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Supplementary Materials for

C9orf72 poly(GR) aggregation induces TDP-43 proteinopathy

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

(available at stm.sciencemag.org/cgi/content/full/12/559/eabb3774/DC1)

Data file S1 (Microsoft Excel format). Raw data for all the quantitative figures where $n < 20$.

Materials and Methods

Reagents

Poly(GR) and Poly(GA) 20-mer dipeptide repeat proteins with a C-terminal FLAG tag were purchased from CSBio and verified by mass spectrometry. Sequences of poly(GR) and poly(GA): Gly-Arg-Gly-Ser-Phe-Glu-Gly-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, and Gly-Ala-Gly-Ala-Gly-Ala-Gly-Ala-Gly-Ala-Gly-Ala-Gly-Ala-Gly-Ala-Gly-Ala-Gly--Ala-Gly-Ala-Gly-Ala-Gly-Ala-Gly-Ala-Gly-Ala-Gly-Ala-Gly-Ala-Gly-Ala-Gly-Ala--Gly-Ala-Gly-Ser-Phe-Glu-Gly-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, respectively. Lyophilized powder was reconstituted in 1× PBS and snap frozen in single use 200 µM aliquots and stored at -80°C.

TDP-43-MBP protein purification

Plasmid encoding human TDP-43 with a C-terminal MBP tag (TDP-43-TEV-MBP-6×His) was purchased from Addgene (Plasmid # 104480). Recombinant TDP-43 was purified as described (*46*). Briefly, protein was expressed in *E. coli* BL21-CodonPlus (DE3)-RIL cells (Agilent). Cell cultures were grown to an OD_{600} of ~0.5-0.7 and then cooled down to 16°C. Protein expression was induced with 1 mM IPTG overnight. Cells were harvested by centrifugation, resuspended in binding buffer (20 mM Tris-HCl pH 8.0, 1 M NaCl, 10 mM imidazole, 10% (v/v) glycerol, 2 mM β-mercaptoethanol) supplemented with complete EDTA-free protease inhibitor cocktail, and lysed with sonication. Cell lysates were clarified by centrifugation, applied to Ni-NTA agarose beads (QIAGEN), then eluted using 20 mM Tris-HCl pH 8.0, 1 M NaCl, 300 mM imidazole, 10% (v/v) glycerol, and 2 mM β-mercaptoethanol. The eluate was further purified over amylose resin (NEB) and eluted using elution buffer (20 mM Tris-HCl pH 8.0, 1 M NaCl, 10 mM imidazole, 10 mM maltose, 10% (v/v) glycerol, and 1 mM DTT). Purified protein was concentrated, flash frozen and stored at -80°C.

In vitro aggregation assay

To measure aggregation kinetics, purified TDP-43 was first thawed and buffer exchanged into 20 mM HEPES-NaOH (pH 7.4), 150 mM NaCl and 1 mM DTT using a Micro Bio-Spin P-6 Gel Column (Bio-Rad). Protein concentration was measured by nanodrop, and TDP-43 was then diluted to a final concentration of 5 µM [in 20 mM HEPES (pH 7.0), 150 mM NaCl, 1 mM DTT], with addition of 2 µM poly(GR), poly(GA) or equivalent volume of 1× PBS as a control. At time = 0 minutes, aggregation was initiated by cleavage of the MBP tag using 1 µg/ml TEV protease, and monitored via turbidity measurements at an absorbance of 395 nm using a TECAN M1000 plate reader. Values were normalized to TDP-43 + TEV protease alone to determine the relative extent of aggregation. The area under each turbidity curve was calculated using GraphPad Prism. To visualize aggregate formation, aggregation was initiated by adding 10

μg/ml TEV protease to samples. After 30 minutes, 10 μl of sample was mounted onto a glass slide and imaged by differential interference contrast (DIC) microscopy.

In vitro sedimentation assay

At the end point of the turbidity assay described above (t = 16 hours), samples were sedimented by centrifugation for 10 minutes at 21,130 x g. Pellet and supernatant were immediately separated and equal volumes of each fraction were analyzed by SDS-PAGE. Proteins were visualized by Coomassie stain.

Transmission electron microscopy

Transmission electron microscopy (TEM) was performed as previously described (*45*). Briefly, at the start of the aggregation assay described above (t = 0), and at the end point of the turbidity assay (t = 16 hours), 10 μl of each sample was adsorbed onto a 300-mesh Formvar/carbon-coated copper grids (Electron Microscopy Sciences) and stained with 2% (w/v) uranyl acetate. Excess uranyl acetate was washed with water and the grids were allowed to air dry. Samples were viewed and imaged using JEOL 1010 transmission electron microscope. Electron micrographs were quantified using ImageJ. Images were inverted to have a black background. Scale was set to 110 pixels/μm, based on the scale bar of the micrographs. Threshold was used to determine the region of interest (ROI), which was considered as the total area occupied by TDP-43 aggregates. Total area (μm^2), mean gray value (a.u.), and integrated density were measured, limited to thresholded area. The integrated density, which is the product of the area of the ROI of TDP-43 aggregates and mean gray value of pixel intensity, was reported. For each condition, 9 representative micrographs were quantified, collected from 3 independent experiments.

Generation of plasmids

To generate GFP-$(GR)_{200}$ and mCherry-$(GR)_{100}$ plasmids, a gene fragment containing 50 repeats of the dipeptide GR was synthesized by GeneArt and used as a template for PCR to generate fragments containing one or two Type IIS restriction enzyme sites. These fragments were ligated together sequentially to generate 100 or 200 GR repeats. The coding sequence for EGFP was cloned into the modified AAV packaging vector [pAM/CBA-pl-WPRE-BGH ("pAAV")] containing the CMV-enhanced chicken β-actin promoter, and $(GR)_{200}$ was ligated in frame downstream of EGFP to generate pAAV EGFP-$(GR)_{200}$. The mCherry coding sequence was amplified using PCR and cloned into the AgeI and HindIII sites of pEGFP-C1, creating the vector mCherry-C1. A $(GR)_{100}$ fragment was ligated in frame downstream of the mCherry to generate mCherry-$(GR)_{100}$. The sequence of GFP-$(GR)_{200}$ and mCherry-$(GR)_{100}$ are listed in **Table S1,** respectively. To generate TDP-43-Myc plasmids, complementary DNA (cDNA) from constructs for wild-type TDP-43 or for TDP-43 with mutations in the nuclear localization signal (NLS) and/or the RNA recognition motifs (RRMs) (*47*, *48*) was used as the PCR template to generate TDP-43 fragments. These fragments were cloned into the HindIII and XhoI sites of

pAG3-Myc vector. The sequences of all plasmids were verified by sequence analysis.

Cell culture and treatments

HEK293T cells were grown in Opti-Mem plus 10% FBS and 1% penicillin–streptomycin. Cells grown in 6-well plates or on glass coverslips in 24-well plates were transfected with the indicated plasmids using Lipofectamine 2000 (Thermo Fisher Scientific). Cells were harvested or fixed for Western blot and immunofluorescence staining, respectively, 24 hours post-transfection.

Immunofluorescence staining and quantification in cultured cells

Fixed cells were permeabilized with 0.5% Triton X-100 for 10 minutes, blocked with 5% nonfat dry milk in PBS for 1 hour, then incubated with primary antibody (**Table S2)** overnight at 4°C. After washing, cells were incubated with corresponding Alexa Fluor 488-, 568- or 647-conjugated donkey anti-species antibodies (1:500 or 1:1000, Molecular Probes) for 2 hours. Hoechst 33258 (1 μg/ml, H3569, Thermo Fisher Scientific) was used to stain cellular nuclei. Images were obtained on a Zeiss LSM 880 laser scanning confocal microscope. To quantify the percentage of cells with cytoplasmic TDP-43 inclusions in GFP- or poly(GR)-positive cells, the number of TDP-43-positive cells containing diffuse or aggregated TDP-43 was counted in a blinded fashion from 3 independent experiments (~500–1000 cells were counted for each group in each independent experiment). To quantify the percentage of cells with cytoplasmic TDP-43 inclusions in poly(GA)-positive cells, the number of TDP-43-positive cells containing diffuse or aggregated TDP-43 was counted in a blinded fashion from 3 independent experiments (~110–190 poly(GA) inclusions were counted for each group in each independent experiment).

Preparation of cell lysates

Cell pellets were lysed in co-immunoprecipitation (co-IP) buffer (50 mM Tris–HCl, pH 7.4, 300 mM NaCl, 1% Triton X-100, 5 mM EDTA) plus 2% SDS, and both protease and phosphatase inhibitors, sonicated on ice, and then centrifuged at 16,000 × g for 20 minutes. Supernatants were saved as cell lysates. The protein concentration of lysates was determined by BCA assay (Thermo Fisher Scientific), and samples were then subjected to Western blot analysis.

Western blot analysis

Cell lysates were diluted with 2× SDS-loading buffer at a 1:1 ratio (v/v), and then heated at 95°C for 5 minutes. Afterwards, equal amounts of protein were loaded into 10-well 4–20% Tris-glycine gels (Novex). After transferring proteins to PVDF membranes, membranes were blocked with 5% nonfat dry milk in TBS plus 0.1% Tween 20 (TBST) for 1 hour, and then incubated with primary antibody (**Table S2**) overnight at 4°C. Membranes were washed in TBST and incubated with donkey anti-rabbit or anti-mouse IgG antibodies conjugated to horseradish peroxidase (1:5000; Jackson ImmunoResearch) for 1 hour. Protein expression was

visualized by enhanced chemiluminescence treatment and exposure to film. The intensity of bands was quantified by FUJI FILM MultiGauge Software, and then normalized to the corresponding controls.

Proximity ligation assay (PLA)

HEK293T cells grown in an 8-well chamber slide (ibidi) were transfected with the indicated plasmids using Lipofectamine 2000 (Life Technologies). Twenty-four hours after transfection, cells were fixed for the PLA study using the Duolink In Situ kit (DUO92004, DUO92002, Sigma-Aldrich) per the manufacturer's protocol. In brief, fixed cells were permeabilized with 0.1% Triton X-100 for 10 minutes, blocked using Duolink blocking buffer for 1 hour at room temperature, and then incubated with rabbit polyclonal anti-GR (Rb7810, 1:2000) (*10*) and mouse monoclonal anti-Myc (MA1-980, 1:1000, Invitrogen) overnight at 4°C. After washing with PBS plus 0.05% Tween 20, cells were incubated with the PLA probes MINUS and PLUS (1:5 dilution) for 1 hour at 37°C. After washing in 1× wash buffer A, cells were incubated with the ligase (1:40 dilution) in ligation buffer for 30 minutes at 37°C. Cells were washed with 1× wash buffer A, and then inoculated with polymerase (1:80 dilution) in amplification buffer for 90 minutes at 37°C. Cells were washed in 1× wash buffer B, followed by 0.01× wash buffer B. Hoechst 33258 (1 µg/ml, H3569, Thermo Fisher Scientific) was used to stain cellular nuclei. Images were obtained on a Zeiss LSM 880 laser scanning confocal microscope. To quantify the percentage of cells with PLA signal, the number of GFP-$(GR)_{100}$-positive cells containing PLA signal was counted in a blinded fashion from 14-18 images.

Immuno- electron microscopy

To examine the ultrastructure of poly(GR) aggregates, immuno- electron microscopy (IEM) was performed as previously described (*21*). Rabbit polyclonal anti-poly(GR) antibody (7810, 1:20) (*10*) was used as a primary antibody and goat anti-rabbit IgG conjugated with 18 nm colloidal gold particles (1:20, Jackson ImmunoResearch Laboratories) was used as the secondary antibody. Thin sections stained with uranyl acetate and lead citrate were examined with a Philips 208S electron microscope (FEI) fitted with a Gatan 831 Orius CCD camera (Gatan).

Human tissues

Post-mortem hippocampal and frontal cortical tissues from frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) patients with the *C9orf72* repeat expansion were obtained from the Mayo Clinic Florida Brain Bank. Information on human patients is provided in **Table S3**. Written informed consent was obtained before study entry from all subjects or their legal next of kin if they were unable to give written consent, and biological samples were obtained with Mayo Clinic Institutional Review Board (IRB) approval.

Animal studies

All procedures using mice were performed in accordance with the National Institutes of

Health Guide for Care and Use of Experimental Animals and approved by the Mayo Clinic Institutional Animal Care and Use Committee (IACUC).

Virus production

rAAV9 virus was produced as previously described (*13*, *14*, *29*). Briefly, AAV vectors expressing GFP, GFP-$(GR)_{200}$, $(G_4C_2)_2$ or $(G_4C_2)_{149}$ were co-transfected with helper plasmids in HEK293T cells using polyethylenimine (23966, Polysciences, Inc.). Cells were harvested forty-eight hours following transfection, and lysed in the presence of 0.5% sodium deoxycholate and 50 Units/mL Benzonase (Sigma-Aldrich) by freeze-thawing. The virus was isolated using a discontinuous iodixanol gradient. The genomic titer of each virus was determined by qRT-PCR, and AAV solutions were diluted in sterile phosphate-buffered saline (PBS).

Neonatal viral injections

Intracerebroventricular (ICV) injections of virus were performed as previously described (*13*, *14*, *29*). Briefly, 2 µl (1×10^{10} genomes/µl) of AAV-GFP, AAV-GFP-$(GR)_{200}$, AAV-$(G_4C_2)_2$ or AAV-$(G_4C_2)_{149}$ solution was manually injected into each lateral ventricle of cryoanesthetized C57BL/6J mouse pups on postnatal day 0 (P0). Pups were allowed to recover from cryoanesthesia on a heating pad, and were then returned to the home cage with the mother.

Antisense oligonucleotide (ASO) injections and sample collection

PBS or c9ASO was injected into the central nervous system of 3 month-old AAV-$(G_4C_2)_2$ or AAV-$(G_4C_2)_{149}$ mice by means of stereotactic ICV injection, as previously described (*34*) with some minor modifications. The c9ASO targeting the G_4C_2 repeat expansion (CGGCCCCGGCCCCGGC), developed and provided by Ionis Pharmaceuticals, was aMOE-gaper ASO with 16 nucleotides in length, wherein the central gap segment comprising eight 2′-deoxyribonucleotides (DNA) that are flanked on the 5′ and 3′ wings by four 2'-O-methoxyethyl (MOE) modified nucleotides. Internucleotide linkages are phosphorothioate interspersed with phosphodiester, and all cytosine residues are 5′-methylcytosines. Specifically, 10 µl of PBS or c9ASO solution (corresponding to 350 µg ASOs) were delivered into the right lateral ventricle using the coordinates: 0 mm anterior and 1.0 mm lateral to the right from bregma, and 1.9-2.0 mm deep as measured from the brain surface. PBS-treated AAV-$(G_4C_2)_2$ mice (n = 17) or AAV-$(G_4C_2)_{149}$ mice (n = 18), and c9ASO-treated AAV-$(G_4C_2)_{149}$ (n = 12) mice were compared.

Tissue processing

For protein, immunostaining and RNA analyses, the mice were euthanized by CO_2 or ketamine/xylazine through intraperitoneal injection. Blood samples were then collected by cardiac puncture, and mice euthanized by exsanguination followed by transcardial perfusion with saline. Then, brains were harvested and cut sagittally across the midline. The brain was rapidly removed and hemisected. Sagittal half brains were immersion fixed in in 4% paraformaldehyde, embedded in paraffin, sectioned (5 µm thick), and then mounted on glass slides for immunofluorescence or immunohistochemistry staining. The other half brains were dissected

and frozen (cortex, hippocampus, subcortex, midbrain, brainstem, and cerebellum frozen separately).

Immunohistochemistry staining

Sagittal half brains fixed in 4% paraformaldehyde were embedded in paraffin, sectioned at 5 μm, and mounted on positively-charged glass slides. After drying overnight, paraffin sections were deparaffinized in xylene, and rehydrated through a series of ethanol solutions, followed by washing in dH2O. Antigen retrieval was performed by steaming slides in dH2O or Tris-EDTA (DAKO), pH 9.0 for 30 minutes followed by a 5 minute incubation in Dako Peroxidase Block (S2001, DAKO) to block endogenous peroxidase activity. To detect the sense DPR proteins, sections were immunostained with primary antibody (**Table S2)** using the DAKO Autostainer (Universal Staining System) and the DAKO+HRP system. To detect ataxin-2 or NeuN, slides were blocked with Dako Protein Block Serum-Free (X0909, DAKO) for 1 hour, and incubated with primary antibody (**Table S2)** for 45 minutes. After washing, sections were incubated for 30 minutes in Dako Envision-Plus anti-rabbit (K4003, DAKO) or anti-mouse (K4001, DAKO) labeled HRP polymer, respectively. Peroxidase labeling was visualized with the Liquid DAB + Substrate Chromogen System (K3468, DAKO).

To detect pTDP-43 in AAV-$(G_4C_2)_{149}$ mice or poly(GR) in AAV-GFP-$(GR)_{200}$ mice, slides were deparaffinized and rehydrated as described above, and antigen retrieval was performed by steaming in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0) for 30 minutes. After cooling and washing with dH2O, slides were incubated with Dako Dual Endogenous Enzyme Block (DAKO), and subsequently washed in PBS. Sections were then blocked with 2% normal goat serum for 1 hour, followed by an overnight incubation with primary antibody (**Table S2**) at 4ºC. The next day, slides were washed with PBS, incubated with biotinylated goat anti-rabbit or rabbit anti-rat secondary (1:200) for 2 hours, and again washed in PBS. Slides were then incubated with avidin-biotin complex solution for 30 minutes, washed in PBS, and reacted with 3,3'-diaminobenzidine (Acros Organics) activated with hydrogen peroxide, with the reaction stopped by rinsing slides in dH2O.

Following labeling, all sections were counterstained with hematoxylin (Thermo Fisher Scientific), dehydrated through ethanol and xylene washes, and cover-slipped with Cytoseal mounting medium (Thermo Fisher Scientific). Slides were scanned with a ScanScope AT2 (Leica Biosystems), and representative images taken with ImageScope software (v12.1; Leica Biosystems).

Immunofluorescence staining in mouse and human brains

Paraffin sections (5 μm) of mouse and human brain tissues were deparaffinized, rehydrated, steamed for 30 minutes in Dako antigen retrieval solution, blocked with Dako All Purpose Blocker for 1 hour, and incubated with primary antibody (**Table S2**). After washing, sections were incubated with corresponding Alexa Fluor 488-, 568- or 647-conjugated donkey

anti-species (1:500, Molecular Probes) for 2 hours. Hoechst 33258 (1 μg/ml, Thermo Fisher Scientific) was used to stain cellular nuclei. Images were obtained on a Zeiss LSM 880 laser scanning confocal microscope.

Quantification of neuropathology

To quantify diffuse and aggregated poly(GR), high resolution digitized images of immunostained slides were obtained by using a ScanScope AT2 (Leica Biosystems). The cortex was annotated on mid-sagittal serial sections. The number of diffuse and aggregated poly(GR) was quantified manually in a blinded fashion. To quantify pTDP-43 inclusions, high resolution digitized images of immunostained slides were obtained by using a ScanScope AT2 (Leica Biosystems). The cortex was annotated on mid-sagittal serial sections. The number of pTDP-43 inclusions was quantified manually in a blinded fashion. To quantify TDP-43, KPNA2 and NUP98 pathology, non-transduced (NT), diffuse poly(GR) or aggregated poly(GR) cells exhibiting TDP-43, KPNA2 and NUP98 pathology were counted in a unblinded fashion in the cortex of 2-week-old mice expressing GFP-$(GR)_{200}$ (TDP-43: ~180–250 cells were counted per mouse; KPNA2: ~150–240 cells were counted per mouse; NUP98: ~140–170 cells were counted per mouse). To quantify abnormal co-localization of NPC with either TDP-43 or eIF3η, non-transduced (NT), diffuse poly(GR) or aggregated poly(GR) cells exhibiting abnormal co-localization of NPC with either TDP-43 or eIF3η were counted in a unblinded fashion in the cortex of 2-week-old mice expressing GFP-$(GR)_{200}$ (NPC and eIF3η: ~170–220 cells were counted per mouse; NPC and TDP-43: ~180–220 cells were counted per mouse). To quantify co-localization of poly(GA) with TDP-43 and eIF3η in the cortex of 3-month-old mice expressing GFP-$(GA)_{50}$, ~90–120 poly(GA) inclusions were counted per mouse. To quantify co-localization of TDP-43 with poly(GA) or poly(GR), the total number of poly(GA) or poly(GR) inclusions, as well as the number of inclusions that were also positive for TDP-43 were counted in a blinded fashion in the hippocampus of c9FTD/ALS patients [poly(GA): ~90–470 inclusions were counted per patient; poly(GR): ~35–180 inclusions were counted per patient].

RNA Fluorescence In Situ Hybridization (FISH)

The RNA FISH protocol was performed as previously described (*13*). Briefly, tissue sections were deparaffinized and rehydrated through a series of xylene and ethanol solutions, permeabilized with ice cold 2% acetone/1× DEPC-PBS, washed with DEPC-H_2O, and then dehydrated in ethanol. To detect sense RNA foci, sections were incubated with pre-hybridization buffer [50% formamide (Midsci), 10% dextran sulfate (Millipore), 2× saline-sodium citrate buffer (SSC), 50 mM sodium phosphate buffer pH 7.0] for 20–30 minutes at 66°C, and then hybridized for 24 hours at 66°C in a dark, humidified chamber with a fluorescently-labeled locked nucleic acid (LNA) probe (*5*) [TYE563-(CCCCGGCCCCGGCCCC); Exiqon product number 500150, design id: 283117] diluted to a final concentration of 40 nM. Next, sections were washed with 2× SSC/0.1% Tween-20 at room temperature for 5 minutes, and then washed

twice with pre-warmed 0.2× SSC at 60°C for 10 minutes in the dark. Following these washes, slides were coverslipped using Vectashield mounting media with DAPI (Vector Laboratories). Representative images of sense RNA foci in the cortex were taken with an AxioImager Z1 fluorescent microscope (Carl Zeiss MicroImaging). RNA foci burden was quantified in a blinded fashion by calculating the percentage of cells in the motor cortex (from 300–400 cells total) containing sense RNA foci.

RNA extraction, reverse transcription and qPCR

For RNA extraction, frozen hippocampi were homogenized in Trizol LS (250 μL), and total RNA extracted using the Direct-zol RNA MiniPrep kit (Zymo Research) according to manufacturer's instructions. cDNA was then obtained following reverse transcription of 250 ng of the extracted RNA with random primers and the High Capacity cDNA Transcription Kit (Applied Biosciences). qRT-PCR was performed in triplicate for all samples using the SYBR green assay (Life Technologies) on an ABI Prism 7900HT Fast Real-Time PCR System (Applied Biosystems). As the AAV-$(G_4C_2)_{149}$ vector contains 119 base pairs of human flanking sequence 5' of the G_4C_2 repeat, primers were designed targeting this region to assess mRNA expression of exogenous, AAV-derived G_4C_2-containing transcripts. Endogenous *Gapdh* and *Rplp0* mRNA were also quantified. The sequences of the primers used for this study are listed in **Table S4**. Relative mRNA expression of the G_4C_2 5' flanking sequence was normalized to the geometric mean of the endogenous transcript controls, *Gapdh* and *Rplp0*.

Immunoassay analysis of poly(GR), poly(GA), and poly(GP)

Frozen cortex samples were homogenized in co-immunoprecipitation buffer (50 mM Tris–HCl, pH 7.4, 300 mM NaCl, 1% Triton X-100, 5 mM EDTA) containing protease and phosphatase inhibitors. After sonication, the lysates were centrifuged at 16,000 × g for 20 minutes, and BCA assay performed on the supernatant to determine protein concentration. Previously characterized Meso Scale Discovery (MSD) sandwich immunoassays were then used to detected poly (GR) (*29*), or poly(GP) and poly(GA) (*49*). In brief, lysates were diluted in Tris-buffered saline (TBS) and an equal amount of protein for all samples was tested in duplicate wells. Response values corresponding to the intensity of emitted light upon electrochemical stimulation of the assay plate using the MSD QUICKPLEX SQ120 were acquired and background corrected using the average response from lysates obtained from control $(G_4C_2)_2$ mice.

Detection of neurofilament light (NfL) in plasma

Blood samples from mice were collected in EDTA tubes, and centrifuged to obtain plasma. Plasma NfL concentrations were then determined using a Simoa NF-Light Advantage Kit (102258) run on the automated HD-1 Analyzer (Quanterix) per the manufacturer's protocol. Briefly, plasma samples were diluted 1:4 at the bench, and subsequently transferred to 96-well plates along with calibrators, two quality control samples, and five interassay controls with a

range of known NfL concentrations. NfL concentrations were then interpolated from the standard curve using a 4 parameter logistic curve fit (1/y2 weighted).

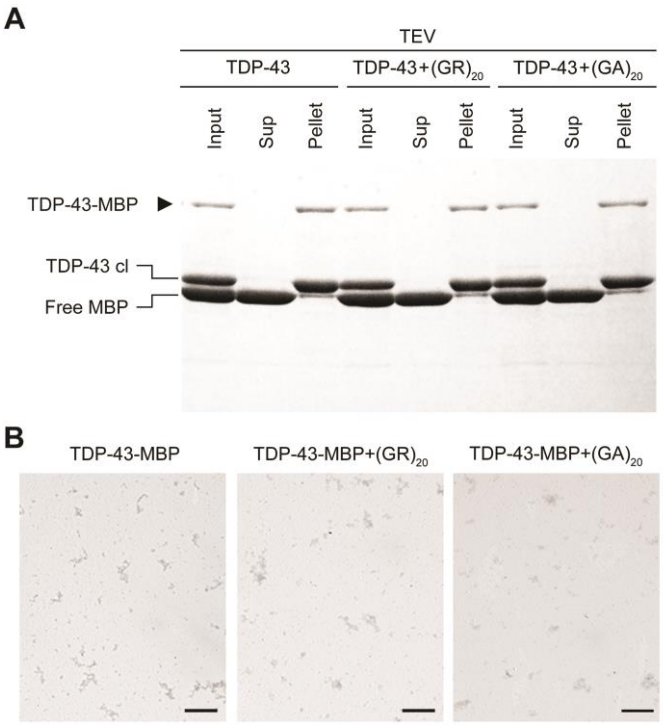

Fig. S1. Poly(GR) accelerates and enhances TDP-43 aggregation. (**A**) Sedimentation of TDP-43 (5 μM) in the absence or presence of 2 μM poly(GR) or poly(GA). Samples were sedimented at the end-point of the aggregation assay (t = 16 hours). (**B**) Representative electron micrographs of TDP-43 (5 μM) in the absence or presence of 2 μM poly(GR) or poly(GA), without TEV protease. Scale bar, 2 μm.

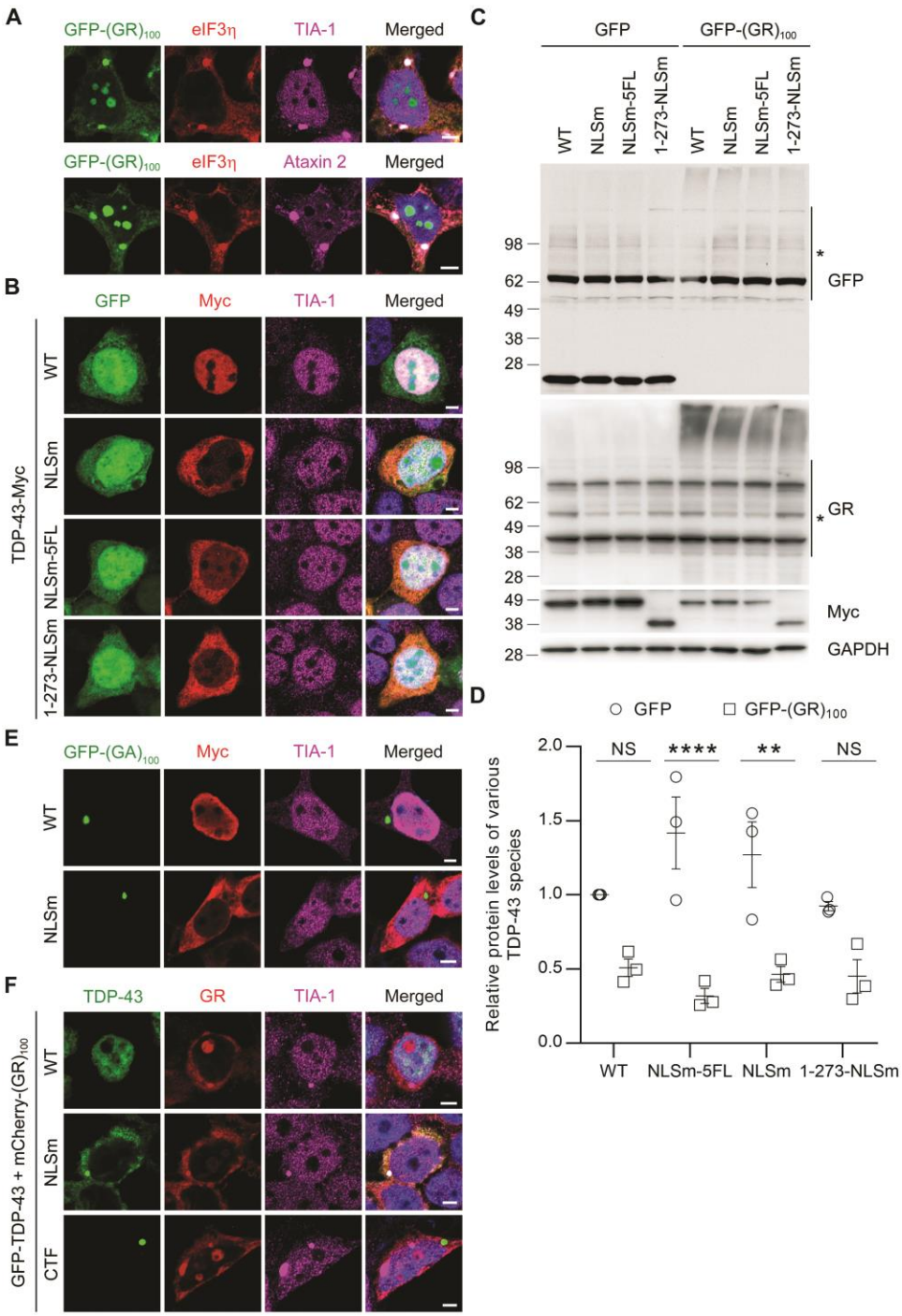

Fig. S2. Poly(GR) mediates sequestration of cytosolic full-length TDP-43 into the inclusions. (**A**) Triple-immunofluorescence staining for GFP-$(GR)_{100}$, eIF3η and TIA-1 or Ataxin 2 in HEK293T cells expressing GFP-$(GR)_{100}$. Scale bars, 5 μm. (**B**) Triple-immunofluorescence staining for GFP, TDP-43-Myc and TIA-1 in HEK293T cells expressing GFP and Myc-tagged TDP-43 species. Scale bars, 5 μm. (**C**) Western blot confirming expression of GFP, GFP-$(GR)_{100}$ and TDP-43-Myc in HEK293T cells co-transfected with either GFP or GFP-$(GR)_{100}$ and various

Myc-tagged TDP-43 species. GAPDH was used to control for protein loading. * Indicates non-specific bands. (**D**) Densitometric analysis of Myc-tagged TDP-43 species in HEK293T cells co-expressing various Myc-tagged TDP-43 constructs with either GFP or GFP-$(GR)_{100}$ (n = 3 independent experiments). (**E**) Triple-immunofluorescence staining for poly(GA), TDP-43 and TIA-1 in HEK293T cells expressing GFP-$(GA)_{100}$ and Myc-tagged TDP-43 constructs [including wild-type (WT) and nuclear localization signal mutant (NLSm)] (n = 3 independent experiments). Scale bars, 5 μm. (**F**) Triple-immunofluorescence staining for GFP-TDP-43, mCherry-$(GR)_{100}$, and TIA-1 in HEK293T cells co-transfected with mCherry-$(GR)_{100}$ and GFP-tagged TDP-43 species [including WT, NLSm, and the C-terminal fragment (CTF)]. Scale bars, 5 μm. Data shown as the mean ± SEM. $^{**}P = 0.0016$, $^{****}P < 0.0001$, NS (left to right) $P = 0.0590$, and $P = 0.0726$, two-way ANOVA, Tukey's post hoc analysis.

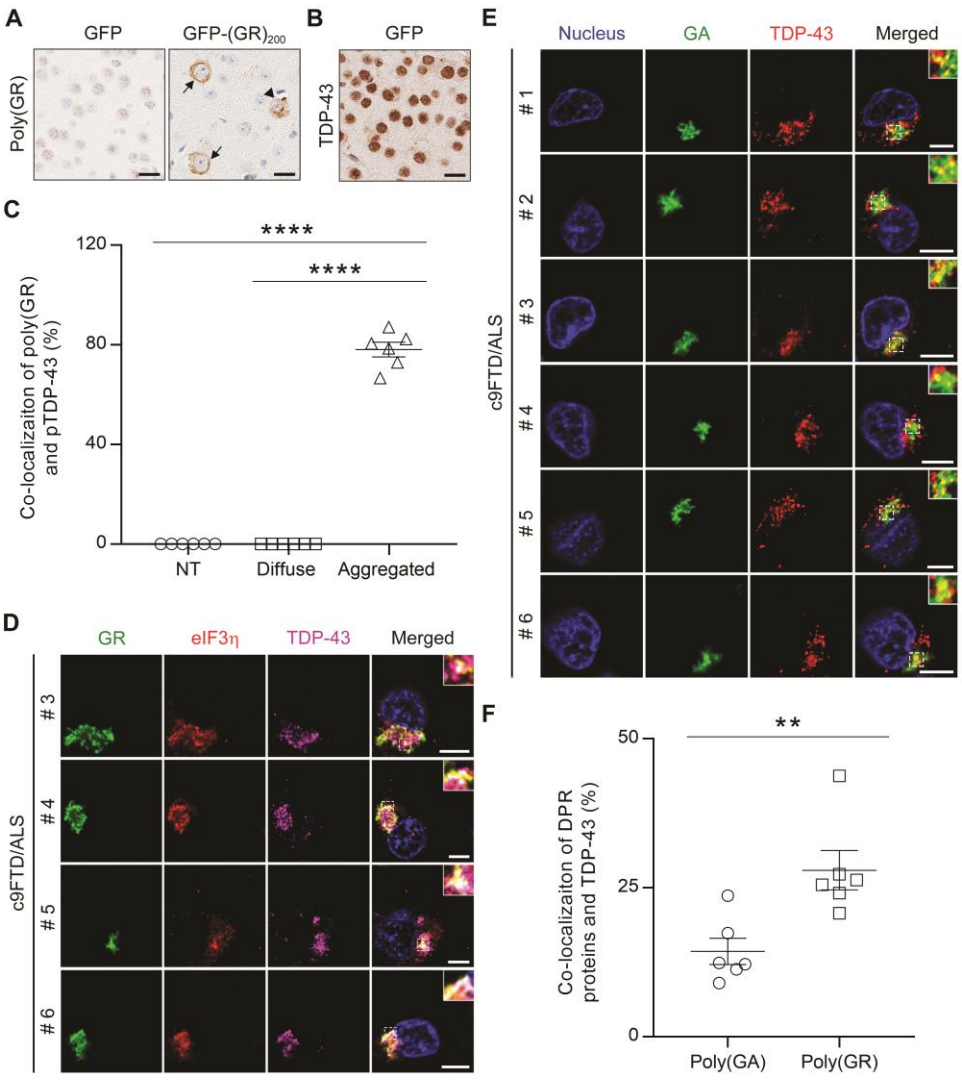

Fig. S3. TDP-43 and DPR burden in mouse and human. (**A**) Representative images of immunohistochemical analysis of poly(GR) in the cortex 3-month-old GFP or GFP-$(GR)_{200}$ mice (diffuse labeling noted by black arrows, aggregates indicated by black arrowheads). Scale bar, 20 µm. (**B**) Representative images of immunohistochemical analysis of TDP-43 in the cortex of 2-week-old GFP mice. Scale bar, 20 µm. (**C**) Quantification of the percentage of co-localization between poly(GR) and pTDP-43 in either non-transduced (NT) or transduced cells with diffuse or aggregated poly(GR) (n =6). (**D**) Triple-immunofluorescence staining for poly(GR), eIF3η, and TDP-43 in the hippocampus of c9FTD/ALS patients (see Table S3 for patient information). Scale bars, 5 µm. (**E**) Double-immunofluorescence staining for poly(GA) and TDP-43 in the hippocampus of c9FTD/ALS patients (see Table S3 for patient information). Scale bars, 5 µm. (**F**) Quantification of the percentage of co-localization between TDP-43 and either poly(GA) or poly(GR) in c9FTD/ALS patient tissue. Data shown as the mean ± SEM. In (B), **** $P <$ 0.0001, one-way ANOVA, Tukey's post hoc analysis. In (F), ** $P = 0.0064$, two-tailed unpaired t test.

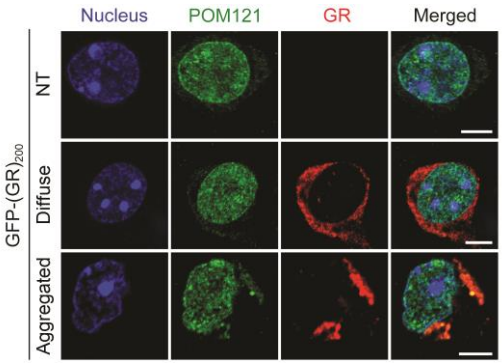

Fig. S4. Poly(GR) aggregates sequester nuclear pore POM121 protein in vivo. Double-immunofluorescence staining for poly(GR) and POM121 in the cortex of 2-week-old GFP-$(GR)_{200}$ mice (n = 6). Scale bars, 5 μm.

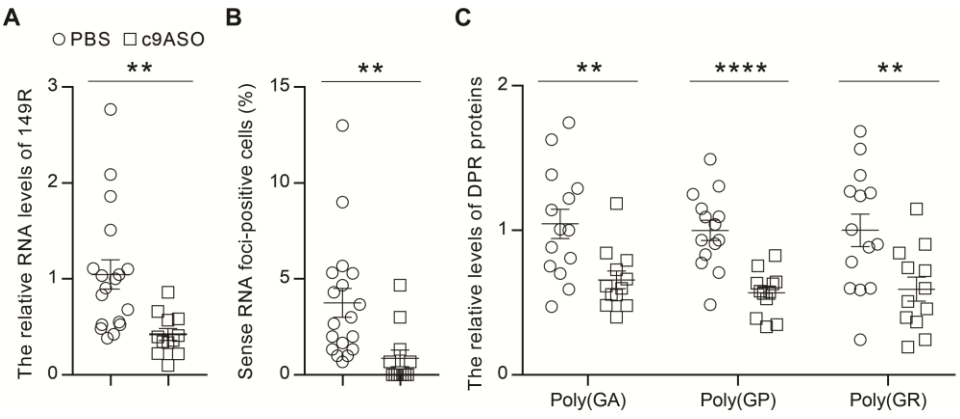

Fig. S5. c9ASO reduces G_4C_2 repeat–containing RNA and sense DPR protein burden. (**A**) qRT-PCR analysis of exogenous G_4C_2 repeat RNA transcripts in $(G_4C_2)_{149}$ mice using human-specific primers 5' of the repeat targeting sequence present in the AAV vector. Exogenous G_4C_2 repeat mRNA expression was normalized to the geometric mean of the endogenous controls *Gapdh* and *Rplp0* (PBS, n = 18; c9ASO, n = 12). (**B**) Percentage of cells containing sense RNA foci in the motor cortex of PBS (n = 19) or c9ASO-treated (n = 12) $(G_4C_2)_{149}$ mice. (**C**) Poly(GA), poly(GP) and poly(GR) concentrations in brain lysates from PBS (n = 14) or c9ASO-treated (n = 12) $(G_4C_2)_{149}$ mice were measured by immunoassay. Data presented as the mean ± SEM. In (A), ** $P = 0.0033$, unpaired two-tailed *t*-test. In (B), ** $P = 0.0066$, unpaired two-tailed *t*-test. In (C), ** (left to right) $P = 0.0045$, $P = 0.0089$ and **** $P < 0.0001$, unpaired two-tailed *t*-test.

Table S1. cDNA sequence of the GFP-$(GR)_{200}$ and mCherry-$(GR)_{100}$ plasmids.

GFP-$(GR)_{200}$ plasmid

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGC
TGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCG
ATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCC
CGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCC
GCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGG
CTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGC
GCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCA
TCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAA
CAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAAC
TTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACC
AGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCT
GAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTC
CTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACA
AGTCCGGACTCAGATCTCGAGCTCAAGCTTCGGGCCGCGGCCGTGGTCGCGGTCG
TGGACGTGGCCGTGGCCGTGGCCGAGGTCGCGGTCGGGGACGTGGCCGTGGTCGT
GGCCGAGGTCGAGGTCGCGGACGTGGACGTGGTCGAGGTCGGGGACGTGGACGA
GGCCGTGGTCGTGGCCGAGGTCGCGGACGTGGGCGAGGTCGCGGTCGAGGCCGGG
GACGTGGCCGTGGTCGTGGACGGGGACGGGGTCGGGGACGCGGTCGAGGCCGTG
GACGTGGACGGGGTCGAGGACGTGGACGTGGTCGTGGACGTGGCCGTGGACGTGG
ACGCGGCCGCGGCCGTGGTCGCGGTCGTGGACGTGGCCGTGGCCGTGGCCGAGGT
CGCGGTCGGGGACGTGGCCGTGGTCGTGGCCGAGGTCGAGGTCGCGGACGTGGAC
GTGGTCGAGGTCGGGGACGTGGACGAGGCCGTGGTCGTGGCCGAGGTCGCGGACG
TGGGCGAGGTCGCGGTCGAGGCCGGGGACGTGGCCGTGGTCGTGGACGGGGACG
GGGTCGGGGACGCGGTCGAGGCCGTGGACGTGGACGGGGTCGAGGACGTGGACG
TGGTCGTGGACGTGGCCGTGGACGTGGACGCGGCCGCGGCCGTGGTCGCGGTCGT
GGACGTGGCCGTGGCCGTGGCCGAGGTCGCGGTCGGGGACGTGGCCGTGGTCGTG
GCCGAGGTCGAGGTCGCGGACGTGGACGTGGTCGAGGTCGGGGACGTGGACGAG
GCCGTGGTCGTGGCCGAGGTCGCGGACGTGGGCGAGGTCGCGGTCGAGGCCGGGG
ACGTGGCCGTGGTCGTGGACGGGGACGGGGTCGGGGACGCGGTCGAGGCCGTGG
ACGTGGACGGGGTCGAGGACGTGGACGTGGTCGTGGACGTGGCCGTGGACGTGGA
CGCGGCCGTGGTCGCGGTCGTGGACGTGGCCGTGGCCGTGGCCGAGGTCGCGGTC
GGGGACGTGGCCGTGGTCGTGGCCGAGGTCGAGGTCGCGGACGTGGACGTGGTCG
AGGTCGGGGACGTGGACGAGGCCGTGGTCGTGGCCGAGGTCGCGGACGTGGGCG
AGGTCGCGGTCGAGGCCGGGGACGTGGCCGTGGTCGTGGACGGGGACGGGGTCG
GGGACGCGGTCGAGGCCGTGGACGTGGACGGGGTCGAGGACGTGGACGTGGTCG
TGGACGTGGCCGTGGACGTGGACGGTAG

The $(GR)_{200}$ sequence is highlighted in yellow

mCherry-$(GR)_{100}$ plasmid

ATGGTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCGCT
TCAAGGTGCACATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGA
GGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAA
GGGTGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCT

CCAAGGCCTACGTGAAGCACCCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTC
CCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTGA
CCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCT
GCGCGGCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGC
TGGGAGGCCTCCTCCGAGCGGATGTACCCCGAGGACGGCGCCCTGAAGGGCGAGA
TCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGCTGAGGTCAAGA
CCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCTACAACGTCAACAT
CAAGTTGGACATCACCTCCCACAACGAGGACTACACCATCGTGGAACAGTACGAA
CGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGGAAGCTT
CGGGCCGCGGCCGTGGTCGCGGTCGTGGACGTGGCCGTGGCCGTGGCCGAGGTCG
CGGTCGGGGACGTGGCCGTGGTCGTGGCCGAGGTCGAGGTCGCGGACGTGGACGT
GGTCGAGGTCGGGGACGTGGACGAGGCCGTGGTCGTGGCCGAGGTCGCGGACGTG
GGCGAGGTCGCGGTCGAGGCCGGGGACGTGGCCGTGGTCGTGGACGGGGACGGG
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CCGTGGCCGTGGCCGAGGTCGCGGTCGGGGACGTGGCCGTGGTCGTGGCCGAGGT
CGAGGTCGCGGACGTGGACGTGGTCGAGGTCGGGGACGTGGACGAGGCCGTGGTC
GTGGCCGAGGTCGCGGACGTGGGCGAGGTCGCGGTCGAGGCCGGGGACGTGGCC
GTGGTCGTGGACGGGGACGGGGTCGGGGACGCGGTCGAGGCCGTGGACGTGGAC
GGGGTCGAGGACGTGGACGTGGTCGTGGACGTGGCCGTGGACGTGGACGGTAG

The $(GR)_{100}$ sequence is highlighted in yellow.

Table S2. Primary antibodies for Western blot, immunohistochemistry, and immunofluorescence staining.

Western blot				
Antibody	**Species**	**Dilution**	**Number**	**Company**
anti-GR	rabbit	1:2000	Rb7810[a]	
anti-GFP	rabbit	1:4000	A-6455	Life Technologies
anti-Myc	mouse	1:1000	MA1-980	Invitrogen
anti-GAPDH	mouse	1:5000	H86504M	Meridian Life Science
Immunohistochemistry				
Antibody	**Species**	**Dilution**	**Number**	**Company**
anti-GA	rabbit	1:50000	Rb9880[a]	
anti-GP	rabbit	1:10000	Rb5823[a]	
anti-GR	rabbit	1:2500	Rb7810[a]	
anti-pTDP-43	rabbit	1:1000	Rb3655[b]	
anti-TDP-43	rabbit	1:1000	MC2079[c]	
anti-GR	rat	1:250	MABN778	EMD Millipore
anti-Ataxin 2	rabbit	1:500	21776-1-AP	Proteintech
Immunofluorescence				
Antibody	**Species**	**Dilution**	**Number**	**Company**
anti-Myc	mouse	1:1000	MA1-980	Invitrogen
anti-TIA-1	rabbit	1:2000	ab40693	Abcam
anti- eIF3η	goat	1:200	sc-16377	Santa Cruz Biotechnology
anti-GR	rabbit	1:2000	Rb7810[a]	Cosmo Bio
anti-GR	rabbit	1:500	MABN778	EMD Millipore
Anti-pTDP-43	rabbit	1:1000	CAC-TIP-PTD-P02	Cosmo Bio
anti-Ataxin 2	rabbit	1:500	21776-1-AP	Proteintech
anti-NPC	mouse	1:100	ab24609	Abcam
anti-NUP98	rat	1:200	ab50610	Abcam
anti-POM121	rabbit	1:100	PA5-36498	Invitrogen
anti-RanGAP1	rabbit	1:100	sc-25630	Santa Cruz Biotechnology
anti-GA	mouse	1:500	MABN889	EMD Millipore
anti-GFP	mouse	1:1000	33-2600	Invitrogen
anti-TDP-43	rabbit	1:1000	MC2079[c]	
Importin α5	rabbit	1:100	18137-1-AP	Proteintech
KPNA2	rabbit	1:100	0819-1-AP	Proteintech

[a, d]Antibody described in: T. F. Gendron *et al.*, *Acta Neuropathol* **126**, 829-844 (2013).
[b]Antibody described in: J. Chew *et al.*, *Science* **348**, 1151-1154 (2015).
[c]Antibody described in: Y .J. Zhang *et al.*, *Proc Natl Acad Sci U S A.* **106**, 7607-7612 (2009).

Table S3. Characteristics of patients with c9FTD/ALS.

Case #	Pathological Diagnosis	Gender	Age at Onset	Age at death	Disease Duration	*C9orf72* repeat expansion
1	FTLD/ALS	F	60.4	61.4	1.0	Yes
2	FTLD	M	62	73.0	11.0	Yes
3	FTLD/ALS	F	52	60.3	8.3	Yes
4	FTLD/ALS	M	57	62.2	5.2	Yes
5	FTLD	M	68	73.9	5.9	Yes
6	FTLD/ALS	M	57	62	6.0	Yes

FTLD, frontotemporal lobar degeneration; ALS, amyotrophic lateral sclerosis

Table S4. Primers for qPCR.

Model	Target	Primers
mouse brain	5’flanking sequence	5’-TAGTACTCGCTGAGGGTGAAC-3’
		5’-CTACAGGCTGCGGTTGTTTC-3’
mouse brain	*Gfap*	5’-CATGGCCTTCCGTGTTCCTA-3’
		5’-CCTGCTTCACCACCTTCTTGAT-3’
mouse brain	*Rplp0*	5’-ACTGGTCTAGGACCCGAGAAG-3’
		5’-CTCCCACCTTGTCTCCAGTC-3’

Data file S1. Raw data for all the quantitative figures where n < 20.
Provided as a separate Excel file.