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Supplemental Information

Optic Atrophy 1-Dependent Mitochondrial Remodeling Controls Steroidogenesis in Trophoblasts

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Inventory of Supplementary Data:

Supplementary Figure 1 – related to Figure 2

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Supplementary Experimental Procedures

Supplementary References

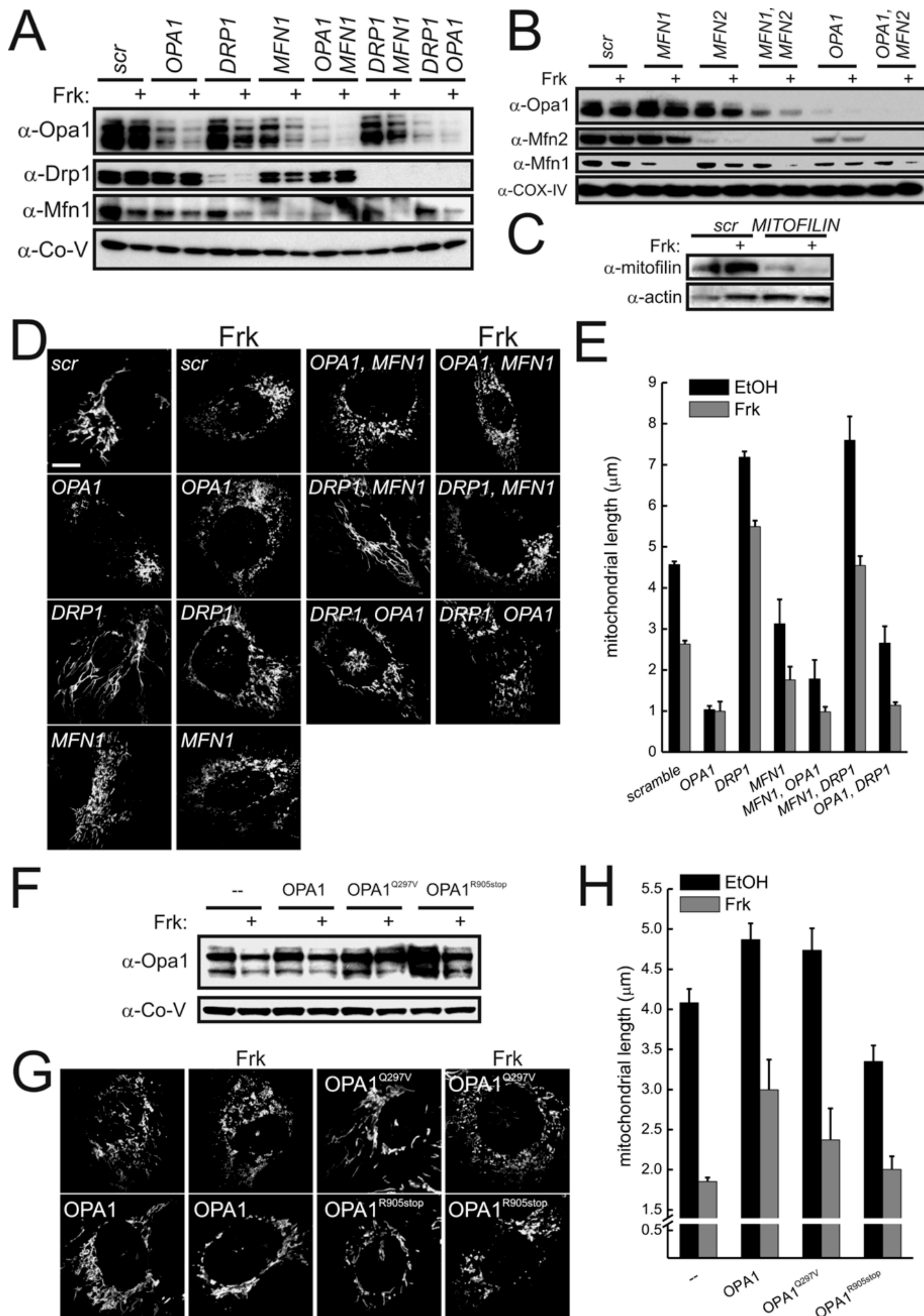

Figure S1. Mitochondria-shaping proteins control remodeling of mitochondria during differentiation of BeWo cells.

(A) BeWo-mtYFP cells were transfected with the indicated siRNA and after 48 hrs where indicated with Frk for further 48 hrs. Cells were then lysed and equal amounts of proteins (20 µg) were separated by SDS-PAGE and immunoblotted with the indicated antibodies.
(B) Experiments performed as in (A).
(C) Experiments performed as in (A).
(D) Representative confocal images of BeWo-mtYFP treated as in (A). Scale bar, 20 µm.
(E) Morphometric analysis of mitochondrial length in BeWo-mtYFP cells. Experiments were as in (A). Data represent mean ± SEM of 6 independent experiments.
(F) BeWo-mtYFP cells were transfected with pMSCV containing the indicated cDNA and after 48 hrs differentiation was initiated by treating cells where indicated with Frk for 48 hrs. Cells were lysed and equal amount of proteins (20 µg) were separated by SDS-PAGE and immunoblotted with the indicated antibodies.
(G) Representative confocal images of BeWo-mtYFP treated as in (F). Scale bar, 20 µm.
(H) Morphometric analysis of mitochondrial length in BeWo-mtYFP cells. Experiments were as in (F). Data represent mean ± SEM of 6 independent experiments.

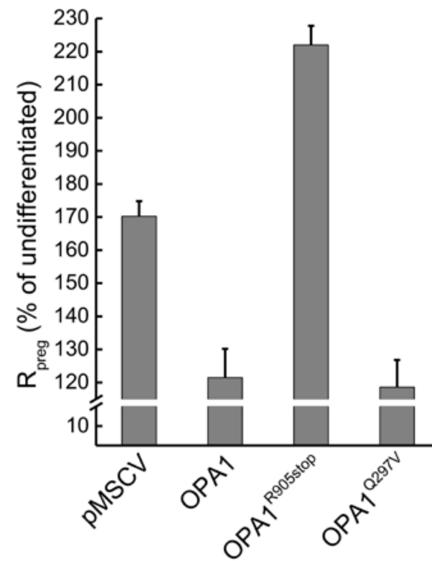

Figure S2. Opa1 controls efficiency of steroidogenesis.
R_{preg} was determined in BeWo cells transfected as indicated and treated where indicated with Frk for 48h. Data represent mean ± SEM of 5 independent experiments.

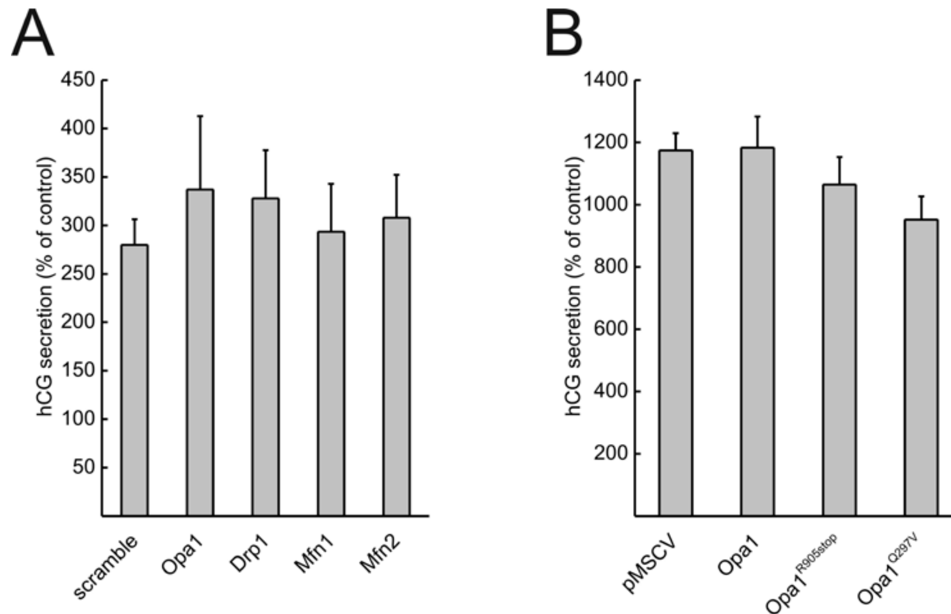

Figure S3. Secretion of hCG during differentiation of BeWo cells is not affected by levels of mitochondria-shaping proteins.
(A,B) BeWo cells were transfected with the indicated siRNA (A) or pMSCV containing the indicated cDNA (B), treated with vehicle or Frk for 48h and the secretion of hCG was assessed as described. Data are normalized to cells transfected and treated for 48h with vehicle and they represent mean ± SEM of 4 independent experiments.

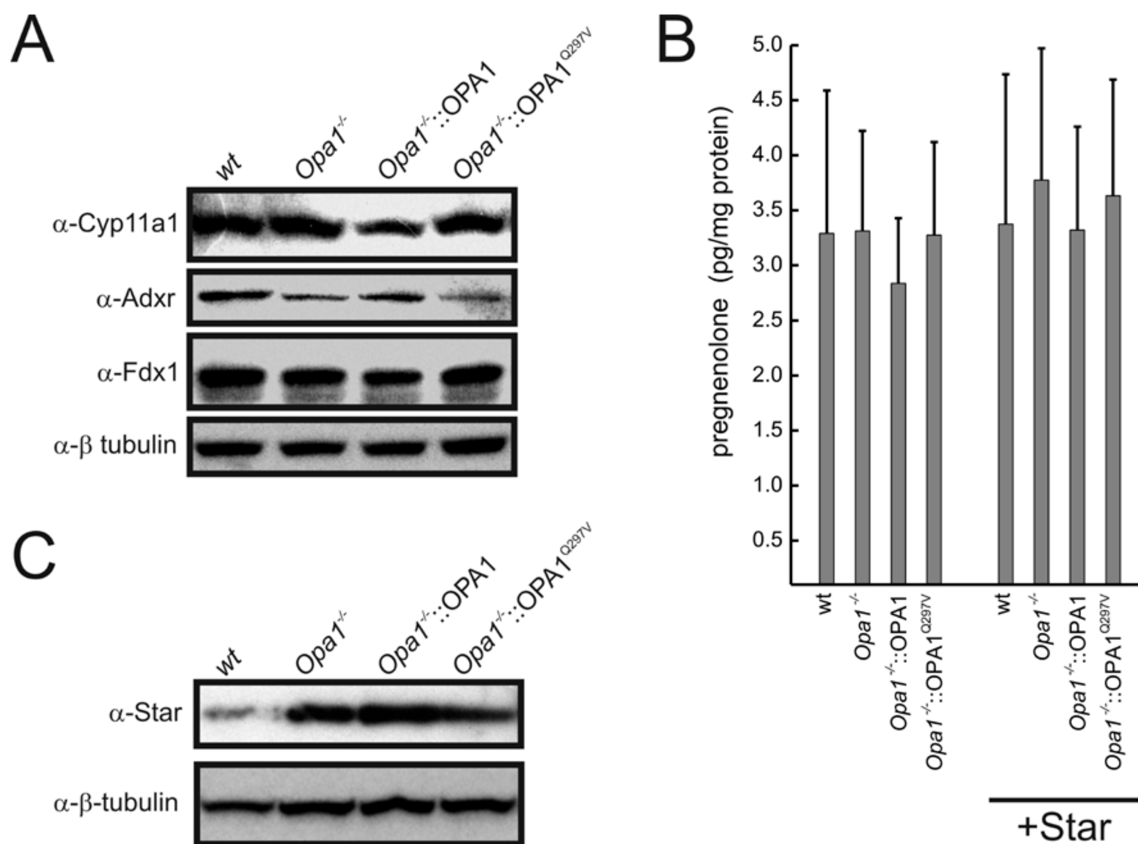

Figure S4. Efficient reconstitution of a minimal mitochondrial pregnenolone biosynthetic pathway in MEFs.

(A) MEFs of the indicated genotype were transfected with plasmids encodingAdxr, Fdx1, cytochrome $P450_{scc}$ (Cyp11a) and after 24h lysed. Equal amounts of protein (20µg) were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

(B) Maximal rate of pregnenolone biosynthesis was determined as described in MEFs of the indicated genotype transfected with Adxr, Fdx1, Cyp11a and where indicated with Star. Data represent mean ± SEM of 6 independent experiments.

(C) Experiment was as in (A) except that cells were transfected with a plasmid encoding Star.

Supplemental Experimental Procedures

Molecular biology

pEYFP-Mito (mtYFP), pMSCV-Opa1 containing murine OPA1 cDNA (GenBank™ accession number AB044138 corresponding to human transcript variant 1), pMSCV-Opa1^{R905stop}, pMSCV-Opa1^{Q297V} were described previously [1-3]. In order to clone Fdx1, Adxr, Cyp11a1 (NM_0041019.3, NM_024417.1 and NM_00781.1 respectively; OriGene) and Star (BC_010550; imaGenes) into pMSCV*Puro,* CDS were amplified uing the following pairs of primers: Fdx1 5'-ATGGCTGCCGCTGGGGGC-3' and 5'-TCAGGAGGTCTTGCCCACATC-3', Adxr 5'-ATGGCTTCGCGCTGCTGGC-3' and 5'-TCAGTGGCCCAGGAGGCGC-3', Cyp11a1 5'-ATGCTGGCCAAGGGTCTTC-3' and 5'-TCACTGCTGGGTTGCTTCCTGG-3', Star 5'-ATGCTGCTAGCGACATTCAAGC-3' and 5'-TCAACACCTGGCTTCAGAGGC-3'. The transcripts were cloned into pCR®2.1-TOPO® (TOPO TA Cloning®; Invitrogen) following manufacturer's instructions and then subcloned into the EcoRI restriction site of the MCS of pMSCV*Puro* and the orientation of inserts was confirmed by sequencing.

For silencing, the following siRNAs were used: Opa1, 5'-AAGUUAUCAGUCUGAGCCAGGUU-3'; Drp1, 5'-UCCGUGAUGAGUAUGCUUU-3'; Mfn1, 5'-CCAGAUGAACCUUUUAACA-3'; Mfn2, 5'-GAGACACAUGGCUGAGGUG; mitofilin, .

Cell culture

SV40-transformed *Opa1*$^{-/-}$ and wild-type MEFs were cultured as described [4]. Transfection of MEFs with DNA was carried out using Transfectin (Biorad) or Neon (Invitrogen).

BeWo choriocarcinoma cells (ATCC) were cultured in Kaighn's Modification of Ham's F12 Medium, supplemented with 10% non-heat inactivated fetal bovine serum, 1% penicillin/streptomycin. Transfection of BeWo cells with DNA and siRNA was carried out

using Neon (Invitrogen). BeWo::mtYFP cells were generated by transfection with pEYFP-Mito and selection with G418 (250 µg/ml). Differentiation of BeWo cells was achieved by the addition of 50 µM Frk to the complete culture medium for 48h.

Transduction of MEFs

Retroviral vectors were generated by co-transfecting HEK293T cell line with pMSCV*Puro*, pMSCV-Opa1 or pMSCV-Opa1^{Q297V} and the helper plasmid pIK. The supernatants collected after 24h were used to transduce MEFs which were then grown in medium supplemented with puromycin (5 µg/ml).

Cell Imaging

For confocal microscopy imaging of live cells, 2×10^5 cells seeded onto 24-mm round glass coverslips, treated as indicated were placed on the stage of a Nikon Eclipse TE300 inverted microscope equipped with a PerkinElmer Ultraview LCI confocal system, a piezoelectric z - axis motorized stage (Pifoc, Physik Instrumente) and an Orca ER 12-bit CCD (charge-coupled device) camera (Hamamatsu Photonics). Cells expressing mtYFP were excited using the 488 nm line of a HeNe laser (PerkinElmer) with a 60X, 1.4 NA Plan Apo objective (Nikon). Quantitative analysis of mitochondrial morphology was performed as described. Length of 5 randomly selected mitochondria per cell was measured using the Freehand Line tool in Image J. Data from at least 50 cells per condition per experiment were collected.

Imaging of human placenta

Placentas from elective terminations of pregnancy (15 and 20 weeks old) were collected and dissected as previously described [5,6] and kept in Dulbecco's modified Eagle's medium-F12

medium (DMEM-F12; 1:1) supplemented with 20% fetal bovine serum (Fisher Scientific), 1% L-glutamine and 1% penicillin/streptomycin (Invitrogen). Several very small fragments of live dissected tissue (size of ~4-10 separate villi) were cut off and incubated in culture media in a 1.5ml Eppendorf tube containing 0.5 µM nonyl acridine orange (Sigma) for 30 minutes in a tissue culture incubator (37°C and 5% CO_2) and briefly rinsed in fresh media. Each small fragment was briefly rinsed in fresh media, placed onto a glass slide with some media, the villi spread out with forceps and then by pushing a coverslip on top. Then the chamber was sealed with heated VALAP (1:1:1 ratio vaseline, lanolin, paraffin) and immediately imaged.

Live placenta tissue was imaged with a Yokagawa CSU22 Spinning Disk Confocal (Solamere Technology Group) mounted on a Nikon TI-E motorized inverted microscope located at the Nikon Imaging Center (UCSF). Imaging was performed using a 100x/1.4 NA oil Plan APO VC or a 40x/ 0.95NA air Plan Apo objective. Images were acquired on an Evolve EMCCD (Photometrics) camera with its gain set to 200.

Immunofluorescence microscopy

Cells (1×10^4) seeded onto 13-mm round glass coverslips and treated as indicated were fixed for 30 min at room temperature with 3.7% (w/v) formaldehyde, permeabilized for 20 min at room temperature with 0.1% Triton X-100 and incubated with anti-E-cadherin (30 µg/ml; R&D Systems), goat anti-mouse fluorescein isothiocyanate-conjugated IgG (1:150; Invitrogen) and Hoechst 33342 (1:10000; Invitrogen). Staining was revealed with using a laser scanning microscope (TCS SP5, Leica) and a ×40, HCX PL Apo objective (Leica).

Electron microscopy

BeWo or MEFs treated as indicated were fixed with 1.25% (v/v) glutaraldehyde in 0.1 M sodium cacodylate at pH 7.4 for 1 h at room temperature. Electron microscopy was carried out as described previously [7]. Mitochondrial cristae were quantified by measuring the number of cristae in each mitochondrion analyzed using ImageJ (NIH).

Pregnenolone assay

Cells (BeWo, 1×10^4; MEFs, 6×10^4) were seeded onto 24-well plate and treated as indicated. To assess basal rate of synthesis of pregnenolone, cells were incubated for 1h (BeWo) or 24h (MEFs) in culture medium containing trilostane (2 µg/ml; AK Scientific). Then, to assess maximal synthesis, cells were incubated for 30 min (BeWo) or 1h (MEFs) with a new culture medium containing trilostane (2 µg/ml; AK Scientific) and 22(R)-hydroxy-cholesterol (0.5 µg/ml; Sigma). In order to verify inhibition of Cyp11a1 by DL-aminoglutethimide (AMG) BeWo cells were pretreated with AMG (1 mM) for 2 h before switching them to the medium containing trilostane. Cells were lysed in 0.1 M NaOH and protein concentration was determined by Bradford analysis. Pregnenolone concentration in culture medium was determined by ELISA (Alpha Diagnostics International) following manufacturer's instructions. In order to calculate efficiency of pregnenolone synthesis, the basal rate was divided by the maximal rate.

hCG assay

Cells (1×10^4) were seeded onto 24-well plate, treated as indicated and incubated for 1h with a fresh culture medium. The medium was collected and hCG concentration was determined

by ELISA (hCG ELISA kit; GenWay), following manufacturer's instructions. Cells were lysed in 0.1 M NaOH and protein concentration was determined by Bradford analysis.

Cell lysis

Cells (10^6) were collected and disrupted in RIPA buffer (150 mM NaCl, 1% Nonidet P40, 0.25% sodium deoxycholate, 100 mM EDTA, 65 mM Tris-HCl at pH 7.4) in the presence of PMSF (1 mM, Fluka). Protein concentration was determined by BCA assay (Pierce).

Immunoblotting

The indicated amounts of proteins were separated by 4–12% Bis-Tris, 12% Bis-Tris or 7% Tris-acetate gels (NuPAGE; Invitrogen) and transferred onto polyvinylidene difluoride (PVDF; BioRad) membranes. The following antibodies were used: anti-Opa1 (1:500; BD Transduction), anti-Mfn1 (1:1000; Abcam), anti-Mfn2 (1:1000; Abcam), anti-Drp1 (1:1000; BD Transduction), anti-Fis1 (1:1000; Alexis), anti-Cyp11a1 (1:500; Abnova), anti-Adxr (1:200; Santa Cruz Biotechnology), anti-Fdx1 (1:500; Abnova), anti-Star (1:500; Santa Cruz Biotechnology), anti-Cox IV (1:5000; MitoSciences), anti-Tom20 (1:5000; Santa Cruz Biotechnology), anti-β-tubulin (1:500; Santa Cruz Biotechnology), anti-ATPase, α subunit (1:10000; MitoSciences), anti-Complex III, core 2 (1:5000; MitoSciences).

Isolation of mitochondria

Cells plated in 500 cm^2 plates were treated as indicated after 24 h and mitochondria were isolated as described previously [8]. Protein concentration was determined by Bradford.

Loading of isolated mitochondria with NBD-cholesterol

Complexes of NBD-cholesterol (Invitrogen) with BSA were prepared as described previously [9]. Briefly, 200 µl of deionized water was added dropwise on vortex to an equal volume of 1% NBD-cholesterol in DMF. The resulting milk-like solution was centrifuged at 10000 g for 10 min and the pellet was re-suspended in 200 µl of a buffer containing 250 mM sucrose, 1 mM EDTA, 10 mM Tris-MOPS pH 7.3. Eighty mg of bovine serum albumin (BSA; Sigma) was added and mixed on vortex until the solution clarified, followed by centrifugation at 12000 g for 30 min at 4°C. The supernatant was further centrifuged at 7000 g for 10 min at 4°C until no pellet was retrieved. NBD-cholesterol-BSA complexes were stored at 4°C. Isolated mitochondria (0.5 mg) were incubated in 100 µl of isolation buffer (IB; 200 mM sucrose, 1mM EGTA, 10 mM Tris-MOPS at pH 7.4) supplemented with 20% (v/v) NBD-cholesterol-BSA for 5 min on ice. Mitochondria were then diluted with 3 volumes of IB, pelleted at 7000 g for 10 min at 4°C and re-suspended in 400 µl of IB. Washing was repeated twice to remove traces of NBD-cholesterol-BSA and the final pellet was re-suspended in 100 µl of IB. Protein concentration was determined by Bradford.

Preparation of mitoplasts

Mitochondria loaded with NBD-cholesterol were incubated in IB enriched in 0.2% digitonin (Sigma) for 30 min at 4°C. The suspension was then centrifuged at 10000 g for 10 min at 4°C. The pellet was re-suspended in IB and centrifuged again. Protein concentration was determined by Bradford. Purity of mitoplasts was assessed by Western Blot.

Extraction of lipids

Up to 50 µl of mitoplasts or mitochondria were mixed with 200 µl of ice cold chloroform:methanol (1:2), vortexed for 1 min at 4°C and centrifuged at 1200g for 5 min at 4°C. Aliquots of supernatant were preserved at -20°C for the assessment of NBD-cholesterol.

NBD-cholesterol assay

Aliquots of lipid extract were diluted in chloroform:ethanol (1:2) and fluorescence was read using 460 nm as excitation and 525 nm as emission wavelengths in 1 cm quartz cuvettes using a RF-5301PC spectrofluorophotometer (Shimadzu).

Statistical Analysis

In each graph, unless noted, data represent mean ± SEM. If indicated, statistical significance has been calculated by a two-tailed Student t-test between the indicated samples. P values are indicated in the legends.

Supplemental Acknowledgments

MW and LS conceived research, analyzed data and wrote the manuscript. MW, MS, SMR performed experiments and analyzed data. JR and AIB analyzed data.

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