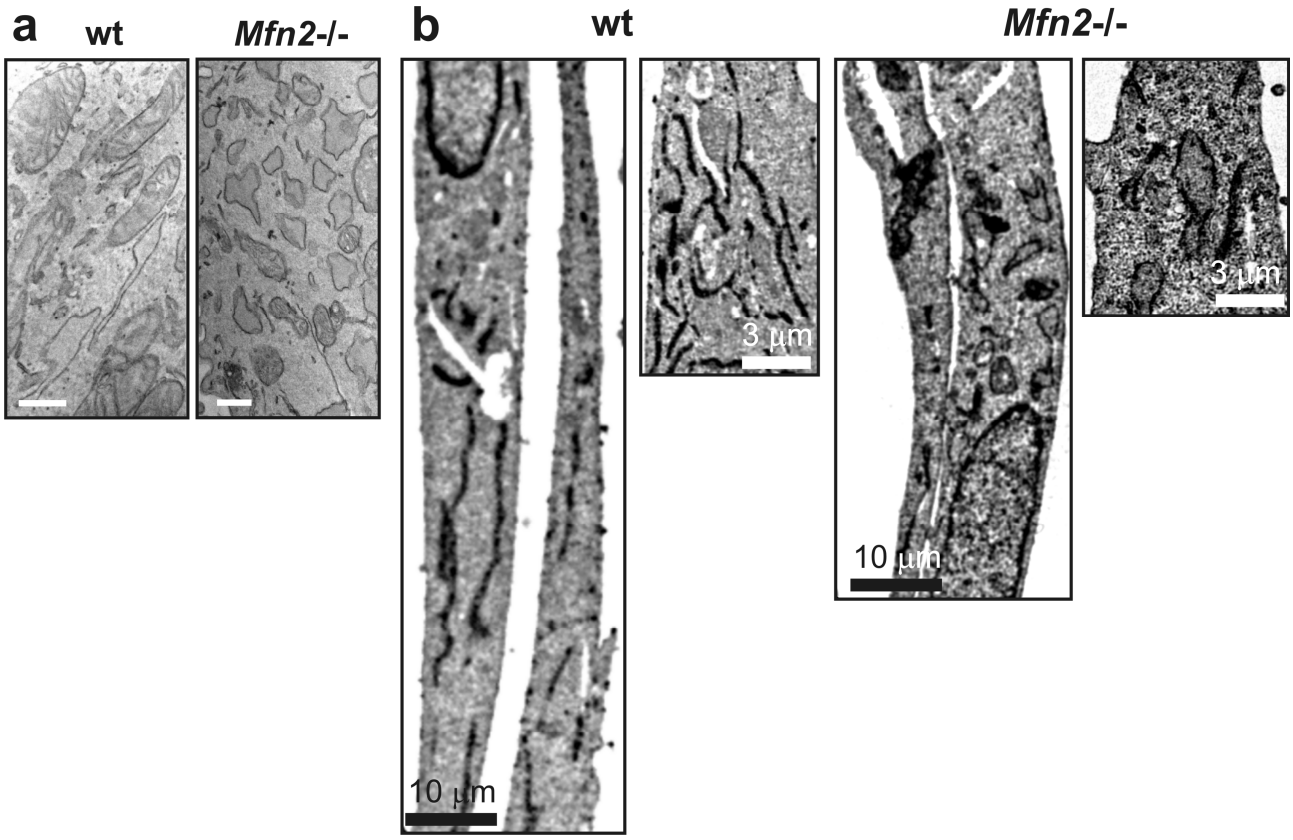
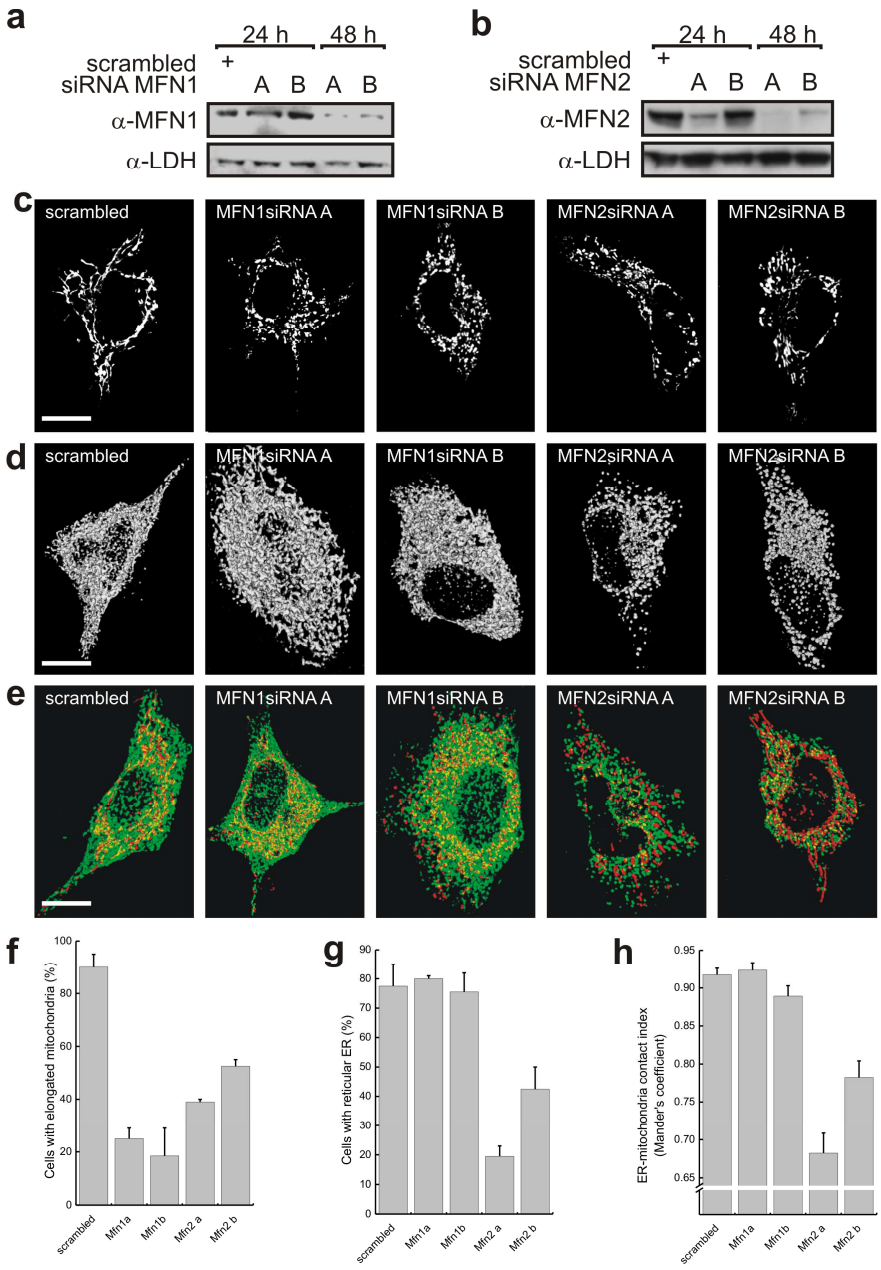
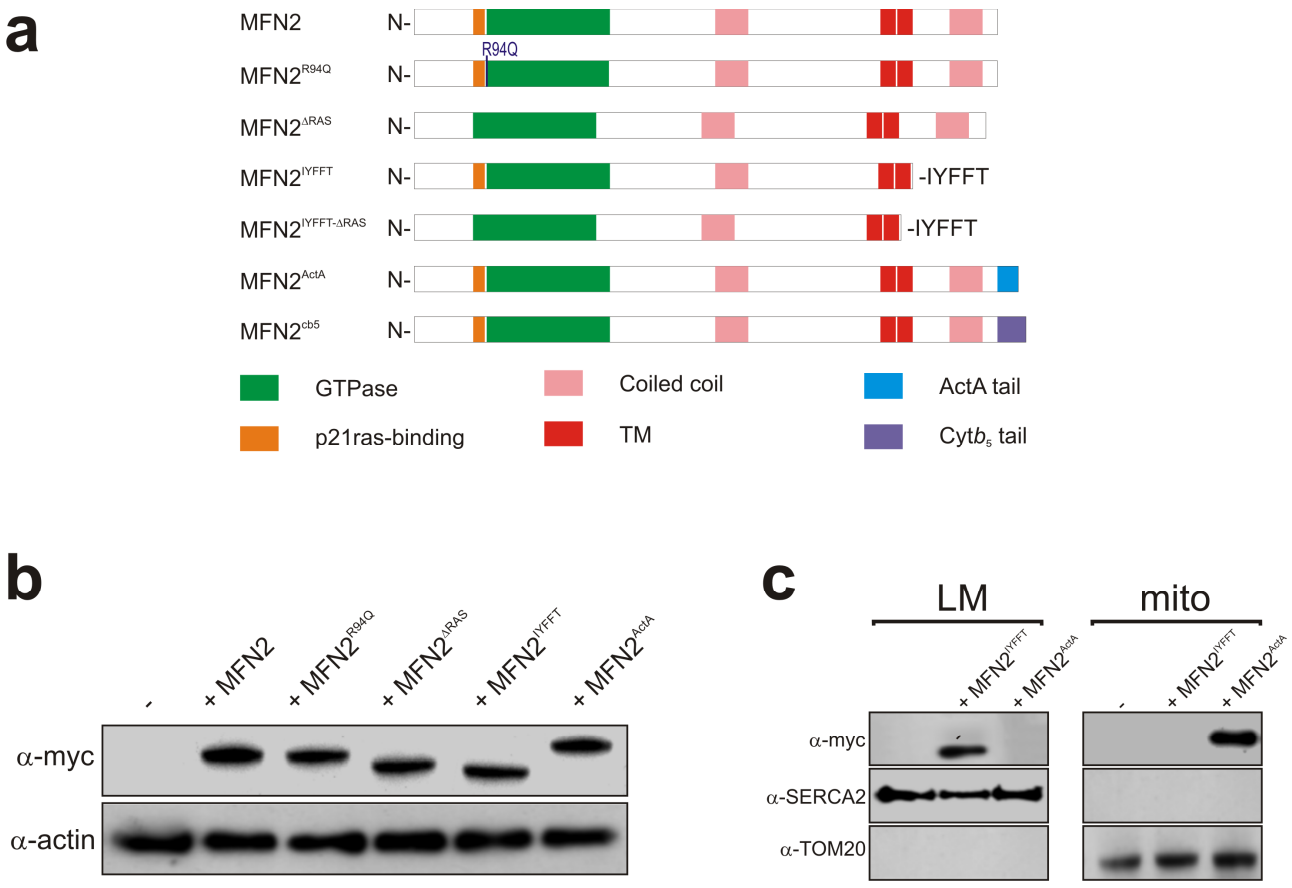
Supplementary Figures



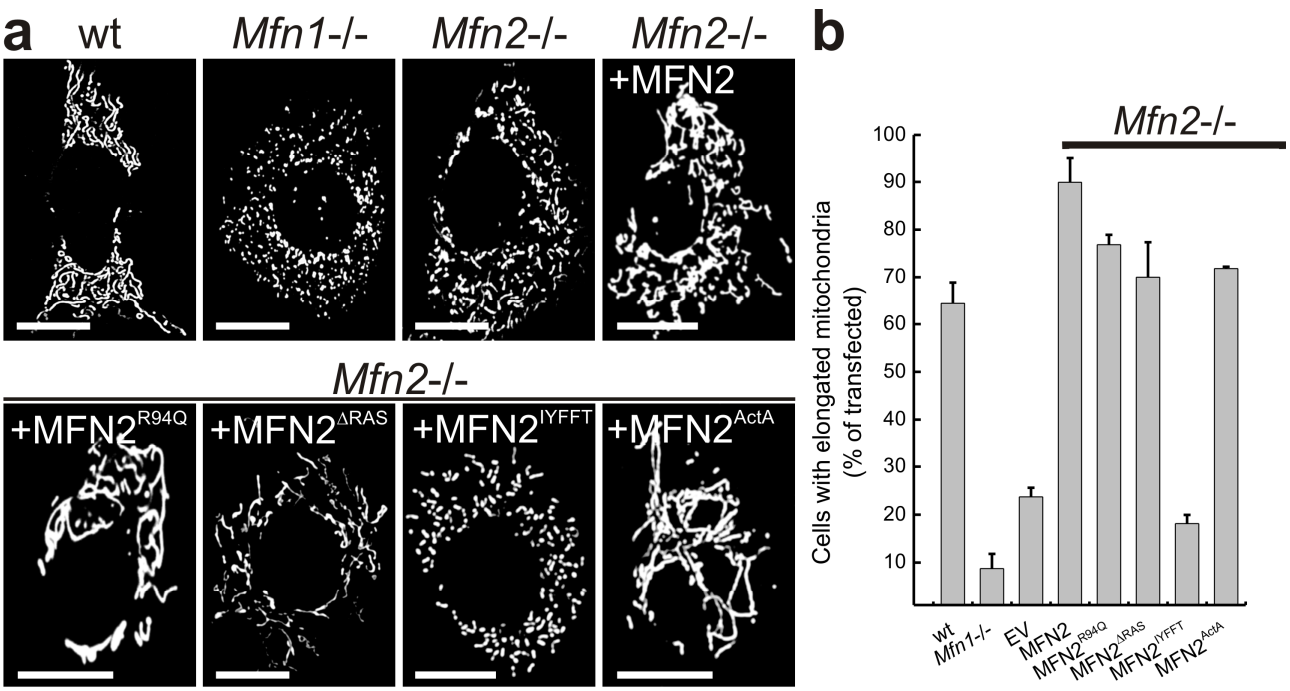
Supplementary Figure 1 – **Electron microscopy of wt and *Mfn2*$^{-/-}$ MEFs**.
a, Transmission electron microscopy of wt and *Mfn2*$^{-/-}$ MEFs. Scale bars, 500 nm. **b**, Transmission electron microscopy of wt and *Mfn2*$^{-/-}$ MEFs stained for glucose-6-phosphatase activity as described. Positive structures appear dark.



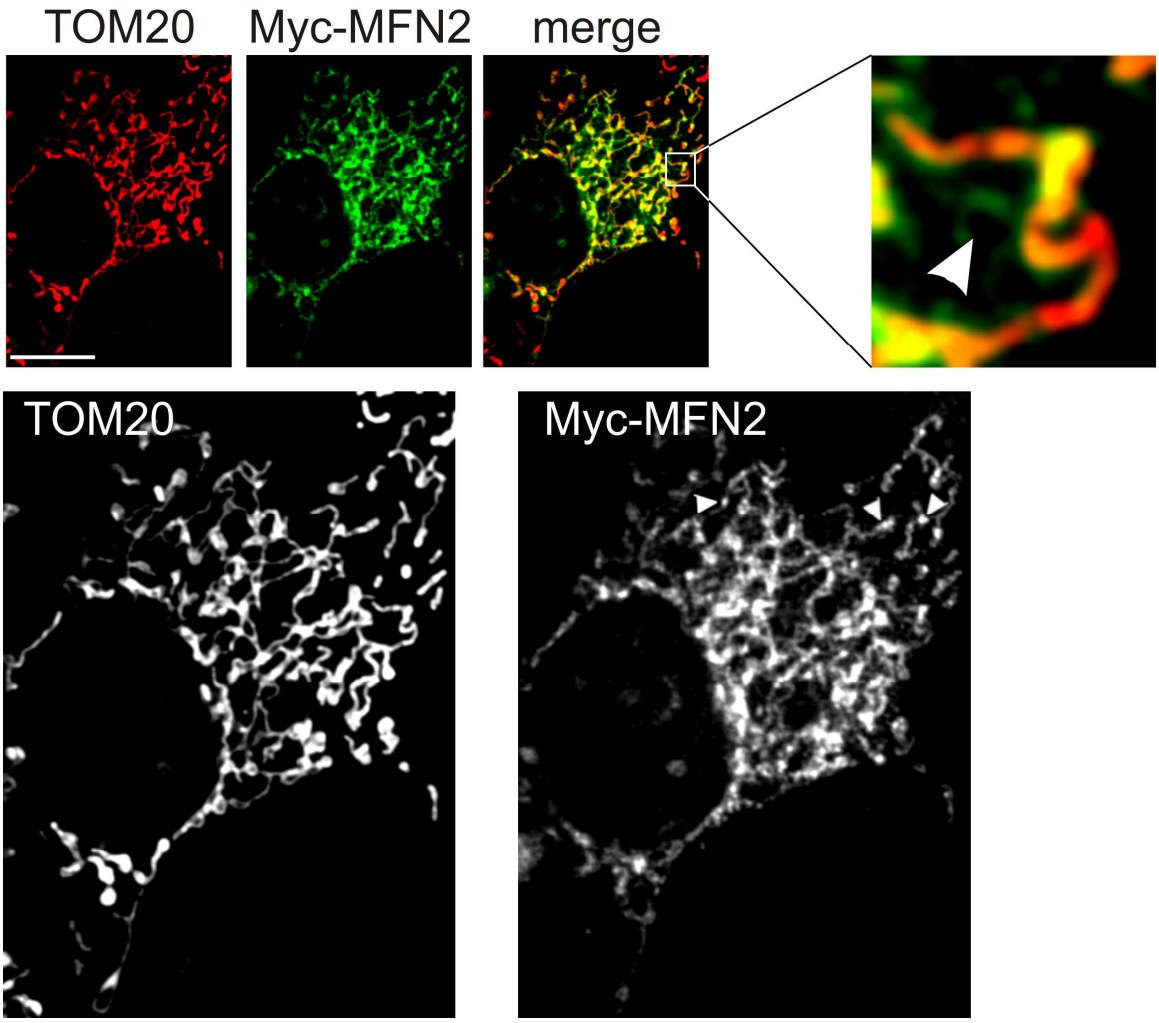
Supplementary Figure 2 – **Down-regulation of *MFN2* but not *MFN1* disrupts ER morphology and reduces ER to mitochondria interaction in HeLa cells.**
a, b, HeLa cells were transfected with the indicated siRNA. After the indicated time, cells were lysed and equal amounts of proteins (50 µg) were separated by SDS-PAGE and immunoblotted using the indicated antibodies. **c,** Representative randomly selected confocal images of mitochondrial shape in HeLa cells transfected with the indicated siRNA and with mtRFP. Scale bar, 18 µm. **d,** Representative volume rendered 3D reconstructions of randomly selected z-axis stacks of confocal erYFP images of HeLa cells transfected with the indicated siRNA and with erYFP. Scale bar, 18 µm. **e,** Representative volume rendered 3D reconstructions of randomly selected *z*-axis stacks of confocal erYFP and mtRFP images in Hela cells transfected with the indicated siRNA and with erYFP, mtRFP. Yellow indicates that organelles are closer than ~270 nm. Scale bar, 18 µm. **f,** Quantitative analysis of mitochondrial shape. Thirty images per condition were acquired as in **c** and classified as described. Data are means ± SE of 3 independent experiments. **g,** Morphometric analysis of ER shape. Thirty images were acquired and classified as described in **d**. Data represent means ± SE of 3 independent experiments. **e,** Quantitative analysis of mitochondria-ER interaction. Experiments were performed exactly as in **h**. Twenty randomly selected confocal stacks per condition were acquired, reconstructed and rendered as described and MCC was calculated. Data are mean ± SE of 3 independent experiments.



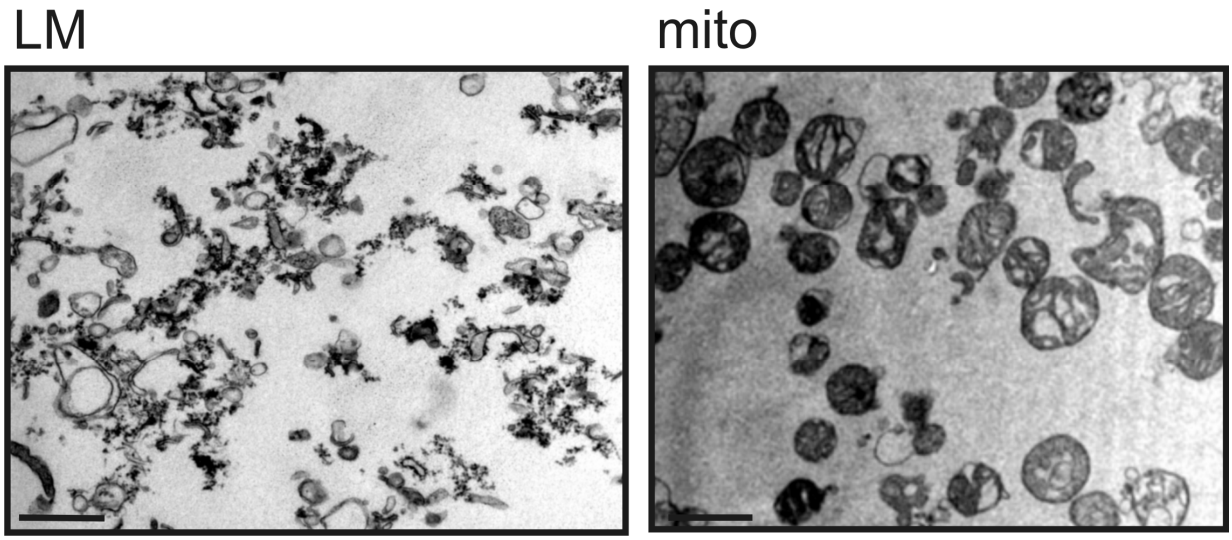
Supplementary Figure 3 - **Construction and analysis of MFN2 mutants.**
a, Primary structure of Mfn2 mutants used in this study. All the mutants are Myc-tagged at their N-terminus. Abbreviations: CC: coiled coil domain; TM: transmembrane domain. **b-c,** *Mfn2*$^{-/-}$ MEFs were transfected with the indicated vectors. Equal amounts of total lysates (50 µg) or the indicated subfractions (20 µg) were separated by SDS-PAGE and immunoblotted using the indicated antibodies.



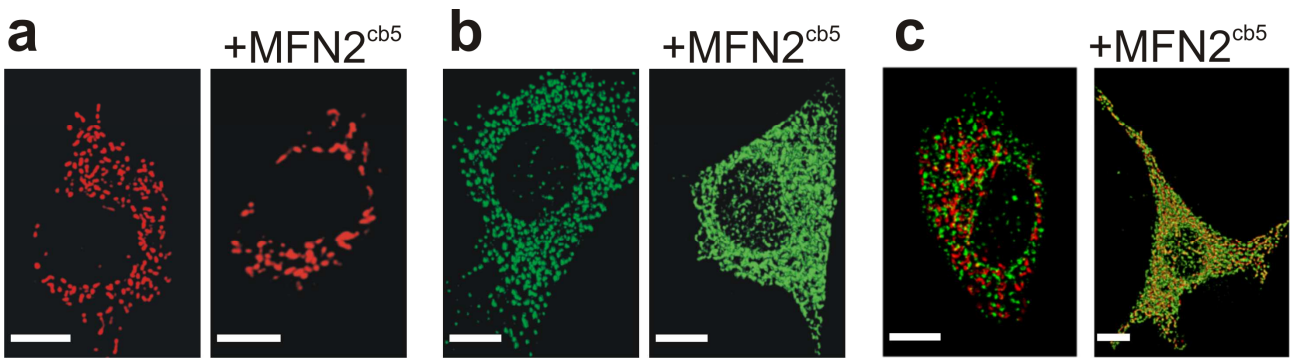
Supplementary Figure 4 – **Genetic analysis of MFN2 regulation of mitochondrial shape**.
a, Representative randomly selected confocal images of mitochondrial shape in MEFs of the indicated genotype cotransfected with mtRFP and the indicated plasmid. Scale bar, 18 μm. **b,** Morphometric analysis of mitochondrial shape. Fifty images per condition were acquired as described in (**a**) and classified as described. Data are means ± SE of 8 independent experiments.



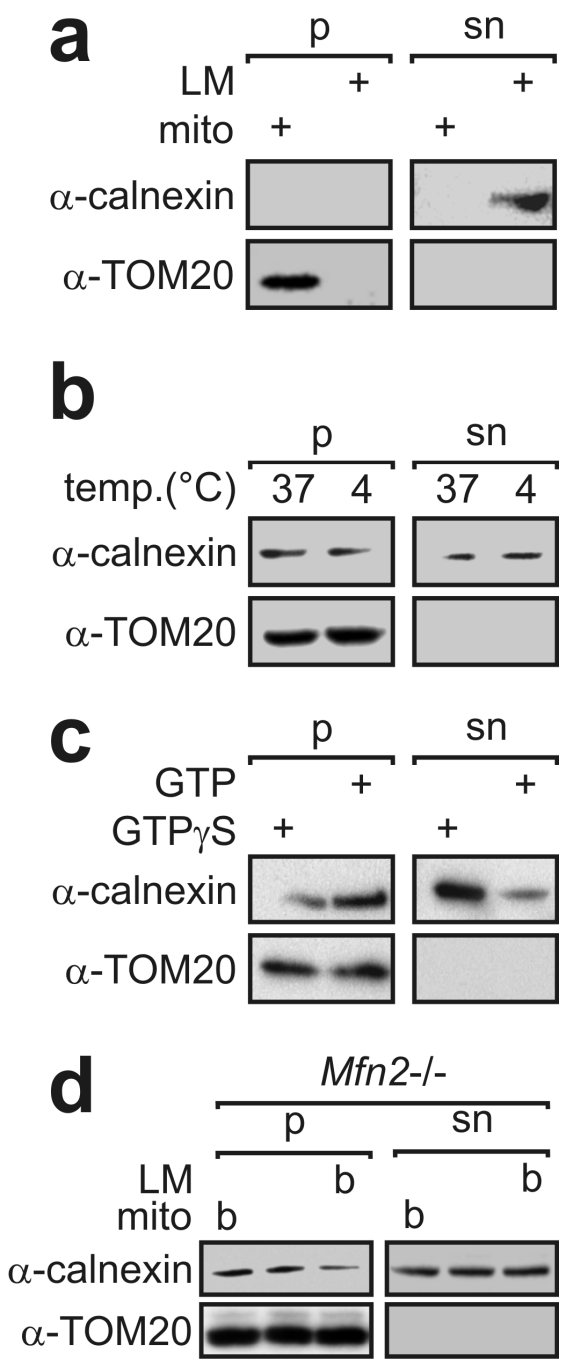
Supplementary Figure 5 –**Confocal imaging of subcellular localization of re-expressed Mfn2.** *Mfn2*$^{-/-}$ MEFs were transfected with Myc-MFN2, fixed and immunostained using an anti-Myc antibody and an anti-TOM20 antibody. Isotype matched, FITC (green, reacting with anti-Myc) or TRITC (red, reacting with anti TOM20) secondary antibodies were used to reveal immunodecoration by primary antibodies. Yellow indicates colocalization of Myc-MFN2 with the mitochondrial marker. Scale bar, 15 μm. The boxed area in the merged image was magnified 9 times to show detailed aspects of reticular pattern of Myc staining not colocalizing with mitochondria (arrowhead). The single channel images were magnified 2 times and are presented in grayscale in the bottom row to show diffuse staining of Myc vs. the much more defined TOM signal.



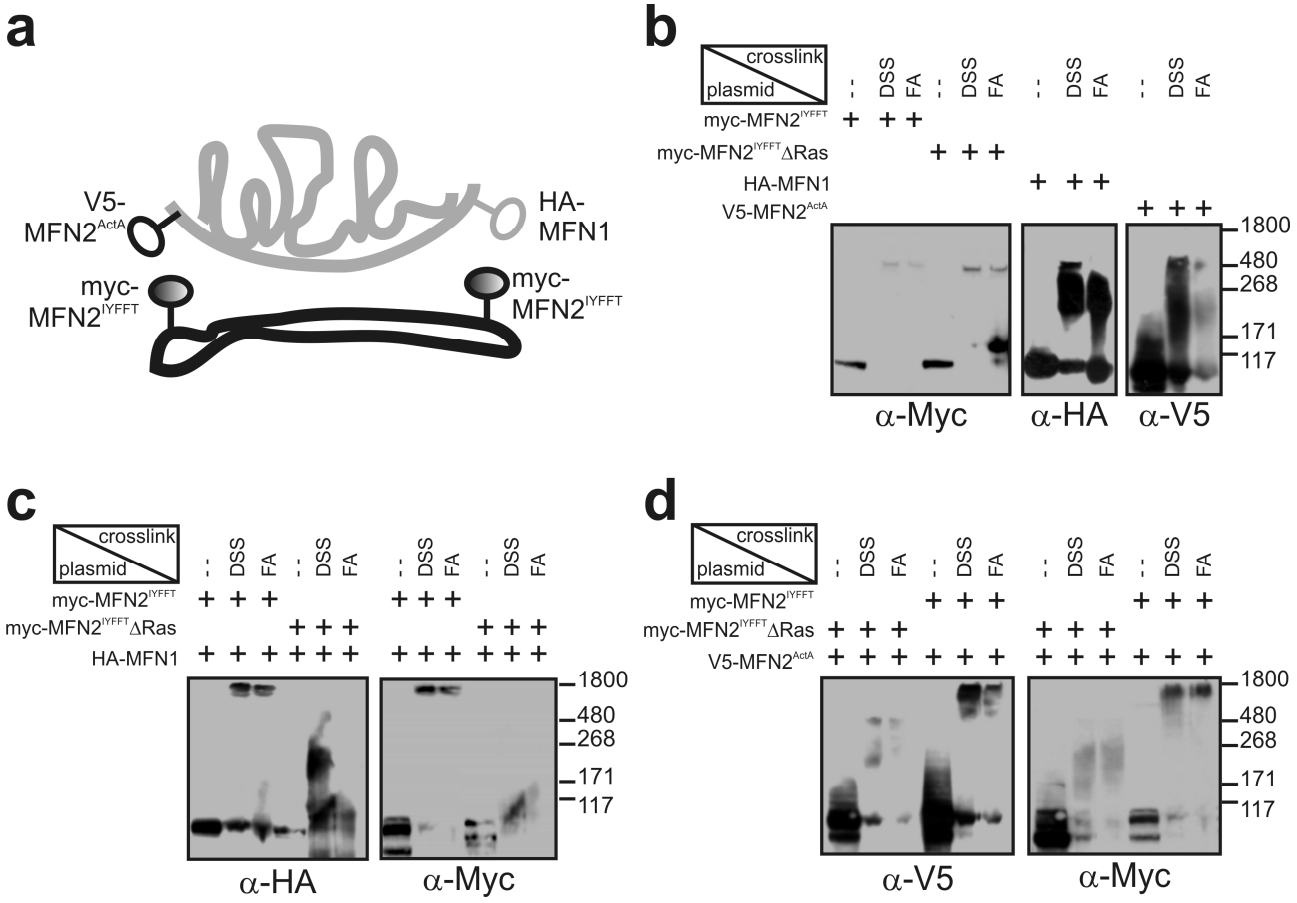
Supplementary Figure 6 – **Electron microscopy of mitochondrial and LM fractions**. Subcellular fractions were Percoll-purified from wt MEFs as described, fixed, processed and analyzed by TEM. Bars, 2 µm.



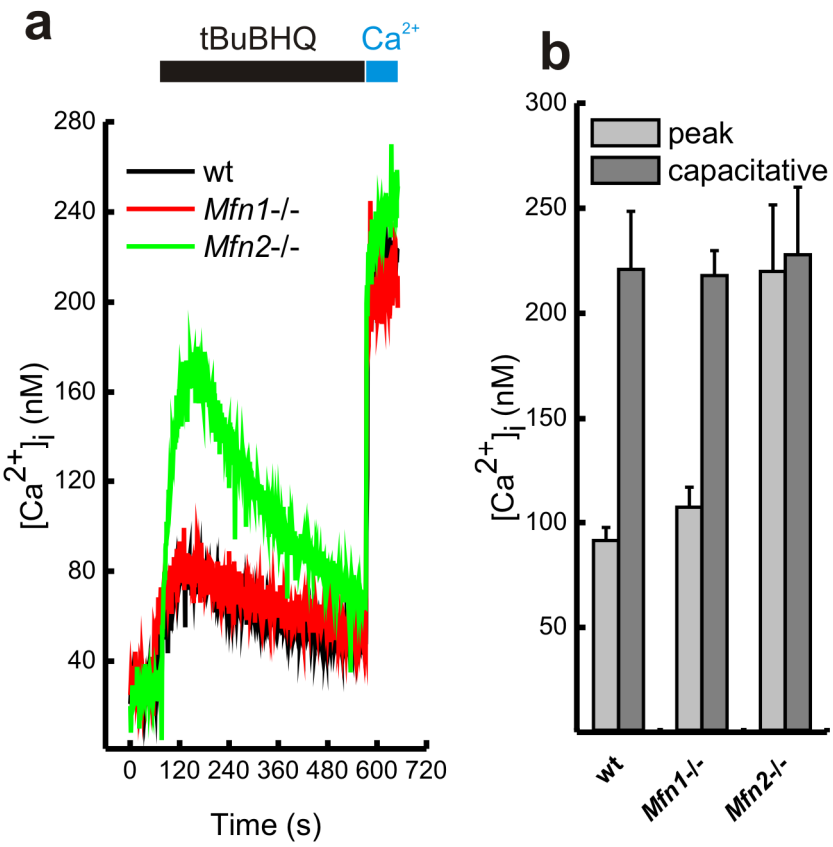
Supplementary Figure 7 - **MFN2^{cb5} corrects ER morphology and tethering to mitochondria in *Mfn2*$^{-/-}$ MEFs.** **a,** Representative randomly selected confocal images of mitochondrial shape in *Mfn2*$^{-/-}$ MEFs cotransfected with mtRFP and where indicated MFN2^{cb5}. Bars, 15 µm. **b,** Representative volume rendered 3D reconstructions of randomly selected z-axis stacks of confocal erYFP images in *Mfn2*$^{-/-}$ MEFs cotransfected with erYFP and where indicated MFN2^{cb5}. Bars, 15 µm. **c,** Representative volume rendered 3D reconstructions of randomly selected z-axis stacks of confocal erYFP and mtRFP images in *Mfn2*$^{-/-}$ MEFs cotransfected with erYFP, mtRFP and where indicated MFN2^{cb5}. Yellow indicates that organelles are closer than ~270 nm. Bars, 15 µm.



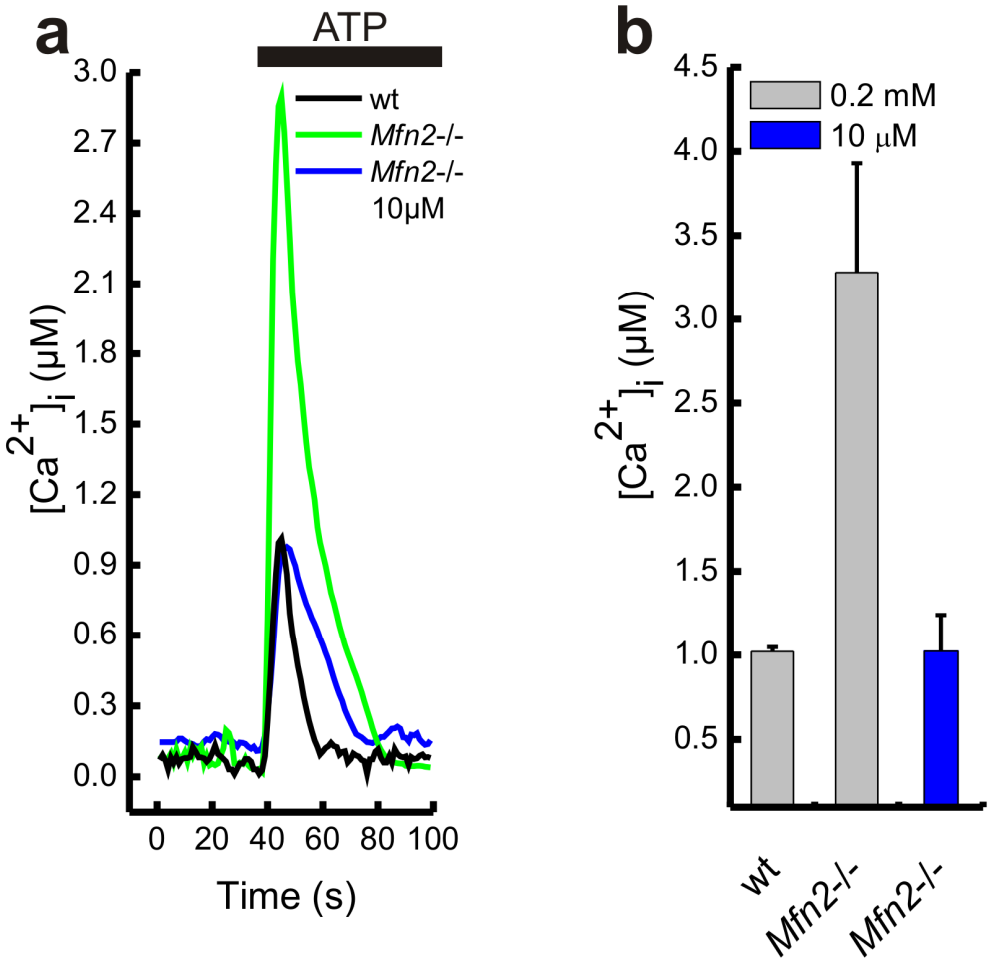
Supplementary Figure 8 - ***In vitro*** **assay of ER-mitochondria interaction.**
a, LM are not pelleted following a 5000*g* centrifugation. Equal amounts (20 µg) of purified mouse liver microsomes or mitochondria were centrifuged at 5000g. Pellet (p) and supernatant (sn) were recovered and proteins were separated by SDS-PAGE and immunoblotted using the indicated antibodies. **b, c**, Interaction between mitochondria and LM depends on temperature and GTP hydrolysis. Equal amounts (20 µg) of purified mouse liver microsomes were co-incubated with equal amounts (20 µg) of purified mouse liver mitochondria at the indicated temperature (**b**), or in the presence of the indicated nucleotide (0.5 mM GTP, 2.5 mM GTPγS, **c**). Following sedimentation, proteins in the pellet (p) and supernatant (sn) were separated by SDS-PAGE and immunoblotted using the indicated antibodies. **d,** Residual *In vitro* interaction between *Mfn2*$^{-/-}$ ER and mitochondria is unspecific. Experiments were as in **(b)**, except that organelles were purified from *Mfn2*$^{-/-}$ MEFs and that where indicated proteins in LM and mitochondria were denatured by boiling the samples for 10 min at 95°C.



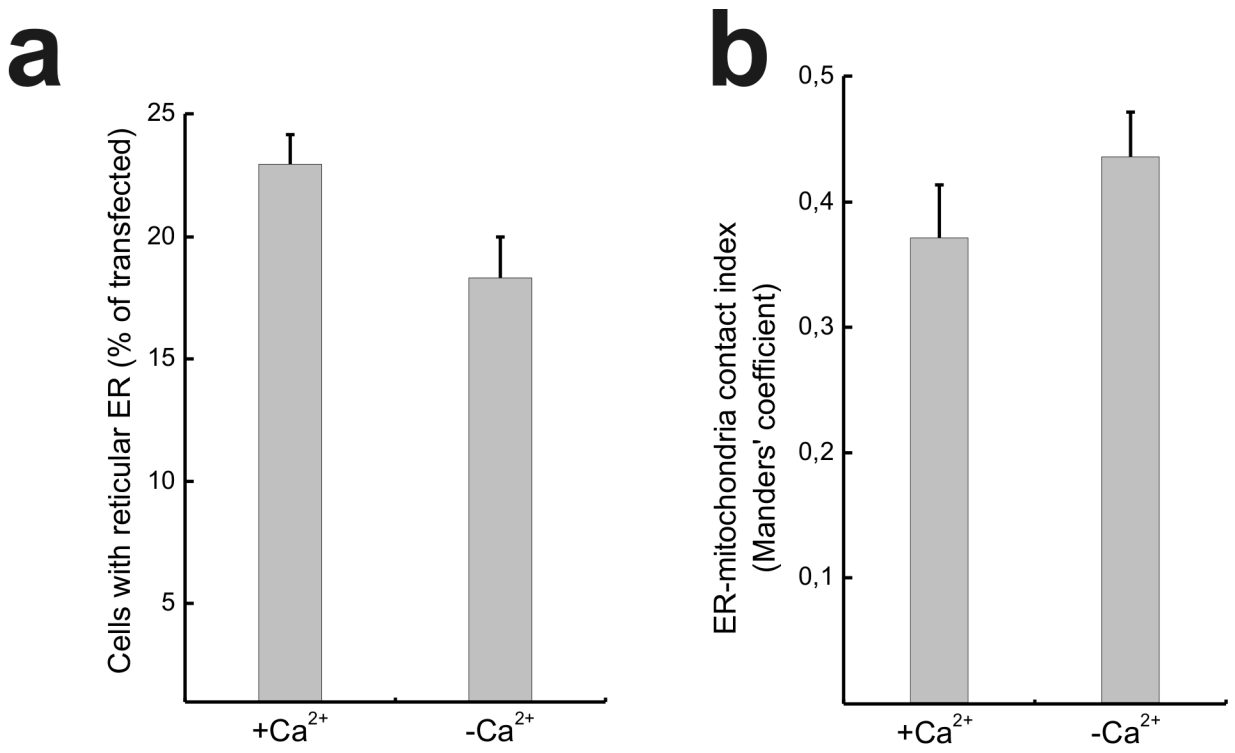
Supplementary Figure 9 - **er-MFN2 needs the p21ras-binding domain to engage in homo- or heterotypic interactions with mitochondrial MFNs.**
Mfn2$^{-/-}$ cells were transfected with the indicated plasmids and fixed using the indicated cross-linkers. Samples were lysed and equal amounts of proteins (50 µg) were separated by SDS-PAGE and immunobloted using the indicated antibodies.



Supplementary Figure 10 – **Capacitative Ca^{2+} entry is not altered in *Mfn2*$^{-/-}$ MEFs.**
a, Representative recordings of $[Ca^{2+}]_i$ in *Mfn2*$^{-/-}$ MEFs loaded with Fura2 and incubated in Ca^{2+}-free KRB. Where indicated, cells were treated with tBuBHQ (0.1 mM) and Ca^{2+} (0.5 mM final concentration) was reintroduced. **b**, Mean ± SEM (n=5) of data from **a**.



Supplementary Figure 11 – **Cytosolic Ca^{2+} transients in wt and *Mfn2*$^{-/-}$ MEFs in response to ATP.** **a,** Representative traces of cytosolic Ca^{2+} changes in wt and *Mfn2*$^{-/-}$ cells transfected with cytAEQ. Where indicated, cells were perfused with ATP (0.2 mM). Where noted, ATP was used at a concentration of 10 μM. **b,** mean ± SEM (n=6) of $[Ca^{2+}]_i$ data from **a**. Cells were perfused with the indicated concentration of ATP.



Supplementary Figure 12 – **ER Ca^{2+} levels do not influence shape of the organelle and its tethering to mitochondria.**
a, *Mfn2*$^{-/-}$ MEFs grown for 12 hrs in regular DMEM (final Ca^{2+} concentration 1.8 mM, +Ca^{2+}) or in Ca^{2+}-free DMEM (final Ca^{2+} concentration 100 μM, - Ca^{2+}) were transfected with erYFP and morphometric analysis of the shape of the ER was performed as described on 15 randomly selected volume rendered 3D reconstructions of *z*-axis stacks of confocal erYFP images. $[Ca^{2+}]_{er}$ determined at the steady state by erAEQ was 198.2 ± 25.3 in MEFs grown in Ca^{2+}-rich medium vs. 102.4 ± 12.2 in Ca^{2+}-free medium, n=3 independent experiments. Data represent mean ± SE of 3 independent experiments. **b,** Experiment was as in (**a**), except that *Mfn2*$^{-/-}$ MEFs were cotransfected with erYFP and mtRFP. Fifteen randomly selected confocal stacks per condition were acquired, reconstructed and rendered and Manders' colocalization coefficient was calculated as described. Data are mean ± SE of 3 independent experiments

Supplementary Methods.

Molecular Biology.

pEYFP-ER (erYFP), fused to the calreticulin targeting and to the KDEL retention sequences for ER, was purchased from BD-Clontech. dsRED fused to the cleavable targeting sequence of subunit VIII of cytochrome c oxidase (mitochondrially targeted dsRED, mtRFP) was a kind gift from M. Zaccolo (Venetian Institute of Molecular Medicine, Padova, Italy). pcB6-Myc-MFN1, pCB6-Myc-Mfn2 and pCB6-Myc-Mfn2^{IYFFT} were kindly provided by M. Rojo (Groupe Hospitalier Pitié-Salpêtrière, Paris, France). pCI-HA-MFN1 was a gift from J.-C. Martinou (U. of Geneva, Geneva, Switzerland). prcCMV-Bcl2-ActA and prcCMV-Bcl2-cb5 were a kind gift of D. Andrews (McMaster University, Hamilton, Ontario, Canada)[1]. Cytosolic (cyt), endoplasmic reticulum (er), and mitochondrial (mt) aequorin (AEQ) were described previously[2].

pCB6-Myc-Mfn2^{R94Q} (MFN2^{R94Q}) was derived by site specific mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagen) with the following primers: 5'-CAG GGT AGG CAT TAT GGA CAT GGC TGA AGT CC-3' and 5'- GGA CTT CAG CCA TGT CCA TAA TGC CTA CCC TG-3'. pCB6-Myc-Mfn2$^{\Delta RAS}$ (MFN2$^{\Delta RAS}$) and pCB6-Myc-MFN2$^{IYFFT\Delta RAS}$ (MFN2$^{IYFFT\Delta RAS}$) were generated from pCB6-Myc-Mfn2 or pcB6-Myc-Mfn2^{IYFFT} by inverse PCR using the following primers 5'-CAG AAC CTG TTC TTC TGT GGT AA-3' and 5'-CTG GCT CGG AGG CAC ATG AAA-3'. The resulting mutants lack aminoacids 77-91, corresponding to the p21Ras-binding domain of MFN2[3].

To subclone MFN2 into prcCMV-Bcl2-ActA, Myc-MFN2 was amplified by PCR using the following primers: 5'- ATG GGA GGT ACC GAA CAA AAA-3' and 5'-TCT GCT GGG CTG CAG GTA CTG-3'. prcCMV-Bcl2-ActA was amplified by inverse PCR using the forward primer 5'- TGC CAG CAT GGC GAG AAT CAG-3' and 5'- GAA TAA ACG CTC AAC TTT GGC AG-3'. The obtained PCR products were blunt-ligated to generate prcCMV-MycMfn2-ActA.. To subclone MFN2 into prcCMV-Bcl2-cb5, Myc-MFN2 was amplified by PCR by

using the primers described above. prcCMV-Bcl2-cb5 was amplified by inverse PCR using the following primers: 5'- GAG TTC GAT TCA ACG GTA CTG AT-3' and 5'- GAA TAA ACG CTC AAC TTT GGC AG-3'. The obtained PCR products were blunt-ligated to generate prcCMV-MycMfn2-cb5. The cDNA corresponding to Myc-Mfn2-ActA and Myc-Mfn2-cb5 was subsequently cloned into the HindIII and BamHI sites of pcDNA3.1Zeo(+) to generate pcDNA3.1-Myc-Mfn2-ActA ($MFN2^{ActA}$) and pcDNA3.1-Myc-Mfn2-cb5 ($MFN2^{cb5}$). Orientation and correctness of all inserts was confirmed by DNA sequencing.

Two siRNA against MFN1 were synthesized from the following sequences 5'-GGCGATTACTGCAATCTTT-3' and 5'-CCAGATGAACCTTTTAACA-3' and against MFN2 from 5'-GAGACACATGGCTGAGGTG-3' and 5'-GGAGAGGGCCTTCAAGCGC-3'. The scrambled control was used at the same final concentration. All siRNA were obtained from Ambion (UK).

Cell culture.

SV40-transformed wt, *Mfn1*$^{-/-}$ and *Mfn2*$^{-/-}$ and *Mfn1*$^{-/-}$ *Mfn2*$^{-/-}$ double-knockout (DMF) mouse embryonic fibroblasts (MEFs) were cultured as described before [4,5]. Transfection was performed using Transfectin (Biorad) according to the manufacturer's instructions.

HeLa cells were a kind gift from C. Montecucco (University of Padova, Italy). Transfection of HeLa cells with siRNAs and plasmid DNA was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. When indicated in the figure legend, cells were transfected with siRNA 24 hrs after seeding on glass coverslips and with mtRFP/erYFP 48hrs after plating.

Imaging.

For confocal imaging, cells seeded onto 24-mm round glass coverslips, incubated in Hanks balanced salt solution (HBSS) supplemented with 10 mM Hepes were placed on

the stage of a Nikon Eclipse TE300 inverted microscope equipped with a spinning-disk PerkinElmer Ultraview LCI confocal system, a piezoelectric *z*-axis motorized stage (Pifoc, Physik Instrumente, Germany), and a Orca ER 12-bit charge-coupled device camera (Hamamatsu Photonics, Japan). Cells expressing erYFP or mtRFP were excited using the 488 nm or the 543 nm line of the HeNe laser (PerkinElmer) with exposure times of 50 msec by using a 60x 1.4 NA Plan Apo objective (Nikon).

For confocal *z*-axis stacks, stacks of 50 images separated by 0.2 µm along the *z*-axis were acquired. Total acquisition time for each stack was 1.1 sec to minimize reconstruction artifacts caused by movement of mitochondria and/or ER. 3D reconstruction and volume rendering of the stacks were performed with the appropriate plugins of ImageJ (National Institutes of Health, Bethesda).

Immunofluorescence.

$2x10^4$ MEFs of the indicated genotype grown on 13 mm round coverslips, were transfected as indicated and fixed after 24h.

For ER staining, cells were fixed for 15 min at room temperature with 4% ice-cold paraformaldehyde in PBS (pH 7.4), permeabilized for 10 sec with ice-cold methanol and incubated for 10 min with 0.38% glycine and 0.27% NH_4Cl in PBS. Cells were blocked by incubation with 3% BSA in PBS for 30 min and subsequentially incubated for 2 h at 25℃ with a rabbit polyclonal anti-Calreticulin (1:500, Upstate).

For anti-MFN2 staining, cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) at RT for 30 min, permeablized with 0.15% TritonX-100 in PBS for 15 min and blocked with 10% BSA in PBS at RT for 45 min. Incubation with anti-MFN2 (rabbit 1:100 or 1:1000, kind gift of R Youle) and with anti-calnexin (goat, 1:100, Stressgen) or with anti-TOM20 (mouse, 1:100 Santa Cruz) was performed in 10% BSA in PBS at RT for 1 h.

For anti-Myc immunofluorescence, cells were fixed with 3.7% formaldehyde in PBS (pH 7.4) for 30 min, permeabilized with 0.01% Nonidet P40 for 20 min at RT and blocked with 0.5% BSA for 15 min. Incubation with anti-Myc (mouse, 1:100, Roche) and with anti-TOM20 (rabbit, 1:200, Santa Cruz Biotechnology) was performed in PBS at RT for 1h.

Staining of the primary antibody was revealed incubating for 60 min at RT with an anti-rabbit, anti-mouse or anti-goat IgG conjugated to the fluorochrome indicated in the figure legend. For detection, green channel images were acquired using the appropriate color channel on the detector assembly of a Nikon Eclipse E600 microscope equipped with a Biorad MRC-1024 laser scanning confocal imaging system with 488± 25 nm bandpass and 525LP filter setting, and with a 60X 1.4 NA oil immersion objective (Nikon).

Morphometric and Contact Analysis.

Morphometric analysis was performed with Imagetool 3.0 (University of Texas Health Science Center, San Antonio). Images of cells expressing mtRFP or erYFP were thresholded by using the automatic threshold function. For morphometric analysis of mitochondria, the major axis length and the roundness index of each identified object were calculated. Cells were scored with elongated mitochondria when >50% of the objects in the image (*i.e.*, mitochondria) displayed a major axis longer than 3 µm and a roundness index below 0.3 (maximum value is 1). For morphometric analysis of ER, major axis length and the elongation index of each identified object were calculated. Cells were scored with reticular ER when major axis was longer than 5 µm and the elongation index was above 4 of more than 50% of the identified objects.

For analysis of the mitochondria to ER interaction, images of cells expressing mtRFP or erYFP were thresholded using the automatic threshold function of ImageJ, followed by deconvolution, 3D reconstruction and surface rendering using the VolumeJ plugin of

ImageJ. Analysis of the interaction between ER and mitochondria was performed using Manders' colocalization coefficient (MCC)[6].

Subcellular Fractionation.

Mouse liver or 10^9 MEFs of the genotype indicated in the figure legends were washed once with PBS, suspended in isolation buffer (IB, 200 mM sucrose, 1mM EGTA-Tris, and 10 mM Tris-MOPS, pH 7.4), and then disrupted by dounce homogenization. The homogenate was spun at 800*g* for 10 min; the supernatant was recovered and further centrifuged for 10 min at 8000*g*. The resulting pellet (mitochondrial fraction) was collected while the supernatant was further spun for 30 min at 100,000*g*. The resulting pellet (LM fraction) and supernatant (cytosolic fraction) were spun again at 100,000*g* to further purify the fractions. The mitochondrial fraction was further purified by centrifuging twice at 8000*g* for 10 minutes. The obtained pellet was purified by centrifugation at 95000*g* for 30 minutes on a 30% Percoll gradient in IB. The obtained mitochondrial layer was washed free of Percoll and resuspended in IB. Alternatively, subcellular fractions were obtained by differential centrifugation from mouse liver as described[7]. Mitochondrial associated membranes (MAM) were identified as an intermediate layer between the light membranes and the mitochondrial fraction on the Percoll gradient as previously described[8]. Protein concentration was determined by bichinconnate assay (Pierce) and 25 µg of protein were separated by SDS-PAGE and immunoblotted as indicated in the figure legends.

In vitro ER-mitochondria interaction assay.

Subcellular fractions were purified as described above and resuspended in reaction buffer (RB, 150 mM KCl, 1 mM KH_2PO_4, 5 mM $MgCl_2$, 10 mM sodium succinate, 10 mM Tris, pH 7.4). Twenty µg of mitochondrial and 20 µg of LM protein were incubated for 5 min at 37℃. Subsequently the reaction was centrifuged for 5 min at 5,000 g at 4℃. The pellet

contained mitochondria and the LM fraction interacting with mitochondria, while the supernatant contained the remaining LM fraction.

Where indicated *in vitro* interaction was performed on mitochondria that were previously boiled (10 min at 95℃). For digestion of mitochond rial outer membrane proteins, 0.1 µg/ml of mitochondrial protein was digested with 100 µg/ml proteinase K for 30 min on ice or with 5 µg/ml of trypsin for 15 min at RT. Reactions were inhibited by the addition of 0.25 mM PMSF. Mitochondria were subsequently pelleted and resuspended in RB. Where indicated, MAMs from mouse liver hepatocytes (20 µg) were added

Total proteins in pellet and supernatant were separated by SDS-PAGE and immunoblotted as detailed in the figure legends.

Immunoprecipitation.

For immunoprecipitation, 100 µg of total cellular extract was dissolved in CHAPS buffer [1% CHAPS, 100 mM KCl, 50 mM Hepes pH 7.5, 1 mM EGTA, complete protease-inhibitor mixture (Sigma)] (for immunoprecipitation with anti-Ras) or in NP40 lysis buffer [1% NP-40, 0.15M NaCl, 5 mM Tris, pH 7.4)] (for immunoprecipitation with anti-Myc) and pre-cleared by centrifugation at 16,000*g* . Cleared extracts were then incubated with the indicated antibody (anti-p21Ras, Oncogene, 1:100; anti-Myc, Roche, 1:50) for 2 hours, and then adsorbed on 50 µl agarose beads conjugated with protein-G for 1 hour (anti-Ras) or 12 hrs (anti-Myc) at 4℃. Following 3 washes, be ads were boiled in NuPage loading buffer (Invitrogen). Protein was separated by SDS-PAGE and immunoblotted using the indicated antibodies.

Cross-linking.

For protein crosslinking, $2x10^5$ cells were washed in PBS and then were treated with 2.5 mM DSS (Pierce) or 10% Formaldehyde in PBS for 15 min at 37ºC. Crosslinker was

quenched by the addition of 25 mM Glycine for 15 min at 37℃. Samples were spun for 10 min at 5000g at 4ºC, and pellets were then washed with PBS supplemented with 1% BSA. After a further centrifugation, samples were dissolved in NuPage loading buffer (Invitrogen), sonicated to disrupt DNA and membranes and centrifuged to remove the unsoluble material, proteins were separated by SDS-PAGE.

Cell lysates for immunobloting.

Twenty-four hours after transfection, cells (10^6) were harvested and disrupted in RIPA buffer (150 mM NaCl, 1% Nonidet P-40/0, 0.25% deoxycholate, 1 mM EDTA, 50 mM Tris, pH 7.4) in the presence of complete protease-inhibitor mixture (Sigma).

Immunoblotting.

Extracted proteins (50 µg) were separated by 4-12% SDS-PAGE (NuPAGE, Invitrogen), transferred onto polyvinylidene difluoride (PVDF, BioRad) or nitrocellulose membranes (Amersham). Membranes were probed using the following antibodies:; anti-actin (1:2000, Chemicon); anti-Calreticulin (1:500, Upstate); anti-MnSOD (manganese superoxide dismutase, 1:2000, Stressgen); anti-MFN2 (1:1000[9]; Abcam; Sigma or Abnova); anti-MFN1 (1:500[9] or Abcam); anti-COX (oxidative phosphorylation Complex IV subunit II; 1:1000, Molecular Probes); anti-Tom20 (translocase of the outer membrane 20 kDa , 1:2000, Santa Cruz Biotechnology); anti-SERCA3 (sarcoplasmic endoplasmic reticulum Ca^{2+} ATPase 3,1:100, ABR); anti-IP3RI (inositol-3-phosphate receptor type I, 1:700, ABR), anti-LDH (lactate dehydrogenase, 1:5000, Rockland); anti-FACL4 (fatty acid-CoA ligase 4, 1:1000, Abgent or 1:1000, Santa Cruz Biotechnology); anti-Calnexin (1:1000; Stressgen); anti-Myc (1:1000, BDPharmingen or 1:1000, Roche); anti-V5 (1:1000, Invitrogen); anti-HA (1:2, kindly provided by I. Mellman, Yale University, New Haven[10]).

Transmission electron microscopy and electron tomography.

Cells were fixed for 1 h at 25℃ using 1% glutaraldehyde in 0.2 M Hepes, embedded in plastic, sectioned, and stained with uranyl acetate and lead citrate.

Thin sections were imaged on a Tecnai-12 electron microscope (Philips-FEI) at the Telethon EM Core Facility (TeEMCoF, Istituto Mario Negri Sud). For tomography, colloidal gold particles were applied to one side of 200-nm-thick sections as alignment markers. Tilt series of 122 images were recorded on the Tecnai-12 electron microscope, operated at an acceleration voltage of 120 kV. The images were recorded around one tilt axis, each over an angular range of 120° with a 1° tilt interval. The tilt images were aligned as previously described[11] and tomographic reconstructions were made using the weighted back-projection method[12]. Image processing was done using the SGI system. The volumes of the whole tomograms had dimensions of 512 x 512 x 80-100 pixels depending on section thickness, with a pixel size range of 2.5-4.1 nm. Surface-rendered models of selected areas of the different tomograms were produced using Recontruct software[13] to trace the volume and render it.

For staining with glucose-6-phosphatase, cells were fixed with 1.25% gluteraldehyde in PIPES buffer (0.1M PIPES pH 7.0; 5 % sucrose) for 30 min on ice, incubated for 2 h at 37℃ in reaction buffer (60 mM glucose-6-phosphate, 1% lead nitrate, 80 mM TRIS-Maleate pH 6,5) and the reaction was stopped by the addition of Tris-Maleate buffer (80mM Tris-Maleate pH 6.5). Samples were washed with cacodylate buffer (0.1 M cacodylate, 5% sucrose, 5 mM $CaCl_2$, 5 mM $MgCl_2$), fixed again with 1% gluteraldehyde in cacodylate buffer for 30 min at RT and further embedded, sectioned and stained as described above[14]

Ratiometric measurement of cytosolic Ca^{2+} concentration

MEFs (10^6) were incubated at 37℃ in Krebs Buffer (KRB, 125 mM NaCl, 5 mM KCl, 1 mM Na_3PO_4, 1 mM $MgSO_4$, 5.5 mM glucose, 20 mM HEPES, pH 7.4) containing 1 mM Ca^{2+}, 10% FBS and 1 µM Fura2-AM (Molecular Probes). After 45 min, cells were washed free of Fura2-AM and resuspended in Ca^{2+}-rich or -free KRB at a density of 10^6/ml. Ratiometric Ca^{2+} measurements were performed using λ_{ex} 340±5 and 380±5, and λ_{em} 510±15 nm at 37℃ in a RF-5301 PC fluorimeter (Shimadzu) equippe d with fast filter and magnetic stirring. Fura2-AM ratio values were converted into Ca^{2+} concentrations, using a K_d for Ca^{2+} of 225 nM[15].

***Aequorin* measurement of Ca^{2+} concentration.**

Cells grown on 13 mm round glass coverslips at 50% confluence were cotransfected with cyt, mt or erAEQ and the plasmids indicated in figure legends. Cyt and mtAEQ reconstitution, measurement and calibration were performed as described[16] in KRB containing 1 mM Ca^{2+}. For reconstituting with erAeq, the luminal [Ca^{2+}] of the ER was first reduced by incubating cells for 5 min at 37℃ with KRB supplemented with 1% FBS, 1mM EGTA, 10 µM ionomycin and 0.1 mM tBuBHQ. Cells were then washed 3 times with KRB containing 1 mM EGTA and 5% FBS and reconstituted for 3 h at 4℃ with KRB supplemented with 0.1 mM tBuBHQ, 1 mM EGTA and 5 µM Coelentarazine N. All AEQ measurements were carried out in KRB. Agonists and other drugs were added to the same medium.

In experiments with permeabilized cells, a cytosolic buffer (intracellular buffer, ICB: 130 mM KCl, 10 mM NaCl, 1 mM K_3PO_4, 1 mM $MgSO_4$, 2 mM sodium succinate, 20 mM Hepes, pH 7.05, 37℃, calculated [Ca^{2+}] by atomic absorption spectrometry was 8 µM) was used for perfusion. When indicated, IB was supplemented with 100 mM EGTA or 0.001%) digitonin.

Fluorescence Recovery After Photobleaching (FRAP).

Cells plated and transfected with erYFP as described above, after 24 hrs were incubated in HBSS and placed on the stage of a laser scanning microscope (TCS SP5, Leica). Using the LasAF software (Leica), 16 μm^2-regions were manually defined to be bleached. To bleach the YFP fluorescence, 1 z-plane was bleached for a total of 3 s using 100% laser power of the 488 nm laser line with a 63X, 1.4NA objective. The post-bleaching images were taken at 1 s intervals for a total of 150 s. Intensities of the photobleached regions were measured and normalized to the intensities of the same region before photobleaching (photobleaching effect) and the neighbouring regions at the same time point (auto-bleaching effect during the recording).

Supplementary Notes

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