Appendix e-1. Supplemental methods

Analyses of C9orf72 expression at the RNA and protein levels

To do the expression analysis of *C9orf72* transcripts, we extracted total RNA from tissues of 9686 and 9548 using the QIAzol plus RNeasy Mini Kit (QIAGEN) and reverse transcribed to cDNA using oligo dT primers and the AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies). RNA integrity was checked on an Agilent 2100 Bioanalyzer and only samples with an RNA integrity number >7.0 were used. Quantitative RT-PCR was performed on a StepOneTM Real-Time PCR system (ThermoFischer Scientific). We used inventoried TaqMan expression assays for total C9orf72 (MIM: 614260, Hs00376619_m1) and three housekeeping genes including HPRT1 (MIM: 308000; Hs99999909_m1), UBC (MIM: 191340; Hs00824723_m1) and B2M (MIM: 109700; Hs99999907_m1) (Applied Biosystems) as previously reported [1, 2]. Samples were run in triplicate. Relative quantification was calculated with the ddCt method after normalization to the corresponding reference genes using the StepOne software V2.3 (ThermoFischer Scientific). Gene expression levels were normalized to the level of C9orf72 expression in corresponding tissues of 9548. C9orf72 expression in blood was assessed for 9686, 9548 and 9697 PED25 family members and normalized to the expression level of carrier of wild-type alleles (9697).

Western blotting was done as previously described [3]. Approximately 100mg of frontal cortex and cerebellar tissue was homogenized using a Tenbroek Tissue Homogenizer in 2.5ml of High Salt-Triton (HST) buffer (50mM Tris pH 7.5, 750mM NaCl, 5mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100). Homogenates were centrifuged at 20,000g for 20 minutes at 4°C. Supernatant was saved as HST fraction. Pellets were resuspended in high salt buffer containing 30% sucrose and centrifuged again using the same settings. Following centrifugation, floating myelin and supernatant were removed and the pellet was solubilized with 300µl urea buffer (7M urea, 2M thiourea, 4% CHAPS, 30mM Tris-HCl pH 8.5). Buffers were supplemented with protease inhibitors before homogenization.

Western blotting was done as previously described [3]. Briefly, sample buffer (3X; 187.5mM Tris-HCl pH 6.8, 6% SDS, 30% glycerol, 0.03% bromophenol blue and 15% β-mercaptoethanol) was added to samples before heating at 95°C for 5 minutes. Samples (approximately 40-50µg protein) were electrophoresed on 10% sodium dodecyl sulphate polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% (w/v) skimmed milk powder in Tris-buffered saline (TBS; 50mM Tris-HCl pH 7.6, 150mM NaCl) at room temperature for one hour followed by overnight incubation in primary antibodies (rabbit polyclonal anti-C9-L: 1:3000, in-house, rabbit anti-C9-S: 1:3000 in-house, mouse anti-β-actin: 1:2000 ab8226 Abcam) at 4°C. Following washes with TBS containing 0.05% (w/v) Tween-20, membranes were incubated with anti-mouse/rabbit horseradish peroxidase secondary antibodies (NA931 and NA934, respectively: 1:5000

VWR International Radnor, PA) in blocking solution for one hour at room temperature. Antibody labelling was detected using Western Lightning Plus ECL (PerkinElmer, Waltham, MA) and visualized with a film processor (SRX-101A; Konica Minolta, Japan). Densitometry was performed using ImageJ software (National Institute of Health, Bethesda, MD). Densitometric values for C9-S and C9-L were normalized to β-Actin.

RNA foci analyses

RNA fluorescence in-situ hybridization (FISH) was carried out on 6μm sections of formalin fixed paraffin-embedded CNS and peripheral tissues. Sections were deparaffinized at 60°C for 25 minutes before 2 x 5-minute incubations in xylene, and 5-minute incubations in 50% xylene-50% ethanol, 100% ethanol, 90% ethanol, 70% ethanol, 50% ethanol, RNase-free water, then 20 minutes in phosphate buffered saline (Ambion) with 0.1% (w/v) Triton X-100, and 2X saline-sodium citrate (SSC) buffer (Sigma). For pre-hybridization, slides were incubated in 2X SSC containing 50% (v/v) formamide for 30 minutes at 60°C, followed by incubation for 30 minutes at 60°C with hybridization solution: 50% formamide, 10% (w/v) dextran sulfate salt, 2X SSC buffer, 0.2% (w/v) bovine serum albumin, 2mM vanadyl ribonucleoside complex (Ambion), 1mg/ml tRNA (Sigma) and 1mg/ml single stranded DNA from salmon sperm (Sigma). Hybridization solution with Cy3-conjugated locked nucleic acid probes recognizing either the sense (probe: $CCCCGG_3$) or antisense (probe: $GGGGCC_3$) strand of the expansion (purchased from Exiqon) was denatured for 5 minutes at 85°C before dilution in hybridization solution to 40nM. Slides were incubated with probe overnight at 60°C. Following 3 x 20-minute washes in 2X SSC with 50% (v/v) formamide at 60°C, slides were washed with 2X SSC for 3 x 10 minutes at room temperature. Slides were cover-slipped with ProLong Gold containing DAPI. Z-stack images were acquired (0.5μm per slice) using a Leica DMI6000B fluorescence microscope and Volocity software, using the 63x objective.

For quantification, investigators were blinded and random fields were chosen from each region: cerebellum Purkinje cells and molecular layer (n=10), frontal cortex layers II-VI (n=25), motor cortex layers II-VI (n=25), hippocampal dentate gyrus (n=15), and spinal cord motor neurons were individually identified; the number of RNA foci-positive nuclei was expressed as a percentage of total nuclei. The number of RNA foci per positive neuron was also quantified. Three non-consecutive slides per region, per case were used. For the sense probe, the total number of nuclei (all cell types) counted for each group was: cerebellum >550, frontal cortex >900, motor cortex >1350, hippocampus >2000, cervical spinal cord >29, and lumbar spinal cord >35. For the antisense probe, the total number of nuclei counted for each group was: cerebellum >650, frontal cortex >1200, motor cortex >1600, hippocampus >2000, cervical spinal cord >40, and lumbar spinal cord >40.

Immunohistochemistry analyses of DPRs

Six-micrometer sections of formalin fixed paraffin embedded tissue (cerebellum, frontal cortex, hippocampus, motor cortex, spinal cord, liver) were deparaffinized at 60°C for 25 minutes before being incubated in 2 x 5-minute washes of xylene and rehydrated through a series of graded ethanol and finally in water. Antigen retrieval was carried out using a pressure cooker at 110°C for 15 minutes, with either TE9 buffer (10mM of Trizma base, 1mM EDTA, 0.005% (w/v) Tween-20, pH 9.0) or distilled water. Slides were allowed to cool to room temperature before being rinsed with distilled water for 20 minutes.

For 3,3'-diaminobenzidine (DAB) staining, slides were incubated with 3% (v/v) H_2O_2 for 20 minutes at room temperature to deplete endogenous peroxidases. Following 3 x 5-minute washes in TBS with 0.1% (w/v) Tween-20, slides were blocked for 30 minutes in 2.5% (v/v) normal horse serum. Primary antibodies: mouse anti-GA (1:5000; MABN889, Millipore), rabbit anti-GP (1:100000; NBP2-25018, Novus), rat anti-GR (1:2000; MABN778, Millipore), rabbit anti-PA (1:2500; Rb8604) and rabbit anti-PR (1:100; Rb8736) were diluted in Dako antibody diluent (S0809, Agilent Technologies, Santa Clara, CA) and incubated overnight at 4°C. After 3 x 5-minute washes in TBS with 0.1% Tween-20, slides were incubated in secondary antibody (ImmPRESS peroxidase polymer anti-mouse MP-7402, anti-rabbit MP-7401, anti-rat MP-7444; Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. ImmPACT DAB peroxidase substrate kit (SK-4105, Vector Laboratories) was used to develop staining visualized under a light microscope. Slides were then rinsed with distilled water for 5 minutes before counterstaining with Hematoxylin Solution Gill No. 1 (GHS132, Sigma-Aldrich) for 2 minutes, followed by another 5-minute wash. Slides were dehydrated (5 minute washes in 50% ethanol, 75% ethanol, 95% ethanol, 100% ethanol, 50% ethanol-50% xylene and 100% xylene) and mounted using cytoseal 60 (8310-16, Thermo Fisher Scientific, Waltham, MA). Images were captured using a Leica DM6000B microscope with Volocity software (Perkin Elmer).

To quantify DPR burden, investigators were blinded and random images were taken from each region cerebellum (granule cell layer; n=10), frontal cortex (layers II-VI; n=25), motor cortex (layers II-VI; n=25), hippocampus (dentate gyrus; n=15) using a 40x objective and the average number of inclusions scored per field. In the frontal cortex and motor cortex of 9686, nonspecific nuclear staining was evident with the GA and GR antibodies, so only cytoplasmic inclusions were compared in these regions.

Table e-4. *C9orf72* repeat size in multiple tissues of small expansion carriers according to previous studies. NA represents not available.

Studies[#]	Sample ID	Pheno -type	Age of onset, years	Duration, years	Blood	Frontal cortex	Temporal cortex	Cerebellum	Spinal cord	Occipital	Patietal lobe	Spleen	Heart	Muscle	Kidney	Dura Mater	Cauda Equina	RNA foci	DPR	TDP43
Van Blitterswijk et al. 2013 [10]	P20	MND-FTD	NA	NA	small	large(smear)	Large (smear)	large (dominant)	small/ large(smear)	small/ large(smear)	small/ large	small/ medium	Small	small/ medium						
Fratta et al. 2015 [11]	61776	ALS	57	6	small (90)	small/large		large (dominant)	small/large							small	small	SF and ASF: FC, HC, CBL, HC	HC, CBL	SC
Gijselink et al. 2016 [12]	DR439.1	FTLD	54	15	small (56)	small(weak)/ large(smear)	small(weak)/ large(smear)	large (dominant)											FC, HC, CBL	HC, FC
Nordin et al. 2015 [13]	P9	ALS	63	2	small (61)	small(weak)/ large		large (dominant)	small/large		small/ large	small/ large	small	small	small					
Nordin et al. 2015 [13]	P11	MND	75	5	small (92)	small(weak)/ large		large (dominant)	small(weak)/la rge					small						

[#] Only those studies that had both CNS and non-CNS tissues were included.

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