# Supporting Information

## Sood et al. 10.1073/pnas.1408061111

### 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 a Olympus FV1000 confocal scanning microscope fitted with a 60× objective and 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 coverslips were counted for each condition. Results are representative of three independent experiments.

**Constructs.** The human Opa1 (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 UCU U; #2, GCG GUC GAC UUU GAC AAU UUC GUU U).

**Antibodies.** The antibodies used in this work were as follows: mouse monoclonal anti-Opa1 (1:500; clone 18, raised against amino acid 708–830 of Opa1; 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 54D2; Cell Signaling), mouse monoclonal anti-DLP1 (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 16B12; Covance), mouse monoclonal anti-GAPDH (1:5,000; clone 6C5; Ambion), mouse monoclonal anti-Hsp60 (1:1,000; Clone LK1; 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*$^{-/-}$ and *Mfn2*$^{-/-}$), Carlos López-Otín, University of Oviedo, Oviedo, Spain, (*Oma1*$^{-/-}$), and Bart De Strooper, Vlaams Instituut voor Biotechnologie Center for the Biology of Disease, Leuven, Belgium, (*Parl*$^{-/-}$). 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 mitochondria 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 $IB_{liver\text{-}1}$ (225 mM mannitol, 75 mM sucrose, 0.5% BSA, 0.5 mM EGTA, 10 μM AEBSF, 1 μM pepstatin, 1 μM aprotinin, and 30 mM Tris·HCl pH 7.4), and $IB_{liver\text{-}3}$ (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 $IB_{liver\text{-}1}$. An aliquot of 1 g was transferred in a 5-mL glass/Teflon Potter Elvehjem homogenizer containing 4 mL of ice-cold $IB_{liver\text{-}1}$. 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 $IB_{liver\text{-}2}$ (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 mitochondria preparations were resuspended to a final protein concentration of 30 μg/μL 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 PACT2) 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), durcupan 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.

1. Wieckowski MR, Giorgi C, Lebiedzinska M, Duszynski J, Pinton P (2009) Isolation of mitochondria-associated membranes and mitochondria from animal tissues and cells. *Nat Protoc* 4(11):1582–1590.

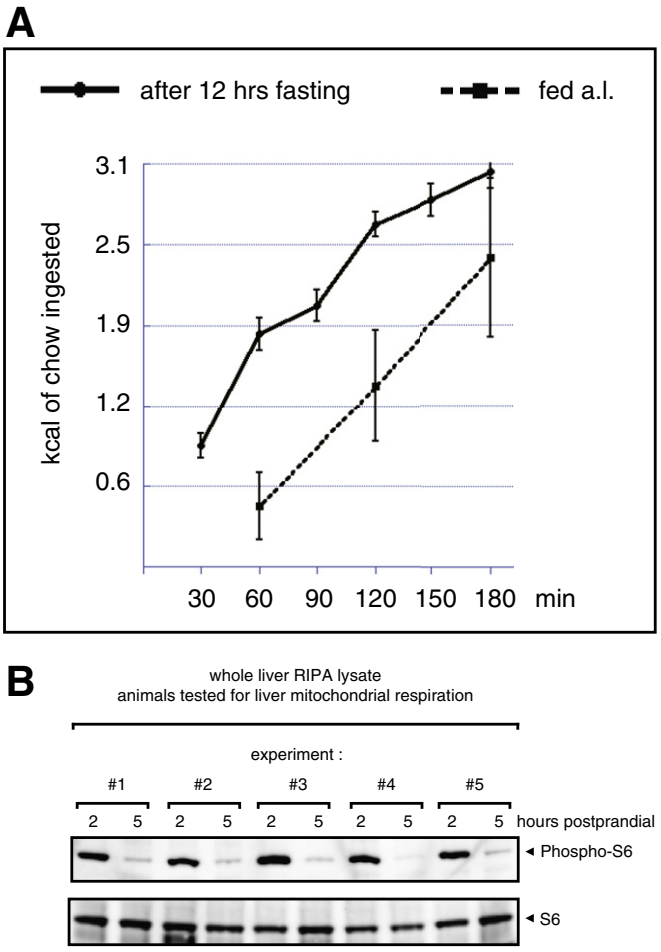


**Fig. S1.** (*A*) A graph comparing the amount of chow (and relative calories) ingested by mice fed ad libitum (a.l.) versus fasted from 8:00 PM to 8:00 AM. Note the reproducibility in the amount of food ingested after overnight fasting. Also, after feeding to 1.9 kCal, the animals seem to have reached 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. 2*D* and Fig. S2*F*).

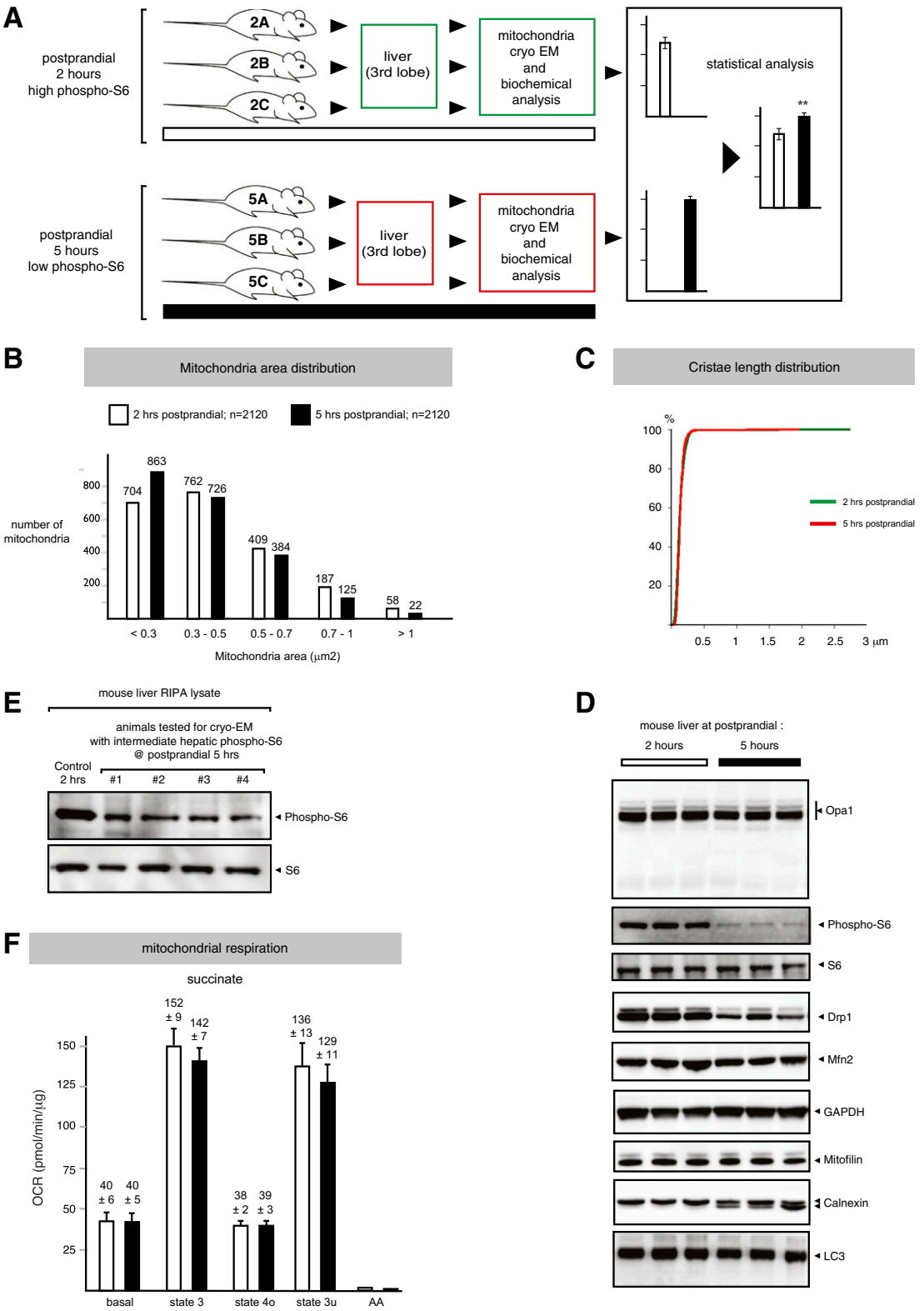


**Fig. S2.** (*A*) An illustration of the experimental setup used for this study. Note that three mice were used for each postprandial condition. (*B*) Mitochondria area distribution of the organelles analyzed in Fig. 1 *G* and *H*. Note the shifts toward smaller organelles at 5 h postprandial. The intervals 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.

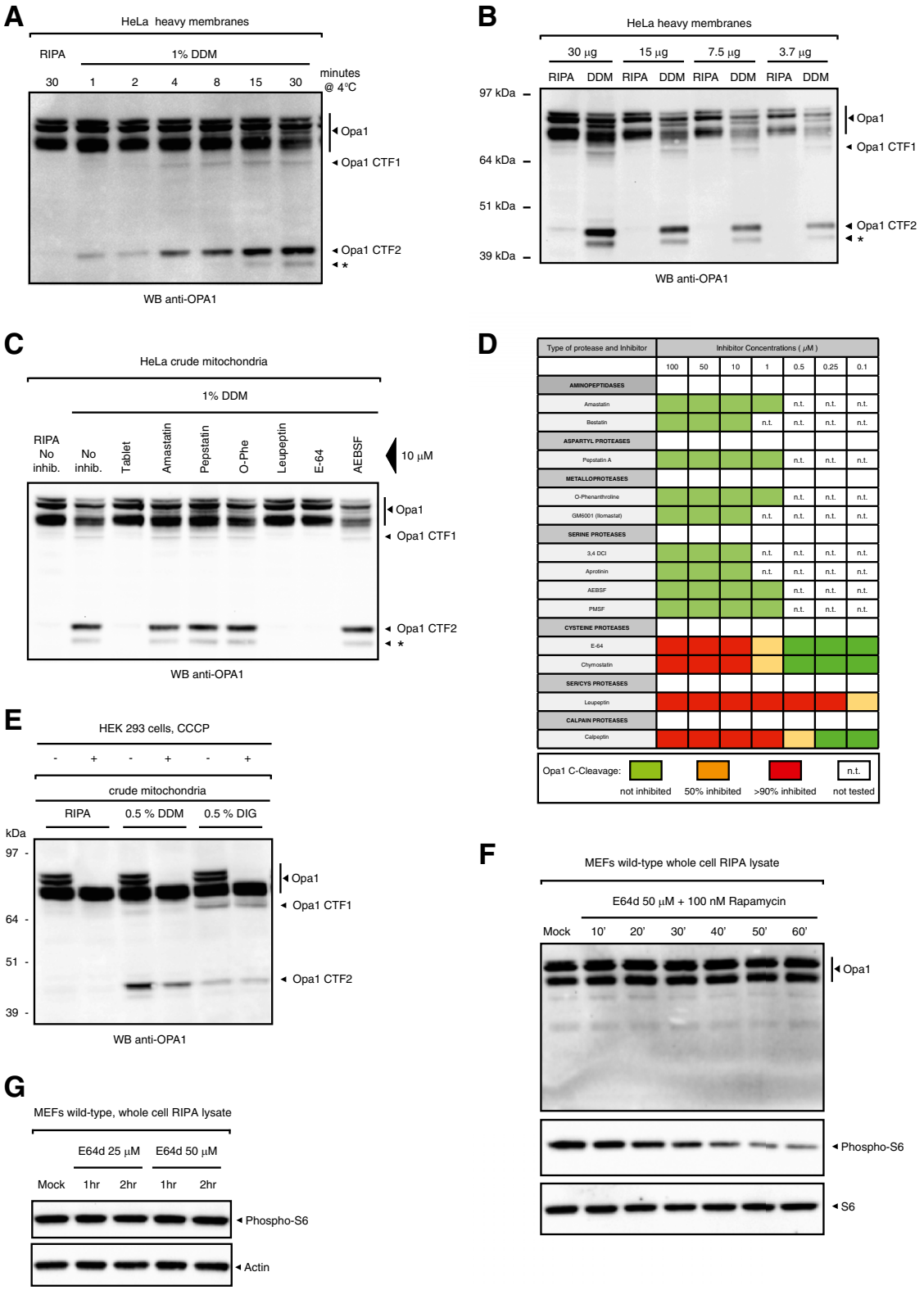


**Fig. S3.** (*A*) Kinetic of the in vitro Opa1 C-cleavage assay. Note the appearance of CTF-2 within minutes after mitochondria were treated with DDM. Asterisk denotes an unspecific product of the in vitro C-cleavage. (*B*) Opa1 C-cleavage assay is effective over a broad range of Opa1 concentrations 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).

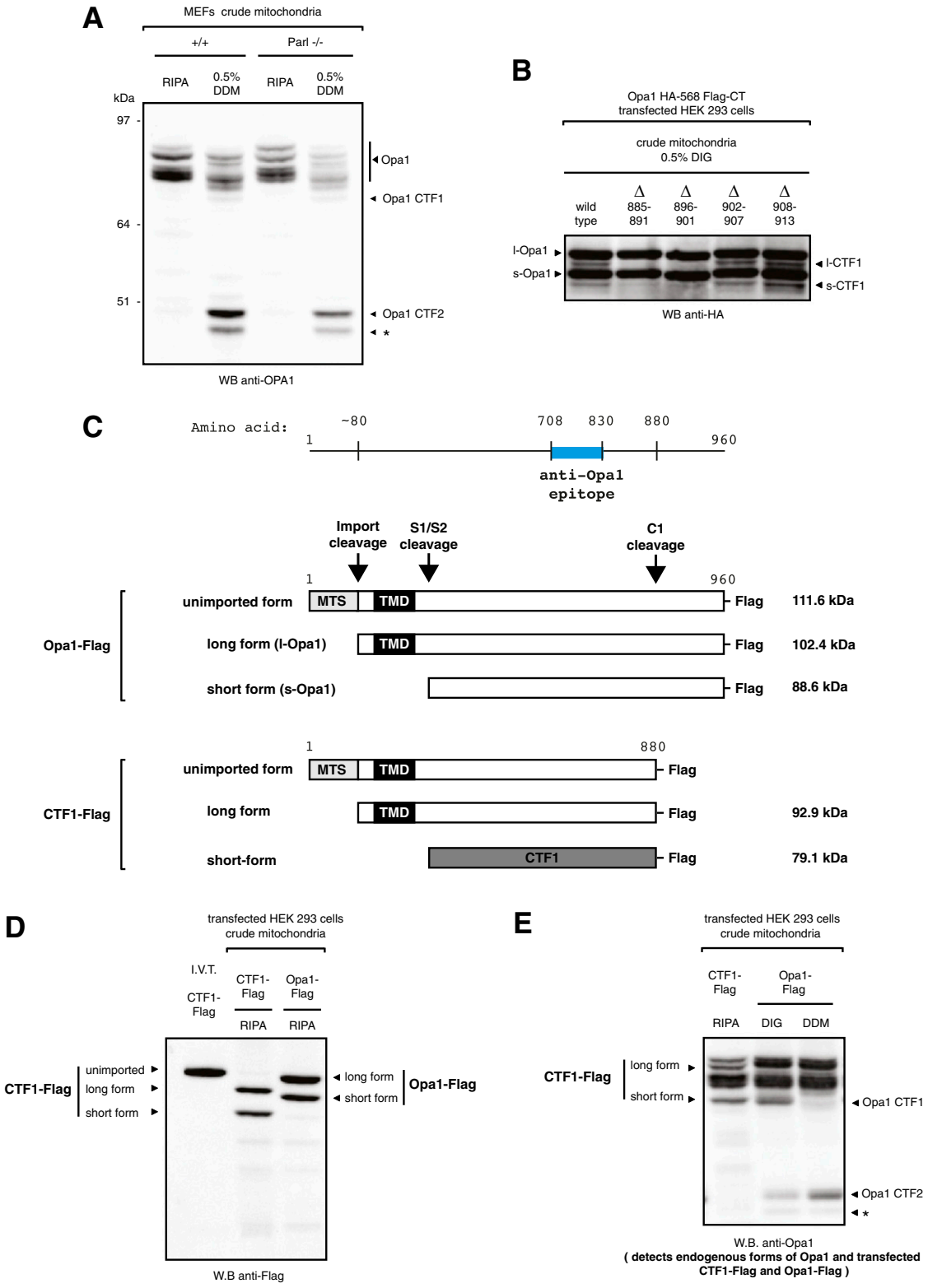


**Fig. S4.** (*A*) Genetic ablation of *PARL* does not eliminate Opa1 C-cleavage. (*B*) Deletion of the amino acids encompassing the putative C1 cleavage site, $_{885}$RMLAITA$_{891}$, blocks the generation of CTF-1. (*C*) Schematic representation of the construct used to express CTF-1–Flag in the mitochondrial 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.

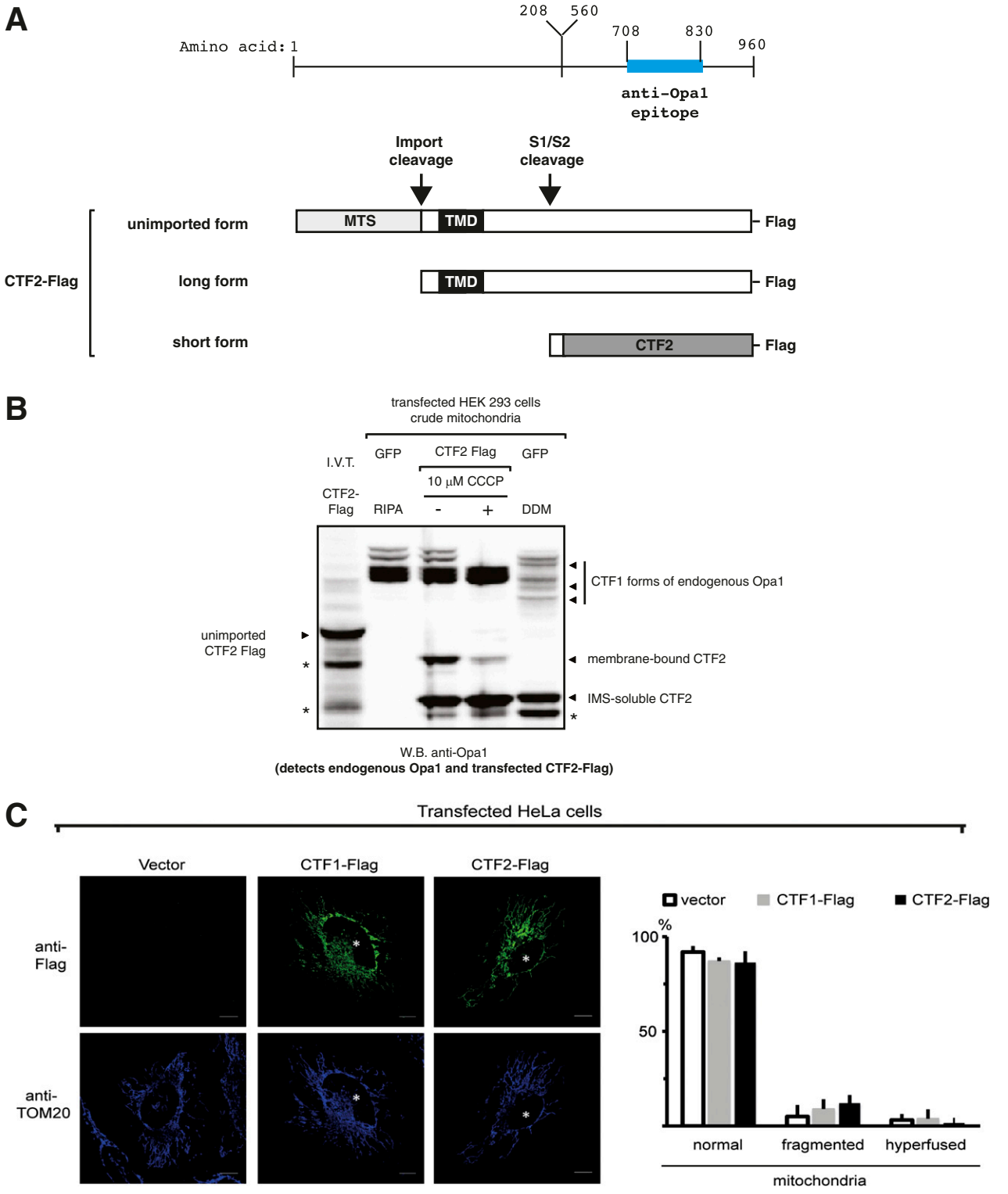


**Fig. S5.** (*A*) Schematic representation of the construct used to express CTF-2–Flag in the mitochondrial IMS. (*B*) Immunoblot analysis showing that CTF-2–Flag is imported in the mitochondria and has the same electrophoretic mobility of the CTF-2 that is generated from in vitro C-cleavage of endogenous 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.

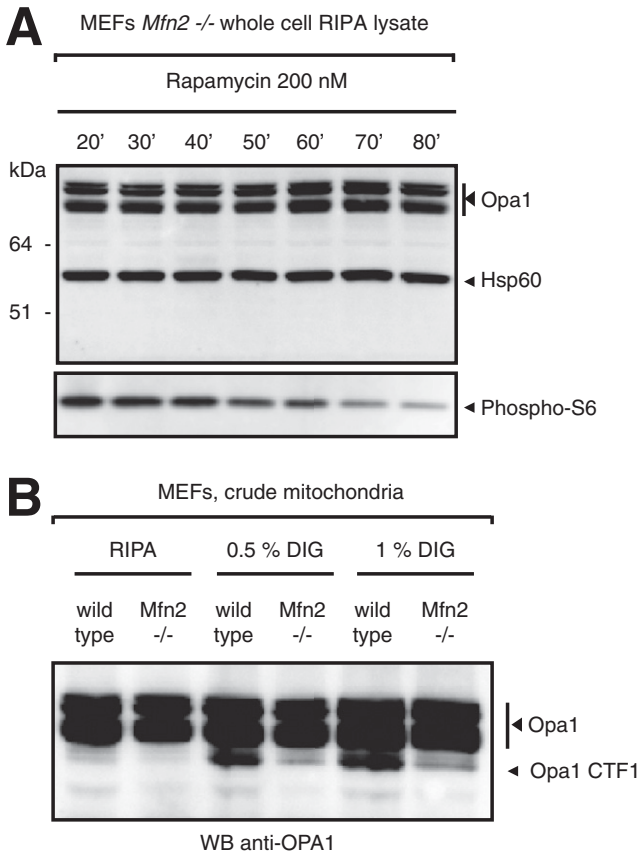


**Fig. S6.** (*A*) *Mfn2*$^{-/-}$ MEFs challenged with a high dose (200 nM) of rapamycin over an extended period fail to activate the generation of Opa1–CTFs. (*B*) Mitochondria isolated from Mfn2$^{-/-}$ MEFs do not respond to DIG-induced activation of Opa1 C-cleavage and CTF-1 generation.