Mitochondrial Cristae Shape Determines Respiratory Chain Supercomplexes Assembly and Respiratory Efficiency

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SUMMARY

Respiratory chain complexes assemble into functional quaternary structures called supercomplexes (RCS) within the folds of the inner mitochondrial membrane, or cristae. Here, we investigate the relationship between respiratory function and mitochondrial ultrastructure and provide evidence that cristae shape determines the assembly and stability of RCS and hence mitochondrial respiratory efficiency. Genetic and apoptotic manipulations of cristae structure affect assembly and activity of RCS in vitro and in vivo, independently of changes to mitochondrial protein synthesis or apoptotic outer mitochondrial membrane permeabilization. We demonstrate that, accordingly, the efficiency of mitochondria-dependent cell growth depends on cristae shape. Thus, RCS assembly emerges as a link between membrane morphology and function.

INTRODUCTION

Mitochondria are key organelles in intermediate cellular metabolism, energy conversion, and calcium homeostasis (Dimmer and Scorrano, 2006). They also integrate and amplify apoptosis induced by intrinsic stimuli, releasing cytochrome c and other proapoptotic factors required for the activation of caspases (Green and Kroemer, 2004). Cytochrome c release is regulated by proteins of the BCL-2 family that control the permeabilization of the outer membrane (OMM) (Dimmer and Korsmeyer, 2004).

Energy conversion occurs at the inner mitochondrial membrane (IMM) that can be further divided into two subcomponents: the so-called “boundary membrane” and the cristae, separated from the former by narrow tubular junctions (Frey and Mannella, 2000). The cristae shape is dynamic: upon activation of mitochondrial respiration, “orthodox” mitochondria become “condensed,” with an expanded cristae space (Hackenbrock, 1966). During apoptosis, the curvature of the cristae membrane is inverted in a remodeling process required for the complete release of cytochrome c, normally confined in the cristae (Scorrano et al., 2002; Frezza et al., 2006; Yamaguchi et al., 2008). Cristae remodeling occurs in response to proapoptotic BH3-only BCL-2 family members, such as BID, BIM-S, and BNIP3, and independently of the outer membrane multidomain BCL-2 family members BAX and BAK (Scorrano et al., 2002; Cipolat et al., 2006; Yamaguchi et al., 2008). Whether changes in morphology of the cristae, where respiratory chain complexes (RCCs) mainly localize (Vogel et al., 2006), affect oxidative phosphorylation efficiency, as originally predicted (Hackenbrock, 1966), is unclear. This issue is further complicated by the assembly of RCC in supercomplexes (RCS) (Schägger, 1995; Acín-Pérez et al., 2008), quaternary supramolecular structures that, by channeling electrons among individual RCCs, allow the selective use of RCC subsets for nicotine adenine dinucleotide (NADH)- or flavin adenine dinucleotide-derived electrons (Lapuente-Brun et al., 2013). Such a supramolecular organization is common in cristae: also, the mitochondrial ATP synthase is assembled into dimers with greater adenosine triphosphatase (ATPase) activity (Campanella et al., 2008; Gomes et al., 2011). Interestingly, cristae shape and ATPase dimers are linked: in yeast mutants where the ATPase cannot dimerize, cristae are disorganized (Paumard et al., 2002; Minaura-Sanmiguel et al., 2005; Strauss et al., 2008), whereas in mammalian cells, increased cristae density favors ATPase dimerization during autophagy (Gomes et al., 2011). On the contrary, despite their importance in mitochondrial bioenergetics,
the relationship between RCS and cristae shape remains unclear.

Mitochondrial morphology and ultrastructure depend on “mitochondria-shaping” proteins that regulate organelar fusion and fission (Gripapic and van der Bliek, 2001). Mitofusins (MFN) 1 and 2, highly homologous dynamin-related proteins of the OMM, orchestrate fusion (Santel and Fuller, 2002; Legros et al., 2002; Chen et al., 2003; Santel et al., 2003). MFN1 primarily participates in fusion, cooperating with the IMM dynamin-related protein optic atrophy 1 (OPA1) (Cipolat et al., 2004), whereas MFN2 also tethers mitochondria to the endoplasmic reticulum (de Brito and Scorrano, 2008). Mitochondrial fission is regulated by the cytoplasmic dynamin-related protein 1 that, upon calcineurin-independent dephosphorylation, translocates to mitochondria (Yoon et al., 2001; Smirnova et al., 2001; Cereghetti et al., 2008). Genetic depletion of OPA1 leads to disorganization of the cristae (Frezza et al., 2006), and oligomers that contain a soluble and a membrane-bound form of OPA1 keep the cristae junctions tight, independently from OPA1 role in fusion (Frezza et al., 2006; Cipolat et al., 2006). During apoptosis, these oligomers are early targets of BID, BIM-S, and BNIP3, as well as of intrinsic death stimuli (Frezza et al., 2006; Yamaguchi et al., 2008; Landes et al., 2010; Costa et al., 2010). Whereas our knowledge of the molecular determinants of cristae shape and their role in apoptosis is increasing, the relationship between cristae morphology and mitochondrial function remains unexplored. We therefore set out to genetically dissect whether and how cristae shape regulates mitochondrial respiration. We show that cristae morphology determines assembly and stability of RCS and hence optimal mitochondrial respiratory function during life and death of the cell.

RESULTS

Genetic Dissection of Outer Membrane Permeabilization from Cristae Remodeling

Whether apoptotic cristae remodeling that maximizes cytochrome c release from mitochondria affects mitochondrial function is unclear, mainly because it occurs around the same time as outer membrane permeabilization (Scorrano et al., 2002). In order to genetically dissociate the two processes, we inspected the primary structure of the prototypical cristae remodeling inducer BCL-2 family BID for homology with peptides known to perturb the mitochondrial inner membrane, like mastoparan, a 14 amino acid wasp venom component (Pfeiffer et al., 1995). Interestingly, BID membrane inserting α6 helix as well as the transmembrane domains of Bnip3 and BimS that also remodel cristae (Yamaguchi et al., 2008; Landes et al., 2010) displayed homology to mastoparan (Figures S1A and S1B available online). To exploit the role of this homologous sequence in cristae remodeling, we mutagenized the two highly conserved 157 and 158 Lys H. sapiens BID residues to Ala (BIDKKAA) (Figure S1C). Because this mutation did not impair caspase-8 cleaved recombinant BID (cBID) integration in purified mouse liver mitochondria (MLM) (Wei et al., 2000; Figure S1D), we could measure its biological activity using an established quantitative, specific cytochrome c release ELISA (Scorrano et al., 2002). cBID efficiently released cytochrome c from purified mitochondria, whereas a BH3 domain G94E mutant was, as expected, inactive (Wei et al., 2000) and the cBIDKKAA mutant released ~25%–30% more cytochrome c than the baseline (Figure 1A), a figure close to the amount of free intermembrane space cytochrome c (Scorrano et al., 2002). BAK oligomerization was superimposable in cBID or cBIDKKAA–treated mitochondria (Figure 1B); conversely, two established assays of intramitochondrial cytochrome c redistribution, the cytochrome b$_{5}$–dependent extramitochondrial NADH oxidation and the ratio of ascorbate-driven over tetramethyl-p-phenylenediamine (TMPD)-driven respiration (Scorrano et al., 2002), indicated that cBIDKKAA mobilized the cristae cytochrome c pool less efficiently than cBID (Figures 1C and 1D). Indeed, cBIDKKAA was unable to remodel mitochondrial cristae, as indicated by morphometric analysis of electron micrographs of mitochondria treated with the BID mutants (Figures 1E and 1F) (Scorrano et al., 2002). Cristae remodeling is associated with the disruption of high molecular weight (HMW) OPA1 oligomers (Frezza et al., 2006). Western blots of blue native gel electrophoresis (BNGE) of mitochondrial proteins revealed four major OPA1-containing complexes. Upon treatment with cBID, OPA1 rapidly disappeared from ~720 kDa HMW complexes (Figures 1G, S1E, and S1F). These HMW forms of OPA1 were similarly targeted by cBIDG94E but significantly less by cBIDKKAA, as determined by BNGE (Figure 1H, quantification in [I]). Chemical crosslinking experiments (Frezza et al., 2006) further confirmed that the OPA1-containing oligomer is disrupted by the mutants of cBID able to induce cristae remodeling (Figures S1G and S1H). Finally, we measured the killing efficiency of these truncated BID (tBID) mutants expressed in mouse embryonic fibroblasts (MEFs). Only tBID efficiently killed MEFs: tBIDKKAA and tBIDG94E elicited comparable low levels of cell death, whereas the double tBIDKKAA,G94E mutant appeared completely ineffective (Figure 1J), suggesting that both outer membrane permeabilization and mitochondrial cristae remodeling are required for BID-induced apoptosis. In conclusion, BIDKKAA is deficient in cristae remodeling, cytochrome c release, and induction of apoptosis.

RCS Disassembly during Cristae Remodeling

The BIDKKAA mutant dissociates outer membrane permeabilization from cristae remodeling and can be used to investigate the relationship between the latter and mitochondrial function. We therefore measured the effect of the BID mutants on the respiratory control ratio (RCR), an index of respiratory efficiency, of mitochondria incubated with excess exogenous cytochrome c and NADH (to compensate for the potential effects of inner membrane or outer membrane [OM] permeabilization), cBID reduced RCR only when mitochondria were energized with substrates for complex I (glutamate/malate) but not when they were fed with substrates entering the electron transport chain at complex II (succinate) or complex IV (ascorbate + TMPD) (Figure 2A; data not shown). Interestingly, these changes were recapitulated by the BH3 domain mutant cBIDG94E that does not permeabilize the OM but not by the cristae remodeling-deficient mutants cBIDKKAA and cBIDG94E (Figure 2B). Maximal (uncoupled) respiration was similarly affected by the cBID mutants tested, ruling out that BID alters RCR, because it affects ATPase or activity of ATP/ADP exchange (Figure S2A). These experiments
suggest that cristae remodeling causes complex I-dependent changes in mitochondrial bioenergetics.

Complex I is further assembled in quaternary functional RCS with complexes III and IV (I + III and I + III + IV), whereas most complex II is not found in RCS (Acín-Pérez et al., 2008). Thus, the reduction in complex I-supported respiration could be a consequence of specific inhibition of complex I or of issues in RCS function. Even after 30 min of acute BID treatment, the specific complex I NADH-ubiquinone reductase activity of purified mitochondria was unaltered (data not shown), prompting us to investigate RCS assembly and stability in situ. We therefore took advantage of Bax$^{-/-}$, Bak$^{-/-}$ (DKO) MEFs, resistant to mitochondrial permeabilization, cytochrome c release, and apoptosis triggered by expression of tBID (Wei et al., 2001).

Upon transduction of metabolically labeled DKO MEFs with tBID but not with tBID$^{K6A}$ that does not cause cristae remodeling (Figure S2B), the RCS radioactivity signal as well as the RCS/complex V radioactivity ratio were reduced (Figures 2C and 2D), and we observed a reduction in the autoradiographic signal of cytochrome $b$ retrieved in RCS compared to that in free complex III (Figures 2E and 2F). Whereas this result could suggest that complex III was incorporated less efficiently into RCS, immunoblotting for the complex I subunit NDUFA9 revealed that RCSs were also destabilized in DKO cells (Figure 2G). Functionally, only cBID and cBID$^{G94E}$ that cause cristae remodeling but not cBID$^{K6A}$ reduced glutamate-supported RCR in DKO mitochondria (Figure 2H). Thus, BID destabilizes RCS and selectively reduces glutamate-dependent RCR.

Figure 1. Two Conserved Lys in BID s6 Helix Are Required for Cristae Remodeling

(A) Mitochondria were treated for the indicated times with the indicated mutants of cBID, and cytochrome c release was measured by ELISA. Data represent average ± SEM of five independent experiments.

(B) Mitochondria treated with the indicated mutants of cBID for the indicated min were cross-linked with 1 mM BMH for 30 min, spun, and the pellets were separated by SDS-PAGE and immunoblotted using anti-BAK antibody. The asterisks denote BAK oligomers.

(C) Mitochondria were treated as indicated (Ca$^{2+}$, 200 μM), and cytochrome b$_{5}$-dependent NADH fluorescence changes were measured. a.u., arbitrary units.

(D) Mitochondria were treated for 15 min with the indicated BID mutants, transferred into the chamber of a Clark’s type O$_{2}$ electrode, and the ascorbate/TMPD-driven respiration ratio was determined. Data represent average ± SEM of four independent experiments.

(E) Representative electron microscopy fields of mitochondria treated for 15 min with the indicated cBID mutants. Arrows indicate class II and III mitochondria. The scale bar represents 450 nm.

(F) Blind morphometric analysis of randomly selected EM of mitochondria treated with the indicated cBID mutants (as in [E]). Mitochondria were assigned to morphological classes I–III as in Scorrano et al. (2002). Data represent average ± SEM of three independent experiments. The asterisk denotes p < 0.05 in a paired sample Student’s t test versus untreated.

(G and H) BNGE analysis of OPA1 oligomers in MLM treated for 30 min (G) or for the indicated minutes (H), as indicated. The boxed area indicates the HMW complexes of OPA1.

(I) Densitometric analysis of OPA1 HMW complexes. Experiments were as in (H). Data represent average ± SEM of four independent experiments.

(J) MEFs were transfected with the pMIG plasmid containing the indicated insert and after 48 hr cell death was determined cyt fluorimetrically as the percentage of Annexin-V+, GFP+ cells. Data represent average ± SEM of four independent experiments. The asterisk denotes p < 0.05 in a paired sample Student’s t test versus tBID. See also Figure S1.
Conditional Ablation of Opa1 Alters Cristae Shape and RCS Assembly

To verify whether RCS disorganization was a general consequence of altered cristae shape, we turned to cells lacking Opa1, a key regulator of cristae morphology (Frezza et al., 2006). However, chronic Opa1 depletion impairs mitochondrial DNA (mtDNA) levels and translation (Figures S3A and S3B), complicating the analysis of the relationship between Opa1 and RCS and calling for a model of conditional Opa1 ablation.

We produced, by homologous recombination, C57BL6/J...
embryonic stem cells with loxP sites inserted in the Opa1 gene (Opa1flx/flx), which were then microinjected in C57BL6/J blastocysts to generate Opa1flx/flx mice. Following Cre-mediated recombination, the deletion of exons 2 and 3 resulted in an aberrant exon1–exon4 transcript with a stop codon immediately after exon 1, producing a predicted 10 amino acid (aa) residual protein (Figures 3A and 3B). Chimerism and germ-line transmission of the offspring was tested by PCR, and germ-line transmittants were bred to homozygosity (Figure 3C). Fibroblasts isolated from the diaphragm of homozygous Opa1flx/flx 7-week-old male mice (MAFs) were immortalized and used for subsequent analysis. OPA1 was completely ablated 24 hr after adenoviral delivery of Cre recombinase (Figure 4A) and, as expected, mitochondria were fragmented (Figures 4B and 4C) with defects in cristae shape (Figures 4D and 4E). Four days after Cre-mediated Opa1 ablation, mtDNA copy number (Figure 4F) and translation (Figures 4G and 4H) were unaffected, allowing us to specifically address the role of OPA1 and cristae shape in RCS assembly using an assay based on the incorporation of radiolabeled mtDNA-encoded proteins into RCC and RCS (Acín-Pérez et al., 2008). Upon acute Opa1 ablation, the assembly of mtDNA-encoded subunits into RCC was not affected (data not shown). We therefore followed the RCS assembly rate (measured as the ratio between RCS and complex V radioactivity throughout the chase period) that resulted ~8-fold slower when Opa1 was ablated from Opa1flx/flx MAFs (Figures 4I and 4J). A similar reduction in the RCS assembly rate was observed in Opa1flx/+/MEFs (Figure S3C), suggesting that, in absence of Opa1, RCC are less superassembled, irrespective of their initial levels. To test if acute Opa1 ablation altered RCS in vivo, we tail vein-injected Cre-expressing adenoviruses in Opa1flx/flx animals. After 72 hr, OPA1 levels in liver mitochondria were reduced by ~50% (Figure S4A), cristae morphology was abnormal (Figure S4B), RCS were reduced (Figure S4C), and glutamate/malate RCR was impaired (Figure 4K). These experiments of conditional ablation of Opa1 identify a role for cristae shape in RCS assembly in vitro and in vivo.
Overexpression of Opa1 Increases RCS Assembly

The model linking cristae shape to RCS predicts that higher OPA1 levels should favor RCS assembly. To verify this hypothesis, we wanted to generate a mouse model of Opa1 overexpression. Very high OPA1 levels are, however, toxic, causing paradoxical mitochondrial fragmentation. To overcome this issue, we generated a mouse model of Opa1 overexpression. Very high OPA1 levels are, however, toxic, causing paradoxical mitochondrial fragmentation.
Mitochondria-Supported Cell Growth Is Controlled by Cristae Shape

We next wished to address if cristae shape affects mitochondrial-dependent cell growth. We therefore measured the growth of DKO cells (resistant to BID-induced outer membrane permeabilization, caspase-dependent mitochondrial damage, and apoptosis) in galactose media, where most of cellular ATP comes from the respiratory chain (Acín-Pérez et al., 2004). WT and G94E TBid impaired growth in galactose, whereas cells transduced with TBidK64A that does not cause cristae remodeling did not display any defect (Figure 6A).

We next turned to genetic models of cristae shape changes. Growth in galactose-containing media was impaired upon acute ablation of Opa1 in MAFs, whereas it was normal for fusion-deficient Mfn1+/−, Mfn2+/− MEFs (Figures 6B and 6C), where mitochondrial fusion is also impaired. In Mfn1−/−, Mfn2−/− MEFs, mtDNA copy number was reduced (Figure S6A), but cristae shape (Figures S6B and S6C), mtDNA translation (Figure S6D), RCS stability (Figure S6E), and assembly (Figure S6F) were not affected. Thus, the galactose growth defect is not the consequence of impaired fusion but correlates with altered cristae shape and RCS. Finally, Opa1flx/flx MAFs grew faster than their WT counterparts in galactose media (Figure 6D), further confirming the link between cristae shape, RCS levels, and mitochondria-dependent cellular growth. In conclusion, cristae shape correlates with the efficiency of mitochondria-dependent cell growth.

DISCUSSION

Respiratory chain supercomplexes have been considered BNGE artifacts until direct respirometric experiments on purified RCS identified them as the functional mitochondrial respiratory units (Acín-Pérez et al., 2008). Since then, RCS have been directly visualized in intact cristae by electron tomography (Davies et al., 2011), complex IV assembly factors that favor RCS formation have been identified (Chen et al., 2012; Vukotic et al., 2012; Strogolova et al., 2012; Lapuente-Brull et al., 2013), and the role of RCS in mitochondrial utilization of reducing equivalents has been demonstrated (Lapuente-Brull et al., 2013). However, the relationship between cristae shape and RCS, as well as between RCS and mitochondrial function, remained obscure. Our results demonstrate that cristae shape regulates respiratory chain supercomplexes stability and assembly, impacting on respiratory efficiency and respiratory cell growth.

To dissect the role of cristae shape in RCS structure and function, we genetically ablated the master cristae shape regulator Opa1. Individual respiratory chain units associate with OPA1 (Zanna et al., 2008), and mitochondrial metabolism is deranged in dominant optic atrophy caused by OPA1 mutations (Lodi et al., 2004). However, the deficit in ATP production in OPA1 haploinsufficient cells was unexplained: OPA1 is not essential for assembly of respiratory chain complexes and mtDNA levels as well as activities of individual respiratory chain complexes are normal in dominant optic atrophy (Zanna et al., 2008). Conversely, the reduction in mtDNA copy number has been invoked to explain the mitochondrial dysfunction of fusion-deficient cells from Mfn1−/−, Mfn2−/− mice (Chen et al., 2010). Our results challenge this hypothesis: upon acute Opa1 ablation, mtDNA levels are normal, whereas cristae shape, RCS, complex-I-dependent respiration, and respiratory growth are impaired. Conversely, in Mfn1−/−, Mfn2−/− cells, mtDNA copy number is reduced, but cristae shape, RCS, and respiratory growth are normal. Thus, RCS disorganization shall be regarded as a key mechanism of mitochondrial dysfunction accompanying altered organelle morphology.

The role of OPA1 and cristae shape in RCS organization is further supported by mouse models of Opa1 conditional ablation and mild overexpression. The first tool allowed us to dissociate cristae biogenesis from mtDNA maintenance: whereas chronic Opa1 depletion reduces mtDNA copy number and translation, upon acute Opa1 ablation, mtDNA levels are normal, but cristae are disorganized, impacting on RCS assembly and respiratory function and growth. Thus, mtDNA reduction appears to be a consequence of chronic fusion inhibition in Opa1−/− (double Mfn−/−) cells. We can therefore predict that the Opa1heterocells will be useful to elucidate how prolonged inhibition of mitochondrial fusion results in mtDNA levels reduction. Opa1 mild overexpression lends further support to the model linking RCS organization to cristae shape: RCS assembly and respiratory function and growth are increased in Opa1flx cells without any measurable change in mtDNA levels and translation. The Opa1flx mouse will be instrumental to investigate the role of Opa1 and cristae shape in vivo.

Apoptotic cristae remodeling further supports the relationship between cristae shape and RCS. The role and mechanisms of cristae remodeling in apoptosis are controversial (Scorrano et al., 2002; Germain et al., 2005; Frezza et al., 2006; Yamaguchi et al., 2008; Merkwirth et al., 2008; Costa et al., 2010). Despite that OPA1-mediated stabilization of cristae shape inhibits...
intrinsic apoptosis (Frezza et al., 2006; Yamaguchi et al., 2008; Costa et al., 2010), cristae remodeling has been reckoned as a mere feedback mechanism in situ, occurring after caspase activation (Sun et al., 2007). Our results suggest that, in addition to its role in cytochrome c release, cristae remodeling also impairs mitochondrial function to precipitate apoptosis. The BID \( \alpha 6 \) mutant generated here, which does not induce cristae changes and cytochrome c mobilization but permeabilizes the outer

**Figure 5. Transgenic Overexpression of OPA1 Increases RCS Assembly**

(A) Equal amounts of proteins (20 \( \mu g \)) from MAFs of the indicated genotypes were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

(B) Representative confocal micrographs of mitochondrial morphology in WT and Opa1\(^{tg} \) MAFs. Mitochondria were visualized by anti-TOM20 immunostaining.

The scale bar represents 20 \( \mu M \).

(C) Average mitochondrial major axis length. Experiments were as in (B). Data represent average \( \pm \) SEM of four independent experiments (five mitochondria per cell, at least 50 cells/experiment). The asterisk denotes \( p < 0.05 \) in a paired sample Student’s \( t \) test versus WT.

(D) Electron micrographs of MAFs of the indicated genotype. The scale bars represent 2 \( \mu M \) (top) and 200 nm (bottom).

(E) Morphometric analysis of cristae width in 40 randomly selected mitochondria of MAFs of the indicated genotype. Data represent average \( \pm \) SEM of three independent experiments. The asterisk denotes \( p < 0.05 \) in a paired sample Student’s \( t \) test versus WT.

(F) Mitochondrial copy number quantification. mtDNA was amplified by RT-PCR from total DNA of MAFs of the indicated genotype. Data are normalized to WT MAFs and represent the average \( \pm \) SEM of four independent experiments.

(G) mtDNA translation assay. MAFs of the indicated genotype were metabolically labeled in presence of emetine and lysed after 30 min. Protein samples (40 \( \mu g \)) were separated by SDS-PAGE, and the radioactivity was detected in the fixed and dried gels for 3 days. The mtDNA-encoded proteins are indicated.

(H) Densitometric analysis of the mtDNA-encoded proteins. Experiments are as in (G). Data represent average \( \pm \) SEM of four independent experiments.

(I) RCS assembly assay. MAFs of the indicated genotype were metabolically labeled for 2 hr and then chased for the indicated times. Equal amounts of protein (100 \( \mu g \)) were separated by BN PAGE, and radioactivity was detected in the fixed and dried gels for 1 week. Individual complexes and supercomplexes of the respiratory chain are indicated.

(J) Densitometric analysis of the incorporation rate of radioactivity into RCS. Values are normalized for the autoradiographic signal of complex V. Data represent average \( \pm \) SEM of three independent experiments performed as in (H).

(K) RCR of mitochondria isolated from livers of mice of the indicated genotype energized with 5 mM/2.5 mM GLU/MAL or 10 mM SUCC. Data represent mean \( \pm \) SEM of four independent experiments. The asterisk denotes \( p < 0.05 \) in a paired sample Student’s \( t \) test versus WT.

See also Figure S5.
membrane, can be a useful tool to dissect in vivo the involvement of cristae remodeling in developmental and homeostatic apoptosis. We think that cristae remodeling influences RCS by targeting OPA1 (Frezza et al., 2006), not by altering membrane potential that is normal during cristae remodeling (Scorrano et al., 2002) or by inhibiting mtDNA translation and insertion of mtDNA-encoded subunits that similarly appear normal in DKO cell-expressing BID (data not shown).

Our work unravels a role for cristae shape in RCS assembly and stability, mitochondrial respiratory efficiency, and respiratory growth, suggesting that shape of biological membranes can influence membrane protein complexes. Moreover, our data highlight the importance of RCS in respiration by complex I-feeding substrates. Finally, we unveil how OPA1 regulates mitochondrial respiratory efficiency. The pathogenesis of dominant optic atrophy where OPA1 is mutated (Alexander et al., 2000) or of other mitochondrial diseases where OPA1 is degraded (Duvezin-Caubet et al., 2006) could also depend on this unexpected OPA1 function. In these latter settings, stabilization of OPA1 could correct RCS and therefore mitochondrial dysfunction, opening novel therapeutic perspectives for currently intractable diseases.

**EXPERIMENTAL PROCEDURES**

**Generation of Opa1flx/flx and Opa1tg Mice**

To generate Opa1flx/flx mice, a mouse Bac clone containing the Opa1 gene was isolated from the C57BL/6J ES BAC clone library. An 11 kb HpaI DNA restriction fragment containing the 5 kb upstream-exon3 was subcloned in a pUC-8 vector. The OPA1 fragment was excised with EcoRV and Xmal to generate blunt ends and inserted into a pKO4.4a-LoxP cut with XhoI and SalI. A LoxP site was introduced between intron1 and exon2 of Opa1 and a phosphoglycerate kinase (PGK) promoter-driven neomycin resistance gene, flanked by two FRT sequences and with one LoxP sequence downstream, was inserted in intron3. The targeting vector was linearized and electroporated into C57BL6 embryonic stem cells (ESCs). Neomycin-resistant ESC clones were tested for homologous recombination. Three mutated ESC lines were microinjected into C57BL6 blastocytes and implanted in host mice to obtain chimeric mice, which were then bred with C57BL6 mates and their offspring tested by PCR for germline transmission. Colonies were established in a C57BL6 background.

To generate Opa1tg mice, the human b-actin promoter was extracted from pDRIVE-h-b-ACTIN (InvivoGen) using SpeI and NcoI and cloned in pENTRY. The complementary DNA of mouse isoform 1 Opa1 and polyA extracted from pcDNA3.1-OPA1 (Cipolat et al., 2004) using Nhe1 and EciI was ligated into pENTRY using Quick Ligase (Ozyme). The transgene was then inserted by homologous recombination in a pDEST vector containing part of the human hypoxanthine phosphoribosyltransferase locus. The resulting vector was
linearized using PvuI and electroporated into C57BL6 BPEs cells by Nucleics (France). Homologous recombinants were selected on stringent hypoxanthine aminopterin-thymidine-supplemented medium. Three positive ESC recombinant clones were microinjected into C57BL6 blastocysts and implanted into host pseudopregnant female C57BL6 to obtain chimeric mice. Six chimeras (identified by fur agouti color) were bred with C57BL6 mates, and germline transmission was verified by fur color and PCR. Colonies were established in a C57BL6 and in a Sv129 background by crossingbreeding. Details on mouse genotyping and handling can be found in the Extended Experimental Procedures.

BNGE, 2D BN/BNGE, and 2D BN/SDS PAGE
Mitochondria (10 mg/ml) were suspended in buffer D (1 M 6-aminohexanoic acid, 1.25% V/V digitonin, 50 mM Bis-Tris-HCl pH 7) and centrifuged. The supernatant was collected, and 5% Serva Blue G dye in 1 M 6-aminohexanoic acid was added to 1/3 of the final sample volume. Equal amounts (100 µg) of mitochondrial proteins were separated by 3%–13% gradient BNGE (Schläger, 1995). For RCS detection, the concentration of digitonin in buffer D was 4% (V/V).

For two-dimensional (2D) Blue Native (BN)/BNGE, the lane cut from the first-dimension BNGE was casted on top of a native 3%–14% gradient gel in 1% (V/V) agar, 1995). For RCS detection, the concentration of digitonin in buffer D was substituted with 0.9 mg/ml galactose. Ecotropic viruses were generated as described (Cheng et al., 2001), WT, Opa1flx/flx, Opa1fl/C, and MAFs SV40 transformed cell lines were generated from the diaphragm of the respective 7-week-old mouse killed by cervical dislocation. Details on the procedure can be found in the Extended Experimental Procedures. Acute Opa7 ablation in Opa1flx/flx MAFs was obtained by infection with adenoviruses expressing cytomegalovirus (CMV)-Cre-GFP (ad-CRE; 300 pfu/cell; Vector Biolabs). CMV-GFP (ad-EV)-expressing adenoviruses were used as control.

Cell growth was determined by counting viable cells for the indicated time. Opa1flx/flx MAFs were infected and DKO MEFs were transduced 24 or 16 hr before the growth was assessed. Apoptosis was measured by flow cytometric detection (FACS Calibur) of the Annexin-V-PE positive events in the GFP-positive population. Details can be found in the Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Extended Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.08.032.

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