Mitochondrial Fusion Is Required for mtDNA Stability in Skeletal Muscle and Tolerance of mtDNA Mutations

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SUMMARY

Mitochondria are highly mobile and dynamic organelles that continually fuse and divide. These processes allow mitochondria to exchange contents, including mitochondrial DNA (mtDNA). Here we examine the functions of mitochondrial fusion in differentiated skeletal muscle through conditional deletion of the mitofusins Mfn1 and Mfn2, mitochondrial GTPases essential for fusion. Loss of the mitofusins causes severe mitochondrial dysfunction, compensatory mitochondrial proliferation, and muscle atrophy. Mutant mice have severe mtDNA depletion in muscle that precedes physiological abnormalities. Moreover, the mitochondrial genomes of the mutant muscle rapidly accumulate point mutations and deletions. In a related experiment, we find disruption of mitochondrial fusion strongly increases mitochondrial dysfunction and lethality in a mouse model with high levels of mtDNA mutations. With its dual function in safeguarding mtDNA integrity and preserving mtDNA function in the face of mutations, mitochondrial fusion is likely to be a protective factor in human disorders associated with mtDNA mutations.

INTRODUCTION

Mitochondrial fusion and fission have emerged as important processes that govern mitochondrial function from yeast to mammals (Detmer and Chan, 2007a; Suen et al., 2008; Okamoto and Shaw, 2005; Hoppins et al., 2007). In mammalian cells, three large GTPases are important for mitochondrial fusion, which requires the coordinated fusion of the outer and inner membranes. The mitofusins, Mfn1 and Mfn2, are located on the mitochondrial outer membrane and are involved in early steps in membrane fusion (Koshiba et al., 2004; Meeusen et al., 2004; Song et al., 2009). The dynamin-related protein OPA1 is associated with the inner membrane and is essential for inner mitochondrial proliferation, and muscle atrophy. Mutant mice have severe mtDNA depletion in muscle that precedes physiological abnormalities. Moreover, the mitochondrial genomes of the mutant muscle rapidly accumulate point mutations and deletions. In a related experiment, we find that disruption of mitochondrial fusion strongly increases mitochondrial dysfunction and lethality in a mouse model with high levels of mtDNA mutations. With its dual function in safeguarding mtDNA integrity and preserving mtDNA function in the face of mutations, mitochondrial fusion is likely to be a protective factor in human disorders associated with mtDNA mutations.
mixing in heteroplasmic cells might underlie the functional complementation of pathogenic mtDNA genomes (Nakada et al., 2001, 2009). However, the proposed role of mitochondrial fusion in functional complementation remains to be experimentally tested.

Other fundamental issues concerning mitochondrial fusion remain to be explored. Most studies on mammalian mitochondrial dynamics have been limited to cultured cells and neurons (Detmer and Chan, 2007a; Suen et al., 2008). These studies show that mitochondrial fusion is highly associated with mitochondrial transport along the cytoskeleton because mitochondria fuse when they collide end-to-end or end-to-side. However, little is known about the role of mitochondrial dynamics in most tissues in vivo, including skeletal muscle fibers, which are highly metabolically active and are often affected in diseases of mitochondrial dysfunction. Elegant ultrastructural studies have documented that mitochondria in skeletal muscle are tightly packed and arranged in a stereotypic manner in relation to the sarcomeric unit (Ogata and Yamasaki, 1997). This unique organization and apparently limited mobility raises the issue of whether mitochondrial dynamics is important in this cell type.

To address these issues, we examined mice lacking mitofusin function in skeletal muscle and found that these mice develop a lethal mitochondrial myopathy. In addition, we found that mitochondrial fusion protects mtDNA function through several distinct mechanisms. It maintains mtDNA levels, preserves mtDNA fidelity, and enables cells to tolerate high levels of mtDNA mutations.

RESULTS

A Role of Mitofusins Mfn1 and Mfn2 in Skeletal Muscle

Given the unique arrangement of mitochondria in skeletal muscle, we examined whether mitochondrial fusion plays an important role in this cell type. Using MLC-Cre transgenic mice, we specifically disrupted the mitochondrial fusion genes Mfn1 and Mfn2 in skeletal muscle (Bothe et al., 2000). The MLC1f promoter that drives Cre recombinase is well-characterized to express in differentiated fast-twitch muscle cells and not in myocytes (Lyons et al., 1990). Surprisingly, such double-mutant mice (MLC-Cre/dm) are severely runted, reaching only 30%–50% the weight of control littermates (Table 1) before dying by 6–8 weeks of age. In contrast, mice of other genotypic combinations (including Mfn1+/−/Mfn2+/− and Mfn1+/− Mfn2−/−) survive and grow to normal size.

MLC-Cre/dm mice show severe physiological aberrations. They display low blood glucose levels under fasting and nonfasting conditions and reduced body temperatures (Table 1). Moreover, we found high levels of lactate in the serum of MLC-Cre/dm mice, most pronounced after an exercise regimen but also under resting conditions. These profound metabolic aberrations suggest a rapid turnover of glucose without adequate energy production, as would result from the predominant use of glycolysis instead of oxidative phosphorylation.

Mitochondrial Defects in Mitofusin-Deficient Muscle

On gross examination of muscle, it is readily apparent that the MLC-Cre/dm mice have smaller muscles that are deeper red than control muscles (Figure 1A), suggesting an increase in mitochondrial myopathies caused by mtDNA mutations (DiMauro and Schon, 2003). Furthermore, both interfibrillar and subsarcolemmal mitochondria show classic ultrastructural signs of dysfunction: they are swollen and contain few of the densely packed cristae membranes characteristic of wild-type mitochondria (Figures 1E and 1F). Quantiﬁcation of the micrographs indicates that in MLC-Cre/dm muscle, the mitochondria occupy several-fold more area than

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Double-Mutant</th>
<th>Control</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (females)</td>
<td>11 ± 2.6 g, n = 2</td>
<td>23 ± 2.1 g, n = 6</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Weight (males)</td>
<td>10 ± 2.4 g, n = 11</td>
<td>30 ± 3.6 g, n = 11</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Body temperature</td>
<td>35 ± 1.1 °C, n = 10</td>
<td>37 ± 0.3 °C, n = 20</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Nonfasting blood glucose</td>
<td>50 ± 14 mg/dL, n = 2</td>
<td>143 ± 13 mg/dL, n = 7</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Fasting blood glucose</td>
<td>26 ± 2.8 mg/dL, n = 2</td>
<td>78 ± 12 mg/dL, n = 10</td>
<td>0.99992</td>
</tr>
<tr>
<td>Resting serum lactate</td>
<td>5.7 ± 1.7 mmol/l, n = 7</td>
<td>3.9 ± 1.2 mmol/l, n = 9</td>
<td>0.02</td>
</tr>
<tr>
<td>Post-exercise lactate</td>
<td>9.5 ± 2.3 mmol/l, n = 5</td>
<td>4.7 ± 0.65 mmol/l, n = 5</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Measurements are given as value ± standard deviation. p values were calculated using the unpaired t test. Controls are mixed genotype littermates of double-mutant animals. The number of animals (n) for each measurement is indicated.
in wild-type muscle (Figure 1B). In contrast, muscles carrying just one Mfn allele show much milder histological defects (Figures 1B, 1G, and 1H), with occasional patches of abnormal mitochondria particularly evident in Mfn1+/− Mfn2+/− muscle (Figure 1G).

Given the physiological and ultrastructural evidence for mitochondrial dysfunction, we used histochemical staining for cytochrome c oxidase (COX, complex IV, brown stain) and succinate dehydrogenase (SDH, complex II, blue stain) activity to directly assess the function of respiratory complexes. In 1- and 2-week-old wild-type mice, the muscle sections show uniform COX staining of all the muscle fibers (Figures 2A and 2C). Over the next several weeks, this gradually matures into the brown checkerboard appearance of adult muscle, due to the differentiation of specific muscle fiber types with varying mitochondrial activity (Figures 2E and 2G). In MLC-Cre/dm muscle, the staining pattern is normal at week 1, but a mildly abnormal pattern emerges at week 2 with the appearance of some fibers that stain faintly blue, indicating increased SDH and low COX activity (Figure 2D, arrows). This abnormal histological pattern becomes highly pronounced at 4 and 7 weeks, at which time many deeply blue fibers are apparent (Figures 2F and 2H). In human mitochondrial encephalomyopathies, this characteristic blue histological pattern is often found in cases of respiratory dysfunction due to mtDNA defects (DiMauro and Schon, 2003; Taylor and Turnbull, 2005), because SDH activity is encoded solely by the nuclear genome, whereas COX activity is dependent on the mitochondrial genome. The increased SDH staining is consistent with the increased mitochondrial accumulation observed by EM (Figures 1B, 1D, and 2J). In addition, double-mutant muscle fibers have notably smaller diameters at 4 and 7 weeks. This small fiber size accounts for the overall decreased muscle size because no reduction in fiber number was found (data not shown). In contrast, Mfn1+/− Mfn2+/− and Mfn1+/− Mfn2−/− muscles demonstrate normal COX/SDH staining patterns, normal fiber size, and no systemic indicators of respiratory deficiency (Figures 2I and 2J). Therefore, the severe defects found in MLC-Cre/dm mice are not due to a specific function of Mfn2 versus Mfn1.

Loss of mitofusins also resulted in a marked shift in the composition of skeletal muscle fiber types. MLC-Cre/dm tibialis anterior muscles show an increase in the proportion of fibers positive for myosin heavy chain 2A, a marker for oxidative type.
IIA fibers, and a corresponding decrease in the proportion of fast-twitch type IIB fibers (Figure S2). These results are consistent with reports that mitochondrial biogenesis can drive muscle fiber type switching (Arany et al., 2007; Lin et al., 2002).

Mitofusins Are Important for mtDNA Stability and Fidelity

Given the histological and physiological features of mitochondrial myopathy in MLC-Cre/dm mice, we investigated the relationship between mitochondrial fusion and mtDNA copy number. Remarkably, we found that 7- to 8-week-old muscle from double-mutant mice contains only ~250 copies of mtDNA per nuclear genome, whereas muscle from age-matched controls contains ~3500 copies of mtDNA per nuclear genome (Figure 3A). Mice with single alleles of either Mfn1 or Mfn2 show normal levels of mtDNA at 7–8 weeks (Figure 3A) and later (Figure S3A).

To determine the temporal relationship between the mtDNA depletion and the muscle fiber defects, we analyzed mtDNA levels in younger MLC-Cre/dm muscle (Figure 3B). Even at the earliest time point, 1 week of age, we measured a significantly lower level of mtDNA in MLC-Cre/dm muscle compared to control littermates. The discrepancy in mtDNA levels between MLC-Cre/dm and wild-type muscle enlarges over the next several weeks due to the rapid increase in mtDNA content in wild-type muscle. In contrast, mtDNA in mutant muscle fails to expand during postnatal development. Because this mtDNA depletion becomes evident prior to histological evidence for gross mitochondrial dysfunction (Figure 2), it is likely that reduced mtDNA levels in the mutants underlie, at least in part, the muscle pathology observed later.

We made similar observations of mtDNA loss in mouse embryonic fibroblasts (MEFs) that lack both Mfn1 and Mfn2 (Figure 3C), consistent with the impaired respiratory capacity of these cells (Chen et al., 2005). In contrast, MEFs with only a partial defect in fusion (Mfn1−/− or Mfn2−/− cells) harbor normal levels of mtDNA (Figure 3C) and respire normally (Chen et al., 2005). Importantly, overexpression of either Mfn1 or Mfn2 in Mfn-double null MEFs rapidly restores mtDNA to wild-type levels (Figure 3D), again emphasizing that the shared function of mitochondrial fusion by Mfn1 and Mfn2 is responsible for the defects.

Moreover, we found that OPA1 null cells, which are deficient for fusion of the inner mitochondrial membrane, but proficient for outer membrane fusion (Song et al., 2009), also display mtDNA depletion (Figure 3C), suggesting that lack of mixing of the matrix compartment is ultimately responsible for loss of mtDNA in fusion-deficient cells.

To further explore the connection between mitochondrial fusion and mtDNA, we interrogated the fidelity of the mitochondrial genome by screening mtDNA for point mutations and deletions. We found that muscle from 7- to 8-week-old MLC-Cre/dm mice harbors a 5-fold increase in mtDNA point mutations (Figure 4A) and a 14-fold increase in deletions (Figure 4C) compared to wild-type animals. We confirmed both of these measurements by interrogating additional loci in the mitochondrial genome (Figures 4B and 4D). Most deletions occurred between short homologous repeats in both double-mutant and wild-type tissue, whereas there was a shift in the spectrum of single base pair substitutions in double-mutant mice (Figures S4B and S4C).

Because mitochondrial mutations accumulate with age (Vermulst et al., 2007, 2008a), we also examined mtDNA from older animals carrying single alleles of Mfn1 or Mfn2. Remarkably, we found that muscle tissue from 8- to 13-month-old Mfn1−/− Mfn2−/− mice exhibits an ~80-fold increase in mtDNA deletions (2.3 × 10−15 genome) compared to control muscle (2.8 × 10−17 genome) (Figure 4C). This increase was confirmed by analysis at an independent site (Figure 4D). However, we...
found no significant change in the frequency of point mutations or mtDNA copy number of Mfn1+/-- Mfn2+/-- or Mfn1+/- Mfn2-- muscle (Figures S3A and S4A), suggesting that the mechanism underlying the generation of mtDNA deletions is more sensitive to a reduction in mitochondrial fusion.

To further confirm this finding of increased mtDNA deletions in a genome-wide, unbiased manner, we analyzed with Solexa sequencing mtDNA from 10-month wild-type and Mfn1+/-- Mfn2+/-- muscle. For each sample, we obtained 75-nucleotide sequence reads that, in aggregate, cover 367.5 and 442.5 million base pairs of mtDNA. Both ends of each sequence read were mapped onto the mtDNA genome, and sequences with ends that mapped to different locations were selected as candidates for deletions. To minimize the chances of artifactual deletions, we only considered deletions that were identified by four or more independent sequence reads (Figure 4E). In wild-type muscle, we found no such deletions. In contrast, the Mfn1+/-- Mfn2-- sample contained nine distinct deletions that were identified by 62 independent sequence reads, and therefore represent 62 deletion events. In each case, the breakpoint junction occurred between repeats of 6–14 nucleotides (Table S1), in agreement with our earlier sequence analysis of deletions breakpoints (Figure S4C). These nine deletions may comprise particularly vulnerable regions in the mtDNA genome of mitofusin-deficient mice.

Previous results indicate that fusion-defective cells have mitochondrial populations that are heterogeneous for membrane potential and the presence of mtDNA nucleoids (Chen et al., 2005, 2007). To extend this finding, we examined whether Mfn-double null cells have a generalized heterogeneity in protein content. An unbalanced mitochondrial proteome could lead to mtDNA instability because precise stoichiometries of proteins involved in mtDNA metabolism are likely to be important for mtDNA stability and fidelity. Therefore, we quantitatively analyzed wild-type and Mfn-double null MEFs immunostained for cytochrome c and hsp60, two abundant mitochondrial proteins (Figures S3B and S3C). In the mutant MEFs, the signal intensity ratios for cytochrome c versus hsp60 showed substantially greater variance within the mitochondrial population than in wild-type cells (Figures S3D and S3E). This increased variance was highly significant and resulted in lower correlation coefficients for the two fluorophores in double-mutant MEFs (p = 0.0001, n = 10). Similar heterogeneity was found upon expression of three fluorescent proteins to the mitochondrial matrix (data not shown). These observations imply that mitochondrial proteomes may become unbalanced in the absence of content mixing.

**Synthetic Lethality between an Error-Prone mtDNA Polymerase and Loss of Mfn1**

We expanded our study to examine a separate issue concerning the relationship between mitochondrial dynamics and mtDNA mutations. It has been proposed that mitochondrial fusion might underlie the remarkable ability of mammalian cells to tolerate
high loads of pathogenic mtDNA (Nakada et al., 2001, 2009). To test this idea, we generated Mfn1/C0/C0 mice carrying homozygous alleles of PolgA257A. The PolgA257A knockin allele encodes a mitochondrial DNA polymerase with a deficient proofreading domain, causing cells to accumulate mtDNA mutations at an accelerated rate (Kujoth et al., 2005; Trifunovic et al., 2004). Remarkably, whereas single mutants for either Mfn1 or PolgA survive well into adulthood, the combination of the two mutations leads to a synthetic neonatal lethality (Table 2). Moreover, PolgA257A heterozygous mice, which also have an increased mutation burden (Vermulst et al., 2007), show reduced viability in the absence of Mfn1 as well. The synthetic lethality of PolgA257A/D257A Mfn1/C0/C0 mice is not due to an increased mutation rate because we detected no significant difference in mutation frequency between PolgA257A/D257A and PolgA257A/D257A Mfn1/C0/C0 embryos (Figure S5). It would be interesting to test for a similar synthetic interaction between PolgA257A and deletion of Mfn2. However, this interaction is more difficult to assess because loss of Mfn2 causes a severe neurodegenerative disorder that leads to early lethality (Chen et al., 2007).

To examine this synergism at a cellular level, we established MEFs and measured their respiratory capacity and ATP production. Oxygen polarography demonstrated a profound decrease in the endogenous (4.5% of wild-type) and maximum rates (3.7% of wild-type) of oxygen consumption in PolgA257A/D257A Mfn1/C0/C0 cells compared to controls (Figure 5A). These oxygen consumption rates were tightly correlated with kinetic measurements of ATP production (Figure 5B), with PolgA257A/D257A Mfn1/C0/C0 cells producing only 2.7% the amount of ATP present in wild-type cells. To determine whether specific segments of the respiratory chain are affected, we measured substrate-driven oxygen consumption rates. The most profound defect was found in respiratory complex I (20-fold reduction), with less severe reductions in complexes III (4-fold) and IV (2-fold) (Figure 5C). The severe reduction in complex I is likely to be largely responsible for the overall respiratory decline because the majority of the respiration in these cells occurs via complex I and is sensitive to the complex I inhibitor rotenone. The severity of the complex I defect in PolgA257A/D257A Mfn1/C0/C0 cells may reflect the fact that seven mtDNA-encoded subunits are present in complex I, far more than for any of the other respiratory complexes. Complex I is therefore more likely than the other complexes to be inactivated by an mtDNA mutation due to

### Table 2. Survival of Mice Carrying the PolgA257A Allele in an Mfn1-Deficient Background

<table>
<thead>
<tr>
<th>PolgA Genotype</th>
<th>Mfn1 Genotype</th>
<th>Actual Number of Mice</th>
<th>Actual Frequency (%)</th>
<th>Expected Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/-</td>
<td>+/-</td>
<td>48</td>
<td>7</td>
<td>6.25</td>
</tr>
<tr>
<td>+/-</td>
<td>+/-</td>
<td>81</td>
<td>12</td>
<td>12.5</td>
</tr>
<tr>
<td>+/-</td>
<td>+/-</td>
<td>31</td>
<td>5</td>
<td>6.25</td>
</tr>
<tr>
<td>D257A/+</td>
<td>+/-</td>
<td>99</td>
<td>15</td>
<td>12.5</td>
</tr>
<tr>
<td>D257A/+</td>
<td>+/-</td>
<td>207</td>
<td>32</td>
<td>25</td>
</tr>
<tr>
<td>D257A/+</td>
<td>+/-</td>
<td>51</td>
<td>8</td>
<td>12.5</td>
</tr>
<tr>
<td>D257A/D257A</td>
<td>+/-</td>
<td>34</td>
<td>5</td>
<td>6.25</td>
</tr>
<tr>
<td>D257A/D257A</td>
<td>+/-</td>
<td>102</td>
<td>16</td>
<td>12.5</td>
</tr>
<tr>
<td>D257A/D257A</td>
<td>+/-</td>
<td>1</td>
<td>0</td>
<td>6.25</td>
</tr>
</tbody>
</table>

Mice of the indicated genotypes are tabulated. A Chi-square test was used to compare observed and expected populations. *p = 0.0003, **p < 0.0001.
error-prone PolgA<sup>D257A</sup> function. These results underscore the importance of an intermixing mitochondrial network to ameliorate the damaging effects of mtDNA mutations.

**DISCUSSION**

Our studies provide insight into the in vivo functions of mitochondrial fusion in mammals. Mitochondrial fusion plays an essential role in skeletal muscle fibers, in spite of its compact subcellular architecture and precise placement of mitochondria. In the absence of mitochondrial fusion, a pathological profile emerges that bears striking similarities to human mitochondrial myopathies, particularly those associated with mtDNA depletion syndromes (Copeland, 2008).

Our studies indicate that mitochondrial fusion safeguards mtDNA function through three distinct pathways. First, mitochondrial dynamics maintains cellular health by stabilizing mtDNA copy number. In budding yeast, loss of the mitofusin Fzo1 results in rapid and complete loss of mtDNA (Hermann et al., 1998; Rapaport et al., 1998), resulting in absence of respiratory function. In contrast, deletion of mammalian mitofusins or OPA1 results in loss of mtDNA nucleoids from only a subpopulation of mitochondria (Chen et al., 2007). Here, we show that this unequal distribution of nucleoids is caused, at least in part, by long-term mtDNA depletion. The dramatic reduction of mtDNA levels to 7% of wild-type levels at 2 months is likely a key cause for the mitochondrial myopathy observed in mitofusin-deficient mice. Consistent with a causative role for mtDNA depletion, the reduction in mtDNA levels is found prior to overt histological evidence for muscle fiber dysfunction. Moreover, whereas wild-type muscle shows a rapid postnatal increase in mtDNA from weeks 1 to 7, mitofusin-deficient muscle fails to show increased mtDNA levels (Figure 3), suggesting a possible defect in mtDNA replication. For comparison, MLC-Cre/Tfam mice—which lack a key DNA-binding protein involved in mtDNA packaging, transcription, and replication—retain greater than 30% mtDNA levels at 4 months of age (Wredenberg et al., 2002). The MLC-Cre/dm mice therefore provide an animal model for human mtDNA depletion syndromes associated with mitochondrial myopathy (Copeland, 2008). We do not, however, think that mtDNA depletion solely accounts for the mitochondrial dysfunction in mutant muscle; it is likely that the inability to mix mitochondrial contents further undermines mitochondrial function.

Second, we discovered that loss of mitochondrial fusion in skeletal muscle leads to an increase in mtDNA point mutations and deletions. In double-mutant mice, the absolute levels of mutations are too low to account for the severe physiological defects. For comparison, double-mutant mice have a several-fold increase in mtDNA point mutations, whereas <em>PolgA<sup>D257A/D257A</sup></em> mutator mice have a 2–3 orders-of-magnitude increase (Vermulst et al., 2007). In old <em>Mfn1</em> mutant mice, mtDNA deletion levels are comparable to those of <em>PolgA<sup>D257A/D257A</sup></em> mice (Vermulst et al., 2008a). Although their relationship to the physiological phenotypes is unclear, these substantial increases in point mutations and deletions demonstrate the importance of mitochondrial fusion for mtDNA fidelity. It is interesting to note that some cases of dominant optic atrophy caused by dysfunction of the mitochondrial fusion gene OPA1 are associated with respiration-deficient muscle fibers and accumulation of mtDNA deletions (Amati-Bonneau et al., 2008; Hudson et al., 2008).

This accumulation of mtDNA mutations could be driven by multiple mechanisms. In principle, the accumulation of mtDNA mutations can be due to an increase in mtDNA damage, a failure to repair damaged mtDNA, or perhaps a failure to clear mitochondria with damaged mtDNA. Our finding that Mfn-double null cells have great protein heterogeneity, from one mitochondrion to another, provides a plausible mechanism that may contribute to each of these processes. If protein stoichiometries...
are improperly balanced, protein complexes essential for mtDNA replication, maintenance, repair, and clearance may operate inefficiently or with lower fidelity. We anticipate that additional mechanisms leading to mtDNA instability will be uncovered with future studies.

The demonstration of a link between mitochondrial fusion and mtDNA fidelity raises the issue of whether mitochondrial fusion plays a protective role in conditions associated with mtDNA mutations. For instance, age-related neurodegeneration and muscle atrophy are closely associated with mtDNA mutations (Chomyn and Attardi, 2003; Krishnan et al., 2007). It will be interesting to examine whether mitochondrial fusion in humans is a modifying factor that affects the rate at which mtDNA mutations occur.

Finally, our finding that loss of Mfn1 is incompatible with an error-prone mtDNA polymerase suggests that mitochondrial fusion can dynamically dampen the deleterious effects of pre-existing mtDNA mutations to preserve respiratory function. The synthetic lethality of mice containing existing mtDNA mutations to preserve respiratory function. The error-prone mtDNA polymerase suggests that mitochondrial factor that affects the rate at which mtDNA mutations occur.

Cre, Mfn1loxP/loxP, Mfn2loxP/loxP, PolgAD257A/loxP/loxP mice. The Mfn1 mutant (Chen et al., 2003, 2007), Mfn2 mutant (Chen et al., 2003, 2007), PolgAD257A mutant (Kujoth et al., 2005), MLC-Cre (Bothe et al., 2000), and Mecox2-Cre mice (Tallquist and Soriano, 2000) have been described previously.

Physiological measurements were performed on 7- to 8-week-old mice. Body temperature was measured with a rectal probe, using the Thermalert Monitoring Thermometer, TH-5 (Braintree Scientific). Blood glucose levels were measured using one drop of blood from the tail vein with the OneTouch Ultra2 Blood Glucose Monitoring System (LifeScan). To obtain lactate measurements, eye bleeds were performed on mice anesthetized with avertin, and the blood was analyzed in CG4+ cartridges with the iSTAT system (Abbott). Mice were exercised by swimming for 20 min at ~37°C before being anesthetized. All experiments were approved by the Institutional Animal Care and Use Committee at Caltech.

Histological and EM Analysis
TA muscle was used for histological analysis, as this muscle contains a high percentage of fast-twitch fibers that express MLC-Cre. For COX/SDH staining, freshly dissected muscle was embedded in optimal Cutting Temperature (OCT) compound (Tissue-Tek) and frozen in liquid nitrogen. Slides were stained for COX activity, washed in water, stained for SDH activity, washed, and mounted in GelMount (Biomeda). Staining protocols were obtained from the Washington University Neuromuscular Disease Center website (http://www.neuro.wustl.edu/neuromuscular/index.html).

For EM, mice were perfused with 3% paraformaldehyde, 1.5% glutaraldehyde, 100 mM cacodylate (pH 7.4), and 2.5% sucrose. The TA was immobilized in an outstretched position by tying onto a toothpick splint prior to excision. The splint-attached muscle was postfixed for 1 hr and then processed and imaged as described previously (Chen et al., 2007).

For analysis of muscle fiber types, fresh-frozen TA muscle was transversely cryosectioned and immunostained with mouse IgG1 anti-MHC2A (ATCC) and 525 goat-anti-mouse (Jackson ImmunoResearch) or Alexa Fluor 555 goat-anti-mouse IgG (Invitrogen) were used as secondary antibodies.

Cell Culture and Respiration Measurements
Previously established MEFs were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with either 10% fetal bovine serum (Mfn-double null and OPAT null cells) or 10% bovine calf serum (wild-type, Mfn1-/-, and Mfn2-/- cells). MEFs for polyomavirus were derived from embryonic day (E) 12.5 embryos, infected with a retrovirus expressing SV40 large T antigen to promote immortalization, and maintained in DMEM, 10% fetal bovine serum, 1 mM pyruvate, and 50 μg/ml uridine. Oxygen consumption was measured in intact cells using a Clark oxygen electrode (Oxygraph, Hansatech Instruments), as described previously (Chen et al., 2005; Villani and Attardi, 2001). Substrate-driven oxygen consumption rates were measured essentially as described (Duan et al., 2003) with the following modifications. The first wash in Respiration buffer I was omitted, succinate and glyceral-3-phosphate were used to drive respiration through complex II, and N.N.N.’-tetramethyl-p-phenyldiamine dihydrochloride (TMPD)-driven respiration was measured in Respiration buffer II instead of Respiration buffer I. The substrate and inhibitor concentrations were as follows: 5 mM glutamate, 5 mM malate, 200 nM rotenone, 5 mM succinate, 5 mM glycerol-3-phosphate, 12 nM antimycin, 400 μM TMPD, and 10 mM ascorbate.

For measurement of ATP production, cells were grown to 80% confluency, harvested, permeabilized with 50 μg/ml digitonin for 1 min, and resuspended in reaction buffer (150 mM KCl, 25 mM Tris-HCl, 0.4 mM EDTA, 0.1% BSA, 10 mM potassium phosphate, 0.5 mM MgCl₂, and 0.15 mM P1,P5-di(adeno-sine) pentaphosphate). After addition of 0.25 mM Tris-acetate, 40 nM luciferin, and 0.25 mM ATP, the luminescence was measured in a Tecan Infinite M200 luminescence meter with a ×10 optical cells per measurement, in the presence of 1 mM malate and 1 mM pyruvate, with and without 2 μg/ml rotenone. The ATP measurements in the presence of rotenone were subtracted to give ATP production via complex I.

Experimental Procedures

Mouse Breeding and Physiological Experiments
MLC-Cre/dm mice and littermate controls were bred by crossing MLC-Cre,Mfn1loxP/loxP,Mfn2loxP/loxP mice. PolgA,Mfn1-

animals were generated by crossing PolgAD257A-/+ , Mecox2-Cre,Mfn1loxP/loxP mice to PolgAD257A-/+ , Mfn1loxP/loxP mice. The Mfn1 mutant (Chen et al., 2003, 2007), Mfn2 mutant (Chen et al., 2003, 2007), PolgAD257A mutant (Kujoth et al., 2005), MLC-Cre (Bothe et al., 2000), and Mecox2-Cre mice (Tallquist and Soriano, 2000) have been described previously.
DNA Isolation
To isolate mitochondrial DNA, cells or tissue were first homogenized and a mitochondrial fraction was isolated according to previously published protocols (Frezza et al., 2007). Mitochondria were then lysed in the presence of 0.5% SDS and 0.2 mg/ml protease K in 10 mM Tris-HCl, 0.15M NaCl, and 0.005M EDTA (Vermulst et al., 2008b). mtDNA was then purified by phenol/chloroform extraction and ethanol precipitation. Total DNA was isolated using standard protocols.

mtDNA Quantitation
To quantify the amount of mtDNA present on each nuclear genome, we used the following primers:
- mtDNA forward primer, CTTACATCCTTGGGATCATAT
- mtDNA reverse primer, GAAGCTTGCTGTCCTGAC

To quantify nuclear DNA, we used a primer set that detects the Pecam gene on chromosome 6:
- nuclear DNA forward primer, ATGGAAAGCCTGCACTGAT
- nuclear DNA reverse primer, TCCTGTGCTGCAGCATCAC

Quantification of relative copy number differences was carried out using both analysis of the difference in threshold amplification between mtDNA and nuclear DNA (delta delta Ct) method and analysis with a standard curve of a reference template. Both methods provided identical results.

mtDNA Mutation Assays
The random mutation capture assays were performed as previously described (Vermulst et al., 2008b). Briefly, mtDNA was digested with TaqI for 5 hr, with the addition of 100 units of TaqI every hour. mtDNA was then diluted in a 96 well format and probed with primers flanking the TaqI restriction site in order to detect mtDNA genomes that contain a mutation in the TaqI restriction site. A second pair of primers was used to determine the amount of mtDNA genomes that was interrogated. PCR steps were as follows: step 1, 37°C for 10 min; step 2, 95°C for 10 min; step 3, 95°C for 30 s; step 4, 58/60°C for 1 min; step 5, 72°C for 1.5 min; step 6, go to step 4 35 times; step 7, 72°C for 4 min; step 8, melting curve from 65°C to 95°C; step 9, hold at 4°C indefinitely. PCR reactions were carried out in 25 μl reactions using the Brilliant SYBR-green I master mix (Stratagene), containing 10 pM of forward and reverse primer and 1 unit of uracil DNA glycosylase. The following primers were used for mtDNA amplification:
- mtDNA control primer forward, CCTATCACCCTTGGGATCATAT
- mtDNA control primer reverse, GAAGCTTGCTGTCCTGAC

mtDNA deletion reverse site 1, GCTTCCGAATGCTAGGCGTT
- mtDNA deletion forward site 1, CAAGGCCACCACACTCCTAT
- mtDNA primer flanking Taq634 forward, ACTCAAAGGACTTGGCGGTA
- mtDNA primer flanking Taq634 reverse, AGCCCATTTCTTCCCATTTC
- mtDNA control primer reverse, GAGGCTGTTGCTTGTGTGAC
- mtDNA control primer forward, CCTATCACCCTTGGGATCATAT
- mtDNA primer flanking Taq7667 forward, AATTTCATCTGAAGACGTCCTC
- mtDNA primer flanking Taq7667 reverse, AACGCTCTTAGCTTCATAGTGA
- mtDNA deletion reverse site 2, GAGAGATTTTATGGGTGTAATGC
- mtDNA deletion forward site 2, ACTGACTTCCAATTAGTAGATTCTG

Solexa sequencing was performed at the Caltech Genomics Facility. Libraries of mtDNA were constructed according to the manufacturer’s instructions, and paired-end sequence reads of 75 nucleotides were obtained. To identify candidate deletions, each sequence read was trimmed to 70 nucleotides, and the 15 nucleotides at each end were mapped using the program Bowtie to the mouse mtDNA genome. Sequence reads with ends mapping to separate regions of the mtDNA genome were retrieved as candidate deletions. With this algorithm, deletions occurring within the central 40 nucleotide region of a sequence read could be identified. The candidate deletions were then manually verified. To minimize the false-positive rate, only deletions identified by four or more independent reads were included in the analysis.

SUPPLEMENTAL INFORMATION
Supplemental Information includes five figures and one table and can be found with this article online at doi:10.1016/j.cell.2010.02.026.

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Figure S1. Mitochondrial Defects in Four-Week-Old MLC-Cre/dm Muscle, Related to Figure 1
Electron micrographs of longitudinal TA muscle from control (A) and MLC-Cre/dm mice (B). Areas of abnormal mitochondrial proliferation are labeled in (B). m, mitochondria.
Figure S2. Analysis of Fiber Type Identity, Related to Figure 2

Transverse frozen sections from wild-type and MLC-Cre/dm tibialis anterior muscle were stained with anti-MHC2A or anti-MHC2B monoclonal antibodies to detect oxidative type IIA fibers and fast-twitch type IIB fibers, respectively.
Figure S3. Quantitative Analysis of mtDNA from 8- to 13-Month TA Muscle and Protein Heterogeneity in Fusion-Deficient Cells, Related to Figure 3

(A) Mitochondrial copy number per nuclear genome. Genotypes are indicated in the legend. Error bars indicate standard deviations. No significant differences were found.

(B–E) Protein heterogeneity in Mfn-double null cells. Wild-type (B) and Mfn-double null (C) cells were immunostained against cytochrome c (Alexa Fluor 488, green) and hsp60 (Alexa Fluor 647, red). Nuclei are stained with DAPI (blue). Representative images are shown. In (D) and (E), each pixel in (B) and (C), respectively, is plotted to display the correlation between the cytochrome c (Channel 1) and hsp60 (Channel 2) signal intensities. Note that pixels in Mfn-double null cells are less tightly correlated: in the sector labeled 3, the spread of pixels located on both sides of the yellow line (where pixels with equal intensities in the two channels would lie) is greater. The plots in (D) and (E) were generated with the colocalization module in the imaging software Zen (Carl Zeiss Microimaging). The axes have arbitrary units.
Figure S4. Mutational Analysis of Wild-Type and Mutant Mice, Related to Figure 4

(A) Point mutation frequency per base pair. Error bars indicate standard deviations. No significant differences were found.

(B) Mutational spectrum compiled from sequence analysis of mutations from TA muscle of the indicated genotypes. Single molecules containing an mtDNA-point mutation were PCR-amplified and directly sequenced. The predominant mutation in all genotypes is a C to T transition. 108 mutations from wild-type mice, and 81 mutations from MLC-Cre/dm mice were sequenced and tabulated. Indel: either small insertion or deletion.

(C) Single molecules containing an mtDNA-deletion were PCR-amplified, and the breakpoints were sequenced. Most deletions recovered from each genotype had breakpoints that contained more than 6 base pairs of homology. This class includes the 4 kb common deletion. The number of sequences of each genotype are as follows: WT, 26; Mfn1+/−Mfn2+/−, 45; Mfn1+/−Mfn2+/−, 41; dm, 24.
Figure S5. Quantitative Mutational Analysis of Embryos, Related to Figure 5

Point mutation frequencies per base pair were measured in embryos of the indicated genotypes. Error bars indicate standard deviations from three experiments. No significant difference was found between the PolgA<sup>D257A/D257A</sup> and PolgA<sup>D257A/D257A</sup> Mfn1<sup>−/−</sup> samples.