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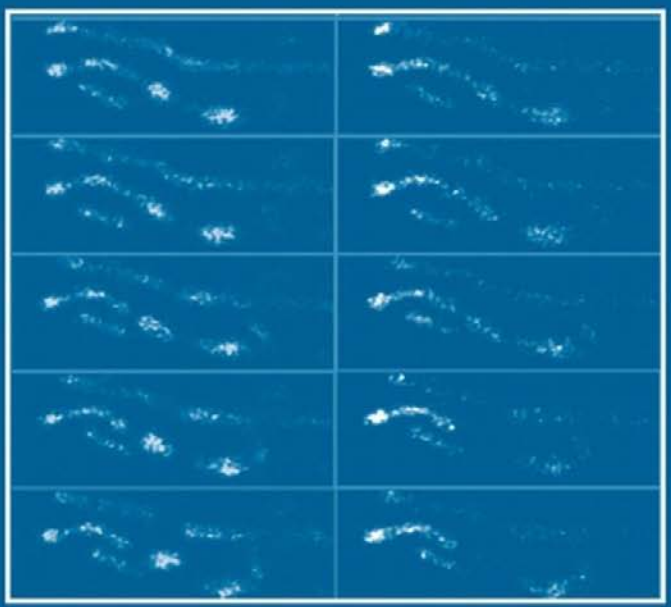

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CHAPTER ONE

Mitochondrial Dynamics

Jürgen Bereiter-Hahn *and* Marina Jendrach

Contents

Abstract

Mitochondrial dynamics is a key feature for the interaction of mitochondria with other organelles within a cell and also for the maintenance of their own integrity. Four types of mitochondrial dynamics are discussed: Movement within a cell and interactions with the cytoskeleton, fusion and fission events which establish coherence within the chondriome, the dynamic behavior of cristae and their components, and finally, formation and disintegration of mitochondria (mitophagy). Due to these essential functions, disturbed mitochondrial dynamics are inevitably connected to a variety of diseases. Localized ATP gradients, local control of calcium-based messaging, production of reactive oxygen species, and involvement of other metabolic chains, that is, lipid and steroid synthesis, underline that physiology not only results from biochemical reactions but, in addition, resides on the appropriate morphology and topography. These events and their molecular basis have been established recently and are the topic of this review.

Key Words: Mitochondria, Mitochondrial movements, Mitochondrial morphology, Mitochondrial fusion and fission, Calcium, Cytoskeleton.

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Abbreviations

AD	Alzheimer's disease
Drp1	dynamin-related protein 1
ER	endoplasmic reticulum
Fis1	fission protein 1
FRAP	fluorescence recovery after photobleaching
HUVEC	human umbilical vein endothelial cells
IMM	inner mitochondrial membrane
KIF	kinesin superfamily
MEF	mouse embryonic fibroblast
Mfn	mitofusin
mtDNA	mitochondrial DNA
mtFP	mitochondrial fluorescent proteins
MTR	MitoTracker Red CMXRos
OMM	outer mitochondrial membrane
Opa1	optic-nerve-atrophy 1
PAGFP	photoactivatable GFP
PD	Parkinson's disease
PGC-1	peroxisome proliferator-activated receptor coactivator 1
ROS	reactive oxygen species
$\Delta\psi_{mit}$	mitochondrial membrane potential

1. Introduction

The production of ATP is the most important function of mitochondria. ATP production is made possible by the concerted action of redox systems forming the core of four electron transport complexes which generate a proton gradient across the inner mitochondrial membrane (IMM). Electron flow via the electron transport chain is a powerful source of reactive oxygen species (ROS), which stimulate cell proliferation and also cause mutations (Klaunig and Kamendulis, 2004) and are supposed to be the main cause for loss of proliferative activity and aging, as stated first by Harman (1956) and Harmann (1972). The proton gradient drives a molecular machine, the ATP-synthase, also termed complex V, phosphorylating ADP to ATP. Using the proton gradient, mitochondria can accumulate other cations such as K^+ and they act as powerful regulators of cytoplasmic Ca^{2+} (Gunter et al., 2000; Malli and Graier, 2010; Parekh, 2008; Werth and Thayer, 1994) and via calcium

signaling influence a wide variety of physiological processes (Ryu et al., 2010; Valero et al., 2008). Among those is the participation of mitochondria as a key component in the glucose-sensing machinery controlling insulin secretion by pancreatic β-cells (Lowell and Shulman, 2005; Maassen et al., 2004; Maechler and Wollheim, 2001). Their general association with the endoplasmic reticulum (ER) reflects the interaction of these two endomembrane systems in control of free cytoplasmic calcium. Among synthetic activities of mitochondria, those of steroids are the most important. Opening a mitochondrial transition pore causing release of cytochrome *c* and its interaction with proteins of the Bcl and Bac/Bax family comprise a central mechanism for apoptosis. Only a small fraction of mitochondrial proteins are encoded within mitochondrial DNA (mtDNA), and most of the genes for mitochondrial proteins are located in the nucleus. This renders mitochondria sensitive to genetic disturbances within the nucleus and within the mitochondria as well. Considering these many functions, mitochondrial integrity is a key requirement for cell performance. Being the main site of ROS production, mitochondria are the primary targets for oxidative damage, in particular because of very limited DNA-repair mechanisms as compared to the nucleus (Bohr and Dianov, 1999; Bohr et al., 1998). The dynamic nature of mitochondria provides quality control mechanisms stabilizing mitochondrial function.

Mitochondria appear spherical and ovoid or are elongated structures within cells. However, mitochondria continuously fuse and divide, and by this, the single mitochondrion represents only a transient manifestation of a reticulum termed chondriome. The nature of single mitochondria has been challenged (Park et al., 2001; Rizzuto et al., 2001) but has to be discussed in relation to the high mitochondrial dynamics (see Section 3.2.2). In a variety of vertebrate cells in culture, fusion and fission events are very frequent and take place within a few minutes per mitochondrion (Bereiter-Hahn and Vöth, 1994; Chen et al., 2003; Jendrach et al., 2005; Legros et al., 2002; Malka et al., 2005; Mattenberger et al., 2003; Twig et al., 2008, 2010) and exchange matrix (Liu et al., 2009; Twig et al., 2006), inner and outer membrane proteins (Busch et al., 2006; Muster et al., 2010), and mtDNA (Bereiter-Hahn and Vöth, 1996; Gilkerson, 2009). The same is true for plant cells (Arimura et al., 2004) and yeast (Hoppins and Nunnari, 2009). Therefore, it does not matter whether single mitochondria are devoid of nucleoids or not, because the next fusion process again can provide them with genetic material and allows for recombination of mtDNA (D'Aurelio et al., 2004; Kraytsberg et al., 2004; Legros et al., 2004; Nakada et al, 2001, 2009; Ono et al., 2001; Sato et al., 2009; Zinn et al., 1987). This overwhelming dynamics is accompanied by shape changes, branching and bending. In many cell types, mitochondria continuously change their position by saltatoric movements. They migrate along microtubules or other cytoskeletal elements. This locomotion as well as fusion and fission are essential for the physiological integrity

of neurons. Disturbances of mitochondrial dynamics are closely related to degenerative diseases like Alzheimer's, Huntington's, or Parkinson's Disease (Chen and Chan, 2009; Cox and Spradling, 2009; Gunawardena and Goldstein, 2001; Gunawardena et al., 2003; Hurd and Saxton, 1996; Reddy et al., 2009). But also in other cell types, mitochondrial dynamics is a prerequisite for functional integrity (Liesa et al., 2009; Zorzano et al., 2009), and if impaired, interrupts cell cycle progression (Karbowski et al., 2000) and leads to loss of mtDNA (Parone et al., 2008). Summing up, we can distinguish four types of mitochondrial dynamics:

- Movement within a cell which is strongly connected to associations with other organelles and at least in part depends on interactions with the cytoskeleton. These interactions may also be responsible for shape changes as are branching, extension, retraction, and bending.
- Fusion and fission events which provide a huge mixing machine establishing coherence within the chondriome.
- Dynamical behavior of cristae, nucleoids, and diffusion of proteins and lipids within the mitochondria.
- Formation and disintegration of mitochondria (mitophagy).

After a short introduction on how to visualize mitochondrial properties, we will discuss these levels of mitochondrial dynamics in relation to their function. It is not intended to review the older literature (roughly before 1990) because of two reviews of the same author summarizing the structure and appearance of mitochondria as seen by light and electron microscopy (Bereiter-Hahn, 1990; Bereiter-Hahn and Vöth, 1994). The more recent work focuses on the molecular basis of mitochondrial dynamics on which we will emphasize in this review.

2. How to Visualize and Quantify Mitochondrial Dynamics

Visualization of mitochondria in living cells became an easy task (Fuller and Arriaga, 2003). Either small fluorochromes staining selectively mitochondria or fluorescent proteins are used. Uptake of small fluorescent molecules mostly depends on $\Delta\psi_{mit}$, for example, rhodamine 123, TMRME, DASPMI, JC-1 and JC-4, and mitotracker red (MTR). Others indicate mitochondrial pH or pCa^{2+} in the mitochondrial matrix, or the factors guiding dye accumulation are not really known (e.g., mitotracker green, NAO). MTR is very special among these dyes because its uptake is driven by $\Delta\psi_{mit}$, but then covalent binding to proteins stabilizes dye incorporation which now survives chemical fixation. Mitochondria themselves are fluorescent structures: Flavoprotein and NADH fluorescence

monitor redox states and can be quantified (Kajstura and Bereiter-Hahn, 1988; Mayevsky and Barbiro-Michaely, 2009).

Fluorescent proteins targeted to mitochondria opened a new area for research (Rizzuto et al., 1995) because these fluorochromes can be targeted not only to the matrix, rather all complexes of the respiratory chain and F_1F_0-ATP-synthase, and outer mitochondrial membrane (OMM) proteins have been labeled successfully. Multiple labeling either by transfecting with more than one fluorescent protein or combining fluorescent protein labeling with $\Delta\psi_{mit}$—sensitive dyes have been performed. All the mitochondrial fluorescent proteins (mtFP) vectors use the targeting sequence of cytochrome *c* oxidase subunit VIII to achieve mitochondrial matrix localization; IMM-PAGFP is the mitochondrial targeting sequence of ABCB10 fused with PAGFP (Twig et al., 2006).

Difficulties arise with plant mitochondria where a novel targeting model for protein import to mitochondria has been identified (Chatre et al., 2009). Therefore and because of interspecies differences, a different strategy for labeling with fluorescent proteins has to be followed (Logan and Leaver, 2000), replacing introduction of the GFP gene into the nuclear genome; this strategy is the use of a chimeric gene carrying appropriate regulatory sequences and a mitochondrial targeting signal (Köhler et al., 1997).

High-resolution light microscopy as 4pi microscopy provides excellent 3D images of the overall shape of mitochondria (Egner and Hell, 2005; Schmidt et al., 2008) but not of their inner membrane arrangements. Further advanced systems, that is, STED-microscopy or STORM (Huang et al., 2008) at the moment do not allow visualization of mitochondrial dynamics because of the relatively long duration needed to acquire single images. But this situation will change in future. In mitochondria with large distances between cristae, for example, in megamitochondria (e.g., Karbowski et al., 1999) or in some HeLa cell mitochondria, the fluorescence pattern of IMM-bound FPs comes close to the visibility of cristae and their dynamics. Electron microscopy has been used to show mitochondrial dynamics indirectly by correlation with light microscope observations (e.g., Bereiter-Hahn and Vöth, 1994).

Thus, visualization of mitochondria in living cells is performed almost exclusively by fluorescence techniques. The high contrast, that is, the grey level difference between the organelle of interest and its environment is a big advantage over previous contrasting procedures, because quantification of dynamics by a computer-assisted image analysis has become a realistic task today.

Because of the significance of mitochondrial dynamics for aging and a variety of diseases (see Brenner and Mak, 2009; Graham et al., 2010; Guglielmotto et al., 2010; Seo et al., 2010; Wallace, 2005), statistical methods have to be applied to monitor and to quantify these dynamics in response to different physiological and pathological conditions. Shape changes, intracellular trafficking, and membrane potential modulations are the main tasks for quantification. Classifications like "fragmented/spherical, swollen, elongated, and interconnected" are widely

used to characterize the overall mitochondrial shape (Jendrach et al., 2008; Mai et al., 2010; Michiorri et al., 2010). This approach is not very precise and contains subjective elements. These problems have been overcome by quantifying shape changes by determining the aspect ratio (ratio between the long axis and the extension vertical to it; deVos et al., 2005).

Short time dynamics can be estimated by adding up a time series of pictures yielding a single 2D image of the total area covered by mitochondria within the chosen time range. Normalization of area summation is reached by division of this area by the area covered by mitochondria as determined within a short time, excluding motion. The result provides a measure of shape changes plus locomotion. A more detailed automated analysis of 3D stacks of confocal images of human skin fibroblasts was presented by simultaneous measurements of mitochondrial morphology (number, length, and branching), mass, and $\Delta\psi_{mit}$ comparing fibroblasts from CI-deficient patients with those from healthy donors (Koopman et al., 2006, 2008). Mitochondria were stained with rhodamine 123, which allowed, at the same time, analysis of morphology and membrane potential. Furthermore, the analysis was extended to follow Ca^{2+} dynamics within the cells (Koopman et al., 2008; Willems et al., 2009). This elegant method, however, is restricted to very well spread out cells with almost no overlap of mitochondria.

The time course of $\Delta\psi_{mit}$ can be followed by fluorometry of mitochondria stained with the appropriate potential sensitive dyes which can easily exchange between mitochondria and the cytoplasm or extracellular space. In case the probes remain within the cytoplasm, measurements are limited to those compounds changing their quantum yield when becoming incorporated into the IMM (e.g., DASPMI; Ramadass and Bereiter-Hahn, 2008), otherwise $\Delta\psi_{mit}$ changes would be masked by the fluorescence increase in the cytoplasm. Using TMRE loading of mitochondria, the group of Aon and Cortassa succeeded in demonstrating $\Delta\psi_{mit}$ modulations in cardiomyocytes *in situ* as well after isolation, distinct from random behavior, with dynamics covering a range of at least three orders of magnitude (from milliseconds to minutes) under physiological conditions. The frequency distribution obeys a homogenous power law with a spectral exponent, $\beta = 1.74$ (Aon et al., 2006a,b). This type of computational analysis is remarkably powerful for the description of complex dynamics.

3. Levels of Mitochondrial Dynamics and their Functional Significance

3.1. Localization and movements of mitochondria within cells

In well spread out animal cells in culture and in neurons, some mitochondria permanently move toward the periphery and again back to the perinuclear region. There, most of the mitochondria remain almost stationary because they

are more integrated into reticular superstructures (Bereiter-Hahn et al., 2008; Yaffe et al., 2003). Wrapping of a mitochondrial reticulum around a nucleus is a widespread phenomenon from animal cells to plant cells (e.g., apical shoot cells of *Arabidopsis*), to green algae (Atkinson et al., 1974; Blank and Arnold, 1981; Calvayrac et al., 1972; Hermann and Shaw, 1998; Sequí-Simarro et al., 2008; Yaffe, 1999, 2003; Zadworny et al., 2007). Also, tethering of mitochondria to the nuclear envelope and membrane bridges between the organelles have been described (Prachař, 2003). This morphology favors the support of mitochondria by nucleus-encoded proteins (see Section 3.1.1) and mutual control of gene expression according to external signals and functional requirements (Ryan and Hoogenraad, 2007). Similar to the role of ER–mitochondria associations, also those close to the nuclei can be involved in regulation of nuclear Ca^{2+} changes (Alonso et al., 2006). Close apposition of nuclei and mitochondria was speculated to be a mechanism to expose the nucleus to hypoxia and thus to protect nuclear DNA against ROS (Skulachev, 2001). This hypothesis is not immediately convincing because $\Delta\psi_{mit}$ in perinuclear mitochondria is often higher than of peripheral mitochondria, showing that hypoxia is not in a range-reducing respiration. But using isolated cytoplasm, Niethammer et al. (2008) demonstrated that $\Delta\psi_{mit}$ can still be high (close to its maximum) also at low oxygen concentrations, corresponding to our previous findings that inhibition of respiration in living cells does not necessarily perturb $\Delta\psi_{mit}$ (Bereiter-Hahn et al., 1983). Despite high $\Delta\psi_{mit}$, ROS production can still be high (Guzy and Schumaker, 2006; Niethammer et al., 2008), $\Delta\psi_{mit}$ alone does not determine ROS production levels (e.g., Dikov et al., 2010). Finally, reduced oxygen levels paired with high $\Delta\psi_{mit}$ might well provide a mechanism for protection against ROS by mitochondria accumulated around nuclei, but experimental evidence is still missing. Two additional reasons for accumulation of mitochondria in the perinuclear area have to be mentioned, a potential high energy demand of the nucleus and dysfunctional motor molecules. Trapping of mitochondria by structures with high energy demand, for example, plasma membrane with high activities in ion transport or signaling, is a well-established phenomenon in many cell types, as revealed by electron microscopic studies (Bereiter-Hahn, 1990; Fawcett, 1981; Germer et al., 1998a, 1998b; Hollenbeck and Saxton, 2005; Munn, 1974; Riva et al., 1999), and may result from immobilization by increased ADP concentrations (Bereiter-Hahn and Vöth, 1983) or from interactions with cytoskeletal elements (e.g., Perkins et al., 2010; Sung et al., 2008; see Section 3.1.2). Loss of $\Delta\psi_{mit}$ renders mitochondria immobile and promotes their accumulation in the cell center. This can also be achieved by factors interfering with the cytoskeleton and motor molecules including members of the dynamin family (Pitts et al., 1999) or the CLU1 gene product in Dictyostelium discoideum and *Saccharomyces cerevisiae* (Fields et al., 1998). However, just an accumulation of immobile organelles in the central part of a cell, aside the nucleus, has to be distinguished from mitochondria wrapping around the nucleus with extensive branching and high $\Delta\psi_{mit}$.

Slow dislocations are often accompanied by the formation of branches or shape changes making mitochondria creep through the cytoplasm like worms (Bereiter-Hahn, 1990; Bereiter-Hahn and Vöth, 1994), but also these deformations may result from the interaction of mitochondria with cytoskeletal elements and be driven by the activity of motor molecules connecting the OMM to cytoskeletal fibrils. Small spherical mitochondria can reach a speed up to 0.5 μm/s without any visible shape changes. In neurons, speed might even be higher, as Misgeld et al. (2007) determined peak velocities of 1.02 μm/s for anterograde movements and 1.41 μm/s for retrograde movements in mouse neurons. Elongated mitochondria only rarely reach these high speeds, probably because of simultaneous activity of motor molecules driving into different directions. Velocities up to 2 μm/s have been determined for the saltatory movements along microtubule tracks in the filamentous fungus *Neurospora crassa* (Steinberg and Schliwa, 1993). In budding yeast, mitochondria extend from the mother cell to a growing bud at a speed of 49 $\pm$ 21 nm/s (Simon et al., 1995).

Neurons represent the most extended cell types in animals. Therefore, mitochondrial locomotion is of utmost significance in these cells. Perturbation of mitochondrial locomotion impairs fusion and fission of mitochondria and inevitably induces neurodegenerative diseases, as mentioned before (Gunawardena and Goldstein, 2004). In developing neurons, mitochondria accumulate near growth cones (Morris and Hollenbeck, 1993) and locate to synaptic terminals in dendritic spines as well as axonal buttons (Chada and Hollenbeck, 2004; Li et al., 2004; Rowland et al., 2000; Shepherd and Harris, 1998; Spacek and Harris, 1998; Spirou et al., 1998) where they are required to maintain synaptic transmission (Chan, 2006; Stowers et al., 2002). Remodeling of the actin cytoskeleton on neuron depolarization by *N*-methyl D-aspartate (NMDA) induces the Wiskott–Aldrich syndrome protein verprolin homologous-1 (WAVE1) to bind to mitochondria where it promotes fission and trafficking from dendritic protrusions to dendritic spines and filopodia, and thus stimulates spine morphogenesis and enhances synaptic plasticity (Sung et al., 2008). The tension applied to mitochondria by motor molecules may promote recruitment of Drp1 to form the scission ring (Ingerman et al., 2005) which then can move back and forth along the mitochondrion (Bereiter-Hahn, 1990), similar to tension-induced dynamin assembly (Roux et al., 2006). WAVE1 targeted to mitochondria becomes part of a complex of other cAMP-regulated signaling molecules, including glucokinase and BH3 pro-apoptotic Bcl-2 homolog (BAD; Danial et al., 2003) impacting on organelle network and respiration (Carlucci et al., 2008).

In embryos, mitochondria accumulate around the nuclei or germinal vesicles, and a second group stays in the subcortical cytoplasm (Barnett et al., 1996; Bavister and Squirrel, 2000). Another type of multiple mitochondrial aggregates has been found during *Drosophila* oogenesis in functionally

impaired mutants of the genes *clu* and *park*, and thus establishing *Drosophila* mutants as models for Parkinson's Disease (PD; Cox and Spradling, 2009).

In plant cells, mitochondria appear to be carried along with other organelles within the streaming cytoplasm, but motion analysis showed that the motions of different mitochondria and of other vesicular organelles are not synchronized, thus more specific interactions have to be considered. In filamentous hyphae of fungi, vivid mitochondrial movements occur back and forth and make them accumulate at the growing tip (Fuchs et al., 2002; Scheckhuber et al., 2007; Suelmann and Fischer, 2000), while in the spherical cells of budding yeast, slow extensions of mitochondria participate in bud formation (Altmann et al., 2007, 2008; Drubin et al., 1993).

3.1.1. Interactions of mitochondria with endoplasmic reticulum

Among the organelles interacting with mitochondria and contributing to their dynamics, the ER is of utmost significance because of the delicate interplay in Ca^{2+}-messaging (e.g., Pizzo and Pozzan, 2007; Rizzuto and Pozzan, 2006; Willems et al., 2009). Thus, an ubiquitous occurrence of ER–OMM interactions can be assumed. This interaction was shown in a large variety of developing oocytes (Dumollard et al., 2006) as well as in many metazoan cells in culture. In well spread out heart endothelial cells and other vertebrate cells in culture, mitochondria are never found in areas devoid of ER, and mitochondria exclusively move along ER traits (Bereiter-Hahn and Vöth, 1983). Joint trafficking of ER and mitochondria has also been found in HEK cells stimulated by carbachol (Brough et al., 2005). This points to rather stable or at least continuing connections.

The existence of special ER areas tethered to mitochondria was suggested by cosedimentation of membranes from both organelles in cell lysates (Ardail et al., 1993; Camici and Corazzi, 1995; Gaigg et al., 1995; Shiao et al., 1995; Vance, 1990; Zinser et al., 1991). These membranes were termed "mitochondria associated membranes" (MAM). Electron microscopy revealed a distance between 10 and 60 nm between ER and OMM (Achleitner et al., 1999). The same group estimated 80–110 contacts of the ER with mitochondria in a yeast cell, considerably more than to any other organelle. About 20% of mitochondrial surface may be in direct contact with the ER (Rizzuto et al., 1998) which was confirmed by electron tomography (Mannella et al., 1998; Renken et al., 2009). Although both organelle systems are highly dynamic, MAM–mitochondria interactions seem to be stable for at least some minutes, otherwise copurification should not be possible (Lebiedzinska et al., 2009). Improper spacing between the MAM and OMM alters their function, that is, small spaces increase the susceptibility to mitochondrial Ca^{2+} overload and may elicit opening of the mitochondrial permeability transition pore, while widening of the may reduce Ca^{2+}-sequestration by mitochondria (Csordás et al., 2006). On the OMM, interactions can take place with the voltage-dependent anion

channel 1 (porin) (Linden and Karlsson, 1996) and with the mitochondrial heat shock protein mtHSP70 (Szabadkai et al., 2006a,b), Drp1 has early been identified as a further molecule mediating tethering (Pitts et al., 1999), but its action might be restricted to cases when fusion/fission equilibrium is shifted toward fission. Overexpression of the Drp1 K38A mutant which lost its GTPase activity causes the formation of big aggregates containing this protein bound to ER and shaping it to tubules (Yoon et al., 2001). Among other tethering proteins are Mfn2 and S-100 within the macromolecular complex (De Brito and Scorrano, 2008; Hayashi et al., 2009). Components of the tethering complex are functionally connected not only to calcium signaling sites but also to phospholipid biosynthesis (Kornmann et al., 2009).

The intimate association of mitochondria with the ER is a key element in the regulation of cellular Ca^{2+} responses by fast Ca^{2+} uptake into mitochondria and consecutive slow release as well as fueling SERCA with ATP (Willems et al., 2009). As a consequence, ER properties close to mitochondria differ from those devoid of this association, but mitochondria may take up Ca^{2+} also without special connections to the ER (Szanda et al., 2006). Several excellent reviews summarized the structural and functional aspects of the complex interplay between ER and mitochondria recently (Celsi et al., 2009; Garcia-Dorado et al., 2008; Giorgi et al., 2009; Griffiths and Rutter, 2009; Hayashi et al., 2009; Lebiedzinska et al., 2009; Ryu et al., 2010; Santo-Domingo and Demaurex, 2010). The description of these topics is beyond the scope of the present review, the role of Ca^{2+} controlling interactions with the cytoskeleton and mitochondrial locomotion is discussed in the following chapter.

3.1.2. Interactions of mitochondria with cytoskeleton

3.1.2.1. Complex interplay of mitochondria with microtubules and actin fibrils In animal cells, mitochondria move along microtubules; whether they are using the actomyosin system for locomotion is not clear yet. Locomotion depends on intact electron transport (Bereiter-Hahn and Vöth, 1983), therefore involvement of OMM at the sites of ADP/ATP exchange (Viitanen et al., 1984) seems reasonable; however, this part of the complex has not been studied till today, but porin-enriched areas have been identified as binding sites to cytoskeletal elements (Leterrier et al., 1994; Linden and Karlsson, 1996). Also in developing sperm cells, mitochondria become attached to the axonemes by distinct areas, however, not chemically identified (Ho and Wey, 2007).

Mitochondria and the intermingling networks of the cytoskeleton share the property of penetrating the whole cytoplasm in a complicated and well-ordered manner. Because of the high density of cytoskeletal elements, close apposition of different organelle systems is unavoidable, thus specific interactions can only be distinguished from mere appositions by functional relationships and knowledge of molecules mediating mutual binding. In animal cells, as are fibroblasts, epithelial cells or neuronal cells, the interaction

of mitochondria with microtubules and their role in mitochondrial motion has been well established (e.g., by Aufderheide, 1979, 1980; Caviston and Holzbaur, 2006; Heggeness et al., 1978; Hollenbeck, 1996; LaFountain, 1972; Leterrier et al., 1994; Linden et al., 1989; Summerhayes et al., 1983). More recently, high-resolution light microscopy revealed images of extreme clarity showing the very intimate contact of mitochondria to microtubules in BS-C1 cells (Huang et al., 2008). In those cells obviously microtubules represent the guiding structures for intracellular displacements of mitochondria (Ligon and Steward, 2000; Morris and Hollenbeck, 1995). Microtubule disrupting drugs have been found to induce mitochondrial fragmentation (Heggeness et al., 1978; Kedzior et al., 2004), the same was reported for cytochalasin treatment (Rojo et al., 2002). Whether this is a direct consequence of microtubule/actin disruption or results from subsequent induction of apoptotic processes by these drugs remains unclear. We exposed HeLa cells as well as chicken embryonic fibroblasts for hours to the microtubule disrupting drug nocodazole. Morphology was changed insofar mitochondria had a strong tendency to form loops (Legros et al., 2002) which finally gave rise to rings by internal fusion (Fig. 1.1), but other fusion and fission processes no longer took place. These rings are not to be confused with cup- or disc-shaped mitochondria typical for exposure to a large variety of drugs inhibiting respiratory activities. They are also different from Drp1-based fragmentation of mitochondria (deVos et al., 2005). In human umbilical vein endothelial cells (HUVEC), movements of mitochondria ceased in the presence of nocodazole (Fig. 1.1). This observation is consistent with observations on an osteosarcoma line by Karbowski et al. (2000) and after β-tubulin knockdown what might cause some swelling and disorganization of mitochondrial arrangement but no fission (Tang et al., 2008).

In animal cells, microtubules preferentially emanate from the perinuclear area with the centrosome (minus end of microtubules) toward the cell periphery. Trafficking of membranous organelles along these microtubule rails in general is driven either by a member of the dynein family (for retrograde transport) or a member of the kinesin superfamily (KIF; for anterograde direction). Leterrier et al. (1994) proposed a control model releasing static binding of mitochondria to microtubules via MAP2 by ATP and thus providing free binding sites for kinesin which then drive mitochondrial motion along the microtubules. Porin-rich areas in the OMM have been identified as sites for attachment of mitochondria to microtubules and neurofilaments (Leterrier et al., 1994). More recently, by electron tomography of cat axons, cristae structure was found polarized in that their junctions to the inner boundary membrane were aligned with a complex cytoskeletal superstructure and occurred at higher density in mitochondrial areas facing the presynaptic membrane (Perkins et al., 2010).

Several members of the KIF, the kinesins-1 (KIF5B) and kinesins-3 (KIF1B), localize preferentially to mitochondria (Boldogh and Pon, 2007;

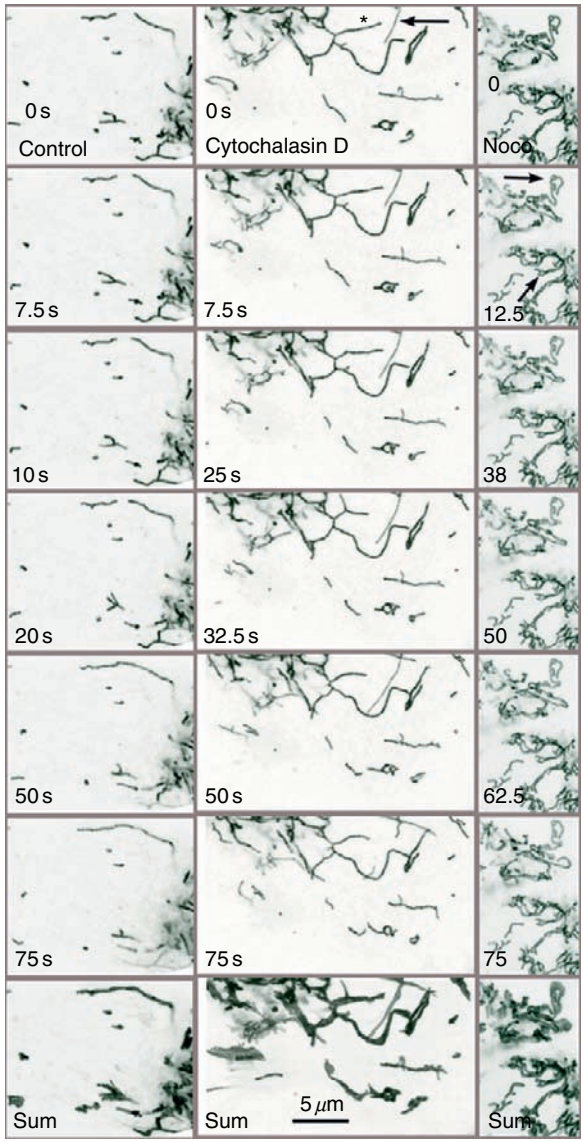

Figure 1.1 Influence of cytoskeleton disruption on mitochondrial dynamics: HUVEC were treated for 90 min either with 25 μM nocodazole or 2 μg/mL cytochalasin D. Untreated cultures served as controls. Mitochondria were stained using Mitotracker Deep Red (25 nM for 20 min). A series of pictures of the same position is shown at different time points as indicated. The end of each row shows the sum of maximum intensities over 30 frames (= 75 s) as a measure of total dynamics including movements and shape changes. The most prominent movements, extensions, and retractions of mitochondria occur after CD treatment (arrow in 0 s image marks an extension, * marks a retraction followed by branching). In nocodazole-treated cells, lateral, "vibrational" movements prevail, mitochondria no longer move, branch, extend, or retract. Typical shapes are ring formations within single mitochondria (arrows in 12.5 s image). Control mitochondria show an intermediate behavior. (Confocal laser scanning microscopy series taken by M. Vöth.)

Hirokawa and Takemura, 2005; Nangaku et al., 1994; Pereira et al., 1997). Disruption of the heavy chain of KIF5B clustered mitochondria in the perinuclear region in mouse embryonic cells (Tanaka et al., 1998). Mitochondrial movements in both anterograde and retrograde directions may depend on kinesins as was shown for larval *Drosophila* axons where inhibition of kinesin-1 causes depletion of axons from mitochondria. The role in retrograde motion, however, could be a modulating one rather than providing the driving force (Pilling et al., 2006). This assumption is supported by yeast two hybrid findings that the intermediate chain of dynein binds to the kinesin light chains 1 and 2 (Ligon et al., 2004). In a similar manner, dysfunction of dynein also impairs movements in both directions (Varadi et al., 2004). Motor molecules mostly are linked via adaptor-proteins to the OMM. Syntabulin links mitochondria to KIF5B and thus mediates anterograde trafficking along axonal processes (Cai et al., 2005). It binds to the kinesin-1 heavy chain (KHC) but possibly also loosely associates with phospholipids of the OMM via its hydrophobic tail (Cai et al., 2005). Little is known how kinesin and dynein motors interact and how many motor molecules bind per mitochondrial length (Gross et al., 2007).

Considering the high significance of mitochondrial trafficking, redundant mechanisms driving these organelles can be expected. Such a further mechanism is represented by the Miro–Milton complex which connects mitochondria to kinesins (Glater et al., 2006; Guo et al., 2005; Stowers et al., 2002) and controls displacements via its Ca^{2+} sensitivity (Liu and Hajnóczky, 2009; Wang and Schwarz, 2008). Milton has been detected as a protein involved in the transport of mitochondria in *Drosophila melanogaster* photoreceptors (Stowers et al., 2002) and can recruit kinesin-1 via its heavy chain to the OMM, as shown for HEK293T and Cos-7 cells (Glater et al., 2006). Miro is a rho-like GTPase and an OMM integral protein also required for both anterograde and retrograde axonal mitochondrial transport in *Drosophila* axons (Russo et al., 2009).

Retrograde trafficking of mitochondria in nerve axons is mediated by the dynein–dynactin complex (Waterman-Storer et al., 1997) including MAP2 (Linden et al., 1989); however, disruption of dynein function in HeLa cells by overexpression of the dynactin subunit dynamitin (p50) resulted in perinuclear accumulation of mitochondria, their elongation, and intensive branching (Varadi et al., 2004). Elongation of mitochondria can be explained by impairment of the dynein-driven translocation of the fission protein Drp1 to mitochondria (which in addition, is controlled by G-actin and phosphorylation at S100). Aggregation of mitochondria around the nucleus by inhibition of the retrograde transporter dynein coincides with the same type of localization on disruption of microtubules by nocodazole (Smirnova et al., 1998). However, Cos-7 cells transfected with the dominant negative mutant of Drp1-spread mitochondria on disruption of microtubules. Cross-regulation of dynein and kinesin activity would

explain cessation of mitochondrial trafficking (Martin et al., 1999; Pilling et al., 2006) but not the retrograde dislocation and its reversal in presence of the dominant-negative Drp1. Retrograde movement could be due to cytoplasmic bulk flow induced by contraction of the actomyosin system resulting from activation of myosin light chain kinase following impairment of microtubule-based fluxes (Karl and Bereiter-Hahn, 1998; Kolodney and Elson, 1995). The spread of mitochondria in cells expressing dominant-negative Drp1 may be a consequence of the actin-binding capacity of Drp1, counteracting actomyosin-based contraction. This hypothesis has to be proved experimentally.

Microtubule-associated proteins other than motor molecules are also involved in mitochondrial trafficking, in neurons in particular. Among those, tau protein plays a significant role by stabilizing microtubules and retarding the transport of neurofilament proteins and of membrane-bound organelles, including mitochondria, toward the cell periphery, probably by interfering with the binding of kinesin-like motors (Ebneth et al., 1998). Overexpression of the microtubule-associated protein tau almost fully blocks anterograde transport of mitochondria and renders more than 50% immobile (20% in controls) in chick retinal ganglion cells (Stamer et al., 2002).

Are mitochondrial movements via microtubules or actin fibrils? The molecular machinery underlying mitochondrial distribution within cells can be very different even in closely related organisms (Westermann and Prokisch, 2002; Yaffe, 1999). Mitochondrial transport in budding yeast is actin dependent (Boldogh et al., 2001b; Hermann and Shaw, 1998; Jensen et al., 2000; Simon et al., 1995, 1997), while in fission yeast, it depends on microtubules (Yaffe et al., 1996).

Myo2, a class V myosin, modulates distribution of mitochondria in budding yeast and has been shown to act as a motor driving mitochondria (Altmann et al., 2008). The binding of mitochondria to F-actin is ATP sensitive (Lazzarino et al., 1994; Simon et al., 1995) and mediated by Mdm10p (Sogo and Yaffe, 1994) as well as Myo2 (Altmann et al., 2008) on the outer membrane side. This binding seems to be an integral part of a regulatory network involved in the control of ROS production, premature aging, apoptosis, actin dynamics, and the Ras-cAMP pathway (Breitenbach et al., 2005; Gourlay and Ayscough, 2005a,b). *In vitro* experiments with immobilized isolated mitochondria allowed actin cables to move across the organelles on the addition of ATP, as to be expected for actomyosin-driven motions (Simon et al., 1995). This is in line with the role of Myo2p in the control of mitochondrial transmission and retention to the bud (Altmann et al., 2008) which is controlled by a small G-protein Jsn1p (Boldogh et al., 2004). However, deletion of yeast myosin genes did not affect mitochondrial movement (Simon et al., 2005) excluding myosin as the only motor driving mitochondria into the bud. Retention within the bud, however, is

mediated by Myo2- and Jsn1p interaction (Boldogh et al., 2004). Evidence was brought forward that actin polymerization and interaction via the Arp2/3 complex drives anterograde movement of mitochondria (Boldogh and Pon, 2007; Boldogh et al., 2001a, 2004). When F-actin cables are destroyed (e.g., by latrunculin), no more mitochondrial trafficking takes place in budding yeast (Drubin et al., 1993). This reaction is to be expected for myosin-driven movements along actin fibrils as well as for polymerization-driven displacements. Boldogh et al. (2001a) stabilized F-actin using an actin mutant and by this impeded motion. In a second experimental approach, they kept the overall F-actin pattern constant by latrunculin, counteracting excessive polymerization. Under these conditions, half the mitochondria moved and their speed was reduced by about 1/3. Interpretation of this result is not easy, because a large amount of short actin fibrils could increase cytoplasmic viscosity and slow down movements. Thus, interpretation of these results remains ambiguous. Lee and Liu (2008) proposed a model for actin polymerization-driven organelle movement, which does not require regular actin cables and shows that the speed for dislocation directly depends on Arp2/3 concentration. In several systems, WASP and WAVE-AbiI-based protein complexes act in concert with Arp2/3 to drive endocytotic vacuoles, organelles, or bacteria within the cytoplasm (Innocenti et al., 2005; Welch, 1999). Cytoplasmic motility driven by actin polymerization without any involvement of myosin is a wide-spread phenomenon (Pollard, 2007; Pollard and Borisy, 2003) and may represent an evolutionarily preserved self-organizing system for the maintenance of cytoplasmic integrity by repair (Henson et al., 2002) which still acts on small membranous structures. Proteins similar to Arp2/3 have also been found in plants, but their role for organelle movement remains unclear (McKinney et al., 2002).

Retrograde mitochondrial movement in budding yeast follows the retrograde flow of actin (Fehrenbacher et al., 2004) as is well known from retrograde flow of cortical actin networks and actin at the front part of leading lamellae. Two types of protein complexes are involved in driving force generation and linking cargo to the cytoskeletal track, the mitochore and the Arp2/3 complex, which is recruited to the OMM by Isn1p/Puf1p to drive anterograde motion. Linking the Arp2/3 complex to the mitochore via Puf3p downregulates anterograde movement (Fig. 1.2; Boldogh and Pon, 2007).

In the fission yeast, *Schizosaccharomyces pombe*, mitochondria become tethered to microtubules and bound to the mitotic spindle which moves them by microtubule polymerization (Yaffe et al., 2003). Their migration is not influenced by kinesins but is disturbed if microtubule dynamics is impaired. Mitochondria can bind to plus ends of microtubules via Mmd1p, a cytoplasmic protein stabilizing microtubules (Boldogh and Pon, 2007). In addition, the microtubule-stabilizing protein CLASP

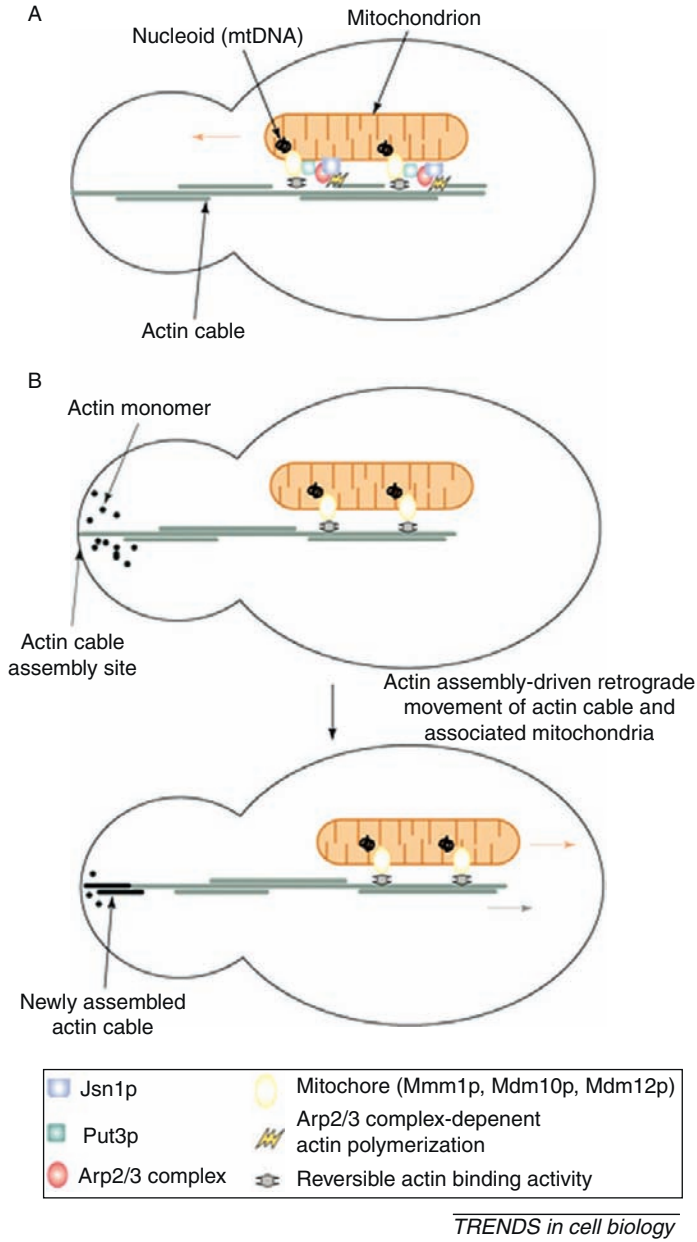

Figure 1.2 Model for bidirectional transport of mitochondria driven by actin in budding yeast. Bundles of actin fibrils align along the mother–bud axis. A multiprotein complex, termed mitochore, mediates reversible binding of mitochondria and mt nucleoids to actin cables. (A) Anterograde movement is driven by the Arp2/3 complex which is recruited to the mitochondrial surface by Jsn1p/Puf1p and stimulates force generation for bud-directed movement via actin polymerization at the location of Arp2/3. (B) Retrograde mitochondrial movement is driven by the retrograde flow of actin cables, resulting from the push exerted by formin-stimulate actin polymerization at the bud-directed end of the actin cables and the pull of type II myosins. (From Boldogh and Pon, 2004, with permission.)

(Bratman and Chang, 2008) connecting microtubules to the cell cortex is also involved in intracellular distribution of mitochondria in fission yeast (Ambrose and Wasteneys, 2008; Chiron et al., 2008).

Among filamentous fungi, *Neurospora* is a good example for microtubule-based mitochondrial trafficking (Fuchs et al., 2002; Prokisch et al., 2000; Steinberg and Schliwa, 1993, 1995), while in *Aspergillus*, exclusively microfilaments are involved (Oakley and Rinehart, 1985; Suelmann and Fischer, 2000). In *Neurospora*, the Unc104/KIF1-related members of the kinesin family, Nkin2 and Nkin3, bind to mitochondria. Nkin2 mediates interactions of mitochondria with microtubules. Only in mutants lacking functional Nkin2, Nkin3 is recruited to mitochondria and rescues from the motility defect (Fuchs and Westermann, 2005).

In plant cells, mitochondrial displacements originally were considered as bulk movements within cytoplasmic streaming. Skillful observations of organelle trafficking warrant some revision of this concept: simultaneous analysis of the trafficking patterns and velocities of Golgi stacks, peroxisomens, and mitochondria in *Nicotiana benthamiana* (*Solanaceae*) cells failed to show coordinated organelle movements or preferential direction patterns, the movements were saltatory rather than continuous (Avisar et al., 2008; Nebenfuhr et al., 1999). Thus, single organelles can be supposed to be driven along actin cables (Watanabe et al., 2007) by motor molecules attached to them, their locomotion drives that of the cytosol by viscous friction. However, in cases of elongated cells with linearly organized actin fibrils (Hepler et al., 2001; Smith and Oppenheimer, 2005), such as vascular epidermis or root hairs (Peremyslov et al., 2008) or *Chara* internodal cells (Foissner, 2004), the single organelle-based movements may add up to the observed directional flow. In these cases, the concept of directional flow is compatible with single organelle-based locomotion. These actin-guided motions require myosins as motor molecules (Sheahan et al., 2005). Among the two types of myosins present in higher plant cells, myosins VIII and myosins XI, only myosin XI-K is involved in trafficking of mitochondria (Li and Nebenführ, 2007), Golgi stacks, and peroxisomes. Myosins XI-2 and XI-F exert only marginal effects (Avisar et al., 2008). This has been extended to XIE as a motor for these organelles in tobacco leaf epidermal cells by overexpression of dominant negative tails of myosins XI lacking the actin binding domain (Sparkes et al., 2008). The members of the myosin XI—family are closely related to myosin V responsible for vesicular trafficking in animal cells and in fungi (e.g., Reisen and Hanson, 2007). Thirteen different myosin XI genes have been found in *Arabidopsis* (Reddy, 2001). The tail domains of other than myosin XI-K bind to unidentified vesicular structures but are not targeted to mitochondria or Golgi stacks (Reisen and Hanson, 2007). Also in plant cells, the GTPase Miro 1 is a player in mitochondrial trafficking. This protein is ubiquitous in plant cells. In *Arabidopsis* mutant, Miro 1 impairs pollen tube development,

disrupts the movement of mitochondria, and results in abnormally enlarged mitochondria (Yamaoka and Leaver, 2008).

In Characean internodal cells, mitochondria are supposed to be passively transported within the streaming endoplasm but also may become attached to microtubules and actin-cables (Foissner, 2004). As stated earlier, in plant cells, mitochondrial movements are driven by interaction with actin, disruption of microtubules does not inhibit this motility, rather it seemed to promote. Therefore, mitochondria in plant cells are immobilized by microtubules and mobilized by actin fibrils (Van Gestel et al., 2002) and thus behave inversely than mitochondria in animal cells (Fig. 1.1).

Neurons are the mammalian cell type attracting most attention on mitochondrial displacement along axons and dendrites. Mitochondria move along microtubules in both directions without major shape changes or fission and fusion processes. At the distal end of an axon, the growth cone, mitochondria are supposed to switch the track and the motor molecule being trapped by actin fibrils (Morris and Hollenbeck, 1995) in a way similar to the transport of melanosomes in melanophores (Wu et al., 1998). Further work is needed to test whether neurons use such a mechanism to retain mitochondria at their extremities (Rube and van der Bliek, 2004).

Disruption of F-actin in CV1-4A cells (mammalian cells with endothelial appearance) does not change the mitochondrial aspect ratio (ratio between long and short axis), but it attenuates fission in mitochondria because actin is involved in recruiting the fission protein Drp1 to the OMM (deVos et al., 2005). In addition, branching of mitochondria is strongly activated by the addition of cytochalasin D, a drug severing actin fibrils (Fig. 1.1). This type of mitochondrial shape changes has not been resolved at the molecular level till today.

Ample evidence is in favor of suppressed mitochondrial motion by interaction with F-actin in animal cells. Some examples are a slowdown in trafficking in senescent cells stuffed with actin cables (Jendrach et al., 2005) or immobilization of mitochondria by cortical F-actin at the NGF-receptor binding sites in chick sensory neurons (Chada and Hollenbeck, 2004). Interactions with actin not only result in motility changes, F-actin binding also enhances the sensitivity to apoptotic stimuli (Gourlay and Ayscough, 2005b; Tang et al., 2006), while G-actin protects against apoptosis by closing the voltage-dependent anion channel and thus retarding the release of cytochrome *c* (Kusano et al., 2000).

3.1.2.2. Interactions with intermediate filaments Specific interactions between intermediate filaments and mitochondria are more difficult to establish than those to microtubules or actin cables, because agents influencing microtubule organization also alter intermediate filament arrangement, in particular that of vimentin filaments which are extensively cross-linked to microtubules (Hirokawa, 1982). Among intermediate filaments, vimentin

plays an important role in preserving mitochondria from degeneration via premature aging of the cell (Tolstonog et al., 2005). The protection mechanism, however, has not yet been understood. More direct evidence of vimentin-mitochondria interactions was brought forward by subcellular fractionation, immune precipitation and immunofluorescence of Cos-7 and 3T3 cells by Tang et al. (2008). Disruption of the vimentin network by vimentin siRNA induced mitochondrial fragmentation and swelling (Tang et al., 2008).

In mouse, cardiomyocytes desmin organizes filament assembly and also mitochondrial localization (Milner et al., 1996). The function of this interaction may be stabilization of mitochondrial position in highly motile cells. Proteins cross-linking different cytoskeletal elements can be assumed to act in concert, as plectin, a powerful cytoskeletal cross-linker is binding to mitochondrial outer membrane as well (Reipert et al., 1999) or MAP2, which connects microtubules to the mitochondrial porin voltage gated anion channel (Linden and Karlsson, 1996) or the intermediate fibril-microfilament cross-linker dystonin, which is also involved in mitochondrial positioning (Daipe et al., 1999). Intermediate fibril association with mitochondria exceeds a function in positioning as shown by decreased respiratory activity of mitochondria in desmin-null soleus or cardiac fiber bundles. Desmin knockout can produce extensive mitochondrial proliferation under load conditions (Milner et al., 2000). Intermediate filaments bind a large variety of molecules, including ribosomes and RNA, therefore the lack of these filaments or their disorganization has been hypothesized, causing the release of factors involved in regulation of outer membrane permeability for adenine nucleotides (Kay et al., 1997; Saks et al., 1995, 1998). Mdm1p, which is involved in mitochondrial transport to the bud in budding yeast, shows some relationship to intermediate filaments and thus might be related to some evolutionary precursors of this filament type in eukaryotes (Fuchs, 1994).

3.2. Functional significance of mitochondrial movements

On the function of anterograde and retrograde movement of mitochondria in animal cells, we only can speculate. These movements are a prerequisite for fusion and fission of mitochondria and thus an important factor in mitochondrial quality control (Mouli et al., 2009). Those organelles that remain static no longer come into contact for fusion and cannot be eliminated after fission if they are dysfunctional. Neuronal degenerations are the consequence of impaired trafficking as is evident for some neurodegenerative diseases summarized as tauopathies (Buee et al., 2000; Ishihara et al., 1999; Stamer et al., 2002).

Only in muscle cells containing a high density of closely apposed mitochondria, fusion and fission may take place without locomotion (Goncalves

et al., 2009). These mitochondria obviously are static, and muscle cells may have developed other protective mechanisms to maintain mitochondrial functionality despite continuous ROS production. This possibility is impressively shown in senescent cells, where also mitochondrial trafficking is reduced (Jendrach et al., 2005; Mai et al., 2010). Extensive reticulum formation of mitochondria and altered gene expression substitute for reduction in locomotion (Mai et al., 2010). Mitochondria trapped at sites of high energy demand, for example, in dendritic spines of axonal buttons, are small and neither seem to fuse or divide, but they may become mobilized again and then move retrogradely. Most probably the interaction with the complex cytoskeletal arrangements will control whether they stay or become mobilized for retrograde movements (Perkins et al., 2010).

Association of mitochondria with structures of high energy demand implies the existence of intracellular metabolic gradients, that is, ATP/ADP ratio, localized synthesis of proteins, or local differences of cytoplasmic Ca^{2+}. The ability of mitochondria to sense such gradients has impressively been demonstrated by Niethammer et al. (2008). Such an ATP-sensing mechanism also seems to be involved in the regulation of mitochondrial RNA transcription (Amiott and Jaehning, 2006). Another reason for trafficking in both directions could be the need for mitochondria to become refreshed with proteins encoded by nuclear DNA and being primarily synthesized in the perinuclear zone and thus less available in the cell periphery. The presence of metabolic gradients in extended cells is suggested by observations of lower $\Delta\psi_{mit}$ in the cell periphery (Bereiter-Hahn et al., 1983; Liu et al., 2009; Twig et al., 2008). This corresponds to higher $\Delta\psi_{mit}$ in neuronal mitochondria moving in an anterograde direction than those moving retrogradely (Miller and Sheetz, 2004). However, the speed of motion is not directly correlated with membrane potential; only after prolonged inhibition of the respiratory chain or uncoupling, the motion ceases (Loew et al., 1993). Because of lower volume fraction of mitochondria in the cell periphery they have to supply more cytoplasm with ATP and therefore the ATP/ADP ratio may decrease and attract mitochondria or act as a transient local trap for mitochondria. The possibility for the existence of localized ATP/ADP concentrations has been shown for isolated Xenopus ooplasm (Niethammer et al., 2008), and both elevated ADP and ATP concentrations immobilize mitochondria, as was revealed by microinjection experiments (Bereiter-Hahn and Vöth, 1983). However, no specific tropism of mitochondria has ever been reported. Association of mitochondria with energy consuming structures, that is, the ER, is in accord with this interpretation as well as microcompartmentation of adenine nucleotides, as shown for the fueling of the actomyosin machinery by *local* ATP supply (Tillmann and Bereiter-Hahn, 1986; van Horssen et al., 2009). 3D reconstruction of a variety of plant cells revealed a particular close association between mitochondria, peroxisomes, and chloroplasts, which also seem to represent a physiological triad (Zellnig et al., 2004, 2009).

Increased Ca^{2+} concentration reversibly stops mitochondrial motion and induces severe changes in their structure (Han et al., 2008; Stolz and Bereiter-Hahn, 1987; Fig. 1.3). Ca^{2+}-induced fission of mitochondria results from phosphorylation of Drp1, increasing its affinity for Fis1 and thus translocating it from the cytoplasm to the OMM (Han et al., 2008). Intracellular Ca^{2+} gradients indeed exist, as has been shown after histamine stimulation (Szabadkai et al., 2004), or are induced by fertilization (Dumollard et al., 2006). In cold-blooded vertebrates, kreatine kinase is compartmentalized in a Ca^{2+}-dependent manner, and by this, influences oxygen consumption by mitochondria (Birkedal and Gesser, 2006).

In yeast, locomotion along cytoskeletal elements is required for appropriate distribution toward the forming bud (Boldogh and Pon, 2007). In green algae, in plant cells as well as in animal cells, this distribution occurs during cytokinesis, most probably just by separating two populations in the two compartments forming the daughter cells without any specific arrangements. This type of separation requires mitochondria which are not too long as to interfere with the cytokinetic furrow. Increased mitochondrial fissions have been attributed to phosphorylation of Drp1by cyclin B-dependent kinase, producing small mitochondria which facilitate their distribution to the daughter cells (Taguchi et al., 2007). This process may be part of a complicated signaling net and thus does not evoke uniform morphotypes as was shown for mitochondria in mitotic HUVEC which mostly remain elongated (Bereiter-Hahn et al., 2008). Mitochondrial nucleoids, however, seem to become smaller and more uniform than in interphase cells (Bereiter-Hahn and Vöth, 1996). The opposite principle is realized in some plant cells (e.g., *Arabidopsis* shoot meristematic cells) by forming a giant reticular mitochondrion which then wraps around the mitotic spindle and becomes distributed to the two daughter cells before it falls apart in cytokinesis (Sequí-Simarro et al., 2008). A still unresolved problem is the presence of mitochondria forming a collar in parallel to the spindle fibers which then remain for a prolonged time within the meiotic cleavage furrow of some insect spermatoblasts.

Accumulation of organelles in the perinuclear area is a very general process for animal cells and is a consequence of dynein-based retrograde movement along microtubules. This affects endosomal vesicles as well as mitochondria. In contrast to the anterograde movements, perinuclear aggregation occurs independent of the presence of $\Delta\psi_{mit}$, thus granular mitochondria lacking membrane potential gather around the nucleus and then no longer are transported along microtubules. Depending on the quality of mitochondria, this accumulation might either support interaction of dysfunctional mitochondria with autophagosomal elements, or in the case of active mitochondria, enhance protein import from an environment enriched with mRNA. Rapid organelle trafficking is a characteristic of all green plants, but its functional significance remains unclear. Some mitochondria

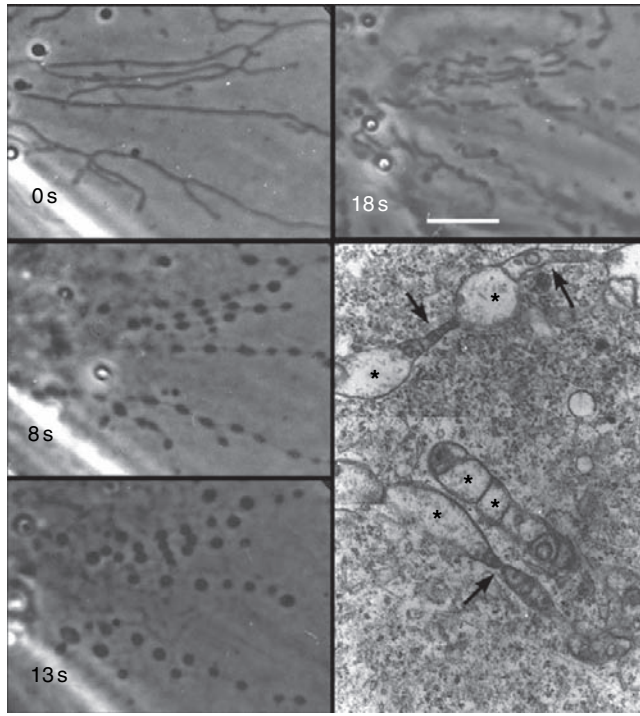

Figure 1.3 Reversible granulation of mitochondria in a living Xenopus endothelial cell (XTH2), injected with Ca^{2+}. Slow injection was done by microiontophoresis (8 nA for 11 s) via a capillary electrode filled with 1 mM $CaCl^{2+}$ solution. The bright zones at the lower left corner of the phase contrast images in the left row show a part of the capillary electrode with its tip close to the nucleus. Within seconds after the onset of Ca^{2+} injection, the long and branched mitochondria start "granulation": frames at 0 s (before injection), 8 s after the start of injection, 16 s (16 s after start and 5 s after stop of Ca^{2+} release), and 18 s all show the same cellular area. Eight seconds after the onset of injection, mitochondria look like beads on a string, the connecting parts are lost subsequently (16 s). After this short challenge, recovery of mitochondria is fast, but because of extensive fission, a larger number of tubular mitochondria are formed which are shorter than those they originated from (18 s). In the 18 s image, cytoplasmic contraction can be seen. The electron micrographs represent a state corresponding that after 8 s injection. The intracristael spaces are extremely enlarged (black stars), corresponding to the swollen areas in the phase image, these zones are separated by constricted areas, containing thin, lamelliform cristae. These zones (arrow in electron micrograph) are prone to fission most probably because of the formation of Drp1-evoked fission sites, as described by Hom et al. (2007) for thapsigargin-treated cells. The swelling of the intracristael spaces forming blown-up vesicular structures indicate mitochondrial Ca^{2+} uptake, most probably followed by water and K+. Bar in the 18 s frame: 5 μm (adapted from Stolz and Bereiter-Hahn, 1987).

might remain immobile and closely associated with chloroplasts (e.g., Foissner, 2004), others migrate very rapidly. Avisar et al. (2008) assume that rapid trafficking might facilitate delivery of secreted macromolecules and cytoplasmic stirring as a replacement of cytoplasmic streaming in higher plants.

3.2.1. Heterogeneity of mitochondria in single cells

In many cells, single mitochondria transiently behave as single organelles and in fact represent one possible state within the chondriome. Elements within the chondriome are continuously exchanging their constituents by fusion and fission (Busch et al., 2006; Liu et al., 2009; Muster et al., 2010; Twig et al., 2008). So far, a homogenous population is to be expected, just varying in the degree of network formation versus single spherical or tubular elements. Transient changes of $\Delta\psi_{mit}$ in *single* mitochondria, as revealed by dye fluorescence, represent a basic form of mitochondrial heterogeneity within a single cell. Such changes have been reported repeatedly (e.g., Belousov et al., 2001; Buckman and Reynolds, 2001; Collins and Bootmann, 2003; Krippeit-Drews et al., 2000) and may occur even within different zones of a single long mitochondrion (Bereiter-Hahn and Vöth, 1998; Smiley et al., 1991). This individual behavior could be an internal property of the mitochondria or just a consequence of different energy demands within a given cytoplasmic volume fraction, for example, at contact sites with the plasma membrane or the ER. Oscillations of $\Delta\psi_{mit}$ of mitochondria isolated from rat brain, however, showed that they can occur independently from cytoplasmic influences, and that mitochondria with stable $\Delta\psi_{mit}$ differ from the oscillating ones in their response to external factors to raised Ca^{2+}, and thus were considered to represent an intermediate activity state (Vergun et al., 2003; Vergun and Reynolds, 2004).

Occasionally, electrical coupling among a large group of mitochondria was found in human skin fibroblasts and neonatal cardiac myocytes (Amchenkova et al., 1988) and in Cos7 cells (De Giorgi et al., 2000). Whether electric coupling exists in addition to fusion remains an open question (Collins et al., 2002; Zorov et al., 2000). $\Delta\psi_{mit}$ oscillations were not related to opening of the mitochondrial permeability transition pore. In cardiomyocytes, TMRE-stained mitochondria have been shown by power spectral analysis to act as a highly correlated network of oscillators (Aon et al., 2006a,b, 2009). Besides $\Delta\psi_{mit}$, also NADH and ROS production oscillated in isolated cardiomyocytes (Aon et al., 2003). Disturbance of these coordinated oscillations, and thus development of spatial and temporal mitochondrial heterogeneity, may render them very sensitive for any perturbations by ROS and then cause cardiac arrhythmias (Aon et al., 2006b). According to studies using inhibitors, coordination is related to oscillating activities of the IMM anion channel modulating the mitochondrial benzodiazepine receptor (Aon et al., 2003, 2008, 2009) and not necessarily result

from synapse type connections or formation of a homogenous network of fused mitochondria. Nevertheless, large areas of fused mitochondria may exist in cells (Park et al., 2001; Rizzuto et al., 1998) and can be easily proved following the distribution of photoactivable fluorochromes (Molina and Shirihai, 2009; Twig et al., 2006). Such an interconnected chondriome is typical for some postmitotic cells (Mai et al., 2010). However, the existence of single mitochondria is well proved by laser-induced photodamage of single mitochondria without affecting their immediate neighbors (Bereiter-Hahn and Vöth, 1998) or by ablation of single mitochondria with the same result (Shen et al., 2005).

Nevertheless, mitochondria can be of considerable heterogeneity within a single cell (Collins et al., 2002, 2003; Diaz et al., 1999; Kuznetsov et al., 2004, 2006; Wikstrom et al., 2007), reflecting the different demands of the surrounding cytoplasm. An impressive example is the coexistence of mitochondria with lammeliform cristae in the subplasmalemmal region and those with tubular cristae in the interfibrillar spaces of human cardiomyocytes, reflecting differences in respiratory activities and physiological functions (Hoppel et al., 2009; Riva, 2005).

Also shifting between fused networks and single organelles does not exclude the fact that mitochondria being dislocated toward the cell periphery face metabolic conditions that differ from those in the perinuclear area, thus giving rise to metabolic heterogeneity of mitochondria within a given cell. If the chondriome falls apart into two or more populations which do not fuse with each other, then real and lasting heterogeneity will emerge. Both these situations have been described. Kuznetsov et al. (2004) described a heterogenous redox response of mitochondria to the addition of dihydroxyacetone as a substrate in liver cells. The differences in flavin oxidation were not related to certain sites within the cells, the heterogeneously responding populations appeared spatially mixed. In HL-1 cells, mitochondria within a single cell considerably differed in their sensitivity against photooxidative stress (Kuznetsov et al., 2009). These variations correspond to the increased heterogeneity of mitochondria in senescent (Hagen et al., 1997; Jendrach et al., 2005) and in ROS-exposed cells (Jendrach et al., 2008) and in apoptotic cells (Krysko et al., 2001).

Positional heterogeneity of mitochondria, as revealed by differences in $\Delta\psi_{mit}$, has been reported repeatedly for cardiomyocytes (Jahangir et al., 1999; Kuznetsov et al., 2006; Skulachev, 2001) including size differences (Unverferth et al., 1981), for skeletal muscle (Battersby and Moyes, 1998; Lombardi et al., 2000), for endothelial cells (Bereiter-Hahn et al., 1983; Jendrach et al., 2005), and for fibroblast-like cells (Liu et al., 2009; Tolstonog et al., 2005; Twig et al., 2008). In the cerebellar cortex of adult rats, the size of mitochondria within single cells was found to be inversely correlated with their cytochrome *c* oxidase activity (Bertoni-Freddari et al., 2003) by means of a histochemical quantification; however, more detailed

studies failed to reveal direct relationships between the size of mitochondria and their metabolic activities (Benard et al., 2007), although the fusion proteins, Mfn1 and Mfn2, as well as Opa1, stimulate respiration and increase $\Delta\psi_{mit}$ (Chen et al., 2005; Ryan and Hoogenraad, 2007; Zorzano et al., 2009). In addition, $\Delta\psi_{mit}$ of mitochondria (HeLa, HUVEC, cardiomyocyte) oscillates independently throughout a cell without any preference for intracellular localization (Collins and Bootmann, 2003). Differences in matrix density of mitochondria within a single cell are a frequent phenomenon, but despite the fact that high matrix density indicates a low ATP/ADP ratio, this is difficult to comprehend if two mitochondria with different matrix density are very close together (e.g., Shepard et al., 2000).

Mitochondria which lost their membrane potential due to exposure to ROS, ageing or other stressors accumulate in the perinuclear region and become excluded from fusion processes (Jendrach et al., 2005, 2008; Liu et al., 2009; Mai et al., 2010), and finally will be degraded by mitophagy. In fact, this also represents different mitochondrial populations within a single cell. If a time delay occurs between the exclusion of impaired organelles from the rest of the chondriome and their degradation via mitophagy, at least some of the heterogeneities described can be comprehended as states within the life cycle of mitochondria. Intracellular differences in the ability for Ca^{2+} sequestration have been reported for CHO and HeLa cells (Collins et al., 2002; Rutter et al., 1996) as well as pancreatic acinar cells (Park et al., 2001) and chromaffin cells (Montero et al., 2002).

Genetic heterogeneity is a matter of debate (Howell et al., 2000). In a somatic cell, up to 5000 mtDNA molecules are supposed to be present. If they are all the same, the condition is termed homoplasmy, if two or more mtDNA sequence variants are present, the cell (tissue, organ or organism) is said to be heteroplasmic. In practice, it will be difficult to distinguish whether mitochondria differ from cell to cell or within a single cell; however, some of the disease-related mitochondrial mutations are clearly heteroplasmic (Fan et al., 2008; Howell et al., 2000; Salvioli et al., 2008; Stewart et al., 2008).

Segregation of mitochondria into two different populations is a characteristic for oocytes and zygotes of many organisms (amphibians, fishes, insects, planarians, chaetognaths, nematodes as well as mammals) (Dumollard et al., 2006). After fertilization, two populations of mitochondria can be found in the zygote. Mitochondria of male origin become destroyed by mitophagy (e.g., Cummins, 2000) or their components are ubiquitinylated and degraded via proteasomes (Sutovsky et al., 1996, 2003). Proteasome activity seems to represent a general mechanism for maintenance of mitochondrial integrity acting in animal cells (Carlucci et al., 2008) as well as in plant cells, as shown for Picea pollen tube growth (Sheng et al., 2010). In the Medaka, recombination between male and female mtDNA obviously can be avoided by fast degradation of male mtDNA, even if the mitochondria themselves might still

be present (Nishimura et al., 2005). Mitochondria of female origin accumulate in the perinuclear area of zygotes (Bavister and Squirrel, 2000). Just in the developing oocytes of *Drosophila* as well as of vertebrates, two lines can be distinguished, one accumulating into a special structure (the Balbiani body) segregating to the germ line (Cox and Spradling, 2003; Kloc et al., 2004) and the rest of the mitochondria becoming scattered in the whole zygote and getting transmitted to the somatic lineage. Movement of mitochondria forming the Balbiani body is regulated by the Miro/Milton complex (Cox and Spradling, 2006), as has been described for other types of mitochondrial trafficking as well. At later stages of embryonic development, oxidative capacity of mitochondria may change according to the tissue-specific requirements, as was shown for developing germs of the sea urchin *Paracentrotus lividus* (Morici et al., 2007). In budding yeast, after fusion of haploid cells of opposite mating type, mitochondrial proteins from the two parent cells mix rapidly after fusion, mtDNA, however, remained distinctly localized to one half of the zygotic cell (Nunnari et al., 1997).

3.2.2. Fusion and fission of mitochondria

The complementary processes of mitochondrial fission and fusion regulate the morphology of the mitochondria. Dependent on cell type and the physiological situation, mitochondria display different morphotypes: (a) small and rounded (fragmented), (b) elongated, tubular, and (c) interconnected networks. In mammalian cells, fusion is regulated by the GTPases, *mitofusin* 1 and 2 (Mfn1 and Mfn2), situated in the OMM, and *optic-nerve-atrophy* 1 (Opa1), which is localized in the IMM. In yeast, Fzo1 is the ortholog of Mfn1/2 and Mgm1 is an ortholog of Opa1 (Westermann, 2008). Fusion of both mitochondrial membranes can occur separately (e.g., Meeusen and Nunnari, 2007), and fusion of the inner membrane is dependent on an intact mitochondrial membrane potential (Malka et al., 2005).

Mutations of Mfn2 or Opa1 cause neurological disorders: loss of functional Mfn2 elicit *Charcot-Marie-Tooth type 2A disease*, which results in the degeneration of axons in the distal extremities (Zuchner et al., 2004). In correlation with this phenotype, Mfn2 interacts with the transport-associated proteins, Miro1 and Miro2, as well as with Milton, indicating an additional role of Mfn2 in the kinesin-driven transport of mitochondria in axons (Misko et al., 2010). Mutations of *OPA1* cause autosomal dominant *optic atrophy*, where degeneration of retinal ganglia cells results in the loss of fibers of the optic nerve system (Alexander et al., 2000; Delettre et al., 2000). Mfn1, Mfn2, or Opa1 knockout mice are embryonically lethal, and the mitochondria in MEF isolated from these mice exhibited the expected strongly fragmented morphotype (Alavi et al., 2007; Chen et al., 2003; Davies et al., 2007).

As additional fusion factors in mammalian cells, *Stomatin-like protein* 2 (SLP-2) and MARCH-V are discussed. SLP-2 interacts with Mfn2 (Hajek et al., 2007), and a downregulation of SLP-2 by shRNA resulted

in a loss of stress-induced hyperfusion (Tondera et al., 2009). Also, the human *membrane-associated RING-CH-V* (MARCH-V) interacts with Mfn2, and overexpression of MARCH-V shifts the equilibrium toward mitochondrial fusion, while mutations of this protein induce mitochondrial fission (Nakamura et al., 2006). A knockdown of MARCH5 induces senescence of Chang cells, mediated by increase of Mfn1 expression and/or inhibition of fission factor Drp1 (Park et al., 2010).

In yeast, another GTPase, Mgm1, resides in the intermembrane space and the inner membrane and mediates together with Opa1 the fusion of the IMM (Meeusen et al., 2006). The outer membrane, Ugo1, is part of a complex containing the yeast fusion factors, Fzo1 and Mgm1 (Wong et al., 2003), and is required for fusion of the outerand the inner membrane in yeast (Hoppins et al., 2009).

The proteins, *dynamin-related protein 1* (Drp1) or *dynamin-like protein* (Dlp1) (in yeast Dnm1) and *fission protein 1* (Fis1) (in yeast Fis1), are the main mitochondrial fission factors in mammalian cells. Drp1 is localized in the cytoplasm and translocates to the mitochondria to initiate their division (Frank et al., 2001; Han et al., 2008). In yeast, Dnm1 interacts with the outer membrane-based protein Fis1, which serves as an anchor for Drp1 (Mozdy et al., 2000; Tieu et al., 2002). In mammalian cells, overexpression of Fis1 shortens mitochondria, while downregulation of Fis1 elongates them, but a direct interaction between Fis1 and Drp1 is still under discussion as Drp1 translocates also in the absence of Fis1 to the mitochondria (Lee et al., 2004). On the other hand, pull-down experiments revealed binding of Drp1 to Fis1 depending on its phosphorylation at Ser-600 (Han et al., 2008). For the actual process of division, Drp1 molecules are predicted to form a ring-structure similar to the Dynamin-mediated processes during endocytosis (Youle and Karbowski, 2005). Drp1 knockout mice are embryonically lethal and mitochondria and peroxisomes are accordingly elongated in these mice (Wakabayashi et al., 2009). Furthermore, transgenic mice with a depletion of Drp1 in neuronal cells die shortly after birth, due to loss of neurons and an inhibition of synapse formation (Ishihara et al., 2009). Apart from modulations of the expression level, the activity of Drp1 can also be stimulated through SUMOylation via the *SUMO E3 ligase mitochondrial-anchored protein ligase* (MAPL; Braschi et al., 2009).

Other mitochondrial fission factors identified in mammalian cells are Mff (Baxter et al., 2002), *ganglioside-induced differentiation activated protein* 1 (GDAP1; Niemann et al., 2005; Pedrola et al., 2005), *mitochondrial protein 18 kDa* (MTP18; Tondera et al., 2004, 2005), and *mitochondrial targeting GxxxG motif protein* (MTGM; Zhao et al., 2009). The first three are mitochondrial outer membrane proteins, while MTGM resides in the IMM. Overexpression of each of these factors results in mitochondrial fragmentation, while silencing causes increased mitochondrial fusion. In yeast, the proteins, Mdv1 (Tieu et al., 2002) and Caf4 (Griffin et al., 2005), are found in

the cytoplasm as well as in the OMM, and both proteins can act as adaptor proteins between Dnm1 and Fis1 after translocation of Dnm1 from the cytoplasm to the future mitochondrial fission sites (Westermann, 2008).

Fusion requires mitochondria coming in close contact to one another. This can either be achieved by intracytoplasmic locomotion or just by close apposition as is the case in skeletal muscle and cardiomyocytes. Fission is more closely related to the action of motor molecules tugging on different regions along a mitochondrion. Therefore, disassembly of microtubules by nocodazole inhibits both fusion and fission (Fig. 1.1); however, after disassembly of F-actin, mitochondria become more mobile again and can fragment (Fig. 1.4). Spherical mitochondria lacking membrane potential gather around the nucleus and are no longer transported along microtubules. Also in senescent human endothelial cells (HUVEC), mitochondria become arrested and fusion and fission cease (Jendrach et al., 2005). In addition, actin as well as dynein are involved in recruiting the fission protein Drp1 to the OMM (deVos et al., 2005; Varadi et al., 2004).

Despite the ongoing fission and fusion activity, the overall mitochondrial morphology for a given cell type remains quite stable. The overall constant morphotype originates from an equal rate of fission and fusion. Under physiological conditions, fusion and fission are carefully balanced (Jendrach et al., 2005; Twig et al., 2008). Within a given cell, up to half of the mitochondria may be devoid of mtDNA (Bereiter-Hahn and Vöth, 1996). Frequent fusions are a mechanism to ensure continuous supply with mRNA derived from mtDNA also to those mitochondria lacking it. This hypothesis is supported by the observation that about 80% of fusion processes of mitochondria in vertebrate cells in culture are followed by fission within short time intervals (Bereiter-Hahn and Vöth, 1996; Liu et al., 2009). Mitochondrial dynamics allow the complementation of mtDNA mutations *in vitro* and *in vivo* (Legros et al., 2004; Nakada et al., 2001, 2009; Ono et al., 2001; Sato et al., 2004, 2009; Zinn et al., 1987), which supports the hypothesis that mitochondrial fusion and fission dynamics play a role in the mitochondrial quality control system (Bossy-Wetzel et al., 2003; Gilkerson, 2009; Mai et al., 2010; Tatsuta and Langer, 2008).

The equilibrium between fission and fusion and thus the mitochondrial morphology is changed toward fusion during a process called *hyperfusion*. In reaction to different stressors, mitochondria undergo a strongly increased fusion, starting 2–3 h after the addition of the respective stress. *Hyperfusion* is mediated by the proteins, SLP2, Mfn1, and Opa1, and seems to act as a prosurvival mechanism (Tondera et al., 2009). A similar *hyperfusion* occurs in the flight muscle mitochondria of a mosquito (*Aedes aegypti*) upon blood-feeding due to increased expression of Mfn and Opa1; in parallel, a reduction of respiration and ROS production was observed (Goncalves et al., 2009). In normal rat kidney (NRK) cells, the transition from G1 to S phase

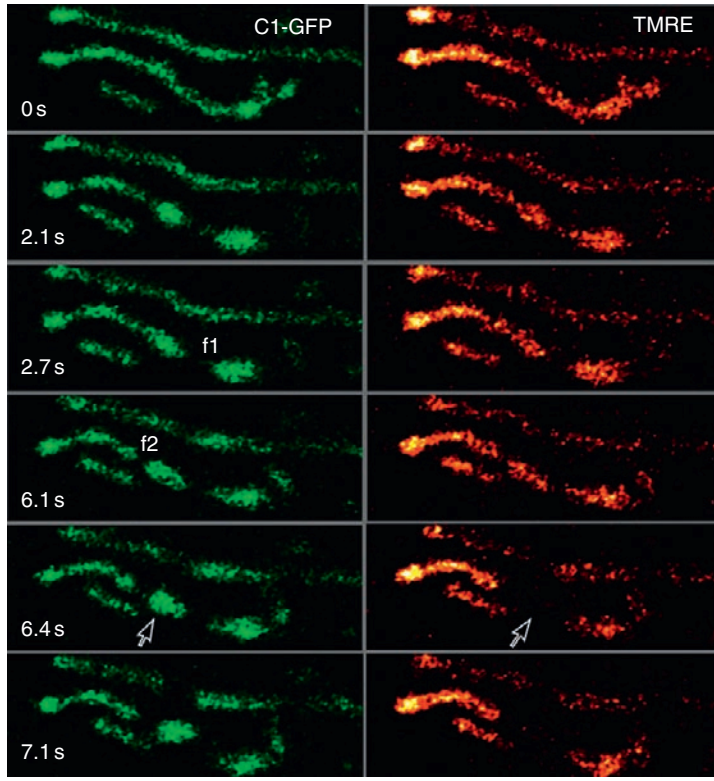

Figure 1.4 Segregation of a mitochondrial part finally lacking $\Delta\psi_{mit}$. HeLa cells were treated with 25 μM nocodazole for 30 min and then 2 μg/mL cytochalasin D was added for another 20 min. Transfection with GFP labeled 30 kDa subunit of the respiratory complex C1 labeled IMM selectively. Counter staining by 100 nM TMRE for 20 min revealed $\Delta\psi_{mit}$. Despite disassembled microtubules and actin fibrils, mitochondria change shape and divide, but they do not move any more. In two fission steps (f1 and f2), a small mitochondrion is released from the central area of the elongated mitochondrion. Within less than 300 msec after the second fission step, TMRE fluorescence is lost from the fragment (arrow in C1-GFP frame 6.4 s). This proves that this part is not functional. Before the second fission step, the functional parts of the mitochondrion kept $\Delta\psi_{mit}$ and TMRE was distributed over the full length on the mitochondrion. After the second fission step, each of the three mitochondrial fragments had to maintain its own $\Delta\psi_{mit}$. A general decrease of fluorescence intensity is a consequence of bleaching. Each single frame measures 10.25 $\times$ 3.7 μm. (Confocal laser scanning series taken by Daniel Dikov.)

is also characterized by *hyperfusion,* indicating a putative link between mitochondrial dynamics and cell cycle control (Mitra et al., 2009).

In many senescent cells, mitochondria display an extensive elongation and/or network formation of mitochondria (Mai et al., 2010; Navratil et al.,

2008; Unterluggauer et al., 2007; Yoon et al., 2006; Zottini et al., 2006). In Chang cells, experimental downregulation of the fission factor Fis1 resulted in premature senescence (Lee et al., 2007a,b), leading to the proposal that elongation of mitochondria induces senescence. Taken together, the appearance of elongated or interconnected or giant mitochondria is characteristic of many senescent cell types and thus it seems possible that this distinct morphotype has a functional role in senescent cells. This hypothesis is supported by the fact that experimental elongation of mitochondria extended the life span of the fungus *Podospora anserina* and of the yeast *Saccharomyces cerevisae* (Scheckhuber et al., 2007). Life time is shortened when the fission gene PaDnm1 is overexpressed (Scheckhuber et al., 2008). Furthermore, the elongation of mitochondria in cell culture models conferred resistance to apoptotic stimuli (Jahani-Asl et al., 2007; Lee et al., 2004; Sugioka et al., 2004), and recent data indicate that mitochondria of senescent HUVEC are more resistant against mitochondria-directed stress, thus indicating indeed a functional role of elongated and interconnected mitochondria in senescent cells (Mai et al., 2010). In the same context, the suppression of fission resulted in protection against ischemia/reperfusion damage (Ong et al., 2010). Enhanced recombination of mtDNA was identified as an alternative pathway to cope with an age-related increase of impaired proteins and mtDNA in *P. anserina* (van Diepeningen et al., 2010).

The protective mechanisms of long mitochondria are starting to emerge: in an extensive mitochondrial network damaging molecules as e.g., ROS as well as damaged molecules can be rapidly diluted respectively replaced (Mai et al., 2010). Furthermore, the reduction of fission in *rat insulinoma cells* (INS1 cells) either by Fis1 RNAi or overexpression of a dominant negative mutant of Drp1 led to a reduction of the maximum respiratory capacity (in the presence of the uncoupler FCCP), and correspondingly, the ROS production was reduced; however, the overall carbonylation of mitochondrial proteins increased (Twig et al., 2008).

In contrast to the morphological changes described earlier, several other stimuli promote mitochondrial fission. In HeLa cells, fragmentation of mitochondria occurs before cytokinesis due to Cdk1/cyclin B-driven phophorylation of Drp1 (Taguchi et al., 2007). A SUMO protease, SenP5, deSUMOylates Drp1 in COS-7 cells after translocation to the mitochondria at the end of the G2 phase, and by this, probably contributes to mitochondrial fragmentation (Zunino et al., 2009). In contrast, HUVEC exhibit only a slight shortening of mitochondria before cell division, and also, *Xenopus tadpole heart endothelial cells* (XTH2 cells) retain their long tubular mitochondria even during cytokinesis (Bereiter-Hahn et al., 2008).

Several pathological conditions also result in increased fission. Recently elevated hydrostatic pressure was shown to evoke mitochondrial fission in retinal cells (Ju et al, 2007). Excessively increased [$Ca2^{+}$] causes fragmentation of mitochondria (Hom et al., 2007; Stolz and Bereiter-Hahn, 1987;

Fig. 1.3). During oxidative stress/increased ROS levels (Jendrach et al., 2008; Mai et al., 2010; Pletjushkina et al., 2006) and after uncoupling of the respiration from the ATP production by addition of 2,4-*p*-dinitrophenol (DNP; Lyamzaev et al., 2004), mitochondrial fragmentation is observed. In correlation with these data, prolonged exercise resulted in elevated ROS levels, and decreased expression of Mfn1 and Mfn2, respectively increased Fis1 expression in rat skeletal muscle (Ding et al., 2010). *Hyperglycemia*, a condition, which induces transient hyperpolarization, also induces fragmentation of mitochondria that is paralleled by increased ROS production but is absent under mild uncoupling conditions (Edwards et al., 2010; Yu et al., 2006). Recent data demonstrate that overexpression of Fis1 and/or Drp1 result in muscle atrophy (Romanello et al., 2010). Furthermore, increased fission takes place during apoptosis which is also mediated by Drp1 (Frank et al., 2001). This thread-granular transition was termed mitoptosis by Skulachev et al. (2004), but the exact function of mitochondrial fragmentation in the apoptotic cascade is still under discussion (Perfettini et al., 2005; Scorrano, 2009).

Also, pathological disorders, such as Alzheimer's disease (AD), PD, and Huntington's disease, alter mitochondrial dynamics. The PD and Huntington's disease-associated protease Omi/HtrA2 interacts with OPA1, and loss of Omi/HtrA2 resulted in elongated mitochondria and lowered membrane potential (Kieper et al., 2010). In the brain of AD patients, axons exhibited a loss of mitochondria due to relocalization and expression of Mfn1, and Mfn2, OPA1, as well as Drp1 were reduced, while Fis1 demonstrated increased levels (Wang et al., 2009a,b).

The loss of the Parkinson-associated proteins, PINK1 and Parkin, causes increased fission and severe mitochondrial dysfunction in *D. melanogaster* (Cui et al., 2010; Deng et al., 2008; Park et al., 2006; Poole et al., 2008, 2009; Yang et al., 2008) and in correlation Mfn1 interacts with Parkin in *D. melanogaster* (Ziviani et al., 2010). In human cell models, the role of PINK1 in mitochondrial dynamics remains controversial: there is evidence that PINK1 is a fission factor (Yang et al., 2008) as well as a fusion factor (Dagda et al., 2009; Exner et al., 2007). The later hypothesis is supported by recent publications, which link altered mitochondrial fission with Drp1 (Lutz et al., 2009; Sandebring et al., 2009). In contrast, no (Gispert et al., 2009) or only a slight elongation (Gautier et al., 2008) of mitochondria was observed in PINK1 knockout mouse models.

At the moment, these opposing data cannot be explained satisfyingly. A possible explanation could be related to the expression levels of PINK1. While Opa1 normally acts as a fusion factor and increased levels of Opa1 cause mitochondrial elongation and network formation (Cipolat et al., 2004), overexpression of Opa1 sometimes results in mitochondrial fragmentation (Griparic et al., 2004; Misaka et al., 2002). These data indicate that the level of the expressed protein can influence its function. Another

possible explanation could be that the mitochondrial alteration observed in human cells after PINK1 knockdown or overexpression are in fact secondary effects caused by mitochondrial dysfunction. In mouse models, most likely compensatory mechanisms would counteract extreme mitochondrial morphology changes (Lutz et al., 2009), thus leading to a certain morphotype. Taken together, these data indicate a role of altered fission and fusion activity in the development of neurodegenerative diseases.

Fission processes may either yield two or more mitochondria or elongated mitochondria "fall into many pieces". Reversible mitochondrial fragmentation by increasing Ca^{2+} concentration is an example for the latter (Fig. 1.3). If a mitochondrion separates into two, these either may be equivalent in terms of membrane potential or one of them has very low $\Delta\psi_{mit}$. Therefore, fission is supposed to be a mechanism for mitochondria getting rid of dysfunctional components. Double staining of mitochondria using a fluorescent protein in combination with TMRE as a measure for $\Delta\psi_{mit}$, indeed, showed that a mitochondrion formed by fusion of two organelles with approximately the same membrane potential can undergo a subsequent fission process yielding two mitochondria differing in membrane potential (Liu et al., 2009). This internal segregation process is even more pronounced in elongated mitochondria falling into three pieces, a short one containing the dysfunctional components, as shown by loss of $\Delta\psi_{mit}$, and two other parts, both exhibiting the same TMRE fluorescene (Fig. 1.4). As a loss of membrane potential activates autophagy of the respective mitochondrion, at least one function of fission sorts damaged mitochondrial material (see Section 3.4). The usual proteins related to fusion and fission have not been identified in plant cells; however, also in plant cells, the mostly short or granular mitochondria are in a continuous process of material exchange by fusion and fission (Arimuara et al., 2004), and genetic recombination is frequent (Sequí-Simarro et al., 2008).

3.2.3. Morphological changes within mitochondria

Mitochondria consist of several compartments, the OMM, the cristae and the inner boundary membrane enclosing a matrix. This tripartite structure, that is, the differentiation between inner boundary membrane and cristae, is revealed by differences in protein composition (Wurm and Jakobs, 2006). Cristae are not simple infoldings of the IMM. They also represent a special membrane compartment which is connected to the inner boundary membrane by junctions with a diameter between 12 and 40 nm, described first by Sjøstrand (1953) and then made visible in 3D reconstructions from electron tomographic images (Frey and Mannella, 2000; Frey et al., 2002; Mannella, 2006; Mannella et al., 1994; Nicastro et al., 2000; Perkins et al., 1997, 1998). Slot-like and tubular-type junctions have been described (Perkins et al., 2001). In healthy mammalian cells, only the tubular junctions are found.

These junctions reduce the continuity between the intracristael space and the space between IMM and OMM, thus limiting the mobility of cytochrome *c* (85% of it are located in the intracristael space) and of protons required for fueling ATP synthase (Kroemer et al., 2007). In addition, all small molecules as well as membrane proteins are restricted in their diffusibility by the junctional complexes (Mannella, 2006; Scorrano, 2005; Sukhorukov and Bereiter-Hahn, 2009). They are hypothesized to limit movement of enzymatic substrates into the cristae (Mannella et al., 2001; Perkins et al., 1997; Vogel et al., 2006). In mouse liver mitochondria, widening of the junctional complexes removes the diffusion barrier and is a prerequisite for the mobilization of cytochrome *c* from the intracristael space to the intermembrane space (Scorrano et al., 2002) supporting apoptosis. The shape of individual cristae is prone to alterations by varying physiological conditions (Bereiter-Hahn and Vöth, 1983; Mannella, 2006; Zick et al., 2009), including apoptosis (e.g., Scorrano et al., 2002).

3.2.3.1. Metabolism-related crista and matrix morphology The tripartite organization of cristae is the basis of two types of motions, resulting in changes of cristae morphology by swelling and shrinking of the intracristael space mostly in conjunction with counteracting density changes of the mitochondrial matrix. A second type of crista motility is related to fusion and fission processes involving both membranes (see Section 3.2.2). Small intracristael spaces ("orthodox state" according to Hackenbrock, 1972) have been suggested to be caused by matrix expansion compressing the tubular or cisternal structures. In these cases, IMM junctions are small (diameter ca. 10 nm), the small intracristael volume is in favor of a large difference in proton concentration between matrix (high pH and pK^+), while breakdown or reduction of the $\Delta\psi_{mit}$ increases pH and pK^+ within the cristae space, causing water to flow from the matrix toward the intracristael space and thus increasing matrix density and swelling of cristae ("condensed state" according to Hackenbrock, 1972), as is the case of high cytoplasmic ADP concentrations challenging respiratory chain proton pumping activity (Bereiter-Hahn and Vöth, 1983; Shepard et al., 2000). In addition to the adenine nucleotide status, pCa^{++} plays a pivotal role in the regulation of oxidative phophosphorylation and ion transport activities (Hayashi et al., 2009), resulting in volume shifts between intracristael space and matrix (Fig. 1.3). These intramitochondrial volume shifts can take place merely by osmotic shifts without the involvement of any "mitoskeletal" elements, as was shown by Ponnuswamy et al. (2005), and they may be comparable to the budding of tubular structures from giant phospholipid vesicles (Kralj-Iglič et al., 2005). Ponnuswamy et al. (2005) calculated an osmotic difference of about 0.2 bar between intracristael space and matrix in the orthodox state, that is, in the presence of high cytoplasmic ATP levels. Furthermore, cristae shape depends on the distribution of its components, proteins as well as lipids determine the radius of curvature.

Model calculations by Ponnuswamy et al. (2005) showed that the natural respective fraction of phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) esters in the IMM of 27.7% and 44.5% represent an optimal ratio to minimize the differences between the entropic contribution to the free energy and the bending energy savings obtained by distributing the molecules according to shape. In zones of smallest radii of curvature, a shape-based redistribution of the lipid molecules occurs, which causes a 7% shift between the inner and the outer leaflet of the membrane, revealing the dominant role played by the entropic contribution to the free energy at normal physiological temperatures. IMM contain an extremely high protein fraction, therefore, proteins are the main factors determining the overall mitochondrial shape and cristae shape. All these features do not explain the large variety of cristae shapes, which may be flat, tubular, prismatic, vesicular or of mixed appearance (e.g., Fawcett, 1981; Munn, 1974) or forming concentric sheets as is evoked by some mutant proteins (see Section 3.2.3.2).

3.2.3.2. Proteins determining shape of cristae and position of nucleoids The arrangement of three groups of proteins forms the basis of cristae morphology (Fig. 1.5). The connection of cristae to the inner boundary membrane via junctions and their specific junction protein Fcj1 (Rabl et al., 2009), the strong curvature at the apex formed by rows of dimeric ATP-synthase (Strauss et al., 2008), and the respiratory chain complexes building the mass of the cristae. Respiratory chain complexes are arranged as supercomplexes (Schäfer et al., 2006, 2007; Schägger, 2002; Schägger and Pfeiffer, 2000; Wittig et al., 2006), thus stabilization of cristae shape will result. If supercomplex formation is impaired by knockdown of the mitochondrial protein LETM1 (a homologue of the yeast Mdm38), then mitochondria swell and cristae disorganize (Tamai et al., 2008).

A crucial role is played by the rows of dimeric complexes of ATP synthase which are located at the apex of the cristae (Allen et al., 1989; Strauss et al., 2008). Subunits, e and g, are inevitably involved in dimerization (Arnold et al., 1998) and thus appropriate cristae formation (Paumard et al., 2002). Deletion of these dimer-specific subunits results in defective oligomerization of the complex and as a consequence, the positive curvature of the crests or tips of cristae cannot be formed anymore. Cristae extend throughout a mitochondrion, branch and may be connected to the inner boundary membrane at several sites (Rabl et al., 2009). Artificial formation of tetrameric instead of dimeric ATP synthase complexes turns cristae to multiple concentric membrane sheets (Gavin et al., 2004) as occurs in yeasts with monomeric ATP synthase as well (Velours et al., 2009). Rows of dimeric ATP synthase form regions of high membrane curvature which is elicited by these rows (Dudkina et al., 2005, 2006). The same radius of curvature ($\sim$17 nm) is found at the crest regions of flat cristae as in tubular cristae (Nicastro et al., 2000). Simulations of electric field strength over cristae surface indicate a 3.5-fold increase in surface

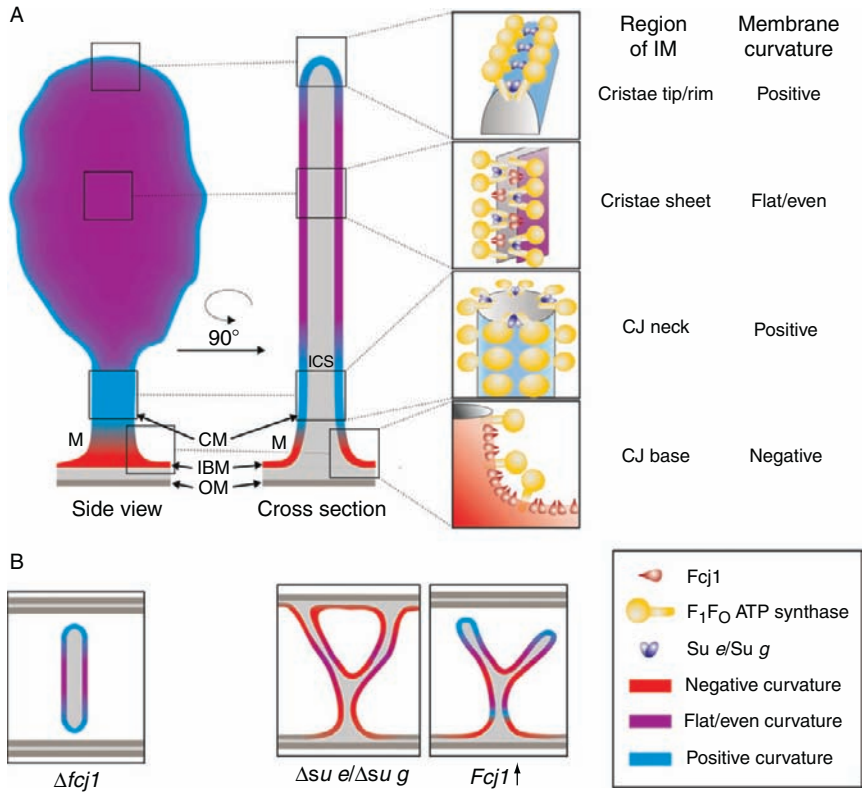

Figure 1.5 Model for shaping cristae. (A) Schematic representation of membrane curvatures at distinct regions of the crista membrane as related to the localization of Fcj1, F_1F_0-ATP synthase, and its subunits, Su e and Su g, responsible for dimerization of the ATP synthase. A representative crista is shown in side view and in cross view, the tentative molecular arrangement at the base of the IMM to inner boundary membrane junction, the junction neck, the flat crista sheet, and the strongly curved rim at the tip of a crista are shown on the right side. (B) Cristae shape evoked by the indicated mutants. In case of fcj1 depletion, no more junctions are formed, cristae are not connected to the outer boundary membrane (negative membrane curvature is missing), while upregulated fcj1 increases the presence of negative curvatures by inducing branching (formation of "excessive junctions"). Inhibition of F_1F_0-ATP synthase dimerization by depletion of Su e and Su g results in enlargement of cristae diameter, positive curvatures as exhibited by crista tips are missing, thus cristae extend from the one side outer boundary membrane to the opposite side. (From Rabl et al., 2009; with permission.)

charge density at the apex, this corresponds to a locally steeper pH gradient of about 0.5 units (Strauss et al., 2008). Thus, cristae shape favors ATP synthesis in the case of flat or tubular morphology. However, under proton-limited conditions, cristae become vesicular, they increase their radius of curvature (see Section 3.2.3.1). Whether they also lose the connections to the inner boundary

membrane is an open question. Therefore, the arrangement of ATP synthase under different physiological conditions still has to be investigated.

Intact cristae junctional protein (termed Fcj1 "formation of cristae junctions" by Rabl et al., 2009) is required for cristae formation. Fcj1 is required to elicit negative curvatures typical for the transition between the inner boundary membrane and cristae. In the case of defective mutants, concentric, onion-shaped inner membrane layers appear, similar to thylakoids in cyanobacteria. Overexpression of the junction protein Fcj1 in yeast increases the mean diameter of the junctions and is accompanied by extensive branching of cristae (Rabl et al., 2009). Thus, the overall shape of cristae seems to depend on local accumulations of Fcj1 proteins and dimerized ATP-synthase at either junction or crest positions. The flat body of cristae would arise from an equilibrium between dispersed Fcj1 and monomeric F_1F_0 ATP-synthase (Rabl et al., 2009; Fig. 1.5).

Mgm1/Opa1 (these are orthologs in yeast and in animal cells) are further proteins involved in maintenance of cristae structure and fusion of IMM by tethering them together (Frezza et al., 2006; Meeusen et al., 2006; Voeltz and Prinz, 2007). Mutations rendering one of these proteins nonfunctional result in the formation of atypical cristae which have lost their connection to the inner boundary membrane (Griparic et al., 2004; Olichon et al., 2003), OPA1 knockdown elicits mitochondrial fragmentation, dissipation of $\Delta\psi_{mit}$, and expression of m-AAA protease paraplegin, probably initiating apoptosis (Ishihara et al., 2006). Because only the L-isoform of OPA1 is fusion competent, the same morphotype can be evoked by destroying the prohibitin complex which processes Opa1 in the IMM (Ishihara et al., 2006; Merkwirth et al., 2008).

The overall shape of mitochondria, whether they appear elongated tubular or "fragmented" spherical, is not only determined by the fusion and fission proteins described earlier; a series of OMM proteins tethered to IMM proteins and thus acting from the outside of a mitochondrion toward the interior influence the cristae shape and nucleoid position and stability (Jensen, 2005). The outer membrane proteins, Mmm1p, Mmm2p, Mdm10p, and Mdm32p (Berger et al., 1997; Burgess et al., 1994; Sogo and Yaffe, 1994; Youngman et al., 2004), and the inner membrane proteins, Mdm31p, Mdm32p, and Mdm38p (Dimmer et al., 2002, 2005, 2007), are required to maintain tubular mitochondria in *S. cerevisiae*. By constructing GFP-fusion proteins, Mmm1p, Mmm2p, Mdm10p, and Mdm12p were found to form small patches ($\sim$0.3 μm in diameter) along mitochondrial tubules They appear to be part of large complexes with loose interactions (Boldogh et al., 2003; Hobbs et al., 2001; Youngman et al., 2004) at the contact sites of IMM and OMM (Kondo-Okamoto et al., 2003). Lack of Mmm2 (Youngman et al., 2004) or the fusion-related large GTPase Fzo1p (Hermann and Shaw, 1998; Rapaport et al., 1998) and Ugo1p cause loss of mtDNA (Sesaki and Jensen, 2001).

Alterations in mitochondrial length may result from shape changes, but primarily they are the products of fusion and fission processes. Long mitochondria have been attributed to higher resistance against stress and as a means to distribute proton gradients throughout large cells which might have spatially limited access to oxygen only where mitochondria can act as cables (Skulachev, 2001). This idea is in accord with observations on reverse relationship of mitochondrial length and cytochrome-oxidase activity (Bertoni-Freddari et al., 2003). Short and granular mitochondria are supposed to facilitate autophagosomal degradation (Section 3.4). On the other hand, local differences in mitochondrial membrane potential (Bereiter-Hahn and Vöth, 1998; Smiley et al., 1991) and the existence of microdomains with different Ca^{2+} dynamics in rat brain capillary endothelial cells (Gerencser and Adam-Vizi, 2005) revealed some limitations of the cable character.

3.2.3.3. Protein mobility (diffusion) As far as is deduced from light microscopic investigations, mitochondria are flexible structures, otherwise they could neither fuse and mix their constituents nor divide. These processes require propagation of constituents along the mitochondria. The functional coupling resulting from fusion is represented by electric coupling, by fast diffusion within the matrix (Liu et al., 2009; Verkman, 2002), and by the diffusion of membrane proteins (Hoppins and Nunnari, 2009; Ishihara et al., 2003; Ono et al., 2001; Twig et al., 2008). OMM and IMM protein mobility is a prerequisite for segregation of impaired mitochondrial part (Fig. 1.4), a mechanism far from being understood. IMM protein mixing after fusion is around six times slower than matrix component mixing (Liu et al., 2009). The mixing of OMM proteins after fusion of mitochondria has first been shown by Ono et al. (2001). Quantitative data, however, are very rare. Indirect evidence for mitochondrial membrane fluidity increase by decreased Drp1 levels in HeLa cells was provided by fluorescence anisotropy of a lipophilic probe incorporated in the membranes (Benard et al., 2007).

Protein diffusion in mitochondrial membranes and within the matrix has been measured using FRAP. Because of the limited size and complex structure of IMM and its junctions to the inner boundary membrane, the recovery process has to be modeled appropriately (Sukhorukov and Bereiter-Hahn, 2009). Linear diffusion of DsRed in the matrix of HeLa cells was calculated to be in the range of about 1 μm/s (Collins and Bootmann, 2003; Sukhorukov et al., 2010). Fluorescence recovery measurements of bleached areas containing many single mitochondria (Collins and Bootmann, 2003) in fact do not reveal molecular mobilities rather than exchange of mitochondria by fusion and trafficking into and out of this area, or after prolonged time, synthesis of new proteins. Critical analysis of IMM protein diffusion as calculated from FRAP measurements at small spots

($0.24\ \mu m^2$) on a single mitochondrion revealed the presence of a mobile and immobile fraction. These proteins were tagged with fluorescent proteins and have been incorporated into the respiratory complexes at least in part. Proteins not integrated into supercomplexes diffuse much faster in the mitochondrial inner or outer membranes than within the plasma membrane. This can be attributed to the lack of membrane supporting cytoskeletal fibers, as they are supporting plasma membranes and restrict movements exceeding the mesh size of the fibrillar net. However, incorporation in the respiratory supercomplexes renders a large fraction of mitochondrial IMM proteins immobile. Mitochondrial heterogeneity is reflected by very high variability of fluorescence recovery after photobleaching (FRAP) of a small membrane area of a single mitochondrion (Sukhorukov et al., 2010). The presence and high diffusibility of the mobile fraction is in good agreement with the relatively fast mixing also of mitochondrial membrane components after fusion (Hoppins and Nunnari, 2009; Twig et al., 2008), as is the patchy localization of proteins of different origin (Busch et al., 2006) with the existence of an immobile fraction.

3.3. Generation of mitochondria

The main regulators of the transcription of mitochondrial proteins are peroxisome *proliferator-activated receptor γ coactivator 1α* and *1β* (PGC-1α and PGC-1β), although the signaling pathways can differ between the various tissues. Further transcription factors involved in mitochondrial biogenesis are *Nuclear Respiratory Factor 1* and *2* (NRF1 and NRF2), *Estrogen-related receptor α* (ERRα), *Stimulatory protein* 1 (Sp1), and the *Yin Yang 1* transcription factor (YY1) (Scarpulla, 2008). Recent data indicate a direct cooperation of ERRα and the *PGC-1-related coactivator* (PRC; Mirebeau-Prunier et al., 2010). PGC1α coactivates NFR1, which in turns activates that transcription of the transcription factor TFAM, thus inducing the synthesis of mtDNA (Puigserver et al., 1998). Interestingly, PGC-1α also regulates positively the expression of the mitochondrial fission factor Mfn2 (Soriano et al., 2006). In yeast, Puf3p negatively influences mitochondrial biogenesis by binding mRNAs that code for mitochondrial proteins (García-Rodríguez et al., 2007).

Biogenesis of mitochondria occurs during the progression of the cell cycle to ensure the heredity of mitochondria to the daughter cells. Lee et al. (2007a, b) observed an increase of mitochondrial mass as well of the mtDNA amount starting in the G1 phase and progressing through S and G2 phase together with an increase of NRF1. Exercise is a strong inducer of mitochondrial biogenesis in skeletal muscle fibers mediated by PGC-1α (Booth and Baldwin, 1997; Geng et al., 2010; Hood et al., 2006; Olesen et al., 2010), and PGC-1α overexpression protects against muscle atrophy (Sandri et al.,

2006). Interestingly, also dieting (caloric restriction) results in PGC1α-mediated mitochondrial synthesis (Lin et al., 2005; Lopez-Lluch et al., 2006; Nisoli et al., 2005). Furthermore, after oxidative damage, a significant increase of PGC-1α levels was observed, indicating the replacement of damaged mitochondria by newly synthesized ones (Jendrach et al., 2008). During aging, the expression of PGC-1α and NFR1 is significantly reduced, indicating an impaired mitochondrial biogenesis in old animals (Viña et al., 2009).

3.4. Mitophagy

All organelles, including mitochondria, as well as some long-lived proteins are degraded by macroautophagy, in the following called autophagy/mitophagy. Autophagy is regulated and executed by the proteins of the ATG family, at the moment, 29 of these proteins are known (Periyasamy-Thandavan et al., 2009). The autophagic process is started by the encircling of a mitochondrion in an isolation membrane/preautophagosomal structure (PAS)/phagophore, resulting in the autophagosome. Recent data indicate that at least part of the isolation membrane comes from the OMM (Hailey et al., 2010). The autophagosome fuses with late endosomes and lysosomes, resulting in an autophagolysosome wherein lysosomal hydrolases at an acidic pH digest the indigested content, thus supplying the cell with amino acids (Cuervo, 2004; Mizushima et al., 2002).

Two different situations can evoke degradation of mitochondria by autophagy, starvation, and impaired mitochondrial function. Autophagy is inhibited by high amounts of amino acids and insulin signaling via mammalian target of Rapamcycin (mTOR). Consequently, one way to activate degradation of mitochondria is the lack of amino acids (*starvation*), thus allowing the continued supply of the necessary amino acids. This is in line with shortening of mitochondrial turnover time in mouse liver under conditions of dietary restrictions (Miwa et al., 2008).

The second reason for induction of autophagy is the degradation of impaired mitochondria (Elmore et al., 2001; Mouli et al., 2009; Priault et al., 2005). Recently, the signals tagging defective mitochondria for autophagy have started to emerge. Twig et al. (2008) followed the fate of mitochondrial fission products with low membrane potential which no longer undergo fusion in INS1 cells. They found that reduction of membrane potential preceded autophagic degradation by hours; however, after autophagy has started, removal of mitochondria seemed to be a very fast process and the turnover rate of autophagic vacuoles is high. The delay between the onset of autophagy and membrane potential reduction may be related to OPA1 reduction, which is typical for mitochondria being prone to mitophagy (Mouli et al., 2009). The loss of membrane potential induces a

translocation of the ubiquitin ligase Parkin to the mitochondria (Narendra et al., 2008). Fragmentation of mitochondria, possibly mediated by Fis1, seems to facilitate autophagy (Gomes and Scorrano, 2008; Twig et al., 2008); however, it is not essential for Parkin translocation (Narendra et al., 2008). Stress-induced Parkin translocation requires the mitochondrial kinase PINK1 which is stabilized on defective mitochondria (Geisler et al., 2010; Matsuda et al., 2010; Narendra et al., 2010; Vives-Bauza et al., 2010). As defects in the Parkin (PARK2) and PINK1 gene (PARK6) result in PD, it can be hypothesized that a defect in the degradation of damaged mitochondria contributes to the pathogensis of PD.

4. Concluding Remarks

Mitochondria first have been recognized because of their ATP-producing capacity fitting well into a concept of an endosymbiont exchanging nutrients with the host. Looking a bit closer, many other functions of extreme physiological significance became apparent. Investigations on mitochondrial dynamics revealed the multifunctional character of motor molecules and those engaged in fusion and fission. Controlled by phosphorylation and/or binding to cytoskeletal proteins or to membranous organelles, fusion and fission factors traffick between the cytoplasm and mitochondria, and thus embed mitochondrial dynamics in the complex networks of the overall cellular control mechanisms, including formation of organelles, quality control, and degradation. Structural interdependence of IMM morphology on outer membrane-bound molecules became apparent and the role protein supercomplexes play in cristae shape, mitochondrial integrity, or proapoptotic cytochrome *c* and calcium release. Mitochondrial nucleoids contain several copies of DNA, whether and how they could be exchanged between different nucleoids for recombination is one of the many open questions. Comprehending the key role of mitochondria in many cellular processes, they received attention for aging and many neurodegenerative diseases. Also, these events result from the accumulating difference between noxious influences and repair or regulatory mechanisms where mitochondria are only one member in the chain, however, a very important one.

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CHAPTER TWO

Ribosome Biogenesis: From Structure to Dynamics

Barbara Cisterna *and* Marco Biggiogera

Contents

Abstract

In this chapter we describe the status of the research concerning the nucleolus, the major nuclear body. The nucleolus has been recognized as a dynamic organelle with many more functions than one could imagine. In fact, in addition to its fundamental role in the biogenesis of preribosomes, the nucleolus takes part in many other cellular processes and functions, such as the cell-cycle control and the p53 pathway: the direct or indirect involvement of the nucleolus in these various processes makes it sensitive to their alteration. Moreover, it is worth noting that the different nucleolar factors participating to independent mechanisms show different dynamics of association/disassociation with the nucleolar body.

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Key Words: Nucleolus, Ribosomal subunits, Ribosomal dynamics, Preribosome export, Ribosomal proteins.

1. Introduction

The nucleolus is the most prominent feature of the cell nucleus. When Fontana (1781) noticed it first, he described a specific spot inside the nucleus; however, he ignored that he was observing the only point where one can exactly localize the presence of specific genes without any special techniques.

Ribosomal genes are present in many repeats which are differently amplified in different species (Cmarko et al., 2008) up to a point where it has become generally accepted to assert that two genomes existed, the nuclear genome and the nucleolar one. These two genomes work together and mutually influence each other; as it has become more and more clear in these last years, some nucleolar functions are strictly related to nuclear functions (Martin et al., 2009; Pederson and Tsai, 2009).

Many reviews have been devoted to the nucleolus, both in animal and in plant cells, and this organelle has been studied and observed under many different aspects. In this chapter, we will mainly consider the nucleolus and its activity in view of the dynamics connected to ribosome biogenesis, integrating new concepts into an old story.

2. Nucleolar Structure and Dynamics

2.1. Morphology and cytochemistry

Structure and function are, as usual, strictly related and the nucleolus is no exception.

At light microscopy, it has long been studied and some of its inner structures described; some were more or less corresponding to reality, such as the "nucleolini" described as prominent inside nucleoli and later recognized to be fibrillar centers (FCs; Love and Wildy, 1963). The nucleolar body is clearly identified in the nucleus when stained by a vital fluorescent probe such as SYTO RNASelect (S32703, Molecular Probes, Eugene, USA, Huang et al., 2006), discriminating preferentially RNA (Fig. 2.1).

However, the morphology of the nucleolus has been characterized mainly by electron microscopy (EM).

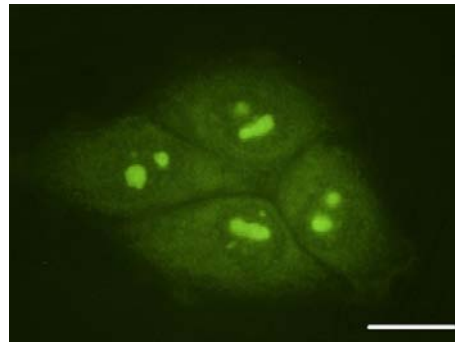

Figure 2.1 HeLa cells stained by the vital fluorescent-probe SYTO which preferentially binds to RNA. The image shows the cospicuous amount of RNA concentrated in the organelle, in addition to a homogeneous staining in the cytoplasm. Bar represents 50 μm.

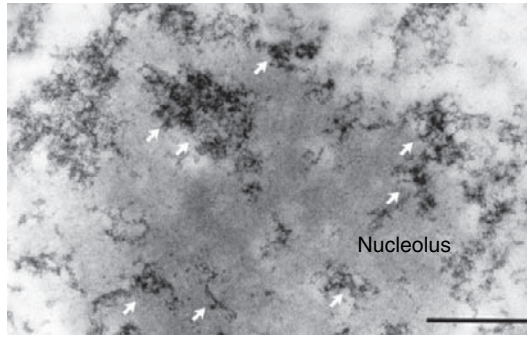

Figure 2.2 A micrograph of a nucleolus is shown. The ultrathin sections of HeLa cells have been stained with osmium ammine complex (OAC) to reveal cytochemically the DNA. The arrows indicate the nucleolar-associated chromatin (NAC) surrounding and penetrating the nuclear organelle. Bar represents 2.5 μm.

Since several decades (Marinozzi and Bernhard, 1963) EM has devoted a huge amount of studies to the morphology of this organelle, describing thus its subdivision into the components we know also today. In a typical nucleolus, four major components are present; for an interesting evolutionary study see Thiry and Lafontaine (2005).

Several models have been presented through the years of the arrangements of the nucleolar components (Hozák, 1995; Thiry, 1993). In our model (Biggiogera et al., 2001), the primal one is represented by the chromatin which will become the nucleolus-associated chromatin (NAC; Fig. 2.2). Within this mass of chromatin, the ribosomal genes are contained, and loosen into an area of DNA distributed into a sort of "cloud" or almost

spherical structure. These clouds represent the interphase counterpart of the nucleolus organizer region (NOR) present on the mitotic chromosomes. The cloud is composed by ribosomal genes, arranged in tandem repeats and in a conformational state which could better be defined as "almost ready."

The center of the cloud constitutes the FC, a clear, fibrillar area, less dense than the surrounding nucleolus. Around it, the dense fibrillar component (DFC) is present as a thick layer which encircles (although usually not completely) the FC. The last part is the granular component (GC), which can be found among the different DFC/FC or generally around them in case of the presence of a single DFC/FC complex. Morphology by itself may hint to function, and at least the maturation of ribosomes was correctly attributed to GC even at the beginning of EM studies (Allfrey, 1963; Wang, 1963). However, it was only with the EM cytochemical studies (Bernhard, 1969; Monneron and Bernhard, 1969) that more data could be collected about the function of the different nucleolar components.

Pioneering studies by Derenzini et al. (1983) showed the presence of tufts of DNA emerging from chromatin clumps in the region supposed to be the FC. This DNA was shown, by means of osmium ammine staining (Cogliati and Gautier, 1973), in an elongated form, that is, more ready to be transcribed. Later, the same authors showed that this DNA extended also within the region of the DFC (Mosgöller et al., 1993). The presence of DNA within FC was taken as a marker of the place where transcription occurred (Derenzini et al., 1990). However, different points of view were expressed by several other groups, leading to a decade-long scientific debate. Briefly, the different position can be summarized as follows: (a) transcription takes place in the FC, (b) transcription takes place in the DFC, (c) transcription takes place at the boundary between FC and DFC. The arguments in favor of each of these hypotheses were many, obtained by several cytochemical and immunocytochemical techniques; unfortunately the same could be said for the counter-arguments, often obtained by the same technique on the same cell model (Mosgöller et al., 1993; Thiry, 1993).

Nowadays, the generally accepted idea is that transcription occurs in the DFC, where nascent transcripts are formed and immediately associated with several specific proteins (Brown and Shaw, 2008; Henras et al., 2008) taking care of elongation, splicing, and leading to the formation of the processome (Osheim et al., 2004; Schneider et al., 2007). In this view, the immuno-electron microscope demonstration of the presence of RNA pol I (Scheer and Rose, 1984) in the FC should be reconsidered as indicating a sort of reservoir of the enzyme, in its inactive form. FC, moreover, was found labeled for different proteins, such as AgNOR, UBF, Nopp140, and several others (Casafont et al., 2007; Schwarzacher and Mosgoeller, 2000). Presently, it is difficult to establish whether FCs represent accumulation sites for all these proteins or their role can be linked to this structure.

Most interesting, on the other hand, were the early autoradiographic data on ^{3}H-uridine incorporation (Granboulan and Granboulan, 1965; Lacour and Crawley, 1965). Despite the relatively low resolution of this technique, even after a 30 s pulse, nascent RNA was constantly found within DFC (Fakan and Bernhard, 1971) and this was confirmed by later findings of Br-uridine (Br-UTP) labeling, with a higher resolution (Cmarko et al., 2000).

A possible model was proposed by Biggiogera et al. (2001) which put together FC and DFC in a single unit at which periphery (the DFC) transcription occurred. The model took into account the possibility of reducing or enlarging these structures as they occur, for instance, during the night–day circadian rhythm or after reduction of transcription (Pebusque and Seite, 1981). Several points remain to be clarified, in any case. When nucleoli are isolated and spread in order to show the transcription units, the so-called Christmas trees (Miller and Beatty, 1969), the structures obtained are difficult to reconcile with the *in situ* nucleolar components (Jordan, 1991; Shaw and Jordan, 1995); Christmas trees are larger, longer, and numerous, so that one must imagine to have several of these units fit into DFC. One problem for all, simply packing the unit into the DFC would create a steric hindrance we cannot, so far, overcome. On the other hand, most of the facts pointing to equivalence between DFC and Christmas trees are there: presence of nascent RNA, proteins involved in transcription, elongation, and splicing, and the density of the structure itself.

The least complicated component, apparently, is the GC. It has long been recognized as formed by ribosome subunits, although only the major one is recognizable on thin sections. Earlier studies have shown (Granboulan and Granboulan, 1965) that pulse-chase experiments with titrated uridine resulted in labeling of the GC after 10 min, thus indicating a movement of RNA from the DFC region to a place where ribosome maturation occurs.

Two open questions are where really begins the assembly of ribosomal proteins (r protein) to RNA and why only the major subunit seems to be present in the GC (see Section 4). Some proteins, such as P1P2, L7 are already present on the DFC in mouse Sertoli cells (Biggiogera et al., 1990). On nucleolar spreads, L7 can be found on Christmas trees (Biggiogera, unpublished data) not far from the terminal knob. The presence of nucleolar proteins has interesting developments. Lam et al. (2007) have shown that the amount of ribosomal proteins is by far larger than that required for the typical rate of ribosome subunit production.

The balance is kept by continual degradation of unassembled proteins in the nucleoplasm where the effector is the proteasome (Scharf et al., 2007). As a consequence, the building of ribosomes is a very expensive activity (Granneman and Tollervey, 2007) which is justified by the importance of the final goal. The presence and function of these proteins in this special location will be discussed later in Section 4.

2.2. Nucleolus in living cells

The possibility to observe subnuclear structures in living cells has been opened years ago with the use of GFP (green fluorescent protein), and additional imaging techniques, such as FRAP (fluorescence recovery after photobleaching), FRET (fluorescence resonance energy transfer), and FLIP (fluorescence loss in photobleaching), have added new data to our knowledge. These techniques have given interesting results, such as the observed colonization of a second nucleolus by protein from another in the same cell (Politz et al., 2003). However, a certain caution should be exercised since the overexpression of a given protein (sometimes necessary to have a significant signal) could lead to results not corresponding to reality.

Another possibility lies in detecting unstained structures with infrared LED imaging (Szucs et al., 2008), and coherent phase microscopy (Tychinsky et al., 2008). The latter seems capable of detecting early changes in nucleolar dynamics and offers quantitative analysis in real-time. Especially considering the development in the microscopy techniques for the *in vivo* analysis, the nucleolus has surely been, so far, an active character of studies which use these methods. If we consider the interesting aspect of the dynamics of exchange of nucleolar factors between the nucleolus and the nucleoplasm (Phair and Misteli, 2001), the fluorescent fusion proteins, which are observable in living cells, have become essential (Janicki and Spector, 2003).

In this connection, it is worth underlining that nucleolar proteins rapidly associate with nucleolar components, and then disassociate by different dynamics in function of their specific role played in the ribosomal biosynthesis process (see Chapter 4).

Therefore, the values of the diffusion coefficients, obtained by FRAP approach, of fluorescently tagged proteins such as fibrillarin involved in early stages of the pre-rRNA transcription (Chen and Huang, 2001; Dundr et al., 2002; Phair and Misteli, 2000; Snaar et al., 2000) and B23 and Nop52 engaged, instead, later in the preribosome processing (Chen and Huang, 2001; Louvet et al., 2005), are lower in the nucleolus in comparison to those in the nucleoplasm. These values reflect the residency time of these proteins that are committed to in carrying out their functions, in the nucleolus. The same techniques have provided interesting evidence on the recovery rates of ribosomal proteins which, once in the nucleolus, are assembled in the ribosomal subunits: the slower and complex process of preribosome assembly (compared with the transcriptional mechanism) justifies a more stable association of ribosomal proteins with pre-rRNAs, and hence, their consequent quite low recovery rate value (Chen and Huang, 2001; Lam et al., 2007).

If, on one hand, FRAP allows to *in vivo* follow both the traffic of a specific protein and its association/disassociation with nuclear compartments, thus highlighting the dynamic nature of the nucleolus, on the other hand, the FRET technique has been used to determine the existence

of a potential colocalization and hence, interaction between two factors. The study of such an interaction within specific pathways should provide basic information on which are the characters active in a specific process and which role is played by each partner in this mechanism.

An example is the analysis of proteins associating in prenucleolar bodies (PNBs): after mitosis, the pre-RNAs localize to these structures (Dousset et al., 2000), whose formation is a cycle-dependent process, as confirmed by their absence in interphase. It has been hypothesized that the rRNAs function as network on which the processing factors could be nucleated (Dousset et al., 2000; Dundr and Olson, 1998; Dundr et al., 2000; Pinol-Roma, 1999) and the PNBs, indeed, may be an assembly platform for the formation of some processing complexes, such as the B23–Nop52 complex. These two proteins, in fact, show positive FRET in PNBs from the beginning of the telophase and later, in the nucleoli, suggesting their assembly in the former one and a common release from this structure. However, the association of B23 and its necessary partner in 60S biogenesis Nop52 does not seem to be permanent, as shown by a new approach which uses a photoactivable GFP (PAGFP) to visualize and track a pool of fluorescently tagged proteins after activation by a laser beam. In interphase cells with two nucleoli, Muro et al. (2008) analyzed the migration of PAGFP–B23 and PAGFP–Nop52 from the activated nucleolus to the neighboring one, finding that the two factors have different dynamics of traffic. B23 redistributes between the nucleoli within 2 min, while the same time is not sufficient for Nop52.

Summarizing, these techniques have given and continue to provide interesting data on the dynamics of movement of proteins in living cells, and the results obtained display a rather animate situation.

2.3. Nucleolar evolution

2.3.1. *In vitro* reconstitution

Several attempts have been made at reconstituting *in vitro* the nucleolus. In *Xenopus* egg nuclei, reconstituted from DNA or chromatin, prenucleolar bodies were observed but not functional nucleoli (Bell et al., 1992). It seems that the nuclei assembled *in vitro* are capable of initiating early steps of nucleologenesis but that the resulting PNBs are unable to fuse with each other. This could be due to the absence of a functional nucleolus organizer. Moreover, PNBs seem to be primary assembly structures which contribute to the formation of both nucleoli and coiled bodies (CBs) and thus offer an explanation for the frequently observed structural association of CBs with nucleoli (Bell and Scheer, 1997).

However, the *in vitro* reconstitution strategy has been very limited from the beginning, and probably doomed as well, since it is worth remembering that, among the inherent complexity of the nucleolar functions, several nonribosomal functions are carried out there. Consequently, it seems better

and more rewarding to follow the dynamics of disassembly and reassembly of the nucleolus *in vivo* during mitosis.

2.3.2. *In vivo* reassembly

During mitosis, nucleoli disassemble during prophase and reassemble in telophase (Sirri et al., 2008). The nucleolus has been described as "an organelle formed by the act of building a ribosome" (Mélèse and Xue, 1995) and when transcription is repressed its components in part stay associated to rDNA in the NOR (Roussel et al., 1996) and in part migrate as chromosomal passengers (Hernandez-Verdun and Gautier, 1994).

At the moment of rDNA transcription restart, nucleoli are again formed via PNB formation (Dundr et al., 2000) via a progressive recruitment of proteins involved in early and late processing. PNBs, with their content of nucleolar processing proteins, pre-rRNAs and small nucleolar RNAs (snoRNA), play a role that has not yet been completely clarified. Moreover, it seems clear that proteins with a different functional role leave the PNBs at different moments. Recently, Muro et al. (2010) have demonstrated that fibrillarin passes from one incipient nucleolus to another without stopping in PNBs, while other proteins like B23 shuttle between PNBs and nucleoli. The difference in this traffic would suggest a way of regulating the assembly first of the DFC and then of the GC, and this mechanism would involve the Cajal bodies.

Several factors are probably involved in the rebirth of a nucleolus. Transcription itself is not sufficient to start the event (Section 2.3.1) but nucleolar assembly can start independently of rDNA transcription (Dousset et al., 2000). Apparently a paradox: transcription arrest means disassembly, reassembly does not mean transcription restart. Other factors, such as CDK, may intervene to regulate both transcription and processing (Sirri et al., 2008). The final assembly is rather rapid and very probably a "prenucleolar" interaction of processing proteins is required.

If one considers the incredible amount of proteins that disassemble and reassemble during mitosis, and that most of them redistribute at different locations and then are recruited to PNBs, it is not clear what could be the driving force behind. Diffusion is the easy answer for the movements, and indeed a part of nonribosomal proteins show a nucleolar localization signal (NLS), but not all of them possess this feature (Jacobson and Pederson, 1998).

Diffusion can account for a series of movements, although mediated by signal recognizing sequences, but necessity of order and time might involve other mechanisms. It is known that some proteins are recruited from PNBs in a specific, sequential order (Louvet et al., 2008). It is difficult in this case to imagine diffusion as the only mechanism. As described for other nucleolar functions such as transcription (Dundr et al., 2002) or ribosome subunit movement (Cisterna et al., 2006, 2009) there could be place for motor proteins to give directionality (impossible in diffusion mechanisms), time schedule (also possible only in active mechanisms), and releasing order, if any.

The coordination found in the movements of nucleolar proteins suggests that they can maintain their interaction during mitosis; however, the mechanisms behind the interactions are still not clear. The interaction has been clearly shown by FRET analysis (Angelier et al., 2005).

2.3.3. Nucleolus in mammalian embryos

In the zygote until the stage of morula (depending on the species), the nucleolus is not present with the same features of interphase cells. Already in the late stages of oogenesis, the nucleolus undergoes a sort of segregation, which leaves a final structure with spherical form, the nucleolus-like body (NLB; Kopecný et al., 1995), and no evident features, with the exception of one or few lenticular, less electrondense bulges. These structures correspond to FCs and were shown to contain also AgNOR proteins (Fléchon and Kopecný, 1998).

The dense sphere, however, has no internal structure; it has been shown to contain mostly proteins and no DNA (Fakan and Odartchenko, 1980; Hughes et al., 1979). Among the proteins described in mouse, bovine, caprine, and pig embryos (Kopecny et al., 1996) of particular interest are the heterogeneous nuclear ribonucleoproteins (hnRNP) and the small nuclear ribonucleoproteins (snRNP), which normally are not associated with nucleoli or nucleolar activity. Moreover, cytochemical studies have shown that RNA is present within these structures, and in large amount; the origin of this RNA has not yet been clarified, but it seems clear that it is not rRNA (Biggiogera et al., 1994).

The NLB are modified during early embryogenesis; first a thin rim of DNA starts surrounding the sphere, then the rim thickens, and transcription occurs. At the same time some proteins like B23 or nucleolin, which were present inside the dense structure, colonize the external rim, together with fibrillarin. The differentiation of the nucleolar structure has been described by Fléchon and Kopecný (1998) and shown in Fig. 2.3. In mammalian embryos, the nucleolar size and morphology in the different species may vary, as well as the timing of their development into fully functional nucleoli. Two definitely different types of nucleolar precursor bodies were described as the mouse-type and the cow-type. In the mouse, the dense compact material is still detectable in the early functional nucleolus (Biggiogera et al., 1990) with a functional nucleolonema at its periphery. The sphere begins to break up, a sort of nucleolonema linking the various pieces is now well visible; at this point, the labeling for hnRNPs and snRNPs decreases on the remnants of the sphere; the cytochemically detectable RNA decreases as well. Later on, between morula and blastocyst stage, the remnants of the sphere completely disappear, leaving an active nucleolus. In the cow, the whole NLB is readily transformed into a functional nucleolus, and interestingly, rRNA synthesis may occur deep inside the structure, demonstrating that NLB becomes a fully functional structure

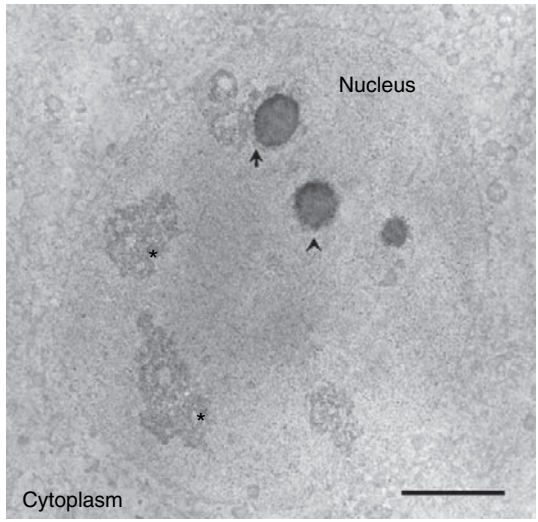

Figure 2.3 Ultrastructural image of the nucleus from a mouse embryo at 4–8 blastomere stage, characterized by the typical differentiation of the nucleolar structures. Arrowhead points to the dense core structure typical of early embryos. A beginning of nucleolonema (arrow) can be seen to develop around the dense structure, finally giving rise to an active nucleolus (asterisk). Bar represents 0.5 μm.

in toto. It is tempting to consider the NLB as a sort of reservoir given by the oocyte to the zygote; this happens with the cytoplasmic content, since almost everything is enough to last for a couple of cell divisions, that is, until the embryo is self-sustaining (Johnson, 2009). In this view, the NLB contains nucleolar as well as nuclear proteins and RNA which will serve for the nuclear purposes of the developing cell.

Another explanation would be to consider at least some, not all, NLB as HERDS (Biggiogera et al., 1997a,b). HERDS are formed when a balance problem occurs between RNA synthesis and the amount of protein involved in RNA production (Biggiogera and Pellicciari, 2000). In several cases, the cell constitutes a storage point which can be used when the stimulus is over, as it happens, for instance, at the end of hibernation (Malatesta et al., 2001). In this view, the NLB will serve this purpose and it could well represent a safe way to transit RNA from the oocyte through fertilization to the embryos.

Interestingly, a good model to understand the property of the nucleolus, or NLB, has been found in experimental models of nuclear transfer. It has been suggested (Laurincik and Maddox-Hyttel, 2007) that rRNA gene activation might be used as a marker for evaluating the potential of embryos after nuclear transfer: many nucleolar proteins, including fibrillarin, B23, and nucleolin, are associated to the nucleolar anlage (Fléchon and Kopecný,

1998) over several cell cycles. In the nuclear transfer embryos, however, some blastomeres show aberrations in protein localization. This seems to be an indication of underlying aberrations in genomic reprogramming.

The factors contained in NLBs can also be found within oocyte nucleoli; in this view, it is significant that nucleoli from growing oocytes can substitute nucleoli from full-grown oocytes during early embryonic development. Enucleated oocytes are capable of reaching the blastocyst stage (Kyogoku et al., 2010).

2.4. Nonribosomal functions

One important point to be considered is that the nucleolus, although apparently compact, is nevertheless very permeable (Handwerger et al., 2005). This property can influence its relationship with the nucleus which apparently lies far beyond our knowledge. Proteomics assays (Andersen et al., 2002, 2005) have shown that more than 50% of proteins from purified HeLa nucleoli were not nucleolar proteins at all.

So far, few data are available on this subject. One of the first was the discovery that the machinery involved in the assembly of the signal recognizing particle (SRP) was detected in transit through the nucleolus (Jacobson and Pederson, 1998; Politz et al., 2000). Other so-called nucleolar visitors include U2 and U6 modifiers (Politz et al., 2006), Cdc phosphatase, or nucleostemin (Pederson and Tsai, 2009; Tsai and Mckay, 2005). The latter is known to interact with several proteins, among which p53, ARF, and B23 (Ma and Pederson, 2008) known to be involved both in cell cycle regulation and ribosome processing. In this view, a sort of regulation of the cell cycle by the nucleolus can be envisaged. Data are present in the literature which show this connection (Gaulden and Perry, 1958) and it is worth considering that the nucleolus is deeply modified by the cell cycle. As discussed later, the nucleolus disassemble during mitosis, its main components are dispersed or rejoin the chromosomes. The change is so dramatic that is difficult not to imagine a direct relationship between the nucleolus and the cycle, in order to reach and maintain a sort of internal modulation of the cell activity.

A number of microRNAs (miRNA) has been found in the nucleoli of rat myoblasts (Politz et al., 2009) and one of them in relevant concentration. In some cases, these miRNAs do not show any analogy with any snoRNAs, so that the reason of their presence is unknown, maybe related to nonribosomal functions. The presence of a population of miRNA inside the nucleoli of HeLa cells do correspond to those found in myoblast suggesting that this localization is a general phenomenon, whether implying regulatory or processing functions is still unclear.

A possible subcompartmentalization of the nucleolus has been proposed as the basis of these new nonribosomal functions. Politz et al. (2005) have found by electron spectroscopic imaging that some regions of the GC were

very poor in nucleic acids and very rich in proteins. In these areas was concentrated nucleostemin, and these findings made the authors to suggest that the RNA-free zones might serve other functions. If so, a fifth area of the nucleolus, which could be indicated as RFC (RNA-free component) could well be the structural support for yet unknown functions.

3. Form and Function Interplay

3.1. rRNA transcription and processing in an *ideal nucleolus*

3.1.1. Extra situm

Nucleolar transcription has been studied *extra situm* since 1969 when Miller and coworkers devised a technique for spreading nucleoli. The surprising result was the presence of the transcription units in the form of Christmas trees. It was soon realized that along the DNA axis each RNA branch developed from a small knob, which was recognized to be the RNA polymerase I, while at the distal terminus of the branch, a terminal knob is present. This knob has been shown to be constituted by U3 snRNPs and since U3 is actively involved in splicing, this has been one of the first demonstration that splicing can occur cotranscriptionally (Thiry and Goessens, 1996). Spreading technique has its inherent limitations, such as the difficulty to reconcile an enlarged structure with the *in situ* morphology, but offers also several advantages. Figure 2.4 is an example of nucleolar spread, followed by an immunolabeling for the large subunit (LSU) protein L7.

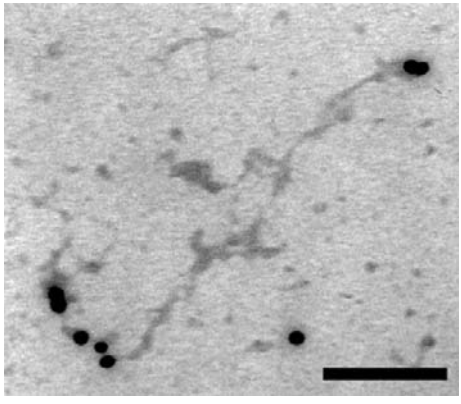

Figure 2.4 Nucleolar spread immunolabeled by antiribosomal protein L7 and stained with PTA. The large subunit protein is already present during transcription. Bar represents 0.1 μm.

Christmas trees represent a map of transcription where is possible to pinpoint the different rRNA and the process ongoing. Osheim et al. (2004) have described the processome, a small particle, visible only on spread preparation, where splicing and partial association with several proteins occur.

In a short period of time, the nascent pre-rRNA must be spliced, the internal transcribed spacer (ITS) removed, and the remaining rRNAs associated with proteins and finally accompanied to the adjacent GC zone. Since it is difficult to imagine that Christmas trees may enter as such into the DFC or FC, we could hypothesize that the branches are somehow folded around the axis and develop in a spiral surrounding the center. However, it is not easy to see all the machinery involved in these processes move smoothly in this overpackaged environment, and this problem has haunted a decade at least of nucleolar research (Jordan, 1991; Raska et al., 2006; Thiry and Goessens, 1996). Probably, if we consider the problem from a strictly dynamic point of view, the necessity to speed up the transcription/processing transit time have made so that the completion of a preribosome (including transcription, processing, folding of rRNA, association of proteins) is a matter of seconds. When we look at a micrograph, we have the idea of a static form of these processes. A process at this molecular level, however, might take place also in a folded structure, such as in the processome (Osheim et al., 2004) and thus the possibility to have a Christmas tree strictly folded within the DFC is not unreasonable.

3.1.2. In situ

An interesting suggestion for the link between form and function comes from the recent observation that lamin B1 can maintain the functional plasticity of nucleoli (Martin et al., 2009). Depletion of this protein leads to structural changes and finally nucleolar dispersion. Moreover, lamin B1 interacts with B23, a protein known to have many functions, among which some nonribosomal functions. An additional mechanism of continued DNA and RNA surveillance has been recently proposed. The apurinic/apyrimidinic endonuclease 1 (APE1) has been shown to interact with rRNA and NPM1 in the nucleolus as well as with proteins involved in ribosome assembly such as RLAO and RSSA (Tell et al., 2010). Shin et al. (2009) have demonstrated that a single methylation at the level of arginines plays a critical role for the behavior of rpS3. The unmethylated protein, in fact, cannot be imported into the nucleus and consequently cannot be incorporated into the small subunit (SSU). On the other hand, rpS5 is not phosphorylated when assembled into the SSU but phosphorylated as free form (Matragkou et al., 2009). Its phosphorylation becomes indispensable for its transport to the nuclei.

3.2. Experimenting with drugs and stress

The nucleolus is by now recognized as a multitasking organelle in the cell, since it is involved in numerous cellular processes such as the cell cycle regulation, control of aging, signal recognition particle assembly, modification of small nuclear RNPs, nuclear export pathways and telomerase function, in addition to be active in the viral replication (Olson et al., 2002). Therefore, a possible nucleolar response to a stress condition, it is either physiological or genotoxic, seems obvious.

The nucleolus, in addition to being capable of adapting its morphology to the stimulus, guarantees the inside–outside dynamics of specific factors in specific conditions. When the ribosomal subunit biogenesis is impaired, for instance by treatment with low doses (0.01–0.04 μg/ml) of actinomycin (AMD), the nucleolus reorganizes (Gébrane-Younès et al., 2005): FCs and DFC condense and migrate toward the nucleolar periphery promoting a segregation of the components in a central body (containing GC-derived proteins) associated with caps where factors related to RNA polymerase I transcription machinery are accumulated (Hadjiolova et al., 1995; Sirri et al., 2008).

If an inhibition of rRNA synthesis may trigger a reorganization of the structure and function of the nucleolus, the nucleolar changes are drastic in an upregulation context as well. Therefore, the cell proliferation process, during which the cycling cells are characterized by a higher biosynthetic demand (Derenzini and Ploton, 1991), appears to be closely coordinated with the nucleolar function. The same relationship can be found in cancer cells, where changes in the tumor suppressor and proto-oncogene expression can upregulate the ribosome biogenesis, thus promoting the cell proliferation typical of the tumor. In addition to evident modifications in nucleolar structure, the upregulation of rRNA synthesis and processing would support an increase in cell growth rate (David-Pfeuty, 2006; Sulić et al., 2005).

It is worth underlining that qualitative and quantitative defects in ribosome biogenesis and the consequent alterations of the nucleolar organization may be due to a neoplastic transformation, but also be involved in the tumorigenesis. However, although for the latter idea an increasing amount of findings have been accumulating, the cancer cell mechanisms leading to a restructuring of the ribosome biogenesis have been mainly investigated. The results principally refer to factors such as the retinoblastoma tumor suppressor protein (pRB) and p53, normally involved in the cell cycle.

While in the hypophosphorylated form, pRB inhibits rRNA transcription by binding UBF (Cavanaugh et al., 1995; Ciarmatori et al., 2001; Hannan et al., 2000; Voit et al., 1997; White et al., 1996), thus blocking its interaction with the rRNA gene promoter along with polymerase I (Grummt, 2003), the progressive phosphorylation of pRB from G1 to G2 phase (Donjerkovic and Scott, 2000) induces a progressive increase of

rRNA synthesis rate with an enlargement of the nucleolar size. In human cancer, genetic changes on the pRB pathway can cause a strongly reduced negative control over rRNA transcription.

Mutations pertaining to p53 are as much as frequent in tumor (Vogelstein et al., 2000; Vousden and Lane, 2007); in fact, p53 undergoes in tumor important modifications, triggering various unbalancing effects, among which an enhanced ribosome biogenesis. p53 can act as negative regulator of ribosome biogenesis, since its accumulation inhibits polymerase I transcription hindering the UBF-SL1 complex formation (Zhai and Comai, 2000), which is essential for the recruitment of the enzyme to the rRNA gene promoter (Grummt, 2003). When mutated, p53 is no longer capable of restraining rRNA transcription and hence cell proliferation.

Moreover, it is worth taking into account that the nucleolus plays an intringuing role in the modulation of the p53 levels (Mayer and Grummt, 2005; Pestov et al., 2001; Rubbi and Milner, 2003), responding to various exogenous stress (UV irradiation, nucleotide depletion, heat shock, hypoxia, and also aberrant ribosome biogenesis); under these conditions, scavenging proteins such as B23, ARF, and the ribosomal proteins L5, L11, L23 are released from the nucleolus and associate in the nucleoplasm with the p53 negative regulator ubiquitin ligase MDM2. In normal cells, when its activity is not inhibited, MDM2 interacts with p53 (Olson, 2004), regulating its turnover by ubiquitination. The release of nucleolar proteins such as ARF (previously sequestered in the nucleolus by B23) and their association in the nucleoplasm with MDM2 leads to the consequent sequestration of the latter in the nucleolus (Gallagher et al., 2006; Gjerset, 2006; Sherr, 2001; Tembe and Henderson, 2007) triggering the stabilization and upregulation of p53 activity. Therefore, a nucleolar damage may induce an increase in p53 levels not only in presence of a DNA damage. Nevertheless, a relationship between a DNA injury and the inhibition of the ribosome biogenesis is clearly existent. In this connection, Kruhlak et al. (2007) have shown how a break in the DNA double-strand can affect the ribosomal gene synthesis. A DNA break, induced by ionizing radiations, leads to a polymerase I rRNA transcription inhibition, which is transient as demonstrated by the restored FluoroUridine (FUrd) incorporation after 60 min of treatment. Moreover, the authors conclude that the polymerase I activity is only inhibited in proximity of the DNA damage since the transcription is blocked in microirradiated nucleoli, but not in the neighboring ones. This underlines that, although the nucleoli of a cell share a common structural organization, their activity may be modulated for each one differently.

It must be mentioned that a possible alteration in the ribosome biogenesis may induce changes in the mRNA translation, with a possible consequent unbalance in the levels of those proteins (proto-oncogenes, antiapoptotic factors, growth factors) that would support a neoplastic transformation, as already shown with the overexpression of the eIF4E

eukaryotic initiation factor (Montanaro and Pandolfi, 2004). Preribosome biogenesis and the mRNA synthesis and translation are tightly bound. In this connection, it is known that the treatment with the casein kinase (ck2) inhibitor 5,6-dichloro1-ribofuranosylbenzimidazole (DRB) induces a specific inhibition of the RNA polymerase II activity, also promoting a physical discontinuity between fibrillar (rDNA transcription sites) and granular (late rRNA processing areas) components (Hernandez-Verdun, 2006). The suppression of the transcriptional activity is confirmed by a decreased incorporation of the RNA precursor BrU and the reorganization of the nucleolar components is shown by antifibrillarin immunolabeling. The cause of this reorganization is not clear yet; the nucleolus seems to react to the decrease in the protein synthesis, provoked by the reduction in the mRNA amount, reorganizing its components.

It is worth considering that, under normal condition, a balance exists among the structures of the nucleus deriving from the dynamic interaction of many different components (Marshal, 2002; Stein et al., 2003). When this stable balance is disturbed, a reorganization of the nuclear constituents occurs, among which the nucleolus has been observed and characterized (Biggiogera and Pellicciari, 2000; Biggiogera et al., 1997a,b, 1999, 2004). Under unbalanced conditions, the ribonucleoprotein (RNP)-containing nuclear structures segregate in large heterogeneous aggregates which are characterized by central dense masses of amorphous material (Biggiogera et al., 1997a,b). These aggregates, which were called HERDS, acronym of Heterogeneous Ectopic RNP-Derived Structures (Biggiogera and Pellicciari, 2000) are visible in many different physiological (terminal differentiation of the erythroblast, spermiogenesis, apoptosis) and drug-induced (DRB, AMD) conditions.

As far as the HERDS function is concerned, Biggiogera et al. (2004) hypothesized that the formation of these ribonucleoprotein-containing clusters may be related to an impairment or arrest of the transcription mechanism. Therefore, HERDS might represent a storage site for RNP-associated proteins, whenever their intranuclear amount exceeds the amount of the newly synthesized hnRNA on which they should function. Taking into account that RNPs have a relatively long half-life (Billings and Martin, 1978; Martin et al., 1979) and that RNP complexes are rather resistant to proteolytic cleavage, the authors suggest that HERDS may serve as a ready-to-use reservoir of proteins needed for RNA processing if and as soon as the transcription is resumed. However, although in some conditions (low doses of drugs such as DRB and AMD, physiological or drug-induced hypometabolism; Biggiogera et al., 2004; Malatesta et al., 1994), when the inducer is removed, HERDS dissolve with the restore of the transcriptional and processing mechanisms, HERDS can have an irreversible nature, whether marked by the presence of nucleolar antigens in the aggregates (Biggiogera et al., 2004).

The nuclear structures initially involved are always IGCs (interchromatin granule cluster, also known as nuclear speckles), PFs (perichromatin fibrils) and PG (perichromatin granule); IGCs and PG especially can be morphologically identified, mainly at the periphery of the heterogeneous structures, where probably the RNP constituents have not undergone any modification yet. If and when nucleolar antigens, such as fibrillarin or the $P_0P_1P_2$ components of the ribosomal subunits (detected by immunocytochemistry, Biggiogera et al., 1997a,b) join HERDS, these structures become irreversibly stable and the cell is committed to terminally differentiate (erythrocytes and spermatids) or dye (apoptotic cells). Therefore, independently whether nucleolar proteins reach the HERDS in the form of morphologically distinct and segregated nucleolar components, as seen in apoptotic thymocytes, the presence in HERDS of factors originating from the nucleolus would establish the fate of the clusters and of the cell.

As mentioned above, Biggiogera and colleagues observed that the HERDS formation may be a reversible process: when the cells are treated with D-Ala-D-Leu-Enkephalin (DADLE, capable of mimicking the effects produced by the Hibernation Induction Trigger, HIT), enter a hypometabolic state characterized by a decrease of the transcriptional and processing mechanisms and the formation of RNP-clusters. These effects are concomitant with an alteration in the NAC which seems to increase penetrating in the organelle. This nucleolar modification represents a morphological signal of a reduced nucleolar function, as also confirmed at EM by a decreased immunolabeling against the rRNA precursor BrU and against the polymerase I active in the synthesis of the pre-rRNA (Vecchio et al., 2006). After a period of recovery, the resumption may be complete, underlining how the nucleolus is an extremely dynamic constituent of the cell, continuously rearranging itself in response to the changes in the metabolic conditions (Emmott and Hiscox, 2009; Lindström and Zhang, 2008; Mongelard and Bouvet, 2007; Stark and Taliansky, 2009; Wanzel et al., 2008; Yang et al., 2008).

4. Ribosome Dynamics

The proof of the nucleolar dynamism is represented by the continuous exchange of molecules between the organelle and the nucleoplasm in response to specific cellular needs. In this transfer of proteins, obviously leading to a temporary specific distribution of a factor, distinct signals or pathways should exist to determine the retention or the release of a factor from the nucleolus.

Emmott and Hiscox (2009) propose the idea that the nucleolus is assembled on the basis of a core of nucleolar proteins constituting the blocks around the rDNA repeats (Hernandez-Verdun et al., 2002). These proteins

would behave as Hub proteins, known to be capable of binding to multiple (10 or more) distinct proteic partners (Krasowski et al., 2008). The Hub proteins, recognizing the potential partner by the identification of a structural or sequence-based motif, retain that protein within a specific subcellular localization. It is worth noting that, in the absence of protein–RNA interactions, the nucleolar Hub factors are believed responsible for much of the nucleolar localizations. Nucleolin and B23 have been recognized as Hub proteins in the nucleolus, as confirmed by their ablation by short interfering RNA (siRNA), which is sufficient to disrupt the organelle structure (Amin et al., 2008; Ma et al., 2007; Ugrinova et al., 2007).

The role played by the nucleolus in the processing of the spliceosomal RNP complex justifies, for instance, the migration of U snRNPs in the nucleolus, though they are not accumulated here. Indeed, the nucleolus may function as nuclear maturation site (Lange and Gerbi, 2000; Lyon et al., 1997): into the nucleolus, U6 snRNA undergoes the 2-O-methylation and pseudoridylation of defined nucleotides by guide snoRNAs (Lange and Gerbi, 2000) and U2, U4, and U5 snRNAs are modified as well (Gerbi and Lange, 2002).

Besides spliceosome components, several other factors are imported and associate with the nucleolus, such as SMN, telomerase, and obviously all the nucleolar factors involved in the preribosome synthesis.

A series of data exists underlining the different dynamics which these factors have in function of the activity they carry out in the nucleolus. In this connection, it has been also suggested that nucleolar proteins, fundamental for the structural and functional organization of the organelle, may be active component of other nucleoplasmic structures. Snaar et al. (2000) have shown that fibrillarin, in addition to being involved in many post-transcriptional processes including pre-rRNA processing, pre-rRNA methylation, and preribosome assembly (Tollervey et al., 1993), presents specific signal sequences targeting the molecule not only to its known site of action, the nucleolus, but also to CBs.

In order to verify the assignment role of the signal domains, the authors constructed a number of truncated fibrillarin mutants, coexpressed with GFP. It seems that the COOH-terminal (α) domain targets fibrillarin to CBs, while the second spacer domain along with the RNP domain targets the protein to the nucleolar transcriptional site. The finding of fibrillarin in CBs opened questions on its possible function in this nuclear structure. If a model exists proposing CB as originated from nucleoli (Matera, 1999), it may be more plausible to believe that either the CBs have acted as carriers (Boudonck et al., 1999; Malatesta et al., 2004), thus justifying the association with fibrillarin, or fibrillarin may be committed in performing one or more specific roles in this nuclear substructure (Sleeman et al., 1998). Fibrillarin is not the only nucleolar protein to be found in the CBs. Others, such as nucleolin and B23 (Bell et al., 1992; Raska et al., 1991), are

contained in this nuclear body, thus confirming the idea, proposed above, of a continuous interplay between the nucleolus and the nucleoplasmic components. The observation that the CBs are involved in key steps for the relocation of nucleolar proteins have suggested that this nuclear component could have also a role in the nucleoplasmic transport of ribosomal proteins (Cisterna et al., 2006).

4.1. Protein import

4.1.1. The nucleoplasmic road toward the nucleolus

Once synthesized in the cytoplasm and entered the nucleus through the nuclear pore complex (NPC), a ribosomal protein will move into an environment characterized by the presence of compartments, more or less structured and committed to a series of basic functions, which are underlined by a constant movement and exchange of "material."

This suggests the possibility that a ribosomal protein as well may be involved in such a complex dynamics.

We previously provided evidence at EM that, following the movements of the ribosomal protein S6 from the cytoplasm to the nucleolus where it is assembled in the small ribosomal subunit (SSU), at least two nucleoplasmic structures participate as transient storage site, IGCs and CBs. The former is known to contain several proteins from both subunits (Saitoh et al., 2004) and, taken into account its role as modification site (Misteli and Spector, 1997), it has been impossible to exclude that the IGCs may be engaged in r protein modifications before they reach the nucleolus. Nevertheless, it is worth considering that the double-immunolabeling with the anti-S6 and the anti-PANA (P 105 IG marker, Clevenger and Epstein, 1984) antibodies highlighted that, although some colocalizations exist confirming the presence of the ribosomal protein in some nuclear speckles, they are partial. This suggested that other nucleoplasmic structures may take part in the S6-import pathway. An interesting candidate seems to be the dense fibrillar structures previously described as IGAZ (interchromatin granules-associated zone, Puvion-Dutilleul et al., 1995) found labeled with the anti-S6 antibody at EM, and likely corresponding to the nuclear paraspeckles (Cardinale et al., 2007).

As far as CB is concerned, we hypothesized that it may be committed as carrier of ribosomal proteins, transporting them from IGCs to the nucleolus, where it is known to associate and sequester/release proteins (Jimenez-Garcia et al., 1994; Platani et al., 2002).

The role carried out by the cited nuclear components in the ribosomal protein import has not been exactly defined, and it is not clear whether all the ribosomal proteins follow the same pathway. Intriguingly, by time course evaluation, Ko et al. (2006) observed that, once entered nucleus, the human tagged rpL7 associates with nucleoplasmic microbodies. Such an association, for which is registered a peak at 12 h, decreases with time and at

24 h most of the transfected cells have the ribosomal protein located into the nucleolus. This, hence, would confirm the hypothesis that at least a part of the ribosomal proteins interacts with other structures in the nucleus before being assembled in the subunits in the nucleolus.

4.1.2. Different dynamics for different roles

The transcription and processing of rRNA involve numerous characters, which are active in coordinated processes. What is particularly interesting is that early and late processing factors present different dynamics of recruitment to the transcription and assembly sites (Fomproix et al., 1998; Savino et al., 1999). A clear example is the rearrangement that the processing factors undergo during mitosis, when the inactivated rDNA transcription machinery remains associated with or close to the rDNA (Roussel et al., 1996) whereas the nucleolar proteins concentrate around the chromosomes, migrating with them (Hernandez-verdun, 2006). When in telophase the inactivation is removed, the nucleolar processing factors assemble in the so-called PNBs moving from the chromosomes, to be then progressively recruited in the nucleolus. The early processing factors associate with the PNBs only for few seconds, while the late ones leave the bodies slower (Hernandez-Verdun, 2006).

Moreover, in interphase it has been observed that nucleolar factors involved in different phases of preribosome production do not show similar intervals of retention in the nucleolus, thus suggesting a different dynamics related to specific functional activities (Chen and Huang, 2001). Nucleolar proteins such as UBF1, nucleolin, fibrillarin, and B23, which are involved in the rRNA transcription and processing, showed rapid movements between nucleolus and nucleoplasm characterized by short time-period of association with the first one. Furthermore, immunoprecipitation of nucleolin can coimmunoprecipitate both B23 and fibrillarin (Pinol-Roma, 1999), suggesting that a common complex exists and that it does not dissociate by cycling in the nucleoplasm. This implies that factors, involved in different steps of the biogenesis of the ribosomal subunits, are not stationary in the nucleolus, but probably cycle more than once between the two compartments. It has been proposed (Chen and Huang, 2001) that a nucleolar protein may be assembled in an active complex in the nucleolus and, there, it carries out its function before dissociating from the complex and being inactivated. At this point, the molecule may exchange with the nucleoplasmic pool to be then reactivated, probably by a phospho/dephosphorylation mechanism. It has been also taken into account the option that a dissociation of the proteins does not necessarily take place after each functional act, but that some modification mechanisms recharge the complexes to make them ready again in activities of the nucleolus.

By contrast, proteins committed to be assembled in the ribosomal subunits (for instance S5 in SSU and L9 in LSU) once in the nucleolus have a

slower rate of mobility: the assembly of the subunits is a complex process not as fast as the transcriptional and processing mechanism.

Recently Lam et al. (2007) have shown, by mass spectrometry (MS)-based proteomics as well as live-cell fluorescence imaging, the differences in mobility and nucleolar association existing among the proteins that would have been part of the ribosomal subunits. The authors showed that, after being translated in the cytoplasm, r proteins are imported in the nucleus reaching the nucleolus quicker than any other nucleolar factor. Even more intriguing is that the 60S r proteins move to the site of preribosome synthesis slower than the 40S r proteins; this would confirm the existence of two distinct pathways of both assembly and export for the large and small ribosomal subunits. (Granneman and Baserga, 2004).

Lam et al. (2007) even hypothesized that, once the r proteins reach the nucleolus, these could move in and out of the organelle mostly in the form of either free proteins or small protein complexes. At this regard, it is quite difficult to imagine that, during the basic process of biosynthesis, intermediates complexes could escape from such a machinery and leave the nucleolus to the nucleoplasm. However, the r proteins, reaching the nucleolus, become tethered in slow-mobility nucleolar complexes, possibly correlated with ribosomal subunits, and their association rate depends on transcriptional activity. In fact, the treatment with AMD, an inhibitor of the rRNA synthesis, induces a reduced recall of r proteins with a consequent decrease in their relative amount, with a larger reduction for the SSU proteins (Andersen et al., 2005). An unexpected result was also that a significant fraction of r proteins is degraded and not assembled into ribosomal subunits. This finding may explain why a higher amount of r proteins is found in entrance than going out from the nucleus in preribosomes. As already observed for other proteins (Rockel et al., 2005), Lam et al. (2007), indeed, demonstrated the presence of a nucleoplasmic degradation mechanism of the r proteins by action of proteasomes. A proteasome-mediated demolition can be an efficient method to control preribosome production.

It is worth underlining that in the nucleolus several proteins involved in the ribosome biogenesis are ubiquitinated (Chen et al., 2002; Itahana et al., 2003; Peng et al., 2003; Sato et al., 2004), and, therefore, targets for a proteasomic degradation. Whereas an inhibition of the proteasomes, using for instance epoxamin, results in both an increase (about 80%) of the nuclear level of r protein RPL27-GFP, as Lam et al. mentioned (2007), and can induce specific molecular changes in the nucleolar structure, Stavreva et al. (2006) have shown the effects of another proteasome inhibitor (MG-132) on the morphology of the nucleolus and the preribosome synthesis. The data provide evidence that the ubiquitin–proteasome system (UPS) influences both early and late ribosomal subunit processing, either working as control element preventing the assembly of a nonfunctional factor in the preribosome particle (in the nucleoplasm, Chen and Huang, 2001; Dundr et al., 2002), or

being potentially a character in the subunit maturation in the nucleolus (Peters et al., 1994; Song and Wu, 2005; Wojcik and DeMartino, 2003).

The authors' experimental conditions highlight the difference in the mobility and consequently in the distribution of factors again involved in the pre-rRNA processing, further underlining how specific dynamics are associated with precise roles. Therefore, if the MG-132 treatment does not significantly affect some processing factors, it may seriously modify the mobility of others such as the late processing factor B23.

It is to mention that, during the biogenesis of the subunits, some factors both in the 60S and 40S preribosomes are decorated with SUMO (Panse et al., 2006). Although the sumoylation does not lead to a degradation of the substrate, as occurs in ubiquitination pathway, it is however capable of affecting the protein functions and their subcellular localization (Johnson and Blobel, 1999; Melchior, 2000; Seeler and Dejean, 2003). Therefore, it is not surprising that mutants of factors involved in sumoylation are characterized, for instance, by accumulation of rpL25-eGFP, suggesting, indeed, a possible role of this process in the presubunit processing and transport. In addition to ubiquitination and sumoylation, Caldarola et al. (2009) reviewed another modification of the ribosomal proteins, the NEDDylation (Xirodimas et al., 2008), involved in the increase of their stability. Taken together these data show how both the ribosomal proteins and the factors active in the ribosomal subunit biogenesis undergo a series of modifications defining their specificity of action.

4.2. Preribosome assembly

As can be imagined from the amount of characters engaged in the process, the preribosome assembly is a dynamic process which involves a series of coordinated mechanisms driven by specific factors (Fig. 2.5). Once synthesized and folded, the nascent rRNA undergoes specific modifications to be then processed by exo- and endonucleases and bound to ribosomal proteins. However, it is by now clear that this set of processes cannot be thought as independently carried out, and data demonstrate that at least some of them are contemporary (Granneman and Baserga, 2005; Pandit et al., 2008); moreover, this is confirmed by both the cotranscriptional association of several ribosomal biogenetic factors with rRNA (Dragon et al., 2002; Gallagher et al., 2004) and the precocious interaction of ribosomal proteins with the nascent rRNA. Therefore, the nascent ribosomal subunit is likely a macromolecular structure in dynamic evolution, which reaches its mature and functional form during its traffic from the nucleolus, through the nucleoplasm to the cytoplasm.

In the nucleolus, the assembly of the pre-rRNA precursors 5S (synthesized by RNA polymerase III in the nucleoplasm) and 5.8S, 18S, 28S (synthesized by RNA polymerase I) with ribosomal and nonribosomal

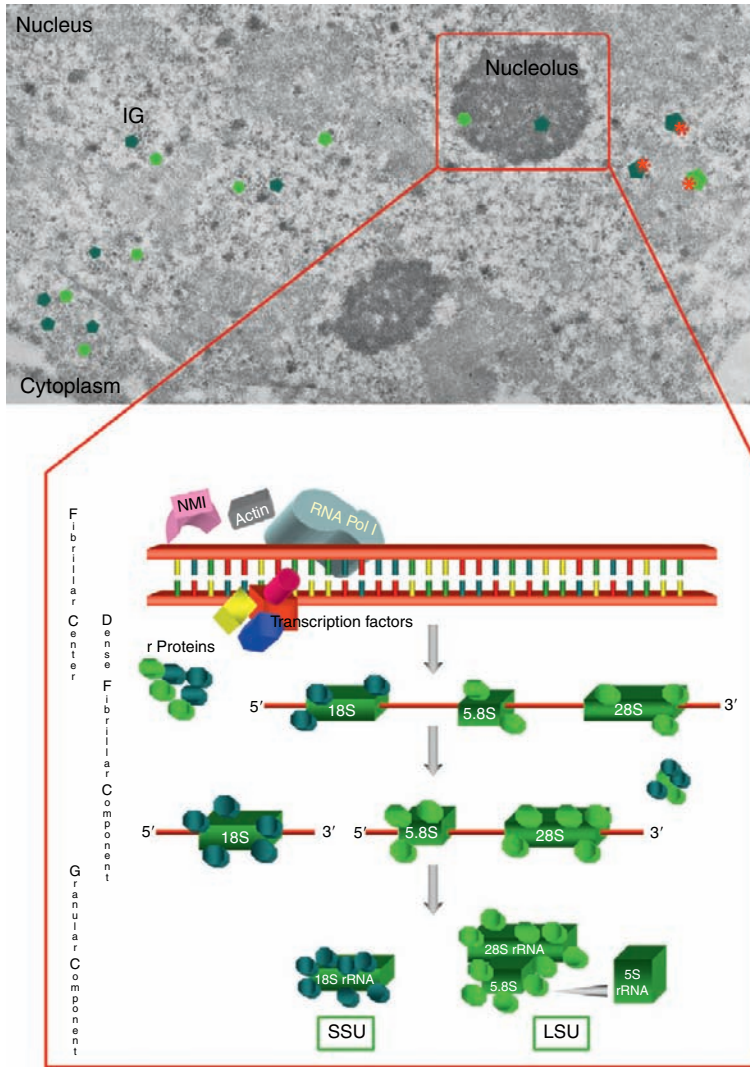

Figure 2.5 A scheme of the pathway followed by a ribosomal protein from the cytoplasm to the nucleolus to be integrated into a ribosomal subunit.

proteins in addition to small nucleolar RNP (snoRNP) yields 90S preribosome particles. These are then split in the pre-60S and pre-40S subunits, then modified and finally exported in the nucleoplasm. Intriguingly, although the 40S and 60S preribosomes share some factors (for instance the Rrp5p and Rrp12p, Venema and Tollervey, 1999; Oeffinger et al., 2004), they follow separate maturation routes (Johnson et al., 2002): 40S

processing factors assemble cotranscriptionally onto the 35S pre-rRNA when the future 18S rRNA is transcribed (Fromont-Racine et al., 2003; Gallagher et al., 2004; Granneman and Baserga, 2004). The 60S processing machinery, instead, is recruited later, after the release of the 40S precursor from the 90S particle and the completion of the rRNA transcription.

However, preassembly subcomplexes have been recognized as stable entities (Dosil and Bustelo, 2004; Grandi et al., 2002; Granneman et al., 2003; Watkins et al., 2000; Wehner et al., 2002); following the subunit maturation (Henras et al., 2008; Staley and Woolford, 2009; Tang et al., 2008) each one is characterized by the presence of specific factors, hierarchically associated and both temporally and spatially disassembled in subsequent steps (Pérez-Fernández et al., 2007). The association and dissociation of the factors committed in the preribosome assembly follow different pathways in the SSU and LSU maturation. It is in fact intriguing that the majority of the 90S components is required for the SSU 40S synthesis (Henras et al., 2008). In eukaryotes, the latter consists of three domains (head, body, and platform) besides the additional features called beak, shoulder, and feet (Frank et al., 1981, 1982; Spahn et al., 2001).

Comparing the pre-40S particle and the mature subunit, Schäfer et al. (2006) obtained evidence on the evolution of the 40S structure and about the factors regulating the maturation of the SSU. The pre-40S subunit lacks the beak structure which is a protrusion of the 18S rRNA helix 33 in close vicinity to RpS3. This ribosomal protein undergoes a cycle of phospho/dephosphorylation before being stably incorporated in mature 40S: the kinase Hrr25 phosphorylates RpS3 (in addition to the 40S synthesis factor Enp1) promoting its dissociation from the preribosome. The subsequent dephosphorylation of the protein induces the formation of the beak structure. The data suggest that the beak may remain flexible (while RpS3 is not tightly integrated into 40S) allowing the passage of the preribosome through the NPC, to finally become rigid in the cytoplasm in concomitance with the definitely stable association of RpS3.

Other factors are fundamental in the maturation of the 40S subunit, taking also into account that an order in the addition of the ribosomal proteins during preribosome assembly exists, at least *in vitro* (Talkington et al., 2005). Such an order defines the formation of folding intermediates which serve as trigger and quality control to allow the proceeding of a correct ribosome biogenesis. In this connection, Ferreira-Cerca et al. (2007) suggest for the preribosome factors a role as organizer of the assembly and export of the subunits: the ribosomal particles are retained in the nucleolus by some factors if not properly structurated, or, on the contrary, helped by others to overcome first the nucleolar retention and secondly the nuclear one.

On the basis of an eubacterial map for the assembly order (Mizushima and Nomura, 1970; Nierhaus and Wilson, 2004; Nomura and Erdmann, 1970; Talkington et al., 2005), Ferreira-Cerca et al. (2007) have considered

the effects of the depletion of the ribosomal protein RpS5 *in vivo* on the formation of the head structure, required to release efficiently preribosomes toward cytoplasm. RpS5 seems to be essential for the head structuring (Brodersen et al., 2002; Mizushima and Nomura, 1970; Nierhaus and Wilson, 2004; Talkington et al., 2005) as its eubacterial homolog RpS7 (Bubunenko et al., 2006); the RpS5 depletion, in fact, leads in addition to an accumulation of early rRNA precursors (35S, 23S, Ferreira-Cerca et al., 2005) due to the delay in the early processing events, even to both an incapability of 20S of being translocated to the cytoplasm (Ferreira-Cerca et al., 2005) and to a weakened interaction of 20S with some ribosomal proteins. This would suggest that such proteins require RpS5 for being stably incorporated in SSU precursor. Although other homologies with the eubacterial map have been highlighted (RpS15 is homolog of RpS19 which is the second binder of the SSU head domain), fifteen 40S proteins do not have procariotic homologs.

As far as the LSU is concerned, Nissan et al. (2002) isolated seven distinct 60S preribosome intermediates characterized by an increased semplification in the protein composition during the movement of the 60S particles from their site of synthesis toward the cytoplasm. Interestingly, the final 60S particles are associated with many ribosomal proteins (L proteins) but few nonribosomal factors, although the ribosomal protein composition in earlier and later 60S particles is quite similar. This suggests that most L proteins are already incorporated onto the early pre-60S complexes. Moreover, findings indicated that the maturation of the 25S rRNA is largely or fully completed before the subunits leave the nucleolus. Furthermore, differently from the final maturation of the 7S to 5.8S rRNA occurring in the nucleoplasm, the 5S rRNA, along with its binding partner RpL5, joins an early pre-60S in the nucleolus, as demonstrated by its detection in each of 60S particles analyzed.

In yeast, 5S rRNA forms a subcomplex with the assembly factors Rpf2 (which gives the name to the subcomplex), Rrs1, and the ribosomal proteins RpL5 and RpL11, giving rise to a stable neighborhood within the 90S particles (Zhang et al., 2007); although it has not been clarified yet whether the components enter 90S preribosome as either an already formed subcomplex or assembling once into 90S particle (Dosil and Bustelo, 2004; Harnpicharnchai et al., 2001; Krogan et al., 2004; Lebreton et al., 2006; Miles et al., 2005; Nissan et al., 2004; Rosado et al., 2007), evidence exists that the subcomplex joins the pre-rRNP at early maturation steps. However, the Rpf2 neighborhood carried its function out in later steps, processing the 27 SB pre-rRNA and promoting the proper release of pre-60S particle from the nucleolus (Zhang et al., 2007). The Rpf2 subcomplex presence at early stages of the pre-rRNP processing may serve to recruit ribosomal proteins or assembly factors and to establish and maintain the local required rRNP architecture. As for the former option, an example could be the mediated stable association of the ribosomal protein RpL10, indeed for

the nuclear export of the nascent 60S subunit (Gadal et al., 2001): a depletion of one of the Rpf2 subcomplex proteins, in fact, provokes an instable interaction of RpL10 with the preribosome (Zhang et al., 2007). In addition, the depletion may also induce the absence of the assembly factor Nog2 in the preribosomes. Nog2 is a putative GTPase required for processing of 27SB pre-rRNA (Saveanu et al., 2001, 2003).

In this connection, it is interesting that both early and late 60S subunits are associated with putative GTPases (Bassler et al., 2001; Becam et al., 2001; Jensen et al., 2003; Karbstein, 2007; Saveanu et al., 2001). GTPases, containing RNA-binding domain (Bassler et al., 2006; Hang and Zhao, 2003; Karbstein, 2007; Karbstein et al., 2005; Sato et al., 2005), can directly be bound to preribosomes. They seem to be involved in the assembly of both large and small subunits (Karbstein, 2007) and may have function as (I) provider of energy for binding or dissociation of proteins to nascent preribosomes, (II) promoter of a conformational rearrangement within the ribosomal particles, (III) place holder preventing a premature protein binding.

4.3. Subunit export

Although the biogenesis and consequent maturation pathways for the large and the small subunits proceed independently, at least a part of the pool of factors involved in their export (Rodriguez et al., 2004) is shared. However, not all the molecules engaged in the preribosome export have been identified yet. It is interesting that the binding of essential export factors at specific sites in correctly assembled preribosomes would coordinate the export mechanism with the late-maturation events (Johnson et al., 2002). Therefore, the complex process of the biosynthesis of the ribosomal subunits is contemporary to the "preparation" for their export.

Both SSUs and LSUs can be indisputably considered sizeable cargoes and, as most proteins and RNAs, their transport through the NPC (Macara, 2001; Mattaj and Englmeier, 1998) is mediated by karyopherines (Fried and Kutay, 2003; Gorlich and Kutay, 1999; Kohler and Hurt, 2007; Weis, 2003) among which the exportin Crm1 (Pemberton and Paschal, 2005). The karyopherines recognize as binding sites the short hydrophobic FG repeats of the nucleoporins, constituents of the NPCs (Denning et al., 2003; Tran and Wente, 2006).

Ribbeck and Gorlich (2002) assumed that multiple export receptors could be necessary for an efficient transport of a large cargo, to possibly cover the large hydrophilic surface of the cargo to pass through the hydrophobic walls of the NPCs. In this connection, it is not surprising that, in yeast, at least three export receptors have been identified simultaneously working for LSU export: the heterodimeric mRNA transporter Mex67/Mtr2 (Yao et al., 2007), the nucleus–cytoplasm shuttling protein Arx1, which directly binds to nucleoporins and the subunit, and the nuclear export receptor Crm1,

whose interaction with the substrate depends on the formation of a ternary complex with RanGTP (Lund and Dahlberg, 2001). RanGTP stabilizes the interaction of exportin Crm1 to its cargo and the directionality of the transport is driven by its concentration gradient (Hurt et al., 1999; Johnson et al., 2001; Moy and Silver, 1999; Stage-Zimmermann et al., 2000).

The export of the pre-60S subunits is mediated by the adapter molecule Nmd3, missing in SSU export pathway, which provides the nuclear export signal (NES) binding to the particle. NES is recognized by Crm1 (Gadal et al., 2001; Ho et al., 2000; Johnson et al., 2001, 2002), without having a direct interaction with the adapter Nmd3. This suggests that other factors participate to the mechanism. Although so far Crm1 has been believed to be a fundamental receptor for the pre-60S subunit export, Lo and Johnson (2009) have given evidence that in yeast Crm1 may be replaced by any of the other known receptors. Although, indeed, no specific requirement of Crm1 exists, the functions of Arx1 and Mex67/mtr2 do not appear to be conserved in higher eukaryotes (Bradatsch et al., 2007; Yao et al., 2007), casting some doubts on their interchangeability.

However, the possible existence of a replacing protein and, moreover, the lack of a specific requirement for a given export factor would allow ample flexibility for the evolution of different export pathways in different eukaryotic lineages (Lo and Johnson, 2009). This idea is supported by the fact that an absolute requirement of Nmd3 as NES adapter does not even exist (Johnson et al., 2002; Lo and Johnson, 2009). The export, in fact, can be accomplished even if NES is provided in *cis* by a ribosomal protein integrally associated with the subunit (Johnson et al., 2002; Lo and Johnson, 2009). The conservation of Nmd3 as adapter throughout the eukaryotes (Thomas and Kutay, 2003; Trotta et al., 2003), however, suggest its active role in a greater control of regulation of pre-60S subunit export, and in the initial joining of the large and small subunits in the cytoplasm. In fact, Nmd3 has been seen stably associated with a free mature 60S subunit (Ho et al., 2000), but it has not been detected in the 80S ribosome (Ho and Johnson, 1999), suggesting that it must be displaced during the initiation of the translation to be replaced in the free 60S particle at the end of the process (Ho and Johnson, 1999).

Differently, Crm1 is immediately released when 60S particle enters the cytoplasm (Lund and Dahlberg, 2001) to be reimported in the nucleoplasm. The export receptor Crm1, along with Ran GTP, is also involved in the small subunit transport outside the nucleoplasm (Gleizes et al., 2001; Hurt et al., 1999; Moy and Silver, 1999), although it is not clear how and exactly when it can bind to the 40S subunit (Rouquette et al., 2005). It has been shown, in fact, that the inhibition of this exportin, by treating HeLa cells with leptomycin B (LMB), induces a decrease in the 18S precursor amount, indicating that Crm1 may be a necessary factor for the preribosome biogenesis prior the nuclear export (Rouquette et al., 2005). Moreover, the LMB treatment leads to an accumulation of an undescribed intermediate (26S)

which appears to result from the cleavage at position G1643 in 5′-ETS (resembling to A0 site in budding yeast, Gerbi and Borovjagin, 2004). The cleavage at A0 is normally coordinated with processing at site A1 and A2 and involves U3 snoRNA (Borovjagin and Gerbi, 2001; Venema and Tollervey, 1999). The evidence that, on the one hand, a mutation of U3 enables to block the processing at site 1 and 2 (Borovjagin and Gerbi, 2001; Hughes and Ares, 1991) and, on the other hand, Crm1 is required for targeting U3 snoRNP to the nucleolus (Boulon et al., 2004) has suggested that the alteration of the 18S production in LMB-treated HeLa cells may result from the mislocation of U3 to the nucleolus (Rouquette et al., 2005).

However, Rouquette et al. (2005) have found, in normal conditions, the existence of a precursor, which they called 18S-E pre-rRNA, whose conversion to mature 18S-rRNA takes place in the cytoplasm with the involvement of the human structural homolog of yeast hRio2. Before this processing, when the pre-40S particle is still in the nucleoplasm, the preribosome interacts with several nonribosomal factors (see pre-assembly chapter) and it must undergo a series of modifications to become competent for the export. The same occurs to the immature LSUs.

In this connection, Nissan et al. (2002, 2004) identified a pre-60S intermediate and named it Rix1-containing pre-60S particle. This particle predominantly associates with Rix1 complex components (Rix1; Ipi1, Ipi3, Krogan et al., 2004) and with Rea1. This latter is a putative AAA-type ATPase capable, as such, of changing their own conformation and promoting a potential dissociation of protein–protein interaction, depending on the bound nucleotide (Vale, 2000). When observed at EM, the Rix1-containing pre-60S particle shows a typical tadpole-like structure with a globular head domain (belonging to the 60S) and a highly elongated tale domain surely occupied by Rea1 ATPase. Being distantly related to the motor protein dynein, which is an AAA-type ATPas with a microtubule motor activity (Garbarino and Gibbons, 2002), it has been hypothesized for Rea1 an involvement in the intranuclear movement and in the export of the pre-60S particles to the cytoplasm (Bassler et al., 2001; Gadal et al., 2001; Nissan et al., 2004).

The nature of Rea1 as molecular motor for the LSU movement opens the question of the mechanisms which mediate the transport of both the ribosomal subunits from the nucleolus, through the nucleoplasm, toward the cytoplasm. Politz et al. (2003) provided data for the existence of an LSU export driven by an anomalous diffusion mechanism. This type of diffusion is called anomalous because, although the LSUs move from the nucleolus freely and follows a random distribution in the nucleoplasm, a verified possibility exists of a slowing down of the 60S diffusion, due to its collisions and retention within nuclear barriers or structures such as chromatin. The authors used oligo d(T) hybridizing to different regions of the 28S RNA, to then analyze the parameters of its mobility at both 37 and 23 °C, finding values suggestive of a diffusive movement.

However, it is evident that, so far, the mechanism involved in the transport of the ribosomal subunits has not been completely clarified. Moreover, considering the idea of a coexistence of diffusive and active processes, it is not clear yet which role is played by such mechanisms and how a diffusive and an energy consuming transport may efficiently cooperate. In order to address these questions, it is opportune to underline that in the last years some groups have been interested in the presence of nuclear myosin and actin associated with the sites of transcription. The existence of motor proteins in the nucleus suggests their action in active processes.

In this connection, a 120-kDa protein has been identified in the nucleus. It is characterized by the typical features of the myosin superfamily of proteins: K^+-EDTA ATPase activity, ATP, and actin binding, in addition to a calmodulin-binding capacity (Nowak et al., 1997). On the basis of the results obtained, Nowak et al. (1997) recognized the 120-kDa protein as a previously undescribed myosin I isoform (NMI) that localizes in the nucleus and, as shown later, containing a unique 16-aminoacid aminoterminal extension (Pestic-Dragovich et al., 2000). Furthermore, the colocalization of such a myosin I molecule and actin in concomitance of the suggested interaction of the latter one with small ribonucleoproteins during the processing and transport of RNA (Sahlas et al., 1993), prompted to hypothesize the involvement of the motor proteins in an energy-consuming mechanism of transcription. In fact, electron microscope immunostaining has demonstrated that actin and nuclear myosin I colocalize in the nucleolus. In particular, at high magnification, NMI has been observed concentrated mainly in DFC, where the transcription of the rDNA takes place, whereas actin localized in FCs surrounding the DFC. This suggests a cooperation of these two motor proteins in the rRNA transcription.

The model proposing that NMI and actin participate to transcriptional mechanisms would support the idea of their possible role played in other basic processes in the nucleus. It would be interesting to investigate whether the motor proteins may participate in an active and consequently modulable mechanism of movement of the ribosomal subunits from the nucleolus and throughout the nucleoplasm toward the cytoplasm. Without ruling out the idea of an anomalous diffusion (Politz et al., 2003), we however, suggested that an energy-consuming mechanism, mediated by nuclear myosin and actin, exists for the transport of at least a subset of the small ribosomal subunits (Cisterna et al., 2006, 2009); it would be a potential partner of a predominant diffusive process. Such a hypothesis has been initially based on the series of data obtained at EM, which point out that the ribosomal protein S6, associated with a ribonucleoprotein particle as a part of the SSU, colocalizes in the nucleoplasm with nuclear myosin I and actin, with a frequency of about 10–20% of the total SSUs.

Moreover, we have provided further evidence for the existence of such an independent, energy-consuming mechanism by means of the blocking of

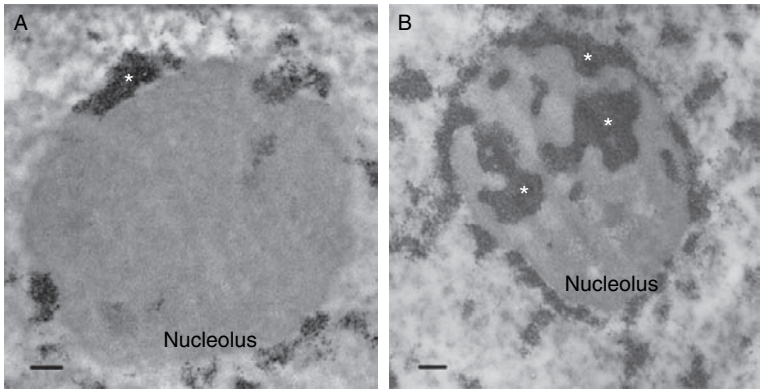

Figure 2.6 The nucleolar-associated chromatin (NAC, asterisk), which in control condition (A) surrounds the nucleolar body, increases after the ATP depletion penetrating in the nucleolus (B). DNA is specifically stained by OAC. Bar represents 0.5 μm.

de novo synthesis of ATP (Cisterna et al., 2009) and *in vivo* incorporation (*via* lysophosphatidylcholine, LPC) of antibodies against motor proteins (Cisterna et al., 2006, 2009). Both the treatments induce a substantial reorganization of the nucleolus, which shows, on one hand, the signals of a reduced activity, such as the increase of the NAC (Fig. 2.6) and the decrease of the area percentage occupied by the fibrillar components (FC, DFC) active in the production of the ribosomal subunits, and on the other hand, an increase in the GC, site of late maturative processing and storage of the produced subunits (Cisterna et al., 2009). The decrease in the available ATP and the motor protein antibody-blockade as well, indeed, would lead to a reduced production of ribosomal subunits which are, however, less efficiently exported and accumulated in GC (as confirmed by flow cytometry and western immunoblotting, Cisterna et al., 2009). At this moment, the question is clearly still open to define the roles played by the diffusive and active mechanisms in the subunit transport and the involvement of motor proteins in this latter. The interaction of NMI and actin engaged in transcription mechanism would, in fact, suggest their cooperation also in a transport process, although the few data on a possible interaction pathway of these two proteins in the nucleoplasm. By now, it has been ascertained the existence in the nucleoplasm of actin both in monomeric form (Vartiainen et al., 2007) and as assembled actin (McDonald et al., 2006; Ye et al., 2008), in addition to the presence of actin-binding and actin-related proteins (Dingová et al., 2009; Hubert et al., 2008).

Intriguingly, Ye et al. (2008) demonstrated that filamentous nuclear actin and NMI act together to regulate polymerase I-mediated transcription in the nucleolus and Chuang et al. (2006) have shown the role of acto-NMI complex in intranuclear transport of activated chromosome regions. Data suggested that

NMI, capable of directly binding to both DNA throughout its positively charged domain in the tail and actin by the actin-binding domain in the head, can translocate a gene along the actin filaments from a heterochromatin space to a less dense region of euchromatin where transcription takes place.

In this connection, one can suggest a similar interaction between NMI and actin involved in the ribosomal subunits movement, as already in part confirmed by our results (Cisterna et al., 2006, 2009). However, it must be taken into account that, in addition to NMIβ, other myosins from several classes have been found in the nucleus (Cameron et al., 2007; deLanerolle et al., 2005; Salamon et al., 2003; Vreugde et al., 2006). For instance, myosin Va (MVa) is an active motor generating force and movement on actin filaments (Mehta et al., 1999), which may participate in the translocation of molecules between nuclear speckles and nucleoli (Pranchevicius et al., 2008). The labeling with a monoclonal antibody recognizing the phospho-Serine^{1650} globular tail of the MVa (9E6, Pranchevicius et al., 2008) has shown a strong signal concentrated in large, irregular punctate structures in the nucleoplasm of several cell line (Espreafico et al., 1992; Nascimento et al., 1997; Rudolf et al., 2003). These dots have been recognized as nuclear speckles on the basis of the colocalization of 9E6 with an antibody against the splicing factor SC35. Therefore, MVa in its phosphorylated form is associated with the IGCs, nuclear compartments active in the storage, assembly and modification of factors involved in the transcriptional and processing mechanisms where the nuclear actin has been also found (Sahlas et al., 1993; Saitoh et al., 2004). After 2 h of an AMD treatment, the phospho-Serine^{1650} MVa undergoes a redistribution to the nucleoli, which are devoid of labeling in a control condition, and to IGC neighboring speckles, which may resemble the so-called nuclear paraspeckles recognized by EM as IGAZ (Cardinale et al., 2007). These series of data provided by Pranchevicius et al. (2008) underline further how several myosins exist in the nucleus and how, often, their presence is in some ways associated with the nucleolus. It is unclear yet which role they exactly play, but the evidence of their interaction with the nuclear actin by now, seems to confirm a cooperation between motor proteins within an acto-myosin mechanism involved in basic nuclear processes such as transcription (Vreugde et al., 2006) and transport (Cisterna et al., 2006, 2009).

5. Concluding Remarks

The nucleolus is a fascinating structure, and after more than 200 years since its discovery still retains many secrets. Perhaps the most intriguing characteristics are its plasticity, and the dynamics behind. These features not only involve the spatial organization of the nucleolus but also its functions.

Besides being the ribosomal factory of the cell, the nucleolus has probably several other functions and involvements, and researchers have just begun to unveil some of these roles. The next direction of the studies in the nucleolar field will probably be linked to a better understanding of the delicate balance between ribosomal and nonribosomal functions in the context of the cellular and nuclear behavior. Such knowledge will be of help in several issues, including the control of cell proliferation and cancer.

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CHAPTER THREE

What Causes a Broken Heart—Molecular Insights into Heart Failure

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Abstract

Our understanding of the molecular processes which regulate cardiac function has grown immeasurably in recent years. Even with the advent of β-blockers, angiotensin inhibitors and calcium modulating agents, heart failure (HF) still remains a seriously debilitating and life-threatening condition. Here, we review the molecular changes which occur in the heart in response to increased load and the pathways which control cardiac hypertrophy, calcium homeostasis, and immune activation during HF. These can occur as a result of genetic mutation in the case of hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM) or as a result of ischemic or hypertensive heart disease. In the majority of cases, calcineurin and CaMK respond to dysregulated calcium signaling and adrenergic drive is increased, each of which has a role to play in controlling blood pressure, heart rate, and left ventricular function. Many major pathways for pathological remodeling converge on a set of transcriptional regulators such as myocyte enhancer factor 2 (MEF2), nuclear factors of activated T cells (NFAT), and GATA4 and these are opposed by the action of the natriuretic peptides ANP and BNP. Epigenetic modification has emerged in recent years as a major influence cardiac physiology and histone acetyl transferases (HATs) and histone deacetylases (HDACs) are now known to both induce and antagonize hypertrophic growth. The newly emerging roles of microRNAs in regulating left ventricular dysfunction and fibrosis also has great potential for novel therapeutic intervention. Finally, we discuss the role of the immune system in mediating left ventricular dysfunction and fibrosis and ways this can be targeted in the setting of viral myocarditis.

Key Words: Cardiac function, Heart failure, Carciac hypertrophy, Ca^{+} signaling, Natriuretic peptides, Myocarditis.

1. Introduction

Heart failure (HF) is one of the leading causes of mortality in the western world. HF affects 1–2% of the general population and accounts for 5% of all adult hospital admissions (Ellery et al., 2006). The major causes of HF are ischemic or hypertensive heart disease, dilated cardiomyopathy (DCM), and valvular heart disease; contributing factors include diabetes, dyslipidaemia, genetic predisposition, viruses, and environmental toxins. Despite advances in the medical management of HF, the 5-year mortality rate remains at almost 50% (Lloyd-Jones et al., 2002). Thus, there is a need for greater understanding of the molecular mechanisms underpinning this debilitating condition in the hope of devising novel clinical interventions.

HF can be classified as either systolic or diastolic. When the heart is in systolic failure, the ventricles cannot contract properly and stroke volume and cardiac output are compromised. Diastolic HF is characterized by stiff ventricles which cannot fully relax and thus do not fill completely, this is common in elderly hypertensive patients. HF manifests in a variety of symptoms including dyspnea, angina, exercise intolerance, and pulmonary or peripheral oedema. HF is classified according to the New York Heart Association (NYHA) Functional Classification. It ranges from Class I, where patients have cardiac disease which does not impact on physical activity to Class IV, where patients have clear symptoms of HF and any physical activity results in discomfort (Hurst, 2006).

Current therapeutic options for HF include diuretics, β-blockers, angiotensin modulating agents, aldosterone antagonists, vasodilators and ionotropic agents, or a combination of the above. A plethora of clinical trials has shown significant improvement in mortality outcomes for all of these agents. In severe HF cases, left ventricular assist devices (LVADs) significantly improve cardiac function and may be used as a bridging measure while patients are awaiting heart transplant or as so called destination therapy in patients who are ineligible to receive a transplant.

2. Pathological Cardiac Remodeling

A hallmark of the failing heart is the presence of remodeling which in the advanced stage greatly contributes to pathogenesis. Defects in systolic function due to pressure or volume overload, cardiac myocyte loss

following myocardial infarction or myocarditis leads to hypertrophic growth of the remaining myocytes. Although a small percentage of the total myocytes can be seen to proliferate following stress, cardiac enlargement occurs primarily as a result of growth of resident myocytes (Yi et al., 2010). Myocytes enlarge by two means; concentric hypertrophy proceeds following the addition of sarcomeres in parallel, thus increasing myocytes thickness, eccentric hypertrophy on the other hand is due to the addition of sarcomeres series, causing the myocyte to elongate.

2.1. Concentric hypertrophy

Hypertension or aortic stenosis causes pressure overload and if left unchecked, the increased stress put on the ventricle walls would adversely affect cardiac output. According to Laplace's law, which states that wall tension = pressure × radius/2 × wall thickness, the increased pressure is balanced by myocyte hypertrophy and increased wall thickness which eventually equalize the wall stress (Grossman et al., 1975). This results in a concentric form of hypertrophy where the ventricle walls increase in thickness but chamber volume in unaffected, commonly seen in patients with hypertrophic cardiomyopathy (HCM). The increase in hypertrophy is proportional to increase in systolic pressure and though wall stress is normalized, there is a greater oxygen demand on the heart due to the increased cardiac mass.

2.2. Eccentric hypertrophy

In contrast, volume overload brought on by mitral regurgitation or valvular insufficiency causes an eccentric growth pattern, where chamber volume is increased with little or no effect on wall thickness; this is typified by DCM. Ventricular dilation allows for increased pressure necessary for ejection, however in this case wall stress is not normalized. In the case of mitral regurgitation for example, LV dilation increases the stroke volume to maintain normal levels of ejection, however over time decompensation occurs and volume overload will ultimately decrease cardiac output and increase end-systolic and end-diastolic volumes (Opie et al., 2006). Prolonged aortic stenosis leads to a drop in cardiac output, elevated end-diastolic pressure and dilation of the left ventricle, suggesting that concentric hypertrophy may ultimately deteriorate into a dilated eccentric growth pattern (Opie et al., 2006). Myocardial infarction results in both pressure and volume stress on the heart and thus a mixed form of hypertrophic growth is evident.

2.3. Hypertrophy as a compensatory process

Hypertrophy is initially a beneficial compensatory process as it decreases wall stress and increases cardiac output and stroke volume. However, hypertophic growth in the context of disease is ultimately maladaptive since it regularly progresses to decompensation, contractile dysfunction, and ultimately HF; indeed hypertrophy is associated with an increased risk of mortality (Levy et al., 1990). The premise that hypertrophy is an absolute requirement for equalizing wall stress and restoring cardiac output has been challenged in recent years. For example, mice genetically engineered to be resistant to hypertrophy were found to maintain cardiac function following pressure overload, even in the absence of normalized wall stress (Esposito et al., 2002). Since these mice had improved hemodynamics compared to wild type mice, it argues that hypertrophy and normalization of wall stress may not always be necessary to maintain cardiac function in the setting of chronic hemodynamic burden.

It is evident that hypertrophy is a nuanced physiological process which includes aspects of beneficial adaption to stress and deleterious biochemical changes; this can be viewed as a spectrum of physiological changes with a transition from adaption to failure. Many factors play a role in this transition, including loss of myocytes through apoptosis and necrosis, dysregulated calcium homeostasis, altered contractile response, and adrenergic receptor (AR) desensitization. Another important contributing factor in hypertrophic remodeling and transition to HF is the fibrotic changes brought about by fibroblast proliferation and increased extracellular matrix turnover (Diez, 2007). The presence of interstitial fibrosis affects the stiffness of the heart and impairs both contraction and relaxation.

3. Physiological Hypertrophy

In contrast to pathological hypertrophy, physiological hypertrophy occurs during postnatal development, pregnancy, or in response to sustained exercise. Physiological cardiac growth can be brought about by eccentric hypertrophy due to increased venous return during aerobic exercise or by concentric hypertrophy due to weight training (Bernardo et al., 2010). In contrast to the pathological situation however, wall thickness increases proportionally to chamber volume and so normal cardiac structure and function is maintained.

3.1. Insulin signaling

The molecular factors which control physiological hypertrophy are distinct from that of pathological setting and appear to be mediated largely through insulin signaling. It has long been appreciated that heart size is related to both

body mass and nutritional intake and this relationship may depend on serum insulin levels (DeBosch and Muslin, 2008). Both insulin and insulin-like growth factor-1 (IGF-1) stimulate cardiac protein synthesis and inhibit cardiac protein degradation, indeed infusion of IGF-1 leads to directly to myocyte hypertrophy (Fuller et al., 1992; McMullen et al., 2004). Moreover, cardiac IGF-1 receptor (IGF1R) transgenic mice displayed a 40% increase in cardiac mass with a proportional increase in chamber size and wall thickness, without any evidence of pathology (McMullen et al., 2004). Likewise, transgenic studies have shown that phosphoinositine-3-kinase (PI3K), which is activated by IGF1R, is necessary and sufficient for cardiac growth and importantly while PI3K transgenic mice have larger hearts, they do not exhibit cell death, fibrosis, or altered cardiac output (Shioi et al., 2000). The cardiomegaly in IGF1R transgenic mice was abolished when they were crossed onto a dominant negative PI3K background, which confirms that the hypertrophic activity of IGF-1 is mediated mainly through PI3K (McMullen et al., 2004). Importantly, both PI3K and IGF1R deficient mice were resistant to swimming induced hypertrophy but responded normally to pressure overload, demonstrating that IGF-1/PI3K is critical for the adaption of the heart to exercise (Kim et al., 2008a; Luo et al., 2005; McMullen et al., 2003). Serum levels of IGF-1 are enhanced following exercise and indeed like exercise, the IGF-1/PI3K pathway has been shown to exert a beneficial effect in the setting of HF, prolonging survival and improving cardiac function (Koziris et al., 1999; McMullen et al., 2007; Welch et al., 2002).

3.2. PI3K/Akt

The serine/threonine kinase Akt lies downstream of PI3K and mediates many of its cellular effects. Forced expression of Akt rescued the small heart phenotype of mice with cardiac deletion of the insulin receptor, demonstrating that Akt signaling regulates insulin-dependent cardiac growth (Shiojima et al., 2002). Deletion of Akt1 recapitulated the phenotype of PI3K deficient mice in terms of a blunted hypertrophic response to exercise but showed more severe hypertrophy in response to pressure overload (DeBosch et al., 2006). This result supports the concept that IGF-1 and PI3K are beneficial in a HF setting and suggests that their signaling may be mediated in a large part through Akt. Recently, the F-box protein atrogin-1 has been implicated in suppressing Akt-mediated cardiac hypertrophy. In culture, atrogin-1 overexpression was found to inhibit both insulin and IGF-1 dependent increases in cardiac myocyte cell size (Li et al., 2007). This is achieved through atrogin-1 dependent activation of the forkhead transcription factors Foxo1 and Foxo3a, which are known suppressors of hypertrophic growth (Li et al., 2007; Sandri et al., 2004; Skurk et al., 2005). Moreover, atrogin-1 deficient mice display increased myocyte hypertrophy following exercise training suggesting that it may be part of an important counter-regulatory mechanism in suppressing excess physiological hypertrophy.

Over the past number of years it has become evident that physiological hypertrophy occurs through a molecular program completely distinct from that of pathological cardiac growth. The recent findings that activation of the factors which promote physiological hypertrophy can also confer protection from HF have given a molecular insight into the well-appreciated benefits of exercise on cardiac health. Indeed a more detailed understanding of the precise control of physiological hypertrophy may yet yield novel therapeutic opportunities for the treatment of HF.

4. Genetic Cardiomyopathies

Genetic mutation of a host of cardiac genes can lead to abnormal cardiac function and ventricular remodeling. This commonly manifests as HCM and DCM, which are clinically heterogeneous in presentation. Early clinical diagnosis is often confounded by age-related penetrance, with many patients remaining asymptomatic until middle age. Most cardiomyopathy associated mutations are inherited in an autosomal dominant fashion, thus placing first degree relatives are at high risk of developing disease; genetic testing of family members is therefore advisable to allow clinical follow-up of affected individuals.

4.1. Hypertrophic cardiomyopathy

HCM is caused by mutations in genes encoding components of the sarcomere, it affects 1 in 500 individuals and manifests in HF, arrhythmias, and often in sudden cardiac death (Ackerman, 2005). There are over 300 mutations currently described, the most commonly affected genes being β-MHC (Myh7) and myosin binding protein C which together account for approximately 80% of cases; other affected genes include troponin I (TnI), troponin T (TnT), actin, α-tropomyosin, titin, and myosin light chain (Richard et al., 2003). When such mutant proteins get incorporated into the sarcomere they cause myocyte disarray, alter the contractile force and push the heart toward hypertrophic remodeling.

The myosin enzyme located in the thick filament of the sarcomere is the driving force behind muscle contraction. Myosin binds tightly to actin in the thin filament and uses ATP hydrolyses to power movement of actin filaments, causing the thick and thin filaments to slide past each other (Metzger and Westfall, 2004). This contractile force is transmitted to adjacent sarcomeres via the Z-disk and to nearby myocytes through intercalated disks. Mutations in the heavy chain of myosin (MHC) are among the most clinically severe and have been shown to affect ATPase activity, thermostability, and protein folding, causing marked ventricular hypertrophy with

onset around late adolescence (Cuda et al., 1997; Richard et al., 2003). These clinical phenotypes have been faithfully recapitulated in transgenic mouse models which contain the corresponding human mutation. For example, the R403Q mutation in MHC caused enhanced ATPase activity, increased speed of actin sliding and increased Ca^{2+} signaling which ultimately resulted in diastolic dysfunction (Walsh et al., 2010). Mouse models such as the myosin R403Q and R453C mutants have shown that DCM-associated mutations appear to increase the force generating capacity of myosin, placing a supraphysiological strain on sarcomeres which may be a driving force in hypertrophic remodeling (Debold et al., 2007).

4.2. Dilated cardiomyopathy

Like HCM, DCM is genetically heterogeneous and manifests clinically with an increase in myocardial mass, thin and stretched ventricular walls, and ultimately depressed contractility and diminished cardiac output (Luk et al., 2009). The 5-year survival rate of DCM is poor at around 50% due to complications of congestive HF and arrhythmias (Michels et al., 2003). Familial disease can account for up to 30% of DCM cases, however clinical presentation of DCM may be due to a spectrum of underlying conditions including myocarditis, peripartum cardiomyopathy, and ischemic heart disease (Karkkainen and Peuhkurinen, 2007). Genetic mutations causing DCM have generally been separated into those involved in force generation or force propagation (Luk et al., 2009). In the former case, mutations in myosin and troponins adversely affect actin–myosin contraction while in the latter, mutations in actin, α-tropomyosin, desmin, and δ-sarcoglycan diminish the propagation of contraction energy from the sarcomere to the sarcolema (Li et al., 1999; McConnell et al., 1999; Murphy et al., 2004; Olson et al., 1998; Regitz-Zagrosek et al., 2000; Tsubata et al., 2000).

4.3. Ca^{2+} homeostasis and cardiomyopathy

In both HCM and DCM, altered sarcomeric function is associated with dysregulated Ca^{2+} signaling and therefore alterations in sarcoplasmic reticulum (SR) Ca^{2+} homeostasis may be a direct contributing factor to hypertrophic remodeling. For example, mice harboring an Arg403Gln α-MHC protein have dysregulated SR Ca^{2+} levels and treatment with an L-type Ca^{2+} channel (LTCC) inhibitor could resolve the pathological hypertrophy induced by this mutation (Semsarian et al., 2002). Moreover, it seems that HCM associated mutations lead to increased Ca^{2+} sensitivity while DCM-associated mutations decrease Ca^{2+} sensitivity. This can result in disparate clinical outcomes for mutations on the same gene, for example, the R21C mutation in toponin I increases Ca^{2+} sensitivity and manifests as HCM whereas the A2V mutation reduces Ca^{2+} dependent myocyte contraction and leads to DCM (Gomes et al., 2005; Murphy et al., 2004). In recent

years, studies such as these have contributed to a greater understanding of the genetics underpinning inherited cardiomyopathy and in certain cases allowed clinicians to predict disease course and devise tailored treatment options for patients based on the specific mutations which they harbor.

5. Calcium Signaling and Hypertrophic Growth

One of the great questions in the biology of excitable cells is how a simple cation such as Ca^{2+} can orchestrate such a wide range of biological responses. Nowhere is this more evident than in the understanding of HF, where changes in the normal regulation of calcium can have profound pathological consequences. Ca^{2+} exerts its effect in two main ways; through the association and dissociation with target proteins and the generation of electrical currents. Cytosolic Ca^{2+} concentration is maintained at a very low level relative to the high Ca^{2+} found in the extracellular space which allows for dramatic changes in intracellular Ca^{2+} concentration ($[Ca]_i$) necessary to regulate cardiac myocyte function.

5.1. How Ca^{2+} regulates cardiac myocyte contraction

Cardiac muscle contraction and heartbeat is regulated by a process known as excitation–contraction coupling (ECC). During systole, depolarization of the plasma membrane opens LTCCs, causing an influx of a small amount of Ca^{2+} into the cell. This in turn induces release of a large amount of Ca^{2+} from the SR via the ryanodine receptor in what is known as Ca^{2+}-induced Ca^{2+} release. Soon after, intracellular Ca^{2+} levels rise 10-fold (from 100 nM to 1 μM) to the levels needed to bind toponin C and induce the conformational change necessary to promote actin–myosin crossbridge cycling (Frank et al., 2003; Maclennan and Kranias, 2003). At the start of diastolic relaxation, Ca^{2+} dissociates from troponin C, followed by reuptake into the SR through the SR Ca2+-ATPase 2 (SERCA2) and transsarcolemmal removal via the Na^{2+}/Ca^{2+} exchanger (NKX; Chakraborti et al., 2007). Heart rate is also regulated through the SERCA2 inhibitor phospholamban (PLB) which is a major target of adrenergic signaling (Maclennan and Kranias, 2003). Thus a tightly controlled cycle of Ca^{2+} entry and release precisely regulates beat to beat oscillations in cardiac cells.

5.2. Dysregulated Ca^{2+} level in heart failure

The concept of altered Ca^{2+} handling in HF has been with us for over 20 years, however the debate as to cause and effect has lingered on. In the late 1980s, it was demonstrated that cardiac muscle biopsies from patients with HCM and DCM displayed prolonged Ca^{2+} transients, resulting in a delay in

relaxation (Gwathmey et al., 1987). This suggested that abnormal calcium handling and an inability to maintain normal calcium homeostasis might be involved in the progression of HF. In animal studies, treatment with adrenergic agonists was found to elevate intracellular Ca^{2+} levels and likewise, increasing Ca^{2+} concentration through mechanical stretch, stimulation with Ca^{2+} ionophores or Ca^{2+} channel agonists all lead to the induction of hypertrophy (Iaccarino et al., 1999; Sadoshima et al., 1995). It is now appreciated that dysregulated calcium homeostasis is a prominent feature in the transition from compensatory hypertrophy to HF. Increased intracellular Ca^{2+} concentrations, reduced amplitude of systolic calcium transients, and altered SR calcium handling during diastole all play a part in cardiac dysfunction, arrhythmia, and ultimately HF (Kaye et al., 2008).

As discussed above, SERCA2 regulates the uptake of Ca^{2+} into SR stores and it is now well appreciated that reduced SERCA2 levels are commonplace in the failing heart and represent a major cause of dysregulated Ca^{2+} signaling (Frank et al., 2003; Kogler et al., 2006; Meyer et al., 1995). In experimental HF, transgenic overexpression or viral delivery of SERCA2 restores contractility, reduces LV remodeling and improves survival after aortic banding (del et al., 2001; Kawase et al., 2008; Muller et al., 2003; Sakata et al., 2007). Correspondingly, SERCA2 hemizygous mice have decreased SR Ca^{2+} uptake, rendering them far more sensitive to compromised cardiac function following pressure overload (Schultz et al., 2004). Hypophosphorylation of PLB has also been documented in HF patients, this further compromises SERCA2 function and some studies have highlighted PLB inhibition as a possible therapeutic strategy for HF (Hoshijima et al., 2002; Minamisawa et al., 1999; Sato et al., 2001).

During systole, the LTCC regulates levels of extracellular Ca^{2+} influx into the cell following membrane depolarization, thus controlling the duration of contraction. LTCC levels are reduced in the failing heart and LTCC blockers have shown efficacy in experimental HF models (Chen et al., 2002; Kumamoto et al., 1999; Shimada et al., 1998). Transgenic mice overexpressing the α_1-subunit of LTCC have elevated peak Ca^{2+} currents and display pronounced hypertrophy, cardiomegaly, and interstitial fibrosis by 8 months of age, with the majority of mice dying of HF within 1 year (Muth et al., 2001).

Pharmacological agents which alter the cardiac response to Ca^{2+} are now in widespread clinical use. Classic ionotropes improve contractility by increasing the intracellular Ca^{2+} concentration and are useful in treating acute end-stage HF but unfortunately they increase the incidence of arrhythmias. Ca^{2+} sensitizers such as levosimendan on the other hand enhance Ca^{2+} sensitivity of myofibrils by increasing the affinity of troponin C for Ca^{2+}, thus improving the contractile force during systole (Kota et al., 2008). Ca^{2+} sensitizers have clear benefit in terms of hemodynamics and clinical outcome may be superior to the commonly used β_1-adrenergic agonist dobutamine in some patients (Follath et al., 2002; Mebazaa et al., 2007).

It is not entirely clear how changes in Ca^{2+} concentration are sensed by intracellular signaling factors within the background of normal fluctuating $[Ca^{2+}]_i$ during ECC. With such large continuous changes in cytosolic Ca^{2+} levels, it would not make physiological sense for normal ionotropic activity to regulate distinct calcium-sensitive signaling intermediates. One theory suggests that separate microdomains of Ca^{2+} which are somehow buffered or sequestered from the main ECC pool of Ca^{2+} may account for activation of Ca^{2+}-dependent factors such as calcineurin, PKC, and Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII; Fowler and Smith, 2009). One way this may be achieved is through local Ca^{2+} release at the nucleus, for example endothelin-1 causes the local release of Ca^{2+} at the nuclear envelope via inositol 1,4,5-trisphosphate receptors, leading to distinct hypertrophic signaling (Wu et al., 2006a). Importantly this signaling cascade was not activated by normal ECC Ca^{2+} transients, thus revealing a method of decoupling global Ca^{2+} signaling involved in heartbeat regulation from local Ca^{2+} signaling necessary to induce physiological changes during hypertrophy. Discrete packets of Ca^{2+} sequestered in membrane microdomains may therefore allow activation of signaling complexes distinct from that involved in Ca^{2+}-mediated contraction (Fig. 3.1).

5.3. Integrators of Ca^{2+} signaling—CaMKII

CaMKII is a serine/threonine kinase which phosphorylates several proteins key to calcium handling and ECC, including ryanondine receptors (RyRs), phospholamban, and the LTCC (Swulius and Waxham, 2008). CaMKII is responsive to β-adrenergic mediated increases in intracellular Ca^{2+} but unlike calcineurin, which is activated by sustained activity of low amplitude Ca^{2+} transients, CaMKII is activated by high amplitude Ca^{2+} waves (Baltas et al., 1997; Dolmetsch et al., 1997).

CaMKII activity is induced by experimental HF and in failing human hearts, where it induces the activity of the hypertrophic transcription factors NFAT and GATA4 (Kirchhefer et al., 1999; MacDonnell et al., 2009; Netticadan et al., 2000; Passier et al., 2000). CaMKII exists in several isoforms transcribed from distinct genes, however predominant cardiac isoform is CaMKIIδ which has two splice variants, the nuclear restricted δ_b splice variant and the cytoplasmic δ_c. Transgenic overexpression of either isoform results in cardiac hypertrophy, left ventricular dilatation, and abnormal Ca^{2+} handling (Zhang et al., 2002c, 2003). The perturbed Ca^{2+} homeostasis by in part be due to CaMKII dependent phosphorylation of RyR which increases SR Ca^{2+} leakage resulting in arrhythmia (Maier et al., 2003; Sag et al., 2009).

The recent generation of CaMKIIδ deficient mice by two separate groups has greatly increased our understanding of the crucial role of CaMKII in cardiac hypertrophy. Both the Brown and Olson groups found that CaMKIIδ-deficient mice had normal cardiac function under unstressed

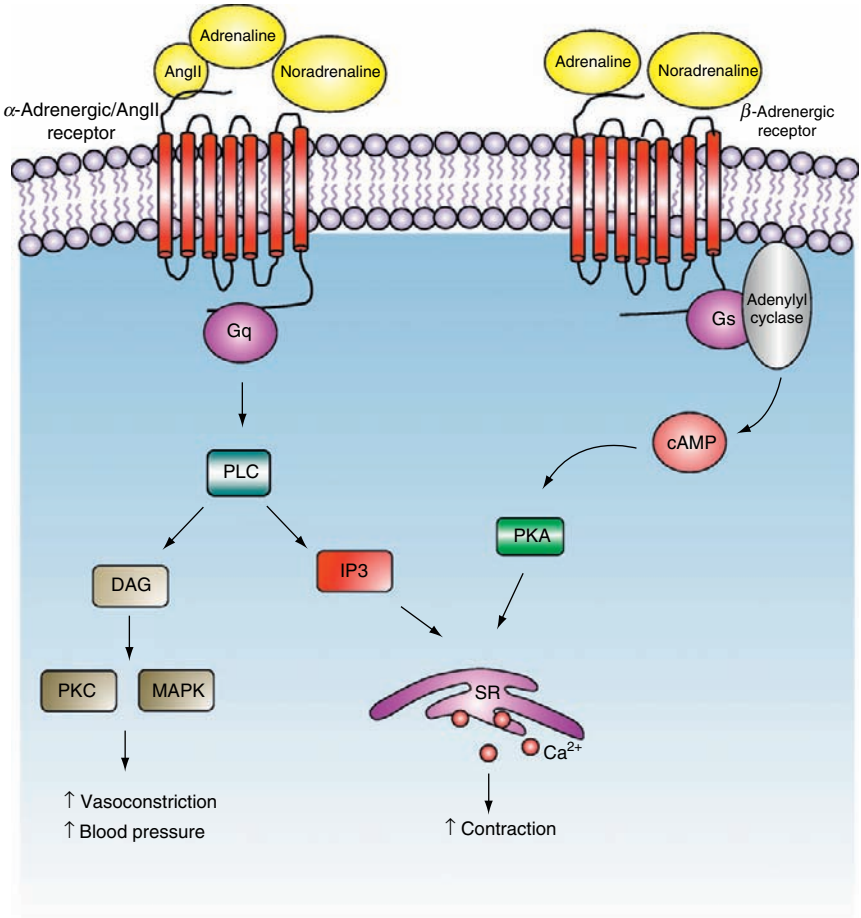

Figure 3.1 Signaling through adrenergic and angiotensin receptors increases heart rate and blood pressure. Catecholamines such as adrenaline and noradrenaline bind to α- and β-adrenergic receptors while angiotensin II (AngII) binds to its α-receptor. The heterodimeric G protein G_q is coupled to α-adrenergic receptors and induces phospholipase C (PLC) activity. PLC hydrolyses phosphadityl 4,5 bisphosphate to release diacylglycerol (DAG), and inositol 1,4,5-triphosphate (IP_3). DAG in turn activates protein kinase C (PKC) and mitogen activated protein kinases (MAPK) which increase vasoconstriction and blood pressure. β-Adrenergic receptors are coupled to G_s which induces adenylyl cyclase activity and downstream activation of the second messenger cAMP and subsequently PKA. Both IP_3 and PKA cause the release of Ca^{2+} from the sarcoplasmic reticulum (SR) altering the rate of cardiac contraction.

conditions but had greatly altered responses to HF stimuli (Backs et al., 2009; Ling et al., 2009). Ling et al. showed that CaMIIKδ was not necessary for the initial development of pressure overload-induced hypertrophy,

however CaMKII$\delta^{-/-}$ mice showed reductions in LV dilation, pulmonary oedma, and fibrosis following long-term aortic banding (Ling et al., 2009). This led to increased survival in CaMKIIδ deficient mice demonstrating a requirement for CaMKIIδ in the transition from overload-induced hypertrophy to HF. Likewise, Backs et al. found diminished levels of hypertrophy and fibrosis and an inhibition of the fetal gene program after 3 weeks of pressure overload in CaMKII$\delta^{-/-}$ mice (Backs et al., 2009). Although both studies clearly show that CaMIIKδ functions as a prohypertrophic kinase, the effect of CaMKIIδ deletion on Ca^{2+} homeostasis is less clear; Ling et al. found that CaMKIIδ deletion prevented the increase in SR Ca^{2+} leak normally associated with pressure overload while Backs et al. did not find any alterations in Ca^{2+} handling. These differences however may simply stem from the level of stenosis used in the banding models to induce pressure overload. The protective effect of CaMKIIδ deletion may be related to the preserved expression of RyR and SERCA2 which are normally downregulated during HF.

It appears that accurate splicing of CaMKIIδ may be necessary for the normal regulation of Ca^{2+} handling, a finding which was hit upon in a seemingly unrelated study. Xu et al. were investigating the function of the splicing factor ASF/SF2 and found that gene targeting of ASF/SF2 in mice leads to DCM by 6 weeks of age accompanied with marked hypertrophy and fibrosis (Xu et al., 2005). All of the ASF/SF2 null mice died within 6–8 weeks of birth due to defects in the contractile apparatus and cardiac myocytes from these mice had dysregulated Ca^{2+} handling with a marked increase in peak Ca^{2+}, increased frequency of Ca^{2+} sparks and transient local release of Ca^{2+} from the SR due to brief opening of the RyR (Cheng and Lederer, 2008; Xu et al., 2005). They found that this Ca^{2+} handling defect was due to a deficiency in postnatal splicing leading to retention of the CaMKIIδ_A neuronal splice variant which is normally switched off in the heart between 1 and 2 months of age. Therefore, CaMKII is a major sensor of increased Ca^{2+} levels which it translates into increased hypertrophy and alterations in Ca^{2+} homeostasis, in addition to the activity status of CaMKIIδ, aberrant splicing may also play a role in hypertrophy.

5.4. Integrators of Ca^{2+} signaling—calcineurin

Calcineurin (Cn) is a Ca^{2+}/calmodulin-dependent phosphatase which has been implicated in immunity, learning and muscle development. Calcineurin exists as a heterodimer of the catalytic subunit CnA which is expressed from three genes (CnAα, CnAβ, and CnAγ) and the regulatory subunit CnB expressed from two genes (CnB1 and CnB2). The catalytic subunit is further divided into three functional domains, the catalytic domain, the autoinhibitory domain, and the calcium/calmodulin binding domain and calcineurin becomes active when calmodulin displaces an

autoinhibitory domain from the active site of CnA (Aramburu et al., 2004). One of the principle targets of calcineurin is the NFAT family of transcription factors and following dephosphorylation by calcineurin, NFATs translocate to the nucleus where they exert wide ranging transcriptional effects.

In 1998, huge interest was garnered in the role of calcineurin in hypertrophy following the publication of a seminal paper by Jeffery Molkentin and Eric Olson. They were searching for novel GATA4 binding proteins which might mediate certain aspects of the hypertrophic program and indentified a GATA4–NFATc4 interaction (Molkentin et al., 1998). They found that calcineurin could enhance the cooperativity between NFATc4 and GATA4 in regulating the brain natriuretic peptide (BNP) promoter, a common marker of hypertrophy. Overexpression of calcineurin *in vivo* led to dramatic increases in cardiac hypertrophy, ventricular dilatation, cardiac fibrosis, and pulmonary oedema which resulted in premature death due to HF (Molkentin et al., 1998). This was reinforced by the discovery of increased calcineurin, NFAT, and GATA4 activity in samples of failed human hearts (Diedrichs et al., 2004; Lim and Molkentin, 1999). Later, the calcineurin/NFAT dyad was shown to be specific for pathological forms of ventricular growth, as hypertrophic growth induced by exercise did not lead to activity of calcineurin and NFAT (Wilkins et al., 2004).

5.4.1. Calcineurin inhibition prevents hypertrophy

Inhibition of calcineurin activity is responsible for the effects of the widely used immunosuppressive drugs cyclosporine (CsA) and FK506, which block T cell proliferation by abrogating Ca^{2+}-dependent IL-2 expression (Clipstone and Crabtree, 1992; O'Keefe et al., 1992). Early studies demonstrated that inhibition of calcineurin with cyclosporine A (CsA) could both inhibit and reverse hypertrophy in pressure overload and genetic models (Lim et al., 2000; Molkentin et al., 1998; Sussman et al., 1998). The antihypertrophic effect of calcineurin inhibition was subsequently confirmed by several groups using models of aortic banding, postinfarction hypertrophy, genetic hypertension, and adrenergic stimulation (Goldspink et al., 2001; Hill et al., 2000; Kato et al., 2000; Oie et al., 2000; Shimoyama et al., 1999, 2001; Takeda et al., 2002). Although some studies have reported negative results with calcineurin inhibitors, it is now widely accepted that inhibition of calcineurin can reduce the development of hypertrophy, at least in animal models (Ding et al., 1999; Luo et al., 1998; Zhang et al., 1999).

It is well recognized however that drugs such as CsA and FK506 exhibit toxicity and off-target effects which may confound the results of some cardiac studies. For example, CsA is known to associate with cyclophilin D, part of the mitochondrial permeability transition pore, and FK506 also regulates ryanondine receptor activity (Bandyopadhyay et al., 2000; Kieffer et al., 1993). To overcome these limitations, several genetic models of

calcineurin inhibition were developed, lending significant support the supposition that calcineurin inhibition is unequivocally antihypertrophic. Mice which overexpress the calcineurin inhibitors Cain/Cabin-1, A-kinase anchoring protein 79 (AKAP79), regulator of calcineurin 1 (RCAN1) or dominant negative CnA were all found to exhibit reduced heart weights following isoprotenol infusion or aortic banding (De Windt et al., 2001; Rothermel et al., 2001; Zou et al., 2001). In agreement with these studies, CnAβ deficient mice also have diminished cardiac hypertrophy in response to adrenergic drive or pressure overload (Bueno et al., 2002).

Despite the promising results from several animal studies, some investigators have argued that inhibition of hypertrophy in this manner may not be entirely beneficial. For example, one study reported that the lack of compensatory hypertrophy in CsA treated mice resulted in decompensation and premature death since the heart could not adequately deal with the elevated levels of after-load (Meguro et al., 1999). Moreover, a cautionary note has also been sounded with regard to the use of calcineurin inhibitors in DCM. In the MLP (muscle LIM protein) knockout mouse, which serves as a model of human DCM, genetic inhibition of CnAβ resulted in early lethality accompanied with ventricular dilation, reduced fractional shortening, increased cardiac cell death, and fibrosis (Heineke et al., 2009). This suggests that the underlying cause and stage of HF may have to be taken into account when exploring options for calcineurin inhibition as a treatment for HF.

Calcineurin may also have a cardio-protective role in some instances, for example calcineurin protects cardiac myocytes from oxidative stress and glucose deprivation and CnAβ deficient mice are more sensitive to I/R induced cell death (Bueno et al., 2004; De Windt et al., 2000; Kakita et al., 2001). This is also borne out on studies of CnB1 deficient mice which exhibit systolic and diastolic dysfunction leading premature death by 7 months of age (Schaeffer et al., 2009). Interestingly, by 3 months of age these mice developed significant atrial dilatation in the absence of ventricular dilatation. In addition, the atria exhibited marked fibrosis and thrombosis which was thought to be due to elevated atrial pressure resulting from noncompliant ventricles.

On balance, calcineurin inhibition does show clear therapeutic promise for the treatment of HF and the outcome of clinical trials is eagerly awaited. If calcineurin modulation is to be fully realized as a HF treatment however, more selective inhibitors will need to be developed, as both CsA and FK506 induce hypertension and as such may not be suitable for use in HF patients (Taler et al., 1999). There is some clinical evidence to suggest that antihypertensive drugs such as angiotensin converting enzyme (ACE)-inhibitors and angiotensin receptor blockers may actually lower calcineurin activity and thus any future calcineurin inhibitors could be used in combination with standard therapy (Zhang et al., 2009).

5.4.2. Ca^{2+} dependent regulation of calcineurin activity

It is important to note that in contrast to the activation of other regulators of hypertrophy such as CaMKII and MAPK, calcineurin is relatively insensitive to transient increases in $[Ca^{2+}]i$ but rather is activated by sustained elevated levels of Ca^{2+} (Dolmetsch et al., 1997). Calcineurin-mediated NFAT nuclear translocation has been proposed to be dependent on the amplitude, duration, and frequency of Ca^{2+} spikes and while the influence of individual Ca^{2+} transients may be minor, prolonged frequency of Ca^{2+} oscillations has an additive effect on molecular signaling (Berridge et al., 2003; Colella et al., 2008). The source of intracellular Ca^{2+} responsible for calcineurin activation has been the subject of much debate and it is currently thought that cellular microdomain pools of Ca^{2+} may have a role to play in calcineurin activity. There are also several Ca^{2+} channels which may be involved in calcineurin activation, including transient receptor potential canonical (TRPC) channels, LTCCs, and T-type calcium channels, each of which will be discussed in turn.

5.4.3. L-type calcium channels

Transit through the voltage gated LTCC (LTCC/$Ca_v1.2$) constitutes the major pathway for Ca^{2+} influx into cardiac myocytes and influences ECC and action potential repolarization. Ca^{2+} entry via LTCC induces calcineurin activity and physical interaction between calcineurin and $Ca_v1.2$ has been shown to enhance L-type Ca^{2+} currents (Graef et al., 1999; Tandan et al., 2009). Calcineurin localization to the sarcolemma at the site of Ca^{2+} entry lends credence to the theory that calcineurin is triggered by distinct Ca^{2+} microdomains; however, the precise mechanisms whereby calcineurin is activated at the cell membrane, the Z-disk and in the nucleus are not yet fully understood (Buch et al., 2005).

5.4.4. T-type calcium channels

Another candidate for Ca^{2+}-dependent calcineurin activation are the T-type Ca^{2+} channels (TCCs) which are characterized by low voltage, transient Ca^{2+} type channels in contrast to the high voltage, longer lasting LTCCs (Ono and Iijima, 2010). In cardiac myocytes, TCCs are normally expressed during embryonic development and are not expressed in adult cells. Following pressure overload *in vivo* or adrenergic stimulation *in vitro* however, T-type currents can be measured from cardiac myocytes (Ferron et al., 2003; Lalevee et al., 2005; Martinez et al., 1999). Mice deficient in the TCC $Ca_v3.2$ deficient displayed reduced NFAT activity and diminished hypertrophy in response to pressure overload or AngII (Chiang et al., 2009). Similarly, treatment with the T-type Ca^{2+} channel blocker ethosuximide prevented pressure overload-induced hypertrophy and blocked calcineurin activity (Horiba et al., 2008).

5.4.5. Transient receptor potential canonical channels

Store-operated Ca^{2+} entry, where depletion of Ca^{2+} stores in the SR induces Ca^{2+} influx from the extracellular space, is controlled by a family of nonselective cation channels called TRPC channels. There are seven members (TRPC1-7) and TRPC1, TRPC3, and TRPC6 are all activated downstream of G-protein coupled receptors (GPCRs) and upregulated in response to hypertrophic stimuli. Silencing of TRPC1 reduced AngII-mediated Ca^{2+} oscillations and NFAT activity, while overexpression of TRPC3 or TRPC6 increased calcineurin/NFAT responses and hypertrophic growth, suggesting that all three channels influence Ca^{2+}-dependent calcineurin activity (Bush et al., 2006; Kuwahara et al., 2006; Nakayama et al., 2006; Seth et al., 2009).

5.4.6. Spatial regulation of calcineurin

Since calcineurin activation is such a potent driving force of cardiac hypertrophy, it is not surprising that there exists multiple levels of control. In cardiac myocytes, calcineurin is tethered to the Z-disks, via the bridging calsarcin proteins, which in turn interact with the Z-disk proteins α-actinin and cipher (Frey and Olson, 2002; Frey et al., 2000). Localization of calcineurin at the Z-disks brings it into close proximity with both NFAT and LTCCs, both of which have been shown to reside there. Calcineurin is regulated *in situ* at the Z-disks through binding of atrogin-1, an F-box protein which targets calcineurin for ubiquitin-mediated degradation (Li et al., 2004). As expected, atrogin-1 transgenic mice display diminished left ventricular size following pressure overload, due to reduced levels of CnA. Calcineurin activity at the Z-disk is also inhibited by casarcin-1 and PICOT (protein kinase C (PKC)-interacting cousin of thioredoxin) which block its interaction with MLP, thereby displacing calcineurin from the Z-disk (Frank et al., 2007; Jeong et al., 2008). Hypertrophic stimuli increase nuclear translocation of CnA and targeting this transit using a peptide which impedes the binding of CnA to importin-β_1 completely blocks AngII-mediated hypertrophy (Hallhuber et al., 2006). Interestingly, this peptide had no effect on calcineurin phosphatase activity but could still block NFAT transcriptional responses. This suggests that calcineurin is active at distinct cellular sites and regulation of calcineurin activity at the Z-disk may be distinct from its activity in the nucleus.

5.4.7. RCAN inhibition

The recently renamed RCAN has in the past been known as MCIP (modulatory calcineurin interacting protein), DSCR (Down's syndrome critical region gene), and calcipressin. RCAN1 has been shown to bind to the catalytic subunit of calcineurin and inhibit its activity, possibly by promoting increased calcineurin degradation via the proteasome (Fuentes

et al., 2000; Genesca et al., 2003). In addition, RCANs compete with NFAT for the same binding site in calcineurin, thus cells doubly deficient in RCAN1 and RCAN2 exhibited diminished nuclear accumulation and transcriptional activation of NFAT in response to Ca^{2+} or AngII (Martinez-Martinez et al., 2009; Sanna et al., 2006). Interestingly, the RCAN1 gene harbors an alternative promoter which contains several NFAT binding sites, rendering it responsive to calcineurin and suggesting the existence of a feedback loop between calcineurin and RCAN1 (Yang et al., 2000). In agreement with a role for RCANs in blunting calcineurin responses, mice overexpressing RCAN1 were protected from pressure overload and adrenergic stimulation, while mice lacking RCAN1 exhibit exaggerated hypertrophy and reduced cardiac function in response to CnA overexpression (Hill et al., 2002; Rothermel et al., 2001). The concept of RCANs as *bona fide* inhibitors of calcineurin signaling has been cast into doubt however with the unexpected finding that RCANs can both augment as well as diminish calcineurin activity. Antithetically, both RCAN1 and RCAN 2 deficient mice exhibit significantly reduced hypertrophy in response to pressure overload and develop a neurological phenotype similar to that of CnAβ deficient mice (Sanna et al., 2006; Vega et al., 2003). These data suggest that RCAN-dependent calcineurin regulation is more complex than initially thought and RCANs may both augment or restrain calcineurin activity depending on the nature and strength of the stimulus.

6. G-Protein Coupled Receptors and Adrenergic Drive

ARs are GPCRs which bind catecholamines such as noradrenaline and adrenaline and mediate their chronotropic and ionotropic effects. Increased α-adrenergic activity constricts the arterioles and increases blood pressure while increased β-adrenergic activity causes the heart to beat faster.

ARs function by binding to heterodimeric G-proteins. α-ARs are coupled to Gq, an inducer of phospholipase C (PLC) activity which hydrolyses phosphadityl 4,5 bisphosphate to release inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 promotes Ca^{2+} release from the SR, thereby stimulating myocyte contraction (Volpe et al., 1985). DAG on the other hand activates the MAPKs and PKC, which in turn phosphorylate hypertrophic effector proteins such as NFAT, MEF2, and GATA4. In contrast, β-adrenergic receptors are coupled to G_s which stimulates adenylyl cyclase activity and production of the second messenger cyclic adenosine monophosphate (cAMP). This in turn activates protein kinase A (PKA) which regulates several factors involved in myocyte contraction, including LTCCs, RyR, phospholamban, and troponins (Marian, 2006).

6.1. Adrenergic drive and receptor desensitization

Patients suffering from HF have elevated circulating levels of catecholamines, which inversely correlate with survival (Lohse et al., 2003). The increased adrenergic drive during cardiac stress is initially an adaptive response since it increases contractility and stabilizes blood pressure, however it ultimately proves detrimental. Thus transgenic overexpression of β-ARs causes premature HF in mice and a β-AR gain of function polymorphism in humans increases the risk of cardiomyopathy (Engelhardt et al., 1999; Mason et al., 1999). Although adrenergic drive is increased in the early stages of HF, counterintuitively, in the failing heart chronic exposure to catecholamines actually reduces adrenergic signaling due to receptor internalization and degradation. β_1 receptors become desensitized through reduced mRNA expression and increased production of β-adrenoreceptor kinase (βARK) which phosphorylates the receptor and causes it to uncouple from G -proteins (Lohse et al., 1990). This may form part of as mechanism to protect the heart from the detrimental effects of chronic adrenergic drive, an idea reinforced by studies showing that mice which downregulated β-ARs in response to chronic adrenergic stimulation suffered less ventricular dysfunction than a strain which did not (Faulx et al., 2005). While the majority of cardiac β-adrenergic receptors is of the β_1 type, β_2-ARs are also present and couple to G_s and G_i (Katada et al., 1984). Desensitization however occurs through specific repression of β_1-ARs, resulting in an increase in the ratio of β_2-AR to β_1-AR in failing hearts. Adrenergic production of cAMP appears to be spatially regulated in cardiac myocytes; β_1-ARs are distributed along the plasma membrane, generating cAMP which can diffuse into the sarcomeres and regulate contraction, in contrast β_2-ARs are confined to the T-tubules at the cell surface (Dorn, 2010; Nikolaev et al., 2010). Intriguingly, in failing hearts β_2-ARs relocate to cell membrane and produce cAMP in a diffuse manner similar to β_1-ARs, thus taking over their pathological activity (Nikolaev et al., 2010).

β-Blockers such as bisoprolol, carvedilol, metoprololare, and nebivolol are now one of the most widely used drug classes in HF treatment and while they were initially thought to simply compete with catecholamines for binding to β-ARs, it is now known that they also promote receptor resensitization (El-Armouche and Eschenhagen, 2009). Although seemingly contradictory, it is thought that the protective versus maladaptive effects of catecholamines may be a matter of the strength and timing of stimulation. Thus, the dose and timing of β-blocker therapy may be a critical factor in the optimum response for HF patients.

6.2. The rennin–angiotensin-system

The rennin–angiotensin-system (RAS) is an important regulator of cardiac physiology, promoting hypertension, and LV hypertrophy. Angiotensin II (AngII) is released from the heart in response to increased wall stress or

mechanical load and binds to the angiotensin II type 1 receptor (AT1R). AngII is a well-established inducer of cardiac hypertrophy and angiotensin converting enzyme (ACE) inhibitors and AT1R blockers significantly reduce morbidity and mortality in HF patients and alongside β-blockers are now a mainstay in HF treatment (Pitt et al., 1997).

A confounding factor in understanding how the AngII receptor (AT1R) promotes hypertrophy is the inability to separate global effects such as increased blood pressure from local effects which directly impact on the myocardium. However, overexpression of AT1R specifically in the heart caused increased hypertrophy and death from HF in the absence of blood pressure changes (Paradis et al., 2000). Ainscough et al. (2009) also circumvented this problem by generating transgenic mice which overexpress the N111G mutant of the human AT1R in the heart. This mutant is fully active when stimulated with the AngII derivative AngIV in the absence of wild type receptor activity. These mice exhibited left ventricular hypertrophy and fibrotic remodeling in the absence of hypertension, clearly indicating that angiotensin acts directly on the myocardium. The AngII receptor along with receptors for endothelin 1 and noradrenaline are couple to G_q. Overexpression of G_q in mice leads to decompensated HF following transverse aortic constriction (TAC), possibly due to increased myocyte apoptosis and this may have an important role to play in the transition to a decompensated state (Adams et al., 1998; Sakata et al., 1998). Similarly, deletion of G_q rendered mice refractory to pressure overload-induced hypertrophy (Wettschureck et al., 2001).

7. Transcriptional Master Regulators of Hypertrophy

The transcriptional networks which regulate hypertrophic growth have been intensively studied in experimental HF models. Notwithstanding that transcriptional control of cardiac growth is a highly complex process, a small number of key transcription factors represent the driving force which bring about well-characterized cellular changes. In addition, epigenetic modification by histone acetyl transferases (HATs) and histone deacetylases (HDACs) regulates the access of transcription factors to target promoters. While our knowledge of these factors in a large part derives from animal models, an important recent study has shown that these same transcription factors are key components in driving pathological hypertrophy in HF patients. Hannenhalli et al. searched for enriched transcription factor binding sites in the promoters of genes which were differentially expressed in both idiopathic and DCM. They found that the most overrepresented transcription factor binding sites included NFAT, MEF2, GATA, Nkx, Foxo, C/EBP, and IRF families (Hannenhalli et al., 2006). Importantly,

studies such as this give confidence that the considerable body of work pertaining to transcriptional responses in experimental murine HF has direct relevance to human cardiac pathology.

7.1. Epigenetic modification of chromatin

Heritable regulation of gene modulation through modification of chromosomal structural proteins or additions to the nucleic acid without a change in the gene sequence is collectively termed epigenetics. Such modifications include methylation of genomic DNA itself in addition to a variety of modifications to the core histone proteins, including acetylation, methylation, phosphorylation, ubiquitination, and SUMOylation. Many excellent studies have demonstrated that epigenetic "marks" or changes have a crucial role in the development and propagation of cardiac hypertrophy, that is, dysregulated histone acetylation status is directly linked to an impaired contraction (Gupta et al., 2008a; Olson et al., 2006).

7.1.1. Histone acetyl transferases

Together, histone proteins and DNA collectively constitute the nucleosomes, the structural units of chromatin that are essential for DNA packaging in eukaryotic cells. Acetylation of lysine residues in the tail regions of histones leads to the relaxation of chromatin structure, creating a permissive environment for transcriptional activation. HATs are the enzymes which govern histone acetylation and have been shown to alter the expression of hypertrophic genes in cardiac myocytes (Davidson et al., 2005).

The HAT enzymes p300 and CBP are structurally related transcriptional coactivators which facilitate transcriptional responses through binding to both transcription factors and the basal transcriptional machinery. p300 and CBP serve as coactivators remodeling including GATA4, MEF2, SRF, and AP-1 (Dai and Markham, 2001; Ma et al., 2005; Yanazume et al., 2003b). Inhibition of CBP or p300 abolishes adrenergic mediated increases in cell size, protein:DNA ratio, and ANP expression in cardiac myocytes (Gusterson et al., 2002, 2003). Importantly, overexpression of CBP or p300 alone is sufficient to drive hypertrophy in cardiac myocytes and this is dependent on a fully functional HAT domain (Gusterson et al., 2003; Yanazume et al., 2003a). In addition to acetylating histones, HATs can directly acetylate other proteins. A salient example of this is the requirement of p300 for GATA4 acetylation, a modification necessary for maximal GATA4 transcriptional activity and induction of hypertrophic genes such as ANP, β-MHC, and ET-1 (Dai and Markham, 2001; Yanazume et al., 2003a). Indeed a mutant GATA4 protein which cannot be phosphorylated by p300 was shown to function as a dominant negative factor in adrenergic-induced hypertrophy (Takaya et al., 2008).

Cardiac p300 protein expression is significantly elevated following experimental pressure overload and in patients with failing hearts (Yanazume et al., 2003b). This is thought to have direct consequences for cardiac growth since p300 transgenic mice exhibit left ventricular eccentric dilatation, increased myocyte hypertrophy, and reduced cardiac function (Wei et al., 2008; Yanazume et al., 2003a). Taken together, studies clearly show that the HAT enzymes are critical factors in the control of LV remodeling and regulate hypertrophy though a combination of elevated GATA4 activity and antagonism of the repressive effects of HDACs on MEF2 (Wei et al., 2008). Interestingly, the natural polyphenol compound curcurmin, responsible for the yellow color of the spice turmeric, has recently been demonstrated to inhibit p300-GATA4 association and has shown promising results as an antihypertrophic agent (Morimoto et al., 2008).

7.1.2. Histone deacetylases

HDACs are enzymes which remove acetyl groups from lysine residues in the tail region of histones. Through this mechanism, HDACs play a critical role in the modulation of chromatin architecture and govern the expression of oncogenes, tumor suppressors, and inflammatory genes (de Ruijter et al., 2003). The development of HDAC inhibitors has therefore emerged as an important strategy in the design of new drugs to treat chronic disease including cancer, immune and inflammatory diseases and, more recently cardiovascular disease (Davis et al., 2005; Lu et al., 2000a; Townsend et al., 2007).

The HDAC enzyme family comprises four subclasses. Class I HDACs are nuclear proteins whose tissue expression is ubiquitous. By comparison, Class II and IV HDACs are found in both the nucleus and the cytoplasm and exhibit greater tissue-specific expression (Witt et al., 2009). Class I, II, and IV HDACs are all zinc-dependent enzymes and are the exclusive focus of this chapter; the Class III HDACs, or sirtuins, constitute a structurally distinct subfamily of NAD^+-dependent enzymes and will not be discussed herein.

Elegant work by McKinsey, Olson, and colleagues has uncovered that Class II HDACs (HDAC4, -5, -7, and -9) can suppress cardiac hypertrophy, in part by inhibiting the activity of MEF2 (Lu et al., 2000b; Zhang et al., 2002b). This is readily evident in HDAC9 deficient mice, which are sensitized to hypertrophic signals (Zhang et al., 2002a). However, we and others have shown that HDAC inhibitors prevent hypertrophy and sarcomere organization in cultured cardiac myocytes (Antos et al., 2003; Davidson et al., 2005), suggesting a positive role for HDACs in cardiac hypertrophy. These disparate observations may be reconciled by considering the specificity of HDAC inhibitors and it is now recognized that Class I HDACs promote while Class II HDACs antagonize hypertrophic responses. Thus there is a clear benefit to the development of Class I HDAC specific inhibitors as therapeutic agents which would not interfere

with the Class II dependent inhibition of prohypertrophic pathways (Barry et al., 2008). The clinical use of HDAC inhibitors may allow the control of key hypertrophic genes, and would provide a novel molecular and therapeutic approach.

7.1.3. HDAC inhibitors: Utility beyond cancer

As mentioned, to date, the overwhelming majority of drug development activity in the HDAC inhibitor arena has focused on cancer. However, there is emerging evidence that HDAC inhibitors may have potential in other therapeutic areas, most particularly in neurodegenerative diseases and, of relevance to this discussion, cardiovascular disease (Shuttleworth et al., 2010). HDAC inhibitors developed to date fall into four main structural classes: carboxylates, hydroxamic acids, benzamides, and cyclic peptides. All HDAC inhibitors contain common structural features, including a zinc-binding group, a linker which occupies the channel leading to the active site and a surface recognition "cap" motif. For representative examples of each structural type (Shuttleworth et al., 2010). All of these compounds display distinct patterns of HDAC subtype selectivity, the therapeutic significance of which remains the source of considerable discussion (Witt and Lindemann, 2009). The development of HDAC inhibitors as experimental therapeutic agents for the treatment of cancer has progressed well, although most of these agents are not isoform-specific. By contrast, the bicyclic tetrapeptide HDAC inhibitors FK228 and Spiruchostatin A appear to possess selectivity toward Class I HDACs (unpublished data; Yurek-George et al., 2007), an activity profile which may make them particularly attractive as starting points to develop novel therapies for cardiovascular disease.

7.2. Myocyte enhancer factor 2

MEF2 is a member of the MADS-box family of transcription factors and has prominent roles in muscle generation, neuronal survival, lymphoid cell development, and tumorigenesis (Potthoff and Olson, 2007). MEF2 binds DNA as a combination of homo- and heterodimers of four separate MEF2 factors (MEF2A-D) which drive the expression of several cardiac genes including α-MHC, ANP, troponins, Serca2, Mlc2v, and desmin. MEF2 is recruited to many promoters indirectly through binding other transcription factors such as the GATA and HAND families, which lead to synergistic induction of target genes (Morin et al., 2000, 2005; Zang et al., 2004). MEF2 represents a major transcriptional regulator of a number of hypertrophic genes and elevated levels of MEF2 DNA binding activity can be seen in cardiac myocytes following mechanical stress, adrenergic stimulation, or calcineurin activation (Molkentin and Markham, 1993).

7.2.1. Regulation of MEF2 activation by HDACs

MEF2 is regulated at several levels including alternative splicing, association with transcriptional repressors, modulation of DNA binding activity, and caspase-mediated cleavage (Potthoff and Olson, 2007). The major point of regulation of MEF2 factors however appears to be their association with HDACs. MEF2 transcriptional activity is repressed by the association with Class II HDACs and only becomes active in the presence of HDAC kinases which promote HDAC nuclear export (Lu et al., 2000b; Miska et al., 1999). For example, the HDAC kinase CaMK phosphorylates HDAC5, thereby unmasking a docking site for the intracellular chaperone 14-3-3 (McKinsey et al., 2000a,b). The subsequent 14-3-3 dependent nuclear export disrupts the HDAC5-MEF2C association, relieving the inhibitory pressure and allowing robust MEF2 activation. Likewise, induced trafficking of HDACs to the nucleus also plays a role in MEF2 regulation. For example, the corepressor SMRT (silencing mediator for retinoic acid and thyroid hormone receptors) promotes the trafficking of HDAC4 and HDAC5 from the cytosol to subnuclear domains, resulting in reduced MEF2 activity (Dressel et al., 2001). Interestingly it seems that most HDACs do not actually require deacetylase activity to mediate repression, HDAC3 is the exception to this as it alone appears to acetylate MEF2 (Gregoire et al., 2007).

Given the well-documented repressive activity of HDACs toward MEF2, it is not surprising that HDAC mediated modulation of MEF2 plays an important role in regulating the hypertrophic response. Pressure overload or calcineurin activation results in phosphorylation and nuclear export of HDAC4, -5, and -9 and nonphosphorylatable forms of these Class II HDACs inhibit the development of myocyte hypertrophy (Zhang et al., 2002a). This is clearly evident in HDAC9 deficient mice which show exaggerated cardiac enlargement and elevated MEF2 expression after pressure overload (Zhang et al., 2002a). Endogenous regulators of the MEF2/HDAC dyad are also involved in shaping hypertrophic growth. For example, protein kinase D1 (PKD1) antagonizes the MEF2/HDAC relationship by phosphorylating Class II HDACs, deficiency in PDK1 thus leads to a reduction in hypertrophy and fibrosis in response to pressure overload (Fielitz et al., 2008). The HDAC–MEF2 axis also regulates hypertrophy of vascular smooth muscle cells (VSMCs). Thus stimulation of VSMCs with AngII led to PDK-1 dependent HDAC5 phosphorylation and MEF2 dependent cellular hypertrophy (Xu et al., 2007).

MEF2 phosphorylation also regulates its activity and indeed MEF2 is the target of several kinases including p38, ERK5, PKA, casein kinase II, and Cdk5 (Du et al., 2008; Gregoire et al., 2006; Nadruz, Jr. et al., 2003; Zhao et al., 2002). In the case of Cdk5, it has been shown that phosphorylation of MEF2D on Ser-444 leads to sumoylation at Lys-439 and subsequent transcriptional inhibition (Gregoire et al., 2006). Both phosphorylation and sumoylation are enhanced by HDAC4 and antagonized by calcineurin, demonstrating the multiple levels of MEF2 transcriptional control.

7.2.2. MEF2 transgenic studies

MEF2 β-galactosidase reporter mice shown enhanced staining in hypertrophied hearts when crossed with CaMKIV or calcineurin transgenic mice (Passier et al., 2000). Genetic inhibition of MEF2 activity downstream of calcineurin rescued the marked ventricular dilation and contractility defects characteristic calcineurin transgenic mice (van Oort et al., 2006). Intriguingly, heart weights were the same in both mouse lines and therefore despite the improvements in ventricular dilation and ejection fraction, there was little effect on the overall hypertrophic response to calcineurin. Thus MEF2 may be involved in a genetic program leading DCM but not in the initial phase of hypertrophic remodeling. This is supported by findings from MEF2A and MEF2C transgenic mice which exhibit eccentric hypertrophy and DCM secondary to sarcomeric disorganization and compromised cardiac function (Xu et al., 2006). Lack of MEF2D however, leads to reduced left ventricular dilation, myocyte area, and fibrosis in response to pressure overload, while transgenic overexpression of MEF2D is sufficient to induce severe pathological remodeling (Kim et al., 2008b). Interestingly, silencing MEF2C expression by administration of siRNA via the jugular vein resulted in a significant reduction in pressure-dependent increases in left ventricular wall thickness, cardiac myocyte diameter, and fibrosis (Pereira et al., 2009). In contrast to MEF2A and MEF2C deficient mice, mice lacking MEF2D are viable and phenotypically normal.

7.2.3. Integration of Ca^{2+} signaling and MEF2 activity

Not surprisingly, the levels of Ca^{2+} in the cell greatly affect the status of MEF2 activity. In the presence of Ca^{2+}, calmodulin binds to the repressor protein Cabin1/Cain causing it to be displaced from MEF2; Cabin1/Cain normally keeps MEF2 in a repressed state through the recruitment of HDAC1 and -2 (Youn and Liu, 2000; Youn et al., 1999). The C-terminal domain of MEF2C is also dephosphorylated by calcineurin and this may expose a nuclear localization signal and allow MEF2C nuclear translocation (Lynch et al., 2005). Calreticulin (Crt) lies upstream of calcineurin and controls agonist dependent Ca^{2+} release from the ER. MEF2 nuclear translocation is compromised in $Crt^{-/-}$ cells and this can be restored by calcineurin transfection or treatment with a Ca^{2+} ionophore (Lynch et al., 2005). There appears to be an elegant feedback mechanism whereby MEF2C controls the expression of calreticulin and thus may regulate the availability of Ca^{2+} needed to sustain its own levels via Ca^{+}-dependent calcineurin activation. Intracellular Ca^{2+} also appears to control the competitive binding of HDAC4 and the coactivator p300 to MEF2; in the presence of $Ca2^{+}$, calmodulin displaces HDAC4 from MEF2, thus facilitating the association of p300 (Youn et al., 2000). MEF2 also binds to NFAT downstream of calcineurin activation and indeed maximal MEF2

activity is achieved in cardiac myocytes after addition of NFAT, calcineurin, and p300 (van Oort et al., 2006).

7.2.4. MEF2 and energy demand

MEF2 may also be important in regulating the increased energy demands experienced by cardiac myocytes undergoing hypertrophy. The transcriptional coactivator PGC-1α (peroxisome proliferator activated receptor-γ coactivator 1), a master regulator of mitochondrial biogenesis and fatty acid oxidation, is a transcriptional target of MEF2 (Czubryt et al., 2003). Silencing of MEF2C blocked the increase in myocardial mitochondrial proliferation induced by pressure overload and this was accompanied by reduced expression of PGC-1α (Pereira et al., 2009). Consistent with this, MEF2A deficient mice have reduced numbers of viable mitochondria with a high level of postnatal mortality (Naya et al., 2002). Taken together MEF2 integrates signals from HDACs, MAPKs, and Ca^{2+} and once MEF2 inhibition is relieved, it is a major player in the upregulation of hypertrophic effectors and regulation of mitochondrial function during HF.

7.3. Nuclear factor of activated T cells

Early studies from Jerry Crabtree's group identified NFAT as an essential mediator of T cell activation and antigen receptor dependent IL-2 expression (Shaw et al., 1988). They subsequently showed that nuclear translocation of NFAT was dependent on calcinueurin activity and thus responsible for the immunosuppressive effect of the widely used transplant rejection drugs cyclosporine A and FK506 (Clipstone and Crabtree, 1992; Flanagan et al., 1991; Shaw et al., 1988). The NFATs have wide ranging tissue distribution and consist of five isoforms, NFATc1, NFATc2, NFATc3 (NFAT4), NFATc4 (NFAT3), and NFAT5. All NFATs share a conserved DNA binding domain known as the Rel-homology region, due to its homology with the Rel (NF-κB) family of transcription factors. In addition they harbor an NFAT homology region which contains a transactivation domain and a series of 13 serine residues that are dephosphorylated by calcineurin, leading to exposure of a nuclear localization signal and subsequent nuclear transport (Okamura et al., 2000). Phosphorylation of NFAT on the other hand exposes a nuclear export signal which leads to Crm-1 dependent nuclear export (Zhu and McKeon, 1999). Several serine/threonine protein kinases regulate NFAT activity and include maintenance kinases such as casein kinase 1 which retain NFAT in an inactive state in the cytosol and export kinases such as GSK3β, JNK, and p38 which actively promote nuclear export (Macian, 2005; Mancini and Toker, 2009). Activity and subcellular localization is also controlled by other posttranslational modifications such as ubiquitination, which leads to NFAT degradation

and SUMOylation which promotes NFAT nuclear retention (Terui et al., 2004; Yoeli-Lerner et al., 2005).

7.3.1. NFAT is obligatory for Ca^{2+}-dependent hypertrophic growth

Binding sites for NFAT can be found in a host of genes commonly upregulated in hypertrophy, while pressure overload significantly enhances NFAT activity *in vivo* (Wilkins et al., 2004). *In vitro* data clearly demonstrates that inhibition of NFAT activity in cardiac myocytes completely blocks hypertrophy induced by calcineurin, endothelin-1, or CT-1 (van Rooji et al., 2002).

Transgenic mice expressing a constitutively active form of NFAT, which translocates to the nucleus in the absence of calcineurin, displayed a similar phenotype to calcineurin transgenic mice, with cardiomegaly, fibrosis, and myofiber disarray (Molkentin et al., 1998). Thus calcineuin dependent activation of NFAT represents a major point of control for the hypertrophic program. NFAT-dependent hypertrophy seems to be largely mediated through the NFATc2 and NFATc3 isoforms, since mice deficient in these two factors (but not NFATc4) exhibited reduced CnA-dependent hypertrophy (Bourajjaj et al., 2008; Wilkins et al., 2002). The scaffold protein mAKAPβ appears to be obligatory for calcineurin dependent NFAT activation and thus the cellular localization of both calcineurin and NFAT have a major role to play induction of the hypertrophic response (Li et al., 2009a).

NFATs can bind DNA as homodimers or heterodimers but are more commonly found in association with other transcription factors such as AP1, GATA4, MEF2, MafA, EGR1, Nished, and FoxoP3 (Koltsova et al., 2007; Macian, 2005; Mathew et al., 2004; Wu et al., 2006b). Binding of NFAT to other transcriptional regulators has a major impact on the upregulation of hypertrophic genes. For example, in response to angiotensin II, NFAT interacts with GATA4 at the BNP and endothelin-1 promoters (Morimoto et al., 2001; Wen et al., 2002). This transcriptional cooperation allows for integration of signaling circuits from several cellular sources such Ca^{2+}, MAPKs, and GPCRs and thus fine tunes coordinate regulation of hypertophic gene expression (Hogan et al., 2003).

7.3.2. Crosstalk between MAPKs and NFAT

Not surprisingly, there exists multiple layers of crosstalk between hypertrophic signaling pathways and this has been explored in some detail in the case of MAPK/NFAT crosstalk. MEK1 enhances NFAT transcriptional activity both *in vitro* and *in vivo* and this appears to be mediated through ERK2 dependent phosphorylation of the NFATc3 C-domain, resulting in increased DNA binding (Sanna et al., 2005). Interestingly, ERK2 forms a complex with both CnA and NFATc3 and inhibition of CnA blocked MEK1 dependent hypertrophy and ANP promoter activity. Conversely, inhibition of

ERK1/2 reduced calcineurin mediated increases in myocyte size and ANP activity, thus showing an interdependence between the CnA and MEK-ERK pathways necessary for fully fledged hypertrophy (Sanna et al., 2005). In contrast, p38 and JNK block NFAT transcriptional activity by phosphorylating its N-terminal regulatory domain and inhibiting nuclear translocation (Braz et al., 2003; Liang et al., 2003; Sanna et al., 2005). Thus in mice with defective JNK or p38 activation, NFAT reporter activity is enhanced and the hypertrophic response is increased (Braz et al., 2003; Liang et al., 2003). The crossregulation of the MAPK and NFAT pathways thus allows for integration of signals from both Ca^{2+} sensors and ARs, allowing adaption to the altered stress levels in the cardiac myocyte (Fig. 3.2).

7.3.3. NFAT inhibition as a therapeutic strategy for HF

Direct targeting of NFAT activity has been shown to have some therapeutic potential. An NFAT inhibitor peptide called VIVIT has been developed based on homology to the conserved calcineurin docking site and impedes calcineurin–NFAT binding without affecting calcineurin activity (Aramburu et al., 1999). Administration of a cell-permeable poly-arginine VIVIT peptide to aortic banded rats reduced heart weight, wall thickness, and natriuretic peptide (NP) expression to a similar extent as cyclosporine (Kuriyama et al., 2006). Direct inhibition of NFAT activity may therefore ultimately prove a more attractive therapeutic avenue as it may avoid the harmful side effects of direct calcineurin inhibition using CsA and FK506.

7.4. GATAT4

The Zn finger transcription factor GATA4 is highly expressed in cardiac myocytes and regulates the expression of several important hypertrophic genes including ANP, BNP, α-MHC, β-MHC, and ET-1. Hypertrophic signals such as pressure overload and adrenergic drive lead to elevated GATA4 activity and overexpression of GATA4 both *in vitro* and *in vivo* is sufficient to drive hypertrophy (Liang et al., 2001a). Similarly to other hypertrophic transcription factors, GATA4 regulates its target promoters in combination with other factors including NFAT, MEF2, p300, STATs, Nkx2.5, and SRF and inhibition of these associations has been shown to stifle the hypertrophic response (Dai and Markham, 2001; Morimoto et al., 2001; Pikkarainen et al., 2003) (Fig. 3.3).

7.4.1. Regulation of GATA4 activity

Like all transcription factors, GATA4 is subject to multiple levels of control. Interestingly, GATA4 protein synthesis appears to be regulated at a translational level during hypertrophy. Treatment of cardiac myocytes with the hypertrophic agonist vasopressin was found to increase IRES (internal

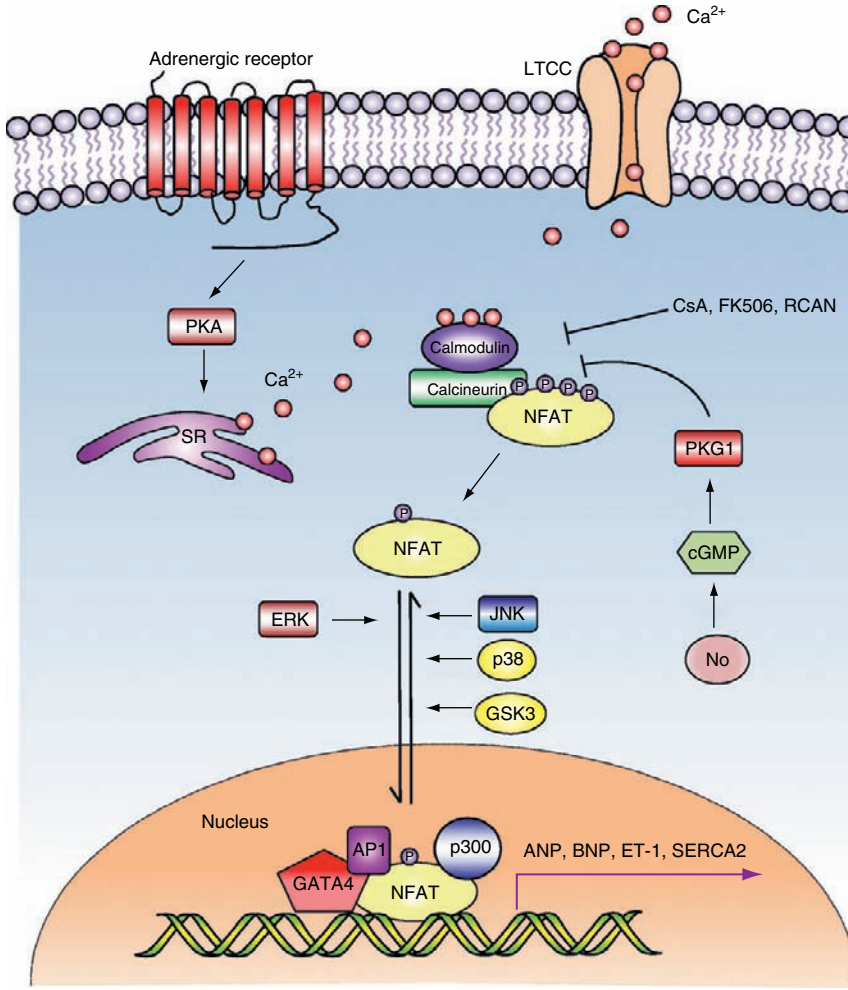

Figure 3.2 The calcineurin/NFAT pathway. Increases in intracellular Ca^{2+} levels via adrenergic mediated release from SR stores or transit through the L-type calcium channel (LTCC) leads to calmodulin-dependent calcineurin activation. Calcineurin dephosphorylates the transcription factor NFAT causing it to translocate to the nucleus where it upregulates hypertrophic genes in conjunction with other transcription factors such as GATA4, NFAT, AP1, and the p300 cofactor. Nuclear translocation is increased by ERK, while NFAT is removed from the nucleus following phosphorylation by JNK, p38, or GSK-3. Calcineurin mediated NFAT activation is inhibited by the nitric oxide (NO) signaling via cGMP and cGMP-dependent protein kinase 1 (PKG1), the endogenous regulator of calcineurin 1 (RCAN1) and the immunosuppressive agents cyclosporine A (CsA) and FK506.

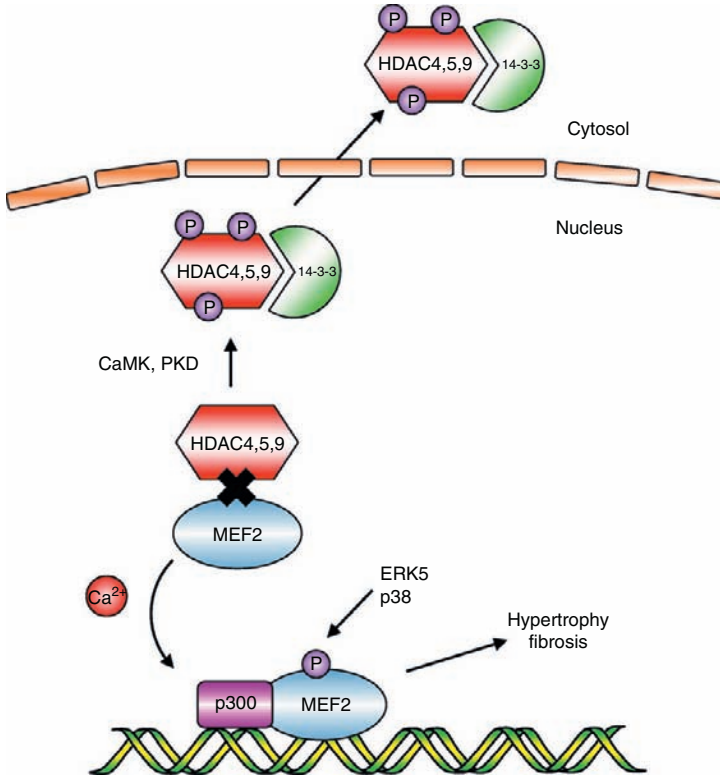

Figure 3.3 Class II HDAC regulation of MEF2 activity. MEF2 is a potent prohypertrophic transcription factor and is kept in an inactive state via binding to the Class II HDACs-4, 5, and 9. Hypertophic signals lead to the activation of HDAC kinases such as Ca^{2+}-calmodulin-dependent protein kinase (CaMK) and protein kinase D which phosphorylate HDACs and promote their binding to the 14-3-3 chaperone and induce their nuclear export. Increased levels of intracellular Ca^{2+} or induction of the MAPKs ERK and p38 increases MEF DNA binding and association with cofactors such as p300.

ribosome entry site) activity in the 5′ UTR of GATA4, causing it to be translated in a 5′ cap-independent manner (Sharma et al., 2007). Once translated, GATA4 can be phosphorylated by ERK, p38, or PKC, all of which increase GATA4 transcriptional responses (Liang et al., 2001b; Saadane et al., 1999; Tenhunen et al., 2004; Wang et al., 2005a). GATA4 is negatively regulated by the basic helix-loop-helix transcription factor Hey2 which inhibits its DNA binding and by FOG-2 (friend of GATA) which competes with GATA4 for the binding of the p300 cofactor (Fischer et al., 2005; Hirai et al., 2004). Thus overexpression of Hey2 or FOG-2 significantly blunts adrenergic dependent increases in myocyte size and induction of fetal genes (Hirai et al., 2004; Xiang et al., 2006).

7.4.2. Requirement of cardiac GATA4 expression for compensation following pressure overload

Myocardial deletion of GATA4 revealed an absolute requirement in compensating for increased wall stress during pressure overload (Oka et al., 2006). Following TAC, $GATA4^{-/-}$ mice had reduced cardiac hypertrophy; however, they exhibited pronounced pulmonary oedema, reduced left ventricular wall thickness, decreased fractional shortening, and elevated levels of myocyte apoptosis (Oka et al., 2006). Although, GATA4 heterozygous mice did not show any difference in heart weight following TAC, the pattern of hypertrophy was altered, with $GATA4^{+/-}$ myocytes displaying an eccentric form of growth (Bisping et al., 2006). Similarly to the study by Oka et al., these mice developed severe LV dysfunction and pulmonary oedema after TAC. These studies not only show that GATA4 is required for correct myocyte hypertrophy but that this hypertrophic growth is necessary to compensate for elevated wall stress and protect the heart from load-induced failure, thus lending strong support to Grossman's original conjecture (Grossman et al., 1975). The benefit of increased GATA4 levels has been elegantly demonstrated by the direct delivery of GATA4 in a preclinical myocardial infarction model. Bian et al. engineered a secreted cell-permeable form of GATA4 which they stably expressed in rat cardiac fibroblasts (Bian et al., 2007). Delivery of these GATA4 producing fibroblasts to the periinfarct zone of rats 1 month after coronary artery ligation led to local cardiac myocyte hypertrophy, improved cardiac function, reduced fibrosis, and an overall increase in survival.

8. MicroRNAs—Emerging Regulators of Heart Failure

MicroRNAs (miRNAs) are short highly conserved noncoding RNAs approximately 22 nucleotides in length which have emerged as potent regulators of gene expression in a diverse array of physiological and disease states. They control target gene expression by base pairing to complementary sequences within the 3′ UTR of mRNA transcripts, promoting degradation and/or inhibiting protein translation. Microarray studies have identified several miRNAs which are differentially regulated in failing human hearts or in experimental HF models and indeed many of these miRNAs are capable of inducing hypertrophic growth when transfected into cardiac myocytes (Sayed et al., 2007; Tatsuguchi et al., 2007; van Rooji et al., 2006). Interestingly, failing human hearts express a set of miRNAs that are similar in profile to fetal hearts and thus the fetal gene program may involve reexpression of miRNAs normally expressed during development (Thum et al., 2007). Evidence for the fundamental role of miRNAs in

maintaining cardiac homeostasis comes from studies of mice deficient in Dgcr8 or Dicer, factors necessary for primary miRNA and pre-miRNA cleavage, respectively. These mice suffer myofibrial disarray, fibrosis, ventricular dysfunction, and premature death from cardiomyopathy, thus highlighting the fundamental importance of miRNAs in maintaining cardiac function (Chen et al., 2008; da Costa Martins et al., 2008; Rao et al., 2009). To date, 11 miRNAs have been directly implicated in the pathology of HF; miR-1, miR-18b, miR-21, miR-23a, miR-23b, miR-24, miR-129, miR-133, miR-195, miR-208, and miR-212; however, the true number of miRNAs which are involved in cardiac pathology is likely to be many times greater (Pan et al., 2010).

8.1. Myocyte-specific miRNAs: miR-1, miR-133, and miR-208

Three miRNAs have been described as muscle specific; MiR-1 appears to be the most abundant miRNA in the heart, representing approximately 40% of sequenced cardiac miRNAs (Rao et al., 2009). miR-1 is downregulated upon HF and has been shown to target key components of the Ca^{2+} mediated hypertrophic signaling cascade, including calmodulin and MEF2 (Ikeda et al., 2009). miR-133 is transcribed together with miR-1 in a bicistronic cluster and is likewise repressed during HF, miR-133 may control myocardial remodeling through inhibition of CTGF (connective tissue growth factor), RhoA, and Cdc42 (Care et al., 2007; Duisters et al., 2009). While inhibition of miR-133 by infusion of an antagomir led to marked left ventricular growth; preventing the normal miR-133 downregulation by transgenic overexpression did not affect cardiac hypertrophy after TAC or adrenergic stimulation (Care et al., 2007; Matkovich et al., 2010). This is somewhat surprising in light of the recent finding that NFAT is a direct target of miR-133 (Li et al., 2010). However, miRNA-133 transgenic mice did have reduced fibrotic changes after TAC, which led to improvements in myocardial stiffness (Matkovich et al., 2010). Repression of both miR-1 and mIR-133 during the early stages of hypertrophy may also contribute to electrical remodeling and arrhythmogenesis which accompanies HF by allowing elevated expression of the hyperpolarization-activated channels HCN2 and HCN4 (Luo et al., 2008).

miR-208a is encoded by an intron in the α-MHC gene and is only expressed in the heart. The expression of miR-208a is sufficient to induce hypertrophy and is obligatory for upregulation of β-MHC, thus miR-208a deficient mice were refractory to pressure overload-induced hypertrophy (van Rooji et al., 2007). The most likely target of miR-208a which accounts for this positive effect on β-MHC expression is Thrap1 (thyroid associated protein 1), a cofactor of the thyroid hormone receptor which is a known inhibitor of β-MHC expression (Callis et al., 2009).

8.2. miRNAs as regulators of cardiac fibrosis

miR-21 was found to be upregulated in fibroblasts during HF, leading to inhibition of sprouty homologue 1 (Spry1) and subsequent increased phosphorylation of its target ERK1/2 (Thum et al., 2008). Elevated MAPK activity in turn facilitated fibroblast survival and promoted interstitial fibrosis during experimental HF. Importantly, it was shown that an miR-21 antagomir could not only prevent hypertrophy, fibrosis, and HF following pressure overload but could also reverse established cardiomyopathy, thus providing a rationale for the use of antagomirs in clinical HF treatment (Thum et al., 2008).

The study of miRNAs in HF is still in its infancy and it is likely that these small RNA species have wide ranging effects on many aspects of cardiac biology. Therapeutic use of miRNAs or inhibitory antagomirs holds great promise for future treatment options in HF. Recently, the use of circulating miRNAs as HF biomarkers has also been mooted. For example, one study has shown that MiR-423-5p levels can accurately discriminate HF patients from healthy controls with an area under the curve of 0.91 and showed promising potential for differentiating HF from non-HF dyspnea patients (Tijsen et al., 2010).

9. The Fetal Gene Program

Shortly after birth, there is a dramatic change in the physiological environment which is accompanied by a shift in the utilization of lactate and glucose to fatty acid oxidation and a postnatal switch from "fetal" to "adult" isoforms of metabolic and other proteins (Taegtmeyer et al., 2010). When the adult heart is subjected to hemodynamic or metabolic stress, one of the initial molecular changes brought about in cardiac myocytes is the reexpression of fetal isoforms normally repressed in the adult myocardium. This includes a change from fatty acid to carbohydrate metabolism, induction of NPs and early response genes such as c-myc and c-fos and isoform switching of contractile proteins such as myosin and actin (Taegtmeyer et al., 2010). This program of changes is necessary to allow for cellular enlargement and adaptation to altered energy demands associated with pathological remodeling but does not occur during physiological hypertrophy.

9.1. Myosin isoform switching

While several genes such as actin and troponis reexpress fetal forms during hypertrophy, the most important and intensively studied example is that of myosin. When the heart is subjected to altered load, as part of the adaption process, the expression of myosin isoforms is altered in order to maintain

contractile force. The myosin protein is formed from two heavy chains and two light chains and regulates myocyte contraction by binding to actin, pulling the Z-bands toward each other and shortening the sarcomeres. Hydrolyses of ATP by the myosin heavy chain (MHC) is required for the formation of the actomyosin crossbridge. Two isoforms of MHC are expressed in the heart; α-MHC has a greater ATPase activity and increased speed of actin displacement, resulting in a 2–3-fold increased velocity of myofilament shortening compared with the slower β-MHC isoform. The ratio of α-MHC:β-MHC mRNA changes from 30:70 in healthy hearts to 5:95 in failing hearts and an increase in β-MHC expression has been found in all models of experimental HF (Izumo et al., 1987; Mahdavi et al., 1984; Palmer, 2005). The α-MHC isoform seems to be more suited to functioning under conditions of low mechanical load and replacement with β-MHC during higher loads allows the heart to maintain force production while using less ATP, thus being more economical (Nakao et al., 1997; Palmer, 2005). However, while expression of β-MHC is energetically favorable, this is at the expense of slower contraction rate and eventual systolic dysfunction. This is supported by studies in transgenic mice expressing mainly the β-MHC isoform, these mice exhibited greater LV dilation and faster cardiac decline following MI (Krenz and Robbins, 2004). Improvement in hypertrophy is also associated with a return of MHC isoform levels to normal, for example, in patients with DCM undergoing β-blocker therapy, recovery was associated with increased α-MHC and decreased β-MHC expression (Lowes et al., 2002).

Thus, the reexpression of fetal genes allows the heart to initially compensate for increased pressure by altering actin–myosin crossbridge dynamics and energy usage. This cannot be sustained in the long-term however and the fetal gene program may eventually be maladaptive.

9.2. Counterbalancing hypertrophy—natriuretic peptides

The NPs are a family of hormones which control the rate of sodium excretion (natriuresis) and urine production (dieresis) from the kidneys and thus are important regulators of fluid homeostasis, vasodilation, and blood pressure. NPs also oppose the action of the rennin–angiotensin–aldostersone system (RAAS). There are three members, atrial natriuretic peptide (ANP), BNP, and C-type natriuretic peptide (CNP). ANP is produced in the atria, BNP is made predominantly in the ventricles with some expression in the atria, and CNP is secreted from the endothelium. ANP and BNP are among the earliest genes which are upregulated in the heart following various mechanical and neurohormonal stress signals leading to their widespread use as markers of hypertrophy and HF. All known hypertrophic stimuli, including elevated wall stress, hypertension, and adrenergic activity result in increased NP release, which forms part of a

compensatory mechanism to reduce pre- and after-load and directly antagonize hypertophic growth.

9.2.1. Natriuretic peptides possess antihypertrophic activity independent antihypertensive action

Work from several studies demonstrates that the NPs have direct antihypertrophic effects independent of their blood pressure lowering activity. For example, transferring $ANP^{-/-}$ mice to a low salt diet successfully ameliorated the hypertension but LV hypertrophy was still evident (Feng et al., 2003). Similarly, Knowles et al. lowered blood pressure in NPR-$A^{-/-}$ mice by treating with a diuretics, ACE inhibitors, or angiotensin receptor antagonists and found that these could not reverse the hypertrophic phenotype (Knowles et al., 2001). Finally, cardiac-specific deletion of NPR-A resulted in increased hypertrophy even though the mice were mildly hypotensive (Holtwick et al., 2003). Therefore, in addition to their diuretic and natriuretic activity, NPs have direct growth effects on cardiac myocytes.

9.2.2. Natriuretic peptide receptors

NPs exert their effects through binding the natriuretic peptide receptors (NPRs), also known as guanylyl cyclase (GC) receptors. Binding to the receptor induces GC activity and subsequent conversion of guanosine triphosphate (GTP) to the second messenger guanosine 3′,5′-cyclic monophosphate (cGMP; Gardner et al., 2007). ANP and BNP bind to NPR-A and CNP binds to NPR-B; an additional receptor NPR-C is responsible for metabolizing NPs and removing them from circulation (Gardner, 2003; Richards, 2007).

Since NPs have well-documented antihypertrophic activity in culture, it was not surprising that deletion of ANP led to elevated basal and pressure overload-induced hypertrophy in mice (Wang et al., 2003). Interestingly, deletion of BNP did not result in a hypertrophic phenotype; however, $BNP^{-/-}$ mice exhibited marked ventricular fibrosis which was further exacerbated by pressure overload (Tamura et al., 2000). This observation may be explained in part by the 10-fold higher affinity for NPR-A shown by ANP. Several groups have shown that deletion of NPR-A leads to pronounced hypertension, hypertrophy, fibrosis, and HF which is further exacerbated by pressure overload (Knowles et al., 2001; Oliver et al., 1997).

9.2.3. Molecular pathways induced by natriuretic peptides

cGMP is the common mediator of NP biological activity and treating myocytes with a cGMP analog or cGMP phosphodiesterase inhibitor has been shown to produce the same effect as NP treatment (Horio et al., 2000). The precise molecular signals downstream of NPR/cGMP have not been fully elucidated; however, ANP has been shown to block phenylephrine induced NFATc3 nuclear translocation in cardiac myocytes (Tokudome

et al., 2005). In support of this, NPR-A deficient mice have significantly elevated cardiac calcineurin activity, NFATc3 nuclear translocation, and GATA4 DNA binding and inhibition of calcineurin could partially reverse the hypertrophic phenotype of NPR-$A^{-/-}$ mice (Tokudome et al., 2005).

NPs are also potent inhibitors of the RAAS system and antagonize the vasoconstrictive and hypertrophic effects on AngII. Hypertrophy and fibrosis in NPR-A deficient mice can thus be rescued by crossing onto an AT1a negative background or by pharmacological inhibition of the AT1 receptor (Li et al., 2009b). cGMP appears to abrogate AngII-dependent signaling by inducing phosphorylation of RGS (regulator of G-protein signaling) proteins, which in turn antagonize Gq which is obligatory for α-adrenergic activity (Klaiber et al., 2010; Tokudome et al., 2008). This appears to be specific for α-adrenergic GPCRs with no documented effects on G_S activity, indeed ANP has been shown to inhibit cardiac myocyte L-type Ca^{2+} currents in response to AngII but not the β-adrenergic agonist isoprotenerol (Klaiber et al., 2010). NPs may also improve cardiac function during HF by promoting vascular regeneration in the heart. ANP and BNP are angiogenic in culture, while deletion of NPR-A in endothelial cells impairs post-TAC angiogenesis and significantly stifles blood flow after hind limb ischemia (Kuhn et al., 2009). Thus NPs are potent antihypertrophic agents which act through both calcineurin and AngII inhibition. This is further evidenced by the clinical effectiveness of synthetic NPs such as nesiritide and carperitide which are now in routine clinical use (Mitrovic et al., 2009).

9.2.4. Natriuretic peptides as biomarkers

NPs are routinely used in clinical medicine to aid in the diagnosis of HF, left ventricular dysfunction and dysnopea and have high predictive value in respect to morbidity and mortality in HF patients (O'Donoghue and Braunwald, 2010). Measurement of the amino terminal fragment of BNP (NT-proBNP) is now a well-established marker for the diagnosis, prognosis, and risk stratification of patients with HF. Circulating levels of NT-proBNP correlate well with NYHA functional class and compared with ejection fraction, allow better risk stratification (Maisel, 2009). A review which included 19 separate studies of HF patients concluded that for every 100 pg/ml increase in BNP levels there was a 35% increases in the relative risk of death (Doust et al., 2005). Similarly, in the recently published PREVEND study of over 8000 patients, it was found that for each doubling of NT-proBNP levels there was a 22% increased risk in all-cause mortality and a 16% increased risk of cardiovascular events (Linssen et al., 2010). Several recent trials have demonstrated that using BNP levels to guide clinical decision making leads to improved outcome for patients. The STARS-BNP trial showed a reduction in HF-related death or hospitalisation from 52% of patients in the standard treatment group to 24% in the BNP-guided group (Jourdain et al., 2007). Likewise, both the TIME-CHF

and BATTLESCARRED trials reported reduced HF hospitalizations and all-cause mortality in BNP-guided patients under 75 years of age (Lainchbury et al., 2009; Pfisterer et al., 2009). While other trials have shown equivocal results, a recent meta-analysis has found that BNP-guided therapy reduced all-cause mortality in patients with chronic HF when compared with normal HF clinical care, especially in patients younger than 75 years (Porapakkham et al., 2010). The improved outcome when patients are managed on the basis of BNP levels is likely due to more frequent alterations to HF treatment regimes, leading to uptitration of ACE inhibitors, β-blockers, and diuretics. Intriguingly, despite increased LV diastolic blood pressure, obese patients have lower circulating levels of NPs and thus a lower diagnostic threshold may be needed for HF diagnosis in this patient group (Beleigoli et al., 2009). Other biomarkers can be used in conjunction with BNP to add to predictive value. For example elevated soluble ST2 receptor combined with elevated BNP are predictive of sudden cardiac death in HF patients (Pascual-Figal et al., 2009).

10. The Immune System and Heart Failure

It has been appreciated for almost 20 years that there is a significant inflammatory component to HF, indeed increased levels of circulating inflammatory cytokines such as TNF-α and IL-6 and the chemokines MCP-1 and MIP-1α are predictive of disease severity and mortality in HF patients (Aukrust et al., 1998; Deswal et al., 2001). Infiltration of inflammatory cells, which include macrophages, dendritic cells, and T cells, is commonly seen when examining cardiac tissue from failing hearts where they are thought to contribute to ongoing dysfunction. Both left and right ventricular hypertrophy is a stimulus for the mobilization of cells from the bone marrow to the heart where they can transdifferentiate into myocytes or myofibroblast-like cells (Endo et al., 2007).

10.1. T cells

Importantly it has been demonstrated that T cells from patients with stable chronic HF express increased levels of TNF-α, IFN-γ, IL-10, FasL, and MIP-1α, whereas no differences in gene expression were found in monocytes from the same patients (Yndestad et al., 2003). T cells which express TNF-α and FasL may contribute directly to cardiac myocyte apoptosis within the failing myocardium and T cell numbers with an anti-CD4 antibody has been shown to inhibit TNF-α dependent hypertrophic growth (Huber et al., 2006).

There is a clear link between T cell activation and HF. $CD4^+$ T helper cells differentiate into several lineages including Th1, Th2 cells based on the initiating stimulus and microenvironmental queues. It has been shown that patients suffering from HF have an altered Th1/Th2 balance, with increased levels of circulating IFN-γ-positive Th1 cells, this correlated with severity of disease but could be reversed by statin treatment (Cheng et al., 2009; Fukunaga et al., 2007a,b; Gage et al., 2004). In agreement with this, patients with end-stage HF have increased levels of Th1-polarizing myeloid dendritic cells (mDCs), with no differences in the levels of Th2-polarizing plasmacytoid DCs (pDCs; Athanassopoulos et al., 2004).

An altered Th1/Th2 balance also occurs in mice following angiotensin II treatment which increases T cell production of IFN-γ and decreases production of IL-4 (Shao et al., 2003). T cells appear to be critical for both the hypertensive and hypertrophic effect of AngII as these were lost in $Rag1^{-/-}$ and SCID mice and could be rescued by adoptive transfer of T cells but not B cells (Crowley et al., 2010; Guzik et al., 2007).

Regulatory T cells (Tregs) express CD25 and Foxp3 and are responsible for restraining exuberant inflammatory responses. Adoptive transfer of Tregs in mice reduced AngII-dependent cardiac hypertrophy, fibrosis, and BNP expression and decreased the incidence of arrhythmias (Kvakan et al., 2009). This was accompanied by reduced inflammatory cell infiltration into the heart. Interestingly, HF patients have been reported to have lower numbers of circulating Treg cells and Treg cells isolated from these patients were compromised in their ability to suppress T cell responses (Tang et al., 2010). Treating patients with ACE inhibitors however can restore the levels of Tregs (Platten et al., 2009).

10.2. Toll-like receptors

Toll-like receptors (TLRs) recognize immunogenic components of pathogens and are necessary for the mounting of a robust immune response. The expression levels of TLR4 on monocytes also appear to be higher in HF patients and $TLR4^{-/-}$ mice exhibited reduced levels of pathological remodeling following MI, which may be due to a combination of decreased ECM degradation, reduced inflammatory cell infiltrate, and decreased cytokine production (Riad et al., 2008; Satoh et al., 2006; Timmers et al., 2008). As a result, the increase in left ventricular size following MI is less pronounced in $TLR4^{-/-}$ mice, allowing them to sustain a relatively normal level of heart function. Interrupting the signaling downstream of TLR4 also provides relief from experimental HF, for example inhibition of the TLR adaptor protein MyD88 has proved beneficial in experimental HF (Ha et al., 2006). Given these results, it is somewhat surprisingly that a recent reported demonstrated that stimulation of TLR4 with LPS or the TB vaccine Bacillus Calmette-Guérin (BCG) reduced aortic-banding mediated

hypertrophy and fibrosis in mice, indeed the effects of BCG administration were potently diminished by coadministration of a TLR4 antagonist (Liu et al., 2008). It is currently unclear how both stimulation and inhibition of TLR4 can produce desirable effects in the context of experimental HF; however, it does suggest that pharmacological modulation of TLR4 activity may prove an interesting therapeutic target. The endogenous stimulator of TLR4 activity during HF is unknown and candidates include the alarmin HMGB1 (high mobility group box 1), which is released from damaged cells and binds to TLR4 (Xu et al., 2010). Interestingly, it has been observed in patients with HF that there are elevated circulating levels of the TLR4 agonist LPS (Peschel et al., 2003; Sharma et al., 2003). This may arise from bacterial transit from the digestive tract due to increased bowel permeability in HF patients caused by mesenteric venous congestion (Anker et al., 1997).

10.3. TNF

The plasma levels of TNF-α are elevated in patients suffering from congestive HF and DCM (Katz et al., 1994). Furthermore, increased levels of TNF-α, soluble TNF receptors are predictive of 2 year mortality in CHF patients (Rauchhaus et al., 2000). TNF-α is known to decrease cardiac contractility, reduce ejection fraction, alter Ca^{2+} transients, perturb ECC, increase myocyte apoptosis, and promote endothelial dysfunction (Meldrum, 1998). Transgenic overexpression of TNF-α in the heart causes DCM and inhibition of TNF-α in rodents has proved quite successful as a means of treating experimental HF (Kubota et al., 1997a,b). Clinical trials of the TNF-α inhibitor etanercept in HF patients have proved disappointing however (Anker and Coats, 2002).

10.4. NF-κB

The transcription factor NF-κB is a downstream target of both TLRs and TNF; its transcriptional activity is induced by adrenergic stimulation and inhibition of NF-κB with a superrepressor revealed an absolute requirement for NF-κB in agonist-mediated hypertrophic growth of cardiac myocytes (Purcell et al., 2001). Genetic deletion of the p50 subunit of NF-κB or transgenic overexpression of an NF-κB superrepressor abrogated the hypertrophic effect of chronic adrenergic drive or MI in mice (Freund et al., 2005; Kawano et al., 2005, 2006). Moreover, NF-κB activity is markedly increased in both peripheral leukocytes and cardiac myocytes from patients suffering from HF (Frantz et al., 2003, 2004). Recently, targeting NF-κB activity by directly injecting lentivirus expressing siRNA against p65 into the heart reversed established hypertrophy in a myotrophin transgenic mouse model (Gupta et al., 2008b). We have also shown in our labs recently that NF-κB and C-Rel have a balancing effect in mediating the

hypertrophic response in human HF and confirmed in mouse models null for NF-κB and C-Rel (Townsend et al., unpublished data).

10.5. IL-6 cytokines

Several IL-6 family cytokines have been shown to possess prohypertrophic properties, including IL-6 itself, leukemia inhibitory factor (LIF), and cardiotrophin-1 (CT-1) all of which are induced by adrenergic drive or pressure overload (Eiken et al., 2001; Tsutamoto et al., 2007). IL-6 may have value as a therapeutic target in reducing LV modeling in HF; blocking IL-6 activity using an IL-6 receptor antibody resulted in reduced LV dilation and prolonged survival following MI in mice (Kobara et al., 2010). IL-6 and CT-1 levels positively correlate with NYHA class and ventricular dysfunction and are predictors of mortality in HF patients (Petretta et al., 2000; Tsutamoto et al., 2007).

IL-6 cytokines utilize a common receptor glycoprotein 130 (gp130) in combination with ligand specific receptors which induce specific combination of the JAK/STAT, MAPK, and PI(3)K pathways (Barry et al., 2007). Both STAT3 and ERK5 appear to play a significant role in the hypertrophic response of IL-6 cytokines. Inhibition of STAT3 abrogates LIF and CT-1 dependent hypertrophy in cardiac myocytes (Kunisada et al., 1998; Railson et al., 2002). Inhibition of the ERK5 activator MEK5 blocks CT-1 and LIF induced eccentric hypertrophy, while MEK5 transgenic mice display an eccentric phenotype closely resembling that of CT-1 or LIF treated mice (Nicol et al., 2001). As well as promoting addition of sarcomeres in series, the LIF/MEK5/ERK5 pathway seems to antagonize the addition of sarcomeres in parallel and thus may serve as a focal point in the decision of myocytes to undergo eccentric or concentric hypertrophy (Nicol et al., 2001). The gp130 receptor represents a common sensor of all IL-6 cytokines, deletion of gp130 renders mice exquisitely sensitive to pressure overload and is instrumental in the transition from compensatory hypertrophy to HF (Hirota et al., 1999).

11. Myocarditis

Myocarditis is defined inflammation of the heart due to viral infection or autoimmunity and is the leading cause of HF in patients under 40 years of age. The precise incidence of myocarditis is difficult to accurately quantify due to subclinical presentation and common misdiagnosis (Esfandiarei and McManus, 2008). The Myocarditis Treatment Trial however has reported that the incidence of myocarditis in patients with unexplained HF is 9.6% (Hahn et al., 1995). Moreover, viral genomes have been detected by PCR in 67% of DCM cases, highlighting a significant role for viral infection in

HF (Kuhl et al., 2005). Myocarditis is a particular problem in young children and has been estimated to be responsible for almost 20% of sudden unexpected deaths in this group (Drory et al., 1991). The overall prognosis for myocarditis is poor with a 5-year survival rate of 50% (Esfandiarei and McManus, 2008). In the initial phase of myocarditis, cardiac myocyte injury leads to activation of the innate and adaptive immune system. In most cases this inflammation is resolved, persistent inflammation however can lead to further myocyte loss, DCM, and ultimately HF.

11.1. Diagnosis of myocarditis

Patients can present with nonspecific flu-like symptoms, dyspnoea, chest pain, and arrhythmias (Leuschner et al., 2009). Diagnosis is usually based on clinical presentation and noninvasive imaging; however, a definitive diagnosis of myocarditis often requires an endomyocardial biopsy. Traditionally, diagnosis is made according to the Dallas criteria which requires inflammatory infiltrate and myocyte necrosis not characteristic of an ischemic event (Aretz et al., 1987). Myocarditis can present as four distinct subgroups; patients with fulminating myocarditis have severe left ventricular dysfunction with multiple foci of active inflammation and patients normally recover or die within 2 weeks of presentation (Cooper, Jr., 2009). Chronic active myocarditis is associated with moderate ventricular dysfunction, persistent inflammation and fibrosis and cardiomyopathy usually develops 2–4 years after presentation (Baughman, 2006). Eosinophilic myocarditis is caused by eosinophilic syndromes or allergic reactions and these patients usually respond well to treatment (Baughman, 2006). Giant cell myocarditis associated with congestive HF, arrhythmias, and heart block has a poor prognosis and although some patients may respond to immunosuppressive therapy, heart transplant is required in many cases (Rosenstein et al., 2000). Although myocarditis thus represents a separate spectrum of cardiomyopathy, the current treatment is standard HF therapy.

11.2. Myocarditis of viral origin

More than 20 common viruses have been identified as causative agents of myocarditis including coxsackievirus B3, paravirus B19, hepatitis C, influenza, adenovirus, herpes simplex, Epstein-Barr and cytomegalovirus and endomyocardial biopsies can now be routinely tested for the presence of viral nucleic acids (Esfandiarei and McManus, 2008; Rose, 2009). Chagas disease is a form of myocarditis prevalent in the developing world due to infection with the protozoon *Trypanosoma cruzi*, which causes myocardial pathology in nearly 80% of infected individuals. Antiviral therapy is currently under investigation as a treatment option and early results suggest that IFN-β may be beneficial in treating myocarditis patients with confirmed viral etiology.

11.3. Autoantibodies in myocarditis patients

Viral damage may also lead to the exposure of self-antigens, resulting in the development of autoimmunity. Indeed, 60% of patients with myocarditis have circulating heart specific IgGs (Neumann et al., 1990). Antiheart antibodies were found to be an independent predictor of DCM in asymptomatic relatives of DCM patients, suggesting that cardiac autoantibodies are a cause of DCM rather than simply an immune response to damaged cardiac cells (Caforio et al., 2007). The most common form of autoantibody associated with myocarditis is α-MHC IgG which has been reported in 86% of patients with DCM (Caforio et al., 1992). Other reported autoantibodies include antibodies against β1-ARs, muscarinergic receptors, laminin, and mitochondrial antigens (Leuschner et al., 2009). Using immunoadsorption to remove circulating antibodies from DCM patients has demonstrated improved hemodynamic function and subsequent increases in antibody titres in these patients is associated with a deterioration of cardiac function (Dorffel et al., 2004; Felix et al., 2000). It has been suggested that infection with viruses such as the coxsackievirus causes damage to cardiac cells, thereby releasing antigen such as cardiac myosin, with the virus itself subsequently serving as an adjuvant (Rose, 2008). Thus, the virus is the trigger which initiates a subsequent autoimmune reaction.

11.4. Mouse models of myocarditis

Much of our understanding of the pathology of human myocarditis comes from murine models which have significantly enhanced our appreciation of the interplay between the immune system and the myocardium. The most widely used mouse models include infection with coxsackievirus B3 (CVB3) or experimental autoimmune myocarditis (EAM), achieved by immunization with α-MHC or troponin I peptides in complete Freud's adjuvant (CFA). Both methods produce a pathology which closely resembles human myocarditis; inflammation eventually resolves with some mice succumbing to left ventricular dilation, fibrosis, and compromised heart function (Goser et al., 2006). Three to four days after inoculation with CVB3 there is viral mediated cardiac myocyte injury with evidence of necrosis and apoptosis. This is followed by local release of proinflammatory mediators and infiltration of immune cells into the myocardium. Myocardial damage occurs in the early phase of viral infection before the infiltration of immune cells and can occur in the absence of T and B cells, suggesting that viruses can directly damage the heart. A second wave of immune cell migration occurs with the release of viral progeny leading to multifocal inflammatory lesions, with eventual clearance of the virus around day 14 (Esfandiarei and McManus, 2008). Immune mediated killing of infected cardiac myocytes by $CD8^+$ T cells and NK cells limits viral replication but has detrimental effects on cardiac

function. Day 15 onward represents the chronic phase of myocarditis, which is accompanied by myocardial hypertrophy, ventricular dilation, and interstitial fibrosis. CVB3 infection also leads to the production of self-reactive IgG antibodies against cardiac proteins such as α-MHC, tropomyosin, desmin, and heat shock proteins (Latif et al., 1999).

11.5. Adaptive immunity in EAM

EAM results in cardiac infiltration of several classes of immune effectors, including $CD4^+$ and $CD8^+$ T cells, NK cells, macrophages, eosinophils, and mast cells (Leuschner et al., 2009). The destructive nature of immune cell mediated pathology is clearly evidenced by the fact that myocarditis can be induced by adoptive transfer of myosin-specific $CD4^+$ T cells or myosin-presenting dendritic cells.

It is well accepted that myocarditis is a T cell driven inflammatory pathology; however, myocarditis appears to develop independently of Th1 or Th2 polarization (Rangachari et al., 2006). Th17 cells have been implicated as the main pathological effector cell in a number of inflammatory conditions including inflammatory bowel disease and arthritis. Recent evidence has also implicated Th17 cells in promoting pathology in myocarditis and several studies have demonstrated that inhibition of IL-17 ameliorates disease symptoms as does inhibition of the Th17 promoting cytokine IL-23 (Rangachari et al., 2006; Valaperti et al., 2008; Yuan et al., 2010). Moreover, adoptive transfer of α-MHC specific Th17 cells but not Th1 cells leads to the development of pronounced myocarditis (Rangachari et al., 2006). Recent studies in IL-17 deficient mice have revealed that IL-17 may actually be dispensable for the initial development of myocarditis but is necessary for the onset of fibrosis, remodeling, and the progression to DCM (Baldeviano et al., 2010). Taken together, IL-17 inhibition may pose a promising therapeutic target in myocardial inflammation.

The Th1 cytokine IFN-γ appears to play a protective role in myocarditis, since mice lacking IFN-γ exhibit a greater inflammatory response and more pronounced fibrosis following CVB3 infection (Fairweather et al., 2004). Interestingly, mice deficient in the Th1 master regulator T-bet developed a more severe pathology of EAM due to reduced IFN-γ expression in $CD8^+$ T cells infiltrating the heart which impaired their capacity to suppress IL-17 driven inflammation (Rangachari et al., 2006). IFN-γ appears to inhibit Th17 cell indirectly on heart infiltrating monocytes which in turn inhibit autoreactive, cardiac damaging Th17 cells.

Mobilization of cells from the bone marrow thus seems to play a significant role in the progression of myocarditis. In the EAM model, following the natural resolution of inflammation, the heart undergoes significant fibrosis which is a significant contributing factor to HF. These cardiac fibroblasts are derived from a $CD133^+$ bone marrow progenitor cell population which

migrate to the heart and undergo differentiation into fibroblasts upon encountering TGF-β (Kania et al., 2009). In addition to monocytes and fibroblasts, CVB3 infection also leads to increased trafficking of myeloid DCs from the bone marrow to the myocardium (Chen et al., 2009).

Patients with myocarditis exhibit increased circulating levels of TNF-α; however while TNF-α is necessary for the development of EAM, clinical trials using the TNF-α blocking antibodies etanercept and infliximab in CHF patients have proved unsuccessful (Anker and Coats, 2002; Chung et al., 2003). Further stratification of patient subsets however might show efficacy of TNF-α inhibition in myocarditis patients confirmed with endomyocardial biopsy.

11.6. Innate immunity in EAM

Lack of TLRs renders mice extremely susceptible to CVB3-mediated cardiac damage due defective macrophage activity and insufficient production of cytokines such as IFN-γ, IL-12, and TNF-α. Mice deficient in TLR3, TLR4, TLR7, and TLR9 have all shown reduced inflammation and decreased transition to HF in myocarditis models (Fairweather et al., 2003; Negishi et al., 2008; Pagni et al., 2010; Riad et al., 2010; Richer et al., 2009).

TLRs utilize the adaptor protein MyD88 to transduce their signals and similarly to TLR knockouts, MyD88$^{-/-}$ mice are resistant to both αMHC and CVB3 induced myocarditis, exhibiting reduced levels of proinflammatory cytokines and increased levels of IFN-β and IRF3 (Fuse et al., 2005; Marty et al., 2006). Using an α-MHC loaded dendritic cell EAM model, Blyszczuk et al. (2009) demonstrated that stimulation of TLRs with CFA led to significant enhancement of EAM mediated fibrosis in wild type but not MyD88$^{-/-}$ or IL-1R$^{-/-}$ mice. Fibrosis was also evident when IL-1β was coadministered in place of CFA, suggesting that TLR mediated IL-1β is a central regulator of inflammatory heart disease associated fibrosis. Moreover, wild type mice reconstituted with MyD88 or IL-1R deficient bone marrow were resistant to fibrosis and HF, whereas reconstitution of MyD88$^{-/-}$ and IL-1R$^{-/-}$ with wild type bone marrow resulted in severe fibrosis and progression to DCM (Blyszczuk et al., 2009). Therefore, IL-1β/MyD88 signaling in the bone marrow is critical for the onset of fibrosis and progression to end-stage HF in experimental myocarditis.

11.7. Future treatment strategies in myocarditis

Adrenergic stimulation appears to worsen the symptoms of myocarditis and treatment with β2 AR antagonists has shown promise in experimental models (Wang et al., 2005b; Yuan et al., 2005). Treatment with statins has also shown promise in ameliorating EAM and a phase II clinical trial using IFN-β demonstrated viral clearance and improved left ventricular

function (Azuma et al., 2004; Kuhl et al., 2003). Currently anti-T cell antibodies and cyclosporine are under trial for treatment of myocarditis. With the significant recent expansion in our understanding of innate and adaptive immune responses in myocarditis, these and other treatments aimed at specific immunomodulation may hold significant promise.

12. Conclusion

Clearly, the molecular processes and regulation that regulate heart and cardiovascular pathophysiology is complex but strides have been made that provide some insight in to understanding HF and heart disease. We have provided an overview of heart biology, response to load and cardiac hypertrophy signaling. Moreover, it is increasingly evident that the calcium ion and its homeostasis is critical in regulating cardiac form and function. Recently, inflammation regulation and immune activation play a significant role in maintaining cardiac health. The role and influence of genetics and the dysregulation of signaling and adrenergic drive have been shown to have significant roles in mediating cardiovascular function. The biochemical and cellular influence of transcription factors (for example, MEF2, NFAT), epigenetic modulation mediated through histone "marks" that is acetylation and methylation and newly identified molecular controls, miRNAs, are exciting avenues for possible HF treatment.

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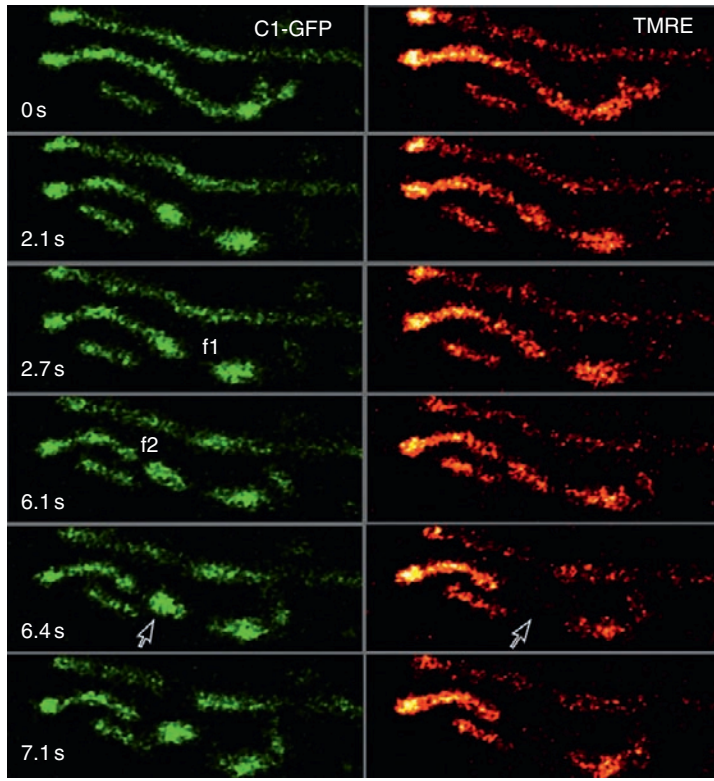
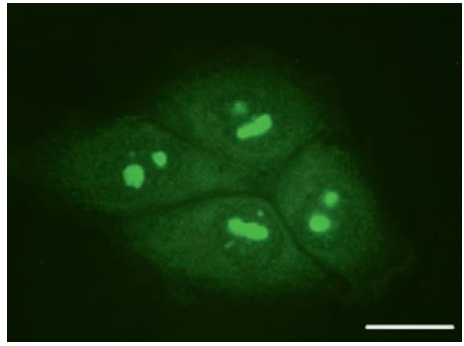
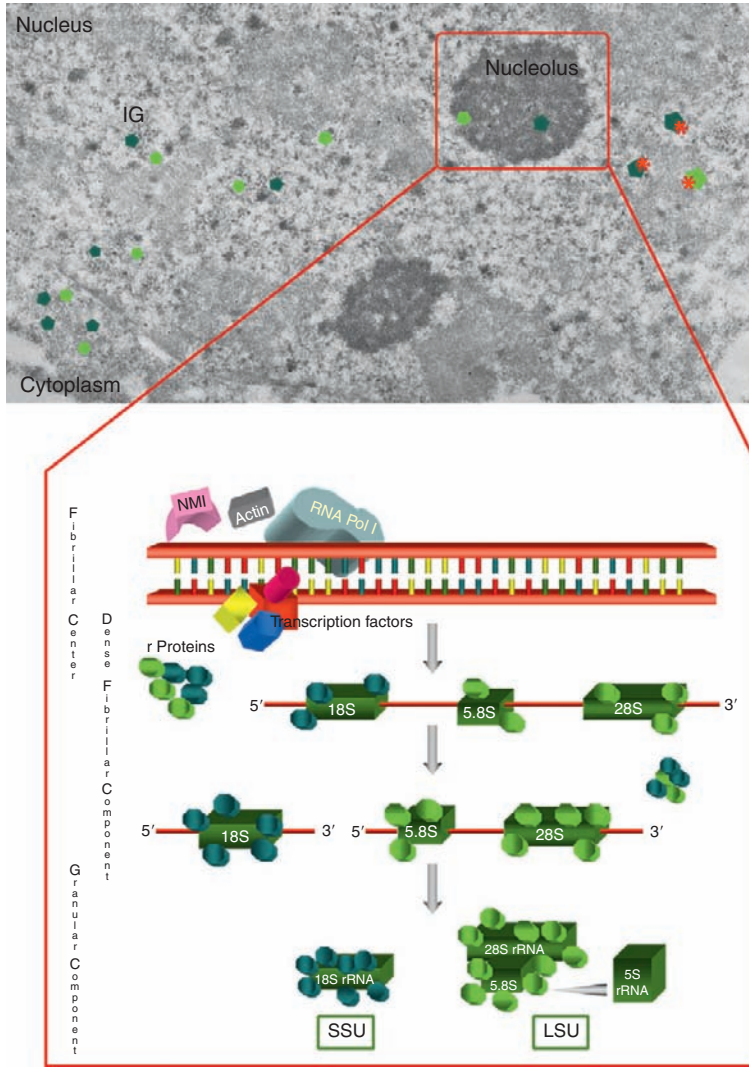


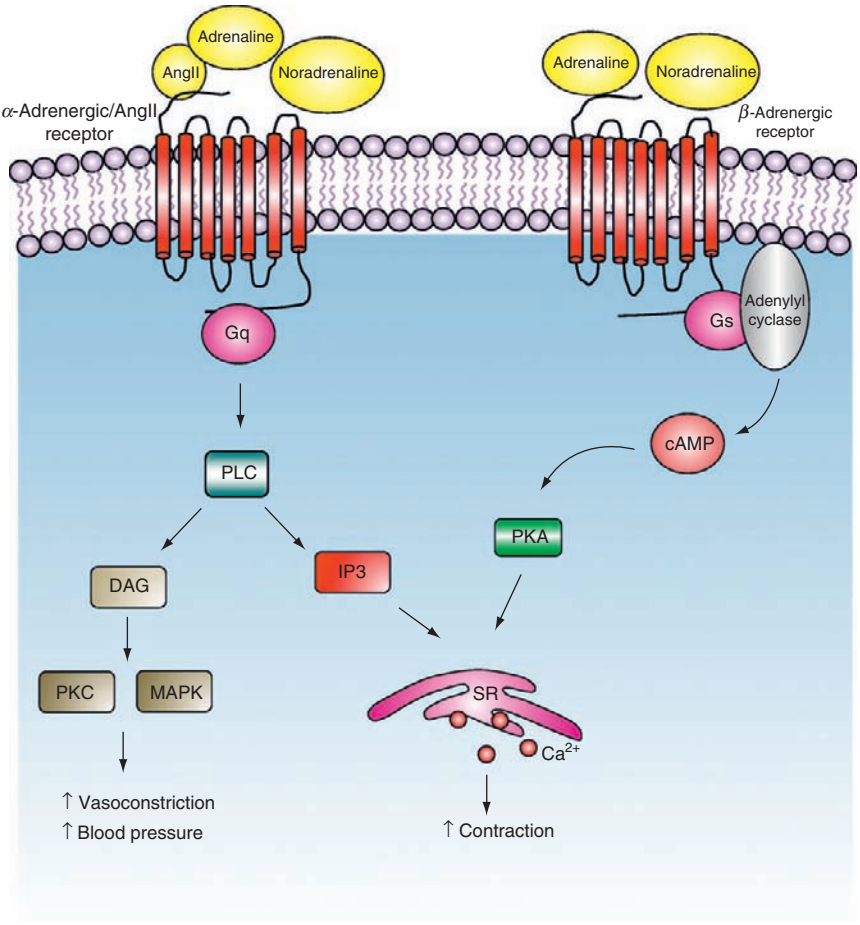
Jürgen Bereiter-Hahn and Marina Jendrach, Figure 1.4 Segregation of a mitochondrial part finally lacking $\Delta\psi_{mit}$. HeLa cells were treated with 25 μM nocodazole for 30 min and then 2 μg/mL cytochalasin D was added for another 20 min. Transfection with GFP labeled 30 kDa subunit of the respiratory complex C1 labeled IMM selectively. Counter staining by 100 nM TMRE for 20 min revealed $\Delta\psi_{mit}$. Despite disassembled microtubules and actin fibrils, mitochondria change shape and divide, but they do not move any more. In two fission steps (f1 and f2), a small mitochondrion is released from the central area of the elongated mitochondrion. Within less than 300 msec after the second fission step, TMRE fluorescence is lost from the fragment (arrow in C1-GFP frame 6.4 s). This proves that this part is not functional. Before the second fission step, the functional parts of the mitochondrion kept $\Delta\psi_{mit}$ and TMRE was distributed over the full length on the mitochondrion. After the second fission step, each of the three mitochondrial fragments had to maintain its own $\Delta\psi_{mit}$. A general decrease of fluorescence intensity is a consequence of bleaching. Each single frame measures 10.25 $\times$ 3.7 μm. (Confocal laser scanning series taken by Daniel Dikov.)



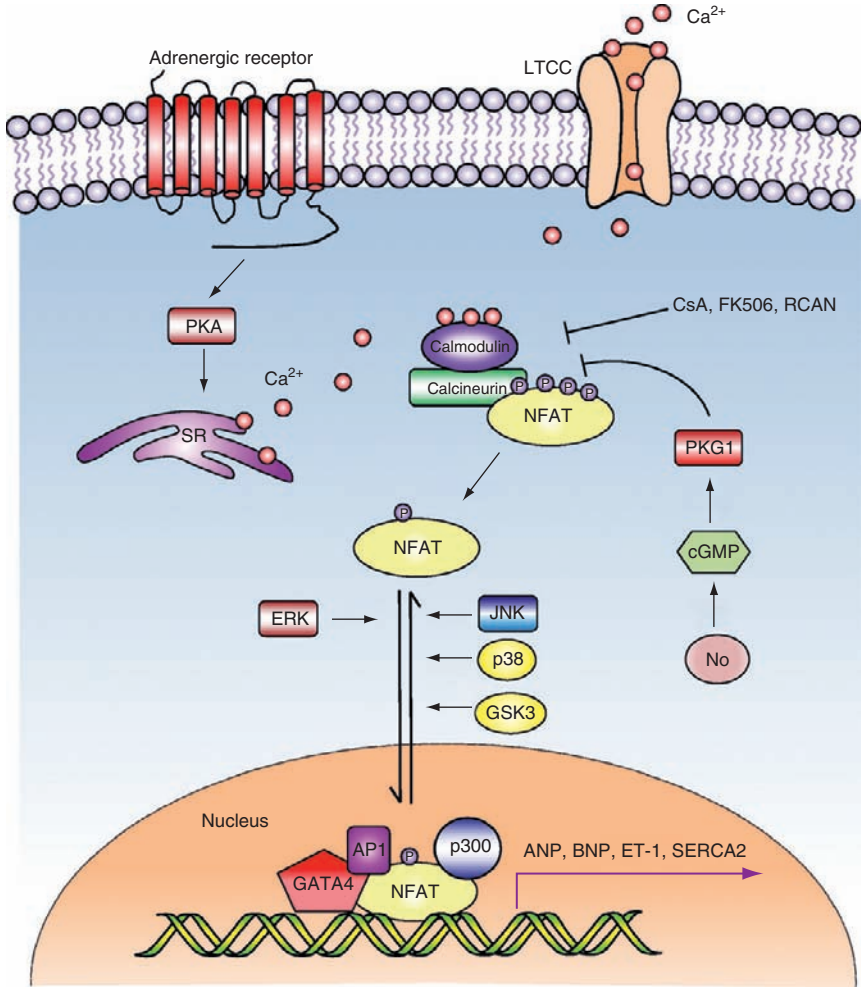
Barbara Cisterna and Marco Biggiogera, Figure 2.1 HeLa cells stained by the vital fluorescent-probe SYTO which preferentially binds to RNA. The image shows the cospicuous amount of RNA concentrated in the organelle, in addition to a homogeneous staining in the cytoplasm. Bar represents 50 μm.



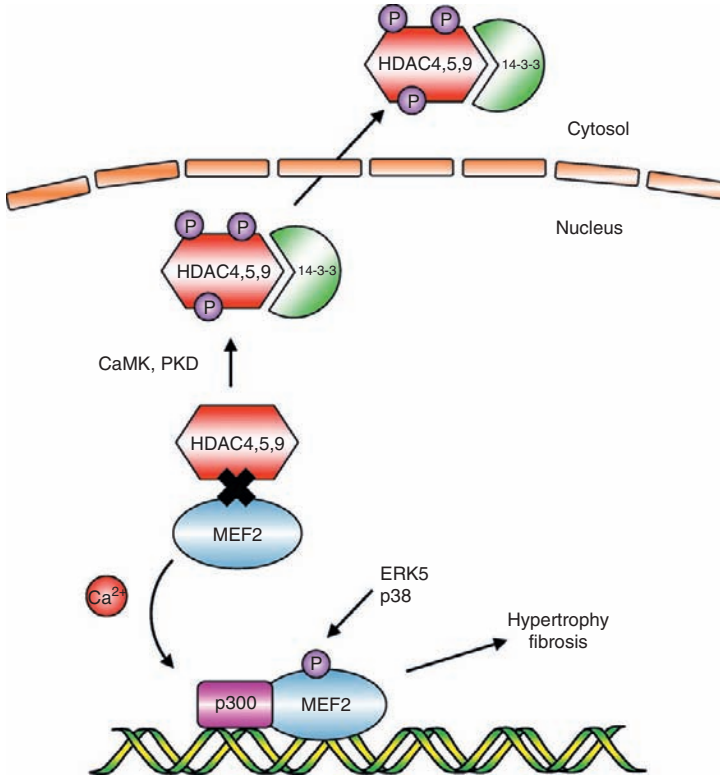
Barbara Cisterna and Marco Biggiogera, Figure 2.5 A scheme of the pathway followed by a ribosomal protein from the cytoplasm to the nucleolus to be integrated into a ribosomal subunit.



Seán P. Barry and Paul A. Townsend, Figure 3.1 Signaling through adrenergic and angiotensin receptors increases heart rate and blood pressure. Catecholamines such as adrenaline and noradrenaline bind to α- and β-adrenergic receptors while angiotensin II (AngII) binds to its α-receptor. The heterodimeric G protein G_q is coupled to α-adrenergic receptors and induces phospholipase C (PLC) activity. PLC hydrolyses phosphadityl 4,5 bisphosphate to release diacylglycerol (DAG), and inositol 1,4,5-triphosphate (IP_3). DAG in turn activates protein kinase C (PKC) and mitogen activated protein kinases (MAPK) which increase vasoconstriction and blood pressure. β-Adrenergic receptors are coupled to G_s which induces adenylyl cyclase activity and downstream activation of the second messenger cAMP and subsequently PKA. Both IP_3 and PKA cause the release of Ca^{2+} from the sarcoplasmic reticulum (SR) altering the rate of cardiac contraction.



Seán P. Barry and Paul A. Townsend, Figure 3.2 The calcineurin/NFAT pathway. Increases in intracellular Ca^{2+} levels via adrenergic mediated release from SR stores or transit through the L-type calcium channel (LTCC) leads to calmodulin-dependent calcineurin activation. Calcineurin dephosphorylates the transcription factor NFAT causing it to translocate to the nucleus where it upregulates hypertrophic genes in conjunction with other transcription factors such as GATA4, NFAT, AP1, and the p300 cofactor. Nuclear translocation is increased by ERK, while NFAT is removed from the nucleus following phosphorylation by JNK, p38, or GSK-3. Calcineurin mediated NFAT activation is inhibited by the nitric oxide (NO) signaling via cGMP and cGMP-dependent protein kinase 1 (PKG1), the endogenous regulator of calcineurin 1 (RCAN1) and the immunosuppressive agents cyclosporine A (CsA) and FK506.



Seán P. Barry and Paul A. Townsend, Figure 3.3 Class II HDAC regulation of MEF2 activity. MEF2 is a potent prohypertrophic transcription factor and is kept in an inactive state via binding to the Class II HDACs-4, 5, and 9. Hypertophic signals lead to the activation of HDAC kinases such as Ca^{2+}-calmodulin-dependent protein kinase (CaMK) and protein kinase D which phosphorylate HDACs and promote their binding to the 14-3-3 chaperone and induce their nuclear export. Increased levels of intracellular Ca^{2+} or induction of the MAPKs ERK and p38 increases MEF DNA binding and association with cofactors such as p300.