Molecular Visualization of Neuronal TDP43 Pathology *In Situ*

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Materials and Methods

Primary Neuron Cell Culture

Cortices from embryonic day (E)19-20 Long-Evans rat embryos were dissected and disassociated, and primary neurons were plated at a density of $6x10^5$ cells/mL on coverslips and incubated at 37˚C and 5% CO_2. At *in vitro* day (DIV) 7, RNAse-free 1X Tris EDTA (TE) buffer (Corning, 46-009-CM) and 2µM Cy5-labeled $(GU)_6$ resuspended in 1X TE buffer or RNAse-free water were added to the primary neurons in Neurobasal Complete Media (Neurobasal (Gibco 21103-049), 1X B27 Supplement (Gibco, 17504-044), 1X Glutamax (Gibco 35050-061), 100 units/mL Penicillin/Streptomycin (Gibco, 15140163)) for 1h, 6h, 24h, and 48h followed by immunocytochemistry.

Ethics statement

All vertebrate animal work was approved by the Committee on the Use and Care of Animals (UCUCA) at the University of Michigan (UM), and all experiments were performed in accordance with UCUCA guidelines. Rats (*Rattus norvegicus*) used for primary neuron collection were housed singly in chambers equipped with environmental enrichment. All studies were designed to minimize animal use and suffering. Rats were cared for by the UM Unit for Laboratory Animal Medicine. All individuals caring for animals were trained and approved to care for and maintain rodent colonies in the long term, in accordance with the NIH-supported Guide for the Care and Use of Laboratory Animals. All personnel handling the rats and administering euthanasia were properly trained in accordance with the UM Policy for Education and Training of Animal Care and Use. Euthanasia was entirely consistent with the recommendations of the Guidelines on Euthanasia of the American Veterinary Medical Association. Brains from individual pups in each litter were pooled to maximize cell counts prior to plating; as a result, primary cortical neurons used for all studies include an even mix of cells from both male and female pups.

Maintenance of iPSCs

iPSCs were maintained in TeSR-E8 media (StemCell Technologies, 05990) on 60 mm dishes (Falcon, 353004) coated with 1:100 vitronectin (Gibco, A14700) in Dulbecco's Mg^{2+}/Ca^{2+} free phosphate buffered saline (DPBS, Gibco, 14190144) and passaged every 4–5 days using 0.5 mM EDTA (Sigma-Aldrich, E7889) dissolved in PBS followed by gentle trituration in TeSR-E8 media with a P1000 pipet. All lines were verified mycoplasma-free monthly. A doxycycline-inducible cassette for induced expression of NGN1/2 was integrated into the *CLYBL* safe harbor locus of each line, enabling directed differentiation into forebrain-like glutamatergic iNeurons(*41*). iPSCs were grown in 60mm dishes in TeSR-E8 media supplemented with 2 µg/mL doxycycline (Sigma-Aldrich, D3447) for two days. TeSR-E8 + doxycycline media was replaced daily. Cells were removed from the plate by washing with Mg^{2+}/Ca^{2+} free DPBS and incubated in Accutase (Sigma-Aldrich, A6964) for 5 min at 37˚C and resuspended in DMEM/F12 media (Gibco, 11330032). Cell

slurry was centrifuged at 300*g* for 5 min, and the supernatant was aspirated. The pellet was resuspended in TeSR-E8 media with 5μM ROCK inhibitor (Cayman Chemical, 10005583) and triturated 20 times gently to achieve single cells. Neural progenitor cells (NPCs) were counted with a hemocytometer (Hausser Scientific), diluted to the appropriate volume and density, and frozen in TeSR-E8 media with 10% DMSO (Fisher Scientific, D1391). NPCs were stored in liquid nitrogen until further use. These frozen vials were thawed and plated, as detailed below.

Differentiation into Forebrain-like Neurons (iNeurons)

35 mm dishes (MatTek Life Sciences, P35G-1.5-14-C), 6-well plates (Costar, 3506), and 96-well plates (Perkin-Elmer, 6055300) were coated overnight at room temperature with 100 μg/mL poly-L-ornithine (PLO, Sigma-Aldrich, P3655) prepared in filter-sterilized (0.22μm filter) 0.1M borate buffer at pH 8.4. Borate buffer was prepared with 100mM boric acid (Sigma-Aldrich, B6768), 25mM sodium tetraborate (Sigma-Aldrich, 221732), and 75mM sodium chloride (Sigma-Aldrich, S7653). The following steps were performed subsequently.

Day 1: PLO was removed by washing with sterile water 4 times and air-dried for at least 1h. 35 mm dishes, 6-well plates, and 96-well plates were coated with 10ug/mL laminin (Corning, 354232) diluted in DPBS (Gibco, 14190144) and incubated for 1h at 37˚C, 5% CO_2. NPCs were plated directly into TeSR-E8 media supplemented with 5μM ROCK inhibitor and 2μg/mL doxycycline at the following concentrations: $1x10^5$ cells/well in a 35 mm dish, 1×10^6 cells/well in a 6-well plate, and $7x10^3$ cells/well in a 96-well plate and incubated at 37˚C overnight.

Day 2: TeSR-E8 media was removed and replaced by transition media consisting of 1X N2 Supplement (Gibco, 17502048), 1X NEAA Supplement (Gibco, 11140050), 10 ng/mL BDNF (PeproTech 450-02), 10 ng/mL NT3 (PeproTech 450-03), 0.2 μg/mL laminin (Sigma-Aldrich, L2020), and 2 μg/mL doxycycline in 1:1 TeSR-E8 and DMEM/F12 media (Gibco 11320-033).

Day 3: Transition media was removed and changed to B27 media (1X B27 Supplement (Gibco, 17504-044), 1X GlutaMAX supplement (Gibco 35050-061), 10 ng/mL BDNF, 10 ng/mL NT3, 0.2 μg/mL laminin, 2 μg/mL doxycycline, and 1X CultureOne Supplement (Gibco, A3320201) in Neurobasal-A (Gibco, 12349015). Cells were sustained in the same B27 culture medium until Day 6.

Day 6: An equal volume of B27 media without CultureOne Supplement was added to each well. Cells were sustained in the same B27 culture medium until treatment with 2μM $(GU)_6$ RNA on Day 7 or 8 for immunocytochemistry and *in situ* cryo-ET experiments.

Immunocytochemistry

Primary neurons were fixed on 12 mm glass coverslips (Fisher, 12-545-80P) in a 12-well plate (Corning, 3513) or in 96-well plates (Corning, 07-200-90). iNeurons and U2OS cells were fixed in 96-well plates (Perkin-Elmer, 6055300) with 4% paraformaldehyde (Electron Microscopy Sciences, 15710) for 10 min at room temperature, rinsed with PBS, and permeabilized with 0.1%

Triton X-100 (Bio-Rad, 161-0407) for 20 min in PBS. Subsequently, neurons were treated with 1mM glycine (Fisher BioReagents, BP3811) in PBS for 20 min, rinsed with PBS, and then incubated in blocking solution (0.1% Triton X-100, 2% fetal calf serum (Sigma-Aldrich, F4135), and 3% bovine serum albumin (Fisher, BP9703-100) at room temperature for 1h.

After blocking, cells were incubated overnight at 4˚C in primary antibody diluted in blocking buffer (Table S1 - Antibody). Cells were washed 3 times with PBS and incubated for 1h at room temperature with a secondary antibody diluted in blocking buffer (Table S1 – Antibody). Cells were washed 5 times for 5 min with PBS and imaged by fluorescence microscopy. For primary neurons, coverslips were mounted with Prolong Gold Antifade Mountant with DAPI (Invitrogen, P36935) on microscope slides (Fisher, 1255400) and imaged using a Nikon Eclipse Ti inverted microscope equipped with a PerfectFocus3a 20x objective lens and an Andor iXon3 897 EMCCD camera.

For TDP43 nuclear-cytoplasmic ratio (N/C) analysis, primary neurons and U2OS cells were imaged on an AXR NSPARC confocal system (Nikon Instruments Inc.) with a 20x and 40x S Plan Fluor ELWD DIC N1 objective with a working distance of 7.40mm and 3.11mm, respectively. Regions of interest (ROIs) were manually drawn around the nuclei and cells in Fiji to determine the mean fluorescence intensity of nuclear and cytoplasmic DAPI and TDP43. For each ROI, the TDP43 nuclear and cytoplasmic intensity were normalized by the mean DAPI nuclear and cytoplasmic intensity to account for regional variation in intensity. Normalized TDP43 N/C values were plotted using SuperPlotsOfData (https://huygens.science.uva.nl/SuperPlotsOfData/)(*88*). For U2OS TDP43 N/C data, a Welch's t-test was performed within SuperPlotsOfData to assess statistical significance. The percentage of primary rat neurons with mislocalized TDP43 was determined by calculating the TDP43 N/C values below 50% of the median TDP43 N/C for untreated neurons for each replicate and timepoint.

To assess statistical differences in primary neuron TDP43 N/C values between treatments over time, a generalized estimating equations (GEE) model was fit to the mean normalized TDP43 N/C data across 1h, 6h, and 24h timepoints after $(GU)_6$ treatment using the *statsmodel* library in Python 3.9.7. The outcome was the log-transformed normalized intensity with an identity link, and the predictors were continuous time, treatment, and interaction. The model used an exchangeable correlation structure to account for the correlation between cells in the same well. The parameters and standard errors were estimated using the Liang and Zeger sandwich estimator to consider the potential misspecification of the correlation structure(*35*).

For iNeuron experiments, high-resolution Z-stacks were obtained on an AXR NSPARC confocal system (Nikon Instruments Inc.) with a 60x NA1.42 Oil/DIS Plan Apochromat Lambda D objective with a working distance of 1.5mm. Denoising and 3D deconvolution were accomplished through NIS-Elements software (Nikon Instruments Inc.).

HEK293T and U2OS Cell Culture

Human embryonic kidney (HEK) 293T cells were cultured in DMEM (Corning, 10-013-CV), supplemented with 10% fetal bovine serum (FBS, Gibco, 10082147) and 100 units/mL Penicillin/Streptomycin (Gibco, 15140163) at 37˚C in 5% CO2. Cells were passaged as required. U2OS cells were cultured in DMEM (Corning, 10-013-CV), supplemented with 10% fetal bovine serum (FBS, Gibco, 10082147), 100 units/mL Penicillin/Streptomycin (Gibco, 15140163), and 1X Glutamax (Gibco 35050-061) at 37˚C in 5% CO2.

Cryptic Splicing Assay

4.0×10^5 U2OS cells/well and 1×10^6 WT (1021 474B 1077H) NPCs/well were plated in 6-well plates (Corning, 3506). U2OS cells grown to 60% confluence and treated with one of the following conditions: 6µM Endo-Porter (GeneTools, OT-EP-PEG-1) and 6µM Endo-Porter with 2µM $(GU)_6$ RNA for 4h and 24h. DIV9 iNeurons were treated for 4h and 24h with 2µM $(GU)_6$ RNA. The RNeasy Mini Kit (QIAGEN, 74106) was used to extract RNA from the treated U2OS cells and iNeurons. RNA concentrations were measured using a NanoDrop (Thermo Scientific, 13400525), and 500-1000ng of RNA was used for reverse transcription. cDNA synthesis was performed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814). Gene expression analysis was performed by qPCR using PowerUp SYBR Green Master Mix (Applied Biosystems, A25742) with *UNC13A* and *GAPDH* primers (Table S1 - Oligo) on a QuantStudio 3 Real-Time PCR system (Applied Biosystems) and quantified using the $\Delta\Delta C_t$ method(*89*). A Welch's t-test was performed using GraphPad Prism version 10.1.2 to assess statistical significance.

HCR-FISH

HCR-FISH was performed according to the manufacturer's protocol(*90*). 7×10^3 WT (1021 474B 1077H) NPCs/well were plated in a 96-well plate (Table S1 – Cell Lines). DIV9 iNeurons were treated with 2µM $(GU)_6$ RNA for 24h and fixed with 4% paraformaldehyde (Electron Microscopy Sciences, 15710) for 10 min at room temperature, then washed three times in PBS. Fixed iNeurons were permeabilized with 70% ethanol overnight at -80˚C. Ethanol was removed and the iNeurons were permeabilized with 0.25% Triton X-100 in PBS for 10 min, followed by immunocytochemistry analysis as described above. Fixed iNeurons were incubated in a warm probe hybridization buffer (Molecular Instruments) for 20 min at 37˚C. 10nM of custom probes targeting native exons in *UNC13A* (Molecular Instruments; Table S1 - Oligos) were added to iNeurons in probe hybridization buffer and hybridized overnight at 37˚C. Samples were washed three times for 5 min with the warm probe wash buffer. Subsequently, samples were washed three times for 5 min with 5X saline sodium citrate + 0.1% Tween 20 (SSCT) (Molecular Instruments) at room temperature. Samples were preincubated with an amplification buffer (Molecular Instruments) for 30 min at room temperature. B2h1-488 and B2h2-488 hairpin amplifiers (Molecular Instruments) were heated at 95˚C for 90s, cooled to room temperature for 30 min, and then 60nM hairpin amplifiers in the amplification buffer were added to samples at room

temperature overnight. Samples were washed five times for 5 min with 5X SSCT buffer and then stored at 4˚C until imaged. iNeurons were imaged on an AXR NSPARC confocal system (Nikon Instruments Inc.) with a 40x CFI Apochromat LWD Lambda S objective with a working distance of 0.30mm. A custom Fiji macro was used to quantify the number of puncta corresponding to native *UNC13A* transcripts within each neuron. A Welch's t-test was performed using GraphPad Prism version 10.1.2 to assess statistical significance.

Gradient Fractionation

Isolation of autophagosomes and lysosomes from cell lines

Dendra2-LC3B HEK293T cells were grown to 80% confluency (~20 million cells) in a 150 mm dish (Thermo Scientific, 130183). Cells were treated with one of the following conditions: 6µM Endo-Porter for 4h (control), 250nM Torin 1 (Tocris, 4247) for 1h, 2µM A_{13} RNA with 6 µM Endo-Porter for 4h, or 2µM $(GU)_6$ RNA with 6 µM Endo-Porter for 4h. Dendra2-LC3B cells were washed with ice-cold PBS, collected by scraping, and pelleted at 500*g* for 5 min at 4˚C. The pellet was resuspended in ice-cold lysis buffer (0.25M sucrose, 1mM EDTA, 10mM HEPES pH 7.4, fresh protease inhibitors: 1mM PMSF (Sigma-Aldrich, P7626), 1X Protease Inhibitor Cocktail (APExBIO, K1010) and centrifuged again at 500*g* for 5 min at 4˚C. The following lysis and fractionation steps were all performed with ice-cold buffers and centrifuges pre-cooled to 4˚C. The cell pellet (P_{500}) was resuspended in fresh lysis buffer and lysed with 7 strokes of a pre-chilled Dounce homogenizer with a tight-fitting pestle (Kimble "B") for 20 strokes on ice before fractionation by centrifugation on a benchtop fixed-angle centrifuge. The lysate was centrifuged at 1000*g* for 5 min to pellet nuclei and unbroken cells (P_{1K}). The supernatant (S_{1K}) was collected, and the P_{1K} fraction was resuspended and dounced again to ensure complete lysis. After nuclei were cleared a second time, the S_{1K} fractions were combined and centrifuged at 5000*g* for 10 min to separate the heavy mitochondrial fraction (P_{5K}). The resulting supernatant (S_{5K}) was collected and spun at 21,000*g* for 20 min to pellet the autophagic fraction (P_{21K}; contains autophagosomes and lysosomes). The supernatant was discarded, and the P_{21K} fraction was resuspended in 140uL of 51% Nycodenz (Accurate Chemical, AN1002423) in gradient dilution buffer (0.125M sucrose, 1mM EDTA, 10mM HEPES pH 7.4, fresh protease inhibitors) on ice. The resuspended P_{21K} fraction was overlaid with equal volumes of 40%, 30%, 25%, 20%, 15%, and 0% Nycodenz (all prepared in gradient dilution buffer on ice) in an ultracentrifuge tube (Beckman Coulter, 344090). The resulting step gradient was centrifuged for 3h at 105,000*g* in a Beckman SW 50.1 rotor at 4˚C. The gradient was collected from the top down in 12 fractions on ice. The floated vesicles formed a visible, discrete band at 15% and 20% Nycodenz interface (fraction 4) and the 20% and 25% Nycodenz interface (fraction 6). These fractions were used immediately for cryo-CLEM analysis. All gradient fractions were stored at -80˚C for subsequent Western blot analysis.

Isolation of lysosomes from patient tissue

Lysosome-enriched fractions were also derived from post-mortem brain tissue using the protocol described above but with minor modifications. Briefly, 1 gram of frozen frontal cortex tissue was

coarsely chopped to 1-5 mm^2 pieces before digesting for 20 min in 7 mL of warm Hibernate A media (Gibco, A1247501) containing 20 U/mL papain (Worthington, LK003178). Papain digest was terminated by the addition of ice-cold 5 µg/mL leupeptin (Sigma, L2884), 5 µg/mL antipain dihydrochloride (Thermo Scientific, AAJ63680LB0), 5 µg/mL pepstatin A (Sigma, P4265), 1mM PMSF (Sigma, P7626), and 1 µM E64 (Tocris Bioscience, 52-081-0) in 13 mL Hibernate A media. Digested tissue was spun at 300*g* for 10 min, and the pellet was resuspended in ice-cold lysis buffer. The resuspended pellet was Dounce homogenized and centrifuged as explained above to pellet unwanted nuclear and mitochondrial cellular components. The resulting supernatant was collected and centrifuged at 21,000*g* for 20 min to pellet the autophagic fraction (P_{21K}; contains autophagosomes and lysosomes). Following ultracentrifugation, the autophagic fraction was resuspended in ice-cold 15% OptiPrep iodixanol solution (Cosmo Bio, NC1059560) in the gradient dilution buffer. The resuspended autophagic fraction was overlaid on top of equal volumes of 30%, 27%, 23%, 20%, and 17% OptiPrep (all prepared in gradient dilution buffer on ice) in an ultracentrifuge tube (Beckman Coulter, 344059). The resulting step gradient was centrifuged for 3h at 145,000*g* in a Beckman SW 41 rotor at 4˚C. The gradient was collected from the top down in 6 equal fractions on ice. The floated vesicles formed a visible, continuous band from 15% Optiprep to the 17%-20% OptiPrep interface (fraction 1-3). These fractions were used fresh for further cryo-ET analysis. All gradient fractions were stored at -80˚C before Western blot analysis.

Immunoblotting

Samples were thawed and resuspended in a final working concentration of 1X Laemmli buffer (Bio-Rad, 161-0747) and boiled at 95˚C for 5 min. Samples were run on a Bio-Rad Precast protein gel at 200 V and transferred onto a PVDF membrane using a Bio-Rad Trans-Blot Turbo Transfer System. Membranes were dried for 15-30 min and then rehydrated with methanol. LI-COR Revert™ 700 Total Protein Stain and Wash Solution Kit was used to stain for total protein. After staining for total protein, membranes were blocked in 5% milk in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% w/v Tween 20) for 1h at room temperature. Subsequently, the membranes were incubated in primary antibodies (Table S1 – Antibody) for 2h at room temperature or 4˚C overnight. Membranes were washed 3 times for 10 min each in TBST, then incubated with secondary antibodies (Table S1 – Antibody) for 1h at room temperature. Membranes were again washed three times for 10 min each in TBST, then developed using Clarity Western ECL substrate (Bio-Rad, 1705061) and imaged on a Bio-Rad ChemiDoc Imaging System for gradient fractionation experiments. For autophagy flux assays, membranes were washed 5 times for 5 min in TBST, then imaged on a LI-COR Odyssey Fc imaging system.

Blots were quantified in ImageJ software by drawing a square around each band and measuring the integrated intensity(*91*). For gradient fractionation experiments, TDP43 band density values were normalized to the total protein stain levels (calculated by drawing a square in each lane and measuring the mean intensity). Then, fractions 4 and 6 values were normalized to the input and plotted for three replicates. For autophagy flux experiments, TDP43, LC3B-II, and p62 band density values were normalized to the GAPDH band density values. A one-way ANOVA with

posthoc Tukey's multiple comparison tests was performed using GraphPad Prism version 10.1.2 to assess statistical significance.

Autophagy Flux Assay

1×10^6 HaloTag-LC3B NPCs/well plated in a 6-well plate (Costar, 3506) were differentiated to DIV9 iNeurons and treated with one of the following conditions: no treatment (control), 50mM NH_4Cl (Sigma-Aldrich, A9434), 50mM NH_4Cl + 2µM $(GU)_6$, 2µM A_{13} RNA, and 2µM $(GU)_6$ RNA for 4h. The cells were then collected in cold PBS and centrifuged at 2000*g* for 2 min. The cell pellet was resuspended in RIPA buffer (Thermo Scientific, 89901) with cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich, 11836170001) on ice for 30 min. Resuspended cells were sonicated (Fisher Scientific, FB50) at 10% amplitude with 5s on and 5s off for 1 min.

Total protein was measured with Bio-Rad Protein Assay Dye reagent concentrate (Bio-Rad, 5000006). Normalized lysates were mixed with 4X Lithium Dodecyl Sulfate (LDS) Sample buffer (GenScript, M00676) containing 2.5% 2-mercaptoethanol (Gibco, 21985023). Samples were heated at 95˚C for 5 min. 10µg of protein from each condition was analyzed by immunoblotting.

Cryo-CLEM of Autophagosomes and Lysosomes Isolated from HEK293T Cells

10 nm gold beads (Cell Microscopy Core University Medical Center Utrecht, FG 10 nm) at a dilution 1:7 were added to isolated lysosomes and autophagosomes (fractions 4 and 6 from Nycodenz gradient fractionation, respectively). R2/2 grids (Quantifoil GmBH) were glow-discharged at 5mA 30s (Pelco), and 3µL of the sample was added before plunge-freezing into liquid ethane with the Vitrobot Mark IV (Thermo Fisher Scientific) with the following specifications: *blot time: 2.5s, blot force: 1, temperature: 4˚C, humidity: 90%.* The plunge-frozen grids were clipped into AutoGrids (Thermo Fisher Scientific) and stored in liquid nitrogen until further use. The location of Dendra2-LC3B positive isolated organelles on grids was determined using a STELLARIS 5 cryo-confocal laser scanning microscope (Leica Microsystems) equipped with a cryo-stage and 50x/0.9-NA objective. Fluorescence stacks of the grid were acquired in the green (excitation, 488nm/emission, 500-550nm) channel using hybrid detectors, with an x/y pixel size of 110 nm and a z step size of 498 nm. Reflected light data was collected simultaneously to visualize the position of the holey-carbon film holes for 2D registration and correlation. Subsequent correlation between confocal and TEM images was performed on the cross-platform correlative software Maps 3.20 (Thermo Fisher Scientific) using the holey-carbon film to guide tomography tilt-series data collection. Grids were imaged on a 300kV Titan Krios G4i transmission electron microscope (Thermo Fisher Scientific) equipped with a K3 direct electron detector and an imaging filter (Gatan Inc.) operated in counting mode. Dose-symmetric tilt-series(*92*) were collected from –60˚ to +60˚, starting at 0˚ at a 3˚ interval, at correlated areas using SerialEM software(*93*). The magnification was set to a pixel size of 3.315 Å/pixel, and the total dose per tilt-series was $120e^-/Å^2$. Data were collected with a 20eV slit width. Tilt-series were

aligned by gold fiducial tracking and reconstructed by weighted back projection implemented in IMOD(*94*). 15 iterations of a 'SIRT-like' filter were applied. A Gaussian blur of 2 was applied to the fluorescent signal in Fiji for the figure overlay on the TEM image(*91*).

Preparation of HEK293T Sarkosyl-insoluble Fractions

Dendra2-LC3B HEK293T cells were grown to 80% confluency in a 15cm dish (Thermo Scientific, 130183). Cells were treated with 6µM Endo-Porter for 24h, 500nM MG132 for 24h, and 6µM Endo-Porter with 2µM $(GU)_6$ RNA for 4h. Cells were washed once with ice-cold PBS and then collected with ice-cold PBS. Cells were pelleted by spinning at 500*g* for 5 min at 4˚C. The pellet was resuspended in 1mL extraction buffer (10 mM Tris-HCl, 0.8 M NaCl, 10% sucrose, 1 mM EGTA at pH 7.5) and then sonicated twice (Virsonic 100 probe sonicator) at power setting 2 for 10s on, 50s rest. A 25% sarkosyl solution was added to the homogenates to create a final concentration of 2% sarkosyl. Homogenates were incubated for 1hr at 37˚C with orbital shaking (INCU-SHAKER™ 10LR) at 250rpm. Next, homogenates were transferred to thick-walled centrifuge tubes (Beckman Coulter NC9495303) and spun at 27,000*g* for 10 min with the TLA120.2 Rotor (Beckman). The supernatant was transferred to a new centrifuge tube and spun at 166,000*g* for 20 min with a TLA120.2 rotor (Beckman). Finally, the resulting pellets were resuspended in 30-50 µL of 20 mM Tris-HCl at pH 7.4 and 150 mM NaCl by sonication (Virsonic 100 probe sonicator) for 30s at 100% amplitude. Additional sonication, if necessary, was performed with a water bath sonicator (Branson 2800) for 30s. The sarkosyl-insoluble fraction was then analyzed with immunogold labeling and immunoblotting.

Immunogold Labeling of HEK293T Sarkosyl-insoluble Fractions

300 mesh carbon-coated copper grids (Electron Microscopy Sciences, CF300-CU-50) were glow discharged for 30s at 5mA (Pelco). 3µL of the sarkosyl-insoluble fraction was applied to the grids, incubated for 1 min, and then blotted off using Whatman 1 filter paper (Cytiva, 1001090). Blocking buffer (PBS, pH 7.4, 0.1% w/v cold water fish skin gelatin (Aurion, CFWS Gelatin) was applied to the grids and incubated for 10 min, then blotted with filter paper. Subsequently, TDP43 antibody 10782-AP diluted 1:20 in blocking buffer was applied to the grids and incubated for 1h. Following blotting, grids were washed five times with a blocking buffer, and excess solution was blotted off between each wash. 12 nm Colloidal Gold AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, 111-205-144) diluted 1:4 in blocking buffer was applied to the grids and incubated for 1h. Grids were washed five times with Milli-Q water, and excess liquid was blotted off between washes. Finally, grids were stained with 3µL of 0.75% uranyl formate (Electron Microscopy Sciences, 16984-59-1) for 1 min, washed two times with Milli-Q water, and then the excess solution was blotted off with filter paper. The grids were imaged using a Morgagni transmission electron microscope (Thermo Fisher Scientific) at an acceleration voltage of 100kV. The images were recorded with a Gatan Orius SC200 CCD camera with a resolution of 2.1 Å/pixel and Digital Micrograph software (Gatan Inc).

High-pressure Freezing (HPF), freeze-substitution, and resin embedding of HEK293T cells

Dendra2-LC3B HEK293T cells were grown to 80% confluency in a 15cm dish (Thermo Scientific, 130183). Cells were treated with 6µM Endo-Porter (Gene Tools OT-EP-PEG-1) for 24h and 2µM $(GU)_6$ RNA and 6µM Endo-Porter for 4 and 24 h. Cells were trypsinized with 0.05% trypsin (Corning, MT25052CV) and then spun at 500*g* for 5 min at 4˚C to pellet the cells. The cell pellet was resuspended in 100µL DMEM (Corning, 10-013-CV), supplemented with 10% FBS and 100 units/mL Penicillin/Streptomycin (Gibco, 15140163) to a final concentration of ~80 million cells/mL. The cell suspensions were sandwiched between one 200µm type A and the flat side of type B 3mm aluminum planchette and then frozen with the EM ICE High-Pressure Freezer (Leica Microsystems) and stored in liquid nitrogen until further use. Subsequently, the frozen samples were taken out from liquid nitrogen and freeze substituted in Leica AFS2 with 0.1% UA (Merck KGaA) + 5% water in acetone (VWR International GmBH), followed by Lowicryl resin infiltration and UV polymerization. For low-temperature embedding, LR-Gold samples were substituted from −90 to −20 °C for 16h, washed twice with pre-cooled acetone, infiltrated for 2h with 50 % (v/v) LR-Gold in acetone, and 1h with 100 % LR-Gold. 70 nm thick sections were cut using a DiATOME 45° knife and a Leica UC7 ultramicrotome. Sections were collected on Formvar carbon-coated 200 mesh hexagonal nickel grids for subsequent immunolabelling and staining.

Immunogold Labeling of HEK293T Cells

Grids with ultrathin sections were floated, section-side down, on a 50µL droplet of pH 7.5 phosphate buffer with 0.1% Tween 20 (PBST) on clean parafilm over a flat surface for 10 min at room temperature. The grids were blotted with filter paper (Whatman 1, Corning) and then floated on a blocking solution containing 2.5% BSA (in PBST) for 1 h at room temperature. Following this, grids were floated on primary TDP43 antibody (10782-2-AP) at 1:20 in PBST overnight at 4˚C and an additional hour at room temperature the following day. Grids were washed with PBST three times for 5 min each, followed by incubation with 12 nm Colloidal Gold AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, 111-205-144) diluted 1:20 for 1h at room temperature. Grids were washed with PBST thrice for 10 min each, followed by a 5 min fixation with 2% glutaraldehyde in PBST. Subsequently, grids were washed 5 times for 2 min each with PBST and then 5 times for 2 min each with filtered water. Grids were then positively stained with 0.75% uranyl formate (Electron Microscopy Sciences, 16984-59-1) for 10 min. Next, the grids were washed an additional 10 times by floating on water droplets and blotting the excess water in between washes. The grids were allowed to dry and imaged on the transmission electron microscope (T12, Technai) operated at an acceleration voltage of 120kV. The images were recorded with a Gatan Rio9 CMOS Detector with a pixel size of 5.68892 Å/pixel and a defocus of -8 µm using SerialEM(*93*). The number of gold beads within single membrane-bound compartments was manually counted for six cells from each treatment and compared between

treatments. Data was plotted using GraphPad Prism version 10.1.2. A Welch's t-test was performed using GraphPad Prism version 10.1.2 to assess statistical significance.

In situ Cryo-correlative Light and Electron Microscopy of iNeurons

Grid preparation

Gold grids, 200 mesh, with holey R1/4 SiO_2 film (Quantifoil Micro Tools GmbH) were glow-discharged for 30s at 5 mA using an EasiGlow system (Pelco). To encourage cell growth in the centers of grid squares, grids were coated with poly-L-lysine (Sigma Aldrich, P6282) and mPEG-SVA (Laysan Bio). Coated grids were then photosensitized with the addition of PLPP gel (Alvéole) and photo-micropatterned using a DMi8 microscope (Leica Microsystems) equipped with an Alvéole PRIMO 2 to generate circular patterns with a diameter of 60 µm at the centers of grid squares. Regions of interest were identified, and patterns were selected using the Leonardo photopatterning software(*95*). Grids were next coated with Poly-L-Ornithine (PLO) and laminin in a 35 mm glass bottom dish (MatTek Life Sciences) as described earlier.

Sample preparation

1×10^5 HaloTag-TDP43 and mEGFP-LC3B (AICS30 + 474-1) NPCs in TeSR-E8, doxycycline, and ROCK inhibitor were grown in 35 mm glass bottom dishes containing micropatterned Au SiO_2 R1/4 200 mesh grids (Quantifoil GmBH) coated with PLO and laminin. NPCs were differentiated to DIV7 iNeurons as described above and then treated with 2µM Cy3 or Cy5-labeled $(GU)_6$ oligo for 4 or 24h. For HaloTag-TDP43 iNeurons, 50nM JF635 Halo ligand (kind gift of L. Lavis, Janelia Research Campus) was added to the 35 mm dish 2h before plunge-freezing in liquid ethane. 1.0µm blue-green (430/465) (Invitrogen, F13080) or crimson (625/645) fluorescent FluoSpheres (Invitrogen, F8816) were sonicated in a water bath sonicator for 10s and then diluted 1:75 in B27 media. iNeurons on grids were kept in a 37˚C incubator during the plunging process. Grids were dipped into warm B27 media to wash off free Halo ligand, and cell debris. Subsequently, 3µL of FluoSpheres were added to the grid, the grid back-side blotted with calcium-free filter paper (Subangstrom, LFP01) and plunge-frozen into liquid ethane at -185 ºC using a GP2 (Leica Microsystems) automatic grid plunger with the following specifications: *blot time*: 8s, *temperature* - 37 ºC and *humidity*: 80%. The plunge-frozen grids were clipped into CryoFIB AutoGrids (Thermo Fisher Scientific) and stored in liquid nitrogen until further use.

Cryo-fluorescence microscopy

The quality of cells and grids was assessed in widefield mode on a Stellaris-5 cryo-confocal laser scanning microscope equipped with a cryo-stage and 50x/0.9 numerical aperture objective (Leica Microsystems). Z-stacks of desired regions were acquired in confocal-scan mode with a 488 nm (500-540 nm emission), 547 nm laser line (565-610 nm emission), and 635 nm (665-680 nm emission), 0.5 µm spacing spanning ± 7 µm of the focus plane and a *x/y* pixel size of 110 nm. Additionally, a reflection image was simultaneously acquired at each Z plane to position the fluorescent signal relative to the holey-carbon film for targeted tomography acquisition. Maximum

intensity projections of Z-stacks were generated using the built-in processing tools package (LAS X 4.5.0.25531). Subsequent correlations between confocal and TEM images were performed on the cross-platform correlative software Maps 3.20 (Thermo Fisher Scientific) using the holey-carbon film to guide FIB-milling and tomography tilt-series positioning.

Cryo-FIB milling

Clipped grids were loaded onto the stage of an Aquilos 2 FIB-SEM (Thermo Fisher Scientific) maintained at ≤ -185ºC. Grids were coated with a layer of organometallic platinum using a gas injection system for 30 s and then sputter-coated with inorganic platinum to form a protective/conductive surface for FIB milling. Initial correlation between confocal and SEM images was performed in Maps 3.20 to roughly identify regions to mill lamellae. Next, 3D Correlation Toolbox (3DCT) was used to correlate the Z-stack confocal data, SEM, and FIB data with the location of the FluoSpheres and precisely pinpoint where to mill the lamellae(*96*). Initial cuts with the FIB to make tension-relief trenches(*97*) and 5µm lamella were milled with a beam current of 1 nA at a milling angle of 10-15º. Subsequent cuts to make rough (1µm) lamella were done with beam currents of 100-500 pA. Milling progress and lamella integrity were monitored via SEM imaging. Upon completion of all rough lamella on a grid, each lamella was revisited and polished to a fine lamella (~200 nm) with the FIB at a beam current of 50 pA.

Tilt-series data acquisition and tomogram reconstruction

Cryo-FIB-milled grids containing iNeuron lamellae were imaged on a 300kV Titan Krios G4i transmission electron microscope (Thermo Fisher Scientific) equipped with a K3 direct electron detector and an imaging filter (Gatan Inc.) operated in counting mode. Dose-symmetric tilt-series(*92*) were collected from –60º to +60º, starting at 0º at a 3º interval, at correlated areas using SerialEM software(*93*). The magnification was set to a pixel size of 2.654 Å/pixel, and the total dose per tilt-series was 120e^{-}/Å^2. Data were collected with a 20eV slit width. Tilt-series were aligned by patch tracking and reconstructed by weighted back projection implemented in IMOD(*94*). 15 iterations of a 'SIRT-like' filter were applied.

Post tilt-series acquisition fluorescence microscopy

After the acquisition of the tilt-series, the lamellae were imaged in the Stellaris to cross-check the locations of regions where the tilt-series were collected. Z-stacks of lamellae were acquired in confocal-scan mode with a 488 nm (500-550 nm emission), 547 nm laser line (565-610 nm emission), and 635 nm (665-680 nm emission), 0.498 µm spacing spanning ± 12 µm of the focus plane and an *x/y* pixel size of 110 nm.

Subtomogram Averaging

Warp (version 1.1.0)(*98*) was used for motion correction and CTF estimation of tilt-series data. Subsequently, motion-corrected tilts were combined into an unaligned tilt-series. These tilt-series were imported to IMOD for patch-tracking. The aligned tilt-series was then imported into Warp for tomogram reconstruction at 4X binning (5.472 Å/pixel). The tomogram and a deconvolved

version were imported into Dynamo (version 1.1.514)(*99*). Particle picking was performed on the deconvolved version using "Filament (crop along axis)" models. Subvolumes were then extracted from the original tomogram at a box size of 32 pixels. An initial template was created by averaging a random subset of 250 particles. The first round of alignment was conducted with a limited search cone of 60˚, no azimuth rotation (in-plane rotation), and limited particle shift (4 pixels in any direction). The second round of alignment allowed azimuthal rotation of up to 180º. Coordinates and Euler angles for aligned particles were converted into Warp format using the dynamo2warp scripts from the warp2dynamo package(*100*). The filament number was added using the Starparser package (https://github.com/sami-chaaban/starparser). Following this, 8298 subtomograms were extracted in Warp at 5.47 Å/ pixel, with a box size of 64 pixels. The subtomograms were imported into Relion 3.1(*101*) for 3D classification and 3D refinement using helical symmetry. 3D Classification was first performed using a featureless cylinder as the reference (generated using *relion_helix_toolbox*), on aligned particles imported from Dynamo, using an initial twist of 1.4˚ and a rise of 4.8Å. 3D refinement was performed separately on the two most abundant classes (class 2 and class 8). The structure (corresponding to class 2) was imported into ChimeraX(*102*), and PDB models for Type A TDP43 filaments 8CG3 (variant 1), 8CGG (variant 2), and 8CGH (variant 3), and for Type B TDP43 filament 7PY2 were fit into the map(*25*, *26*).

Segmentation

The tomograms were reconstructed at 4X or 6X binning. The TomoSegMemTV and the v-10 pre-trained model of MemBrain v2 were used to help detect organelle membranes and TDP43 filaments(*103*, *104*). Subsequently, the tomograms were manually segmented using Amira (Thermo Fisher Scientific). For tomograms represented in Figure 4, tomograms from WARP were deconvolved, and the warp2dynamo package was used to obtain the coordinates of ribosomes in the tomograms(*100*). These coordinates were then used to place the ribosome map (EMD-15636). Microtubules were segmented using the Cylinder Correlation feature in Amira. TDP43 was manually segmented in Amira (Thermo Fisher Scientific).

Filament Measurements

Filament widths and lengths for HEK293T, iNeuron, and ALS/FTLD patient TDP43 filaments were measured with contours in 3dmod. Data was plotted using GraphPad Prism version 10.1.2.

Immunohistochemistry and Immunofluorescence of Patient Brain Tissue Sections

Duplex immunohistochemistry and duplex immunofluorescence were performed on a Ventana Discovery Ultra stainer (Ventana/Roche). Slides were dewaxed, rehydrated, and subjected to heat-induced epitope retrieval on the stainer. Slides were subjected to sequential incubation with either LC3B antibody for 16 min or LAMP1 antibody for 60 min, polymer goat anti-rabbit IgG conjugated to HRP for 16 min (Table S1 – Antibody) and then developed with Discovery Green

chromogen (Ventana/Roche, 760-271) for immunohistochemistry and with Akoya Opal 570 (Akoya Biosciences, NC1601878) for immunofluorescence. After an additional round of heat-induced epitope retrieval to remove the LC3B or LAMP1 primary antibody-secondary antibody complex, the slides were stained with TDP43 antibody for 20 min and polymer goat anti-rabbit IgG conjugated to HRP for 16 min (Table S1 – Antibody) and developed with Discovery Red chromogen (Ventana/Roche, 760-228) for immunohistochemistry and with Akoya Opal 690 (Akoya Biosciences, NC1605064) for immunofluorescence.

For immunohistochemistry, slides were counterstained with hematoxylin (Ventana/Roche, 760-2021) and coverslipped. Images were acquired on an Olympus BX51 light microscope equipped with a UPlanSApo100x oil objective with a numerical aperture of 1.40 and a working distance of 0.12 mm. Image deconvolution and analysis were performed using Fiji(*91*).

Slides were counterstained and coverslipped for immunofluorescence using Prolong Gold containing DAPI (Invitrogen, P36931). High-resolution Z-stacks were acquired on an AXR NSPARC confocal system (Nikon Instruments Inc.) with a 60x NA1.42 Oil/DIS Plan Apochromat Lambda D objective with a working distance of 1.5mm.

Cryo-ET of ALS/FTLD-TDP Patient-derived Lysosomes

Fraction 1 of the OptiPrep gradient used to isolate lysosomal enriched fractions from the patient-brain was plunge-frozen in liquid ethane as described earlier (see section ‘Cryo-CLEM of Autophagosomes and Lysosomes Isolated from HEK293T Cells’ above*)***.** All the subsequent steps, except for cryo-confocal microscopy, were performed as described above.

Fig. S1 to S11

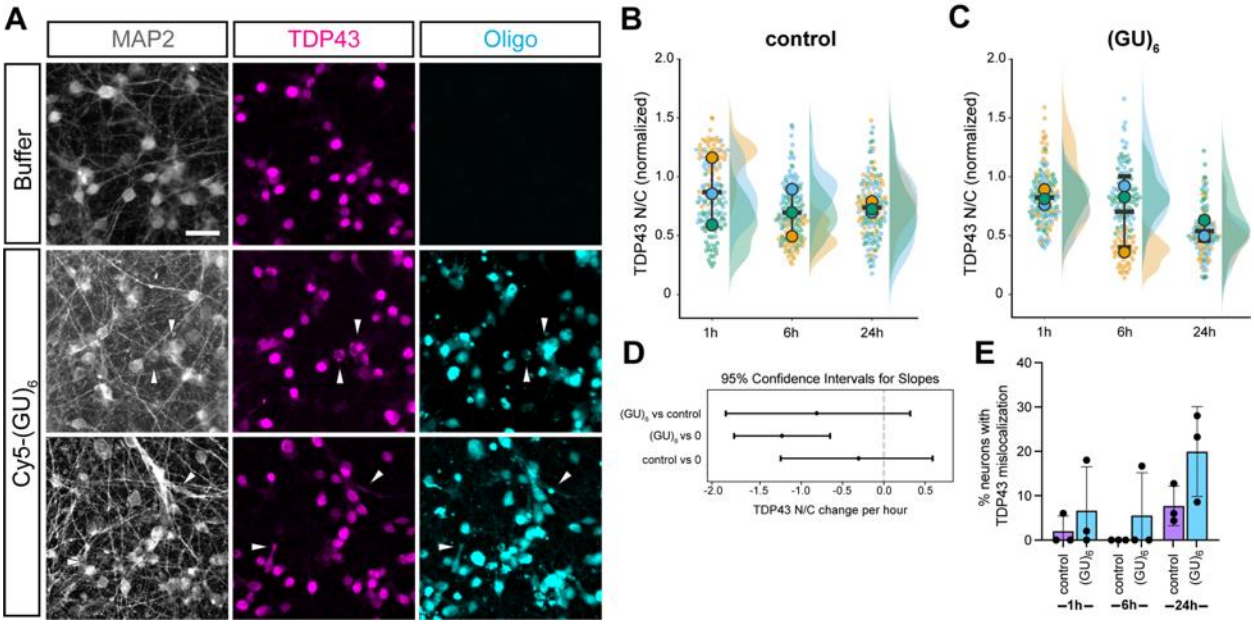

Fig. S1. GU-rich oligonucleotides induce TDP43 nuclear export in primary rat neurons. (**A**) Primary mixed cortical rat neurons treated with 2µM Cy5-$(GU)_6$ or buffer for 48h were fixed and stained for neuronal marker MAP2 (gray) and TDP43 (magenta). Cy5 fluorescence (from labeled oligonucleotides) is depicted in cyan. White arrows indicate mislocalized TDP43 co-localized with Cy5-$(GU)_6$ puncta within intact neurons. Scale bar, 10µm. (**B-C)** Plot showing the normalized TDP43 nuclear/cytoplasmic (N/C) ratio for **(B)** control and **(C)** $(GU)_6$-treated primary rat neurons at 1h, 6h, and 24h after treatment (n>35 for all three biological replicates and timepoints). **(D)** The slope of the change in TDP43 N/C over time. 95% confidence interval (CI) determined by generalized estimating equations. Conditions in which the 95% CI does not overlap with 0 are statistically significant. **(E)** Percentage of primary rat neurons with mislocalized TDP43 (SD of three biological replicates shown).

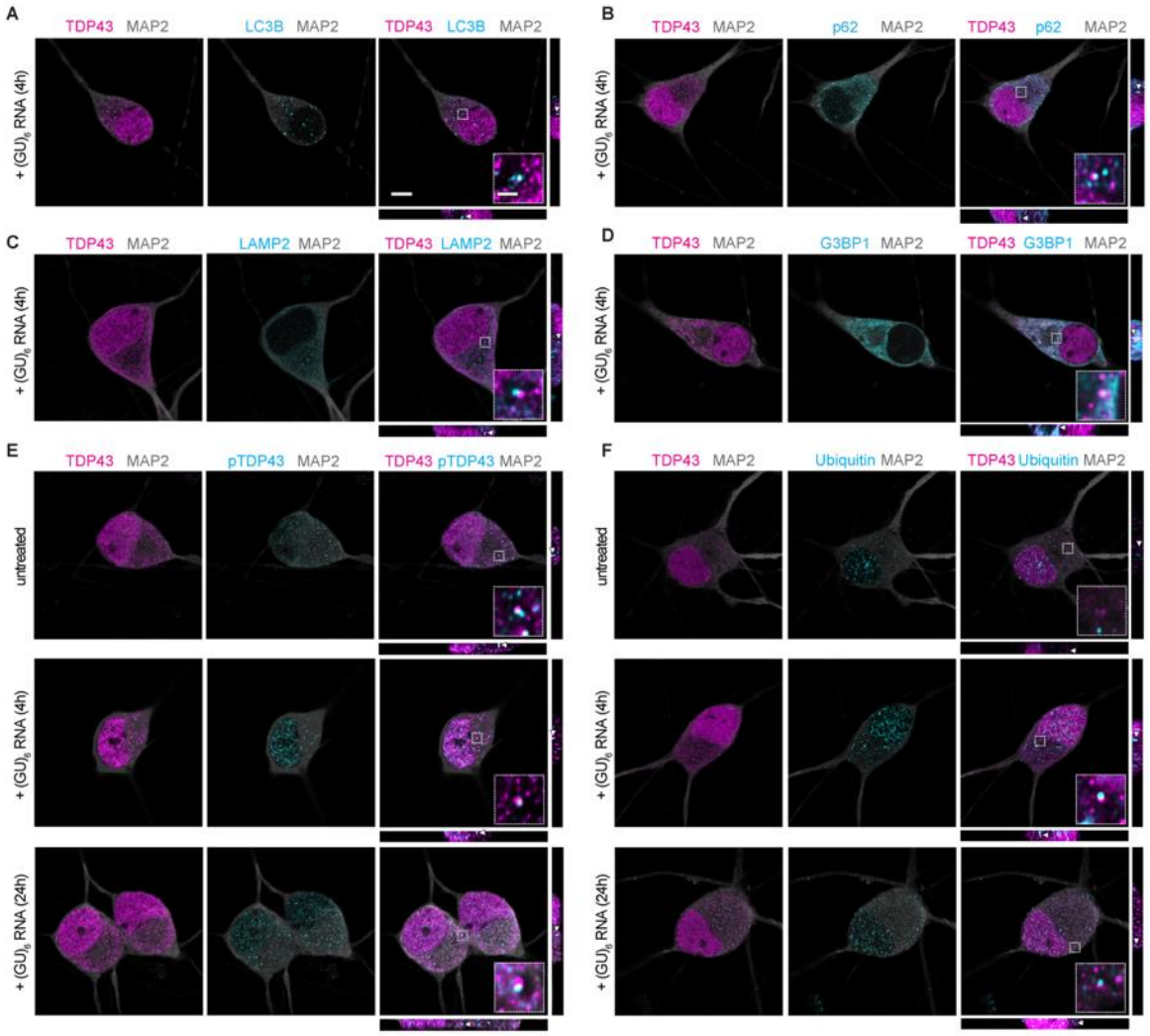

Fig. S2. Mislocalized TDP43 is phosphorylated and ubiquitinated in oligonucleotide-treated iNeurons. (**A-D**) 60x confocal images with xz and yz orthogonal views of $(GU)_6$ RNA-treated DIV10 iNeurons chemically fixed and stained with MAP2 (gray), TDP43 (magenta), and (A) LC3B, (**B**) p62, (**C**) LAMP2, or (**D**) G3BP1 (cyan). Scale bar, 5µm. Insets provide ~4.8x magnification; scale bar, 1µm. White arrowheads point to the inset region in xz and yz orthogonal views. (**E-F**) Confocal images with xz and yz orthogonal views of oligonucleotide-treated or control DIV10 iNeurons fixed and immunostained for MAP2 (gray), TDP43 (magenta), and (**E**) phosphorylated TDP43 (S409/410) or (**F**) ubiquitin. Scale bar, 5µm. Insets provide ~4.8x magnification; scale bar, 1µm. White arrowheads in xz and yz orthogonal views indicate TDP43 puncta present in the inset.

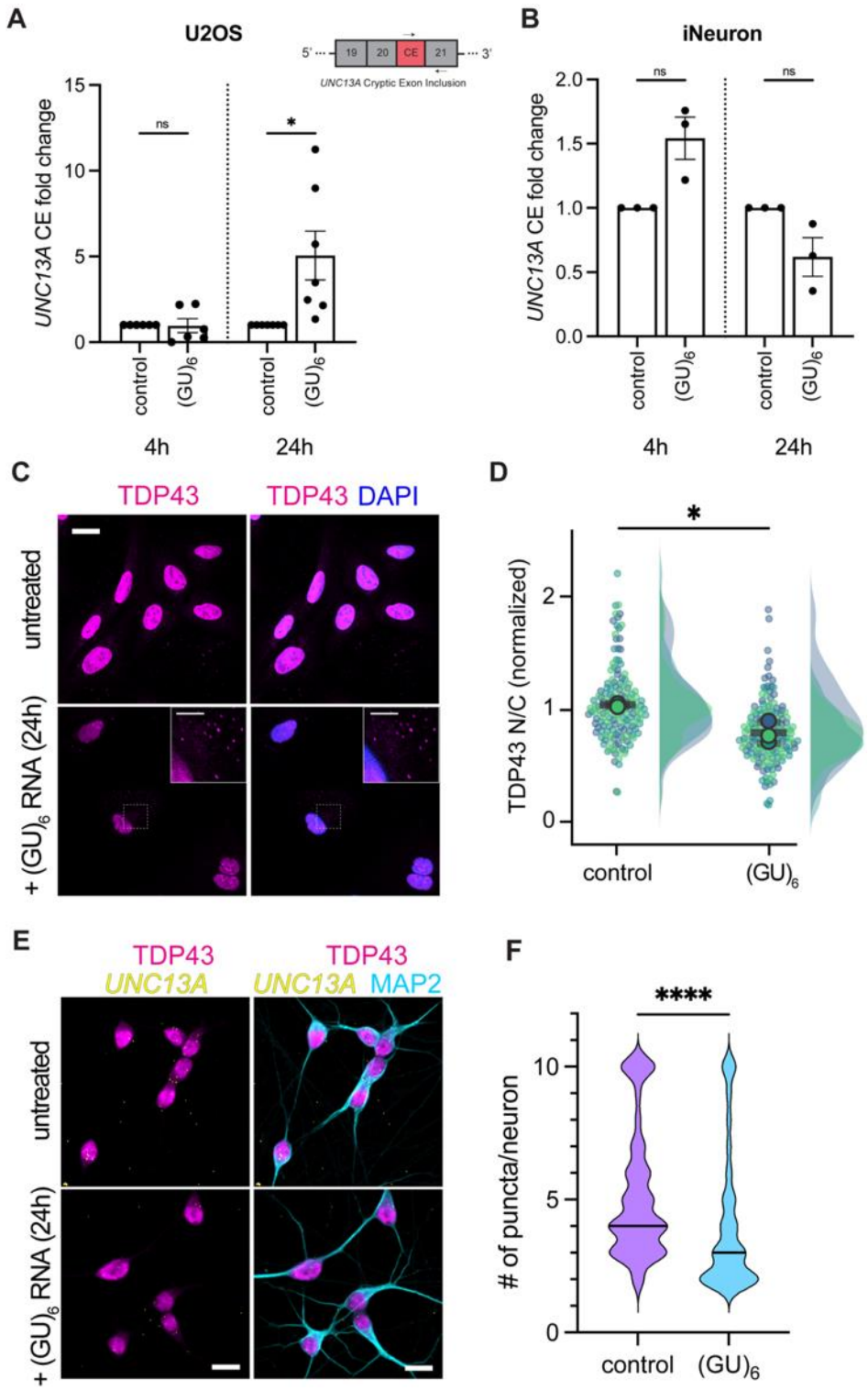

Fig. S3. GU-rich oligonucleotides induce TDP43-regulated *UNC13A* cryptic exon inclusion. **(A-B)** RT-qPCR of *UNC13A* cryptic products in **(A)** U2OS cells (n=6) and **(B)** DIV10 iNeurons (n=3) after $(GU)_6$ oligonucleotide treatment for 4h and 24h (mean ± SEM, Welch's t-test, *p < 0.05). Schematic of *UNC13A* cryptic exon (CE) inclusion shown in upper right corner of (A).

Arrows indicate the primer pair used to detect *UNC13A* cryptic exon (CE) inclusion with RT-qPCR. **(C)** Representative images of $(GU)_6$ RNA-treated U2OS cells chemically fixed and stained with DAPI (blue) and TDP43 (magenta). Scale bar, 20 µm. Insets provide 4x magnification; scale bar, 5 µm. **(D)** TDP43 nuclear/cytoplasmic (N/C) ratio of $(GU)_6$ RNA-treated U2OS cells, normalized to control (mean ± SD for ~50 cells in each replicate, n=3, Welch's t-test, *$p<0.05$). The distribution for each replicate is shown to the right of the scatterplot for each treatment. **(E)** Representative images and **(F)** quantification of HCR-FISH for native *UNC13A* transcripts (yellow puncta) in DIV10 iNeurons treated with $(GU)_6$ RNA for 24h and chemically fixed and stained with TDP43 (magenta) and MAP2 (cyan). The line through the violin plot indicates the median number of puncta per neuron (n=415 for each treatment, ****$p<0.00005$, Welch's t-test). Scale bar, 15µm.

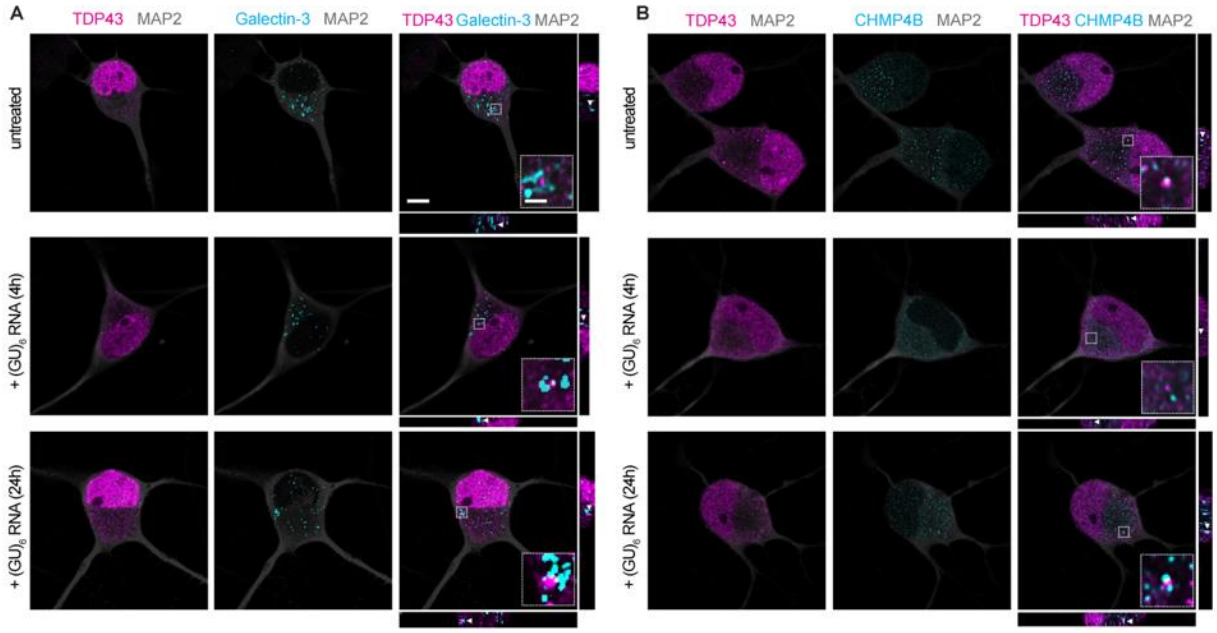

Fig. S4. Mislocalized TDP43 overlaps with markers of lysosomal damage in oligonucleotide-treated iNeurons. (**A-B**) Confocal images with xz and yz orthogonal views of $(GU)_6$-treated DIV10 iNeurons fixed and stained for MAP2 (gray), TDP43 (magenta), and **(A)** Galectin-3 or (**B**) CHMP4B. Scale bar, 5µm. Insets provide ~4.8x magnification; scale bar, 1µm.

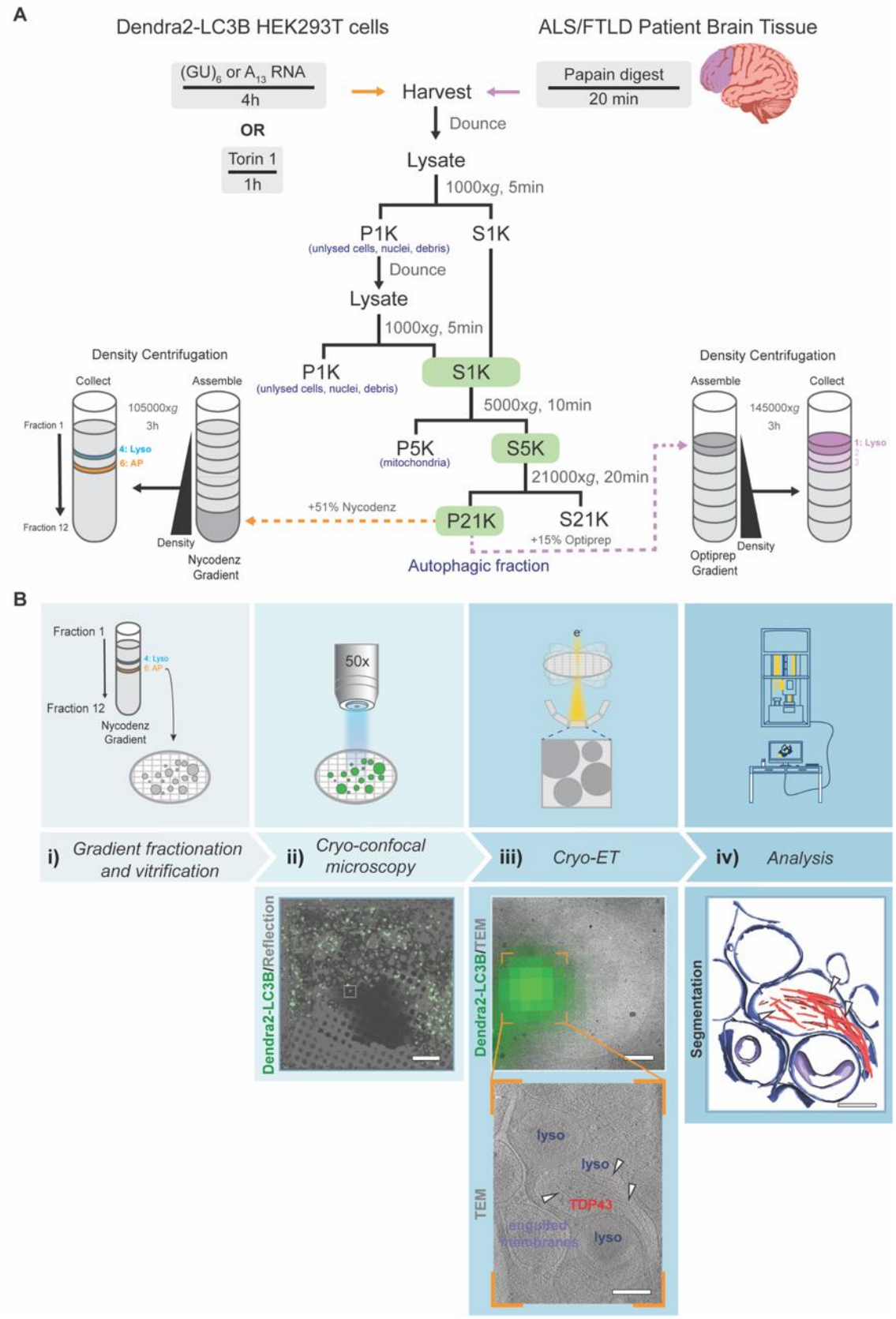
A
Dendra2-LC3B HEK293T cells
ALS/FTLD Patient Brain Tissue
(GU)6 or A13 RNA
4h
OR
Torin 1
1h
Harvest
Papain digest
20 min
Dounce
Lysate
1000xg, 5min
P1K
(unlysed cells, nuclei, debris)
S1K
Dounce
Lysate
1000xg, 5min
P1K
(unlysed cells, nuclei, debris)
S1K
5000xg, 10min
P5K
(mitochondria)
S5K
21000xg, 20min
P21K
S21K
+15% Optiprep
+51% Nycodenz
Autophagic fraction
Density Centrifugation
Collect
Assemble
105000xg
3h
Fraction 1
4: Lyso
6: AP
Fraction 12
Density
Nycodenz Gradient
Density Centrifugation
Assemble
Collect
145000xg
3h
1: Lyso
Optiprep Gradient
Density
B
Fraction 1
4: Lyso
6: AP
Fraction 12
Nycodenz Gradient
50x
e-
i) Gradient fractionation and vitrification
ii) Cryo-confocal microscopy
iii) Cryo-ET
iv) Analysis
Dendra2-LC3B/Reflection
Dendra2-LC3B/TEM
TEM
lyso
lyso
TDP43
engulfed membranes
lyso
Segmentation

Fig. S5. Isolation and cryo-correlative microscopy of autophagosomes and lysosomes from $(GU)_6$-treated HEK293T cells or ALS/FTLD patient postmortem brain tissue. (**A**) Detailed schematic of gradient fractionation protocol to isolate autophagosomes (AP) and lysosomes (Lyso) from HEK293T cells (left) and ALS/FTLD patient postmortem brain tissue (right). (**B**) Schematic (top) and representative images (bottom) of cryo-correlative light and electron microscopy of AP-enriched fraction 6 from $(GU)_6$-treated Dendra2-LC3B HEK293T cells (i-iv). Overlay of the Dendra2-LC3B fluorescence signal with the reflection channel obtained in the cryo-confocal Stellaris 5 microscope (ii). Overlay of the Dendra2-LC3B fluorescence with low magnification cryo-TEM image (iii). The orange box represents a high-resolution slice through the tomogram. White arrowheads highlight TDP43 fibrils. 3D segmentation of TDP43 fibrils in autolysosome (iv). Red, TDP43; blue, autolysosome membrane. Scale bars, 200nm.

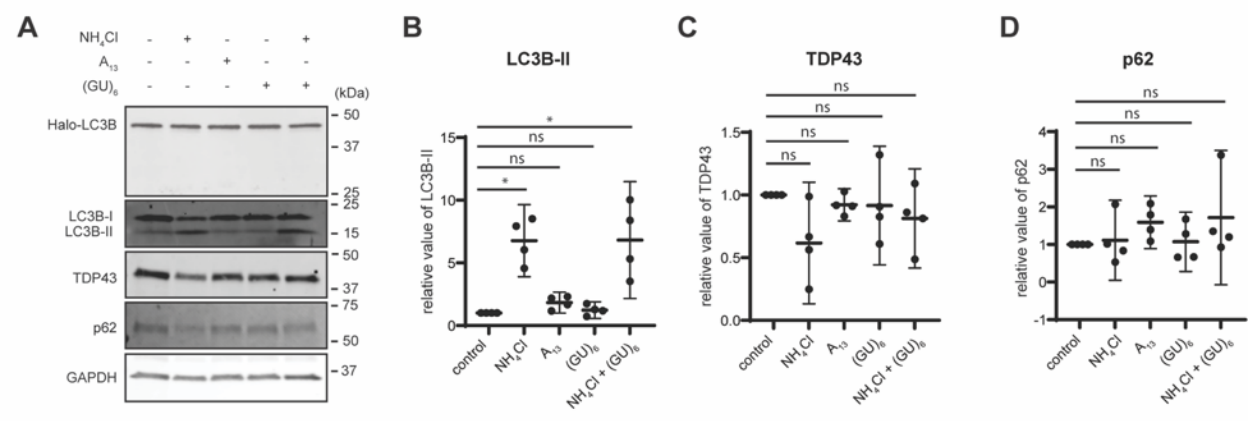

Fig. S6. Oligonucleotide treatment does not increase macroautophagy in iNeurons. (**A**) Representative immunoblots showing Halo-LC3B, LC3B-I/II, TDP43, and GAPDH in total cell lysates from iNeurons treated with oligonucleotides in the presence and absence of 50mM NH_4Cl, n=3. (**B**) Quantification of relative levels of (**B**) LC3B-II, (**C**) TDP43, (**D**) p62 for each condition (mean ± SEM, ANOVA, Tukey's test, *$p < 0.05$).

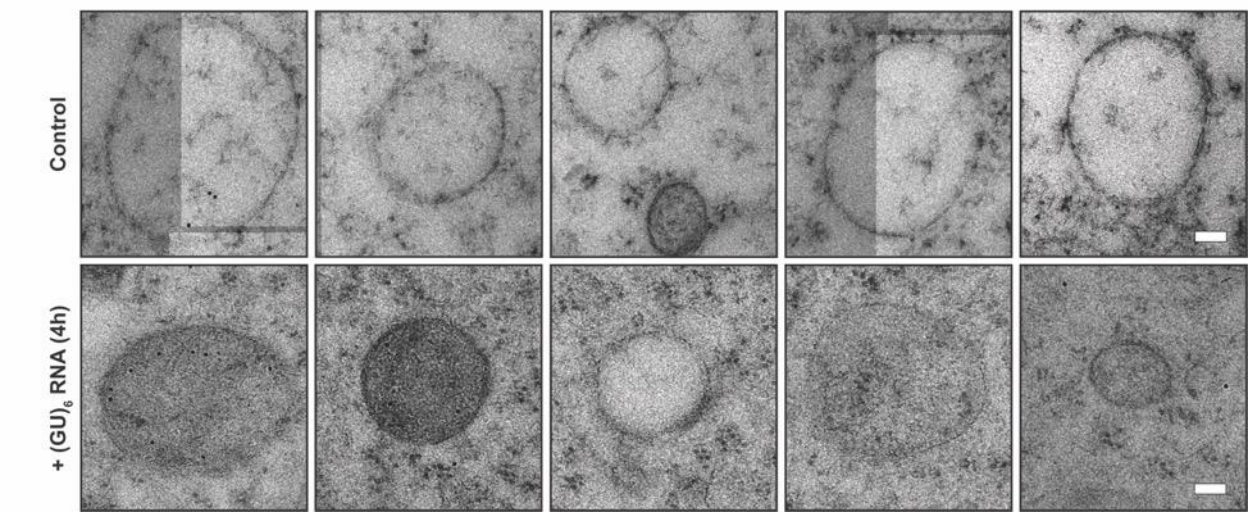

Fig. S7. Immunogold labeling. Anti-TDP43 immunogold labeling on ultrathin sections of $(GU)_6$-treated Dendra2-LC3B HEK293T cells. Representative images of single membrane endolysosomes from control and $(GU)_6$-treated Dendra2-LC3B HEK293T cells are shown. Scale bars, 100nm.

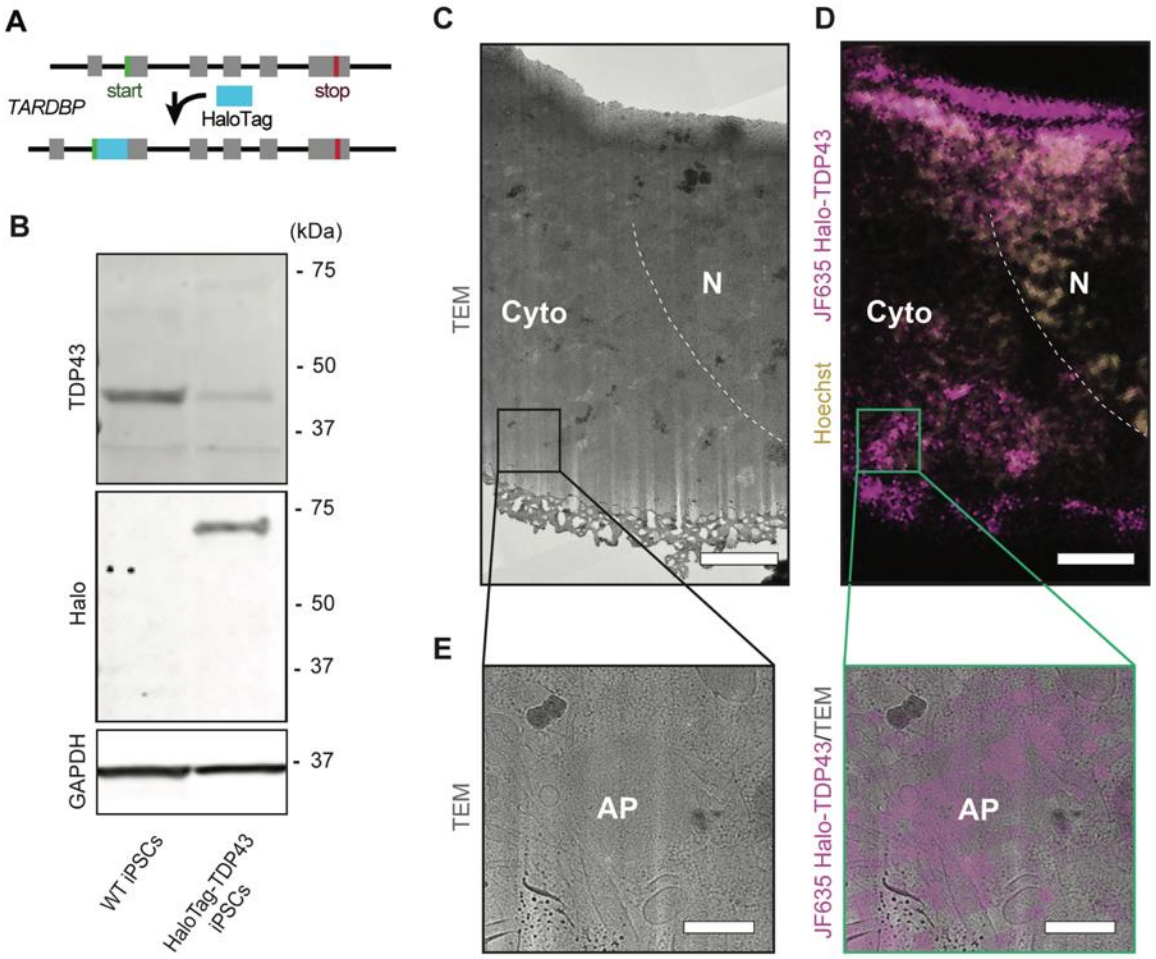

Fig. S8. Visualizing TDP43 architecture in Halo-TDP43 iNeurons. (**A**) Schematic for CRISPR/Cas9-based insertion of the HaloTag open reading frame immediately downstream of *TARDBP* start codon. (**B**) Immunoblot for HaloTag and TDP43 in WT and HaloTag-TDP43 iPSC lysates. (**C**) A medium magnification map (6500x) of cryo-FIB milled lamella. The black square highlights the region where the tilt-series containing the autophagosome in Fig. 4B was collected. Scale bar, 2µm. (**D**) JF635 HaloTag-TDP43 fluorescence image of the cryo-lamella in (**C**). Scale bar, 2µm. (**E**) Zoom of the inset in (**C**) and (**D**) highlighting the area of data collection in Figure 4B (left) and overlay of fluorescence (right). Scale bars, 500 nm. N – nucleus, Cyto – cytoplasm, AP – autophagosome.

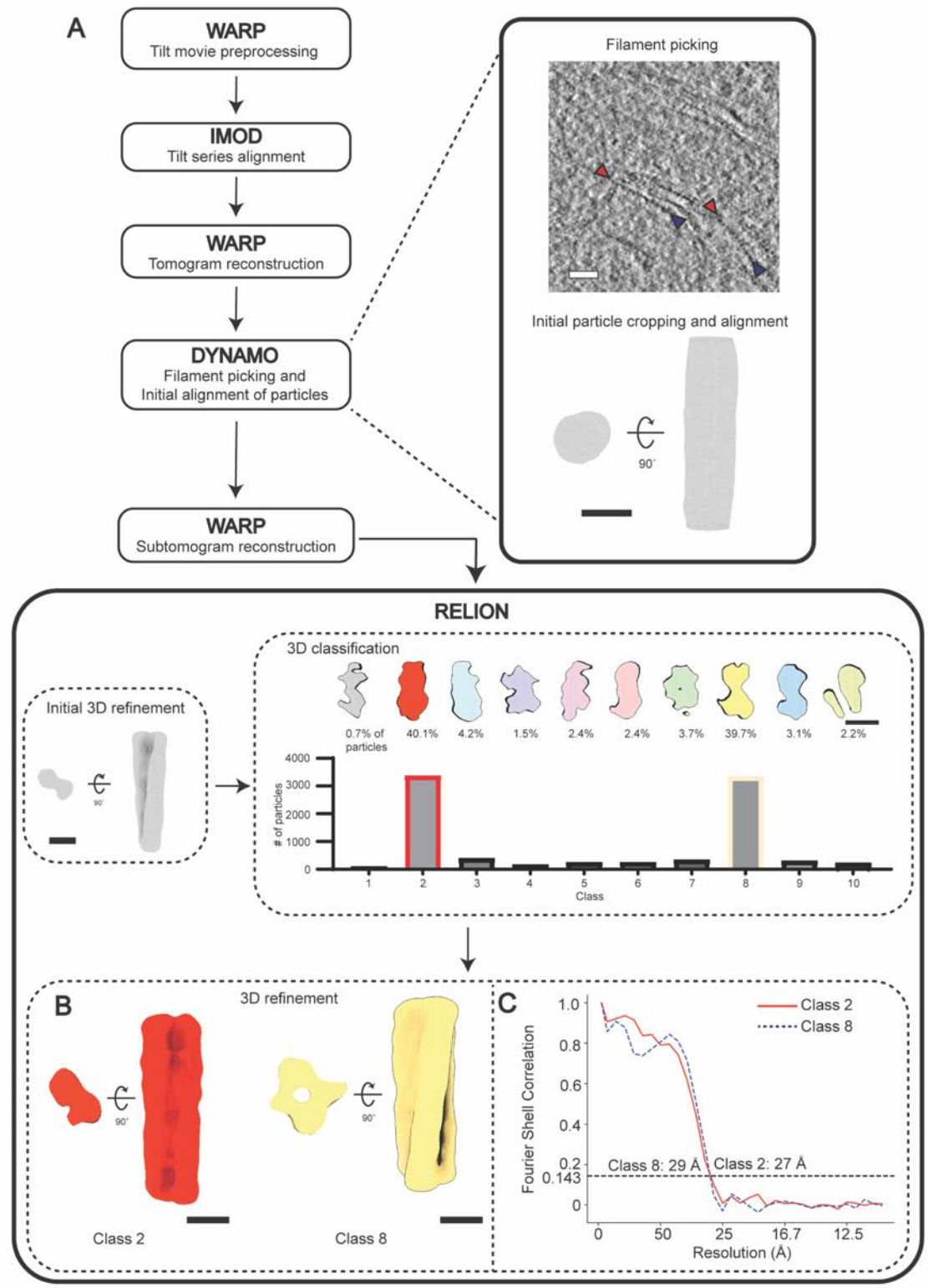

Fig. S9. Cryo-ET data processing workflow. (**A**) Schematic of the data processing pipeline (detailed in Materials and Methods). Tilt-series movies were initially processed in Warp and then exported into IMOD for tilt-series alignment. 4x binned tomograms (5.472 Å/pixel) were reconstructed in Warp and imported into Dynamo to pick TDP43 filaments. A representative slice

through a tomogram with TDP43 filaments is shown. Scale bar, 50 nm. Red and blue arrowheads indicate TDP43 filaments used for subtomogram averaging. 128 filaments were picked, resulting in 8,298 particles. Subvolumes were cropped in Dynamo, and the particles were initially aligned. Scale bar, 5 nm. Particle coordinates were used to reconstruct subtomograms in Warp and perform iterative classification and alignment in RELION 3.1. Scale bars, 5 nm. (**B**) Final refinement maps of the two major classes obtained from 3D classification. Scale bar, 5 nm. (**C**) Fourier shell correlation (FSC) curves for the two independently refined subtomogram averaged half-maps of TDP43 fibrils. The $FSC_{0.143}$ is shown with a dashed line.

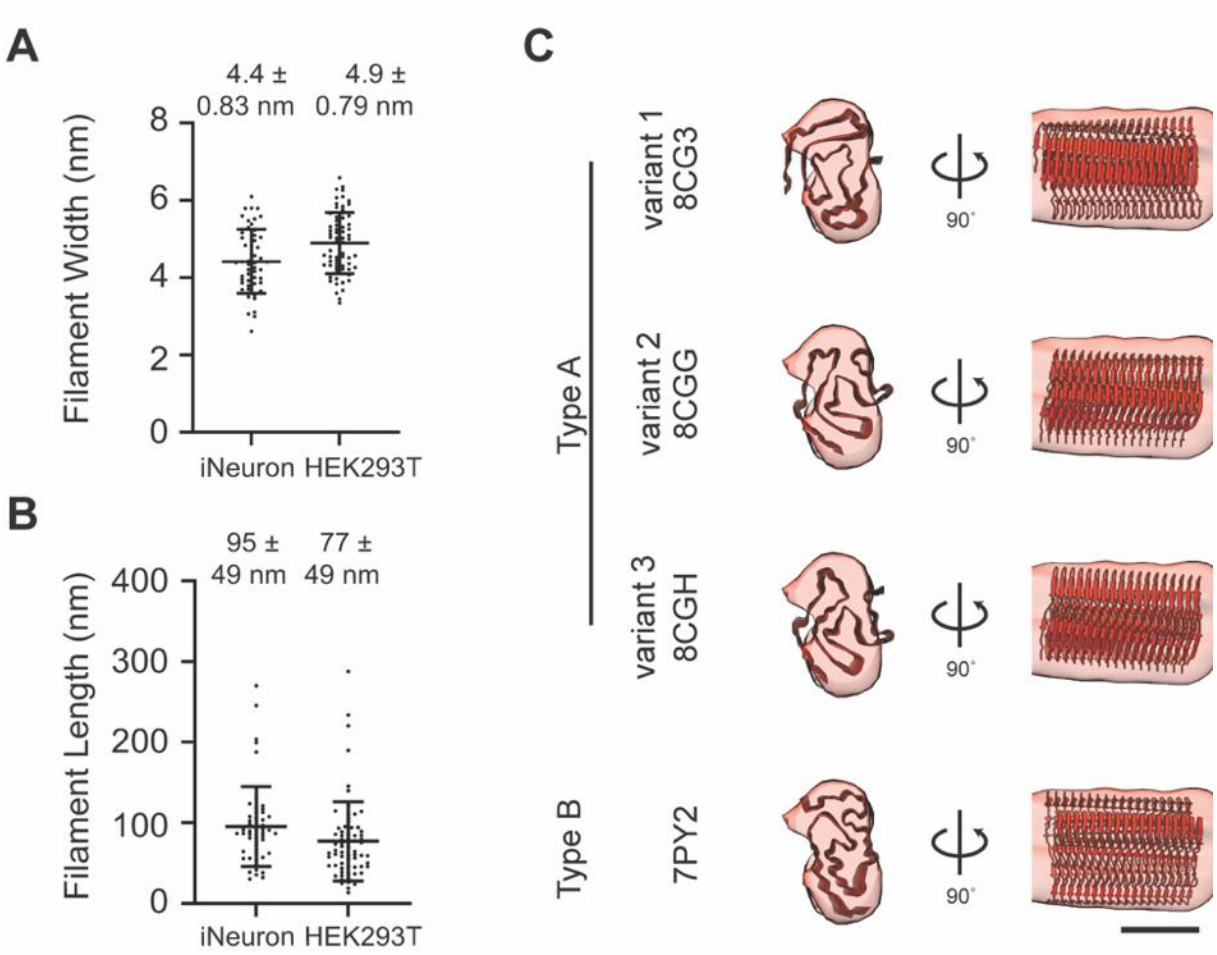

Fig. S10. TDP43 fibrils *in situ* are similar to TDP43 fibrils isolated from ALS/FTLD-TDP postmortem patient tissue. (**A**) Measurement (mean ± SD) of the widths and (**B**) lengths of TDP43 fibrils in iNeurons (n=52) and lysosomes isolated from Dendra2-LC3B HEK293T cells (n=69). (C) Fit of atomic models of TDP43 FTLD-TDP type A variants 1-3 (PDB 8CG3, 8CGG, 8CGH) and FTLD-TDP type B variant 1 (PDB 7PY2) to the subtomogram averaged map of TDP43 fibrils in iNeurons. Scale bar, 5 nm.

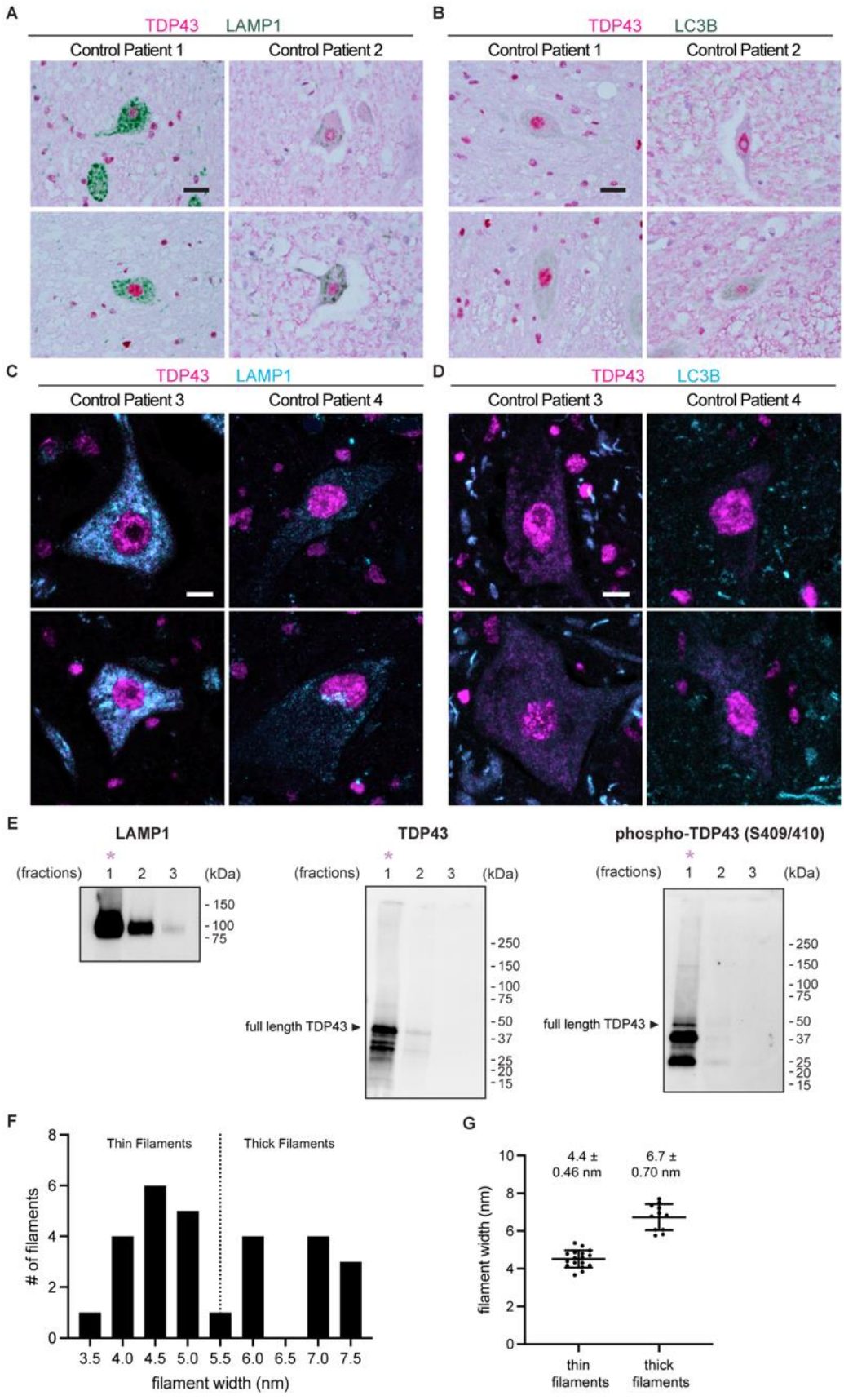
A
TDP43 LAMP1
Control Patient 1
Control Patient 2
B
TDP43 LC3B
Control Patient 1
Control Patient 2
C
TDP43 LAMP1
Control Patient 3
Control Patient 4
D
TDP43 LC3B
Control Patient 3
Control Patient 4
E
LAMP1
(fractions) 1 2 3 (kDa)
150
100
75
TDP43
(fractions) 1 2 3 (kDa)
full length TDP43
250
150
100
75
50
37
25
20
15
phospho-TDP43 (S409/410)
(fractions) 1 2 3 (kDa)
full length TDP43
250
150
100
75
50
37
25
20
15
F
Thin Filaments
Thick Filaments
# of filaments
filament width (nm)
3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0 7.5
G
4.4 ± 0.46 nm
6.7 ± 0.70 nm
filament width (nm)
thin filaments
thick filaments

Fig. S11. Characterizing subcellular localization of TDP43 in patient tissue. (A-B) Immunohistochemistry of TDP43 (magenta) and **(A)** LAMP1 or **(B)** LC3B (green) in the spinal cord of two control patients. Scale bars, 20µm. **(C-D)** Immunofluorescence of TDP43 (magenta) and **(C)** LAMP1 or **(D)** LC3B (cyan) in the spinal cord of two control patients. Scale bars, 10µm. **(E)** Immunoblots of fractions 1-3 from Optiprep density gradient for isolating lysosomes from ALS/FTLD patient brain tissue, detected with anti-LAMP1, -TDP43, and -phospho-TDP43 (S409/410). **(F)** Histogram of the filament widths within lysosomes isolated from ALS/FTLD patient brain tissue. **(G)** Measurement (mean ± SD) of the widths of thin (n=17) and thick (n=11) TDP43 filaments in lysosomes isolated from ALS/FTLD patient brain tissue.

Table S1- S3

Table S1 (separate file). Antibodies, oligonucleotides, and cell lines used in this study. Separate Excel file (Table S1.xlsx)

Table S2. Cryo-EM data collection, refinement, and validation statistics.

	TDP43 fibrils from iNeurons for STA (EMD-45215) **(Fig. 4E, Fig. S9, Fig. S10)**	**TDP43 fibrils from HEK293 lysosomes (Fig. 2E, Fig S6)**	**TDP43 fibrils from iNeurons for segmentation (Fig. 4A-C)**	**TDP43 fibrils from FTD patient brain (Fig. 5, Fig. S11)**
Data collection and processing				
Magnification	64000	42000	33000	42000
Voltage (kV)	300	300	300	300
Electron exposure/tilt (e–/Å^2)	3.27916	3.15388	4.04987	2.62481
Defocus range (μm)	-2.75	-5	-4	-2.5
Pixel size (Å)	1.368	2.12	2.654	2.12
Symmetry imposed	Helical twist of 1.4˚, rise of 4.8 Å			
Initial particle images (no.)	8298			
Final particle images (no.)	3325			
Map resolution (Å), FSC threshold	26.939, 0.143			
Map resolution range (Å)	21.24 to 33.73			

Table S3. Clinicopathological details

	ALS Patient 1	**ALS Patient 2**	**ALS/ FTLD Patient 1**	**Control Patient 1**	**Control Patient 2**	**Control Patient 3**	**Control Patient 4**
Male/female	M	F	M	F	M	F	F
Age (y)	64	76	56	64	72	78	96
Disease duration (y)	UNK	5	6	UNK	7	UNK	UNK
Clinical diagnosis	ALS	ALS	FTD	HD	Lewy Body Dementia	Probable AD	AD
Neuropathological diagnosis	ALS	ALS	FTLD with Motor Neuron Disease-like Inclusions, with Motor Neuron Disease ADNC, low	HD ADNC, low	ADNC, high LATE-NC, stage 1	ADNC, int	ADNC, high LATE-NC, stage 2
Thal phase	NA	0	NA	1	5	3	5
Braak stage	NA	I	NA	II	VI	VI	IV
CERAD score	NA	0	NA	1	3	2	2
Lewy score	NA	N/A	NA	N/A	N/A	N/A	N/A
VonSattel score	N/A	N/A	NA	3	N/A	N/A	N/A

N/A - not applicable
NA - not assessed
HD - Huntington's Disease

ADNC - Alzheimer's Disease Neuropathologic Change
LATE-NC - Limbic predominant age-related TDP-43 encephalopathy neuropathologic change
UNK - unknown