A Mitofusin-2–dependent inactivating cleavage of Opa1 links changes in mitochondria cristae and ER contacts in the postprandial liver

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Hepatic metabolism requires mitochondria to adapt their bioenergetic and biosynthetic output to accompany the ever-changing anabolic/catabolic state of the liver cell, but the wiring of this process is still largely unknown. Using a postprandial mouse liver model and quantitative cryo-EM analysis, we show that when the hepatic mammalian target of rapamycin complex 1 (mTORC1) signaling pathway disengages, the mitochondria network fragments, cristae density drops by 30%, and mitochondrial respiratory capacity decreases by 20%. Instead, mitochondria–ER contacts (MERCs), which mediate calcium and phospholipid fluxes between these organelles, double in length. These events are associated with the transient expression of two previously unidentified C-terminal fragments (CTFs) of Optic atrophy 1 (Opa1), a mitochondrial GTPase that regulates cristae biogenesis and mitochondria dynamics. Expression of Opa1 CTFs in the intermembrane space has no effect on mitochondria morphology, supporting a model in which they are intermediates of an Opa1 degradation program. Using anti in vitro assay, we show that these CTFs indeed originate from the cleavage of Opa1 at two evolutionarily conserved consensus sites that map within critical folds of the GTPase. This processing of Opa1, termed C-cleavage, is mediated by the activity of a cysteine protease whose activity is independent from that of Oma1 and presenilin-associated rhomboid-like (PARL), two known Opa1 regulators. However, C-cleavage requires Mitofusin-2 (Mfn2), a key factor in mitochondria–ER tethering, thereby linking cristae remodeling to MERC assembly. Thus, in vivo, mitochondria adapt to metabolic shifts through the parallel remodeling of the cristae and of the MERCs via a mechanism that degrades Opa1 in an Mfn2-dependent pathway.

The last decade expanded our understanding of the importance of mitochondrial shape, position, and interorganelle interactions in the regulation of cell stress. For example, mitochondrial hyperfusion is a stress response that protects against cell death and autophagic degradation, whereas chronic stress triggers mitochondrial fragmentation and cell death. However, the in vivo implications of mitochondrial plasticity under normal physiologic conditions are still largely unknown. The liver is a key organ responsible for nutrient sensing and the maintenance of whole-body energy homeostasis. Therefore, we considered the liver as a primary model to examine the changes in mitochondrial plasticity that accompanies physiological transitions in feeding and postprandial metabolism (1–4).

The mechanistic target of rapamycin complex 1 (mTORC1) is an evolutionary conserved serine/threonine kinase that plays an important role in regulating metabolism and cell growth in response to anabolic signals (5). Studies indicate that mTORC1, which is activated by growth factors and amino acids, is a key sensor allowing cells and tissues to adapt their metabolism in response to the nutritional state (5). In the liver, it controls the activation of various metabolic processes including lipogenesis (6) and ketogenesis (3). Recent observations indicate that mTORC1 regulates mitochondrial biogenesis and metabolism (7, 8), but the underlying mechanisms remain to be determined.

It has been established that the core machinery that governs mitochondrial shape and ultrastructure is essential. Indeed, genetic ablation of its components, which includes the outer mitochondrial membrane (OMM) fusion GTPase Mitofusin-1 (Mfn1) and Mfn2 (9) as well as the inner mitochondrial membrane (IMM) GTPase Optic atrophy 1 (Opa1) (10), is embryonic lethal. Similarly, the loss of the core fission GTPase Drp1 is also lethal (11). Tissue-specific deletions of these genes, including the liver (12, 13), are now emerging and lead to more complex phenotypes (14); however, these models will not inform us on the adaptive mitochondrial response to metabolic changes.

Mitochondria cristae shape changes from the “orthodox” state, when oxidative phosphorylation is low, to a more “condensed” form in high respiratory conditions. The mechanisms that regulate these changes have emerged in cultured models (15), but have not been followed within tissues in vivo. The inner membrane GTPase Opa1 has been shown to regulate mitochondrial fusion and cristae architecture (16), where loss of Opa1 in cultured mouse embryonic or adult fibroblast cells compromised the function and assembly of the respiratory chain complexes. Further, recent studies have shown that Opa1 ablation in the liver leads to hepatic metabolism requiring mitochondria to adapt their bioenergetic and biosynthetic output to accompany the ever-changing anabolic/catabolic state of the liver cell, but the wiring of this process is still largely unknown. Using a postprandial mouse liver model and quantitative cryo-EM analysis, we show that when the hepatic mammalian target of rapamycin complex 1 (mTORC1) signaling pathway disengages, the mitochondria network fragments, cristae density drops by 30%, and mitochondrial respiratory capacity decreases by 20%. Instead, mitochondria–ER contacts (MERCs), which mediate calcium and phospholipid fluxes between these organelles, double in length. These events are associated with the transient expression of two previously unidentified C-terminal fragments (CTFs) of Optic atrophy 1 (Opa1), a mitochondrial GTPase that regulates cristae biogenesis and mitochondria dynamics. Expression of Opa1 CTFs in the intermembrane space has no effect on mitochondria morphology, supporting a model in which they are intermediates of an Opa1 degradation program. Using an in vitro assay, we show that these CTFs indeed originate from the cleavage of Opa1 at two evolutionarily conserved consensus sites that map within critical folds of the GTPase. This processing of Opa1, termed C-cleavage, is mediated by the activity of a cysteine protease whose activity is independent from that of Oma1 and presenilin-associated rhomboid-like (PARL), two known Opa1 regulators. However, C-cleavage requires Mitofusin-2 (Mfn2), a key factor in mitochondria–ER tethering, thereby linking cristae remodeling to MERC assembly. Thus, in vivo, mitochondria adapt to metabolic shifts through the parallel remodeling of the cristae and of the MERCs via a mechanism that degrades Opa1 in an Mfn2-dependent pathway.

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a loss of cristae (13). However, Opa1 function is complex and can also promote mitochondrial fragmentation (17). Upon mitochondrial dysfunction or depolarization, an inner membrane protease called Oma1 becomes activated, cleaving Opa1 into a short, soluble form that then promotes mitochondrial fragmentation (18, 19). Interestingly, mice lacking Oma1 are not embryonic lethal; rather, they show a metabolic phenotype where they become obese, accompanied by hepatic steatosis when placed on a high fat diet (20). This result is of critical importance because it hints at a major role of Opa1 proteolysis in metabolic adaptation and connects it to the emerging notion that central regulators of mitochondria—ER tethering, like Mfn2, might also be central to this process (12). To shed light on this possibility, in this study we have used an in vivo mouse model to link changes in the structure of the mitochondria–ER contacts (MERCs) to that of the cristae. Our findings support a model where mitochondria adapt to the postprandial loss of mTORC1 signaling by activating an Mfn2-dependent degradation program of Opa1. Thus, mitochondria adapt to postprandial metabolic transitions by coupling the machineries that organize cristae architecture and MERC assembly, which were previously thought to operate independently of each other.

**Results**

**Postprandial Loss of Hepatic mTORC1 Signaling Is Accompanied by Mitochondrial Fragmentation, Loss of Cristae, and Expansion of the MERCs.** The period between meals is called the postprandial state. Postprandial mouse models are widely accepted to study in vivo hepatic mTORC1 signaling (1–4); in this tissue, mTORC1 is active when the ribosomal protein S6 is phosphorylated at residues Ser240/244 (hereby referred as phospho-S6) and is inactive when these two amino acids are not phosphorylated (3). For this study, we developed a postprandial protocol (Fig. 1A and SI4) where hepatic phospho-S6 typically drops between 4 and 5 h postprandial (Fig. 1B and C) without, however, leading to the activation of the autophagy program (Fig. S2D).

We used this postprandial model to assess in vivo whether mitochondrial mass and morphology change during the metabolic shift that culminates in loss of hepatic phospho-S6. To stratify the samples based on the state of mTORC1 activity, we performed cryo-EM analysis on the livers of two groups of mice (Fig. 1D, n = 14 per group). In the first group, the animals were killed at 2 h postprandial and had high hepatic phospho-S6; in the second group, the animals were killed at 5 h and had low hepatic phospho-S6 (Fig. 1B and Fig. S2A).

In these livers, the total number of mitochondria was calculated as the average sum of organelles present in cryo-EM images of 660 μm² each (Fig. 1D; n = 14 per group). We found that at 5 h the mitochondrial population increases by ~25% (120 vs. 152 per image of 660 μm²; P < 0.05; Fig. 1E). The total mitochondrial area does not change between the two groups of animals (Fig. 1F, Left); however, at 5 h postprandial, the area of the individual mitochondrion decreases by ~10% (0.42 vs. 0.38 μm²;  P < 0.001; Fig. 1F, Right, and Fig. S2B). This change is due to a shift in mitochondria size distribution toward rounder (Fig. 1G), shorter organelles (Fig. 1H), thereby indicating the occurrence of mitochondrial fragmentation. Noteworthy, with respect to the size class distribution of the organelles, at 5 h postprandial, intermediate levels of hepatic phospho-S6 (Fig. S2E) result in an intermediate mitochondrial phenotype (Fig. 1H), supporting a model where mitochondria morphology remodels in parallel to the metabolic transition linked to the loss of mTORC1 signaling.

In a subset of mitochondria analyzed above (n = 54), we then measured the number, density, and length of the cristae (Fig. 2A). Data showed that at 5 h postprandial cristae density drops ~30% (5.2 vs. 3.7 μm²/μm;  P < 0.001; Fig. 2B). This corresponds to a similar decrease in the number of cristae (41 vs. 28 μm²/μm; P < 0.001; Fig. 2C), but not to a change in the average length of each cristae (Fig. S2C), indicating a loss in cristae number rather than reduced cristae extension. Consistent with this finding, we found that mitochondria isolated from livers at 5 h postprandial and energized with pyruvate/maltate have ~20% less state 3 respiration than at 2 h postprandial (95 vs. 78 pmol/min/μg;  P < 0.05). However, basal respiration remained similar (Fig. 2D), indicating that the loss of cristae that accompanies the disengagement of the mTORC1 pathway reduces the overall respiratory capacity of the organelle. Noteworthy, at 2 and 5 h postprandial, liver mitochondria energized with succinate did not show a significant difference in state 3 respiration, a finding that is consistent with a recent study reporting that Opa1-mediated changes in cristae architecture do not affect respiration driven by this substrate (13).

In the same liver cryo-EM samples in which we performed the cristae analysis, we then analyzed MERC number, size, and mitochondrial surface coverage. Here, MERCs were manually
Fig. 2. Postprandial loss of mTORC1 signaling is accompanied by decreased cristae density and respiration and augmented MERC length. (A) Representative image and analysis of liver mitochondria used to study cristae number and density. (B and C) Effect of the change in phospho-S6 level on mitochondria cristae number and density. (D) Oxygen consumption rate (OCR) of liver mitochondria energized with 10 mM pyruvate + 2 mM malate. Data represent mean ± SEM of five independent experiments. (E) Representative image of the MERCs analyzed in this study. Arrows indicate the two extremities of the MERC, which are defined as the structures where the cytosolic side of the OMM interfaces the ribosome-free ER membrane with a gap of 9–15 nm between these two membranes (21). (F–I) Effect of the change in phospho-S6 level on MERC number, size density, and mitochondria coverage. Data represent mean ± SEM. *P < 0.05; ***P < 0.001.

identified according to two well-established criteria: being ribosome-free and having a gap of 9–15 nm between the OMM and the ER (21) (Fig. 2E). Data showed that the level of hepatic phospho-S6 does not impact the number of MERCs per mitochondria (MERC density, 1 in 4 mitochondria had one MERC; Fig. 2F). However, with low phospho-S6, the average MERC length nearly doubles (145 ± 18 nm; Fig. 2G) due to a sweeping increase in the length of each MERC (Fig. 2H). As a result, the percentage of the mitochondrial perimeter covered by MERCs increases by 140%, shifting from 4.7% to 11.2% (P < 0.001; Fig. 2F).

To corroborate the role of the mTORC1 signaling in MERC remodeling, we next investigated the length of the MERCs in the livers of four mice that, at 5 h postprandial, showed an intermediate level of phospho-S6 signal (Fig. S2E). In these samples, the average MERC length was in between that observed with a high and low phospho-S6 signal, respectively (Fig. 2G and H). All together these findings indicate that mitochondria adapt to changes in nutrient availability through an extensive and parallel remodeling of the cristae and of the MERCs.

Expression of Opa1 CTF-1 and CTF-2 upon Loss of mTORC1 Signaling. Given the role of Opa1 in cristae biogenesis and remodeling (13, 16), we investigated the pattern of expression of the GTPase in the livers analyzed by cryo-EM at 2 and 5 h postprandial. In these samples, Opa1 appears to remain substantially unchanged (Fig. S2D). This observation raised the possibility that transient changes in Opa1 expression might have occurred before the drop in cristae density that accompanies the postprandial inhibition of the hepatic mTORC1 pathway (Fig. 2B). To address this possibility, we investigated Opa1 in the liver of the large cohort of animals on which we previously performed the phospho-S6 analysis (Fig. 1C).

To this goal, we used a panel of anti-Opa1 antibodies that included a well-established monoclonal antibody (13, 19, 22) whose epitope maps within amino acid 708–830 of the protein.

Data showed two new forms of Opa1 in seven of the 25 mice with low or no hepatic phospho-S6 at 5 h postprandial (Fig. 3A and B); we named them C-terminal fragment-1 and -2, respectively (CTF-1, ~78 kDa; CTF-2, ~48 kDa). Importantly, Opa1 CTFs were never observed in the livers of mice with high phospho-S6, whether at 2, 4, or 5 h postprandial (62 animals, in total; Figs. 1C and 3B). These findings suggested that Opa1 CTFs expression is transient and linked to the inhibition of the mTORC1 pathway. To test this possibility, we turned to a cultured model of mouse embryonic fibroblasts (MEFs) in which we pharmacologically inhibited mTORC1 with rapamycin. Data showed the transient appearance of Opa1 CTF-1 10 mins after rapamycin started to reduce the phospho-S6 signal (Fig. 3C), thereby linking the expression of these forms of Opa1 to mTORC1 inhibition.

In the mouse liver, only four Opa1 mRNAs are expressed, none of which encodes CTF-1 or -2 (23). Further, analysis of the murine genomic and EST databases did not show evidence of cryptic promoter(s) and alternative splicing sites that could
justify the expression of these forms of Opa1. We conclude that CTF-1 and -2 derive from proteolytic processing of Opa1, which we name C-cleavage.

Opa1 C-cleavage could be reconstituted in vitro using an assay that consisted of incubating crude mitochondria with 0.5–1% of either digitonin (DIG) or n-Dodecyl-β-D-Maltoside (DDM), two mild nonionic detergents that are also used to permeabilize mitochondria and isolate active IMM-bound ATP synthase complexes. Here, Opa1 proteolysis occurs within a few minutes at low temperature (Fig. S3A), yielding fragments that have the same electrophoretic mobility of endogenous liver and rapamycin-stimulated CTFs (Fig. 4A and B). Interestingly, whereas DIG mostly yields CTF-1, DDM preferentially generates CTF-2 (Fig. 4A); the reason for this, however, is unclear. DDM also yields a low amount of a smaller ~44-kDa Opa1 fragment that, however, was never detected in vivo and is, therefore, unspecific (Fig. 4A). Importantly, the appearance of CTF-1 and -2 is accompanied by the loss of long and short forms of endogenous Opa1 (Fig. S3A and B), indicating that, in vitro, C-cleavage occurs via a high-affinity proteolytic reaction that attacks all forms of the GTPase. This includes the short-Opa1 (s-Opa1) form that is generated following carbonyl cyanide m-chlorophenyl hydrazone (CCCP)-induced loss of membrane potential by Oma1 (Fig. S3B).

To screen a large number of protease inhibitors over a broad range of concentrations. Data showed that classical aspartyl-, metallo-, and serine-protease inhibitors do not affect Opa1 C-cleavage; instead, cysteine protease inhibitors like E64 (24), calpeptin, leupeptin, and chymostatin blocked the generation of CTF-1 and -2 (Fig. 4C and Fig. S3 C and D). The cell-permeable inhibitor E64d also blocked the generation of CTF-1 induced by rapamycin in MEFs (Fig. S3 F and G). We conclude that Opa1 C-cleavage is mediated by a cysteine protease. Noteworthy, Opa1 C-cleavage remains unaffected by genetic ablation of the Opa1 proteases that actuate the role of the GTPase in mitochondria cristae and morphology remodeling (25) (Fig. 4E and Fig. S4A). Similarly, silencing of Calpain-1 (CAPN1), which previous studies have claimed to be localized in the intermembrane space (IMS) (26, 27), does not affect the generation of Opa1 CTFs (Fig. 4D). Thus, Opa1 C-cleavage is mediated by a still unknown cysteine protease whose activity is independent from that of major Opa1-actuating proteases.

We named C1 and C2 the sites that generate CTF-1 and CTF-2, respectively. To locate them, we used an epitope mapping
approach in combination with the in vitro C-cleavage assay on mitochondria prepared from cells expressing Opal1 with an HA tag inserted at position 568 and a Flag tag at the C terminus (Opal1-HA-568 Flag-CT; Fig. 5A). Altogether, data revealed that the C1 site maps downstream of the HA-568 tag and upstream of the Flag tag (Fig. 5A); instead, the C2 site maps upstream of the HA-568 tag (Fig. 5B). Additional mutagenesis analysis identified the C2 cleavage site to be located in proximity of residue 545. Indeed, deletion of residues 340-540 (Fig. S5A) but not of its neighboring sequences (530NSKLL534, 535KAHQV539, 545SLAVS549), abolished the in vitro generation of CTF-2 (Fig. 5E). Using this information we performed an in silico analysis to identify a potential C1-cleavage site in the C-terminal region of Opal1. Data showed the existence of a conserved potential consensus sequence at position 885 (885RMHSL587; Fig. 5D). Deletion of the residues encompassing this potential C1-cleavage site (885RMLAIA891) eliminated CTF-1 generation (Fig. S4B), supporting a role for the RxL consensus sequence in Opal1 C-cleavage.

Cleavage at either the C1 or C2 site is bound to inactivate the dynamin-like activity of Opal1 because it dissociates from the GTPase domain in the middle domain as well as the GTPase-effector domain (Fig. 5C). To validate this analysis, we developed constructs that express in the IMS CTF-1 (Fig. S4 C–E) and CTF-2 (Fig. S5 A and B) and performed a quantitative imaging analysis of the mitochondrial network. Data showed that overexpression of either construct in COS7 and HeLa cells had no effect on mitochondrial morphology (Fig. 5F and Fig. S5C). Thus, C-cleavage inactivates Opal1 without generating fragments that interfere with mitochondrial dynamics. Whether this processing is conserved in evolution remains unknown.

**Opal1 C-Cleavage Requires Mfn2 but Not Mfn1.** The parallel remodeling of the cristae and of the MERCs at postprandial 5 h (Fig. 2B and G) hints at the possibility that Opal1 elimination via C-cleavage might be coupled to the activity of the mitochondria-ER tethering GTPase Mfn2. To address this possibility, we challenged Mfn2−/− or Mfn1−/− MEFs with either 100 or 200 nM of rapamycin over an extended period (up to 80 min). Data showed that, unlike wild-type MEFs, Mfn2−/− cells do not respond to this TORC inhibitor by expressing CTF-1 (Fig. 5D and Fig. S6A). To further support this observation, we returned to the in vitro C-cleavage assay on crude mitochondria isolated from Mfn1−/− and Mfn2−/− MEFs. Results showed that genetic ablation of Mfn2, but not of Mfn1, markedly impairs CTF-1 and -2 generation (Fig. 4F and Fig. S6B). These findings are consistent with recent studies showing that liver-specific ablation of Mfn2 in mice disrupts hepatic metabolism (12) and correlate with the data showing a parallel *crisustae* and MERC remodeling during the postprandial metabolic shift.

**Discussion**

In this study, we investigated the adaptive responses of the mitochondrial reticulum to the metabolic changes that occur in the liver in the hours that follow feeding. More specifically, we investigated how loss of hepatic mTORC1 signaling during the postprandial state relates to mitochondria morphology, *crisustae* structure, and MERC remodeling. To our knowledge, this is the first comparative

![Diagram](image-url)

**Fig. 5.** Opal1 C-cleavage destroys Opal1 integrity and produces fragments that are not competent for mitochondrial dynamics. (A and B) Epitope mapping of the Opal1 C-cleavage site that yields CTF-1 (C1 site) and CTF-2 (C2 site). The diagrams depict the epitope organization of the double-tagged Opal1 protein and the predicted products of its cleavage at the C1 and C2 site. (C) Top) Sequence homology of the putative C1 and C2 cleavage sites in Opal1. (Bottom) Diagram depicting the protein domain organization of Opal1 and the location where all of the known sites of Opal1 proteolysis occur. (D) Evolutionary conservation of the putative C-cleavage consensus sequences in Opal1 homologs. (E) Deletion of five amino acids encompassing the putative C2 cleavage site, 540TRNL544, blocks the generation of CTF-2. (F) Expression of either CTF-1-Flag or CTF-2-Flag in the IMS of COS7 cells does not affect mitochondria morphology.
quantitative description of the ultrastructural changes that occur to mitochondria in vivo under normal physiological conditions.

Loss of hepatic mTORC1 activity in the postprandial liver is majorly associated with a fragmentation of the mitochondrial reticulum and to a loss of cristae density and respiratory capacity. These major changes are associated with cleavage of Opa1, the major regulator of mitochondria cristae biogenesis and a critical component of the mitochondrial fusion machinery of the organelle. The underlying processing eliminates Opa1 from the organelle through the activation of a still unknown cysteine protease that operates downstream of presenilin-associated rhomboid-like (PARL) and Oma1. The raison d'être of this not unforeseeable degradation program within the broad context of the postprandial metabolism remains unclear. However, because at 5 h postprandial Opa1 levels remain unchanged (Fig. S2D), newly imported Opa1 could replace the fraction that, through mTORC1 inactivation, is eliminated.

Mitochondria Respiration Assay. Respiration measurements on isolated liver mitochondria were performed as described (28) using the XF24 Extracellular Flux Analyzer (Seahorse Bioscience).

In vitro, Opa1 C-cleavage does not require the known Opa1 proteases PARL and Oma1, but depends on the expression of Mfn2. This latter result is unexpected but important because it connects for the first time the machineries that govern the structure of the cristae and of the MERCs. Notably, this finding parallels the observation that loss of cristae density accompanies a large increase in MERC length and is consistent with the report that liver-specific ablation of Mfn2 in mice leads to numerous metabolic abnormalities (12). It would appear, therefore, that mitochondria adapt to metabolic transitions by remodeling their cristae architecture through a mechanism that is linked to MERC assembly. It is tempting to speculate that the loss of the cristae could allow the IMM to distort and juxtapose under the MERC, to allow efficient calcium, lipid, and metabolite transfer across both mitochondrial membranes.

Materials and Methods

Postprandial Protocol. Male C57BL6 mice 9–12 wk in age (~18 g) were trained to handling and to the postprandial protocol described here to minimize variability linked to stress and food consumption. Mice were left unfed for 12 h (8:00 PM–8:00 AM). In the morning, they were individually transferred to an empty cage and given 1.9 kcal of normal chow (~20% of daily caloric intake) for 1 h. After feeding, the animals were moved to a clean cage (with bedding) for the time indicated in the experiment. Liver biopsy and/or harvest followed. The animal committee of the Université Laval approved these studies.

Cryo-EM and Statistical Analysis. A description of the cryo-EM protocols is provided in SI Materials and Methods. Low-magnification EM images of the liver samples each measured 660 μm². On these images, every mitochondria and cristae was identified manually, and the perimeter was outlined with an optical pen using the ImageJ software. The shape of each mitochondria was defined by the axis ratio of the oval that best fits the area of the organelle (ImageJ software). Cristae and MERCs were identified manually from high-resolution, high-magnification cryo-EM images, and they were outlined using an optical pen in ImageJ to calculate their length. Data are expressed as means ± SEM. The statistical significance of differences was assessed with a one- or two-sample Student t test (for Fig. 3B the χ² test was used). For the cumulative probability plots, the Kolmogorov-Smirnov test was used to determine if the values between conditions were significantly different.

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Supporting Information

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SI Materials and Methods

Immunofluorescence and Mitochondrial Morphology Analysis. Cos-7 and HeLa cells grown on glass coverslips in a 24-well dish were transfected with the appropriate constructs with Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). Twenty-four hours later, cells were fixed with 5% (vol/vol) prewarmed paraformaldehyde in PBS for 15 min at 37 °C. After fixation, cells were washed three times with PBS, and autofluorescence was quenched by incubation with 50 mM NH4Cl for 15 min. After three washes in PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min and washed again three times. Cells were blocked with 10% FBS in PBS for 30 min and then incubated with anti–FLAG-M2 (1:750; Sigma) and anti–Tom20 (1:2,000; Santa Cruz) antibodies in 5% FBS in PBS for 1 h at room temperature (RT). Cells were washed three times in 5% FBS in PBS and incubated with Alexa Fluor secondary antibodies (1:1,000; Molecular Probes) in PBS with 5% FBS for 1 h at RT. Cells were finally washed three times in PBS, and coverslips were mounted with fluorescent mounting medium (Dako). Images were obtained on an Olympus IX81 inverted microscope with appropriate lasers, using an Olympus FV1000 confocal scanning microscope fitted with a 60× objective and a N.A. 1.4. Mitochondrial morphology was categorized into “normal,” “fragmented,” or “hyperfused” mitochondria. More than 100 transfected cells were analyzed per condition, and three replications were counted for each condition. Results are representative of three independent experiments.

Constructs. The human Opal (Uniprot O60313) was expressed using the mammalian expression vector pcDNA3 (Invitrogen). Mutants were obtained using the Q5 site-directed mutagenesis kit (New England Biolabs) and validated by DNA sequence analysis. Human CAPN1 protein expression was silenced by transfecting HeLa cells with Stealth siRNA (Invitrogen) directed against the cognate target mRNA (#1, CCG UAC ACU UGA AGC GUG ACU UC(U); #2, GGC GUC GAC UUU GAC AAU UUC GUU U).

Antibodies. The antibodies used in this work were as follows: mouse monoclonal anti-Opal (1:500; clone 18), raised against amino acid 708–830 of Opal; BD Biosciences), mouse monoclonal anti-Mfn2 antibody (1:1,000; clone 4H8; Abnova), rabbit monoclonal antiphospho (Ser240/244)-S6 ribosomal protein (1:1,000; clone D68F8 XP; Cell Signaling), mouse monoclonal anti-S6 ribosomal protein (1:500; clone 5D2; Cell Signaling), mouse monoclonal anti-DP1 (1:1,000; BD Biosciences), mouse monoclonal anti-Flag HRP-conjugated (1:1,000; clone M2; Sigma), mouse monoclonal anti-HA (1:1,000; clone 6E12; Covance), mouse monoclonal anti-GAPDH (1:5,000; clone 6C5; Ambion), mouse monoclonal anti-Hsp60 (1:1,000; Clone LKI; Sigma), mouse monoclonal anti-ATP 5A synthase subunit alpha (1:500; clone 15H4C4; Abcam), mouse monoclonal anti-CAPN1 (1:500; clone 2H2A7C2; Abcam), and mouse monoclonal anti-TOM20 (1:2,000; Santa Cruz).

Cell Culture. The MEFs used in this study were a kind gift of David C. Chan, California Institute of Technology, Pasadena, CA, (Mfn1<sup>+/--</sup> and Mfn2<sup>+/--</sup>), Carlos Lopez-Otín, University of Oviedo, Oviedo, Spain, (Oma1<sup>+/--</sup>), and Bart De Strooper, Vlaams Institut voor Biotechnologie Center for the Biology of Disease, Leuven, Belgium, (Par<sup>+/--</sup>). HEK 293, HeLa, and COS7 cells were purchased from American Type Culture Collection. All cell lines were maintained under standard cell culture conditions and transfected with either Transfectin (BioRad), Lipofectamin 2000 (Invitrogen), or GenJet (SignaGen).

Whole Liver Lysates and Crude Mitochondria Preparation. Crude mitochondrial preparations were obtained as described (1). Briefly, the animal was anesthetized with isoflurane and decapitated. The liver was immediately removed and the gallbladder eliminated. It was then thoroughly washed in ice-cold IB<sub>bver-1</sub> (225 mM mannitol, 75 mM sucrose, 0.5% BSA, 0.5 mM EGTA, 10 μM AEBSF, 1 μM pepstatin, 1 μM aprotime, and 30 mM Tris·HCl pH 7.4), and IB<sub>bver-3</sub> (225 mM mannitol, 75 mM sucrose, and 30 mM Tris·HCl pH 7.4). The liver was then cut in small pieces using a razor blade and washed again in IB<sub>bver-1</sub>. An aliquot of 1 g was transferred in a 5-mL glass/Teflon Potter Elvehjem homogenizer containing 4 mL of ice-cold IB<sub>bver-1</sub>. Tissue was homogenized with a variable speed motor drive homogenizer operated at 4,000 rpm using 4 strokes (#1234R92, Thomas Scientific, Swedesboro, NJ), transferred to a 50-mL Falcon tube, and centrifuged at 600 × g for 10 min at 4 °C. The supernatant was collected; for whole liver lysates, 200 μL were added to one volume of lysis buffer (130 mM Tris base, 300 mM NaCl, 2% Nonidet P-40, 0.5% Na deoxycholate, 0.2% SDS, 2 mM EDTA, pH 7.4, protease inhibitor mixture, 20 units RNaseA) and incubated at 4 °C for 30 min. For crude mitochondria preparation, the supernatant was centrifuged at 7,000 × g for 10 min at 4 °C, and the resulting pellet was washed twice in 500 μL of IB<sub>bver-2</sub> (225 mM mannitol, 75 mM sucrose, 0.5% BSA, 30mM Tris·HCl pH 7.4). The pellet was resuspended in 200 μL of MRB (250 mM mannitol, 0.5 mM EGTA, and 5 mM Hepes pH 7.4). The protein concentration was estimated by Bradford analysis, and the mitochondrial preparations were resuspended to a final protein concentration of 30 μg/mL in MRB. Crude mitochondrial preparations from the indicated cell line were obtained from three to six large Petri dishes as described above.

Cryo-EM. Animals were anesthetized first with isoflurane and then with ketamine/xylazine. Liver was quickly biopsied using Rapid Transfer System (Leica). All protocols were approved by the Animal Protection Committee of the Université Laval. High-pressure freezing (Leica EM PAC2) was used for cryofixation of the samples. Freeze substitution was performed with the Leica automatic freeze substitution (AFS) chamber. The substitution fluid was acetone containing 1% OsO4 and 0.1% uranyl acetate. The procedure started at −90 °C for 8 h and warmed up to −60 °C at the speed of 5 °C/h. Substitution medium was replaced with pure acetone after the temperature reached 0 °C. Samples were embedded in Araldite/Epon/Dodecenylsuccinic anhydride (DDSA) and 2,4,6-tris (dimethylaminomethyl) phenol (DMP-30) mixture [araldite/epon stock, epoxy 41% (wt/wt), duracon Araldite casting resin M (ACM) 54% (wt/wt), dibutylphthalate 5% (wt/wt), araldite/epon complete formulation, araldite/epon stock 49% (wt/wt), hardener DDSA 49% (wt/wt), and accelerator DMP-30 2% (wt/wt)]. Procedure was performed stepwise: 33% resin in water-free acetone for 4 h, 66% resin in water-free acetone for hours, 100% resin overnight, and one 100% resin change before polymerization. All samples were polymerized at 58 °C for at least 48 h. Samples were cut at 50 nm and put on single-slot copper grids using a Leica Ultramicrotome. After counterstaining with lead citrate, samples were viewed on a Tecnai-12 by Philips with a Megaview camera using the Analysis software.

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**Fig. S1.**  (A) A graph comparing the amount of chow (and relative calories) ingested by mice fed ad libitum (a.l.) versus fasted from 8:00 PM to 8:00 AM. Note the reproducibility in the amount of food ingested after overnight fasting. Also, after feeding to 1.9 kCal, the animals seem to have reached satiety because they typically stop ingesting more chow. (B) Immunoblot analysis of lysates prepared from the same livers on which mitochondrial oxygen consumption rates were analyzed (Fig. 2D and Fig. S2F).
Fig. S2. (A) An illustration of the experimental setup used for this study. Note that three mice were used for each postprandial condition. (B) Mitochondria area distribution of the organelles analyzed in Fig. 1 G and H. Note the shifts toward smaller organelles at 5 h postprandial. The intervals of the classes were chosen arbitrarily. (C) The cristae length of the mitochondria analyzed in Fig. 2 B and C remains the same at 2 h (green line) and 5 h postprandial (red line). This is evidenced by the nearly perfect overlap of the lines that indicate the cristae length cumulative distribution. (D) Pattern of expression of Opa1 and of additional mitochondria morphology regulators in the mouse livers on which the cryo-EM analysis shown in Fig. 2 was conducted. (E) Immunoblot analysis of lysates prepared from the same mouse livers used for the cryo-EM analysis shown in Fig. 2 G and H (gray bar/line). These samples were chosen for their intermediate level of hepatic phospho-S6 expression at 5 h postprandial. (F) Oxygen consumption rate of liver mitochondria energized with 10 mM succinate + 2 μM rotenone. Data represent mean ± SEM of five independent experiments.

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Fig. S3. (A) Kinetic of the in vitro Opa1 C-cleavage assay. Note the appearance of CTF-2 within minutes after mitochondria were treated with DDM. Asterisk denotes an unspecific product of the in vitro C-cleavage. (B) Opa1 C-cleavage assay is effective over a broad range of Opa1 concentrations and depletes all forms of endogenous Opa1. (C and D) Protease inhibitor screening showing that Opa1 C-cleavage is inhibited by E-64 and calpeptin, two known inhibitors of cysteine proteases of the calpain family. (E) In vitro Opa1 C-cleavage on mitochondria prepared from CCCP-treated HEK 293 cells. Note that Oma1-generated s-Opa1 can be subjected to C-cleavage. (F and G) The cell-permeable cysteine protease inhibitor E64d blocks the generation of Opa1 CTF-1 in wild-type MEFs treated with rapamycin without, however, affecting the response of these cells to the TORC inhibitor (phospho-S6).
Fig. S4. (A) Genetic ablation of PARL does not eliminate Opa1 C-cleavage. (B) Deletion of the amino acids encompassing the putative C1 cleavage site, RM_RMLAITAAS, blocks the generation of CTF-1. (C) Schematic representation of the construct used to express CTF-1–Flag in the mitochondrial IMS. A schematic representation of Opa1-Flag is also shown to highlight the differences and similarities with CTF-1–Flag. MTS, mitochondrial targeting sequence; TMD, transmembrane domain. (D) Immunoblot analysis showing that CTF-1–Flag is imported in the mitochondria. The in vitro transcribed and translated protein is denoted as I.V.T. and corresponds to the form that is not imported in the mitochondria (unimported). (E) Immunoblot data showing that the short form of CTF-1–Flag has the same electrophoretic mobility of the CTF-1 fragment that is generated by in vitro C-cleavage of Opa1-Flag. Asterisk denotes an unspecific product of the in vitro C-cleavage.
Fig. S5. (A) Schematic representation of the construct used to express CTF-2–Flag in the mitochondrial IMS. (B) Immunoblot analysis showing that CTF-2–Flag is imported in the mitochondria and has the same electrophoretic mobility of the CTF-2 that is generated from in vitro C-cleavage of endogenous Opa1 (mitochondria were prepared from GFP-transfected HEK 293 cells). The in vitro transcribed and translated protein is denoted as I.V.T. and corresponds to the form that is not imported in the mitochondria (unimported). Note that cells expressing CTF-2–Flag and challenged with CCCP convert the membrane-bound form of CTF-2–Flag into its IMS-soluble form. This indicates that this chimeric Opa1 construct is imported and inserted in the organelle like the wild-type protein. Asterisks denote unspecific products. (C) Expression of either CTF-1–Flag or CTF-2–Flag in the IMS of HeLa cells does not affect mitochondria morphology.
Fig. 56. (A) Mfn2−/− MEFs challenged with a high dose (200 nM) of rapamycin over an extended period fail to activate the generation of Opa1–CTFs. (B) Mitochondria isolated from Mfn2−/− MEFs do not respond to DIG-induced activation of Opa1 C-cleavage and CTF-1 generation.