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Disruption of Fusion Results in Mitochondrial Heterogeneity and Dysfunction*[S]

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Mitochondria undergo continual cycles of fusion and fission, and the balance of these opposing processes regulates mitochondrial morphology. Paradoxically, cells invest many resources to maintain tubular mitochondrial morphology, when reducing both fusion and fission simultaneously achieves the same end. This observation suggests a requirement for mitochondrial fusion, beyond maintenance of organelle morphology. Here, we show that cells with targeted null mutations in *Mfn1* or *Mfn2* retained low levels of mitochondrial fusion and escaped major cellular dysfunction. Analysis of these mutant cells showed that both homotypic and heterotypic interactions of Mfns are capable of fusion. In contrast, cells lacking both Mfn1 and Mfn2 completely lacked mitochondrial fusion and showed severe cellular defects, including poor cell growth, widespread heterogeneity of mitochondrial membrane potential, and decreased cellular respiration. Disruption of *OPA1* by RNAi also blocked all mitochondrial fusion and resulted in similar cellular defects. These defects in Mfn-null or OPA1-RNAi mammalian cells were corrected upon restoration of mitochondrial fusion, unlike the irreversible defects found in *fzo*Δ yeast. In contrast, fragmentation of mitochondria, without severe loss of fusion, did not result in such cellular defects. Our results showed that key cellular functions decline as mitochondrial fusion is progressively abrogated.

Mitochondrial dynamics involves the two opposing processes of fusion and fission (1, 2). In cultured cells, an equilibrium is achieved between the two processes, and thus a relatively stable but dynamic morphology is maintained. In mouse embryonic fibroblasts (MEFs),[1] for example, a tubular network of mitochondria normally predominates. If fusion is inhibited, however, unopposed fission leads to mitochondrial fragmentation (3).

In mammalian cells, the large mitochondrial GTPases Mitofusin 1 (Mfn1), Mitofusin 2 (Mfn2), and OPA1 play important roles in mitochondrial fusion (3–8). In humans, mutations in *Mfn2* cause Charcot-Marie-Tooth neuropathy type 2A (9), and mutations in *OPA1* cause dominant optic atrophy (10, 11). Mice lacking either of the mitofusins die as embryos, with Mfn2-null mice showing placental insufficiency (3). To understand these diverse tissue-specific phenotypes, it will be critical to elucidate the relative importance of Mfn1, Mfn2, and OPA1 in specific cell types. In particular, Mfn1 and Mfn2 appear to play similar roles in mitochondrial fusion, but the degree of redundancy between these homologs and whether they can cooperate in mitochondrial fusion remains to be determined.

On a cellular level, the best known function of mitochondrial fusion is to maintain mitochondrial morphology (12, 13). Given the highly ordered arrangement of mitochondrial networks in some cell types (14), it is likely that mitochondrial shape indeed has important consequences for mitochondrial function. Nevertheless, there are clues that mitochondrial fusion has additional functions beyond regulation of morphology. First, the *in vivo* rates of mitochondrial fusion and fission far exceed those necessary to maintain tubular mitochondrial morphology. Cells with greatly reduced rates can have tubular mitochondria, as long as the fusion and fission rates are appropriately balanced (3). Second, in Mfn1-null or Mfn2-null cells, a small subset of mitochondria lose their membrane potential ($\Delta\Psi$) (3). We have hypothesized that mitochondrial dynamics protects mitochondria by ensuring that stochastic depletion of materials, such as metabolic substrates or mitochondrial DNA (mtDNA), is transient. Without fusion, however, these deficiencies persist, accumulate, and result in cellular dysfunction.

In this study, we examined the dependence of mitochondrial fusion on Mfn1, Mfn2, and OPA1 and demonstrated that cells lacking all mitochondrial fusion show defects in key cellular functions. Fusion-defective cells have dramatically reduced growth rates, reduced respiration, and a highly heterogeneous mitochondrial population prone to loss of $\Delta\Psi$.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture—To generate Mfn-dm cells, MEFs were first derived from *Mfn1*−/−, *Mfn2*-/conditional embryos (carrying one copy of a *loxP*-flanked conditional allele of *Mfn2*), as described previously (3). Transient transfection with a Cre-enhanced GFP expression plasmid, followed by cell sorting for Cre-GFP expression, was used to isolate Mfn-dm cells. The genotypes of all mutant cells were confirmed by PCR analysis. Mfn-dm and OPA1-RNAi lines were derived and maintained in rich medium (Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum, 1 mM pyruvate, 50 μg/ml uridine). All other cell lines were maintained in standard medium (Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum). All experiments involving retroviral infection were conducted on both freshly infected bulk cultures and multiple clonal lines.

Mitochondrial $\Delta\Psi$ was monitored, as described previously (3), except that mitochondria were detected by expression Su9-GFP (enhanced GFP with the mitochondrial presequence of subunit 9 of the F_0-

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[1] The abbreviations used are: MEF, mouse embryonic fibroblast; Mfn, mitofusin; Mfn-dm, mitofusin double mutant; OPA1-OE, OPA1 overexpressing; RNAi, RNA interference; $\Delta\Psi$, membrane potential; mtDNA, mitochondrial DNA; shRNA, short hairpin RNA; GFP, green fluorescent protein; PEG, polyethylene glycol.

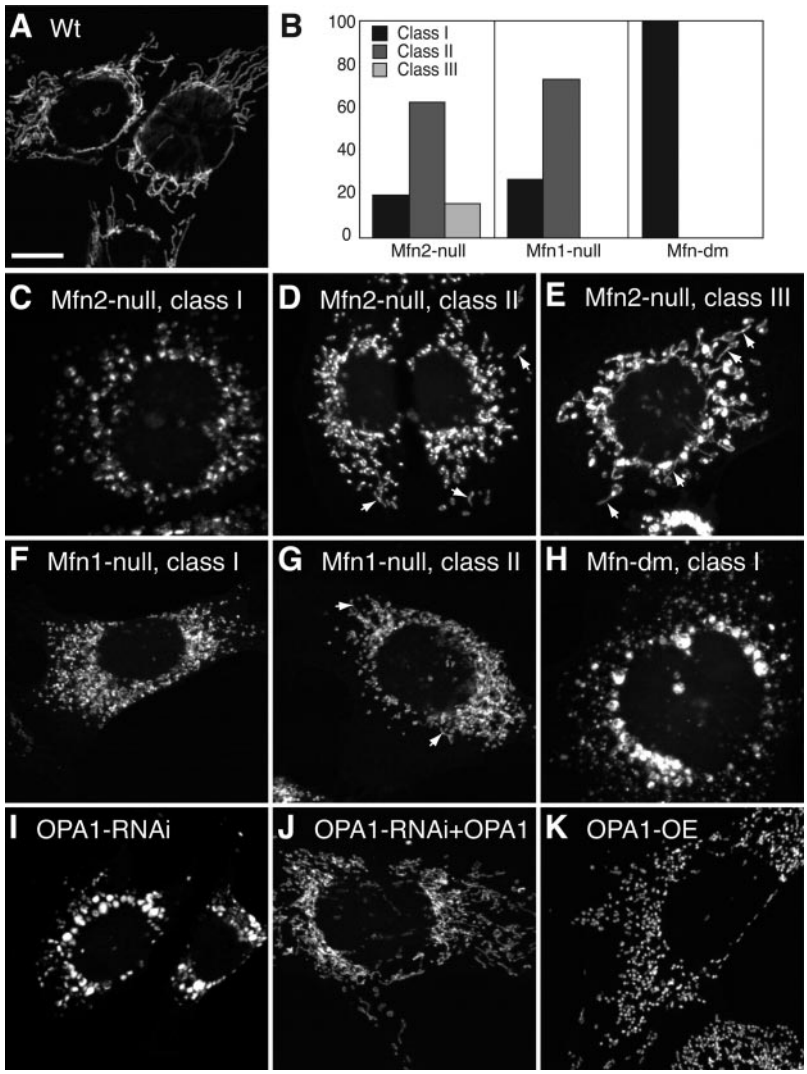

FIG. 1. **Mitochondrial morphology.** *A*, confocal image of GFP-labeled mitochondria in wild-type cells. *B*, bar graph showing differences in mitochondrial morphologies in Mfn2-null, Mfn1-null, and Mfn-dm cells. 200 cells were counted for each genotype. *C–K*, mitochondrial morphology in Mfn2-null (*C–E*), Mfn1-null (*F* and *G*), Mfn-dm (*H*), OPA1-RNAi (*I*), OPA1-RNAi cells expressing an RNAi-resistant construct of OPA1 (*J*), and OPA1-OE (*K*) cells. *Scale bar* represents 10 μm.

ATPase). Z-stacks of images acquired on a Zeiss Axiovert 200 M microscope were deconvolved using the iterative algorithm in AxioVision 4.2 and presented as maximum intensity projections.

For cell growth measurements, parental control cultures were split in two and either mock-infected or infected with the experimental virus. Cells were then plated onto two 6-well plates, at 6×10^4 cells/well in their respective growth media. 4 h later, the cells were counted, in triplicate, to constitute the day 0 score. At this time, the medium in the remaining 9 wells of each cell line was changed to standard medium. The cells were then counted, in triplicate, on days 2, 3, and 4.

Short Hairpin RNA (shRNA) Construction—For RNA interference (RNAi) studies, we constructed retroviral vectors expressing short hairpin RNAs from the human H1-RNA promoter. The H1-RNA promoter was cloned into pBKSII (Stratagene), as described previously (15). The pMND-BANSHEE vector carrying GFP (gift of Drs. G. Hernandez-Hoyos and J. Ila-Alberola, California Institute of Technology) and pMND-BANSHEE vector carrying Neo(R) (gift of Dr. J. Rossi, City of Hope, CA) were digested with BglII and StuI. The H1 promoter was then inserted as a BamHI/HincII fragment to make the retroviral vectors pBG and pBN, respectively. shRNA constructs were then inserted as annealed oligonucleotides into BglII/HindIII sites. An RNAi-resistant *OPA1* construct was made by altering codons for residues 13–18 (from gtc tgc caa tcc tta gtg to gtg tgt cag tcg ttg gta). Retrovirus was prepared from these plasmids and used to infect MEFs, as described previously (3). The following oligonucleotides were used: anti-OPA1-f, 5′-gatccccgtctgtcaatccttagtgattcaagagatcactaaggattggcagacttttggaaa-3′; anti-OPA1-r, 5′-agcttttccaaaaagtctgccaatccttagtgatctcttgaatcactaaggattgacagacgggg-3′; anti-Mfn1-f, 5′-gatccccggtatctctactgaagcattcaagagatgcttcagtggagatacgtttttggaaa-3′; anti-Mfn1-r, 5′-agcttttccaaaaacggtatctccactgaagcatctcttgaatgcttcagtagagataccgggg-3′.

Antibodies—Standard Western analysis and immunofluorescence were conducted using the following antibodies: anti-OPA1 (gift of Drs. L. Griparic and A. van der Bliek, UCLA), anti-Mfn1 (3), anti-β-actin (Sigma, clone AC-15), and anti-Myc (9E10 hybridoma).

PEG Fusion Assay—PEG fusion was performed, as described previously (3), except that cells were plated and grown in rich medium. Cell fusion was allowed to proceed for 7 h in the presence of cycloheximide. Pre-incubation with cycloheximide for 1–8 h did not affect the fusion results. To evaluate outer membrane fusion in OPA1-RNAi cells, cells expressing the mitochondrial outer membrane marker Mfn1(K88T)-Myc (3) were used. The marker was detected by immunofluorescence against the Myc epitope. Mfn1(K88T)-Myc is a non-functional Mfn1 that has no dominant negative effects on mitochondrial morphology. The cells were imaged with a Plan NeoFluar 63× objective on a Zeiss 410 laser confocal microscope using AxioVision software (Carl Zeiss MicroImaging, Inc.).

Oxygen Electrode Polarography—Oxygen consumption was measured in intact cells using a Clark oxygen electrode (Oxygraph, Hansatech Instruments), as described previously (16). In brief, 2×10^6 cells in 500 μl of TD buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 25 mM Tris-HCl, pH 7.4) were added to the chamber, and mixing was started. After 1 min of equilibration, the chamber was stoppered. Endogenous respiration was measured as the average oxygen consumption over a period of 1 min, starting 2 min after cap closure. At ~4.5 min, 2,4-dinitrophenol (83 mM final concentration) was injected into the chamber, and the maximum respiration rate was measured for 1 min starting 0.5 min after injection. Substrate-driven respiration rates were measured in digitonin-permeabilized cells, as described previously (17).

RESULTS

Comparison of Mitochondrial Morphologies in Cells Lacking Mitofusins or OPA1—Using targeted mutagenesis, we previ-

TABLE I
PEG fusion assay
250 cell fusion events were counted for each genotypic pair. Percentages are shown in parentheses.

Cells	Full fusion	Partial fusion	No fusion
Wild-type and wild-type	242 (97)	7 (3)	1 (0)
Mfn-dm and Mfn-dm	0 (0)	0 (0)	250 (100)
Mfn1-null and Mfn1-null	1 (0)	235 (94)	14 (6)
Mfn2-null and Mfn2-null	36 (14)	212 (85)	2 (1)
Mfn2-null and Mfn1-null	16 (6)	230 (92)	4 (2)
OPA1-RNAi and OPA1-RNAi	0 (0)	0 (0)	250 (100)
OPA1-OE and OPA1-OE	245 (98)	5 (2)	0 (0)

ously reported that Mfn1-null, Mfn2-null, and Mfn double mutant (Mfn-dm) cells have fragmented mitochondria, because of perturbations in mitochondrial fusion (3, 18). However, it remains unclear to what degree these homologs are functionally redundant and whether they cooperate to mediate normal levels of mitochondrial fusion.

To address these issues, we performed a comparative analysis of mitochondrial morphology and fusion in the mutant cell lines. Although MEFs of all three genotypes (Mfn1-null, Mfn2-null, and Mfn-dm) showed extensive mitochondrial fragmentation, they could be readily distinguished. We found that all mutant cells could be scored into three categories. Class I cells showed complete fragmentation resulting in only mitochondrial spheres. Class II cells showed extensive fragmentation but contained some very short mitochondrial rods (<5 μm in length). Cells with any medium-length mitochondrial tubules (>5 μm in length) were placed in Class III, regardless of whether accompanying fragmentation was present. Representative images of these classes are shown in Fig. 1 for each of the mutant MEFs. None of these three phenotypic classes approaches the tubular network typically found in wild-type cells (Fig. 1*A*).

Mfn2-null cells (Fig. 1, *B–E*) showed all three phenotypic classes. 20% showed no mitochondrial rods (Class I). Most (>60%) contained a few short rods in addition to mitochondrial spheres (Class II), and 16% contained medium-length tubules (Class III). In contrast, Mfn1-null MEFs (Fig. 1, *B*, *F*, and *G*) showed more severe mitochondrial fragmentation, with 27% of cells showing only small mitochondrial spheres (Class I). Mfn1-null cells of Class I could be distinguished from Mfn2-null cells of Class I by the smaller diameter of the Mfn1-null mitochondrial spheres. The majority (73%) of Mfn1-null cells showed a combination of small spheres and very short rods (Class II). No Mfn1-null cells contained Class III morphology. The presence of more rod-shaped mitochondria in Mfn2-null cells suggests that cells containing only Mfn1 retain more fusion activity than cells containing only Mfn2.

Notably, all Mfn-dm cells (Fig. 1, *B* and *H*) belonged to Class I and never showed significant mitochondrial rods or tubules. However, in contrast to Mfn1-null or Mfn2-null cells of Class I, Mfn-dm cells showed prominent heterogeneity in the mitochondrial population, characterized by a mixture of large and small spheres. Fig. 1 summarizes the scoring of these mutant cells and shows how even cells scored into the same class have distinctive mitochondrial morphologies, depending on their genotype.

We also analyzed the mitochondrial morphology of cells with perturbed expression of OPA1, another protein implicated in mitochondrial dynamics (4–6). OPA1-deficient cells (termed OPA1-RNAi) were created by stably infecting wild-type MEFs with retrovirus encoding short hairpin RNA (shRNA) directed against *OPA1*. This shRNA sequence targets all isoforms of OPA1, and indeed, OPA1-RNAi cells had <10% of wild-type OPA1 protein levels (supplemental Fig. S1). Wild-type cells overexpressing a Myc-tagged OPA1 were also established (termed OPA1-OE). Similar results were obtained with untagged OPA1.[2]

As previously described, both overexpression and underexpression of OPA1 lead to mitochondrial fragmentation (4–6). However, we found that the mitochondrial morphologies of the two types of cells could nevertheless be easily distinguished. OPA1-OE cells (Fig. 1*K*) had uniform, small mitochondrial spheres that were spread throughout the cytoplasm. In contrast, OPA-RNAi cells (Fig. 1*I*) contained mitochondria that were highly heterogeneous in size, a phenotype similar to that of Mfn-dm cells. Both OPA1-RNAi and Mfn-dm cells typically had some very large mitochondrial spheres (several microns in diameter) accompanied by a scattering of very small mitochondrial fragments. For both Mfn-dm (18) and OPA1-RNAi cells (Fig. 1*J*), this mitochondrial fragmentation was reversible, because re-expression of the corresponding gene restored the tubular network of mitochondria.

Requirements for OPA1 and Mitofusins in Fusion—The different types of mitochondrial fragmentation observed could be explained by differences in the degree of residual mitochondrial fusion. To test this hypothesis, we used the PEG cell fusion technique to quantitate mitochondrial fusion in our mutant cell lines (Table I). In each case, 250 cell hybrids were scored for mitochondrial fusion. Consistent with previous results (18), wild-type cells demonstrated complete fusion of all their mitochondria in the vast majority (97%) of cell fusion events after 7 h (Table I; Fig. 2*A*), whereas Mfn-dm cells showed absolutely no fusion (Table I), even after 24 h.[2]

In contrast, we detected extensive, although incomplete, fusion in cells containing only Mfn1 or Mfn2. Cell hybrids containing only Mfn2 (Mfn1-null cells; Table I) usually showed unambiguous and extensive partial fusion (94% of cell hybrids), although full fusion was found in only one hybrid of 250. Most cell hybrids containing only Mfn1 (Mfn2-null cells; Table I) showed partial fusion (85%), but a significant fraction even showed full fusion (14%). These residual levels of fusion activity likely explain the presence of mitochondrial rods in Mfn1-null and Mfn2-null cells and again suggest that, in fibroblasts, Mfn1 has a more prominent role in mitochondrial fusion than Mfn2. Thus, the mitofusins are partially redundant in that a single Mfn homolog is capable of mediating substantial levels of fusion. Because mitofusins have been shown to be required on adjacent mitochondria to mediate fusion (18), these results indicate that homotypic Mfn interactions (Mfn1-Mfn1 and Mfn2-Mfn2) can promote fusion.

Notably, cell hybrids between Mfn1-null and Mfn2-null cells also had significant fusion, with 92% showing partial fusion and 6% showing full fusion (Table I; Fig. 2*B*). This result indicates, for the first time, that heterotypic Mfn interactions (Mfn1-Mfn2) can promote fusion at endogenous expression levels.

A previous OPA1 RNAi study shows that a reduction of

[2] H. Chen and D. Chan, unpublished results.

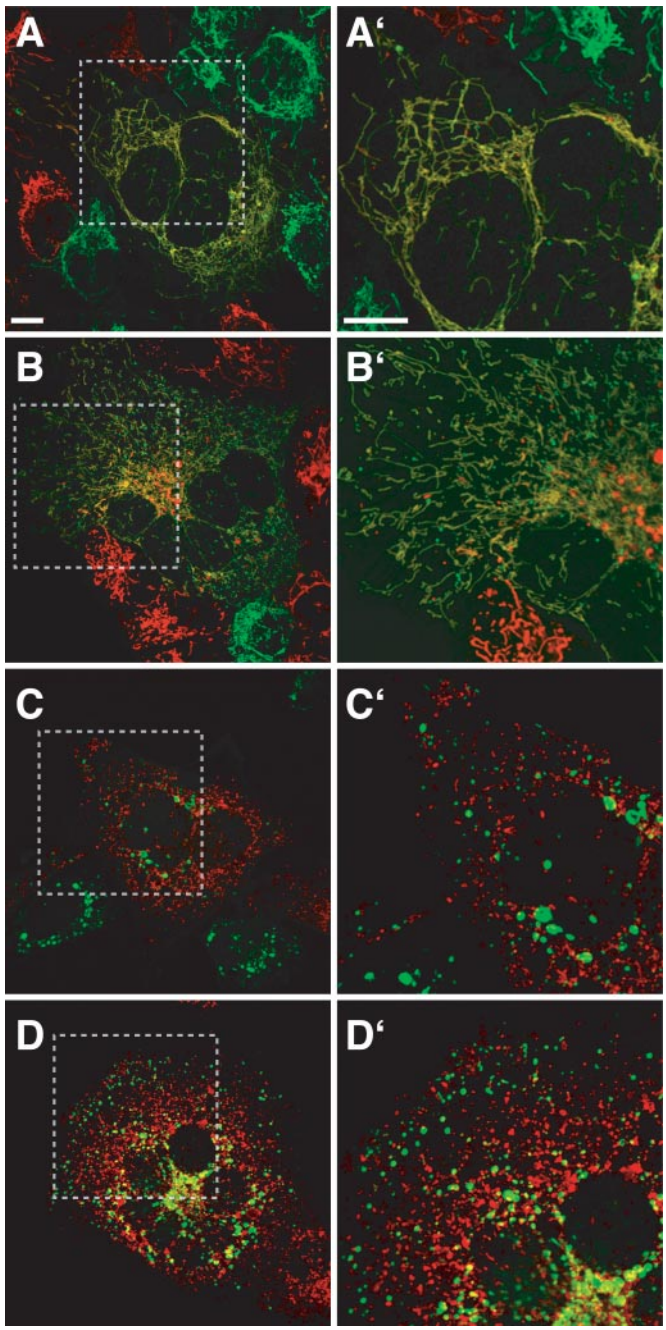

FIG. 2. **Mitochondrial fusion in mutant cells.** PEG fusion of cells containing mitochondrially targeted red fluorescent protein from *Discosoma* (DsRed) and GFP. Cell hybrids were formed between cells with the following genotypes: wild-type to wild-type (*A*), Mfn2-null to Mfn1-null (*B*), OPA1-RNAi to OPA1-RNAi (*C*), OPA1-RNAi (outer membrane marker, *red*) to OPA1-RNAi (matrix marker, *green*) (*D*). *A′–D′*, magnified view of corresponding *boxed area* in the *left panel*. *Scale bars* represent 10 μm.

OPA1 levels to 30% of wild-type results in reduction of mitochondrial fusion by 33% (6). Because of the inefficiency of this RNAi experiment, it was not possible to conclude whether OPA1 is absolutely essential for mitochondrial fusion or simply enhances the efficiency of fusion. We addressed this issue with our OPA1-RNAi cells, which had barely detectable levels of OPA1 (supplemental Fig. S1). In PEG fusion assays, OPA1-RNAi cells were devoid of any mitochondrial fusion (Table I; Fig. 2*C*), even after 24 h.[2] This complete fusion defect likely explains the similar morphological defects in mitochondria in OPA1-RNAi and Mfn-dm cells. Moreover, these results definitely demonstrated that OPA1, like mitofusins, are strictly required for mitochondrial fusion.

Because our standard PEG fusion assay relied on mitochondrial matrix markers, we also used an outer mitochondrial membrane marker to test whether outer membrane fusion is abolished in OPA1-RNAi cells. Again, no mitochondrial fusion was observed (Fig. 2*D*), indicating that both outer and inner membrane fusion require OPA1.

In contrast, OPA1-OE cells fused their mitochondria as efficiently as wild-type cells (Table I), indicating that the mitochondrial fragmentation observed in these cells was not due to the lack of fusion. We also found normal levels of fusion at earlier time points (supplemental Table S1), eliminating the possibility that OPA1-OE cells have a subtle fusion defect. In previous studies of mitochondrial fragmentation by OPA1 overexpression (5, 19), it has been unclear whether fragmentation is because of reduced fusion or increased fission. Our findings suggest the latter possibility. Consistent with this possibility, we found that mitochondrial fragmentation due to OPA1 overexpression was blocked when the fission molecule Drp1 was down-regulated by RNAi.[2]

Inhibition of Cell Growth—In addition to defects in mitochondrial morphology, we noticed that both OPA1-RNAi and Mfn-dm cells grow significantly more slowly than wild-type MEFs. Because cell growth is a general indicator of cell function, we quantitated this growth defect by monitoring cell proliferation over the course of 4 days. Mfn2-null cells depleted of Mfn1 by shRNA (supplemental Fig. S1) grew to only 18% of the parental Mfn2-null control (Fig. 3*A*). Likewise, Mfn-dm cells also showed very slow growth. This growth defect was reversible, as demonstrated by the growth rescue of such cells upon re-introduction of Mfn1 and Mfn2 (Fig. 3*B*). Interestingly, cells lacking only Mfn2 or Mfn1 showed no growth defect,[2] suggesting that the residual levels of fusion documented above are sufficient to prevent severe disruption of cell function.

Similar to Mfn-dm cells, OPA1-RNAi cells also grew much more slowly and attained only 15% of the density of mock-infected control cells (Fig. 3*C*). Finally, OPA1-OE cells, with fusion-competent but fragmented mitochondria, grew at the same rate as wild-type controls (Fig. 3*D*).

Loss of Mitochondrial $\Delta\Psi$—The generalized cell growth problem in cells deficient in mitochondrial fusion led us to investigate what specific mitochondrial defects might exist. To monitor mitochondrial $\Delta\Psi$, cells were stained with MitoTracker Red, a mitochondrial dye dependent on $\Delta\Psi$. This staining was compared with Su9-GFP, a marker that localizes to the mitochondrial matrix even in cells with low mitochondrial $\Delta\Psi$ (20). For the studies described below, identical results were obtained when a mitochondrial outer membrane marker (GFP-OMP25) was used instead of Su9-GFP.[2] Consistent with our previous study (3), we found that both Mfn1-null and Mfn2-null cells have mild heterogeneity in mitochondrial $\Delta\Psi$.[2] Most Mfn1-null cells showed some mitochondria with weak MitoTracker Red staining, but this was restricted to only a few mitochondria (1–10/cell). With Mfn2-null cells, only a minority of cells showed heterogeneity in MitoTracker staining, and again this defect was restricted to a few mitochondria.

In contrast, 100% of Mfn-dm cells showed widespread heterogeneity of mitochondrial $\Delta\Psi$, with a large fraction of the mitochondrial population failing to stain well with MitoTracker Red (Fig. 4, *A–C*). The loss of mitochondrial $\Delta\Psi$ was reversible with complete restoration of $\Delta\Psi$ in Mfn-null cells overexpressing Mfn1.[2] Depletion of OPA1 by RNAi similarly led to widespread loss of mitochondrial $\Delta\Psi$ (Fig. 4, *D–F*). Cells overexpressing OPA1 showed no $\Delta\Psi$ defects (Fig. 4, *G–I*). Taken together with the PEG fusion data, these results indicate that

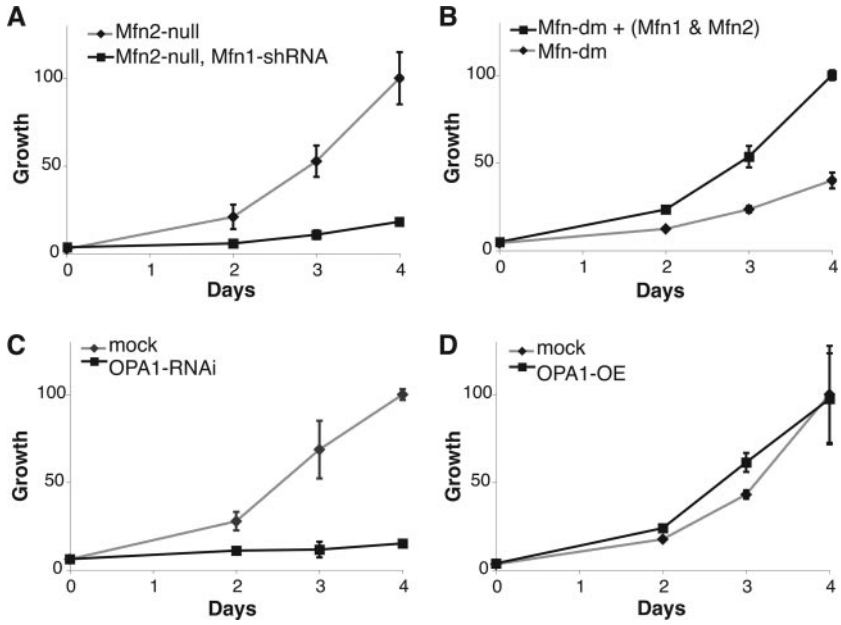

FIG. 3. **Growth defects in Mfn-dm and OPA1-RNAi cells.** *A*, Mfn2-null cells, uninfected or infected with *Mfn1*-shRNA. *Mfn1*-shRNA was used so that a direct comparison to its parental *Mfn2*-null cell line could be made. Similar growth defects are seen in Mfn-dm cells. *B*, Mfn-dm cells, uninfected or overexpressing Mfn1 and Mfn2. *C*, wild-type cells, mock-infected or infected with *OPA1*-shRNA. *D*, wild-type cells, mock-infected or overexpressing OPA1. Cells were counted in triplicate, and the mean number of control cells on day 4 was set to 100%. *Error bars* represent standard deviation.

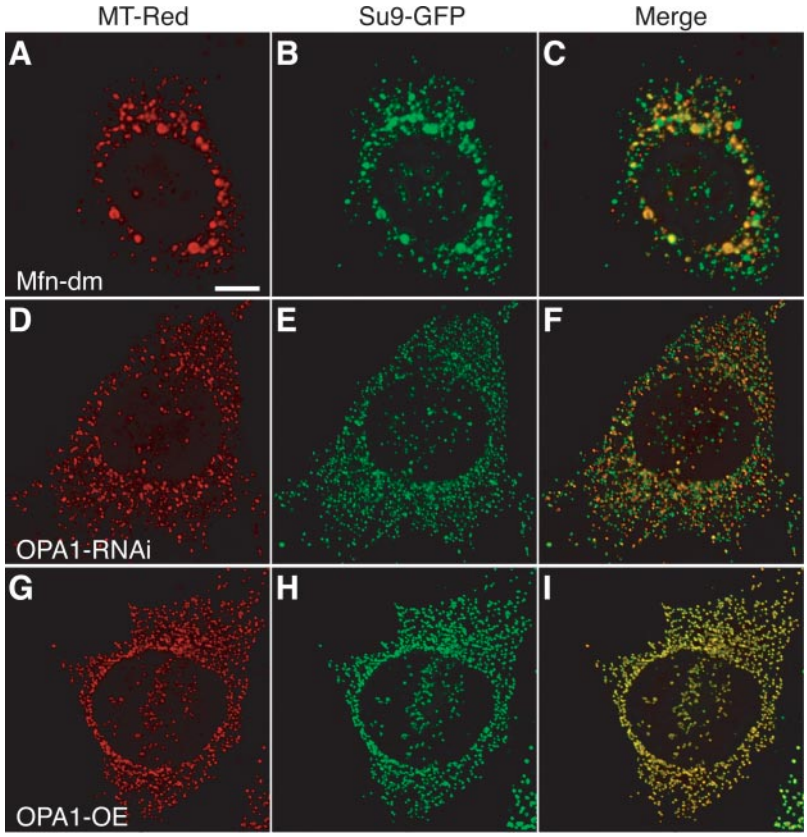

FIG. 4. **ΔΨ loss in mutant cells.** Mitochondrial ΔΨ was examined by MitoTracker Red staining (*left panels*) and compared with Su9-GFP (*middle panels*) in the merged images (*right panels*). *A–C*, Mfn-dm. *D–F*, OPA1-RNAi. *G–I*, OPA1-OE. *Scale bar* represents 10 μm.

the severity of mitochondrial ΔΨ loss correlates with the severity of fusion deficiency.

Reduced Mitochondrial Respiration—To examine the respiratory capacity of mutant cells, we used oxygen electrode studies to measure the rate of oxygen consumption in intact cells. We first measured the endogenous respiration rate and then added a proton ionophore (2,4-dinitrophenol) to induce the uncoupled respiration rate. Upon dissipation of the proton gradient, a compensatory increase in electron transport ensued, resulting in respiration at maximal capacity.

Wild-type MEFs had a strong endogenous respiration rate that was capable of increasing 40–70% upon dissipation of the proton gradient (Fig. 5*A*). A similar profile was observed in OPA1-OE cells (Fig. 5*B*). In contrast, Mfn-dm cells had reduced endogenous respiration, and respiration failed to increase upon the addition of ionophore (Fig. 5*C*). OPA1-RNAi cells reproducibly displayed an even more severe reduction in endogenous respiration and again no increase upon the addition of ionophore (Fig. 5*E*). The more severe defect found in OPA1-RNAi cells, as compared with that of Mfn-dm cells, was highly reproducible between multiple trials (Fig. 5*G*).

To further dissect the respiration defect in our fusion-deficient cell lines, we measured substrate-driven respiration in permeabilized cells. Specifically, we used glutamate and malate to measure complex I activity, succinate/glycerol-3-

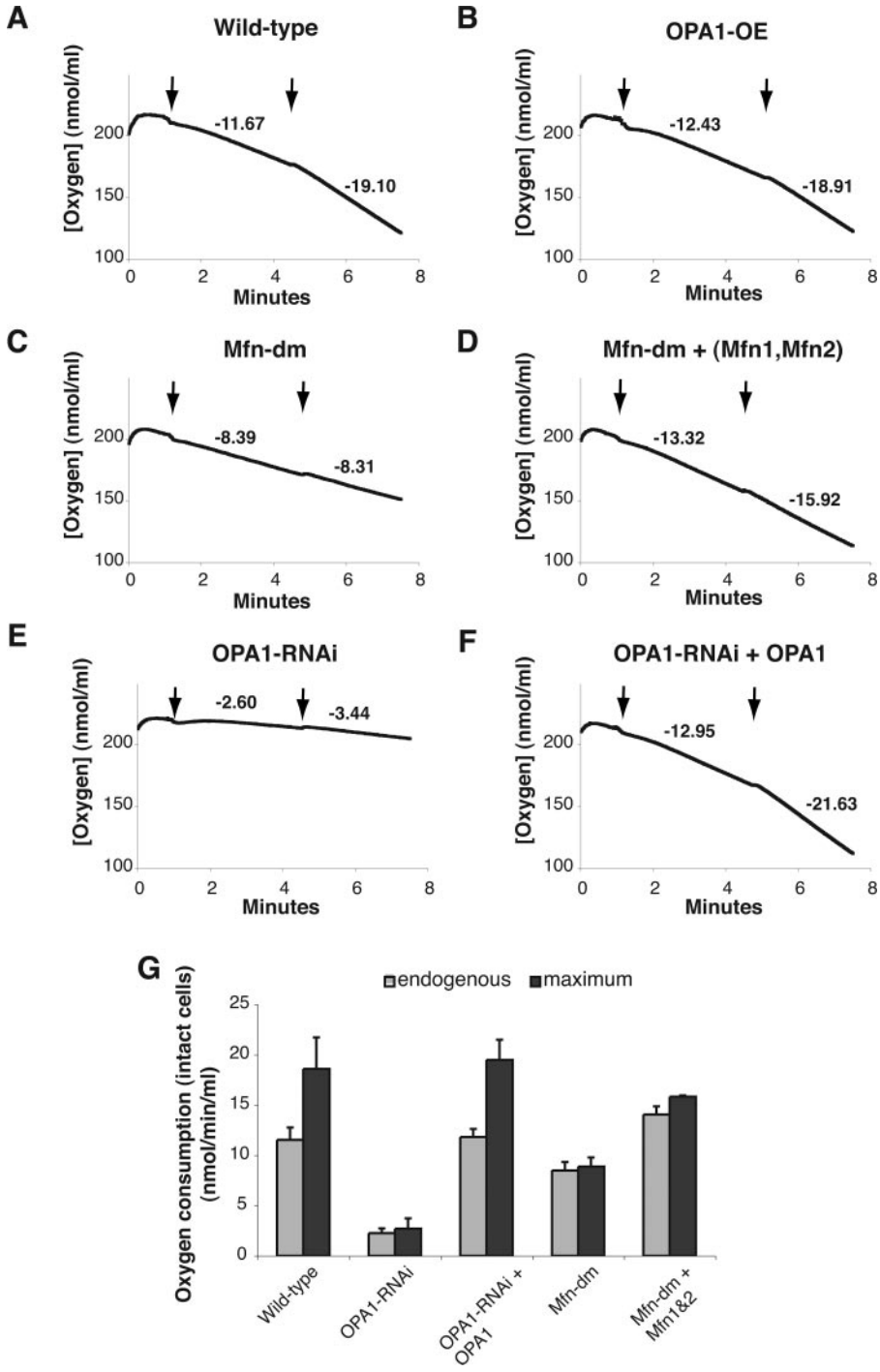

FIG. 5. **Reversible respiratory defects in mutant cells.** Representative respiration profiles are shown for the following cells: wild-type cells (*A*), OPA1-OE cells (*B*), Mfn-dm cells (*C*), Mfn-dm cells expressing Mfn1 and Mfn2 (*D*), OPA1-RNAi cells (*E*), and OPA1-RNAi cells expressing an RNAi-resistant construct of OPA1 (*F*). *Arrows* denote when the cell chamber was closed (*left*) and when 2,4-dinitrophenol was added to obtain the maximum respiration rate (*right*). Rates of oxygen depletion (nmol/ml/min) are shown above their respective time intervals. *G*, bar graph showing reversible respiratory defects in OPA1-RNAi and Mfn-dm cells. *Error bars* represent standard deviation.

phosphate for complex III, and *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine/ascorbate for complex IV. Respiratory capacity was attenuated for all three complexes in the mutant lines (Fig. 6).

An important aspect of the respiration defects was that they were reversible. Mfn-dm cells had a restored respiration profile upon expression of exogenous Mfn1 and Mfn2 (Fig. 5, *D* and *G*). Even more strikingly, expression of OPA1 in OPA1-RNAi cells completely restored normal respiration (Fig. 5, *F* and *G*), despite the severe defect in OPA1-RNAi cells. This reversibility indicates that, unlike in yeast, respiration defects in mammalian cells are not secondary to a complete loss of mtDNA, and suggests that lack of fusion itself affects respiration. It is interesting to note that cells lacking only Mfn1 or Mfn2 had no gross defect in cell growth or bulk respiration,[2] implying that low levels of mitochondrial fusion are sufficient to prevent at least some of the functional defects shown here.

DISCUSSION

Requirement of Mfns and OPA1 in Mitochondrial Membrane Fusion—There is some evidence that Mfn1 and Mfn2 may have distinct activities (6, 21), but they nevertheless have similar roles in mitochondrial membrane fusion. Because these two mitofusin homologs have overlapping expression patterns, it is important to clarify whether they play redundant roles in tissues where they are co-expressed and whether they can cooperate to mediate mitochondrial fusion. Our study showed that both Mfn1 and Mfn2 play a major role in maintaining normal levels of mitochondrial fusion. In the absence of either homolog, mitochondrial fragmentation results from a drastic reduction

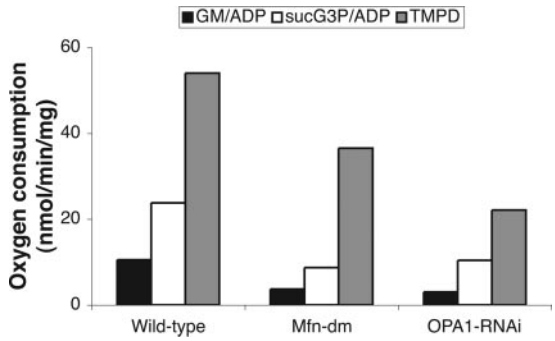

FIG. 6. **Defects in multiple respiratory complexes.** Oxygen consumption was measured in permeabilized cells under conditions of substrate-driven respiration. Shown are GM/ADP (glutamate, malate, and ADP (complex I)); sucG3P/ADP (succinate, glycerol-3-phosphate, ADP (complex III)); and TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine, ascorbate (complex IV)).

in the extent of mitochondrial fusion, as observed by the PEG fusion assay. Nevertheless, a single mitofusin is sufficient to generate residual mitochondrial fusion that can be readily detected. In mouse fibroblasts, Mfn1 appears to play a more prominent role than Mfn2, as evidenced by the greater loss of mitochondrial fusion and more severe fragmentation found in Mfn1-null MEFs.

Recent evidence suggests that mitofusins act in *trans* to mediate mitochondrial tethering and fusion (18). Our PEG fusion assays, using various combinations of Mfn1-null and Mfn2-null cells, further suggest that mitochondrial fusion, *in vivo*, can be mediated by three distinct complexes acting in *trans*: Mfn1 homotypic complexes, Mfn2 homotypic complexes, and Mfn1/Mfn2 heterotypic complexes.

Our results clarify the puzzling observation reported here and by others (4–6, 19) that overexpression as well as depletion of OPA1 causes mitochondrial fragmentation. We find that OPA1 deficiency abolishes all mitochondrial fusion. In contrast, fragmentation due to overexpression of OPA1 does not result from a fusion defect but is Drp1-dependent. The basis for this fragmentation remains to be resolved. One possibility is that overexpression of OPA1 can lead to an imbalance in OPA1 isoforms. A similar phenomenon is observed in yeast. Pcp1p is the rhomboid protease responsible for cleavage of Mgm1p (23, 24). The proper balance of long and short isoforms of Mgm1p is critical for maintenance of tubular mitochondrial morphology (25, 26). Yeast lacking Pcp1p produce only the long form of Mgm1p and have fragmented mitochondria that are fusion-competent (25).

Additional Functions of Mitochondrial Fusion—Previous studies have clearly substantiated the role of mitochondrial fusion and fission in maintenance of mitochondrial morphology (3, 12, 13). It is likely that, depending on the cell type, the shape of mitochondria can directly influence their functional properties. For example, it has been hypothesized that the filamentous and interconnected structure of mitochondria in some cells may facilitate the delivery of energy or membrane potential to specific regions of the cell (27).

In addition to this role in regulation of organelle shape, our studies suggest that mitochondrial fusion *per se* is important for mitochondrial function. Mfn1-null and Mfn2-null MEFs contain highly fragmented mitochondria but retain enough residual mitochondrial fusion to escape major cellular dysfunction. In contrast, Mfn-dm cells and OPA1-RNAi cells lack any detectable mitochondrial fusion. These cells grow poorly, show widespread heterogeneity in mitochondrial $\Delta\Psi$, and suffer reduced respiratory capacity. In polarography measurements, these fusion-incompetent cells respire at maximal rates in the absence of an uncoupler, and these rates are severely depressed compared with wild-type cells. Because these cells have increased heterogeneity in their mitochondria, this phenomenon may reflect a compensatory mechanism for the reduced number of functional mitochondria. Furthermore, the mutant cells have little or no reserve capacity for increased respiration.

These functional deficits do not appear to be secondary to mitochondrial fragmentation, because OPA1-overexpressing cells have severely fragmented mitochondria without loss of mitochondrial fusion and do not suffer cellular defects. Also of note is that the cell growth and respiratory defects are not due to an irreversible loss of mtDNA as seen in yeast, because reintroduction of the relevant protein restores normal cell function.

It remains a formal possibility that the severe functional defects observed in Mfn-dm and OPA1-RNAi cells may be due to as yet unknown functions of Mfns or OPA1, apart from fusion. This issue is more of a concern with OPA1, because this protein is still poorly understood and has been proposed to play a role in inner membrane remodeling (4). Such additional functions may explain why the respiration defect in OPA1-RNAi cells is significantly more severe than in Mfn-dm cells. However, we favor the explanation that the functional defects observed in Mfn-dm and OPA1-RNAi cells are specifically because of the lack of mitochondrial fusion. Mitofusins are becoming increasingly well understood and appear to play a direct role in mediating mitochondrial membrane fusion (18). In addition, the functional deficits in Mfn-dm and OPA1-RNAi cells are very similar, consistent with the hypothesis that they arise from a common defect.

A major challenge for future studies will be to identify the pathway through which loss of fusion in mammalian cells results in mitochondrial dysfunction. In some experimental systems, mitochondrial fusion has been associated with protective effects on mitochondrial function. Cell hybrids between parental cells containing mutant mtDNA molecules result in restoration of respiratory activity (28), indicating that complementation of mtDNA products can occur in fused mitochondria. Moreover, mice containing pathogenic mtDNA mutations are protected from mitochondrial dysfunction until a critical load of mutant mtDNA molecules is reached (22). These results again suggest that mitochondrial fusion can allow interaction of mtDNA gene products and enable protection of mitochondrial function.

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