Opa1 Overexpression Ameliorates the Phenotype of Two Mitochondrial Disease Mouse Models

Highlights
- Opa1 overexpression improves the phenotype of Ndufs4−/− and Cox15sm/sm mice
- O2 consumption rate and respiratory chain activities are increased in double mutants
- Mitochondrial ultrastructure is corrected by Opa1 overexpression in Cox15sm/sm
- Respiratory complexes and supercomplexes are stabilized in Cox15sm/sm::Opa1tg

Authors
Gabriele Civiletto, Tatiana Varanita, ..., Luca Scorrano, Massimo Zeviani

Correspondence
mdz21@mrc-mbu.cam.ac.uk (M.Z.), luca.scorrano@unipd.it (L.S.)

In Brief
Although mitochondrial diseases have diverse causes, they are all characterized by defective oxidative phosphorylation. Civiletto et al. show that overexpression of the mitochondria-shaping protein OPA1, which improves respiratory chain efficiency, improves the phenotypes of two pre-clinical models of defective mitochondrial bioenergetics.

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Opa1 Overexpression Ameliorates the Phenotype of Two Mitochondrial Disease Mouse Models

Gabriele Civiletto,1,2,5 Tatiana Varanita,3,5 Raffaele Cerutti,2 Tatiana Gorletta,1 Serena Barbaro,1 Silvia Marchet,1 Costanza Lamperti,1 Carlo Viscomi,1,2 Luca Scorrano,3,4,* and Massimo Zeviani1,2,*

1Fondazione IRCCS Istituto Neurologico “C. Besta,” Milan, Italy
2MRC-Mitochondrial Biology Unit, Cambridge, UK
3Dulbecco Telethon Institute, Venetian Institute of Molecular Medicine, Padova, Italy
4Department of Biology, University of Padova, Padova, Italy
5Co-first author
*Correspondence: mdd21@mrc-mbu.cam.ac.uk (M.Z.), luca.scorrano@unipd.it (L.S.)
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SUMMARY

Increased levels of the mitochondria-shaping protein Opa1 improve respiratory chain efficiency and protect from tissue damage, suggesting that it could be an attractive target to counteract mitochondrial dysfunction. Here we show that Opa1 overexpression ameliorates two mouse models of defective mitochondrial bioenergetics. The offspring from crosses of a constitutive knockout for the structural mitochondrial bioenergetics. The offspring from crosses of a constitutive knockout for the structural complex I component Ndufs4 (Ndufs4−/−), and of a muscle-specific conditional knockout for the complex IV assembly factor Cox15 (Cox15sm/sm), with Opa1 transgenic (Opa1tg) mice showed improved motor skills and respiratory chain activities compared to the naive, non-Opa1-overexpressing, models. While the amelioration was modest in Ndufs4−/−::Opa1tg mice, correction of cristae ultrastructure and mitochondrial respiration, improvement of motor performance and prolongation of lifespan were remarkable in Cox15sm/sm::Opa1tg mice. Mechanistically, respiratory chain supercomplexes were increased in Cox15sm/sm::Opa1tg mice, and residual monomeric complex IV was stabilized. In conclusion, cristae shape amelioration by controlled Opa1 overexpression improves two mouse models of mitochondrial disease.

INTRODUCTION

Mutations in mitochondrial DNA (mtDNA), and in the vast repertoire of nuclear genes that converge on the formation and function of the mitochondrial respiratory chain (MRC), are responsible for primary “mitochondrial disorders,” a group of highly heterogeneous conditions, hallmarked by faulty oxidative phosphorylation (OXPHOS), that can affect any organ, at any age, and by any mode of transmission (Koopman et al., 2012). When taken as a whole, mitochondrial disorders are among the most frequent genetic diseases, affecting >1 in 5,000 individuals in the European population (Elliott et al., 2008). Despite substantial progress in mitochondrial medicine, the complexity of mitochondrial biology and genetics still constitutes a major challenge for understanding the mechanistic basis of mitochondrial disorders and explains, at least in part, their huge clinical and biochemical variability, which is also a major hurdle toward effective treatment. However, the development of “general” therapeutic strategies, extendable to diverse disease-associated OXPHOS defects, is now a realistic goal, based on rapidly expanding knowledge of the molecular mechanisms underpinning mitochondrial biogenesis, quality control, and signaling pathways. Several of these mechanisms are related to the control of mitochondrial shape and organization of the mitochondrial network.

Mitochondria are highly dynamic organelles that fuse and divide to adapt their structure and morphology to the energetic needs of the cell (Kasahara and Scorrano, 2014). Dynamin-related GTPases located on the inner and outer mitochondrial membranes (IMMs and OMMs) control the fission and fusion processes (Griparic and van der Bliek, 2001). Mitochondrial fission and fusion regulate a number of cellular processes, including organelle distribution during cell proliferation, bioenergetics proficiency, mitochondrial calcium flux, mitochondrial apoptosis, autophagy, and even complex morphogenetic processes such as the formation of dendritic spines (Kasahara and Scorrano, 2014). Fission is regulated by Dynamin-Related Protein 1 (DRP1) and by its OMM partners Fission 1 (FIS1), Mitochondrial Fission Factor (MFF), and Mitochondrial Division (MID) 49 and 51; fusion is controlled by the OMM proteins Mitofusins 1 and 2 (MFN1 and 2) and by the IMM protein Optic Atrophy 1 (OPA1). In humans, eight OPA1 isoforms are produced by alternative splicing of a single gene (Delettre et al., 2001), which are further processed by at least three proteases to form several long (L) and short (S) forms of OPA1 (Anand et al., 2014; Ehses et al., 2009; Griparic et al., 2007; Head et al., 2009; Ishihara et al., 2006; Song et al., 2007), which oligomerize to form functionally active quaternary structures (Frezza et al., 2006). In addition to its role in mitochondrial fusion, OPA1 controls the cristae remodeling arm of mitochondrial apoptosis (Frezza et al., 2006) and the physical and functional organization of the MRC complexes in MRC supercomplexes (Cogliati et al., 2013). These functional quaternary structures increase electron flow channeling through MRC complexes during respiration, thus minimizing
electron leaks (Acín-Pérez and Enriquez, 2014; Enriquez and Lennaz, 2014), and stabilize individual MRC complexes such as Complex III, CIII (Acín-Pérez et al., 2004). Consequently, OPA1 levels directly affect mitochondrial respiratory efficiency (Cologläti et al., 2013) and proteolytic inactivation of OPA1 has been shown to occur in cells from mitochondrial disease patients (Duvezin-Caulet et al., 2007). However, the role of OPA1 in vivo and the potential of its stabilization as a strategy to combat mitochondrial dysfunction and disease remains unclear. Genetic ablation of Opa1 is lethal during mouse embryonic development (Rahn et al., 2013) and causes massive dysfunction also in postmitotic tissues (L.S., unpublished data). On the other hand, high levels of OPA1 overexpression are toxic in cells, leading to paradoxical consequences in subsequent measurements, with the Ndufs4+/− groups showing consistently better than the Ndufs4−/− group in weeks 5 (latency to fall: 556 ± 39 versus 388 ± 46 s) and 6 (latency to fall: 384 ± 56 versus 123 ± 50 s). At week 7, both groups were virtually unable to perform the test at any rate, due to collapse of neurological conditions or death (Figure 1A).

**RESULTS**

**Generation of Ndufs4−/+::Opa1tg and Cox15sm/sm::Opa1tg Mouse Lines**

All procedures on animals were conducted under the UK Animals (Scientific Procedures) Act, 1986, and approved by the local ethical review committee. To obtain transgenic overexpression of Opa1 in OXPHOS defective mouse lines, we crossed the Opa1tg mouse with either Ndufs4−/− or Cox15sm/sm mice. The constitutive Ndufs4−/+ mouse shows severe CI deficiency, and the accumulation of a catalytically inactive 830 kDa CI assembly intermediate, associated with the onset of a rapidly progressive syndrome, dominated by neurological impairment starting approximately 40 days after birth (Calvaruso et al., 2012; Kruse et al., 2008). The Cox15sm/mm mouse shows profound skeletal muscle COX deficiency, leading to muscle wasting, severe motor performance impairment, and markedly reduced survival (Visconi et al., 2011).

**Ndufs4−/+::Opa1tg Mice Live Longer and Show Better Motor Coordination than Ndufs4−/− Mice**

Western blot analysis on two animals for each genotype showed tissue-specific increases in the levels of long and short forms of OPA1 in brain (Figure S1A), skeletal muscle (Figure S1B), and heart (Figure S1C) in Opa1tg and Ndufs4−/+::Opa1tg relative to their Ndufs4−/− and wild-type (WT) littermates. Motor coordination, measured as latency to fall by weekly performed rotorad test, showed steady downhill in both Ndufs4−/−::Opa1tg and Ndufs4−/+ groups, each composed of six animals. However, the scores, which were identical in the two groups at the beginning of the observation (week 4 after birth), showed significant differences in subsequent measurements, with the Ndufs4−/+::Opa1tg group performing consistently better than the Ndufs4−/− group in weeks 5 (latency to fall: 556 ± 39 versus 388 ± 46 s) and 6 (latency to fall: 384 ± 56 versus 123 ± 50 s). At week 7, both groups were virtually unable to perform the test at any rate, due to collapse of neurological conditions or death (Figure 1A). Furthermore, Kaplan-Meier analysis showed that survival probability was moderately, but significantly, prolonged in Ndufs4−/+::Opa1tg versus Ndufs4−/− littermates (survival median: 62.0 versus 53.5 days; log-rank test p = 0.015) (Figure 1B). The amelioration in motor coordination and prolongation of the lifespan was associated with a ~1.5-fold increase in glutamate-malate-dependent state-3 oxygen consumption rate of isolated brain mitochondria (reflecting CI-driven respiration), which nevertheless remained well below the values obtained in brain mitochondria of WT and Opa1tg littermates (Figure 1C). Accordingly, CI activity, measured by spectrophotometric assay (Figure 1D), was ~2.5-fold increased (p < 0.05) in Ndufs4−/+::Opa1tg versus Ndufs4−/− isolated brain mitochondria. Several MRC complex subunits were analyzed by western blotting on SDS-PAGE of isolated brain mitochondria. Notably, the two tested CI subunits (NDFU9A and NDFUFB8), as well as the UQCR22 subunit of CIII, were increased ~1.3-fold in Ndufs4−/+::Opa1tg versus Ndufs4−/− brain mitochondria (Figure 1E). The p values of two-tail, unpaired Student’s t test of densitometric analysis obtained from four independent samples/genotype was p = 0.02 for NDFU9A, p = 0.04 for NDFUFB8, and p = 0.01 for UQCR22. Contrariwise, no significant change was observed for SDHA (CII), COX1 (CIV), and ATP5A (CV) subunits. Blue native gel electrophoresis (BNGE)-based in-gel activity of CI and CI-containing supercomplexes (sc) showed hardly any reactive band in both Ndufs4−/+::Opa1tg double mutants and Ndufs4−/− littermates (Figure S1D). Western blot analysis using antibodies against NDFUB8 (specific to CI), UQCR1 (specific to CIII), and COX1 (specific to CIV) showed, in the same genotypes, a catalytically active 200 kDa assembly intermediate as well as the catalytically inactive 830 kDa CI and 830 kDa+CIII2 assembly intermediates previously reported in the Ndufs4−/− mouse model (Calvaruso et al., 2012), but neither...
CI holocomplex nor CI supercomplexes containing CI+CIll2 or CI+CIll2+CIIV (Figure S1E). However, electron microscopy analysis of forebrain (Figure 1F) and cerebellum (not shown) suggested amelioration in cristae architecture, with no change in mitochondrial shape and dimensions. These findings suggest that moderate Opa1 overexpression partially rescues the phenotype of a CI-deficient mouse model by improving cristae morphology.

Survival and Functional Improvement in the Double Mutant Cox15sm/sm::Opa1tg Mice

Similar to Cox15sm/sm::Opa1tg, male and female Cox15sm/sm::Opa1tg mice were smaller than WT or Opa1tg littermates (p < 0.005; n = 4 for each genotype; data not shown). To test muscle performance, we monthly monitored motor endurance by a standard treadmill test starting at 2 months of age. Albeit still significantly lower than WT (n = 8) and Opa1tg (n = 9) littermates, the scores of the Cox15sm/sm::Opa1tg double mutant group (n = 8) were ~2- to 3-fold higher than those of the Cox15sm/sm group (n = 10) (p < 0.005 at each time point; Figure 2A). On average, the distance covered by Cox15sm/sm::Opa1tg animals was three times that of the Cox15sm/sm littermates (169 ± 20 versus 58 ± 6 m, p = 2.59 × 10^-5) (Figure S2A). This improvement was recorded in both homzygous females and hemizygous males, with no significant gender difference (Figure S2B). Interestingly, Opa1tg performed better than WT littermates (890 ± 50 versus 803 ± 27 m, p < 0.001; Figure S2A). We followed the animals up to 6 months of age. Notably, Cox15sm/sm::Opa1tg double mutants showed a remarkable increase in survival probability compared to their Cox15sm/sm littermates (log-rank test p = 0.0003). Only 1/17 (7%) Cox15sm/sm::Opa1tg animal died during our observation period, versus 12/23 (50%) Cox15sm/sm littermates (Figure 2B).

These findings indicate that controlled Opa1 overexpression ameliorates life expectancy and motor function of a mouse model of muscle CIV deficiency.

Amelioration of Mitochondrial Morphology and Biochemistry in Cox15sm/sm::Opa1tg Muscle

H&E staining of the mid-portion of gastrocnemius muscle (Figure 3A) showed numerous centralized nuclei in both Cox15sm/sm and Cox15sm/sm::Opa1tg, the proportion of which did not significantly differ between the two groups (Figure 3B). However, the average cross-sectional area (CSA) of muscle fibers was doubled in the Cox15sm/sm::Opa1tg relative to Cox15sm/sm muscles (p < 0.05), although still smaller (~50%) than the areas measured in WT and Opa1tg muscles (p < 0.05) (Figure 3C). We also tested whether the muscle hypertrophic and dystrophic features of the Cox15sm/sm mice were associated with increased apoptosis or altered autophagy. TUNEL staining in muscle sections did not reveal hardly any positive fiber in either Cox15sm/sm or Cox15sm/sm::Opa1tg, similar to WT and Opa1tg (not shown), suggesting that apoptosis is not a major player in the pathogenesis of Cox15sm/sm myopathy. Immuno-staining of muscle sections with Lamp1 and P62 antibodies revealed increased reactions in Cox15sm/sm samples compared to WT and Opa1tg, which, however, did not change in Cox15sm/sm::Opa1tg (not shown). These data indicate that apoptosis and autophagy are unlikely to contribute to the amelioration observed in Cox15sm/sm::Opa1tg mice.

Next, we performed ultrastructural analysis of muscle fibers (Figure 4). Examination of tibialis anterior muscle revealed that the number of mitochondria in both Cox15sm/sm::Opa1tg and Cox15sm/sm were smaller than WT or Opa1tg littermates (log-rank test p = 0.005; n = 4 for each genotype; data not shown). To test muscle performance, we monthly monitored motor endurance by a standard treadmill test starting at 2 months of age. Albeit still significantly lower than WT (n = 8); blue outline: Opa1tg (n = 5); red outline: Ndufs4–/–:Opa1tg (n = 6). The asterisks represent the significance levels calculated by unpaired, two-tail Student’s t-test *p < 0.05. (B) Kaplan-Meier survival probability. Significance was assessed by the log rank test. Solid blue: Ndufs4–/– (n = 16); red solid: Ndufs4–/–:Opa1tg (n = 9).

(C) Oxygen consumption measurements (nmol O2/min/mg of protein). Solid blue: WT (n = 5); blue outline: Opa1tg (n = 5); red outline: Ndufs4–/–:Opa1tg (n = 6). The asterisks represent the significance levels calculated by unpaired, two-tail Student’s t-test *p < 0.05. (D) MRC activities (nmol/min/mg of protein) in isolated brain mitochondria. Solid blue: WT (n = 8); blue outline: Opa1tg (n = 5); red solid: Ndufs4–/– (n = 7); red outline: Ndufs4–/–:Opa1tg (n = 8). Error bars represent SEM. The asterisks represent the significance levels calculated by unpaired, two-tail Student’s t test *p < 0.05.
marked improvement of mitochondrial and myofibrillar morphology in Cox15\textsuperscript{sm/sm}::Opa1\textsuperscript{Tg} compared to Cox15\textsuperscript{sm/sm}. While Cox15\textsuperscript{sm/sm} muscle mitochondria showed profound cristae disorganization and prominent vacuolar degeneration of the inner mitochondrial compartment, the Cox15\textsuperscript{sm/sm}::Opa1\textsuperscript{Tg} cristae were organized in an ordered and parallel array and were tightly folded, similar to those observed in WT and Opa1\textsuperscript{Tg} littermate specimens (Figure 4A). No hyperfused organelles were observed in Opa1\textsuperscript{Tg} muscle. Likewise, myofilbrils, which in Cox15\textsuperscript{sm/sm} appeared disorganized and separated from each other by the accumulation of membranous and granular material, showed a compact orderly array in Cox15\textsuperscript{sm/sm}::Opa1\textsuperscript{Tg}, again resembling those of WT and Opa1\textsuperscript{Tg} specimens (Figure 4A). Quantification of area, perimeter, length, and width of muscle mitochondria showed significantly increased values (1.5- to 5-fold) in Cox15\textsuperscript{sm/sm}::Opa1\textsuperscript{Tg} samples, indicating mitochondrial ballooning. These parameters were partially corrected in Cox15\textsuperscript{sm/sm}::Opa1\textsuperscript{Tg} samples (1.3- to 1.8-fold versus WT) (Figure 4B).

We then tested whether the improvements in motor performance and muscle morphology observed in Cox15\textsuperscript{sm/sm}::Opa1\textsuperscript{Tg} animals were associated with ameliorated biochemical parameters. Glutamate-malate dependent state-3 oxygen consumption rate in isolated muscle mitochondria (reflecting CI-driven respiration) was decreased in Cox15\textsuperscript{sm/sm} animals (128 ± 37 nmol/min/mg protein), but increased significantly in Cox15\textsuperscript{sm/sm}::Opa1\textsuperscript{Tg} (190 ± 48 nmol/min/mg; p < 0.05), up to lower normal values, not significantly different from those obtained in WT (250 ± 84 nmol/min/mg) and Opa1\textsuperscript{Tg} (276 ± 101 nmol/min/mg) samples (Figure 5A). Similar results were obtained by measuring succinate- and TMPD/ascorbate-dependent state-3 oxygen consumption rate (reflecting CI- and CIV-driven respiration, respectively) (Figure 5A). We also analyzed the activity of the single respiratory complexes by spectrophotometric assays. The specific activity of CIV normalized to that of citrate synthase (CS) was significantly increased (p < 0.05) in Cox15\textsuperscript{sm/sm}::Opa1\textsuperscript{Tg} (16 ± 3) versus Cox15\textsuperscript{sm/sm} (10 ± 4) muscle homogenates, albeit still much lower than that of WT (47 ± 18) or Opa1\textsuperscript{Tg} (44 ± 7) muscles (Figure 5B). These results were qualitatively confirmed by histochemical COX staining, which showed reduced COX-negative fibers in COX/SDH double reaction in Cox15\textsuperscript{sm/sm}::Opa1\textsuperscript{Tg} versus Cox15\textsuperscript{sm/sm} muscle fibers (Figure 5C).

Overall, these data indicate that the ameliorated Cox15\textsuperscript{sm/sm}::Opa1\textsuperscript{Tg} mitochondrial ultrastructure is accompanied by improved respiration and CIV activity.

### Mitochondrial Protein and Transcriptional Analysis in Cox15\textsuperscript{sm/sm}::Opa1\textsuperscript{Tg} Mice

In order to investigate the molecular bases of the observed correction of mitochondrial respiration in Cox15\textsuperscript{sm/sm}::Opa1\textsuperscript{Tg} animals, we first analyzed the amount of subunits specific to each MRC complex by western blotting analysis of muscle homogenates separated by SDS-PAGE (Figure 6A). COX1, the mtDNA-encoded largest CIV subunit, which contains the two heme-a moieties and the Cux center, and COX4 (isofrom 1), a nucleus-encoded early-assembled CIV subunit, were both reduced in Cox15\textsuperscript{sm/sm} compared to WT and Opa1\textsuperscript{Tg} samples, but did increase in those from Cox15\textsuperscript{sm/sm}::Opa1\textsuperscript{Tg} animals (Figure 6B). In both Cox15\textsuperscript{sm/sm} and Cox15\textsuperscript{sm/sm}::Opa1\textsuperscript{Tg} samples, subunits of other MRC complexes were generally increased, particularly the UQRCR2 subunit of CIII, suggesting compensatory activation of MRC biogenesis induced by CIV deficiency. Interestingly, analysis of mitochondrial and nuclear transcripts of CIV subunits revealed a specific, marked reduction of mt-Cox1 mRNA in Cox15\textsuperscript{sm/sm} samples, which was fully rescued in Cox15\textsuperscript{sm/sm}::Opa1\textsuperscript{Tg} samples (Figure 6C). Conversely, other mtDNA- (mt-Cox2) and nucleus-encoded (Cox4I1 and Cox5a) transcripts were unchanged in Cox15\textsuperscript{sm/sm} versus WT mice and were not affected by Opa1 overexpression (Figure 6C). We detected no quantitative change in any group of animals for transcripts Ppargc1a and Tfam, encoding PGC-1α and TFAM, respectively (Figure 6C). The mtDNA copy number, which was increased in Cox15\textsuperscript{sm/sm}, returned to WT levels in Cox15\textsuperscript{sm/sm}::Opa1\textsuperscript{Tg} (Figure S3A), suggesting normalization of the homeostatic control on mtDNA maintenance (Scarpulla \textit{et al.}, 2012). Importantly, the Cox15 transcript level measured in skeletal muscle of Cox15\textsuperscript{sm/sm} was ~9% of that present in WT and Opa1\textsuperscript{Tg} littermates and did not change significantly in Cox15\textsuperscript{sm/sm}::Opa1\textsuperscript{Tg} (Figure S3B), ruling out a direct effect of Opa1 overexpression on Cox15 transcription.

In order to analyze the composition and amount of OPA1, we performed a western blot experiment using an anti-OPA1
antibody that specifically recognizes five OPA1 species in skeletal muscle, namely isoforms 1 and 7, and short forms 1–3, resulting from proteolytic cleavage (Figure 6A). Densitometric quantification showed a significant increase (~1.4-fold) in the amount of all five detected OPA1 species in Opa1tg muscle samples relative to WT. The amount of OPA1 species was further increased (~1.3- to 1.8-fold) in Cox15sm/sm::Opa1tg versus WT and significantly more so in Cox15sm/sm::Opa1tg (~1.5- to 2-fold), particularly for the short forms 1–3 (Figure S3C).

These data suggest that Opa1 overexpression leads to stabilization in CI protein and transcript levels.

MRC Supercomplexes Are Increased in Cox15sm/sm::Opa1tg Mice

Individual stability of MRC complexes depends on their assembly in respiratory chain supercomplexes (sc) (Acín-Pérez et al., 2004). We therefore analyzed CI and CIV BNGE in-gel activities in digitonin-treated isolated muscle mitochondria. In Cox15sm/sm samples, the intensity of the band corresponding to individual CI supercomplex was markedly reduced, and hardly any reaction was detected in the high-molecular-weight area harboring sc. However, individual CIV in-gel activity was increased, and CIV reactive high-molecular-weight bands were clearly detected in Cox15sm/sm::Opa1tg mitochondria (Figure 7A), indicating stabilization of CIV holocomplex and CIV-containing sc. Likewise, several high-molecular-weight CI-reactive bands, corresponding to CI- and CIV-containing sc, were virtually absent in the same Cox15sm/sm samples but were clearly, albeit weakly, visible in the Cox15sm/sm::Opa1tg samples (Figure 7B). Western blot analysis on BNGE samples using an anti-COX1-specific antibody confirmed the results of in-gel activity: a band corresponding to CI holocomplex was increased in Cox15sm/sm::Opa1tg compared to Cox15sm/sm and high-molecular-weight Cox1-reactive bands, which were detected in WT and an anti-Ndufa9 antibody showed slight increased Ndufa9 amount in Cox15sm/sm::Opa1tg versus Cox15sm/sm (Figure 7D). These results indicate that increased OPA1 levels stabilize RCS and residual CIIV in Cox15sm/sm mice.

DISCUSSION

The moderate overexpression of Opa1 achieved in the Opa1tg mouse model is well tolerated and compatible with normal development, fertility, and lifespan. No obvious detrimental effects were noted during the 6-month period of our experimental observation; although, as mentioned in the accompanying paper by Varanita et al. (2015), prolonged Opa1 overexpression may lead to increased strain-specific prevalence of cancer, a somehow expected result given the antiapoptotic role of OPA1. However, increased OPA1 amount in key tissues, including skeletal and cardiac muscle, brain, and liver, confer remarkable protection against a wide spectrum of experimental tissue damage models (accompanying paper by Varanita et al., 2015). Here, we demonstrate that this effect can be exploited to improve the motor performance, as well as biochemical and molecular phenotypes of two mouse models of genetically determined OXPHOS failure.

The Ndufs4−/− mouse is characterized by the complete absence of a structural component of CI. In the current map of mammalian CI, the NDUF54 18 kDa subunit has been modeled into a density in a cleft between the 75 kDa subunit and the 49 kDa, 30 kDa, and NDUF8 (TYKY) subunits (Vinothkumar et al., 2014). This localization may explain why disease mutations in the 18 kDa subunit lead to accumulation of late-stage intermediates lacking the NADH-dehydrogenase module (Calvaruso et al., 2012). The indispensable role of NDUF54 in preserving the CI structure and redox activity can also explain the limited improvement in motor coordination,
measured by rotarod, and biochemical phenotypes observed in Ndufs4\textsuperscript{−/−}::Opa1\textsuperscript{Tg} double mutants. Rapamycin-induced inhibition of mTOR, which was recently shown to alleviate the phenotype, also failed to correct the CI defect of Ndufs4\textsuperscript{−/−} mice (Johnson et al., 2013). However, the cristae-centric approach used here to treat CI deficiency improved survival to levels comparable to those achieved by every-other-day rapamycin administration (Johnson et al., 2013). It would be interesting to verify if the combination of Opa1 overexpression and mTOR inhibition is additive and can achieve even better amelioration. We also cannot exclude that OPA1-driven correction of OXPHOS failure may be less amenable in brain than in other tissues, e.g., skeletal muscle.

The Cox15\textsuperscript{sm/sm} mouse model is based on muscle-specific ablation of Cox15, dependent on the activity of the Cre recombinase under the control of the skeletal-muscle-specific actin promoter. Although Cox15\textsuperscript{sm/sm} animals display early-onset progressive mitochondrial myopathy, characterized by profound muscle weakness and wasting leading to early death, the genetic lesion underpinning this phenotype can be considered as a hypomorphic mutant allele, the effects of which on COX activity and MRC proficiency are severe but partial. Accordingly, Cox15 transcript was drastically decreased, but not absent, in Cox15\textsuperscript{sm/sm} and Cox15\textsuperscript{sm/sm}::Opa1\textsuperscript{Tg} muscles, explaining the presence of some residual COX activity in muscle of adult animals and the “mosaic”-like distribution of the histochemical reaction to COX, which is preserved in scattered muscle fibers (Figure 5C). The molecular Cox15\textsuperscript{sm/sm} defect resembles that of the majority of mitochondrial disorders (i.e., partial rather than complete biochemical impairment) due to decreased but not abolished function of the mutant gene product. Incidentally, this category of mitochondrial diseases also includes the few COX15 mutant patients reported in the literature (Alfadhel et al., 2011; Antonicka et al., 2003; Bugiani et al., 2005). These considerations may explain the marked and persistent improvement of the motor performance observed in Cox15\textsuperscript{sm/sm}::Opa1\textsuperscript{Tg} double mutants and suggest that OXPHOS impairment due to decreased, but not abolished, function of the mutant gene product is amenable to correction by potentiating the effects of OPA1 on mitochondrial cristae shape and MRC function.

The Cox15\textsuperscript{sm/sm}::Opa1\textsuperscript{Tg} animals displayed not only a dramatic increase in motor endurance but also a remarkable extension of survival up to, and in most cases well beyond, the 6-month period of observation of our experimental protocol. These effects were associated with robust increase of muscle mitochondrial respiration accompanied by a milder but significant increase of muscle COX activity. The discrepancy between the nearly complete reversion to normal in the respiratory rate and the modest increment of COX activity may be explained by increased stabilization of CIV holocomplex and CIV-associated supercomplexes, as indicated by results of BNGE in-gel activity and immunoblotting. The recovery in the motor performance and biochemical proficiency of the Cox15\textsuperscript{sm/sm}::Opa1\textsuperscript{Tg} double mutants was associated with marked correction of the profound ultrastructural alteration in cristae morphology associated with Cox15 ablation. This effect is likely to be directly connected to the role of OPA1 in cristae shape and may be correlated to partial stabilization of the CIV holocomplex and supercomplex organization in the cristae membrane. Accordingly, both nucleus- and mitochondria-encoded MRC subunits were increased in Cox15\textsuperscript{sm/sm}::Opa1\textsuperscript{Tg} versus Cox15\textsuperscript{sm/sm} muscles, while the corresponding transcripts were unchanged, with the notable exception of mt-COX1 mRNA. COX1 is the mtDNA-encoded catalytic CIV subunit that contains the heme-α moieties essential for COX1 stabilization (Bareth et al., 2013). The concordant decrease of both COX1 protein and mt-COX1 mRNA in Cox15\textsuperscript{sm/sm} was completely reverted in Cox15\textsuperscript{sm/sm}::Opa1\textsuperscript{Tg}. Taken together, these results substantiate the possibility that in mammals, as in Saccharomyces cerevisiae, COX1 biosynthesis is regulated by a feedback mechanism linking translation and post-translation processing to transcription (Hermann et al., 2013). In yeast, a key role in this homeostatic loop is carried out by the mt-COX1-specific translation activators MSSS1 and PET309 (Hermann et al., 2013). The mammalian ortholog of PET309 has been identified as the leucine-rich pentatricopeptide repeat-containing (LRPPRC) protein, a RNA binding factor that is mutated in French-Canadian, COX defective Leigh-like syndrome in humans (Mootha et al., 2003). Interestingly, LRPPRC interacts with OPA1, at least in D. melanogaster (Banerjee and Chinthapalli, 2014). However, as there is no obvious mammalian
ortholog of MSS51, and the 5’ UTR sequence of the yeast Cox1 mRNA, to which MSS51 binds, is virtually absent in the mammalian transcript, these feedback mechanisms are probably different in their molecular details between yeast and mammalian systems.

Finally, while apoptosis seems not to play a significant role in Cox15sm/sm myopathy and is not increased in Cox15sm/sm::Opa1tg samples, immunoanalysis suggested dysregulated autophagy in response to Cox15 ablation, but whether this is relevant to the pathogenesis of the disease remains to be further investigated.

Our study demonstrates that OPA1-dependent mitochondrial cristae and sc stabilization is effective in correcting mitochondrial disease conditions characterized by partial, however severe, MRC defects. OPA1 can even partially improve defects caused by complete lack of mitochondrial CI via an unknown mechanism. Our results indicate OPA1 as a new target for effective therapy of primary mitochondrial disorders.
translational point of view, future work is warranted to elucidate the molecular details underpinning these remarkable effects, extend the experimental observation to additional OXPHOS-defective models, and eventually transfer this set of proof-of-principle observations into effective therapeutic approaches by, for example, selecting compounds or bioreactors able to control the expression or stability of OPA1 in suitable mammalian cell systems and animal models.

**EXPERIMENTAL PROCEDURES**

**Reagents and Materials**

Antibodies (COX1, COX4, UQCRG2, NDUFA9, ATP5a, and SDHA) were from Mitoscience, GAPDH were from Millipore, P62 was from Sigma, LC3-I/I was from Cell Signaling, Lamp1 was from Sigma, and OPA1 was from BD Biosciences.

**Animal Work**

All procedures were conducted under the UK Animals (Scientific Procedures) Act, 1986, approved by Home Office license (PPL: 7538) and local ethical review. The mice were kept on a C57Bl6/129Sv mixed background, and WT littermates were used as controls. The animals were maintained in a temperature- and humidity-controlled animal-care facility with a 12 hr light/dark cycle and free access to water and food and were sacrificed by cervical dislocation.

**Behavioral Analysis**

A treadmill apparatus (Columbus Instruments) was used to measure motor exercise endurance according to the number of falls in the motivational grid during a gradually accelerating program with speed initially at 6.5 m/min and increasing by 0.5 m/min every 3 min. The test was terminated by exhaustion, defined as >10 falls/min into the motivational grid.

A rotarod apparatus (Ugo Basile) was used to assess coordination skills. After two acclimation sessions, the mice underwent three trial sessions at least 20 min apart, using a standard acceleration protocol pre-set by the constructor.

**Oxygen Consumption Studies**

Mouse brains were homogenized in 0.075 M sucrose, 0.225 M mannitol, 1 mM EGTA, and 0.01% fatty-acids-free BSA (pH 7.4); skeletal muscle were homogenized in 150 mM sucrose, 50 mM Tris-HCl, 10 mM EDTA, 0.2% BSA (pH 7.4), and subtilisin 1 mg/ml of muscle (Frezza et al., 2007). Mitochondria isolated by differential centrifugation and resuspended 25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 0.05 mM EDTA, 5 mM MgCl2, 10 mM Tris-HCl (pH 7.4), and 10 mM H3PO4 (pH 7.4) (Fernández-Vizarra et al., 2002).

For oxygraphic measurements, 250–500 μg of mitochondrial proteins were incubated in a buffer containing 225 mM sucrose, 75 mM mannitol, 10 mM Tris-HCl (pH 7.4), 10 mM KCl, 10 mM KH2PO4, 5 mM MgCl2, and 1 mg/ml fatty-acids-free BSA (pH 7.4). Oxygen consumption was evaluated by a Clark oxygen electrode (Hansatech, Instruments), using the following substrates and inhibitors concentrations: 5 mM glutamate and 2.5 mM malate for cl-dependent respiration, 5 mM succinate and 2 μM rotenone for cI-dependent respiration, 6 mM ascorbate, and 300 μM TMPD and antimycin A 0.25 μg/ml for cIV-dependent respiration. 100 μM ADP was added to stimulate ATP-coupled oxygen consumption. 100 μM NaCN was added to completely inhibit respiration (Frezza et al., 2007).

**BNGE**

For BNGE analysis, 250 μg of mitochondria isolated as described above were resuspended in native page buffer (Invitrogen), protease inhibitors, and 4% digitonin and incubated for 1 hr on ice before centrifuging at 20,000 × g at 4°C. 5% Coomassie G250 was added to the supernatant. 30 μg were separated by 3%–12% gradient BNGE and either stained with for in-gel activities or electroblotted on PVDF membranes for immunodetection (Nijtmans et al., 2002).

**Morphological Analysis**

For histochemical analysis, tissues were frozen in liquid-nitrogen-pre-cooled isopentane, 8-μm-thick sections were stained for COX and SDH, as described (Sciacco and Bonilla, 1996). Analysis of centralized nuclei and analysis was performed on H&E-stained sections using ImageJ on four samples/genotype (600 fibers/sample). For ultrastructural studies, samples were fixed with 2.5% glutaraldehyde in 10 mM phosphate buffer (pH 7.4). Quantification of mitochondrial morphometry (area, perimeter, long axis, and short axis) in electron micrographs was performed using ImageJ on 200 mitochondria from WT and Opa1Tg mutant and 500 mitochondria from Cox15sm/sm::Opa1tg muscles.

**Biochemical Analysis of MRC Complexes**

Brain and skeletal muscle samples were snap-frozen in liquid nitrogen and homogenized in 10 mM phosphate buffer (pH 7.4). The spectrophotometric activity of CI, CII, CIII, and CIV, as well as Cox, was measured as described in Bugiani et al. (2004).

**Real-Time PCR**

MtDNA content and transcripts analysis was carried out by SYBR Green real-time PCR, as described in Viscomi et al. (2011).

**Western Blot Analysis**

Mouse tissues were homogenized in ten volumes of 10 mM potassium phosphate buffer (pH 7.4). Mitochondrial-enriched fractions were collected after centrifugation at 800 × g for 10 min in the presence of protease inhibitors.
and frozen and thawed three times in liquid nitrogen. Protein concentration was determined by the Lowry method. Aliquots, 70 µg each, were run through a 4%–12% SDS-PAGE and electroblotted onto a nitrocellulose membrane, which was then immunodecorated with different antibodies.

Statistical Analysis
All numerical data are expressed as mean ± SEM. Student’s unpaired two-tailed t test and Kaplan-Meier distribution were used for statistical analysis. Differences were considered statistically significant for p < 0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this manuscript.

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REFERENCES


Supplemental Information

Opa1 Overexpression Ameliorates the Phenotype of Two Mitochondrial Disease Mouse Models

Gabriele Civiletto, Tatiana Varanita, Raffaele Cerutti, Tatiana Gorletta, Serena Barbaro, Silvia Marchet, Costanza Lamperti, Carlo Viscomi, Luca Scorrano, and Massimo Zeviani
Supplemental Material Online

Figure S1, related to figure 1. Analysis of the *Ndufs4*−/− and *Ndufs4*−/−::*Opa*<sup>Tg</sup> mouse models.

A) Western blot immunovisualization (upper panel) and densitometric analysis (lower panel) of OPA1 long (L) and Short (S) isoforms in brain mitochondria. Solid blue: WT; blue outline: *Opa1*<sup>Tg</sup>; solid red: *Cox15*<sup>sm/sm</sup>; red outline: *Cox15*<sup>sm/sm</sup>::*Opa1*<sup>Tg</sup>. Error bars represent SEM. VDAC was used as a loading control.

B) Western blot immunovisualization (upper panel) and densitometric analysis (lower panel) of OPA1 forms in skeletal muscle mitochondria of *WT*, *Opa*<sup>Tg</sup>, *Ndufs4*−/−, *Ndufs4*−/−::*Opa*<sup>Tg</sup> mice. Solid blue: WT; blue outline: *Opa1*<sup>Tg</sup>; solid
red: Cox15$^{sm/sm}$; red outline: Cox15$^{sm/sm,:Opa1^Tg}$. Error bars represent SEM. VDAC was used as a loading control.

C) Western blot immunovisualization (upper panel) and densitometric analysis (lower panel) of OPA1 forms in heart mitochondria of WT, Opa$^{Tg}$, Ndufs4$^{−/−}$, Ndufs4$^{−/−,:Opa1^Tg}$ mice. Solid blue: WT; blue outline: Opa1$^{Tg}$; solid red: Cox15$^{sm/sm}$; red outline: Cox15$^{sm/sm,:Opa1^Tg}$. Error bars represent SEM. VDAC was used as a loading control.

D) BNGE-in-gel activities of MRC CI from BNGE of digitonin-treated isolated mitochondria of WT, Opa$^{Tg}$, Ndufs4$^{−/−}$, Ndufs4$^{−/−,:Opa1^Tg}$ samples. See main text for details.

E) Western-blot immunovisualization of MRC CI, CIII, and CIV from BNGE of digitonin-treated isolated mitochondria of WT, Opa$^{Tg}$, Ndufs4$^{−/−}$, Ndufs4$^{−/−,:Opa1^Tg}$ samples. See main text for details. Anti-COX1, anti-NDUFB8 and anti-UQCRC1 antibodies were used for CIV, CI, and CIII.
Figure S2, related to figure 2.
In vivo phenotypic characterization of $\text{Cox15}^{\text{sm/sm}}$ and $\text{Cox15}^{\text{sm/sm}}::\text{Opa1}^{\text{Tg}}$ mouse models

A) Means of weekly-performed treadmill tests over 5 weeks. Solid blue: WT (n=8); blue outline: $\text{Opa1}^{\text{Tg}}$ (n=9); solid red: $\text{Cox15}^{\text{sm/sm}}$ (n=9); red outline: $\text{Cox15}^{\text{sm/sm}}::\text{Opa1}^{\text{Tg}}$ (n=10). Error bars represent SEM. Statistical significance was calculated by unpaired, 2-tail Student’s t test: **$p<0.01$: ***$p<0.005$.

B) Motor performance by treadmill tests in males (continuous lines) and females (dashed lines). Red lines: $\text{Cox15}^{\text{sm/sm}}::\text{Opa1}^{\text{Tg}}$; blue lines: $\text{Cox15}^{\text{sm/sm}}$ individuals. Asterisks refer to significant differences between $\text{Cox15}^{\text{sm/sm}}::\text{Opa1}^{\text{Tg}}$ vs $\text{Cox15}^{\text{sm/sm}}$ calculated by unpaired, 2-tail Student’s t test: **$p<0.01$. Values between genders were not significantly different for either genotype. Error bars represent SEM.
Figure S3, related to figure 6.
Quantitative analyses in skeletal muscle

A) Quantification of Cox15 transcript. Solid blue: WT (n=4); blue outline: Opa1tg (n=4); solid red: Cox15sm/sm (n=4); red outline: Cox15sm/sm::Opa1tg (n=4). Error bars represent SEM. Statistical significance was calculated by unpaired, 2-tail Student’s t test: ***p<0.005.

B) Quantification of mtDNA copy number. Solid blue: WT (n=4); blue outline: Opa1tg (n=4); solid red: Cox15sm/sm (n=4); red outline: Cox15sm/sm::Opa1tg (n=4). Error bars represent SEM. Statistical significance was calculated by unpaired, 2-tail Student’s t test: **p<0.01; ***p<0.005.

C) Densitometric analysis of Opa1 isoforms in skeletal muscle on n=4 samples for each genotype. Error bars represent SEM. Statistical significance was calculated by unpaired, 2-tail Student’s t test: *p<0.05; **p<0.01; ***p<0.005.