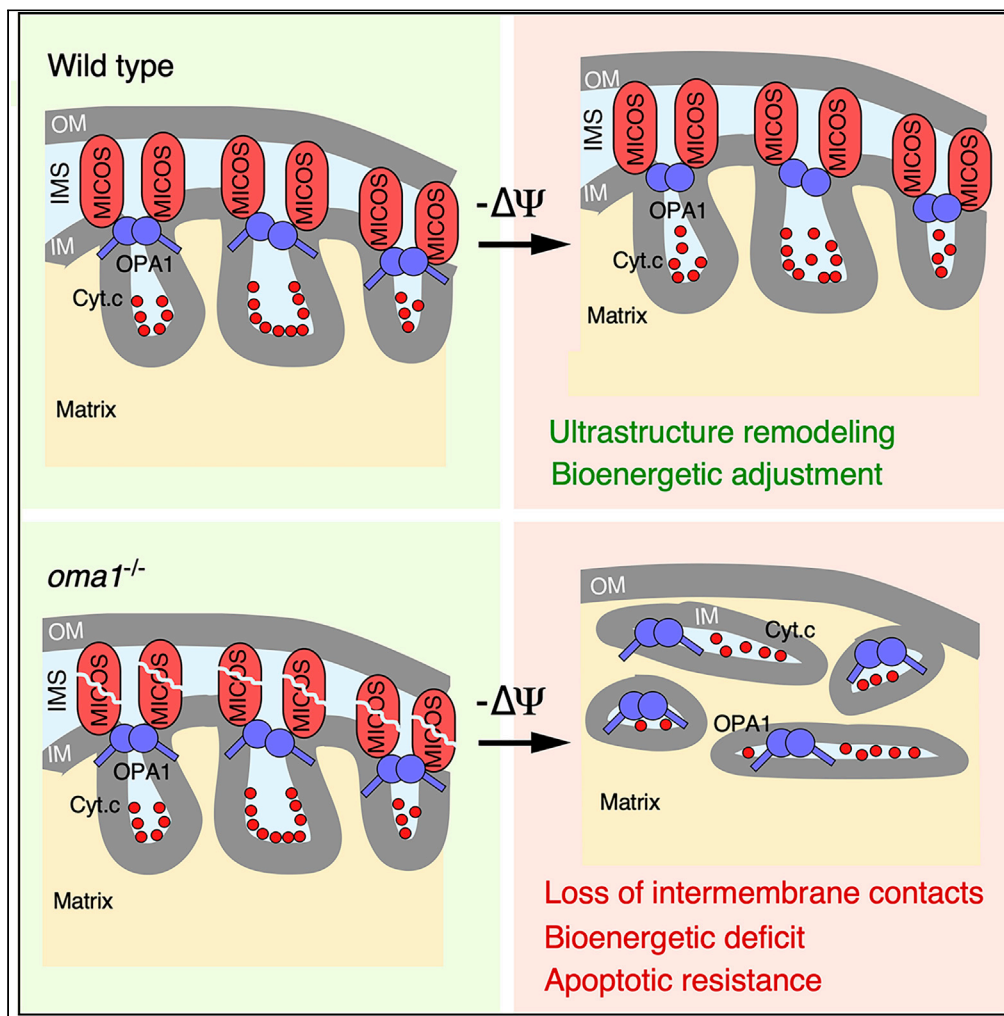


Article

Protease OMA1 modulates mitochondrial bioenergetics and ultrastructure through dynamic association with MICOS complex



Martonio Ponte Viana, Roman M. Levytsky, Ruchika Anand, Andreas S. Reichert, Oleh Khalimonchuk

okkhalimonchuk2@unl.edu

HIGHLIGHTS

Metalloprotease OMA1 is physically associated with MICOS complex

OMA1-MICOS association is important for intermembrane connectivity

Membrane bridging alleviates bioenergetic and apoptotic defects in *oma1^{-/-}* cells

Previously unrecognized role of OMA1 in mitochondrial physiology is revealed

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Article

Protease OMA1 modulates mitochondrial bioenergetics and ultrastructure through dynamic association with MICOS complex

Martonio Ponte Viana,^{1,6} Roman M. Levytsky,^{1,6,7} Ruchika Anand,² Andreas S. Reichert,² and Oleh Khalimonchuk^{1,3,4,5,8,*}

SUMMARY

Remodeling of mitochondrial ultrastructure is a process that is critical for organelle physiology and apoptosis. Although the key players in this process—mitochondrial contact site and cristae junction organizing system (MICOS) and Optic Atrophy 1 (OPA1)—have been characterized, the mechanisms behind its regulation remain incompletely defined. Here, we found that in addition to its role in mitochondrial division, metallopeptidase OMA1 is required for the maintenance of intermembrane connectivity through dynamic association with MICOS. This association is independent of OPA1, mediated via the MICOS subunit MIC60, and is important for stability of MICOS and the intermembrane contacts. The OMA1-MICOS relay is required for optimal bioenergetic output and apoptosis. Loss of OMA1 affects these activities; remarkably it can be alleviated by MICOS-emulating intermembrane bridge. Thus, OMA1-dependent ultrastructure support is required for mitochondrial architecture and bioenergetics under basal and stress conditions, suggesting a previously unrecognized role for OMA1 in mitochondrial physiology.

INTRODUCTION

Mitochondria are dynamic organelles with distinctive and complex architecture, wherein two disparate membranes, termed the outer mitochondrial membrane (OM) and the inner mitochondrial membrane (IM), form specific sub-compartments—the mitochondrial matrix and the intermembrane space (IMS). Unlike the OM, the IM is topologically heterogeneous and comprises two distinct regions: the inner boundary membrane (IBM), a planar membrane located in the vicinity of the OM, and the inner cristae membrane (ICM)—the region of IM that forms large invaginations termed cristae (Frey and Manella, 2000; Zick et al., 2009). These tubular- or lamellar-like structures are highly enriched in respiratory complexes and stabilized by structures known as cristae junctions (Glickerson et al., 2003; Zick et al., 2009; Cogliati et al., 2016). Such architecture is at least in part mediated by a large protein complex known as MICOS (for mitochondrial contact site and cristae junction organizing system), which has been shown to have a crucial role in the formation and maintenance of cristae junctions and OM-IM contacts (Rabl et al., 2009; Harner et al., 2011; Hoppins et al., 2011; Van der Laan et al., 2016; Wollweber et al., 2017; Pfanner et al., 2014). MICOS resides in cristae junctions and encompasses at least six evolutionary conserved subunits: MIC60/Mitofilin, MIC19/CHCHD3, MIC25/CHCHD6, MIC27/APOOL, MIC26/APOO, and MIC10/MINOS1 (Van der Laan et al., 2016). Other factors important for cristae ultrastructure include F₁F₀-ATP synthase (Davies et al., 2012; Daum et al., 2013) and Optic Atrophy 1, OPA1 (Frezza et al., 2006; Yamaguchi et al., 2008; Cogliati et al., 2013; Patten et al., 2014; Varanita et al., 2015). The latter is an IM-resident dynamin-like GTPase related to OM-associated GTP hydrolases DRP1 and Mitofusins 1 and 2, which have been shown to reciprocally regulate division and fusion of the mitochondrial network, respectively (Smirnova et al., 2001; Yoon et al., 2001; Chen et al., 2003; Song et al., 2009). OPA1 exists as a nearly stoichiometric mix of IM-anchored long (L-OPA1) and soluble IMS-localized short (S-OPA1) forms (Duvezin-Caubet et al., 2006; Ishihara et al., 2006). In addition to its well-established role as a pro-fusion factor in the maintenance of the mitochondrial network (Ishihara et al., 2006; Song et al., 2009; MacVicar and Langer, 2016), OPA1 variants form oligomers that are believed to connect cristae junctions, thereby segregating cristae content from the IMS and stabilizing respiratory supercomplexes (RSCs) (Cogliati et al., 2013; Patten et al., 2014; Yoon et al., 2001). Interestingly, OPA1 and MICOS oligomers appear to be in physical contact and have been proposed to

¹Department of Biochemistry, University of Nebraska, Lincoln, NE 68588, USA

²Institute of Biochemistry and Molecular Biology I, Medical Faculty, Heinrich Heine University of Dusseldorf, Dusseldorf 40225, Germany

³Nebraska Redox Biology Center, University of Nebraska, Lincoln, NE 68588, USA

⁴Center for Integrated Biomolecular Communication, University of Nebraska, Lincoln, NE 68588, USA

⁵Fred & Pamela Buffett Cancer Center, Omaha, NE 68198, USA

⁶These authors contributed equally

⁷Present address: Exelixis, Inc., 1851 Harbor Bay Parkway, Alameda, CA 94502, USA

⁸Lead contact

*Correspondence: okhalimonchuk2@unl.edu
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regulate cristae shape in a semi-cooperative manner (Darshi et al., 2011; Barrera et al., 2016; Schweppe et al., 2017; Glytsou et al., 2016).

Changes in cellular metabolic demands or homeostatic insults are known to correlate with changes in the mitochondrial network and ultrastructure (Hackenbrock 1966; Cogliati et al., 2013; Patten et al., 2014; Plecита-Hlavata et al., 2016). A number of studies implicated OPA1 as a central component behind these alterations (Cogliati et al., 2013; Mishra et al., 2014; Patten et al., 2014; Zhang et al., 2011). Although the significance of the L- and S-OPA1 variants in these events remains debated (Mishra et al., 2014; Anand et al., 2014; Lee et al., 2017), the OPA1-dependent IM remodeling is recognized as pivotal to several vital mitochondrial functions such as energy conversion and apoptosis (Frezza et al., 2006; Yamaguchi et al., 2008; Tondera et al., 2009; Mishra et al., 2014; Anand et al., 2014; Korwitz et al., 2016). Two IM proteases, the i-AAA protease YME1L and the stress-activated peptidase OMA1, are critical regulators of IM dynamics in higher eukaryotes (Rainbolt et al., 2016; MacVicar and Langer, 2016; Levytsky et al., 2017). They modulate the mitochondrial network via proteolytic processing of L-OPA1 variants under basal and stress conditions, respectively (Head et al., 2009; Ehses et al., 2009; Baker et al., 2014; Zhang et al., 2014; Anand et al., 2014; Wai et al., 2015). In the latter case, rapid proteolytic conversion of all available L-OPA1 to the S-OPA1 form by OMA1 promotes fragmentation of the mitochondrial network, a critical event required for several downstream mechanisms such as apoptosis or mitophagy (Frezza et al., 2006; Yamaguchi et al., 2008; Rambold et al., 2011; Anand et al., 2014; MacVicar and Lane, 2016). As such, preservation of L-OPA1 variant through its overexpression or OMA1 depletion has been shown to stabilize the mitochondrial network, maintain normal cristae structure, and exert anti-apoptotic effects (Anand et al., 2014; Varanita et al., 2015). On the other hand—despite normal mitochondrial ultrastructure—OMA1-deficient mouse embryonic fibroblasts (MEFs) are bioenergetically compromised under conditions that demand maximal respiratory output (Quiros et al., 2012; Korwitz et al., 2016; Bohovych et al., 2015). OMA1 is also known to perform protein quality control functions and mediate the stability of RSCs, thus influencing mitochondrial energy metabolism (Desmurs et al., 2015; Bohovych et al., 2015). An important unresolved question is regarding mechanistic aspects and the functional connection of these activities.

In the present study, we show that in addition to its role in mitochondrial fission, OMA1 is required for the organelle's ultrastructural organization through its association with MICOS machinery. This interaction is likely mediated by the MIC60-MIC19 subcomplex of MICOS and influences the recruitment of other MICOS proteins without significantly impacting mitochondrial fission/fusion machinery. We show that OMA1-mediated MICOS stabilization is particularly important for optimal mitochondrial performance in response to cellular insults such as mitochondrial depolarization. Remarkably, in the absence of OMA1, these activities can be partially compensated for by an artificial tether protein that bridges the OM and IM, thereby emulating the tunable MICOS complex.

Our results demonstrate that OMA1-mediated support of mitochondrial intermembrane contacts is required for the modulation of mitochondrial bioenergetics in response to homeostatic challenges and suggest a previously unrecognized role for this protease in mitochondrial physiology.

RESULTS

OMA1 associates with MIC60 component of MICOS complex

In our effort to gain new insight into the physical interactome of OMA1, we screened for its physiologically relevant interacting partners using the recently developed BioID method (Roux et al., 2013). This non-biased approach involves fusing the protein of interest to promiscuous BirA biotin ligase, which specifically modifies proteins in the immediate vicinity to the bait. HEK293T cells expressing OMA1-BirA or a topologically matched SURF1-BirA fusion construct (non-specific binding control) were cultured to allow the modification, lysed, and subjected to affinity purification of biotinylated proteins followed by their identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Figure S1A). Subsequent analysis of candidate hits eliminated known contaminants and non-specific interactors. One prey protein that withstood our elimination analysis was Mitofilin/MIC60, a core component of the MICOS complex. To validate this interaction, we used HEK293T cells expressing C-terminally Myc-tagged MIC60. Immunoprecipitation of epitope-tagged MIC60 also led to specific co-purification of endogenous MIC60 and OMA1, but not the abundant m-AAA protease subunit AFG3L2 (Figure 1A). We have also detected some amounts of OPA1, which has been previously reported to interact with MIC60 (Glytsou et al., 2016; Barrera et al., 2016). Reciprocally, endogenous MIC60 co-precipitated with the GFP-tagged OMA1 (Figure S1B). Consistent with

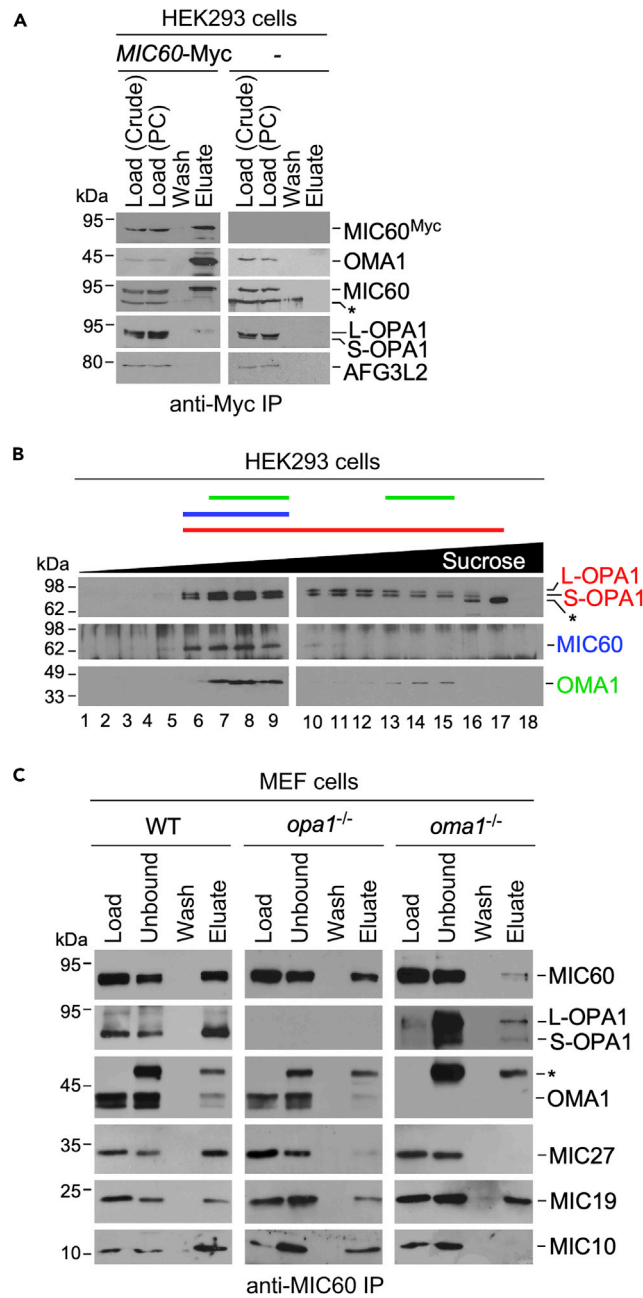


Figure 1. Evolutionarily conserved interaction between OMA1 and MIC60

(A) HEK293T cells were transfected with MIC60-Myc construct or mock transfected. Mitochondrial lysates from the respective cells were pre-cleared with IgG beads and then immunoprecipitated with anti-Myc magnetic beads. Precipitated proteins from the initial sample [load (crude)], the sample following pre-clearing [load (PC)], the last wash, and the eluate were analyzed by western blot with indicated antibodies. The asterisk indicates a non-specific band.

(B) Digitonin-solubilized mitochondria from HEK293T cells were fractionated in a 12%–50% sucrose density gradient by centrifugation. Collected fractions were analyzed by immunoblotting with antibodies against indicated proteins. The asterisk indicates a non-specific band.

(C) Mitochondrial lysates from indicated MEF cell lines were incubated with agarose beads-coupled anti-MIC60 antibodies. Immunoprecipitated proteins from the initial sample (load), unbound fraction (unbound), last wash (wash), and eluate were analyzed by western blot with indicated antibodies. The asterisk indicates a non-specific band.

these results, OMA1 and MIC60 high-molecular-weight pools co-fractionated on sucrose density gradient (Figure 1B). We also observed partial co-fractionation of these proteins with OPA1 signal.

Because OPA1 is the substrate of OMA1, and OPA1 and MIC60 are known to interact physically, we wondered if OPA1 might be mediating OMA1-MIC60 interaction. To better understand how these proteins are interrelated, we carried out MICOS co-purifications in mitochondrial lysates from wild-type (WT), *opa1*^{-/-}, and *oma1*^{-/-} MEFs. Similar to HEK293T mitochondria, immunosorbition of MIC60 resulted in co-precipitation of both OMA1 and OPA1 along with MIC19, MIC10, and MIC27 MICOS subunits in WT mitochondrial lysates (Figure 1C and data not shown). Importantly, OMA1-MIC60 interaction remained largely unperturbed in *opa1*^{-/-} mitochondria, indicating that OPA1 is dispensable for OMA1 association with MICOS. Similarly, we found that OPA1 retained its ability to interact with MIC60 in *oma1*^{-/-} cells (Figure 1C). We also noted that loss of OMA1, and to a lesser extent OPA1, negatively impacted MICOS as reflected by impaired MIC60-MIC27 interaction in both *opa1*^{-/-} and *oma1*^{-/-} mitochondria. Interestingly, larger amount of MIC19 co-precipitated with MIC60 in *oma1*^{-/-} mitochondria, likely reflecting more efficient binding of the former protein to MIC60 in the absence of OMA1. Additionally, we noticed lower recovery of MIC60 bait in *oma1*^{-/-} mitochondria. At present, the basis for such phenomenon is unclear, but we speculate that this may be due to changes in epitope accessibility and consequently less efficient binding of the protein to anti-MIC60 beads. We conclude that OMA1 is associated with MICOS machinery independently of OPA1.

Loss of OMA1 impacts mitochondrial structure upon uncoupling

Our finding that OMA1 is physically associated with MICOS prompted us to revisit mitochondrial morphology and ultrastructure in OMA1-deficient cells. We first examined mitochondrial network morphology in WT and *oma1*^{-/-} MEFs. Consistent with previous reports (Quiros et al., 2012; Anand et al., 2014; Korwitz et al., 2016), deletion of OMA1 did not significantly affect mitochondrial tubulation under basal conditions (Figure 2A). We then assessed the morphology of the mitochondrial network under mild uncoupling, the condition upon which *oma1*^{-/-} MEFs are known to exhibit bioenergetic deficit (Bohovich et al., 2015). As expected under this condition, control MEFs showed rapid fragmentation of mitochondrial network, reflecting OMA1-mediated proteolysis of L-OPA1. The *oma1*^{-/-} cells predictably retained tubular mitochondria upon uncoupling. We noticed, however, that the mitochondrial network morphology was different from that observed in unstressed *oma1*^{-/-} MEFs. Mitochondrial tubules were shorter and tended to form clump-like clusters in CCCP-treated *oma1*^{-/-} cells (Figure 2A). Steady-state levels of the OM fusion- and fission-mediating proteins were unchanged in these cells (Figure 2B). As expected, in contrast to the WT cells, the CCCP-treated *oma1*^{-/-} cells retained the long form of OPA1 (Figure 2C).

We next sought to test if these changes may be related to OMA1-MIC60 interaction. We examined stability of MICOS high-mass complexes and steady-state levels of key MICOS subunits in cells lacking OMA1. Surprisingly, blue native-PAGE analysis revealed marked attenuation of high-mass MICOS complex in *oma1*^{-/-} cells, irrespective of CCCP treatment (Figures 2D and S2), indicating that MICOS may be more labile under blue native gel conditions in the absence of OMA1. Consistent with this notion, *oma1*^{-/-} cells exhibited increased accumulation of a lower-mass MIC27-containing complex (Figure S2B), suggesting that the MIC60-MIC19 subcomplex is likely affected by OMA1 loss. We then examined steady-state levels of key MICOS subunits and observed a slight decrease in the levels of MIC60 in *oma1*^{-/-} MEFs under both basal and stress conditions, which suggests this protein is likely not a substrate of OMA1 (Figure 2E). The steady-state levels of MIC27 subunit were unaltered. By contrast, steady-state levels of MICOS subunits MIC19, MIC13, and MIC10 were decreased, but not completely lost, in *oma1*^{-/-} cells relative to WT control in both basal and CCCP-uncoupled conditions (Figure 2E).

MIC60 has been shown to participate in the contacts with the OM (Barrera et al., 2016). We thus hypothesized that MICOS impeding seen in *oma1*^{-/-} cells may be affecting the architecture of IBM and/or IBM-OM contact sites (Figure S2A), thereby contributing to the altered mitochondrial morphology seen in stressed *oma1*^{-/-} cells (Figure 3A). To test this postulate, we examined mitochondrial ultrastructure in normal and CCCP-stressed *oma1*^{-/-} MEFs by transmission electron microscopy (TEM). In line with previous reports (Quiros et al., 2012; Anand et al., 2014; Korwitz et al., 2016), we found that about half of unstressed *oma1*^{-/-} cells displayed mitochondrial ultrastructure similar to that of WT MEFs. Interestingly, however, in our hands a significant fraction of analyzed *oma1*^{-/-} MEFs (~51%) exhibited defective cristae ultrastructure

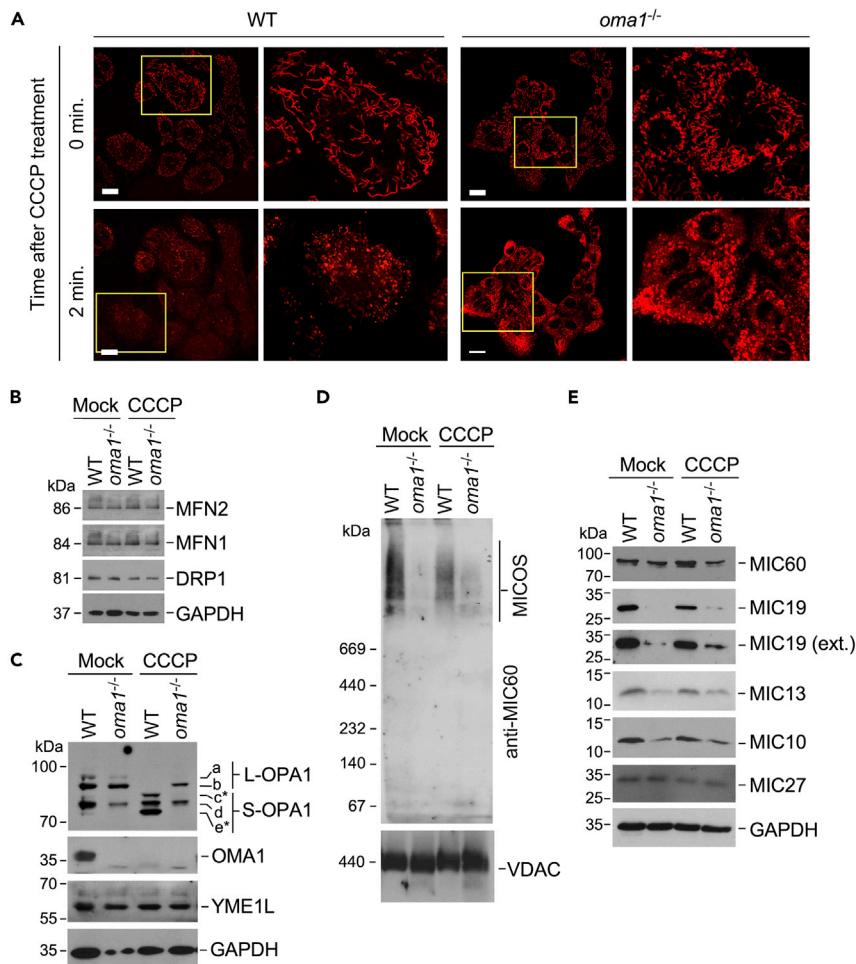


Figure 2. Mitochondrial morphology in normal and stressed *oma1*^{-/-} cells

(A) *In vivo* confocal microscopy of the mitochondrial network in wild-type (WT) and *oma1*^{-/-} MEF cells. Fibroblasts were treated with 2 μ M CCCP and incubated for indicated times to induce mild uncoupling, stained with the mitochondrial dye MitoTracker Deep Red, and analyzed by confocal fluorescent microscopy. Representative images are shown. Scale bars, 20 μ m.

(B and C) Immunoblot analysis with indicated antibodies of cell lysates from the same samples as in (A). In (C), letters a–e denote OPA1 isoforms, with a and b representing the long form of OPA1, L-OPA1, and c–e representing the short form, S-OPA1. Asterisks denote S-OPA1 forms generated via OMA1-mediated cleavage of L-OPA1.

(D) Mitochondrial lysates were subjected to one-dimensional blue native gel electrophoresis and analyzed by western blot with antibodies against MIC60 and the outer mitochondrial membrane protein VDAC (loading control).

(E) Steady-state levels of indicated proteins in mitochondrial lysates from cells described above, analyzed by western blot. Note that MICOS subunits such as MIC19 are not completely lost in *oma1*^{-/-} mitochondria; substantial amount of the protein can be detected upon extended exposure.

(Figure 3B). Of note, we also observed a small fraction (~14%) of WT cells exhibiting altered cristae morphology, likely due to sample preparation and sectioning conditions. Furthermore, TEM analysis of CCCP-treated *oma1*^{-/-} MEFs revealed that unlike in WT, ~70% of mitochondria in OMA1-deleted cells displayed defective cristae ultrastructure—including short, swollen, vesicular, and onion-like cristae—upon uncoupling (Figure 3C). One particularly pronounced alteration included the appearance of onion-like cristae that were fully detached from the OM. Such ultrastructural changes are comparable to those described in cells depleted for MICOS subunits (Figure S3, Anand et al., 2016; Glytsou et al., 2016) or harboring catalytically dead OPA1 (Lee et al., 2017) and are consistent with our predicted model outlined in Figure 3A. Altogether, these results suggest that OMA1-MICOS association is required for cristae positioning and/or remodeling, particularly during homeostatic insults.

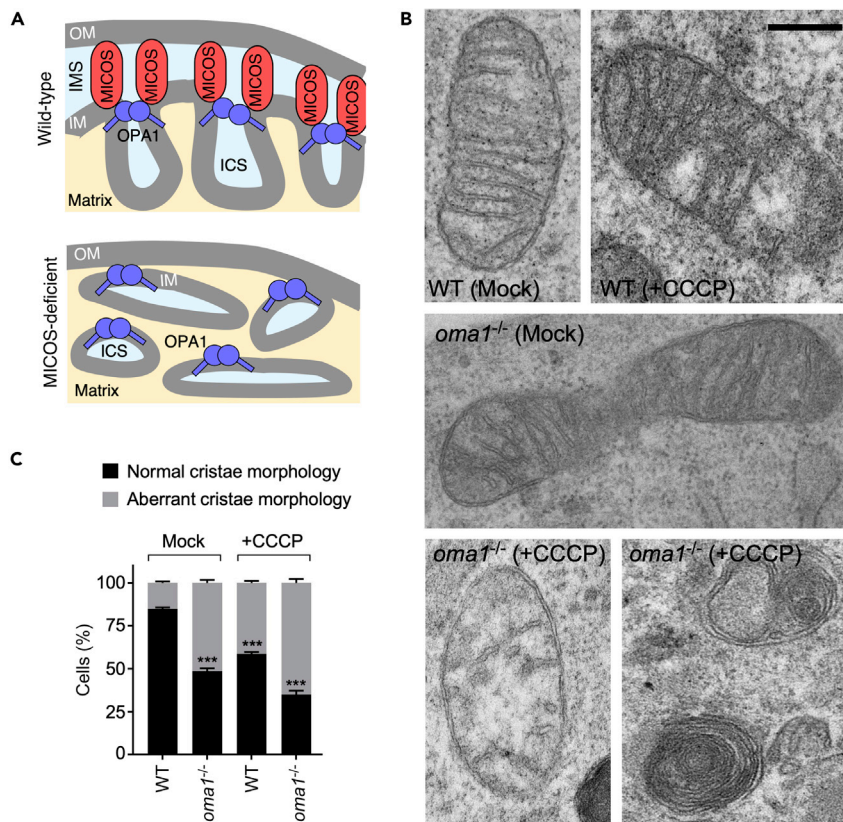


Figure 3. Stressed *oma1*^{-/-} cells exhibit altered mitochondrial ultrastructure.

(A) Schematic depiction of the predicted ultrastructure defect in MICOS-deficient cells. A situation wherein mitochondrial cristae junctions remain closed but the IM-OM contacts are impeded would result in the appearance of vesicle-like cristae detached from the OM.

(B) Transmission electron microscopic imaging of mitochondrial ultrastructure in wild-type (WT) and *oma1*^{-/-} MEF cells incubated with (+CCCP) or without (Mock) 2 μ M CCCP. Representative images are shown. Scale bars, 0.4 μ m.

(C) The number of cells bearing mitochondria with aberrant cristae from (B) was quantified. Error bars, mean \pm SD of $n = 3$ biological replicates with 100 cells per replicate; *** $p < 0.001$ by t test compared with WT without CCCP.

Emulation of OM-IM contact sites preserves mitochondrial architecture in stressed *oma1*^{-/-} cells

Our data suggest that integrity of OM-IM contacts may be critical for stress-induced cristae remodeling. To validate this idea, we engineered a chimeric construct comprising the N-terminal portion of the IM protein SCO1 (residues 1–161), an unstructured 12 amino-acids-long linker region, and the transmembrane segment of the OM protein TOMM20 (residues 1–24), followed by a GFP tag (Figure 4A). This construct, which we called mitoT, is designed to bridge the OM and IM, thereby emulating the structural function of MICOS at the IBM sites. Indeed, a similar chimeric tether has been previously shown to partially compensate for the loss of MICOS in yeast (Aaltonen et al., 2016). We generated stable *oma1*^{-/-} MEFs expressing mitoT at levels that were well tolerated by the cells in question (Figures 4B and 4C) and showed that this construct was exclusively localized to mitochondria (Figure 4B). The expression of mitoT neither had appreciable effect on the survival or proliferation of *oma1*^{-/-} cells nor did it influence L-OPA1 processing (Figure S4A). Of note, in WT, but not in *oma1*^{-/-} cells, the mitoT protein displayed a slightly faster migration pattern, suggesting that OMA1 might be involved in its processing (Figure S4B).

Next, we examined the ultrastructure of *oma1*^{-/-} [mitoT] cells under basal and stress conditions by TEM. MitoT expression resulted in fewer disorganized cristae: 18% in *oma1*^{-/-} [mitoT] versus 50% in *oma1*^{-/-} MEFs under basal conditions and 45% in *oma1*^{-/-} [mitoT] versus 70% in *oma1*^{-/-} MEFs in CCCP-treated cells (Figures 4D and 4E). These data indicate that mitoT is at least in part able to maintain OM-IM contacts

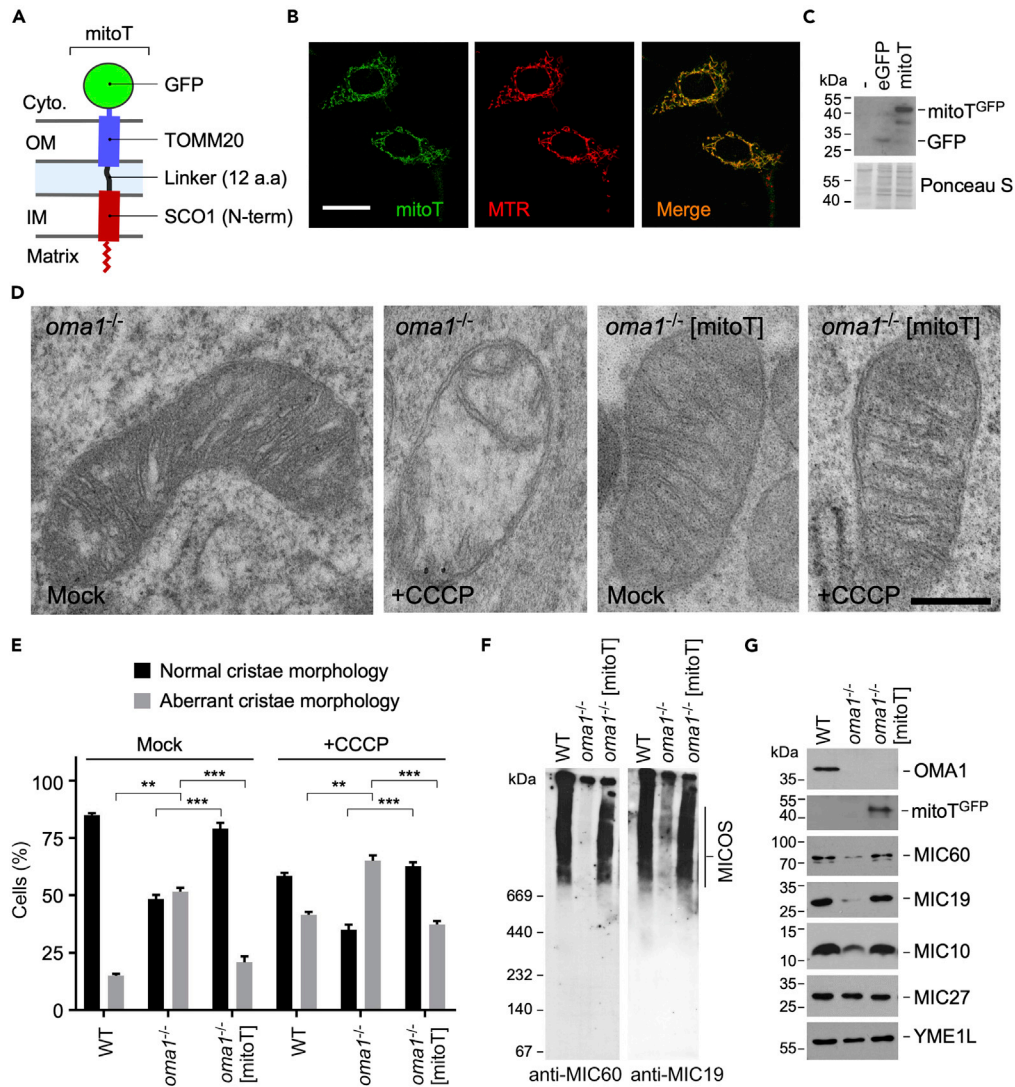


Figure 4. Mitochondrial membranes-bridging chimera partially corrects ultrastructure defects in stressed *oma1*^{-/-} cells

(A) Schematic depiction of the mitoT artificial tether construct.

(B) *In vivo* confocal microscopy of the mitochondrial network and mitoT-GFP signal in *oma1*^{-/-} MEF cells stably expressing mitoT construct. Representative images are shown for the GFP signal from the tagged mitoT construct (mitoT) and MitoTracker Deep Red (MTR). Scale bar, 20 μ m.

(C) Mitochondrial lysates from cells expressing GFP-tagged mitoT and control cells were analyzed by western blotting with anti-GFP antibodies.

(D) Representative TEM micrographs of mitochondria from control and CCCP-treated *oma1*^{-/-} MEF cells expressing mitoT. Scale bar, 0.4 μ m.

(E) The number of cells bearing mitochondria with normal and aberrant cristae from indicated cell lines was quantified. Error bars, mean \pm SD of n = 3 biological replicates with 100 cells per replicate; **p < 0.01, ***p < 0.001 by t test.

(F) Mitochondrial lysates from indicated cell lines were analyzed by blue native-PAGE and immunoblotting with antibodies against MICOS subunits MIC60 and MIC19.

(G) Steady-state levels of indicated proteins in the respective cell lines analyzed by western blot.

in OMA1-deficient mitochondria. Biochemically, the expression of mitoT in *oma1*^{-/-} cells stabilized the high-mass MICOS complex (Figure 4F) and restored normal steady-state levels of MIC60, MIC19, and MIC10 MICOS subunits (Figure 4G), further suggesting that mitoT-mediated permanent contacts between the IM and OM may act as a scaffold to support MICOS, thus resulting in stabilization of mitochondrial architecture in OMA1-deficient cells.

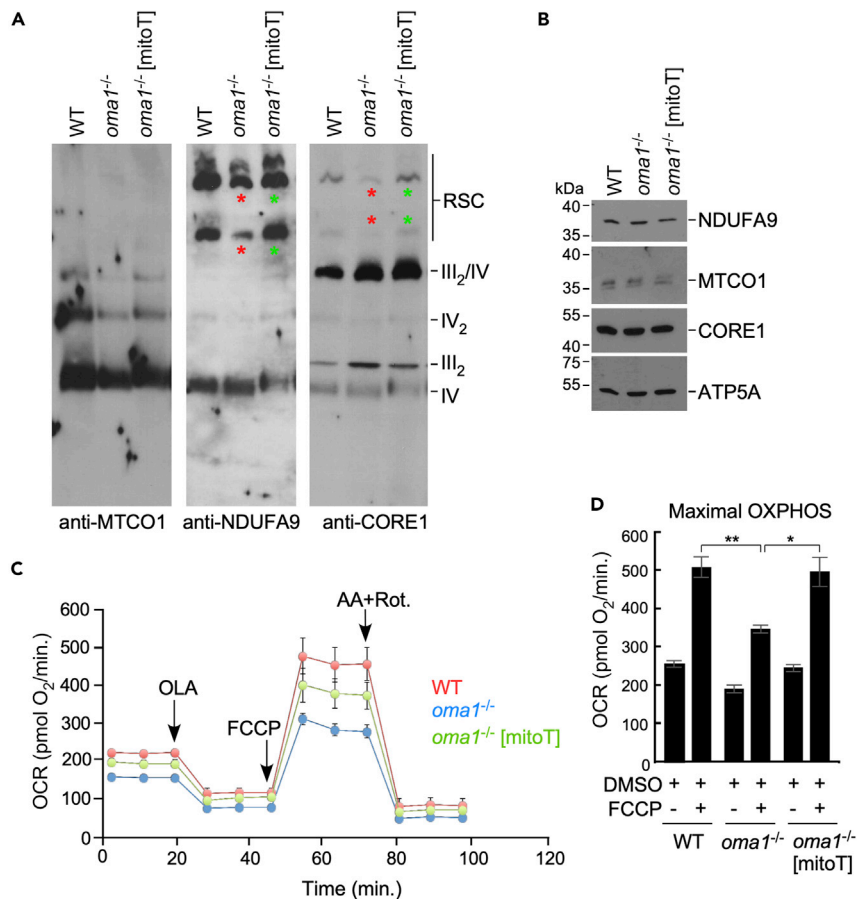


Figure 5. MitoT expression stabilizes respiratory complexes and mitigates bioenergetic deficit in OMA1-deficient cells

(A) Mitochondrial lysates from the respective cells were subjected to native gel electrophoresis and analyzed by western blot with indicated antibodies. Red asterisks highlight more labile respiratory supercomplexes, and green asterisks highlight stabilized respiratory supercomplexes.

(B) Steady-state levels immunoblot analysis with indicated antibodies of mitochondrial lysates from the same samples as in (A).

(C and D) *In vivo* oxygen consumption rates (OCR) of indicated cell lines (50,000 cells/well) under basal, oligomycin A (OLA)-, FCCP-, and antimycin A + rotenone-stimulated conditions were assessed by extracellular flux analysis. Cells were cultured in medium containing 10 mM galactose. Representative OCR graph is shown in (C). (D) focuses on FCCP-stimulated maximal respiration. Error bars, mean \pm SE of $n = 3$ biological replicates; * $p < 0.05$, ** $p < 0.01$ by t test.

MitoT-mediated OM-IM contacts stabilize respiratory complexes and alleviate bioenergetic deficit in *oma1*^{-/-} cells

Mitochondrial ultrastructure is important for the stability and optimal performance of RSCs (Cogliati et al., 2013; Lee et al., 2017). We therefore wondered if the impaired stability of RSCs and bioenergetic deficit seen in *oma1*^{-/-} cells (Bohovych et al., 2015) could be a consequence of impaired OM-IM connectivity. To test this scenario, we examined mitochondrial lysates from WT, *oma1*^{-/-}, and *oma1*^{-/-} [mitoT] MEFs by blue native gel electrophoresis. Consistent with our previous observations, we observed a reproducible trend wherein the abundance and stability of higher-order RSCs was slightly decreased in *oma1*^{-/-} MEFs under the conditions tested (Figure 5A). By contrast, we found that mitoT expression stabilized the RSCs in these cells (Figure 5A). Steady-state levels of the representative complex subunits remained unchanged in all cell lines tested (Figure 5B).

We next evaluated the physiological relevance of mitoT-mediated RSCs stabilization by measuring *in vivo* oxygen consumption rates (OCR) in WT, *oma1*^{-/-}, and *oma1*^{-/-} [mitoT] cells cultured in galactose. This

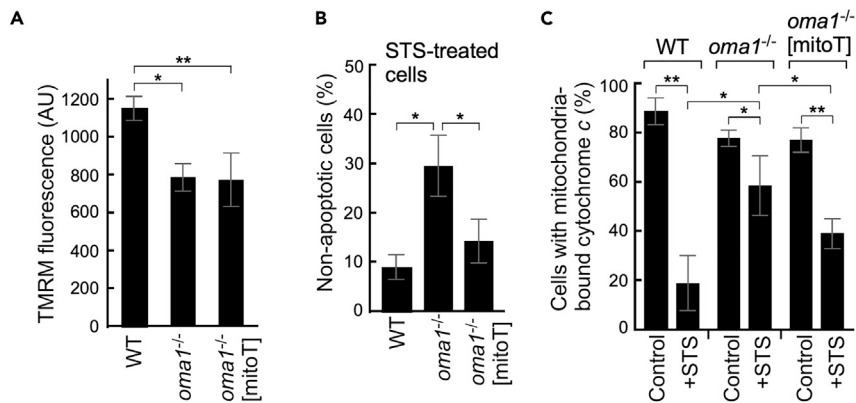


Figure 6. MitoT expression ablates apoptotic resistance in *oma1*^{-/-} cells

(A) Mitochondrial membrane potential in the indicated cell lines was assessed by flow cytometry analysis of tetramethylrhodamine (TMRM)-stained cells. Error bars, mean \pm SD of $n = 3$ biological replicates; * $p < 0.05$, ** $p < 0.01$, by t test.

(B and C) Flow cytometry analysis of indicated cell lines that were treated with or without 1 mM staurosporine to assess the percentage of viable/apoptosis-resistant (Q3: 7-AAD negative, Annexin V-APC negative) cells (B) or cells with mitochondria-retained cytochrome c (C). Error bars, mean \pm SE of $n = 3$ biological replicates; * $p < 0.05$, ** $p < 0.01$, by t test.

culture condition forces cells to rely on oxidative metabolism (Gohil et al., 2010), and OMA1-deficient MEFs exhibit signs of bioenergetic deficit when cultured in galactose-supplemented medium (Bohovych et al., 2015). This condition does not significantly alter viability of these cells (Figure S5A). In line with our previous report (Bohovych et al., 2015), we found that *oma1*^{-/-} MEFs were unable to maximize their bioenergetic output in response to CCCP treatment (Figures 5C and 5D). In contrast, the mitoT-expressing *oma1*^{-/-} cells exhibited a nearly WT bioenergetic profile, most notably being able to efficiently maximize their OCR in response to mild uncoupling (Figures 5C and 5D). The observed effect does not appear to be merely due to an excess of the L-OPA1 form, as expression of the L-OPA1 Δ S1 variant lacking OMA1 cleavage site (Mishra et al., 2014; Lee et al., 2017) in *oma1*^{-/-} MEFs did not mitigate bioenergetic deficit in these cells (Figures S5B and S5C). In fact, we found that such genetic manipulation reduced basal OCR of *oma1*^{-/-} cells (Figure S5C). These data indicate that an additional mechanism is at play in OMA1-mediated regulation of bioenergetic output.

Finally, we used tetramethylrhodamine (TMRM) staining to examine basal mitochondrial membrane potential in the respective cells. We found that OMA1-depleted cells exhibited lower TMRM fluorescence thereby reflecting decreased mitochondrial membrane potential (Figure 6A). Interestingly, mitoT expression did not improve mitochondrial polarization in these cells, indicating that membrane tethering via mitoT may not fully replace some functions related to mitochondrial ultrastructure and/or MICOS complex that are compromised in OMA1-deficient MEFs.

Collectively, these results suggest that impaired RSC stability and bioenergetic deficit in OMA1-deficient cells arise from impaired OM-IM contacts and altered mitochondrial ultrastructure.

Anti-apoptotic effect of OMA1 deletion is linked to impaired membrane contacts

Previous reports established that loss of OMA1 renders cells resistant to apoptotic stimuli and attributed this effect to attenuated mitochondrial fission and cytochrome c release due to impaired L-OPA1 cleavage (Jiang et al., 2014; Varanita et al., 2015; Korwitz et al., 2016). Our findings prompted us to revisit this model and test whether impaired OM-IM connectivity may also be contributing to an anti-apoptotic effect in *oma1*^{-/-} cells. We thus examined Annexin V presentation and cytochrome c release—two key steps in the commencement of a classical apoptotic program—in WT, *oma1*^{-/-}, and *oma1*^{-/-} [mitoT] MEFs treated with the apoptosis-inducing drug staurosporine. In line with previous reports (Jiang et al., 2014; Varanita et al., 2015; Korwitz et al., 2016), we observed that *oma1*^{-/-} cells accumulated significantly less externalized Annexin V, relative to WT control (Figures 6B and S6A). Likewise, staurosporine-treated *oma1*^{-/-} MEFs displayed significant impairment in cytochrome c release when compared with WT cells (Figures S3B and 6C).

Remarkably, mitoT expression partially restored both staurosporine-induced Annexin V presentation and cytochrome *c* release in *oma1*^{-/-} MEFs (Figures 6B and 6C). The expression of mitoT had no appreciable effect on mitochondrial fragmentation in the cells of interest (Figure S7). We conclude that reversal of apoptotic resistance in *oma1*^{-/-} [mitoT] cells is due to stabilized IM-OM contacts rather than enhanced mitochondrial fragmentation.

These findings further suggest that OM-IM connectivity is important for stress-associated membrane remodeling and initiation of the apoptotic program. Moreover, our data expand the current model of how OMA1 inactivation may relate to cellular apoptotic resistance and warrant further investigation of this issue.

DISCUSSION

Remodeling of mitochondrial membranes is now being recognized as an integral part of multiple physiological processes in eukaryotic cells. Although the key players involved in sculpturing and dynamic rearrangement of the OM and IM have been identified, their coordinated function and the factors that orchestrate these mechanisms remain largely unclear. Here, we show that IM-anchored metallopeptidase OMA1 is dynamically associated with the MICOS complex, thereby stabilizing IM-OM contact sites and facilitating their remodeling in response to homeostatic insults. Mechanistically, this association appears to be mediated by OMA1's interaction with the MIC60 subunit of MICOS, which we identified by proximity labeling, direct co-purification, and co-fractionation analyses. Interestingly, similar to the recently reported MIC60-OPA1 association (Glytsou et al., 2016; Barrera et al., 2016), this interaction has previously eluded identification by the recent high-throughput proteomic analysis of MICOS (Guarani et al., 2015; Schweppe et al., 2017), probably due to the low cellular abundance of OMA1. Of note, we have also observed limited interaction between MIC60 and OPA1. However, the results of co-purifications in *opa1*^{-/-} and *oma1*^{-/-} MEF cells indicate that OMA1 and OPA1 associate with MICOS independently of one another.

What is the effect of OMA1 loss with respect to MICOS? Consistent with previous reports (Quiros et al., 2012; Anand et al., 2014; Korwitz et al., 2016), we observed that OMA1 deletion in MEFs did not result in any appreciable mitochondrial morphology under basal conditions. As expected, unlike the WT cells, *oma1*^{-/-} MEFs did not exhibit fragmented mitochondrial networks upon mild uncoupling due to impaired L-OPA1 processing and preservation of OPA1 oligomers. We found, however, that stressed OMA1-deficient mitochondria tended to form tubules that were morphologically distinct from those observed in unperturbed cells. Further analyses revealed that high-mass MICOS complexes were less stable, that is, susceptible to destabilization, under native gel electrophoresis conditions in *oma1*^{-/-} mitochondria even if cells were not challenged with an uncoupler. Accumulation of lower-mass MIC27-containing complexes (Figure S2) likely reflects impaired association of MIC60-MIC19 subcomplex with other MICOS subunits known as the MIC10 subcomplex. Our data indicate a general nature of this defect, but suggest that it becomes physiologically significant upon conditions of mitochondrial stress. In line with this notion are the outcomes of our mitochondrial ultrastructure analysis. We reproducibly observed that about half of *oma1*^{-/-} MEF cells exhibited aberrant mitochondrial ultrastructure; this might be in part due to harsh EM sample preparation conditions, which we believe have helped to reveal this subtle phenotype. More importantly, mitochondria in uncoupled *oma1*^{-/-} MEFs displayed gross ultrastructure alterations wherein the majority of cristae appeared to be dislocated from the OM and formed vesicle- or onion ring-like structures. Remarkably, the latter alterations strongly resemble ultrastructure defects observed in MICOS-depleted cells (Plecita-Hlavata et al., 2016; Glytsou et al., 2016 and Figure S3). Altogether, these data suggest that OMA1-MICOS association is required for efficient ICM and/or IBM rearrangement in response to homeostatic insults. Although neither MIC60 nor other core MICOS subunits seem to be a *bona fide* substrate of OMA1, and the exact mechanistic aspect of this association awaits an in-depth investigation, our results indicate that in the absence of OMA1, MICOS complex appears to be more labile and thus impeded in its ability to efficiently reorganize OM-IM contact sites in response to homeostatic insults. This model is in line with our observation that mitochondrial ultrastructure is relatively mildly affected in unstressed *oma1*^{-/-} cells. The model is also consistent with our finding that expression of the OM-IM bridging mitoT substantially ameliorated uncoupling-induced ultrastructure abnormalities in *oma1*^{-/-} cells. A possibility exists that some of the aforementioned effects might also be connected to the inability of *oma1*^{-/-} mitochondria to rapidly produce S-OPA1. However, previous reports showing that L-OPA1 alone is sufficient to mediate mitochondrial dynamics and ultrastructure (Ishihara et al., 2006; Tondera et al., 2009; Anand et al., 2014; Lee et al., 2017) and that cells expressing a non-cleavable L-OPA1 variant do not appear to exhibit vesicular cristae (Lee et al., 2017) argue against this scenario.

What is the physiological role of OMA1-MICOS association? Results of our experiments with the mitoT membrane bridge indicate that this association is required for stabilization of RSCs and optimal respiratory output in response to physiological challenges. These data are consistent with a model wherein remodeling of both IBM and ICM ultrastructure and the maintenance of OM-IM connectivity are required to reorganize and/or stabilize respiratory complexes, thereby optimizing their performance to meet changing bioenergetic demands in response to physiological or stress stimuli (Mishra et al., 2014; Patten et al., 2014; Lee et al., 2017). Alternatively, by facilitating the intermembrane connectivity, mitoT may promote formation of metabolic domains (metabolons) to stimulate, e.g., coenzyme Q synthesis or phospholipid transport as has been demonstrated in yeast (Aaltonen et al., 2016; Subramanian et al., 2019); ultimately this may provide better substrate accessibility for RSCs. MitoT-fortified intermembrane domains may act as a scaffold to support MICOS, thereby rendering it more stable. Our results also offer a plausible explanation as to how loss of OMA1 could impair RSCs' stability and cause bioenergetic deficit in response to mild uncoupling (Bohovich et al., 2015; Quiros et al., 2012). These models are in line with the previously reported bioenergetic deficit in MICOS mutant cells (Darshi et al., 2012; Ott et al., 2012, 2015; Ding et al., 2015; Genin et al., 2016; Guarani et al., 2015).

Finally, our findings indicate that OMA1-MICOS-mediated intermembrane connectivity is important for commencement of the apoptotic program. Earlier reports established that preservation of L-OPA1 oligomers via L-OPA1 overexpression (Varanita et al., 2015) or OMA1 depletion (Quiros et al., 2012; Jiang et al., 2014; Korwitz et al., 2016; Wai et al., 2015) results in an anti-apoptotic effect, presumably due to the maintenance of tight cristae junctions and subsequent obstruction of cytochrome c release from the intercrisae space in response to apoptotic stimuli (Frezza et al., 2006; Yamaguchi et al., 2008; Varanita et al., 2015). Our data expand this model of apoptotic resistance of *oma1*^{-/-} cells as follows. First, we observed that physiological insults to OMA1-deficient cells promoted formation of OM-disconnected yet locked cristae that are likely to encapsulate and retain the majority of available cytochrome c pools. Indeed, these results were consistent with the elevated resistance of *oma1*^{-/-} MEFs to the apoptosis-inducing drug staurosporine, as indicated by the significant decrease in Annexin V-stained cells and enhanced retention of mitochondria-bound cytochrome c. Second, we found that enforcing OM-IM contacts in OMA1-deficient MEFs through mitoT tether expression was sufficient to curtail the cells' resistance to staurosporine-induced apoptosis. In line with this finding we observed that mitoT-expressing *oma1*^{-/-} MEFs exhibited increased cytochrome c release upon staurosporine treatment when compared with untransformed cells. Collectively, these results suggested an updated model wherein not only cristae junction tightness but also IM-OM connectivity is important for molecular events preceding apoptosis initiation. Overall, these data deepen our mechanistic understanding of OMA1's role in mitochondrial architecture, bioenergetics, and cell death.

At the time of writing this article a report appeared demonstrating that MIC60 and MIC19 MICOS subunits are connected to the SAM50 core component of the OM sorting and assembly machinery (SAM) complex via the MIC19 subunit to dynamically shape cristae architecture (Tang et al., 2020). Tang et al. showed that under certain homeostasis-challenging conditions, OMA1 protease can mediate the N-terminal cleavage of MIC19, thereby destabilizing SAM-MICOS association and impacting mitochondrial architecture. Although these data are generally in line with our results described in the present study, we were unable to obtain any evidence for OMA1-mediated MIC19 cleavage, likely due to differences in sample isolation procedures (whole-cell lysates versus purified mitochondria) and/or types of antibodies and cell lines used. Therefore, the exciting new mechanism by which MICOS is dynamically regulated by OMA1 awaits further mechanistic investigation.

Limitations of the study

One obvious limitation of this study is that the analyses that we have used do not fully address ultrastructural alterations in OMA1-deficient cells. A more thorough assessment of mitochondrial ultrastructure in the future will likely help to develop stronger models for how exactly OMA1 depletion impacts ultrastructural features of the IM and how these features are corrected by the mitoT-mediated membrane bridging. Future work will also likely examine the degree of OMA1 involvement in the regulation of intermembrane contacts in other cell lines to address potential cell type-specific differences.

Resource availability

Lead contact

All requests for additional information, resources, and reagents should be directed to the Lead Contact, Dr. Oleh Khalimonchuk (okhalimonchuk2@unl.edu).

Materials availability

All reagents generated in this study are available from the Lead Contact upon request and with completed materials transfer agreement.

Data and code availability

The published article includes all BioID LC-MS/MS data generated or analyzed during this study.

METHODS

All methods can be found in the accompanying [Transparent Methods supplemental file](#).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.isci.2021.102119>.

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AUTHOR CONTRIBUTIONS

R.M.L., M.P.V., and O.K. designed experiments. R.M.L. and O.K. wrote the paper with the input from all authors. R.M.L. and M.P.V. conducted the experiments and analyzed data. R.A. and A.S.R. provided critical data and reagents and helped with data interpretation.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

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Supplemental Information

**Protease OMA1 modulates mitochondrial
bioenergetics and ultrastructure through
dynamic association with MICOS complex**

Martonio Ponte Viana, Roman M. Levytsky, Ruchika Anand, Andreas S. Reichert, and Oleh Khalimonchuk

SUPPLEMENTAL INFORMATION

SUPPLEMENTAL FIGURES

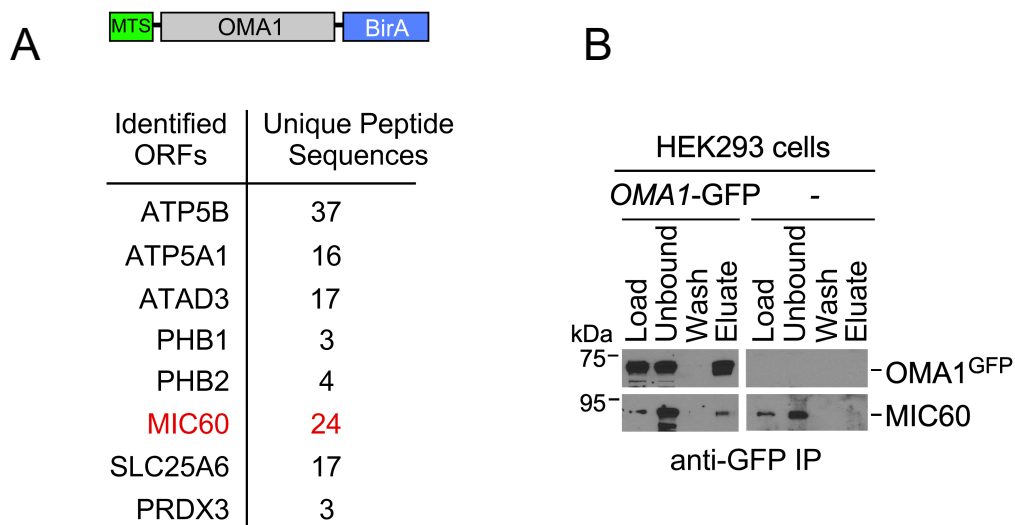


Figure S1. BioID analysis of OMA1 physical interactome, Related to Figure 1.

(A) Top, Schematic depiction of OMA1-BirA construct used in BioID proximity labeling analysis. Bottom, List of relevant proteins identified by BioID proteomic analysis. **(B)** HEK293T cells were transfected with OMA1-GFP construct or mock transfected. Mitochondrial lysates from the respective cells were subjected to immunoprecipitation with anti-GFP magnetic beads. Precipitated proteins from the initial sample (Load), unbound fraction (Unbound), last wash (Wash), and eluate were analyzed by western blot with indicated antibodies.

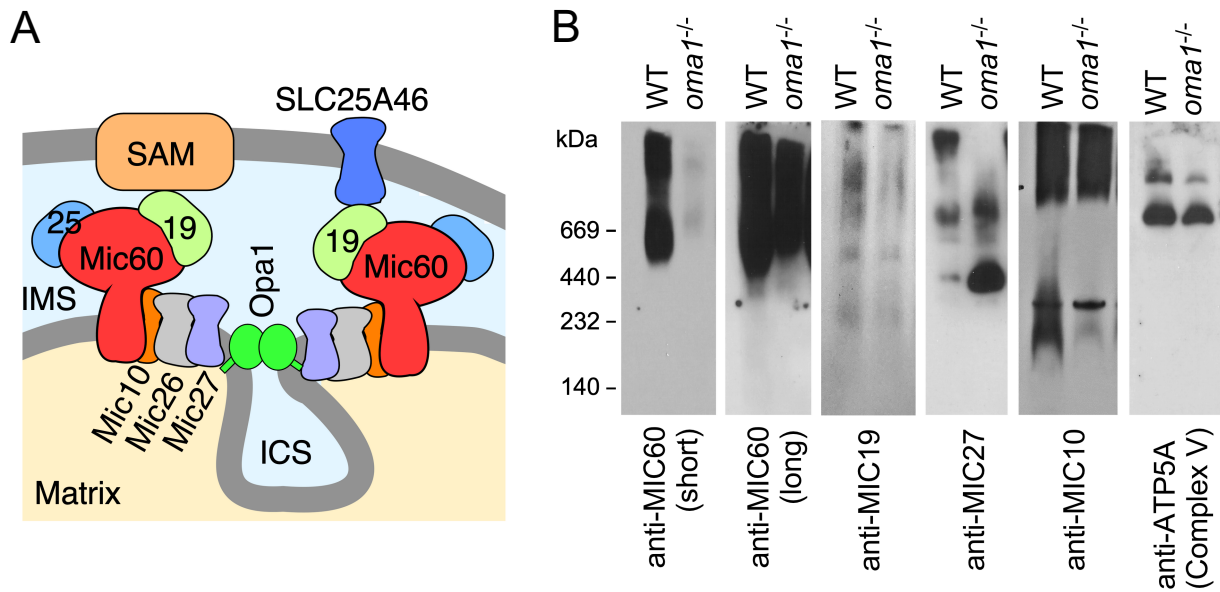


Figure S2. Stability of high-mass MICOS complexes is compromised in *oma1*^{-/-} cells, Related to Figure 2. (A) Schematic depiction of MICOS machinery and its partner protein complexes forming an IM-OM contact site. Some proteins are not shown for simplicity. (B) Mitochondrial lysates from WT and *oma1*^{-/-} fibroblasts were analyzed by blue-native gel electrophoresis and immunoblotting with antibodies against indicated MICOS subunits as well as respiratory complex V subunit ATP5A (loading control).

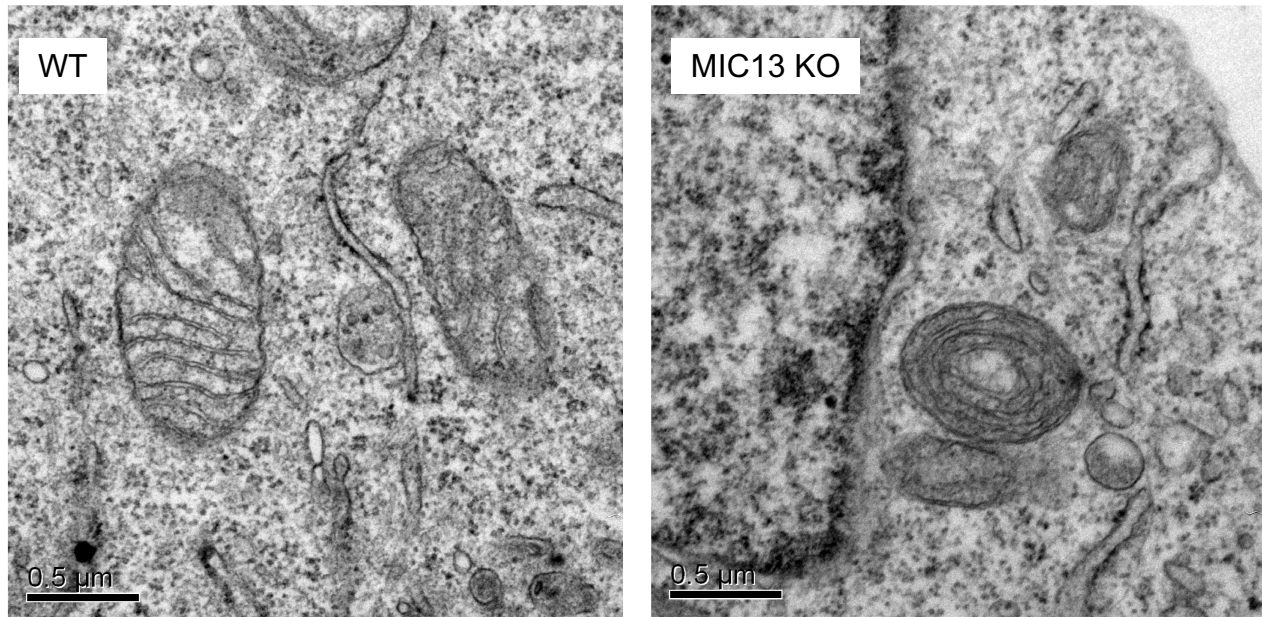


Figure S3. MICOS-deficient cells exhibit altered cristae morphology characterized by concentric membrane stacks and lack of cristae junctions, Related to Figure 3. Representative TEM images of mitochondria in WT (left) and MIC13 knockout (right) HEK293T cells. Scale bars, 0.5 μm.

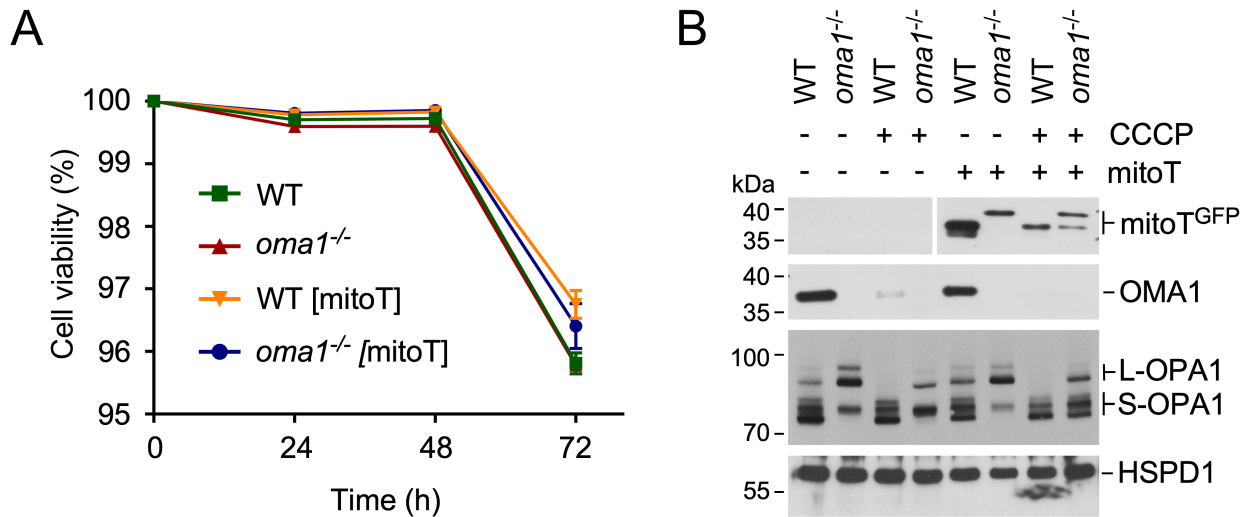


Figure S4. Quantitative assessment of cell survival by flow cytometry, Related to Figure 4.

(A) Viability over indicated periods of time of WT and *oma1*^{-/-} fibroblasts with or without stable mitoT expression. Cells (500,000) were seeded per well in a 6-well plate. Growth was monitored for 3 days until over-confluence was reached. Events (10,000) were counted using Hoechst 33342 and propidium iodide staining parameters; healthy cells were PI-negative and Hoechst 33342-positive. Hoechst 33342-negative cells were not accounted for. Error bars, mean \pm S.D. of n=3 biological replicates. **(B)** Steady-state levels of mitoT-GFP, OMA1, OPA1 and HSPD1 (loading control) in indicated cells that were incubated with or without 2 μ M CCCP. Note that mitoT undergoes additional processing in the WT MEFs.

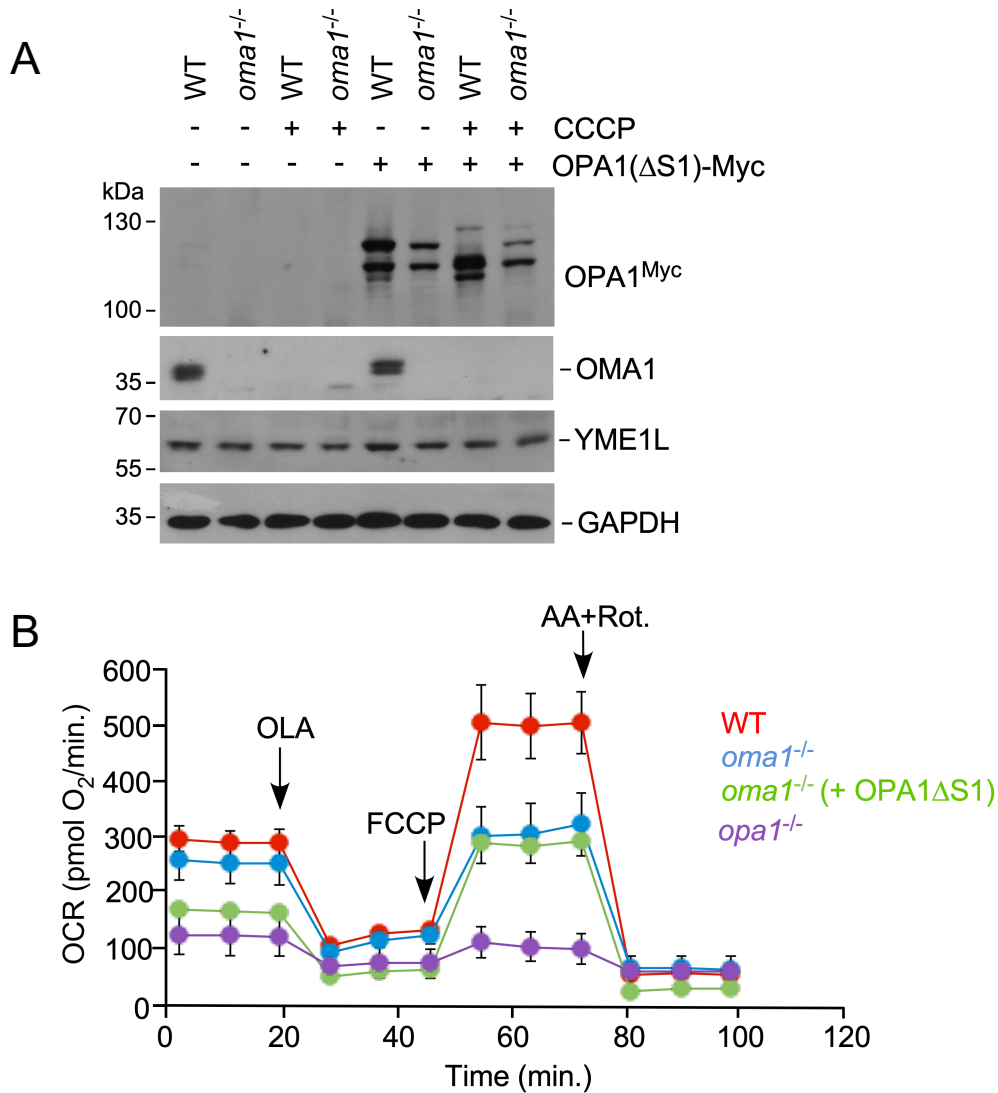


Figure S5. Bioenergetic deficit in *oma1*^{-/-} cells is not directly related to defective OPA1 processing, Related to Figure 5.

(A) Steady-state levels immunoblot analysis of OMA1, YME1L, GAPDH and Myc-tagged OPA1 variant lacking S1 processing site (OPA1 Δ S1) with relevant antibodies in mitochondrial lysates from indicated cells, with and without CCCP treatment. **(B)** Extracellular flux analysis of *in vivo* oxygen consumption rates in indicated MEF cells. Cells were cultured in medium containing 10 mM galactose, then transferred into standard Seahorse XF medium and analyzed at 50,000 cells/well under basal, oligomycin A (OLA), FCCP and antimycin A + rotenone stimulated conditions. Error bars, mean \pm S.E. of n=4 biological replicates.

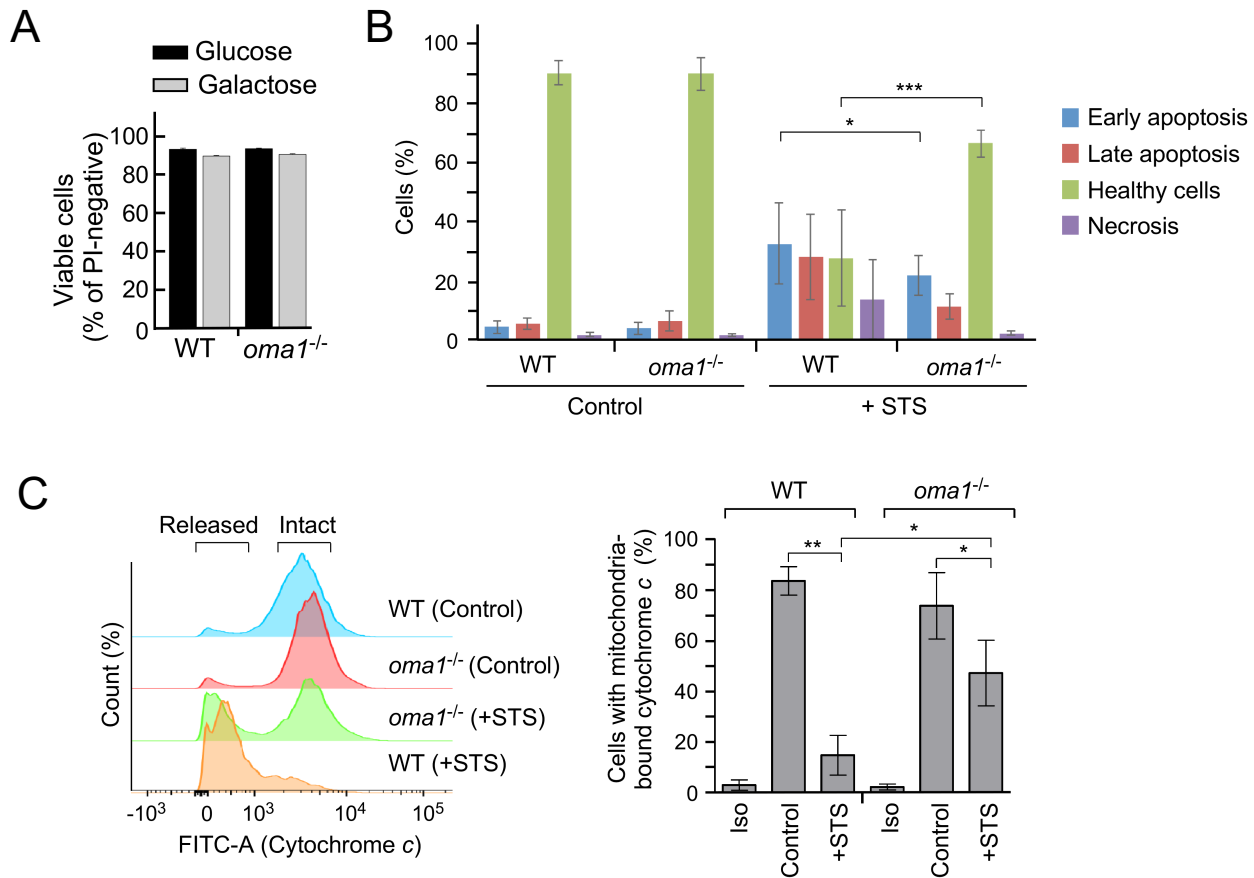


Figure S6. Apoptotic resistance of *oma1^{-/-}* cells, Related to Figure 6.

(A) Quantitative assessment of cell survival by flow cytometry. Indicated cells were cultured in 10 mM glucose or 10 mM galactose-supplemented medium and stained with propidium iodide (PI). Healthy cells were identified as PI-negative. Data represents means \pm S.E. of 3 biological replicates. **(B)** Flow cytometry analysis of healthy (Q3: 7-AAD negative, Annexin V-APC negative), early apoptotic (Q1: 7-AAD negative, Annexin V-APC positive), late apoptotic (Q2: 7-AAD positive, Annexin V-APC positive) and necrotic (Q4: 7-AAD negative, Annexin V-APC positive) cells. Data are presented as mean \pm S.E., $n=3$; $*p<0.05$, $***p<0.001$ by *t*-test. **(C)** Left: Representative flow cytometry histograms assessing cytochrome *c* release in healthy (Control) and 1 mM staurosporin-treated (+STS) WT and *oma1^{-/-}* fibroblasts. Iso, isotype control used to monitor antibody specificity. 50,000 cells were analyzed in each experiment. Right: Quantitative analysis of mitochondria-bound cytochrome *c* in the cells of interest. Data are presented as mean \pm S.E. of 3 independent experiments; $*p<0.05$, $**p<0.01$, $***p<0.001$ by *t*-test.

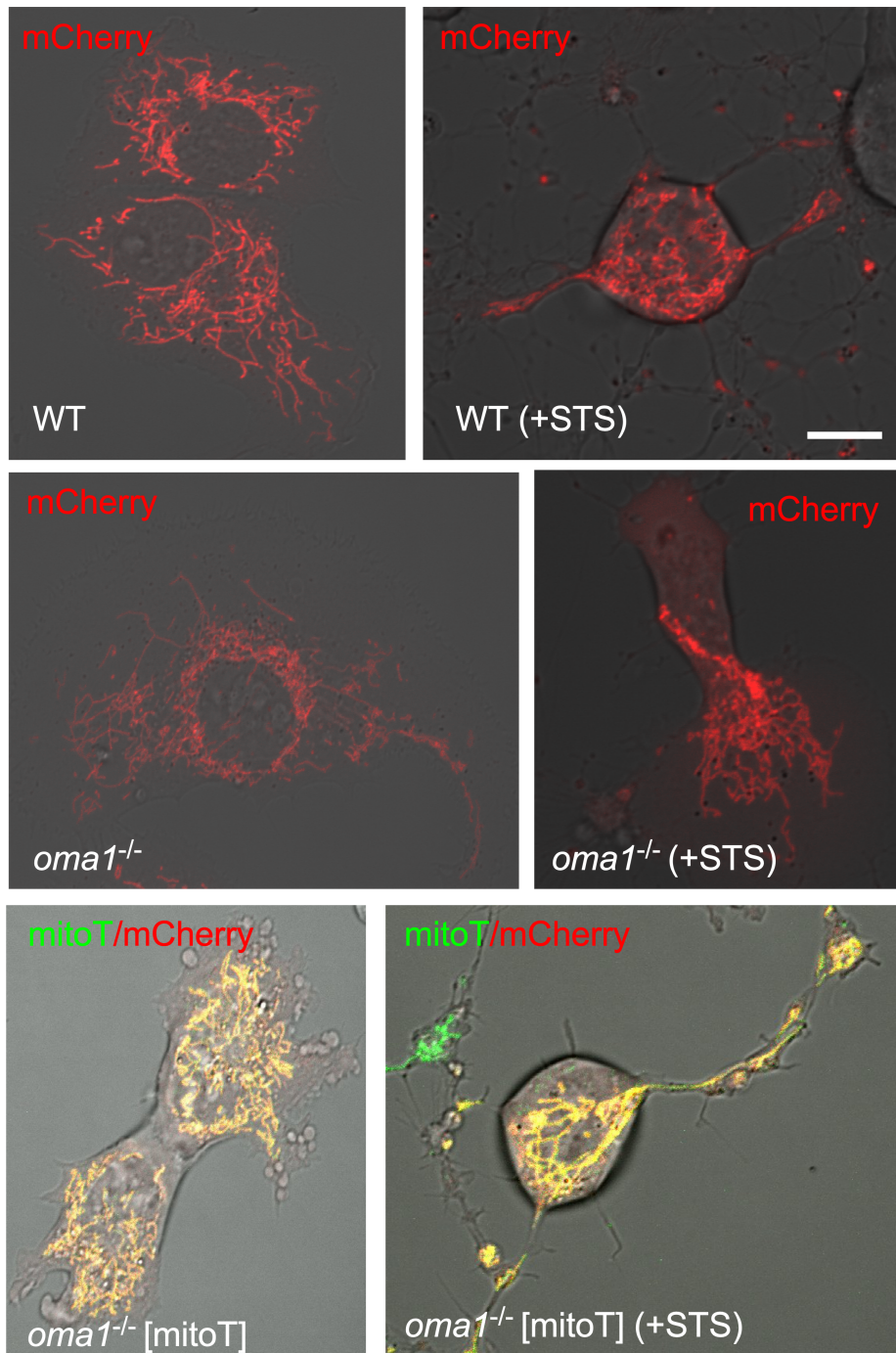


Figure S7. Expression of mitoT does not induce mitochondrial fragmentation in *oma1*^{-/-} cells, Related to Figure 6. Representative merged *in vivo* confocal microscopy images of mitochondrial network (visualized with mCherry, red signal) and mitoT-GFP (green signal) in indicated cells. Yellow color indicates signal overlap. Scale bar, 20 μ m.

400 xg, resuspended in fresh PBS, and measured on a Cytex DXP10 flow cytometer within 30 minutes. TMRM fluorescence was measured using 561 nm excitation, and 580/20 nm emission filters.

Annexin V presentation and cell viability were assessed after an 8-hour exposure to 1 μ M staurosporin or mock-treatment using 7-AAD APC-Annexin Kit (BioLegend) according to manufacturer's recommendations.

Cytochrome *c* release was assessed after an 8-hour exposure to 1 mM staurosporin or mock using a cytochrome *c* release kit (EMD Millipore) per manufacturer's recommendations. Upon necessity to avoid spectral overlap with the GFP fluorescence, the FITC-labeled anti-cytochrome *c* antibody supplied in the kit was substituted with Alexa Fluor 647-labeled anti-cytochrome *c* antibody (BioLegend).

Imaging Techniques

For confocal microscopy imaging, cells were stained in 1 cm glass bottom dishes (MatTek Corp.) with 5 nM Hoechst 33342 (Thermo Fisher Scientific) and/or 5 μ M Mitotracker DeepRed (Thermo Fisher Scientific) dyes in Phenol Red-free DMEM medium for 20 minutes in the dark, washed 2x with PBS, and supplemented with 2 ml of Phenol Red-free DMEM medium. The imaging was conducted using a Nikon A1R-Ti2 confocal system (Nikon, Japan). Multicolor images were generated using ImageJ software (NIH) by merging appropriate channels after assigning colors to each of them (green for GFP channel, blue for Hoechst 33342, and red for MitoTracker DeepRed).

For TEM, cells exposed to 2 μ M CCCP or mock for 1 h were trypsinized, washed 3x with PBS, fixed in 100 mM sodium cacodylate buffer pH=7.4 with 2.5% glutaraldehyde, and processed as previously described (Graham and Orenstein, 2007). TEM was performed on a Hitachi H7500 TEM microscope. Pictures were taken at 10,000x and 25,000x magnification from three different grids per sample. Mitochondria on each photo were assessed for ultrastructure type, which was classified as abnormal/vesicular or normal. The number of mitochondria of each type was counted in each grid, and percent of total for each type was calculated in each sample.

Biochemical Assays

For cell lysis preparation 2 million cells per sample were lysed by tumbling at 4°C for 2 hours in NP-40 lysis buffer containing 1x protease inhibitors cocktail and 2 mM PMSF freshly added prior to the lysis. The lysates were supplemented with 6x Laemmli buffer containing 5% β -mercaptoethanol and after boiling for 5 min at 100°C were loaded into a 10% polyacrylamide gel for subsequent immunoblot analysis. For IP, cells were grown to 90% confluence, and 24 hours after transfection were trypsinized, washed 3x with PBS, and lysed. Anti-Myc magnetic beads (Thermo Fisher Scientific, 88842), anti-GFP magnetic beads (MBL International, D153-9), and isotype IgG magnetic beads (MBL International, M076-11) were used with the lysates according to manufacturer's recommendations. Sucrose gradient separation of mitochondrial proteins was performed as previously described (Khalimonchuk et al., 2010) and analyzed by western blotting with relevant antibodies. Mitochondria (200 μ g) were lysed using 20 μ M HEPES buffer pH 7.4 with 100 mM NaCl and 1% digitonin on ice for 15 minutes. The lysate was centrifuged at 20000 $\times g$ for 10 min. at 4°C. 150 μ l of the supernatant was added to 40 μ l of anti-Mic60 agarose beads containing 1500 μ l of 20 μ M HEPES buffer pH 7.4 with 10 mM NaCl and 0.1% digitonin and was kept rotating at 4°C for 16h. Proteins were eluted from the beads using 2x Laemmli buffer without any reducing agent.

For blue native gel electrophoresis of mitochondria, Native PAGE Novex Bis-Tris Gel System and Native PAGE Novex 3-12% Bis-Tris Gels (Thermo Fisher Scientific) were used along with other reagents according to manufacturer's recommendations for organelle protocols. When necessary, we also used high-resolution home-made 3-15% Bis-Tris gels as described previously (Khalimonchuk et al., 2010). Mitochondria were extracted from MEF cells as described previously (Chen et al., 2012), solubilized in a sample buffer containing 0.5% digitonin and used as samples according to the manufacturer's protocol. Proteins from the resulting gels were transferred to PVDF membranes (Thermo Fisher Scientific) and analyzed by western blotting with relevant antibodies.

Immunoblotting

Proteins were detected using the primary antibodies indicated in the Key Resources Table. Relevant protein bands were visualized using secondary horseradish peroxidase-coupled secondary goat-anti-mouse (Jackson ImmunoResearch) and goat-anti-rabbit (Cell Signaling Technologies) secondary antibodies and chemiluminescent reagents (Thermo Fisher Scientific).

BioID Analysis

The pcDNA3.1-mycBioID plasmid was a kind gift from K. Roux (Addgene #35700). Human *OMA1* and human *SURF1* (matched negative control) were amplified using Phusion High Fidelity DNA Polymerase (Thermo Fisher

Scientific) from HEK293T cDNA library and subcloned into the plasmid using NheI and EcoRI restriction sites to create BirA fusion proteins. The plasmids were expressed in HEK293T cells, and proximity interactions were analyzed according to a previously described protocol (Roux et al., 2013). Briefly, 48 hours after transfection, 20 million transfected cells were lysed and tumbled overnight with Streptavidin magnetic beads (Thermo Fisher Scientific), after which the beads were washed 3x with lysis buffer, boiled, and subjected to SDS-PAGE. Abundant protein bands were extracted out of the gel and subjected to mass spectrometry detection on a QSTAR XL Hybrid LC/MS/MS (Applied Biosystems). Results were analyzed using Scaffold 4 (Proteome Software).

Extracellular Flux Analysis

The cells were seeded in parallel in two identical Seahorse cell culture plates (Agilent Technologies), one of which was used for the actual experiment, while the other was used for cell count for normalization purposes. Extracellular Flux experiments were performed on a Seahorse XFe24 Analyzer (Agilent Technologies) using XF Cell Mito Stress Test and XF Glycolysis Stress Test kits (Agilent Technologies) per manufacturer's protocols. Data were analyzed using manufacturer's calculation templates for Microsoft Excel 365. Summary bar graphs were plotted in Microsoft Excel 365 based on the data from the original analysis templates.

Quantification and Statistical Analysis

Unless stated otherwise, the data were analyzed in Microsoft Excel 365 using the Data Analysis package. All data are shown as mean \pm S.D., unless indicated differently. *P* values were calculated using paired two-tailed *t*-tests when analyzing paired samples and unpaired two-tailed *t*-tests when analyzing unpaired samples. *P* values less than 0.05 were considered significant. Violin graphs were plotted in GraphPad Prism 4.

For image quantification, images on X-ray films were digitalized and analyzed using Image J software.

For BioID LC-MS/MS data analysis, the following stringency criteria for candidate protein acceptance were applied. First, common background proteins were discarded. Second, candidate proteins with less than three spectral counts were considered as low-confidence hits and not pursued further. Third, candidate hits were checked against the CRAPome contaminant repository database (<https://www.crapome.org>) and unrelated mitochondrial control (SURF1) BioID analysis data to further eliminate nonspecific binding partners.

KEY RESOURCES TABLE

REAGENT / RESOURCE	SOURCE	IDENTIFIER
<i>Antibodies</i>		
OMA1	Santa Cruz	Cat.# sc-515788
OPA1	BD Biosciences	Cat.# 612606
OPA1	Novus Biologicals	Cat.# NB110-5529
MIC60	Protein Tech	Cat.# 10179-1-AP
MIC27	Aviva Systems Biology	Cat.# OAAF03292
MIC19	Protein Tech	Cat.# 10179-1-AP
MIC10	Aviva Systems Biology	Cat.# ARP44801_P050
MIC13	Proteintech	Cat.# 25514-1-AP
VDAC	Abcam	Cat.# ab14734
HSPD1	Santa Cruz	Cat.# sc-13966
YME1L	Protein Tech	Cat.# 11510-1-AP
GAPDH	Protein Tech	Cat.# 60004-1-Ig
GAPDH	GeneTex	Cat.# GTX627408
NDUFA9	Abcam	Cat.# ab14713
CORE1	Abcam	Cat.# ab110252
ATP5A	Abcam	Cat.# ab14748
AFG3L2	Abcam	Cat.# ab68023
AFG3L2	Aviva Systems Biology	Cat.# ARP4678055
MTCO1	Abcam	Cat.# ab14705
GFP	Invitrogen	Cat.# a6455
GFP	Santa Cruz	Cat.# sc-9996
Myc	Roche	Cat.# 11667149001
MFN1	Abcam	Cat.# ab57602
MFN2	Abcam	Cat.# ab124773
DRP1	Santa Cruz	Cat.# sc-271583
MIC60-agarose beads	Santa Cruz	Cat.# sc-390707 AC
Sterptavidin-magnetic beads	Thermo Fisher Scientific	Cat.# 88816
Myc-magnetic beads	Thermo Fisher Scientific	Cat.# 88842
GFP-magnetic beads	MBL International	Cat.# D153-9
IgG-magnetic beads	MBL International	Cat.# M076-11
2 ^o HRP-anti mouse	Jackson ImmunoResearch	Cat.# 115-035-068
2 ^o HRP-anti rabbit	Cell Signaling Technologies	Cat.# 7074S
<i>Cell Lines</i>		
HEK293T	ATCC	Cat.# CRL-3216
Wild-type MEF	ATCC	Cat.# CRL-2991
<i>opa1</i> ^{-/-} MEF	ATCC	Cat.# CRL-2995
Wild-type MEF	Quiros <i>et al.</i> , 2012	NA
<i>oma1</i> ^{-/-} MEF	Quiros <i>et al.</i> , 2012	NA
Wild-type MEF [mitoT]	This study	NA
<i>oma1</i> ^{-/-} MEF [mitoT]	This study	NA
<i>Media, Chemicals and Reagents</i>		
DMEM medium	Hyclone	Cat.# SH30243.01
L-glutamine	Gibco	Cat.# 25030081
Glutamax	Gibco	Cat.# 35050061
Sodium Pyruvate	Gibco	Cat.# 11360070
FBS, natural source	Hyclone	Cat.# SH30396.03
Gentamicin	Gibco	Cat.# 15750060
Penn/Strep mix	Hyclone	Cat.# 15070063
Trypsin	Hyclone	Cat.# SH30236.02
Lipofectamine 3000	Gibco	Cat.# L3000015
Opti-MEM I medium	Gibco	Cat.# 31985070
FluoroBrite DMEM medium	Gibco	Cat.# A1896702

Halt protease inhibitor	Thermo Fisher Scientific	Cat.# 87785
Mitotracker DR-FM dye	Invitrogen	Cat.# M22426
Hoechst 33342 dye	Invitrogen	Cat.# H3570
Propidium Iodide	Invitrogen	Cat.# P3566
Seahorse XF DMEM medium	Agilent	Cat.# 103575-100
Digitonin	Sigma	Cat.# D141
NP-40	Sigma	Cat.# I3021
NativePage 4-16% Bis-Tris precast gels	Invitrogen	Cat.# BN1002BOX
<i>Commercial Kits/Assays</i>		
Super Signal West Dura Substrate	Thermo Fisher Scientific	Cat.# 34075
XF Cell Mitostress Kit	Agilent	Cat.# 103015-100
XFe24 FluxPack	Agilent	Cat.# 100867-100
<i>Software</i>		
Image J	NIH	https://imagej.nih.gov/ij/
KaleidaGraph v.4.02	Synergy Software	NA
GraphPad Prism 4	GraphPad Software	NA
Scaffold 4	Proteome Software	NA
FlowJo 10	FlowJo Inc.	NA
Microsoft Excel 365	Microsoft	NA

SUPPLEMENTAL REFERENCES

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