



Supplementary Materials for

ER-mitochondria tethering by PDZD8 regulates Ca²⁺ dynamics in mammalian neurons

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Material and Methods

Animals

All animals were housed and handled according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Columbia University. Time-pregnant CD1 females were purchased from Charles River (Wilmington MA, USA).

Constructs

pCAG::mito (CoxVIII)-mTAGBFP2 was previously described (53). C-terminal HA-tagged mouse PDZD8 was cloned with from cDNA library obtained from C57/B16 mouse brain by PCR. pCAG PDZD8HA was created by inserting the mouse PDZD8 coding sequence at XhoI-NotI sites of pCIG2 vector. pCAG PDZD8HA resistant to shPDZD8 was created by primers having silent mutations at the shPDZD8 targeting site. pLKO1.5 shPDZD8 (TRCN0000431056) was from Sigma Aldrich. Control vector used in shRNA experiments is the pLKO1 vector from TRC library (expressing small RNA sequence CCGCAGGTATGCACGCGT). pCAG::Calnexin-YFP was created by replacing mitochondria targeting sequence of pCAG::mito-YFP (53) with Calnexin coding sequence. pCAG::Calnexin-mCherry was created by replacing YFP sequence with mCherry sequence. Synthetic ER-mitochondria tethering construct was created by replacing mitochondria targeting sequence of pCAG-mito-mtagBFP2 (53) with DNA oligomers coding a mitochondria targeting sequence (AKAP) and inserting DNA oligomers coding ER targeting sequence (UBC6) at the 3' end of mtagBFP2 at the BsrGI site (28). pCMV::CEPIA3mt, pCMV::R-CEPIA1er, and pCMV::G-CEPIA1er were gifts from Masamitsu Iino (Addgene plasmid # 58219, 58216, 58215, respectively). For cytosolic RCaMP1h, we inserted RCaMP1h from pRSET-RCaMP1h (Addgene plasmid #42874) to pCIG2 by replacing IRES-GFP. pCAG::mito-RCaMP1h was generated by swapping GCaMP5G with RCaMP1h in pCAG::mito-GCaMP5G (54). pCAG::G-CEPIA1er and pCAG::CEPIA3mt were created by inserting G-CEPIA1er and CEPIA3mt into pCAG vector, respectively.

Generation of Pdzd8-Venus knock-in Neuro2a cell line

For CRISPR-Cas9 plasmid, CRISPR guide RNA was designed using Optimized CRISPR Design tool (<http://crispr.mit.edu>) and cloned into pSpCas9(BB)-2A-Puro (PX459) V2.0 (a gift from Feng Zhang; Addgene plasmid # 62988) as previously described (55). For the donor vector, 5' arm sequence was amplified from pCAG-PDZD8HA with primers containing silent mutations at the CRISPR target site. 3'UTR sequence was cloned from a bacterial artificial chromosome (RP23-373G10, BACPAC resources). These sequences, together with Venus, P2A, and Neo sequences, were cloned into the XhoI-NotI restriction enzyme sites of pCIG2 with In-Fusion HD cloning kit (Takara Bio). Neuro2a cells were transfected with CRISPR-Cas9 plasmid and the donor vector by Jetprime (Polyplus-transfection). Cells were replated after 24 hours and treated with puromycin (2 μ g/ml) to select transfected cells for 2 days. The selected cells were then cultured with Geneticin (G418 sulfate, 400 μ g/ml; Sigma-Aldrich A-1720) to select successfully recombined cells.

Super-resolution imaging (3D-SIM)

Neuro2a-PDZD8-Venus-KI cells were plated on a High-performance cover glass (D =

0.17, Carl Zeiss Microscopy) and transfected with pCAG Canx-mCherry by Jetprime and cultured for two days. Transfected cells were fixed with 4% PFA for 10' followed by fixation with ice cold methanol for 6min. Cells were then treated with 3% BSA/PBS for 30' followed by an incubation with primary antibodies against GFP (1:2000, GFP-1020; Aves labs), RFP (1:1000, ab62341; Abcam), and anti-OxPhos Complex (1:500, Thermo Fisher Scientific) overnight at 4°C. After treated with secondary antibodies anti-mouse IgG Alexa 405, anti-chicken IgG Alexa 488, and anti-rabbit IgG Alexa 546 (Thermo Fisher Scientific), the coverslip was mounted on a slide glass with ProLong® Diamond Antifade Mountant (Thermo Fisher Scientific). Cells were imaged with Nikon Ti Eclipse inverted microscope equipped with a SIM illuminator, an iXon Ultra 897 camera (Andor), and a × 100 plan apochromat immersion oil objective (NA 1.49). Image reconstruction and analysis were performed with the NIS-Elements software (Nikon).

Cell Culture

Neuro2a (ATCC), HeLa cells (ATCC), PDZD8 KO HeLa cells (gift from Dr. Joseph Sodroski) and NIH3T3 cells (ATCC) were maintained with Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific) supplemented with 10% FBS (Gemini Bio-products) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific) under 5% CO₂.

Neuronal Culture

Embryonic mouse cortices (E15.5) were *ex utero* electroporated following the injection of plasmids to ventricles, then dissected in Hank's Buffered Salt Solution (HBSS) supplemented with HEPES (10mM, pH7.4). Tissues were incubated in HBSS containing papain (Worthington; 14U/ml) and DNase I (100 µg/ml) for 15min at 37°C. Then, samples were washed 3 times with HBSS containing DNase I, and dissociated by gentle pipetting. Dissociated cells (8.5×10^4) were plated on poly-D-lysine (1mg/ml, Sigma)-coated glass bottom dishes (MatTek, 35mm dishes with 14mm glass diameter) in Neurobasal media (Invitrogen) containing B27 (1x), Glutamax (1x), FBS (2.5%) and penicillin/streptomycin (0.5x) (all supplements were from Invitrogen). After 5 to 7 days, media were changed with FBS-free supplemented Neurobasal medium.

Structural modeling

Structural homology search for, and corresponding structural homology modeling based on PDB structures mapping to PDZD8 and Mmm1 was performed through the Phyre2 web portal (56). Structural analysis, tertiary alignments and visualizations of the E-syt2 (4P42) and Mdm12 (5gyk) PDB structures and SMP-domain homology models generated for PDZD8 and Mmm1 was carried out with PyMOL Molecular Graphics System (Version 1.8 Schrödinger, LLC).

Yeast strain construction

S. cerevisiae strains for *mmm1Δ*-rescue experiments were derived from the heterozygous *mmm1Δ/MMM1* diploid strain YLL006W (BY4743; MAT α/α , *his3Δ1/his3Δ1*, *leu2Δ0/leu2Δ0*, *LYS2/lys2Δ0*, *met15Δ0/MET15*, *ura3Δ0/ura3Δ0*) from the Yeast Knockout Collection (GE Healthcare Dharmacon Inc.). All other strains were derived from from wild-type BY4741 (MAT α *his3Δ0*, *leu2Δ0*, *met15Δ0* and *ura3Δ0*) from Open

Biosystems (Huntsville, AL). C-terminal tagging of yeast endogenous proteins with fluorescent proteins was performed through homologous recombination as previously described (57). Briefly, PCR-fragments with 40bp-flanking regions homologous to 40bp upstream and downstream of pertinent stop-codons, containing mCherry or mTFP1 ORFs followed by suitable selectable markers were generated from plasmids pCY3080-07 (Addgene) and pFA6:mTFP1:kanMX (available upon request). Transformations were performed using the lithium acetate method (ref). Transformants were selected on suitable auxotrophic medium or medium containing 300 $\mu\text{g ml}^{-1}$ Hygromycin B (for hphMX4) (Life Technologies, Carlsbad, CA).

Sporulation and tetrad dissection to derive haploid strains from *mmm1 Δ :KanMX6/MMM1*, *CIT1-mCherry*: hphMX4/*CIT1* diploid strains containing pRESC with a pertinent test-construct was carried out according to previously published protocols (58). Briefly, cells were incubated in sporulation medium (SPO) on a rotating wheel for 7 days. Asci were digested in 1M sorbitol with 2.5mg/ml zymolase (Seikagaku Corp., Tokyo Japan) for 7 min and tetrad dissection was carried out on SE -URA dropout plates containing 200 $\mu\text{g ml}^{-1}$ Geneticin (for KanMX6) (Sigma-Aldrich) or 300 $\mu\text{g ml}^{-1}$ Hygromycin B (for hphMX4). Haploid status and correct segregation of genomic alleles and plasmids was confirmed by PCR. Plasmids were cured on 5-FOA plates (SC supplemented with 50 $\mu\text{g ml}^{-1}$ Uracil and 1% 5-Fluoroorotic acid (5-FOA; Sigma-Aldrich, St Louis, MO)). Single colonies were picked and grown in 5-FOA liquid medium.

Yeast growth conditions

Yeast strains were grown and handled as previously described (59). All cells were grown at 30 °C (shaking for liquid cultures) in synthetic complete (SC) medium, unless otherwise stated. For strains requiring selection, appropriate SC dropout medium was used. All imaging was carried out during logarithmic phase.

pRESC construction

pRESC was derived from p416TEF (ATCC® 87368™) by replacing the TEF-promoter with 1000bp directly upstream of the MMM1-ORF. Rescue inserts were derived by PCR or synthesized by GENEWIZ® (South Plainfield, NJ).

Staining of mitochondrial DNA using DAPI

mtDNA was visualized by DAPI staining in live cells in SC containing 1 $\mu\text{g/ml}$ DAPI. Cells were stained for 10 min shaking at room temperature, washed three times, and imaged immediately.

Yeast microscopy and image analysis

Fluorescent microscopy was carried out one of the following microscopes: an Axioskop 2 microscope with 100x/1.4 Plan-Apochromat objective (Zeiss, Thornwood, NY) and an Orca ER cooled CCD camera (Hamamatsu) or an inverted AxioObserver.Z1 microscope with a 100x/1.3 oil EC Plan-Neofluar objective (Zeiss, Thornwood, NY) and Orca ER cooled CCD camera (Hamamatsu, Bridgewater, NJ). Images were collected through the entire cell depth (21 z-sections at 0.3- μm intervals) and deconvolved using an iterative

restoration algorithm (Volocity, Perkin-Elmer, Waltham, MA). Budding cells stained with DAPI were scored for presence of mitochondrial DNA that co-localizes with mitochondria in 3-dimensional reconstructions. To avoid errors due to incidental co-localization of mitochondria with nuclei in dividing cells, cells with less than three individual DAPI foci co-localizing with mitochondria were scored as ρ^0 . Mitochondrial inheritance dynamics was analyzed in budding cells expressing Cit1p-mCherry with daughter-cell diameters smaller than two-third of the mother-cell (small-to-medium buds). Mitochondrial morphology was analyzed by classifying mitochondrial objects in three categories based on geometric parameters. To this end, mitochondrial objects were detected, and mitochondrial volumes and skeletons were derived in Volocity using inbuilt algorithms. The tubularity of mitochondrial objects was determined by calculating the tubular radius (d) of an ideal cylinder (**Fig. S5A**) based on the determined volume (V) and skeletal length (h) as:

$$\sqrt{\frac{V}{\pi h}} = r$$

Mitochondrial objects were defined as *tubular* if the skeletal length $h \geq 2.5r$ and *spherical* if $h < 2.5r$ (**Fig. S5A-C**). As this simple classifier fails for very small objects (**Fig. S5C**), mitochondria were categorized as *fragmented* regardless of tubularity, if their skeletal length was less than $0.325\mu\text{m}$. During normal mitochondrial inheritance in WT cells, mitochondria accumulate at the daughter-cell tip and form aggregates that would be classified as spherical (**Fig. S5D**). To avoid this confounding factor, mitochondrial morphology was analyzed in mother cells only.

Western blotting

Cells were lysed with RIPA buffer (50mM Tris-HCl pH7.2, 500mM NaCl, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS, 10mM MgCl_2) supplemented with the cocktail of protease inhibitors (Roche), and Benzonase (EMD Millipore) at 37°C for 30min. Samples were loaded onto SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membrane (Amersham). After transfer, membranes were blocked for 1 h with blocking buffer (Li-Cor technology), followed by incubation with primary antibodies overnight at 4°C. The membranes were washed four times with 0.1% TritonX100/PBS and incubated with fluorescent secondary antibodies (Li-Cor technology) for 1 h at room temperature, followed by four times washes with 0.1% TritonX100/PBS. The membranes were scanned by Odyssey® CLx Imaging System (Li-Cor technology). Primary antibodies are rabbit anti-PDZD8 (1:500, in **Fig. 1E** and **Fig. S7A**) (24), rabbit antiserum against a peptide corresponding to a.a. 1103-1119 of mouse PDZD8 (DQHSKKNKYADDTEED) raised by Covance (1:500, in **Fig. 6B**), chicken anti-GFP (1:2000, GFP-1020; Aves labs), mouse anti-HA (1:2000, clone 16B12, BioLegend), mouse anti-actin (1:5000, Millipore), rabbit anti-VDAC1 (1:500, #4866, Cell Signaling Technology), rabbit anti-MCU (1:500, HPA016480, Sigma Aldrich), and rabbit anti-PTPIP51 (1:1000, A5820, ABclonal).

Quantitative RT-PCR

Cortical neuron culture was infected with lentivirus carrying either the control or shPDZD8 at 4 DIV. RNA was isolated at 13DIV with NucleoSpin® RNA (MACHEREY-NAGEL) according to the instructions of the manufacturer. cDNA was reverse transcribed using SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR (Life Technologies) according to the directions of the manufacturer. The resulting cDNA was analyzed using quantitative PCR with Power SYBR® Green PCR Master Mix (Life Technologies) in an Eppendorf Realplex using the following primers:

Actin

5'- GGCTGTATTCCCCTCCATCG -3'

5'- CCAGTTGGTAACAATGCCATGT-3'

Mouse PDZD8

5'- TTGACATAGAAGCCTGCCATAG -3'

5'- GACATCCTAAAGCCACTTCCTC -3'

Organelle fractionation

Isolation of ER, mitochondria, and MAM from HEK293T cells and PDZD8-Venus knock-in Neuro2a cells was performed following the previously described protocols (3, 60, 61). HEK293T cells or Neuro2a cells were harvested from 50 dishes (10cm) at 90-100% confluence and homogenized in isolation buffer (225mM mannitol, 75mM sucrose, 0.1mM EGTA, 30mM Tris-HCl pH7.4) using a Teflon pestle until 80-90% of cells were broken. Then, the homogenate was centrifuged at 600g for 10min twice for removing nuclei and debris. Collected supernatant was centrifuged for 15min at 7000g for obtaining crude mitochondria. For ER isolation, this step was repeated with the supernatant until a pellet is no longer visible, and the supernatant was centrifuged at 20,000g for 30min. Then, the mitochondria-free supernatant was centrifuged again at 100,000g for 1h and the pellet was resuspended for the ER fraction and the supernatant was kept for the cytosolic fraction. For pure mitochondria and MAM fraction, the crude mitochondria pellet was resuspended in 4x-5x volume resuspension buffer (250mM mannitol, 5mM HEPES pH7.4, 0.5mM EGTA), and the fraction was added on the top of 30% percoll medium (225mM mannitol, 25mM HEPES pH7.4, 1mM EGTA, 30% percoll (v/v)) in a centrifuge tube. Centrifuge was performed at 95,000g for 30min, and then the upper (MAM) and lower layer (pure mitochondria) were collected with a Pasteur pipette. Both fractions were diluted with 10x volume resuspension buffer and centrifuged at 6,300g for 15min. Then, the pure mitochondria fraction was obtained from the pellet after one more wash with the same condition. The supernatant containing the MAM was centrifuged at 100,000g for 1h and the resulting pellet was resuspended in resuspension buffer. Western blot was performed using chicken anti-GFP (1:2000, GFP-1020; Aves labs), rabbit anti-PDZD8 (1:500), mouse anti-Calnexin (1:500, 610523; BD bioscience), and mouse anti-cytochrome C (1:1000, 556433; BD bioscience) antibodies.

Confocal imaging

Samples were imaged with either a 20x (0.75 NA) air objective or 60x (1.4 NA) oil objective on a Nikon Ti-e inverted microscope equipped with a Nikon A1 confocal.

Immunohistochemistry

Following anesthesia, mice were heart perfused with 5ml of phosphate buffered saline (PBS 0.1M) and subsequently with 40ml of the fixative solution containing 2% paraformaldehyde (EM grade, EMS) and 0.025% of Glutaraldehyde (GA, EMS) diluted in PB. Brains were then dissected out and post-fixed in the same fixative solution for 3 hours. After embedded in low melting agarose (3%, MP Biomedicals), brains were sectioned at 100 μ m thickness with a Leica VT1200S vibratome. Sections were treated with ice cold methanol for 6' and blocked with 2% goat serum/PBS for 30 min followed by incubation with anti-GFP at 4°C overnight. Sections were then washed with PBS followed by treatment with anti-chicken Alexa-488 and mounted with Aqua-Poly/Mount (Polysciences).

Electron microscopy

HeLa cells and PDZD8 KO HeLa cells were fixed with 2% glutaraldehyde (EMS) in 0.1M Phosphate buffer, pH7.4 for 1h at room temperature. After washing with 0.1M Phosphate buffer, cells were scraped and collected with 0.2% BSA/0.1M phosphate buffer followed by centrifugation at 3000 rpm. The samples were post-fixed with 1% OsO₄, 1.5% potassium ferricyanide in 0.1M Phosphate buffer for 1 hour. After 3 times rinse with distilled water, cells were stained with 10% thiocarbohydrazide for 5min followed by incubation with 1% aqueous uranyl acetate for 30 min at room temperature. The samples were rinsed in water, dehydrated in an ethanol series and then infiltrated and embedded in Epon resin (62).

The sample block was trimmed then mounted on a SEM sample holder using double-sided carbon tape (EMS). Colloidal silver paint (EMS) was used to electrically ground the exposed edges of the tissue block. The entire surface of the specimen was then sputter coated with a thin layer of gold/palladium. The tissue was imaged using back scattered electron (BSE) mode in a FEI Helios Nanolab 650. Images were recorded after each round of ion beam milling using the SEM beam at 2keV and 100pA with a working distance of 4.0 mm. Data acquisition occurred in an automated way using the Auto Slice and View G3 software. The raw images were 2,048x1,768 pixels, with 20 nm slices viewed at a -38 degree cross-sectional angle. Each raw image had a horizontal field width (HFW) of 15 μ m with an XY pixel size of 7-9 nm and 20 nm Z slices. These images were then aligned using the image processing programs in IMOD (63).

Analysis of 3D-SEM imaging

3D-SEM images were manually segmented with a Fiji plug-in Track-EM2. Segmented images were imported to Imaris (Bitplane) and analyzed with Surface-Surface contact plug-in. In Imaris, surface-area and volume were calculated using the Statistics feature. The area of ER analyzed was chosen based on localization in region containing the mitochondrial network which was also reconstructed systematically.

ER Three-way Junction Quantification

Three-way junctions in the 3D-SEM images of ER were quantified using a Fiji plug-in Track-EM2. Three-way junctions were identified at points where three distinct ER segments branched from. Quantification of three-way junctions was normalized by the

surface area of the ER that was analyzed. The area of ER analyzed was chosen based on localization to the mitochondrial network.

Calcium imaging

NIH3T3 cells were plated and transfected with plasmids with Jetprime or FugeneHD (Promega). Cells were replated on glass bottom dishes (MatTek) at $1 \times 10^4/\text{cm}^2$ density and incubated for 1 day. The medium was replaced with Hank's buffered salt solution (HBSS) supplemented with HEPES (pH7.4; 2.5mM), CaCl_2 (2mM, Sigma), MgSO_4 (1mM, Sigma), NaHCO_3 (4mM, Sigma), and D-glucose (30mM, Sigma). Cells were stimulated with $200\mu\text{M}$ ATP (Sigma Aldrich, A1852) and imaged with an inverted Nikon Ti-E microscope equipped with EMCCD camera (Andor, iXon3-897), 20x objective (0.75NA). 470nm, 555nm, and 360nm Spectra X LED lights (Lumencor) were used for the light source, and a custom quad-band excitation/mirror/emission cube (based off Chroma, 89400) followed by clean up filters (Chroma, ET525/50, ET600/50, ET435/26) were applied for excitation and emission.

For experiment with permeabilized cells (**Fig. S9**), cells were treated with $2.5\mu\text{M}$ digitonin (Sigma-Aldrich) and $20\mu\text{M}$ cyclopiazonic acid (CPA; Tocris) in intracellular buffer (130mM KCl, 10mM NaCl, 2mM K_2HPO_4 , 5mM succinic acid, 1mM MgCl_2 , 20mM HEPES) for 3min before imaging. After 10s of imaging, cells were stimulated by application of a solution containing 1mM CaCl_2 and imaged for another 20s. To confirm the Ca^{2+} uptake is mediated by MCU, cells were treated with MCU inhibitor $10\mu\text{M}$ RU360 (Calbiochem).

For neuronal calcium imaging, electroporated cortical neurons were imaged using the same microscope (40x objective NA 0.95) with an EMCCD (Andor, iXon3-897) camera at 19-22DIV. 470nm and 555nm LED lights were applied for the light source, and the same cube and clean up filters (Chroma, ET525/50, ET600/50) were used for dual color imaging. We imaged neurons in modified normal tyrode solution (145mM NaCl, 2.5mM KCl, 10mM HEPES pH7.4, 2mM CaCl_2 , 1mM MgCl_2 , 10mM glucose) at 37°C (Tokai Hit Chamber). Electrical stimulation is triggered by 1ms current injections with a concentric bipolar electrode (FHC) placed 50-100 μm away from transfected neuronal cell bodies for stimulating nearby axons. We applied 20APs (10Hz) with 30V using the stimulator (Model 2100, A-M systems) and imaged with 500ms interval (2Hz) during 90sec for G-CEPIA1er, mito-RCaMP1h, and cyto-RCaMP1h signals. For CEPIA3mt imaging, 100ms interval (10Hz) was applied during 40s. For blocking SERCA pump, NMDAR, or mGluR1, cyclopiazonic acid (CPA, $30\mu\text{M}$ for 5min; Tocris), APV ($50\mu\text{M}$ for 3min; Tocris), or LY367385 ($100\mu\text{M}$ for 10min; Sigma-Aldrich) were incubated, respectively.

Images were analyzed using the NIS Elements software (Nikon) or a Fiji (Image J) plug-in, Time Series Analyzer (v3.0). One small area of each Hela cell is selected by circular ROI by NIS Elements, and average intensities were measured with background correction. 2-3 dendritic segments (10 μm) from a single neuron and nearby backgrounds were selected by ROIs and average intensities were measured by the plug-in. After intensities were corrected for background subtraction, ΔF values were calculated from $(F-F_0)$. F_0 values were defined by averaging 10 frames before stimulation, and used for normalization. For F_{max} values, ionomycin ($5\mu\text{M}$) were applied after stimulation and average intensity of 10 frames from a plateau was employed for normalization.

TMRM imaging

NIH3T3 cells were plated and transfected with pCAG-mitoYFP and either control plasmid (pLKO1) or pLKO1 vector expressing shRNA targeting *Pdzd8* plasmids with EugeneHD (Promega). Cells were replated on glass bottom dishes (MatTek) at $1 \times 10^4/\text{cm}^2$ density and incubated for 1 day. After treatment with 20nM TMRM for 20min, the medium was replaced with Hank's buffered salt solution (HBSS) supplemented with HEPES (pH7.4; 2.5mM), CaCl_2 (2mM, Sigma), MgSO_4 (1mM, Sigma), NaHCO_3 (4mM, Sigma), and D-glucose (30mM, Sigma) and 5nM TMRM. Cells were imaged with 60x (1.4 NA) oil objective on a Nikon Ti-e inverted microscope equipped with a Nikon A1 confocal. To obtain eliminate mitochondrial membrane potential, cells were treated with $0.3\mu\text{M}$ FCCP (Enzo Life Sciences) and $1\mu\text{g}/\mu\text{l}$ Oligomycin (Calbiochem).

***In utero* cortical electroporation**

The *in utero* electroporation method was performed as previously described. Briefly, a mixture of the vectors pCAG Canx-YFP and pCAG mito (CoxVIII)-mtagBFP2 together with either control pLKO vector or pLKO-shPDZD8 were injected into the neocortical lateral horn of embryos obtained from a timed-pregnant CD1 mouse female at E15.5 with Picospritzer III (Parker, Hollis NH, USA). The cDNA mixtures were then electroporated into the neural progenitor cells resided in the ventricular zone by 5 electric pulses (50ms) of 38V using Tweezer-type platinum electrode (diameter 3mm; NEPA GENE) and an electroporation system ECM 830 (BTX, Holliston MA, USA).

***In situ* hybridization**

In situ hybridization was performed as previously described (64). The probes for PDZD8 was generated with the following primers;

5'- GCGAATTAACCCTCACTAAAGGGTTCCTTCAGAAAGTTCTGCTCC -3'

5'- GCGTAATACGACTCACTATAGGGGTCTATTCGCCTCTCAGTTGCT -3'

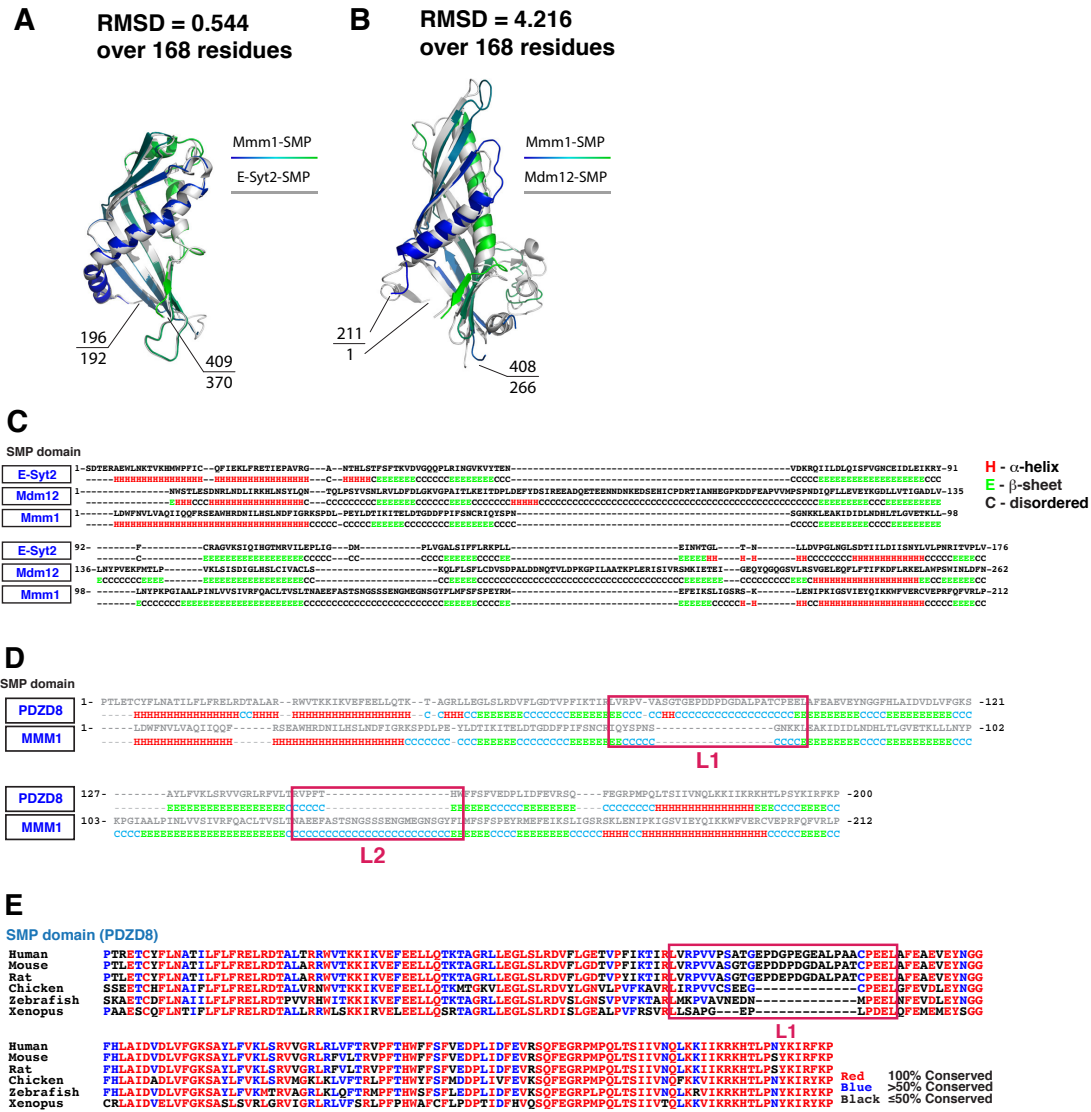


Fig. S1 Tertiary, secondary and primary sequence alignment between the SMP domain of PDZD8 and other SMP domains.

(A, B) Superimposition of Phyre2 homology models for the SMP domains of Mmm1 (green-blue in A,B) over templates from the crystal structures of either E-Syt2 (white in A, 192-370 a.a.; PDB code 4P42) or Mdm12 (white in B, 1-266 a.a.; PDB code 5gyk). (C) Predicted secondary structure of the SMP domain of Mmm1 (*S. cerevisiae*) aligned with the secondary structure of E-Syt2 (human) and Mdm12 (*S. cerevisiae*), another SMP domain-containing protein found in the ERMES complex. (D) Secondary structural alignment of a.a. sequences corresponding to the SMP domains of yeast MMM1 and mouse Pdzd8. Predicted secondary structures are shown below the a.a. sequence. Loop regions (L1 and L2) are highlighted by red boxes and show absence of conservation between the two sequences. (E) Primary a.a. sequences of PDZD8 SMP domains from indicated species are aligned. Red, blue, and black characters indicate 100%, >50%, and ≤50% conservation, respectively.

≤50% conservation of the a.a. residues among corresponding species, respectively. Note that L1 loop region (box) is not conserved across the vertebrate species.

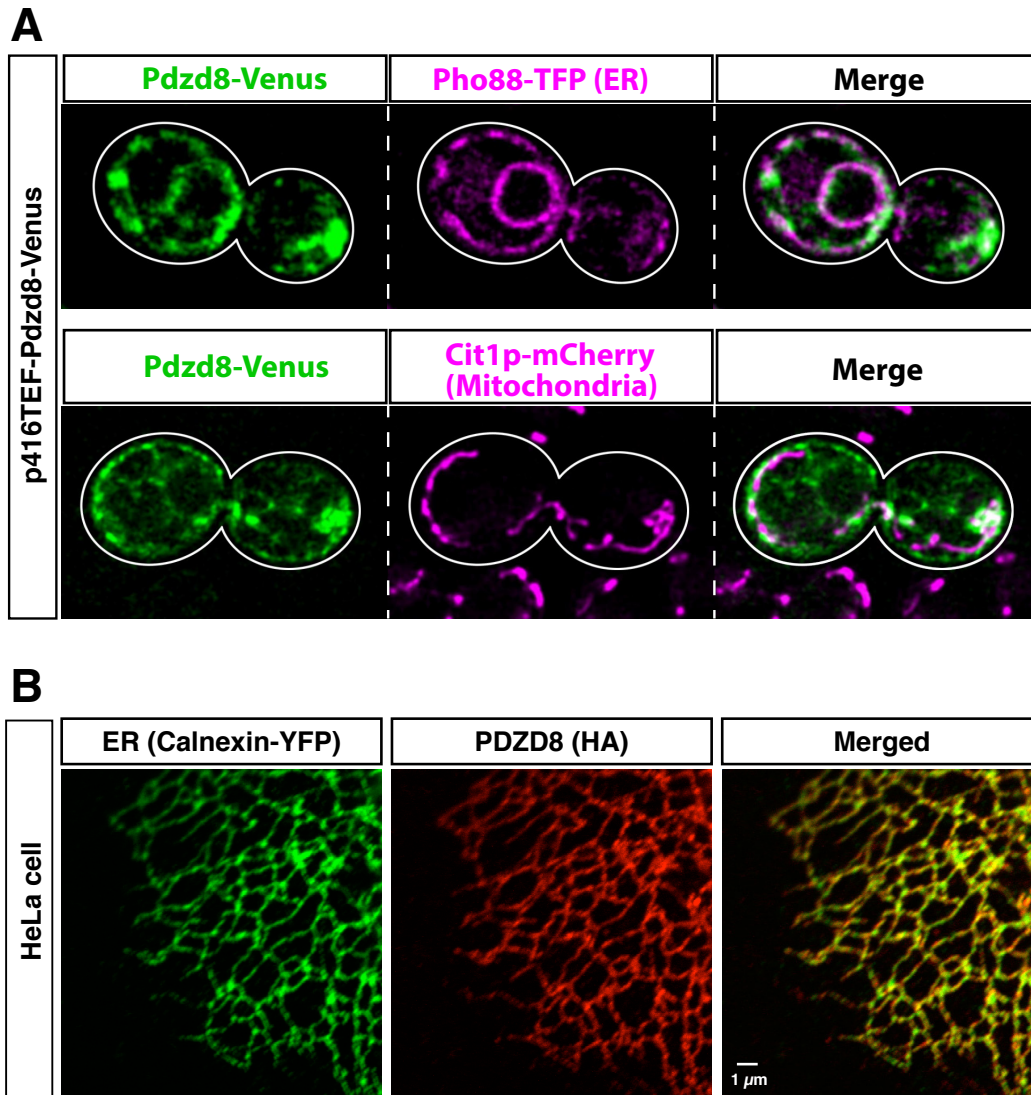


Fig. S2 PDZD8 is localized at ER in yeast and human cells.

(A) Plasmid-borne PDZD8-Venus co-localizes with the ER-marker Pho88-TFP, but not the mitochondrial-matrix marker Cit1p-mCherry, both expressed from their endogenous loci. (B) Overexpressed HA-tagged PDZD8 localizes at the ER in HeLa cell co-transfected with a plasmid expressing Canx-YFP (ER marker) and stained with anti-GFP and anti-HA antibodies.

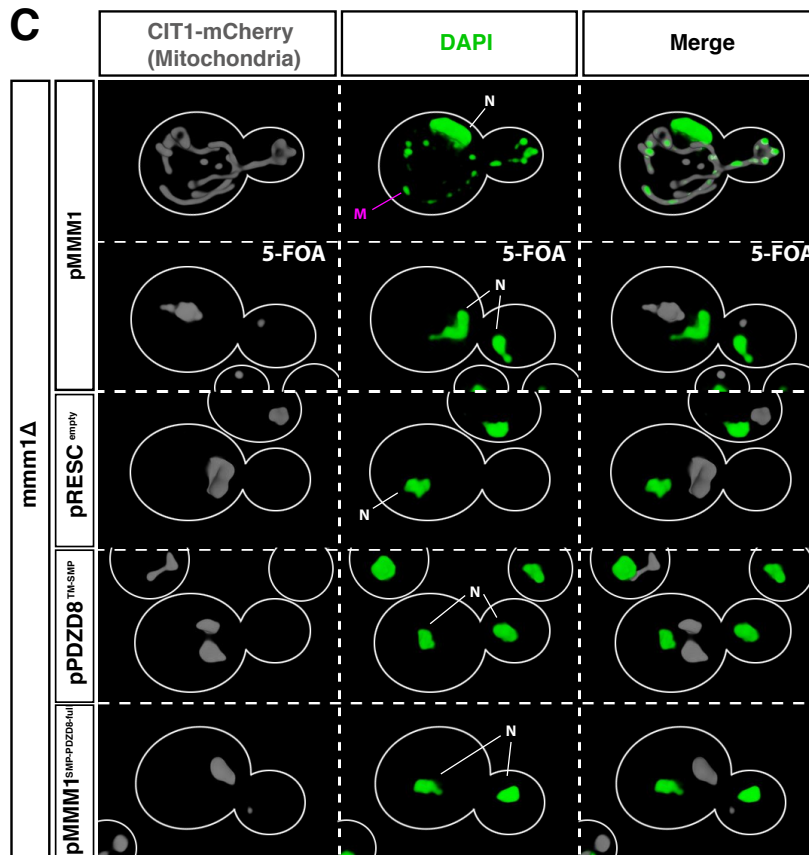
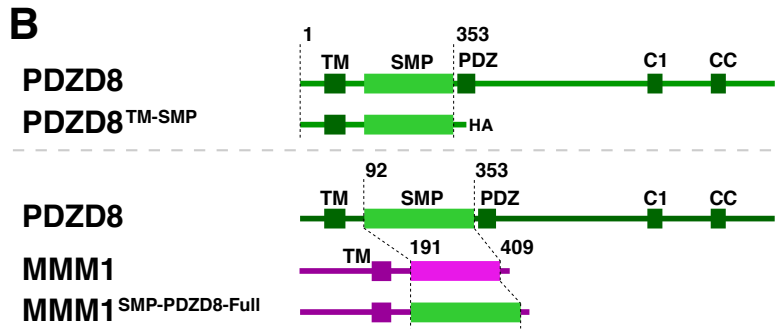
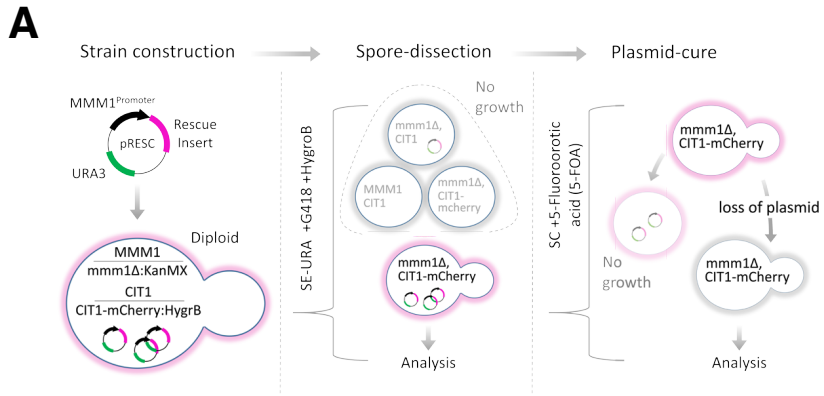


Fig. S3 Original SMP domain of PDZD8 is not sufficient to restore the yeast *mmm1Δ* defects.

(A) Diagram detailing the experimental strategy. Diploid heterozygotes for *mmm1Δ:KanMX* and *CIT1-mCherry:Hygromycin-B* (for visualizing mitochondria) alleles were transformed with plasmids (pRESC) containing a *URA3*-selection marker and the endogenous *MMM1*-promoter sequence followed by particular cDNA rescue inserts. Tetrad dissection was performed and colonies selected for co-segregation of *mmm1Δ*, *CIT1-mCherry* and pRESC, and analyzed. pRESC was then shuffled out from rescue strains by growth on media containing 5-Fluoroorotic acid (5-FOA), which is toxic to *URA3* expressing strains, and assessed for reversion to *mmm1Δ* phenotype. (B) Diagrams detailing particular rescue constructs (*Mmm1*-sequence in magenta shading). (C) Rescue of *mmm1Δ* phenotypes by several pRESC constructs was assessed. Representative images (3D opacity rendering) of nuclear (N in white) and mitochondrial (M in magenta) DNA (DAPI) and mitochondria (Cit1p-mCherry) in *mmm1Δ* strains containing pRESC expressing full-length *MMM1* (p*MMM1*), p*MMM1* cells after 5-FOA plasmid cure, empty pRESC plasmids; pRESC expressing an N-terminal fragment of PDZD8 containing its TM and SMP domain (pPDZD8^{TM-SMP}), and *MMM1* containing PDZD8-SMP domain (p*MMM1*^{SMP-PDZD8-Full}). Cell outlines are shown in white.

Representative images (3D opacity rendering) of nuclear (N) and mitochondrial (M) DNA (DAPI) and mitochondria (Cit1p-mCherry) in WT, *mmm1* Δ , *mmm1* Δ rescue strains containing p*MMMI-SMP*^{PDZD8+L1L2}, or p*MMMI-SMP*^{Mdm12+L1} plasmids and the same strain following plasmid removal with 5-FOA. Cell-outlines are shown in white. Mitochondria falling into *Tubular* (T), *Spherical* (S) and *Fragment* (F) morphology categories are indicated. (E) Proportion of cells (n>100) containing mtDNA (rho⁺) as identified by DAPI staining, and (F) mitochondrial inheritance dynamics as the proportion of small-to-medium sized buds (n>60) containing mitochondrial Cit1-mCherry signal. (G) Quantitation of the proportion of mitochondrial objects in budding mother cells (n>100) falling into each mitochondrial morphological category as defined in Experimental Procedures. Statistical significance was determined by Fisher's Exact Test for Count Data. * p<0.05, ** p<0.01, ***p<0.005.

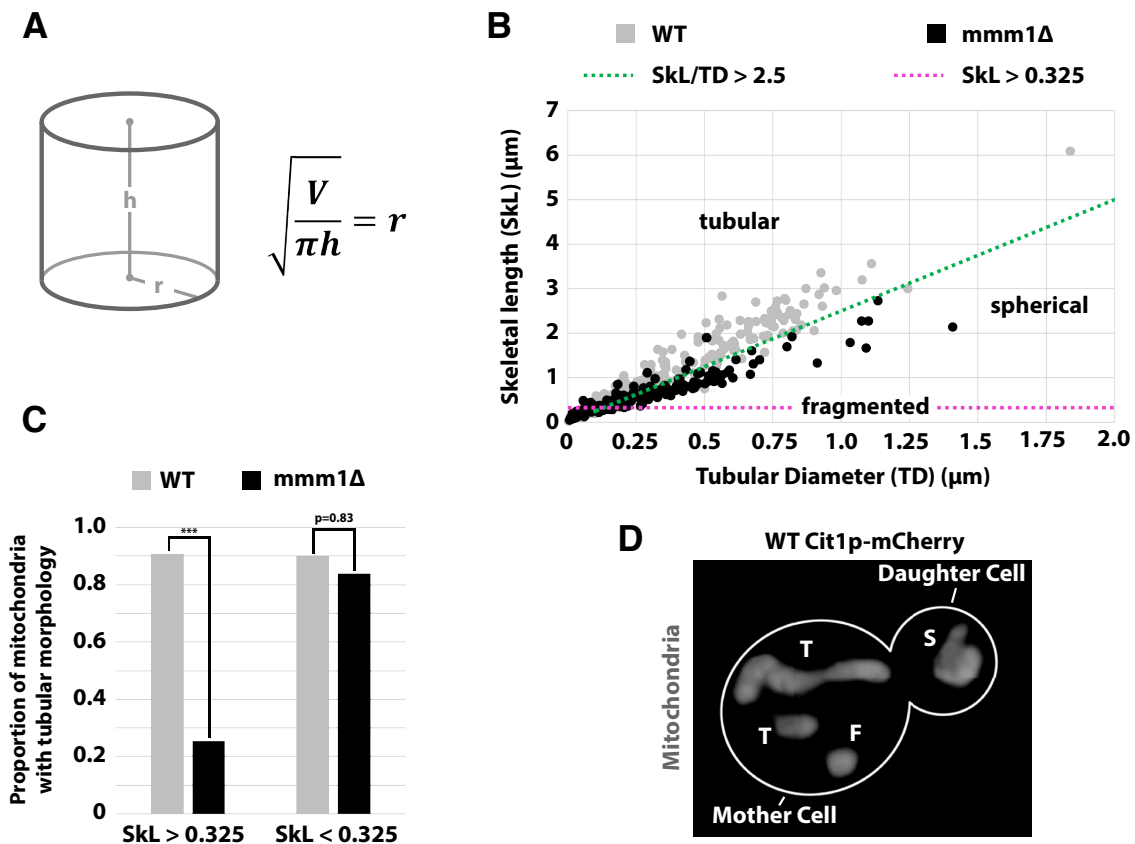


Fig. S5 Quantitative approaches for measurement of mitochondrial morphology in yeast

(A) Volume and skeletal length (SkL) of mitochondrial objects in budding mother-cells were used to calculate the diameter (d) of an ideal cylinder with equal volume (V) and SkL as height (h). Based on this (d) was defined as the tubular diameter (TD) and (B) individual mitochondrial objects from WT and *mmm1Δ* cells were classified as *tubular* for $SkL/TD > 2.5$ and as *spherical* for $SkL/TD < 2.5$. (B,C) The classifier is unable to distinguish objects from WT and *mmm1Δ* cells with $SkL < 0.325 \mu m$ according to *tubular* and *spherical* categories and small objects are classified as *fragmented*. (D) Representative WT cell expressing Cit1p-mCherry containing examples of all three categories; *tubular* (T), *spherical* (S), *fragmented* (F). Cell outlines are shown in white. Statistical significance was determined by Fisher's Exact Test for Count Data. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

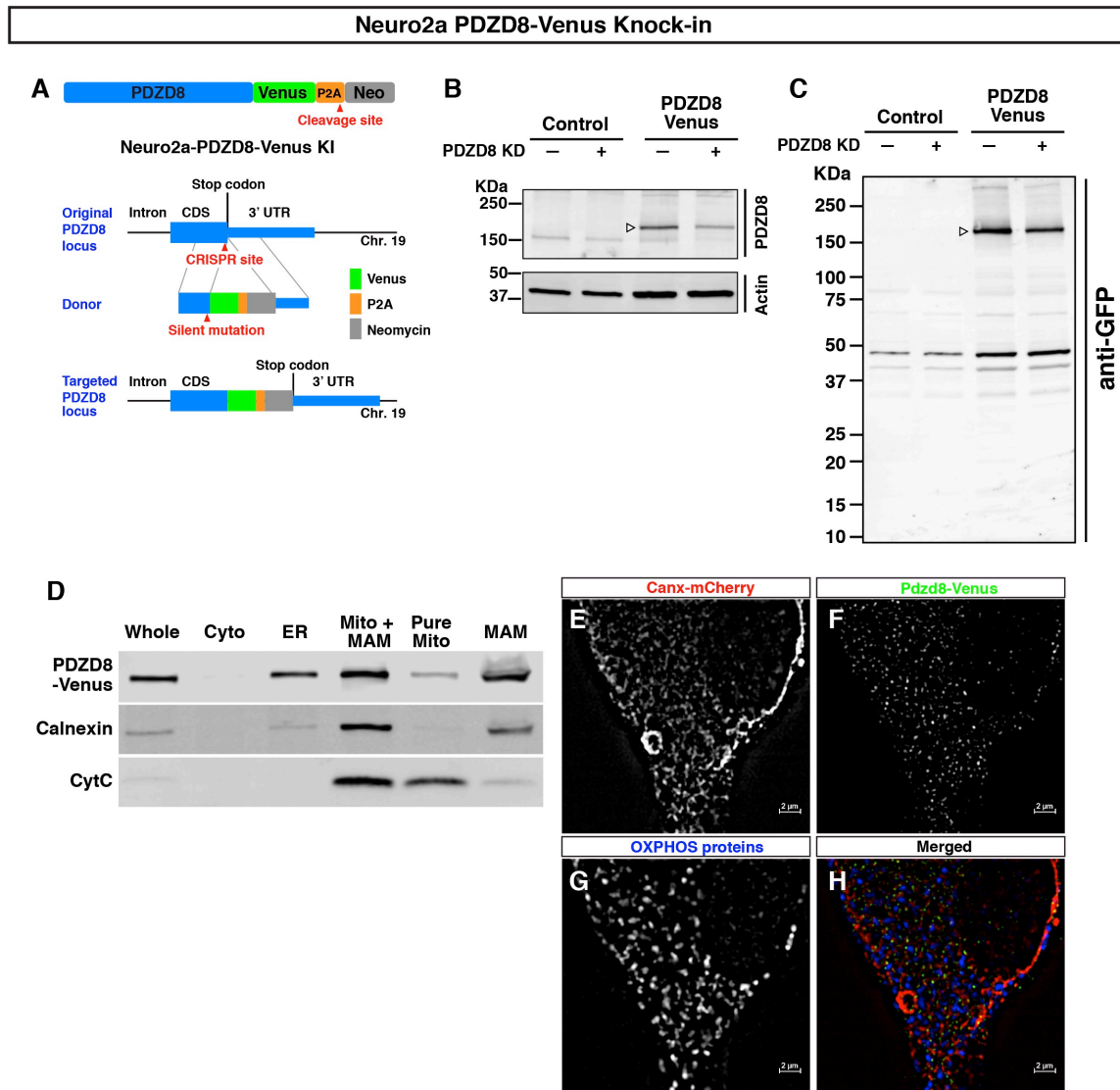


Fig. S6 Validation and analysis of Pdzd8-Venus knock-in Neuro2a cell line

(A) Diagram describing the strategy for making Neuro2a-PDZD8-Venus cell line. (B-C) Western blot analysis using anti-PDZD8 antiserum (B) or GFP antibody (C) in PDZD8-Venus-KI-Neuro2a cell line. Cell lysates were collected from control or PDZD8-Venus-KI Neuro2a cells transfected with plasmids expressing either control or *Pdzd8* shRNA. Arrowhead indicates bands corresponding to PDZD8-Venus fusion protein. (D) Immunoblot of subcellular fractionation from PDZD8-Venus knock-in Neuro2a (N2a) cells demonstrates the presence of PDZD8 in ER and MAM fractions. MAM fraction was isolated using a Percoll gradient, and immunoblot was performed with anti-GFP for PDZD8-Venus, anti-Calnexin for ER and MAM fraction, and anti-cytochrome C for mitochondrial fraction. (E-H) Larger field of view for images detailed in Fig. 1F-I. PDZD8-Venus knock-in N2a cells were transfected with a plasmid expressing Calnexin-mCherry (ER marker) and stained with anti-RFP, anti-GFP antibody, and anti-OXPPOS complex antibody (mitochondrial marker). The stained cells were imaged with 3D N-SIM.

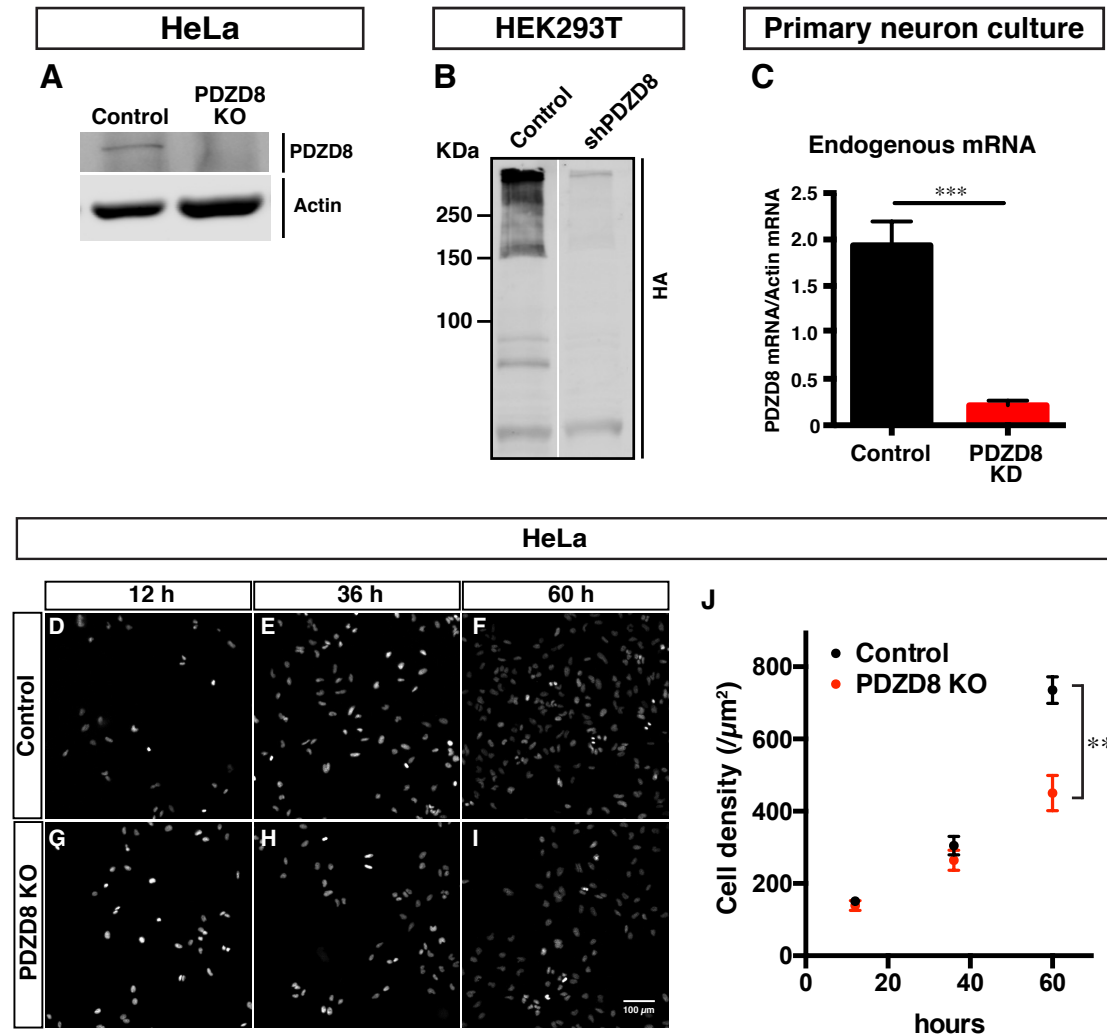


Fig. S7 Validation of *Pdzd8* shRNA knockdown efficacy and PDZD8 knockout HeLa cells.

(A) PDZD8 is absent in the PDZD8 KO HeLa cells. Cell lysates collected from control or PDZD8 KO HeLa cells were subjected to Western blotting with anti-PDZD8 and anti-Actin antibodies. (B) Efficacy of knockdown using shRNA against mouse *Pdzd8* in HEK293 cells measured by Western blot analysis (C). Cultured mouse cortical neurons were infected at 4DIV with lentivirus expressing either control or *Pdzd8* shRNA and total RNA was extracted at 13DIV. The *Pdzd8* mRNA amounts were quantified by quantitative RT-PCR and normalized by *Actin* mRNA levels. (D-J) PDZD8 KO decreases the rate of HeLa cell growth. Control or PDZD8 KO HeLa cells were plated at 7.9×10^3 cells/cm². (D-I) Representative images of the cells with nuclear staining are shown. (J) Cell density i.e. number of cells per region of interest was quantified at 12, 36 and 60 hours after plating following longitudinal imaging from the same dishes (n=3). **p<0.005 *** p<0.001, Student's t-test.

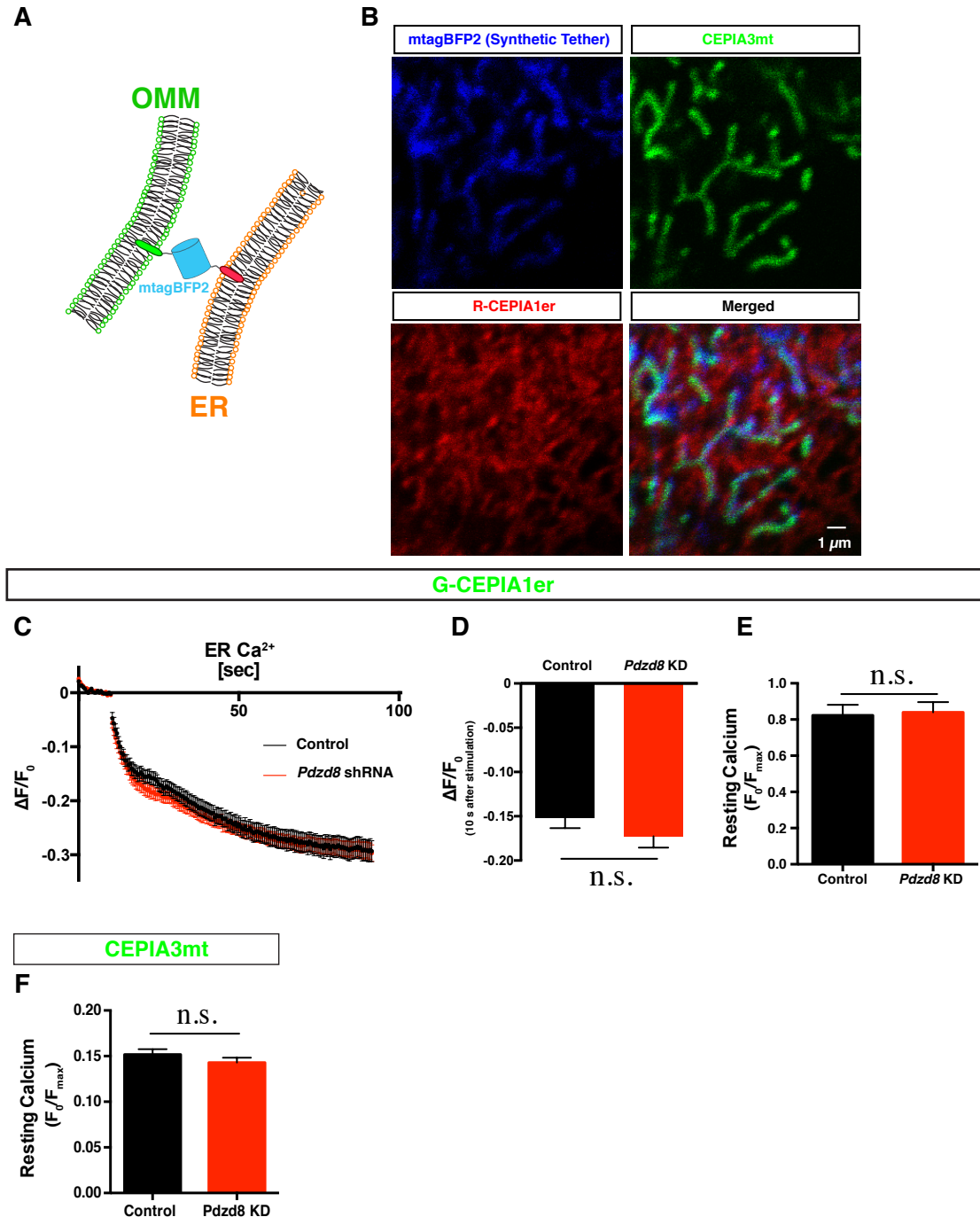


Fig. S8 Localization of synthetic ER-mitochondria tethering construct and measurement of ER Ca^{2+} load and release in NIH3T3 cells.

(A) Schematic of the synthetic tethering protein. Green oval; mitochondria targeting domain of mouse AKAP1, Red oval; ER targeting domain of yeast UBC6, Grey line; linker protein. OMM; outer mitochondrial membrane. (B) Subcellular localization of the synthetic tethering protein (mTagBFP2; blue) used in Figure 3 along with CEPIA3mt (green), and R-CEPIA1er (red) in NIH3T3 cells was assessed by confocal microscopy

and reveals strong overlap with both mitochondria and ER. **(C-D)** NIH3T3 cells were transfected with plasmids encoding ER-targeted GECI (G-CEPIA1er) together with either a control plasmid (Control) or a *Pdzd8* shRNA plasmid. Cells were stimulated with 200 μ M ATP to induce ER Ca^{2+} release and changes in fluorescence from G-CEPIA1er were measured. **(C)** Changes of G-CEPIA1er fluorescence (ΔF) from normalized by basal signals before ATP stimulation (F_0) are shown. **(D)** Peak intensities of $\Delta F/F_0$ 10 seconds after ATP stimulation are not significantly different between the control and *Pdzd8* KD cells. n= 37 (Control), 23 (*Pdzd8* KD). **(E-F)** Resting ER **(E)** and mitochondrial **(F)** Ca^{2+} levels are measured from basal signals (F_0 i.e. prior to ATP stimulation) normalized by F_{max} obtained following ionomycin (5 μ M) treatment which allows normalization for potential cell-to-cell variation in GECI expression levels. Resting Ca^{2+} levels are not significantly changed in *Pdzd8* KD NIH3T3 cells compared to control cells. **(E)** n= 13 (Control), 16 (*Pdzd8* KD), **(F)** n= 35 (Control), 25 (*Pdzd8* KD). **(D, F)** n.s. $p > 0.05$ according to Unpaired t-test with Welch's correction. **(E)** n.s. $p > 0.05$ according to Unpaired t-test.

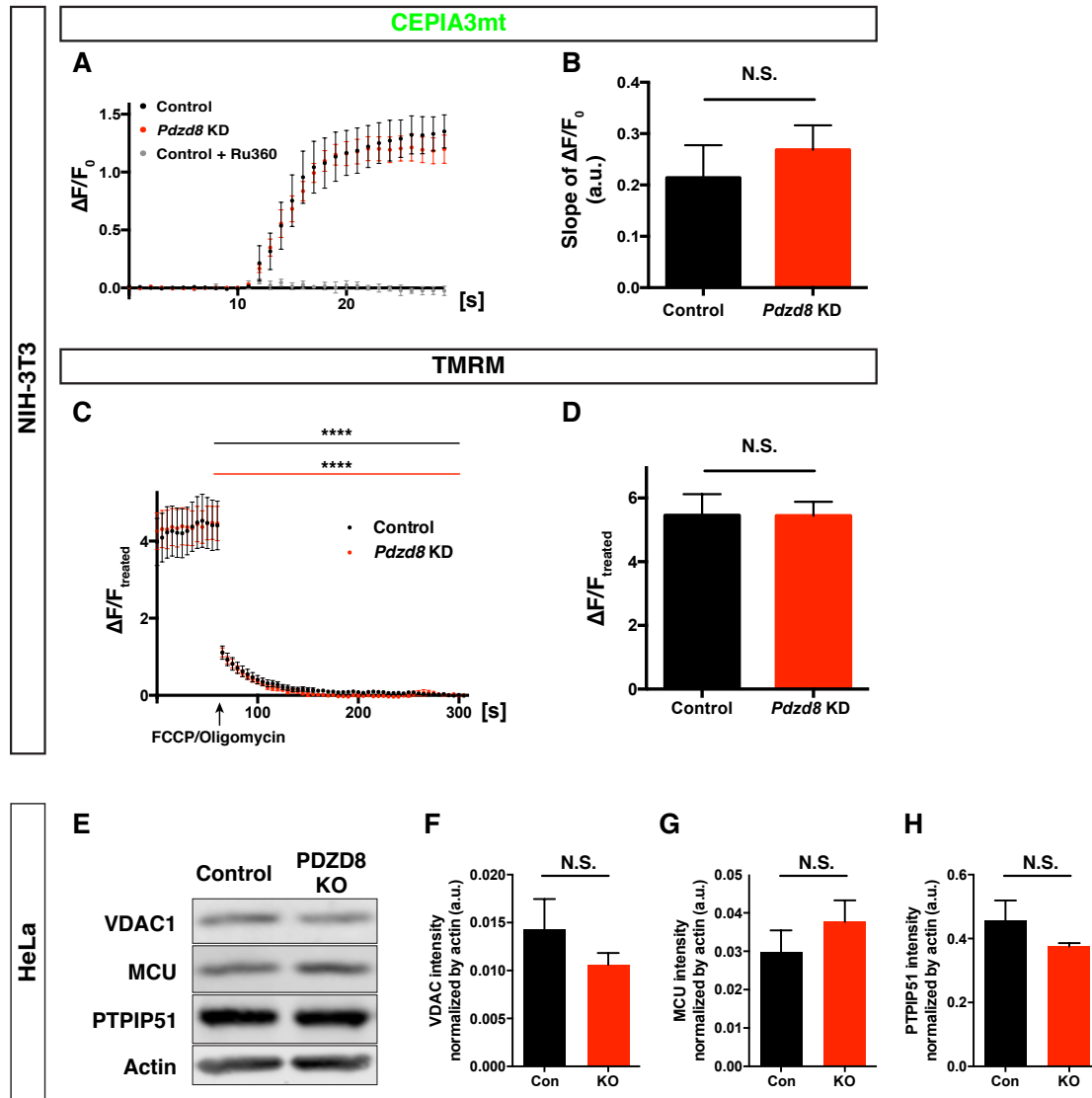


Fig. S9 Mitochondrial Ca^{2+} import machinery, membrane potential and other MAM proteins are not altered in PDZD8-depleted mammalian cells.

(A-B) Mitochondrial Ca^{2+} uptake itself is intact in PDZD8 KD NIH3T3 cells. NIH3T3 cells expressing CEPIA3mt were permeabilized with 2.5 μ M digitonin and treated with SERCA inhibitor (CPA 20 μ M) for 3 min allowing to measure directly mitochondrial Ca^{2+} uptake following application of 1mM Ca^{2+} (at 10sec). This uptake is purely dependent of MCU because it is completely abolished by application of MCU inhibitor (Ru360, 10 μ M). Slope of $\Delta F/F_0$ increase in the linear phase was calculated as an indicator for the speed of mitochondrial Ca^{2+} uptake which is dependent on combination of MCU expression level and MCU channel gating properties. The uptake speed was not significantly different between the control and *Pdzd8* KD cell. n= 9 (Control), 12 (*Pdzd8* KD). Mann-Whitney test. (C-D) Mitochondrial membrane potential is maintained in *Pdzd8* KD NIH3T3 cells. NIH3T3 cells expressing mito-YFP and either control or sh*Pdzd8* plasmids were loaded with 20 nM TMRM for 20 min and were imaged for 1min

in the presence of 5 nM TMRM. Then, cells were treated with 0.3 μ M FCCP (electron transport chain uncoupler) and 1 μ g/ μ l Oligomycin (ATPase blocker) for complete elimination of mitochondrial potential (used as baseline fluorescence for normalization). (C) Changes of TMRM fluorescence (ΔF). **** $p < 0.0001$ according to paired t-test. (D) Mitochondrial TMRM signals normalized by baseline show no difference between control and *Pdzd8* KD cells. n= 17 (Control), 16 (*Pdzd8* KD). Mann-Whitney test. (E-H) Expression of mitochondrial Ca^{2+} regulatory proteins and other tethering proteins are not changed in PDZD8 KO HeLa cells. Immunoblot of control and PDZD8 KO HeLa cells shows no significant difference in expression level of mitochondrial Ca^{2+} uptake proteins (VDAC1 and MCU) and other ER/mitochondria tethering components (PTPIP51 and VDAC1). n=3 blots, Mann-Whitney test.

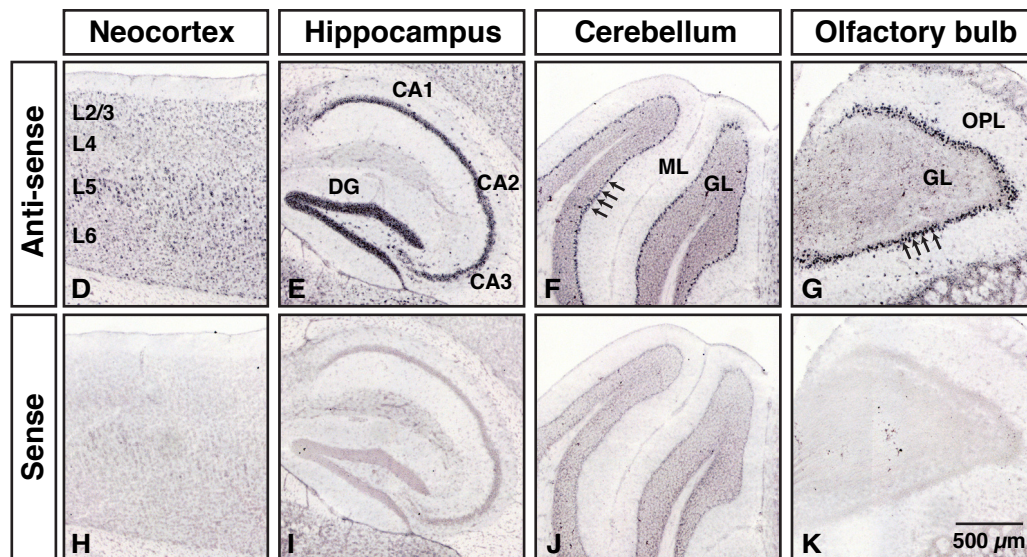
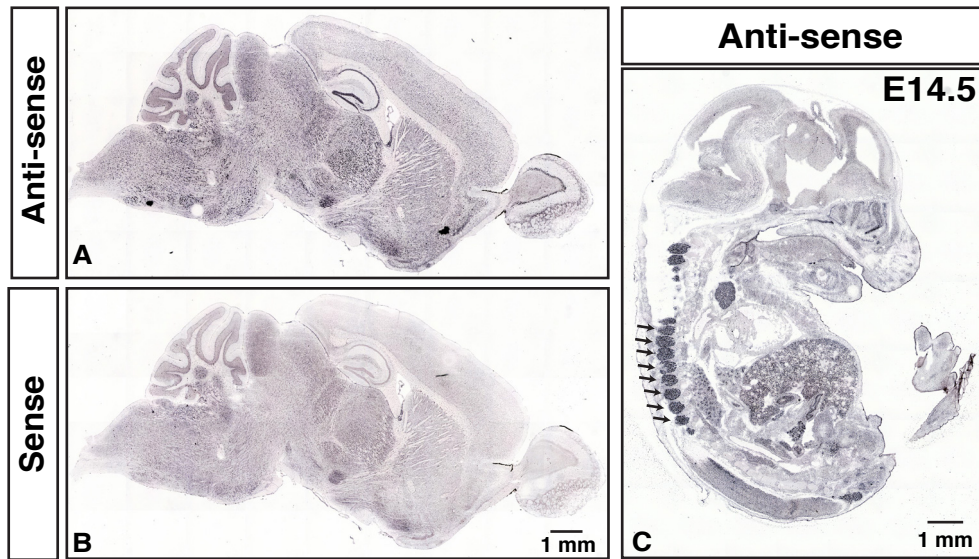


Fig. S10 *In situ* hybridization for *Pdzd8* mRNA in the embryonic and adult mouse brain.

Sections from brains of adult (A, B, D-K) or E14.5 C57Bl6 mouse embryos (C) were hybridized with either antisense (A, C, D-G) or sense probes (negative control, B, H-K) for mouse *Pdzd8* mRNA. (A, B) Sagittal sections of adult brains. (C) Sagittal section of E14.5 embryo. (D-K) Regions of brain indicated. Arrows indicate dorsal root ganglia in (C), Purkinje cells in (F), and mitral cells in (G).

Abbreviations: L, Layer; DG, Dentate gyrus; ML, Molecular layer; GL, Granule layer; OPL, Outer Plexiform Layer.

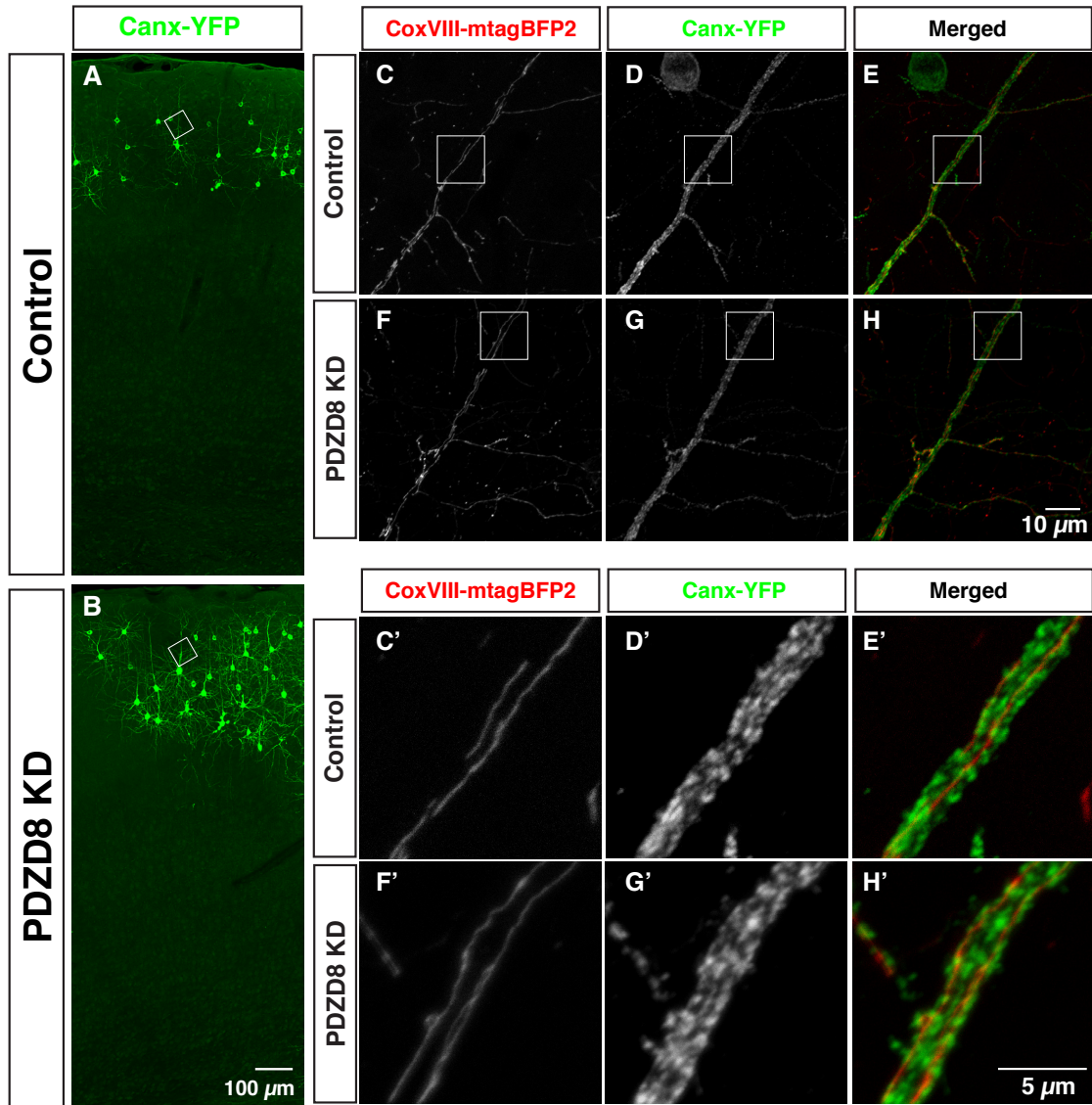


Fig. S11 Normal structure and distribution of mitochondria and ER in dendrites of control and PDZD8-depleted layer 2/3 pyramidal neurons *in vivo*.

Plasmids expressing COXVIII-mtagBFP2 (mitochondrial marker) and Canx-YFP (ER marker) with either control shRNA or *Pdzd8* shRNA were electroporated into the neocortex using *in utero* cortical electroporation at E15.5 and examined by confocal microscopy at P21. The apical dendrites of layer 2/3 neurons in the primary somatosensory cortex (S1) were imaged (A, B). The squares in panels A-B indicate the regions shown at high magnification in C-H. The squares in C-H indicate the regions shown at high magnification in C'-H'. Maximum projection images from 7.2 μm (C-E), 3.5 μm (C'-E'), 13.6 μm (F-H), and 4.25 μm (F'-H') thick Z-stacks are shown.

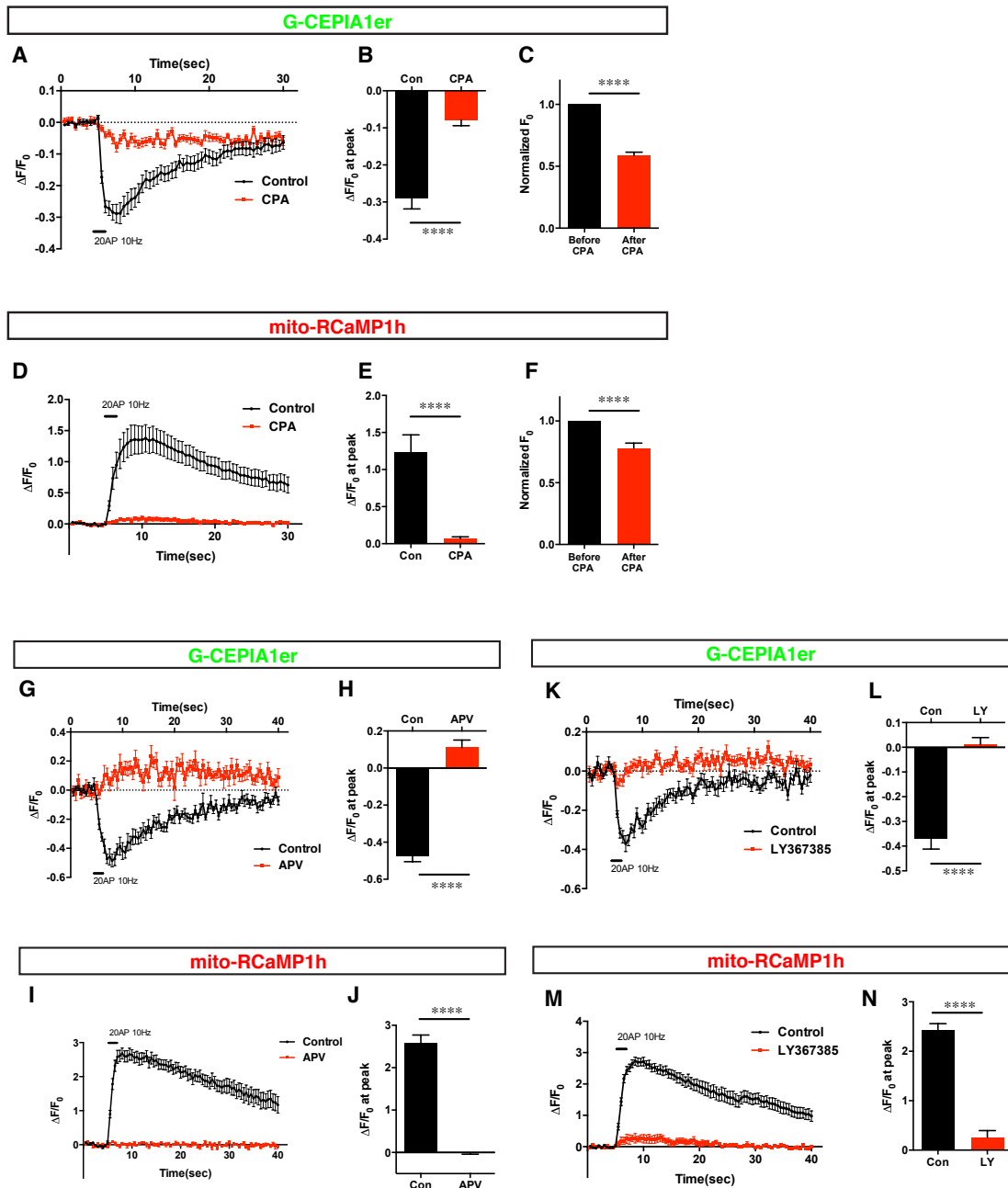


Fig. S12 Pharmacological dissection of ER-mitochondria Ca^{2+} exchange evoked by synaptic stimulation in cortical pyramidal neurons.

(A-F) ER Ca^{2+} -depletion disrupts mitochondrial Ca^{2+} elevation during repetitive stimulation with 20APs at 10Hz. ER and mitochondrial Ca^{2+} dynamics were monitored using G-CEPIA1er and mito-RCaMP1h from the same cortical neurons (19-24DIV) before and after cyclopiazonic acid treatment (CPA, $30\mu\text{M}$ over 5min), which inhibits sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA). Inhibition of ER Ca^{2+} uptake blocks the replenishment of ER Ca^{2+} amount. Basal level of ER and mitochondrial Ca^{2+} is decreased after CPA treatment (C, F). This suggests that CPA treatment did not indirectly saturate mitochondrial Ca^{2+} concentration through store-operated Ca^{2+} entry (SOCE) in

dendrites. The modest but significant decreased in basal mitochondrial Ca^{2+} levels following CPA treatment cannot explain the almost complete disappearance of stimulation evoked mitochondrial Ca^{2+} increase observed in A-B. $n=27$ dendritic segments from 9 neurons. **** $p<0.0001$, Wilcoxon matched pairs signed rank test. **(G-J)** NMDA receptor-dependent Ca^{2+} influx is required for triggering ER Ca^{2+} release and mitochondrial Ca^{2+} influx upon stimulation with 20APs-10Hz. Neurons were imaged before and after NMDA receptor blocker, APV, application ($50\mu\text{M}$). APV treatment abolished ER Ca^{2+} release (G, H) and mitochondrial Ca^{2+} increase (I, J). $n=16$ dendritic segments from 5 neurons. **** $p<0.0001$, Paired t test. **(K-N)** Acute inhibition of mGluR1 by LY367385 ($100\mu\text{M}$ applied 10 min before and during imaging) disrupts ER Ca^{2+} release and mitochondrial Ca^{2+} uptake during synaptic stimulation (20APs at 10Hz). $n=22$ dendritic segments from 8 neurons. **** $p<0.0001$, Wilcoxon matched pairs signed rank test.

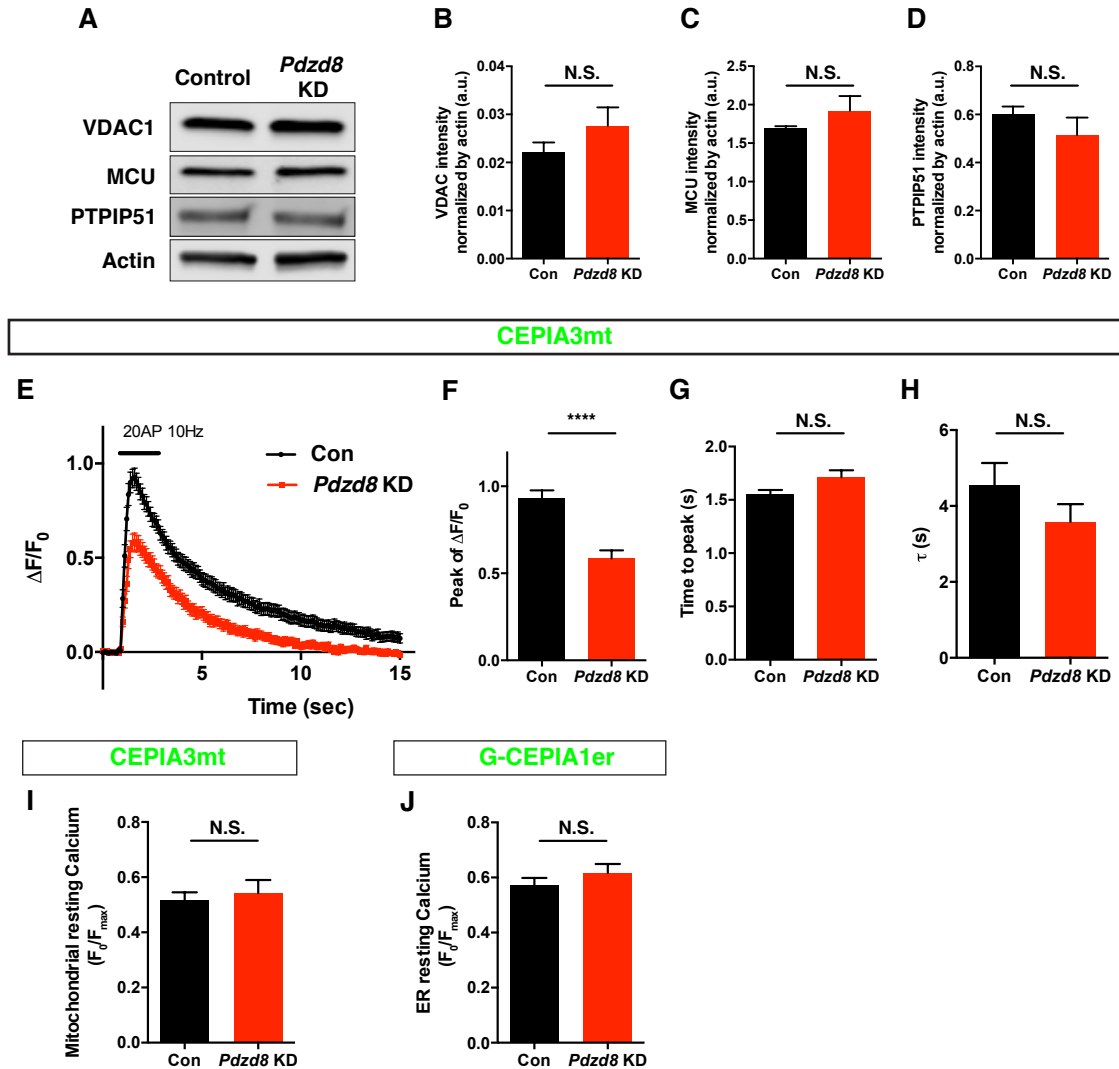


Fig. S13 Mitochondrial Ca^{2+} import machinery and other tethering components are not altered in *Pdzd8* knockdown cortical neurons.

(A-D) Expression of mitochondrial Ca^{2+} regulatory proteins and other tethering proteins are not changed in *Pdzd8* KD neurons. Cultured neurons were infected at 4DIV with lentivirus expressing either control shRNA or *Pdzd8* shRNA and cells were lysed at 14DIV. Immunoblot of control and PDZD8 KO neurons shows no difference in expression level of mitochondrial Ca^{2+} uptake proteins (VDAC1 and MCU) and other ER-mitochondria tethering components (PTPIP51 and VDAC1; n=3 independent immunoblots). (E-H) Mitochondrial Ca^{2+} uptake and extrusion kinetics are not affected by *Pdzd8* KD. Mitochondrial Ca^{2+} dynamics was monitored using CEPIA3mt at 21-22DIV with 20APs. We employed CEPIA3mt for measuring more precisely mitochondrial Ca^{2+} kinetics because of its lower Ca^{2+} affinity ($K_d=11\mu M$) compared to mito-RCaMph1 ($K_d=1.3\mu M$), and thus to minimize saturation in some cells following synaptic stimulation. Images were captured with 100ms interval. Time to peak and decay time constant (τ) are not altered in dendrites of *Pdzd8* KD neurons compared to control

neurons, but peak of the CEPIA3mt signals is significantly reduced (by ~40%). n= 57 dendritic segments from 26 neurons for control, and 67 dendritic segments from 27 neurons for *Pdzd8* KD. **(I-J)** Resting level Ca^{2+} of mitochondria and ER are not changed in *Pdzd8* KD neurons. Somatic CEPIA3mt and dendritic G-CEPIA1er signals were measured before (for F_0) and after ionomycin treatment (for F_{max}). Normalized baseline signals (F_0/F_{max}) showed no significant difference between control and PDZD8-deficient neurons. n=7 neurons for control and 9 neurons for *Pdzd8* KD in somatic CEPIA3mt analysis. n=38 dendritic segments from 14 neurons for control and 32 dendritic segments from 13 neurons for *Pdzd8* KD in dendritic G-GCEPIA1er analysis. Statistical significance: n.s. $p>0.05$, **** $p<0.0001$ according to non-parametric Mann-Whitney test.

Captions for Movies S1-S9

Movie S1. Serial z-stack images of *Pdzd8*-venus knock-in N2a cells.

The cell shown in Figures 1 and S6 (E-H) was imaged using 3D N-SIM (step size 0.12 μm , 20 sections) showing predominant colocalization of PDZD8-Venus with ER (red, Calnexin-mCherry). See legend of Figure 1 for details.

Movies S2 and S3. 3D FIB-SEM image series and animation showing reconstructed ER, mitochondria and ER-mitochondria contacts of control HeLa cells.

Serial EM images (20 nm interval, 100 sections for Movie 2, 83 sections for Movie 3) from areas enriched with sheet-like ER (**Movie 2**) or tubular ER (**Movie 3**), obtained by FIB-SEM of control HeLa cells are shown. The mitochondria and ER were labeled with green and magenta, respectively. This is merged with the 3-dimensional distribution of mitochondria (green) and ER (magenta), followed by display of only ER-mitochondria contact sites labeled in magenta on the last frames. See text for details.

Movies S4 and S5. 3D-SEM image series and animation showing reconstructed ER, mitochondria and ER-mitochondria contacts in PDZD8 KO HeLa cells.

Serial FIB-SEM images (20 nm interval, 38 sections for Movie 4, 75 sections for Movie 5) from areas enriched with sheet-like ER (**Movie 4**) or tubular ER (**Movie 5**) obtained by FIB-SEM of PDZD8-KO HeLa cells are shown. The mitochondria and ER were labeled with green and magenta, respectively. This is merged with the 3-dimensional distribution of mitochondria (green) and ER (magenta), followed by display of only ER-mitochondria contact sites labeled in magenta on the last frames. See text for details.

Movie S6. ATP-induced Ca^{2+} release from ER and transfer into mitochondria in control NIH3T3 cells.

Representative time-lapse images of ER and mitochondrial Ca^{2+} dynamics before and after ATP stimulation. Cells are displayed in ratio view normalized by basal signals (F_0) of each probe using R-CEPIA1er and G-CEPIA3mt. F/F_0 values are plotted for the duration of the acquisition. See text for details.

Movie. S7 ATP-induced Ca²⁺ release from ER and transfer into mitochondria in *Pdzd8* KD NIH3T3 cells.

Representative time-lapse images of ER and mitochondrial Ca²⁺ dynamics before and after ATP stimulation. Color range displayed as ratio of fluorescence value (F) normalized by basal signals (F₀) of each probe (R-CEPIA1er and CEPIA3mt). Bottom panels: F/F₀ values are plotted for the duration of the acquisition. See text for details.

Movie. S8 Ca²⁺ transfer from ER to mitochondria upon synaptic stimulation in proximal dendrites of cortical pyramidal neuron expressing control shRNA.

Representative time-lapse images of ER and mitochondrial Ca²⁺ dynamics before and after synaptic stimulation (20AP, 10Hz) in cortical neurons expressing control shRNA. Color range displayed as ratio of fluorescence values (F) normalized by basal signals (F₀) for each probe (G-CEPIA1er and mito-RCaMP1h). Bottom panels: F/F₀ values are plotted for the duration of the acquisition. See text for details.

Movie. S9 Ca²⁺ transfer from ER to mitochondria upon synaptic stimulation in proximal dendrite of cortical pyramidal neuron expressing *Pdzd8* shRNA.

Representative time-lapse images of ER and mitochondrial Ca²⁺ dynamics before and after synaptic stimulation (20AP, 10Hz) expressing *Pdzd8* shRNA. Color range displayed as ratio view normalized by basal signals (F/F₀) for each probe (G-CEPIA1er and mito-RCaMP1h). Bottom panels: F/F₀ values are plotted for the duration of the acquisition. See text for details.

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