into the outer membrane

2.1.1. Structure and functions of the TOM complex

The translocase of the outer membrane of mitochondria mediates the initial steps of the import of preproteins into the organelle. The TOM complex is involved in the import of almost all mitochondrial precursor proteins characterized so far. It has the capacity to promote insertion of outer membrane proteins into the lipid bilayer or to translocate precursors destined for other compartments across the outer membrane.

The TOM holo complex in fungi is composed of seven different subunits. Tom20 and Tom70 are the *primary receptors* whereas the subunits

Tom40, Tom22, Tom7, Tom6, and Tom5 form the stable TOM core complex also called *the general insertion pore* (Ahting *et al.*, 1999; Dekker *et al.*, 1998; Künkele *et al.*, 1998). The TOM holo complex was estimated by size exclusion chromatography to have a molecular mass of 490-600 kDa. In contrast, the TOM core complex has an estimated molecular weight of 400–450 kDa according to size exclusion chromatography and blue native PAGE. Electron microscopic analysis of the holo complex revealed the presence of two to three pores. Removal of Tom20 and Tom70 by detergent or isolating the TOM complex from a Tom20-deficient strain result in a complex that forms mainly two pores (Ahting *et al.*, 1999; Model *et al.*, 2002). The size of these pores was estimated to be 20–25 Å based on electron microscopy (Künkele *et al.*, 1998; Model *et al.*, 2002), conductance through TOM-related ion channels (Hill *et al.*, 1998; Künkele *et al.*, 1998), and size exclusion studies (Schwartz and Matouschek, 1999).

Except of Tom40, all Tom components are anchored to the outer membrane by a single α-helical transmembrane segment (TMS). Theoretical predictions and secondary structure determinations suggest that *Tom40* traverses the mitochondrial outer membrane as a series of antiparallel β-strands which form a β-barrel (Ahting *et al.*, 2001; Hill *et al.*, 1998; Mannella *et al.*, 1996). The native basic structural unit of Tom40 is a dimer (Dekker *et al.*, 1998; Rapaport *et al.*, 1998). Upon *in vitro* reconstitution, both purified native Tom40 and recombinant Tom40 form ion channels in lipid bilayers (Ahting *et al.*, 2001; Hill *et al.*, 1998), strongly suggesting that Tom40 forms the protein conducting channel of the TOM complex. The importance of Tom40 for the biogenesis of mitochondria is underscored by its requirement for the viability of yeast and *N. crassa* cells.

The *Tom22* subunit connects the primary receptors to the core complex and functions itself as a receptor (van Wilpe *et al.*, 1999). Although Tom22 is essential in *N. crassa* (Nargang *et al.*, 1995), it has been reported that under special genetic conditions a Δ*tom22* yeast strain is viable, however, with severe growth defects (van Wilpe *et al.*, 1999). All the other Tom subunits are not essential for viability in yeast or *N. crassa* although combinations of double deletions lead often to synthetic lethality.

Tom6 and *Tom7* are two small subunits that play a structural role in the organization of the TOM complex. Tom6 forms the link between Tom40 and Tom22, while Tom7 destabilizes the association of the various subunits of the TOM complex (Alconada *et al.*, 1995; Honlinger *et al.*, 1996).

2.1.2. Topologies of mitochondrial outer membrane proteins

Proteins of the outer membrane span the lipid bilayer once, twice, or as multispanning proteins. Based on their membrane topology, the outer membrane proteins can be subdivided into several groups. One group includes those containing a single TMS at their N-terminus, like Tom20, Tom70, and OM45 (Fig. 5.1, example 1). These *signal-anchored proteins* are

present in the outer membrane in an orientation, where the bulk of the polypeptide is exposed to the cytosol and only a small N-terminal segment crosses the outer membrane. *Tail-anchored proteins* such as Fis1, Tom5, and VAMP-1B form another distinct group of outer membrane proteins (Fig. 5.1, example 2). These proteins have a single membrane insertion sequence at their C-terminus and display their large N-terminal portion to the cytosol (Wattenberg and Lithgow, 2001). Mim1/Tom13 and Tom22 represent yet another group of proteins (Fig. 5.1, example 3), namely those that span the membrane once and expose an N-terminal domain to the cytosol and a soluble C-terminal domain to the intermembrane space. Representatives of the fourth group are characterized by more than one TMS (Fig. 5.1, examples 4 and 5). Fzo1 is currently the only known example of a protein which spans the outer membrane twice (Fig. 5.1, example 4). This protein exposes both termini to the cytosol and has only a small loop in the intermembrane space (Fritz *et al.*, 2001; Rojo *et al.*, 2002). Ugo1 was recently demonstrated to transverse the outer membrane at least three times (Fig. 5.1, example 5) (Coonrod *et al.*, 2007). Finally, the outer membrane harbors proteins which are predicted to traverse the outer membrane as a

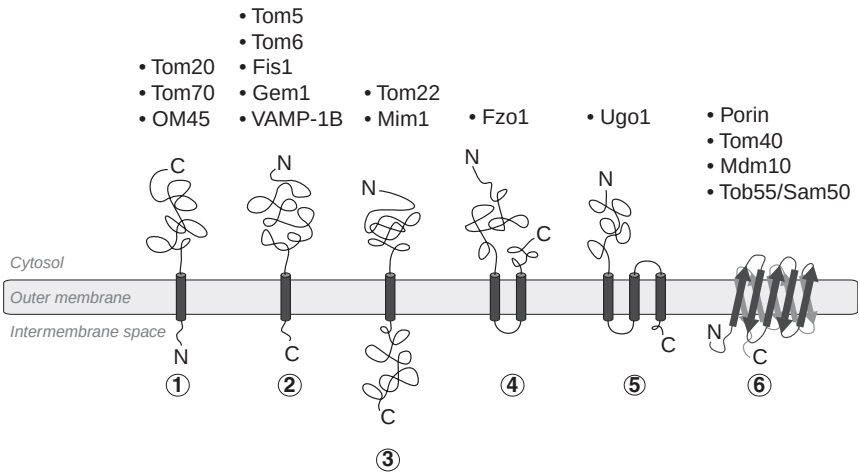

Figure 5.1 Topologies of proteins residing in the outer membrane of mitochondria. The outer membrane proteins can be subdivided into six groups based on their membrane topology. Group 1 contains the signal-anchored proteins while group 2 is composed of the tail-anchored proteins. Mim1 and Tom22 which belong to group 3 span the membrane once and expose an N-terminal domain to the cytosol and a soluble C-terminal domain to the intermembrane space. The fourth and fifth groups are those spanning the outer membrane twice or at least three times, respectively. Finally, the outer membrane harbors proteins which traverse the membrane as a series of antiparallel β-strands which form a membrane-embedded β-barrel structure (group 6). The N- and C-termini of the proteins are presented as N and C, respectively.

series of antiparallel β-strands which form a membrane-embedded β-barrel structure (Fig. 5.1, example 6) (Mannella *et al.*, 1996). Prominent members of this latter group are Tom40, Tob55/Sam50, and porin (also called *v*oltage-*d*ependent *a*nion *c*hannel, VDAC, in mammalian cells).

All these proteins, like the vast majority of mitochondrial proteins, are encoded by nuclear DNA and synthesized on cytosolic ribosomes. However, in contrast to most matrix proteins, all proteins of the outer membrane contain noncleavable targeting and sorting signals. In the following sections, we summarize the current knowledge on the signals which target mitochondrial outer membrane proteins to their correct intracellular location. Furthermore, the mechanisms by which these signals are decoded by the mitochondria and by which the proteins are inserted into the membrane are discussed.

2.1.3. Targeting and membrane insertion of signal-anchored proteins

A number of mitochondrial outer membrane proteins is characterized by one single transmembrane segment at their N-terminus. They are called *''signal-anchored'' proteins* since their TMS together with its flanking regions serve both as an intracellular sorting signal and as an anchor to the membrane (Fig. 5.1, example 1). The known members of this group are Tom20, Tom70, OM45, and the outer membrane isoform of Mcr1. Tom20 and Tom70 function as receptor proteins that recognize precursor proteins on the surface of mitochondria. Tom20 is involved in the recognition of most protein precursors, in particular those with N-terminal targeting signals (Harkness *et al.*, 1994; Lithgow *et al.*, 1995), whereas Tom70 forms a binding site for a more restricted set of preproteins, most notably the mitochondrial carrier family (Brix *et al.*, 1999; Schlossmann *et al.*, 1994). OM45, another member of this group, is a very abundant protein in the yeast mitochondrial outer membrane whose function, however, is unknown (Yaffe *et al.*, 1989). The outer membrane isoform of Mcr1 functions as a cytochrome *b*5 NADH-dependent reductase (Hahne *et al.*, 1994).

These proteins do not share any sequence similarity in their signal-anchor domain. Thus, their *targeting information* is encoded probably in the structural characteristics of the signal-anchor domain rather than in a specific primary sequence. Initial results have revealed that the targeting information in yeast Tom70 lies in a linear sequence that includes the predicted TMS (residues 11–29), together with amino acid residues 1–10 which comprise a hydrophilic, positively charged segment (Shore *et al.*, 1995). The TMS is required for both targeting to mitochondria and membrane anchoring (a ''signal-anchor'' function) while the positively charged residues cooperate with the TMS to increase the overall rate of import (McBride *et al.*, 1992).

Studies with Tom20 and Tom70 as substrate proteins revealed that signal-anchor sequences need to be of moderate hydrophobicity and flanked by positively charged residues in order to be inserted into the mitochondrial outer membrane. When these positive charges are removed or when the hydrophobicity of the TMS is increased, the proteins are mistargeted to the ER (Kanaji *et al.*, 2000; Suzuki *et al.*, 2002; Waizenegger *et al.*, 2003).

The mechanisms by which these signals are deciphered by the mitochondria are only partially understood. The targeting of precursors of both Tom20 and Tom70 to mitochondria was found to be independent of protease-accessible surface receptors (Schlossmann and Neupert, 1995; Schneider *et al.*, 1991; Suzuki *et al.*, 2002). Similarly, the membrane insertion of the outer membrane isoform of Mcr1 does not require import receptors (Meineke *et al.*, 2008). In addition, antibodies against the receptors Tom20 and Tom70 did not inhibit the membrane integration of newly synthesized Tom20 molecules (Schneider *et al.*, 1991). Therefore, the import receptors are obviously not essential for the biogenesis of signal-anchored proteins.

In contrast to the receptor subunits, Tom40 is critical for the import of Tom20. However, currently it is unresolved at which stage Tom40 interacts with precursor molecules of Tom20. Tom40 may contribute to the initial recognition of precursors of Tom20 whereas the insertion step per se could be unassisted. Independently of this putative recognition function, Tom40 can interact with precursor molecules of Tom20 upon their (partial) insertion into the membrane thereby trapping them within the membrane. An unassisted membrane insertion could be followed by a trapping step within the membrane. Such a putative trapping step would ensure the complete insertion of precursors of Tom20 and would prevent backsliding out of the membrane.

Mim1 is another outer membrane protein which is required for the membrane insertion of Tom20 (Waizenegger *et al.*, 2005). Mim1 is not a member of the TOM or TOB/SAM complexes and its role in the biogenesis of Tom20 is ill-defined. Further experiments are required to dissect the membrane integration process of this protein.

Tom20 is a component of the TOM complex and as such may follow a unique pathway. Thus, the involvement of Tom40 and Mim1 in the biogenesis of Tom20 does not have to apply to other N-terminally anchored proteins. A recent study with the outer membrane isoform of Mcr1 suggests that both Tom40 and Mim1 are not involved in the membrane insertion of this signal-anchor protein (Meineke *et al.*, 2008). Hence, the TOM complex seems to be not generally involved in the import of signal-anchored proteins. The mechanism of insertion of the outer membrane isoform of Mcr1 into the membrane can be based on a trapping process which could be mediated by interactions with lipids or with other, yet unknown, proteins.

2.1.4. Membrane integration of mitochondrial tail-anchored proteins

Tail-anchored (TA) proteins have a single membrane insertion sequence at their C-terminus and expose their large N-terminal portion to the cytosol (Fig. 5.1, example 2) (Borgese *et al.*, 2007; Wattenberg and Lithgow, 2001). All TA proteins are imported post-translationally from the cytosol into their corresponding organelle by a mechanism which is not well resolved. The mitochondrial outer membrane harbors a distinct set of TA proteins. Among them are the three small subunits (Tom5, Tom6, Tom7) of the TOM complex (Allen *et al.*, 2002; Beilharz *et al.*, 2003), Fis1 which promotes the fission of mitochondria (Mozdy *et al.*, 2000), regulators of apoptosis belonging to the Bcl-2 family (Cory and Adams, 2002), the mitochondrial isoform of cytochrome *b*5 (D'Arrigo *et al.*, 1993), an alternatively spliced isoform of the vesicles-associated membrane protein, VAMP-1B (Isenmann *et al.*, 1998), and a synaptojanin-binding protein, OMP25 (Nemoto and De Camilli, 1999). At least for some of these proteins it was demonstrated that the tail-anchor domain is necessary and sufficient for targeting to mitochondria (Allen *et al.*, 2002; Beilharz *et al.*, 2003; Dembowski *et al.*, 2001; Egan *et al.*, 1999; Kemper *et al.*, 2008; Nguyen *et al.*, 1993). Similar to the signal anchor domains in "signal-anchored" proteins, the tail regions of TA proteins are of moderate hydrophobicity and flanked by positive charges. It seems that the relative contribution of each of these two structural features varies from protein to protein (Borgese *et al.*, 2007; Rapaport, 2003).

The mechanisms by which the TA proteins are recognized at the mitochondrial surface and subsequently inserted into the membrane are still largely unresolved. Conflicting reports exist regarding the requirements for surface receptors, external energy, and cytosolic chaperones (Fig. 5.2). On the one hand, the targeting of VAMP-1B was reported to rely on surface receptors (Lan *et al.*, 2000), and the import of Bcl-2 precursor into yeast mitochondria was proposed to involve the import receptor Tom20 (Motz *et al.*, 2002). On the other hand, mitochondrial targeting of tail-anchored proteins in mammalian cells was proposed recently to be independent of protease-sensitive proteins and of the TOM complex (Setoguchi *et al.*, 2006). Importantly, these authors reported that mitochondrial targeting and integration of the analyzed TA proteins shared a common pathway that did not depend on the hydrolysis of cytosolic ATP (Setoguchi *et al.*, 2006). Similarly, a recent study on the insertion pathway of the yeast tail-anchored protein, Fis1, revealed that the TOM complex seems not to be involved in the membrane integration process (Fig. 5.2) (Kemper *et al.*, 2008). The role of cytosolic factors in delivering the TA proteins to the surface of mitochondria remains to be investigated. Another open question is whether the membrane integration of the TA proteins is mediated by proteins in the outer membrane (Fig. 5.2, option 1) or if they are inserted directly into the membrane in an unassisted manner (Fig. 5.2, option 2).

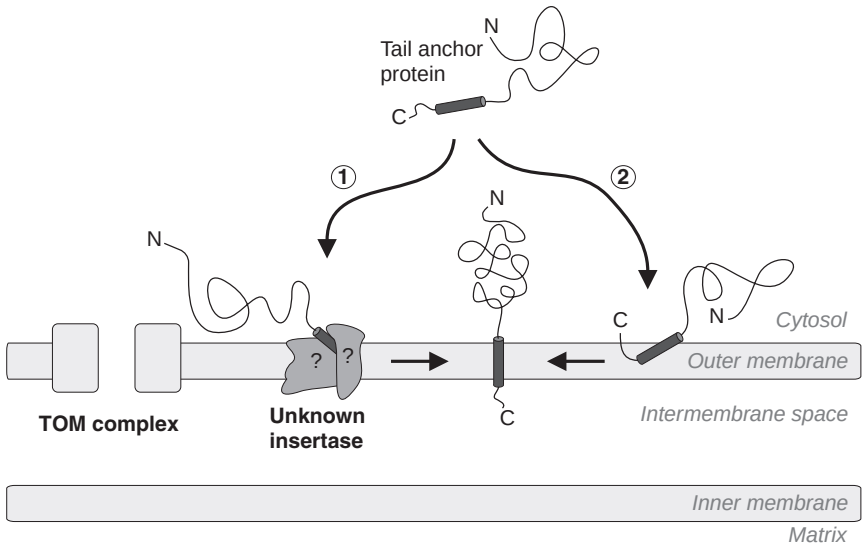

Figure 5.2 Membrane insertion of tail-anchored proteins. The precursors of tail-anchored proteins are inserted without an apparent involvement of the TOM complex. It is yet an open question whether other membrane-embedded proteins (depicted here as "unknown insertase") are promoting the process (option 1) or the membrane integration occurs directly into the lipid bilayer in an unassisted manner (option 2).

2.1.5. Biogenesis of β-barrel membrane proteins

Mitochondria and chloroplasts contain β-barrel proteins in their outer membranes (Gabriel *et al.*, 2001; Rapaport, 2003; Schleiff *et al.*, 2003). These proteins cross the outer membrane by multiple antiparallel β-strands consisting each of 9–11 amino acid residues (Wimley, 2003). The only other biological membrane known to harbor β-barrel proteins is the outer membrane of Gram-negative bacteria (Tamm *et al.*, 2001; Wimley, 2003). This situation is believed to reflect the evolutionary origin of mitochondria and chloroplasts from endosymbionts that belong to the class of Gram-negative bacteria. Despite their central role in bacterial and organelle biogenesis very little is known about how newly synthesized β-barrel proteins are sorted within the cell, integrated into lipid bilayers and assembled into oligomeric structures.

In the case of mitochondria, two β-barrel membrane proteins have been known for a long time, porin and Tom40, both channel-forming oligomeric proteins. More recently discovered β-barrel mitochondrial membrane proteins are Mdm10 and Mmm2 which have a role in the maintenance of the morphology of mitochondria (Sogo and Yaffe, 1994; Youngman *et al.*, 2004). Another β-barrel membrane protein is Tob55/Sam50, the key component of the TOB/SAM complex (Fig. 5.1, example 6).

The biogenesis of β-barrel proteins was studied in detail only in recent years. Precursors of these proteins are initially recognized by the import receptors Tom20 and Tom70. They are then translocated via the general import pore of the TOM complex (Fig. 5.3, step 1) (Krimmer *et al.*, 2001; Model *et al.*, 2001; Rapaport, 2002; Rapaport and Neupert, 1999; Schleiff *et al.*, 1999). From the TOM complex, β-barrel precursors are transferred to the TOB/SAM complex which mediates the topogenesis of these proteins in the outer membrane (Fig. 5.3, steps 2 and 3). On their way from the TOM to the TOB/SAM complex, β-barrel precursors are exposed to the intermembrane space where they were reported to interact with small Tim components residing in this compartment (Fig. 5.3, step 2) (Hoppins and Nargang, 2004; Wiedemann *et al.*, 2004). In the following sections, we will discuss the various stages in the biogenesis of these proteins in more detail.

2.1.5.1. The targeting information of mitochondrial β-barrel proteins The targeting information of β-barrel proteins, such as porin and Tom40, is spread throughout different regions of the sequence. In the case of *N. crassa* Tom40, deletion of the N- or the C-terminus did not affect protein targeting to

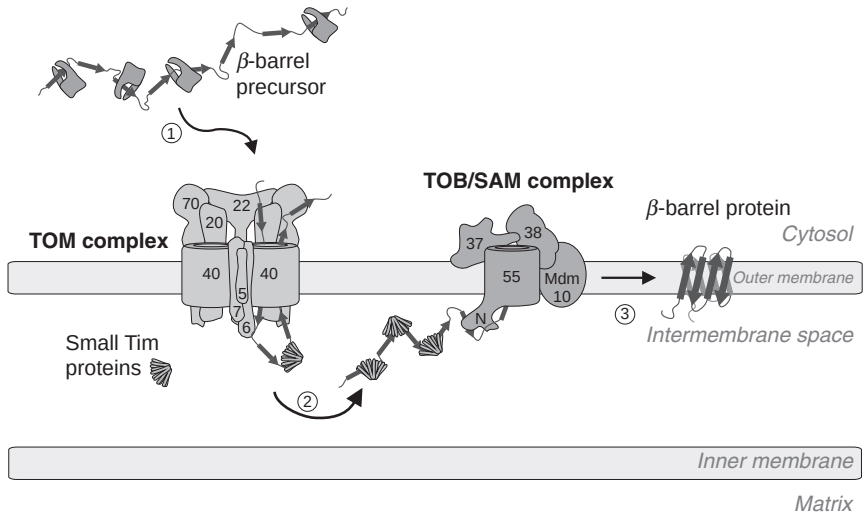

Figure 5.3 The biogenesis of mitochondrial β-barrel membrane proteins. The precursors of β-barrel proteins are probably attached to cytosolic chaperones before their recognition by import receptors of the TOM complex (step 1). The precursor proteins are subsequently translocated across the outer membrane and passed on to the TOB/SAM complex. Small Tim chaperones in the intermembrane space facilitate the transfer of the precursor from the TOM complex to the TOB/SAM machinery (step 2). Finally, the β-barrel precursors are inserted into the outer membrane by the TOB/SAM complex (step 3). The numbers refer to the respective subunits of the translocases.

mitochondria, indicating that the termini do not carry the necessary targeting information (Rapaport and Neupert, 1999). Studies on yeast porin demonstrated that deletions of various segments in the N-terminal half of the protein abrogate the import process (Hamajima *et al.*, 1988; Smith *et al.*, 1995). The import efficiency is also decreased when specific lysine residues are mutated to a neutral or negatively charged amino acid (Smith *et al.*, 1995). In contrast, it appears that *N. crassa* porin contains targeting, import, and/or assembly information at its C-terminus, rather than at the N-terminus (Court *et al.*, 1996). In the case of yeast Tob55/Sam50, the deletion of the N-terminal domain did not affect the targeting and assembly of the precursor (Habib *et al.*, 2007). Taken together, these studies suggest that the targeting information in β-barrel proteins is not included in a linear sequence but rather may be encoded by a structural element that is composed of different regions of the protein (Rapaport, 2003).

2.1.5.2. Structure and function of the TOB/SAM complex After crossing the outer membrane via the TOM complex, precursors of β-barrel proteins are engaged by the TOB/SAM complex. The major component of the TOB/SAM complex is *Tob55/Sam50* (Gentle *et al.*, 2004; Kozjak *et al.*, 2003; Paschen *et al.*, 2003). Its sequence is similar to that of the highly conserved bacterial protein Omp85/YaeT, which was proposed to mediate the insertion of β-barrel proteins into the bacterial outer membrane (see below) (Voulhoux *et al.*, 2003; Wu *et al.*, 2005). Furthermore, Tob55/Sam50 apparently has homologous proteins throughout the entire eukaryotic kingdom and was found to be essential for viability in yeast and *N. crassa* cells (Hoppins *et al.*, 2007). Depletion of Tob55/Sam50 or the growth of a conditional mutant at nonpermissive temperature leads to reduced levels of β-barrel membrane proteins in mitochondria. Furthermore, isolated mitochondria with reduced amounts of Tob55/Sam50 or harboring a mutated Tob55/Sam50 are unable to import β-barrel membrane proteins. Taken together, Tob55/Sam50 plays a specific role in the biogenesis of mitochondrial β-barrel membrane proteins.

Tob55/Sam50 is a 55-kDa protein which is composed of two parts: the membrane-integrated β-barrel domain in its C-terminal region and the predicted hydrophilic N-terminal domain which is exposed to the intermembrane space. The N-terminus contains a POTRA (*po*lypeptide-*t*ransport-*a*ssociated) domain which is assumed to have a chaperone-like function (Gentle *et al.*, 2005; Pfanner *et al.*, 2004; Sanchez-Pulido *et al.*, 2003). Recently, this domain was shown to interact with β-barrel precursors thereby facilitating their transfer from the TOM complex to the TOB/SAM machinery (Habib *et al.*, 2007).

Tob55/Sam50 is the main component of a complex of $\sim$250 kDa, termed *TOB/SAM complex*. The TOB/SAM complex in yeast contains two further proteins, *Mas37/Sam37/Tom37* and *Tob38/Sam35/Tom38*

(Ishikawa *et al.*, 2004; Milenkovic *et al.*, 2004; Waizenegger *et al.*, 2004; Wiedemann *et al.*, 2003). These latter two components are associated with Tob55/Sam50 at the cytosolic side of the outer membrane. The role of Mas37/Sam37/Tom37 in the biogenesis of mitochondrial β-barrel membrane proteins has not yet been identified. Mas37/Sam37/Tom37 is essential for viability of yeast cells only at elevated temperatures. In its absence, a functional TOB/SAM core complex is present, yet imported β-barrel precursors accumulate as TOB/SAM-bound species (Paschen *et al.*, 2003; Wiedemann *et al.*, 2003). A recent study points to a possible function of Mas37/Sam37/Tom37 in the release of β-barrel precursors from the TOB/SAM complex (Habib *et al.*, 2005).

Tob38/Sam35/Tom38 is, like Tob55/Sam50, an essential component for the viability of yeast. It is tightly bound to Tob55/Sam50 and the levels of Tob38/Sam35/Tom38 are strongly reduced in Tob55/Sam50-depleted cells. Thus, Tob38/Sam35/Tom38 is most probably anchored to mitochondria via Tob55/Sam50. The depletion of Tob38/Sam35/Tom38 has a severe effect on the import of β-barrel precursors, but not on the import of precursor proteins destined to other mitochondrial subcompartments. Together with Tob55/Sam50, Tob38/Sam35/Tom38 forms a functional TOB/SAM core complex even in the absence of Mas37/Sam37/Tom37. Since the extraction of Tob38/Sam35/Tom38 destabilizes the structure of the membrane-embedded Tob55/Sam50 (Habib *et al.*, 2005), it was concluded that Tob38/Sam35/Tom38 is required for the stability and assembly of the TOB/SAM complex. A subcomplex of Mas37/Sam37/Tom37 with Tob55/Sam50 on depletion of Tob38/Sam35/Tom38 was not detected.

A further protein which was suggested to be a component of the TOB/SAM complex is *Mdm10*. This outer membrane protein is predicted to be a β-barrel protein and uses the TOB/SAM complex for its topogenesis (Paschen *et al.*, 2003; Wiedemann *et al.*, 2003). It was demonstrated to play an important role in mitochondrial division and morphology (Sogo and Yaffe, 1994). A fraction of Mdm10 was found in association with the TOB/SAM complex and a function in the import of Tom40 precursor was proposed (Meisinger *et al.*, 2004). Recently, also the TOM component *Tom7* and the morphology proteins, Mdm12 and Mmm1, were suggested to play such a dual role (Meisinger *et al.*, 2006, 2007).

2.1.5.3. The assembly of the TOB/SAM complex The TOB/SAM complex is composed of both, membrane-embedded and membrane-associated subunits. The import pathway of Tob55/Sam50 shares in principle the conserved route of other β-barrel proteins. Initially, Tob55/Sam50 is recognized by the mitochondrial surface receptors Tom70 and Tom20. Then, it is translocated via the general import pore of the TOM complex and transferred to the TOB/SAM complex. The small Tim protein

complexes Tim8-Tim13 and Tim9-Tim10 seem to be involved in this process. The TOM complex was shown to be involved in the import of most mitochondrial proteins analyzed so far. In contrast, Mas37/Sam37/Tom37 was shown to follow a unique association pathway, where it is directly recognized by and assembled into the TOB/SAM core complex (Habib *et al.*, 2005). Since Tob38/Sam35/Tom38 is also peripherally attached to the TOB/SAM complex, it presumably associates with the outer membrane in a similar way as Mas37/Sam37/Tom37. Upon depletion of Tob55/Sam50 from yeast cells, significantly reduced amounts of Tob38/Sam35/Tom38 were detected in the mitochondria and no accumulation of Tob38/Sam35/Tom38 in the cytosolic fractions was observed (unpublished data). This observation suggests that Tob55/Sam50 is most likely the docking site on the outer membrane for newly synthesized Tob38/Sam35/Tom38 molecules. In the absence of this docking site Tob38/Sam35/Tom38 molecules were degraded in the cytosol.

2.1.5.4. How does the TOB/SAM complex mediate its function? At present, one can only speculate how the TOM/SAM complex might function. Tob55/Sam50 is predicted to be a 12-stranded β-barrel with an N-terminal extension in the intermembrane space. This domain, the POTRA domain, is suggested to have chaperone-like qualities. The POTRA domain of Tob55/Sam50 is involved in recognition and binding of the unfolded β-barrel precursors which emerge from the TOM complex. At this stage, the precursors might already start to fold and are accessible to different folding catalysts of the intermembrane space (Fig. 5.3, step 2). In the absence of this domain, the translocation across the TOM complex and the subsequent membrane integration of β-barrel precursors is impaired (Habib *et al.*, 2007). Thus, it appears that the translocation of β-barrel precursor proteins across the outer membrane and their recognition by the TOB/SAM complex are coupled processes. Currently, the molecular mechanism of this coupling is not clear.

Electron microscopy analysis of recombinant and purified Tob55/Sam50 revealed ring-shaped assemblies with an outer diameter of ~15 nm and an inner diameter of ~7–8 nm. The central pore measured ~4–5 nm and should be large enough to accommodate a β-barrel of 16–22 β-strands (Paschen *et al.*, 2003). Therefore, two possibilities for TOB/SAM-mediated membrane insertion seem likely: (1) the TOB/SAM-mediated membrane integration of β-barrel proteins involves initial insertion of newly synthesized precursors into the pore and then lateral release into the lipid phase of the membrane; (2) the Tob55/Sam50 subunit serves as a scaffold for the β-barrel precursors. In such a mechanism, assembly can follow a concerted partitioning of the bulky β-barrel into the membrane. As the first mechanism would require major structural rearrangements of the β-barrel and

disruption of many hydrogen bonds (Gabriel *et al.*, 2001), we favor the second alternative.

Why is the biogenesis of the β-barrel proteins so complex? When the amino acid sequence of Tob55/Sam50 was determined, it became immediately clear that there are homologues of Tob55/Sam50 in Gram-negative bacteria. Depletion of the bacterial homologue, Omp85 in *N. meningitides* and later in *E. coli* led to accumulation of β-barrel membrane proteins in the periplasm and loss of cell viability (Voulhoux *et al.*, 2003; Wu *et al.*, 2005). Thus, Omp85 appears to be essential for the insertion of β-barrel proteins into the bacterial outer membrane. This function has apparently been conserved during evolution of mitochondria from bacteria. In bacteria, β-barrel membrane proteins are first secreted by the Sec machinery into the periplasm and then inserted into the outer membrane. In mitochondria, these proteins are produced in the cytosol as their genes have been transferred from the endosymbiont's genome into the nucleus. Therefore, these precursors see the mitochondrial outer membrane from the "wrong" side. Thus, they have to be translocated first to the other side of the outer membrane from which they can be inserted by the TOB/SAM complex that has been inherited from the prokaryotic ancestors. Membrane insertion of these β-strand proteins into lipid membranes represents a protein folding reaction of considerable complexity which was likely optimized during evolution of prokaroytic life. Apparently, it was easier during evolution to make the precursors use the TOM translocation machinery rather than to develop a novel insertion pathway starting at the cytosolic surface. Thus, the insertion pathway of β-barrel proteins is an example of "conservative sorting" and a reflection of the evolutionary origin of the mitochondria.

2.1.5.5. Topogenesis of the mitochondrial β-barrel protein Tom40 The import pathway of Tom40 has been studied in some detail. It can serve as a paradigm since its topogenesis can be followed up to its assembly into the well-characterized TOM complex. The initial steps of import of precursors of Tom40 involve the TOM complex (Keil *et al.*, 1993; Model *et al.*, 2001; Rapaport and Neupert, 1999). Recent studies allowed the dissection of the complex import pathway into different stages like targeting, translocation, and membrane insertion. It was demonstrated that the Tom40 precursor traverses the TOM complex via the general import pore after interaction with both import receptors. For example, blocking the TOM channel by arresting matrix-targeted precursors within the TOM translocation pore, impaired the import of β-barrel proteins suggesting that both types of precursor proteins compete on the same TOM elements (Paschen *et al.*, 2003; Wiedemann *et al.*, 2003). Next, the precursor of Tom40 is released into the intermembrane space before it is passed on to the TOB/SAM complex. Evidence for a passage through the intermembrane space was

obtained among others by demonstrating the involvement of the small Tim protein complexes, Tim9–Tim10 and Tim8–Tim13, in the biogenesis of β-barrel proteins (Habib *et al.*, 2005; Hoppins and Nargang, 2004; Wiedemann *et al.*, 2004). These small proteins were suggested to function as general chaperones for precursors of the hydrophobic carrier proteins on their passage from the TOM complex to the TIM22 complex in the inner membrane (see below). It is envisaged that the small Tim proteins play a similar role in the biogenesis of β-barrel proteins by guiding them through the intermembrane space.

Although β-barrel precursors are exposed to the intermembrane space on their insertion route, when these proteins were imported into Tob55/Sam50-depleted mitochondria, they did not accumulate as import intermediates in the intermembrane space. This suggests that the translocation across the outer membrane via the TOM complex is probably coupled to the interaction with the TOB/SAM complex. Furthermore, it was shown that the interaction of β-barrel precursor proteins with the TOM complex is required for their proper and efficient recognition by the TOB/SAM complex. (Habib *et al.*, 2007).

The import of Tom40 involves a number of further intermediate stages after transfer into the intermembrane space. These were characterized by the determination of their apparent native molecular masses, their kinetics of appearance and disappearance, and subunits composition (Model *et al.*, 2001; Rapaport and Neupert, 1999). The discovery of the TOB/SAM complex allowed detailed characterization of these intermediates. An early intermediate of the fully imported Tom40 has an apparent molecular mass of around 250–300 kDa and represents a Tom40 precursor interacting with the TOB/SAM complex. Comparable intermediates were also shown for other β-barrel proteins (Hoppins *et al.*, 2007; Ishikawa *et al.*, 2004; Milenkovic *et al.*, 2004; Paschen *et al.*, 2003; Waizenegger *et al.*, 2004; Wiedemann *et al.*, 2003). *Mim1/Tom13*, a small integral outer membrane protein, was shown to be involved in the assembly process of the Tom40 precursor in a step downstream of the 250 kDa intermediate. Mim1/Tom13 was found to be required after the interaction of the Tom40 precursor with the TOB/SAM complex (Ishikawa *et al.*, 2004; Waizenegger *et al.*, 2005). The 250 kDa intermediate is followed by an intermediate of $\sim$100 kDa, which probably consists of a Tom40 dimer and may also contain Tom5 (Model *et al.*, 2001). This intermediate apparently has undergone insertion into the lipid bilayer of the outer membrane and appears to be an assembly intermediate that eventually leads to the formation of a new TOM complex. Mdm10 appears to play an undefined role in the subsequent assembly stages of Tom40 precursor (Meisinger *et al.*, 2004). The final assembly stages of Tom40 involve interactions with Tom6, Tom7, Tom22, and Tom20 but these steps have not been resolved so far.

2.2. Translocation across and into the inner membrane

2.2.1. The TIM23 complex

The TIM23 complex is one of two import complexes of the inner membrane and the major translocase for precursor proteins. It facilitates mitochondrial import of presumably all matrix proteins and of most inner membrane proteins. The TIM23 complex consists of two different parts: (1) four membrane-embedded subunits which are critical for the recognition of precursor proteins and the formation of a membrane potential-triggered translocation pore; (2) a number of matrix proteins which are more or less tightly associated with the translocation pore and which constitute the import motor that drives protein translocation in an ATP-dependent process.

2.2.1.1. The membrane sector of the TIM23 complex The membrane sector of the TIM23 complex is composed of four subunits: Tim17, Tim21, Tim23, and Tim50. Tim17 and Tim23 are multispanning membrane proteins which form the central translocation pore of the TIM23 complex. Tim21 and Tim50 are anchored to the inner membrane and expose soluble domains into the intermembrane space which are critical for the interaction with the TOM complex and with incoming preproteins (Emtage and Jensen, 1993; Geissler *et al.*, 2002; Maarse *et al.*, 1994; Mokranjac *et al.*, 2003a; Ryan *et al.*, 1998; Yamamoto *et al.*, 2002).

Tim23 consists of a hydrophobic core domain embedded in the inner mitochondrial membrane and an N-terminal hydrophilic domain localized in the intermembrane space. This intermembrane space domain comprises roughly 100 amino acid residues and features a bipartite structure. The N-terminal half of this hydrophilic domain was found to penetrate the outer membrane (Donzeau *et al.*, 2000) thereby positioning the TIM23 complex in close proximity to the outer membrane (Vogel *et al.*, 2006). The C-terminal half of the intermembrane space domain of Tim23 was shown to be responsible for the dimerization of Tim23 and for the recognition of presequences (Bauer *et al.*, 1996). The hydrophobic region of the protein consists of four membrane spanning domains and has the ability to form voltage-gated pores in artificial membranes (Truscott *et al.*, 2001). Tim23 is essential for the formation of the translocation channel which presumably consists of a twin pore structure of two equally sized pores which open and close cooperatively (Lohret *et al.*, 1997; Martinez-Caballero *et al.*, 2007).

Tim23 is directly associated in a 1:1 stoichiometry with *Tim17* via hydrophobic interactions (Blom *et al.*, 1995; Moro *et al.*, 1999; Ryan *et al.*, 1998). Within their hydrophobic domains, Tim23 and Tim17 share 46% sequence identity but despite this high degree of sequence conservation, these domains are not functionally exchangeable. Whether Tim17 is directly involved in channel formation is not clear but it plays an essential

role in the regulation of the pore (Martinez-Caballero *et al.*, 2007). Ionic interactions between charged residues on the intermembrane space side of Tim17 appear to control pore opening (Meier *et al.*, 2005a). It was speculated that binding of positively charged residues in mitochondrial presequences interferes with these ionic interactions and thereby triggers the opening of the translocation channel.

Like Tim17 and Tim23, *Tim50* is essential for viability in yeast. Tim50 does not directly contribute to the formation of the import channels but is critical for their regulation (Chacinska *et al.*, 2003; Geissler *et al.*, 2002; Meinecke *et al.*, 2006; Mokranjac *et al.*, 2003a; Yamamoto *et al.*, 2002). Upon fractionation of mitochondria, only a fraction of Tim50 can be recovered with the TIM23 complex suggesting a dynamic association of Tim50 with the other subunits of the TIM23 translocase. Tim50 exposes a large C-terminal domain into the intermembrane space which is critical for the interaction of the TIM23 translocase with the TOM complex (Chacinska *et al.*, 2003) and for the closure of TIM23 channels in the absence of preproteins (Meinecke *et al.*, 2006). Thus, Tim50 might serve as regulator of the import channel which allows the dynamic formation of import pores as soon as presequences emerge from the TOM channel.

The fourth component of the membrane sector, *Tim21*, was only recently identified (Chacinska *et al.*, 2005; Mokranjac *et al.*, 2005). Like Tim50, Tim21 contains an N-terminal membrane anchor and a soluble domain in the intermembrane space. It dynamically interacts with the TIM23 complex and with the Tom22 subunit of the TOM complex. It was proposed that Tim21 thereby is critical for the communication between both translocases and serves as an important regulator of the translocation machinery of both membranes. The deletion of Tim21, however, does not cause significant growth defects and Tim21 therefore is dispensable for protein translocation per se. In addition, Tim21 might interact with the bc_1 complex of the respiratory chain. The recruitment of this proton pumping enzyme might ensure a high local membrane potential at the import site to allow efficient translocation even under nutrition-limiting conditions (van der Laan *et al.*, 2006).

2.2.1.2. The import motor The membrane sector of the TIM23 complex forms a voltage-gated pore which, in principle, is sufficient for the translocation of positively charged presequences across the inner membrane in a strictly membrane potential-driven process (van der Laan *et al.*, 2007). However, the translocation of most matrix and inner membrane proteins requires the activity of the import motor. The import motor complex is composed of at least six subunits: Tim44, Tim16/Pam16, Tim14/Pam18, Pam17, mtHsp70, and Mge1.

The yeast *mtHsp70* is essential for cell viability. It was linked to the mitochondrial import machinery almost two decades ago when the protein

was shown to transiently bind precursor proteins (Craig *et al.*, 1987; Kang *et al.*, 1990; Manning-Krieg *et al.*, 1991; Scherer *et al.*, 1990). mtHsp70 recognizes incoming polypeptides as soon as they emerge on the matrix side and its binding prevents their backsliding across the inner membrane. The binding to substrates releases mtHsp70 from the TIM23 complex so that the Brownian motion of the incoming polypeptides promotes their further translocation into the matrix. Sequential rounds of mtHsp70 binding will finally complete the import reaction. It was speculated that mtHsp70 might undergo a conformational switch in order to actively pull the polypeptides in but evidence for this ''pulling hypothesis'' is still lacking (Neupert and Brunner, 2002).

A tight association of mtHsp70 with the TIM23 complex is essential for efficient trapping of the incoming polypeptides. The recruitment of mtHsp70 to the TIM23 complex is mediated by the *Tim44* subunit. This membrane-associated matrix protein functions as a docking site for mtHsp70 on the TIM23 complex (Horst *et al.*, 1993; Maarse *et al.*, 1992; Scherer *et al.*, 1992; Schneider *et al.*, 1994).

Substrate binding and release by Hsp70 chaperones is regulated by DnaJ-like proteins (Walsh *et al.*, 2004). The substrate binding of Tim44-bound mtHsp70 is regulated by a DnaJ subunit of the import motor, *Tim14/Pam18* (D'Silva *et al.*, 2003; Mokranjac *et al.*, 2003b; Truscott *et al.*, 2003). Tim14/Pam18 forms a subcomplex together with *Tim16/Pam16*, the second DnaJ-like subunit of the import motor. In contrast to Tim14/Pam18, Tim16/Pam16 lacks the essential histidine-proline-aspartate signature of the active center and therefore is unable to stimulate the ATPase activity of mtHsp70 (D'Silva *et al.*, 2005; Frazier *et al.*, 2004; Kozany *et al.*, 2004). Tim14/Pam18 and Tim16/Pam16 form a 1:1 complex in which both subunits might alternatively interact with mtHsp70 and thereby switch between an activating and inhibiting state. The relevance of the inhibiting state induced by Tim16/Pam16 is not clear but it was speculated that Tim16/Pam16 prevents the unproductive idling of the import motor (Mokranjac *et al.*, 2006; Slutsky-Leiderman *et al.*, 2007).

Another essential component of the import motor is *Mge1*, a soluble matrix protein which transiently binds to mtHsp70. Mge1 functions as nucleotide exchange factor which stimulates the release of ADP from mtHsp70 to allow its conversion into the ATP-bound state (Bolliger *et al.*, 1994; Ikeda *et al.*, 1994; Laloraya *et al.*, 1994). The most recently identified subunit of the import motor is *Pam17*. Pam17 is not essential for protein import and its functional role in the import process is not clear yet.

Five of the subunits of the TIM23 complex were only discovered during the last five years (Tim50, Tim21, Tim16/Pam16, Tim14/Pam18, and Pam17) and it seems likely that more subunits which interact permanently or transiently with the TIM23 translocase will be identified in the next years.

2.2.1.3. The mechanism of TIM23-mediated translocation The TIM23 complex is responsible for the translocation of proteins with matrix-targeting sequences. These sequences are initially recognized by receptors of the TOM complex and transported through the TOM pore across the outer membrane after which they interact with the intermembrane space domains of Tim50 and Tim23. Tim21 presumably facilitates the release from binding sites at the TOM complex (Fig. 5.4, step 1). Thereby precursor proteins are directed to the translocation pore formed by Tim17 and Tim23. Concomitantly, the positively charged presequences interact with Tim17, which is responsible for the voltage gating of the translocation pore of the TIM23 complex. This interaction triggers the opening of the protein-conducting channel followed by the translocation of the presequences across the inner membrane in a *membrane potential-dependent* manner (Fig. 5.4, step 2). Following its translocation into the matrix, the

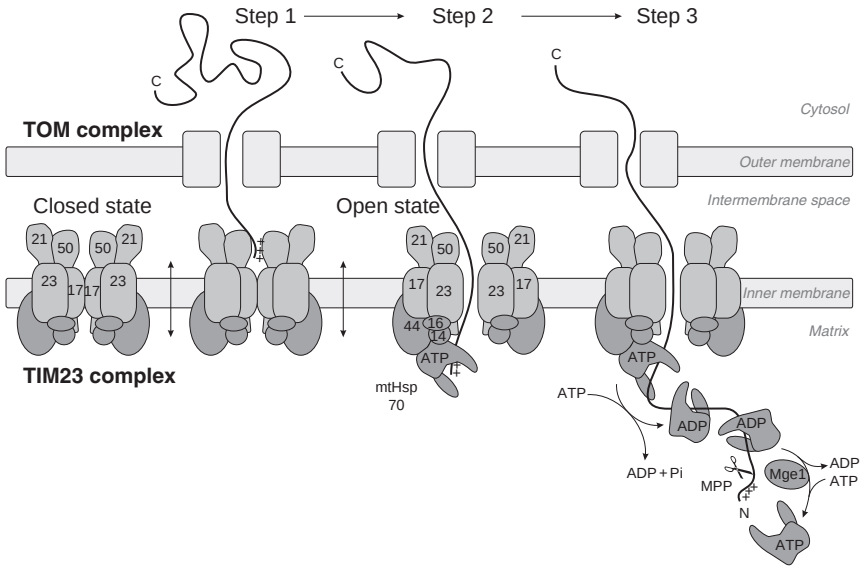

Figure 5.4 TIM23-mediated protein translocation across the inner mitochondrial membrane. In a first step, incoming polypeptides contact the intermembrane space domains of the TIM23 complex and are directed to the protein-conducting channel (step 1). This triggers the opening of the voltage-gated TIM23 pore and initiates the translocation of presequences in a membrane-potential ($\Delta\Psi$)-dependent manner (step 2). Following passage of presequences across the TIM23 channel, sequential ATP-dependent interactions with mtHsp70 chaperones drive the translocation of the entire preprotein into the matrix. The presequences are cleaved by the mitochondrial processing peptidase (MPP) and the mature proteins can fold into their native functional conformations (step 3). Membrane subunits of the TIM23 complex are depicted in medium gray. Components of the motor complex are shown in dark gray. The TOM complex is depicted as a light gray box.

presequences are bound by mtHsp70 which is positioned at the protein-conducting channel by Tim44. The DnaJ-like subunits, Tim16/Pam16 and Tim14/Pam18, coordinate the binding of the polypeptides by mtHsp70. mtHsp70 is maintained at the TIM23 complex in the ATP-bound form which represents the open state that allows efficient binding to incoming polypeptides. Upon precursor binding, mtHsp70 dissociates from Tim44 and ATP is converted to ADP leading to a tight association of the chaperone to substrates. The bound chaperone prevents the backsliding of polypeptides in the import pore whereas it can move inward due to the Brownian motion. Subsequent cycles of binding and release of mtHsp70 then lead to the complete translocation of the polypeptide chain into the matrix in an *ATP-dependent* manner (Fig. 5.4, step 3). Concomitantly with the translocation of the polypeptide chains, the presequences are cleaved by the mitochondrial processing peptidase (MPP). The nucleotide exchange factor Mge1 stimulates the exchange of ADP for ATP in mtHsp70 and thereby causes the opening of the substrate-binding cleft of the chaperone. Thus, upon release of the mtHsp70 proteins, the imported proteins can fold into their final conformations.

2.2.2. Insertion pathways of inner membrane proteins

The inner membrane harbors a large number of integral membrane proteins of different topologies. These proteins are inserted basically via three distinct pathways which are sketched in Fig. 5.5:

The Stop-Transfer Pathway: Most single spanning membrane proteins reach the inner membrane by the stop-transfer route which is a deviation of the TIM23-mediated import pathway. These proteins are first arrested in the TIM23 complex (stop) and then laterally released into the lipid bilayer of the inner membrane (transfer).

The Conservative Sorting Pathway: Multispanning membrane proteins can be inserted via the conservative sorting pathway which also employs the TIM23 complex. In this case, however, the proteins are initially imported into the matrix and insert from the matrix into the inner membrane. Since the direction of protein insertion resembles that in the prokaryotic ancestors of mitochondria, this route is called conservative.

TIM22-Mediated Insertion Pathway: Members of the carrier proteins and some Tim subunits have pairs of transmembrane domains which are inserted in a loop-like fashion into the inner membrane by the TIM22 translocase. The insertion takes place from the intermembrane space and appears to be restricted to substrates which are not of prokaryotic origin.

2.2.3. Insertion via the TIM23 complex—The Stop Transfer Pathway

2.2.3.1. Import of single-spanning membrane proteins of N_{in}–C_{out} topology All inner membrane proteins that are synthesized with matrix-targeting signals are targeted to the TIM23 complex. Substrates of the

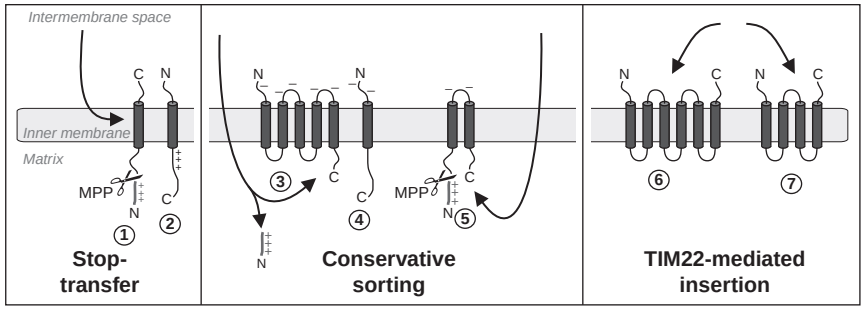

Figure 5.5 Topogenesis of inner membrane proteins. Proteins are directed to the inner membrane via three distinct pathways. *The Stop-Transfer Pathway*: Many single-spanning inner membrane proteins are imported by matrix-targeting signals and deviate from the general import pathway at the level of the TIM23 complex. Members of this group can adopt both N_{in}–C_{out} and N_{out}–C_{in} topologies (examples 1 and 2, respectively). *The Conservative Sorting Pathway*: Complex polytopic inner membrane proteins can also be synthesized with matrix targeting signals. These direct the proteins initially into the matrix from where they integrate into the inner membrane (examples 4 and 5). This integration step resembles the membrane insertion in bacteria. *The TIM22-Mediated Insertion Pathway*: Carrier proteins (example 6) and some Tim subunits (example 7) do not contain matrix targeting signals and are imported by an alternative inner membrane translocase, the TIM22 complex. Insertion occurs from the intermembrane space in a membrane-potential-dependent manner.

Stop-Transfer Pathway contain one internal transmembrane domain which is arrested at the level of the TIM23 translocase and inserted into the inner membrane (Daum *et al.*, 1982; Van Loon and Schatz, 1987). Most substrates of this mode of protein insertion are inner membrane proteins with an N_{in}–C_{out} topology (Fig. 5.5, example 1). The import process of a number of stop-transfer proteins was studied in some detail and the presumably best characterized example is subunit 5a of the cytochrome oxidase (*Cox5a*) of *Saccharomyces cerevisiae*. Like matrix-targeted proteins, Cox5a has a presequence and is imported by the cooperative action of the TOM and the TIM23 complexes. Cox5a is inserted into the inner membrane during protein import, a process which was found to depend on its hydrophobic TMS (Glaser *et al.*, 1988, 1990; Meier *et al.*, 2005b). The import of Cox5a depends on the membrane potential and successive cycles of mtHsp70 binding and release driven by ATP hydrolysis, basically like that of soluble matrix proteins. When the matrix domain of Cox5a was deleted so that the TMS followed shortly after the matrix-targeting sequence, mtHsp70 and ATP became dispensable for import and insertion of Cox5a (Miller and Cumsky, 1993). In this case insertion might irreversibly trap the protein in the inner membrane, efficiently preventing a backsliding out of the mitochondria. It is assumed that lateral movements of the polypeptide driven by Brownian motion finally pull the C-terminal domain completely into the intermembrane space. However, experimental evidence for such a

sliding-driven import reaction is still lacking. Further examples of stop-transfer proteins of N_{in}–C_{out} topology are *Cbp4*, *Cox11*, *Cyc2*, *Dld1*, *Hem14*, the *MWFE* subunit of complex I, *Mia40*, *Oms1*, *Tim21*, *Tim50*, *Yme1*, and *Yme2*.

2.2.3.2. Sorting signals in stop-transferred proteins Not all hydrophobic stretches serve as arresting signals since conservatively sorted proteins are translocated into the matrix of mitochondria despite the presence of hydrophobic transmembrane domains. Detailed analysis of the arrested and transferred membrane proteins suggested that at least three different characteristics of these proteins are responsible for their respective sorting at the level of the TIM23 complex.

First of all, the *length and hydrophobicity* of the transmembrane segments plays a critical role for protein sorting. Detailed experiments with variants of D-lactate dehydrogenase (Dld1) showed that shortening of the TMS favors a mis-sorting of this stop-transferred protein into the matrix, whereas an extension improved the arrest of the protein (Rojo *et al.*, 1998). Moreover, the insertion of hydrophilic residues into hydrophobic arresting signals strongly perturbs their sorting by the TIM23 complex (Beasley *et al.*, 1993). In comparison to conservatively sorted proteins, transmembrane domains arrested at the level of the inner membrane show in general a higher degree of hydrophobicity which contributes to a stop in protein translocation (Meier *et al.*, 2005b). It should be noted that transmembrane domains of conservatively sorted proteins are still uncharged and hydrophobic; two crucial requirements in order to be tolerated in the lipid bilayer of the inner membrane. However, they are enriched in moderately hydrophobic residues like alanine, glycine, or serine and show a relative low content of valine, leucine, and isoleucine residues (Meier *et al.*, 2005b).

Second, proteins sorted by the stop-transfer mechanism often have *clusters of charged amino acid residues C-terminally to their hydrophobic domain*. These charged clusters were initially found for Dld1 but are also present in Sco1, Sco2, and Cox5a which are also inserted into the inner membrane by the stop-transfer mechanism (Rojo *et al.*, 1998). In the case of Dld1, these charges are essential to arrest the translocation of the imported protein. Whether these clusters are recognized by the TIM23 complex or physically slow down the translocation due to their stronger hydration is not known.

The third determinant for a stop-transfer signal is the *absence of proline residues* in the transmembrane domain. Many hydrophobic domains of conservatively sorted proteins contain proline residues which were shown by mutation experiments to actively counteract their insertion by the TIM23 translocase (Meier *et al.*, 2005b). The TIM23 complex might have a preference to insert straight helical structures into the inner membrane

which are not formed when proline residues are placed in central regions of the hydrophobic domain.

2.2.3.3. Import of single-spanning membrane proteins of N_{out}–C_{in} topology Some membrane proteins of N_{out}–C_{in} topology (Fig. 5.5, example 2) also use the stop-transfer mechanism to integrate into the inner membrane. Experimentally characterized examples are *Bcs1*, *Tim14/Pam18*, and *Mdj2* (Fölsch *et al.*, 1996; Mokranjac *et al.*, 2003b; Westermann and Neupert, 1997). Bcs1 is an AAA ATPase with one transmembrane span and a large N-terminal domain exposed to the intermembrane space. The integral membrane domain is C-terminally followed by a short positively charged, amphipathic segment which was found to function as an internal mitochondrial targeting signal. It was suggested that the internal targeting signal together with the TMS forms a hairpin-like loop which mimicks the structural features of a matrix-targeting sequence with one hydrophobic and one positively charged side. This signal integrates into the TIM23 translocase and upon further translocation of the C-terminal hydrophilic segment, the protein acquires its N_{out}–C_{in} topology (Fölsch *et al.*, 1996). Thus, these proteins presumably are imported by the same principles as stop-transferred proteins which expose their N-termini to the matrix.

2.2.4. Insertion via the Conservative Sorting Pathway

Presumably as a consequence of their prokaryotic origin, mitochondria insert many proteins from the matrix into the inner membrane in a process which resembles protein insertion in bacteria (Fig. 5.5, examples 3–5) (Hartl *et al.*, 1986). Well-characterized examples that employ the conservative insertion pathway are *Oxa1* (Herrmann *et al.*, 1997) and *Cox18/Oxa2* (Funes *et al.*, 2004) (Fig. 5.5, example 3), *subunit 9* of the F_0F_1-ATPase of *N. crassa* (Rojo *et al.*, 1995, 1999) (Fig. 5.5, example 4), and *Mrs2* and *Yta10* (Baumann *et al.*, 2002) (Fig. 5.5, example 5). These proteins are synthesized with matrix-targeting signals. They reach the inner membrane in a two-step process: First, they are imported into the mitochondrial matrix through the protein-conducting channels of the TOM and TIM23 complexes. Some of the proteins even accumulate transiently in the matrix as sorting intermediates which are bound by mtHsp70. The association with this chaperone prevents the aggregation of these hydrophobic polypeptides. In a second step, the proteins are inserted from the matrix side into the inner membrane (Fig. 5.6A). The process by which these proteins integrate into the membrane is not well understood. Efficient insertion of some proteins requires the inner membrane protein Oxa1 which plays a crucial role in membrane insertion of mitochondrial translation products (see below). However, Oxa1 is dispensable for a number of conservatively sorted proteins like Mrs2 or Yta10 and it is completely unclear how these proteins make their way into the inner membrane. The insertion reaction appears to be

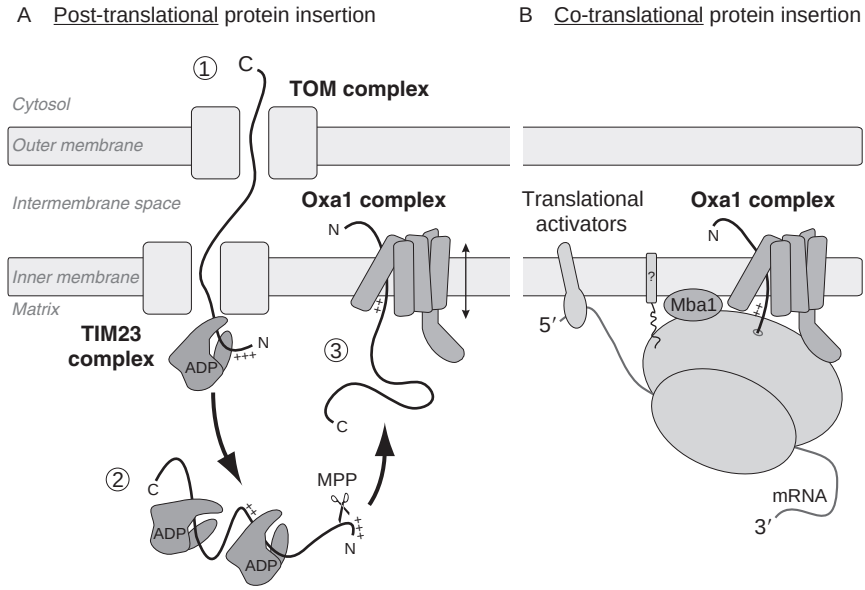

Figure 5.6 Oxa1-dependent protein insertion. The Oxa1 complex of the inner membrane facilitates the insertion of two groups of inner membrane proteins. First, nuclear encoded proteins that use the *Conservative Sorting Pathway* can embark on an Oxa1-mediated insertion route to insert from the matrix into the inner membrane in a post-translational manner (Part A). Second, mitochondrial translation products interact with the Oxa1 complex and are integrated by Oxa1 into the inner membrane in a *co-translational insertion* reaction (Part B). Oxa1 not only facilitates the membrane insertion of nascent chains but also directly recruits ribosomes to the insertion sites at the inner membrane. The membrane association of mitochondrial ribosomes is further stabilized by additional tethering factors like Mba1, Mdm38, and Cox11. In addition, the binding of mitochondrial mRNAs to membrane-associated translational activators might contribute to the recruitment of translation complexes to the inner membrane. Substrates of the post-translational and co-translational insertion adhere to the ''positive inside rule,'' and especially negatively charged regions are translocated to the intermembrane space side of the membrane. For simplicity, the TOM and TIM23 complexes are depicted as light gray boxes.

driven by the membrane potential and relatively high potentials are required for efficient insertion. For example, under moderate membrane potential values, like when protein import reactions are carried out *in vitro* in the absence of NADH, conservatively sorted proteins are still efficiently imported into the matrix but accumulate there until the membrane potential is further increased (Herrmann *et al.*, 1995, 1997). The domains which are translocated from the matrix into the intermembrane space show consistently a negative net charge and the translocation reaction therefore is presumably *driven by an electrophoretic effect* (Herrmann and Bonnefoy, 2004).

2.2.5. Insertion via the TIM22 complex

The TIM22 translocase represents the second protein import complex in the inner membrane. This complex is responsible for the insertion of a number of polytopic membrane proteins, namely of members of the carrier family and of Tim subunits (Fig. 5.5, examples 6 and 7). It does not mediate the import of proteins into the matrix of mitochondria. Substrates of this pathway do not carry matrix-targeting signals but rather more complex targeting signatures that are scattered along the entire sequence.

2.2.5.1. The architecture of the TIM22 complex The TIM22 complex consists of the three membrane proteins Tim22, Tim54, and Tim18 and of the three hydrophilic intermembrane space proteins Tim9, Tim10, and Tim12 (Fig. 5.7). *Tim22* is sequence related to Tim17 and Tim23 (Sirrenberg *et al.*, 1996). It represents the essential core of the complex that can mediate the insertion of carrier proteins even in the absence of Tim54 and Tim18 although at strongly reduced levels. Tim22 forms oligomers, presumably dimers, which function as voltage-activated and signal-gated channels (Kovermann *et al.*, 2002). These Tim22 channels are ''twin pores,'' that is, they consist of two neighboring pores which might merge to

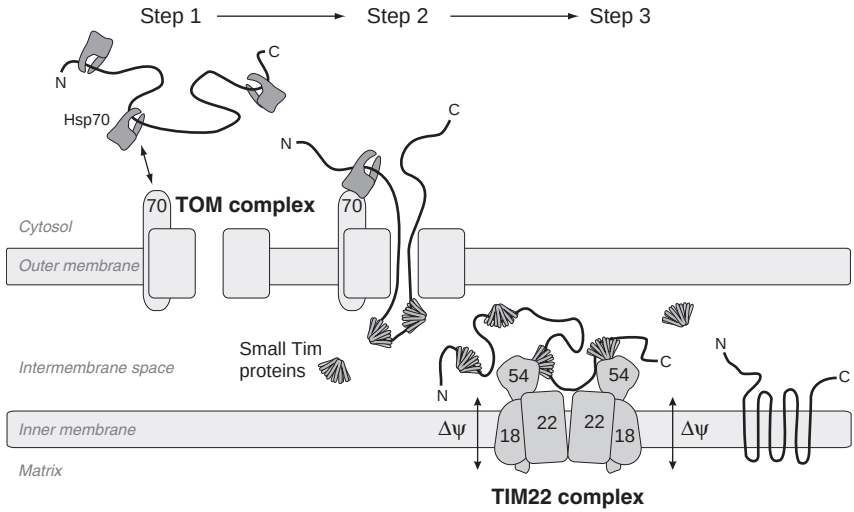

Figure 5.7 **TIM22-mediated protein insertion. Carrier proteins as well as several Tim subunits are synthesized in the cytosol. They are recognized by chaperones (Hsp70, Hsp90) and escorted to the import receptor Tom70 of the TOM complex (step 1). The precursor proteins traverse the TOM pore presumably in a hairpin-like conformation (step 2). Bound by small Tim proteins, the carrier proteins are further transported to the TIM22 complex which mediates their final insertion into the inner membrane in a reaction which depends on the membrane potential (ΔΨ) (step 3). For simplicity, the TOM complex is depicted as a light gray box.**

one joint channel during the translocation process. How this channel mediates the lateral insertion of its substrates is not known.

Tim54 and *Tim18* are important, though not essential, components of the TIM22 complex and their precise functions are not clear (Kerscher *et al.*, 1997, 2000; Koehler *et al.*, 2000; Kovermann *et al.*, 2002).

A hexameric complex consisting of the *Tim9*, *Tim10*, and *Tim12* is permanently tethered to the intermembrane space side of the TIM22 complex and might play an important role in the perception of the hydrophobic substrate proteins (Murphy *et al.*, 2001; Sirrenberg *et al.*, 1998). Tim9, Tim10, and Tim12 belong to the "small Tim proteins," a family of chaperone-like factors which usher the substrates of the TIM22 complex across the intermembrane space. For more details see recent reviews (Koehler, 2004b; Tokatlidis *et al.*, 2000; Wiedemann *et al.*, 2006).

2.2.5.2. Sorting signals in TIM22 substrates Proteins that employ the Tim22 complex significantly differ from other inner membrane proteins by the fact that they lack matrix-targeting signals and comprise always pairs of transmembrane domains which insert from the intermembrane space side in loop-like modules. Examples of TIM22 substrates are members of the solute carrier family (Fig. 5.5, example 6) and the polytopic Tim subunits Tim17, Tim22, and Tim23 (Fig. 5.5, example 7).

Members of the solute carrier protein family mediate the transport of metabolites, nucleotides, and ions across the mitochondrial inner membrane. Even in the simple eukaryote *S. cerevisiae*, more than 30 different carrier proteins exist in the inner membrane. They are related in sequence and share a common architecture. All members consist of three pairs of transmembrane segments forming sequence-related internal repeats or modules (Endres *et al.*, 1999; Pebay-Peyroula *et al.*, 2003; Wiedemann *et al.*, 2001). The regions around the hydrophobic transmembrane domains are preferentially recognized by the outer membrane receptor Tom70 (Brix *et al.*, 1999, 2000) and by the small Tim proteins in the intermembrane space (Curran *et al.*, 2002; Vasiljev *et al.*, 2004). Each of the modules appears to contribute to these two initial recognition events, and each module might in principle contain the necessary information to be targeted into mitochondria (Brandner *et al.*, 2005; Endres *et al.*, 1999). However, only certain modules appear to contain by themselves sufficient information which allows them to interact productively with the TIM22 complex so that they are inserted into the inner membrane. The molecular nature of the insertion signals that are recognized by the TIM22 complex are only partially known.

2.2.5.3. TIM22-mediated protein insertion The import of carrier proteins into mitochondria was studied in great detail and is covered by a number of excellent review articles (Koehler, 2004a; Neupert and

Herrmann, 2007; Pfanner and Geissler, 2001; Rehling *et al.*, 2004, 2003). The import reactions comprise a series of consecutive steps. Initially, precursors of carrier proteins are targeted to the Tom70 receptor of the TOM complex with help of *cytosolic chaperones*, namely of Hsp90 (in mammals) and Hsp70 (in mammals and fungi) (Young *et al.*, 2003, 2004) (Fig. 5.7, step 1). The precursor proteins further traverse the TOM complex as loops which are bound by hexameric complexes of *small Tim proteins* in the intermembrane space (Fig. 5.7, step 2). Small Tim proteins are polypeptides of 8–12 kDa which are characterized by a central ''twin cysteine-XXX-cysteine'' motif, that is, two short stretches each comprising a pair of cysteine residues which are separated by three amino acid residues. Six of these small proteins form together structures of ~70 kDa in size which form a ring-like core surrounded by the 12 flexible α-helical termini of the subunits (Lu *et al.*, 2004; Vergnolle *et al.*, 2005; Webb *et al.*, 2006). They resemble jellyfish-like structures with 12 flexible tentacles. It was suggested that these arms accommodate the hydrophobic regions of carrier proteins in the intermembrane space thereby shielding them from unproductive interactions (Webb *et al.*, 2006).

After the precursors reached *the membrane-embedded TIM22 complex*, they are inserted into the inner membrane in a *membrane potential-dependent* reaction (Fig. 5.7, step 3). The insertion into the inner membrane is not well understood. Electrophysiological analyses of recombinant Tim22 as well as of purified TIM22 complexes revealed the presence of hydrophilic voltage-dependent channels that respond to the addition of peptides corresponding to internal targeting signals as they are found in carriers. Whereas reconstituted Tim22 on its own forms only one channel, two coupled channels are present in the endogenous TIM22 complex (Peixoto *et al.*, 2007). In agreement with the presence of two channels, electron microscopy of negatively stained TIM22 complexes showed two stain-filled centers. Successful reconstitution experiments with purified TIM22 complexes might path the way to study this insertion process at high spatial and temporal resolution.

3. Mitochondrially Encoded Proteins

Mitochondria contain a strongly reduced genome which encodes a small set of proteins. The number of genes on the mitochondrial genome varies between different eukaryotes: The most complex mitochondrial genomes are found among plants and protists where up to 67 protein-encoding genes are present. The most reduced genomes are present in mitochondria of some parasites like *Plasmodium* where the mitochondrial genome encodes for not more than three proteins (Gray *et al.*, 1999; Lang *et al.*, 1997).

In most animals and fungi, a characteristic set of about one dozen of proteins is encoded in mitochondria which are almost without exception hydrophobic membrane-embedded core subunits of complexes I, III, IV, and V of the respiratory chain. The human mitochondrial genome contains 13 protein-coding genes, that of *S. cerevisiae* only 8 due to the complete loss of complex I in this fungus (Attardi, 1981; Borst, 1977; Borst and Grivell, 1978). It appears likely that the high tendency of these polypeptides to form insoluble aggregates in aqueous environment forced the cell to maintain a translation system that allows their co-translational insertion into the inner membrane. It was indeed shown that at least some of these proteins cannot be imported into mitochondria as they misfold in the cytosol and clog the import channels (Claros *et al.*, 1995).

3.1. Insertion via the Oxa1 complex

Seven of the eight proteins encoded by the mitochondrial genome of *S. cerevisiae* are highly hydrophobic inner membrane proteins (Cox1, Cox2, Cox3 of the cytochrome oxidase, cytochrome *b* of the bc_1 complex and Atp6, Atp8, and Atp9 of the F_1F_0-ATPase). All seven proteins show a characteristic charge distribution: the matrix-exposed segments contain predominately positively charged residues while domains exposed to the intermembrane space carry a negative net charge (Gavel and von, 1992). The same charge distribution was found for proteins of the bacterial inner membrane and—though not as strictly—for the ER (Higy *et al.*, 2004; von Heijne, 1989; White and von Heijne, 2004). In contrast to bacteria and to the ER, membrane insertion in mitochondria is not facilitated by the Sec translocase. Rather, the inner membrane contains a relatively simply structured "protein insertase," the *Oxa1 complex*.

Oxa1 (for *Ox*idase *a*ssembly mutant *1*) was initially identified in two independent genetic studies as a component involved in the assembly of cytochrome oxidase in *S. cerevisiae* (Bauer *et al.*, 1994; Bonnefoy *et al.*, 1994). The protein is conserved throughout the prokaryotic and eukaryotic kingdoms (Yen *et al.*, 2001). All members of the so-called *YidC/Oxa1/Alb3 protein family* are implicated in the insertion of membrane proteins and share a conserved hydrophobic core domain consisting of five transmembrane domains.

The yeast Oxa1 has a molecular mass of 36 kDa. It is an integral protein of the inner membrane with an N_{out}–C_{in} topology which forms homo-oligomeric complexes (Herrmann *et al.*, 1997). Upon solubilization with mild detergents, the Oxa1 complex migrates in size exclusion chromatography with an apparent molecular mass of 200-250 kDa. Taking into account the contribution of the detergent molecules this size is compatible with a homotetrameric structure (Hell *et al.*, 1998; Nargang *et al.*, 2002). Since the contribution of bound lipids is not known, also a smaller, for example, dimeric nature of the Oxa1 complex is possible.

Oxa1 is nuclearly encoded and the protein is sorted into the inner membrane via a conservative sorting route employing the TOM and TIM23 complexes. The insertion of Oxa1 precursor into the inner membrane is strongly stimulated by preexisting Oxa1 complexes in the membrane and, at least *in vitro*, Oxa1-deficient strains fail to insert newly imported Oxa1 protein into the inner membrane (Herrmann *et al.*, 1997).

Oxa1 plays a pivotal role in membrane insertion of mitochondrial translation products (Fig. 5.6 B). In *oxa1* mutants, newly synthesized membrane proteins accumulate unproductively in the matrix and are rapidly degraded (Fiumera *et al.*, 2007; He and Fox, 1997; Hell *et al.*, 1997, 2001; Preuss *et al.*, 2001). However, even in the absence of Oxa1 a basic insertion capacity is still retained, indicating that Oxa1 is not absolutely essential for membrane insertion. How Oxa1 catalyses the insertion of proteins into lipid bilayers is not known. Biochemical studies with the purified bacterial homologue of Oxa1, YidC, showed an activity as ''insertase'' which even in the absence of other components stimulates membrane insertion of simple membrane proteins (Serek *et al.*, 2004; van der Laan *et al.*, 2004).

The synthesis and membrane insertion of mitochondrial translation products are kinetically, and presumably also functionally, coupled. Mitochondria lack signal recognition particles which facilitate co-translational protein insertion in the cytosol of eukaryotes and bacteria and in chloroplasts. Instead, *mitochondrial ribosomes* are physically and, at least in yeast, permanently tethered to the inner membrane. Membrane binding of mitochondrial ribosomes is achieved by several membrane-associated interacting partners of the ribosome. One of these is Oxa1 which binds with its C-terminus the large subunit of the ribosome in close proximity to the polypeptide exit tunnel (Jia *et al.*, 2003; Szyrach *et al.*, 2003). This binding is important to position the site of protein synthesis near the site of membrane insertion, and in addition might facilitate efficient translocation of newly synthesized polypeptides across the membrane by a ''ribosomal pushing'' effect. Direct experimental evidence for such a ribosomal pushing of nascent chains does not exist. However, in contrast to the post-translational insertion of conservatively sorted proteins, the co-translational insertion of nascent chains does not depend on the presence of a membrane potential and therefore must be driven by another energy source. Thus it seems reasonable, that ongoing protein translation generates a kind of pushing force which contributes to the translocation of nascent polypeptides chains across the inner membrane.

In addition to its interaction with Oxa1, the ribosome is bound to the inner membrane by *Mba1*, a membrane-associated matrix protein (Ott *et al.*, 2006; Preuss *et al.*, 2001; Rep and Grivell, 1996). Mba1 also binds to the large subunit and can be efficiently crosslinked to even very short nascent chains. Mba1 collaborates with Oxa1 in the insertion of membrane proteins and the simultaneous deletion of Mba1 and the ribosome-binding domain

of Oxa1 causes severe synthetic defects. Thus, Oxa1 and Mba1 may act in concert to bind and position the ribosome and hence the nascent chain to the site of protein insertion (Ott *et al.*, 2006).

Recently two further membrane anchors of mitochondrial ribosomes were identified, *Mdm38* (Frazier *et al.*, 2006) and *Cox11* (Khalimonchuk *et al.*, 2005). Whether these proteins play a direct role in membrane integration of nascent chains is not known, but they might also contribute to the positioning of mitochondrial ribosomes at the inner membrane.

In addition to its role in membrane insertion, Oxa1 presumably facilitates the *folding and/or assembly of membrane proteins*. For example, Oxa1 forms a complex with newly synthesized Atp9 and stimulates its assembly into the F_1F_0 ATPase (Jia *et al.*, 2007). In humans, Oxa1 might even be permanently associated with the ATPase complex (Stiburek *et al.*, 2007). An assembly role was most convincingly shown for the bacterial Oxa1 homologue YidC which contributes to the post-translational assembly of lactose permease (Nagamori *et al.*, 2004).

Mitochondria contain a close relative of Oxa1, named *Oxa2* or *Cox18*, which might be specialized in a post-translational assembly of the cytochrome oxidase complex (Funes *et al.*, 2004; Saracco and Fox, 2002; Souza *et al.*, 2000). Oxa2/Cox18 lacks the C-terminal ribosome-binding domain and interacts with newly synthesized translation products after they were integrated into the inner membrane by Oxa1 (Funes *et al.*, 2004). The precise molecular function of Oxa2/Cox18 is not yet clear.

3.2. Alternative insertion routes

The Oxa1 complex is critical for the insertion of a number of proteins from the matrix side of the inner membrane. However, several lines of evidence point towards an alternative, Oxa1-independent route for the insertion of other proteins from the matrix side of the inner membrane. First of all, residual activities of the cytochrome bc_1 complex and of the ATPase are still retained in *oxa1*-deficient cells. This observation is in agreement with the fact that only the insertion of Cox2 is strictly Oxa1 dependent, whereas the remaining six mitochondrially encoded inner membrane proteins are inserted even in the absence of Oxa1, albeit less efficient (Hell *et al.*, 2001; Herrmann and Bonnefoy, 2004). In addition, some nuclear encoded proteins like Mrs2 and Yta10 are conservatively sorted even in the absence of Oxa1 (Baumann *et al.*, 2002). Moreover, the subunit Atp8 of the F_1F_0-ATPase can be inserted into inverted inner mitochondrial membrane vesicles *in vitro*. As Atp8 was found to integrate into these vesicles even after protease treatment, it was suggested that Atp8 can insert into the inner membrane even in a ''spontaneous,'' protein-independent manner (Ii and Mihara, 2001). Finally, several Oxa1 suppressor mutants have been identified that rescue a functional respiratory chain in Oxa1 deletion strains

(Hamel *et al.*, 1998; Lemaire *et al.*, 2000, 2004; Saint-Georges *et al.*, 2001). Therefore, Oxa1 can be considered as important but not essential for membrane insertion. Currently, it is not known how this Oxa1-independent protein insertion is mediated and whether so far unidentified insertion components are involved in these reactions.

4. Future Perspectives

Despite the considerable progress in our understanding of the biogenesis of mitochondrial membrane proteins, many open questions remain. The subproteomes of the two mitochondrial membranes are still only partially known. Future studies will be necessary to identify further components of the import machinery, gain detailed insights on the targeting of known substrates, and for the identification of novel substrate proteins. Such knowledge in turn will definitely help us to analyze the various biogenesis pathways of these proteins. In addition, a main current problem is the lack of information on the atomic structures of the translocases which are involved. Determination of these structures will provide an essential foundation for future studies and will allow us to design and perform experiments which will unravel the molecular mechanism of these protein machineries. Much more emphasis has to be attributed to the understanding of the interplay between the mitochondrial proteins and the lipid molecules in which they are embedded. Specifically, it will be important to address the relevance of the specific lipid composition of the mitochondrial membranes for protein targeting and topogenesis. As currently close to nothing is known about the regulation of the targeting and insertion processes, efforts should be made to unravel how cells can adapt the rate of protein translocation into mitochondria to their physiological needs. Finally, future experiments will probably extend our understanding on the correlation between post-translational modification of mitochondrial membrane proteins and their biogenesis.

REFERENCES

Ahting, U., Thun, C., Hegerl, R., Typke, D., Nargang, F. E., Neupert, W., and Nussberger, S. (2006). The TOM core complex: The general protein import pore of the outer membrane of mitochondria. *J. Cell Biol.* **147,** 959–968.

Ahting, U., Thieffry, M., Engelhardt, H., Hegerl, R., Neupert, W., and Nussberger, S. (2001). Tom40, the pore-forming component of the protein-conducting TOM channel in the outer membrane of mitochondria. *J. Cell Biol.* **153,** 1151–1160.

Alconada, A., Kübrich, M., Moczko, M., Hönlinger, A., and Pfanner, N. (1995). The mitochondrial receptor complex: The small subunit Mom8b/Isp6 supports association of

receptors with the general insertion pore and transfer of preproteins. *Mol. Cell. Biol.* **15,** 6196–6205.

Allen, R., Egan, B., Gabriel, K., Beilharz, T., and Lithgow, T. (2002). A conserved proline residue is present in the transmembrane-spanning domain of Tom7 and other tail-anchored protein subunits of the TOM translocase. *FEBS Lett.* **514,** 347–350.

Attardi, G. (1981). Organisation and expression of the mammalian mitochondrial genome: A lesson in economy. *Trends Biochem. Sci.* **6,** 86–89, 100–103.

Bauer, M., Behrens, M., Esser, K., Michaelis, G., and Pratje, E. (1994). *PET1402*, a nuclear gene required for proteolytic processing of cytochrome oxidase subunit 2 in yeast. *Mol. Gen. Genet.* **245,** 272–278.

Bauer, M. F., Sirrenberg, C., Neupert, W., and Brunner, M. (1996). Role of Tim23 as voltage sensor and presequence receptor in protein import into mitochondria. *Cell* **87,** 33–41.

Baumann, F., Neupert, W., and Herrmann, J. M. (2002). Insertion of bitopic membrane proteins into the inner membrane of mitochondria involves an export step from the matrix. *J. Biol. Chem.* **277,** 21405–21413.

Beasley, E. M., Muller, S., and Schatz, G. (1993). The signal that sorts yeast cytochrome b2 to the mitochondrial intermembrane space contains three distinct functional regions. *EMBO J.* **12,** 2303–2311.

Beilharz, T., Egan, B., Silver, P. A., Hofmann, K., and Lithgow, T. (2003). Bipartite signals mediate subcellular targeting of tail-anchored membrane proteins in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **278,** 8219–8223.

Blom, J., Dekker, P., and Meijer, M. (1995). Functional and physical interactions of components of the yeast mitochondrial inner-membrane import machinery (MIM). *Eur. J. Biochem.* **232,** 309–314.

Bolliger, L., Deloche, O., Glick, B. S., Georgopoulos, C., Jeno, P., Kronidou, N., Horst, M., Morishima, N., and Schatz, G. (1994). A mitochondrial homolog of bacterial GrpE interacts with mitochondrial hsp70 and is essential for viability. *EMBO J.* **13,** 1998–2006.

Bonnefoy, N., Chalvet, F., Hamel, P., Slominski, P. P., and Dujardin, G. (1994). *OXA1*, a *Saccharomyces cerevisiae* nuclear gene whose sequence is conserved from prokaryotes to eukaryotes controls cytochrome oxidase biogenesis. *J. Mol. Biol.* **239,** 201–212.

Borgese, N., Brambillasca, S., and Colombo, S. (2007). How tails guide tail-anchored proteins to their destinations. *Curr. Opin. Cell Biol.* **19,** 368–375.

Borst, P. (1977). Structure and function of mitochondrial DNA. *Trends Biochem. Sci.* **2,** 31–34.

Borst, P., and Grivell, L. A. (1978). The mitochondrial genome of yeast. *Cell* **15,** 705–723.

Brandner, K., Rehling, P., and Truscott, K. N. (2005). The carboxyl-terminal third of the dicarboxylate carrier is crucial for productive association with the inner membrane twin-pore translocase. *J. Biol. Chem.* **280,** 6215–6221.

Brix, J., Rudiger, S., Bukau, B., Schneider-Mergener, J., and Pfanner, N. (1999). Distribution of binding sequences for the mitochondrial import receptors Tom20, Tom22, and Tom70 in a presequence-carrying preprotein and a non-cleavable preprotein. *J. Biol. Chem.* **274,** 16522–16530.

Brix, J., Ziegler, G. A., Dietmeier, K., Schneider-Mergener, J., Schulz, G. E., and Pfanner, N. (2000). The mitochondrial import receptor Tom70: Identification of a 25 kDa core domain with a specific binding site for preproteins. *J. Mol. Biol.* **303,** 479–488.

Chacinska, A., Rehling, P., Guiard, B., Frazier, A. E., Schulze-Specking, A., Pfanner, N., Voos, W., and Meisinger, C. (2003). Mitochondrial translocation contact sites: Separation of dynamic and stabilizing elements in formation of a TOM-TIM-preprotein supercomplex. *EMBO J.* **22,** 5370–5381.

Chacinska, A., Lind, M., Frazier, A. E., Dudek, J., Meisinger, C., Geissler, A., Sickmann, A., Meyer, H. E., Truscott, K. N., Guiard, B., Pfanner, N., and Rehling, P. (2005). Mitochondrial presequence translocase: Switching between TOM tethering and motor recruitment involves Tim21 and Tim17. *Cell* **120,** 817–829.

Claros, M. G., Perea, J., Shu, Y. M., Samatey, F. A., Popot, J. L., and Jacq, C. (1995). Limitations to *in vivo* import of hydrophobic proteins into yeast mitochondria—The case of a cytoplasmically synthesized apocytochrome b. *Eur. J. Biochem.* **228,** 762–771.

Coonrod, E. M., Karren, M. A., and Shaw, J. M. (2007). Ugo1p is a multipass transmembrane protein with a single carrier domain required for mitochondrial fusion. *Traffic* **8,** 500–511.

Cory, S., and Adams, J. M. (2002). The Bcl2 family: Regulators of the cellular life-or-death switch. *Nat. Rev. Cancer* **2,** 647–656.

Court, D. A., Kleene, R., Neupert, W., and Lill, R. (1996). Role of the N- and C-termini of porin in import into the outer membrane of *Neurospora* mitochondria. *FEBS Lett.* **390,** 73–77.

Craig, E. A., Kramer, J., and Kosic-Smithers, J. (1987). SSC1, a member of the 70 kDa heat shock protein multigene family of *Saccharomyces cerevisiae*, is essential for growth. *Proc. Natl. Acad. Sci. USA* **84,** 4156–4160.

Curran, S. P., Leuenberger, D., Oppliger, W., and Koehler, C. M. (2002). The Tim9p-Tim10p complex binds to the transmembrane domains of the ADP/ATP carrier. *EMBO J.* **21,** 942–953.

D'Arrigo, A., Manera, E., Longhi, R., and Borgese, N. (1993). The specific subcellular localization of two isoforms of cytochrome b5 suggests novel targeting pathways. *J. Biol. Chem.* **268,** 2802–2808.

Daum, G., Böhni, P. C., and Schatz, G. (1982). Import of proteins into mitochondria: Cytochrome b_2 and cytochrome c peroxidase are located in the intermembrane space of yeast mitochondria. *J. Biol. Chem.* **257,** 13028–13033.

Dekker, P. J., Ryan, M. T., Brix, J., Müller, H., Hönlinger, A., and Pfanner, N. (1998). Preprotein translocase of the outer mitochondrial membrane: Molecular dissection and assembly of the general import pore complex. *Mol. Cell Biol.* **18,** 6515–6524.

Dembowski, M., Kunkele, K. P., Nargang, F. E., Neupert, W., and Rapaport, D. (2001). Assembly of Tom6 and Tom7 into the TOM core complex of *Neurospora crassa*. *J. Biol. Chem.* **276,** 17679–17685.

Donzeau, M., Kaldi, K., Adam, A., Paschen, S., Wanner, G., Guiard, B., Bauer, M. F., Neupert, W., and Brunner, M. (2000). Tim23 links the inner and the outer mitochondrial membranes. *Cell* **101,** 401–412.

D'Silva, P. D., Schilke, B., Walter, W., Andrew, A., and Craig, E. A. (2003). J protein cochaperone of the mitochondrial inner membrane required for protein import into the mitochondrial matrix. *Proc. Natl. Acad. Sci. USA* **100,** 13839–13844.

D'Silva, P. R., Schilke, B., Walter, W., and Craig, E. A. (2005). Role of Pam16's degenerate J domain in protein import across the mitochondrial inner membrane. *Proc. Natl. Acad. Sci. USA* **102,** 12419–12424.

Egan, B., Beilharz, T., George, R., Isenmann, S., Gratzer, S., Wattenberg, B., and Lithgow, T. (1999). Targeting of tail-anchored proteins to yeast mitochondria *in vivo*. *FEBS Lett.* **451,** 243–248.

Emtage, J. L., and Jensen, R. E. (1993). MAS6 encodes an essential inner membrane component of the yeast mitochondrial protein import pathway. *J. Cell Biol.* **122,** 1003–1012.

Endres, M., Neupert, W., and Brunner, M. (1999). Transport of the ADP/ATP carrier of mitochondria from the TOM complex to the TIM22.54 complex. *EMBO J.* **18,** 3214–3221.

Fiumera, H. L., Broadley, S. A., and Fox, T. D. (2007). Translocation of mitochondrially synthesized Cox2 domains from the matrix to the intermembrane space. *Mol. Cell Biol.* **27,** 4664–4673.

Fölsch, H., Guiard, B., Neupert, W., and Stuart, R. A. (1996). Internal targeting signal of the BCS1 protein: A novel mechanism of import into mitochondria. *EMBO J.* **15,** 479–487.

Frazier, A. E., Dudek, J., Guiard, B., Voos, W., Li, Y., Lind, M., Meisinger, C., Geissler, A., Sickmann, A., Meyer, H. E., Bilanchone, V., Cumsky, M. G., *et al.* (2004). Pam16 has an essential role in the mitochondrial protein import motor. *Nat. Struct. Mol. Biol.* **11,** 226–233.

Frazier, A. E., Taylor, R. D., Mick, D. U., Warscheid, B., Stoepel, N., Meyer, H. E., Ryan, M. T., Guiard, B., and Rehling, P. (2006). Mdm38 interacts with ribosomes and is a component of the mitochondrial protein export machinery. *J. Cell Biol.* **172,** 553–564.

Fritz, S., Rapaport, D., Klanner, E., Neupert, W., and Westermann, B. (2001). Connection of the mitochondrial outer and inner membranes by Fzo1 is critical for organellar fusion. *J. Cell Biol.* **152,** 683–692.

Funes, S., Nargang, F. E., Neupert, W., and Herrmann, J. M. (2004). The Oxa2 protein of *Neurospora crassa* plays a critical role in the biogenesis of cytochrome oxidase and defines a ubiquitous subbranch of the Oxa1/YidC/Alb3 protein family. *Mol. Biol. Cell* **15,** 1853–1861.

Gabriel, K., Buchanan, S. K., and Lithgow, T. (2001). The alpha and the beta: Protein translocation across mitochondrial and plastid outer membranes. *Trends Biochem. Sci.* **26,** 36–40.

Gavel, Y., and von, H. G. (1992). The distribution of charged amino acids in mitochondrial inner-membrane proteins suggests different modes of membrane integration for nuclearly and mitochondrially encoded proteins. *Eur. J. Biochem.* **205,** 1207–1215.

Geissler, A., Chacinska, A., Truscott, K. N., Wiedemann, N., Brandner, K., Sickmann, A., Meyer, H. E., Meisinger, C., Pfanner, N., and Rehling, P. (2002). The mitochondrial presequence translocase. An essential role of Tim50 in directing preproteins to the import channel. *Cell* **111,** 507–518.

Gentle, I., Gabriel, K., Beech, P., Waller, R., and Lithgow, T. (2004). The Omp85 family of proteins is essential for outer membrane biogenesis in mitochondria and bacteria. *J. Cell Biol.* **164,** 19–24.

Gentle, I. E., Burri, L., and Lithgow, T. (2005). Molecular architecture and function of the Omp85 family of proteins. *Mol. Microbiol.* **58,** 1216–1225.

Glaser, S., Trueblood, C., Dircks, L., Poyton, R., and Cumsky, M. (1988). Functional analysis of mitochondrial protein import in yeast. *J. Cell Biochem.* **36,** 275–287.

Glaser, S. M., Miller, B. R., and Cumsky, M. G. (1990). Removal of a hydrophobic domain within the mature portion of a mitochondrial inner membrane protein causes its mislocalization to the matrix. *Mol. Cell. Biol.* **10,** 1873–1881.

Gray, M. W., Burger, G., and Lang, B. F. (1999). Mitochondrial evolution. *Science* **283,** 1476–1481.

Habib, S. J., Waizenegger, T., Lech, M., Neupert, W., and Rapaport, D. (2005). Assembly of the TOB complex of mitochondria. *J. Biol. Chem.* **280,** 6434–6440.

Habib, S. J., Neupert, W., and Rapaport, D. (2007). Analysis and prediction of mitochondrial targeting signals. *Methods Cell Biol.* **80,** 761–781.

Hahne, K., Haucke, V., Ramage, L., and Schatz, G. (1994). Incomplete arrest in the outer membrane sorts NADH-cytochrome b5 reductase to two different submitochondrial compartments. *Cell* **79,** 829–839.

Hamajima, S., Sakagushi, M., Mihara, K., Ono, S., and Sato, R. (1988). Both amino- and carboxyterminal portions are required for insertion of yeast porin into the outer mitochondrial membrane. *J. Biochem.* **104,** 362–367.

Hamel, P., Lemaire, C., Bonnefoy, N., Brivet-Chevillotte, P., and Dujardin, G. (1998). Mutations in the membrane anchor of yeast cytochrome c_1 compensate for the absence of Oxa1p and generate carbonate-extractable forms of cytochrome c_1. *Genetics* **150,** 601–611.

Harkness, T. A., Nargang, F. E., van der Klei, I., Neupert, W., and Lill, R. (1994). A crucial role of the mitochondrial protein import receptor MOM19 for the biogenesis of mitochondria. *J. Cell Biol.* **124,** 637–648.

Hartl, F. U., Schmidt, B., Wachter, E., Weiss, H., and Neupert, W. (1986). Transport into mitochondria and intramitochondrial sorting of the Fe/S protein of ubiquinol-cytochrome c reductase. *Cell* **47,** 939–951.

He, S., and Fox, T. D. (1997). Membrane translocation of mitochondrially coded Cox2p: Distinct requirements for export of N and C termini and dependence on the conserved protein Oxa1p. *Mol. Biol. Cell* **8,** 1449–1460.

Hell, K., Herrmann, J., Pratje, E., Neupert, W., and Stuart, R. A. (1997). Oxa1p mediates the export of the N- and C-termini of pCoxII from the mitochondrial matrix to the intermembrane space. *FEBS Lett.* **418,** 367–370.

Hell, K., Herrmann, J. M., Pratje, E., Neupert, W., and Stuart, R. A. (1998). Oxa1p, an essential component of the N-tail protein export machinery in mitochondria. *Proc. Natl. Acad. Sci. USA* **95,** 2250–2255.

Hell, K., Neupert, W., and Stuart, R. A. (2001). Oxa1p acts as a general membrane insertion machinery for proteins encoded by mitochondrial DNA. *EMBO J.* **20,** 1281–1288.

Herrmann, J. M., and Bonnefoy, N. (2004). Protein export across the inner membrane of mitochondria: The nature of translocated domains determines the dependence on the Oxa1 translocase. *J. Biol. Chem.* **279,** 2507–2512.

Herrmann, J. M., Koll, H., Cook, R. A., Neupert, W., and Stuart, R. A. (1995). Topogenesis of cytochrome oxidase subunit II—Mechanisms of protein export from the mitochondrial matrix. *J. Biol. Chem.* **270,** 27079–27086.

Herrmann, J. M., Neupert, W., and Stuart, R. A. (1997). Insertion into the mitochondrial inner membrane of a polytopic protein, the nuclear encoded Oxa1p. *EMBO J.* **16,** 2217–2226.

Higy, M., Junne, T., and Spiess, M. (2004). Topogenesis of membrane proteins at the endoplasmic reticulum. *Biochemistry* **43,** 12716–12722.

Hill, K., Model, K., Ryan, M. T., Dietmeier, K., Martin, F., Wagner, R., and Pfanner, N. (1998). Tom40 forms the hydrophilic channel of the mitochondrial import pore for preproteins. *Nature* **395,** 516–521.

Honlinger, A., Bomer, U., Alconada, A., Eckerskorn, C., Lottspeich, F., Dietmeier, K., and Pfanner, N. (1996). Tom7 modulates the dynamics of the mitochondrial outer membrane translocase and plays a pathway-related role in protein import. *EMBO J.* **15,** 2125–2137.

Hoppins, S. C., and Nargang, F. E. (2004). The Tim8-Tim13 complex of *Neurospora crassa* functions in the assembly of proteins into both mitochondrial membranes. *J. Biol. Chem.* **279,** 12396–12405.

Hoppins, S. C., Go, N. E., Klein, A., Schmitt, S., Neupert, W., Rapaport, D., and Nargang, F. E. (2007). Alternative splicing gives rise to different isoforms of the *Neurospora crassa* Tob55 protein that vary in their ability to insert beta-barrel proteins into the outer mitochondrial membrane. *Genetics* **177,** 137–149.

Horst, M., Jeno, P., Kronidou, N. G., Bolliger, L., Oppliger, W., Scherer, P., Manning, K. U., Jascur, T., and Schatz, G. (1993). Protein import into yeast mitochondria: The inner membrane import site protein ISP45 is the MPI1 gene product. *EMBO J.* **12,** 3035–3041.

Ii, M., and Mihara, K. (2001). Insertion of mitochondrial DNA-encoded F1F0-ATPase subunit 8 across the mitochondrial inner membrane in vitro. *J. Biol. Chem.* **276,** 24704–24712.

Ikeda, E., Yoshida, S., Mitsuzawa, H., Uno, I., and Toh-e, A. (1994). YGE1 is a yeast homologue of *Escherichia coli* grpE and is required for maintenance of mitochondrial functions [published erratum appears in *FEBS Lett.* 1994 Apr 25;**343**(2):181]. *FEBS Lett.* **339,** 265–268.

Isenmann, S., Khew-Goodall, Y., Gamble, J., Vadas, M., and Wattenberg, B. W. (1998). A splice isoform of vesicle-associated membrane protein-1 (VAMP-1) contains a mitochondrial targeting signal. *Mol. Biol. Cell* **9,** 1649–1660.

Ishikawa, D., Yamamoto, H., Tamura, Y., Moritoh, K., and Endo, T. (2004). Two novel proteins in the mitochondrial outer membrane mediate beta-barrel protein assembly. *J. Cell Biol.* **166,** 621–627.

Jia, L., Dienhart, M., Schramp, M., McCauley, M., Hell, K., and Stuart, R. A. (2003). Yeast Oxa1 interacts with mitochondrial ribosomes: The importance of the C-terminal hydrophilic region of Oxa1. *EMBO J.* **22,** 6438–6447.

Jia, L., Dienhart, M. K., and Stuart, R. A. (2007). Oxa1 directly interacts with Atp9 and mediates its assembly into the mitochondrial F1Fo-ATP synthase complex. *Mol. Biol. Cell* **18,** 1897–1908.

Kanaji, S., Iwahashi, J., Kida, Y., Sakaguchi, M., and Mihara, K. (2000). Characterization of the signal that directs Tom20 to the mitochondrial outer membrane. *J. Cell Biol.* **151,** 277–288.

Kang, P. J., Ostermann, J., Shilling, J., Neupert, W., Craig, E. A., and Pfanner, N. (1990). Requirement for hsp70 in the mitochondrial matrix for translocation and folding of precursor proteins. *Nature* **348,** 137–143.

Keil, P., Weinzierl, A., Kiebler, M., Dietmeier, K., Sollner, T., and Pfanner, N. (1993). Biogenesis of the mitochondrial receptor complex. Two receptors are required for binding of MOM38 to the outer membrane surface. *J. Biol. Chem.* **268,** 19177–19180.

Kemper, C., Habib, S. J., Engl, G., Heckmeyer, P., Dimmer, K. S., and Rapaport, D. (2008). The integration of tail-anchored proteins into the mitochondrial outer membrane does not require the known import components. *J. Cell Sci.* doi: 10.1242/jcs.024034.

Kerscher, O., Holder, J., Srinivasan, M., Leung, R. S., and Jensen, R. E. (1997). The Tim54p-Tim22p complex mediates insertion of proteins into the mitochondrial inner membrane. *J. Cell Biol.* **139,** 1663–1675.

Kerscher, O., Sepuri, N. B., and Jensen, R. E. (2000). Tim18p is a new component of the Tim54p-Tim22p translocon in the mitochondrial inner membrane. *Mol. Biol. Cell.* **11,** 103–116.

Khalimonchuk, O., Ostermann, K., and Rödel, G. (2005). Evidence for the association of yeast mitochondrial ribosomes with Cox11p, a protein required for the Cu(B) site formation of cytochrome *c* oxidase. *Curr. Genet.* **47,** 223–233.

Koehler, C. M. (2004a). New developments in mitochondrial assembly. *Annu. Rev. Cell Dev. Biol.* **20,** 309–335.

Koehler, C. M. (2004b). The small Tim proteins and the twin Cx3C motif. *Trends Biochem. Sci.* **29,** 1–4.

Koehler, C. M., Murphy, M. P., Bally, N. A., Leuenberger, D., Oppliger, W., Dolfini, L., Junne, T., Schatz, G., and Or, E. (2000). Tim18p, a new subunit of the TIM22 complex that mediates insertion of imported proteins into the yeast mitochondrial inner membrane. *Mol. Cell Biol.* **20,** 1187–1193.

Kovermann, P., Truscott, K. N., Guiard, B., Rehling, P., Sepuri, N. B., Muller, H., Jensen, R. E., Wagner, R., and Pfanner, N. (2002). Tim22, the essential core of the

mitochondrial protein insertion complex, forms a voltage-activated and signal-gated channel. *Mol. Cell* **9,** 363–373.

Kozany, C., Mokranjac, D., Sichting, M., Neupert, W., and Hell, K. (2004). The J domain-related cochaperone Tim16 is a constituent of the mitochondrial TIM23 preprotein translocase. *Nat. Struct. Mol. Biol.* **11,** 234–241.

Kozjak, V., Wiedemann, N., Milenkovic, D., Lohaus, C., Meyer, H. E., Guiard, B., Meisinger, C., and Pfanner, N. (2003). An essential role of Sam50 in the protein sorting and assembly machinery of the mitochondrial outer membrane. *J. Biol. Chem.* **278,** 48520–48523.

Krimmer, T., Rapaport, D., Ryan, M. T., Meisinger, C., Kassenbrock, C. K., Blachly-Dyson, E., Forte, M., Douglas, M. G., Neupert, W., Nargang, F. E., and Pfanner, N. (2001). Biogenesis of porin of the outer mitochondrial membrane involves an import pathway via receptors and the general import pore of the TOM complex. *J. Cell Biol.* **152,** 289–300.

Künkele, K.-P., Heins, S., Dembowski, M., Nargang, F. E., Benz, R., Thieffry, M., Walz, J., Lill, R., Nussberger, S., and Neupert, W. (1998). The preprotein translocation channel of the outer membrane of mitochondria. *Cell* **93,** 1009–1019.

Laloraya, S., Gambill, B. D., and Craig, E. A. (1994). A role for a eukaryotic GrpE-related protein, Mge1p, in protein translocation. *Proc. Natl. Acad. Sci. USA* **91,** 6481–6485.

Lan, L., Isenmann, S., and Wattenberg, B. W. (2000). Targeting and insertion of C-terminally anchored proteins to the mitochondrial outer membrane is specific and saturable but does not strictly require ATP or molecular chaperones. *Biochem. J.* **349,** 611–621.

Lang, B. F., Burger, G., O'Kelly, C. J., Cedergren, R., Golding, G. B., Lemieux, C., Sankoff, D., Turmel, M., and Gray, M. W. (1997). An ancestral mitochondrial DNA resembling a eubacterial genome in miniature. *Nature* **387,** 493–497.

Lemaire, C., Hamel, P., Velours, J., and Dujardin, G. (2000). Absence of the mitochondrial AAA protease Yme1p restores F_0-ATPase subunit accumulation in an *oxa1* deletion mutant of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **275,** 23471–23475.

Lemaire, C., Guibet-Grandmougin, F., Angles, D., Dujardin, G., and Bonnefoy, N. (2004). A yeast mitochondrial membrane methyltransferase-like protein can compensate for oxa1 mutations. *J. Biol. Chem.* **279,** 47464–47472.

Lithgow, T., Glick, B. S., and Schatz, G. (1995). The protein import receptor of mitochondria. *Trends Biochem. Sci.* **20,** 98–101.

Lohret, T. A., Jensen, R. E., and Kinnally, K. W. (1997). Tim23, a protein import component of the mitochondrial inner membrane, is required for normal activity of the multiple conductance channel, MCC. *J. Cell Biol.* **137,** 377–386.

Lu, H., Allen, S., Wardleworth, L., Savory, P., and Tokatlidis, K. (2004). Functional TIM10 chaperone assembly is redox-regulated *in vivo*. *J. Biol. Chem.* **279,** 18952–18958.

Maarse, A. C., Blom, J., Grivell, L. A., and Meijer, M. (1992). MPI1, an essential gene encoding a mitochondrial membrane protein, is possibly involved in protein import into yeast mitochondria. *EMBO J.* **11,** 3619–3628.

Maarse, A. C., Blom, J., Keil, P., Pfanner, N., and Meijer, M. (1994). Identification of the essential yeast protein MIM17, an integral mitochondrial inner membrane protein involved in protein import. *FEBS Lett.* **349,** 215–221.

Mannella, C., Neuwald, A., and Lawrence, C. (1996). Detection of likely beta-strand regions in sequences of mitochondrial pore proteins using the Gibbs sampler. *J. Bioenerg. Biomembr.* **28,** 163–169.

Manning-Krieg, U. C., Scherer, P. E., and Schatz, G. (1991). Sequential action of mitochondrial chaperones in protein import into the matrix. *EMBO J.* **10,** 3273–3280.

Martinez-Caballero, S., Grigoriev, S. M., Herrmann, J. M., Campo, M. L., and Kinnally, K. W. (2007). Tim17p regulates the twin pore structure and voltage gating of the mitochondrial protein import complex TIM23. *J. Biol. Chem.* **282,** 3584–3593.

McBride, H. M., Millar, D. G., Li, J. M., and Shore, G. C. (1992). A signal-anchor sequence selective for the mitochondrial outer membrane. *J. Cell Biol.* **119,** 1451–1457.

McDonald, T., Sheng, S., Stanley, B., Chen, D., Ko, Y., Cole, R. N., Pedersen, P., and Van Eyk, J. E. (2006). Expanding the subproteome of the inner mitochondria using protein separation technologies: One- and two-dimensional liquid chromatography and two-dimensional gel electrophoresis. *Mol. Cell Proteomics* **5,** 2392–2411.

Meier, S., Neupert, W., and Herrmann, J. M. (2005a). Conserved N-terminal negative charges in the Tim17 subunit of the TIM23 translocase play a critical role in the import of preproteins into mitochondria. *J. Biol. Chem.* **280,** 7777–7785.

Meier, S., Neupert, W., and Herrmann, J. M. (2005b). Proline residues of transmembrane domains determine the sorting of inner membrane proteins in mitochondria. *J. Cell Biol.* **170,** 881–888.

Meinecke, M., Wagner, R., Kovermann, P., Guiard, B., Mick, D. U., Hutu, D. P., Voos, W., Truscott, K. N., Chacinska, A., Pfanner, N., and Rehling, P. (2006). Tim50 maintains the permeability barrier of the mitochondrial inner membrane. *Science* **312,** 1523–1526.

Meinecke, B., Engl, G., Kemper, C., Vasiljev-Neumeyer, A., Paulitschke, H., and Rapaport, D. (2008). The outer membrane form of the mitochondrial protein Mcr1 follows a TOM-independent membrane insertion pathway. *FEBS Lett.* **582,** 855–860.

Meisinger, C., Rissler, M., Chacinska, A., Szklarz, L. K., Milenkovic, D., Kozjak, V., Schonfisch, B., Lohaus, C., Meyer, H. E., Yaffe, M. P., Guiard, B., Wiedemann, N., *et al.* (2004). The mitochondrial morphology protein Mdm10 functions in assembly of the preprotein translocase of the outer membrane. *Dev. Cell* **7,** 61–71.

Meisinger, C., Wiedemann, N., Rissler, M., Strub, A., Milenkovic, D., Schonfisch, B., Muller, H., Kozjak, V., and Pfanner, N. (2006). Mitochondrial protein sorting: Differentiation of beta-barrel assembly by Tom7-mediated segregation of Mdm10. *J. Biol. Chem.* **281,** 22819–22826.

Meisinger, C., Pfannschmidt, S., Rissler, M., Milenkovic, D., Becker, T., Stojanovski, D., Youngman, M. J., Jensen, R. E., Chacinska, A., Guiard, B., Pfanner, N., and Wiedemann, N. (2007). The morphology proteins Mdm12/Mmm1 function in the major beta-barrel assembly pathway of mitochondria. *EMBO J.* **26,** 2229–2239.

Milenkovic, D., Kozjak, V., Wiedemann, N., Lohaus, C., Meyer, H. E., Guiard, B., Pfanner, N., and Meisinger, C. (2004). Sam35 of the mitochondrial protein sorting and assembly machinery is a peripheral outer membrane protein essential for cell viability. *J. Biol. Chem.* **279,** 22781–22785.

Mileykovskaya, E., Zhang, M., and Dowhan, W. (2005). Cardiolipin in energy transducing membranes. *Biochemistry (MOSC)* **70,** 154–158.

Millar, A. H., Whelan, J., and Small, I. (2006). Recent surprises in protein targeting to mitochondria and plastids. *Curr. Opin. Plant Biol.* **9,** 610–615.

Miller, B. R., and Cumsky, M. G. (1993). Intramitochondrial sorting of the precursor to yeast cytochrome c oxidase subunit Va. *J. Cell Biol.* **121,** 1021–1029.

Model, K., Meisinger, C., Prinz, T., Wiedemann, N., Truscott, K. N., Pfanner, N., and Ryan, M. T. (2001). Multistep assembly of the protein import channel of the mitochondrial outer membrane. *Nat. Struct. Biol.* **8,** 361–370.

Model, K., Prinz, T., Ruiz, T., Radermacher, M., Krimmer, T., Kuhlbrandt, W., Pfanner, N., and Meisinger, C. (2002). Protein translocase of the outer mitochondrial membrane: Role of import receptors in the structural organization of the TOM complex. *J. Mol. Biol.* **316,** 657–666.

Mokranjac, D., Paschen, S. A., Kozany, C., Prokisch, H., Hoppins, S. C., Nargang, F. E., Neupert, W., and Hell, K. (2003a). Tim50, a novel component of the TIM23 preprotein translocase of mitochondria. *EMBO J.* **22,** 816–825.

Mokranjac, D., Sichting, M., Neupert, W., and Hell, K. (2003b). Tim14, a novel key component of the import motor of the TIM23 protein translocase of mitochondria. *EMBO J.* **22,** 4945–4956.

Mokranjac, D., Popov-Celeketic, D., Hell, K., and Neupert, W. (2005). Role of Tim21 in mitochondrial translocation contact sites. *J. Biol. Chem.* **280,** 23437–23440.

Mokranjac, D., Bourenkov, G., Hell, K., Neupert, W., and Groll, M. (2006). Structure and function of Tim14 and Tim16, the J and J-like components of the mitochondrial protein import motor. *EMBO J.* **25,** 4675–4685.

Mootha, V. K., Bunkenborg, J., Olsen, J. V., Hjerrild, M., Wisniewski, J. R., Stahl, E., Bolouri, M. S., Ray, H. N., Sihag, S., Kamal, M., Patterson, N., Lander, E. S., *et al.* (2003). Integrated analysis of protein composition, tissue diversity, and gene regulation in mouse mitochondria. *Cell* **115,** 629–640.

Moro, F., Sirrenberg, C., Schneider, H.-C., Neupert, W., and Brunner, M. (1999). The TIM17·23 preprotein translocase of mitochondria: Composition and function in protein transport of mitochondria. *EMBO J.* **18,** 3667–3675.

Motz, C., Martin, H., Krimmer, T., and Rassow, J. (2002). Bcl-2 and porin follow different pathways of TOM-dependent insertion into the mitochondrial outer membrane. *J. Mol. Biol.* **323,** 729–738.

Mozdy, A. D., McCaffery, J. M., and Shaw, J. M. (2000). Dnm1p GTPase-mediated mitochondrial fission is a multi-step process requiring the novel integral membrane component Fis1p. *J. Cell Biol.* **151,** 367–380.

Murphy, M. P., Leuenberger, D., Curran, S. P., Oppliger, W., and Koehler, C. M. (2001). The essential function of the small Tim proteins in the TIM22 import pathway does not depend on formation of the soluble 70-kilodalton complex. *Mol. Cell Biol.* **21,** 6132–6138.

Nagamori, S., Smirnova, I. N., and Kaback, H. R. (2004). Role of YidC in folding of polytopic membrane proteins. *J. Cell Biol.* **165,** 53–62.

Nargang, F. E., Künkele, K. P., Mayer, A., Ritzel, R. G., Neupert, W., and Lill, R. (1995). 'Sheltered disruption' of *Neurospora crassa* MOM22, an essential component of the mitochondrial protein import complex. *EMBO J.* **14,** 1099–1108.

Nargang, F. E., Preuss, M., Neupert, W., and Herrmann, J. M. (2002). The Oxa1 protein forms a homooligomeric complex and is an essential part of the mitochondrial export translocase in *Neurospora crassa*. *J. Biol. Chem.* **277,** 12846–12853.

Nemoto, Y., and De Camilli, P. (1999). Recruitment of an alternatively spliced form of synaptojanin 2 to mitochondria by the interaction with the PDZ domain of a mitochondrial outer membrane protein. *EMBO J.* **18,** 2991–3006.

Neupert, W., and Brunner, M. (2002). The protein import motor of mitochondria. *Nat. Rev. Mol. Cell Biol.* **3,** 555–565.

Neupert, W., and Herrmann, J. M. (2007). Translocation of proteins into mitochondria. *Annu. Rev. Biochem.* **76,** 723–749.

Nguyen, M., Millar, D. G., Yong, V. W., Korsmeyer, S. J., and Shore, G. C. (1993). Targeting of Bcl-2 to the mitochondrial outer membrane by a COOH-terminal signal anchor sequence. *J. Biol. Chem.* **268,** 25265–25268.

Ott, M., Prestele, M., Bauerschmitt, H., Funes, S., Bonnefoy, N., and Herrmann, J. M. (2006). Mba1, a membrane-associated ribosome receptor in mitochondria. *EMBO J.* **25,** 1603–1610.

Paschen, S. A., Waizenegger, T., Stan, T., Preuss, M., Cyrklaff, M., Hell, K., Rapaport, D., and Neupert, W. (2003). Evolutionary conservation of biogenesis of beta-barrel membrane proteins. *Nature* **426,** 862–866.

Pebay-Peyroula, E., Dahout-Gonzalez, C., Kahn, R., Trezeguet, V., Lauquin, G. J., and Brandolin, G. (2003). Structure of mitochondrial ADP/ATP carrier in complex with carboxyatractyloside. *Nature* **426,** 39–44.

Peixoto, P. M., Grana, F., Roy, T. J., Dunn, C. D., Flores, M., Jensen, R. E., and Campo, M. L. (2007). Awaking TIM22, a dynamic ligand-gated channel for protein insertion in the mitochondrial inner membrane. *J. Biol. Chem.* **282,** 18694–18701.

Pfanner, N., and Geissler, A. (2001). Versatility of the mitochondrial protein import machinery. *Nat. Rev. Mol. Cell Biol.* **2,** 339–349.

Pfanner, N., Wiedemann, N., Meisinger, C., and Lithgow, T. (2004). Assembling the mitochondrial outer membrane. *Nat. Struct. Mol. Biol.* **11,** 1044–1048.

Preuss, M., Leonhard, K., Hell, K., Stuart, R. A., Neupert, W., and Herrmann, J. M. (2001). Mba1, a novel component of the mitochondrial protein export machinery of the yeast *Saccharomyces cerevisiae*. *J. Cell Biol.* **153,** 1085–1096.

Rapaport, D. (2002). Biogenesis of the mitochondrial TOM complex. *Trends Biochem. Sci.* **27,** 191–197.

Rapaport, D. (2003). Finding the right organelle. Targeting signals in mitochondrial outer-membrane proteins. *EMBO Rep.* **4,** 948–952.

Rapaport, D., and Neupert, W. (1999). Biogenesis of Tom40, core component of the TOM complex in mitochondria. *J. Cell Biol.* **146,** 321–331.

Rapaport, D., Künkele, K.-P., Dembowski, M., Ahting, U., Nargang, F., Neupert, W., and Lill, R. (1998). Dynamics of the TOM complex of mitochondria during binding and translocation of preproteins. *Mol. Cell. Biol.* **18,** 5256–5262.

Rehling, P., Pfanner, N., and Meisinger, C. (2003). Insertion of hydrophobic membrane proteins into the inner mitochondrial membrane—A guided tour. *J. Mol. Biol.* **326,** 639–657.

Rehling, P., Brandner, K., and Pfanner, N. (2004). Mitochondrial import and the twin-pore translocase. *Nat. Rev. Mol. Cell Biol.* **5,** 519–530.

Rep, M., and Grivell, L. A. (1996). *MBA1* encodes a mitochondrial membrane-associated protein required for biogenesis of the respiratory chain. *FEBS Lett.* **388,** 185–188.

Rojo, E. E., Stuart, R. A., and Neupert, W. (1995). Conservative sorting of F_0-ATPase subunit 9: Export from matrix requires delta pH across inner membrane and matrix ATP. *EMBO J.* **14,** 3445–3451.

Rojo, E. E., Guiard, B., Neupert, W., and Stuart, R. A. (1998). Sorting of D-lactate dehydrogenase to the inner membrane of mitochondria: Analysis of topogenic signal and energetic requirements. *J. Biol. Chem.* **273,** 8040–8047.

Rojo, E. E., Guiard, B., Neupert, W., and Stuart, R. A. (1999). N-terminal tail export from the mitochondrial matrix. Adherence to the prokaryotic "positive-inside" rule of membane protein topology. *J. Biol. Chem.* **274,** 19617–19622.

Rojo, M., Legros, F., Chateau, D., and Lombes, A. (2002). Membrane topology and mitochondrial targeting of mitofusins, ubiquitous mammalian homologs of the transmembrane GTPase Fzo. *J. Cell Sci.* **115,** 1663–1674.

Ryan, K. R., Leung, R. S., and Jensen, R. J. (1998). Characterisation of the mitochondrial inner membrane translocase complex: The Tim23p hydrophobic domain interacts with Tim17p but not with other Tim23p molecules. *Mol. Cell. Biol.* **18,** 178–187.

Saint-Georges, Y., Hamel, P., Lemaire, C., and Dujardin, G. (2001). Role of positively charged transmembrane segments in the insertion and assembly of mitochondrial inner-membrane proteins. *Proc. Natl. Acad. Sci. USA* **98,** 13814–13819.

Sanchez-Pulido, L., Devos, D., Genevrois, S., Vicente, M., and Valencia, A. (2003). POTRA: A conserved domain in the FtsQ family and a class of beta-barrel outer membrane proteins. *Trends Biochem. Sci.* **28,** 523–526.

Saracco, S. A., and Fox, T. D. (2002). Cox18p is required for export of the mitochondrially encoded *Saccharomyces cerevisiae* Cox2p C-tail and interacts with Pnt1p and Mss2p in the inner membrane. *Mol. Biol. Cell* **13,** 1122–1131.

Scherer, P. E., Krieg, U. C., Hwang, S. T., Vestweber, D., and Schatz, G. (1990). A precursor protein partly translocated into yeast mitochondria is bound to a 70 kDa mitochondrial stress protein. *EMBO J.* **9,** 4315–4322.

Scherer, P. E., Manning, K. U., Jeno, P., Schatz, G., and Horst, M. (1992). Identification of a 45-kDa protein at the protein import site of the yeast mitochondrial inner membrane. *Proc. Natl. Acad. Sci. USA* **89,** 11930–11934.

Schleiff, E., Silvius, J. R., and Shore, G. C. (1999). Direct membrane insertion of voltage-dependent anion-selective channel protein catalyzed by mitochondrial Tom20. *J. Cell Biol.* **145,** 973–978.

Schleiff, E., Eichacker, L. A., Eckart, K., Becker, T., Mirus, O., Stahl, T., and Soll, J. (2003). Prediction of the plant beta-barrel proteome: A case study of the chloroplast outer envelope. *Protein Sci.* **12,** 748–759.

Schlossmann, J., and Neupert, W. (1995). Assembly of the preprotein receptor MOM72/MAS70 into the protein import complex of the outer membrane of mitochondria. *J. Biol. Chem.* **270,** 27116–27121.

Schlossmann, J., Dietmeier, K., Pfanner, N., and Neupert, W. (1994). Specific recognition of mitochondrial preproteins by the cytosolic domain of the import receptor MOM72. *J. Biol. Chem.* **269,** 11893–11901.

Schmitt, S., Prokisch, H., Schlunck, T., Camp, D. G., 2nd, Ahting, U., Waizenegger, T., Scharfe, C., Meitinger, T., Imhof, A., Neupert, W., Oefner, P. J., and Rapaport, D. (2006). Proteome analysis of mitochondrial outer membrane from *Neurospora crassa*. *Proteomics* **6,** 72–80.

Schneider, H., Sollner, T., Dietmeier, K., Eckerskorn, C., Lottspeich, F., Trulzsch, B., Neupert, W., and Pfanner, N. (1991). Targeting of the master receptor MOM19 to mitochondria. *Science* **254,** 1659–1662.

Schneider, H.-C., Berthold, J., Bauer, M. F., Dietmeier, K., Guiard, B., Brunner, M., and Neupert, W. (1994). Mitochondrial Hsp70/MIM44 complex facilitates protein import. *Nature* **371,** 768–774.

Schwartz, M. P., and Matouschek, A. (1999). The dimensions of the protein import channels in the outer and inner mitochondrial membranes. *Proc. Natl. Acad. Sci. USA* **96,** 13086–13090.

Serek, J., Bauer-Manz, G., Struhalla, G., Van Den Berg, L., Kiefer, D., Dalbey, R., and Kuhn, A. (2004). *Escherichia coli* YidC is a membrane insertase for Sec-independent proteins. *EMBO J.* **23,** 294–301.

Setoguchi, K., Otera, H., and Mihara, K. (2006). Cytosolic factor- and TOM-independent import of C-tail-anchored mitochondrial outer membrane proteins. *EMBO J.* **25,** 5635–5647.

Shore, G. C., McBride, H. M., Millar, D. G., Steenaart, N. A. E., and Nguyen, M. (1995). Import and insertion of proteins into the mitochondrial outer membrane. *Eur. J. Biochem.* **227,** 9–18.

Sickmann, A., Reinders, J., Wagner, Y., Joppich, C., Zahedi, R., Meyer, H. E., Schonfisch, B., Perschil, I., Chacinska, A., Guiard, B., Rehling, P., Pfanner, N., *et al.* (2003). The proteome of *Saccharomyces cerevisiae* mitochondria. *Proc. Natl. Acad. Sci. USA* **100,** 13207–13212.

Sirrenberg, C., Bauer, M. F., Guiard, B., Neupert, W., and Brunner, M. (1996). Import of carrier proteins into the mitochondrial inner membrane mediated by Tim22. *Nature* **384,** 582–585.

Sirrenberg, C., Endres, M., Fölsch, H., Stuart, R. A., Neupert, W., and Brunner, M. (1998). Carrier protein import into mitochondria mediated by the intermembrane proteins Tim10/Mrs11p and Tim12/Mrs5p. *Nature* **391,** 912–915.

Slutsky-Leiderman, O., Marom, M., Iosefson, O., Levy, R., Maoz, S., and Azem, A. (2007). The interplay between components of the mitochondrial protein translocation motor studied using purified components. *J. Biol. Chem.* **282,** 33935–33942.

Smith, M. D., Petrak, M., Boucher, P. D., Barton, K. N., Carter, L., Reddy, G., Blachly-Dyson, E., Forte, M., Price, J., Verner, K., and McCauley, R. B. (1995). Lysine residues

at positions 234 and 236 in yeast porin are involved in its assembly into the mitochondrial outer membrane. *J. Biol. Chem.* **270,** 28331–28336.
Sogo, L. F., and Yaffe, M. P. (1994). Regulation of mitochondrial morphology and inheritance by Mdm10p, a protein of the mitochondrial outer membrane. *J. Cell Biol.* **130,** 1361–1373.
Souza, R. L., Green-Willms, N. S., Fox, T. D., Tzagoloff, A., and Nobrega, F. G. (2000). Cloning and characterization of *COX18*, a *Saccharomyces cerevisiae* PET gene required for the assembly of cytochrome oxidase. *J. Biol. Chem.* **275,** 14898–14902.
Stiburek, L., Fornuskova, D., Wenchich, L., Pejznochova, M., Hansikova, H., and Zeman, J. (2007). Knockdown of human Oxa1l impairs the biogenesis of F(1)F(0)-ATP synthase and NADH:ubiquinone oxidoreductase. *J. Mol. Biol.* **374,** 506–516.
Suzuki, H., Maeda, M., and Mihara, K. (2002). Characterization of rat TOM70 as a receptor of the preprotein translocase of the mitochondrial outer membrane. *J. Cell Sci.* **115,** 1895–1905.
Szyrach, G., Ott, M., Bonnefoy, N., Neupert, W., and Herrmann, J. M. (2003). Ribosome binding to the Oxa1 complex facilitates cotranslational protein insertion in mitochondria. *EMBO J.* **22,** 6448–6457.
Tamm, L. K., Arora, A., and Kleinschmidt, J. H. (2001). Structure and assembly of beta-barrel membrane proteins. *J. Biol. Chem.* **276,** 32399–32402.
Tokatlidis, K., Vial, S., Luciano, P., Vergnolle, M., and Clemence, S. (2000). Membrane protein import in yeast mitochondria. *Biochem. Soc. Trans.* **28,** 495–499.
Truscott, K. N., Kovermann, P., Geissler, A., Merlin, A., Meijer, M., Driessen, A. J., Rassow, J., Pfanner, N., and Wagner, R. (2001). A presequence- and voltage-sensitive channel of the mitochondrial preprotein translocase formed by Tim23. *Nat. Struct. Biol.* **8,** 1074–1082.
Truscott, K. N., Voos, W., Frazier, A. E., Lind, M., Li, Y., Geissler, A., Dudek, J., Muller, H., Sickmann, A., Meyer, H. E., Meisinger, C., Guiard, B., *et al.* (2003). A J-protein is an essential subunit of the presequence translocase-associated protein import motor of mitochondria. *J. Cell Biol.* **163,** 707–713.
van der Laan, M., Bechtluft, P., Kol, S., Nouwen, N., and Driessen, A. J. (2004). F_1F_0 ATP synthase subunit c is a substrate of the novel YidC pathway for membrane protein biogenesis. *J. Cell Biol.* **165,** 213–222.
van der Laan, M., Wiedemann, N., Mick, D. U., Guiard, B., Rehling, P., and Pfanner, N. (2006). A role for Tim21 in membrane potential-dependent preprotein sorting in mitochondria. *Curr. Biol.* **16,** 2271–2276.
van der Laan, M., Meinecke, M., Dudek, J., Hutu, D. P., Lind, M., Perschil, I., Guiard, B., Wagner, R., Pfanner, N., and Rehling, P. (2007). Motor-free mitochondrial presequence translocase drives membrane integration of preproteins. *Nat. Cell Biol.* **9,** 1152–1159.
Van Loon, A. P., and Schatz, G. (1987). Transport of proteins to the inner membrane: The "sorting" domain of the cytochrome c_1 presequence is a stop-transfer sequence for the mitochondrial inner membrane. *EMBO J.* **6,** 2441–2448.
van Wilpe, S., Ryan, M. T., Hill, K., Maarse, A. C., Meisinger, C., Brix, J., Dekker, J. T., Moczko, M., Wagner, R., Meijer, M., Guiard, B., Hönlinger, A., *et al.* (1999). Tom22 is a multifunctional organizer of the mitochondrial preprotein translocase. *Nature* **401,** 485–489.
Vasiljev, A., Ahting, U., Nargang, F. E., Go, N. E., Habib, S. J., Kozany, C., Panneels, V., Sinning, I., Prokisch, H., Neupert, W., Nussberger, S., and Rapaport, D. (2004). Reconstituted TOM core complex and Tim9/Tim10 complex of mitochondria are sufficient for translocation of the ADP/ATP carrier across membranes. *Mol. Biol. Cell* **15,** 1445–1458.
Vergnolle, M. A., Baud, C., Golovanov, A. P., Alcock, F., Luciano, P., Lian, L. Y., and Tokatlidis, K. (2005). Distinct domains of small Tims involved in subunit interaction and substrate recognition. *J. Mol. Biol.* **351,** 839–849.
Vogel, F., Bornhovd, C., Neupert, W., and Reichert, A. S. (2006). Dynamic subcompartmentalization of the mitochondrial inner membrane. *J. Cell Biol.* **175,** 237–247.

von Heijne, G. (1989). Control of topology and mode of assembly of a polytopic membrane protein by positively charged residues. *Nature* **341,** 456–458.

Voulhoux, R., Bos, M. P., Geurtsen, J., Mols, M., and Tommassen, J. (2003). Role of a highly conserved bacterial protein in outer membrane protein assembly. *Science* **299,** 262–265.

Waizenegger, T., Stan, T., Neupert, W., and Rapaport, D. (2003). Signal-anchor domains of proteins of the outer membrane of mitochondria: Structural and functional characteristics. *J. Biol. Chem.* **278,** 42064–42071.

Waizenegger, T., Habib, S. J., Lech, M., Mokranjac, D., Paschen, S. A., Hell, K., Neupert, W., and Rapaport, D. (2004). Tob38, a novel essential component in the biogenesis of beta-barrel proteins of mitochondria. *EMBO Rep.* **5,** 704–709.

Waizenegger, T., Schmitt, S., Zivkovic, J., Neupert, W., and Rapaport, D. (2005). Mim1, a protein required for the assembly of the TOM complex of mitochondria. *EMBO Rep.* **6,** 57–62.

Walsh, P., Bursac, D., Law, Y. C., Cyr, D., and Lithgow, T. (2004). The J-protein family: Modulating protein assembly, disassembly and translocation. *EMBO Rep.* **5,** 567–571.

Wattenberg, B., and Lithgow, T. (2001). Targeting of C-terminal (tail)-anchored proteins: Understanding how cytoplasmic activities are anchored to intracellular membranes. *Traffic* **2,** 66–71.

Webb, C. T., Gorman, M. A., Lazarou, M., Ryan, M. T., and Gulbis, J. M. (2006). Crystal structure of the mitochondrial chaperone TIM9★10 reveals a six-bladed alpha-propeller. *Mol. Cell* **21,** 123–133.

Westermann, B., and Neupert, W. (1997). Mdj2p, a novel DnaJ homolog in the mitochondrial inner membrane of the yeast *Saccharomyces cerevisiae*. *J. Mol. Biol.* **272,** 477–483.

White, S. H., and von Heijne, G. (2004). The machinery of membrane protein assembly. *Curr. Opin. Struct. Biol.* **14,** 397–404.

Wiedemann, N., Pfanner, N., and Ryan, M. T. (2001). The three modules of ADP/ATP carrier cooperate in receptor recruitment and translocation into mitochondria. *EMBO J.* **20,** 951–960.

Wiedemann, N., Kozjak, V., Chacinska, A., Schonfisch, B., Rospert, S., Ryan, M. T., Pfanner, N., and Meisinger, C. (2003). Machinery for protein sorting and assembly in the mitochondrial outer membrane. *Nature* **424,** 565–571.

Wiedemann, N., Truscott, K. N., Pfannschmidt, S., Guiard, B., Meisinger, C., and Pfanner, N. (2004). Biogenesis of the protein import channel Tom40 of the mitochondrial outer membrane: Intermembrane space components are involved in an early stage of the assembly pathway. *J. Biol. Chem.* **279,** 18188–18194.

Wiedemann, N., Pfanner, N., and Chacinska, A. (2006). Chaperoning through the mitochondrial intermembrane space. *Mol. Cell* **21,** 145–148.

Wimley, W. C. (2003). The versatile beta-barrel membrane protein. *Curr. Opin. Struct. Biol.* **13,** 404–411.

Wu, T., Malinverni, J., Ruiz, N., Kim, S., Silhavy, T. J., and Kahne, D. (2005). Identification of a multicomponent complex required for outer membrane biogenesis in *Escherichia coli*. *Cell* **121,** 235–245.

Wurm, C. A., and Jakobs, S. (2006). Differential protein distributions define two subcompartments of the mitochondrial inner membrane in yeast. *FEBS Lett.* **580,** 5628–5634.

Yaffe, M. P., Jensen, R. E., and Guido, E. C. (1989). The major 45-kDa protein of the yeast mitochondrial outer membrane is not essential for cell growth or mitochondrial function. *J. Biol. Chem.* **264,** 21091–21096.

Yamamoto, H., Esaki, M., Kanamori, T., Tamura, Y., Nishikawa, S., and Endo, T. (2002). Tim50 is a subunit of the TIM23 complex that links protein translocation across the outer and inner mitochondrial membranes. *Cell* **111,** 519–528.

Yen, M. R., Harley, K. T., Tseng, Y. H., and Saier, M. H., Jr. (2001). Phylogenetic and structural analyses of the oxa1 family of protein translocases. *FEMS Microbiol. Lett.* **204,** 223–231.

Young, J. C., Hoogenraad, N. J., and Hartl, F. U. (2003). Molecular chaperones Hsp90 and Hsp70 deliver preproteins to the mitochondrial import receptor Tom70. *Cell* **112,** 41–50.

Young, J. C., Agashe, V. R., Siegers, K., and Hartl, F. U. (2004). Pathways of chaperone-mediated protein folding in the cytosol. *Nat. Rev. Mol. Cell Biol.* **5,** 781–791.

Youngman, M. J., Hobbs, A. E., Burgess, S. M., Srinivasan, M., and Jensen, R. E. (2004). Mmm2p, a mitochondrial outer membrane protein required for yeast mitochondrial shape and maintenance of mtDNA nucleoids. *J. Cell Biol.* **164,** 677–688.

Zahedi, R. P., Sickmann, A., Boehm, A. M., Winkler, C., Zufall, N., Schonfisch, B., Guiard, B., Pfanner, N., and Meisinger, C. (2006). Proteomic analysis of the yeast mitochondrial outer membrane reveals accumulation of a subclass of preproteins. *Mol. Biol. Cell* **17,** 1436–1450.

CHAPTER SIX

Molecular Biology of Gibberellins Signaling in Higher Plants

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Contents

Abstract

Gibberellins (GAs), a large family of tetracyclic, diterpenoid plant hormones, play an important role in regulating diverse processes throughout plant development. In recent years, significant advances have been made in the isolation of GA signaling components and GA-responsive genes. All available data have indicated that DELLA proteins are an essential negative regulator in the GA

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signaling pathway and GA derepresses DELLA-mediated growth suppression by inducing degradation of DELLA proteins through the ubiquitin-26S proteasome proteolytic pathway. Identification of *GID1*, a gene encoding an unknown protein with similarity to hormone-sensitive lipases, has revealed that GID1 acts as a functional GA receptor with a reasonable binding affinity to biologically active GAs. Furthermore, the GID1 receptor interacts with DELLA proteins in a GA-dependent manner. These results suggest that formation of a GID1–GA–DELLA protein complex targets DELLA protein into the ubiquitin-26S proteasome pathway for degradation.

Key Words: Plant hormone, Gibberellin, DELLA protein, GID1 receptor, $SCF^{SLY1/GID2}$ ubiquitin ligase.

1. Introduction

Bioactive gibberellins (GAs) are plant hormones that modulate a wide range of processes involved in plant growth, organ development, and environmental responses. These include seed germination, stem elongation, leaf expansion, flowering time, and the development of flowers, fruit, and seeds (Davies, 2004). The perception of GA and its signal transduction pathway convert the GA signal into alternations in gene expression and plant morphology. Significant progress has been made so far in identifying upstream GA signaling components and transcription factors that directly regulate downstream GA-responsive genes. These studies have revealed that GA-regulated growth is governed by the GA-dependent derepression of a growth-repressive factor (DELLA protein) and that GA-dependent proteolysis of the DELLA protein is a core molecular mechanism in the GA signaling pathway.

Recently, one of the longstanding issues in GA signaling has been solved. A GA receptor, GIBBERELLIN INSENSITIVE DWARF1 (GID1), was identified by a combination of biochemical and genetic techniques (Ueguchi-Tanaka *et al.*, 2005). The biochemical properties of GID1 are very attractive and greatly improve our understanding how GA triggers changes in gene expression. This review highlights the newly discovered molecular mechanism of GA perception by GID1, GA-dependent proteolysis of DELLA protein, and additional factors that regulate transcription of GA-responsive genes in higher plants.

2. DELLA Protein, a Repressor of GA Signaling

2.1. What is DELLA protein?

DELLA protein is an evolutionally conserved protein that acts as a negative regulator of GA signaling. Genes encoding DELLA proteins have been isolated from various plant species including *Arabidopsis* (GAI, RGA,

RGL1, RGL2, RGL3; Lee *et al.*, 2002; Peng *et al.*, 1997; Silverstone *et al.*, 1998; Wen and Chang, 2002), rice (SLR1; Ikeda *et al.*, 2001), barley (SLN1; Chandler *et al.*, 2002), maize (d8; Peng *et al.*, 1999), wheat (Rht; Peng *et al.*, 1999), and grape (VvGAI; Boss and Thomas, 2002). The DELLA family members are categorized as a subfamily of the GRAS family of proteins based on their sequence similarities. The GRAS family of putative transcription factors is a plant-specific protein family. Within the completed Arabidopsis genome sequence, over 30 GRAS family members have been identified. All of the GRAS members contain some conserved motifs in the C-terminal portion, namely, leucine heptad repeats (LHR), VHIID, PFYRE, and SAW (Pysh *et al.*, 1999). However, the N-termini of GRAS family members are highly divergent, probably because of their distinct roles in different cellular pathways. The DELLA proteins contain two conserved and unique domains, DELLA (hence its name) and TVHYNP, both near the N-terminus (Fig. 6.1). Several studies using GFP fusion proteins have demonstrated that DELLA proteins are localized in plant cell nuclei (Dill *et al.*, 2001; Gubler *et al.*, 2002; Itoh *et al.*, 2002; Olszewski *et al.*, 2002; Sun and Gubler, 2004). However, since DELLA proteins do not have a clearly identified DNA-binding domain, it has been speculated that they function as coactivators or corepressors by associating with other transcription factors.

The biological function of DELLA protein has been deduced from genetic studies based on mutants. Dominant mutations at the Arabidopsis *GIBBERELLIN INSENSITIVE* (*GAI*), wheat *Reduced height* (*Rht-B1/Rht-D1*), and maize *Dwarf8* (*D8*) loci confer a dwarf phenotype with a GA-unresponsive nature (Harberd and Freeling, 1989; Koornneef *et al.*, 1985; Winkler and Freeling, 1994). Molecular cloning of Arabidopsis *GAI* first demonstrated that GAI acts as a repressor in GA signaling and showed that an in-frame deletion of the N-terminal DELLA domain occurs in the *gai-1* mutant (Peng *et al.*, 1997). On the basis of their results, Peng *et al.* (1997) proposed that the GAI product represses GA-regulated growth and that repression of GAI can be relieved by the GA signal. In this context, *gai-1* product lacking an N-terminal DELLA domain acts as a constitutive

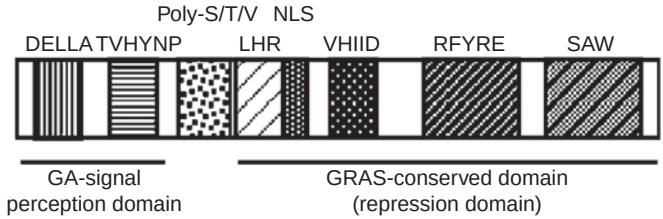

Figure 6.1 **Schematic representation of DELLA proteins. Each domain is indicated by different shading. GA signal perception and GRAS-conserved (repression) domains are indicated by underlines. Poly-S/T/V, homopolymeric Ser/Thr-rich domain; LHR, Lue heptad repeat; NLS, nuclear localization signal.**

gain-of-function repressor that cannot be derepressed by GA. Sequence analysis of wheat *Rht-1B/Rht-1D* and maize *D8* alleles confirmed that their N-terminal deletion or truncation leads to a GA-insensitive dwarf phenotype in those species (Peng *et al.*, 1999). If the repressive function of DELLA protein is essential for GA-regulated growth in plants, a loss-of-function mutant of *GAI* should show a GA-overdosed slender phenotype even in the absence of GA. However, *gai-t6*, a T-DNA insertion allele of *gai-1*, did not show an obvious GA-related phenotype under normal growth condition (Peng *et al.*, 1997). The importance of the repressive function of DELLA protein in the GA signaling pathway has been demonstrated by the isolation of rice *slr1* and barley *sln1* mutants. The loss-of-function alleles of rice *SLENDER RICE1* (*SLR1*) show a constitutive GA-responsive slender phenotype. The *slr1* mutation completely masks the effect of a GA biosynthetic inhibitor on leaf and stem elongation. Also, GA-induced α-amylase production occurs constitutively in embryoless half seeds of the *slr1* mutant, with or without GA application. Moreover, the *slr1* mutant has a reduced level of bioactive GA in comparison to that of wild-type rice (Ikeda *et al.*, 2001). A loss-of-function allele of barley *SLENDER1* (*SLN1*) also showed a constitutive GA-overdosed slender phenotype similar to the rice *slr1* mutant (Chandler *et al.*, 2002). Thus, the phenotype of the loss-of-function mutants of DELLA genes in monocots clearly demonstrates that DELLA proteins act as an essential repressor for GA-regulated growth in plants. These results also indicate that GA functions to trigger derepression of DELLA protein.

Why did the loss-of-function *gai-t6* allele not show any significant GA-overdose phenotypes? Subsequent genetic analyses demonstrated that the step regulated by DELLA protein is governed redundantly in *Arabidopsis*. There are four additional members of the DELLA gene family in the Arabidopsis genome (see above), and they are highly related to each other. For example, Repressor of *ga1–3* (RGA) shares 82% identity with the amino acid sequence of GAI. Loss-of-function *rga* alleles partially suppress the dwarf phenotype conferred by GA-deficient mutation *ga1–3*, except for seed germination and flower development (Silverstone *et al.*, 1997, 1998). Genetic analyses using double, triple, and quadruple combinations of null alleles in each DELLA gene have demonstrated the overlapping and distinct functions of these genes in GA-regulated growth in *Arabidopsis*. RGA and GAI act as repressors for GA-induced stem elongation, leaf expansion, and floral initiation (Dill and Sun, 2001; King *et al.*, 2001). RGA-Like2 (RGL2) is the predominant negative regulator of GA-promoted seed germination. *RGL2* transcript levels increase transiently during imbibition of dormant seeds, although GAI, RGA, and RGL1 also have minor roles (Cao *et al.*, 2006; Lee *et al.*, 2002; Tyler *et al.*, 2004; Wen and Chang, 2002). In addition, RGA and RGL2 have been reported to act dominantly in floral development, and RGL1 functions as a minor repressor

(Tyler *et al.*, 2004; Yu *et al.*, 2004). In contrast to the functional redundancy reported in *Arabidopsis*, the complete rice genome sequence has revealed that rice has only one gene encoding a protein orthologous to GAI/RGA/RGLs in its genome (Itoh *et al.*, 2002); consequently, the rice *slr1* mutants provide clear genetic evidence of the critical role of DELLA protein in GA signaling.

2.2. GA-dependent degradation of DELLA protein is a key event in GA signaling

As described above, genetic analyses of gain- and loss-of-function mutants of DELLA family genes have indicated that the GA signal somehow suppresses the repressive function of DELLA proteins. To investigate this molecular mechanism, similar analyses were performed using Arabidopsis RGA and rice SLR1. These proteins were each fused with GFP and expressed in transgenic plants. In the absence of GA, the GFP-fused RGA and SLR1 were localized in nucleus. However, after treatment with GA, these fusion proteins rapidly disappeared from the nucleus and GA response occurred (Dill *et al.*, 2001; Gubler *et al.*, 2002; Itoh *et al.*, 2002; Silverstone *et al.*, 2001). These results strongly suggest that the GA signal triggers rapid degradation of DELLA protein to relieve the repressive state of downstream GA response.

The finding of GA-dependent degradation of DELLA proteins led us to speculate that DELLA proteins possess at least two domains for the expression or regulation of its function: one that functions as a repression domain against the GA response and another that functions in GA signal perception. The uniqueness of the N-terminal DELLA motif leads us to speculate that the region might be involved in perception of upstream GA signals. Several lines of evidence demonstrate that the DELLA motif acts as the GA signal perception domain to trigger DELLA protein degradation in response to the GA signal, and that deletion of this motif confers a GA-insensitive dwarf phenotype through resistance to GA-induced proteolysis. First, as described above, the gain-of-function *gai-1* allele contains an in-frame deletion in the *GAI* gene, which results in the loss of 17 amino acids encompassing the DELLA motif (Peng *et al.*, 1997). Second, artificially introduced in-frame deletions (*rga-*Δ*17* and Δ*DELLA-SLR1*) of DELLA domains in Arabidopsis RGA and rice SLR1, respectively, or spontaneous point mutation of barley SLN1, *sln1d*, confer a GA-insensitive severe dwarf phenotype (Chandler *et al.*, 2002; Dill *et al.*, 2001; Ikeda *et al.*, 2001; Itoh *et al.*, 2002). In this context, rga-Δ17 and ΔDELLA-SLR1 mutant proteins were stably localized in nucleus even in the presence of GA, although wild-type RGA and SLR1 were degraded in response to GA (Dill *et al.*, 2001; Itoh *et al.*, 2002). Functional domain analysis of SLR1 revealed that another N-terminal conserved region located near the DELLA motif, TVHYNP, also functions

as a part of the GA signal perception: Transgenic plants overexpressing a mutated DELLA protein with an in-frame deletion of the TVHYNP region also showed a GA-insensitive dwarf phenotype (Itoh *et al.*, 2002). Functional domain analysis of SLR1 also revealed several functional modules that are essential for SLR1 to be a functional repressor of GA signaling. For example, a homopolymeric Ser, Thr, and Val (poly S/T/V) domain located just behind the N-terminal GA signal perception domain may function as a regulatory domain for repressor activity of SLR1 (Itoh *et al.*, 2002). Glycosylation and phosphorylation might be involved in this type of regulation because the target sites for known kinase or glycosidase were observed in this region (see below; Silverstone *et al.*, 1998). The LHR, which may mediate protein–protein interaction, is essential for repressive function of SLR1- and GA-induced degradation. The other C-terminal regions such as the VHIID, RFYRE, and SAW domains, which are shared with other GRAS family genes, are essential for the suppression function of SLR1 (Itoh *et al.*, 2002).

2.3. 26S proteasome-dependent degradation of DELLA protein is targeted by $SCF^{GID2/SLY1}$ E3 ubiquitin ligase

Rapid degradation of specific protein through the ubiquitin-26S proteasome-mediated proteolytic pathway plays a critical step in cellular signaling pathways in yeast and mammals. The involvement of 26S proteasome-mediated proteolysis in GA-dependent degradation of DELLA protein was first suggested by the observation that the degradation of barley SLN1 protein by GA application was blocked by the presence of a 26S proteasome inhibitor, MG-132 (Fu *et al.*, 2002).

Recessive mutations of *GIBBERELLIN INSENSITIVE DWARF2* (*GID2*) in rice and *SLEEPY1* (*SLY1*) in *Arabidopsis* confer GA-insensitive dwarf phenotypes, indicating that the wild-type genes normally act as positive regulators of GA signaling (Sasaki *et al.*, 2003; Steber *et al.*, 1998). The *gid2* and *sly1* mutants accumulate much higher levels of the SLR1 and RGA proteins, respectively, and the accumulated DELLA proteins do not disappear upon application of GA. The *slr1* mutation completely suppresses the *gid2* dwarf phenotype in the *slr1–11 gid2–1* double mutant, indicating that the elevated DELLA protein levels caused the GA-insensitive dwarf phenotype of these mutants and, furthermore, that DELLA proteins function downstream of *GID2* and *SLY1* in the GA signaling pathway. Positional cloning of these mutated genes revealed that *GID2* and *SLY1* are orthologous genes that encode F-box containing proteins (McGinnis *et al.*, 2003; Sasaki *et al.*, 2003). F-box proteins occur widely throughout the eukaryote kingdom, in organisms ranging from yeast to humans, and they function as receptors that recruit proteins as substrates for ubiquitin-26S proteasome proteolysis (Deshaies, 1999; Hershko and Ciechanover, 1998).

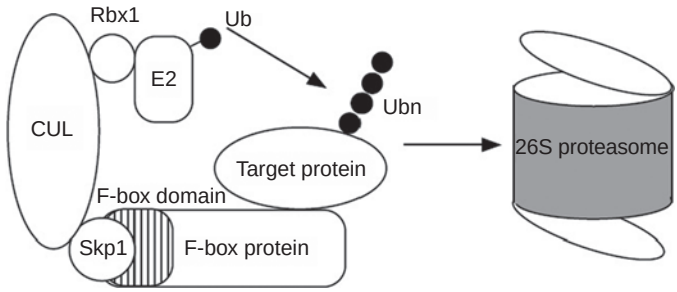

Figure 6.2 Key steps in the pathway of polyubiquitination by the SCF E3 ubiquitin ligase complex. The SCF–E3 complex (Skp1, CUL, F-box protein, and Rbx1) catalyzes the transfer of ubiquitin (Ub) from E2 ubiquitin-conjugating enzyme to the target protein. Formation of a polyubiquitin chain (Ubn) on the target protein directs it for degradation by the 26S proteasome.

F-box proteins associated with Skp1, Cullin (CUL), and Rbx1 proteins form an E3 ubiquitin ligase, known as the SCF complex, through interaction between the F-box domain and Skp1 proteins (Bai *et al.*, 1996; Deshaies, 1999). The SCF complex promotes the transfer of ubiquitin from an ubiquitin-conjugating enzyme (E2) to a target protein (Fig. 6.2). Co-immunoprecipitation experiments using transgenic plants overexpressing a hemagglutinin (HA)-tagged GID2 gene demonstrated that GID2 associated with OsSkp15 (a rice ortholog to Arabidopsis Skp1) and OsCUL1 to form a SCF^{GID2} complex *in vivo* (Gomi *et al.*, 2004). Complementary data showing the importance of the F-box domain of GID2 in SCF complex formation comes from the result that a mutant version of GID2 with an internal deletion of the F-box domain is unable to restore the *gid2* mutant phenotype when it is overexpressed in rice plants (Gomi *et al.*, 2004). Domain analysis of GID2 also revealed that C-terminal conserved motifs (GGF and LSL) between GID2 and SLY1 are necessary to express its function. In *Arabidopsis*, there is one homolog of SLY1 in its genome named *SEEZY* (*SNZ*). Overexpression of *SNZ* can rescue the *sly1–10* dwarf phenotype and restore normal RGA protein level, indicating that the SNZ F-box protein can replace SLY1 function in GA-induced proteolysis of RGA (Strader *et al.*, 2004). Rice also has *SNZ*-related sequence in its genome, suggesting that the function of SNZ may be conserved among dicots and monocots. However, the biological function of the SNZ homolog in rice has not been elucidated.

Physical interaction between SLY1 and RGA or GAI has been demonstrated by yeast two-hybrid (Y2H) and pull-down assays using GST fused-SLY1 recombinant protein (Dill *et al.*, 2004; Fu *et al.*, 2004). The interaction between GID2 and SLR1 in rice also has been demonstrated by *in vitro* pull-down assays with GST–GID2 (Gomi *et al.*, 2004). Dill *et al.* (2004) and Fu *et al.* (2004) also demonstrated that interaction between GAI

and SLY1 requires the GRAS domain of GAI, suggesting that GA signal perception in the DELLA domain may induce conformational change of DELLA protein enabling the $SCF^{GID2/SLY1}$ complex to recognize the DELLA protein as a substrate. In addition, the *GAR2* gene, which had been identified as a suppressor of the dwarf phenotype of the *gai-1* mutant, was isolated. It was revealed that the dominant *gar2* mutation is a gain-of-function allele of *SLY1*, which results in a single amino acid substitution in one of conserved motifs, LSL. This mutation causes a reduction in the levels of wild-type and degradation-resistant versions of DELLA protein by an increased affinity between the *gar2* F-box protein and its substrates (DELLA proteins), leading to increased ubiquitination and subsequent degradation by the 26S proteasome (Dill *et al.*, 2004; Fu *et al.*, 2004).

Studies of SCF-mediated signaling in yeast and mammals have shown that posttranslational modifications of substrate proteins, such as phosphorylation, glycosylation, and hydroxylation, trigger the interaction with the SCF complex (Deshaies, 1999; Ivan *et al.*, 2001; Yoshida *et al.*, 2003). There are some observations to support that phosphorylation of DELLA proteins is required for its GA-dependent degradation. Treatments with protein Tyr kinase inhibitors, such as genistein and tyrophostin B46, blocked the GA-induced degradation of SLN1 in barley seedlings and heterologously expressed Arabidopsis RGL2 in tobacco BY2 cells (Fu *et al.*, 2002; Hussain *et al.*, 2005). In rice *gid2* seedlings, the level of phosphorylated SLR1 increased in response to GA, and phosphorylated SLR1, isolated by means of gel filtration chromatography, could bind to recombinant GST–GID2 (Gomi *et al.*, 2004; Sasaki *et al.*, 2003). On the other hand, more recent observations suggest that phosphorylation of DELLA proteins is not directly involved in GA-dependent degradation (Itoh *et al.*, 2005). Rice callus cells contain a very low level of bioactive GA and represent a suitable system for studying GA signaling. SLR1 protein exists in both phosphorylated and nonphosphorylated forms in wild-type callus and both forms are degraded by GA treatment with a similar half-life (Itoh *et al.*, 2005). In addition, both phosphorylated and nonphosphorylated SLR1s are able to interact with GST–GID2 (Itoh *et al.*, 2005). Furthermore, when the function of *SPY*, another negative regulator of GA signaling (see below), is suppressed by transformation with RNAi or an antisense construct, the phosphorylation state of SLR1 is altered without any change in the total amount of SLR1 (Shimada *et al.*, 2006). This finding supports the idea that the amount of SLR1 is not regulated by its phosphorylation state. Another experiment shows that Ser/Thr phosphatase inhibitors effectively block RGL2 degradation in tobacco BY2 cells, but Ser/Thr kinase inhibitors have no detectable effect, suggesting that dephosphorylation of serine/threonine may constitute a prerequisite step for degradation of RGL2 via the 26S proteasome pathway (Hussain *et al.*, 2005). To make sense of these conflicting observations, the phosphorylated residue(s) in the DELLA

protein must first be identified. It must then be determined whether phosphorylation (or dephosphorylation) occurs in response to GA. However, since the stability of DELLA protein is affected by other plant hormone signaling and environmental stimuli including auxin, ethylene, and salinity (Achard *et al.*, 2003, 2006; Fu and Harberd (2003)), it is possible that the phosphorylation (or dephosphorylation) status of DELLA protein might be affected at a secondary or tertiary levels by kinase or phosphatase that is activated in GA-independent manner. This possibility means that regulation of phosphorylation of DELLA protein could be independent from the interaction between GID2/SLY1 and DELLA protein, an idea supported by the finding of GID1 (see Section 3.1).

3. Identification of a GA Receptor, GID1

3.1. Characterization of *gid1* mutants in rice reveals the function of GID1

The loss-of-function *gid1* alleles do not exhibit any GA-responsive phenotypes such as elongation of the second leaf sheath or induction of α-amylase activity in seeds. Another characteristic of GA-insensitive mutants is that they overaccumulate bioactive GA because GA signaling homeostatically inhibits GA biosynthesis and promotes GA catabolism. GA_1, a major bioactive GA of vegetative tissue in rice, accumulates in *gid1* mutants up to over 100-fold the level of GA_1 in wild-type plants. The *gid1–1 slr1–1* double mutant exhibits the *slr1–1* phenotype, indicating that GID1 and SLR1 function in the same GA signaling pathway and that GID1 acts upstream of SLR1. Immunoblot analysis and microscopic observation of SLR1 and SLR1–GFP fusion protein also revealed that GA treatment does not diminish the amount of SLR1 and that SLR1–GFP stably localizes in the nucleus of *gid1–1* plants (Ueguchi-Tanaka *et al.*, 2005).

Although the phenotype of *gid1* is a typical feature of GA-insensitive mutants, the dwarfism produced by *gid1* is more severe than that by seen in *gid2* mutants. Interestingly, in contrast to their phenotypic similarity, the amount of SLR1 protein in *gid1* mutants is much less than in *gid2* mutants. Because it is expected that the interaction between DELLA proteins and GID2/SLY1 F-box protein requires GA signal perception of DELLA protein (Dill *et al.*, 2004; Sasaki *et al.*, 2003), these contradicting results led us to speculate that GID1 may act to trigger GA-dependent SLR1 degradation via the $SCF^{GID2/SLY1}$ complex. This idea was supported by the observation that *gid1* mutants resemble the phenotype of a GA-deficient *cps* mutant (defective in the gene for copalyl diphosphate synthase gene, an enzyme catalyzing an early step of GA biosynthesis) (Sakamoto *et al.*, 2004), except that bioactive GA overaccumulates in the *gid1* mutant.

The *GID1* gene was cloned by positional cloning and encodes an unknown protein with similarity to hormone-sensitive lipases (HSL). GID1 contains motifs conserved in HSL, namely HGG and GXSXG (Osterlund, 2001). The importance of the GXSXG motif is highlighted by the fact that a single amino acid substitution of the first G to D in the motif of *gid1–1* causes the severe dwarf phenotype. Although three conserved amino acids are essential to form the catalytic triad in the HSL family, GID1 lacks one of these conserved residues, His, and a recombinant GID1 protein does not hydrolyze an artificial substrate for HSL. Consequently, GID1 is not considered to be a lipase. GID1–GFP fusion protein is localized mainly in nuclei, and its localization is not affected by GA treatment (Ueguchi-Tanaka *et al.*, 2005).

3.2. GID1 protein has GA-binding activity and forms complexes with DELLA protein in a GA-dependent manner

GID1 binds to ^{3}H-labeled 16,17-dihydro-GA_4 in a saturable manner. The ligand specificity of GID1, which is estimated by competition assays between ^{3}H-labeled 16,17-dihydro-GA_4 and GAs with differing biological activity, is generally consistent with the physiological activity. That is, biologically active GAs have at least tenfold higher activity than inactive GAs. These results strongly indicate that GID1 is a functional GA receptor. However, in this assay, GID1 preferentially binds to GA_4 (K_d: 1×10^{-7} M) in comparison with GA_3 (K_d: 4×10^{-5} M), although the physiological activity of GA_4 in the rice second-leaf-sheath elongation assay is lower than that of GA_3 (Ueguchi-Tanaka *et al.*, 2005). Further analysis indicates that the discrepancy could be due to stability of these GAs *in vivo*. The GA-dependent degradation of DELLA protein occurs within 30 min or less after GA application (Gubler *et al.*, 2002; Sun and Gubler, 2004; Ueguchi-Tanaka *et al.*, 2007; Zentella *et al.*, 2007). In this response, GA_4 treatment effectively triggers GA-dependent degradation of SLR1 at a lower concentration than does GA_3 (Ueguchi-Tanaka *et al.*, 2007), and this selectivity corresponds to the GA selectivity predicted by biochemical analysis. The result also suggests that GA_4 is not the most effective GA for leaf sheath elongation because, compared to GA_3, GA_4 is preferentially metabolized by GA-inactivating enzymes *in vivo* (Sakamoto *et al.*, 2001).

On the other hand, the preferential binding of rice GID1 receptor to GA_4 is curious since GA_4 is not the major bioactive GA in every plant species. While quantitative analysis of GA compounds in *Arabidopsis* demonstrated that *Arabidopsis* synthesizes GA_4 as a major bioactive GA throughout its life cycle, there are two types of bioactive GAs in rice, and their biosynthesis is differentially regulated in a developmental stage-dependent manner (Itoh *et al.*, 2001; Kobayashi *et al.*, 1988). GA_1 is the

major bioactive GA during the vegetative stage in rice; however, the amount of endogenous GA_4 is temporally and spatially regulated during the reproductive stage. Because the timing of GA_4 synthesis in rice coincides with dramatic GA requirements, such as for the rapid elongation of internodes and pollen tubes that occurs during the reproductive stage, rice may use the most effective GA at this stage and use GA_1 for ordinary GA-regulated growth at the vegetative stage.

The first evidence that the GID1 receptor is directly involved in DELLA-mediated GA signaling was obtained from a GA-dependent interaction between GID1 and SLR1 in a Y2H assay (Ueguchi-Tanaka *et al.*, 2005). The GA-dependent interaction between GID1 and SLR1 *in planta* has also been demonstrated by co-immunoprecipitation assays of rice callus cells overexpressing GID1–GFP and bimolecular fluorescence complementation (BiFC) experiments in *Nicotiana benthamiana* (Ueguchi-Tanaka *et al.*, 2007). Further domain analysis of SLR1 using various deletion constructs in Y2H assays revealed that the N-terminal DELLA and TVHYNP domains, which together have been defined as the GA signal perception domain, are essential and sufficient for the GA-dependent interaction between GID1 and SLR1 (Ueguchi-Tanaka *et al.*, 2007). Thus, these results clearly demonstrate that direct protein–protein interaction between GID1 and DELLA proteins directs DELLA proteins to rapid degradation via $SCF^{GID2/SLY1}$. Studies on Arabidopsis GID1 and DELLA proteins (GAI and RGA) have also shown that DELLA–GID1 interaction requires an N-terminal GA signal perception domain in the DELLA proteins (Griffiths *et al.*, 2006). However, analysis of interacting domain(s) of the Arabidopsis DELLA proteins with AtGID1a using Y2H leads to a different conclusion. In RGA, both DELLA and VHYNP (the same region as TVHYNP in rice) domains are necessary for interaction with AtGID1a (Griffiths *et al.*, 2006), whereas the VHYNP domain is not essential for the interaction between GAI and AtGID1a (Willige *et al.*, 2007). Although further studies, including *in vitro* and *in vivo* binding assays, will be necessary to verify the domain(s) of DELLA protein required for GID1 interaction in *Arabidopsis*, it is interesting that there might be two different types of DELLA proteins in plants: a one-motif requirement type (GAI) and a two-motif requirement type (RGA and SLR1).

A molecular model for the formation of the GA-dependent GID1–SLR1 complex has been proposed (Fig. 6.3; Ueguchi-Tanaka *et al.*, 2007). By analyzing many different mutant versions of GID1 (such as the spontaneous mutants of GID1, N- or C-terminal truncated GID1 as well as artificially mutagenized GID1 proteins with conserved amino acids replaced with Ala), the important motifs or amino acids for GA binding and SLR1 interaction have been mapped. The amino acid residues that are important for SLR1 interaction completely overlap the residues required for GA binding that are scattered throughout the GID1 molecule. The predicted

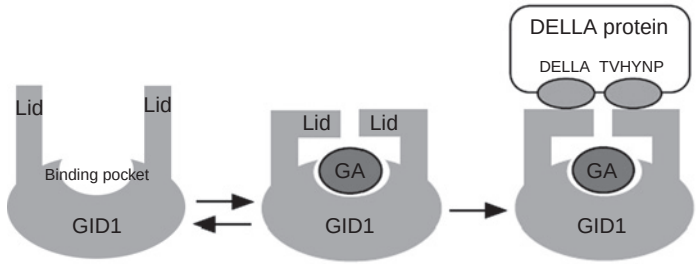

Figure 6.3 Model for formation of the GID1–GA–SLR1 complex. GID1 binds GA within the binding pocket in cooperation with two lid structures. Both the lids and the binding pocket holding GA are necessary for interaction with SLR1. As a result of SLR1 binding, the GID1–GA complex is stabilized.

secondary structure of GID1 is quite similar to the structure of AFEST (*Archaeoglobus fulgidus* esterase), whose secondary and tertiary structure has been characterized by X-ray diffraction analysis (De Simone *et al.*, 2001), indicating that GID1 has the canonical α/β hydrolase fold with lid structures similar to HSL-group proteins. The amino acid residues important for GA binding and SLR1 interaction are positioned around the region corresponding to the substrate-binding pocket and lid region in HSL. Similar to the mechanism for substrate binding in other HSLs, the distribution of important amino acid residues indicates that GID1 binds GA within the binding pocket with the aid of the lid, and SLR1 interacts with the GA-bound GID1 at its N-terminal DELLA and TVHYNP domains (Fig. 6.3). Since the GA-binding activity of recombinant GID1 is enhanced approximately threefold in the presence of SLR1 in an *in vitro* binding assay (Ueguchi-Tanaka *et al.*, 2007), the interaction of SLR1 with GA–GID1 might close the lid, thereby ensuring that GA will be held in the substrate pocket. The stabilized complex of GA, GID1, and SLR1 might be recognized by GID2, an F-box protein, leading to its degradation by 26S proteasome through ubiquitination catalyzed by the SCF^{GID2} complex. Recent results from Y3H assays demonstrated that the GA–GID1 complex promoted the interaction between an Arabidopsis DELLA protein, RGA, and an F-box protein, SLY1 (Griffiths *et al.*, 2006), supporting the above idea.

3.3. GID1 is a common GA receptor in two model plants: Rice and *Arabidopsis*

In Arabidopsis genome, there are three *GID1* homologous genes, designated *GID1a, GID1b*, and *GID1c* (Nakajima *et al.*, 2006). When these genes were expressed in *Escherichia coli*, all three Arabidopsis GID1 proteins bound to biologically active GAs with affinities similar to rice GID1

protein. In Y2H assays, these three GID1s interacted with five members of the Arabidopsis DELLA protein family in a GA-dependent manner. Furthermore, overexpression of individual Arabidopsis *GID1* genes rescued the GA-insensitive dwarf phenotype of the rice *gid1* mutant, implying that a common GA signal transduction pathway operates in rice and *Arabidopsis* (Nakajima *et al.*, 2006).

The existence of three homologous genes for GID1 in *Arabidopsis* indicates that they perform functionally redundant roles, and indeed they show overlapping expression profiles (Griffiths *et al.*, 2006; Nakajima *et al.*, 2006). The functional redundancy of Arabidopsis GID1 proteins was defined by characterizing T-DNA and transposon insertion mutants for each gene. Although only the *gid1b* single mutant showed a reduced germination rate when treated with a GA biosynthetic inhibitor, uniconazol, during imbibition and chilling (Iuchi *et al.*, 2007), none of the single mutants exhibit pronounced phenotypic defects when grown under standard growth conditions. However, combinations of mutants show that there are some functional differences among the three GID1s. For example, *gid1a gid1c* double mutants have a dwarf phenotype with reduced silique length. The *gid1a gid1b* double mutants have a lower yield of seeds per silique than the other mutants. Since artificial self-pollination can restore the defect observed in *gid1a gid1b* double mutants, this suggests that the reduced yield of seeds in *gid1a gid1b* is not caused by lower pollen fertility, but is probably due to a defect in filament elongation (Griffiths *et al.*, 2006; Iuchi *et al.*, 2007; Willige *et al.*, 2007). Also, in *gid1a gid1b* double mutants, the hexagonal pattern of the seed surface is severely disarranged similar to GA-deficient mutants, *ga1–3* and *ga3ox* knockout line, indicating that GID1a and GID1b have roles for GA perception primarily at the seed maturation stage (Iuchi *et al.*, 2007). None of the double mutants displayed an obvious defect in root growth and seed germination, suggesting that these developmental processes are regulated by the trio of GID1 proteins.

The *gid1a gid1b gid1c* triple mutant displays a dwarf phenotype more severe than that of the extreme GA-deficient mutant *ga1–3* (Griffiths *et al.*, 2006; Iuchi *et al.*, 2007). Triple-mutant Arabidopsis plants showed complete insensitivity to GA at various GA-regulated growth stages, including seed germination, root and stem elongation, leaf expansion, and flowering time, indicating that GID1 receptors are responsible for all aspects of GA-regulated growth in *Arabidopsis*. Similar to the rice *gid1* mutant, the Arabidopsis triple *gid1* mutant is completely GA insensitive to GA-regulated gene expression. For example, feedback regulation of the *GA3ox* gene by GA is not observed (Griffiths *et al.*, 2006; Iuchi *et al.*, 2007). In addition, global expression analysis with the Arabidopsis ATH1 gene chip demonstrates that expression of the 148 genes regulated by GA in *ga1–3* is not affected by GA treatment in the triple mutant (Willige *et al.*, 2007). GA insensitivity in the triple mutant is tightly linked to the level of RGA, and

GA-dependent degradation of RGA is completely blocked. The *rga-28 gid1a gid1b gid1c* quadruple mutant suppresses many of the growth defects of the *gid1a gid1b gid1c* triple mutant including stem elongation and leaf expansion (Griffiths *et al.*, 2006; Willige *et al.*, 2007), demonstrating that three GID1s are responsible for promoting GA-regulated growth in *Arabidopsis* through their regulation of GA-dependent degradation of DELLA proteins. It is likely that the developmental defects still observed in the *rga-28 gid1a gid1b gid1c* mutant are caused by the accumulation of other DELLA members (GAI, RGL1, RGL2, and RGL3) in *Arabidopsis*. However, in the case of GAI, GA-mediated degradation of GAI is completely blocked in the triple mutant, whereas accumulation of GAI is not observed (Willige *et al.*, 2007). Although further analysis will be needed to clarify whether this is also the case for the other RGL members, some members of the Arabidopsis DELLA gene family may be under the control of feedback regulation through the repressive state of GA signaling in cells.

3.4. Is GID1 the only GA receptor in higher plants?

Although phenotypic analysis of rice *gid1* mutant plants indicates that GID1 could be the only GA receptor, controlling all aspects of GA-regulated response in rice, previous genetic and molecular biological analyses have suggested that a plasma membrane-bound GA receptor is involved in GA-inducible α-amylase expression in cereal aleurone layers.

Genetic evidence suggesting the involvement of a membrane-bound GA receptor has come from the analysis of rice *dwarf1* (*d1*) mutant plants. The rice *D1* gene encodes the α-subunit of heterotrimeric G protein (Ashikari *et al.*, 1999; Fujisawa *et al.*, 1999). Although *d1* mutant plants show pleiotropic defects throughout the rice life cycle, the loss-of-function *d1* allele clearly results in lower expression of the GA-inducible α-amylase gene in endosperm than is found in wild-type endosperm. Genetic analysis using *d1 slr1* double mutants demonstrates that D1 acts upstream of SLR1 in the regulation of GA-inducible α-amylase expression (Ueguchi-Tanaka *et al.*, 2000). Since G-protein signaling is activated by the binding of ligand to plasma membrane-bound G-protein-coupled receptor (GPCR) in animal systems (Gilman, 1987), we can hypothesize a membrane-bound GA receptor in aleurone cells responsible for GA signal transduction mediated by heterotrimeric G-protein. Cell biology experiments have supported the idea that membrane-bound GA receptor is required for GA-inducible α-amylase expression. For example, GA covalently bound to Sepharose beads induces the expression of α-amylase genes in oat aleurone protoplasts but not in cells with an intact wall (Hooley *et al.*, 1991). Further, GA microinjected into barley aleurone cells did not trigger GA responses, whereas extracellular applied GA did (Gilroy and Jones, 1994).

Can we incorporate the above results into the GID1 system? The same question has been asked by Hartweck and Olszewski (2006). It is unlikely that GID1 is a plasma membrane-associated protein since GID1–GFP fusion protein expressed in rice cells was localized mainly in the nucleus (Ueguchi-Tanaka *et al.*, 2005). This finding indicates that GA would interact with the GID1 receptor intracellularly to activate α-amylase gene expression. To date, a plausible explanation has not been made for the identity of the membrane-associated protein. However, studies using aleurone protoplast cells might give us a clue to the answer. One of the reported rapid GA responses is an increase in the concentration of cytosolic calcium, $[Ca^{2+}]_i$, which is initiated within 2–5 min of GA stimulation in wheat aleurone protoplasts (Bush, 1996; Sun and Gubler, 2004). DELLA protein degradation cannot be required for this response because the GA-dependent degradation of SLN1 in barley aleurone cells occurs at 5–10 min after GA treatment (Gubler *et al.*, 2002). In this context, the precise relationship between the kinetics of calcium fluxes and disappearance of DELLA protein, the role (if any) of GID1 in the early $[Ca^{2+}]_i$ change stimulated by GA, are the next important questions to address.

4. Additional Regulators of GA Signaling

4.1. *O*-GlcNAc transferase is involved in GA signaling

The *spindly* (*spy*) mutant was originally identified from screening for mutants that germinate even in the presence of the GA biosynthetic inhibitor pacrobutrazol (Jacobsen and Olszewski, 1993). Additional *spy* alleles have been isolated as suppressors of the extreme GA-deficient mutant *ga1–3* and the GA-insensitive mutant *gai-1* (Jacobsen and Olszewski, 1993; Peng *et al.*, 1997; Silverstone *et al.*, 1997). The *spy* mutant alleles partially rescue the nongerminating, male-sterile dwarf phenotype of *ga1–3* and the dwarf phenotype of *gai-1*, indicating that SPY acts as a negative regulator of GA signaling. All the known *spy* mutant alleles exhibit some of the phenotypes seen in wild-type plants repeatedly treated with GA, including pale-green foliage, fewer leaves, and early flowering (Jacobsen and Olszewski, 1993). However, additional phenotypes observed in *spy* mutant plants, such as cotyledon movement and altered phyllotaxy, are not associated with the GA-overdosed phenotype. Recent biochemical and genetic studies show that SPY regulates the circadian clock and cytokinin signaling (Greenboim-Wainberg *et al.*, 2005; Tseng *et al.*, 2004). Thus, SPY plays an important role not only in GA signaling but also in various cellular signaling pathways in *Arabidopsis*. There is another *SPY*-related gene in the Arabidopsis genome, *SECRET AGENT* (*SEC*). Although the single *sec* mutant has no obvious phenotype, analysis of *spy sec* double mutants indicate that these proteins

have overlapping functions during gametogenesis and embryogenesis (Hartweck *et al.*, 2002, 2006).

The negative regulation of SPY in GA signaling has also been investigated in barley and rice. HvSPY, a SPY ortholog in barley, can repress GA-induced α-amylase expression when transiently expressed in GA-treated barley aleurone cells (Robertson *et al.*, 1998). In rice, a reverse-genetics approach has been taken to obtain plants suppressing OsSPY function by using RNAi or antisense constructs of *OsSPY* (Shimada *et al.*, 2006). Although suppression of OsSPY function did not cause any GA-overdosed phenotype in a wild-type background, it partially rescued the dwarf phenotype of the extreme GA-deficient *cps* and GA-insensitive mutants *gid1* and *gid2*. The elevated expression of the $GA_2$0-oxidase gene ordinarily observed in GA-related mutants is canceled in the partially rescued transgenic plants, indicating that inhibition of OsSPY function released the strongly suppressed state of GA signaling in these mutants (Shimada *et al.*, 2006). Thus, all available data indicates that SPY has an evolutionarily conserved role for GA signaling pathway in higher plants and that SPY acts as a negative regulator upstream of the DELLA proteins. In addition to the GA-related phenotypes, *OsSPY* antisense and RNAi plants show increased lamina joint bending [a brassinosteroid (BR)-related response in rice], and a low but detectable increase in both BR and sterol levels is detected in *OsSPY* RNAi plants (Shimada *et al.*, 2006). As described previously, it is possible that SPY function may act as an important mediator at the cross-talk point between GA, BR, and cytokinin signaling.

SPY encodes a protein with similarity to *O*-linked N-acetylglucosamine (GlcNAc) transferase (OGT) from human, rat, *C. elegans* (Jacobsen *et al.*, 1996), and SPY heterologously expressed in insect cells possesses OGT activity (Thornton *et al.*, 1999). Like other OGT proteins reported in animals, SPY contains the tetratricopeptide repeats (TPRs) near its N-terminus and a catalytic domain at its C terminus. TPR motifs are highly degenerate 34-amino acid repeats with eight loosely conserved residues, these motifs mediate interaction with a number of proteins (Blatch and Lassle, 1999). The TPR repeats of SPY mediate protein–protein interactions that are thought to be important for both substrate specificity and enzyme assembly. Ectopic expression of the SPY TPR domain alone confers a *spy*-like GA-overdosed phenotype in transgenic *Arabidopsis* and petunia, suggesting that overexpression of TPRs alone may inhibit SPY function by preventing active homodimerization of SPY and/or by capturing the target proteins of SPY (Izhaki *et al.*, 2001; Tseng *et al.*, 2001). Furthermore, recent phenotypic characterization of 12 *spy* alleles reveals that mutations in TPRs 6, 8, and 9 affect GA signaling in floral induction and fertility (Silverstone *et al.*, 2007). Thus, protein interaction at specific TPR domains is likely to be important for GA signaling.

OGTs transfer a GlcNAc monosaccharide in *O*-linkage to Ser or Thr of a target protein (Love and Hanover, 2005). In animal systems,

O-GlcNAc modification affects the nuclear localization, protein stability, and/or activity of target proteins (Thornton *et al*., 1999; Wells *et al*., 2001). As described above, loss-of-function *spy* alleles suppress the repressive state of DELLA protein in GA signaling, suggesting that SPY may activate the DELLA protein by *O*-GlcNAc modification. The DELLA proteins contain sequences that are rich in Ser and Thr residues, predicted as target sites for *O*-GlcNAc modification, between the GA signal perception domain and the LHR, supporting the hypothesis that the DELLA protein may be an *O*-GlcNAc protein in plants. Furthermore, this region in DELLA protein was predicted to be a regulatory domain of its repressive activity, because plants overexpressing a *ΔpolyS/T/V-SLR1* gene had more severe dwarf phenotypes than those overexpressing the intact form of SLR1 even though no changes were seen in GA-dependent degradation (Itoh *et al*., 2002). Until now, there has been no experimental demonstration of *O*-GlcNAc modification of DELLA proteins by SPY; however, recent studies have suggested that SPY may enhance the repressive activity of DELLA protein by competing with kinase. Antagonistic regulation of protein activity by *O*-GlcNAc modification and phosphorylation has been demonstrated in some cellular signaling pathways in animal systems (Comer and Hart, 2001; Love and Hanover, 2005; Slawson and Hart, 2003; Wells *et al*., 2001). Phosphorylation of DELLA proteins has been shown in rice SLR1 and Arabidopsis GAI and RGL2 (Fu *et al*., 2004; Hussain *et al*., 2005; Itoh *et al*., 2005; Sasaki *et al*., 2003). Especially in rice, expression of *ΔpolyS/T/V-SLR1* in *gid2* mutants indicates that the homopolymeric Ser/Thr stretch contains the primary target sites for phosphorylation of SLR1 (Itoh *et al*., 2005). Importantly, differences in the phosphorylation status of SLR1 were observed between *gid2* plants and *OsSPY RNAi/gid2* plants having a mild dwarf phenotype. Analysis by 2-D gel electrophoresis reveals that SLR1 in the *OsSPY*RNAi plants was more acidic than that in nontransgenic plants, indicating that suppression of OsSPY causes a pI shift in SLR1 through an increase in its phosphorylation status (Shimada *et al*., 2006). These results indicate that *O*-GlcNAc modification of the poly-S/T/V region by SPY may activate (or maintain) the repressive activity of DELLA proteins. Thus, if SPY function is lost, the sites of *O*-GlcNAc modification would be phosphorylated by an unknown kinase; as a result, the repressive function of DELLA protein would be reduced. Another example of the effect of *spy* mutations on the physical state of DELLA protein is the case of RGA in *Arabidopsis*. When *rga-Δ17* is expressed in plants, an extra protein band (designated form II) with a slower mobility than nascent rga-Δ17 (form I) is detected. This additional band has the characteristics of a phosphorylated protein (Dill *et al*., 2001; Silverstone *et al*., 2007). Although the nature of the modification has not been identified, form II increases dramatically in *spy* mutants (Silverstone *et al*., 2007).

4.2. Transcription factors directly regulate GA-responsive gene expression

4.2.1. Myb transcription factor (GAMyb)

GA-regulated α-amylase in cereal aleurone cells is one of the best-characterized GA-regulated responses in higher plants. In germinating cereal grains, the aleurone layer functions to synthesize and secrete hydrolytic enzymes to digest the starchy endosperm reserves (Sun and Gubler, 2004). GA triggers the expression of genes encoding various hydrolytic enzymes. Importantly, the aleurone layer itself does not synthesize bioactive GA at all: The GA response in aleurone cells is triggered by GA provided by the embryo. Thus, GA-regulated response in the cells of isolated aleurone layers is totally dependent on exogenously applied GA. Taking advantage of this dependency, a transient assay using isolated aleurone protoplasts has been established to explore molecular mechanisms of GA-inducible gene expression. These studies identified a *cis*-regulatory element, GA-response element (GARE), that confers GA responsiveness to a minimal 35S promoter (Skriver *et al.*, 1991). GARE is the critical element in the GA-response complex (GARC), which consists of GARE in combination with other *cis*-regulatory elements including the pyrimidine box, the TATCCAC box, the CAACTC box, and the Box1/O2S-like element (Gubler and Jacobsen, 1992; Lanahan *et al.*, 1992). The coupling of GARE with other regulatory elements is thought to quantitatively and qualitatively regulate GA-inducible hydrolase gene expression in aleurone cells.

GAMYB is a GA-regulated MYB transcription factor whose expression is rapidly increased by GA treatment in barley aleurone layers (HvGAMYB; Gubler *et al.*, 1995). HvGAMYB binds specifically to the TAACAAA element, a highly conserved sequence within GARE in the hydrolase gene, and activates α-amylase and other GA-responsive hydrolase gene promoters in the absence of GA (Gubler *et al.*, 1995, 1999). Further expression analysis using the barley *sln1* mutant has demonstrated that HvGAMYB is a positive regulator of GA signaling acting downstream of the DELLA protein SLN1. Reporter gene expression of the *HvGAMYB* promoter fused with a GUS gene is higher in loss-of-function *sln1a* mutant aleurone cells than in wild-type cells (Gomez-Cadenas *et al.*, 2001). Conversely, GA-induced *HvGAMYB* expression is repressed in the gain-of-function *sln1d* mutant (Gubler *et al.*, 2002). In addition to SLN1-dependent transcriptional regulation by GA, posttranslational regulation of HvGAMYB protein, such as protein stability and phosphorylation, has also been suggested (Gubler *et al.*, 2002; Woodger *et al.*, 2003).

GAMYB is an evolutionally conserved protein. Orthologous proteins have been identified not only in other grasses including rice, wheat, and *Lolium temulentum*, but also in *Arabidopsis* (Gubler *et al.*, 1997, Kaneko *et al.*, 2004; Millar and Gubler, 2005; Murray *et al.*, 2003; Tsuji *et al.*, 2006).

Expression analysis of *GAMYB* and *GAMYB*-like genes from *Arabidopsis*, rice, and barley shows similar expression patterns that are specifically high in seeds (aleurone cells) and flowers but moderate in meristematic regions (Kaneko *et al.*, 2004; Millar and Gubler, 2005; Murray *et al.*, 2003; Tsuji *et al.*, 2006). Recent genetic analyses using knockout mutants of *OsGAMYB* in rice and *AtMYB33* and *AtMYB65* in *Arabidopsis* highlighted an important role of GAMYB in floral organ development. In addition to the expected lack of GA-inducible α-amylase expression in aleurone cells, rice *osgamyb* mutants showed varying levels of floral abnormalities that might depend on physical (environmental) conditions, and they consistently produce shrunken anthers without pollen grains. Anatomical analysis of *osgamyb* anthers showed that microspore mother cells are not in contact with the tapetal layers at an early stage of anther development. As a result, microspore mother cells start degenerating at the premeiotic cell division stage and expansion of the tapetal cells occurs at a later stage (Kaneko *et al.*, 2004). These observations indicate that OsGAMYB is essential for the adherence of microspore mother cells to the tapetal layer and the subsequent meiotic division of these cells during anther development. *AtMYB33* and *AtMYB65* are two of three *GAMYB*-like genes of *Arabidopsis* and they are able to substitute for HvGAMYB in transactivating the α-amylase promoter in barley aleurone cells (Gocal *et al.*, 2001). Double-knockout mutants of *AtMYB33* and *AtMYB65* show a developmental arrest of the microspore mother cells just before meiosis, and the tapetum undergoes hypertrophy (Millar and Gubler, 2005). Therefore, the role of GAMYB in anther development appears to be highly conserved between rice and *Arabidopsis*.

A new layer of transcriptional regulation of *GAMYB* and *GAMYB-like* genes, via a microRNA-regulated pathway, has recently been proposed. MicroRNAs can regulate gene expression by directing cleavage or inhibiting translation of target transcripts (Llave *et al.*, 2002). MiR159 is an evolutionally conserved sequence in *Arabidopsis*, rice, tobacco (*Nicotiana benthamiana*), and barley (Achard *et al.*, 2004, Tsuji *et al.*, 2006). The miR159 sequence is also complementary to an internal region of several *GAMYB* mRNAs from *Arabidopsis*, rice, barley, wheat, and *L. temulentum*. The cleavage of mRNA of the *GAMYB*-like, *AtMYB33* by miR159 has been demonstrated using an *Agrobacterium*-mediated delivery system to coexpress miR159 and *AtMYB33* target mRNA in *N. benthamiana* leaf tissue (Achard *et al.*, 2004). The involvement of *GAMYB-like* genes in plant development has been illustrated by phenotypic characterization of transgenic plants overexpressing miR159 in *Arabidopsis* and rice. For example, in *Arabidopsis*, in contrast to the phenotypic abnormalities observed in *myb33 myb65* double-knockout mutants, the developmental arrest of anthers observed in *35S:miR159a* lines occurs at a different stage and the resultant anther morphology also differ. In addition, the flowering time of *35S:miR159a* lines is specifically delayed under short-day conditions

(Achard *et al.*, 2004; see below). In rice, although *osgamyb* mutants show only the developmental arrest of flowers, transgenic plants overexpressing miR159 have severely-blocked elongation of the topmost internode, and some flowers do not develop stamen and pistil primordia (Tsuji *et al.*, 2006). Although it has been suggested that GA regulates miR159 levels via the DELLA-dependent GA signaling pathway in *Arabidopsis*, recent studies on rice miR159 and Arabidopsis *MYB33*, one of three target genes of miR159, indicates that developmental regulation of miR159 expression maybe largely independent of GA signaling. Expression of *mMYB33*, a cleavage-resistant version of *AtMYB33*, induces various growth-retarding effects that appear contradictory to the proposed role in mediating GA signals (Millar and Gubler, 2005). Furthermore, miR159 expression in rice is not affected by GA application during flower development but it is developmentally downregulated as flower development proceeds (Tsuji *et al.*, 2006). Since miR159 expression is undetectable in rice aleurone cells, microRNA-mediated transcriptional regulation of *GAMYB* and *GAMYB-like* gene expression could be required at a stage later than germination. For example, it has been reported that the GA-dependent degradation of SLN1 and subsequent induction of *HvGAMYB* gene expression occurs in barley anthers (Murray *et al.*, 2003). Thus, it will be very interesting to further understand how GA signaling and microRNA-mediated pathways integrate to regulate anther development through the regulation of *GAMYB* and *GAMYB-like* gene expression.

Tsuji *et al.* (2006) reported, based on microarray analysis, that almost all of the genes regulated by GA in aleurone cells are regulated by SLR1–OsGAMYB pathway (Fig. 6.4). Furthermore, comparative study of the gene expression between aleurone cells and anthers reveals that OsGAMYB regulates different sets of genes in a tissue-specific manner, indicating that OsGAMYB might require different cooperator(s) to confer its tissue-specific function. In fact, *in silico* analysis of the OsGAMYB-dependent genes expressed in anthers revealed that conserved sequences such as the pyrimidine box and TATCACC box (located around the GARE region in the hydrolase gene promoter, which is expressed in aleurone layers) are not found in the promoter region of the OsGAMYB-dependent genes expressed in anthers. To support the above hypothesis, further studies are necessary to identify the factor(s) that cooperate with OsGAMYB and the *cis*-acting elements of OsGAMYB-regulated genes in anthers.

In addition to the possible biological functions of GAMYB in aleurone cells and anthers, some studies have suggested that GAMYB may also be involved in floral initiation. The promoter of the Arabidopsis gene *LEAFY* (*LFY*), a floral integrator, is GA responsive. Analysis of the *LFY* promoter indicates that the regulatory sequence conferring GA responsiveness contains a MYB protein target sequence (Blazquez and Weigel, 2000; Blazquez *et al.*, 1998). Furthermore, in the long-day plant *L. temulentum, LtGAMYB*

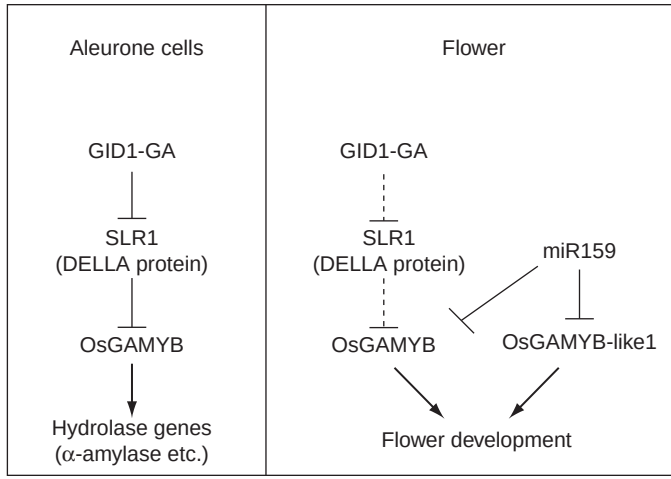

Figure 6.4 **Model of OsGAMYB function in rice. OsGAMYB regulates the expression of almost all GA-regulated genes in aleurone cells (left). In later growth stages, *OsGAMYB* and *OsGAMYB-like1* genes are essential for flower development. At this stage, *miR159* begins to be expressed, which downregulates *OsGAMYB* and *OsGAMYB-like1* expression in a GA-independent manner (right). Dashed lines represent unclear relationships.**

expression is induced in the shoot apex in response to GA and long-day treatment (Gocal *et al.*, 1999). In addition to the perturbation of anther development, transgenic Arabidopsis plants overexpressing miR159a are delayed in flowering under a short-day photoperiod that is also associated with a reduction in the levels of *LFY* transcripts (Achard *et al.*, 2004). These results suggest that GA-dependent floral transition, mediated by the *LFY* product under short-day conditions, is activated by *GAMYB-like* protein.

4.2.2. Transcription factors involved in the feedback regulation of GA homeostasis

The function of the GA-response pathway is tightly linked to the feedback regulation controlling GA biosynthesis and catabolism (Hedden and Phillips, 2000; Olszewski *et al.*, 2002). $GA_2$0-oxidase ($GA_2$0ox) and GA_3-oxidase (GA_3ox) are enzymes that catalyze the penultimate and last steps for synthesis of bioactive GA, respectively, while GA_2-oxidases are responsible for GA deactivation (Hedden and Phillips, 2000). The concept of feedback regulation of GA biosynthesis via its signaling pathway has come from expression analyses of GA biosynthetic genes in GA-response mutants. In constitutive GA-response mutants such as rice *slr1*, barley *sln1*, and *Arabidopsis rga gai-t6*, bioactive GAs and/or transcripts encoding GA biosynthetic enzymes (*$GA_2$0ox* and/or *GA_3ox*) are detected at lower levels than in wild-type plants (Croker *et al.*, 1990; Dill *et al.*, 2001; Ikeda *et al.*, 2001). In contrast,

GA-insensitive dwarf mutants, including rice *gid1* and *gid2*, maize *D8*, and *Arabidopsis gai-1* and *sly1*, accumulate significantly higher amounts of bioactive GAs and have higher GA_2ox and GA_3ox mRNA levels (Fujioka *et al.*, 1988; McGinnis *et al.*, 2003; Peng *et al.*, 1997; Sasaki *et al.*, 2003; Ueguchi-Tanaka *et al.*, 2005). Recently, two transcription factors that bind to the promoter regions of GA_3ox genes have been identified (Dai *et al.*, 2007; Matsushita *et al.*, 2007). Overexpression of the rice *YABBY1* (*YAB1*) gene causes a semidwarf phenotype, an increased level GA_{20}, and reduced level of GA_1 (Dai *et al.*, 2007). Further analyses show that $OsGA_3ox2$ expression is specifically reduced in the *YAB1*-overexpressing plants and that YAB1 protein binds to a promoter region of $OsGA_3ox2$. These results suggest that YAB1 directly represses $OsGA_3ox2$ gene expression; curiously, however, this effect is only seen in an indica rice background (Dai *et al.*, 2007). The observed effect of rice *YAB1* on $OsGA_3ox2$ expression may be due to a neomorph derived from seven amino acid sequence differences between japonica and indica YAB1. A second recently identified factor is AGF1, an AT-hook protein in *Arabidopsis*. AGF1 was identified from yeast one-hybrid screening using a regulatory sequence for feedback regulation in the $AtGA_3ox1$ promoter (Matsushita *et al.*, 2007). AGF1 binds a *cis*-regulatory sequence that is necessary for the induction of $AtGA_3ox1$ expression under GA-deficient conditions. Since constitutive expression of *AGF1* conferred both hyper upregulation of $AtGA_3ox1$ under reduced GA levels and resistance to downregulation of $AtGA_3ox1$ by excess GA, AGF1 could be a transcriptional activator specific to GA-negative feedback. Demonstration that AGF1 binds the $AtGA_2ox$ promoter would be necessary to show its general role in GA negative-feedback regulation.

In contrast to the factors described above, DELLA proteins are strong candidates for the master controllers of feedback regulation of GA biosynthetic genes by GA. This idea is further supported by the report that two Arabidopsis GRAS proteins, SHR and SCR, are associated with the promoter sequences of their target genes *in vivo* (Cui *et al.*, 2007; Levesque *et al.*, 2006). The strength of feedback regulation of GA_3ox and GA_2ox is tightly correlated with the amounts of DELLA proteins in the nucleus (McGinnis *et al.,* 2003; Sasaki *et al.*, 2003; Ueguchi-Tanaka *et al.*, 2005). In the *ga1–3* mutant, RGA accumulates in nuclei, and transcript levels of $AtGA_3ox1$ and $AtGA_2ox2$ are also high. After GA treatment, the levels of $AtGA_3ox1$ and $AtGA_2ox2$ transcripts rapidly decrease, and changes are noticeable in as early as 15 min, under these same GA treatment conditions, –90% of the RGA protein disappears within 10 min (Zentella *et al.*, 2007). Furthermore, transcript levels of $AtGA_3ox1$ and $AtGA_2ox2$ accumulated in a DEX-dependent manner when the degradation-resistant mutant rga-Δ17 was expressed under the control of a DEX-inducible promoter. These results indicate that $AtGA_3ox1$ and $AtGA_2ox$ may be direct target genes of DELLA proteins. The requirement of additional DELLA-associated

transcription factor(s) for DELLA-dependent regulation in the expression of GA biosynthetic genes has also been suggested by Zentella *et al.* (2007), because of the lack of significant enrichment of *AtGA$_2$0ox2* and *AtGA$_3$ox1* promoters detected using ChIP-qPCR analysis.

In addition to the feedback regulation of GA biosynthetic gene expression, recent studies in *Arabidopsis* have revealed that expression of *GID1* (GA receptor) genes is also under feedback regulation. Transcripts of all three Arabidopsis *GID1* genes are decreased upon GA treatment (Griffiths *et al.*, 2006). In addition, *GID1* transcript levels are accumulated in *ga1–3* and P_{RGA}:(*rga-*Δ*17*) transgenic plants. In the *ga1 rga gai* triple and *ga1 rga gai rgl1 rgl2* quintuple null mutants, transcript levels are similar to those in wild-type plants (Cao *et al.*, 2006; Griffiths *et al.*, 2006). Direct association of DELLA protein and the *GID1a* and *GID1b* promoter regions has been demonstrated by ChIP-qPCR assays using RGA–TAP as bait (Zentella *et al.*, 2007). Thus, DELLA proteins modulate GA homeostasis by upregulating expression of GA biosynthetic genes and GA receptor genes at the same time.

5. A Model of GA Signaling

A revised model of the GA signaling pathway in plants is shown in Fig. 6.5. This model is constructed mainly from molecular genetic studies of model plants (rice and *Arabidopsis*) and molecular biological studies using transient assays of cereal aleurone cells. All available data indicates that the repressive function of DELLA proteins is the center of the GA signaling pathway. Once GID1 binds to GA, GID1–GA directs DELLA protein to $SCF^{GID2/SLY}$-mediated proteolysis via direct protein–protein interaction between GID1–GA and DELLA proteins. Since GA-binding ability of GID1 is enhanced in the presence of SLR1 (Ueguchi-Tanaka *et al.*, 2007), this interdependent association of GID1–GA–DELLA protein may minimize undesirable deactivation of DELLA protein in the absence of GA.

Based on the genetic analysis of *spy* mutants in *Arabidopsis*, SPY may catalyze the *O*-GlcNAc modification of DELLA proteins. So far, it has been proposed that the *O*-GlcNAc modification of DELLA protein increases its repressive activity and that *O*-GlcNAc modification competes with phosphorylation, similar to the mechanism reported in mammalian systems (Comer and Hart, 2001; Love and Hanover, 2005; Slawson and Hart, 2003; Wells *et al.*, 2001). It has also been suggested that *O*-GlcNAc modification could facilitate nuclear localization or increase protein stability in mammalian systems (Thornton *et al.*, 1999; Wells *et al.*, 2001), but molecular biological study of RGA protein indicates that such regulation is not the case for DELLA proteins (Silverstone *et al.*, 2007). Furthermore, a competition model of *O*-GlcNAc and phosphorylation has been supported by the observation that the

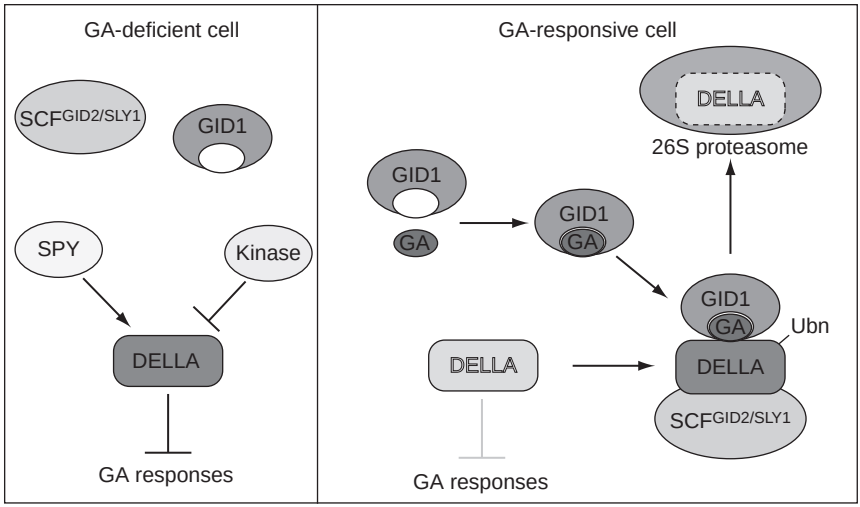

Figure 6.5 **Model of GA-dependent degradation of the DELLA protein and GA signaling. Under GA-deficient conditions (left), DELLA protein stably represses GA responses. Genetic analysis suggests that DELLA protein is modified with *O*-GlcNAc by SPY and that this modification increases the repressive activity of DELLA protein. *O*-GlcNAc modification might compete with phosphorylation by an unknown kinase to reduce the repressive function of DELLA protein. Once GA is received by GID1, GA binding of GID1 is stabilized by subsequent interaction with DELLA protein. Formation of the GID1–GA–DELLA protein complex triggers recruitment of DELLA protein to the SCF$^{GID2/SLY1}$ complex, leading to degradation of DELLA protein by 26S proteasomes.**

phosphorylation status of SLR1 is dramatically promoted when OsSPY function is suppressed by RNAi in rice plants (Shimada *et al.*, 2006).

The transcriptional factor GAMYB is a positive regulator of GA signaling that acts downstream of DELLA protein in cereal aleurone cells to induce the expression of almost all of the GA-induced genes (Tsuji *et al.*, 2006). Genetic analyses using the knockout mutant of *osgamyb* in rice and the *atmyb33 atmyb65* double mutant of *Arabidopsis* has highlighted the function of GAMYB in anther development (Kaneko *et al.*, 2004; Millar and Gubler, 2005; Tsuji *et al.*, 2006). Because GA-dependent induction of GAMYB expression is observed in barley anthers, it is possible that the DELLA–GAMYB pathway controls GA-regulated anther development. However, it should be noted that GAMYB-mediated anther development is also under the control of a *miR159*-mediated RNA interference pathway probably independent of GA signaling (Fig. 6.4, Achard *et al.*, 2004; Millar and Gubler, 2005; Tsuji *et al.*, 2006).

Additional elements also need to be incorporated into the GA signaling model. ChIP-qPCR assays using RGA–TAP indicate that transcriptional regulation of *GA$_3$ox* and *GA$_2$0ox* in feedback regulation is mediated by RGA-associated protein with DNA-binding activity (Zentella *et al.*, 2007).

Based on transient assays using barley aleurone cells, it has been hypothesized that there is an intermediate repressor protein downstream of DELLA protein and upstream of GAMYB because there is a time lag (–70 min) between the GA-dependent degradation of DELLA protein and the induction of *GAMYB* by GA (Sun and Gubler, 2004). Further, it has also been suggested that the antagonistic effect of ABA on α-amylase induction by GA may target a repressor acting downstream of DELLA protein (Gubler *et al.*, 2002; Zentella *et al.*, 2002). Genetic analysis of the rice *d1* mutant leads us to postulate a membrane-bound GA receptor in GA signaling of aleurone cells; this question must be answered to explain why mutation of the intracellular GID1 receptor leads to shut-down of GA-dependent α-amylase expression in the same cell.

6. Concluding Remarks

Identification of the GA receptor GID1 has greatly improved our understanding of how GA induces the transcription of its target genes. However, there are still missing elements in our current model of the GA signaling pathway. These elements include possible roles for a membrane-bound GA receptor, an unidentified kinase, and transcriptional regulators with DNA-binding activity. Whole-genome expression profiling and proteomic technology will be powerful tools to facilitate the identification of such elements. In addition to building up the complete picture of GA signaling pathway, X-ray and NMR analyses of GID1 will be important for understanding the 3D structure of the GA receptor. The 3D-structure of GID1, alone and interacting with GA, will provide clues to the molecular mechanism of GID1–GA–SLR1 complex formation.

REFERENCES

Achard, P., Verizen, W. H., Van Der Straeten, D., and Harberd, N. P. (2003). Ethylene regulates Arabidopsis development via the modulation of DELLA protein growth repressor function. *Plant Cell* **15,** 2816–2825.

Achard, P., Herr, A., Baulcombe, D. C., and Harberd, N. P. (2004). Modulation of floral development by a gibberellin-regulated microRNA. *Development* **131,** 3357–3365.

Achard, P., Cheng, H., De Grauwe, L., Decat, J., Schoutteten, H., Moritz, T., Van Der Straeten, D., Peng, J., and Harberd, N. P. (2006). Integration of plant responses to environmentally activated phytohormonal signals. *Science* **311,** 91–94.

Ashikari, M., Wu, J., Yano, M., Sasaki, T., and Yoshimura, A. (1999). Rice gibberellin-insensitive dwarf1 mutant gene *Dwarf1* encodes the α-subunit of GTP-binding protein. *Proc. Natl. Acad. Sci. USA* **96,** 10284–10289.

Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J. W., and Elledge, S. J. (1996). SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. *Cell* **86,** 263–274.

Blatch, G. L., and Lassle, M. (1999). The tetratricopeptide repeat: A structural motif mediating protein-protein interactions. *Bioessays* **21,** 932–939.

Blazquez, M. A., and Weigel, D. (2000). Integration of floral inductive signals in Arabidopsis. *Nature* **404,** 889–892.

Blazquez, M. A., Green, R., Nilsson, O., Susmen, M. R., and Weigel, D. (1998). Gibberellins promotes flowering of Arabidopsis by activating the *LEAFY* promoter. *Plant Cell* **10,** 791–800.

Boss, P. K., and Thomas, M. R. (2002). Association of dwarfism and floral induction with a grape 'green-revolution' mutation. *Nature* **416,** 847–850.

Bush, D. S. (1996). Effects of gibberellic acid and environmental factors on cytosolic calcium in wheat aleurone cells. *Planta* **199,** 89–99.

Cao, D., Cheng, H., Wu, W., Soo, H. M., and Peng, J. (2006). Gibberellin mobilizes distinct DELLA-dependent transcriptomes to regulate seed germination and floral development in Arabidopsis. *Plant Physiol.* **142,** 509–525.

Chandler, P. M., Marion-Poll, A., Ellis, M., and Gubler, F. (2002). Mutation at the *Slender1* locus of barley *cv* Himalaya. Molecular and physiological characterization. *Plant Physiol.* **129,** 181–190.

Comer, F. I., and Hart, G. W. (2001). Reciprocity between *O*-GlcNAc and O-phosphate on the carboxyterminal domain of RNA polymerase II. *Biochemistry* **40,** 7845–7852.

Croker, S. J., Hedden, P., Lwnton, J. R., and Stoddart, J. L. (1990). Comparison of gibberellins in normal and slender barley seedlings. *Plant Physiol.* **94,** 194–200.

Cui, H. C., Levesque, M. P., Vermoux, T., Jung, J. W., Paquette, A. J., Gallagher, K. L., Wang, J. Y., Blilou, I., Scheres, B., and Benfey, P. N. (2007). An evolutionarily conserved mechanism delimiting SHR movement defines a single layer of endodermis in plants. *Science* **316,** 421–425.

Dai, M., Zhao, Y., Ma, Q., Hu, Y., Hedden, P., Zhang, Q., and Zhou, D. X. (2007). The rice *YABBY1* gene is involved in the feedback regulation of gibberellin metabolism. *Plant Physiol.* **144,** 121–133.

Davies, P. J. (2004). ''Plant Hormones,'' 3rd edn. Kluwer Academic Publishers, The Netherlands.

Deshaies, R. J. (1999). SCF and Cullin/RingH2-based ubiquitin ligases. *Annu. Rev. Cell Dev. Biol.* **15,** 435–467.

De Simone, G., Menchise, V., Manco, G., Mandrich, L., Sorrentino, N., Lang, D., Rossi, M., and Pedone, C. (2001). The crystal structure of a hyper-thermophilic carboxylesterase from the archaeon *Archaeoglobus fulgidus*. *J. Mol. Biol.* **314,** 507–518.

Dill, A., and Sun, T.-P. (2001). Synergistic derepression of gibberellin signaling by removing RGA and GAI function in Arabidopsis thaliana. *Genetics* **159,** 777–785.

Dill, A., Jung, H. S., and Sun, T.-P. (2001). The DELLA motif is essential for gibberellin-induced degradation of RGA. *Proc. Natl. Acad. Sci. USA* **98,** 14162–16167.

Dill, A., Thomas, S. G., Hu, J., Steber, C. M., and Sun, T.-P. (2004). The Arabidopsis F-box protein SLEEPY1 targets gibberellin signaling repressors for gibberellin-induced degradation. *Plant Cell* **16,** 1392–1405.

Fu, X., and Harberd, N. P. (2003). Auxin promotes Arabidopsis root growth by modulating gibberellin response. *Nature* **421,** 740–743.

Fu, X., Richards, D. E., Ait-Ali, T., Hynes, L. W., Ougham, H., Peng, J., and Harberd, N. P. (2002). Gibberellin-mediated proteasome-dependent degradation of the barley DELLA protein SLN1 repressor. *Plant Cell* **14,** 3191–3200.

Fu, X., Richards, D. E., Fleck, B., Xie, D., Burton, N., and Harberd, N. P. (2004). The Arabidopsis mutant sleepy^{gar2-1} protein promotes plant growth by increasing affinity of SCF^{SLY1} E3 ubiquitin ligase for DELLA protein substrates. *Plant Cell* **16,** 1406–1418.

Fujioka, S., Yamane, H., Spray, C. R., Katsumi, M., and Phinney, B. O. (1988). The dominant non-gibberellin-responding dwarf mutant (*D8*) of maize accumulates native gibberellins. *Proc. Natl. Acad. Sci. USA* **85,** 9031–9035.

Fujisawa, Y., Kato, T., Ishikawa, A., Kitano, H., Sasaki, T., Asahi, T., and Iwasaki, Y. (1999). Suppression of the heterotrimeric G protein causes abnormal morphology, including dwarfism, in rice. *Proc. Natl. Acad. Sci. USA* **96,** 7575–7580.

Gilman, A. G. (1987). G proteins: Transducer of receptor-generated signals. *Annu. Rev. Biochem.* **56,** 615–649.

Gilroy, S., and Jones, R. L. (1994). Perception of gibberellin and abscisic acid at the external face of the plasma membrane of barley (*Hordeum vulgare* L.) aleurone protoplasts. *Plant. Physiol.* **104,** 1185–1192.

Gocal, G. F., Poole, A. T., Gubler, F., Blundell, C., and King, R. W. (1999). Long-day up-regulation of a *GAMYB* gene during *Lolium temulentum* inflorescence formation. *Plant Physiol.* **119,** 1271–1278.

Gocal, G. F., Sheldon, C. C., Gubler, F., Moritz, T., Bagnall, D. J., MacMillan, C. P., Li, S. F., Parish, R. W., Dennis, E. S., Weigel, D., and King, R. W. (2001). *GAMYB-like* genes, flowering, and gibberellin signaling in Arabidopsis. *Plant Physiol.* **127,** 1682–1693.

Gomez-Cadenas, A., Zentella, R., Walker-Simmons, M. K., and Ho, T.-H. D. (2001). Gibberellin/abscisic acid antagonism in barley aleurone cells: Site of action of the protein kinase PKABA in relation to gibberellin signaling molecules. *Plant Cell* **13,** 667–679.

Gomi, K., Sasaki, A., Itoh, H., Ueguchi-Tanaka, M., Ashikari, M., Kitano, H., and Matsuoka, M. (2004). GID2, an F-box subunit of the SCF E3 complex, specifically interacts with phosphorylated SLR1 protein and regulates the gibberellin-dependent degradation of SLR1 in rice. *Plant J.* **37,** 626–634.

Greenboim-Wainberg, Y., Maymon, I., Borochov, R., Alvarez, J., Olszewski, N. E., Ori, N., Eshed, Y., and Weiss, D. (2005). Cross talk between gibberellin and cytokinin: The Arabidopsis GA response inhibitor SPINDLY plays a positive role in cytokinin signaling. *Plant Cell* **17,** 92–102.

Griffiths, J., Murase, K., Rieu, I., Zentella, R., Zhang, Z. L., Pwers, S. J., Gong, F., Phillips, A. L., Hedden, P., Sun, T. P., and Thomas, S. G. (2006). Genetic characterization and functional analysis of the GID1 gibberellin receptors in Arabidopsis. *Plant Cell* **18,** 3399–3414.

Gubler, F., and Jacobsen, J. V. (1992). Gibberellin-responsive elements in the promoter of a barley high-pI a-amylase gene. *Plant Cell* **4,** 1435–1441.

Gubler, F., Kalla, R., Roberts, J. K., and Jacobsen, J. V. (1995). Gibberellin-regulated expression of a *myb* gene in barley aleurone cells: Evidence for Myb transactivation of a high-pI α-amylase gene promoter. *Plant Cell* **7,** 1879–1891.

Gubler, F., Watts, R. J., Kalla, R., Mattews, P., Keys, M., and Jacobsen, J. V. (1997). Cloning of a rice cDNA encoding a transcription factor homologous to barley GAMYB. *Plant Cell Physiol.* **38,** 362–365.

Gubler, F., Reventos, D., Keys, M., Watts, R., Mundy, J., and Jacobsen, J. V. (1999). Target genes and regulatory domains of the GAMYB transcriptional activator in cereal aleurone. *Plant J.* **17,** 1–9.

Gubler, F., Chandler, P. M., White, R. G., Llewellyn, D. J., and Jacobsen, J. V. (2002). Gibberellin signaling in barley aleurone cells: Control of SLN1 and *GAMYB* expression. *Plant Physiol.* **129,** 191–200.

Harberd, N. P., and Freeling, M. (1989). Genetics of dominant gibberellin-insensitive dwarfism in maize. *Genetics* **121,** 827–838.

Hartweck, L. M., and Olszewski, N. E. (2006). Rice GIBBERELLIN INSENSITIVE DWARF1 is a gibberellin receptor that illuminates and raises questions about GA signaling. *Plant Cell* **18,** 278–282.

Hartweck, L. M., Scott, C. L., and Olszewski, N. E. (2002). Two *O*-linked N-acetylglucosamine transferase genes of *Arabidopsis thaliana* L. Heyn. have overlapping functions necessary for gamete and seed development. *Genetics* **161,** 1279–1291.

Hartweck, L. M., Ganger, R. K., Gray, W. M., and Olszewski, N. E. (2006). SECRET AGENT and SPINDLY have overlapping role in the development of *Arabidopsis thaliana* L. Heyn. *J. Exp. Bot.* **57,** 865–875.

Hedden, P., and Phillips, A. L. (2000). Gibberellin metabolism: New insights reveals by the genes. *Trends Plant Sci.* **5,** 523–530.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. *Annu. Rev. Biochem.* **67,** 425–479.

Hooley, R., Beale, M. H., and Smith, S. J. (1991). Gibberellin perception at the plasma membrane of *Avena fatua* aleurone protoplasts. *Planta* **183,** 274–280.

Hussain, A., Cao, D., Cheng, H., Wen, Z., and Peng, J. (2005). Identification of the conserved serine/threonine residues important for gibberellin-sensitivity of Arabidopsis RGL2 protein. *Plant J.* **44,** 88–99.

Ikeda, A., Ueguchi-Tanaka, M., Sonoda, Y., Kitano, H., Koshioka, M., Futsuhara, Y., Matsuoka, M., and Yamaguchi, J. (2001). *Slender rice*, a constitutive gibberellin response mutant is caused by a null mutation of the *SLR1* gene, an ortholog of the height-regulating gene *GAI/RGA/RHT/D8*. *Plant Cell* **13,** 999–1010.

Itoh, H., Ueguchi-Tanaka, M., Sentoku, N., Kitano, H., Matsuoka, M., and Kobayashi, M. (2001). Cloning and functional analysis of two gibberellin 3beta-hydroxylase genes that are differently expressed during the growth of rice. *Proc. Natl. Acad. Sci. USA* **98,** 8909–8914.

Itoh, H., Ueguchi-Tanaka, M., Sato, Y., Ashikari, M., and Matsuoka, M. (2002). The gibberellin signaling pathway is regulated by the appearance and disappearance of SLENDER RICE1 in nuclei. *Plant Cell* **14,** 57–70.

Itoh, H., Sasaki, A., Ueguchi-Tanaka, M., Ishiyama, K., Kobayashi, M., Hasegawa, Y., Minami, E., Ashikari, M., and Matsuoka, M. (2005). Dissection of the phosphorylation of DELLA protein, SLENDER RICE1. *Plant Cell Physiol.* **46,** 1392–1399.

Iuchi, S., Suzuki, H., Kim, Y. C., Iuchi, A., Kuromori, T., Ueguchi-Tanaka, M., Asami, T., Yamaguchi, I., Matsuoka, M., Kobayashi, M., and Nakajima, M. (2007). Multiple loss-of-function of Arabidopsis gibberellin receptor AtGID1s completely shuts down a gibberellin signal. *Plant J.* **50,** 958–966.

Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S., and Kaelin, W. G., Jr. (2001). HIF α targeted for VHL-mediated destruction by proline hydroxylation: Implications for O-2 sensing. *Science* **292,** 464–468.

Izhaki, A., Swain, S. M., Tseng, T., Borochov, A., Olszewski, N. E., and Weiss, D. (2001). The role of SPY and its TPR domain in the regulation of gibberellin action throughout the life cycle of *Petunia hybrida* plants. *Plant J.* **28,** 181–190.

Jacobsen, S. E., and Olszewski, N. E. (1993). Mutation at the *SPINDLY* locus of Arabidopsis alter gibberellin signal transduction. *Plant Cell* **5,** 887–896.

Jacobsen, S. E., Binkowski, K. A., and Olszewski, N. E. (1996). SPINDLY, a tetratricopeptide repeat protein involved in gibberellin signal transduction in Arabidopsis. *Proc. Natl. Acad. Sci. USA* **93,** 9292–9296.

Kaneko, M., Inukai, Y., Ueguchi-Tanaka, M., Itoh, H., Izawa, T., Kobayashi, Y., Hattori, T., Miyao, A., Hirochika, H., Ashikari, M., and Matsuoka, M. (2004). Loss-of-function mutations of the rice *GAMYB* gene impair α-amylase expression in aleurone and flower development. *Plant Cell* **16,** 33–44.

King, K. E., Moritz, T., and Harberd, N. P. (2001). Gibberellins are not required for normal stem growth in *Arabidopsis thaliana* in the absence of *GAI* and *RGA*. *Genetics* **159,** 767–776.

Kobayashi, M., Yamaguchi, I., Murofushi, N., Ota, Y., and Takahashi, N. (1988). Fluctuation and localization of endogenous gibberellins in rice. *Agric. Biol. Chem.* **52,** 1189–1194.

Koornneef, M., Elgersma, A., Hanhart, C. J., von Loenen-Martinet, E. P., van Rign, L., and Zeevaart, J. A. D. (1985). A gibberellin insensitive mutant of *Arabidopsis thaliana*. *Physiol. Plant*. **65,** 33–39.

Lanahan, M. B., Ho, T.-H. D., Rogers, S. W., and Rogers, J. C. (1992). A gibberellin response complex in cereal alpha-amylase gene promoters. *Plant Cell* **4,** 203–211.

Lee, S., Cheng, H., King, K. E., Wang, W., He, Y., Hussain, A., Lo, J., Harberd, N. P., and Peng, J. (2002). Gibberellin regulates Arabidopsis seed germination via RGL2, a GAI/RGA-like gene whose expression is up-regulated following imbibition. *Genes Dev*. **16,** 646–658.

Levesque, M. P., Vornoux, T., Busch, W., Cui, H., Wang, J. Y., Blilou, I., Hassan, H., Nakajima, K., Matsumoto, N., Lohmann, J. U., Scheres, B., and Benfey, P. N. (2006). Whole-genome analysis of the SHORT-ROOT developmental pathway in Arabidopsis. *PloS Biol*. **4,** e143.

Llave, C., Xie, Z., Kasschau, K. D., and Carrington, J. C. (2002). Cleavage of *Scarecrow-like* mRNA targets directed by a class of Arabidopsis miRNA. *Science* **297,** 2053–2056.

Love, D. C., and Hanover, J. A. (2005). The hexosamine signaling pathway: Deciphering the "O-GlcNAc code". *Sci. STKE* **2005:** re13.

Matsushita, A., Furumoto, T., Ishida, S., and Takahashi, Y. (2007). AGF1, an AT-hook protein, is necessary for the negative feedback of *AtGA3ox1* encoding GA 3-oxidase. *Plant Physiol*. **143,** 1152–1162.

McGinnis, K. M., Thomas, S. G., Soule, J. D., Strader, L. C., Zale, J. M., Sun, T. P., and Steber, C. M. (2003). The Arabidopsis *SLEEPY1* gene encodes a putative F-box subunit of an SCF E3 ubiquitin ligase. *Plant Cell* **15,** 1120–1130.

Millar, A. A., and Gubler, F. (2005). The Arabidopsis *GAMYB-like* genes, *MYB33* and *MYB65*, are microRNA-regulated genes that redundantly facilitate anther development. *Plant Cell* **17,** 705–721.

Murray, F., Kalla, R., Jacobsen, J. V., and Gubler, F. (2003). A role for HvGAMYB in anther development. *Plant J*. **33,** 481–491.

Nakajima, M., Shimada, A., Takashi, Y., Kim, Y. C., Park, S. H., Ueguchi-Tanaka, M., Suzuki, H., Katoh, E., iuchi, S., Kobayashi, M., Maeda, T., Matsuoka, M., *et al.* (2006). Identification and characterization of Arabidopsis gibberellin receptor. *Plant J*. **46,** 880–889.

Olszewski, N. E., Sun, T.-P., and Gubler, F. (2002). Gibberellin signaling: Biosynthesis, catabolism, and response pathways. *Plant Cell* **14**(Suppl.), S61–S80.

Osterlund, T. (2001). Structure-function relationships of hormone-sensitive lipase. *Eur. J. Biochem*. **268,** 1899–1907.

Peng, J., Carol, P., Richards, D. E., King, K. E., Cowling, R. J., Murphy, G. P., and Harberd, N. P. (1997). The Arabidopsis *GAI* gene defines a signaling pathway that negatively regulates gibberellin responses. *Genes Dev*. **11,** 3194–3205.

Peng, J., Richards, D. E., Hartley, N. M., Murphy, G. P., Devos, K. M., Flintham, J. E., Beales, J., Fish, L. J., Worland, A. J., Pelica, F., Sudhakar, D., Christou, P., *et al.* (1999). 'Green revolution' genes encode mutant gibberellin response modulators. *Nature* **400,** 256–261.

Pysh, L. D., Wysocha-Diller, J. W., Camilleri, C., Bouchez, D., and Benfy, P. N. (1999). The *GRAS* gene family in Arabidopsis: Sequence characterization and basic expression analysis of the *SCARECROW-LIKE* genes. *Plant J*. **18,** 111–119.

Robertson, M., Swain, S. M., Chandler, P. M., and Olszewski, N. E. (1998). Identification of a negative regulator of gibberellin action, HvSPY, in barley. *Plant Cell* **10,** 995–1007.

Sakamoto, T., Kobayashi, M., Itoh, H., Tagiri, A., Kayano, T., Tanaka, H., Iwahori, S., and Matsuoka, M. (2001). Expression of a gibberellin 2-oxidase gene around shoot apex is related to phase transition in rice. *Plant Physiol*. **125,** 1508–1516.

Sakamoto, T., Miura, K., Itoh, H., Tatsumi, T., Ueguchi-Tanaka, M., Ishiyama, K., Kobayashi, M., Agrawal, G. K., Takeda, S., Abe, K., Miyao, A., Hirochika, H., *et al.* (2004). An overview of gibberellin metabolism enzyme genes and their related mutants in rice. *Plant Physiol.* **134,** 1642–1653.

Sasaki, A., Itoh, H., Gomi, K., Ueguchi-Tanaka, M., Ishiyama, K., Kobayashi, M., Jeong, D. H., An, G., Kitano, H., Ashikari, M., and Matsuoka, M. (2003). Accumulation of phosphorylated repressor for gibberellin signaling in an F-box mutant. Science **299,** 1896–1898.

Shimada, A., Ueguchi-Tanaka, M., Sakamoto, T., Fujioka, S., Takatsuto, S., Yoshida, S., Sazuka, T., Ashikari, M., and Matsuoka, M. (2006). The rice *SPINDLY* gene functions as a negative regulator of gibberellin signaling by controlling the suppressive function of the DELLA protein, SLR1, and modulating brassinosteroid synthesis. *Plant J.* **48,** 390–402.

Silverstone, A. L., Mak, P. Y., Martinez, E. C., and Sun, T.-P. (1997). The new *RGA* locus encodes a negative regulator of gibberellin response in *Arabidopsis thaliana*. *Genetics* **146,** 1087–1099.

Silverstone, A. L., Ciampaglio, C. N., and Sun, T.-P. (1998). The Arabidopsis *RGA* gene encodes a transcriptional regulator repressing the gibberellin signal transduction pathway. *Plant Cell* **10,** 155–169.

Silverstone, A. L., Jung, H. S., Dill, A., Kawaide, H., Kamiya, Y., and Sun, T.-P. (2001). Repressing a repressor: Gibberellin-induced rapid reduction of the RGA protein in Arabidopsis. *Plant Cell* **13,** 1555–1566.

Silverstone, A. L., Tseng, T. S., Swain, S. M., Dill, A., Jeong, S. Y., Olszewski, N. E., and Sun, T. P. (2007). Functional analysis of SPINDLY in gibberellin signaling in Arabidopsis. *Plant Physiol.* **143,** 987–1000.

Skriver, K., Olse, F. L., Rogers, J. C., and Mundy, J. (1991). *cis*-acting DNA elements responsive to gibberellin and its antagonist abscisic acid. *Proc. Natl. Acad. Sci. USA* **88,** 7266–7270.

Slawson, C., and Hart, G. W. (2003). Dynamic interplay between *O*-GlcNAc and O-phosphate: The sweet side of protein regulation. *Curr. Opin. Struct. Biol.* **13,** 631–636.

Steber, C. M., Cooney, S., and McCourt, P. (1998). Isolation of the GA-response mutant *sly1* as a suppressor of *ABI1-1* in *Arabidopsis thaliana*. *Genetics*. **149,** 509–521.

Strader, L. C., Ritchie, S., Soule, J. D., McGinnis, K. M., and Steber, C. M. (2004). Recessive-interfering mutation in the gibberellin signaling gene *SLEEPY1* are rescued by overexpression of its homologue, *SNEEZY*. *Proc. Natl. Acad. Sci. USA* **101,** 12771–12776.

Sun, T.-P., and Gubler, F. (2004). Molecular mechanism of gibberellin signaling in plants. *Annu. Rev. Plant Biol.* **55,** 197–223.

Thornton, T. M., Swain, S. M., and Olszewski, N. E. (1999). Gibberellin signal transduction presents . . . the SPY who *O*-GlcNAc'd me. *Trends Plant Sci.* **4,** 424–428.

Tseng, T. S., Swain, S. M., and Olszewski, N. E. (2001). Ectopic expression of the tetratricopeptide repeat domain of SPINDLY causes defects in gibberellin response. *Plant Physiol.* **126,** 1250–1258.

Tseng, T. S., Salome, P. A., McClung, C. R., and Olszewski, N. E. (2004). SPINDLY and GIGANTEA interact and act in *Arabidopsis thaliana* pathways involved in light responses, flowering, and rhythms in cotyledon movements. *Plant Cell* **16,** 1550–1563.

Tsuji, H., Aya, K., Ueguchi-Tanaka, M., *et al.* (2006). GAMYB controls different set of genes and is differentially regulated by microRNA in aleurone cells and anthers. *Plant J.* **47,** 427–444.

Tyler, L., Thomas, S. G., Hu, J., Dill, A., Alonso, J. M., Ecker, J. R., and Sun, T. P. (2004). Della proteins and gibberellin-regulated seed germination and floral development in Arabidopsis. *Plant Physiol.* **135,** 1008–1019.

Ueguchi-Tanaka, M., Fujisawa, Y., Kobayashi, M., Ashikari, M., Iwasaki, Y., Kitano, H., and Matsuoka, M. (2000). Rice dwarf mutant *d1*, which is defective in the α-subunit of heterotrimeric G protein, affects gibberellin signal transduction. *Proc. Natl. Acad. Sci. USA* **97,** 11638–11643.

Ueguchi-Tanaka, M., Ashikari, M., Nakajima, M., Itoh, H., Katoh, E., Kobayashi, M., Chow, T. Y., Hsing, Y. I., Kitano, H., Yamaguchi, I., and Matsuoka, M. (2005). *GIBBERELLIN INSENSITIVE DWARF1* encodes a soluble receptor for gibberellin. *Nature* **437,** 693–698.

Ueguchi-Tanaka, M., Nakajima, M., Katoh, E., Ohmiya, H., Asano, K., Saji, S., Hongyu, X., Ashikari, M., Kitano, H., Yamaguchi, I., and Matsuoka, M. (2007). Molecular interaction of a soluble gibberellin receptor, GID1, with a rice DELLA protein, SLR1, and gibberellin. *Plant Cell* **19,** 2140–2155.

Wells, L., Vosseller, K., and Hart, G. W. (2001). Glycosylation of nucleocytoplasmic proteins: Signal transduction and *O*-GlcNAc. *Science* **291,** 2376–2378.

Wen, C. K., and Chang, C. (2002). Arabidopsis *RGL1* encodes a negative regulator of gibberellin responses. *Plant Cell* **14,** 87–100.

Willige, B. C., Ghosh, S., Nill, C., Zourelidou, M., Dohmann, E. M. N., Maier, A., and Schwechheimer, C. (2007). The DELLA domain of GA INSENSITIVE mediates the interaction with the GA INSENSITIVE DWARF1A gibberellin receptor of Arabidopsis. *Plant Cell* **19,** 1209–1220.

Winkler, R. G., and Freeling, M. (1994). Physiological genetics of the dominant gibberellin-nonresponsive maize dwarf, *Dwarf8* and *Dwarf9*. *Planta* **193,** 341–348.

Woodger, F. J., Miller, A., Murray, F., Jacobsen, J. V., and Gubler, F. (2003). The role of GAMYB transcription factors in GA-regulated gene expression. *J. Plant Growth Regul.* **22,** 176–184.

Yoshida, Y., Tokunaga, F., Chiba, T., Iwai, K., Tanaka, K., and Tai, T. (2003). Fbs2 is a new member of the E3 ubiquitin ligase family that recognizes sugar chains. *J. Biol. Chem.* **278,** 43877–43884.

Yu, H., Ito, T., Zhao, Y., Peng, J., Kumar, P., and Meyerowitz, E. M. (2004). Floral homeotic genes are targets of gibberellin signaling in flower development. *Proc. Natl. Acad. Sci. USA* **101,** 7827–7832.

Zentella, R., Yamauchi, D., and Ho, T.-H. D. (2002). Molecular dissection of the gibberellin/abscisic acid signaling pathways by transiently expressed RNA interference in barley aleurone cells. *Plant Cell* **14,** 2289–2301.

Zentella, R., Zhang, Z. L., Park, M., Thomas, S. G., Endo, A., Murase, K., Fleet, C. M., Jikumaru, Y., Nanbara, E., Kamiya, Y., and Sun, T. P. (2007). Global analysis of DELLA direct targets in early gibberellin signaling in Arabidopsis. *Plant Cell* **19,** 3037–3057.

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CHAPTER SEVEN

Oocyte Quality and Maternal Control of Development

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Contents

Abstract

The oocyte is a unique and highly specialized cell responsible for creating, activating, and controlling the embryonic genome, as well as supporting basic processes such as cellular homeostasis, metabolism, and cell cycle progression in the early embryo. During oogenesis, the oocyte accumulates a myriad of

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factors to execute these processes. Oogenesis is critically dependent upon correct oocyte–follicle cell interactions. Disruptions in oogenesis through environmental factors and changes in maternal health and physiology can compromise oocyte quality, leading to arrested development, reduced fertility, and epigenetic defects that affect long-term health of the offspring. Our expanding understanding of the molecular determinants of oocyte quality and how these determinants can be disrupted has revealed exciting new insights into the role of oocyte functions in development and evolution.

Key Words: Oocyte quality, Epigenetics, Oogenesis, Maternal mRNA, Mitochondria, Preimplantation embryo, Meiosis.

1. Introduction

The traditional Korean Um-Yang concept of nature posits the existence of opposite and complimentary forces that, when in balance, provide for harmonious existence. The feminine, or Um, component is associated with the properties of combining or gathering to establish functionality. This concept applies very well to the oocyte. The oocyte is uniquely endowed with the ability to combine its own components with those of the incoming sperm, and direct the creation of a functional embryo. The oocyte is a highly differentiated, molecularly complex product of gametogenesis, despite its outwardly simple morphological appearance. During oogenesis, the oocyte must accumulate the components that are needed to support early embryo metabolism and physiology, as well as components needed to complete meiosis, initiate cell cycle progression, and direct early developmental events, such as the establishment of the primary embryonic body axes in many species. The oocyte must combine the two haploid genomes into a single embryonic genome, activate transcription of that genome at the correct time, and activate the appropriate array of genes to be transcribed. Additionally, the oocyte must maintain essential epigenetic information, while simultaneously remodeling chromatin and modifying certain other kinds of epigenetic information. In short, the successful development of all embryos is predicated upon correct and efficient execution of many crucial processes by the oocyte to create a functional embryo.

In this review, we will discuss key aspects of oogenesis, oocyte biology, and oocyte components that are responsible for creating a healthy, functional embryo. We will also discuss situations where these processes are disrupted, resulting in poor quality oocytes that are deficient in supporting correct embryo development. Indeed, it is becoming increasingly clear that the phenotype of offspring is largely defined by the quality of the oocytes from which they are derived. This applies to adult phenotypes as well as

embryonic characteristics, and indeed both maternal and grandmaternal effects on development and phenotype can be realized. Accordingly, the review will encompass recent discoveries that link maternal nutrition to defects in organogenesis and physiology in adult stage offspring.

2. Oogenesis

2.1. Stages of oogenesis

There are five stages of oogenesis in mammals, beginning with primordial germ cell (PGC), colonization of the developing gonad and continuing through formation of oogonia, primary oocytes, secondary oocytes, and mature eggs (Fig. 7.1). PGCs are diploid precursors of eggs and sperm, and exist transiently in the embryo before establishing close associations with the somatic cells of the gonad (Hogan, 2001). They can be visualized by alkaline phosphatase staining at 4 weeks of gestation in human embryos. In the mouse embryo, the PGCs are first detectable at day 7.25 postcoitum (dpc) as a small population of alkaline phosphatase-expressing cells in the extraembryonic mesoderm near the base of the allantois (Chiquoine, 1954; Ginsburg *et al.*, 1990). Over several days, the PGCs proliferate and migrate to the genital ridge, the gonadal anlage. By 13.5 dpc in the mouse, PGC proliferation is complete (Chiquoine, 1954; Mintz and Russell, 1957; Ozdzenski, 1967; Tam and Snow, 1981). Upon arrival in the female genital ridge, germ cells, now referred to as gonocytes, give rise to oogonia. The population of oogonia expands through a predetermined, species specific, number of mitotic divisions until the cells enter meiosis and become oocytes (Gosden and Bownes, 1995). For example, oogonia in the mouse ovary undergo ~4 mitotic cycles before entering meiosis between days 14 and 16 of a 20-day gestation period. In comparison, for humans as for other large mammalian species, there are many more rounds of mitotic division over a period of several months until shortly before birth (reviewed by Picton, 2000). In humans, the oogonia begin to differentiate in the 9th week of fetal life (Motta *et al.*, 1995, 2003). Clusters of oocytes or "nests" will break down, some oocytes will degenerate (apoptosis), and primordial follicles will form (Barnett *et al.*, 2006, for an in-depth review of genetic regulation of this process). The oocytes remain arrested in prophase I of meiosis until the female becomes sexually mature.

The primary oocytes synthesize an extracellular coat and cortical granules. Within a few days after birth in rats and mice, the primordial stage oocytes are surrounded by a single layer of flattened cells, also known as pregranulosa cells (Fortune, 2003; Fortune *et al.*, 2000). During the transition from the primary to the tertiary follicle, the granulosa cells (GCs)

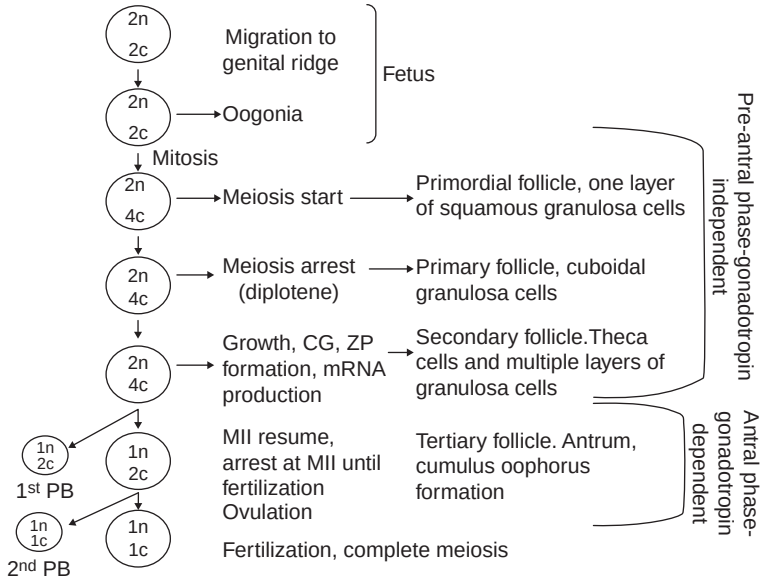

Figure 7.1 **The stages of mouse oogenesis. Primordial germ cells (PGCs) migrate to the genital ridge during fetal development and form oogonia. Oogonia proliferate by mitotic divisions, transitioning to primary oocytes as the first meiotic division initiates, and primordial follicles form with one layer of squamous granulosa cells (GCs) before or shortly after birth. Ploidy (*n*) is defined here on the basis of centromere number. DNA content is indicated as "c." After birth, primary oocytes remain meiotically arrested at prophase I and primary follicles form, with oocytes surrounded by cuboidal GCs. Secondary follicle formation proceeds with theca cells and multiple layers of GCs. The stages through secondary follicle formation are Gonadotropin independent. Oocytes continue to grow, synthesizing cortical granules (CGs), zona pellucida (ZP) and mRNA production. After puberty, under the influence of Gonadotropins, secondary follicles proceed to tertiary follicles. At this stage, antrum and cumulus oophorus formation occurs. Cells stop differentiation. Meiosis resumes and nuclear and cytoplasm maturation occurs. Secondary (mature) oocytes extrude the first polar body (PB), which has the haploid number of chromosomes (*n*). Meiosis arrests at metaphase II (MII), waiting for fertilization. Upon fertilization, oocyte activation occurs and meiosis is completed with extrusion of the second polar body.**

undergo a transition from a flattened epithelial to a cuboidal epithelial cell morphology, and with further proliferation produce a multilayered collection of cells surrounded by an outer layer of thecal cells and a basal membrane. *In vivo*, follicles with some flattened and some cuboidal GC are frequently observed, and can be very prolonged (Fortune *et al.*, 2000). An extensive network of gap junctions is established, which is essential for folliculogenesis (Ackert *et al.*, 2001). The formation of an antrum inside the GCs leads to the formation of an antral follicle. Within the antral follicle, the oocyte is surrounded by specialized cumulus cells. The antrum is lined by

the follicular epithelium. The whole follicle is shaped by the surrounding connective tissue. The layer of GCs does not contain blood vessels. The next step in oocyte development occurs when the oocyte is stimulated by hormones to resume meiosis as a prelude to ovulation. At this stage, several events occur such as chromosome condensation, nuclear envelope breakdown, and formation of the first polar body and large secondary oocyte. In mammals, oocyte maturation proceeds to metaphase II and then arrests until fertilization.

2.2. Oocyte–follicle cell interactions

The interaction between oocyte and follicular cells has been investigated extensively. Complex bidirectional communication between the oocyte and its surrounding somatic cells is essential for the coordinated development of both germ cell and somatic cell compartments (Fig. 7.2). The oocyte plays an active role in cumulus expansion (Epigg, 2001). The growth and development of the oocyte and the somatic components of the follicle occur in a highly coordinated manner. Previously it was thought that the

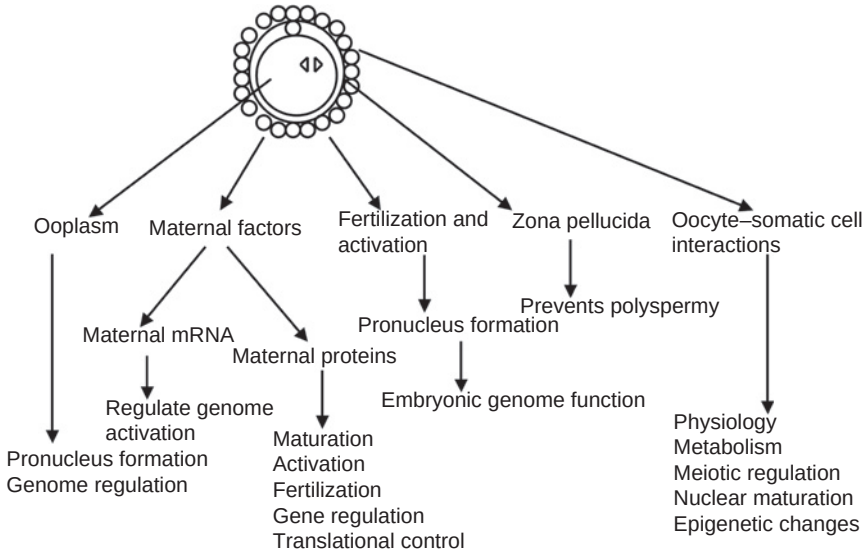

Figure 7.2 **Determinants of oocyte quality. The processes affecting oocyte quality are outlined. Ooplasm affects pronucleus formation and embryonic genome activation. Factors in the ooplasm, such as maternal mRNA and proteins, regulate genome activation in the absence of transcription. Bidirectional oocyte–somatic cell interactions help in the proper early development of the oocyte by affecting physiology, metabolism, nuclear maturation, and meiotic regulation.**

somatic components of the follicle control the oocyte and its development. Pincus and Enzmann (1934) found that fully grown oocytes removed from antral follicles underwent a spontaneous, gonadotropin-independent resumption of meiosis in culture, and concluded that follicular somatic cells maintain oocytes in meiotic arrest. But recent experiments indicate that the oocyte orchestrates follicular development. Oocytes regulate their own maturation and also affect the functions of the neighboring somatic cells (e.g., cumulus cell expansion) and ovulation rate (Juengel and McNatty, 2005). Follicular somatic cells in turn regulate oocyte transcription (De La Fuente and Eppig, 2001) and promote oocyte competence to undergo fertilization and preimplantation embryogenesis (cytoplasmic maturation) (Buccione *et al.*, 1990). GCs participate in the global suppression of transcription in oocytes that occurs before nuclear maturation. This highly coordinated development requires constant intercommunication between the oocyte and somatic cells. During follicular development, factors from GCs such as Kit ligand (KITL) promote oocyte development, whereas factors from the oocyte influence GC development and function. These interactions, in concert with gonadotropins and other factors, promote the transition to antral follicle. In antral follicles, oocyte-derived factors, such as growth differentiation factor 9 (GDF9), promote the development of the cumulus cell phenotype by suppressing expression of the mural granulosa cell (MGC) phenotype (Elvin *et al.*, 2000; Eppig, 2001; Matzuk *et al.*, 2002; McNatty *et al.*, 2003). GDF9 in culture can promote many of the changes in GC gene expression brought about by oocytes. Interestingly, the oocyte influence is developmentally regulated, with growing oocytes more able to promote GC development than fully grown oocytes (Latham *et al.*, 2004). Bone morphogenetic protein 15 (BMP15), another oocyte-derived factor, is mitogenic for somatic cells and stimulates GC proliferation (Di Pasquale *et al.*, 2004; Otsuka *et al.*, 2000; Shimasaki *et al.*, 2004). Oocytes regulate cumulus cell metabolism (Sugiura *et al.*, 2005) and BMP15 and fibroblast growth factors (FGFs) cooperatively promote increased glycolysis rates in cumulus cells (Sugiura *et al.*, 2007). BMP15-deficient mice demonstrate reduced ovulation, fertilization and developmental potential (Yan *et al.*, 2001). Humans demonstrate a mutation in *Bmp15* that leads to hypergonadotropic ovarian failure in women (Di Pasquale *et al.*, 2004), indicating an important role of BMP15 in follicular development and ovulation. Oocytes also secrete a potent mitogenic factor that promotes mural granulosa and cumulus cell DNA synthesis and cell proliferation (Li *et al.*, 2000; Gilchrist *et al.*, 2001, 2003). Oocytes modulate follicle stimulating hormone (FSH)-induced progesterone and estradiol synthesis by mural and cumulus GCs (Li *et al.*, 2000) and suppress FSH-induced luteinizing hormone receptor (*Lhcgr*) mRNA expression (Eppig *et al.*, 1997). They also regulate the differentiation of the GCs toward the cumulus cell phenotype, which is markedly distinct from the MGC phenotype. The cumulus cells have very

low *Lhcgr* expression compared to MGCs, and possess the capacity to secrete hyaluronic acid and undergo mucification/expansion while MGC do not (Eppig *et al.*, 1997; Li *et al.*, 2000). Mucification of the cumulus cells is needed for fertilization to be successful. With the microsurgical removal of the oocytes from the cumulus–oocyte complex (COC), the cumulus cells display a phenotype similar to MGC, evidenced by less DNA synthetic activity and increased secretion of progesterone (Buccione *et al.*, 1990). Culture of the oocytectomized complex with denuded oocytes restores cumulus cell characteristics. The oocyte also enables cumulus cell expansion in response to epidermal growth factor (EGF) (Diaz *et al.*, 2006). Oocyte-mediated signaling via MAPK1/3 (ERK1/2) and SMAD2/3 within cumulus cells promotes the cumulus cell phenotype (Diaz *et al.*, 2007; Su *et al.*, 2003).

The development of the oocyte and the somatic cells occurs simultaneously and this is responsible for ensuring an ovulated oocyte ready for fertilization. Disruption of this communication will result in oocyte developmental failure. Oocytes can regulate the rate of development of the somatic cells. This was demonstrated by a set of elegant experiments conducted by Eppig *et al.* (2002). Oocytes from 12-day-old mice were combined with somatic cells from newborn mouse ovaries. Upon grafting, oocytes were surrounded by one or two layers of GCs typical of primary to secondary follicle development. Nine days after grafting, large antral follicles were formed, whereas in the control, which contained oocytes and cumulus cells both from neonatal ovaries, follicles developed at the normal rate, that is, 19–20 days. The rate of development of the follicles was approximately doubled by the presence of later stage oocytes. This demonstrated that oocytes coordinate the development of mammalian ovarian follicles and that the rate of follicular development is based on a developmental program intrinsic to the oocyte (Eppig *et al.*, 2002). Follicles are also regulated by extraovarian factors such as gonadotropin hormones, and require FSH and receptor to become large antral preovulatory follicles (Dierich *et al.*, 1998; Kumar *et al.*, 1997).

2.3. Relevance to oocyte quality

2.3.1. *In vitro* culture effects on oocyte quality

Changes in oocyte development are normally coordinated with follicular differentiation. This coordination, however, can be changed abruptly by accelerating follicular development with exogenous gonadotropins, or other factors. Early recruitment of small antral follicles into the pool of preovulatory follicles results in the ovulation of oocytes incapable of normal maturation, fertilization, or embryogenesis (Eppig *et al.*, 1992a; Hunter, 1998; Schramm and Bavister, 1996a). Many studies in oocyte growth and development *in vitro* were done by the laboratory of John Eppig, employing

cultures of oocyte–GC complexes isolated from the preantral follicles of 12-day-old mice (mid-growth phase) by collagenase digestion. Theca cells were removed and the basal lamina enclosing the GCs and oocytes was degraded. The oocytes at this stage were incompetent to resume meiosis without further development. Between 200 and 300 oocyte–GC complexes were cultured on a collagen-impregnated membrane for 10 days, a period that spans the time of antrum formation and the acquisition of competence to resume meiosis and undergo fertilization and preimplantation development by oocytes *in vivo*. Initially 5% fetal bovine serum was used (Eppig and Schroeder, 1989), and this was later replaced by the serum protein fetuin, which was able to reduce zona hardening (Eppig *et al.*, 1992b, 1996). Serum-free culture medium was supplemented with insulin, transferrin, and selenium (ITS) to promote healthy development of the complexes (Eppig *et al.*, 1992b). The culture system yielded oocytes that could undergo maturation, be fertilized, and develop, but with lower rates compared to *in vivo* oogenesis. Thus, oocyte growth *in vitro* was not equivalent to growth *in vivo* (Eppig and O'Brien, 1996; Eppig and Schroeder, 1989), and some important follicular factors were missing, thus compromising oocyte quality.

Oocytes were also grown *in vitro* from the primordial stage. In the initial studies, the success rate was very low (0.5%) and the first mouse produced from these oocytes displayed abnormal adult characteristics (Eppig and O'Brien, 1996). Two dimensional protein gel analysis revealed a number of proteins altered in expression in GCs and cumulus cells by *in vitro* culture (Latham *et al.*, 1999). Additional effects of FSH and insulin were also seen, and these effects may contribute to differences in oocyte quality and developmental competence. Cultured oocyte–GC complexes fail to undergo appropriate transcriptional regulation (De La Fuente and Eppig, 2001), further indicating the importance of the correct follicular environment for establishing the correct oocyte phenotype. Subsequently, these and other observations were employed to develop a revised culture protocol, differing in the duration of treatment with FSH, and this yielded much greater success in the production of developmentally competent oocytes (O'Brien *et al.*, 2003). This success highlights the critical and dynamic nature of oocyte–somatic cell interactions and the importance of follicular environment in the production of high quality oocytes that can support development and yield healthy offspring.

Other evidence for the importance of these oocyte–follicle cell interactions in establishing oocyte quality comes from studies in the rhesus monkey using *in vitro* oocyte maturation protocols. In rhesus monkeys, *in vitro* maturation remains very inefficient, and has not led to the birth of live young. In unprimed monkeys, oocytes subjected to *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) form blastocysts at an efficiency of less than 1% (Schramm and Bavister, 1996a). Prior stimulation with FSH

elevates the blastocyst formation rate to as high as 40% (Schramm and Bavister, 1996b; Schramm *et al.*, 1994). Interestingly, co-culture of maturing oocytes from nonstimulated females with GCs from FSH stimulated females enhances blastocyst formation by more than tenfold (Schramm and Bavister, 1996a).

2.3.2. Genetic control of oocyte quality

Estrogens extend the action of FSH on GCs by promoting their proliferation and increasing their expression of FSH receptors (Ireland and Richards, 1978). Estrogen signaling is mediated via binding to estrogen receptors (ESRs), which are ligand-dependent transcription factors. Two ESR subtypes exist in humans, ESR1 (ERα) (Menasce *et al.*, 1993; Walter *et al.*, 1985) and ESR2 (ERβ) (Enmark *et al.*, 1997; Mosselman *et al.*, 1996), coded by *ESR1* and *ESR2* genes, respectively. In the ovary, ESR1 is mostly located in the thecal layer, whereas ESR2 can be found in GCs of growing follicles at all developmental stages (Pelletier and El-Alfy, 2000). ESR1, is most abundant, and found in all human reproductive tissues. Studies on male and female *Esr1* knockout (ERKO) mice showed complete infertility (Matthews and Gustafsson, 2003). A study by Altmäe *et al.* (2007) determined the associations between genetic variations in estrogen receptor *ESR1* and *ESR2* genes and etiology of female infertility. They analyzed the influence of these variations on controlled ovarian hyperstimulation (COH) outcome—the quantity and quality of oocytes retrieved. *ESR1 PvuII* T/C (rs2234693) and *XbaI* A/G (rs9340799) single-nucleotide polymorphisms (SNPs) and $(TA)_n$ microsatellite polymorphism, as well as *ESR2 RsaI* G/A (rs1256049) SNP and $(CA)_n$ microsatellite polymorphism were determined in 159 IVF patients. Their results suggested that variations in the *ESR1* gene, in addition to the age of a woman, may predict the COH outcome in IVF.

Follistatin plays an important role in female physiology, regulating FSH levels through blocking activin actions. Failure to regulate FSH correctly has been implicated as a potential cause of premature ovarian failure. Follistatin has been associated with polycystic ovary syndrome (PCOS) in women through genetic linkage studies (Urbanek *et al.*, 2000). PCOS is an endocrine disorder characterized by reduced fertility, hyperandrogenism, and chronic anovulation (Calvo *et al.*, 2001; Liao *et al.*, 2000; Urbanek *et al.*, 1999, 2000). Ovarian failure in women may be diagnosed by high concentrations of the gonadotropins, FSH and luteinizing hormone (LH), and a low concentration of estradiol. A rise in FSH is the most sensitive and the best early marker for ovarian failure (Conway, 2000). Mice lacking follistatin show a drastic reduction in the number of follicles, a reduction in the pool of available oocytes, poor oocyte quality, a failure to ovulate, and increased FSH levels, indicating premature ovarian failure (Jorgez *et al.*, 2004). Women with premature ovarian failure who undergo attempts to

induce ovulation using different regimens have reduced ovulation rates (Johnson and Peterson, 1979; Nelson *et al.*, 1992; van Kasteren *et al.*, 1995).

A long list of genes has been experimentally demonstrated by mutations in mice to control folliculogenesis and oocyte quality. Mice homozygous for a null allele at the *Gdf9* locus (Dong *et al.*, 1996) or for a hypomorphic *Kitl* allele (Donovan and de Miguel, 2001) exhibit female infertility as a result of blocks in follicular development preceding formation of secondary follicles. Females deficient in pentraxin-related gene 3 ($Ptx3^{-/-}$) are subfertile due to defects in the integrity of the cumulus cell–oocyte complex that are reminiscent of $Bmp15^{-/-}/Gdf9^{+/--}$ double mutant and BMP type IB receptor mutant mice. The zona pellucida plays an important role in oocyte–cumulus cell communication as well as controlling sperm penetration and providing physical protection to the embryo. Mice lacking ZP2 and ZP3 proteins are deficient in early antral and preovulatory follicle COC formation and ovulation, and blastocysts from their eggs fail to complete development after transfer to the pseudopregnant mice (Rankin *et al.*, 1996, 2001; Zhao and Dean, 2002). Gap junctions are important for oocyte–granulosa cell communication. Defects in meiotic maturation are evident in mice lacking the GC oocyte junction protein GJA4 (connexin 37) (Carabatsos *et al.*, 2000).

Collectively, these observations further highlight the importance of oocytes in promoting GC proliferation and differentiation (Eppig, 2001), with the oocyte in turn depending on somatic cells to support its growth and development (Brower and Schultz, 1982), regulate meiosis (Chesnel *et al.*, 1994), and modulate global transcriptional activity in the oocyte genome (De La Fuente and Eppig, 2001). The genetic studies to date reveal a wide array of genes that control oogenesis, emphasizing the complexity of the process and the many opportunities for genetic disruption of oocyte quality and subsequent effects on embryogenesis.

2.3.3. Environmental factors affecting oocyte quality

Environmental factors that disrupt follicular function also affect oocyte quality. The potential effects on reproduction by chemicals with hormone-like activity is a growing concern. A number of chemicals in the environment are estrogenic and can disrupt the endocrine system of wildlife and humans by binding to and activating the ESR. These environmental estrogens have the potential to perturb sensitive hormone pathways that regulate reproductive function. Most phytoestrogens and synthetic compounds bind to estrogen receptor ESR1 and ESR2 with relatively low affinity (Kuiper *et al.*, 1997). Diethylstilbestrol (DES) is a synthetic estrogen. It has been used as an estrogen supplement before its carcinogenic effect was recognized (Herbst *et al.*, 1971). In mouse oocytes, DES interferes with centrosomes and microtubule dynamics (Can and Semiz, 2000).

Another important endocrine disruptor is bisphenol A (4,4′-isopropylidene-2-diphenol; BPA). This is a high-volume diphenylalkane used for the production of polycarbonate plastics, epoxy and phenolic resins, polyesters, and polyacrylates, which have wide industrial applications including the manufacture of plastic wares, dental resins, and food can lining. It is proposed to have estrogenic activity (Krishnan *et al.*, 1993; Kuiper *et al.*, 1998; Metcalfe *et al.*, 2001; Welshons *et al.*, 2006). BPA is a widespread contaminant of the aquatic environment (Kawahata *et al.*, 2004; Vethaak *et al.*, 2005). Fish exposed to BPA show testes growth inhibition and maturation delay, altered male sex cell types, lower semen quality, reduced percentage of ovulation in females, altered ovulation timing, and occurrence of ovo-testes (Hassanin *et al.*, 2002; Kang *et al.*, 2002; Lahnsteiner *et al.*, 2005; Mandich *et al.*, 2007; Sohoni *et al.*, 2001; Tabata *et al.*, 2001; Tokumoto *et al.*, 2005). In mice, BPA effects on the oocyte have been revealed by Hunt *et al.* (2003), including a sudden, spontaneous increase in meiotic disturbances, such as aneuploidy coinciding with the accidental exposure of animals to an environmental source of bisphenol (Hunt *et al.*, 2003). In a recent study by Susiarjo *et al.*, (2007), the effect of BPA on meiosis of fetal ovaries was assessed by exposing pregnant mice in their mid-gestation stage to BPA. Oocytes from exposed female fetuses displayed gross aberrations in meiotic prophase, while in mature females BPA exposure increased aneuploidy in oocytes and embryos. In another study (Can *et al.*, 2005), BPA induced cell cycle delay and altered centrosome and spindle microtubular organization in oocytes during meiosis.

The endogenous metabolite of 17β-estradiol (E_2), 2-methoxyestradiol (2-ME), is a natural component of follicular fluid produced by the GCs (Dehennin *et al.*, 1984; Hammond *et al.*, 1986; Spicer *et al.*, 1987). Concentrations of 2-ME can be increased by exposure to environmental pollutants that activate the expression of enzymes in the metabolic pathway from 17β-estradiol to 2-ME. Exogenous estradiol does not affect maturation, whereas its metabolite 2-ME impairs the acquisition of full developmental competence (Lattanzi *et al.*, 2003). There are marked alterations in the spindle assembly of oocytes exposed to 2-ME during maturation, leading to gross chromosomal aberrations after fertilization and subsequent developmental arrest at the morula stage (Lattanzi *et al.*, 2003). Another recent study by Eichenlaub-Ritter *et al.* (2007) found that a high concentration of 2-ME produces a minor increase in GV-arrested dictyate-stage oocytes, blocked progression from first to second meiotic metaphase, caused delays in the formation of a typical bipolar spindle at meiosis I, induced severe spindle abnormalities and aberrant spindle pole organization at metaphase II, disrupted centrosome integrity, and induced nondisjunction and aneuploidy. Other chemicals, such as triclorofon (Cukurcam *et al.*, 2004; Yin *et al.*, 1998), chloral hydrate (Eichenlaub-Ritter and Betzendahl, 1995), nocodazole (Eichenlaub-Ritter and Boll, 1989; Everett and Searle, 1995;

Shen *et al.*, 2005; Sun *et al.*, 2005), mancozeb (Rossi *et al.*, 2006), diazepam (Sun *et al.*, 2001), okadaic acid (de Pennart *et al.*, 1993; Zernicka-Goetz and Maro, 1993), and taxol (Mailhes *et al.*, 1999), affect the meiotic spindle as well. Such effects on the oocyte can affect embryonic development by compromising genome integrity.

3. Oocyte Activation

Immediately after fertilization, egg activation leads to a complex series of events associated with blocks to polyspermy and additional downstream events that culminate in cleavage and eventually activation of gene transcription to complete the oocyte to embryo transition. The activation stimulus normally arises via actions of sperm-derived components (Fujimoto *et al.*, 2004; Stice and Robl, 1990). Artificial activation of oocytes can be achieved through a variety of treatments, including brief ethanol treatment, exposure to calcium ionophores, electrical pulses, treatment with strontium chloride, and treatment with the protein phosphorylation inhibitor 6-Dimethylamino purine (DMAP). Artificial oocyte activation is an essential component of experimental procedures such as nuclear transfer (NT) and parthenogenesis to produce potentially useful stem cell lines, and may also be advantageous in combination with clinical procedures involving subnormal sperm. Considerable interest has thus arisen in understanding oocyte activation and how it relates to embryo quality. An optimized activation protocol could enhance reprogramming of NT embryos, and could enhance the quality of embryos obtained by artificial activation.

3.1. Molecular mechanisms

Egg activation is a complex process involving multiple mechanisms. One key mechanism is calcium signaling. At fertilization, a massive increase in intracellular calcium abundance occurs in the egg, which leads to the formation of pronuclei and resumption of cell divisions (Miyazaki and Ito, 2006). Calcium is released from the endoplasmic reticulum by the 1,4,5-triphosphate receptor (Miyazaki, 2006). This calcium wave starts at the site of sperm entry and travels across the whole egg, thus activating it. Swann (1994) injected sperm head extract into eggs, which resulted in oocyte activation. This led to the hypothesis that there is a factor called Ca^{2+} oscillation-inducing protein (COIP) in the sperm responsible for egg activation. Phospholipase C (PLC) family members have been prime candidates for this factor. Most PLCs fail to induce calcium oscillations upon injection. Saunders *et al.* (2002) found that PLC-zeta, which is specifically expressed in sperm, induces calcium oscillations upon injection of its mRNA into

oocytes, implicating it as a putative COIP protein. Fujimoto *et al.* (2004) demonstrated that PLC-zeta is a sperm-borne oocyte activation factor associated with the perinuclear matrix in the postacrosomal region. Once oocyte activation is initiated by the sperm, additional activation-associated processes are executed by the egg in response to oscillations in intracellular free calcium ion.

3.2. Comparative effects of natural and artificial activation on development

While the sperm provides the natural stimulus for oocyte activation, a variety of artificial stimuli can trigger oocyte activation and initiate development. The occurrence of one or more transient increases in free calcium ion is the key trigger of meiotic resumption during fertilization, and a wide range of procedures for artificial oocyte activation have been established to elicit calcium transients, including mechanical, chemical, and physical stimuli that elicit one or several Ca^{2+} transients in the oocyte. Chemical activation can be induced by exposure to the Ca^{2+} ionophore (Kline and Kline, 1992), ionomycin (Loi *et al.*, 1998), 7% ethanol (Presicce and Yang, 1994), strontium chloride (Cuthbertson *et al.*, 1981), phorbol ester, and thimerosal. Ionophore A23187 promotes the release of intracellular Ca^{2+} stores but also facilitates the influx of extracellular Ca^{2+} ions (Kline and Kline, 1992). When a combination of calcium ionophore A23187 and puromycin is used, the activation rate is ~90% and the proportion of parthenotes displaying one pronucleus with extrusion of the second polar body is ~80% in mouse oocytes and human aged oocytes (Nakagawa *et al.*, 2000; Sengoku *et al.*, 2004; Yamano *et al.*, 2000). Ionomycin is another potent Ca^{2+} ionophore currently used in NT protocols (Cibelli *et al.*, 1998; Wells *et al.*, 1999). It mobilizes intracellular Ca^{2+} from Ca^{2+} stores. Ionomycin has been used widely with human intracytoplasmic sperm injection (ICSI) as well as in bovine NT experiments. Exposure of matured oocytes to 7% ethanol for 5–7 min induces successful activation and pronucleus formation by promoting the formation of inositol triphosphate (IP_3) and the influx of extracellular Ca^{2+} (Presicce and Yang, 1994). Strontium chloride induces multiple Ca^{2+} transients, probably by displacing bound Ca^{2+} in the oocyte (Whittingham and Siracusa, 1978), but also by inducing intracellular Ca^{2+} release (Kline and Kline, 1992). Strontium chloride has been used successfully to activate mouse oocytes after NT (Chung *et al.*, 2002; Gao *et al.*, 2003, 2004; Vassena *et al.*, 2007a,b; Wakayama *et al.*, 1998). Phorbol ester, which mimics endogenous diacylglycerol, activates the calcium- and phospholipid-dependent protein kinase C (Nishizuka, 1984) and induces calcium oscillations and pronucleus formation in mouse oocytes (Cuthbertson and Cobbold, 1985). However, the activation rate is lower when compared to calcium ionophore (Uranga *et al.*, 1996). This compound

has not been used in other mammalian oocytes. Thimerosal, a sulfhydryl-oxidizing agent that induces repetitive Ca^{2+} oscillations, has been used successfully for the activation of bovine oocytes (Fissore *et al.*, 1992, 1995). However, the peak and the duration of the calcium oscillations induced by thimerosal are shorter than those of the first rise induced by spermatozoa during fertilization (Nakada and Mizuno, 1998). Electrical stimulation is an alternative to chemical activation to induce Ca^{2+} influx through the formation of pores in the plasma membrane. The success of this procedure depends on the size of the pores formed and the ionic content of the medium. Moreover, the time to restore membrane integrity depends on the temperature, which affects the fluidity of lipids and proteins in the plasma membrane (Zimmermann *et al.*, 1985). Periodically repeated electrical stimulation mimics the pattern of oscillations observed during fertilization (Ozil, 1990). The single Ca^{2+} rise recorded after electrical stimulation is dependent on the presence of extracellular Ca^{2+} ions. However, when rabbit oocytes are pulsed in the presence of lithium (which prevents the production of IP_3), oocyte activation is inhibited (Ozil, 1990). This suggests that electrical stimulation induces the production of IP_3 that leads to intracellular Ca^{2+} release. Electroporation of IP_3 in a calcium- and magnesium-free medium followed by incubation in 6-DMAP has been used to activate parthenogenetic and NT rabbit embryos (Mitalipov *et al.*, 1999). Another physical stimulus used for oocyte activation is the exposure of oocytes to room temperature before NT (Stice *et al.*, 1994).

The number and pattern of calcium oscillations control essential early processes, such as cortical granule exocytosis, cell cycle resumption, and gene transcription (Ducibella *et al.*, 2002, Ozil *et al.*, 2005). The pattern of calcium oscillations affects gene expression and developmental potential (Ozil *et al.*, 2006). Too few oscillations can alter expression of 20% of genes analyzed, preferentially affecting the expression of mRNAs related to transcription and mRNA processing. Hyperstimulation has a much lesser effect on gene expression, but is associated with effects on progeny growth. Thus, the mode of oocyte activation can have long-term consequences on embryo development.

4. Oocyte Components Controlling Early Development

The oocyte contains a rich supply of macromolecules and organelles that collectively support and regulate vital processes in the early embryo. This includes processes such as ion homeostasis, metabolism, cell cycle progression, DNA repair, apoptosis, transcriptional activation of the embryonic genome, epigenetic modifications and reprogramming of the genomic

material, and in some species early cell fate determinative events that control subsequent morphogenesis and differentiation.

4.1. Spindle formation and function

The establishment of a bipolar spindle is essential for the accurate segregation of chromosomes during mitosis and meiosis. In somatic cells, a centrosome, consisting of a pair of centrioles surrounded by proteins called pericentriolar material (PCM), determines both the location of the microtubule organizing center and the polarity of microtubule arrays with their growing plus ends extending away from the microtubule organizing center. During the S phase, the centrosome is duplicated, and just before mitosis the duplicated centrosomes are separated to enable them to serve as two mitotic spindle poles. Microtubules, which are nucleated from the centrosomes, alternate rapidly between growing and shrinking phases, until some are captured and stabilized by interactions with kinetochores through a "search and capture" mechanism of mitotic spindle formation (Kirschner and Mitchison, 1986). Thereafter, a centrosome together with an accompanying set of chromosomes is distributed into each daughter cell at the end of mitosis, thus ensuring that both the centrosome and chromosome number are accurately conserved through successive cell generations (Mazia, 1987). Meiotic spindles in mammalian oocytes (with the exception of rodents) lack centrioles, which are present only up to the pachytene stage during oogenesis (Szollosi *et al.*, 1972), and have to be inherited from fertilizing sperm (Schatten, 1994). In mouse oocytes, antibodies to PCM react with the acentriolar meiotic spindle poles as well as in the cytoplasm (Maro *et al.*, 1985).

The faithful transmission of chromosomes during both meiosis and mitosis is fundamental to the survival and reproduction of all living organisms. Errors during this process result in aneuploidy. Whereas sister chromatids segregate from each other during anaphase in mitotic cells, homologous chromosomes do so at the equivalent stage of the first meiotic division. Incorrect segregation of chromosome 21 during human meiosis is the cause of Down's syndrome, whereas that of other chromosomes is the cause of many spontaneous fetal abortions (Hassold and Hunt, 2001). Aneuploidy during mitotic divisions is associated with many forms of human cancer (Sen, 2000). Studies of human embryos have revealed that chromosomal aneuploidies are common (Angell *et al.*, 1986, Magli *et al.*, 2001; Munne *et al.*, 1993; Plachot *et al.*, 1986; Wolstenholme, 1996). The greater the fraction of aneuploid cells, the lower the developmental potential of the embryo (Baltaci *et al.*, 2006; Bielanska *et al.*, 2002). Interestingly, aneuploid cells may be excluded from the inner cell mass (ICM) (Johnson *et al.*, 1993), or cells may lose supernumerary chromosomes (Munne *et al.*, 2005). Aneuploidy rates increase with maternal age (Fujimoto *et al.*, 1978).

Thus, one component of decreased oocyte quality is disruption in spindle function, which can affect long-term embryo development.

The spindle can also control embryo genotype and phenotype by participating in meiotic drive processes. Meiotic drive is a process wherein chromosome segregation occurs in a nonrandom fashion. It is mediated by genetic elements, called segregation distorters that actively bias segregation, resulting in transmission of itself and linked chromosome material to more than half of the functional gametes. The most studied examples affect gamete viability, including *Segregation Distorter* in *Drosophila melanogaster* (fruit fly), the *t* haplotype in *Mus musculus* (mouse), and *spore killer* (*sk*) in *Neurospora* sp. (fungus) (Lyttle, 1991; Ripoll *et al.*, 1985; Turner and Perkins, 1979). Segregation distorters that are present in sexual chromosomes (e.g., the X chromosome in several *Drosophila* species; Atlan *et al.*, 2004) are denominated sex-ratio distorters, as they induce a sex-ratio bias in the offspring of the carrier individual. Selective death of subsets of gametes involves two tightly linked loci, a "Killer" locus and a "Target" locus. The segregation distorter set is composed of a "Killer" allele and "Resistant" allele, while its rival set is composed of "Non-killer" and "Non-resistant" alleles. In *Drosophila*, sperm receiving sensitive alleles of *Responder* (*Rsp*) on chromosome 2 are subject to dysfunction (Houtchens and Lyttle, 2003). The Segregation Distorter locus (also on chromosome 2) has been discovered to encode a mutant RanGAP protein that is mislocalized to the nucleus, diminishing nuclear RanGTP and disrupting nuclear RAN pathway signaling (Kusano *et al.*, 2001).

With respect to spindle function, meiotic drive can also occur by a process that does not involve gamete destruction, but rather relies upon an asymmetry of meiosis in females: the driving allele ends up in the oocyte instead of in the polar bodies with a probability greater than one half. This is termed true meiotic drive, as it does not rely on a postmeiotic mechanism. This form of drive is unique to female meiosis wherein a single functional gamete is produced from amongst the four meiotic products, thereby providing a mechanism whereby nonrandom segregation can direct enhanced transmission via the oocyte. One example of this is the DDK mouse strain. In this system, segregation distortion in favor of DDK alleles of the *Om* locus is due to nonrandom segregation of chromatids at the second meiotic division (Wu *et al.*, 2005). Additionally, nonrandom segregation of Robertsonian translocations occurs during the first meiotic division in humans (Pardo-Manuel de Villena and Sapienza, 2001b,c,d), and during the second division of murine meiosis (Pardo-Manuel de Villena *et al.*, 2001a,b). This is likely a key process contributing to speciation and genome evolution toward predominantly acrocentric or metacentric chromosomes (Pardo-Manuel de Villena and Sapienza, 2001a). Such nonrandom chromosome segregation in the oocyte requires asymmetry in the meiotic spindle and in the manner in which chromosomes associate with

the spindle. Nonrandom chromosome segregation can affect oocyte quality by biasing inheritance of specific alleles of genes linked to the distorter locus.

Spindle defects may play a special role in limited success of cloning by somatic cell nuclear transfer (SCNT). During SCNT, the second meiotic spindle is removed from the ovulated oocyte and replaced with a donor cell nucleus. Western blot analysis revealed that some proteins are depleted slightly by this procedure, but recover in abundance within a matter of hours (Miyara *et al.*, 2006). Despite this, the spindle that forms anew in the oocyte after SCNT is deficient in specific proteins, and this same deficiency is recapitulated at mitotic divisions in the embryo (Miyara *et al.*, 2006). Examination of cloned blastocysts revealed increased rates of aneuploidy and tetraploidy (Booth *et al.*, 2003; Nolen *et al.*, 2005; Shi *et al.*, 2004); however, the fraction of abnormal cells is small, indicating that mitotic errors are not obligatorily coupled to the earliest cleavage divisions. Rather, the spindle deficiencies likely create an enhanced risk of aneuploidy at each cell division.

4.2. Maternal mRNAs

During oogenesis, a high rate of gene transcription leads to the accumulation and storage of mRNAs as message-ribonucleoprotein (mRNP) complexes for use during oocyte maturation and early development. These maternal mRNAs can be released from the mRNP compartment and recruited for translation in a stage-specific manner, thereby providing for a changing array of proteins produced in the embryo during a period of transcriptional quiescence (Fig. 7.2).

4.2.1. Diversity of the maternal mRNA population

The maternal mRNA population is highly diverse, and supports a range of different functions during oocyte maturation and after fertilization. The maternal mRNA population also changes extensively during these periods. During murine oocyte maturation, the total amount of mRNA bearing long poly(A) tails diminishes greatly, as actively translating mRNAs become either degraded or deadenylated (Bachvarova *et al.*, 1985; Paynton *et al.*, 1988). Following fertilization, the amount of poly(A) mRNA in the cell increases dramatically, detectable by a rapid incorporation of radioactive ATP into poly(A) mRNA (Clegg and Piko, 1982, 1983a,b; Piko and Clegg, 1982) as stored maternal mRNAs undergo elongation of their poly(A) tails. This increase in poly(A) mRNA content is insensitive to transcription inhibitors, emphasizing the role of maternal mRNA polyadenylation. The overall effect of this maternal mRNA recruitment is an extensive shift in the protein synthesis pattern during the first cell cycle. Over half (60%) of the proteins being synthesized during the 1-cell stage change by twofold or more (27% by more than fourfold) in rates of synthesis during this period (Latham *et al.*, 1991). This represents a vast amount of change in the protein

synthetic activity, and exceeds the amount of difference observed between such extreme situations as proliferating versus quiescent fibroblast cultures. Moreover, a microarray analysis of polysomal mRNA populations before and after fertilization revealed that 29% of the detected mRNAs undergo twofold or greater changes in translation during this transition (Potireddy *et al.*, 2006). Thus, the recruitment, translation, and degradation of maternal mRNAs after fertilization are major processes that lead to profound changes in the array of proteins being produced in the cell.

The vast change in maternal mRNA recruitment is not chaotic, but rather seems to follow a very carefully orchestrated pattern wherein large groups of maternal mRNAs may be coordinately recruited, translated, and degraded at specific times during the first cell cycle. Cluster analysis of individual protein synthesis patterns (sampling at 3 h time intervals using synchronized groups of embryos) revealed four major patterns of synthesis (Latham *et al.*, 1991). One group represents proteins that cease to be produced, most likely encoded by maternal mRNAs that are degraded. A second group includes proteins that increase in rates of synthesis from the mid 1-cell to late 1-cell stage onward, and most likely reflects maternal mRNAs that are recruited. A third class of proteins peak in synthesis at the mid 1-cell stage, also most likely representing recruited maternal mRNAs that are recruited and then degraded. The fourth class represents proteins that diminish in rates of synthesis progressively until the very end of the 1-cell stage, when they display a transient and dramatic reappearance. This class includes the 32K complex of proteins encoded by *Spin*, a gene that produces multiple transcript variants that are recruited at specific stages and under the control of different combinations of cis regulatory elements (Oh *et al.*, 1997, 1998, 2000).

Evidence that maternal mRNA recruitment during the first two cell cycles is critical for early development has come from studies to elucidate the requirements for transcriptional activation. Cycloheximide treatment at the late 1-cell stage prevents the major genome activation event (Wang and Latham, 1997). Similarly, treatment with cordycepin, a chain terminating analogue that blocks maternal mRNA polyadenylation also prevents genome activation, affecting a wide range of housekeeping genes as well as stage-specific transiently induced genes (Aoki *et al.*, 1997, 2003). Taken together, these observations indicate that maternal mRNAs are recruited for translation at multiple stages after fertilization, that this is essential for development, and that recruitment is carefully orchestrated, most likely in coordination with the cell cycle (Hara *et al.*, 2005).

4.2.2. Functions of maternal mRNAs

One key role played by maternal mRNAs in many species relates to early cell fate determination, with key mRNAs localized to specific regions of oocytes either before or after fertilization. This is seen in a wide range of

vertebrates (e.g., amphibians) and invertebrates (e.g., nematodes, mollusks, insects). Localized messenger ribonucleoprotein complexes contain proteins or mRNAs that encode protein determinants of the germ lineage, such as P granules in *Caenorhabditis elegans* and polar plasm in *Drosophila* and other insects (Amikura *et al.*, 2005; Dworkin and Dworkin-Rastl, 1990; Hird *et al.*, 1996; Illmensee and Mahowald, 1974; Micklem, 1995; Olesnicky and Desplan, 2007; Schisa *et al.*, 2001; Semotok *et al.*, 2005; Strome and Wood, 1982; Strome *et al.*, 1994; Tadros and Lipshitz, 2005; Warn, 1975). In other cases, localization of maternal mRNAs during oogenesis is progressively translated into axial patterning during embryogenesis. This occurs as a result of the localized production of proteins to generate molecular gradients, which in turn define downstream gene expression programs. An ideal example of this is seen with regulation of the *hunchback* (*hb*) gene in *Drosophila*. The maternally produced bicoid mRNA must be localized at the anterior tip of the oocyte, from where translation produces a bicoid protein concentration gradient. Above a certain concentration, *bcd* activates embryonic transcription of the *hb* gene, whilst below that concentration transcription is inhibited (Struhl *et al.*, 1989; Tautz, 1988). Additional proteins, particularly nanos and torso, inhibit *hb* expression posteriorly thereby sharpening the gradient (Irish *et al.*, 1989; Struhl, 1989). Recent studies indicated that in *Drosophila* embryos, concentration gradients of morphogens are established by an mRNP complex, the Nos Response Element (NRE) complex which includes Nanos, Pumilio, and Brain tumor proteins and interacts with the NRE in the 3′ untranslated regions (3′UTRs) of *hb* and *caudal* mRNA (Cho *et al.*, 2006). d4EHP, a cap-binding protein is involved in repression of *caudal* and *hb* mRNA translation. The same protein is also involved in the translation inhibition of *hb* mRNA by interacting with the mRNA 5′ cap structure and brain tumor. Another excellent example is the localization of a variety of mRNAs (e.g., *VG1*, *VEGT*) at the vegetal pole in *Xenopus* embryos (White and Heasman, 2008).

Maternal mRNAs encode a wide range of essential proteins other than determinants of cell fate, too numerous to be covered in a single review. Of particular note amongst the essential functions regulated by maternal mRNAs are control of the cell cycle and control of embryonic genome activation. An excellent example of cell cycle control is regulation of appearance of the protein MOS. In *Xenopus laevis*, the production of MOS protein in response to hormonal stimulation is essential for germinal vesicle breakdown and initiation of meiosis (Sagata *et al.*, 1988), and MOS also controls meiotic progression in the mouse oocyte (Paules *et al.*, 1989), a process that occurs via activation of mitogen-activated protein kinase (MAPK) in a positive regulatory loop (Matten *et al.*, 1996). A protein called *Musashi* (which otherwise functions as a neural stem cell regulator) is involved in translational control of MOS production. The *Musashi* protein

interacts with the *MOS* mRNA. Inhibition of *Musashi* by a dominant inhibitory form prevents meiotic cell cycle progression. Early *Musashi*-dependent translation of *MOS* mRNA is required for late cytoplasmic polyadenylation-dependent (CPE) temporal regulation of maternal mRNAs (Charlesworth *et al.*, 2006).

Maternal mRNA translation is also likely to provide a key timing mechanism to control transcriptional activation of the embryonic genome. The ability of an embryo to undergo the process of gene transcription arises after fertilization. In the mouse embryo, this occurs during the second half of the 1-cell stage, as evidenced by endogenous gene transcription, transgene expression, and the acquired ability to transcribe genes in transplanted cleavage stage nuclei (Latham, 1999). Despite this early acquisition of the ability to transcribe genes, the actual rate of gene transcription can remain quite low until after one or more cleavage divisions. Transcriptional activation is not a single, discrete event, but rather occurs in periodic waves. In rodents, the major embryonic genome activation event occurs at the late 2-cell stage, whereas in other mammalian species this occurs at the 6- to 8-cell stage. This delay in embryonic genome activation is likely attributable to the stage-specific synthesis of transcription regulatory factors encoded by maternal mRNAs. The genome activation event is protein synthesis dependent, and can be inhibited with cycloheximide (Wang and Latham, 1997). Moreover, enhancers first become required for a high rate of transcription at the 2-cell stage (Henery *et al.*, 1995), and a maternal mRNA encoding at least one enhancer-binding protein (TEAD2) is recruited specifically at that stage (Kaneko *et al.*, 1997, 2004; Wang and Latham, 2000). In addition to providing for stage-specific production of essential transcription factors like TEAD2, maternal mRNAs encode chromatin regulators and factors that regulate RNA polymerase II (Zheng *et al.*, 2004). In fact there appears to be a significant transition in chromatin regulatory proteins encoded by maternal versus embryonic transcripts (Zheng *et al.*, 2005), such that changes in the level of gene transcription and the array of genes being transcribed could be driven by a transition in utilization of these two populations of mRNAs.

An in-depth microarray-based analysis of mouse maternal polysomal mRNAs that are recruited for translation either before or after fertilization (Potireddy *et al.*, 2006) revealed substantial differences in the biological functions supported by proteins being produced at the two stages. The mRNAs enriched in the polysomes of ovulated eggs were predominantly related to homeostatic processes, whereas those enriched on 1-cell polysomes were predominantly related to metabolism and biosynthesis. This latter group has many mRNAs- encoding transcription factors, again pointing to regulated maternal mRNA recruitment as playing a key role in controlling transcriptional activation in the early embryo. Interestingly, this transition in the polysomal mRNA population is mirrored at the level of total cellular mRNA (Zeng *et al.*, 2004), indicating that as the relevant

maternal mRNAs are recruited for translation they also become degraded, so that the overall mRNA population changes dramatically.

The correct control of maternal mRNAs is crucial for development. In the rhesus monkey, incorrect regulation of maternal mRNAs is associated with poor oocyte quality and arrested embryo development (Zheng *et al.*, 2005). *In vitro* maturation of oocytes from small antral follicles appears to be associated with precocious recruitment and then degradation of many maternal mRNAs, with the result that cleaving embryos produced by *in vitro* fertilization of these oocytes are deficient in these mRNAs. Cell cycle regulatory mRNAs appear to be particularly susceptible to this mode of disruption, possibly accounting for the early cleavage arrest (Mtango and Latham, 2008).

Another intriguing example of how disruptions in maternal mRNA regulation may compromise development is seen with cloned embryos produced by SCNT. A detailed array analysis of the transcriptomes of mouse 2-cell stage cloned, fertilized, and parthenogenetic embryos revealed that cloned embryos display either aberrant persistence or precocious loss of hundreds of maternal mRNAs (Vassena *et al.*, 2007b). The predominant effect is the persistence of maternal mRNAs, suggesting that many maternal mRNAs are simply not recruited and degraded as required. This is an interesting observation, as it indicates a role for the nucleus in controlling maternal mRNA translation after fertilization, a role that is not readily filled by a somatic cell genome.

It is worth noting that translational control of maternal mRNAs is a widespread and essential component to early development in a wide range of multicellular plants and animals (Benoit *et al.*, 2005; Caldwell and Emerson, 1985; Crosby *et al.*, 1988; Dworkin *et al.*, 1985; Grainger and Winkler, 1987; Harris and Dure, 1978; Kuligowski *et al.*, 1991; Lieberfarb *et al.*, 1996; Lublin and Evans, 2007; Rosenthal and Ruderman, 1987). Thus, understanding how this regulation is achieved is an important question in developmental biology.

4.2.3. Mechanisms of translational regulation

Our understanding of the molecular mechanisms regulating maternal mRNA translation in oocytes and embryos has increased greatly in recent years. The mechanisms can best be considered by examining two principal components: molecular mechanisms that promote silencing and storage of mRNAs, and the regulation of interactions between factors binding to the 5′ and 3′ regions of mRNAs that regulate formation of the translation initiation complex.

Maternal mRNAs are stored in mRNP particles in the oocyte until they are translated. This protects the mRNAs until they are recruited for translation at specific stages. The RNA-associated protein LSM14A, aka RAP55, is localized to mRNP cytoplasmic foci in oocytes, where it also associates

with the protein YBX2 (aka FRGY2, MSY2), a principal component of mRNP particles along with mRNP3, and with other associated proteins including DEAD-box ATPase Xp54, and the protein arginine methyltransferase PRMT1 (Murray *et al.*, 1992; Sommerville and Ladomery, 1996; Tafuri and Wolffe, 1990, 1993; Tanaka *et al.*, 2006; Yang *et al.*, 2006). Other components of mRNPs, include embryonic poly(A)-binding proteins, ePAB and ePABP2 (Cosson *et al.*, 2004; Good *et al.*, 2004; Ladomery *et al.*, 1997; Voeltz, *et al.*, 2001; Weston and Sommerville, 2006). The RAP55 protein also localizes to processing bodies (P bodies) and stress granules in somatic cells under stress conditions (Yang *et al.*, 2006). The Y-box proteins like YBX2 are responsible for the packaging of mRNAs into mRNPs (Matsumoto *et al.*, 2003; Skabkin *et al.*, 2004). The mRNP material, or nuage, in mammalian oocytes also includes the homologues of *Drosophila* germ cell marker VASA (DEAD/H Box 4, DDX4) and the maternal effect gene product Tudor, specifically Tudor repeat domain proteins 1, 6, and 7 (TDRD1, 6, and 7) (Hosokawa *et al.*, 2007). Mice deficient in TDRD1 display only male infertility, even though TDRD proteins localize to the nuage (intermitochondrial cement) in both male and female germ cells, indicating that, while some aspects of mRNP formation are essential and conserved between male and female gametes (e.g., YBX2), other components provide for sex-specific functions in germ cells (Chuma *et al.*, 2006; Hosokawa *et al.*, 2007).

XP54 and its human homologue RCK repress translation *in vitro* and in oocytes (Coller and Parker, 2005; Minshall *et al.*, 2001). XP54 interacts with cytoplasmic polyadenylation element-binding protein (CPEB) in oocytes (see below), associates with nascent transcripts in the nucleus, and is involved in the assembly of storage mRNPs (Smillie and Sommerville, 2002). Furthermore, yeast genetic studies demonstrated that Dhh1p, which is the yeast homologue of XP54, is involved in general translational repression (Coller and Parker, 2005). In aged *C. elegans*, large ribonucleoprotein foci form if fertilization is delayed because of lack of internal sperm. These foci contain RNA-binding proteins and nuclear pore proteins, and maintain the integrity of the oocytes until fertilization (Jud *et al.*, 2007). Recently, Buchet-Poyau *et al.* (2007) demonstrated that human MEX3A and MEX3B colocalize with decapping enzyme DCP1A and EIF2C1 (AGO1) proteins in the P bodies. Mutation in the MEX3B prevents the accumulation of the mRNAs in the P bodies, indicating that interaction with the above protein is necessary for the localization of the mRNAs, where nontranslated transcripts will be degraded or sequestered (Buchet-Poyau *et al.*, 2007).

Stored mRNAs are maintained in an untranslated state by preventing formation of a translation initiation complex. A widely held model of how translation is initiated is the closed loop model, in which the 5′ and 3′ ends of the mRNA are believed to make a contact via respective binding proteins. In this model, the poly(A) tail has a major role in circularizing

the mRNA. The interaction between the 5′ and 3′ ends is mediated by EIF4G and the poly(A)-binding protein, PABP, which binds to the poly(A) tail. This interaction facilitates binding of EIF4G to EIF4E, which is associated with the 5′ mRNA cap. The interaction between EIF4G and EIF4E is critical for forming the translation initiation complex (Hernandez and Vazquez-Pianzola, 2005), and can be regulated by a combination of eIF4E phosphorylation and binding of the inhibitory EIF4E binding proteins EIF4EBP1 and EIF4EBP2 (Clemens, 2004; Feigenblum and Schneider, 1996; Fingar and Blenis, 2004; Fingar *et al.*, 2002; Haghighat *et al.*, 1995; Hernandez and Vazquez-Pianzola, 2005; Huang *et al.*, 1987; Jagus *et al.*, 1992; Mader *et al.*, 1995; Salaun *et al.*, 2003, 2005; Zhou *et al.*, 2005). Circularization is likely also helpful in reinitiating the translation process on the same mRNA once the translation is terminated at the 3′ end and also in protecting the mRNA from degradation (Gingras *et al.*, 1999; Mazumder *et al.*, 2001).

In general, stored mRNAs have short poly(A) tails, and extending the poly(A) tail leads to activation of translation by recruiting PABP molecules and promoting interaction with the 5′ end of the mRNA. Decreasing the length of the poly(A) tail has the opposite effect. Maternal mRNAs are synthesized in the nucleus and polyadenylated under the influence of the nuclear polyadenylation signal AAUAAA. Once the synthesis is completed, they are transported into the cytoplasm where they become deadenylated and stored for later use, or polyadenylated further for immediate translation.

Two elements in mRNAs have well-documented roles in regulating polyadenylation. The cytoplasmic polyadenylation element, CPE, resides in the 3′UTR and regulates which mRNAs are polyadenylated and when this occurs. The CPE function is provided by a diverse array of sequences identified amongst amphibians and mammalian mRNAs that are recruited during oocyte maturation (Oh *et al.*, 2000). The second element is the polyadenylation signal, or hexanucleotide, of the consensus AAUAAA. The CPE and AAUAAA are required for polyadenylation. In developing *Xenopus* oocytes, deadenylation is a default mechanism occurring in the absence of a CPE (Varnum and, Wormington, 1990; Fox and Wickens, 1990). EDEN, the embryonic deadenylation element (Bouvet *et al.*, 1994), is a cis acting element that can promote deadenylation (Paillard *et al.*, 1998). In *Xenopus* oocytes, the AU-rich element (ARE) regulates mRNA deadenylation. The embryonic poly(A)-binding protein (ePAB) binds to the ARE and to the poly(A) tail, and immunodepletion of ePAB accelerates ARE-mediated default deadenylation, with subsequent mRNA degradation, indicating its role in deadenylation and accumulation of maternal mRNAs (Voeltz *et al.*, 2001). SMAUG, a multifunctional posttranscriptional (translational) regulator is conserved from yeast to humans. In *Drosophila*, maternal mRNA regulation by smaug triggers the translational repression and deadenylation of maternal mRNAs by independent mechanisms, and the

yeast homologue *Vts1* stimulates degradation of mRNAs containing the smaug recognition element (SRE) (Baez and Boccaccio, 2005).

The 3′UTRs exert critical control over the translation of maternal mRNAs. The 3′UTR plays a role in a number of processes, including polyadenylation of the mRNA, temporal regulation of mRNA translation by stage-specific polyadenylation, mRNA deadenylation, and mRNA stability. The 3′UTRs contain elements for regulating maternal mRNAs during maturation. Evidence for the role of 3′UTR in translation came from antisense-mediated truncation studies of the 3′UTR, which prevented mRNA activation (Strickland *et al.*, 1988). Two elements of the 3′UTR are involved: one that directs the selective polyadenylation, and the AAUAAA hexanucleotide, which functions as a cleavage and polyadenylation signal in the processing of nuclear transcripts (Proudfoot and Brownlee, 1976; Wickens and Stephenson, 1984). In mammals, the presence of the poly (A) tail is sufficient for mRNA translation (Vassalli *et al.*, 1989). Polyadenylation of tPA mRNA requires active protein synthesis during the first hours after GVBD (Huarte *et al.*, 1987), indicating that synthesis of a new protein is necessary, which could be a poly(A) polymerase or another protein involved in recognition of the mRNA by the polymerase.

Identification of the CPE led to the discovery of the CPE-binding protein, CPEB. CPEB is a 62-kDa protein that contains two RNA recognition motifs, and within this region, it is 62% identical to orb, an oocyte-specific RNA-binding protein from *Drosophila* (Hake and Richter, 1994). CPEB is involved in oocyte maturation and is degraded by prophase of meiosis I in *Xenopus* (Mendez *et al.*, 2002). Injection of a nondegradable form of CPEB into *Xenopus* oocytes interferes with translation of cyclin B1 mRNA and progression to meiosis II (Mendez *et al.*, 2002). CPEB binds to the CPE and also interacts with the protein maskin, which binds to eIF4E. The binding of maskin to eIF4E prevents eIF4E–eIF4G interaction, thereby suppressing translation (Barnard *et al.*, 2004, 2005; Cao and Richter, 2002; de Moor and Richter, 1999; Groisman *et al.*, 2002; Richter, 1999; Richter and Sonenberg, 2005; Stebbins-Boaz *et al.*, 1999). In response to specific stimuli to induce oocyte maturation, phosphorylation of both maskin and CPEB promote polyadenylation and translation of maternal mRNAs. MAPK phosphorylates CPEB on four residues (T22, T164, S184, S248), but not on S174, a key residue for activating CPEB function (Keady *et al.*, 2007) (Fig. 7.3). MAPK may prime CPEB phosphorylation, but the activation of CPEB by phosphorylation of S174 is achieved by another kinase, EG2 (current symbol AURKC, aka Aurora A, AIE2, AIK3, STK13, IPL1-like kinase), which also phosphorylates maskin (Hodgman *et al.*, 2001; Mendez *et al.*, 2000). This induces the recruitment of the cytoplasmic form of cleavage and polyadenylation-specific factor, CPSF (comprised of three subunits of 30, 100, and 160 kDa) to the hexanucleotide. Associated with the CPSF is poly(A) polymerase (PAP), which

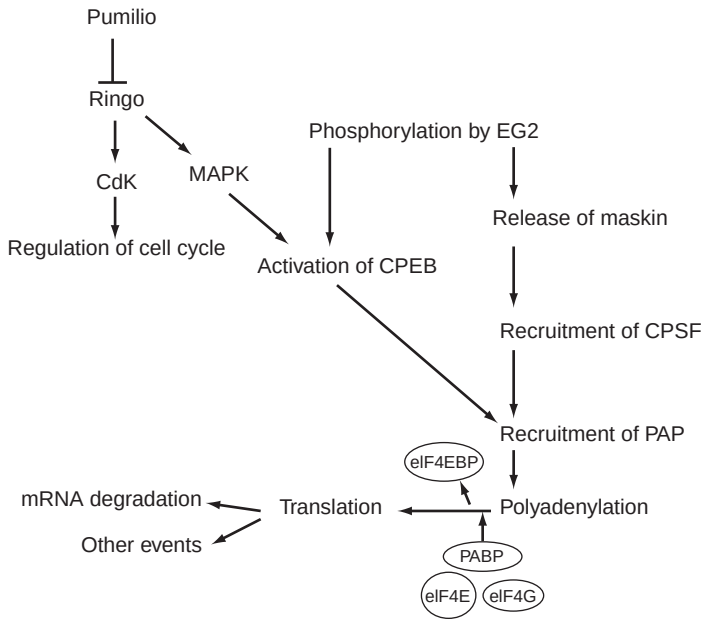

Figure 7.3 Translational regulation of maternal mRNAs. Schematic of the various regulator proteins controlling the translational recruitment. Ringo, a cell cycle regulator binds to the cyclin-dependent kinase inhibitor 1B (CDKN1B, aka p27/Kip1) needed for cytoplasmic polyadenylation element-binding protein (CPEB)-mediated translation. Pumilio 2 protein binds to the Pumilio-binding element in the 3′UTR of RINGO mRNA leading to the repression of its translation. RINGO activates mitogen-activated protein kinase (MAPK), leading to the phosphorylation of CPEB. Activation of CPEB is through phosphorylation by EG2 kinase. EG2 also phosphorylates Maskin, leading to its release from CPEB. Cleavage and polyadenylation specificity factor (CPSF) binds to the AAUAAA and interacts with CPEB on the CPE. This interaction recruits poly(A) polymerase (PAP), leading to polyadenylation of the mRNA. The poly(A) tail is bound by poly(A)-binding protein (PABP), which interacts with the 5′ end of the mRNA, recruiting the various translation initiation factors, including EIF4E, EIF4G, and ribosomes, as EIF4E-binding protein (EIF4EBP) is released. Once translated, the mRNA is degraded.

catalyzes poly(A) tail elongation (Fox *et al.*, 1992). This polyadenylation event results in the translation of *MOS* mRNA, which in turn activates CDC2 kinase to phosphorylate CPEB at multiple sites (Mendez *et al.*, 2000). This leads to dissolution of maskin-EIF4E, which permits binding of ElF4E to ElF4G and the translational activation of CPE-containing mRNAs (Stebbins-Boaz *et al.*, 1999).

RINGO (rapid inducer of G2–M in oocytes)/SPY (speedy) protein, is an activator of cyclin-dependent kinases (cdks), and is required for CPEB-directed translation (Padmanabhan and Richter, 2006). Overexpression of RINGO/SPY induces MOS synthesis, MAPK and MPF activation, and oocyte maturation. RINGO/SPY binds to CDKs and associates with

CDKN1B (p27), a CDK inhibitor, indicating a role in cell cycle regulation (Dinarina *et al.*, 2005; Karaiskou *et al.*, 2001; McAndrew *et al.*, 2007; Porter *et al.*, 2003). RINGO/SPY translation precedes maturation and is required for polyadenylation and translation of Mos mRNA and also for all CPEB-mediated processes (Padmanabhan and Richter, 2006) (Fig. 7.3). In immature oocytes, Pumilio 2 (PUM2) binds to the PUM2-binding elements (PBE) within the RINGO/SPY mRNA 3′UTR and represses its translation. PUM2 also interacts with other proteins, such as deleted in azoospermia-like (DAZL) (Cooke and Elliott, 1997), and embryonic PABP (Voeltz *et al.*, 2001). PUM2 dissociates from the RINGO/SPY mRNA when its translation is activated upon maturation, while DAZL and ePAB still interact with RINGO/Spy mRNA. PUM2 inhibits ePAB activity (Collier *et al.*, 2005) and this impacts EIF4G, EIF4B, or poly(A) interacting protein (PAIP). PUM2 may affect the interaction of EIF4E with EIF4G (Cao and Richter, 2002; Stebbins-Boaz *et al.*, 1999), or EIF4E with the cap (Cho *et al.*, 2005). Irrespective of the mechanism, PUM2-mediated repression of RINGO/SPY RNA during maturation is necessary for controlling translation.

Other elements exist in the 3′UTR to control translation. Cyclin B1 (*CCNB1*) mRNA is regulated by maturation promoting factor signaling (de Moor and Richter, 1997; Howard *et al.*, 1999). In contrast to cyclin B1, *MOS* mRNA translation can be stimulated by the MAPK pathway independently of CDC2 activity (Howard *et al.*, 1999). Aside from the requirement for the CPE in *MOS* mRNA translation (Mendez *et al.*, 2000), another regulatory element exists distinct from CPE. This element links MAPK signaling to the early progesterone-stimulated induction of *MOS* mRNA translation, and is named the PRE, for polyadenylation response element (Charlesworth *et al.*, 2002). Charlesworth *et al.* (2002) reported that the initial translation of the Mos mRNA is mediated by the PRE. In Mos, even though this element overlaps the CPE, the two elements have different roles, so that MAPK signaling targets PRE-directed mRNA translation, whereas cdc2 activation promotes the later CPE-directed mRNA translation.

The scenario outlined above for the regulated deadenylation, storage, polyadenylation, and translation of maternal mRNAs is likely incomplete. Most of what has been learned of maternal mRNA regulation has come from studies of events that occur during oocyte maturation. As described above, however, many maternal mRNAs are likely recruited for translation after fertilization to meet early, stage-specific demands of the embryo. Oocyte maturation requires the synthesis of new proteins (Uzbekova *et al.*, 2008). This new protein synthesis is achieved by the translational recruitment of stored mRNAs in a stage-specific manner, compensating for an absence of transcription. This requires mechanisms to prevent recruitment of subpopulations of maternal mRNAs during oocyte maturation, and

additional mechanisms to promote stage-specific recruitment. Comparison of the mRNA populations undergoing translation at the egg and the zygote stage indicated dramatic differences in the 3′UTRs of the transcripts being preferentially translated at the two stages. Sequence analysis indicates that CPEs are prevalent in transcripts translated preferentially at the MII oocyte stage, with 84% containing known CPEs in the 3′UTRs. By contrast, only 41% of the mRNAs enriched on polysomes at the 1-cell stage possessed known CPEs (Potireddy *et al.*, 2006). This indicates that other novel motifs likely control the process of stage-specific recruitment of maternal transcripts. Additionally, multiple genes encoding CPEBs exist, raising the possibility of further diversity in function and complexity in mRNA translational regulation.

Micro RNAs (miRNAs) and small RNAs (sRNAs) also regulate maternal mRNAs. In zebrafish embryos, miRNAs play a major role in regulating the maternal mRNA pool. Murchison *et al.* (2007) reported that elimination of the oocyte supply of DICER results in arrest during meiosis I, indicating its critical role in eliminating a group of maternal mRNAs that must be degraded in order to accomplish this critical transition (Su *et al.*, 2007). Schier and Giraldez (2006) developed transgenic embryos lacking the enzyme DICER, which is involved in synthesizing miRNAs, so that the embryos are devoid of the miRNAs. In the absence of miRNAs, the embryos showed morphogenetic defects. But with the addition of miR430, maternal mRNAs are subjected to decay through deadenylation. Giraldez *et al.* (2006) also reported that maternal mRNAs are subjected to degradation by the miR430 in zebrafish. This degradation is not the result of nonproductive translation, as repression of translation of the reporter gene with morpholino antisense oligonucleotides does not result in the decay to the same extent as the miRNA-mediated decay. MiR430 is expressed at the onset of zygotic transcription and accelerates the deadenylation and decay of a large set of maternal mRNAs in zebrafish. Lack of miR430 does not block development but results in a mixed maternal–zygotic state. This suggests that miR430 facilitates the developmental transition from maternal to zygotic states. In contrast, however, of 1000 *Drosophila* oocyte proteins examined, only a minor fraction (4%) of maternal mRNAs was increased in the *dicer* mutants (Nakahara *et al.*, 2005).

Another small RNA involved in early development is the PIWI interacting piRNA. Polar granules contain the piRNA-binding protein, PIWI (Megosh *et al.*, 2006). Depleting the levels of PIWI leads to defects in pole plasm maintenance and PGC formation. The mouse PIWI homologue, MIWI, associates with DICER, piRNAs, and mRNAs resident either on polysomes or within mRNPs (Grivna *et al.*, 2006). It is suggested that small RNAs likely play diverse roles in regulating the maternal mRNA population at the level of translation and mRNA stability, contributing to overall oocyte quality.

4.3. Maternal proteins

The foregoing discussion of maternal mRNA regulation makes it clear that the oocyte is endowed with a rich supply of maternal mRNAs to direct a changing array of proteins being synthesized, as well as a network of regulatory proteins to control the translation of these mRNAs in coordination with transit through the cell cycle. The oocyte also contains a vast array of proteins that provide key regulatory functions, such as transcriptional activation, cell cycle control, and many other regulatory processes, as well as proteins to support basic cellular homeostasis and metabolism (Fig. 7.2). The importance of many of the regulatory proteins has been revealed by spontaneous mutations and gene targeting, yielding classical maternal effect phenotypes.

4.3.1. Maternal effect mutations

Many maternal proteins regulate gene transcription. One such maternal factor is tripartite motif containing protein 24 (TRIM24, aka transcription intermediary factor 1α, TIF1A). This protein translocates from the cytoplasm into pronuclei enriched with chromatin remodelers SMARCA4 (BRG1) and SMARCA5 (SNF2H). Inhibition of TRIM24 expression by RNA interference leads to developmental arrest of the embryos at 2- to 4-cell stage (Torres-Padilla and Zernicka-Goetz, 2006). TRIM24 plays a major role in remodeling of the chromatin during the first major wave of transcription. SMARCA4 is also an important maternal factor involved in chromatin remodeling. Conditional mutation of mouse Smarca4 results in oocytes that completed meiosis and undergo fertilization normally, but yields embryos that arrest at the 2-cell stage and show reduced transcription in 30% of expressed genes (Bultman *et al.*, 2006). NLRP5 (aka MATER, for Maternal Antigen that Embryos Require), first identified in mouse, is distributed in the cytoplasm, and is required for the oocyte–embryo transition. Even though the function of NLRP5 still needs to be established, a decrease in transcription in embryos lacking NLRP5 indicates a role of this protein in transcription and the resulting oocyte–embryo transition (Tong *et al.*, 2000). *Nlrp5* null females ovulate normally but produce no litters, whereas homozygous null males and heterozygous females have normal fertility. Fertilization is normal in null females, but the embryos arrest at the 2-cell stage and degenerate. Zygote arrest 1 (*Zar1*) is another maternal effect gene, and is expressed in oocytes and embryos. ZAR1 decreases in abundance by the 2-cell stage and is absent thereafter. Most *Zar1* null embryos arrest at the 1-cell stage, and show suppression of genome activation (Wu *et al.*, 2003). A mouse homolog of *Xenopus* nucleoplasmin (nucleoplasmin 2, NPM2) is also critical for development. Embryos from *Npm2*-null females have defects in nuclei and nucleolar organization specifically evident in the loss of heterochromatin from these organelles (Burns *et al.*, 2003). Although some embryos are able to proceed through the 2-cell stage and some offspring are born to null females, most

embryos show reduced cleavage to the 2-cell stage. Another recent maternal effect factor identified is *Dppa3* (developmental pluripotency-associated 3, aka *Stella*). Embryos from DPPA3-deficient oocytes progress to the 4-cell stage but are defective in further cleavage and in preimplantation development (Bortvin *et al.*, 2004; Payer *et al.*, 2003).

Maternal proteins also provide for correct epigenetic modifications of the embryonic genome. The DNA (cytosine-5)-methyltransferase, DNMT1, is responsible for maintenance of DNA methylation. RNA silencing of the *Dnmt1* leads to activation of the zygotic genome two cycles earlier than normal in *Xenopus* embryos (Stancheva and Meehan, 2000). The methylation patterns in the embryonic genome are programmed and depletion of the DNMT1 leads to disruptions in the programmed changes of the promoter regions. This leads to altered gene expression in the embryos (Stancheva *et al.*, 2002). Another critical role for DNMT1 in the early embryo is maintenance of genomic imprints. Mammalian oocytes possess a specific oocyte form of the protein, termed DNMT1o, and express only very slight amounts of the somatic form (DNMT1s) during preimplantation development (Ratnam *et al.*, 2002). Deficiency for DNMT1o leads to embryonic lethality and loss of genomic imprinting information, even in wild-type nuclei transferred to mutant ooplasm (Howell *et al.*, 2001). Interestingly, cloned embryos produced by SCNT display reduced nuclear localization of DNMT1o, and this correlates with imprinting defects (Chung *et al.*, 2003).

Maternal proteins also play a role in repackaging chromatin. An oocyte-specific form of histone H1, called H1FOO, displays enhanced binding to DNA compared to somatic forms. Within as little as 5 min after sperm injection or SCNT, H1FOO associates with the incoming DNA, and within an hour can completely displace the incoming chromatin bound proteins, resulting in a complete replacement of somatic H1 after SCNT (Gao *et al.*, 2004). The H1FOO is then eliminated by the 2-cell stage in both normal and cloned embryos. These transitions may involve proteolytic events (Gao *et al.*, 2005). The ability of the oocyte to mediate these transitions is developmentally regulated, so that by 2 h after oocyte activation somatic histone removal is inhibited (Gao *et al.*, 2004). One curious aspect of these events is that the mouse 2-cell stage embryo experiences a period during which histone H1 of any kind is sparse, creating a potential for promiscuous gene transcription before the somatic forms become fully assembled on the chromatin (Gao *et al.*, 2004). This may contribute to a transient pattern of gene expression at the 2-cell stage.

4.3.2. Elimination of maternal proteins

While both maternal mRNA and protein play critical roles in early development, it is just as important to accomplish a successful transition from maternal to embryonic control of development. This requires degradation of

maternal mRNAs and proteins. The degradation of the mRNAs is coupled to translational recruitment, as discussed above. The ubiquitin-proteasome pathway forms the major channel of degradation of maternal proteins. In *C. elegans*, degradation of the MEI1 oocyte protein is tightly regulated by phosphorylation mediated by the minibrain kinase homolog, MBK2. MBK2 is tethered to the cortex by EGG3, an oocyte protein required during egg activation. During meiotic divisions, EGG3 is degraded. This causes MBK2 release from the cortex, leading to MEI1 phosphorylation and degradation. The phosphorylated MEI1 is recognized by the MEL26/CUL3 (DeRenzo and Seydoux, 2004). The phosphorylation of MEI1 by MBK2 increases the affinity of the protein to the MEL26/CUL3. The first hint for involvement of cullins came from RFL1, a homolog of UBA3 and a member of the neddylation pathway, which activates cullins. RNAi screening of the cullins led to the identification of CUL3 as an important factor for degradation of the MEI1. MBK2 is also involved in phosphorylation of other proteins such as OMA1, which is degraded after first mitosis with the phosphorylation by the second kinase (Nishi and Lin, 2005). Loss of MBK2 delays and/or blocks the degradation of MEI1, OMA1, and CCCH-finger proteins (Pellettieri *et al.*, 2003; Quintin *et al.*, 2003).

VASA, a regulator of germ cell fate in *Drosophila* is localized to the posterior pole by recruitment by *Oskar* (Hay *et al.*, 1990; Lasko and Ashburner, 1990). The ubiquitin pathway plays a role in Vasa localization to the posterior pole. Localization requires the deubiquitinating enzyme *fat facets* and the SOCS-box protein *Gustavus*. In the absence of the deubiquitinating enzyme, *vasa* is polyubiquitinated and accumulated at lower levels. Even though the exact role of SOCS-box protein in the localization of *vasa* is not known, in general it functions as a substrate-recruitment factor for E3 ubiquitin ligases (Kile *et al.*, 2002), which bind to specific targets and stimulate their ubiquitination and degradation.

Correct elimination of maternal proteins is essential for normal development. Protein degradation eliminates proteins that are useful early in the development but are harmful later. For example, CPEB functions early during oocyte maturation and is degraded later. But use of a nondegradable form of CPEB interferes with the translation of cyclin B1 and progression to meiosis II (Mendez *et al.*, 2002). CPEB is a PEST domain containing protein that is degraded during maturation, and deleting the PEST domain or use of proteasome inhibitors prevents the degradation of CPEB (Reverte *et al.*, 2001).

Studies in the rhesus monkey have revealed complex temporal patterns of regulation of maternal and embryonic mRNAs encoding components of the ubiquitin-proteasome pathway (Mtango and Latham, 2007). Differences in expression of these mRNAs between oocytes of different qualities indicate a role for the ubiquitin-proteasome pathway in determining oocyte quality and embryo development.

4.4. Effects of maternal mitochondria

The maternal mitochondrial population constitutes a major determinant of oocyte quality, via its role in embryonic metabolism and its role in controlling apoptosis. Apoptosis is the process of programmed cell death, either as a part of normal tissue differentiation or as a means of eliminating defective cells. A central regulator and mediator of apoptosis is the caspase family, which consists of cysteine-dependent aspartate-specific proteases. These are divided into two groups, initiator caspases such as caspase-8 and caspase-9 that activate other caspases, and executor caspases such as caspase-3, -6, and -7 that are responsible for degrading cellular proteins. Three general mechanisms are known, and their effects may be interrelated: (i) disruption of electron transport, oxidative phosphorylation, and adenosine triphosphate (ATP) production; (ii) release of proteins that trigger activation of caspase family proteases; and (iii) alteration of cellular reduction–oxidation (redox) potential (Green and Reed, 1998). Mitochondria contain many pro-apoptotic proteins such as apoptosis-inducing factor (AIF), SMAC/DIABLO, and cytochrome *C*. These factors are released from the mitochondria following the formation of pores in the mitochondrial membrane called the permeability transition, or PT pores (Chipuk *et al.*, 2006; Lemasters, 2005; Skommera *et al.*, 2006). These pores are thought to form through the action of the pro-apoptotic members of the *Bcl2* family of proteins, which are activated by apoptotic signals such as cell stress, free radical damage, or growth factor deprivation. Mitochondria also play an important role in amplifying apoptotic signals from the death receptors, with receptor-recruited caspase 8 activating the pro-apoptotic *Bcl2* family protein, BID.

Mitochondria distribution and localization in maturing embryos and oocytes are mediated primarily by microtubules, although there is evidence for actin-based localization as well (Barnett *et al.*, 1996; Haggeness *et al.*, 1978; Hales, 2004; Muggleton-Harris and Brown, 1988; Pozo *et al.*, 1990; Van Blerkom, 1991). The localization is presumably in response to localized energy needs. Available data indicate that correct mitochondrial distribution within the blastomeres is correlated with developmental potential, and hence oocyte and embryo quality (Barnett *et al.*, 1996). The mechanism responsible for this relationship is not entirely known, but may relate to providing local high concentrations of ATP within the cell.

Mitochondrial genome integrity may be sensitive to procedures that may affect oocyte quality. An increase in the incidence of mitochondrial mutations was reported for rhesus monkey oocytes produced by assisted reproduction methods (Gibson *et al.*, 2005). This indicates that unknown processes may affect maintenance of the mitochondria, and long-term health of offspring.

The mammalian oocyte and sperm are designed to achieve strictly matrilineal inheritance of mitochondria. Mitochondria and mitochondrial

DNA (mtDNA) are transmitted through the female germ line (Birky, 1995, 2001; Giles *et al.*, 1980; Hutchinson *et al.*, 1974). Mitochondria cannot be made *de novo*, only elaborated from other mitochondria, and thus derived from the oocytes (Cummins, 2000; Dawid and Blackler, 1972; Jansen, 2000; Shoubridge, 2000). The spermatozoon introduces up to 100 functional mitochondria into the ooplasm at fertilization; however, these are degraded rapidly in the preimplantation embryo via a ubiquitin-dependent process that targets ubiquitinated proteins on the sperm mitochondria (Nishimura *et al.*, 2006; Sutovsky, 2003; Sutovsky *et al.*, 1999, 2000). The mandatory destruction of sperm mitochondria may provide an evolutionary and developmental advantage (Ankel-Simons and Cummins, 1996; Cummins, 1998, 2000), because the paternal mitochondria and their DNA (mtDNA) may be compromised by the action of reactive oxygen species encountered by the sperm during spermatogenesis and fertilization (Aitken, 1994, 1995; Aitken and Fisher, 1994; Aitken *et al.*, 1999). The work on mitochondrial inheritance provided an explanation of how the fertilized egg destroys paternal mitochondrial genes (Sutovsky, 2003; Sutovsky *et al.*, 1999, 2000; Thompson *et al.*, 2003). As a consequence of such selective sperm mitochondrion destruction, mitochondria in humans and other mammals are typically inherited only from the mother. This inheritance pattern allows scientists to determine the lineage of human and animal evolution and to calibrate the evolutionary clock.

The destruction of paternal mitochondria should result in homoplasmic individuals, but there is evidence of sperm mtDNA persistence in human polyploid IVF-generated blastocysts (St. John *et al.*, 2000). The surveillance system that targets paternal mtDNA can apparently be abrogated in interspecific crosses, as shown in studies with mice (Gyllensten *et al.*, 1991; Kaneda *et al.*, 1995), fruit flies (Kondo *et al.*, 1990), sheep (Zhao *et al.*, 2004), and nonhuman primates (St. John and Schatten, 2004).

The coexistence of two or more mtDNA variants within a cell (heteroplasmy) can result from sperm transmission, interspecific crosses, or from supplementation arising through microsurgical procedures such as germinal vesicle transfer (GVT), cytoplasmic transfer (CT), pronulear transfer (PNT), or SCNT. The mixing of diverse nuclear and mtDNA fusion partners could compromise electron transfer channel (ETC) function and be disadvantageous for preimplantation or fetal development, and for survival of the offspring. Heteroplasmy can change amino acid composition, as shown in interspecific NT-offspring (Steinborn *et al.*, 2002; St. John *et al.*, 2005), and thus potentially alter protein conformation of the components of the ETC. Furthermore, as both mitochondrial and chromosomal genes contribute proteins to the ETC, the genetic divergence between the gene products of these genomes could also affect ATP output. The mtDNA can be either inherited from the recipient oocyte only or from both the donor cell and recipient oocyte (heteroplasmy) in NT. Such transmission has been

observed in both NT embryos (Lloyd *et al.*, 2006; Steinborn *et al.*, 1998) and their offspring (Evans *et al.*, 1999; Hiendleder *et al.*, 1999; Meirelles *et al.*, 2001; St. John and Schatten, 2004; Steinborn *et al.*, 2002).

Microsurgical techniques thus contravene the strict mechanism that regulates mtDNA transmission postfertilization. Intraspecific crosses in mice eliminate sperm mitochondria by the late 1-cell stage (Kaneda *et al.*, 1995; Shitara *et al.*, 2001), and before the 8-cell stage in cattle and rhesus macaque via a mechanism that requires mitochondrial protein ubiquitination (Sutovsky *et al.*,1999). With interspecific crosses, however, the ubiquitin-mediated proteolysis is avoided and sperm mitochondria persist at low levels (Gyllensten *et al.*, 1991; Shitara *et al.*, 1998). In cytoplasm transfers, successful human births have been achieved, and there is evidence that infants resulting from "ooplasm transfer" exhibit heteroplasmy (Brenner *et al.*, 2000; Harvey *et al.*, 2007; Van Blerkom *et al.*, 1998). Furthermore, the outcome of IVF in couples who underwent ooplasm transfer was poor due to low oocyte and embryo quality, possibly due in part to mitochondrial dysfunction (Harvey *et al.*, 2007). Some studies in mice failed to reveal detrimental effects of heteroplasmy on mouse development (Levron *et al.*, 1996; Meirelles and Smith, 1997, 1998; Takeda *et al.*, 2000). A recent study by Acton *et al.* (2007), however, screened basic physiological functions for heteroplasmic mice (NZB mtDNA on a BALB/cByJ background). The mice were tested for cardiovascular and metabolic function, hematological parameters, body mass analysis, ovarian reserve, and tissue histological abnormalities over a period of 15 months, and defects were seen in heteroplasmic mice in all tests.

4.5. Effects of the maternal pronucleus

Left unfertilized, oocytes are destined to die. One of the earliest things a fertilized embryo must do is to divert itself from a pathway of death to a pathway of life. Failure to suppress apoptotic events after fertilization leads to blastomere fragmentation, DNA fragmentation, and other typical signs of apoptosis, and indeed it has been suggested that insufficient suppression of these processes may constitute an important quality control mechanism to eliminate abnormal embryos at an early stage (Jurisicova *et al.*, 1998). Numerous recent studies have reported oocyte or blastomere fragmentation and/or apoptosis in a number of different mammalian species, including cow, mouse, and human (Antczak and Van Blerkom, 1999; Bergeron *et al.*, 1998; Bolton *et al.*, 1989; Brewster *et al.*, 2000; Byrne *et al.*, 1999; Casper and Jurisicova, 2000; Erenus *et al.*, 1991; Hardy, 1999; Hardy *et al.*, 1999; Jurisicova *et al.*, 1995, 1996, 1998; Liu and Keefe, 2000; Moley and Mueckler, 2000; Morita *et al.*, 1999, 2000; Otoi *et al.*, 1999; Pampfer, 2000; Perez *et al.*, 2000a,b; Van Blerkom and Davis, 1998; Watson *et al.*, 2000). Clinically, this reduces the number of high-quality embryos available

for establishing pregnancy of human embryos produced by IVF, >80% exhibit some degree of cellular fragmentation, and this propensity appears to be programmed by the 1-cell stage (Jurisicova *et al.*, 1996). The maternal pronucleus appears to play a commanding role in suppressing apoptotic processes. Mouse embryos produced with eggs from C3H/HeJ females display enhanced rates of blastomere fragmentation as compared to those made with eggs from C57BL/6 mothers (Han *et al.*, 2005). An extensive series of maternal pronuclear transfer experiments indicated that a C57BL/6 maternal pronucleus can suppress cytofragmentation regardless of the strain of origin of the ooplasm or paternal pronucleus, and conversely a C3H/HeJ maternal pronucleus can enhance the process (Han *et al.*, 2005). This is an interesting discovery, because other studies revealing greater rates of gene transcription in paternal as compared to maternal pronuclei (Aoki *et al.*, 1997; Henery *et al.*, 1995) indicated that the paternal pronucleus might exert a greater level of control over early development than the maternal pronucleus. However, it appears instead that the maternal pronucleus is endowed with the very important attribute of controlling apoptosis in early embryos, which is in fact the earliest documented effect of the embryonic genome on embryo phenotype. Interestingly, a parental origin effect of the maternal strain has also been observed (Han *et al.*, 2005; Hawes *et al.*, 2001), indicating a possible role for genomic imprinting in controlling apoptosis. This could account for why the maternal pronucleus plays such a predominant role in this early process, which would also make this the earliest known effect of imprinting on embryonic phenotype.

4.6. Effects of the ooplasm on paternal pronucleus function

While the maternal pronucleus itself exerts a commanding influence over early embryo apoptotic processes, a broader effect of the maternal genotype is seen in the potent influences over the early embryo via the ooplasm, particularly controlling the paternal genome. The ooplasm plays a key role in establishing early embryo phenotype by transforming the paternal genome into an integral component of the embryonic genome. Once fertilization occurs, the ooplasm directs the breakdown of the sperm nuclear envelope. The ooplasm then creates a paternal pronucleus (pPN) from the decondensed haploid sperm genome. This process involves extensive restructuring of the chromatin, as histones replace protamines and then subsequently undergo extensive posttranslational modifications in preparation for the onset of embryonic gene transcription (Latham, 1999; Latham and Schultz, 2001). Moreover, the paternal pronucleus undergoes enhanced changes in DNA methylation and histone acetylation, and displays evidence of increased transcriptional activity of injected transgenes as well as endogenous genes, relative to the maternal pronucleus (Adenot *et al.*, 1997; Aoki *et al.*, 1997;

Henery *et al.*, 1995; Lepikhov and Walter, 2004; Santos *et al.*, 2005; Van der Heijden *et al.*, 2005, 2006; Wiekowski *et al.*, 1993; Yeo *et al.*, 2005).

The interactions between the ooplasm and the paternal genome are subject to genetic variation. One example of this is the polar phenotype exerted by the Ovum mutant (*Om*) locus. This locus is responsible for the peculiar incompatibility between the DDK mouse strain and other strains, wherein DDK female X non-DDK male crosses produce 95% lethality by the morula stage, while the reciprocal cross is fully fertile (Wakasugi *et al.*, 1967). The trait is controlled by the *Om* locus on chromosome 11, and appears to consist of multiple genes, at least one of which is expressed as an mRNA and/or protein in the oocyte and at least one other gene that is expressed from the paternal genome and responds to this ooplasmic constituent to yield a nonviable phenotype (Baldacci *et al.*, 1992; Bell *et al.*, 2006; Mann, 1986; Pardo-Manuel de Villena *et al.*, 1997; Renard and Babinet, 1986; Renard *et al.*, 1994; Sapienza *et al.*, 1992).

Another example of ooplasmic control of paternal genome function is seen with androgenetic mouse embryos, which are prepared by microsurgery to contain two paternal pronuclei and no maternal pronuclei. Such embryos display differences in developmental potential to the blastocyst stage depending on the cytoplasm in which the paternal pronucleus forms (Latham, 1994; Latham and Solter, 1991). This reflects an effect of the ooplasm on the paternal genome very early during the 1-cell stage, and is attributed to two independently segregating loci on mouse chromosomes one and two (Latham and Sapienza, 1998; Latham and Solter, 1991). Collectively, these observations indicate that the paternal pronucleus undergoes extensive modifications that affect later paternal genome function and embryo phenotype, and that this is under the control of genes expressed in the oocyte.

A third possible instance of this interaction between ooplasm and paternal pronucleus can be seen with certain genetic combinations of mouse inbred strains C57BL/6 and C3H/HeJ, and outbred strains including CD1. The rate of cytofragmentation can be higher in simple crosses between these strains as compared to intrastrain crosses, so that oocytes from one strain will display enhanced fragmentation when fertilized by the sperm of one strain as compared to another (Jurisicova *et al.*, 1998; Han *et al.*, 2005; Hawes *et al.*, 2001). The genetic basis for this effect has not been revealed. It is possible that this relates to differences in sperm function, sperm components, and the quality of egg activation by the sperm (Ducibella *et al.*, 1993; Kono *et al.*, 1996; Moore *et al.*, 1993; Schultz and Kopf, 1995).

Why do such effects of the ooplasm exist over paternal genome function? One explanation is that genomic imprinting, like any biological process, is subject to genetic variation, and so is not identical between strains (Forejt and Gregorova, 1992). The epigenetic modifications of the paternal genome may require editing or modification in order to ensure

complementarity with the maternal strain. Without such editing, embryos could either over-express or lack expression of imprinted genes. Such effects may thus be revealed only in certain genetic combinations wherein the editing is imperfect, or via the extreme measure of performing pronuclear transfer to create unusual combinations of ooplasm and pronuclear genotypes. With respect to clinical practice, one can only speculate whether certain genetic combinations within couples may create similar incompatibilities, and thereby affect fertility or progeny phenotype.

5. Oocyte Polarity and Development

Across a wide range of animals, localized molecules in the oocyte provide information that directly determines cell fate or participates in the patterning of embryonic axes. Whether such determinants exist in mammalian oocytes has recently become an active area of study and subject of much debate. The mammalian oocyte is indeed polarized, with asymmetric distributions of microvilli and surface proteins, as well as an asymmetrically located meiotic spindle (Van Blerkom and Bell, 1986). The question thus arises whether cleavage occurs in such a way as to distribute materials unequally between daughter cells and whether this alters subsequent cell fates. Recent studies in the mouse involving marking the sperm entry point with lectin, monitoring the relationship between polar body position and blastocoel location, and marking the zona pellucida with oil droplets have led to the suggestion that this may occur (Gardner, 1997, 2001; Piotrowska *et al.*, 2001; Piotrowska and Zernicka-Goetz, 2001). The blastomere inheriting the sperm entry point is believed to divide first and this is believed to affect the fate of cellular progeny (Piotrowska and Zernicka-Goetz, 2001). Additionally, the sperm entry point and position of polar body are believed to define an early embryonic axis that provides an early bias affecting the position of blastocoel formation (Piotrowska and Zernicka-Goetz, 2001). These observations, while remarkable, have been called into question (Alarcon and Marikawa, 2003, 2005; Chroscicka *et al.*, 2004; Hiiragi and Solter, 2004; Louvet-Vallée *et al.*, 2005; Motosugi *et al.*, 2005). For example, upon detailed examination of time lapse videos of immobilized embryos, the positions of surface lectins and polar bodies are seen to change dramatically, and moreover the embryo can rotate within the zona pellucida, invalidating oil droplets in the zona pellucida as landmarks (Hiiragi and Solter, 2004, 2005, 2006). Mechanical forces rather than molecular specialization biases formation of the embryonic–abembryonic axis (Motosugi *et al.*, 2005). Lineage tracing studies in the mouse indicate that both blastomeres at the 2-cell stage contribute equally to the developing embryo (Alarcon and Marikawa, 2005; Chroscicka *et al.*, 2004; Motosugi *et al.*, 2005).

Moreover, twinning by embryo splitting is successful, as is development of demi-embryos when performed either at early cleavage (2-cell or 4-cell) stages or at the morula–blastocyst stage (Allen and Pashen, 1984; Johnson *et al.*, 1995; Matsumoto *et al.*, 1989; Oppenheim *et al.*, 2000; Ozil, 1983; Papaioannou *et al.*, 1989; Robl and First, 1985; Saito and Niemann, 1991; Seike *et al.*, 1991; Tarkowski, 1959a,b; Tsunoda *et al.*, 1984; Voelkel *et al.*, 1985; Yanagimachi, 2002). Two-cell stage blastomeres display equivalent developmental capacities in a substantial number of experimentally separated monozygotic pairs (Mitalipova *et al.*, 2002; Wildasen, 1979). Procedures that eliminate one blastomere at the 2-cell stage within the zona pellucida yield a high efficiency of blastocyst formation (Illmensee *et al.*, 2006; Tojo and Ogita, 1984). Demi-embryos can regulate their size during gestation (Lewis and Riossant, 1982; Papaioannou *et al.*, 1989; Rands, 1986; Tsunoda and McLaren, 1983). These observations that the fates of individual blastomeres are plastic and can be regulated are inconsistent with the idea that localized determinants exist in the mammalian oocyte and play an essential role in controlling early development (Alarcon and Marikawa, 2003).

6. Maternal Nutrition and Diabetes Affecting Oocyte and Embryo Quality

Beyond the need to support early embryogenesis, the importance of oocyte quality in determining future health of the progeny is becoming increasingly appreciated. One of the most startling discoveries in recent years has been that the health and disease status of adult offspring may be determined by maternal food consumption during a brief period at the time of conception. A low protein diet (LPD) given to female rats for the brief period of 4.5 days between fertilization and implantation, followed by a normal diet thereafter, reduces embryonic cell number, affects birth weight, and subsequently can lead to compensatory weight gain, hypertension, and alterations in organ/body weight ratios in adults (Kwong *et al.*, 2000). Periconception LPD (i.e., LPD during the preimplantation period) also can affect expression of imprinted and nonimprinted genes in progeny liver and affects the hypothalamo–pituitary–adrenal axis in fetal sheep (Kwong *et al.*, 2006, 2007). Interestingly, these effects display sex-dependent differences, suggesting either differential sex-dependent sensitivity or sex-dependent compensatory mechanisms. These observations collectively suggest that a range of adult diseases, including hypertension and subsequent kidney and heart disease, in many cases constitute birth defects arising during the first few days of life as a result of seemingly innocuous, short-term variations in maternal diet, a situation that has substantial clinical and societal importance.

The mechanisms by which maternal diet and the preimplantation embryo milieu affect long-term development are likely complex. The best-studied example of this relates to glucose availability. Exposure of embryos to elevated glucose levels (including via maternal hyperglycemia) leads to downregulation of glucose transporters and IGF1 receptors, followed by reduced glucose uptake, altered carbohydrate metabolism, and induction of apoptosis via a p53-dependent mechanism involving cell death effector pathways (Chi *et al.*, 2000a,b, 2002; Gäreskog *et al.*, 2007; Keim *et al.*, 2001; Moley, 1999, 2001; Moley *et al.*, 1996, 1998a,b; Riley and Moley, 2006). Insufficiency for glucose transporters compromises the ability of preimplantation stage embryos to cope with hypoxic stress (Heilig *et al.*, 2003). Reduced amino acid availability leads to reduced rates of cell division and later effects on blastocyst quality and stem cell lineages (Lane and Gardner, 1997). Amino acids are beneficial for blastocyst development and implantation (Biggers *et al.*, 2000; Devreker *et al.*, 1998, 2001; Lane and Gardner, 1997). Peri-conception LPD in the rat leads to a significant increase in maternal serum glucose and decrease in insulin between days 3 and 4 of treatment, returning to normal values by 2 days after return to normal diet. Coincidentally, the LPD led to depletion of six amino acids by day 4 of treatment (Kwong *et al.*, 2000).

The fact that LPD can both elevate glucose and reduce amino acids in maternal serum indicates that the embryos of these mothers may be forced to respond to both adverse conditions. The reduced cell number in both ICM and trophectoderm (TE) of blastocysts from mothers treated with peri-conception LPD could be explicable on the basis of either reduced cell proliferation, increased cellular apoptosis, or a combination of these, might result from the combined effects of elevated glucose and reduced amino acid availability. A slow rate of cleavage is correlated with reduced cell numbers in blastocysts, and reduced developmental potential, either to term (Gonzales *et al.*, 1995; Lonergan *et al.*, 1999; Lundin *et al.*, 2001; Sakkas *et al.*, 1995, 1998; Scott *et al.*, 2007; Wharf *et al.*, 2004) or for the *in vitro* production of embryonic stem (ES) cell lines (Chen *et al.*, 2005). Growth factors that promote cell proliferation and suppress apoptosis can improve development of embryos with reduced cell numbers (Glabowski *et al.*, 2005; Kurzawa *et al.*, 2004; Lin *et al.*, 2003), and can enhance the formation of stem cells (Lin *et al.*, 2003). Subsequently, the sizes of specific stem cell populations arising from downstream development of the ICM can affect the formation of specific organs (Stanger *et al.*, 2007). Thus, the size of the initial ICM population (the forerunner of all embryonic lineages) could significantly affect organogenesis by affecting the numbers of cells available to form critical units (e.g., nephrons). Moreover, a small ICM may contribute to fetal growth retardation and large placenta (Kwong *et al.*, 2000; Lane and Gardner, 1997), so that early effects on the ICM size can greatly affect fetal development, including

direct effects on the fetus itself as well as indirect effects via changes in maternal–fetal waste, nutrient, and oxygen exchange.

Even more striking than the relatively immediate effects of maternal diet during the preimplantation period is the potential for long-term effects even transcending generations—a grandmaternal effect. Maternal undernutrition during pregnancy and lactation can seriously compromise the health of offspring. Effects include low birth weights, obesity, type 2 diabetes, defects in organ development, hypertension, vascular and heart disease, and effects on brain, liver, and muscle function (e.g., Akahoshi *et al.*, 2006; Armitage *et al.*, 2007; Barker, 1997; Cox *et al.*, 2006; Godfrey *et al.*, 1994; Kelly *et al.*, 2005; Lau and Rogers, 2004; Oreffo *et al.*, 2003; Ozaki *et al.*, 2000; Painter *et al.*, 2006; Patera *et al.*, 2006; Petry *et al.*, 2006; Pires *et al.*, 2006; Ravelli *et al.*, 1998, 2005; Sayer and Cooper, 2005; Symonds *et al.*, 2007; Thone-Reineke *et al.*, 2006). Alternatively, modulating maternal diet can ameliorate genetic predispositions to certain diseases, and dietary antioxidants can ameliorate some effects of LPD (Cahill *et al.*, 2007; Sankaran *et al.*, 2006). Maternal diet prior to conception can affect oocyte quality (Adamiak *et al.*, 2005; Cambonie *et al.*, 2007; Hunter *et al.*, 2005). This effect on oocytes affects embryo quality and potentially fetal development and adult health, raising the possibility of effects on oocytes in the next generation, possibly producing further trans-generational effects. The effect of nutrient restriction is also believed to exert transgenerational effects in humans and rodents (Barker *et al.*, 1994).

7. Perspectives and Significance

The results summarized in this review highlight the truly remarkable qualities of the oocyte, along with the remarkable and complex oocyte–somatic cell interactions that endow the oocyte with these qualities. The oocyte is endowed with a rich legacy of proteins, mRNAs, and other macromolecules that direct and regulate early development. This legacy arises through essential biosynthetic processes that are directly affected by interactions with the follicular environment. Disruptions in these interactions can compromise oocyte quality. The creation of high-quality oocytes is thus sensitive to genetic and environmental factors. Additionally, the oocyte and early embryo can respond to exogenous stimuli and stressors, modulating their phenotype accordingly; however, these responses may compromise the ability of the oocyte to respond appropriately to the developmental milieu it encounters at a later stage. Thus, along with genetic and environmental factors, procedural variables employed for assisted reproduction methods in human and nonhuman species have the potential for long-term effects on embryo development and health of the offspring.

Our appreciation is increasing rapidly for the potential long-term effects of oocyte quality on embryo developmental capacity and adult health, as well as potential transgenerational effects that may arise through self-perpetuating rounds of effects of maternal physiology and oogenesis/oocyte quality on each other. As our understanding of the molecular mechanisms that drive early development and control oocyte and embryo responses to their environments improves, so too should we see increases in our understanding of what specific characteristics denote high-quality oocytes, what specific stimuli or factors compromise or enhance oocyte quality, and how the availability of high-quality oocytes can be improved for clinical and applied purposes. The prospects for improved efficiencies in infertility treatment, contraception, bioengineering of domestic species, and species preservation of endangered species are therefore quite favorable. From a basic science perspective, anticipated advances in understanding oocyte control of nuclear function would yield broadly applicable lessons for understanding gene regulation, and for achieving new success in the development of emerging technologies for stem cell derivation and stem cell-based therapeutic applications. The oocyte indeed provides the essential life-generating force for combining gametes to create a new, functional, and viable individual. Continued improvement in understanding the mechanisms at play should provide us with a much greater appreciation for the remarkable fact of our very existence, where we came from, and what lies on the road ahead.

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REFERENCES

Ackert, C. L., Gittens, J. E., O'Brien, M. J., Eppig, J. J., and Kidder, G. M. (2001). Intercellular communication via connexin43 gap junctions is required for ovarian folliculogenesis in the mouse. *Dev. Biol.* **15,** 258–270.

Acton, B. M., Lai, I., Shang, X., Jurisicova, A., and Casper, R. F. (2007). Neutral mitochondrial heteroplasmy alters physiological function in mice. *Biol. Reprod.* **77,** 569–576.

Adamiak, S. J., Mackie, K., Watt, R. G., Webb, R., and Sinclair, K. D. (2005). Impact of nutrition on oocyte quality: Cumulative effects of body composition and diet leading to hyperinsulinemia in cattle. *Biol. Reprod.* **73,** 918–926.

Adenot, P. G., Mercier, Y., Renard, J. P., and Thompson, E. M. (1997). Differential H4 acetylation of paternal and maternal chromatin precedes DNA replication and differential

transcriptional activity in pronuclei of 1-cell mouse embryos. *Development* **124,** 4615–4625.
Aitken, R. J. (1994). A free radical theory of male infertility. *Reprod. Fertil. Dev.* **6,** 19–23.
Aitken, R. J. (1995). Free radicals, lipid peroxidation and sperm function. *Reprod. Fertil. Dev.* **7,** 659–668.
Aitken, R. J. (1999). The amoroso lecture. The human spermatozoon—A cell in crisis? *J. Reprod. Fertil.* **115,** 1–7.
Aitken, R. J., and Fisher, H. (1994). Reactive oxygen species generation and human spermatozoa: The balance of benefit and risk. *Bioessays* **16,** 259–267.
Akahoshi, N., Izumi, T., Ishizaki, Y., and Ishii, I. (2006). Differential induction of cystathionine gamma-lyase in the livers and kidneys of mouse dams during gestation and lactation. *Biol. Pharm. Bull.* **29**(1), 799–802.
Alarcon, V. B., and Marikawa, Y. (2003). Deviation of the blastocyst axis from the first cleavage plane does not affect the quality of mouse postimplantation development. *Biol. Reprod.* **4,** 1208–1212.
Alarcon, V. B., and Marikawa, Y. (2005). Unbiased contribution of the first two blastomeres to mouse blastocyst development. *Mol. Reprod. Dev.* **72,** 354–361.
Allen, W. R., and Pashen, R. L. (1984). Production of monozygotic (identical) horse twins by embryo micromanipulation. *J. Reprod. Fertil.* **71,** 607–613.
Altmäe, S., Haller, K., Peters, M., Hovatta, O., Stavreus-Evers, A., Karro, H., Metspalu, A., and Salumets, A. (2007). Allelic estrogen receptor 1 (*ESR1*) gene variants predict the outcome of ovarian stimulation in *in vitro* fertilization. *Mol. Hum. Reprod.* **13,** 521–526.
Amikura, R., Sato, K., and Kobayashi, S. (2005). Role of mitochondrial ribosome-dependent translation in germline formation in *Drosophila* embryos. *Mech. Dev.* **122,** 1087–1093.
Angell, R. R., Templeton, A. A., and Aitken, R. J. (1986). Chromosome studies in human *in vitro* fertilization. *Hum. Genet.* **72,** 333–339.
Ankel-Simons, F., and Cummins, J. M. (1996). Misconceptions about mitochondria and mammalian fertilization: Implications for theories on human evolution. *Proc. Natl. Acad. Sci. USA* **93,** 13859–13863.
Antczak, M., and Van Blerkom, J. (1999). Temporal and spatial aspects of fragmentation in early human embryos: Possible effects on developmental competence and association with the differential elimination of regulatory proteins from polarized domains. *Hum. Reprod.* **14,** 429–447.
Aoki, F., Worrad, D. M., and Schultz, R. M. (1997). Regulation of transcriptional activity during the first and second cell cycles in the preimplantation mouse embryo. *Dev. Biol.* **181,** 296–307.
Aoki, F., Hara, K. T., and Schultz, R. M. (2003). Acquisition of transcriptional competence in the 1-cell mouse embryo: Requirement for recruitment of maternal mRNAs. *Mol. Reprod. Dev.* **64,** 270–274.
Armitage, J. A., Ishibashi, A., Balachandran, A. A., Jensen, R. I., Poston, L., and Taylor, P. D. (2007). Programmed aortic dysfunction and reduced Na+,K+-ATPase activity present in first generation offspring of lard-fed rats does not persist to the second generation. *Exp. Physiol.* **92,** 583–589.
Atlan, A., Joly, D., Capillon, C., and Montchamp-Moreau, C. (2004). Sex-ratio distorter of *Drosophila* simulans reduces male productivity and sperm competition ability. *J. Evol. Biol.* **17,** 744–751.
Bachvarova, R., De Leon, V., Johnson, A., Kaplan, G., and Paynton, B. V. (1985). Changes in total RNA, polyadenylated RNA, and actin mRNA during meiotic maturation of mouse oocytes. *Dev. Biol.* **108,** 325–331.
Baez, M. V., and Boccaccio, G. L. (2005). Mammalian Smaug is a translational repressor that forms cytoplasmic foci similar to stress granules. *J. Biol. Chem.* **280,** 43131–43140.

Baldacci, P. A., Richoux, V., Renard, J. P., Guenet, J. L., and Babinet, C. (1992). The locus Om, responsible for the DDK syndrome, maps close to Sigje on mouse chromosome 11. *Mamm. Genome* **2,** 100–105.

Baltaci, V., Satiroglu, H., Kabukcu, C., Unsal, E., Aydinuraz, B., Uner, O., Aktas, Y., Cetinkaya, E., Turhan, F., and Aktan, A. (2006). Relationship between embryo quality and aneuploidies. *Reprod. Biomed. Online* **12,** 77–82.

Barker, D. J. (1997). Maternal nutrition, fetal nutrition, and disease in later life. *Nutrition* **13,** 807–813.

Barker, D. J., Gluckman, P. D., and Robinson, J. S. (1994). Conference report: Fetal origins of adult disease—Report of the First International Study Group, Sydney, 29–30 October 1994. *Placenta* **16,** 317–320.

Barnard, D. C., Ryan, K., Manley, J. L., and Richter, J. D. (2004). Symplekin and xGLD-2 are required for CPEB-mediated cytoplasmic polyadenylation. *Cell* **119,** 641–651.

Barnard, D. C., Cao, Q., and Richter, J. D. (2005). Differential phosphorylation controls Maskin association with eukaryotic translation initiation factor 4E and localization on the mitotic apparatus. *Mol. Cell. Biol.* **25,** 7605–7615.

Barnett, D. K., Kimura, J., and Bavister, B. D. (1996). Translocation of active mitochondria during hamster preimplantation embryo development studied by confocal laser scanning microscopy. *Dev. Dyn.* **205,** 64–72.

Barnett, K. R., Schilling, C., Greenfeld, C. R., Tomic, D., and Flaws, J. A. (2006). Ovarian follicle development and transgenic mouse models. *Hum. Reprod. Update* **12,** 537–555.

Bell, T. A., de la Casa-Esperon, E., Doherty, H. E., Ideraabdullah, F., Kim, K., Wang, Y., Lange, L. A., Wilhemsen, K., Lange, E. M., Sapienza, C., and de Villena, F. P. (2006). The paternal gene of the DDK syndrome maps to the Schlafen gene cluster on mouse chromosome 11. *Genetics* **172,** 411–423.

Benoit, B., Mitou, G., Chartier, A., Temme, C., Zaessinger, S., Wahle, E., Busseau, I., and Simonelig, M. (2005). An essential cytoplasmic function for the nuclear poly(A) binding protein, PABP2, in poly(A) tail length control and early development in *Drosophila*. *Dev. Cell* **9,** 511–522.

Bergeron, L., Perez, G. I., McDonald, G., Shi, L., Sun, Y., Jurisicova, A., Varmuza, S., Latham, K., Flaws, J. A., Salter, J., Hara, H., Moskowitz, M. A., *et al.* (1998). Defects in regulation of apoptosis in Caspase-2 deficient mice. *Genes Dev.* **12,** 1303–1317.

Bielanska, M., Tan, S. L., and Ao, A. (2002). Chromosomal mosaicism throughout human preimplantation development *in vitro*: Incidence, type, and relevance to embryo outcome. *Hum. Reprod.* **17,** 413–419.

Biggers, J. D., McGinnis, L. K., and Raffin, M. (2000). Amino acids and preimplantation development of the mouse in protein-free potassium simplex optimized medium. *Biol. Reprod.* **63,** 281–293.

Birky, C. W., Jr. (1995). Uniparental inheritance of mitochondria and chloroplast genes: Mechanism and evolution. *Proc. Natl. Acad. Sci. USA* **92,** 11331–11338.

Birky, C. W., Jr. (2001). The inheritance of genes in mitochondria and chloroplast: Laws, mechanism and models. *Annu. rev. Genet.* **35,** 125–148.

Bolton, V. N., Hawes, S. M., Taylor, C. T., and Parsons, J. H. (1989). Development of spare human preimplantation embryos *in vitro*: An analysis of the correlations among gross morphology, cleavage rates, and development to the blastocyst. *J. In Vitro Fert. Embryo Transf.* **6,** 30–35.

Booth, P. J., Viuff, D., Tan, S., Holm, P., Greve, T., and Callesen, H. (2003). Numerical chromosome errors in day 7 somatic nuclear transfer bovine blastocysts. *Biol. Reprod.* **68,** 922–928.

Bortvin, A., Goodheart, M., Liao, M., and Page, D. C. (2004). Dppa3/Pgc7/stella is a maternal factor and is not required for germ cell specification in mice. *BMC Dev. Biol.* **23,** 2.

Bouvet, P., Omilli, F., Arlot-Bonnemains, Y., Legagneux, V., Roghi, C., Bassez, T., and Osborne, H. B. (1994). The deadenylation conferred by the 3′ untranslated region of a developmentally controlled mRNA in *Xenopus* embryos is switched to polyadenylation by deletion of a short sequence element. *Mol. Cell. Biol.* **14,** 1893–1900.

Brenner, C. A., Barritt, J. A., Willadsen, S., and Cohen, J. (2000). Mitochondrial DNA heteroplasmy after human ooplasmic transplantation. *Fertil. Steril.* **74,** 573–578.

Brewster, J. L., Martin, S. L., Toms, J., Goss, D., Wang, K., Zachrone, K., Davis, A., Carlson, G., Hood, L., and Coffin, J. D. (2000). Deletion of Dad1 in mice induces an apoptosis-associated embryonic death. *Genesis* **26,** 271–278.

Brower, P. T., and Schultz, R. M. (1982). Intercellular communication between granulosa cells and mouse oocytes: Existence and possible nutritional role during oocyte growth. *Dev. Biol.* **90,** 144–153.

Buccione, R., Schroeder, A. C., and Eppig, J. J. (1990). Interactions between somatic cells and germ cells throughout mammalian oogenesis. *Biol. Reprod.* **43,** 543–547.

Buchet-Poyau, K., Courchet, J., Le Hir, H., Séraphin, B., Scoazec, J. Y., Duret, L., Domon-Dell, C., Freund, J. N., and Billaud, M. (2007). Identification and characterization of human Mex-3 proteins, a novel family of evolutionarily conserved RNA-binding proteins differentially localized to processing bodies. *Nucleic Acids Res.* **35,** 1289–1300.

Bultman, S. J., Gebuhr, T. C., Pan, H., Svoboda, P., Schultz, R. M., and Magnuson, T. (2006). Maternal BRG1 regulates zygotic genome activation in the mouse. *Genes Dev.* **20,** 1744–1754.

Burns, K. H., Viveiros, M. M., Ren, Y., Wang, P., DeMayo, F. J., Frail, D. E., Eppig, J. J., and Matzuk, M. M. (2003). Roles of NPM2 in chromatin and nucleolar organization in oocytes and embryos. *Science* **300,** 633–636.

Byrne, A. T., Southgate, J., Brison, D. R., and Leese, H. J. (1999). Analysis of apoptosis in the preimplantation bovine embryo using TUNEL. *J. Reprod. Fertil.* **117,** 97–105.

Cahill, L. E., Peng, C. Y., Bankovic-Calic, N., Sankaran, D., Ogborn, M. R., and Aukema, H. M. (2007). Dietary soya protein during pregnancy and lactation in rats with hereditary kidney disease attenuates disease progression in offspring. *Br. J. Nutr.* **97,** 77–84.

Caldwell, D. C., and Emerson, C. P., Jr. (1985). The role of cap methylation in the translational activation of stored maternal histone mRNA in sea urchin embryos. *Cell* **42,** 691–700.

Calvo, R. M., Villuendas, G., Sancho, J., San Millan, J. L., and Escobar-Morreale, H. F. (2001). Role of the follistatin gene in women with polycystic ovary syndrome. *Fertil. Steril.* **75,** 1020–1023.

Cambonie, G., Comte, B., Yzydorczyk, C., Ntimbane, T., Germain, N., Lê, N. L., Pladys, P., Gauthier, C., Lahaie, I., Abran, D., Lavoie, J. C., and Nuyt, A. M. (2007). Antenatal antioxidant prevents adult hypertension, vascular dysfunction, and microvascular rarefaction associated with in utero exposure to a low-protein diet. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **292,** R1236–R1245.

Can, A., and Semiz, O. (2000). Diethylstilbestrol (DES)-induced cell cycle delay and meiotic spindle disruption in mouse oocytes during *in-vitro* maturation. *Mol. Hum. Reprod.* **6,** 154–162.

Can, A., Semiz, O., and Cinar, O. (2005). Bisphenol-A induces cell cycle delay and alters centrosome and spindle microtubular organization in oocytes during meiosis. *Mol. Hum. Reprod.* **11,** 389–396.

Cao, Q., and Richter, J. D. (2002). Dissolution of the maskin-eIF4E complex by cytoplasmic polyadenylation and poly(A)-binding protein controls cyclin B1 mRNA translation and oocyte maturation. *EMBO J.* **21,** 3852–3862.

Carabatsos, M. J., Sellitto, C., Goodenough, D. A., and Albertini, D. F. (2000). Oocyte-granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence. *Dev. Biol.* **226,** 167–179.

Casper, R. F., and Jurisicova, A. (2000). Protecting the female germ line from cancer therapy. *Nat. Med.* **6,** 1100–1101.

Charlesworth, A., Ridge, J. A., King, L. A., MacNicol, M. C., and MacNicol, A. M. (2002). A novel regulatory element determines the timing of Mos mRNA translation during *Xenopus* oocyte maturation. *EMBO J.* **21,** 2798–2806.

Charlesworth, A., Wilczynska, A., Thampi, P., Cox, L. L., and MacNicol, A. M. (2006). Musashi regulates the temporal order of mRNA translation during *Xenopus* oocyte maturation. *EMBO J.* **25,** 2792–2801.

Chen, H., Qian, K., Hu, J., Liu, D., Lu, W., Yang, Y., Wang, D., Yan, H., Zhang, S., and Zhu, G. (2005). The derivation of two additional human embryonic stem cell lines from day 3 embryos with low morphological scores. *Hum. Reprod.* **20,** 2201–2206.

Chesnel, F., Wigglesworth, K., and Eppig, J. J. (1994). Acquisition of meiotic competence by denuded mouse oocytes: Participation of somatic-cell product(s) and cAMP. *Dev. Biol.* **161,** 285–295.

Chi, M. M., Pingsterhaus, J., Carayannopoulos, M., and Moley, K. H. (2000a). Decreased glucose transporter expression triggers BAX-dependent apoptosis in the murine blastocyst. *J. Biol. Chem.* **275,** 40252–40257.

Chi, M. M., Schlein, A. L., and Moley, K. H. (2000b). High insulin-like growth factor 1 (IGF-1) and insulin concentrations trigger apoptosis in the mouse blastocyst via down-regulation of the IGF-1 receptor. *Endocrinology* **141,** 4784–4792.

Chi, M. M., Hoehn, A., and Moley, K. H. (2002). Metabolic changes in the glucose-induced apoptotic blastocyst suggest alterations in mitochondrial physiology. *Am. J. Physiol. Endocrinol. Metab.* **283,** E226–E232.

Chipuk, J. E., Bouchier-Hayes, L., and Green, D. R. (2006). Mitochondrial outer membrane permeabilization during apoptosis: The innocent bystander scenario. *Cell Death Differ.* **13,** 1396–1402.

Chiquoine, A. D. (1954). The identification, origin, and migration of the primordial germ cells in the mouse embryo. *Anat. Rec.* **118,** 135–146.

Cho, P. F., Poulin, F., Cho-Park, Y. A., Cho-Park, I. B., Chicoine, J. D., Lasko, P., and Sonenberg, N. (2005). A new paradigm for translational control: Inhibition via 5′-3′ mRNA tethering by Bicoid and the eIF4E cognate 4EHP. *Cell* **121,** 411–423.

Cho, P. F., Gamberi, C., Cho-Park, Y. A., Cho-Park, I. B., Lasko, P., and Sonenberg, N. (2006). Cap-dependent translational inhibition establishes two opposing morphogen gradients in *Drosophila* embryos. *Curr. Biol.* **16,** 2035–2041.

Chroscicka, A., Komorowski, S., and Maleszewski, M. (2004). Both blastomeres of the mouse 2-cell embryo contribute to the embryonic portion of the blastocyst. *Mol. Reprod. Dev.* **68,** 308–312.

Chuma, S., Hosokawa, M., Kitamura, K., Kasai, S., Fujioka, M., Hiyoshi, M., Takamune, K., Noce, T., and Nakatsuji, N. (2006). Tdrd1/Mtr-1, a tudor-related gene, is essential for male germ-cell differentiation and nuage/germinal granule formation in mice. *Proc. Natl. Acad. Sci. USA* **103,** 15894–15899.

Chung, Y. G., Mann, M. R., Bartolomei, M. S., and Latham, K. E. (2002). Nuclearcytoplasmic "tug-of war" during cloning: Effects of somatic cell nuclei on culture medium preferences in the preimplantation cloned mouse embryo. *Biol. Reprod.* **66,** 1178–1184.

Chung, Y. G., Ratnam, S., Chaillet, J. R., and Latham, K. E. (2003). Abnormal regulation of DNA methyltransferase expression in cloned mouse embryos. *Biol. Reprod.* **69,** 146–153.

Cibelli, J. B., Stice, S. L., Golueke, P. J., Kane, J. J., Jerry, J., Blackwell, C., Ponce De Leon, F. A. P., and Robl, J. M. (1998). Cloned transgenic calves produced from nonquiescent fetal fibroblast. *Science* **280,** 1256–1258.

Clegg, K. B., and Piko, L. (1982). RNA synthesis and cytoplasmic polyadenylation in the one-cell mouse embryo. *Nature* **295,** 343–344.

Clegg, K. B., and Piko, L. (1983a). Poly(A) length, cytoplasmic adenylation and synthesis of poly(A)+ RNA in early mouse embryos. *Dev. Biol.* **95,** 331–341.
Clegg, K. B., and Piko, L. (1983b). Quantitative aspects of RNA synthesis and polyadenylation in 1-cell and 2-cell mouse embryos. *J. Embryol. Exp. Morphol.* **74,** 169–182.
Clemens, M. J. (2004). Targets and mechanisms for the regulation of translation in malignant transformation. *Oncogene* **23,** 3180–3188.
Coller, J., and Parker, R. (2005). General translational repression by activators of mRNA decapping. *Cell* **122,** 875–886.
Collier, B., Gorgoni, B., Loveridge, C., Cooke, H. J., and Gray, N. K. (2005). The DAZL family proteins are PABP-binding proteins that regulate translation in germ cells. *EMBO J.* **24,** 2656–2666.
Conway, G. S. (2000). Premature ovarian failure. *Br. Med. Bull.* **56,** 643–649.
Cooke, H. J., and Elliott, D. J. (1997). RNA-binding proteins and human male infertility. *Trends Genet.* **13,** 87–89.
Cosson, B., Braun, F., Paillard, L., Blackshear, P., and Osborne, H. B. (2004). Identification of a novel *Xenopus laevis* poly (A) binding protein. *Biol. Cell* **96,** 519–527.
Cox, L. A., Nijland, M. J., Gilbert, J. S., Schlabritz-Loutsevitch, N. E., Hubbard, G. B., McDonald, T. J., Shade, R. E., and Nathanielsz, P. W. (2006). Effect of 30 per cent maternal nutrient restriction from 0.16 to 0.5 gestation on fetal baboon kidney gene expression. *J. Physiol.* **572,** 67–85.
Crosby, I. M., Gandolfi, F., and Moor, R. M. (1988). Control of protein synthesis during early cleavage of sheep embryos. *J. Reprod. Fertil.* **82,** 769–775.
Cukurcam, S., Sun, F., Betzendahl, I., Adler, I. D., and Eichenlaub-Ritter, U. (2004). Trichlorfon predisposes to aneuploidy and interferes with spindle formation in *in vitro* maturing mouse oocytes. *Mutat. Res.* **12,** 165–178.
Cummins, J. (1998). Mitochondrial DNA in mammalian reproduction. *Rev. Reprod.* **3,** 172–182.
Cummins, J. M. (2000). Fertilization and elimination of the paternal mitochondrial genome. *Hum. Reprod.* **15,** 92–101.
Cuthbertson, K. S., and Cobbold, P. H. (1985). Phorbol ester and sperm activate mouse oocytes by inducing sustained oscillations in cell Ca2+. *Nature* **316,** 541–542.
Cuthbertson, K. S., Whittingham, D. G., and Cobbold, P. H. (1981). Free Ca2+ increases in exponential phases during mouse oocyte activation. *Nature* **294,** 754–757.
Dawid, I. B., and Blackler, A. W. (1972). Maternal and cytoplasmic inheritance of mitochondrial DNA in *Xenopus*. *Dev. Biol.* **29,** 152–161.
De La Fuente, R., and Eppig, J. J. (2001). Transcriptional activity of the mouse oocyte genome: Companion granulosa cells modulate transcription and chromatin remodeling. *Dev. Biol.* **229,** 224–236.
de Moor, C. H., and Richter, J. D. (1997). The Mos pathway regulates cytoplasmic polyadenylation in *Xenopus* oocytes. *Mol. Cell Biol.* **17,** 6419–6426.
de Moor, C. H., and Richter, J. D. (1999). Cytoplasmic polyadenylation elements mediate masking and unmasking of cyclin B1 mRNA. *EMBO J.* **18,** 2294–2303.
de Pennart, H., Verlhac, M. H., Cibert, C., Santa Maria, A., and Maro, B. (1993). Okadaic acid induces spindle lengthening and disrupts the interaction of microtubules with the kinetochores in metaphase II-arrested mouse oocytes. *Dev. Biol.* **157,** 170–181.
Dehennin, L., Blacker, C., Reiffsteck, A., and Scholler, R. (1984). Estrogen 2-, 4-, 6- or 16-hydroxylation by human follicles shown by gas chromatography-mass spectrometry associated with stable isotope dilution. *J. Steroid Biochem.* **20,** 465–471.
DeRenzo, C., and Seydoux, G. (2004). A clean start: Degradation of maternal proteins at the oocyte-to-embryo transition. *Trends Cell Biol.* **14,** 420–426.
Devreker, F., Winston, R. M., and Hardy, K. (1998). Glutamine improves human preimplantation development *in vitro*. *Fertil. Steril.* **69,** 293–299.

Devreker, F., Hardy, K., Van den Bergh, M., Vannin, A. S., Emiliani, S., and Englert, Y. (2001). Amino acids promote human blastocyst development *in vitro*. *Hum. Reprod.* **16,** 749–756.

Di Pasquale, E., Beck-Peccoz, P., and Persani, L. (2004). Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene. *Am. J. Hum. Genet.* **75,** 106–111.

Diaz, F. J., O'Brien, M. J., Wigglesworth, K., and Eppig, J. J. (2006). The preantral granulosa cell to cumulus cell transition in the mouse ovary: Development of competence to undergo expansion. *Dev Biol.* **299,** 91–104.

Diaz, F. J., Wigglesworth, K., and Eppig, J. J. (2007). Oocytes determine cumulus cell lineage in mouse ovarian follicles. *J. Cell Sci.* **120,** 1330–1340.

Dierich, A., Sairam, M. R., Monaco, L., Fimia, G. M., Gansmuller, A., LeMeur, M., and Sassone-Corsi, P. (1998). Impairing follicle-stimulating hormone (FSH) signaling *in vivo*: Targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. *Proc. Natl. Acad. Sci. USA* **95,** 13612–13617.

Dinarina, A., Perez, L. H., Davila, A., Schwab, M., Hunt, T., and Nebreda, A. R. (2005). Characterization of a new family of cyclin-dependent kinase activators. *Biochem. J.* **386,** 349–355.

Dong, J., Albertini, D. F., Nishimori, K., Kumar, T. R., Lu, N., and Matzuk, M. M. (1996). Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* **383,** 531–535.

Donovan, P. J., and de Miguel, M. P. (2001). *In* "Transgenics in Endocrinology" (M. M. Matzuk, C. W. Brown, and T. R. Kumar, Eds.), pp. 147–163. Humana, Totowa, NJ.

Ducibella, T., Kurasawa, S., Duffy, P., Kopf, G. S., and Schultz, R. M. (1993). Regulation of the polyspermy block in the mouse egg: Maturation-dependent differences in cortical granule exocytosis and zona pellucida modifications induced by inositol 1,4,5-trisphosphate and an activator of protein kinase C. *Biol. Reprod.* **48,** 1251–1257.

Ducibella, T., Huneau, D., Angelichio, E., Xu, Z., Schultz, R. M., Kopf, G. S., Fissore, R., Madoux, S., and Ozil, J. P. (2002). Egg-to-embryo transition is driven by differential responses to Ca(2+) oscillation number. *Dev. Biol.* **250,** 280–291.

Dworkin, M. B., and Dworkin-Rastl, E. (1990). Functions of maternal mRNA in early development. *Mol. Reprod. Dev.* **26,** 261–297.

Dworkin, M. B., Shrutkowski, A., and Dworkin-Rastl, E. (1985). Mobilization of specific maternal RNA species into polysomes after fertilization in *Xenopus laevis*. *Proc. Natl. Acad. Sci. USA* **82,** 7636–7640.

Eichenlaub-Ritter, U., and Betzendahl, I. (1995). Chloral hydrate induced spindle aberrations, metaphase I arrest and aneuploidy in mouse oocytes. *Mutagenesis* **10,** 477–486.

Eichenlaub-Ritter, U., and Boll, I. (1989). Nocodazole sensitivity, age-related aneuploidy, and alterations in the cell cycle during maturation of mouse oocytes. *Cytogenet. Cell Genet.* **52,** 170–176.

Eichenlaub-Ritter, U., Winterscheidt, U., Vogt, E., Shen, Y., Tinneberg, H. R., and Sorensen, R. (2007). Methoxyestradiol induces spindle aberrations, chromosome congression failure, and nondisjunction in mouse oocytes. *Biol. Reprod.* **76,** 784–793.

Elvin, J. A., Yan, C., and Matzuk, M. M. (2000). Oocyte-expressed TGF-ß superfamily members in female fertility. *Mol. Cell Endocrinol.* **159,** 1–5.

Enmark, E., Pelto-Huikko, M., Grandien, K., Lagercrantz, S., Lagercrantz, J., Fried, G., Nordenskjold, M., and Gustafsson, J. A. (1997). Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *J. Clin. Endocrinol. Metab.* **82,** 4258–4265.

Epigg, J. J. (2001). Oocyte control of ovarian follicular development and function in mammals. *Reproduction* **122,** 829–838.

Eppig, J. J., and O'Brien, M. J. (1996). Development *in vitro* of mouse oocytes from primordial follicles. *Biol. Reprod.* **54,** 197–207.

Eppig, J. J., and Schroeder, A. C. (1989). Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation, and fertilization *in vitro*. *Biol. Reprod.* **41,** 268–276.

Eppig, J. J., Schroeder, A. C., and O'Brien, M. J. (1992a). Developmental capacity of mouse oocytes matured *in vitro*: Effects of gonadotrophic stimulation, follicular origin and oocyte size. *J. Reprod. Fertil.* **95,** 119–127.

Eppig, J. J., Wigglesworth, K., and O'Brien, M. J. (1992b). Comparison of embryonic developmental competence of mouse oocytes grown with and without serum. *Mol. Reprod. Dev.* **32,** 33–40.

Eppig, J. J., Schultz, R. M., and Kopf, G. S. (1996). Maturation of mouse oocytes in serum-free medium. *Hum. Reprod.* **11,** 1139–1140.

Eppig, J. J., Chesnel, F., Hirao, Y., O'Brien, M. J., Pendola, F. L., Watanabe, S., and Wigglesworth, K. (1997). Oocyte control of granulosa cell development: How and why. *Hum. Reprod.* **12,** 127–132.

Eppig, J. J., Wigglesworth, K., and Pendola, F. L. (2002). The mammalian oocyte orchestrates the rate of ovarian follicular development. *Proc. Natl. Acad. Sci. USA* **99,** 2890–2894.

Erenus, M., Zouves, C., Rajamahendran, P., Leung, S., Fluker, M., and Gomel, V. (1991). The effect of embryo quality on subsequent pregnancy rates after *in vitro* fertilization. *Fertil. Steril.* **56,** 707–710.

Evans, M. J., Gurer, C., Loike, J. D., Wilmut, I., Schnieke, A. E., and Schon, E. A. (1999). Mitochondrial DNA genotypes in nuclear transfer-derived cloned sheep. *Nat. Genet.* **23,** 90–93.

Everett, C. A., and Searle, J. B. (1995). Pattern and frequency of nocodazole induced meiotic nondisjunction in oocytes of mice carrying the ''tobacco mouse'' metacentric Rb(16.17)7Bnr. *Genet. Res.* **66,** 35–43.

Feigenblum, D., and Schneider, R. J. (1996). Cap-binding protein (eukaryotic initiation factor 4E) and 4E-inactivating protein BP-1 independently regulate cap-dependent translation. *Mol. Cell Biol.* **16,** 5450–5457.

Fingar, D. C., and Blenis, J. (2004). Target of rapamycin (TOR): An integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. *Oncogene* **23,** 3151–3171.

Fingar, D. C., Salama, S., Tsou, C., Harlow, E., and Blenis, J. (2002). Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. *Genes Dev.* **16,** 1472–1487.

Fissore, R. A., Dobrinsky, J. R., Balise, J. J., Duby, R. T., and Robl, J. M. (1992). Patterns of intracellular Ca2+ concentrations in fertilized bovine eggs. *Biol. Reprod.* **47,** 960–969.

Fissore, R. A., Pinto-Correia, C., and Robl, J. M. (1995). Inositol trisphosphate-induced calcium release in the generation of calcium oscillations in bovine eggs. *Biol. Reprod.* **53,** 766–774.

Forejt, J., and Gregorova, S. (1992). Genetic analysis of genomic imprinting: An Imprintor-1 gene controls inactivation of the paternal copy of the mouse Tme locus. *Cell* **70,** 443–450.

Fortune, J. E. (2003). The early stages of follicular development: Activation of primordial follicles and growth of preantral follicles. *Anim. Reprod. Sci.* **78,** 135–163.

Fortune, J. E., Cushman, R. A., Wahl, C. M., and Kito, S. (2000). The primordial to primary follicle transition. *Mol. Cell Endocrinol.* **163,** 53–60.

Fox, C. A., and Wickens, M. (1990). Poly(A) removal during oocyte maturation: A default reaction selectively prevented by specific sequences in the 3′ UTR of certain maternal mRNAs. *Genes Dev.* **4,** 2287–2298.

Fox, C. A., Sheets, M. D., Wahle, E., and Wickens, M. (1992). Polyadenylation of maternal mRNA during oocyte maturation: Poly(A) addition *in vitro* requires a regulated RNA binding activity and a poly(A) polymerase. *EMBO J.* **11,** 5021–5032.

Fujimoto, S., Sato, C., Yamagami, Y., and Arai, E. (1978). Chromosomal anomalies of preimplantation rabbit blastocysts and human artificial abortuses in relation to maternal age. *Int. J. Fertil.* **23,** 207–212.

Fujimoto, S., Yoshida, N., Fukui, T., Amanai, M., Isobe, T., Itagaki, C., Izumi, T., and Perry, A. C. (2004). Mammalian phospholipase Czeta induces oocyte activation from the sperm perinuclear matrix. *Dev. Biol.* **274,** 370–383.

Gao, S., Chung, Y. G., Williams, J. W., Riley, J., Moley, K., and Latham, K. E. (2003). Somatic cell-like features of cloned mouse embryos prepared with cultured myoblast nuclei. *Biol. Reprod.* **69,** 48–56.

Gao, S., Chung, Y. G., Parseghian, M. H., King, G. J., Adashi, E. Y., and Latham, K. E. (2004). Rapid H1 linker histone transitions following fertilization or somatic cell nuclear transfer: Evidence for a uniform developmental program in mice. *Dev. Biol.* **266,** 62–75.

Gao, S., Han, Z., Kihara, M., Adashi, E., and Latham, K. E. (2005). Protease inhibitor MG132 in cloning: No end to the nightmare. *Trends Biotechnol.* **23,** 66–68.

Gardner, R. L. (1997). The early blastocyst is bilaterally symmetrical and its axis of symmetry is aligned with the animal-vegetal axis of the zygote in the mouse. *Development* **124,** 289–301.

Gardner, R. L. (2001). Specification of embryonic axes begins before cleavage in normal mouse development. *Development* **128,** 839–847.

Gäreskog, M., Cederberg, J., Eriksson, U. J., and Wentzel, P. (2007). Maternal diabetes *in vivo* and high glucose concentration *in vitro* increases apoptosis in rat embryos. *Reprod. Toxicol.* **23,** 63–74.

Gibson, T. C., Kubisch, H. M., and Brenner, C. A. (2005). Mitochondrial DNA deletions in rhesus macaque oocytes and embryos. *Mol. Hum. Reprod.* **11,** 785–789.

Gilchrist, R. B., Ritter, L. J., and Armstrong, D. T. (2001). Mouse oocyte mitogenic activity is developmentally coordinated throughout folliculogenesis and meiotic maturation. *Dev. Biol.* **240,** 289–298.

Gilchrist, R. B., Morrissey, M. P., Ritter, L. J., and Armstrong, D. T. (2003). Comparison of oocyte factors and transforming growth factor-beta in the regulation of DNA synthesis in bovine granulosa cells. *Mol. Cell. Endocrinol.* **201,** 87–95.

Giles, R. E., Blanc, H., Cann, H. M., and Wallace, D. C. (1980). Maternal inheritance of human mitochondrial DNA. *Proc. Natl. Acad. Sci. USA* **77,** 6715–6719.

Gingras, A. C., Raught, B., and Sonenberg, N. (1999). eIF4 initiation factors: Effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu. Rev. Biochem.* **68,** 913–963.

Ginsburg, M., Snow, M. H., and McLaren, A. (1990). Primordial germ cells in the mouse embryo during gastrulation. *Development* **110,** 521–528.

Giraldez, A. J., Mishima, Y., Rihel, J., Grocock, R. J., Van Dongen, S., Inoue, K., Enright, A. J., and Schier, A. F. (2006). Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science* **312,** 75–79.

Glabowski, W., Kurzawa, R., Wiszniewska, B., Baczkowski, T., Marchlewicz, M., and Brelik, P. (2005). Growth factors effects on preimplantation development of mouse embryos exposed to tumor necrosis factor alpha. *Reprod. Biol.* **5,** 83–99.

Godfrey, K. M., Forrester, T., Barker, D. J., Jackson, A. A., Landman, J. P., Hall, J. S., Cox, V., and Osmond, C. (1994). Maternal nutritional status in pregnancy and blood pressure in childhood. *Br. J. Obstet. Gynaecol.* **101,** 398–403.

Gonzales, D. S., Pinheiro, J. C., and Bavister, B. D. (1995). Prediction of the developmental potential of hamster embryos *in vitro* by precise timing of the third cell cycle. *J. Reprod. Fertil.* **105,** 1–8.

Good, P. J., Abler, L., Herring, D., and Sheets, M. D. (2004). *Xenopus* embryonic poly(A) binding protein 2 (ePABP2) defines a new family of cytoplasmic poly(A) binding proteins expressed during the early stages of vertebrate development. *Genesis* **38,** 166–175.

Gosden, R. G., and Bownes, M. (1995). Cellular and molecular aspects of oocyte development. *In* "Reviews in Human Reproduction" (J. G. Grudzinskas and J. L. Yovich, Eds.), pp. 23–53. Cambridge University Press, Gametes. The Oocyte, Cambridge.

Grainger, J. L., and Winkler, M. M. (1987). Fertilization triggers unmasking of maternal mRNAs in sea urchin eggs. *Mol. Cell Biol.* **7,** 3947–3954.

Green, D. R., and Reed, J. C. (1998). Mitochondria and Apoptosis. *Science* **281,** 1309–1312.

Grivna, S. T., Pyhtila, B., and Lin, H. (2006). MIWI associates with translational machinery and PIWI-interacting RNAs (piRNAs) in regulating spermatogenesis. *Proc. Natl. Acad. Sci. USA* **103,** 13415–13420.

Groisman, I., Jung, M. Y., Sarkissian, M., Cao, Q., and Richter, J. D. (2002). Translational control of the embryonic cell cycle. *Cell* **109,** 473–483.

Gyllensten, U., Wharton, D., Josefsson, A., and Wilson, A. C. (1991). Paternal inheritance of mitochondrial DNA in mice. *Nature* **352,** 255–267.

Haggeness, M. H., Simon, M., and Singer, S. J. (1978). Association of mitochondria with microtubules in cultured cells. *Proc. Natl. Acad. Sci. USA* **75,** 3863–3866.

Haghighat, A., Mader, S., Pause, A., and Sonenberg, N. (1995). Repression of cap-dependent translation by 4E-binding protein 1: Competition with p220 for binding to eukaryotic initiation factor-4E. *EMBO J.* **14,** 5701–5709.

Hake, L. E., and Richter, J. D. (1994). CPEB is a specificity factor that mediates cytoplasmic polyadenylation during *Xenopus* oocyte maturation. *Cell* **79,** 617–627.

Hales, K. G. (2004). The machinery of mitochondrial fusion, division, and distribution, and emerging connections to apoptosis. *Mitochondrion* **4,** 285–308.

Hammond, J. M., Hersey, R. M., Walega, M. A., and Weisz, J. (1986). Catecholestrogen production by porcine ovarian cells. *Endocrinology* **118,** 2292–2299.

Han, Z., Chung, Y. G., Gao, S., and Latham, K. E. (2005). Maternal factors controlling blastomere fragmentation in early mouse embryos. *Biol. Reprod.* **72,** 612–618.

Hara, K. T., Oda, S., Naito, K., Nagata, M., Schultz, R. M., and Aoki, F. (2005). Cyclin A2-CDK2 regulates embryonic gene activation in 1-cell mouse embryos. *Dev. Biol.* **286,** 102–113.

Hardy, K. (1999). Apoptosis in the human embryo. *Rev. Reprod.* **4,** 125–134.

Hardy, K., Spanos, S., Becker, D., Iannelli, P., Winston, R. M. L., and Stark, J. (1999). From cell death to embryo arrest: Mathematical models of human preimplantation embryo development. *Proc. Natl. Acad. Sci. USA* **98,** 1655–1660.

Harris, B., and Dure, L., 3rd. (1978). Developmental regulation in cotton seed germination: Polyadenylation of stored messenger RNA. *Biochemistry* **17,** 3250–3256.

Harvey, A. J., Gibson, T. C., Quebedeaux, T. M., and Brenner, C. A. (2007). Impact of assisted reproductive technologies: A mitochondrial perspective of cytoplasmic transplantation. *Curr. Top. Dev. Biol.* **77,** 229–249.

Hassanin, A., Kuwahara, S., Nurhidayat, Y., Tsukamoto, Y., Ogawa, K., Hiramatsu, K., and Sasaki, F. (2002). Gonadosomatic index and testis morphology of common carp (*Cyprinus carpio*) in rivers contaminated with estrogenic chemicals. *J. Vet. Med. Sci.* **64,** 921–926.

Hassold, T., and Hunt, P. (2001). To err (meiotically) is human: The genesis of human aneuploidy. *Nat. Rev. Genet.* **2,** 280–291.

Hawes, S. M., Chung, Y. G., and Latham, K. E. (2001). Genetic and epigenetic factors affecting blastomere fragmentation in preimplantation stage mouse embryos. *Biol. Reprod.* **65,** 1050–1056.

Hay, B., Jan, L. Y., and Jan, Y. N. (1990). Localization of vasa, a component of *Drosophila* polar granules, in maternal-effect mutants that alter embryonic anteroposterior polarity. *Development* **109,** 425–433.

Heilig, C., Brosius, F., Siu, B., Concepcion, L., Mortensen, R., Heilig, K., Zhu, M., Weldon, R., Wu, G., and Conner, D. (2003). Implications of glucose transporter protein type 1 (GLUT1)-haplodeficiency in embryonic stem cells for their survival in response to hypoxic stress. *Am. J. Pathol.* **163,** 1873–1885.

Henery, C. C., Miranda, M., Wiekowski, M., Wilmut, I., and DePamphilis, M. L. (1995). Repression of gene expression at the beginning of mouse development. *Dev. Biol.* **169,** 448–460.

Herbst, A. L., Ulfelder, H., and Poskanzer, D. C. (1971). Adenocarcinoma of the vagina: Association of maternal stilbestrol therapy with tumor appearance in young women. *N. Engl. J. Med.* **284,** 878–881.

Hernandez, G., and Vazquez-Pianzola, P. (2005). Functional diversity of the eukaryotic translation initiation factors belonging to eIF4 families. *Mech. Dev.* **122,** 865–876.

Hiendleder, S., Schmutz, S. M., Erhardt, G., Green, R. D., and Plante, Y. (1999). Trans-mitochondrial differences and varying levels of heteroplasmy in nuclear transfer cloned cattle. *Mol. Reprod. Dev.* **1,** 24–31.

Hiiragi, T., and Solter, D. (2004). First cleavage plane of the mouse egg is not predetermined but defined by the topology of the two apposing pronuclei. *Nature* **430,** 360–364.

Hiiragi, T., and Solter, D. (2005). Mechanism of first cleavage specification in the mouse egg: Is our body plan set at day 0? *Cell Cycle* **4,** 661–664.

Hiiragi, T., and Solter, D. (2006). Fatal flaws in the case for prepatterning in the mouse egg. *Reprod. Biomed. Online* **12,** 150–152.

Hird, S. N., Paulsen, J. E., and Strome, S. (1996). Segregation of germ granules in living Caenorhabditis elegans embryos: Cell-type-specific mechanisms for cytoplasmic localisation. *Development* **122,** 1303–1312.

Hodgman, R., Tay, J., Mendez, R., and Richter, J. D. (2001). CPEB phosphorylation and cytoplasmic polyadenylation are catalyzed by the kinase IAK1/Eg2 in maturing mouse oocytes. *Development* **128,** 2815–2822.

Hogan, B. (2001). Primordial germ cells as stem cells. *In* "Stem Cell Biology" (D. R. Marshak, D. K. Gardner, and D. Gottlieb, Eds.), pp. 189–204. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Hosokawa, M., Shoji, M., Kitamura, K., Tanaka, T., Noce, T., Chuma, S., and Nakatsuji, N. (2007). Tudor-related proteins TDRD1/MTR-1, TDRD6 and TDRD7/TRAP: Domain composition, intracellular localization, and function in male germ cells in mice. *Dev. Biol.* **301,** 38–52.

Houtchens, K., and Lyttle, T. W. (2003). Responder (Rsp) alleles in the segregation distorter (SD) system of meiotic drive in *Drosophila* may represent a complex family of satellite repeat sequences. *Genetica* **117,** 291–302.

Howard, E. L., Charlesworth, A., Welk, J., and MacNicol, A. M. (1999). The mitogen-activated protein kinase signaling pathway stimulates mos mRNA cytoplasmic polyadenylation during *Xenopus* oocyte maturation. *Mol. Cell Biol.* **19,** 1990–1999.

Howell, C. Y., Bestor, T. H., Ding, F., Latham, K. E., Mertineit, C., Trasler, J. M., and Chaillet, J. R. (2001). Genomic imprinting disrupted by a maternal effect mutation in the *Dnmt1* gene. *Cell* **104,** 829–838.

Huang, W. I., Hansen, L. J., Merrick, W. C., and Jagus, R. (1987). Inhibitor of eukaryotic initiation factor 4F activity in unfertilized sea urchin eggs. *Proc. Natl. Acad. Sci. USA* **84,** 6359–6363.

Huarte, J., Belin, D., Vassalli, A., Strickland, S., and Vassalli, J. D. (1987). Meiotic maturation of mouse oocytes triggers the translation and polyadenylation of dormant tissue-type plasminogen activator mRNA. *Genes Dev.* **1,** 1201–1211.

Hunt, P. A., Koehler, K. E., Susiarjo, M., Hodges, C. A., Ilagan, A., Voigt, R. C., Thomas, S., Thomas, B. F., and Hassold, T. J. (2003). Bisphenol, a exposure causes meiotic aneuploidy in the female mouse. *Curr. Biol.* **13,** 546–553.

Hunter, M. G. (1998). Follicular factors regulating oocyte maturation and quality. *Hum. Fertil.* **1,** 69–74.

Hunter, M. G., Brankin, V., Quinn, R. L., Ferguson, E. M., Edwards, S. A., and Ashworth, C. J. (2005). Oocyte-somatic cell-endocrine interactions in pigs. *Domest. Anim. Endocrinol.* **29,** 371–384.

Hutchinson, C. A., Newbold, J. E., and Potter, S. S. (1974). Maternal inheritance of mammalian mitochondrial DNA. *Nature* **251,** 536–538.

Illmensee, K., and Mahowald, A. P. (1974). Transplantation of posterior polar plasm in *Drosophila*. Induction of germ cells at the anterior pole of the egg. *Proc. Natl. Acad. Sci. USA* **71,** 1016–1020.

Illmensee, K., Kaskar, K., and Zavos, P. M. (2006). In-vitro developmental potential of individual mouse blastomeres cultured with and without zona pellucida: Future implications for human assisted reproduction. *Reprod. Biomed. Online* **13,** 284–294.

Ireland, J. J., and Richards, J. S. (1978). Acute effects of estradiol and follicle-stimulating hormone on specific binding of human [125I]iodofollicle-stimulating hormone to rat ovarian granulosa cells *in vivo* and *in vitro*. *Endocrinology* **102,** 876–883.

Irish, V., Lehmann, R., and Akam, M. (1989). The *Drosophila* posterior-group gene nanos functions by repressing hunchback activity. *Nature* **338,** 646–648.

Jagus, R., Huang, W. I., Hansen, L. J., and Wilson, M. A. (1992). Changes in rates of protein synthesis and eukaryotic initiation factor-4 inhibitory activity in cell-free translation systems of sea urchin eggs and early cleavage stage embryos. *J. Biol. Chem.* **267,** 15530–15536.

Jansen, R. P. (2000). Germline passage of mitochondria: Quantitative considerations and possible embryological sequelae. *Hum. Reprod.* **15,** 112–128.

Johnson, T. R., Jr., and Peterson, E. P. (1979). Gonadotropin-induced pregnancy following "premature ovarian failure." *Fertil. Steril.* **31,** 351–352.

Johnson, M. P., Childs, M. D., Robichaux, A. G., 3rd, Isada, N. B., Pryde, P. G., Koppitch, F. C., 3rd, and Evans, M. I. (1993). Viable pregnancies after diagnosis of trisomy 16 by CVS: Lethal aneuploidy compartmentalized to the trophoblast. *Fetal. Diagn. Ther.* **8,** 102–108.

Johnson, W. H., Loskutoff, N. M., Plante, Y., and Betteridge, K. J. (1995). Production of four identical calves by the separation of blastomeres from an *in vitro* derived 4-cell embryo. *Vet. Rec.* **137,** 15–16.

Jorgez, C. J., Klysik, M., Jamin, S. P., Behringer, R. R., and Matzuk, M. M. (2004). Granulosa cell-specific inactivation of follistatin causes female fertility defects. *Mol. Endocrinol.* **18,** 953–967.

Jud, M., Razelun, J., Bickel, J., Czerwinski, M., and Schisa, J. A. (2007). Conservation of large foci formation in arrested oocytes of Caenorhabditis nematodes. *Dev. Genes Evol.* **217,** 221–226.

Juengel, J. L., and McNatty, K. P. (2005). The role of proteins of the transforming growth factor-beta superfamily in the intraovarian regulation of follicular development. *Hum. Reprod. Update* **11,** 143–160.

Jurisicova, A., Varmuza, S., and Casper, R. F. (1995). Involvement of programmed cell death in preimplantation embryo demise. *Hum. Reprod. Update* **1,** 558–566.

Jurisicova, A., Varmuza, S., and Casper, R. F. (1996). Programmed cell death and human embryo fragmentation. *Mol. Hum. Reprod.* **2,** 93–98.

Jurisicova, A., Latham, K. E., Casper, R. F., and Varmuza, S. L. (1998). Expression and regulation of genes associated with cell death during murine preimplantation embryo development. *Mol. Reprod. Dev.* **51,** 243–253.

Kaneda, H., Hayashi, J. I., Takahama, S., Taya, C., LIndahl, K. F., and Yonekawa, H. (1995). Elimination of paternal mitochondria in intraspecific crosses during early embryogenesis. *Proc. Natl. Acad. Sci. USA* **92,** 4542–4546.

Kaneko, K. J., Cullinan, E. B., Latham, K. E., and DePamphilis, M. L. (1997). Transcription factor mTEAD-2 is selectively expressed at the beginning of zygotic gene expression in the mouse. *Development* **124,** 1963–1973.

Kaneko, K. J., Rein, T., Guo, Z. S., Latham, K., and DePamphilis, M. L. (2004). DNA methylation may restrict but does not determine differential gene expression at the Sgy/Tead2 locus during mouse development. *Mol. Cell. Biol.* **24,** 1968–1982.

Kang, I. J., Yokota, H., Oshima, Y., Tsuruda, Y., Oe, T., Imada, N., Tadokoro, H., and Honjo, T. (2002). Effects of bisphenol A on the reproduction of Japanese medaka (*Oryzias latipes*). *Environ. Toxicol. Chem.* **21,** 2394–2400.

Karaiskou, A., Perez, L. H., Ferby, I., Ozon, R., Jessus, C., and Nebreda, A. R. (2001). Differential regulation of Cdc2 and Cdk2 by RINGO and cyclins. *J. Biol. Chem.* **276,** 36028–36034.

Kawahata, H., Ohta, H., Inoue, M., and Suzuki, A. (2004). Endocrine disrupter nonylphenol and bisphenol A contamination in Okinawa and Ishigaki Islands, Japan—Within coral reefs, and adjacent river mouths. *Chemosphere* **55,** 1519–1527.

Keady, B. T., Kuo, P., Martinez, S. E., Yuan, L., and Hake, L. E. (2007). MAPK interacts with XGef and is required for CPEB activation during meiosis in *Xenopus* oocytes. *J. Cell Sci.* **120,** 1093–1103.

Keim, A. L., Chi, M. M., and Moley, K. H. (2001). Hyperglycemia-induced apoptotic cell death in the mouse blastocyst is dependent on expression of p53. *Mol. Reprod. Dev.* **60,** 214–224.

Kelly, J. M., Kleemann, D. O., and Walker, S. K. (2005). The effect of nutrition during pregnancy on the in vitro production of embryos from resulting lambs. *Theriogenology* **63,** 2020–2031.

Kile, B. T., Schulman, B. A., Alexander, W. S., Nicola, N. A., Martin, H. M., and Hilton, D. J. (2002). The SOCS box: A tale of destruction and degradation. *Trends Biochem. Sci.* **27,** 235–241.

Kirschner, M., and Mitchison, T. (1986). Beyond self-assembly: From microtubules to morphogenesis. *Cell* **45,** 329–342.

Kline, D., and Kline, J. T. (1992). Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Dev. Biol.* **149,** 80–89.

Kondo, R., Satta, Y., Matsuura, E. T., Ishiwa, H., Takahata, N., and Chigusa, S. I. (1990). Incomplete maternal transmission of mitochondrial DNA in *Drosophila*. *Genetics* **126,** 657–663.

Kono, T., Jones, K. T., Bos-Mikich, A., Whittingham, D. G., and Carroll, J. (1996). A cell cycle-associated change in Ca2 releasing activity leads to the generation of Ca2 transients in mouse embryos during the first mitotic division. *J. Cell Biol.* **132,** 915–923.

Krishnan, A. V., Starhis, P., Permuth, S. F., Tokes, L., and Feldman, D. (1993). Bisphenol-A: An estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology* **132,** 2279–2286.

Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Häggblad, J., Nilsson, S., and Gustafsson, J. A. (1997). Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and ß. *Endocrinology* **138,** 863–870.

Kuiper, G. G. J. M., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., van der Saag, P. T., van der Burg, B., and Gustafsson, J. A. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor *β*. *Endocrinology* **139,** 4252–4263.

Kuligowski, J., Ferrand, M., and Chenou, E. (1991). Stored mRNA in early embryos of a fern Marsilea vestita: A paternal and maternal origin. *Mol. Reprod. Dev.* **30,** 27–33.

Kumar, T. R., Wang, Y., Lu, N., and Matzuk, M. M. (1997). Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nat. Genet.* **15,** 201–204.

Kurzawa, R., Glabowski, W., Baczkowski, T., Wiszniewska, B., and Marchlewicz, M. (2004). Growth factors protect in vitro cultured embryos from the consequences of oxidative stress. *Zygote* **12,** 231–240.

Kusano, A., Staber, C., and Ganetzky, B. (2001). Nuclear mislocalization of enzymatically active RanGAP causes segregation distortion in *Drosophila*. *Dev .Cell* **1,** 351–361.

Kwong, W. Y., Wild, A. E., Roberts, P., Willis, A. C., and Fleming, T. P. (2000). Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. *Development* **127,** 4195–4202.

Kwong, W. Y., Miller, D. J., Ursell, E., Wild, A. E., Wilkins, A. P., Osmond, C., Anthony, F. W., and Fleming, T. P. (2006). Imprinted gene expression in the rat embryo-fetal axis is altered in response to peri-conceptional maternal low protein diet. *Reproduction* **132,** 265–277.

Kwong, W. Y., Miller, D. J., Wilkins, A. P., Dear, M. S., Wright, J. N., Osmond, C., Zhang, J., and Fleming, T. P. (2007). Maternal low protein diet restricted to the preimplantation period induces a gender-specific change on hepatic gene expression in rat fetuses. *Mol. Reprod. Dev.* **74,** 48–56.

Ladomery, M., Wade, E., and Sommerville, J. (1997). Xp54, the *Xenopus* homologue of human RNA helicase p54, is an integral component of stored mRNP particles in oocytes. *Nucleic Acids Res.* **25,** 965–973.

Lahnsteiner, F., Berger, B., Kletzl, M., and Weismann, T. (2005). Effect of bisphenol A on maturation and quality of semen and eggs in the brown trout, *Salmo trutta f. fario*. *Aquat. Toxicol.* **75,** 213–224.

Lane, M., and Gardner, D. K. (1997). Differential regulation of mouse embryo development and viability by amino acids. *J. Reprod. Fertil.* **109,** 153–164.

Lasko, P. F., and Ashburner, M. (1990). Posterior localization of vasa protein correlates with, but is not sufficient for, pole cell development. *Genes Dev.* **4,** 905–921.

Latham, K. E. (1994). Strain-specific differences in mouse oocytes and their contributions to epigenetic inheritance. *Development* **120,** 3419–3426.

Latham, K. E. (1999). Mechanisms and control of embryonic genome activation in mammalian embryos. *Int. Rev. Cytol.* **193,** 71–124.

Latham, K. E., and Sapienza, C. (1998). Localization of genes encoding egg modifiers of paternal genome function to mouse chromosomes one and two. *Development* **125,** 929–935.

Latham, K. E., and Schultz, R. M. (2001). Embryonic genome activation. *Front. Biosci.* **6,** D748–D759.

Latham, K. E., and Solter, D. (1991). Effect of egg composition on the developmental capacity of androgenetic mouse embryos. *Development* **113,** 561–568.

Latham, K. E., Garrels, J. I., Chang, C., and Solter, D. (1991). Quantitative analysis of protein synthesis in mouse embryos. I. Extensive reprogramming at the one- and two-cell stages. *Development* **112,** 921–932.

Latham, K. E., Bautista, F. D., Hirao, Y., O'Brien, M. J., and Eppig, J. J. (1999). Comparison of protein synthesis patterns in mouse cumulus cells and mural granulosa cells: Effects of follicle-stimulating hormone and insulin on granulosa cell differentiation *in vitro*. *Biol. Reprod.* **61,** 482–492.

Latham, K. E., Wigglesworth, K., McMenamin, M., and Eppig, J. J. (2004). Stage-dependent effects of oocytes and growth differentiation factor 9 on mouse granulosa cell development: Advance programming and subsequent control of the transition from preantral secondary follicles to early antral tertiary follicles. *Biol. Reprod.* **70,** 1253–1262.

Lattanzi, M. L., Santos, C. B., Mudry, M. D., and Baranao, J. L. (2003). Exposure of bovine oocytes to the endogenous metabolite 2-methoxyestradiol during *in vitro* maturation inhibits early embryonic development. *Biol. Reprod.* **69,** 1793–1800.

Lau, C., and Rogers, J. M. (2004). Embryonic and fetal programming of physiological disorders in adulthood. *Birth Defects Res. C Embryo Today* **72,** 300–312.
Lemasters, J. J. (2005). Dying a thousand deaths: Redundant pathways from different organelles to apoptosis and necrosis. *Gastroenterology* **129,** 351–360.
Lepikhov, K., and Walter, J. (2004). Differential dynamics of histone H3 methylation at positions K4 and K9 in the mouse zygote. *BMC Dev. Biol.* **4,** 12.
Levron, J., Willadsen, S., Bertoli, M., and Cohen, J. (1996). The development of mouse zygotes after fusion with synchronous and asynchronous cytoplasm. *Hum. Reprod.* **11,** 1287–1292.
Lewis, N. E., and Riossant, J. (1982). Mechanism of size regulation in mouse embryo aggregates. *J. Embryol. Exp. Morphol.* **72,** 169–181.
Li, R., Norman, R. J., Armstrong, D. T., and Gilchrist, R. B. (2000). Oocyte secreted factors determine functional differences between bovine mural granulosa cells and cumulus cells. *Biol. Reprod.* **63,** 839–845.
Liao, W. X., Roy, A. C., and Ng, S. C. (2000). Preliminary investigation of follistatin gene mutations in women with polycystic ovary syndrome. *Mol. Hum. Reprod.* **6,** 587–590.
Lieberfarb, M. E., Chu, T., Wreden, C., Theurkauf, W., Gergen, J. P., and Strickland, S. (1996). Mutations that perturb poly(A)-dependent maternal mRNA activation block the initiation of development. *Development* **122,** 579–588.
Lin, T. C., Yen, J. M., Gong, K. B., Hsu, T. T., and Chen, L. R. (2003). IGF-1/IGFBP-1 increases blastocyst formation and total blastocyst cell number in mouse embryo culture and facilitates the establishment of a stem-cell line. *BMC Cell Biol.* **4,** 14.
Liu, L., and Keefe, D. L. (2000). Cytoplasm mediates both development and oxidation-induced apoptotic cell death in mouse zygotes. *Biol. Reprod.* **62,** 1828–1834.
Lloyd, R. E., Lee, J. H., Alberio, R., Bowles, E. J., Ramalho-Santos, J., Campbell, K. H., and St. John, J. C. (2006). Aberrant nucleo-cytoplasmic cross-talk results in donor cell mtDNA persistence in cloned embryos. *Genetics* **172,** 2515–2527.
Loi, P., Ledda, S., Fulka, J., Jr., Cappai, P., and Moor, R. M. (1998). Development of parthenogenetic and cloned ovine embryos: Effect of activation protocols. *Biol. Reprod.* **58,** 1177–1187.
Lonergan, P., Khatir, H., Piumi, F., Rieger, D., Humblot, P., and Boland, M. P. (1999). Effect of time interval from insemination to first cleavage on the developmental characteristics, sex ratio and pregnancy rate after transfer of bovine embryos. *J. Reprod. Fertil.* **117,** 159–167.
Louvet-Vallée, S., Vinot, S., and Maro, B. (2005). Mitotic spindles and cleavage planes are oriented randomly in the two-cell mouse embryo. *Curr. Biol.* **15,** 464–469.
Lublin, A. L., and Evans, T. C. (2007). The RNA-binding proteins PUF-5, PUF-6, and PUF-7 reveal multiple systems for maternal mRNA regulation during C. elegans oogenesis. *Dev. Biol.* **303,** 635–649.
Lundin, K., Bergh, C., and Hardarson, T. (2001). Early embryo cleavage is a strong indicator of embryo quality in human IVF. *Hum. Reprod.* **16,** 2652–2657.
Lyttle, T. W. (1991). Segregation distorters. *Annu. Rev. Genet.* **25,** 511–557.
Mader, S., Lee, H., Pause, A., and Sonenberg, N. (1995). The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4 gamma and the translational repressors 4E-binding proteins. *Mol. Cell. Biol.* **15,** 4990–4997.
Magli, M. C., Gianaroli, L., and Ferraretti, A. P. (2001). Chromosomal abnormalities in embryos. *Mol. Cell. Endocrinol.* **183,** S29–S34.
Mailhes, J. B., Carabatsos, M. J., Young, D., London, S. N., Bell, M., and Albertini, D. F. (1999). Taxol-induced meiotic maturation delay, spindle defects, and aneuploidy in mouse oocytes and zygotes. *Mutat. Res.* **423,** 79–90.
Mandich, A., Bottero, S., Benfenati, E., Cevasco, A., Erratico, C., Maggioni, S., Massari, A., Pedemonte, F., and Viganò, L. (2007). In vivo exposure of carp to graded concentrations of bisphenol A. *Gen. Comp. Endocrinol.* **153,** 15–24.

Mann, J. R. (1986). DDK egg-foreign sperm incompatibility in mice is not between the pronuclei. *J. Reprod. Fertil.* **76,** 779–781.

Maro, B., Howlett, S. K., and Webb, M. (1985). Non-spindle microtubule organizing centers in metaphase II-arrested mouse oocytes. *J. Cell Biol.* **101,** 1665–1672.

Matsumoto, K., Miyake, M., Utsumi, K., and Iritani, A. (1989). Production of identical twins by separating two-cell rat embryos. *Gamete Res.* **22,** 257–263.

Matsumoto, K., Tanaka, K. J., Aoki, K., Sameshima, M., and Tsujimoto, M. (2003). Visualization of the reconstituted FRGY2-mRNA complexes by electron microscopy. *Biochem. Biophys. Res. Commun.* **306,** 53–58.

Matten, W. T., Copeland, T. D., Ahn, N. G., and Vande Woude, G. F. (1996). Positive feedback between MAP kinase and Mos during *Xenopus* oocyte maturation. *Dev. Biol.* **179,** 485–492.

Matthews, J., and Gustafsson, J. A. (2003). Estrogen signaling: A subtle balance between ER alpha and ER beta. *Mol. Interv.* **3,** 281–292.

Matzuk, M. M., Burns, K. H., Viveiros, M. M., and Eppig, J. J. (2002). Intercellular communication in the mammalian ovary: Oocytes carry the conversation. *Science* **296,** 2178–2180.

Mazia, D. (1987). The chromosome cycle and the centrosome cycle in the mitotic cycle. *Int. Rev. Cytol.* **100,** 49–92.

Mazumder, B., Seshadri, V., Imataka, H., Sonenberg, N., and Fox, P. L. (2001). Translational silencing of ceruloplasmin requires the essential elements of mRNA circularization: Poly(A) tail, poly(A)-binding protein, and eukaryotic translation initiation factor 4G. *Mol. Cell Biol.* **21,** 6440–6449.

McAndrew, C. W., Gastwirt, R. F., Meyer, A. N., Porter, L. A., and Donoghue, D. J. (2007). Spy1 enhances phosphorylation and degradation of the cell cycle inhibitor p27. *Cell Cycle* **6,** 1937–1945.

McNatty, K. P., Juengel, J. L., Wilson, T., Galloway, S. M., Davis, G. H., Hudson, N. L., Moeller, C. L., Cranfield, M., Reader, K. L., and Laitinen, M. P. (2003). Oocyte-derived growth factors and ovulation rate in sheep. *Reproduction* **61,** 339–351.

Megosh, H. B., Cox, D. N., Campbell, C., and Lin, H. (2006). The role of PIWI and the miRNA machinery in *Drosophila* germline determination. *Curr. Biol.* **16,** 1884–1894.

Meirelles, F., and Smith, L. (1997). Mitochondrial genotype segregation in a mouse heteroplasmic lineage produced by embryonic karyoplast transplantation. *Genetics* **145,** 445–451.

Meirelles, F., and Smith, L. (1998). Mitochondrial genotype segregation during preimplantation development in mouse heteroplasmic embryos. *Genetics* **148,** 877–884.

Meirelles, F. V., Bordignon, V., Watanabe, Y., Watanabe, M., Dayan, A., Lobo, R. B., Garcia, J. M., and Smith, L. C. (2001). Complete replacement of the mitochondrial genotype in a Bos indicus calf reconstructed by nuclear transfer to a Bos taurus oocyte. *Genetics* **158,** 351–356.

Menasce, L. P., White, G. R., Harrison, C. J., and Boyle, J. M. (1993). Localization of the estrogen receptor locus (ESR) to chromosome 6q25.1 by FISH and a simple post-FISH banding technique. *Genomics* **17,** 263–265.

Mendez, R., Hake, L. E., Andresson, T., Littlepage, L. E., Ruderman, J. V., and Richter, J. D. (2000). Phosphorylation of CPE binding factor by Eg2 regulates translation of c-mos mRNA. *Nature* **404,** 302–307.

Mendez, R., Barnard, D., and Richter, J. D. (2002). Differential mRNA translation and meiotic progression require Cdc2-mediated CPEB destruction. *EMBO J.* **21,** 1833–1844.

Metcalfe, C. D., Metcalfe, T. L., Kiparissis, Y., Koenig, B. G., Khan, C., Hughes, R. J., Croley, T. R., March, R. E., and Potter, T. (2001). Estrogenic potency of chemicals

detected in sewage treatment plant effluents as determined by *in vitro* assays with Japanese medaka (Oryzias latipes). *Environ. Toxicol. Chem.* **20,** 297–308.
Micklem, D. R. (1995). mRNA localisation during development. *Dev. Biol.* **172,** 377–395.
Minshall, N., Thom, G., and Standart, N. (2001). A conserved role of a DEAD box helicase in mRNA masking. *RNA* **7,** 1728–1742.
Mintz, B., and Russell, E. S. (1957). Gene-induced embryological modifications of primordial germ cells in the mouse. *J. Exp. Zool.* **134,** 207–234.
Mitalipov, S. M., White, K. L., Farrar, V. R., Morrey, J., and Reed, W. A. (1999). Development of nuclear transfer and parthenogenetic rabbit embryos activated with inositol 1,4,5-trisphosphate. *Biol. Reprod.* **60,** 821–827.
Mitalipova, S. M., Yeomana, R. R., Kuoa, H.-C., and Wolf, D. P. (2002). Monozygotic twinning in rhesus monkeys by manipulation of *in vitro*-derived embryos. *Biol. Reprod.* **66,** 1449–1455.
Miyara, F., Han, Z., Gao, S., Vassena, R., and Latham, K. E. (2006). Non-equivalence of embryonic and somatic cell nuclei affecting spindle composition in clones. *Dev. Biol.* **289,** 206–217.
Miyazaki, S. (2006). Thirty years of calcium signals at fertilization. *Semin. Cell Dev. Biol.* **17,** 233–243.
Miyazaki, S., and Ito, M. (2006). Calcium signals for egg activation in mammals. *J. Pharmacol. Sci.* **100,** 545–552.
Moley, K. H. (1999). Diabetes and preimplantation events of embryogenesis. *Semin. Reprod. Endocrinol.* **17,** 137–151.
Moley, K. H., and Mueckler, M. M. (2000). Glucose transport and apoptosis. *Apoptosis.* **5,** 99–105.
Moley, K. H. (2001). Hyperglycemia and apoptosis: Mechanisms for congenital malformations and pregnancy loss in diabetic women. *Trends Endocrinol. Metab.* **12,** 78–82.
Moley, K. H., Chi, M. M., Manchester, J. K., McDougal, D. B., and Lowry, O. H. (1996). Alterations of intraembryonic metabolites in preimplantation mouse embryos exposed to elevated concentrations of glucose: A metabolic explanation for the developmental retardation seen in preimplantation embryos from diabetic animals. *Biol. Reprod.* **54,** 1209–1216.
Moley, K. H., Chi, M. M., Knudson, C. M., Korsmeyer, S. J., and Mueckler, M. M. (1998a). Hyperglycemia induces apoptosis in pre-implantation embryos through cell death effector pathways. *Nat. Med.* **4,** 1421–1424.
Moley, K. H., Chi, M. M., and Mueckler, M. M. (1998b). Maternal hyperglycemia alters glucose transport and utilization in mouse preimplantation embryos. *Am. J. Physiol.* **275,** E38–E47.
Moore, G. D., Kopf, G. S., and Schultz, R. M. (1993). Complete mouse egg activation in the absence of sperm by stimulation of an exogenous G protein-coupled receptor. *Dev. Biol.* **159,** 669–678.
Morita, Y., Perez, G. I., Maravei, D. V., Tilly, K. I., and Tilly, J. L. (1999). Targeted expression of Bcl-2 in mouse oocytes inhibits ovarian follicle atresia and prevents spontaneous and chemotherapy-induced oocyte apoptosis *in vitro*. *Mol. Endocrinol.* **13,** 841–850.
Morita, Y., Perez, G. I., Paris, F., Miranda, S. R., Ehleiter, D., Haimovitz-Friedman, A., Fuks, Z., Xie, Z., Reed, J. C., Schuchman, E. H., Kolesnick, R. N., and Tilly, J. L. (2000). Oocyte apoptosis is suppressed by disruption of the acid sphingomyelinase gene or by sphingosine-1-phosphate therapy. *Nat. Med.* **6,** 1109–1114.
Mosselman, S., Polman, J., and Dijkema, R. (1996). ER beta: Identification and characterization of a novel human estrogen receptor. *FEBS Lett.* **392,** 49–53.
Motosugi, N., Bauer, T., Polanski, Z., Solter, D., and Hiiragi, T. (2005). Polarity of the mouse embryo is established at blastocyst and is not prepatterned. *Genes Dev.* **19,** 1081–1092.

Motta, P. M., Nottola, S. A., Familiari, G., Macchiarelli, G., Vizza, E., and Correr, S. (1995). Ultrastructure of human reproduction from folliculogenesis to early embryo development. A review. *Ital. J. Anat. Embryol.* **100,** 9–72.

Motta, P. M., Nottola, S. A., Familiari, G., Makabe, S., Stallone, T., and Macchiarelli, G. (2003). Morphodynamics of the follicular–luteal complex during early ovarian development and reproductive life. *Int. Rev. Cytol.* **223,** 177–288.

Mtango, N. R., and Latham, K. E. (2007). Ubiquitin proteasome pathway gene expression varies in rhesus monkey oocytes and embryos of different developmental potential. *Physiol. Genomics*. **31,** 1–14.

Mtango, N. R., and Latham, K. E. (2008). Differential expression of cell cycle genes in Rhesus Monkey oocytes and embryos of different developmental potentials. *Biol. Reprod*. **78,** 254–256.

Muggleton-Harris, A. L., and Brown, J. J. (1988). Cytoplasmic factors influence mitochondrial reorganization and resumption of cleavage during culture of early mouse embryos. *Hum. Reprod*. **3,** 1020–1028.

Munne, S., Lee, A., Rosenwaks, Z., Grifo, J., and Cohen, J. (1993). Diagnosis of major chromosome aneuploidies in human preimplantation embryos. *Hum. Reprod*. **8,** 2185–2191.

Munne, S., Velilla, E., Colls, P., Garcia Bermudez, M., Vemuri, M. C., Steuerwald, N., Garrisi, J., and Cohen, J. (2005). Self-correction of chromosomally abnormal embryos in culture and implications for stem cell production. *Fertil. Steril*. **84,** 1328–1334.

Murchison, E. P., Stein, P., Xuan, Z., Pan, H., Zhang, M. Q., Schultz, R. M., and Hannon, G. J. (2007). Critical roles for Dicer in the female germline. *Genes Dev*. **21,** 682–693.

Murray, M. T., Schiller, D. L., and Franke, W. W. (1992). Sequence analysis of cytoplasmic mRNA-binding proteins of *Xenopus* oocytes identifies a family of RNA-binding proteins. *PNAS* **89,** 11–15.

Nakada, K., and Mizuno, J. (1998). Intracellular calcium responses in bovine oocytes induced by spermatozoa and by reagents. *Theriogenology* **50,** 269–282.

Nakagawa, K., Yamano, S., Nakasaka, H., Hinokio, K., Yoshizawa, M., and Aono, T. A. (2000). Combination of calcium ionophore and puromycin effectively produces human parthenogenones with one haploid pronucleus. *Zygote* **9,** 83–88.

Nakahara, K., Kim, K., Sciulli, C., Dowd, S. R., Minden, J. S., and Carthew, R. W. (2005). Targets of microRNA regulation in the *Drosophila* oocyte proteome. *Proc. Natl. Acad. Sci. USA* **102,** 12023–12028.

Nelson, L. M., Kimzey, L. M., White, B. J., and Merriam, G. R. (1992). Gonadotropin suppression for the treatment of karyotypically normal spontaneous premature ovarian failure: A controlled trial. *Fertil. Steril*. **57,** 50–55.

Nishi, Y., and Lin, R. (2005). DYRK2 and GSK-3 phosphorylate and promote the timely degradation of OMA-1, a key regulator of the oocyte-to-embryo transition in C. elegans. *Dev. Biol*. **288,** 139–149.

Nishimura, Y., Yoshinari, T., Naruse, K., Yamada, T., Sumi, K., Mitani, H., Higashiyama, T., and Kuroiwa, T. (2006). Active digestion of sperm mitochondrial DNA in single living sperm revealed by optical tweezers. *Proc. Natl. Acad. Sci. USA* **103,** 1382–1387.

Nishizuka, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature* **308,** 693–698.

Nolen, L. D., Gao, S., Han, Z., Mann, M. R., Gie Chung, Y., Otte, A. P., Bartolomei, M. S., and Latham, K. E. (2005). X chromosome reactivation and regulation in cloned embryos. *Dev. Biol*. **279,** 525–540.

O'Brien, M. J., Pendola, J. K., and Eppig, J. J. (2003). A revised protocol for *in vitro* development of mouse oocytes from primordial follicles dramatically improves their developmental competence. *Biol. Reprod*. **68,** 1682–1686.

Oh, B., Hwang, S. Y., Solter, D., and Knowles, B. B. (1997). Spindlin, a major maternal transcript expressed in the mouse during the transition from oocyte to embryo. *Development* **124,** 493–503.

Oh, B., Hampl, A., Eppig, J. J., Solter, D., and Knowles, B. B. (1998). SPIN, a substrate in the MAP kinase pathway in mouse oocytes. *Mol. Reprod. Dev.* **50,** 240–249.

Oh, B., Hwang, S., McLaughlin, J., Solter, D., and Knowles, B. B. (2000). Timely translation during the mouse oocyte-to-embryo transition. *Development* **127,** 3795–3803.

Olesnicky, E. C., and Desplan, C. (2007). Distinct mechanisms for mRNA localization during embryonic axis specification in the wasp Nasonia. *Dev. Biol.* **306,** 134–142.

Oppenheim, S. M., Moyer, A. L., Bondurant, R. H., Rowe, J. D., and Anderson, G. B. (2000). Successful pregnancy in goats carrying their genetically identical conceptus. *Theriogenology* **54,** 629–639.

Oreffo, R. O., Lashbrooke, B., Roach, H. I., Clarke, N. M., and Cooper, C. (2003). Maternal protein deficiency affects mesenchymal stem cell activity in the developing offspring. *Bone* **33,** 100–107.

Otoi, T., Yamamoto, K., Horikita, N., Tachikawa, S., and Suzuki, T. (1999). Relationship between dead cells and DNA fragmentation in bovine embryos produced *in vitro* and stored at 4 degrees C. *Mol. Reprod. Dev.* **54,** 342–347.

Otsuka, F., Yao, Z., Lee, T., Yamamoto, S., Erickson, G. F., and Shimasaki, S. (2000). Bone morphogenetic protein-15. Identification of target cells and biological functions. *J. Biol. Chem.* **275,** 39523–39528.

Ozaki, T., Hawkins, P., Nishina, H., Steyn, C., Poston, L., and Hanson, M. A. (2000). Effects of undernutrition in early pregnancy on systemic small artery function in late-gestation fetal sheep. *Am. J. Obstet. Gynecol.* **183,** 1301–1307.

Ozdzenski, W. (1967). Observations on the origin of primordial germ cells in the mouse. *Zool. Pol.* **17,** 367–379.

Ozil, J. P. (1983). Production of identical twins by bisection of blastocysts in the cow. *J. Reprod. Fertil.* **69,** 463–468.

Ozil, J. P. (1990). The parthenogenetic development of rabbit oocytes after repetitive pulsatile electrical stimulation. *Development* **109,** 117–127.

Ozil, J. P., Markoulaki, S., Toth, S., Matson, S., Banrezes, B., Knott, J. G., Schultz, R. M., Huneau, D., and Ducibella, T. (2005). Egg activation events are regulated by the duration of a sustained [Ca2+]cyt signal in the mouse. *Dev. Biol.* **282,** 39–54.

Ozil, J. P., Banrezes, B., Toth, S., Pan, H., and Schultz, R. M. (2006). Ca^{2+} oscillatory pattern in fertilized mouse eggs affects gene expression and development to term. *Dev. Biol.* **300,** 534–544.

Padmanabhan, K., and Richter, J. D. (2006). Regulated Pumilio-2 binding controls RINGO/Spy mRNA translation and CPEB activation. *Genes Dev.* **20,** 199–209.

Paillard, L., Omilli, F., Legagneux, V., Bassez, T., Maniey, D., and Osborne, H. B. (1998). EDEN and EDEN-BP, a cis element and an associated factor that mediate sequence-specific mRNA deadenylation in *Xenopus* embryos. *EMBO J.* **17,** 278–287.

Painter, R. C., de Rooij, S. R., Bossuyt, P. M., Simmers, T. A., Osmond, C., Barker, D. J., Bleker, O. P., and Roseboom, T. J. (2006). Early onset of coronary artery disease after prenatal exposure to the Dutch famine. *Am. J. Clin. Nutr.* **84,** 322–327.

Pampfer, S. (2000). Apoptosis in rodent peri-implantation embryos: Differential susceptibility of inner cell mass and trophectoderm cell lineages—A review. *Placenta* **21,** S3–S10.

Papaioannou, V. E., Mkandawire, J., and Biggers, J. D. (1989). Development and phenotypic variability of genetically identical half mouse embryos. *Development* **106,** 817–827.

Pardo-Manuel de Villena, F., and Sapienza, C. (2001a). Female meiosis drives karyotypic evolution in mammals. *Genetics* **159,** 1179–1189.

Pardo-Manuel de Villena, F., and Sapienza, C. (2001b). Transmission of Robertsonian chromosomes through human female meiosis. *Cytogenet. Cell Genet.* **92,** 342–344.

Pardo-Manuel de Villena, F., and Sapienza, C. (2001c). Transmission ratio distortion in offspring of heterozygous female carriers of Robertsonian translocations. *Hum. Genet.* **108,** 31–36.

Pardo-Manuel de Villena, F., and Sapienza, C. (2001d). Nonrandom segregation during meiosis: The unfairness of females. *Mamm. Genome* **12,** 331–339.

Pardo-Manuel de Villena, F., Naumova, A. K., Verner, A. E., Jin, W. H., and Sapienza, C. (1997). Confirmation of maternal transmission ratio distortion at Om and direct evidence that the maternal and paternal "DDK syndrome" genes are linked. *Mamm. Genome* **8,** 642–646.

Patera, J., Chorostowska-Wynimko, J., Słodkowska, J., Borowska, A., Skopiński, P., Sommer, E., Wasiutyński, A., and Skopińska-Rózewska, E. (2006). Morphometric and functional abnormalities of kidneys in the progeny of mice fed chocolate during pregnancy and lactation. *Folia Histochem. Cytobiol.* **44,** 207–211.

Paules, R. S., Buccione, R., Moschel, R. C., Vande Woude, G. F., and Eppig, J. J. (1989). Mouse Mos protooncogene product is present and functions during oogenesis. *Proc. Natl. Acad. Sci. USA* **86,** 5395–5399.

Payer, B., Saitou, M., Barton, S. C., Thresher, R., Dixon, J. P., Zahn, D., Colledge, W. H., Carlton, M. B., Nakano, T., and Surani, M. A. (2003). Stella is a maternal effect gene required for normal early development in mice. *Curr. Biol.* **13,** 2110–2117.

Paynton, B. V., Rempel, R., and Bachvarova, R. (1988). Changes in state of adenylation and time course of degradation of maternal mRNAs during oocyte maturation and early embryonic development in the mouse. *Dev. Biol.* **129,** 304–314.

Pelletier, G., and El-Alfy, M. (2000). Immunocytochemical localization of estrogen receptors alpha and beta in the human reproductive organs. *J. Clin. Endocrinol. Metab.* **85,** 4835–4840.

Pellettieri, J., Reinke, V., Kim, S. K., and Seydoux, G. (2003). Coordinate activation of maternal protein degradation during the egg-to-embryo transition in C. elegans. *Dev. Cell* **5,** 451–462.

Perez, G. I., Maravei, D. V., Trbovich, A. M., Cidlowski, J. A., Tilly, J. L., and Hughes, F. M., Jr. (2000a). Identification of potassium-dependent and -independent components of the apoptotic machinery in mouse ovarian germ cells and granulosa cells. *Biol. Reprod.* **63,** 1358–1369.

Perez, G. I., Trbovich, A. M., Gosden, R. G., and Tilly, J. L. (2000b). Mitochondria and the death of oocytes. *Nature* **403,** 500–501.

Petry, C. J., Jennings, B. J., James, L. A., Hales, C. N., and Ozanne, S. E. (2006). Suckling a protein-restricted rat dam leads to diminished albuminuria in her male offspring in adult life: A longitudinal study. *BMC Nephrol.* **7,** 14.

Picton, H. M. (2000). Activation of follicle development: The primordial follicle. *Theriogenology* **55,** 1193–1210.

Piko, L., and Clegg, K. B. (1982). Quantitative changes in total RNA, total poly(A), and ribosomes in early mouse embryos. *Dev. Biol.* **89,** 362–378.

Pincus, G., and Enzmann, E. V. (1934). Can mammalian eggs undergo normal development *in vitro*? *Proc. Natl. Acad. Sci. USA* **20,** 121–122.

Piotrowska, K., and Zernicka-Goetz, M. (2001). Role for sperm in spatial patterning of the early mouse embryo. *Nature* **409,** 517–521.

Piotrowska, K., Wianny, F., Pedersen, R. A., and Zernicka-Goetz, M. (2001). Blastomeres arising from the first cleavage division have distinguishable fates in normal mouse development. *Development* **128,** 3739–3748.

Pires, K. M., Aguila, M. B., and Mandarim-de-Lacerda, C. A. (2006). Early renal structure alteration in rat offspring from dams fed low protein diet. *Life Sci.* **79,** 2128–2134.

Plachot, M., Junca, A. M., Mandelbaum, J., de Grouchy, J., Salat-Baroux, J., and Cohen, J. (1986). Chromosome investigations in early life. II. Human preimplantation embryos. *Hum. Reprod.* **1,** 547–551.

Porter, L. A., Kong-Beltran, M., and Donoghue, D. J. (2003). Spy1 interacts with p27Kip1 to allow G1/S progression. *Mol. Biol. Cell* **14,** 3664–3674.

Potireddy, S., Vassena, R., Patel, B. G., and Latham, K. E. (2006). Analysis of polysomal mRNA populations of mouse oocytes and zygotes: Dynamic changes in maternal mRNA utilization and function. *Dev. Biol.* **298,** 155–166.

Pozo, J., Corral, E., and Pereda, J. (1990). Subcellular structure of prenatal human ovary: Mitochondrial distribution during meiotic prophase. *J. Submicrosc. Cytol. Pathol.* **22,** 601–607.

Presicce, G. A., and Yang, X. (1994). Parthenogenetic development of bovine oocytes matured in vitro for 24 hr and activated by ethanol and cycloheximide. *Mol. Reprod. Dev.* **38,** 380–385.

Proudfoot, N. J., and Brownlee, G. G. (1976). 3′ non-coding region sequences in eukaryotic messenger RNA. *Nature* **263,** 211–214.

Quintin, S., Mains, P. E., Zinke, A., and Hyman, A. A. (2003). The mbk-2 kinase is required for inactivation of MEI-1/katanin in the one-cell Caenorhabditis elegans embryo. *EMBO J.* **4,** 1175–1181.

Rands, G. F. (1986). Size regulation in the mouse embryo. II. The development of half embryos. *J. Embryol. Exp. Morphol.* **98,** 209–217.

Rankin, T., Familari, M., Lee, E., Ginsberg, A., Dwyer, N., Blanchette-Mackie, J., Drago, J., Westphal, H., and Dean, J. (1996). Mice homozygous for an insertional mutation in the Zp3 gene lack a zona pellucida and are infertile. *Development* **122,** 2903–2910.

Rankin, T. L., O'Brien, M., Lee, E., Wigglesworth, K., Eppig, J., and Dean, J. (2001). Defective zonae pellucidae in Zp2-null mice disrupt folliculogenesis, fertility and development. *Development* **128,** 1119–1126.

Ratnam, S., Mertineit, C., Ding, F., Howell, C. Y., Clarke, H. J., Bestor, T. H., Chaillet, J. R., and Trasler, J. M. (2002). Dynamics of Dnmt1 methyltransferase expression and intracellular localization during oogenesis and preimplantation development. *Dev. Biol.* **245,** 304–314.

Ravelli, A. C., van der Meulen, J. H., Michels, R. P., Osmond, C., Barker, D. J., Hales, C. N., and Bleker, O. P. (1998). Glucose tolerance in adults after prenatal exposure to famine. *Lancet* **351,** 173–177.

Ravelli, A. C., Bleker, O. P., Roseboom, T. J., van Montfrans, G. A., Osmond, C., and Barker, D. J. (2005). Cardiovascular disease in survivors of the Dutch famine. *Nestle Nutr. Workshop Ser. Pediatr. Program.* **55,** 183–191.

Renard, J. P., and Babinet, C. (1986). Identification of a paternal developmental effect on the cytoplasm of one-cell-stage mouse embryos. *Proc. Natl. Acad. Sci. USA* **83,** 6883–6886.

Renard, J. P., Baldacci, P., Richoux-Duranthon, V., Pournin, S., and Babinet, C. (1994). A maternal factor affecting mouse blastocyst formation. *Development* **120,** 797–802.

Reverte, C. G., Ahearn, M. D., and Hake, L. E. (2001). CPEB degradation during *Xenopus* oocyte maturation requires a PEST domain and the 26S proteasome. *Dev. Biol.* **231,** 447–458.

Richter, J. D. (1999). Cytoplasmic polyadenylation in development and beyond. *Microbiol. Mol. Biol. Rev.* **63,** 446–456.

Richter, J. D., and Sonenberg, N. (2005). Regulation of cap-dependent translation by eIF4E inhibitory proteins. *Nature* **433,** 477–480.

Riley, J. K., and Moley, K. H. (2006). Glucose utilization and the PI3-K pathway: Mechanisms for cell survival in preimplantation embryos. *Reproduction* **131,** 823–835.

Ripoll, P., Pimpinelli, S., Valdivia, M. M., and Avila, J. (1985). A cell division mutant of *Drosophila* with a functionally abnormal spindle. *Cell* **41,** 907–912.

Robl, J. M., and First, N. L. (1985). Manipulation of gametes and embryos in the pig. *J. Reprod. Fertil. Suppl.* **33,** 101–114.

Rosenthal, E. T., and Ruderman, J. V. (1987). Widespread changes in the translation and adenylation of maternal messenger RNAs following fertilization of Spisula oocytes. *Dev. Biol.* **121,** 237–246.

Rossi, G., Palmerini, M. G., Macchiarelli, G., Buccione, R., and Cecconi, S. (2006). Mancozeb adversely affects meiotic spindle organization and fertilization in mouse oocytes. *Reprod. Toxicol.* **22,** 51–55.

Sagata, N., Oskarsson, M., Copeland, T., Brumbaugh, J., and Vande Woude, G. F. (1988). Function of c-mos proto-oncogene product in meiotic maturation in *Xenopus* oocytes. *Nature* **335,** 519–525.

Saito, S., and Niemann, H. (1991). Effects of extracellular matrices and growth factors on the development of isolated porcine blastomeres. *Biol. Reprod.* **44,** 927–936.

Sakkas, D., Manicardi, G., Bianchi, P. G., Bizzaro, D., and Bianchi, U. (1995). Relationship between the presence of endogenous nicks and sperm chromatin packaging in maturing and fertilizing mouse spermatozoa. *Biol. Reprod.* **52,** 1149–1155.

Sakkas, D., Shoukir, Y., Chardonnens, D., Bianchi, P. G., and Campana, A. (1998). Early cleavage of human embryos to the two-cell stage after intracytoplasmic sperm injection as an indicator of embryo viability. *Hum. Reprod.* **13,** 182–187.

Salaun, P., Pyronnet, S., Morales, J., Mulner-Lorillon, O., Belle, R., Sonenberg, N., and Cormier, P. (2003). eIF4E/4E-BP dissociation and 4E-BP degradation in the first mitotic division of the sea urchin embryo. *Dev. Biol.* **255,** 428–439.

Salaun, P., Boulben, S., Mulner-Lorillon, O., Belle, R., Sonenberg, N., Morales, J., and Cormier, P. (2005). Embryonic-stage-dependent changes in the level of eIF4E-binding proteins during early development of sea urchin embryos. *J. Cell Sci.* **118,** 1385–1394.

Sankaran, D., Bankovic-Calic, N., Peng, C. Y., Ogborn, M. R., and Aukema, H. M. (2006). Dietary flax oil during pregnancy and lactation retards disease progression in rat offspring with inherited kidney disease. *Pediatr. Res.* **60,** 729–733.

Santos, F., Peters, A. H., Otte, A. P., Reik, W., and Dean, W. (2005). Dynamic chromatin modifications characterise the first cell cycle in mouse embryos. *Dev. Biol.* **280,** 225–236.

Sapienza, C., Paquette, J., Pannunzio, P., Albrechtson, S., and Morgan, K. (1992). The polar-lethal Ovum mutant gene maps to the distal portion of mouse chromosome 11. *Genetics* **132,** 241–246.

Saunders, C. M., Larman, M. G., Parrington, J., Cox, L. J., Royse, J., Blayney, L. M., Swann, K., and Lai, F. A. (2002). PLC zeta: A sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. *Development* **129,** 3533–3544.

Sayer, A. A., and Cooper, C. (2005). Fetal programming of body composition and musculoskeletal development. *Early Hum. Dev.* **81,** 735–744.

Schatten, G. (1994). The centrosome and its mode of inheritance: The reduction of the centrosome during gametogenesis and its restoration during fertilization. *Dev. Biol.* **165,** 299–335.

Schier, A. F., and Giraldez, A. J. (2006). MicroRNA function and mechanism: Insights from zebra fish. *Cold Spring Harb. Symp. Quant. Biol.* **71,** 195–203.

Schisa, J. A., Pitt, J. N., and Priess, J. R. (2001). Analysis of RNA associated with P granules in germ cells of C. elegans adults. *Development* **128,** 1287–1298.

Schramm, R. D., and Bavister, B. D. (1996a). Granulosa cells from follicle stimulating hormone-primed monkeys enhance the development competence of *in-vitro*-matured oocytes from non-stimulated rhesus monkeys. *Hum. Reprod.* **11,** 1698–1702.

Schramm, R. D., and Bavister, B. D. (1996b). Use of purified porcine follicle-stimulating hormone for ovarian stimulation of macaque monkeys. *Theriogenology* **45,** 727–732.

Schramm, R. D., Tennier, M. T., Boatman, D. E., and Bavister, B. D. (1994). Effects of gonadotropins upon the incidence and kinetics of meiotic maturation of macaque oocytes *in vitro*. *Mol. Reprod. Dev.* **37,** 467–472.

Schultz, R. M., and Kopf, G. S. (1995). Molecular basis of mammalian egg activation. *Curr. Top. Dev. Biol.* **30,** 21–62.

Scott, L., Finn, A., O'Leary, T., McLellan, S., and Hill, J. (2007). Morphologic parameters of early cleavage-stage embryos that correlate with fetal development and delivery: Prospective and applied data for increased pregnancy rates. *Hum. Reprod.* **22,** 230–240.

Seike, N., Sakai, M., and Kanagawa, H. (1991). Development of frozen-thawed demiembryos and production of identical twin calves of different ages. *J. Vet. Med. Sci.* **53,** 37–42.

Semotok, J. L., Cooperstock, R. L., Pin der, B. D., Vari, H. K., Lipshitz, H. D., and Smibert, C. A. (2005). Smaug recruits the CCR4/POP2/NOT deadenylase complex to trigger maternal transcript localization in the early *Drosophila* embryo. *Curr. Biol.* **15,** 284–294.

Sen, S. (2000). Aneuploidy and cancer. *Curr. Opin. Oncol.* **12,** 82–88.

Sengoku, K., Takuma, N., Miyamato, T., Yamauchi, T., and Ishikawa, M. (2004). Nuclear dynamics of parthenogenesis of human oocytes: Effect of oocyte aging *in vitro*. *Gynecol. Obstret. Invest.* **58,** 155–159.

Shen, Y., Betzendahl, I., Sun, F., Tinneberg, H. R., and Eichenlaub-Ritter, U. (2005). Non-invasive method to assess genotoxicity of nocodazole interfering with spindle formation in mammalian oocytes. *Reprod. Toxicol.* **19,** 459–471.

Shi, W., Dirim, F., Wolf, E., Zakhartchenko, V., and Haaf, T. (2004). Methylation reprogramming and chromosomal aneuploidy in *in vivo* fertilized and cloned rabbit preimplantation embryos. *Biol. Reprod.* **71,** 340–347.

Shimasaki, S., Moore, R. K., Otsuka, F., and Erickson, G. F. (2004). The bone morphogenetic protein system in mammalian reproduction. *Endocr. Rev.* **25,** 72–101.

Shitara, H., Hayashi, J., Takahama, S., Kaneda, H., and Yonekawa, H. (1998). Maternal inheritance of mouse mtDNA in interspecific hybrids: Segregation of leaked paternal mtDNA followed by the prevention of subsequent paternal leakage. *Genetics* **148,** 851–857.

Shitara, H., Kaneda, H., Sato, A., Iwasaki, K., Hayashi, J., Taya, C., and Yonekawa, H. (2001). Non-invasive visualization of sperm mitochondria behavior in transgenic mice with introduced green fluorescent protein (GFP). *FEBS Lett.* **500,** 7–11.

Shoubridge, E. A. (2000). Mitochondrial DNA segregation in the developing embryos. *Hum. Reprod.* **15,** 229–234.

Skabkin, M. A., Kiselyova, O. I., Chernov, K. G., Sorokin, A. V., Dubrovin, E. V., Yaminsky, I. V., Vasiliev, V. D., and Ovchinnikov, L. P. (2004). Structural organization of mRNA complexes with major core mRNP protein YB-1. *Nucleic Acids Res.* **32,** 5621–5635.

Skommera, J., Wlodkowica, D., and Deptala, A. (2006). Larger than life: Mitochondria and the Bcl-2 family. *Leuk. Res.* **31,** 277–286.

Smillie, D. A., and Sommerville, J. (2002). RNA helicase p54 (DDX6) is a shuttling protein involved in nuclear assembly of stored mRNP particles. *J. Cell Sci.* **115,** 395–407.

Sohoni, P., Tyler, C. R., Hurd, K., Caunter, J., Hetheridge, M., Williams, T., Woods, C., Evans, M., Toy, R., Gargas, M., and Sumpter, J. P. (2001). Reproductive effects of long-term exposure to bisphenol A in the fathead minnow (*Pimephales promelas*). *Environ. Sci. Technol.* **35,** 2917–2925.

Sommerville, J., and Ladomery, M. (1996). Transcription and masking of mRNA in germ cells: Involvement of Y-box proteins. *Chromosoma* **104,** 469–478.

Spicer, L. J., Walega, M. A., and Hammond, J. M. (1987). Metabolism of [^{3}H] 2-hydroxyestradiol by cultured porcine granulosa cells: Evidence for the presence of a catechol-O-methyltransferase pathway and a direct stimulatory effect of 2-methoxyestradiol on progesterone production. *Biol. Reprod.* **36,** 562–571.

St. John, J. C., and Schatten, G. (2004). Paternal mitochondrial DNA transmission during nonhuman primate nuclear transfer. *Genetics* **167,** 897–905.

St. John, J. C., Sakkas, D., and Barratt, C. L. (2000). A role for mitochondrial DNA and sperm survival. *J. Androl.* **21,** 189–199.

St. John, J. C., Moffatt, O., and D'Souza, N. (2005). Aberrant heteroplasmic transmission of mtDNA in cloned pigs arising from double nuclear transfer. *Mol. Reprod. Dev.* **72,** 450–460.

Stancheva, I., and Meehan, R. R. (2000). Transient depletion of xDnmt1 leads to premature gene activation in *Xenopus* embryos. *Genes Dev.* **14,** 313–327.

Stancheva, I., El-Maarri, O., Walter, J., Niveleau, A., and Meehan, R. R. (2002). DNA methylation at promoter regions regulates the timing of gene activation in *Xenopus laevis* embryos. *Dev. Biol.* **243,** 155–165.

Stanger, B. Z., Tanaka, A. J., and Melton, D. A. (2007). Organ size is limited by the number of embryonic progenitor cells in the pancreas but not the liver. *Nature* **445,** 886–891.

Stebbins-Boaz, B., Cao, Q., de Moor, C. H., Mendez, R., and Richter, J. D. (1999). Maskin is a CPEB-associated factor that transiently interacts with elF-4E. *Mol. Cell* **4,** 1017–1027.

Steinborn, R., Zakhartchenko, V., Jelyazkov, J., Klein, D., Wolf, E., Muller, M., and Brem, G. (1998). Composition of parental mitochondrial DNA in cloned bovine embryos. *FEBS Lett.* **426,** 352–356.

Steinborn, R. P., Schinogl, D. N., Wells, A., Bergthaler, M., Muller, M., and Brem, G. (2002). Coexistence of Bos taurus and B. Indicus mitochondrial DNAs in nuclear transfer-derived somatic cattle clones. *Genetics* **162,** 823–829.

Stice, S. L., and Robl, J. M. (1990). Activation of mammalian oocytes by a factor obtained from rabbit sperm. *Mol. Reprod. Dev.* **25,** 272–280.

Stice, S. L., Keefer, C. L., and Matthews, L. (1994). Bovine nuclear transfer embryos: Oocyte activation prior to blastomere fusion. *Mol. Reprod. Dev.* **38,** 61–68.

Strickland, S., Huarte, J., Belin, D., Vassalli, A., Rickles, R. J., and Vassalli, J. D. (1988). Antisense RNA directed against the 3′ noncoding region prevents dormant mRNA activation in mouse oocytes. *Science* **241,** 680–684.

Strome, S., and Wood, W. B. (1982). Immunofluorescence visualization of germ-line-specific cytoplasmic granules in embryos, larvae, and adults of Caenorhabditis elegans. *Proc. Natl. Acad. Sci. USA* **79,** 1558–1562.

Strome, S., Garvin, C., Paulsen, J., Capowski, E., Martin, P., and Beanan, M. (1994). Specification and development of the germline in Caenorhabditis elegans. *Ciba Found. Symp.* **182,** 31–45.

Struhl, G. (1989). Differing strategies for organizing anterior and posterior body pattern in *Drosophila* embryos. *Nature* **338,** 741–744.

Struhl, G., Struhl, K., and Macdonald, P. M. (1989). The gradient morphogen bicoid is a concentration-dependent transcriptional activator. *Cell* **57,** 1259–1273.

Su, Y. Q., Denegre, J. M., Wigglesworth, K., Pendola, F. L., O'Brien, M. J., and Eppig, J. J. (2003). Oocyte-dependent activation of mitogen-activated protein kinase (ERK1/2) in cumulus cells is required for the maturation of the mouse oocyte-cumulus cell complex. *Dev. Biol.* **263,** 126–138.

Su, Y. Q., Sugiura, K., Woo, Y., Wigglesworth, K., Kamdar, S., Affourtit, J., and Eppig, J. J. (2007). Selective degradation of transcripts during meiotic maturation of mouse oocytes. *Dev. Biol.* **302,** 104–117.

Sugiura, K., Pendola, F., and Eppig, J. J. (2005). Oocyte control of metabolic cooperativity between oocytes and companion granulosa cells: energy metabolism. *Dev Biol.* **279,** 20–30.

Sugiura, K., and Eppig, J. J. (2005). Society for reproductive biology founders' lecture 2005. Control of metabolic cooperativity between oocytes and their companion granulosa cells by mouse oocytes. *Reprod. Fertil. Dev.* **17,** 667–674.

Sugiura, K., Su, Y. Q., Diaz, F. J., Pangas, S. A., Sharma, S., Wigglesworth, K., O'Brien, M. J., Matzuk, M. M., Shimasaki, S., and Eppig, J. J. (2007). Oocyte-derived

BMP15 and FGFs cooperate to promote glycolysis in cumulus cells. *Development* **134,** 2593–2603.

Sun, F., Yin, H., and Eichenlaub-Ritter, U. (2001). Differential chromosome behaviour in mammalian oocytes exposed to the tranquilizer diazepam *in vitro*. *Mutagenesis* **16,** 407–417.

Sun, F., Betzendahl, I., Pacchierotti, F., Ranaldi, R., Smitz, J., Cortvrindt, R., and Eichenlaub-Ritter, U. (2005). Aneuploidy in mouse metaphase II oocytes exposed in vivo and *in vitro* in preantral follicle culture to nocodazole. *Mutagenesis* **20,** 65–75.

Susiarjo, M., Hassold, T. J., Freeman, E., and Hunt, P. A. (2007). Bisphenol A exposure in utero disrupts early oogenesis in the mouse. *PLoS Genet*. **12,** e5.

Sutovsky, P. (2003). Ubiquitin-dependent proteolysis in mammalian spermatogenesis, fertilization, and sperm quality control: Killing three birds with one stone. *Microsc. Res. Tech*. **61,** 88–102.

Sutovsky, P., Moreno, R. D., Ramalho-Santos, J., Dominko, T., Simerly, C., and Schatten, G. (1999). Ubiquitin tag for sperm mitochondria. *Nature* **402,** 371–372.

Sutovsky, P., Moreno, R. D., Ramalho-Santos, J., Dominko, T., Simerly, C., and Schatten, G. (2000). Ubiquitinated sperm mitochondria, selective proteolysis, and the regulation of mitochondrial inheritance in mammalian embryos. *Biol. Reprod*. **63,** 582–590.

Swann, K. (1994). Ca2+ oscillations and sensitization of Ca2+ release in unfertilized mouse eggs injected with a sperm factor. *Cell Calcium* **15,** 331–339.

Symonds, M. E., Stephenson, T., Gardner, D. S., and Budge, H. (2007). Long-term effects of nutritional programming of the embryo and fetus: Mechanisms and critical windows. *Reprod. Fertil. Dev*. **19,** 53–63.

Szollosi, D., Calarco, P., and Donahue, R. P. (1972). Absence of centrioles in the first and second meiotic spindles of mouse oocytes. *J. Cell Sci*. **11,** 521–541.

Tabata, A., Kashiwada, S., Ohnishi, Y., Ishikawa, H., Miyamoto, N., Itoh, M., and Magara, Y. (2001). Estrogenic influences of estradiol-17 beta, p-nonylphenol and bisphenol-A on Japanese medaka (*Oryzias latipes*) at detected environmental concentrations. *Water Sci. Technol*. **43,** 109–116.

Tadros, W., and Lipshitz, H. D. (2005). Setting the stage for development: mRNA translation and stability during oocyte maturation and egg activation in *Drosophila*. *Dev. Dyn*. **232,** 593–608.

Tafuri, S. R., and Wolffe, A. P. (1990). *Xenopus* Y-box transcription factors: Molecular cloning, functional analysis and developmental regulation. *Proc. Natl. Acad. Sci. USA* **87,** 9028–9032.

Tafuri, S. R., and Wolffe, A. P. (1993). Selective recruitment of masked maternal mRNA from messenger ribonucleoprotein particles containing FRGY2 (mRNP4). *J. Biol. Chem*. **268,** 24255–24261.

Takeda, K., Takahashi, S., Onishi, A., Hanada, H., and Imai, H. (2000). Replicative advantage and tissue-specific segregation of RR mitochondrial DNA between C57BL/6 and RR heteroplasmic mice. *Genetics* **155,** 777–783.

Tam, P. P., and Snow, M. H. (1981). Proliferation and migration of primordial germ cells during compensatory growth in mouse embryos. *J. Embryol. Exp. Morphol*. **64,** 133–147.

Tanaka, K. M., Ogawa, K., Takagi, M., Imamoto, N., Matsumoto, K., and Tsujimoto, M. (2006). RAP55, a cytoplasmic mRNP component, represses translation in *Xenopus* oocytes. *J. Biol. Chem*. **281,** 40096–40106.

Tarkowski, A. K. (1959a). Experiments on the development of the viability of mouse embryos containing half the normal number of blastomeres. *Nature* **84,** 1286–1287.

Tarkowski, A. K. (1959b). Experimental studies on regulation in the development of isolated blastomeres of mouse eggs. *Theriologica* **3,** 191–267.

Tautz, D. (1988). Regulation of the *Drosophila* segmentation gene hunchback by two maternal morphogenetic centres. *Nature* **332,** 281–284.

Thompson, W. E., Ramalho-Santos, J., and Sutovsky, P. (2003). Ubiquitination of prohibitin in mammalian sperm mitochondria: Possible roles in the regulation of mitochondrial inheritance and sperm quality control. *Biol. Reprod.* **69,** 254–260.

Thone-Reineke, C., Kalk, P., Dorn, M., Klaus, S., Simon, K., Pfab, T., Godes, M., Persson, P., Unger, T., and Hocher, B. (2006). High-protein nutrition during pregnancy and lactation programs blood pressure, food efficiency, and body weight of the offspring in a sex-dependent manner. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **291,** R1025–R1030.

Tojo, H., and Ogita, Z. (1984). An improved method for destroying mouse blastomeres electrically inside the zona pellucida and the *in vitro* development of the surviving blastomeres. *J. Exp. Zool.* **229,** 475–480.

Tokumoto, T., Tokumoto, M., and Nagahama, Y. (2005). Induction and inhibition of oocyte maturation by EDCs in zebrafish. *Reprod. Biol. Endocrinol.* **9,** 69.

Tong, Z. B., Gold, L., Pfeifer, K. E., Dorward, H., Lee, E., Bondy, C. A., Dean, J., and Nelson, L. M. (2000). Mater, a maternal effect gene required for early embryonic development in mice. *Nat. Genet.* **26,** 267–268.

Torres-Padilla, E., and Zernicka-Goetz, M. (2006). Role of TIF1α as a modulator of embryonic transcription in the mouse zygote. Maria. *J. Cell Biol.* **174,** 329–338.

Tsunoda, Y., and McLaren, A. (1983). Effect of various procedures on the viability of mouse embryos containing half the normal number of blastomeres. *J. Reprod. Fert.* **69,** 315–322.

Tsunoda, Y., Uasui, T., and Sugie, T. (1984). Production of monozygotic twins following transfer of separated half embryos in the goat. *Jpn. J. Zootech. Sci.* **55,** 643–647.

Turner, B. C., and Perkins, D. D. (1979). Spore killer, a chromosomal factor in neurospora that kills meiotic products not containing it. *Genetics* **93,** 587–606.

Uranga, J. A., Pedersen, R. A., and Arechaga, J. (1996). Parthenogenetic activation of mouse oocytes using calcium ionophores and protein kinase C stimulators. *Int. J. Dev. Biol.* **40,** 515–519.

Urbanek, M., Legro, R. S., Driscoll, D. A., Azziz, R., Ehrmann, D. A., Norman, R. J., Strauss, J. F., III, Spielman, R. S., and Dunaif, A. (1999). Thirty-seven candidate genes for polycystic ovary syndrome: Strongest evidence for linkage is with follistatin. *Proc. Natl. Acad. Sci. USA* **96,** 8573–8578.

Urbanek, M., Wu, X., Vickery, K. R., Kao, L. C., Christenson, L. K., Schneyer, A., Legro, R. S., Driscoll, D. A., Strauss, J. F., III, Dunaif, A., and Spielman, R. S. (2000). Allelic variants of the follistatin gene in polycystic ovary syndrome. *J. Clin. Endocrinol. Metab.* **85,** 4455–4461.

Uzbekova, S., Arlot-Bonnemains, Y., Dupont, J., Dalbies-Tran, R., Papillier, P., Pennetier, S., Thelie, A., Perreau, C., Mermillod, P., Prigent, C., and Uzbekov, R. (2008). Spatio-temporal expression patterns of aurora kinases A, B, and C and cytoplasmic polyadenylation-element-binding protein in bovine oocytes during meiotic maturation. *Biol. Reprod* **78,** 218–233.

Van Blerkom, J. (1991). Microtubule mediation of cytoplasmic and nuclear maturation during the early stages of resumed meiosis in cultured mouse oocytes. *Proc. Natl. Acad. Sci. USA* **88,** 5031–5035.

Van Blerkom, J., and Bell, H. (1986). Regulation of development in the fully grown mouse oocyte: Chromosome-mediated temporal and spatial differentiation of the cytoplasm and plasma membrane. *J. Embryol. Exp. Morphol.* **93,** 213–238.

Van Blerkom, J., and Davis, P. W. (1998). DNA strand break and phosphatidylserine redistribution in newly ovulated cultured mouse and human oocytes: Occurrence and relationship to apoptosis. *Hum. Reprod.* **13,** 1317–1324.

Van Blerkom, J., Sinclair, J., and Davis, P. (1998). Mitochondrial transfer between oocytes: Potential applications of mitochondrial donation and the issue of heteroplasmy. *Hum. Reprod.* **13,** 2857–2868.

Van der Heijden, G. W., Dieker, J. W., Derijck, A. A., Muller, S., Berden, J. H., Braat, D. D., van der Vlag, J., and de Boer, P. (2005). Asymmetry in histone H3 variants and lysine methylation between paternal and maternal chromatin of the early mouse zygote. *Mech. Dev.* **122,** 1008–1022.

van der Heijden, G. W., Derijck, A. A., Ramos, L., Giele, M., van der Vlag, J., and de Boer, P. (2006). Transmission of modified nucleosomes from the mouse male germline to the zygote and subsequent remodeling of paternal chromatin. *Dev. Biol.* **298,** 458–469.

van Kasteren, Y. M., Hoek, A., and Schoemaker, J. (1995). Ovulation induction in premature ovarian failure: A placebo controlled randomized trial combining pituitary suppression with gonadotropin stimulation. *Fertil. Steril.* **64,** 273–278.

Varnum, S. M., and Wormington, W. M. (1990). Deadenylation of maternal mRNAs during *Xenopus* oocyte maturation does not require specific cis-sequences: A default mechanism for translational control. *Genes Dev.* **4,** 2278–2286.

Vassalli, J. D., Huarte, J., Belin, D., Gubler, P., Vassalli, A., O'Connell, M. L., Parton, L. A., Rickles, R. J., and Strickland, S. (1989). Regulated polyadenylation controls mRNA translation during meiotic maturation of mouse oocytes. *Genes Dev.* **3,** 2163–2171.

Vassena, R., Han, Z., Gao, S., and Latham, K. E. (2007a). Deficiency in recapitulation of stage-specific embryonic gene transcription in two-cell stage cloned mouse embryos. *Mol. Reprod. Dev.* **74,** 1548–1556.

Vassena, R., Han, Z., Gao, S., Baldwin, D. A., Schultz, R. M., and Latham, K. E. (2007b). Tough beginnings: Alterations in the transcriptome of cloned embryos during the first two cell cycles. *Dev. Biol.* **304,** 75–89.

Vethaak, A. D., Lahr, J., Schrap, S. M., Belfroid, A. C., Rijs, G. B., Gerritsen, A., de Boer, J., Bulder, A. S., Grinwis, G. C., Kuiper, R. V., Legler, J., Murk, T. A., *et al.* (2005). An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of The Netherlands. *Chemosphere* **59,** 511–524.

Voelkel, S. A., Viker, S. D., Johnson, C. A., Hill, K. G., Humes, P. E., and Godke, R. A. (1985). Multiple embryo-transplant offspring produced from quartering a bovine embryo at the morula stage. *Vet. Rec.* **117,** 528–530.

Voeltz, G. K., Ongkasuwan, J., Standart, N., and Steitz, J. A. (2001). A novel embryonic poly(A) binding protein, ePAB, regulates mRNA deadenylation in *Xenopus* egg extracts. *Genes Dev.* **15,** 774–788.

Wakayama, T., Perry, A. C. F., Zuccotti, M., Johnson, K. R., and Yanagimachi, R. (1998). Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* **394,** 369–374.

Wakasugi, N., Tomita, T., and Kondo, K. (1967). Differences of fertility in reciprocal crosses between inbred strains of mice. DDK, KK and NC. *J. Reprod. Fertil.* **13,** 41–50.

Walter, P., Green, S., Greene, G., Krust, A., Bornert, J. M., Jeltsch, J. M., Staub, A., Jensen, E., Scrace, G., and Waterfield, M. (1985). Cloning of the human estrogen receptor cDNA. *Proc. Natl. Acad. Sci. USA* **82,** 7889–7893.

Wang, Q., and Latham, K. E. (1997). Requirement for protein synthesis during embryonic genome activation in mice. *Mol. Reprod. Dev.* **47,** 265–270.

Wang, Q., and Latham, K. E. (2000). Translation of maternal messenger ribonucleic acids encoding transcription factors during genome activation in early mouse embryos. *Biol. Reprod.* **62,** 969–978.

Warn, R. (1975). Restoration of the capacity to form pole cells in u.v.-irradiated *Drosophila* embryos. *J. Embryol. Exp. Morphol.* **33,** 1003–1011.

Watson, A. J., De Sousa, P., Caveney, A., Barcroft, L. C., Natale, D., Urquhart, J., and Westhusin, M. E. (2000). Impact of bovine oocyte maturation media on oocyte transcript levels, blastocyst development, cell number, and apoptosis. *Biol. Reprod.* **62,** 355–364.

Wells, D. N., Misica, P. M., and Tervit, H. R. (1999). Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. *Biol. Reprod.* **60,** 996–1005.

Welshons, W. V., Nagel, S. C., and vom Saal, F. S. (2006). Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure. *Endocrinology* **147,** S56–S69.

Weston, A., and Sommerville, J. (2006). Xp54 and related (DDX6-like) RNA helicases: Roles in messenger RNP assembly, translation regulation and RNA degradation. *Nucleic Acids Res.* **34,** 3082–3094.

Wharf, E., Dimitrakopoulos, A., Khalaf, Y., and Pickering, S. (2004). Early embryo development is an indicator of implantation potential. *Reprod. Biomed. Online* **8,** 212–218.

Whittingham, D. G., and Siracusa, G. (1978). The involvement of calcium in the activation of mammalian oocytes. *Exp. Cell Res.* **113,** 311–317.

White, J. A., and Heasman, J. (2008). Maternal control of pattern formation in *Xenopus laevis*. *J. Exp. Zool. B Mol. Dev. Evol.* **310B**(1), 73–84.

Wickens, M., and Stephenson, P. (1984). Role of the conserved AAUAAA sequence: Four AAUAAA point mutants prevent messenger RNA 3′ end formation. *Science* **226,** 1045–1051.

Wiekowski, M., Miranda, M., and DePamphilis, M. L. (1993). Requirements for promoter activity in mouse oocytes and embryos distinguish paternal pronuclei from maternal and zygotic nuclei. *Dev. Biol.* **159,** 366–378.

Wildasen, S. M. (1979). A method for culture of micromanipulated sheep embryos and its use to produce monozygotic twins. *Nature* **277,** 298–300.

Wolstenholme, J. (1996). Confined placental mosaicism for trisomies 2, 3, 7, 8, 9, 16, and 22: Their incidence, likely origins, and mechanisms for cell lineage compartmentalization. *Prenat. Diagn.* **16,** 511–524.

Wu, X., Viveiros, M. M., Eppig, J. J., Bai, Y., Fitzpatrick, S. L., and Matzuk, M. M. (2003). Zygote arrest 1 (Zar1) is a novel maternal-effect gene critical for the oocyte-to-embryo transition. *Nat. Genet.* **33,** 187–191.

Wu, G., Hao, L., Han, Z., Gao, S., Latham, K. E., de Villena, F. P., and Sapienza, C. (2005). Maternal transmission ratio distortion at the mouse Om locus results from meiotic drive at the second meiotic division. *Genetics* **170,** 327–334.

Yamano, S., Nakagawa, K., Nakasaka, H., and Aono, T. (2000). Fertilization failure and oocyte activation. *J. Med. Invest.* **47,** 1–8.

Yan, C., Wang, P., DeMayo, J., DeMayo, F. J., Elvin, J. A., Carino, C., Prasad, S. V., Skinner, S. S., Dunbar, B. S., Dube, J. L., Celeste, A. J., and Matzuk, M. M. (2001). Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. *Mol. Endocrinol.* **15,** 854–866.

Yanagimachi, R. (2002). Cloning: Experience from the mouse and other animals. *Mol. Cell Endocrinol.* **187,** 241–248.

Yang, W. H., Yu, J. H., Gulick, T., Bloch, K. D., and Bloch, D. B. (2006). RNA-associated protein 55 (RAP55) localizes to mRNA processing bodies and stress granules. *RNA* **12,** 547–554.

Yeo, S., Lee, K. K., Han, Y. M., and Kang, Y. K. (2005). Methylation changes of lysine 9 of histone H3 during preimplantation mouse development. *Mol. Cells* **20,** 423–428.

Yin, H., Cukurcam, S., Betzendahl, I., Adler, I. D., and Eichenlaub-Ritter, U. (1998). Trichlorfon exposure, spindle aberrations and nondisjunction in mammalian oocytes. *Chromosoma* **107,** 514–522.

Zeng, F., Baldwin, D. A., and Schultz, R. M. (2004). Transcript profiling during preimplantation mouse development. *Dev. Biol.* **272,** 483–496.

Zernicka-Goetz, M., and Maro, B. (1993). Okadaic acid affects spindle organization in metaphase II-arrested rat oocytes. *Exp. Cell Res.* **207,** 189–193.

Zhao, M., and Dean, J. (2002). The zona pellucida in folliculogenesis, fertilization and early development. *Rev. Endocr. Metab. Disord.* **3,** 19–26.

Zhao, X., Li, N., Guo, W., Hu, X., Liu, Z., Gong, G., Wang, A., Feng, J., and Wu, C. (2004). Further evidence for paternal inheritance of mitochondrial DNA in the sheep (Ovis aries). *Heredity* **93,** 399–403.

Zheng, P., Patel, B., McMenamin, M., Paprocki, A. M., Schramm, R. D., Nagl, N. G., Jr., Wilsker, D., Wang, X., Moran, E., and Latham, K. E. (2004). Expression of genes encoding chromatin regulatory factors in developing rhesus monkey oocytes and preimplantation stage embryos: Possible roles in genome activation. *Biol. Reprod.* **70,** 1419–1427.

Zheng, P., Patel, B., McMenamin, M., Moran, E., Paprocki, A. M., Kihara, M., Schramm, R. D., and Latham, K. E. (2005). Effects of follicle size and oocyte maturation conditions on maternal messenger RNA regulation and gene expression in rhesus monkey oocytes and embryos. *Biol. Reprod.* **72,** 890–897.

Zhou, L., Goldsmith, A. M., Bentley, J. K., Jia, Y., Rodriguez, M. L., Abe, M. K., Fingar, D. C., and Hershenson, M. B. (2005). 4E-binding protein phosphorylation and eukaryotic initiation factor-4E release are required for airway smooth muscle hypertrophy. *Am. J. Respir. Cell Mol. Biol.* **33,** 195–202.

Zimmermann, U., Vienken, J., Halfmann, J., and Emeis, C. C. (1985). Electrofusion: A novel hybridization technique. *Adv. Biotechnol. Processes* **4,** 79–150.

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