

SUPPLEMENTARY MATERIAL

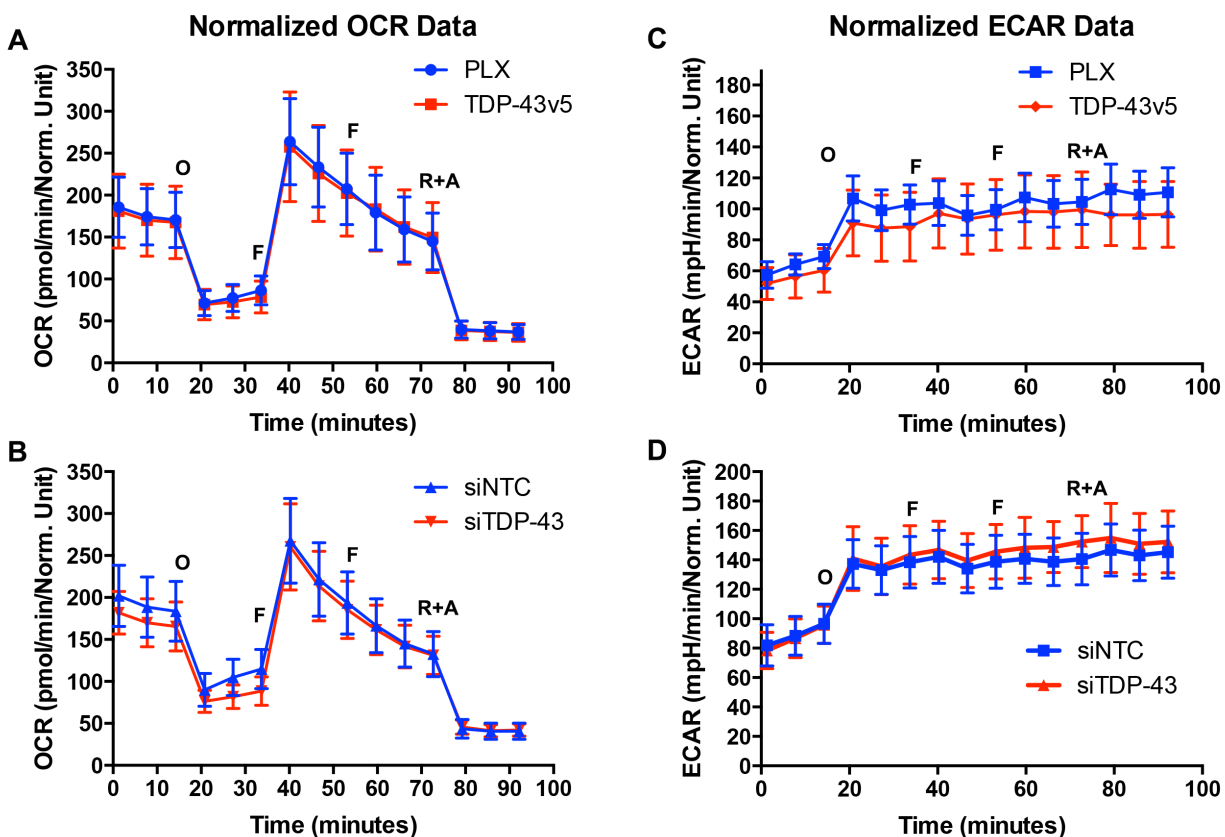


Figure S1. TDP-43 overexpression and knockdown does not significantly affect mitochondrial bioenergetics in HEK293T cells. **A)** Normalized oxygen consumption rate (OCR) data for HEK293T cells transfected with PLX vector or TDP-43v5. **B)** Normalized OCR data for HEK293T cells transfected with siNTC or siTDP-43. **C)** Normalized extracellular acidification rate (ECAR) data for HEK293T cells transfected with PLX vector or TDP-43v5. **D)** Normalized ECAR data for HEK293T cells transfected with siNTC or siTDP-43. Sequential injections of the following compounds were performed at the times indicated in the graphs: oligomycin (O), FCCP (F), and rotenone plus antimycin A (R+A).

Symbol	Protein Description	MW [kDa]	Scores	Validation
Actb	Actin, cytoplasmic 1	41.7	497.9	
Actbl	Beta-actin-like protein 2	42	30	
Aldoa	Fructose-bisphosphate aldolase A	39.3	39.8	
Atp1b1	Sodium/potassium-transporting ATPase subunit beta-1	35.2	94.9	
Atp1b2	Sodium/potassium-transporting ATPase subunit beta-2	33.3	70.3	
Chmp2a	Charged multivesicular body protein 2a	25.1	35.9	
Ckb	Creatine kinase B	42.7	511.3	
Cln5	Ceroid-lipofuscinosis neuronal protein 5 homolog	39.3	43.2	
Cnp	2',3'-cyclic-nucleotide 3'-phosphodiesterase	47.1	245.3	
Coro1a	Coronin-1A	51	33.1	
Ddx39b	DExD-Box Helicase 39B	49	33.8	
Eef1a1	Eukaryotic translation elongation factor 1 alpha 1	50.1	30.5	
Emc6	ER membrane protein complex subunit 6	12	31.8	
Eno1	Enolase 1	47.1	393.2	
Eno2	Enolase 2 (Gamma Enolase)	47.3	415.9	
Flot2	Flotillin 2	47	31.2	
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	35.8	81.5	
Gfap	Glial fibrillary acidic protein	49.9	455	
Glul	Glutamate-ammonia ligase, glutamine synthase	42.1	37.2	
Gpm6a	Neuronal membrane glycoprotein M6-a	31.1	77.3	
Hnrnpd	Heterogeneous nuclear ribonucleoprotein D	38.3	53.4	
Ldha	Lactate dehydrogenase A	36.5	98	
Pgk1	Phosphoglycerate kinase 1	44.5	117.3	
Ppp3ca	Protein phosphatase 3 catalytic subunit alpha	58.6	53.2	Liachko et al. 2016 (36); Davis et al. 2017 (37)
Psmb10	Proteasome subunit beta 10	29	33.3	
Tardbp	TAR DNA-binding protein 43	44.5	185.9	
Tuba1c	Tubulin alpha 1C	49.9	78.6	
Tuba8	Tubulin alpha 8	50	91.2	
Tfe3	Transcription factor binding to IGHM enhancer 3	61.5	55.3	
Ywhae	14-3-3 protein epsilon	29.2	267.6	Volkening et al. 2009 (35)
Ywhaz	14-3-3 protein zeta/delta	27.8	123.9	
Mitochondrial Proteins				
Got2	Aspartate aminotransferase	47.4	138.6	
Aldh2	Aldehyde dehydrogenase, mitochondrial precursor	56.5	49.1	
Mdh1	Malate dehydrogenase 1	35.6	232.6	
Pdha1	Pyruvate dehydrogenase E1 subunit alpha	43.2	31.4	
Phb2*	Prohibitin-2, mitophagy receptor	33.3	32.4	
Uqcrc1	Ubiquinol-Cytochrome C Reductase Core Protein 1	52.7	141.6	
Uqcrc2	Ubiquinol-Cytochrome C Reductase Core Protein 2	48.2	141.4	
Vdac1*	Voltage-dependent anion-selective channel protein 1	32.3	152.6	

Figure S2. Proteomics screen of binding partners from TDP-43 immunoprecipitation from 9-month old wild-type mouse cortex/hippocampus. Asterisk (*) indicates protein interactors that were verified by co-immunoprecipitation in this study.

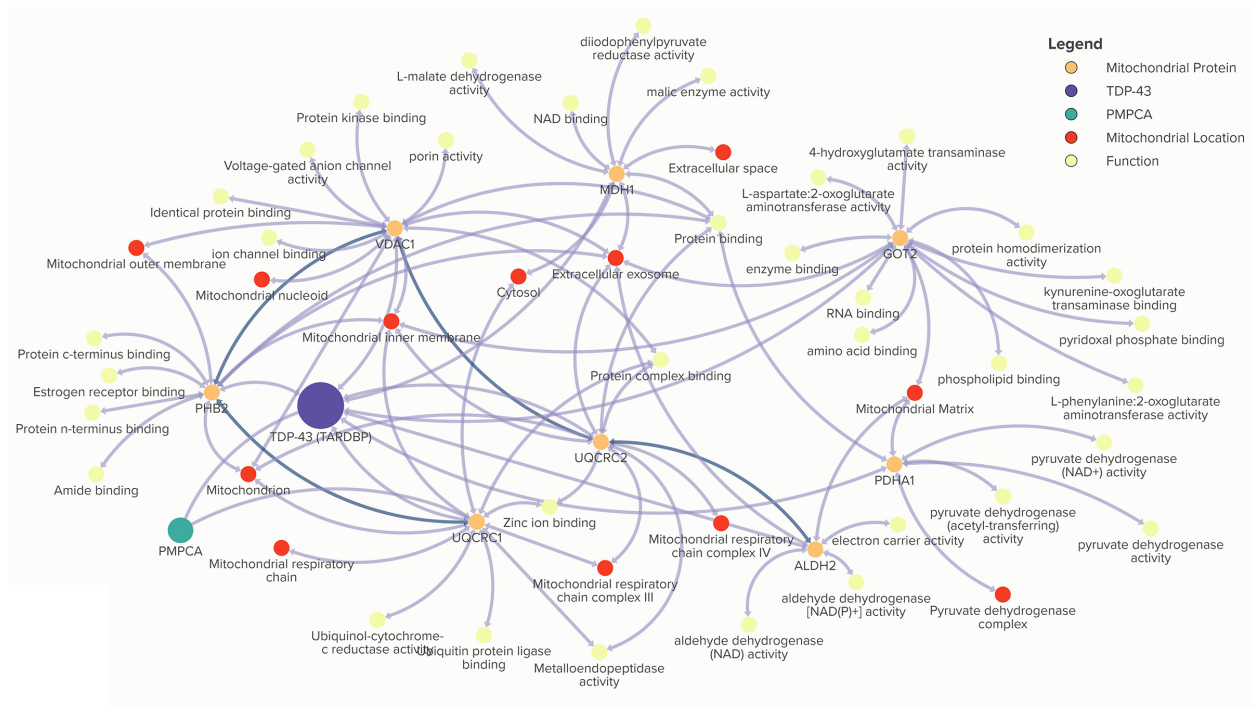


Figure S3. TDP-43 mitochondrial protein interactome map. Using a web-based application for visualizing data and connections (Kumu.io), each identified mitochondrial protein was entered, along with data about each mitochondrial protein from PubMed (<https://www.ncbi.nlm.nih.gov/pubmed/>), including function, pathways, process, and interactions. TDP-43 (purple), peptidase mitochondrial processing alpha subunit, PMPCA (green), specifically identifying each mitochondrial protein (light orange), the mitochondrial locations of each mitochondrial protein (dark orange), known connections/interactions with each other (dark blue connections), and known functions of each mitochondrial protein (yellow). Analysis and visualization of the data indicated that the most common subcellular location of the mitochondrial proteins were the mitochondrial inner membrane along with extracellular exosomes (see review)[29]. Five of the identified mitochondrial proteins have known direct interactions with each other. The interactive map is available online for viewing at <https://kumu.io/-/56707#map-XGtXLJMj>, with real-time updates.

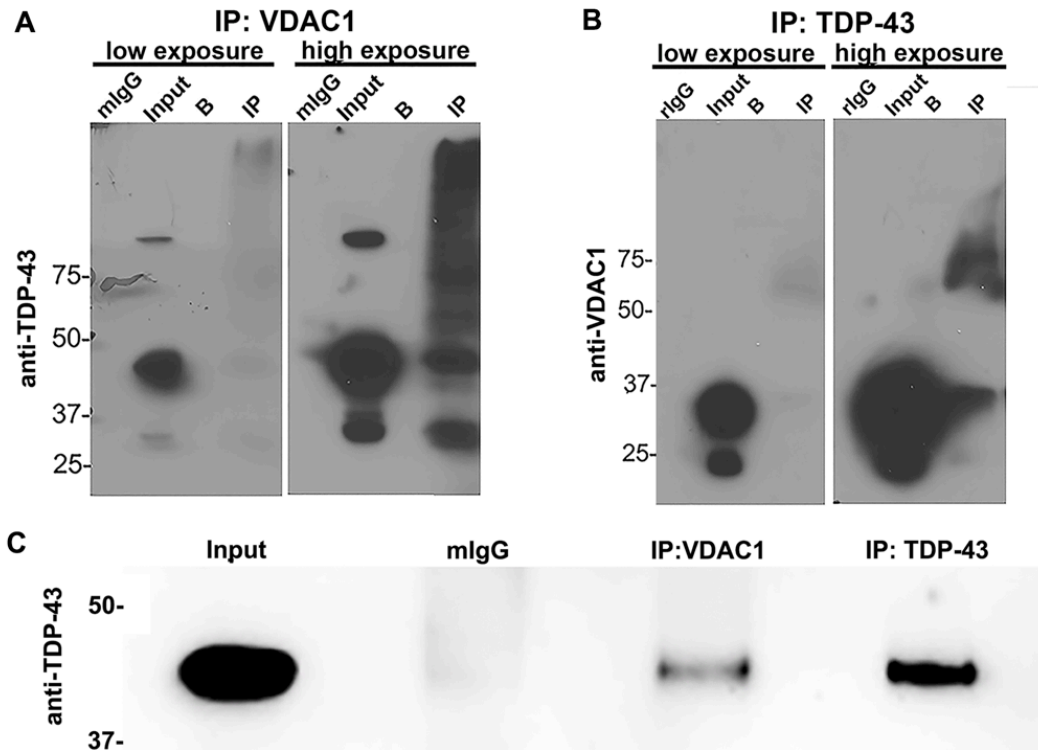


Figure S4. TDP-43 interacts with VDAC1. **A)** Immunoprecipitation anti-VDAC1 probed for TDP-43 (PTG, ProteinTech Group) (short exposure left, long exposure right) IgG control, input (cortex human brain), blank (B), human control, **B)** immunoprecipitation anti-TDP-43 probed with VDAC1 (**bottom**) (short exposure left, long exposure right) **C)** Immunoprecipitation of TDP-43 and VDAC1 probed for TDP-43 in HEK293T cells (covalently linked antibodies, Magnabeads, Invitrogen), input (HEK293T lysate) mouse IgG control (mIgG), immunoprecipitation (IP) VDAC1 and TDP-43.

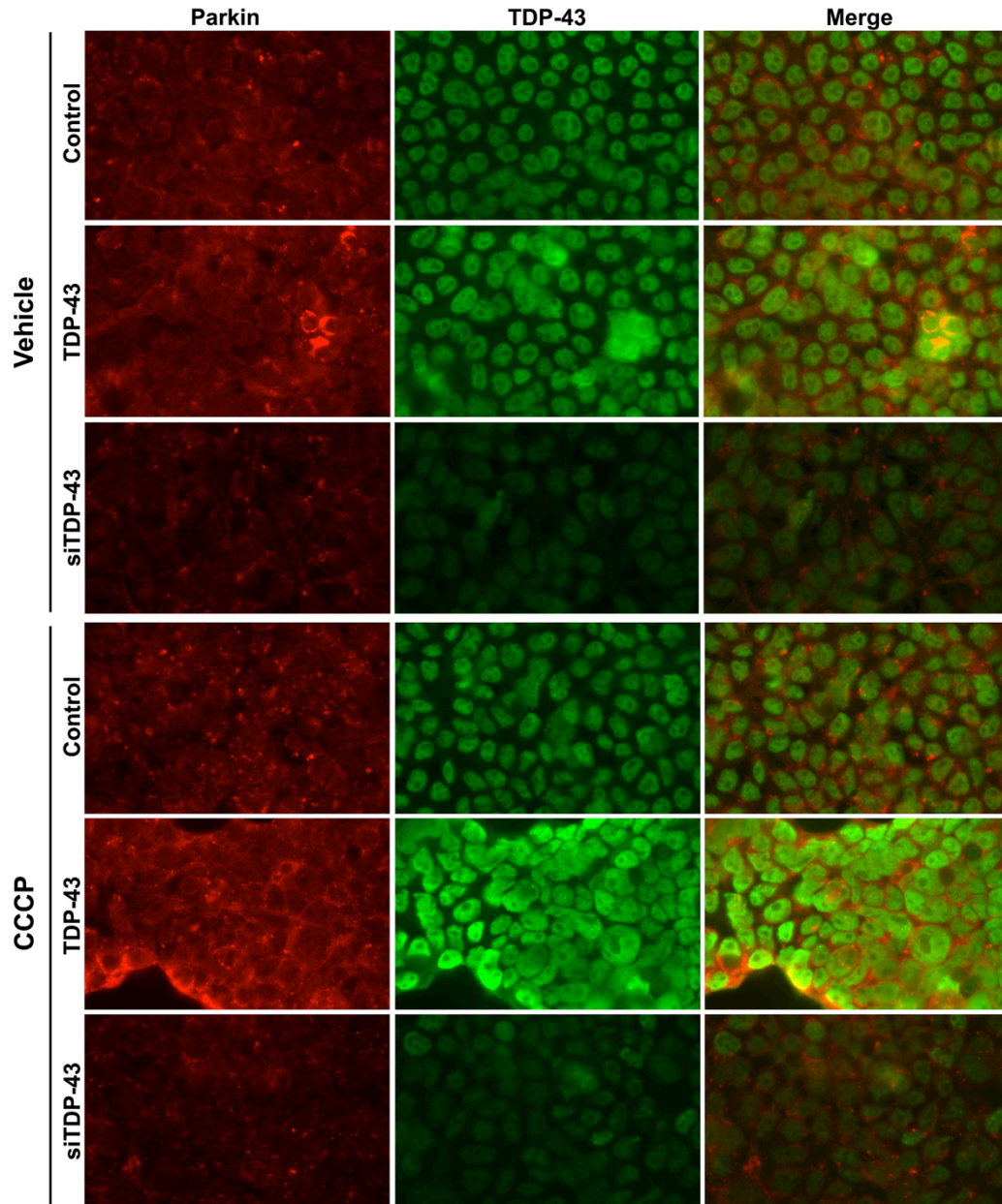


Figure S5. Parkin cytoplasmic localization increases in cells overexpressing TDP-43. Vehicle, TDP-43 overexpression, and siTDP-43 knockdown in HEK293T cells treated with vehicle (0.1% DMSO, top panels) and cells treated with 10 μ M CCCP for 24 hours (bottom panel). Immunofluorescence of TDP-43 (C-terminal, 488nm, green), Parkin (Genscript, 555nm, red), and merge (400x).

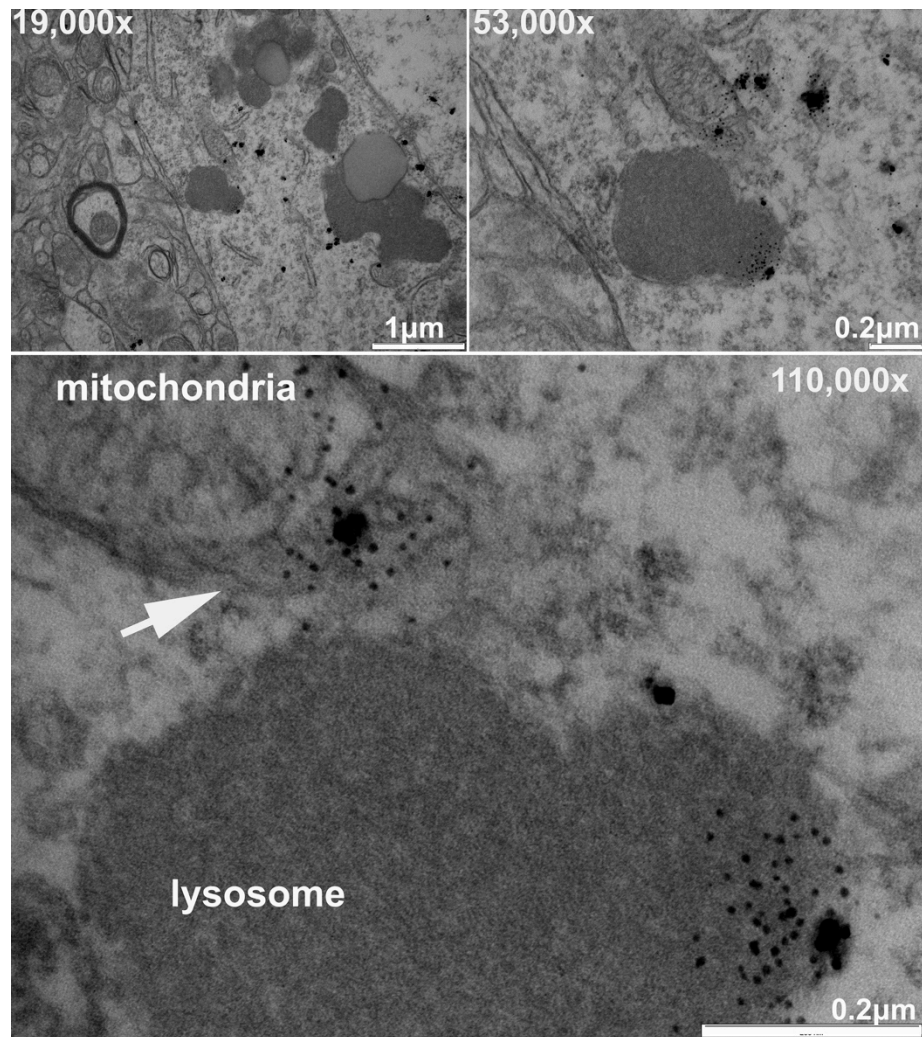


Figure S6. Transmission electron micrograph of endogenous immunogold labeled TDP-43 in the hippocampus of 8-month-old APP/PS1 mice. Immunogold labeled TDP-43 localizes around the mitochondria in APP/PS1 mice (19,500x, 53,000x, & 110,000x magnification). Mitochondria and lysosome labeled TDP-43. Mitochondrial membrane disorganized between mitochondria and lysosome (arrow).

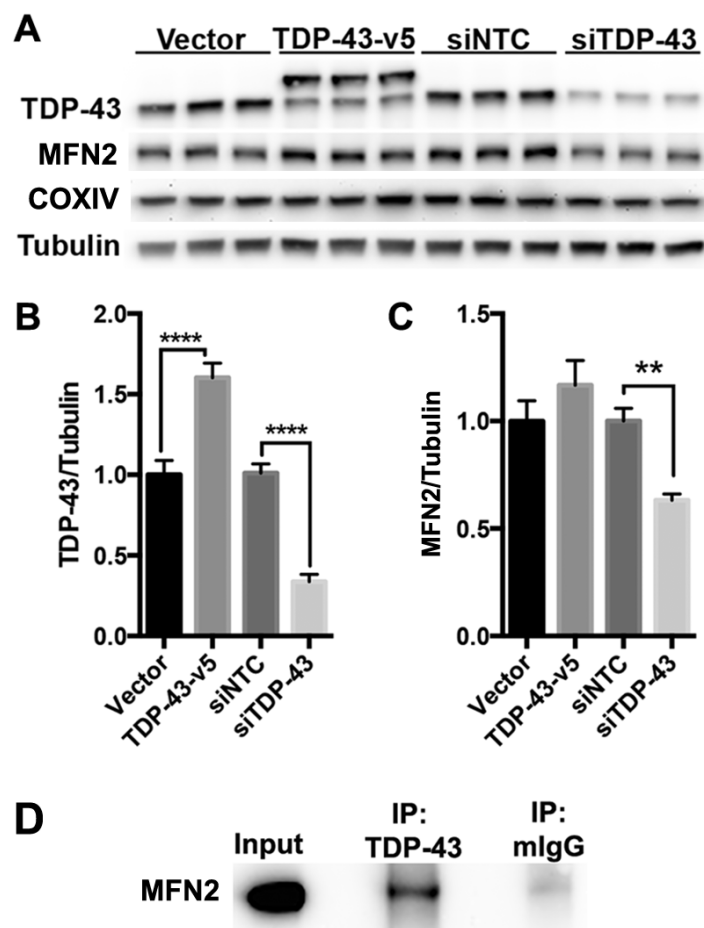


Figure S7. TDP-43 interacts with MFN2. **A)** Western blot for TDP-43 and MFN2 in HEK293T cells transfected with Vector (PLX), TDP-43 (v-5-tagged, TDP-43-v5), siNTC (non-targeting control), or siTDP-43. COXIV and Tubulin were used as loading controls. **B)** Quantitation of TDP-43/Tubulin overexpression and knockdown in HEK293 cells. **C)** Quantitation of MFN2/tubulin Western blot in HEK293T cells. **D)** Immunoblot of mitofusin-2 (MFN2, Cell Signaling) for immunoprecipitated TDP-43 (IP:TDP-43). IP: mIgG, mouse IgG control.

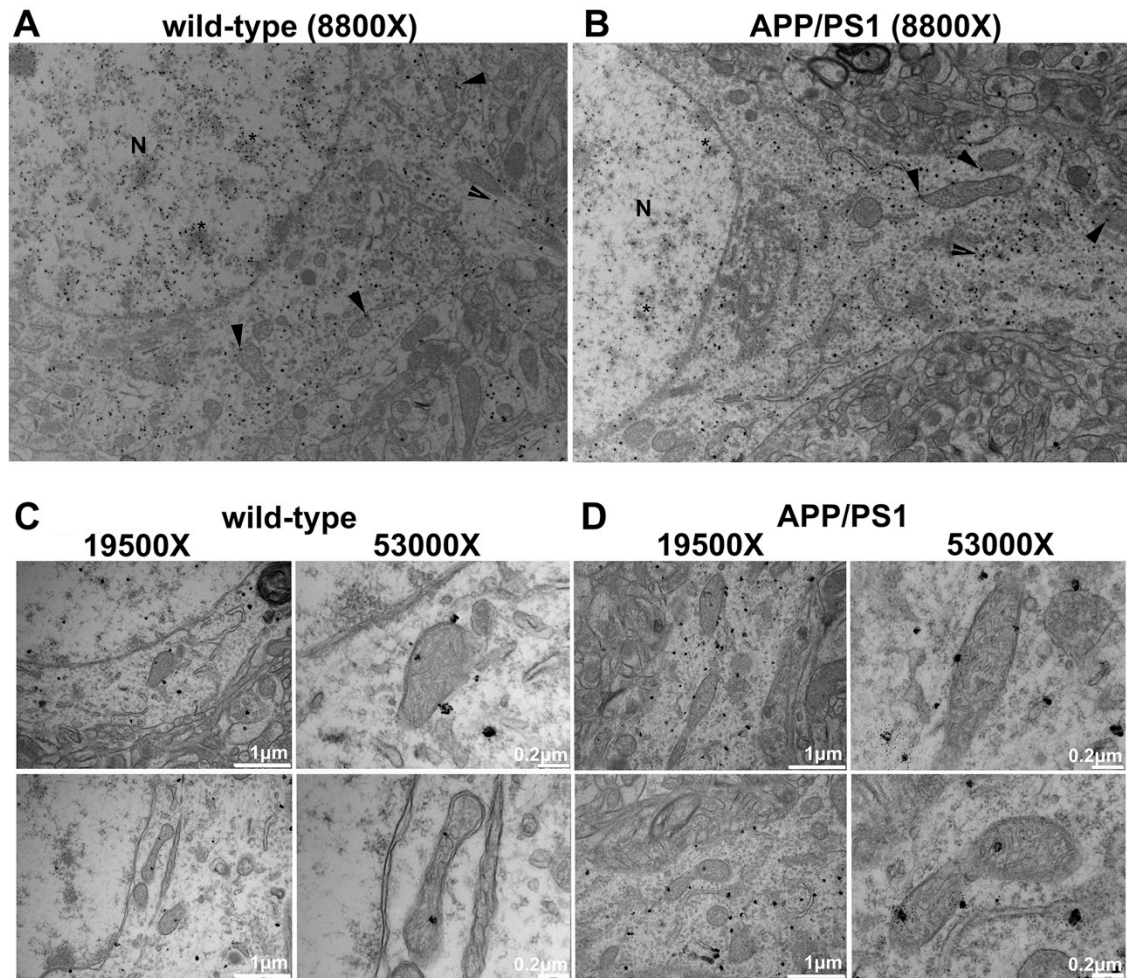


Figure S8. Transmission electron micrograph of immunogold labeled TDP-43 in the hippocampus of 8-month-old wild-type and APP/PS1 mice. **A)** Wild type and **B)** APP/PS1 hippocampus in 8-month old mice transmission electron microscopy immunogold labeled for TDP-43. TDP-43 labels throughout the nucleus (**N**) and cytoplasm in both wild-type and APP/PS1 mice. TDP-43 is labeled in clusters within the nucleus at sites of possible transcription indicated by an asterisk (*). TDP-43 is labeled in 8-month-old wild-type APP/PS1 mice within the cytoplasm. TDP-43 is also present throughout the cytoskeleton (**open arrow**) and localized around mitochondria (**black arrow**) (8,800x magnification). **C & D)** Immunogold labeled TDP-43 localizes around the mitochondria in wild-type and APP/PS1 mice (19,500x and 53,000x).

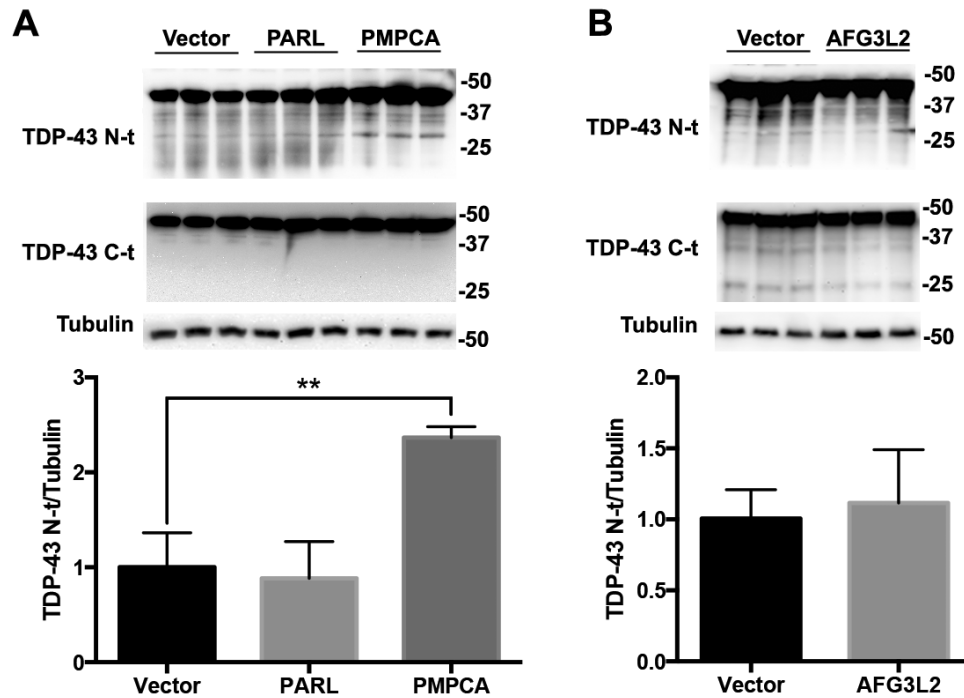


Figure S9. Overexpression of Mitochondrial-processing peptidase subunit alpha (PMPCA) increases 27kDa TDP-43. A) Western blots for N- and C-terminal TDP-43 in SVGP12 cells transfected with vector (PLX), PARL, PMPCA. There was a significant increase in 27kDa N-terminal TDP-43 in cells overexpressing PMPCA. **B)** Western blots for N- and C-terminal TDP-43 in HEK293T cells transfected with vector (PLX) or AFG3L2. There was no significant difference in TDP-43 expression. Tubulin was used as a loading control.

SUPPLEMENTARY METHODS

Cell culture, transfections, and CCCP treatment

Human embryonic kidney cells (HEK293T) cells were maintained in a humidified atmosphere at 95% air and 5% CO₂, and grown in Dulbecco's Modified Eagle Medium supplemented with 1% penicillin/streptomycin and 5% fetal bovine serum. Cells were transfected with either pLX304 vector or pLX-TDP-43-v5 (v5-tagged TDP-43) using Lipofectamine 3000. For knockdown experiments, cells were transfected with TDP-43 siRNA (siTDP-43) or non-targeting siRNA control (IDT, Trifecta RNAi Kit) using Lipofectamine RNAiMAX according to the manufacturer's recommendations. Cells were treated with 10μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) 30 hours post-transfection and lysed 12 hours later.

Analysis of mitochondrial function using Seahorse

HEK293T cells were plated on poly-D-lysine coated Agilent Seahorse XF96 cell culture plates at a density of 8,000 cells per well. Five hours after seeding, cells were transfected with PLX (v5 vector control) or plxTDP-43v5 using Lipofectamine LTX and non-targeting control siRNA or siTDP-43 using RNAiMAX. 48 hours later, cells were incubated in a humidified non-CO₂ incubator at 37°C for one hour in assay medium (Seahorse XF Base Media supplemented with 1mM pyruvate, 2mM glutamine, and 10mM glucose, pH 7.4) and then oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured using the Agilent XF Cell Mito Stress Test Kit and Seahorse XFe96 Analyzer (Agilent Technologies) with Wave 2.4 software. 1μM of oligomycin, two injections of 0.5μM Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), and 0.5μM rotenone/antimycin A were used. Cells were stained with DAPI followed by fixation in 4% PFA for normalization. A SpectraMax5e plate reader was used in fluorescence mode with WellScan feature with plate parameters adjusted to dimensions of the XF96 cell culture plate. Normalization was applied in Wave with a factor of 5000 and graphs were generated using Prism GraphPad.

Mice

APP^{swe}/PSEN1^{dE9} (APP/PS1) (Jackson Lab, stock# 005864), Camk2a-tTA (tet-off, Jackson Lab 007004), TetO-hTDP-43 (developed by M. Gitcho and N. Cairns, Washington University School of

Medicine, St. Louis, MO) mice were maintained on a C57BL/6J background (Jackson Laboratory, stock # 000664) for this study.

Immunofluorescent Staining

HEK293T cells were cultured on poly D-lysine (PDL)-coated coverslips in 24-well plates and then fixed in 4% paraformaldehyde. After washing with PBS, cells were blocked in 5% BSA and 0.3% Triton-X in PBS for one hour at room temperature. Cells were then incubated in primary antibodies (anti-Parkin, Genscript, A01250 and anti-TDP-43 (C-terminal) mouse monoclonal developed by Genscript) for one hour, washed with PBS three times and then incubated in secondary Alexa-fluor antibodies (rabbit 555nm, A10470 and mouse 488nm, A10468, Invitrogen Inc.) for one hour followed by washing with PBS. Coverslips were mounted onto slides using Fluoromount-G mounting media (Southern Biotech).

Proteomics

TDP-43 was immunoprecipitated from wild-type mouse cortex lysate as described above using anti-TDP-43 antibodies (Proteintech Group, 10782-2-AP, Rosemont, IL). Immunoprecipitated proteins were run on a 15% SDS-PAGE gel and stained with SimplyBlue (Invitrogen, 46-5034). Bands between 20 and 70kDa were excised and digested with trypsin. Trypsin digested immunoprecipitates were analyzed by LC-MS/MS. Tryptic peptides were injected onto a Waters Nanoacquity UPLC equipped with a nanoAcquity BEH C18 column (0.075 x 15cm) at a flow rate of 300 nL/min and eluted over 80 min with a 5-40% acetonitrile (+0.1% formic acid) gradient. Peptides were analyzed with a Bruker amaZon 3D ion trap mass spectrometer using data-dependent acquisition. Database searches were performed against the mouse SwissProt database using Mascot (Matrix Science) with a mass accuracy of 0.1 Da for both MS1 and MS2 data.

Immunoprecipitation and Western Blots

For immunoprecipitation (IP), human cortex tissue was lysed in an optimal IP buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 100 μ M sodium orthovanadate, and a protease inhibitor cocktail (Sigma, P8340) with Protein A agarose beads as previously described [11]. For IP validation in HEK293T cells, TDP-43 (C-terminal, monoclonal), control mouse and rabbit IgG (Genscript) antibodies were cross-linked to magnetic beads as per manufacturer's

protocol (ThermoFisher Scientific, 88805) modified to eliminate non-specific binding with two high salt washes (50mMTris-HCl pH 7.5, 500mM NaCl, 0.1% NP-40, 0.05% sodium dexoycholate) followed by two low salt washes (50mMTris-HCl pH 7.5, 0.1% NP-40, 0.05% sodium dexoycholate).

The following antibodies were used for Western blotting: TDP-43 (N-terminal, epitope: MSEYIRVTEDENDIEIPSEDDGTVLLST rabbit polyclonal developed by Genscript), TDP-43 (C-terminal, epitope: FGSSMSDKSSGWGM, mouse monoclonal developed by Genscript) (N- and C-terminal TDP-43 antibodies previously characterized) (1/1000) [9], COXIV (CST, 4850) (1/1000), Histone H3 (CST, 4499) (1/1000), APP (6E10, Covance, SIG-39321) (1/1000), VDAC1 (UC-Davis/NIH NeuroMab Facility, N152B/23) (1/1000), beta tubulin (1/5000) (E7, developed by Michael Klymkowsky and obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City), PHB2 (CST, 140185) (1/1000), PHB2 (Proteintech, 66424-1-Ig) (1/1000), LC3B (CST, 3868S) (1/1000), MFN2 (CST, 9428S) (1/1000), and phospho-DRP1 Ser637 (Sigma, sab4301399). N- and C-terminal TDP-43 antibodies have been validated with siRNA experiments in both mouse and human cell lines and have been used for Western blots, immunostaining, and immunoprecipitation in Davis *et al.*, 2017 [9].

Immunogold labeling transmission electron microscopy

8-month-old wild-type and APP/PS1 mice for were perfused with 4% paraformaldehyde in PBS and hippocampal samples were dissected and immersion fixed in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1M Sorensens phosphate buffer pH=7.4 (PB). Slices were cut sagittally from dissected brain samples using a vibrating microtome (Leica, Germany). All immunolabeling steps were performed using Aurion immunogold pre-embedding products and procedures. To inactivate residual aldehyde groups present after aldehyde fixation, the specimens were incubated with 0.1% NaBH₄ in 20 mM phosphate buffered saline (PBS) buffer for 15-30 minutes then washed with PBS three times for 10 minutes, incubated in 0.05% Triton-X-100 in PBS for 30 min., and then transferred into matching Aurion goat blocking solution for 30 minutes. Samples were washed with a modified Aurion incubation buffer (PBS, 0.1 - 0.2% Aurion BSA-c) twice for 10 minutes. Separate samples were incubated with 5 µg/ml dilutions of TDP-43 (Proteintech Group Inc., Chicago, IL) antibody made up in incubation buffer overnight at 4°C. The specimens were washed with incubation buffer for 6 x 10 minutes and then were transferred into aliquots of F(ab')₂ fragment of Goat-anti-Rabbit IgG (H&L), diluted 1/50 in incubation solution overnight at 4°C.

Specimens were washed with incubation buffer six times for 10 minutes each, washed twice with PBS for 10 minutes each, twice with PB, postfixed in 2% glutaraldehyde in PB for 15 minutes, and finally washed with Aurion Enhancement Conditioning Solution three times for 10 minutes. Silver Enhancement was performed using Aurion R-Gent SE-EM, as described in the kit directions, for 75 minutes. The immunolabeled samples were post-fixed in 0.5% osmium tetroxide, 1% potassium ferrocyanide in 0.1M PB for 1 hour at RT, and rinsed five times in PB. The post-fixed and rinsed samples were dehydrated in a graded series of ethanol (EtOH) in the increasing percentages for 10 minutes and 100% EtOH for 3 x 10 minutes at RT. Fully dehydrated samples were infiltrated in increasing concentrations of PolyBed 812 (Polysciences Inc., Warrington, PA) and Propylene Oxide PO mixtures as previously described. Embedding and polymerization took place in fresh PolyBed 812 for 48 hours at 60°C sandwiched between two 25x75mm Rain-X coated glass microscope slides. Polymerized flat samples were trimmed out and remounted. The samples were sectioned on a Leica EM UC6 ultramicrotome at 90 nm. The sections were collected on Cu, pioloform coated 2x1 oval slot grids (EMS, Hatfield, PA), and post-stained in uranyl acetate and lead citrate. The sectioned samples were viewed at 80kV on a Philips CM120 transmission electron microscope, equipped with MegaView III camera (Olympus Soft Imaging System Lakewood, CO).

Mitochondrial isolation

Cortex and hippocampus were isolated from 12-month-old wild-type and APP/PS1 mice and mitochondria were isolated using Percoll density centrifugation following Method C as described [34].

Statistical analysis

Data analyses were performed using a one-way or two-way analysis of variance (ANOVA) with Tukey correction for type 1 error. For each experiment, the significance levels were determined and data expressed as mean \pm standard deviation (n=3, from separate experiments and *in vivo* studies utilized littermate biological replicates).