APPENDIX SUPPLEMENTARY METHODS:

Antibodies and reagents

Primary antibodies used in this study were: rabbit polyclonal anti-GFP (Life technologies) and monoclonal anti-PDI (Genetex) for IEM; monoclonal anti-Tom20 (BD Bioscience) for immunofluorescence; monoclonal anti-FLAG M2 (Sigma-Aldrich) for immunoblot and immunofluorescence; monoclonal anti-GFP (Roche), anti-β-actin (Abcam), anti-HA (Sigma-Aldrich), anti-α-tubulin, monoclonal IP3R-3 and cytochrome c (BD Biosciences), rabbit polyclonal anti-ORP8 (Genetex), anti-ORP5, anti-VAPA (Sigma-Aldrich) and anti-VAPB (Atlas antibodies) for immunoblot; monoclonal anti-ORP8 (Santa Cruz) for IP.

Plasmids and cDNAclones

The following reagents were kind gifts: mRFP-Sec61βand GFP-Sec61β from T.Rapoport (Harvard University) [1], ssHRP-KDEL from T. Schikorski[2], PTPIP51-HA from C.Miller (King's College London) [3], EGFP-ORP5L389D, EGFP-ORP8L425D [4] and EGFP-VAPB from P. De Camilli. cDNA clones of human ORP8 and ORP5 were obtained fromDharmacon (GE Healthcare) (ORP8: Clone 40029118, Accession BC111728; ORP5: Clone 5769002, Accession BC039579).

Cloning of EGFP-ORP5, EGFP-ORP8 and 3XFLAG-ORP8

cDNAs of ORP8 and ORP5 were amplified by PCR. In all PCR reactions Herculase II fusion DNA polymerase (Agilent) was used. Primers used were (coding sequence shown in lowercase):

5' XhoI-CT-ORP5_Fw CGC *CTCGAG* CT atgaaggaggaggccttcct

3’ HindIII-ORP5_Rv ACCG *AAGCTT*ctatttgaggatgtggttaatga

5’ BglII-ORP8_Fw CGC *AGATCT*atggagggaggtttggcagat

3’ ApaI-ORP8_Rv ACCG *GGGCCC*ctacttgaacatgaagtttattatg

5’ NotI-ORP8_Fw ATAAAGAAT *GCGGCCGC*atggagggaggtttggcagat

3’ XbaI-ORP8_Rv CG *TCTAGA*ctacttgaacatgaagtttattatg

PCR products were ligated between BglII and ApaI for ORP8, and between XhoI and HindIII for ORP5, in the pEGFP-C1 vector (Clontech)to generate EGFP-ORP8 or EGFP-ORP5. ORP8 PCR product was ligated between NotI and XbaI in the p3XFLAGCMV10 vector (SIGMA-Aldrich) to generate 3XFLAG-ORP8.

Cloning of EGFP-tagged ORP5, ORP8 and PTPIP51-HA domain deletion constructs and EGFP-tagged ORP5 ORD domain

ORP5ΔPH (residues 126-243) and ORP8ΔPH (residues 148-265) were generated by site directed mutagenesis using the following primers:

ORP5ΔPH_Fw 5’-cagtaggctagagcagctggggtctgtcag-3 ‘

ORP5ΔPH_Rv5'-ctgacagaccccagctgctctagcctactg-3'

ORP8ΔPH_Fw5'-gctcagtacaatcacagatccttcttgttctagtcttcttaaacg-3'

ORP8ΔPH_Rv5'-cgtttaagaagactagaacaagaaggatctgtgattgtactgagc-3’

ORP5ΔORD (residues 126-243) and ORPΔTM (residues 148-265) were generated by site directed mutagenesis using the following primers:

ORP5ΔORD_Fw: 5’-cagaggagaacaagagtctggaggaccacagcccctgggac-3'

ORP5ΔORD_Rv: 5’-gtcccaggggctgtggtcctccagactcttgttctcctctg-3'

ORP5ΔTM_Fw: 5’-cgactgcagaattcgaagcttctatcgggggctctgcaggagg-3'

ORP5ΔTM_Rv: 5’-cctcctgcagagcccccgatagaagcttcgaattctgcagtcg-3'

PTPIP51ΔTM_Fw: 5’-ccctgggtggtgcccgtgccgactatacgcagacttcagatc-3’

PTPIP51ΔTM_Rv: 5’-gatctgaagtctgcgtatagtcggcacgggcaccacccaggg-3’

For the cloning of the ORP5 ORD domain (residues 331-771) , primers flanking the target region were created and cDNA was amplified by PCR from EGFP-ORP5 using the following primers:

5’ XhoI-CT-ORP5 ORD_Fw$CCG\mathit{CTCGAG}$ CT atggagacccctggggccccggt

3’ HindIII-ORP5 ORD _Rv$AACG\mathit{AAGCTT}$ctactgtggccggagggctggtcg

ORP5 ORD PCR product was ligated between XhoI and HindIII in the pEGFP-C1 vector (Clontech)to generate EGFP-ORP5 ORD.

siRNAs

For knockdowns, HeLa cells were transfected with control or ORP8/5 siRNAoligos by using oligofectamine (Life Technologies) and cultured for 48 hours prior to analysis. Double-stranded siRNAs were derived from the following references:

ORP8 (ON-TARGETplussiRNA Smart pool siRNAs: J-009508-05 and J-009508-06 from GE Healthcare)

ORP5 (ON-TARGETplussiRNA Smart pool siRNAs: J-009274-10 and J-009274-11 from GE Healthcare)

VAPA (siRNA Standard: GEHCF-000031 from GE Healthcare)

VAPB (GE Healthcare)[3]

Control (NC1 negative control duplex from IDT).

Fluorescence Microscopy

Immunofluorescence and Confocal Microscopy

Transfected cells were fixed with 4% PFA/PBS for 15 min at room temperature, washed in PBS and incubated with 50mM NH4Cl/PBS for 15 min at room

temperature. After washing with PBS and blocking buffer (1% BSA/ 0,1% Saponin in PBS), cells were incubated with primary antibodies diluted in blocking buffer for 1h at room temperature and then with fluorescently-labeled secondary antibodies. After washing with blocking buffer and then PBS, coverslips were mounted on microscopy slides and images were acquired on a LSM 780 confocal microscope (Zeiss). Optical sections were recorded 63x/1.4Oil immersion objective and fluorescent pictures were collected with a PMT GaAsP camera. Images from a mid-focal plane are shown. Images were processed using Adobe Photoshop CS6 software. Figure compilation was accomplished using Adobe Illustrator CS6.

By using image J find-peaks plugin we measured the max intensity at PM and cytoplasm line-based profiles (3 for each cell).

TIRF microscopy

Total internal reflection fluorescence (TIRF) microscope was performed around a setup build around an AxioObserver Z1 microscope (Zeiss) equipped with 100X, ON 1.46 objective. Excitation light was provided by 488nm laser coupled to the TIRF illuminator through an optical fiber. Fluorescence was detected with an EMCCD camera iXon 897 (Andor, QE 90%). Fluorescence was analysed off-line using Fiji (http://fiji.sc/wiki/index.php/Fiji).

Electron Microscopy Analysis

HRP Detection

HeLa cells expressing HRP-KDEL were fixed on coverslips with 1.3% glutaraldehyde in 0.1 M cacodylate buffer, washed in 0.1 M ammonium phosphate [pH 7.4] buffer for 1h and HRP was visualized with 0.5 mg/ml DAB and 0.005% H_2O_2 in 0.1 M Ammonium Phosphate [pH 7.4] buffer. Development of HRP (DAB dark reaction

product) took between 5 min to 20 min and was stopped by extensive washes with cold water. Cells were postfixed in 2% OsO_4+1% $K_3Fe(CN)_6$ in 0.1 M cacodylate buffer at 4°C for 1h, washed in cold water and then contrasted in 0.5% uranyl acetate for 2hrs at 4°C, dehydrated in an ethanolseries and embedded in epon as for conventional EM[5]. Ultrathin sections were counterstained with 2% uranyl acetate before observation.

Immunogoldlabelling

HeLa cells were fixed with a mixture of 2%PFA and 0.125% glutaraldehyde in 0.1 M phosphate buffer [pH 7.4] for 2hrs, and processed for ultracryomicrotomy as described previously [6]. Ultrathin cryosections were single- or double-immunogold-labeled with antibodies and protein A coupled to 10 or 15 nm gold (CMC, UMC Utrecht, The Netherlands), as indicated in the legends to the figures. Sections of resin-embedded cells and immunogold-labeledcryosections were observed under a FEI Tecnai 12 microscope equipped with a CCD (SiS 1kx1k keenView) camera.

Quantifications

For the quantification in HRP-stained Epon sections, the total length of plasma membrane or the circumference of each mitochondria and the length of the multiple HRP-positive ER segments closely associated (<30nm) with them were measured by iTEM software (Olympus) on acquired micrographs of HeLa cells for each of 30 cell profiles. Cells were randomly selected for analysis without prior knowledge of transfected plasmid or siRNA.

For the quantification of immunogoldlabeling on ultrathin cryosections, 800 gold particles were counted in randomly selected cell profiles and assigned to non-cortical ER, ER-PM or ER-mitochondria MCS in each of three experiments. All data are presented as mean ± SEM of three experimental replicates.

Biochemical Analysis

Immunoprecipitation of ORPs

Transfected HeLa cells were washed in cold PBS and lysed on ice in lysisbuffer [50 mMTris, 120 mMNaCl, 40 mMHepes, 0,5% digitonin, 0,5% CHAPS, pH 7.36, and protease inhibitor cocktail (Roche) or 50 mMTris, 150 mMNaCl, 0.5% NP40, 10 mM EDTA, pH 7.2, and protease inhibitor cocktail for 3XFLAG-ORP8 immunoprecipitation]. Cell lysates were then centrifuged at 21,000 g for 20 min at 4°C. Supernatants were then incubated with anti-ORP8 (Santa Cruz) or control anti-IgG2A (Sigma-Aldrich) antibodies and protein A/G beads for 2h at 4°C,Chromotek GFP-trap agarose beads (Allele Biotech) or anti-HA agarose beads (Pierce) for 1h at 4°C under rotation. Subsequently beads were washed in 0.1 M phosphate buffer. After extensive washes in cold lysis buffer, immunoprecipitated proteins bound to the beads were processed for Mass Spectrometry analysis (see below) or incubated in sample buffer (containing 2% SDS) and then boiled for 1 min. In the latter case immunoprecipitates were loaded and separated in 10% SDS–PAGE gel and immunoblotting was carried out.

Cell fractionation

HeLa cells were harvested and washed with PBS by centrifugation at 600g for 5min. The cell pellet was resuspended in starting buffer (225 mMmannitol, 75 mM sucrose and 30 mMTris-HCl pH 7.4) and homogenized using a Tissue Grinder Dura-Grind®, Stainless Steel, Dounce (Wheaton). The homogenate was centrifuged three times at 600g for 5 min to remove nuclei and unbroken cells. The crude mitochondria was pelleted by centrifugation at 10 000g for 10 min. To separate MAM and pure mitochondria fractions, the pellet was resuspended in MRB buffer (250 mMmannitol,

5 mM HEPES and 0.5 mM EGTA, pH 7.4) and layered on top of different concentrations of Percoll gradient (225 mMmannitol, 25 mM HEPES, 1 mM EGTA pH 7.4 and 30% or 15% Percoll). After centrifugation at 95 000g for 30 min, two dense bands containing either the pure mitochondria or MAM fraction were recovered and washed twice with MRB buffer by centrifugation at 6300g for 10 min to remove residual Percoll and residual contamination. MAM was pelleted by centrifugation at 100 000g for 1h and resuspendedin Lysis Buffer (50 mMTris, 150 mMNaCl, 1% Triton X-100, 10 mM EDTA, pH 7.2, and protease inhibitor cocktail) and protein concentrations were determined by Bradford assay. Equal amount of proteins were loaded on 4-20% gradient SDS-PAGE gels (Biorad) and immunoblotting was carried out.

Western blot

For immunoblotting, cells were resuspended in lysis buffer, centrifuged at 21000g for 20 min at 4°C. The supernatants were boiled in reducing SDS sample buffer and proteins were separated using 10% SDSPAGE and immunoblot was carried using standard methods [5].

Mass spectrometry analysis

MS analysis was carried out by the proteomics/mass spectrometry platform in IJM (http://www.ijm.fr/plateformes/spectrometrie-de-masse).Briefly, after washes with binding buffer, immunoprecipitations beads were rinsed with 100 µl of NH4HCO3 25 mmol/l. Proteins on beads were digested overnight at 37°C by sequencing grade trypsin (12,5 µg/ml; Promega Madison, Wi, USA) in 20 µl of NH4HCO3 25 mmol/l. Digests were analysed by an Orbitrap Fusion (Thermo Fisher Scientific, San Jose, CA) equipped with a Thermo Scientific EASY-Spray nanoelectrospray ion source and coupled to an Easy nano-LC Proxeon 1000 system (Thermo Fisher Scientific, San

Jose, CA). MS/MS data were processed with Proteome Discoverer 1.4 software (Thermo Fisher scientific, San Jose, CA) coupled to an in house Mascot search server (Matrix Science, Boston, MA ; version 2.4.2). MS/MS datas were searched against SwissProt databases with Homo sapiens taxonomy.

Mitochondrial respiration assay

OXYGEN CONSUMPTION RATE (OCR) was measured using the extracellularflux analyzer XF_p (Seahorse Bioscience Inc.). Hela cells transfected with Ctrl or ORP5 siRNA were plated in a Seahorse XF_p tissue culture plate on day 1. On day 2, the OCR was measured over a period of 2 minutes. 3 consecutive measurements of OCR were done in basal conditions. Next, oligomycin (OM), a selective inhibitor of mitochondrial ATP synthase was injected at a concentration of 8 µM (final concentration in well of 1 µM). The reduction in OCR indicates the amount of oxygen consumption used for the production of ATP. 3 consecutive measurements were done. Next, Antimycin-A (AA), an inhibitor of complex III, was added at a concentration of 5 µM (final concentration in well of 0.5 µM). AA abolishes the electron transport chain activity, abrogating mitochondrial oxygen consumption. This allows for the calculation of basal mitochondrial respiration (OCR_{BAS}). The leftover OCR corresponds to non-mitochondrial oxygen consumption. All mitochondrial inhibitors were from Sigma.

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