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Mitochondrial Dynamics in Mammals

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I. Introduction

Biologists often think of mitochondria as static, kidney bean-shaped organelles that have the mundane chore of providing energy for the cell. However, the mitochondrial population is in fact dynamic (Bereiter-Hahn and Voth, 1994; Rizzuto *et al*., 1998), and the hundreds of mitochondria in a cell can have a range of morphologies, including small spheres, long tubules, and interconnected tubules. This morphological plasticity is based on the ability of mitochondria to undergo both organellar fusion and fission. These opposing processes are readily visualized in living fibroblasts, where individual mitochondrial tubules continually migrate back and forth along their long axes on radial tracks (Fig. 1). Frequently, two mitochondrial tubules encounter each other and rapidly fuse, typically end to end. Conversely, there are normally an equal number of fission events that resolve a single mitochondrial tubule into two. By regulating the relative rates of fusion and fission, however, the morphology of the mitochondrial population can be dramatically altered, with important consequences for mitochondrial function.

Here we review our current molecular understanding of mitochondrial fusion and fission. Several good reviews are available for yeast mitochondrial dynamics (Hermann and Shaw, 1998; Jensen *et al*., 2000; Shaw and

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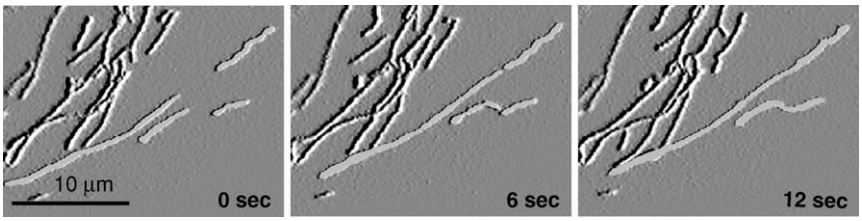

Figure 1 Mitochondrial fusion in mouse embryonic fibroblasts. Time-lapse confocal microscopy was used to image living fibroblasts expressing yellow fluorescent protein targeted to the mitochondrial matrix. The still frames show two pairs of mitochondria, pseudo-colored blue, moving toward each other along their long axes (*left*), approaching (*center*), and then fusing (*right*). Reproduced from the *Journal of Cell Biology*, 2003, 160(2), p. 193 by copyright permission of the Rockefeller University Press (Chen *et al.*, 2003). Videos showing the full movie, including fission events, can be viewed at http://www.jcb.org/cgi/content/full/jcb.200211046/DC1. (See Color Insert.)

Nunnari, 2002; Westermann, 2002). This chapter pays special attention to how these processes might be important in vertebrates. In particular, we discuss the role of mitochondria dynamics in mammalian mitochondrial morphology, mitochondrial function, disease, embryogenesis, and apoptosis.

II. The Central Molecular Players

Significant progress has been made in identifying molecules central to mitochondrial fusion and fission (Table I). The first major discovery was made with the identification of Fzo in *Drosophila*, and much of the later progress has been made in yeast, where powerful genetic screens have identified several key molecules. Some additional promising molecules have been identified but are beyond the scope of this chapter (Dimmer *et al.*, 2002; Fritz *et al.*, 2003; Messerschmitt *et al.*, 2003).

A. Fusion Pathway

1. Fzo/Mfn

The first central molecule in the control of mitochondrial dynamics was discovered through the study of spermatogenesis in *Drosophila* (Hales and Fuller, 1997). Ultrastructural studies documented that during spermatid differentiation in *Drosophila*, mitochondria progress through an unusual but precise set of morphological changes. After meiosis, the mitochondria in spermatids aggregate and fuse into two giant mitochondria that then wrap around each other to form the Nebenkern structure (Fuller, 1993). A

Table I Molecules Involved in Mitochondrial Fusion and Fission

Mammalian protein	Yeast protein	Process
Mfn1,[a] Mfn2[b]	Fzo1[c]	Fusion
OPA1[d]	Mgm1[e]	Fusion
Unknown	Ugo1[f]	Fusion
Drp1[g]/DLP1[h]/DVLP[i]	Dnm1[j]	Fission
Unknown	Mdv1[k]/Fis2[l]/Gag3[m]/Net2[n]	Fission
hFis1[o]	Fis1[p]/Mdv2[q]	Fission

[a]Santel *et al.* (2003); Legros *et al.* (2002); Chen *et al.* (2003).
[b]Santel and Fuller (2001); Rojo *et al.* (2002); Chen *et al.* (2003).
[c]Hermann *et al.* (1998); Rapaport *et al.* (1998).
[d]Delettre *et al.* (2000); Alexander *et al.* (2000).
[e]Shepard and Yaffe (1999); Wong *et al.* (2000).
[f]Sesaki and Jensen (2001).
[g]Smirnova *et al.* (1998); Smirnova *et al.* (2001).
[h]Pitts *et al.* (1999).
[i]Shin *et al.* (1999).
[j]Bleazard *et al.* (1999); Sesaki and Jensen (1999).
[k]Tieu and Nunnari (2000).
[l]Mozdy *et al.* (2000).
[m]Fekkes *et al.* (2000).
[n]Cerveny *et al.* (2001).
[o]James *et al.* (2003); Yoon *et al.* (2003).
[p]Mozdy *et al.* (2000).
[q]Tieu and Nunnari (2000).

cross-section of this Nebenkern structure, when examined by electron microscopy, resembles an onion slice due to the concentric layers of wrapped mitochondria. The *Drosophila fuzzy onions* (*fzo*) mutant has a specific defect in fusion of mitochondria such that the mitochondria undergo movements like aggregation, but fail to fuse (Hales and Fuller, 1997). As a result, multiple mitochondria remain in the spermatid, resulting in an obvious morphological aberration by electron microscopy. A functional defect evidently accompanies this structural change, because the mutant flies are sterile.

Fzo encodes a large transmembrane GTPase, localized to the outer mitochondrial membrane, and is conserved in more complex eukaryotes (Mozdy and Shaw, 2003). The amino terminus of the protein contains a GTPase domain with canonical G1 to G4 motifs. Mutation of the GTPase domain results in a nonfunctional protein that fails to rescue mutant flies (Hales and Fuller, 1997). The transmembrane domain is located toward the carboxyl terminus of the protein and is flanked by two regions with hydrophobic heptad repeats, sequences characteristic of coiled coils (Fig. 2). The presence of coiled-coil and GTPase domains is intriguing, because both of these sequence motifs play important roles in membrane fusion in other systems.

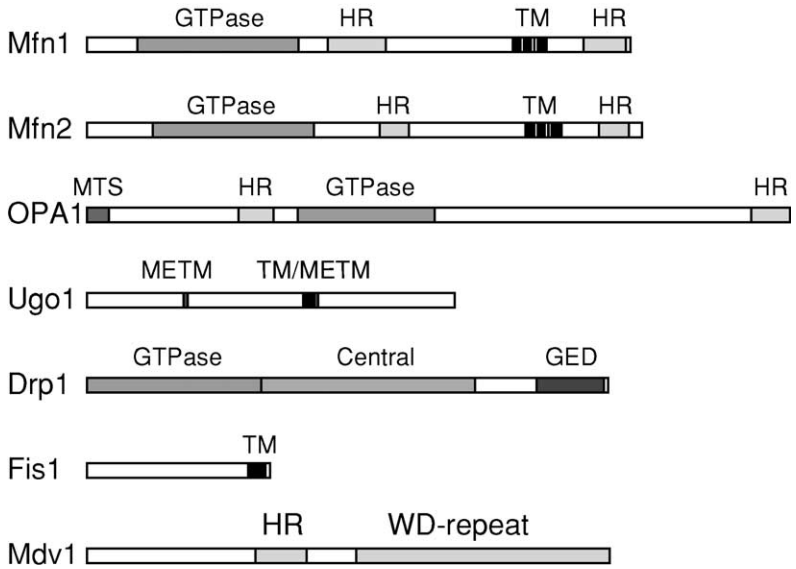

Figure 2 Molecules involved in mitochondrial fusion and fission. Schematic of proteins: Mfn1 (mouse, 742 residues), Mfn2 (mouse, 758 residues), OPA1 (mouse, 960 residues), Ugo1 (yeast, 502 residues), Drp1 (mouse, 712 residues), Fis1 (mouse, 250 residues), and Mdv1 (yeast, 714 residues). Charged residues in the transmembrane segments (TM) of Mfn1 and Mfn2 are indicated by white, vertical lines. In contrast to yeast Mgm1, murine OPA1 has no obvious TM region, and it is unknown whether processing involves cleavage by rhomboid-like proteases. HR, Hydrophobic heptad repeats identified by the program MULTICOIL (Wolf *et al.*, 1997); MTS, mitochondrial targeting sequence; METM, mitochondrial energy transfer motif; Central, homologous to dynamin central domain; GED, GTPase effector domain. (See Color Insert.)

Coiled coils in envelope glycoproteins of certain viruses play a direct role in membrane fusion, including those of the retrovirus, orthomyxovirus, and paramyxovirus families (Eckert and Kim, 2001; Skehel and Wiley, 1998). In intracellular vesicular trafficking, SNARE complexes form similar helical bundles that likely mediate vesicle fusion. Rab GTPases are known to regulate vesicular fusion (Mellman and Warren, 2000). It remains to be determined whether Fzo and its homologs act directly as fusogenic molecules (analogous to viral fusion proteins and SNAREs), or as regulators (analogous to small G proteins), or both.

The budding yeast homolog, Fzo1, also controls mitochondrial fusion. Deletion of *FZO1* results in fragmented, spherical mitochondria instead of the normal tubules observed in wild-type cells (Hermann *et al.*, 1998; Rapaport *et al.*, 1998). In addition, mating-induced mitochondrial fusion is defective (Hermann *et al.*, 1998). Subsequent to the fragmentation of mitochondria, *fzo1*Δ yeast lose mitochondrial DNA (mtDNA) and become

deficient in respiration. The mechanism of mtDNA loss is unknown, and is not observed in Mfn-deficient mammalian cells (Chen *et al*., 2003).

Mammals have two homologs of Fzo, termed mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2). These proteins are 80% similar to each other and are broadly expressed in a wide range of cell types (Rojo *et al*., 2002; Santel *et al*., 2003). Most studies have described a uniform localization of human Mfn1 and Mfn2 to the mitochondrial outer membrane (Rojo *et al*., 2002; Santel and Fuller, 2001; Santel *et al*., 2003), but one report describes a predominantly punctate localization with lower levels throughout the mitochondria (Karbowski *et al*., 2002). Murine Mfn1 and Mfn2 are clearly localized in a uniform manner to mitochondria (Chen *et al*., 2003).

Overexpression studies of human mitofusins have been difficult to understand fully. Overexpression of either human Mfn1 or Mfn2 leads predominantly to perinuclear clustering of mitochondria (Legros *et al*., 2002; Rojo *et al*., 2002; Santel and Fuller, 2001; Santel *et al*., 2003). In the case of Mfn2 overexpression, mitochondrial clustering is not dependent on a functional GTPase (Santel and Fuller, 2001). However, the formation of an interconnected tubular network, through coexpression of Mfn2 with an inhibitor of fission, does depend on a functional GTPase domain (Santel and Fuller, 2001). In the case of Mfn1 overexpression, one study found only perinuclear clustering at high expression levels (Legros *et al*., 2002). In cells with lower levels of expression, long and interconnected mitochondrial tubules emanated from the perinuclear clusters. In a second study, overexpression of Mfn1 induced the formation of ''grapelike'' perinuclear clusters (Santel *et al*., 2003). A GTPase mutation reduced, but did not abolish, the formation of such clusters.

For both Mfn1 and Mfn2, the significance of their ability to cause perinuclear clustering of mitochondria is unclear. Because this effect is not strictly GTPase dependent, it is not necessarily related to their mitochondrial fusion activity, which is known to depend on a functional GTPase domain (Chen *et al*., 2003). It should be noted that overexpression of various mitochondrial membrane proteins, unrelated to fusion, has been reported to nonspecifically cause mitochondrial clustering or aggregation (Yano *et al*., 1997). On the other hand, it is possible that the mitochondrial clustering phenotype reflects a physiological function of mitofusins in mediating mitochondrial adherence, a necessary step in progression to full fusion.

Definitive evidence for the functional roles of Mfn1 and Mfn2 has come from loss-of-function analyses. Disruption of Mfn1 or Mfn2 function results in fragmentation of mitochondria in mouse embryonic fibroblasts, secondary to a severe reduction in mitochondrial fusion (Chen *et al*., 2003). Loss of either Mfn1 or Mfn2 leads to midgestation embryonic lethality. Furthermore, simultaneous loss of Mfn1 and Mfn2 leads to a significantly earlier lethality and more severe developmental delay, indicating

that these related proteins probably act in concert or have some redundant functions.

Interestingly, the mitochondrial phenotypes of cells lacking Mfn1 differ significantly from that of cells lacking Mfn2 (Chen *et al.*, 2003). Cells lacking Mfn1 contain mitochondria that appear as short tubules or small spheres. In contrast, cells lacking Mfn2 have a less uniform mitochondrial population, with both large and small mitochondrial spheres. The mitochondria in both of these mutant cells have aberrations in mitochondrial motility, a phenotype thought to be secondary to the morphological defects. These observations show that Mfn1 and Mfn2 have related but distinct roles in controlling mitochondrial morphology.

In contrast to human mitofusins, overexpression of murine Mfn1 or Mfn2 does not lead to perinuclear clustering (Chen *et al.*, 2003). In addition, overexpression in mouse embryonic fibroblasts does not extend the length of mitochondrial tubules or increase their interconnectivity. However, when the corresponding mitofusin is expressed in mutant cells lacking Mfn1 or Mfn2, the fragmented mitochondria are dramatically restored to long tubules. As expected, this activity requires a functional GTPase domain.

Protease sensitivity experiments on yeast Fzo1 (Fritz *et al.*, 2001) and human Mfn2 (Rojo *et al.*, 2002) indicate that both the amino and the carboxyl termini are oriented toward the cytosol. This membrane topology is supported by analysis of protein fragments expressed in mammalian cells. An Mfn2 fragment, consisting of the transmembrane segment to the carboxyl terminus, contains sufficient information to localize to mitochondria (Rojo *et al.*, 2002; Santel and Fuller, 2001). The carboxyl terminus of this fragment is oriented toward the cytosol, because it is able to interact with a soluble amino-terminal fragment, thereby changing the localization of the amino-terminal fragment from the cytosol to the mitochondria (Rojo *et al.*, 2002).

This topological arrangement indicates that the transmembrane segment must span the outer mitochondrial membrane twice. Indeed, the transmembrane segment has unusual sequence features (Fig. 2). In all Fzo homologs, the transmembrane segment is divided into two or more hydrophobic segments separated by charged residues. This feature has led to suggestions that the two hydrophobic segments each span the outer membrane, with the intervening charged residues located in the intermembrane space, where they may be in a position to interact with intermembrane or inner membrane proteins (Fritz *et al.*, 2001; Santel and Fuller, 2001). This model, however, must accommodate the fact that some of the hydrophobic segments are only 13–15 residues long, shorter than most transmembrane segments that span the membrane as α helices.

Mitofusins probably function as higher order oligomers. Immunoprecipitation experiments clearly show that both Mfn1 and Mfn2 can physically

interact homotypically or heterotypically (Chen *et al.*, 2003). In mouse fibroblasts, both Mfn1 and Mfn2 have important roles in promoting mitochondrial fusion, because disruption of either protein leads to severe mitochondrial fragmentation. However, these defects can be rescued by overexpression of Mfn1 or Mfn2, showing that either protein is functional as a homotypic oligomer. These results suggest a model in which Mfn1 homotypic oligomers, Mfn2 homotypic oligomers, and Mfn1–Mfn2 heterotypic oligomers are all functional complexes that play different roles in maintaining mitochondrial fusion, depending on the cell type.

2. Mgm1/OPA1

Yeast mutants of the dynamin-related GTPase, Mgm1, behave similarly to *fzo1*Δ mutants (Shepard and Yaffe, 1999; Wong *et al.*, 2000). They show mitochondrial fragmentation with loss of mtDNA and an inability to survive on nonfermentable carbon sources. These phenotypes are rescued by the concurrent loss of Dnm1, a protein required for mitochondrial fission (Wong *et al.*, 2000). In addition, *mgm1*Δ mutants are deficient in mating-induced mitochondrial fusion. Interestingly, *mgm1*Δ yeast contain ultrastructural alterations in the mitochondrial inner membrane (Sesaki *et al.*, 2003), raising the possibility that the fusion defects may be secondary to structural changes in the mitochondria. However, *mgm1*Δ *dmn1*Δ yeast contain mitochondrial tubules that do not have such inner membrane alterations, but nevertheless cannot fuse (Sesaki *et al.*, 2003; Wong *et al.*, 2003). Therefore, the fusion defect is most likely the primary deficiency.

Mgm1 is a mitochondrially localized protein with an amino-terminal mitochondrial targeting sequence. The precise subcellular localization of Mgm1 has been controversial, however, with reports attributing it to the mitochondrial outer membrane (Shepard and Yaffe, 1999), intermembrane space with associations with the inner membrane (Wong *et al.*, 2000), or intermembrane space with associations with both the outer and inner membranes (Sesaki *et al.*, 2003). Part of the discrepancy may be due to an unusual proteolytic processing event during maturation of Mgm1. After import into mitochondria, the mitochondrial targeting sequence is removed in the matrix by the mitochondrial processing peptidase (MPP). Then the rhomboid-related transmembrane protease Mdm37/Pcp1/Rbd1 (Herlan *et al.*, 2003; McQuibban *et al.*, 2003) cleaves off a segment near the amino terminus. The region of Mgm1 removed by Mdm37/Pcp1/Rbd1 contains a putative transmembrane anchor, and thus the uncleaved form of Mgm1 may be integrated into a mitochondrial membrane, whereas the mature form may be peripherally associated with a membrane or simply soluble in the intermembrane space.

As with Mgm1, the subcellular localization of its mammalian homolog, OPA1, is unclear. Studies have generally indicated localization to the

intermembrane space, but reports vary as to which membrane it contacts and whether it is integrally embedded in the membrane (Olichon *et al*., 2002; Satoh *et al*., 2003). To complicate matters, *OPA1* encodes eight splicing variants (Delettre *et al*., 2001), the subcellular localizations of which may vary (Satoh *et al*., 2003). Expression studies in mice and humans show that whereas OPA1 can be found in all tissues, expression levels of specific isoforms differ (Delettre *et al*., 2001; Misaka *et al*., 2002). All isoforms contain two predicted coiled-coil domains, one toward the amino terminus and one at the extreme carboxyl terminus (Fig. 2). Alternatively spliced exon 5b also encodes a predicted coiled coil immediately prior to the first universal coiled coil.

The precise function of OPA1 in regulating the morphology of mammalian mitochondria remains to be determined. As expected, RNA interference (RNAi)-mediated knockdown of OPA1 leads to fragmentation of the mitochondrial network (Chen and Chan, unpublished results, 2003; Olichon *et al*., 2003). However, overexpression of OPA1 also changes the normally tubular network of mitochondria into small spheres (Misaka *et al*., 2002). This phenotype could result from either an induction of mitochondrial fission or an inhibition of fusion. The GTPase dependence of this phenomenon is unclear. Overexpression of various GTPase mutants leads to alterations in morphology, but the biochemical effects of these mutations on GTPase activity have not been tested (Misaka *et al*., 2002; Satoh *et al*., 2003). These observations are difficult to fully reconcile without further experiments, but they do suggest that the cellular levels of OPA1 are critical for maintenance of tubular mitochondrial morphology. Either overexpression or underexpression of OPA1 can lead to fragmentation.

3. Ugo1

To identify additional components of the yeast mitochondrial fusion pathway, a genetic screen was used to isolate genes with properties similar to that of *FZO1* (Sesaki and Jensen, 2001). In *fzo1* mutants, mtDNA is lost, resulting in lack of respiratory competence. However, this loss of mtDNA is prevented if Dnm1 activity has been previously disrupted. In a screen for mutants that show this pattern of Dnm1-dependent loss of mtDNA, *UGO1* (as well as *FZO1, MGM1*, and *UGO2*) was identified. Like *fzo1*Δ or *mgm1*Δ mutants, *ugo1*Δ mutants show fragmentation of mitochondria, loss of mtDNA, and lack of mating-induced mitochondrial fusion. Biochemically, Ugo1 behaves as an integral protein embedded within the outer mitochondrial membrane (Fig. 2). On the basis of immunoprecipitation experiments, Ugo1, Mgm1, and Fzo1 appear to be components of a large fusion apparatus (Sesaki *et al*., 2003; Wong *et al*., 2003). However, only a minority of these proteins appears to coassemble into a complex, implying that the associations are weak or transient.

Unlike Fzo1 and Mgm1, there are thus far no known mammalian homologs of Ugo1. Aside from the transmembrane segment, the only notable sequence feature of Ugo1 is the six-residue sequence motif PX(D/E)XX(K/R) (Sesaki and Jensen, 2001), which is found in mitochondrial carrier proteins such as the ATP/ADP carrier and may be involved in mediating electrostatic interactions (Nelson *et al*., 1998).

B. Fission Pathway

1. Dnm1/Drp1

Drp1 controls mitochondrial fission in mammalian cells (Smirnova *et al*., 2001). This dynamin-related protein contains regions homologous to the amino-terminal GTPase domain, central region, and carboxyl-terminal GTPase effector domain (GED) of dynamin (Fig. 2). Much of Drp1 exists as a cytosolic pool, but a fraction localizes to spots on mitochondria that coincide with future fission sites. Overexpression of a dominant-negative variant, Drp1 (K38A), results in collapse of the mitochondrial network into a large perinuclear mass (Pitts *et al*., 1999; Smirnova *et al*., 1998). These perinuclear masses are actually congregations of abnormally long and interconnected mitochondria, which can be more easily visualized by dispersing the masses with the microtubule depolymerizing drug nocodazole (Smirnova *et al*., 2001). Overexpression of dominant-negative Drp1 (Chen *et al*., 2003) or RNAi directed against Drp1 (Chen and Chan, unpublished results, 2003) can also rescue the mitochondrial fragmentation observed in Mfn1- or Mfn2-deficient cells.

As with dynamin (Danino and Hinshaw, 2001), two fundamentally different functions have been proposed for Drp1. Drp1 may act as a mechanochemical enzyme driven by GTP hydrolysis to directly mediate membrane fission. Alternatively, it may act as a signaling molecule to regulate the activity of a separate fission machinery. Consistent with the first model, purified Drp1 can assemble under low-salt conditions into higher order structures that appear as rings (Smirnova *et al*., 2001) and can tubulate liposomes (Yoon *et al*., 2001), suggesting that Drp1 may mediate fission by forming constricting rings around mitochondria. Support for the second model has come from analysis of the GED domain of Dnm1, the yeast Drp1 homolog (Fukushima *et al*., 2001).

Disruption of Dnm1 in yeast results in netlike mitochondria (abnormally interconnected networks) that are thought to result from lack of mitochondrial fission (Bleazard *et al*., 1999; Sesaki and Jensen, 1999). Consistent with this function, loss of *Dnm1* prevents the mitochondrial fragmentation observed in yeast fusion mutants (*fzo1*Δ, *mgm1*Δ, and *ugo1*Δ), indicating

that manipulation of the relative rates of fusion and fission can control the overall morphology of mitochondria (Bleazard *et al*., 1999; Sesaki and Jensen, 1999, 2001; Wong *et al*., 2000). In *Caenorhabditis elegans*, overexpression of dominant-negative Drp1 blocks only fission of the outer mitochondrial membrane, while fission of the inner membrane proceeds (Labrousse *et al*., 1999).

2. Mdv1

Two other components of the fission pathway, Mdv1 and Fis1*, were identified as extragenic suppressors of yeast fusion mutants (Cerveny *et al*., 2001; Fekkes *et al*., 2000; Mozdy *et al*., 2000; Tieu and Nunnari, 2000). Like *dnm1* mutants, these fission mutants result in the formation of mitochondria with netlike morphology, presumably caused by lack of mitochondrial fission.

MDV1 encodes a soluble protein with a coiled-coil motif and WD repeats in the carboxyl terminus (Fig. 2) (Fekkes *et al*., 2000; Mozdy *et al*., 2000; Tieu and Nunnari, 2000). Remarkably, Mdv1 localizes to mitochondria in punctate spots that also contain Dnm1. Consistent with this colocalization, Mdv1 and Dnm1 have been shown to physically interact by two-hybrid assays (Tieu and Nunnari, 2000; Uetz *et al*., 2000). In *dnm1*Δ mutants, Mdv1 is no longer localized to punctate spots, but instead is found uniformly associated with the mitochondrial membrane (Cerveny *et al*., 2001; Tieu and Nunnari, 2000). Further analysis indicates that the WD repeat region of Mdv1 is sufficient to mediate this colocalization with Dnm1 (Cerveny and Jensen, 2003; Tieu *et al*., 2002). Thus far, no mammalian homologs of Mdv1 have been identified.

3. Fis1/hFis1

Fis1 is a mitochondrial outer membrane protein required for mitochondrial fission (Mozdy *et al*., 2000). It is localized uniformly on mitochondria. In *fis1*Δ cells, Dnm1- and Mdv1-positive mitochondrial spots are still present, but their numbers are dramatically decreased (Mozdy *et al*., 2000; Tieu and Nunnari, 2000). In addition, a higher proportion of Mdv1 appears to be dispersed in the cytosol. These observations suggest that Fis1 regulates the formation of these mitochondrial structures, which are potential sites of fission.

Fis1 is a small protein with a single transmembrane segment located near the carboxyl terminus (Fig. 2). The majority of the protein faces the cytosol. By both two-hybrid and immunoprecipitation analyses, the cytosolic region

* For clarity, the ''standard name'' listed in the *Saccharomyces* Genome Database (www.yeastgenome.org) is used in the text; equivalent gene names are listed in Table I.

of Fis1 physically interacts with Mdv1, thus recruiting it to the mitochondria (Cerveny and Jensen, 2003; Tieu *et al*., 2002).

Overexpression of human Fis1, termed hFis1, causes fragmentation of the mitochondrial network (James *et al*., 2003; Yoon *et al*., 2003). Consequently, it has been proposed that the number of hFis1 molecules present on the mitochondria regulates the amount of fission activity. This fission activity is Drp1 mediated because it is blocked by expression of dominant-negative Drp1. A physical interaction between Drp1 and hFis1 has been demonstrated by fluorescence resonance energy transfer and immunoprecipitation experiments (Yoon *et al*., 2003). Interestingly, overexpression of hFis1 is also associated with an increase in apoptosis (James *et al*., 2003).

III. Mitochondrial Dynamics in the Control of Mammalian Mitochondrial Morphology

Several observations indicate that mitochondrial dynamics plays a significant role in vertebrate cells. Time-lapse observations of mammalian cells reveal frequent and constant cycles of mitochondrial fusion and fission (Fig. 1) (Bereiter-Hahn and Voth, 1994; Rizzuto *et al*., 1998). Cell cycle-dependent changes in mitochondrial morphology have been reported, with reticular structures present in G_1 and fragmented structures in S phase (Barni *et al*., 1996; Margineantu *et al*., 2002). In artificially fused cells, mitochondrial fusion has been documented. When murine fibroblasts or HeLa cells are fused with polyethylene glycol (PEG) or a hemagglutinating virus, the resulting cell hybrids show intermixing of mitochondrial matrix contents, providing definitive evidence of mitochondrial fusion (Chen *et al*., 2003; Hayashi *et al*., 1994; Ishihara *et al*., 2003; Legros *et al*., 2002; Mattenberger *et al*., 2003). The fusion of mitochondria requires an intact mitochondrial membrane potential, and dissipation of this potential results in fragmentation (Ishihara *et al*., 2003; Legros *et al*., 2002; Mattenberger *et al*., 2003). By morphological criteria, mitochondrial fusion is largely completed by 8–12 h after cell fusion (Chen *et al*., 2003; Legros *et al*., 2002). Functionally, however, efficient complementation of gene products does not occur until many days later (Ono *et al*., 2001).

The identification of molecules involved in the fusion and fission pathways has allowed an assessment of their relative roles in controlling mitochondrial morphology. It is clear that inhibition of mitochondrial fission, by overexpression of a dominant-negative mutant of Drp1, results in a greater interconnectivity of the mitochondrial network and an elongation of the length of the mitochondrial tubules (Smirnova *et al*., 2001). It is also clear that partial inhibition of the fusion pathway, by deletion of either Mfn1 or Mfn2,

results in dramatic shortening of mitochondrial tubules, such that most mitochondria appear as short rods or spheres (Chen *et al*., 2003). Taken together, these results indicate that the overall mitochondrial network observed in mammalian cells is quite labile. Both fusion and fission must be highly active, and even partial disruption of one process can shift the delicate balance and dramatically alter mitochondrial morphology (Fig. 3).

As expected, then, a defect in the fusion pathway can be at least partially rescued by a compensatory inhibition of the fission pathway. Mitochondrial tubules can be restored to Mfn1- or Mfn2-deficient cells by overexpression of dominant-negative Drp1 (Fig. 3) (Chen *et al*., 2003). These results present an interesting conundrum. If mitochondrial tubules can be maintained by downregulating both the fusion and fission pathways, why have cells developed such sophisticated machineries to maintain high levels of fusion and fission? The answer must be that a dynamic mitochondrial population has advantages over a static one.

IV. Mitochondrial Dynamics and Mitochondrial Function

What specific advantages a dynamic mitochondrial population has are still not certain, but evidence suggests that fusion promotes intermitochondrial cooperation and fission enables compartmentalization. In particular, fission may allow equitable distribution of mitochondria to the daughter cells during cell division, perhaps explaining the cell cycle dependence of mitochondrial morphology in mammalian cells (Barni *et al*., 1996; Margineantu *et al*., 2002). It should be noted, however, that fission is not required for mitochondrial inheritance in yeast, because mitochondrial fission mutants are viable (Bleazard *et al*., 1999; Sesaki and Jensen, 1999). Fission most likely also allows mitochondria in different parts of the cell to perform discrete functions. For example, mitochondria in different regions of the cell show functional heterogeneity, as assayed by membrane potential, calcium sequestration, and membrane permeability (Collins *et al*., 2002). On the other hand, fusion may facilitate the rapid transmission of membrane potential (Skulachev, 2001) or the exchange of mitochondrial contents. These contents could include both membrane-bound or diffusible molecules, substrates or products such as ATP. In addition, mtDNA exchange between mitochondria would raise the possibilities of complementing mtDNA mutations, or repairing them through gene conversion.

The ability to experimentally test for mtDNA exchange was enabled by the development of cell fusion techniques that allow the creation of cybrids, hybrid cells containing one nucleus but two populations of mitochondria (King and Attardi, 1989). Mitochondria containing a recessive mtDNA

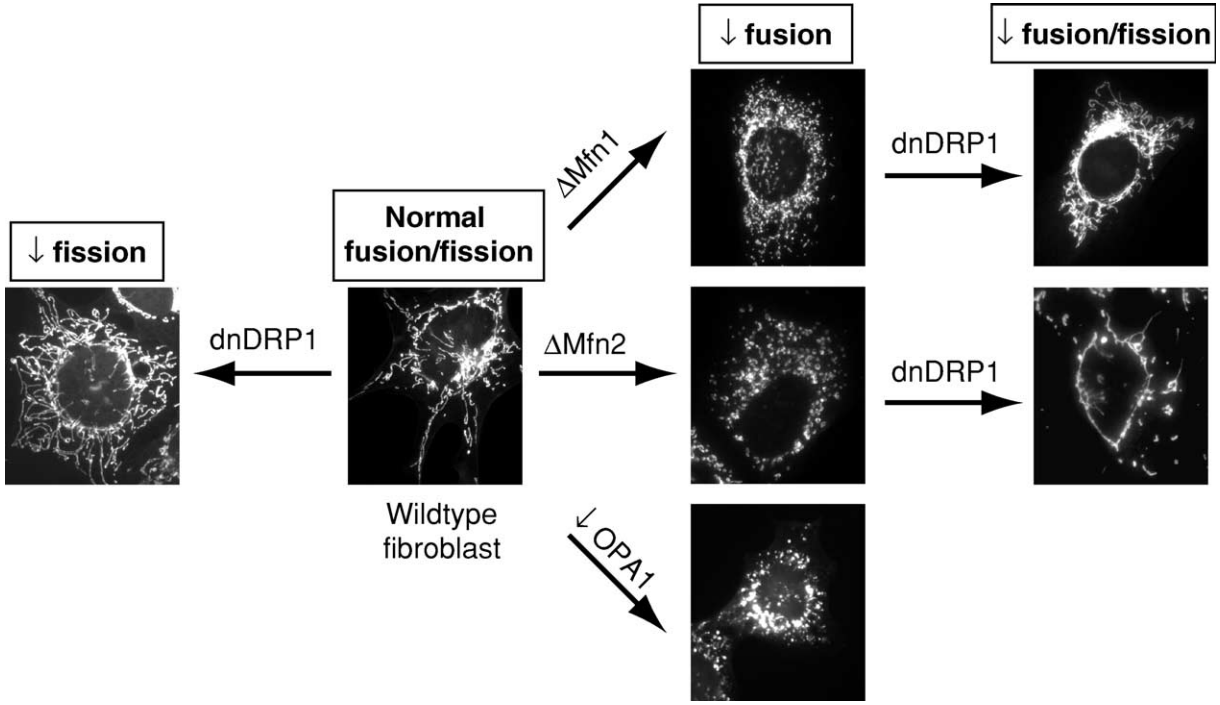

Figure 3 Relative rates of fusion and fission control the morphology of mitochondria. Wild-type fibroblasts have a tubular mitochondria with high rates of fusion and fission. Inhibition of fission (by dominant-negative Drp1) results in more elongated and interconnected tubules, whereas inhibition of fusion (by disruption of Mfn1 or Mfn2 or by RNAi-mediated reduction of OPA1) results in fragmentation. In cells with reduced fusion, subsequent inhibition of fission can restore mitochondrial tubules (far right).

mutation can be introduced into a cell line carrying mitochondria with a separate mtDNA mutation, and complementation can be assessed by growth on medium that selects for respiration-competent cells. In one study, only about 1% of the cybrids exhibited complementation (Enriquez *et al*., 2000). Because mitochondrial fusion results in mitochondria simultaneously carrying both mtDNAs, it was expected that high rates of mitochondrial fusion would lead to efficient complementation. The low levels of complementation observed led to the conclusion that mitochondria (at least in the 143B nuclear background, in which these experiments were performed) are largely autonomous organelles that either fuse rarely or fuse without mixing of mtDNA products.

Analogous experiments using different cell lines yielded different results (Ono *et al*., 2001). In this case, it was observed that formation of cybrids carrying two different recessive mtDNA mutations resulted in highly efficient restoration of respiratory competence. Intriguingly, the cybrids did not regain respiratory competence until 10–14 days after cell fusion. It is unclear why complementation would take such a long time, given that other experiments show fusion of mitochondria occurring within either minutes (Hayashi *et al*., 1994) or hours (Chen *et al*., 2003; Legros *et al*., 2002; Mattenberger *et al*., 2003) after cell fusion. The possible reasons for the discrepancy between the two studies have been discussed (Attardi *et al*., 2002; Hayashi *et al*., 2002), and may involve an unanticipated lag time needed for restoration of respiratory activity or the effect of nuclear genes. Nevertheless, it appears clear that, in at least some cultured cells, extensive fusion of mitochondria does occur, and this fusion protects mitochondrial function.

The same concept is supported by analysis of cells lacking mitofusin function. In either Mfn1- or Mfn2-deficient cells, a subset of mitochondria loses membrane potential, even though the bulk culture is competent for respiration (Chen *et al*., 2003). Therefore, in the absence of mitochondrial fusion, the mitochondria are forced to be autonomous, and transient losses of membrane potential can become prolonged or permanent. In spite of rapid mitochondrial fusion, however, it should not be assumed that the entire mitochondrial population is necessarily homogeneous. At any given time, individual mitochondria are electrically uncoupled and functionally heterogeneous (Collins *et al*., 2002).

The ability of mitochondrial fusion to protect mitochondrial function is not limited to cultured cells. Using transgenic techniques, mice have been generated that contain varying ratios of mitochondria carrying mutant mtDNA and mitochondria carrying wild-type mtDNA (Nakada *et al*., 2001b). When tissues from such mice are stained for cytochrome oxidase (COX) activity (an activity dependent on both mtDNA and nuclear function), all the mitochondria in an individual cell are either homogeneously COX positive or COX negative. That is, any particular cell does not show

a mosaic of mitochondrial phenotypes. In fact, cells containing more than 60% mutant mtDNA still had fully functional, COX-positive mitochondria. These results suggest that mitochondria are not autonomous organelles, but rather cooperate to protect mitochondrial function even when wild-type mtDNA is in the minority.

V. Mitochondrial Dynamics and Human Disease

The results described above, indicating protective effects of mitochondrial cooperation, are relevant to understanding the pathogenesis of human diseases caused by mtDNA mutations (Larsson and Clayton, 1995; Wallace, 1999). It may explain why such diseases typically are not symptomatic until the burden of mutant mtDNA reaches a critical threshold. Furthermore, it may be possible to exploit this phenomenon to develop new therapies (Nakada *et al.*, 2001a). Introduction of a modest amount of mitochondria carrying wild-type mtDNA may be sufficient to rescue the function of the entire mitochondrial population in mutant cells. Technically, this approach may be more feasible than gene therapy with mtDNA.

The importance of mitochondrial dynamics in human physiology is also clearly indicated by the discovery that the most common hereditary form of optic neuropathy, autosomal dominant optic atrophy (DOA) Kjer type, is due to mutations in *OPA1* (Alexander *et al.*, 2000; Delettre *et al.*, 2000, 2002). Estimates of the prevalence of this disease range from 1 in 10,000 to 1 in 50,000. Although the disease has a variable presentation and penetrance, it typically presents in childhood with loss of visual acuity, visual field defects, and optic disc pallor. The pathophysiology appears to be a primary degeneration of retinal ganglion cells. The subsequent atrophy of the optic nerve gives rise to the pallor of the optic discs characteristic on fundoscopic examination. Affected patients in one family show heterogeneous clumping of mitochondria in monocytes (Delettre *et al.*, 2000).

Large-scale sequencing of disease alleles carried out by several groups has uncovered dozens of molecular lesions in *OPA1* leading to autosomal dominant atrophy (Delettre *et al.*, 2002; Thiselton *et al.*, 2002). From these studies, two types of mutations have been commonly found—missense mutations in the GTPase domain and truncation mutations located throughout the protein. There are at least two possible mechanisms through which OPA1 mutations may act. First, haploinsufficiency may be the cause of this dominant autosomal disease. Some of the severely truncated mutations probably act in this manner. Consistent with this model, one family with dominant optic atrophy has been reported to carry a deletion of one *OPA1* allele (Marchbank *et al.*, 2002). In another family, one compound heterozygous patient exhibited a much more severe disease phenotype than any

of her simple heterozygous family members (Pesch *et al*., 2001). Finally, some mutations truncate the protein so severely (including one that causes termination after just one residue) that mechanisms other than haploinsufficiency are unlikely (Delettre *et al*., 2002; Pesch *et al*., 2001). This genetic behavior, along with the OPA1 overexpression and underexpression studies described earlier, suggest that precise cellular levels of OPA1 are critical for maintaining normal mitochondrial morphology and function.

Although haploinsufficiency seems to account for some of the *OPA1* mutations, the biochemical nature of OPA1 suggests an additional mechanism through which other mutations may act. Dynamin and dynamin-related proteins such as Drp1 are able to self-assemble (Shin *et al*., 1999; Smirnova *et al*., 2001; Yoon *et al*., 2001), a biochemical property that is essential for their role in membrane remodeling. As a result, GTPase mutants in dynamin and Drp1 often act as dominant negatives by incorporating into complexes with wild-type protein. It remains to be shown directly that OPA1 forms oligomers, but it would not be surprising if some OPA1 mutants similarly act in a dominant-negative manner by incorporating into wild-type complexes. Also, OPA1 mutants that are incorporated into larger fusion complexes, potentially including Mfn, may act as dominant negatives. There are two regions of OPA1 with coiled/coil motifs that may be involved in protein–protein interactions. Some disease alleles encode truncated OPA1 molecules that retain some of these candidate self-assembly domains. Further biochemical characterization of such alleles will be necessary to resolve whether any of them act in a dominant-negative manner.

Interestingly, a spontaneous semidominant mutation in the mouse, termed *Bst* (Belly Spot and Tail), maps close to the *OPA1* locus and leads to optic nerve atrophy in heterozygous animals. These observations had raised the possibility that *Bst* may be a naturally occurring mouse model for human dominant autosomal optic atrophy (Rice *et al*., 1995). However, more detailed mapping and sequencing studies have established that *Bst* and *OPA1* are actually two distinct loci (Delettre *et al*., 2003). Western analysis of *Bst* homozygous embryo lysates shows no loss of OPA1, and the mitochondria of mutant cells are morphologically indistinguishable from those of wild-type cells (Chen and Chan, unpublished results, 2003).

VI. Mitochondrial Dynamics in Vertebrate Development

Mitochondrial dynamics also appears to be important in tissues other than the retina. Early cytological and ultrastructural studies of mitochondria in vertebrate embryos suggest that mitochondrial morphology in specific tissues is developmentally regulated. For example, mitochondria in rat liver undergo a complex developmental pattern of morphological transitions. In

the early hepatic diverticulum of the embryonic rat, the mitochondria initially appear as minute spheres. Over the next several days, these mitochondria apparently coalesce into beaded filaments that later convert into smooth filaments shortly before birth (Smith, 1931). Although it is not possible to draw definitive mechanistic conclusions from these studies, it is reasonable to hypothesize that these morphological transitions result from upregulation of mitochondrial fusion or downregulation of fission during hepatocyte differentiation. Another striking example occurs during development of skeletal muscle in the rat diaphragm (Bakeeva *et al*., 1978, 1981). During embryonic stages, the muscle cells contain only a few mitochondria that form short tubules without interconnections. During the first two postnatal months, significant mitochondrial biogenesis occurs in association with a dramatic increase in interconnectivity of mitochondria. By 2 months, the muscle cells have developed a highly organized mitochondrial reticulum composed of interconnected mitochondrial tubules. It has been speculated that these long tubules may enable the transport of energy and substrates over long distances in muscle fibers (Bakeeva *et al*., 1978; Skulachev, 2001).

Studies of the mitofusins, Mfn1 and Mfn2, have provided clear evidence for the importance of mitochondrial fusion during mouse development. When either *Mfn1* or *Mfn2* is disrupted by homologous recombination techniques, midgestation embryonic lethality ensues (Chen *et al*., 2003). The cause of lethality in Mfn1-deficient mice is uncertain, but the loss of Mfn1 clearly affects development of the embryo proper. Mutant embryos are much smaller than their wild-type or heterozygous littermates and manifest developmental delay. The degree of delay is not uniform among various tissues, however, and therefore the embryos appear severely deformed (Chen and Chan, unpublished results, 2003). Great variation also exists between embryos, even among littermates.

In the case of *Mfn2* disruption, the lethality has been traced to improper development of the placenta (Chen *et al*., 2003). Anatomically, Mfn2 mutant embryos are quite normal looking, albeit slightly smaller and delayed relative to their littermates. Remarkably, the extraembryonic defect is strikingly cell-type specific—of the three layers of the developing placenta, only the trophoblast giant cell layer shows a severe disruption in structure. The trophoblast giant cells are uniquely situated at the fetal–maternal boundary and are therefore critical for several processes essential for maintenance of the pregnancy, including invasion of the placenta into the maternal decidua, secretion of placental hormones, and development of the fetal–maternal vasculature (Cross, 2000). In trophoblast stem cell cultures, the Mfn2-deficient cells show spherical mitochondria, in contrast to the tubular mitochondria of wild-type cells. Interestingly, ultrastructural studies on preimplantation human embryos describe morphogenetic changes in mitochondrial structure during differentiation and hatching of the blastocyst (Sathananthan and Trounson,

2000). The spherical or oval mitochondria of the oocyte and early embryo dramatically transform into long tubules with increased transverse cristae, an indication of increased metabolic activity. Trophoblast cells, in particular, contain extremely elongated mitochondria. The analysis of conditionally targeted alleles of *Mfn1* and *Mfn2* will allow the identification of additional developmentally regulated fusion events during vertebrate development.

VII. Mitochondrial Dynamics and Apoptosis

Many lines of evidence implicate mitochondria as central regulators of programmed cell death. Under appropriate conditions, mitochondria trigger cell death by the release of apoptosis-promoting proteins sequestered in the intermembrane space (Desagher and Martinou, 2000; Newmeyer and Ferguson-Miller, 2003; Wang, 2001). For example, cytochrome *c*, Smac/DIABLO, and apoptosis-inducing factor (AIF) are released from the mitochondrial intermembrane space to promote later apoptotic events in the cytosol and nucleus.

Clearly, regulation of the permeability of the mitochondrial outer membrane is critical for precise control of cell survival. The mechanism of mitochondrial membrane permeabilization during induction of apoptosis is controversial and has been extensively discussed (Desagher and Martinou, 2000; Martinou and Green, 2001; Newmeyer and Ferguson-Miller, 2003; Wang, 2001; Zamzami and Kroemer, 2001, 2003). Two types of models have been proposed to explain mitochondrial membrane permeabilization. In the first scenario, members of the Bcl-2 family are thought to directly generate protein-conducting channels in the mitochondrial outer membrane. The Bcl-2 family of proteins can have either antiapoptotic (Bcl-2, Bcl-x_L) or proapoptotic (Bid, Bax) functions. Although Bax normally resides in the cytosol, during induction of apoptosis it is activated by a cleaved form of Bid, undergoes a conformational change, and inserts into the mitochondrial outer membrane. It is thought that Bax may generate protein-conducting channels by forming large homotypic complexes. This idea has received support from structural studies revealing similarity between the structure of Bcl-2 family members and that of diptheria toxin and bacterial colicins, both pore-forming proteins (Muchmore *et al*., 1996; Suzuki *et al*., 2000). Furthermore, Bax and Bid can directly permeabilize vesicles *in vitro*, recapitulating some of the features of outer mitochondrial membrane permeabilization (Newmeyer and Ferguson-Miller, 2003; Zamzami and Kroemer, 2003). In this proposed type of membrane permeabilization, there is no immediate catastrophic damage to the mitochondria.

A second proposed mechanism for permeabilization of the mitochondrial outer membrane involves a phenomenon called the mitochondrial

permeability transition (Martinou and Green, 2001; Zamzami and Kroemer, 2001). In this model, a protein channel, called the permeability transition pore (PTP), opens and dissipates the inner membrane potential. The opening of the PTP leads to osmotic swelling of the mitochondrial matrix and consequently to rupture of the outer membrane, due to the much larger surface area of the highly involuted inner membrane surrounding the matrix. Rupture of the outer membrane allows release of apoptotic proteins trapped in the intermembrane space. At sites of contact between the outer and inner mitochondrial membranes, the PTP is composed of the voltage-dependent anion channel (VDAC) in the outer membrane and the adenine nucleotide translocator (ANT) in the inner membrane, along with several other associated proteins. Bcl-2 family members may regulate apoptosis by modulating the opening of this channel. This mechanism would normally result in irreparable damage to the mitochondria, although modified versions of this model can accommodate transient or less severe damage.

Given the importance of mitochondria in many forms of apoptosis, does mitochondrial dynamics play a role in regulating this process? Specifically, do the pathways of mitochondrial fusion or fission intersect with those of apoptosis? These questions have been addressed only recently, and the evidence is intriguing (Karbowski and Youle, 2003). Some forms of programmed cell death are correlated with changes in mitochondrial morphology (Desagher and Martinou, 2000). For example, when COS-7 cells undergo apoptosis from overexpression of proapoptotic Bax or from treatment with compounds such as staurosporine or etoposide, they display concomitant fragmentation of the mitochondrial network into shorter tubules or spheres (Frank *et al.*, 2001). During the fragmentation process, Drp1 relocalizes from a predominantly cytosolic pool to punctate spots on mitochondria. Overexpression of dominant-negative Drp1 in such cells prevents fragmentation and seems to suppress programmed cell death, as measured by mitochondrial membrane depolarization, cytochrome *c* release, and TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling) staining (Frank *et al.*, 2001). Drp1 is also involved in the fragmentation of mitochondria observed during apoptosis induced by stimulation of death receptors (Breckenridge *et al.*, 2003). In this case, activation of caspase 8 causes cleavage of BAP31, an integral membrane protein of the endoplasmic reticulum (ER). One of the BAP31 cleavage products, termed p20, induces Ca^{2+} release from the ER, followed by Drp1-dependent fragmentation of mitochondria. Expression of dominant-negative Drp1 prevents mitochondrial fragmentation and reduces both cytochrome *c* release and downstream caspase activation.

The possible connection of apoptosis with the mitochondrial fission pathway is also suggested by tracking the localization of Bax and Drp1 in apoptotic cells. Bax, normally a cytosolic protein, accumulates in punctate spots on mitochondria on induction of apoptosis (Karbowski *et al.*, 2002).

These puncta of Bax localization colocalize with Drp1, thereby suggesting an association of an apoptotic stimulator with the fission machinery. The significance of these colocalizations, while intriguing, will need to be tested by functional studies.

hFis1, another component of the mitochondrial fission machinery, has also been implicated in apoptotic events. hFis1 overexpression leads to mitochondrial fragmentation (James *et al*., 2003; Yoon *et al*., 2003) and an increase in cytochrome *c* release and cell death (James *et al*., 2003). However, mitochondrial fission and cell death are not necessarily linked, because coexpression of dominant-negative Drp1 inhibited mitochondrial fragmentation (and cytochrome *c* release) without inhibiting cell death. Furthermore, Bcl-x_L blocks apoptosis without affecting mitochondrial fragmentation.

If mitochondrial fission is involved in apoptosis, mitochondrial fusion may protect against cell death. Indeed, two components of the fusion pathway have been associated with apoptosis. Reduction of OPA1 levels by RNAi leads not only to fragmentation of the mitochondrial network but also to characteristics of programmed cell death (Olichon *et al*., 2003). Cells display loss of mitochondrial membrane potential, condensed chromatin, cleavage of poly(ADP-ribose) polymerase (PARP), and cytochrome *c* release. In addition, Mfn2 was found to colocalize with Bax and Drp1 during apoptosis (Karbowski *et al*., 2002). It is unclear what a fusion factor is doing at supposed sites of fission. This result is also perplexing, because other studies have shown that Mfn2 is localized uniformly to the mitochondrial membrane (Chen *et al*., 2003; Rojo *et al*., 2002; Santel and Fuller, 2001).

VIII. Perspectives and Future Challenges

Despite the identification of several important players in the control of mitochondrial dynamics, we still have little understanding of the molecular mechanisms through which mitochondria fuse and divide. The mechanisms will likely be novel, because unlike other well-studied membrane-remodeling events, such as vesicle fusion and viral entry, mitochondrial fusion and fission require the coordination of four separate bilayers. A structural understanding of the known proteins, along with extensive mutational analysis, will be required to gain a detailed model for their mode of action. In particular, it will be important to establish which proteins actually mediate the fusion/fission processes. Some of the known proteins physically interact to form complexes on the mitochondria. Further work will be required to elucidate whether these interactions serve to coordinate the inner and outer membranes. Although many tools are available for the study of mitochondrial dynamics, one serious gap has been the lack of a cell-free, biochemical assay for either fusion or fission. Such an assay would be invaluable for

dissecting the steps involved in both fusion and fission, as has been illustrated for the analysis of intracellular vesicular trafficking (Mellman and Warren, 2000; Wickner, 2002).

Yeast genetics has identified many of the genes involved in regulating mitochondrial dynamics. Some of these yeast genes, such as *DNM1* and *FIS1*, have obvious mammalian homologs that have been shown to affect mammalian mitochondrial dynamics (James *et al*., 2003; Smirnova *et al*., 2001; Yoon *et al*., 2003). Other yeast genes, such as *UGO1* and *MDV1*, have no known homologs, and approaches other than bioinformatics will be required to identify their mammalian counterparts or other novel mammalian components.

To study the role of mitochondrial dynamics in vertebrate development, it is clear that development of animal models will be critical. In the case of the mitofusins, simple knockout alleles lead to midgestation embryonic lethality, precluding the ability to study later developmental effects and adult physiology (Chen *et al*., 2003). In such cases, it is clearly important to develop conditional alleles that allow temporal and spatial control of gene disruption. In the case of Mfns, such conditional alleles are being analyzed (Chen and Chan, unpublished results, 2003).

We are clearly in the beginning stages of understanding mitochondrial dynamics in mammals. It is becoming evident that this process is important in regulating mitochondrial morphology and function, and therefore is essential for embryonic development, but much more remains to be explored. In particular, the possible connections between mitochondrial dynamics and programmed cell death, aging, and mtDNA maintenance remain to be firmly established.

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