Mitofusins Mfn1 and Mfn2 coordinate mitochondrial fusion and are essential for embryonic development

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Mitochondrial morphology is determined by a dynamic equilibrium between organelle fusion and fission, but the significance of these processes in vertebrates is unknown. The mitofusins, Mfn1 and Mfn2, have been shown to affect mitochondrial morphology when overexpressed. We find that mice deficient in either Mfn1 or Mfn2 die in midgestation. However, whereas Mfn2 mutant embryos have a specific and severe disruption of the placental trophoblast giant cell layer, Mfn1-deficient giant cells are normal. Embryonic fibroblasts lacking Mfn1 or Mfn2 display distinct types of fragmented mitochondria, a phenotype we determine to be due to a severe reduction in mitochondrial fusion. Moreover, we find that Mfn1 and Mfn2 form homotypic and heterotypic complexes and show, by rescue of mutant cells, that the homotypic complexes are functional for fusion. We conclude that Mfn1 and Mfn2 have both redundant and distinct functions and act in three separate molecular complexes to promote mitochondrial fusion. Strikingly, a subset of mitochondria in mutant cells lose membrane potential. Therefore, mitochondrial fusion is essential for embryonic development, and by enabling cooperation between mitochondria, has protective effects on the mitochondrial population.

Introduction

Mitochondria are remarkably dynamic organelles. Time-lapse microscopy of living cells reveals that mitochondria undergo constant migration and morphological changes (Bereiter-Hahn and Voth, 1994; Nunnari et al., 1997; Rizzuto et al., 1998). Even in cells with a seemingly “stable” network of mitochondrial tubules, there are frequent and continual cycles of mitochondrial fusion and fission, opposing processes that exist in equilibrium and serve to maintain the overall architecture of these organelles (Bereiter-Hahn and Voth, 1994; Nunnari et al., 1997).

In both yeast and flies, mitochondrial fusion is controlled by the nuclearly encoded mitochondrial transmembrane GTPase, fuzzy onions (Fzo).* In Drosophila, Fzo is specifically and transiently expressed in spermatids. Disruption of Fzo prevents developmentally regulated mitochondrial fusion in postmeiotic spermatids and results in male sterility (Hales and Fuller, 1997). In budding yeast, deletion of FZO1 disrupts the highly branched, tubular mitochondrial network typical of normal cells and results in numerous small spherical mitochondria.

*Abbreviations used in this paper: Drp, dynamin-related protein; e, embryonic day; EYFP, enhanced YFP; Fzo, fuzzy onions; MEF, mouse embryonic fibroblast; Mfn, mitofusin; mtDNA, mitochondrial DNA; PEG, polyethylene glycol; TS, trophoblast stem.

Key words: membrane fusion; mitochondria; GTPase; mice; knockout

fzo1Δ yeast form “petite” colonies that lack mitochondrial DNA (mtDNA) (Hermann et al., 1998; Rapaport et al., 1998). Furthermore, there is a disruption of mating-induced mitochondrial fusion (Hermann et al., 1998). Humans contain two Fzo homologues, termed mitofusin (Mfn)1 and Mfn2, that can alter mitochondrial morphology when overexpressed in cell lines (Santel and Fuller, 2001; Rojo et al., 2002). Both mitofusins are broadly expressed (Rojo et al., 2002), and therefore their functional redundancy is unclear.

Despite our increasing knowledge about the importance of mitochondrial fusion in lower eukaryotes, there is disagreement about its physiological role in vertebrates. Doubts have been raised about the frequency and importance of mitochondrial fusion in cultured mammalian cells (Enriquez et al., 2000). However, several observations suggest that this basic cellular process indeed plays a significant role in vertebrate cells. First, ultrastructural studies of mitochondria show that dramatic transitions occur during the development of certain tissues. For example, the mitochondria of rat cardiac muscle and diaphragm skeletal muscle appear as isolated ellipses or tubules in embryonic stages but then reorganize into reticular networks in the adult (Bakeeva et al., 1978, 1981, 1983). It is likely that these progressive morphological changes occur through mitochondrial fusion. Second, time-lapse fluorescence microscopy of cultured HeLa cells shows that the mitochondria are organized into extensive tubular networks that undergo frequent fusion and fission (Rizzuto et al., 1998). Third, experimentally induced cell hybrids demon-
strate rapid mtDNA mixing (Hayashi et al., 1994) and complementation of mtDNA gene products (Nakada et al., 2001b; Ono et al., 2001). Finally, mitochondrial dynamics have been implicated in the regulation of apoptosis. Induction of cell death is sometimes associated with fragmentation of the mitochondrial network. This fragmentation requires dynamin-related protein (Drp)1, which is involved in mitochondrial fission (Frank et al., 2001).

To assess the physiological role of mitochondrial fusion in vertebrates, we have generated knockout mice for Mfn1 and Mfn2. Our analysis reveals that Mfn1 and Mfn2 are each essential for embryonic development and mitochondrial fusion. These mitofusins can exist as both homotypic and heterotypic oligomers and therefore can cooperate as well as act individually to promote mitochondrial fusion. In addition, our results suggest that mitochondrial fusion functions to allow cooperation between mitochondria, thereby protecting mitochondria from respiratory dysfunction.

Results

Identification of murine Fzo homologues

We identified two murine homologues of fzo from a mouse cDNA library. In accordance with the nomenclature for the human mitofusins (Santel and Fuller, 2001), we designate these murine homologues as Mfn1 and Mfn2. Linkage analysis placed Mfn1 at the proximal end of mouse chromosome 3 (~12–13 cM) in a region syntenic to human 3q25-26. Mfn2 was localized to the distal end of mouse chromosome 4 (~70–80 cM) in a region syntenic to human 1p36.

As with the fzo genes from Drosophila melanogaster (Santel and Fuller, 2001; Hwa et al., 2002), Homo sapiens (Santel and Fuller, 2001), and Saccharomyces cerevisiae (Hermann et al., 1998; Rapaport et al., 1998), each murine Mfn gene encodes a predicted transmembrane GTPase. The transmembrane segment is flanked by two regions containing hydrophobic heptad repeats, hallmarks of coiled-coil regions (Lupas, 1996). Mfn1 and Mfn2 are 81% similar to each other and are both 52% similar to Drosophila Fzo.

Generation of knockout mice deficient in Mfn1 and Mfn2

We constructed gene replacement vectors for Mfn1 and Mfn2 using the neomycin resistance gene for positive selection and the diphtheria toxin subunit A gene for negative selection. In both cases, a stop codon was engineered at the very beginning of the GTPase domain near the NH2 terminus (Fig. 1, A and
In addition, the resulting genomic loci each contain a replacement of the G1 and G2 motifs of the GTPase domain with the neomycin expression cassette. These universal GTPase motifs are crucial for binding of the α and β phosphates of GTP and for Mg$^{2+}$ coordination (Bourne et al., 1991; Sprang, 1997). Genetic analyses in Drosophila and Saccharomyces cerevisiae (Hales and Fuller, 1997; Hermann et al., 1998), as well as our own studies (see Fig. 7 C and Fig. 8 C), demonstrate that an intact GTPase domain is essential for Fzo function. Therefore, the disrupted Mfn1 and Mfn2 alleles described here should be null alleles. Both Southern blot and PCR analysis confirmed germline transmission of the targeted alleles (Fig. 1, B, C, F, and G). Importantly, Western blot analysis using affinity-purified antisera raised against Mfn1 or Mfn2 confirmed loss of the targeted protein in homozygous mutant lysates (Fig. 1, D and H).

**Embryonic lethality in homozygous mutant mice**

Both the Mfn1 and Mfn2 knockouts demonstrate full viability and fertility in heterozygous animals but result in embryonic lethality of homozygous mutants. For the Mfn1 knockout line, normal frequencies of live mutant embryos were obtained up to embryonic day e10.5 (noon of the day a copulatory plug was detected is designated e0.5). However, by e11.5, 20% of the mutant embryos were resorbed, indicating inviability. At e12.5, most identifiable mutant embryos were resorbed (86%), and additional resorptions were so advanced that they could not be genotyped. Although resorptions were not evident until e11.5, by e8.5 all mutant embryos were significantly smaller and showed pronounced developmental delay. In addition, mutant embryos were often deformed.

In the Mfn2 knockout line, normal numbers of live homozygous mutant embryos were recovered up to e9.5. However, starting at e10.5, 29% of homozygous mutant embryos were in the process of resorption. By e11.5, most identifiable mutant embryos were resorbed (86%), and additional resorptions were so advanced that they could not be genotyped. Although resorptions were not evident until e11.5, by e8.5 all mutant embryos were significantly smaller and showed pronounced developmental delay. In addition, mutant embryos were often deformed.

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### Figure 2. Defective giant cell layer of mutant placentae. (A–D) DAPI-stained sections of placentae from e10.5 wild-type (A and C) and mutant (B and D) littermate embryos. The boxed areas of A and B are enlarged in C and D. Arrows and arrowheads indicate trophoblast giant cells. Note that the giant cells in D are sparser and have smaller nuclei. (E and F) Hematoxylin-eosin-stained sections from the placentae above. (G and H) PL-I (giant cell marker) RNA in situ analysis of placentae from e9.5 wild-type (G) and mutant (H) littermates.
observation suggests that Mfn1 and Mfn2 are both required, and may have a cooperative relationship, in a particular developmental event early in embryogenesis.

**Placental defects in Mfn2 mutant mice**

We examined placental development in detail because placental insufficiency is one of the most common causes of midgestation lethality (Copp, 1995). Hematoxylin-eosin–stained histological sections of placentae from wild-type and heterozygous embryos showed the typical trilaminar structure composed of a proximal labyrinthine layer, a middle spongiotrophoblast layer, and a distal, circumferential giant cell layer (Fig. 2 E). Trophoblast giant cells are polyploid cells (derived from endoreplication, a process where DNA replication proceeds repeatedly without associated cytokinesis) that lie at the critical interface between fetal and maternal tissues and play important roles in hormone production, recruitment of blood vessels, and invasion of the conceptus into the uterine lining (Cross, 2000). Deficiencies in these cells lead to midgestation lethality (Kraut et al., 1998; Riley et al., 1998; Hesse et al., 2000; Scott et al., 2000). Strikingly, placentae from Mfn2 mutant embryos reproducibly show an impaired giant cell layer with two defects. The giant cells are deficient in quantity, and the few that are observed contain smaller nuclei, implying a reduction in the number of endoreplication cycles (Fig. 2 F). These observations were confirmed with DAPI staining, which highlights the giant cells because of their high DNA content (Fig. 2, A–D). No defects in placental development were detected in Mfn1 mutant embryos (unpublished data) despite expression of Mfn1 in the placenta (Fig. 1 D).

Using RNA in situ hybridization, we examined the expression of molecular markers specific for each of the three placental layers. With the giant cell marker PL-I (Faria et al., 1991), wild-type placentae showed an intense full ring of giant cell staining with multiple layers of giant cells packed in the region distal to the spongiotrophoblast layer (Fig. 2 G). In contrast, Mfn2 mutant placentae showed an incomplete ring of weakly staining giant cells (Fig. 2 H). Moreover, the giant cell layer was generally only one cell layer thick. Similar results were seen in placentae from e8.5 through e10.5. These observations were confirmed with RNA in situ hybridizations (unpublished data) using the additional giant cell markers, proliferin (Lee et al., 1988) and Hand1 (Riley et al., 1998). Interestingly, our in situ hybridizations also revealed somewhat weaker staining with the ectoplacental cone and spongiotrophoblast marker 4311 (Lescisin et al., 1988), suggesting an additional but more subtle defect in this placental layer (unpublished data). No differences in staining between wild-type and Mfn2 mutant sections were observed using TEF5 (unpublished data), a marker for the labyrinthine layer (Jacquemin et al., 1998). Together, these findings suggest that the Mfn2 mutant embryos are dying in midgestation secondary to an inadequate placenta.

**Both Mfn1- and Mfn2-deficient cells have aberrant mitochondrial morphology**

To examine mitochondrial morphology in Mfn-deficient cells, we derived mouse embryonic fibroblasts (MEFs) from e10.5 embryos. The MEF cultures were infected with a retrovirus expressing mitochondrially targeted enhanced yellow fluorescent protein (EYFP). Wild-type MEFs showed a range of mitochondrial morphologies. The major morphological class, which encompassed >90% of wild-type cells, was characterized by a network of extended wavy tubules distributed in a roughly radial manner throughout the cytoplasm. Such cells had no or only a few spherical mitochondria. The length of these mitochondrial tubules ranged from several microns to >10 μm (Fig. 3, A and B, arrow). A much smaller morphological class, which encompassed only ~6% of wild-type cells, had mitochondria that were mostly spherical and were termed as “fragmented.”

In contrast, Mfn1 mutant MEFs had dramatically fragmented mitochondria (Fig. 3, C and D). Greater than 95%
of these cells contained only severely fragmented mitochondria, whereas only 1–2% of cells contained any significant tubules. Similarly, in 85% of Mfn2 mutant fibroblasts the mitochondria appeared mostly as spheres or ovals (Fig. 3, E and F). Only a small minority of mutant cells (4.5%) contained significant tubules. Neither Mfn1 nor Mfn2 mutant cells ever displayed the networks of long extended tubules that characterize the largest morphological class in wild-type cells. Thin section EM revealed that in spite of their aberrant dimensions the spherical mitochondria in these mutant cells contained both cristae and the typical double membrane (unpublished data). These results are consistent with the hypothesis that Mfn1 and Mfn2 play major roles in mitochondrial fusion.

To determine whether mitochondrial defects underlie the placental insufficiency of Mfn2 mutant embryos, we derived trophoblast stem (TS) cell lines from wild-type and mutant blastocysts. With MitoTracker Red staining, the cells within wild-type TS colonies displayed a network of long mitochondrial tubules (Fig. 3 G). In contrast, Mfn2 mutant TS cells showed only spherical mitochondria (Fig. 3 H).

Although loss of either Mfn1 or Mfn2 results in fragmentation of mitochondrial tubules, the two mutations lead to characteristic mitochondrial morphologies that are readily distinguishable. Loss of Mfn1 leads to a greater degree of fragmentation, resulting in either very short mitochondrial tubules or very small spheres that are uniform in size, with diameters no larger than that of a normal tubule. In contrast, many Mfn2 mutant cells exhibit mitochondrial spheres of widely varying sizes. Interestingly, the diameters of some of the spherical mitochondria in both Mfn2 mutant MEF and TS cells are several times larger than the diameters of mitochondrial tubules in wild-type cells. Because a simple defect in mitochondrial fusion would be expected to result in shorter tubules and small spheres with the diameter of normal tubules, these observations suggest that in addition to controlling mitochondrial fusion, Mfn2 is involved in maintaining tubular shape. Together, these results suggest that even though both Mfn1 and Mfn2 are expressed in fibroblasts, each protein is essential for mitochondrial fusion and may have some distinct functions.

Altered mitochondrial dynamics in Mfn-deficient cells

Because mitochondria are such dynamic organelles, we used time-lapse confocal microscopy to determine whether mutant MEFs display aberrations in mitochondrial dynamics. In wild-type cells, the mitochondria are highly motile, and at least one apparent fusion or fission event was observed for most mitochondria during 20-min recordings (Fig. 4 A; video 1, available at http://www.jcb.org/cgi/content/full/jcb.200211046/DC1). However, in both Mfn1 and Mfn2 mutant cells the ovoid or spherical mitochondria undergo fusion events much less frequently (videos 2 and 3, available at http://www.jcb.org/cgi/content/full/jcb.200211046/DC1), although a few such events can be found (Fig. 4 C).

In addition to reduced fusion, our time-lapse videos revealed striking defects in the mobility of mitochondria in mutant cells. Most mitochondria in wild-type cells were tubular, were directed radially, and moved back and forth along their long axis on radial tracks (Fig. 4 A). This movement along defined tracks is consistent with reports that mitochondria can be anchored along microtubule or actin filaments, depending on the cell type (Nangaku et al., 1994; Morris and Hollenbeck, 1995; Tanaka et al., 1998b). The mitochondria of Mfn-deficient cells display a dramatic alteration in mobility; the spherical or ovoid mitochondria in mutant cells show random “Brownian-like” movements (Fig. 4 B and C). This lack of directed movement is not due to disorganization of the cytoskeleton because staining with an antitubulin mAb or rhodamine-phalloidin revealed no defects (unpublished data; Fig. 3). Strikingly, in Mfn2 mutant cells with both spherical and short tubular mitochondria, the tubular mitochondria can still move longitudinally along radial tracks, whereas the spherical mitochondria do not. However, the two morphological classes are interchangeable because tubules can project out of spheres and subsequently move away (Fig. 4 D), or conversely tubules can merge with spheres and lose directed movement. These observations suggest that there is no intrinsic defect in mobilizing Mfn2-deficient mitochondria. Instead, the hampered mobility of spherical mitochondria is secondary to their altered morphology resulting from reduced fusion.

Decreased mitochondrial fusion rates in cells lacking Mfn1 or Mfn2

To definitively show that lack of fusion is the basis for the fragmented mitochondria in mutant cells, we measured mitochondrial fusion activity using a polyethylene glycol
(PEG) cell fusion assay. A cell line expressing mitochondrially targeted dsRed was cocultured with a cell line expressing mitochondrially targeted GFP, and PEG was transiently applied to fuse the cells. Cycloheximide was included to prevent synthesis of new fluorescent molecules in the fused cells. When wild-type cells were examined 7 h after PEG treatment, all of the fused cells \((n = 200)\) contained extensively fused mitochondria (Fig. 5 A) as demonstrated by colocalization of red and green fluorescent signals. In contrast, when Mfn1 mutant cells were examined 7 h after PEG fusion 57% \((n = 364)\) of the fused cells contained predominantly unfused mitochondria (Fig. 5, B and C) even when red and green mitochondria were dispersed throughout the fused cell. 35% of cells showed extensive mitochondrial fusion, and 8% showed partial fusion. Similarly, 69% \((n = 202)\) of fused Mfn2 mutant cells showed predominantly unfused mitochondria after 7 h (Fig. 5, E and F). 1% showed extensive fusion, and 30% showed partial fusion. Mfn1 and Mfn2 mutant cells with unfused mitochondria were observed even 24 h after PEG treatment (unpublished data). Thus, mutant cells have severely reduced levels of mitochondrial fusion. Interestingly, in 10% of fused Mfn1 mutant cells, the mitochondria did not readily spread throughout the cytoplasm as shown by discrete sectors of red and green fluorescence (Fig. 5 D). Only 1% of fused Mfn2 mutant cells exhibited this sectoring effect. Therefore, it seems that the mobility of Mfn1-deficient mitochondria is impaired to a greater extent than that of Mfn2-deficient mitochondria, perhaps due to their more severely fragmented morphology.

**Stochastic defects in mitochondrial membrane potential**

We tested whether Mfn1 and Mfn2 mutant cells lose mtDNA because there is complete loss of mtDNA in \(fzo1\Delta\) yeast (Hermann et al., 1998; Rapaport et al., 1998). Southern blot and PCR analysis showed that both mutant cell lines contain normal levels of mtDNA (Fig. 6 A; unpublished data). In addition, the mitochondria in mutant cells expressed COX1, a mitochondrial protein encoded by mtDNA (Fig. 6, B and C). Therefore, unlike \(fzo1\Delta\) yeast, Mfn-deficient cells clearly retain mtDNA, and this feature allows a critical assessment of the relationship between respiration and mitochondrial fusion. Like wild-type cells, both Mfn1 and Mfn2 mutant cultures showed high rates of endogenous and coupled respiration (unpublished data), indicating no gross defects in respiration and further confirming that mtDNA products are intact and functional.

Although the mutant cells are capable of respiration, we reasoned that functional defects may exist at the level of individual mitochondria. To test this hypothesis, we used MitoTracker Red, a lipophilic cationic dye which is sensitive to mitochondrial membrane potential, to stain MEFs expressing EYFP targeted to the mitochondrial matrix. In wild-type cells, EYFP-marked mitochondria were typically uniformly stained with MitoTracker Red, resulting in colocalization of the two fluorophores (Fig. 6 D). In 7% of wild-type cells, there existed isolated mitochondria that were labeled with EYFP but failed to sequester MitoTracker Red dye. Such mitochondria were invariably small and spherical as opposed to the tubular mitochondria that predominate in these cells. In Mfn1 mutant cultures, almost every cell contained a subset of mitochondria that showed poor staining with MitoTracker Red (Fig. 6 E). Likewise, in Mfn2 mutant cultures over one third of the cells showed some mitochondria that were marked by EYFP but not by MitoTracker Red (Fig. 6 F). It is likely that the compromised mitochondria detected by this assay initially contained membrane potential because the matrix-targeted EYFP requires an intact membrane potential for import. Because of the high stability of EYFP, mitochondria that subsequently lose or reduce their membrane potential would still contain EYFP fluorescence. These results indicate that while bulk cultures display respiratory activity, the function of individual mitochondria is compromised in the absence of either Mfn1 or Mfn2.

**Rescue of mitochondrial tubules by modulation of fusion or fission**

To unequivocally demonstrate that the mitochondrial morphological defects observed in mutant cells are due to loss of Mfn, we tested whether these defects could be rescued by restoration of Mfn function and whether such rescue depended on an intact GTPase domain. In uninfected Mfn1 mutant cultures or mutant cultures infected with an empty virus, >99% of the cells showed highly fragmented mitochondria that lacked interconnections (Fig. 7, A and F). However, when Mfn1 mutant cultures were infected with a retrovirus expressing Mfn1-Myc >90% of the infected cells showed extensive mitochondrial networks consisting of long tubules (Fig. 7, B...
and F). Similarly, >80% of Mfn2 mutant cells infected with a Mfn2-expressing retrovirus showed predominantly tubular mitochondria (Fig. 8, B and F). In contrast, constructs carrying mutations in the GTPase G1 motif (Mfn1[K88T] and Mfn2[K109A]) had greatly reduced ability to restore tubular structure (Fig. 7, C and F, and Fig. 8, C and F). These results show that both Mfn1 and Mfn2 promote mitochondrial fusion in a GTPase-dependent manner.

Although both Mfn1 and Mfn2 are clearly required for normal mitochondrial tubules in MEFs, we were able to rescue the morphological defects by overexpression of a single mitofusin. Overexpression of Mfn1 in Mfn2-deficient cells was sufficient for rescue of mitochondrial morphology (Fig. 8, D and F). In addition, overexpression of Mfn2 in Mfn1-deficient cells was sufficient to restore mitochondrial tubules (Fig. 7, D and F). These results show that although both Mfn1 and Mfn2 are required in vivo for normal mitochondrial morphology in MEFs, when overexpressed, each protein is capable of acting alone to promote mitochondrial fusion.

First, the thickness of the tubules promoted by Drp1(K38A) were less uniform, often showing regions of thinning attached to thicker knobs. Second, the mitochondrial networks were generally less dispersed, with an increase in density near the cell nucleus that often gave the appearance of circumferential rings. Nevertheless, these results indicate that a reduction in fusion caused by loss of Mfn1 or Mfn2 can be compensated, at least partially, by a reduction in fission.

### Mfn1 and Mfn2 form homotypic and heterotypic oligomers

Because both Mfn1 and Mfn2 are essential for normal mitochondrial morphology, it is possible that they act in concert to promote mitochondrial fusion. To explore this idea, we...

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**Figure 6.** *Stochastic loss of membrane potential in mitochondria of mutant cells.* (A) mtDNA is detected by Southern blot analysis using a COX1 probe. (B and C) COX1 expression in Mfn1 (B) and Mfn2 (C) mutant cells. (D-F) Staining of mitochondria using dyes sensitive to membrane potential. Wild-type (D), Mfn1 mutant (E), and Mfn2 mutant (F) cells expressing mitochondrially targeted EYFP (green) were stained with the dye MitoTracker Red, whose sequestration into mitochondria is sensitive to membrane potential. In these merged images, note that in the mutant cells (E and F) a subset of mitochondria (arrows) stain poorly with MitoTracker Red and thus appear green.
tested whether these two proteins form a complex when expressed in wild-type MEF cells (Fig. 9 A). Our results revealed three types of intermolecular interactions. First, both Mfn1 and Mfn2 can form homotypic complexes. Mfn1-HA is coimmunoprecipitated with Mfn1-Myc; analogously, Mfn2-HA is coimmunoprecipitated with Mfn2-Myc. Second, Mfn1 and Mfn2 form heterotypic complexes. Mfn2-HA is coimmunoprecipitated with Mfn1-Myc; conversely, Mfn1-HA is coimmunoprecipitated with Mfn2-Myc. All of these interactions are specific because no HA-tagged protein is found in the anti-Myc immunoprecipitate when the Myc-tagged protein is omitted (unpublished data) or when a control Drp1-Myc is used.

It is a formal possibility that the homotypic interactions detected might not be strictly homotypic due to the expression of endogenous Mfn1 and Mfn2 in the parental cells. To relieve these concerns, we also performed the immunoprecipitation assay in mutant MEFs (Fig. 9 B). We find that Mfn1-Mfn1 homotypic complexes are formed in Mfn2-deficient cells, thereby showing that this interaction is strictly homotypic. Likewise, we also detect Mfn2-Mfn2 homotypic complexes in Mfn1-deficient cells.

Discussion

An essential role for mitochondrial fusion in mouse development

Although mitochondrial dynamics involving fusion and fission has been previously demonstrated in vertebrates, the physiological importance of these processes has remained unclear. Our analysis of Mfn1 and Mfn2 mutant mice demonstrates an essential role for mitochondrial fusion in vertebrate development. Both lines of mice die in midgestation. For Mfn2-deficient mice, we observe a dramatic disruption in placental development, most obviously in the paucity of trophoblast giant cells. Trophoblast cell lines cultured from Mfn2 mutant blastocysts show fragmentation of mitochondrial tubules, consistent with a defect in mitochondrial fusion. These results show that despite its broad expression pattern (unpublished data) Mfn2 is only required for selective developmental transitions.

Trophoblast giant cells are polyploid cells that arise from endoreplication, a process often associated with highly metabolically active cells (Edgar and Orr-Weaver, 2001). Because of their high metabolic rate, we speculate that, in vivo, trophoblast giant cells may be particularly vulnerable to pertur-
The advantage of maintaining mitochondria in a highly dynamic state is still unclear.

Our analysis of Mfn-deficient cells suggests an answer to this question. Although bulk cultures of Mfn-deficient fibroblasts show normal levels of endogenous and coupled respiration by oxygen electrode measurements, when individual mitochondria are examined, we find that many cells contain a percentage of nonfunctional mitochondria as evidenced by loss of membrane potential. In other cell culture systems, the use of dyes sensitive to mitochondrial membrane potential has revealed occasional and transient losses of membrane potential within small regions of a single mitochondrial tubule (Loew, 1999). We suggest that the dynamic nature of mitochondria protects these organelles by ensuring that regional losses of membrane potential, caused perhaps by local depletion of metabolic substrates or mtDNA, are always transient (Fig. 10 A). Mitochondrial fusion enables intermitochondrial cooperation by allowing exchange of both membrane and matrix contents and therefore may help to re-store local depletions and maintain mitochondrial function (Nakada et al., 2001a). Although there is no gross loss of mtDNA in mutant cells, we currently do not know if the individual, defective mitochondria have lost mtDNA.

A model for the role of mitochondrial fusion in protecting mitochondrial function

It has been paradoxical why eukaryotes have invested in fusion and fission pathways in cells that have “stable” mitochondrial networks. Given that mitochondrial tubules can be maintained by reducing fusion and fission simultaneously (Bleazard et al., 1999; Sesaki and Jensen, 1999; Fekkes et al., 2000; Mozdy et al., 2000; Tieu and Nunnari, 2000), what is the advantage of maintaining mitochondria in a highly dynamic state?

Our analysis of Mfn-deficient cells suggests an answer to this question. Although bulk cultures of Mfn-deficient fibroblasts show normal levels of endogenous and coupled respiration by oxygen electrode measurements, when individual mitochondria are examined, we find that many cells contain a percentage of nonfunctional mitochondria as evidenced by loss of membrane potential. In other cell culture systems, the use of dyes sensitive to mitochondrial membrane potential has revealed occasional and transient losses of membrane potential within small regions of a single mitochondrial tubule (Loew, 1999). We suggest that the dynamic nature of mitochondria protects these organelles by ensuring that regional losses of membrane potential, caused perhaps by local depletion of metabolic substrates or mtDNA, are always transient (Fig. 10 A). Mitochondrial fusion enables intermitochondrial cooperation by allowing exchange of both membrane and matrix contents and therefore may help to re-store local depletions and maintain mitochondrial function (Nakada et al., 2001a). Although there is no gross loss of mtDNA in mutant cells, we currently do not know if the individual, defective mitochondria have lost mtDNA.
Multiple molecular modes of mitofusin action

Given their broad and overlapping expression, it has been unclear why there are two separate mammalian mitofusins. Our results show that the two mitofusins form three distinct molecular complexes that are capable of promoting mitochondrial fusion—Mfn1 homotypic oligomers, Mfn2 homotypic oligomers, and Mfn1-Mfn2 heterotypic oligomers. We propose that the relative importance of each of these modes of mitofusin action can change depending on the cell type (Fig. 10 B). In mouse fibroblasts, all three oligomeric complexes are likely to play important roles because disruption of either Mfn1 or Mfn2 leads to severe mitochondrial fragmentation. Nevertheless, overexpression of Mfn1 homotypic oligomers or Mfn2 homotypic oligomers is sufficient to restore mitochondrial tubules, clearly demonstrating that the homotypic complexes are functional for fusion. In trophoblast giant cells, it appears that Mfn2 homotypic oligomers are most important. As a result, this cell type is affected in Mfn2 deficient embryos but not Mfn1 deficient embryos.

It remains to be determined whether these three complexes function in the same or distinct types of mitochondrial fusion. It is also interesting to note that loss of Mfn2 leads to the formation of both large and small mitochondrial spheres. This phenotype is easily distinguished from loss of Mfn1. The larger mitochondrial fragments may be due to a higher residual fusion activity in Mfn2 mutant cells than in Mfn1 mutant cells. The loss of tubular shape may simply reflect the loss of cytoskeletal interactions as noted in Fig. 4. Alternatively, it may be that Mfn2, and by extension Mfn2 homotypic complexes, play a more direct role in maintaining mitochondrial tubular shape. Thus, although each of the Mfn complexes is involved in fusion, it is possible that they have distinct functions in addition to fusion.

In conclusion, in cells with continual cycles of fusion and fission, the mitochondrial population is essentially functionally homogeneous. However, at any given time point individual mitochondria are functionally distinct entities (Collins et al., 2002). Therefore, without mitochondrial fusion, the stochastic differences between distinct mitochondria can accumulate to affect the well-being of the cell. The mammalian mitofusins, Mfn1 and Mfn2, function in three distinct molecular complexes to promote mitochondrial fusion and thus protect mitochondrial function.

Materials and methods

Cloning of Mfn cDNA and genomic constructs

Homology searches using the Drosophila Fzo sequence (Hales and Fuller, 1997) identified a murine EST (IMAGE Consortium Clone ID 733269) that encodes a highly homologous polypeptide. This cDNA was used to screen a mouse kidney cDNA library, resulting in isolation of cDNAs for both Mfn1 and Mfn2. These sequences are available from GenBank/EMBL/DDBJ under accession nos. AY174062 and AY123975.

Genomic clones of Mfn1 and Mfn2 were retrieved from a lambda 129/SvJ mouse genomic library (Stratagene). Genomic fragments were subcloned into the targeting vector pHGNeo2pAlox2PGK-DTA (a gift from F. Gertler and L. Jackson-Grusby, Massachusetts Institute of Technology, Cambridge, MA) as the right arms. To insert the left arms, genomic fragments were amplified by PCR, which also introduced a stop codon just be-
fore the conserved GKS sequence in each GTPase domain. All constructs were verified by DNA sequence analysis. The sequences of all oligonucleotides used in this study are available from the authors.

To identify the chromosomal locations of Mfn1 and Mfn2, the Jackson Laboratory (C5BL/6J × (C3H/HeJ × SPRET/Ei) F1 × SPRET/Ei) backcross panel was used. Linkage analysis was performed by the Jackson Laboratory Mapping Resource.

Construction of knockout mice

Each gene replacement vector was linearized with SacI and electroporated into mouse embryonic stem cells using established procedures (Chester et al., 1998). Neomycin-resistant colonies were screened by Southern blot analysis. Two independently isolated clones for each targeted allele were injected into C57BL/6 blastocysts to generate chimera mice. Excision of the neomycin cassette had no effect on phenotypes.

Confirmation of targeting event

For Southern blot analysis of targeted alleles, genomic DNAs were digested with XbaI (Mfn1 mutant line) or EcoRI (Mfn2 mutant line) and hybridized with a flanking genomic probe. PCR was used for routine genotyping of offspring. A forward genomic primer, a reverse genomic primer, and a reverse neomycin primer were used to detect both the targeted and wild-type alleles in a single reaction.

For Western blot analysis, chicken antisera was generated against Mfn1 (residues 348–579) or Mfn2 (residues 369–598) fused to an NH2-terminal histidine tag. IgG purified from chicken eggs was affinity purified on a column coupled to a maltose-binding protein fusion protein containing either Mfn1 residues 348–579 or Mfn2 residues 369–598.

In situ hybridization

Individual implantation sites were fixed overnight at 4°C in 4% paraformaldehyde, dehydrated through an ethanol series, treated with xylene, and embedded into paraffin blocks. For staining, 10-μm sections were cut in a transverse plane with respect to the placenta. Slides were processed for hematoyxlin-eosin staining or in situ hybridization as described previously (Mfn2 mutant line) or EcoRI (Mfn2 mutant line) and hybridized with a site-directed mutagenesis (Kunkel et al., 1991). To make the Mfn-HA constructs, Mfn1 and Mfn2 cDNAs were subcloned into a pcDNA3.1(−/−) vector containing a COOH-terminal 3×His tag. Murine Drp1 was amplified from mouse placental RNA, and Drp1(K38A) was constructed by site-directed mutagenesis. The Drp1(K38A) insert was subcloned into a modified pcDNA3.1(−/−) vector containing vector pCDNA3.1(−/−)/Myr-His A (Invitrogen). Myc epitope tag cassettes derived from pMyc-His 3×Myc were then inserted into the BamH1 site to form pMyc-Myc. Mfn1[K88T] and Mfn2[K109A] were constructed by site-directed mutagenesis using Myc- and HA-tagged Mfn1, Mfn2, and Drp1(K38A). Monolayers were transfected with various combinations of retroviruses expressing Myc- and HA-tagged Mfn1, Mfn2, and Drp1(K38A). Monolayers were transfected with various combinations of retroviruses expressing Myc- and HA-tagged Mfn1, Mfn2, and Drp1(K38A). Monolayers were transfected with various combinations of retroviruses expressing Myc- and HA-tagged Mfn1, Mfn2, and Drp1(K38A). Monolayers were transfected with various combinations of retroviruses expressing Myc- and HA-tagged Mfn1, Mfn2, and Drp1(K38A).

Retroviral and plasmid constructs

To construct Mfn1 or Mfn2 with Myc epitope tags, a BamH1 site was introduced immediately before the stop codon. The CDNA was subcloned into the vector pcDNA3.1(−/−)/Myr-His A (Invitrogen). Myc epitope tag cassettes derived from pMyc-His 3×Myc were then inserted into the BamH1 site to form pMyc-Myc. Mfn1[K88T] and Mfn2[K109A] were constructed by site-directed mutagenesis (Kunkel et al., 1991). To make the Mfn-HA constructs, Mfn1 and Mfn2 cDNAs were subcloned into a pcDNA3.1(−/−) vector containing a COOH-terminal 3×His tag. Murine Drp1 was amplified from mouse placental RNA, and Drp1(K38A) was constructed by site-directed mutagenesis using Myc- and HA-tagged Mfn1, Mfn2, and Drp1(K38A). Monolayers were transfected with various combinations of retroviruses expressing Myc- and HA-tagged Mfn1, Mfn2, and Drp1(K38A).

Southern analysis of mtDNA

Southern analysis of mtDNA was performed by linearizing with XhoI and probing with a radio-labeled PCR fragment containing the COX1 gene.

Immunoprecipitation

Cell lines were infected with various combinations of retroviruses expressing Myc- and HA-tagged Mfn1, Mfn2, and Drp1(K38A). Monolayers were transfected with various combinations of retroviruses expressing Myc- and HA-tagged Mfn1, Mfn2, and Drp1(K38A). Monolayers were transfected with various combinations of retroviruses expressing Myc- and HA-tagged Mfn1, Mfn2, and Drp1(K38A).

Analysis of mtDNA

Southern analysis of mtDNA was performed by linearizing with Xhol and probing with a radio-labeled PCR fragment containing the COX1 gene.

Immunoprecipitation

Cell lines were infected with various combinations of retroviruses expressing Myc- and HA-tagged Mfn1, Mfn2, and Drp1(K38A). Monolayers were transfected with various combinations of retroviruses expressing Myc- and HA-tagged Mfn1, Mfn2, and Drp1(K38A). Monolayers were transfected with various combinations of retroviruses expressing Myc- and HA-tagged Mfn1, Mfn2, and Drp1(K38A). Monolayers were transfected with various combinations of retroviruses expressing Myc- and HA-tagged Mfn1, Mfn2, and Drp1(K38A).

Online supplemental materials

Confocal time-lapse videos of EYFP-labeled mitochondria in wild-type (video 1), Mfn1 mutant (video 2), and Mfn2 mutant (video 3) MEFs are available at http://www.jcb.org/cgi/content/full/jcb.200211046/DC1. Videos 1–3 show mitochondrial dynamics in wild-type and mutant MEFs. Video 1 and 3 are 20-min recordings; video 2 is a 10-min recording. The microtubule field is 96 μm × 96 μm in video 1, 68 μm × 68 μm in video 2, and 75 μm × 75 μm in video 3.

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References

Bakeeva, L.E., S. Chentsov Yu, and V.P. Skulachev. 1981. Ontogenesis of mitochondrial fission in yeast. Microsc. Res. Tech. 27:198–219.

Bakeeva, L.E., Y.S. Chentsov, and V.P. Skulachev. 1981. Ontogenesis of mitochondrial fission in yeast. Microsc. Res. Tech. 27:198–219.

Bakeeva, L.E., S. Chentsov Yu, and V.P. Skulachev. 1981. Ontogenesis of mitochondrial fission in yeast. Microsc. Res. Tech. 27:198–219.

Bakeeva, L.E., S. Chentsov Yu, and V.P. Skulachev. 1981. Ontogenesis of mitochondrial fission in yeast. Microsc. Res. Tech. 27:198–219.

Bakeeva, L.E., S. Chentsov Yu, and V.P. Skulachev. 1981. Ontogenesis of mitochondrial fission in yeast. Microsc. Res. Tech. 27:198–219.
Bourne, H.R., D.A. Sanders, and F. McCormick. 1991. The GTPase superfamily: conserved structure and molecular mechanism. Nature. 349:117–127.

Chester, N., F. Kuo, C. Kozak, C.D. O’Hara, and P. Leder. 1998. Stage-specific apoptosis, developmental delay, and embryonic lethality in mice homozygous for a targeted disruption in the murine Bloom’s syndrome gene. Genes Dev. 12:3382–3393.

Collins, T.J., M.J. Berridge, P. Lipp, and M.D. Bootman. 2002. Mitochondria are morphologically and functionally heterogeneous within cells. EMBO J. 21:1616–1627.

Copp, A.J. 1995. Death before birth: clues from gene knockouts and mutations. Trends Genet. 11:87–93.

Cross, J.C. 2000. Genetic insights into trophoblast differentiation and placental morphogenesis. Semin. Cell Dev. Biol. 11:105–113.

Edgar, B.A., and T.L. Orr-Weaver. 2001. Endoreplication cell cycles: more for less. Trends Biochem. Sci. 26:375–382.

Hesse, M., T. Franz, Y. Tamai, M.M. Taketo, and T.M. Magin. 2000. Targeted disruption of mouse I-mfa gene for placental development and skeletal patterning. EMBO J. 19:5060–5070.

Lee, S.J., F. Talamantes, E. Wilder, D.I. Linzer, and D. Nathans. 1988. Trophoblast-specific giant cells of the mouse placenta as the site of proliferin synthesis. J. Cell Biol. 105:297–306.

Lupas, A. 1996. Coiled coils: new structures and new functions. Trends Biochem. Sci. 21:375–382.

Morris, R.L., and P.J. Hollenbeck. 1995. Axonal transport of mitochondria along microtubules and F-actin in living vertebrate neurons. J. Cell Biol. 131:1315–1326.

Moody, A.D., J.M. McCaffrey, and J.M. Shaw. 2000. Dnm1p GTPase-mediated mitochondrial fission is a multi-step process requiring the novel integral membrane component Fis1p. J. Cell Biol. 151:367–380.

Nakada, K., K. Inoue, and J. Hayashi. 2001a. Interaction theory of mammalian mitochondria. Biochem. Biophys. Res. Commun. 288:745–746.

Nakada, K., K. Inoue, T. Ono, K. Isobe, A. Ogura, Y.I. Goto, I. Nonaka, and J.I. Hayashi. 2001b. Inter-mitochondrial complementation: Mitochondria-specific system preventing mice from expression of disease phenotypes by mutant mtDNA. Nat. Med. 7:954–940.

Nakamura, M., R. Sato-Yoshitake, Y. Okada, Y. Noda, R. Takemura, H. Yamazaki, and N. Hirokawa. 1994. KIF1B, a novel microtubule plus end-directed monomeric motor protein for transport of mitochondria. Cell. 79:1209–1220.

Okada, A., R. Lansford, J.M. Weimann, S.E. Fraser, and S.K. McConnell. 1999. Imaging cells in the developing nervous system with retrovirus expressing modified green fluorescent protein. Exp. Neurol. 156:394–406.

Scott, I.C., L. Anson-Cartwright, and J.C. Cross. 1998. The Hand1 SHHL tran-scription factor is essential for placentalisation and cardiac morphogenesis. Nat. Genet. 18:271–275.

Scott, I.C., L. Anson-Cartwright, P. Riley, D. Reda, and J.C. Cross. 2000. The HAND1 basic helix-loop-helix transcription factor regulates trophoblast differentiation via multiple mechanisms. Mol. Cell. Biol. 20:530–541.

Sesaki, H., and R.E. Jensen. 1999. Division versus fusion: Dnm1p and Fzo1p antagonistically regulate mitochondrial shape. J. Cell Biol. 147:699–706.

Smitrovic, E., L. Griscic, D.L. Shurland, and A.M. van Der Bliek. 2001. Dynamics of mitochondrial targeting of mitofusins, ubiquitous mammalian homologs of the Drosophila mitofusin. Mol. Biol. Cell. 12:2245–2256.

Sprang, S.R. 1997. G protein mechanisms: insights from structural analysis. Annu. Rev. Biochem. 66:639–678.

Tani, T. 2002. G protein with a novel subunit topology and function. J. Biol. Chem. 277:13590–13593.