In situ architecture of Opa1-dependent mitochondrial cristae remodeling

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Abstract

Cristae membrane state plays a central role in regulating mitochondrial function and cellular metabolism. The protein Optic atrophy 1 (Opa1) is an important crista remodeler that exists as two forms in the mitochondrion, a membrane-anchored long form (l-Opa1) and a processed short form (s-Opa1). The mechanisms for how Opa1 influences cristae shape have remained unclear due to lack of native three-dimensional views of cristae. We perform *in situ* cryo-electron tomography of cryo-focused ion beam milled mouse embryonic fibroblasts with defined Opa1 states to understand how each form of Opa1 influences cristae architecture. In our tomograms, we observe a variety of cristae shapes with distinct trends dependent on s-Opa1:l-Opa1 balance. Increased l-Opa1 levels promote cristae stacking and elongated mitochondria while increased s-Opa1 levels correlated with irregular cristae packing and round mitochondria shape. Functional assays indicate a role for l-Opa1 in wild-type apoptotic and calcium handling responses, and compromised respiratory function under Opa1 imbalance. In summary, we provide three-dimensional visualization of cristae architecture to reveal relationships between mitochondrial ultrastructure and cellular function dependent on Opa1-mediated membrane remodeling.

Highlights

- *In situ* ultrastructural characterization of mitochondrial cristae with different forms of Opa1.
- Mitochondria with predominantly l-Opa1 show crista stacking, longer cristae, reduced globular cristae and an absence of tubular cristae.
- Mitochondria with mostly s-Opa1 showed irregular cristae packing with wider cristae junctions and narrower cristae.
- l-Opa1 expressing cells with WT-like cristae junction properties, show wild-type apoptotic response and calcium handling.
- Imbalance in Opa1 processing show compromised respiratory function and an increase in amorphous cristae.

Introduction

Mitochondria play essential roles in energy production, metabolism and signaling, which drive diverse biological functions and processes of the cell (Chandel, 2014; Spinelli & Haigis, 2018; Picard & Shirihai, 2022). The organelle undergoes membrane remodeling during homeostatic steady-state conditions through fusion and fission to generate a dynamic and responsive reticulum (Twig *et al*, 2008; Mishra *et al*, 2014; Mishra & Chan, 2016). Mitochondrial ultrastructure is defined by a double-membrane architecture with an outer (OMM) and inner mitochondrial membrane (IMM). The IMM can be divided into three sub-regions, the cristae folds, the inner boundary membrane (IBM) and the cristae junctions (CJ), which separates cristae from the IBM. Cristae are enriched with electron transport chain (ETC) proteins, making them a hub for energy production (Schägger & Pfeiffer, 2000). In metazoans, cristae are mostly lamellar or tubular (Hashimi, 2019) and undergo dramatic rearrangements in response to stress and during initiation of apoptotic cell death (Frey & Mannella, 2000; Mannella, 2006; Zick *et al*, 2009). Despite longstanding interest in mitochondrial morphogenesis and major advances in understanding of mitochondrial structure and function (Friedman & Nunnari, 2014), regulation of cristae remodeling remains poorly understood.

Relevant proteins for cristae remodeling include ATP synthase (Blum *et al*, 2019), optic atrophy protein 1 (Opa1) and the mitochondrial contact site and cristae organizing system (MICOS) (Friedman & Nunnari, 2014; Laan *et al*, 2016). Opa1 is responsible for fusing the IMM during mitochondrial fusion and is also implicated in cristae remodeling. Inner-membrane proteases, such as Oma1, Yme1L, and ParI, process Opa1 to generate a 'soluble' form that lacks the N-terminal transmembrane (TM) segment (s-Opa1) (Song *et al*, 2007; Anand *et al*, 2014). This processing results in two forms of Opa1 in the mitochondria: the soluble short form (s-Opa1) and the unprocessed, full N-terminal TM anchored long form (l-Opa1). We previously showed that stoichiometric levels of both l- and s-Opa1 are required for fast and efficient membrane fusion *in vitro* (Ge *et al*, 2020); however, *in situ* ultrastructural understanding of how cristae shape is maintained by each Opa1 form is lacking.

Previous work used transmission electron microscopy (TEM) imaging of chemically fixed and heavy metal-stained cells to investigate the role of Opa1 in cristae shape. Knocking-down Opa1 induced more disorganized, globular and hyper-convex cristae, whereas over expression of Opa1 resulted in narrower cristae and CJs (Frezza *et al*, 2006). Initial steps of apoptosis involve release of cytochrome c from the cristae lumen, where ~85% of the cellular cytochrome c is stored (Scorrano *et al*, 2002). Expression of wild-type (WT) Opa1 was reported to protect against apoptosis by restricting cytochrome c release, whereas the loss of Opa1 was reported to result in mitochondria fragmentation and enhanced cytochrome c release (Scorrano *et al*, 2002). Addition of pro-apoptotic peptides resulted in wider CJs observed in TEM images. These observations suggest the organelle's inner-membrane

functional state may be sensitive to the levels of Opa1 present, and the membrane architectures supported by Opa1.

To understand the role of s- and l-Opa1 in cristae architecture and remodeling, we investigated the *in situ* state of mitochondrial membranes in mouse embryonic fibroblasts (MEF) with different levels of s- and l-Opa1, by applying cryo-electron tomography (cryo-ET) on cryo-focused ion beam (cryo-FIB) milled MEF. We used defined cell lines that predominantly contained either l- or s-Opa1 to characterize how mitochondrial membrane organization and shape depend on the expression levels and form of Opa1. Here, we present an extensive characterization of the 3D morphological properties of mitochondrial cristae membranes. We observed that l-Opa1 contributes to crista stacking, longer cristae, a reduction of globular cristae, an absence of tubular cristae and the maintenance of cristae junction widths. We found that the presence of s-Opa1 correlates with tubular cristae, wider cristae junctions, narrower cristae, and irregular cristae packing. Using BH3 profiling, we observed WT-like apoptotic responses only in the cells with l-Opa1. We also found WT-like calcium handling responses in cells expressing mainly l-Opa1 and that both forms of Opa1 are important for normal respiratory function. Our work reveals roles for s- and l-Opa1 beyond mitochondrial fusion and demonstrates that both forms of Opa1 play distinct roles in cristae shape maintenance important for mitochondrial function.

Results

In situ morphology of mitochondria with different Opa1 processing.

We generated cryo-electron tomograms of mitochondria from cryo-FIB milled MEF cell lines that differed in the expression levels and processed states of Opa1, to investigate mitochondrial cristae ultrastructure (**Fig. S1**). In this study, we used five MEF cell lines: (i) wild-type (WT) cells, (ii) cells stably overexpressing Opa1 (Opa1-OE), (iii) a Δ*exon5b* CRISPR MEF line with an Oma1$^{-/-}$ background, which restricts Opa1 cleavage and results in the presence of mostly l-Opa1 (referred to as l-Opa1* in this work), (iv) an Opa1 knock-out (Opa1-KO) line stably expressing the Opa1 isoform 5, which is robustly processed and results in the presence of s-Opa1 (referred to as s-Opa1*) and (v) Opa1-KO cells (Mishra *et al*, 2014; Wang *et al*, 2021) (**Fig. S1)**. While previous studies have characterized mitochondrial ultrastructure, these samples were subjected to chemical fixation and heavy metal staining, which are recognized to perturb cellular membrane state and limit resolution (Bäuerlein & Baumeister, 2021). Thus, we vitrified MEF cell lines to characterize mitochondrial membrane architecture under native conditions. To prepare samples for cryo-ET imaging, we generated ~200-350 nm thick lamellae by cryo-FIB milling (Rigort *et al*, 2012; Mahamid *et al*, 2016), following a previously

established imaging pipeline (Navarro *et al*, 2022). A total of 100 tilt-series (WT = 33, Opa1-OE = 7, l-Opa1* = 21, s-Opa1* = 28 and Opa1-KO = 11) were acquired, aligned and three-dimensionally (3D) reconstructed into tomograms (**Table S1**). Cryo-electron tomograms were denoised using Topaz-Denoise to improve mitochondrial membrane visualization (Bepler *et al*, 2020). It is important to note that cryo-electron tomograms generated by cryo-FIB milling cover a section of the mitochondria contained within the thickness of the lamella, thus, all mitochondria were partially imaged in the Z-axis. In some cases, mitochondria are only partially visible in the XY plane due to the trade-off between resolution and field of view (Navarro, 2022) (**Fig. S2a**). Densities corresponding to the IMM and OMM were 3D segmented in yellow and green, respectively (**Fig.1a, Movies 1-5**).

The generated tomograms provide rich 3D information on mitochondrial morphology. By visual morphological analysis, we classified mitochondria into ellipsoidal, round, partial (when partially imaged in the XY plane) or polygon shape categories. The majority of mitochondria in all cell lines are ellipsoidal, except for s-Opa1* cells, where round mitochondria dominate (**Fig. 1b**), correlating with a smaller apparent mitochondrial area (**Fig. 1c**). Mitochondria in WT cells are larger in area (0.34 ± 0.03 μm^2) than in other cell lines; with smaller mitochondria in l-Opa1* cells (0.2 ± 0.02 μm^2) and significantly smaller mitochondria in Opa1-OE (0.14 ± 0.02 μm^2) and s-Opa1* cells (0.18 ± 0.02 μm^2) (**Fig. 1c**). We measured the mitochondrial volume and the volumes of each subcompartment: the matrix, inner membrane space (IMS) and cristae lumen (CL) (**Fig. S3)**. Our analysis shows that imbalance of l- and s-Opa1 levels results in larger CL volume (**Fig. S3c-f**).

At first glance, we observed differences in matrix contrast between mitochondria from different cell lines in our cryo-ET data. To investigate this further, we quantified the grey scale levels in normalized summed projected images from cryo-electron tomograms (**Fig. S3g)**. We observed denser mitochondrial matrices in Opa1-OE, l-Opa1*, and Opa1-KO mitochondria. For the Opa1-OE mitochondria, the darker measured grey scale value can be attributed to the presence of electron dense deposits within the mitochondrial matrix, likely to be calcium phosphate deposits (Wolf *et al*, 2017; Strubbe-Rivera *et al*, 2021) (**Fig. 1a and S2a white arrowheads, Fig. S3h)**.

We analyzed mitochondrial cristae density (number of cristae/μm^2) in the cryo-electron tomograms. No significant difference was observed in the cristae density between mitochondria in WT and Opa1-KO cells (**Fig. S4a**). All other conditions (Opa1-OE, l-Opa1* and s-Opa1*) resulted in a significant increase in cristae density compared to WT. Such variation was not reflected in the total number of cristae per mitochondria (**Fig. S4b**). This can be explained by the reduction in average mitochondria size (**Fig. 1c**). These observations indicate that altered Opa1 levels or processing can impact the steady-state cristae number in a given mitochondrial volume.

131 We next characterized how Opa1 processing influences cristae organization. In l-Opa1* cells,
132 we observed organized and parallel oriented cristae, whereas cristae from s-Opa1* cells do not exhibit
133 such a pattern or organization and frequently cross over one another along the z-axis (**Fig. 1a, S2a and**
134 **S4c**). Additionally, we observed a subpopulation of mitochondria displaying a stacking cristae
135 phenotype, which we defined as three or more lamellar cristae running in parallel to one another into
136 the mitochondrial matrix throughout the tomogram **(Fig. S4c).** This phenotype was observed at similar
137 levels in WT (23.53%) and s-Opa1* cells (27.27%), but slightly more frequently in Opa1-KO (33.33%)
138 cells. Opa1 overexpression or inhibition of Opa1 processing (l-Opa1*) resulted in a dramatic increase
139 (>50%) of mitochondria with stacking cristae (**Fig. S4d**). These results suggest that homeostatic levels
140 of Opa1 processing maintain WT levels of cristae stacking.
141 We also used live-cell fluorescence microscopy (FM) and immunofluorescence to assess whole
142 cell mitochondrial network morphology. Consistent with previous FM reports, we observed an almost
143 equal mixture of short and long tubular mitochondrial networks in WT cells and a dramatically
144 fragmented network in Opa1-KO cells (Song *et al*, 2007; Patten *et al*, 2014; Bocca *et al*, 2018) **(Figure**
145 **S5)**. While fragmented mitochondrial networks were observed in s-Opa1* cells, more elongated tubular
146 networks were observed in l-Opa1* cells correlating with the increased number of round and polygon
147 mitochondria respectively found by cryo-ET (**Fig. 1a-b**). Opa1-OE cells showed longer tubular
148 networks, compared to WT **(Figure S5b)**. These data suggest healthy fusion activity in WT and l-Opa1*
149 cells and corroborate our cryo-ET observations (**Fig. S5, Movie 6**).
150
151 ***In situ* cristae ultrastructure**
152 To address questions related to the specific roles for Opa1 forms in cristae morphology, we
153 sought to characterize the dependence of cristae architecture on the form of Opa1 present. Based on
154 their direction relative to the OMM, we quantified cristae as straight or tilted. For some cristae, no
155 connection to the IMM was captured in the tomogram and thus classified as no attachment observed
156 (NAO) (**Fig. 2a**). This is a consequence of all mitochondria being partially imaged in the z-axis, as
157 mentioned above. In Opa1-KO cells fewer cristae were straight compared to WT (**Fig. 2a**). The
158 presence of either form of Opa1 restores the proportion of straight cristae to WT-like levels, while
159 overexpression of Opa1 results in more straight cristae than WT.
160 Cristae were then classified based on 3D-shape into canonical lamellar, globular, and tubular
161 categories (Harner *et al*, 2016). Across all cell lines, lamellar cristae dominate (**Fig. 2b**). Interestingly,
162 the proportion of lamellar cristae increases in l-Opa1* (81%) and s-Opa1* (77%) cells. Tubular cristae
163 are not observed in l-Opa1* and Opa1-KO cells and are reduced in Opa1-OE (3.24%) and s-Opa1*
164 (2.3%) cells compared to WT (9.6%). Globular cristae are present in all cell lines albeit to a lesser

extent when Opa1 expression levels are altered but increased in the absence of Opa1 **(Fig. 2b)**. The proportion of unusually shaped cristae is similar among WT (27%) and Opa1-OE (30.52%), but reduced in l-Opa1* (17%), s-Opa1* (16.9%) and Opa1-KO (18.9%) cell lines (**Fig. 2b, S2a**).

Unusual cristae were further subclassified into eight defined categories: (i) loop, where cristae curve and connect to IBM via two CJs; (ii) split, where cristae branch into two or more cristae; (iii) straight-across, when cristae are perpendicular and connect to the IBM via two CJs; (iv) amorphous, when cristae display a nebulous morphology; (v) ring, where cristae are circular; (vi) pinched, where cristae show areas where membranes touch; (vii) zipped, when cristae have regions where both membranes merge until both cannot be distinguished; and (viii) vesicular, where material is observed within the cristae (**Fig. S6**). Of these categories only five were observed in WT mitochondria – ring, loop, straight-across, amorphous, and split. The loop phenotype is dominant in WT unusual cristae, but also present in all cell lines (**Fig. S6a**). Pinched and zipped cristae are absent in WT cells but predominant in Opa1-OE cells. Zipped cristae were observed in l-Opa1* cells and only once in s-Opa1* cells, suggesting l-Opa1 may play a role in bridging the two membranes of the cristae for long stretches. In the absence of Opa1, no split or straight across cristae were observed; instead, more cristae fall into the amorphous and vesicular categories. An increase in amorphous cristae were also observed in l-Opa1* and s-Opa1* cells. All cristae shapes were observed in s-Opa1* cells, with roughly a quarter of the unusual cristae falling into the pinched category. Compared to WT, a similar proportion of unusual cristae in s-Opa1* mitochondria fall into the split category. Ring shaped cristae are rare and were not observed in l-Opa1* cells.

To determine the dependence of cristae length and width on the form of Opa1 present, we measured in 3D the length and width of fifty cristae per cell line (**Fig. S7a**). Knocking-out Opa1 results in significantly longer cristae (**Fig. 2c**). This increase in cristae length was not observed in Opa1-OE cells, but was observed in both s-Opa1* and l-Opa1* cells. In s-Opa1* mitochondria, there is a broad distribution of cristae lengths, with a considerable number of longer cristae than WT, which was also observed in Opa1-KO mitochondria. Overexpression of Opa1 does not affect cristae width, but the absence of Opa1 correlates with wider cristae, consistent with a larger proportion of observed globular cristae (**Fig. 2d**). Cristae widths are restored to WT values in l-Opa1* mitochondria, but in s-Opa1* mitochondria cristae are significantly narrower. Though Opa1-OE and l-Opa1* mitochondria have similar average cristae widths to WT mitochondria, a larger variation in cristae widths was observed, with many values falling into 0-5 nm and 9-14 nm ranges (**Fig. 2f**). This suggests that a significant proportion of cristae in Opa1-OE and l-Opa1* mitochondria vary in width, correlating with an increase in the number of zipped or pinched cristae in these cell lines (**Fig. 2e**). Even though cristae width histograms from s-Opa1* and WT mitochondria exhibit a single narrow peak, s-Opa1* mitochondria

199 have tighter cristae (6-15 nm and 9-16 nm, respectively) (**Fig. 2e-f**). In Opa1-KO mitochondria, the
200 cristae width distribution peak ranges from 10-17 nm, with an outlier representing a population of
201 extremely wide cristae also seen in l-Opa1* mitochondria, particularly a subclass of globular cristae
202 (**Fig. 2b, d-f**). These observations suggest the uniformity of cristae widths observed in WT mitochondria
203 is disrupted by changes in Opa1 expression levels and suppression of Opa1 processing.
204
205 **Cristae junction morphology by cryo-ET**
206 To gain insight into the role of each Opa1 form at the cristae junction (CJ), we measured and
207 analyzed CJs from summed projected central slices of tomograms for all cell lines. CJs were defined as
208 the site where the crista membrane joins the boundary region of the IMM (**Fig. S7c)**. Compared to WT
209 mitochondria, we observed wider CJs in Opa1-KO mitochondria, consistent with previous TEM data
210 (Scorrano *et al*, 2002) and unaltered CJ widths in Opa1-OE mitochondria (**Fig. 3a**). Intriguingly, we
211 found that CJs are also significantly wider in s-Opa1* mitochondria compared to WT mitochondria (**Fig.**
212 **3a**). We did not, however, find any significant difference in CJ widths in l-Opa1* mitochondria. These
213 measurements indicate under steady-state conditions complete processing of Opa1 results in larger CJ
214 widths.
215 In our initial inspection of our tomograms, we noticed that a greater proportion of cristae in s-
216 Opa1* mitochondria are tilted, i.e. where the crista length is not perpendicular to the IBM (**Fig. 2a**). To
217 further investigate if this tilt occurs at the beginning of the crista or along the length of the cristae, we
218 quantified the angle at the CJ (**Fig. S7d)**. While most cristae are orthogonal to the OMM, we observed
219 a wide range of CJ angles in s-Opa1* mitochondria. Overall, the CJ angles in s-Opa1* cristae are less
220 perpendicular compared to WT CJs, demonstrating that the tilted cristae observed in these cells occurs
221 at the CJ (**Fig. 2a and 3b-c**).
222 We also found that some cristae have two or more CJs. Most l-Opa1* mitochondria (77%) have
223 at least one crista with multiple junctions, which is considerably higher than WT (35%), Opa1-OE
224 (30%), s-Opa1* (27%), and Opa1-KO (25%) mitochondria (**Fig. S8a**). These multijunction cristae were
225 further classified as straight or loop. In WT cells, there is an equivalent number of straight and loop
226 cristae with more than one CJ and in Opa1-KO mitochondria, the ratio shifts to favor the loop class (**Fig**
227 **S8b**). In Opa1-OE and l-Opa1* mitochondria, the majority of multijunction cristae fall into the straight
228 category, correlating with a greater proportion of straight-across cristae shapes observed in l-Opa1*
229 mitochondria (**Fig. 3b and S8b**).
230
231 **Functional consequences of Opa1 state**

We next examined the consequences of Opa1 levels and processing on mitochondrial function. First, we sought to uncover potential effects of Opa1 perturbation on the intrinsic apoptotic pathway. We used BH3 profiling to compare mitochondrial responses to pro-apoptotic stimuli across the cell lines. BH3 profiling quantifies the percentage of cells that release cytochrome c from their mitochondria, the key commitment step in mitochondrial apoptosis, in response to treatment with BH3 peptides that mimic the activity of pro-apoptotic proteins from the BCL-2 family (Fraser *et al*, 2018). Cells that release cytochrome c in response to moderate or weak pro-apoptotic stimuli are considered to be primed for apoptosis, whereas cells that require strong stimuli to trigger cytochrome c release are unprimed. Apoptotic priming has been previously shown to determine whether healthy and cancerous cells undergo apoptosis in response to cellular stress or damage (Chonghaile *et al*, 2011; Sarosiek *et al*, 2017; Spetz *et al*, 2022). BH3 profiling showed a substantial reduction in apoptotic priming of Opa1-KO cells relative to WT cells as indicated by decreased sensitivity to the BIM BH3 peptide, which can inhibit all pro-survival Bcl-2 family proteins and directly activate BAX and BAK (Kale *et al*, 2018) (**Fig. 4a**). Equal doses of the BIM BH3 peptide induced less cytochrome c release from mitochondria in Opa1-KO cells than WT cells, indicating that stronger pro-death stimuli are required to induce apoptosis in cells lacking Opa1 (**Fig. 4a**). We also tested MEF sensitivity to common apoptosis-inducing agents by quantifying positivity for Annexin V, which binds phosphatidylserine on the surface of cells undergoing early apoptosis, after treatment. Based on the BH3 profiling results, we would expect less apoptosis after treatment of Opa1-KO cells than WT cells. We found that indeed this was the case: Opa1-KO MEF were more resistant to treatment with the topoisomerase inhibitor etoposide, DNA damaging agent doxorubicin, and pan-kinase inhibitor staurosporine at 24 hours (**Fig. 4b**). Similar differences in sensitivity were also detected at 48 and 72 hours, suggesting that retention of cytochrome c durably protected Opa1 KO cells from commitment to apoptosis (**Fig. S9**). Overexpression of Opa1 maintains a WT-level response to apoptotic stimuli. While s-Opa1* cells show levels of apoptotic priming between WT and Opa1-KO cells, the short-form is not sufficient to rescue normal apoptotic priming of MEF. In contrast, l-Opa1* cells behave similarly to the WT cells across most conditions, restoring apoptotic priming (**Fig. 4a**). These results suggest that these lines would not be as resistant to apoptosis-inducing agents as the Opa1-KO cells and is evident in the 24-hour chemosensitivity data for etoposide, doxorubicin, and staurosporine treatment (**Fig. 4b**) as well as data at 48- and 72-hours post-treatment (**Fig. S9**). The presence of a stable l-Opa1 population has been previously shown to be essential in apoptotic resistance (Merkwirth *et al*, 2008, 2012). Our results indicate Opa1 processing is not required for apoptotic priming. In Opa1-KO or s-Opa1* cells, wider CJ at steady-state conditions correlated with cells resistant to apoptosis.

To understand the impact of Opa1-dependent remodeling on mitochondrial calcium homeostasis and mitochondrial permeability transition pore (mPTP) opening, we performed mitochondrial calcium retention capacity (CRC) assay on MEF cell lines. Mitochondria from permeabilized l-Opa1* cells show similar CRC profiles to WT **(Fig. 4c)**. In contrast, s-Opa1* cells and Opa1-KO cells are observed to be more resistant to mPTP opening **(Fig. 4c)** and require higher concentrations of $CaCl_2$ to induce the pore opening transition **(Fig. 4b)**. The presence of vesicular cristae and wider CJs at steady-state conditions correlate with s-Opa1* and Opa1-KO cells resistant to calcium-induced mPTP opening. In contrast, Opa1-OE cells undergo mPTP transition at lower amounts of $CaCl_2$ stimulation. We note an elevated presence of mitochondrial calcium deposits in the Opa1-OE cell line **(Fig. S3H)**.

Next, we evaluated the mitochondrial respiratory fitness of all cell lines to assess how a key cristae-dependent function varies upon Opa1 perturbation. As previously observed, mitochondrial respiration is severely impaired in Opa1-KO cells (Zhang *et al*, 2011) (**Fig. 5a**). Basal OCR levels for Opa1-OE cells are similar to WT. s-Opa1* and l-Opa1* cells both have basal oxygen consumption respiration (OCR) levels significantly lower than WT, as previously reported (Lai *et al*, 2020; Lee *et al*, 2020). Cell lines with lower basal OCRs, also display significantly lower maximal respiration and spare capacity levels. Longer cristae and more amorphous cristae were observed in these cell lines. These results suggest that balanced levels of both forms of Opa1 are required for normal mitochondrial respiration.

Mitochondrial fitness was also measured in the Oma1$^{-/-}$ background in which the l-Opa1* cells were generated (Wang *et al*, 2021). Like l-Opa1* cells, Oma1$^{-/-}$ cells also have WT levels of cytochrome c release upon treatment with BIM **(Fig S10a)**. Additionally, Oma1$^{-/-}$ MEF cells show WT-like propensity for mPTP opening under Ca^{2+} stimulation albeit displaying a lower overall calcium buffering profile than WT and l-Opa1* cells **(Fig S10b)**. OCR values in l-Opa1* cells are significantly lower than in Oma1$^{-/-}$ cells, indicating the *oma1* deletion is not the sole contributor to the impaired respiration observed in l-Opa1* cells **(Fig. S10c-d)**. These data suggest that functional differences in the l-Opa1* mitochondria are dependent on the form of Opa1 present.

Finally, we investigated the effect of Opa1 imbalance on mtDNA maintenance. Mitochondrial fusion is essential for maintaining mtDNA stability (Elachouri *et al*, 2011) and Opa1 depletion has been shown to lead to a decrease in mtDNA number and translation (Chen *et al*, 2010). Consistent with previous reports, we observed robust mtDNA decrease in Opa1-KO MEF quantified by instant Structured Illumination Microscopy (iSIM) imaging **(Fig. S11a-c)** and qPCR **(Fig. S11d)**. Stimulated emission depletion (STED) microscopy studies in HeLa and human fibroblast cells have shown that nucleoids occupy mitochondrial matrix spaces between clusters of cristae (Stephan *et al*, 2019) and

when fusion is inhibited, nucleoids cluster without changes in size and copy number (Ramos *et al*, 2019). We observed higher nucleoid area and puncta in l-Opa1* cells than in WT cells **(Fig. S11b-c)**. No difference in mtDNA copy number was observed in l-Opa1* cells by qPCR **(Fig. S11d)**, suggesting these changes are likely due to mtDNA distribution and organization.

Discussion

Understanding how sophisticated membrane architectures are generated in pleiomorphic organelles poses a challenge and opportunity for structural cell biology. The mitochondrion is an exemplar case where the organelle's morphology can take on a wide range of forms regulated by dynamic protein assemblies (Mageswaran *et al*, 2023). In this work, we used state-of-the-art *in situ* cryo-ET to analyze how Opa1 processing regulates mitochondrial ultrastructure, in particular cristae morphology. Previous analyses of mitochondrial cristae morphology have been informed by conventional TEM micrographs of fixed and heavy metal-stained cells known to perturb membrane structures and introduce imaging artifacts (Scorrano *et al*, 2002; Olichon *et al*, 2003; Frezza *et al*, 2006). In overcoming these limitations, we characterized and quantified a diverse range of cristae morphologies specific to the form of Opa1 present in the cell. Here, we discuss structure/function relationships which emerged from this study (**Fig. 6**).

Our 3D data captures the diversity of cristae morphologies and can distinguish lamellar and tubular cristae. While the majority of cristae are lamellar, tubular cristae were observed in WT mitochondria and a fraction of s-Opa1* cells, but are entirely absent from l-Opa1* cells (**Fig. 2a, b, e; Fig. 6a, b**). Tubular cristae are compatible with reported *in vitro* helical assemblies of the yeast homolog, s-Mgm1 reconstituted on membranes (Faelber *et al*, 2019). Recent single particle cryo-EM helical reconstructions of human s-Opa1 decorating the exterior of membranes tubes show how paddle domain interactions facilitate self-assembly (Malsburg *et al*, 2023; Nyenhuis *et al*, 2023). The increase in globular cristae in the Opa1-KO condition may imply that the presence of either or both forms of Opa1 support lamellar cristae. Indeed, a recent study found knock-down of Opa1 altered the ratio of lamellar to tubular cristae (Suga *et al*, 2023). Stacking cristae phenotype in l-Opa1* as well as increased presence of multijunction cristae may implicate an accumulation of fusion intermediates (**Fig. 6c**). Further integrative structural studies will also be necessary to directly relate how specific protein conformational states influence such morphologies (**Fig. 6c**) (Harner *et al*, 2016).

Visualizing mitochondrial membrane structure using cryo-ET enables us to make accurate measurements of membrane distances. l-Opa1* cells maintained WT-like CJ widths (**Fig. 3a**), which with our BH3 profiling assay suggest that l-Opa1 plays a role in maintaining CJ widths and mediating

333 the response to apoptotic stimulation (**Fig. 6d**). TEM imaging showed that cytochrome c release
334 (stimulated by pro-apoptotic signaling proteins such as BID) results in the widening of cristae junctions
335 and opening of the cristae lumen (Scorrano *et al*, 2002; Frezza *et al*, 2006). In contrast to previous
336 studies, we investigated cristae state prior to any exposure to extracellular stress or apoptotic stimuli
337 (Merkwirth *et al*, 2008, 2012). Building upon previous studies, we observed wider cristae junctions in
338 Opa1-KO and s-Opa1* cells prior to apoptotic initiation (**Fig. 3**). Together, with our apoptotic priming
339 results demonstrating that cells lacking l-Opa1 released less cytochrome c upon apoptosis stimulation,
340 our observations support a specific CJ regulatory role for l-Opa1 in the cytochrome c release transition
341 (**Fig. 6b, c**) (Merkwirth *et al*, 2008).

342 Our findings that Opa1-KO cells are resistant to apoptosis may seem counterintuitive given
343 previous reports of apoptotic resistance in Opa1-overexpressing cells (Frezza *et al*, 2006)**.** However,
344 this apparent discrepancy can be reconciled if considering multiple roles for Opa1 during apoptotic
345 membrane transitions. During apoptosis, Opa1 maintains cristae junctions, which may restrict
346 cytochrome c release in response to pro-apoptotic signals (Frezza *et al*, 2006). However, the
347 importance of rearrangement and disassembly of Opa1 complexes during apoptosis initiation, to
348 facilitate CJ opening, as discussed above, demonstrates that Opa1 also has a direct role in the earlier
349 steps when apoptosis is being initiated (**Fig. 6d**). This view is consistent with our finding that complete
350 knock-out of Opa1 impairs cytochrome c release and can be protective against apoptosis-inducing
351 agents (**Fig. 4a-b**). Our data indicate that apoptotic response is independent of Opa1 processing
352 (Cipolat *et al*, 2006) and cytochrome c release is dependent on initial CJ widths.

353 Our BH3 profiling results are supported by CRC assay results, where l-Opa1 presence in MEF
354 allows for WT-like responses towards increased Ca^{2+} and apoptotic stimulation (**Fig. 4c-d**). These
355 independent functional outcomes are connected by the importance of membrane integrity in mPTP
356 opening (Strubbe-Rivera *et al*, 2021; Bernardi *et al*, 2023) and BAX/BAK-mediated cytochrome c
357 cristae remodeling (Scorrano *et al*, 2002; Renault *et al*, 2015). Consistent with previous reports of
358 calcium phosphate deposits, we observed dense deposits in the mitochondria matrix of most Opa1-OE
359 mitochondria (Wolf *et al*, 2017), which correlates with a lower mPTP transition $CaCl_2$ threshold. Cryo-
360 ET enabled this finding and allows us to attribute differences in density in the mitochondrial matrix to
361 biological material, and not artifacts from chemical agents. We note a less condensed matrix in
362 mitochondria in s-Opa1* cells and a lighter matrix in the Opa1-KO mitochondria, which we speculate
363 may be related to matrix components other than calcium.

364 Our measurements also suggest that l-Opa1 may contribute to maintaining perpendicular CJ, as
365 fewer perpendicular CJs were observed in s-Opa1* cells. Perpendicular CJs can facilitate cristae
366 stacking, which we noticed in a striking number of l-Opa1* cells. Stacking cristae have been previously

observed both under conditions that induce metabolic and endoplasmic reticulum stress upon addition of thapsigargin (Barad *et al*, 2022) and in Oma1$^{-/-}$ cells (Anand *et al*, 2014). We speculate that cristae stacking may have features in common to an intermediate preceding fusion (**Fig. 6c**). Consistent with this role, we also note a correlation between stacked cristae organization in the l-Opa1* cells facilitating mtDNA distribution. While Opa1-KO cells have a dramatic loss of mtDNA number, we still visualize the presence of cristae in these mitochondria. This further supports emerging evidence that the organization of multiple crista contribute to mtDNA maintenance (Stephan *et al*, 2019; Jakubke *et al*, 2021).

We also assessed the effects of Opa1 state on mitochondrial respiration, a central function dependent on cristae shape (Cogliati *et al*, 2016). In short, compromised respiration observed in l-Opa1*, s-Opa1*, and Opa1-KO cells correlates with a larger percentage of amorphous cristae and longer cristae lengths. This suggests both forms of Opa1 play a role in maintaining a defined cristae shape and length. Our work indicates more subtle cristae phenotypes (such as vesicular and amorphous) may also not be optimal for respiration (**Fig. 6**). We speculate disruption of cristae shape could destabilize respiratory complex assembly in the cristae membranes or vice versa. Future studies will be necessary to more directly link *in situ* mitochondrial ultrastructure with mitochondrial dysfunction.

In summary, here we characterize cristae morphologies related to the levels or forms of Opa1 present in the cell. We characterize and quantify cristae morphological differences and define distinct differences specific to each Opa1 form. Notably, we find evidence that l-Opa1 plays important roles in maintaining CJ width and connectivity, which correlates well with WT-like apoptotic and calcium responses. We also demonstrate that both forms of Opa1 are required for maintaining cristae membrane shape and mitochondrial respiration, linking amorphous cristae with mitochondrial dysfunction. This work, in describing cristae morphology and functions, opens new opportunities for understanding mitochondrial ultrastructure and function, and motivates further studies visualizing and dissecting mechanisms underlying cristae heterogeneity by new live cell imaging approaches to gain insights into the spatio-temporal regulation of their lifecycle.

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Declaration of Interests

B.P.K is an inventor on patents and/or patent applications filed by Mass General Brigham that describe genome engineering technologies. B.P.K. is a consultant for EcoR1 capital, and is an advisor to Acrigen Biosciences, Life Edit Therapeutics, and Prime Medicine. L.H.C. is an advisor for Stealth Biotherapeutics. The remaining authors declare that there are no competing financial interests.

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636 **Methods**

637

638 **Cell lines and culture**

639 MEF were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1%
640 penicillin/streptomycin at 37°C and 5% CO_2. Wildtype and Opa1 knock-out MEF cell lines were
641 purchased from ATCC. *Δexon5b/Oma1*$^{-/-}$ cells and Opa1 knock-outs transfected retrovirally with Opa1
642 Isoform 5 (KO Opa1 + Iso5) were kind gifts from David Chan (California Institute of Technology) (Song
643 *et al*, 2007). The KO Opa1 + Iso5 were maintained in the same condition described above and
644 supplemented with puromycin at 1 µg/mL. Cell genotypes were confirmed by Western blotting. To
645 mediate a constitutive overexpression of Opa1 from the safe harbor locus Rosa26 in MEF, mouse
646 Opa1 sequence was cloned into pR26-CMVconst (Addgene plasmid #127373;
647 http://n2t.net/addgene:127373; RRID: Addgene_127373; kind gift by Lance Miller) to generate pR26-
648 CMV-Opa1 (pAH33). Following sequence verification, the construct was co-transfected with pX330-
649 sgR26 (Addgene plasmid #127376; http://n2t.net/addgene:127376; RRID: Addgene_127376; kind gift
650 by Lance Miller) into WT MEF cells using Lipofectamine 3000. Transfected cells were selected with 5
651 µg/mL puromycin starting 24 hours post-transfection for 7 days. Stable clones were expanded for 7
652 days, and their genotypes were confirmed by Western blotting. Opa1-OE MEF cell line was cultured in
653 the same manner described above, with the addition of 2.5 µg/mL puromycin in the culture media.

654

655 Human HEK 293T cells (American Type Culture Collection; ATCC) were cultured in Dulbecco's
656 Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated FBS (HI-FBS) and 1%
657 penicillin-streptomycin. Samples of supernatant media from cell culture experiments were analyzed
658 monthly for the presence of mycoplasma using MycoAlert PLUS (Lonza).
659 To generate a HEK 293T cell line bearing an *OPA1* exon 5b knock-out, SpCas9 sgRNAs were cloned
660 into pUC19-U6-BsmBI_cassette-SpCas9gRNA (BPK1520; Addgene ID 65777) harboring spacer
661 sequences GCTCATTGTGAACTCGTGGCA (plasmid CJT90), GCCAACAGAAGCGCAAGGTGA
662 (plasmid CJT91), GTTCTCCTCATTGTGAACTCG (plasmid CJT92), and
663 GCAGAAGCGCAAGGTGATGGA (plasmid CJT93), each with added 5'Gs. Transfections were
664 performed 20 hours following seeding of $2x10^4$ HEK 293T cells per well in 96-well plates. Transfections
665 contained 70 ng of SpCas9 nuclease plasmid (pCMV-T7-SpCas9-P2A-EGFP (RTW3027; Addgene ID
666 139987) and 15 ng each of two sgRNA expression plasmids mixed with 0.3 µL of TransIT-X2 (Mirus) in
667 a total volume of 15 µL Opti-MEM (Thermo Fisher Scientific), incubated for 15 minutes at room
668 temperature, and distributed across the seeded HEK 293T cells. Cells were grown for approximately 72

669 hours prior to extracting genomic DNA (gDNA) by discarding the media, resuspending the cells in 100
670 µL of quick lysis buffer (20 mM Hepes pH 7.5, 100 mM KCl, 5 mM MgCl2, 5% glycerol, 25 mM DTT,
671 0.1% Triton X-100, and 60 ng/µL Proteinase K (New England Biolabs; NEB)), heating the lysate for 6
672 minutes at 65 ºC, heating at 98 ºC for 2 minutes, and then storing at -20 ºC. Editing efficiency in bulk
673 transfected cells was assessed by next-generation sequencing (NGS) essentially as previously
674 described (Walton *et al*, 2020) using PCR round 1 primers oLLH9-
675 ACACTCTTTCCCTACACGACGCTCTTCCGATCTCATCTGTTCCTTTGTTGCACCCTTGG and
676 oLLH10-GACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGAGTCCATGAACAGATTGAGGTGAC.
677 To create cell lines, HEK 293T cells were seeded and transfected with plasmids RTW3027 and both
678 sgRNAs CJT91 and CJT92. Transfected cells were grown for approximately 72 hours prior to dilution
679 plating into 96-well plates and grown until confluent. Cells were transferred into 24-well plates with
680 some cell mass reserved to extract genomic DNA (gDNA) for genotyping via PCR and NGS to verify
681 biallelic *OPA1* exon 5b deletion between the two SpCas9-sgRNA cleavage sites.
682 To generate a HEK 293T cell line bearing an *OPA1* R194G mutation, SpCas9 sgRNAs were cloned
683 into BPK1520 harboring spacer sequences GCGGCGTTTAGAGCAACAGAT (plasmid CJT87),
684 GCGTTTAGAGCAACAGATCGT (plasmid CJT88), and GCGTTTAGAGCAACAGATCG (plasmid
685 CJT89). Adenine base editor (ABE) plasmids included pCMV-T7-ABE8e-nSpCas9-P2A-EGFP
686 (KAC978; Addgene ID 185910) or pCMV-T7-ABE8e-nSpG-P2A-EGFP (KAC984; Addgene ID 185911).
687 Transfections were performed 20 hours following seeding of $2x10^4$ HEK 293T cells per well in 96-well
688 plates and contained 70 ng of ABE8e plasmid and 30 ng of sgRNA expression plasmid mixed with 0.3
689 µL of TransIT-X2 (Mirus) in a total volume of 15 µL Opti-MEM (Thermo Fisher Scientific). The
690 transfection mixtures were incubated for 15 minutes at room temperature and distributed across the
691 seeded HEK 293T cells. Cells were grown for approximately 72 hours prior to extracting gDNA as
692 described above. Editing efficiency in bulk transfected cells was assessed by NGS using PCR round 1
693 primers oLLH7-
694 ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCTTAGGCTGTTGACATCACTGGAGAATG and
695 oLLH8-GACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCAGAACTGCCACGTAATACCTTGTAC.
696 To create cell lines, HEK 293T cells were seeded and transfected with plasmids KAC984 and CJT88.
697 Transfected cells were grown for approximately 72 hours prior to dilution plating into 96-well plates and
698 grown until confluent. Cells were transferred into 24-well plates with some cell mass reserved to extract
699 genomic DNA (gDNA) for genotyping via PCR and NGS to verify biallelic introduction of *OPA1*-R194G.
700
701 **Live cell epifluorescence microscopy**

702 Confluent MEF cells were harvested, seeded onto 35 mm glass-bottom dishes (MatTek Life Sciences)
703 coated with poly-D-lysine (0.1 mg/mL) and allowed to grow overnight at 37˚C under 5% CO_2. For
704 visualization of mitochondria, cells were stained with 50nM MitoTracker™ Deep Red FM (Thermo
705 Fisher Scientific) at 37˚C for 15 mins. Following three rounds of washes with 1X PBS, cells were placed
706 in Live Cell Imaging Solution (Invitrogen). Imaging relating to figure S5 was performed using Zeiss Axio
707 Observer Z1 Advanced Marianas™ Microscope system, an Alpha Plan-Apochromat 100x/1.46 NA Oil
708 TIRF Objective M27 and Prime 95B scientific CMOS camera (Photometrics). MitoTracker™-stained
709 mitochondria were imaged using "Cy5" filter set (Cy5-4040C, Excitation: 628/40 nm [608-648nm],
710 Emission: 692/40 nm [672-712nm], Dichroic Mirror: 660nm) (Semrock). Temperature, humidity, and
711 CO_2 concentrations were controlled with an Okolab Microscope Stage Incubator System. Image
712 acquisition and processing were done using SlideBook™6 (Intelligent Imaging Innovations, Inc, Denver,
713 CO) and Fiji (Schindelin et al., 2012). Time-lapse videos of stained mitochondria were taken at one
714 frame per 30 seconds for a duration of 5 mins.
715
716 **Fluorescence imaging and quantification**
717 To determine mitochondrial network morphology, MEF cells were seeded onto glass-bottom dishes and
718 grown to ~80% confluency. Cells were washed with PBS and fixed in 4% paraformaldehyde (PFA) in
719 PBS for 20 mins. Following three washes with PBS, cells were permeabilized in 0.1% Triton X-100
720 diluted in PBS for 5 min. Cells were incubated in 2% normal goat serum and 0.05% Triton X-100 in
721 PBS for 1 hour, and subsequently incubated in the same buffer with 1:300 Tom20 Recombinant rabbit
722 antibody (Invitrogen) overnight at 4˚C. After thorough washing with PBS, cells were incubated with
723 1:1000 goat anti-rabbit Alexa Fluor® 488 AffiniPure antibody (Jackson ImmunoResearch) for 1 hour at
724 RT. Cells were washed in PBS and coated with UltraCruz® Aqueous Mounting Medium with DAPI
725 (Santa Cruz) prior to imaging. Images were captured using A1R HD25 point scanning confocal with
726 GaAsP and PMT detectors, equipped with an Apo TIRF 60x/1.49 NA objective lens and Ti2 Z-drive.
727 Cells were manually scored into four morphological classifications (Wang et al. 2021): "Fragmented"
728 refers to cells that contain spherical mitochondrial fragments with two or less short tubules present.
729 "Short tubular" refers to cells containing a mixture of fragmented and short tubular mitochondria. "Long
730 tubular" refers to cells with elongated mitochondria, but not fused into a mitochondrial mesh.
731 "Interconnected" refers to cells with a highly interconnected network of mitochondrial filaments, with few
732 isolated mitochondria present.
733
734 **Cryo-EM specimen preparation**

Cells were prepared following the deposition method. Cells were detached and counted with a hemocytometer. Quantifoil 200 mesh holey carbon R2/2 (EMS) were glow-discharged for 60s or 90s at 20mA or 15mA using a PELCO easiGlow glow discharge system (Ted Pella). ~1000-3000 cells were deposited onto a grid by pipetting 3 µL of detached cells onto the EM grid. Blotting and plunging was performed in a FEI Vitrobot Mark IV (Thermo Fisher Scientific (TFS)) at RT, 100% humidity with a waiting time of 60 seconds, one-side blotting time of 15 seconds and blotting force of 10 or 7. Customized parafilm sheets were used for one-sided blotting. All subsequent grid handling and transfers were performed in liquid nitrogen. Grids were clipped onto cryo-FIB autogrids (TFS).

Cryo-FIB milling

Grids were loaded in an Aquilos 2 Cryo-FIB (TFS). Specimen was sputter coated inside the cryo-FIB chamber with inorganic platinum, an integrated gas injection system (GIS) was used to deposit an organometallic platinum layer to protect the specimen surface and avoid uneven thinning of cells (Wagner *et al*, 2020). Cryo-FIB milling was performed on the specimen using two rectangular patterns to mill top and bottom parts of cells, and two extra rectangular patterns were used to create macro-expansion joints to improve lamellae instability (Wolff *et al*, 2019). Cryo-FIB was performed at a nominal tilt angle of 14-26 which translates into a milling angle of 7-19. Cryo-FIB milling was performed in several steps of decreasing ion beam current ranging from 1 nA to 10 pA and decreasing thickness to obtain 200-400 nm lamellae.

Cryo-electron tomography

All imaging was performed on a FEI Titan Krios (TFS) transmission electron microscope operated at 300KeV equipped with a Gatan BioQuantum K3 energy filter (20eV zero-loss filtering and a Gatan K3 direct electron detector. Prior acquisition, a full K3 gain reference was acquired, and ZLP and BioQuantum energy filter were finely tuned. The nominal magnification for data collection was 33,000x giving a calibrated 4K pixel size of 2.758. Data collection was performed in nanoprobe mode using SerialEM (Mastronarde, 2003) or TFS Tomography 6 software. The tilt range varied depending on the lamella, but generally was from -70 to 70 in 2 steps following the dose-symmetric scheme (Hagen *et al*, 2017). Tilt images were acquired as 8K x 11K super-resolution movies of 6 frames with a set dose rate of 1.5-3 e-/Å/sec. Tilt series were collected at a range of nominal defoci between -3.5 and -5 µm and a target total dose of 100 to 180 e-/Å (Supplementary Table 1).

Cryo-electron tomography image processing

Acquired tilted super-resolution movies were motion corrected and Fourier cropped to 4K x 5K stacks, minimizing aliasing effects using *framealign* from IMOD (Kremer *et al*, 1996). Tilt-series were aligned using etomo in IMOD (Mastronarde & Held, 2017). CTF-estimation was performed in IMOD (Turoňová *et al*, 2017) and/or using customized MATLAB scripts. CTF-correction was performed by *ctfphaseflip* program in IMOD. CTF-corrected unbinned tomograms were reconstructed by weighted back projection with and without a SIRT-like filter and subsequently 2x, 4x and 8x in IMOD. Cryo-electron tomograms were denoised using Topaz (Bepler *et al*, 2020) and summed projection of cryo-tomogram slices were performed in *Dynamo* (Castaño-Díez *et al*, 2012) complemented with customized MATLAB scripts.

3D Segmentation

Segmentation was done in TomoSegMemTV (Martinez-Sanchez *et al*, 2014) to create the first triangulation of mitochondrial membranes. Such triangulation was refined using Amira (TFS) by unbiased semi-automatic approaches. Final triangulated surfaces were remeshed and smooth in Amira for final rendering.

Quantitative analysis of cryo-ET data

Mitochondrial shape

Mitochondria morphology was categorized into ‘ellipsoidal’, ‘round’, ‘heart-shaped’ (when displaying a polygon shape) and ‘partial’ (when mitochondria was out of the XY image) by visual inspection of cryo-electron tomograms.

Mitochondrial size

Mitochondria were outlined in summed projection images of the central slices of cryo-electron tomograms in FIJI using the ‘polygon selection’ tool and pressing the measure key to output the area of outline mitochondria in nm^2.

Mitochondrial coverage

Mitochondrial area in μm^2 obtained from mitochondria size measurements was divided by the total area of the summed projected image.

Matrix density: Mitochondria density was measured in summed projection images of the central slices of cryo-electron tomograms that were all equally grey scale normalized in FIJI by applying the function equalize histogram set at 0.35% for all images. Three lines were drawn in the matrix region of the mitochondria under analysis and their mean grey value was calculated by pressing the measure button in FIJI (Schindelin *et al*, 2012). Three measurements per mitochondria were obtained, thus, the mean was calculated to obtain a single value per mitochondrial matrix.

Cristae density

Number of cristae was quantified in cryo-electron tomogram using the multi-point tool in FIJI. The number of cristae was normalized against area of mitochondria in μm^2.

Mitochondrial volume: Total mitochondria volume was calculated in Amira by summing the volume of cristae lumen (CL), inter membrane spacing (IMS) and matrix volume in μm^3. CL, IMS and matrix volumes was outputted by Amira based on the 3D surface of each compartment segmented and rendered in Amira with the module 'measure surface'. Ratios were calculated by dividing the volume values of the specified mitochondrial compartments.

Cristae directionality

Cristae was classified as 'straight', 'tilted' or 'disconnected' by visual inspection of cryo-electron tomograms.

Cristae shape

Cristae was classified as 'lamellar', 'globular', 'tubular' or 'unusual' by visual inspection of cryo-electron tomograms. Within the category 'unusual' the following classes were defined: 'loop' when cristae present two cristae connection with the IMS and was curved, 'pinching' when cristae membranes presenting punctual touching points, 'straight-across' when cristae present two cristae connection with the IMS just opposite to each other forming a straight septum-like structure across a mitochondrion, 'amorphous' when cristae displayed an irregular polygon shape, 'splitting' when cristae branched into two or more cristae within a giving mitochondrion, 'ring' when cristae formed a circular ring within mitochondria, 'zip' when cristae membranes come close and only one membrane was distinguished that later opens up into regular lamellar cristae, and 'vesicular' when cristae was wide, usually amorphous, but contained electron dense material resembling to membranes.

Cristae length

Cristae length was measured in cryo-electron tomograms by extracting the cristae volumes in *Dynamo* using the 'oblique slices in tomoslice' tool (https://wiki.dynamo.biozentrum.unibas.ch/w/index.php/Oblique_slices_in_tomoslice). Then, length was computing using the length tool in *Dynamo* (https://wiki.dynamo.biozentrum.unibas.ch/w/index.php/Walkthrough_on_GUI_based_tilt_series_alignment_(EMBO2021)#Visualization_matrix).

Cristae width

Subtomogram averaging was performed in *Dynamo*. Particles were manually identified using '*dtmslice*' interface in *Dynamo* (Navarro *et al*, 2018, 2020). Subtomograms with a size of $(1058.8)^3$ Å were extracted from 4x-binned tomograms. An initial reference generated from a random set of particles was used for 3D particle alignment. A total of 12 iterations were used to align particles until convergence, i.e., until no further improvement of alignment parameters was detected by additional iterations, and

then final averages were obtained. Final averages were generated from 222 (WT), 430 (OE-Opa1), 323 l-Opa1*, 653 s-Opa1* and 243 KO-Opa1 subtomograms. EM densities were visualized in UCSF Chimera (Pettersen *et al*, 2004). Cristae width is measured per particle in 3D width measurement was done in *Dynamo* by cross-correlation of each particle against a set of 40 templates displaying a distance range between membranes of 1 to 40 pixels (corresponding to 2.2 to 88 nm distance, https://wiki.dynamo.biozentrum.unibas.ch/w/index.php/Framework_for_estimation_of_membrane_thickness_and https://github.com/NavarroPP/membraneThickness/blob/main/CristaeThickness.m). A cross-correlation peak per particle is outputted corresponding to the distance value between the two cristae membranes defined here as cristae width (Fig. S7).

Cristae junction measurement

Cristae junctions were measured in summed projection images of 10 slices from each tomogram. Each CJ was isolated, and the width was measured using the line tool and measurement function in FIJI (Schindelin *et al*, 2012). The angle of each CJ was measured using the angle tool and measure function in FIJI. If a CJ was visible in multiple and nonoverlapping sections of the tomograms, multiple measurements were made for that CJ and averaged to represent the overall 3D shape of the CJ.

Statistical analysis

Statistical tests performed to assess differences in our quantifications were chosen based on the distribution of the data points. Since most of our data did not follow a normal distribution, we used non-parametric tests as Mann-Whitney test in order to measure significant difference between two data sets (e.g., WT vs Opa1-OE). When both data sets followed normal distribution unpaired t test with Welch's correction was used since standard deviations were different between the compared data sets. Furthermore, additional statistical tests were applied to reassure statistical results outputting very similar p values and same significance. All statistical analyses were performed in Prism 9 GraphPad software.

Mitochondrial respiration assays

Cells were seeded in a Seahorse XFe96 Cell Culture plate in 80µL of DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at a concentration of 30,000 cells/well and left to recover at 37°C in 5% CO_2 for 6 hours. After, cells were then washed three times with prewarmed assay buffer (XF Seahorse DMEM media supplemented with 10mM Seahorse XF Glucose, 1mM Seahorse XF Pyruvate, and 2mM Seahorse XF L-Glutamine), covered with 180µL of assay buffer, and incubated for one hour at 37°C under no atmospheric CO_2. Prior to the assay, the injection ports on the sensor cartridge were loaded with 2.0µM oligomycin, 1µM carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), and 0.5µM of rotenone/antimycin A. Before the injection of drugs, the Seahorse XFe96 Analyzer mixed the

870 assay media for each well for 10 minutes and took three baseline measurements. After the injection of
871 each drug, the analyzer mixed for 3 mins, waited for 1 min, and the measured for 3 mins, three times.
872 Oxygen consumption rates (OCRs) are the average oxygen consumption rate during each three-minute
873 measurement time and were normalized to cells/well. Biological replicates indicate assay performed on
874 different flasks of MEF cell culture grown under the same condition and passage number. Statistical
875 analysis performed to assess differences in our measurements were chosen based on the distribution
876 of the data points. The mitochondrial respiration data followed a normal distribution by the Shapiro-Wilk
877 normalcy test. We used unpaired t test with Welch's correction to evaluate significance as standard
878 deviations were different between the compared data sets. All statistical analyses were performed in
879 Prism 9 GraphPad software.
880
881 **BH3 profiling and chemosensitivity methods**
882 BH3 profiling was conducted by flow cytometry according to published protocols (Fraser *et al*, 2018).
883 Briefly, cells in culture were trypsinized and added to wells of prepared 96 well plates containing the
884 indicated peptide conditions and .001% digitonin in mannitol experimental buffer (MEB; 10 mM HEPES
885 (pH 7.5), 150 mM mannitol, 50 mM KCl, 0.02 mM EGTA, 0.02 mM EDTA, 0.1% BSA, and 5 mM
886 succinate). Peptide treatments were carried out for 60 minutes at 28 degrees C, then cells were fixed
887 for 10 minutes in 2% PFA. Fixation was quenched with N2 buffer (1.7 M tris base and 1.25 M glycine
888 (pH 9.1)), then cells were stained overnight with DAPI and an Alexa Fluor 647-conjugated anti-
889 cytochrome c antibody (Biolegend, clone 6H2.B4). Stained cells were analyzed using an Attune NxT
890 flow cytometer, with gates drawn based on cytochrome c staining in the negative and positive control
891 treatments (PUMA2A and DFNA5 peptide). The percentage of cytochrome c negative cells was
892 reported for each peptide treatment condition.
893 For chemosensitivity assays, cells were plated at 10^4 cells per well in 100 µl culture medium on 96-well
894 flat-bottom plates (Denville). They were treated with the following drugs at specified concentrations:
895 etoposide 10 µM, staurosporine 0.1 µM, and doxorubicin 1 µM. After 24 hours in standard tissue culture
896 conditions, cells were stained with Alexa Fluor 488-conjugated Annexin V in 10× Annexin binding buffer
897 [0.1 M Hepes (pH 7.4), 1.4 M NaCl, and 25 mM $CaCl_2$ solution]. Alexa Fluor 488-conjugated Annexin V
898 was added to the solution at a 1:500 dilution. The staining solution was added to the cells at a 1:10
899 dilution, and the cells were allowed to stain for 20 minutes on ice in the dark. Annexin V positivity was
900 measured by Attune flow cytometer equipped with an autosampler (Thermo Fisher Scientific).
901 Biological replicates indicate assay performed on different flasks of MEF cell culture grown under the
902 same condition and passage number. Statistical analysis was performed using a two-way ANOVA with

903 Holm-Sidak's correction for multiple hypothesis since the data were normally distributed (Fraser *et al*,
904 2022; Singh *et al*, 2023). All statistical analyses were performed in Prism 9 GraphPad software.
905
906 **Mitochondrial Calcium Retention Capacity (CRC) Assays**
907 MEF cells (1 x 10^6) were incubated in 150 µl assay buffer (125 mM KCl, 20 mM HEPES, 2 mM
908 K_2HPO_4, 5 mM glutamate, 5 mM malate, 5 mM succinate, 1 mM $MgCl_2$, 5 µM EGTA, 1 µM Calcium
909 Green 5N, 40 µM digitonin, pH 7.2) supplemented with 1.5 µM thapsigargin (Sigma) for 10 mins.
910 Fluorescence was continuously monitored using a PerkinElmer EnVision plate reader (excitation, 485
911 nm; emission, 535 nm). Sequential 30 µM $CaCl_2$ pulses were administered until Ca^{2+} uptake ceased,
912 and a sudden release of previously taken up Ca^{2+} indicated by a sustained increase in fluorescence
913 reading, consistent with mPTP opening. Biological replicates indicate assay performed on different
914 flasks of MEF cell culture grown under the same condition and passage number.
915
916 **Mitochondrial network and mtDNA imaging and analysis by instant Structured Illumination**
917 **Microscopy (iSIM)**
918 For live-cell imaging, cells were stained with PKMito Orange probe (Spirochrome) and SYBR™ Gold
919 Nucleic Acid Stain (Thermo Fisher Scientific), following manufacturer's instructions. 250 nM PKMito
920 Orange and 1:10 000 SYBR™ Gold Nucleic Acid Stain were the optimal concentrations for the cell
921 type. Cells were allowed to rest for 1-2 hours before imaging. Instant structured illumination microscopy
922 (iSIM) was performed on the custom-built microscope set-up at the EPFL Laboratory of Experimental
923 Biophysics (York *et al*, 2013; Mahecic *et al*, 2020). Time-lapse images were acquired from MEF cells
924 acclimatized in a 37 °C chamber, using 488-nm and 561-nm excitation lasers, a 1.49 NA oil immersion
925 objective (APONXOTIRF; Olympus), and an sCMOS camera (PrimeBSI, 01-PRIME-BSI-R-M-16-C;
926 Photometric), with additional fast vertically scanning piezo actuated mirrors for scanning/illumination
927 pattern homogenization. The raw images were deconvolved using the Richardson Lucy algorithm as
928 implemented by the flowdec Python package (Czech *et al*, 2019) with 10 iterations. Deconvolved iSIM
929 images of stained MEF cells were processed and analyzed using the CellProfiler™ software (Stirling *et*
930 *al*, 2021). Briefly, mitochondria were segmented from the PKMito Orange signal using the Robust
931 Background thresholding algorithm with 2 standard deviations following median filtering, which was
932 subsequently used to mask and segment the nucleoid foci from the SYBR™ Gold image (Gaussian
933 filtering and Robust Background algorithm with 2 standard deviations). The size and shape of each
934 segmented object were then measured, and each cell's values are displayed normalized to the
935 segmented mitochondrial area. These data are not normally distributed, thus we used Mann-Whitney

936 test to measure significant difference between WT and the other cell lines. All statistical analyses were
937 performed in Prism 9 GraphPad software.
938
939 **Quantification of mtDNA by qPCR**
940 Total genomic DNA was extracted using Qiagen DNeasy kit following manufacturer's protocol and 30
941 ng DNA was used for each qPCR reaction. Two mitochondrial probes were each normalized to a
942 nuclear probe to calculate mtDNA copy number relative to nuclear genome copies. This relative mtDNA
943 copy number for all genotypes were then normalized to that of WT MEF. Biological replicates indicate
944 assay performed on MEF cell culture derived from different flasks, grown under the same condition and
945 passage number. Sequences of qPCR primers:
946 MT-16S Fwd: CCGCAAGGGAAAGATGAAAGAC
947 MT-16S Rev: TCGTTTGGTTTCGGGGTTTC
948 MT-ND1 Fwd: CTAGCAGAAACAAACCGGGC
949 MT-ND1 Rev: CCGGCTGCGTATTCTACGTT
950 nuclear HK2 Fwd: GCCAGCCTCTCCTGATTTTAGTGT
951 nuclear HK2 Rev: GGGAACACAAAAGACCTCTTCTGG
952 These data followed a normal distribution by the Shapiro-Wilk normalcy test. We used unpaired t test
953 with Welch's correction to evaluate significance as standard deviations were different between the
954 compared data sets. All statistical analyses were performed in Prism 9 GraphPad software.
955

956

957 **Figure 1 | *In situ* mitochondrial membrane morphology is influenced by Opa1 processing.**
958 Mitochondria with distinguishable inner mitochondrial membrane (IMM) and outer mitochondrial
959 membrane (OMM) visualized by cryo-ET. **(a)** (Right) Summed, projected central slices of cryo-electron
960 tomograms visualizing representative mitochondria in indicated MEF cell lines. (Left) Three-dimensional
961 (3D) rendering of segmented membranes with mitochondria shown across Z slices. Green and yellow
962 surfaces indicate OMM and IMM, respectively. (Bottom right) Schematic of Opa1 forms present in
963 respective cell lines. **(b)** Graph bar representing the relative proportion of different mitochondrial shapes
964 observed. **(c)** Plot of mitochondria size (μm^2) observed in cryo-electron tomograms in MEF lines.
965 Scatter plots show data distribution, the mean is marked by a bold black line. Significance of difference
966 is tested relative to wild type using Mann Whitney test; *p<0.05, **p<0.01, ****p< 0.0001. N refers to
967 number of mitochondria: wild-type = 57, Opa1-OE = 17, l-Opa1* = 39, s-Opa1* = 55, Opa1-KO = 12.
968 Scale bar = 200 nm.

969

970 **Fig. 2 | *In situ* crista ultrastructure. (a)** Graph bars showing the proportions of straight, tilted, and no
971 attachment observed (NAO) crista and **(b)** of lamellar, tubular, globular and unusual crista observed in
972 indicated MEF lines. **(c)** Measured cristae length and **(d)** cristae width across cell lines. **(e)** (Top rows)
973 Computational slices of straight, tilted, disconnected, globular and tubular crista across cell lines and
974 the corresponding 3D renderings (bottom rows) from cryo-electron tomograms (n.a. =not applicable). **(f)**
975 Histograms of crista widths across cell conditions (see Methods). **(g)** Subtomogram averages of
976 mitochondrial cristae membranes with the average width indicated. Scatter plots show data distribution,
977 the mean is marked by a bold black line. Removal of outliers did not change statistical test results (see
978 source data). Significance of difference is tested relative to WT using Mann Whitney test; *p<0.05,
979 ***p<0.001, ****p<0.0001. For (a) and (b): N refers to the number of cristae analyzed: wild-type = 131,
980 Opa1-OE = 166, l-Opa1* = 380, s-Opa1* = 495, Opa1-KO = 112. For (c): N = 50 for all cell lines. For
981 (d) and (f): N refers to the number of cristae subvolumes: WT = 222, Opa1-OE = 430, l-Opa1* = 323, s-
982 Opa1* = 653, Opa1-KO = 243. For (d) mean ± SD in WT = 14.5±5.8; Opa1-OE = 15.2±10.5; l-Opa1* =
983 17.6±15.4; s-Opa1* = 12.9±7.9; Opa1-KO = 20±15.8. Scale bar = 50 nm.

984

985 **Fig. 3 | Quantification of cristae junction (CJ) properties. (a)** Plots of measured cristae junction
986 width and **(b)** angle across cell lines (see Fig. S8c, d and Methods section for measurement methods).
987 **(c)** (Top) Summed, projected central slices of cryo-electron tomograms of representative mitochondria
988 analyzed in (a) and (b) with magnified cristae junction (bottom insets). Scatter plots show data
989 distribution, the mean is marked by a bold black line. Significance of difference is tested relative to wild

990 type using Mann Whitney test; *p<0.05, **p<0.01, ***p<0.001. N refers to number of cristae analyzed:
991 wild-type = 103, Opa1-OE = 33, l-Opa1* = 107, s-Opa1* = 92, Opa1-KO = 34. Scale bar = 100 nm.
992 Inset scale bar = 25 nm.
993
994 **Fig. 4 | l-Opa1* cells show WT apoptotic priming. (a)** BH3 profiling of MEF lines with BIM BH3
995 peptide at indicated concentrations compared with positive control DFNA5 and negative control
996 PUMA2A. N = 3-4 biological replicates (see methods for description). Significance of difference is
997 tested relative to wild-type using the Holm-Sidak's multiple comparison test; *p<0.05, **p<0.01,
998 ***p<0.001, ****p<0.001. **(b)** MEF lines were treated with indicated agents for 24h and apoptosis was
999 detected via flow cytometry after staining with Annexin V. N = minimum 4 biological replicates. **(c)**
1000 Representative traces of mitochondrial calcium retention capacity assays done in indicated MEF lines.
1001 **(d)** Quantification of $CaCl_2$ concentration required to induce mPTP opening in (c). N = 3 biological
1002 replicates.
1003
1004
1005 **Fig. 5 | Defects to mitochondrial functions are observed in cells with altered Opa1 levels. (a)**
1006 OCR plotted against time with the addition of each compound indicated by an arrow for oligomycin
1007 (2μM), FCCP (1 μM), and rotenone/antimycin A (0.5μM). **(b)** Aspects of mitochondrial respiration; basal
1008 respiration rates, the amount of respiration used for ATP production, maximum respiration and spare
1009 capacity, are extracted by the data plotted in (a). N = 3 biological replicates. Significance of difference
1010 is tested relative to WT using Welch's t-test; *p<0.05, **p<0.01, ***p<0.001.
1011
1012 **Fig. 6 | Summary of cryo-ET cristae observations.** Cartoon schematic summarizing mitochondrial
1013 morphological observations dependent on Opa1 state. Mitochondria in l-Opa1* cells displayed WT-like
1014 CJ widths and perpendicularity. The majority of mitochondria displayed a stacking phenotype and multi-
1015 junction cristae. Tubular and vesicular cristae were found in s-Opa1* cells. Summaries of functional
1016 dysfunctions corresponding to observed cristae morphological changes. (a) Respiratory defects
1017 correlate with a large number of unstructured cristae. (b) Defects in cytochrome c (CytC) release
1018 properties (as evaluated by BH3 profiling) and calcium handling correlate with wider CJ and vesicular
1019 cristae. Cartoon schemes with hypothesized modes of action for l-Opa1 in the (c) crista stacking, and
1020 (d) BH3 and calcium handling process.
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1027 **Supplementary Figure S1 | Presence of Opa1 forms per MEF cell line. (a)** (Top) Western blot
1028 detection of Opa1 forms in indicated MEF cell lines using Opa1 antibody. (Bottom) Actin was used as
1029 loading control. **(c)** Genetic schematic and cartoon depictions of Opa1 forms present in MEF cell lines
1030 used in this study.
1031
1032 **Supplementary Figure S2 | Gallery of cryo-ET data. (a)** Summed, projected central slices of cryo-
1033 electron tomograms visualizing mitochondria in wild-type, Opa1-OE, l-Opa1*, s-Opa1* and Opa1-KO
1034 MEF. White arrowheads indicate calcium deposits, blue arrowheads indicate ellipsoidal mitochondria
1035 and purple arrowheads indicate round mitochondria. **(b)** Mitochondria size (μm^2) broken down by shape
1036 per cell line. Scatter plots show data distribution, the mean is shown by a bold black line. Significance
1037 of difference is tested relative to wild type using Mann Whitney; ****$p<0.0001$. For b: N refers to number
1038 of mitochondria: wild-type = 57, Opa1-OE = 17, l-Opa1* = 39, s-Opa1* = 55, Opa1-KO = 12. Scale bar
1039 = 200 nm.
1040
1041 **Supplementary Figure S3 | Mitochondrial subcompartment volumes. (a)** Three-dimensional
1042 renderings of segmented inter-membrane space (IMS, pink surface), cristae lumen (CL, magenta
1043 surface), and matrix (translucent grey surface) volumes. **(b)** Total mitochondrial volume across
1044 indicated cell lines. **(c)** Quantification of IMS volume, **(d)** CL volume and **(e)** matrix volume relative to
1045 total volume of each mitochondrion indicated in (b). **(f)** CL to matrix ratio and **(g)** normalized grey scale
1046 mitochondrial matrix value across cell lines. (**h**) Graph bar representing percentage of cells with
1047 detected calcium deposits in cryo-electron tomograms. Scatter plots show data distribution, the mean is
1048 shown by a bold black line. Significance of difference is tested relative to wild type using Mann Whitney
1049 test in b, d, e, g; *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$; and unpaired t test in (c): **$p<0.01$; N
1050 refers to number of cells, for b-g: N = 5 for all cell lines. For h: N refers to the number of mitochondria:
1051 wild-type = 57, Opa1-OE = 17, l-Opa1* = 39, s-Opa1* = 55, Opa1-KO =12. Scale bar = 200 nm.
1052
1053 **Supplementary Figure S4 | Cristae analysis. (a)** Cristae density (cristae per μm^2) and **(b)** Number of
1054 cristae per mitochondria represented as scatter plots. **(c)** (Top) Summed, projected central slices of
1055 cryo-electron tomograms visualizing mitochondria with stacking crista characteristics, supported by 3D
1056 representations consisting of their sub compartments (bottom) in indicated MEF lines. **(d)** Graph bar
1057 representing percentage of mitochondria with stacking crista formation in each MEF line. For a: N refers
1058 to number of cells, N: wild-type = 33, Opa1-OE = 7, l-Opa1* = 21, s-Opa1* = 28, Opa1-KO = 11. For b:
1059 N wild-type = 51, Opa1-OE = 17, l-Opa1* = 39, s-Opa1* = 55, Opa1-KO = 12. For (c) and (d): N wild-
1060 type = 57, Opa1-OE = 17, l-Opa1* = 39, s-Opa1* = 55, Opa1-KO = 12. Scale bar = 200 nm.

1061
1062 **Supplementary Figure S5 | Mitochondrial network morphology in MEF lines by fluorescence**
1063 **microscopy.** Representative images of mitochondrial morphology in indicated MEF lines labeled with
1064 MitoTracker™ Deep Red FM. Insets show magnified view of regions indicated with dashed boxes.
1065 Scale bar = 10 µm. Inset scale bar = 5 µm. (**b**) Graph bar representing mitochondrial network
1066 morphology scored in indicated MEF lines. N = 100 cells analyzed per cell line.
1067
1068 **Supplementary Figure S6 | Unusual cristae morphology. (a)** Graph bar representing the relative
1069 proportion of unusual cristae morphology observed in indicated MEF lines. Unusual cristae were
1070 categorized into vesicular, zipped, ring, split, amorphous, straight-across, pinched and loop. N refers to
1071 number of cristae analyzed, N: wild-type = 222, Opa1-OE = 430, l-Opa1* = 323, s-Opa1* = 653, Opa1-
1072 KO = 243. **(b)** Summed, projected central slices of cryo-electron tomograms showing examples of
1073 unusual cristae in mitochondria across cell lines in 2D (top) and 3D (bottom). Loop, ring, straight-
1074 across, pinched, vesicular, and amorphous cristae are shown. Scale bar = 200 nm.
1075
1076 **Supplementary Figure S7 | Cristae length, width quantification, junction width, angle.**
1077 **(a)** Cartoon schematics representing sub-tomogram averaging (STA) approach for measuring crista
1078 length and **(b)** width in 3D. **(c)** Cartoon schematic for measurement of cristae junction width and **(d)**
1079 angle. See Methods for details.
1080
1081 **Supplementary Figure S8** | **Multijunction cristae. (a)** Scatter plot showing the percentage of
1082 multijunction cristae per mitochondrion in indicated MEF lines. **(b)** Graph bar representing percentage
1083 of multijunction cristae categorized into straight-across and loop morphology in each MEF line. Scatter
1084 plot shows data distribution, the mean is marked by a bold black line. Significance of difference is
1085 tested relative to wild type using Mann Whitney; ****$p<0.0001$. N refers to number of cristae, for (a), N:
1086 WT = 18, Opa1-OE =5, l-Opa1* = 30, s-Opa1* = 16, Opa1-KO = 3. For (b), N: WT = 26, Opa1-OE = 9,
1087 l-Opa1* = 79, s-Opa1* = 29, Opa1-KO = 4.
1088
1089 **Supplementary Figure S9** | **Cell viability following apoptotic priming.** Assessment of cell viability
1090 by Annexin V staining in MEF cell lines after treatment with the indicated compounds for **(a)** 48 hours
1091 and **(b)** 72 hours. N = minimum 4 biological replicates.
1092
1093 **Supplementary Figure S10** | **Oma1$^{-/-}$ cell functional characterization. (a)** BH3 profiling of WT and
1094 Oma1$^{-/-}$ MEF for sensitizer BIM BH3 and PUMA. N = 3 biological replicates. **(b)** Representative traces
1095 of mitochondrial calcium retention capacity assays done in indicated MEF lines. **(c)** OCR plotted
1096 against time for indicated MEF lines. **(d)** Aspects of mitochondrial respiration; basal respiration rates,
1097 the amount of respiration used for ATP production, maximum respiration, and spare capacity, are

1098 extracted by the data plotted in (c). N = 3 biological replicates. Significance of difference between l-
1099 Opa1* and Oma1$^{-/-}$ is tested using Welch's t-test; *p<0.05, **p<0.01, ***p<0.001.
1100
1101 **Supplementary Figure S11 | mtDNA maintenance characterization. (a)** Representative live iSIM
1102 images of mitochondrial network (PKmito Orange, in green) and nucleoid signal, (SYBR Gold, in
1103 magenta). **(b)** Quantification of mean nucleoid area and **(c)** total nucleoid number per cell, normalized
1104 to mitochondrial area and relative to the experimental controls (median of WT cells imaged on the same
1105 day). Significance of difference is tested relative to wild type using Mann Whitney; *p<0.05, **p<0.01,
1106 ***p<0.001, ****p<0.0001. N refers to the number of quantified cells per MEF line, WT = 83, Opa1-OE
1107 =51, l-Opa1* = 31, s-Opa1* = 55, Opa1-KO = 58. **(d)** qPCR-based determination of mtDNA (RNR2 and
1108 ND1 probes) copy number relative to nuclear genome copies (HK2 probe), normalized to WT cells.
1109 Significance of difference is tested relative to wild type using Welch's t-test; **p<0.001. N refers to 3
1110 biological replicates. Scale bar = 10 μm.
1111

1112 **Supplementary Table 1 | Summary of data acquisition and image processing for cryo-ET data in**
1113 **this study.**

Sample		wt	Opa1-OE	l-Opa1*	s-Opa1*	Opa1-KO
Cryo-FIB milling	**Microscope**	Aquilos Cryo-FIB, FEI – Thermo Fisher Scientific	Aquilos Cryo-FIB, FEI – Thermo Fisher Scientific	Aquilos Cryo-FIB, FEI – Thermo Fisher Scientific	Aquilos Cryo-FIB, FEI – Thermo Fisher Scientific	Aquilos Cryo-FIB, FEI – Thermo Fisher Scientific
Acquisition settings	**Microscope**	Titan Krios Gi3 FEI, Thermo Fisher Scientific	Titan Krios Gi3 FEI, Thermo Fisher Scientific	Titan Krios Gi3 FEI, Thermo Fisher Scientific	Titan Krios Gi3 FEI, Thermo Fisher Scientific	Titan Krios Gi3 FEI, Thermo Fisher Scientific
	Voltage (KeV)	300	300	300	300	300
	Detector	Gatan K3 IS	Gatan K3 IS	Gatan K3 IS	Gatan K3 IS	Gatan K3 IS
	Energy filter	Gatan BioQuantum K3	Gatan BioQuantum K3	Gatan BioQuantum K3	Gatan BioQuantum K3	Gatan BioQuantum K3
	Slit width (eV)	20	20	20	20	20
	Super-resolution mode	Yes	Yes	Yes	Yes	Yes
	Å/pixel	2.076	2.076	2.076	2.076	2.076
	Defocus (µm)	-3.5 to -5.0	-3.5 to -5.0	-3.5 to -5.0	-3.5 to -5.0	-3.5 to -5.0
	Acquisition scheme	-70/70, 2°, Dose-symmetric	-70/70, 2°, Dose-symmetric	-70/70, 2°, Dose-symmetric	-70/70, 2°, Dose-symmetric	-70/70, 2°, Dose-symmetric
	Total dose	~90 - 120	~90 - 180	~90 - 180	~90 - 120	~90 - 120
	Dose rate (e-/Å/sec)	~ 2.5 – 3.5	~ 2.5 – 3.5	~ 2.5 – 3.5	~ 2.5 – 3.5	~ 2.5 – 3.5
	Frame number	6	6	6	6	6
	Number of tomograms	33	7	27	22	11
Image processing	**Frame alignment and dose weighting**	*framealign*, IMOD	*framealign*, IMOD	*framealign*, IMOD	*framealign,* IMOD	*framealign,* IMOD
	Tilt series alignment	IMOD	IMOD	IMOD	IMOD	IMOD
	WBP	IMOD	IMOD	IMOD	IMOD	IMOD
	Denoising	Topaz	Topaz	Topaz	Topaz	Topaz
	3D-segmentation	Amira	Amira	Amira	Amira	Amira
	3D-rendering	Amira	Amira	Amira	Amira	Amira

1114

1115 **Movie 1:** 3D renderings of WT mitochondrial membranes (OMM in green and IMM in yellow) and
1116 subcompartments (IMS in pink and CL in magenta) on tomogram Z slices in XY orientation. Scale bar =
1117 200 nm.
1118
1119 **Movie 2:** 3D renderings of Opa1-OE mitochondrial membranes (OMM in green and IMM in yellow) and
1120 subcompartments (IMS in pink and CL in magenta) on tomogram Z slices in XY orientation. Scale bar =
1121 200 nm.
1122
1123 **Movie 3:** 3D renderings of l-Opa1* mitochondrial membranes (OMM in green and IMM in yellow) and
1124 subcompartments (IMS in pink and CL in magenta) on tomogram Z slices in XY orientation. Scale bar =
1125 200 nm.
1126
1127 **Movie 4:** 3D renderings of s-Opa1* mitochondrial membranes (OMM in green and IMM in yellow) and
1128 subcompartments (IMS in pink and CL in magenta) on tomogram Z slices in XY orientation. Scale bar =
1129 200 nm.
1130
1131 **Movie 5:** 3D renderings of Opa1-KO mitochondrial membranes (OMM in green and IMM in yellow) and
1132 subcompartments (IMS in pink and CL in magenta) on tomogram Z slices in XY orientation. Scale bar =
1133 200 nm.
1134
1135 **Movie 6:** Live-cell fluorescence microscopy of MitoTracker™ Deep Red FM-stained mitochondria in
1136 indicated MEF cell lines. Movies were taken at 30 seconds per frame for 5 mins. Playback at 2 frames
1137 per second (60x real-time). Scale bar = 10 μm.
1138