Supplementary Materials for

Spt4 selectively regulates the expression of *C9orf72* sense and antisense mutant transcripts associated with c9FTD/ALS

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Materials and Methods

Yeast strains and plasmids

Yeast experiments were performed using the wild type haploid S288C-derivative laboratory strain, BY4741 or the wild type halpoid Y7092 strain. Deletion of SPT4 was performed by standard PCR and homologous recombination techniques to replace the ORF with a NatMX antibiotic resistance cassette, creating a null allele, spt4Δ::NatMX (referred to as *spt4Δ*). Clones were selected by growth on Nat-containing plates and further verified by PCR and sequencing. Three independent deletion strains were generated and used for these studies. *C9orf72* hexanucleotide repeats, sense $(GGGGCC)_n$ or antisense $(GGCCCC)_n$, flanked on either end by ~100 nucleotides of human genomic sequence without any upstream ATG start codons, were cloned into galactose inducible yeast expression plasmids ('low copy' = centromeric (CEN), 'high copy' = 2-micron plasmids). Plasmids were introduced into yeast strains using standard lithium acetate transformation. Yeast strains were maintained and cultured using standard selectable media for auxotrophic markers.

Yeast spotting assays

Yeast strains were grown overnight in 2% raffinose-containing media. The following day, cell numbers were normalized using $OD_{600,}$ serially diluted in 96-well plates, spotted onto plates containing either 2% glucose or 2% galactose using a pin frogger, and subsequently grown at 30°C. Pictures were acquired with a standard digital camera after 2-3 days of growth.

Yeast lysate preparation

Overnight yeast cultures grown in raffinose-containing media were diluted into galactose-containing media to allow transgene expression, and were further grown for 18-24 hours with shaking at 30°C. Yeast cells were harvested by centrifugation, resuspended in lysis buffer [50mM Tris pH 8.0, 1% DMSO, 200mM NaCl, 1mM EDTA, 1mM PMSF, 2X Halt Protease Inhibitor Cocktail (ThermoFisher Scientific)], and incubated for 15 minutes on ice. Cell suspensions were lysed by mechanical disruption using acid-washed glass beads and vortexing. Lysates were clarified by centrifugation and soluble lysates were subjected to immunoassays.

Yeast immunoblotting

Protein lysates were quantified using bicinchoninic acid (BCA) assays and equal protein amounts were mixed with 1X NuPAGE LDS sample buffer, denatured for 10 min at 70°C, then subjected to PAGE using 4-12% Bis-Tris gels. Gels were transferred to nitrocellulose membranes using standard semi-dry transfer methods, blocked with Odyssey Blocking Buffer, and probed using the following antibodies: anti-GFP (1:1000, ThermoFisher Scientific A-11122), anti-GAPDH (1:5000, Sigma G8795), and HRP-conjugated secondary antibodies (1:5000).

Yeast RNA fluorescence in situ hybridizations (FISH)

RNA FISH was performed on yeast cells as described (*28, 29*) with modifications using locked-nucleic acid (LNA) probes conjugated to TYE-563 fluorophores (Exiqon): sense GGGGCC probe = 5'-TYE563-CCCCGGCCCCGGCCCC, antisense GGCCCC probe = 5'-TYE563-GGGGCCGGGGCCGGGG. Briefly, yeast cells were harvested from galactose-containing cultures at early log phase and fixed in 4% formaldehyde/10% acetic acid. Cells were washed in potassium sorbitol buffer [1.2M sorbitol, 0.1M potassium phosphate, pH 7.5], then spheroplasted in the same buffer containing 0.1mg oxalyticase, 20mM vanadyl-ribonucleoside complex, 28mM beta-mercaptoethanol, and 0.06mg/ml PMSF for 10 min. at 30°C. Spheroplasts were adhered to poly-l-lysine coated coverslips and incubated overnight in 70% ethanol at -20°C. Following rehydration with potassium sorbitol, cells were hybridized using 100nM sense or antisense LNA probes in hybridization buffer [2X SSC, 50% formamide, 0.02% BSA, 40 µg *E. coli* tRNA] overnight at 37°C. Cells were washed with 0.1X SSC/50% formamide at 50°C, then mounted on coverslips for imaging with Prolong Diamond Antifade Mountant with DAPI. Images were captured using a Leica DMI6000B inverted microscope with an 100X objective. The percentage of yeast cells containing RNA foci was quantified by counting the number of foci-positive cells in >150 cells per coverslip. Multiple coverslips were analyzed across independent experiments (n=4-6 experiments each with >150 cells). Experiments were analyzed using 2 independently generated *spt4Δ* strains.

Yeast qRT-PCR

RNA was extracted from yeast using a MasterPure Yeast RNA Extraction kit (Epicentre), including DNAseI digestions. Equal amounts of RNA were reverse transcribed into cDNA using MultiScribe MuLV reverse transcriptase with random primers, then analyzed by qPCR using custom primer sets and SYBR green reagents. The following primer sets were used (scACT1 Fwd = 5'ATTCTGAGGTTGCTGCTTTGG; scACT1 Rev = 5'TGTCTTGGTCTACCGACGATAG; C9repeat Fwd = 5'AGCTTAGTACTCGCTGAGGGTG; C9repeat Rev = 5'GACTCCTGAGTTCCAGAGCTTG). Control samples without reverse transcriptase were analyzed to rule out contaminating plasmid DNA. Samples without reverse transcriptase (–RT) yielded Ct values on average 14 cycles higher than +RT Ct values, indicating little to no DNA contamination.

Transgenic *C. elegans* strain construction and maintenance

Maintenance and growth of worms was performed as described in (*30*) and all strains were raised at 20°C. All transgenic strains used in this study were created by gonad microinjection of plasmid DNA paired with an independent transformation-marker plasmid followed by integration of DNA array. For clarity, we have used the convention that transgenes generated by transcriptional fusions to *C. elegans* promoters are identified with a '/' (e.g. *snb-1*/SUPT4H1 or *snb-1*/$(G_4C_2)_{66}$). Marker plasmids used were: pCL26 (mtl-2/GFP, constitutive intestinal GFP) and pLen1.8 (mtl-2/mCherry, constitutive

intestinal mCherry). Transgenic worm lines were produced by integration using worms that show heritable extrachromosomal multicopy arrays, containing copies of the experimental and marker plasmids.

C. elegans immunoblotting and FISH

Worm lysates were prepared as described in (*9*). Twenty micrograms of total protein were loaded per lane, and the resultant blots were probed with primary antibodies to detect SUPT4H1 (1:1,000, Cell Signaling) or tubulin (1:4,000, Sigma). Blots were probed with secondary HRP-conjugated antibodies (1:5,000, Jackson) and developed in ECL Plus (Amersham). For FISH, the worms were washed off the plate in S. basal and washed twice. After the final wash the worms were fixed in 4% paraformaldehyde (Electron Mycroscopy Sciences) overnight at 4°C. After fixation the worms were permeabilized in 70% ethanol overnight at 4°C, then washed through a series of ethanol rinses (70%, 90% and 100%). The worms were then hybridized with denatured Cy3-conjugated $(GGCCCC)_4$ probe (2 ng/µl) in hybridization buffer (50% formamide, 10% dextran sulfate, 2xSSC, 50mM sodium phosphate) overnight at 37°C. The following day, worms were washed twice with 2xSSC for 30 min at 37°C, and the nuclei subsequently labeled with 1ug/ul Hoechst diluted in 4xSSC with 0.1% Triton-100x for 10 minutes. After two final washes in 2xSSC for 5 minutes each, worms were mounted on a 2% agarose pad and visualized with Zeiss Axioimager microscope equipped with ApoTome system. Image brightness and contrast were digitally adjusted in Photoshop. To confirm the specificity of the FISH probe for RNA, permeabilized worms were incubated with either RNase A (Sigma) and RNase H (Invitrogen) for 1 hour at 37°C in PBS. RNase-treated worms were washed and probed with Cy3-conjugated $(GGCCCC)_4$ as described above.

RNAi-mediated gene knockdown in *C. elegans*

For RNAi gene knockdown experiments, we applied *E. coli* HT115 to the worms as previously described (*31*). The Spt4 clone was derived from Ahringer library (Source BioScience, Nottingham, UK). Bacteria were spotted on NGM plates containing 1 mM isopropyl-β-D-thiogalactoside and 100 µg ml−1 Carbenicillin. After plates were poured and dried, they were sealed and stored at 4°C. Freshly prepared bacteria were spotted on plates on the previous evening and allowed to dry and settle overnight.

Lifespan analysis of *C. elegans*

Lifespan analysis was performed as described previously (*32*). RNAi treatments were performed continuously from the time of hatching. Eggs were placed on plates seeded with the RNAi bacteria of interest. As a result of variability between experiments, related lifespans were performed concurrently or in overlapping time frames. In all experiments, the prefertile period of adulthood was used as t=0 for lifespan analysis. Animals that ruptured, bagged (i.e., exhibited internal progeny hatching), or crawled off the plates were censored but included in the lifespan analysis as censored worms. GraphPad Prism 6 software was used for statistical analysis and to determine means. In all cases, P values were calculated using the log-rank (i.e., Mantel-Cox) method.

Drosophila lines

Animals were raised and maintained at 25°C on standard fly media. The UAS-(GGGGCC)n transgene is as described ((*33*)). Repeat lengths in individual lines were determined by PCR and subsequent product length analysis using an Agilent bioanalyzer. Animals expressing 6, 29 and 49 GGGGCC repeats were matched for expression level by northern blots. Control (luciferase) shRNA and spt4 shRNA stocks were obtained from the Bloomington *Drosophila* Stock Center.

External and internal retinal analysis in *Drosophila*

1-2d animals were imaged on a Leica Apo16 microscope for external eye analysis. For quantification, a scale was developed for GGGGCC flies with varying levels of an external eye phenotype (see Supplemental Figure). 2-10 animals were quantified/time point. All results have been seen at least three times. For internal sections, heads from 1-2d animals were fixed in Bouin's solution for 6d, then processed and embedded into paraffin for sectioning (8um thick) as described ((*34*)). The tissue depth at the optic chiasm was measured for individual animals. 5-10 animals were used for internal quantification. All results have been seen at least three times. Student t-tests were used to determine significant.

β-galactosidase western blots

Western immunoblot analysis was performed on heads of 3d animals. Tissue was homogenized in 1X NuPAGE LDS sample buffer using a plastic mortar and pestle. Western immunoblots were run by standard protocols, while transfers were done with an Invitrogen iBlot Dry blotting system. Anti-β-galactosidase (Promega #Z3781) and anti-α-tubulin antibodies (DSHB #AA4.3) were used at 1:2000. Anti-mouse-HRP secondary was used at 1:5000. Imaging was performed using an Amersham Imager 600 after incubating immunoblots with Amersham ECL Prime Western Blotting Detection Reagent for 2min. For each sample, 6-12 animals were used, and the assay was performed three times independently.

Lifespan and spt4 qRT-PCR in *Drosophila*

Fly lifespans were done as previously described with some minor changes ((*35*)). Crosses were setup using the daughterless-geneswitch (da-GS) ubiquitous, drug-inducible driver. 2d females were transferred onto vials containing RU486 (40μg/ml). Animals were raised and maintained at 25°C. Log-ranked tests was used to determine significance. For qRT-PCR analysis, RNA was collected from animals on RU486 for 5dys and analyzed for levels of spt4 present in heads only. For lifespan analysis, 202-220 animals were used per genotype.

Fibroblast cell culture and treatments

Human fibroblasts were derived from skin biopsy and were generated by ReGen Theranostics Inc (Rochester, MN) as previously described (*36*). Skin biopsies were obtained from twenty-six individuals, which included eight non-ALS controls, ten *C9orf72*-negative ALS patients, and eight *C9orf72*-positive ALS patients. Information on human fibroblast cells is provided in Supplementary Table 1. Human fibroblast cells were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% FBS and 1% penicillin-streptomycin. To examine the expression of poly(GP) proteins and measure *C9orf72* repeat expansion length*,* cells were grown in a T150 flask to 90% confluency and then harvested. The cell pellet was divided in two for protein and DNA extraction. For the siRNA experiments, fibroblast cells were seeded at a density of 1.2×10^6 cells/10 cm dish or 5×10^4 cells/coverslip in 24-well plates. Two days after seeding, the cells were transfected with a negative control siRNA (DHARMACON, D-001206-13-05), or siRNAs towards SUPT4H1 (DHARMACON, L-012602-00-0005) and/or SUPT5H (DHARMACON, L-016234-00-0005) at a final concentration of 20 nM using Lipofectamine® RNAiMAX Transfection Reagent (Thermo Fisher Scientific, 13778150) according to manufacturer's standard protocol. C9-ASO was directly added into media at final concentration of 5mM. Five days after transfection, the cells were re-treated with siRNA or C9-ASO for an additional five days as described above. Then, lysates were prepared from cells grown in the 10 cm dishes for immunoblot, immunoassay or quantitative real-time PCR analysis. Cells grown on coverslips in 24-well plates were fixed with 4% paraformaldehyde in DEPC-PBS for RNA fluorescence in situ hybridization (FISH) analysis. Toxicity of siRNA treatments was monitored by live cell counts (Scepter 2.0 handheld automated cell counter, EMD Millipore), or lactose dehydrogenase (LDH) assay (G1781, Promega).

Preparation of fibroblast cell lysates

Cell pellets were lysed in co-immunoprecipitation (co-IP) buffer (50mM Tris–HCl, pH 7.4, 300mM NaCl, 1% Triton X-100, 5mM EDTA) plus 2% sodium dodecyl sulfate (SDS) and both protease and phosphatase inhibitors. Lysates were sonicated on ice, and then centrifuged at 16,000g for 20 min. Supernatants were saved as cell lysates and protein concentration was determined by BCA assay (Thermo Scientific).

Immunoblot analysis of fibroblast lysates

Immunoblot analysis was performed as previously described (*4*). In brief, cell lysates were heated in Laemmli's buffer, and equal amounts of protein were loaded into 4–20% Tris-glycine gels (Novex). After transferring, membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Triton X-100 (TBST) for 1 h, then incubated with a rabbit polyclonal SUPT4H1 antibody (Cell signaling, #64828, 1:1,000), rabbit polyclonal SUPT5H (Santa Cruz Biotechnology, sc-28678, 1:500,), or mouse monoclonal GAPDH antibody (Meridian Life Science, H86504M, 1:10,000) overnight at 4°C. After incubating with donkey anti-rabbit or anti-mouse IgG antibodies conjugated to horseradish peroxidase (1:5,000; Jackson ImmunoResearch) for 1 h, 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.

Fibroblast DNA extraction and Southern blot analysis

Genomic DNA was extracted from fibroblast cells using Wizard® Genomic DNA Purification Kit (Promega, A1620) according to the manufacturer's standard protocol, and then subjected to Southern blotting as previously described (*37*). In brief, 10 μg of genomic DNA was digested with XbaI and separated by electrophoresis for 6 h at 100 Volts in a 0.8% agarose gel. DNA was transferred to a positively charged nylon membrane (Roche) and cross-linked by UV irradiation. Following prehybridization in digoxigenin (DIG) EasyHyb solution (Roche), hybridization was performed with a DIG-labeled probe. To detect the probe, an anti-DIG antibody (Roche, 1:10,000) was used, which was visualized with CDP-star substrate (Roche) on X-ray film.

Fibroblast RNA extraction and quantitative real-time PCR (qPCR).

Total RNA was extracted from fibroblast cells using the Direct-zol™ RNA MiniPrep kit (Zymo Research) according to the manufacturer's instructions, combined with an in-column DNase I digestion step. Then, 2 μg of total RNA was used for reverse transcription to synthesize cDNA using random primers and the High Capacity cDNA Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. To quantify mRNA levels of *SUPT4H1, SUPT5H, total C9orf72 or C9orf72* variants, qPCR was conducted in triplicate for all samples using a SYBR green assay (Life Technologies) on an ABI Prism 7900HT Fast Real-Time PCR System (Applied Biosystems). The primers used were: *SUPT4H1*: 5′-GCGGTGTCAGTCACTGGTC-3′ and 5'-CCACTCCTCGACTTTTCAGC-3'; *SUPT5H*: 5'- GTATGAGGACGAGGACCAGTG-3' and 5'-GAACGATCTTCATCCAGGACA-3'; *total C9orf72*: 5'-AACTGGAATGGGGATCGCA-3' and 5'-CTGATCTTCCATTCTCTCTGTGCC-3'; *C9orf72* variant 1: 5'-CCACGTAAAAGATGACGCTTGATA-3' and 5'-TGGGCAAAGAGTCGACATCA-3'; *C9orf72* variant 2: 5'-CGGTGGCGAGTGGATATCTC-3' and 5'-TGGGCAAAGAGTCGACATCA-3'; *C9orf72* variant 3: 5'-GCAAGAGCAGGTGTGGGTTT-3' and 5'-TGGGCAAAGAGTCGACATCA-3'; *GUSB*: 5′-CGCCCTGCCTATCTGTATTC-3′ and 5′-TCCCCACAGGGAGTGTGTAG-3. *RPLP0*: 5′-TCTACAACCCTGAAGTGCTTGAT-3′ and 5′-CAATCTGCAGACAGACACTGG-3′. Relative mRNA expression of examined genes was normalized to *GUSB* or *RPLP0* values, the endogenous transcript controls.

Human fibroblast RNA-sequencing

Primary fibroblast cells from 2 independent population control lines (Coriell Institute, ND38530 and ND29510) were cultured and treated with SUPT4H1 siRNAs for 10 days, as described above. Total RNA was extracted using Ambion PureLink RNA Mini kits, and knockdown of SUPT4H1 mRNA levels was confirmed by qRT-PCR prior to RNA-sequencing analysis. RNA integrity was evaluated using Agilent's 2100

Bioanalyzer, Eukaryote Total RNA Nano assay. mRNA library preparation and sequencing was performed by Centrillion Technologies (Palo Alto, CA). Briefly, mRNA was enriched using polyA selection, and sequencing was performed using an Illumina Hiseq2500, 2x100 paired end reads. Alignment against UCSC hg19 was performed using TopHat 2.1., while differential gene and transcript expression was analyzed using Cufflink 2.2.1 (*38*). Graphs were generated using the R package 'cummeRbund' (http://compbio.mit.edu/cummeRbund).

RNA fluorescence in situ hybridization (FISH) and quantification of RNA foci in fibroblasts

RNA FISH was performed as previously reported (*14*). In brief, fixed fibroblast cells grown on coverslips were permeabilized with 0.2% Triton/DEPC-PBS and then dehydrated with a series of ethanol washes using 70%, 70% and 100% ethanol (1 min per wash). Cells were then incubated with hybridization solution for 30 min at 66°C. In the meantime, LNA probes to detect sense (/5TYE563/CCCCGGCCCGGCCCC) or antisense (/5TYE563/GGGGCCGGGGCCGGGG) *C9orf72* repeats were heated at 80°C for 75 sec, and then diluted to 40 nM with hybridization buffer. After hybridization, the cells were incubated with the diluted LNA probes at 66°C for 24 h. Cells were then washed once with 2× SSC/0.1% Tween-20 for 5 min at room temperature and 3 times with 0.1× SSC for 10 min per wash at 65°C, followed by counterstaining with Hoechst 33258 (1 μg/ml, Thermo Fisher Scientific). Afterward, the cells were dehydrated as described above and coverslips were mounted. Images were obtained on a Zeiss LSM700 laser scanning confocal microscope. To quantify foci-bearing cells, ~200 total cells per coverslip and 3–4 coverslips per treatment were used for counting the number of foci-positive cells. These counts were used to determine the average percentage of foci-positive cells.

Evaluation of associations between SUPT4H1 or SUPT5H and poly(GP) or C9orf72 variant 3 in cerebellar tissue from patients with C9orf72 repeat expansions

For studies involving human tissues, written informed consent was obtained from all participants or their legal next of kin if they were unable to give written consent, and biological samples were obtained with ethics committee approval. Through the Mayo Clinic Jacksonville brain bank, frozen cerebellar tissues were obtained from 59 cases previously identified as *C9orf72* expansion carriers by repeat-primed PCR and immunostaining for poly(GP) pathology.

For poly(GP) analyses, brain homogenates were prepared as previously described (*11, 36*). In brief, approximately 50 mg of cerebellar tissue was homogenized in cold RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, protease and phosphatase inhibitors) and sonicated on ice. Homogenates were cleared by centrifugation at 100,000 g for 30 min at 4°C. The supernatant was collected and the concentration was determined by BCA assay. Poly(GP) levels in lysates were determined using the poly(GP) immunoassay described below.

For measurements of *SUPT4H1*, *SUPT5H*, and *C9orf72* variant 3 mRNA, total RNA was extracted as previously described (*39*) and 500 ng of RNA with RNA integrity values higher than 7, measured in an Agilent Bioanalyzer, was used for reverse transcription to synthesize cDNA, as described above for patient fibroblasts. Quantification of mRNA levels of *SUPT4H1, SUPT5H*, and *C9orf72* variant 3 was conducted as described for the patient fibroblasts, and relative mRNA expression of examined genes was normalized to *GAPDH* and *RPLP0* values, the endogenous transcript controls. Associations between *SUPT4H1* or *SUPT5H* and *C9orf72* variant 3 or poly(GP) were evaluated using a Spearman's test of correlation.

Poly(GP) immunoassays

Poly(GP) levels in lysates were measured using a previously described sandwich immunoassay that utilizes Meso Scale Discovery (MSD) electrochemiluminescence detection technology (*11, 36*). Lysates were diluted to the same concentration using Tris-buffered saline (TBS) and tested in duplicate wells. Serial dilutions of recombinant $(GP)_8$ in TBS were used to prepare the standard curve. Response values corresponding to the intensity of emitted light upon electrochemical stimulation of the assay plate using the MSD QUICKPLEX SQ120 were acquired and used to interpolate poly(GP) levels using the standard curve.

Differentiation of iPSC-derived neurons and treatments

Four iPSC lines derived from 3 patients with *C9ORF72* repeat expansions were used in this study. Patient 1 (Carrier 1 in Almeida et al., 2013) as well as Patient 2 (29iC9-ALS in ref. 14) and Patient 3 (30iC9-ALS in ref. 14) have been previously described. Neuronal differentiation into cortical neurons was performed as described in (*13*) with the addition of kinase inhibitors and a sonic hedgehog antagonist as reported in (*40*). In these cultures, most neurons are $VGluT1^+$ excitatory neurons (*40*). Briefly, iPSC colonies were lifted after incubation with 1:2 accutase/PBS and grown as embryoid bodies (EBs) in suspension for 4 days in basic fibroblast growth factor-free iPSC medium containing dorsomorphin and A-83. EBs were cultured for two additional days in neuronal induction medium supplemented with cyclopamine and then allowed to attach and form neural tube-like rosettes. Fifteen-day-old rosettes were collected and grown in suspension as neurospheres. Neurospheres were dissociated after 3 weeks, and the cells were seeded on plates coated with poly-D-lysine and laminin. Half of the medium was replaced twice a week for 8 weeks to mature the neurons. After 8 weeks, neurons were transduced with lentiviruses expressing a shRNA against *SUPT4H1* or a non-coding control shRNA (Dharmacon). Six days later, neurons were collected for RNA extraction and GP levels measurements. Experiments were performed in neuron cultures from three independent differentiations.

iPSC-neuron RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was isolated using the RNeasy Kit (Qiagen) and 500 ng of RNA were reverse transcribed using random primers and the TaqMan Reverse Transcription

Reagents kit (Applied Biosystems), following the manufacturer's instructions. Quantitative PCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems) using SYBR Select Master Mix (Applied Biosystems) and the following forward and reverse primers: *SUPT4H1* forward – 5'-TTGCGATGATGAGTCCAGAG and reverse – 5'-TGGATTTGTAGGCCACTCCT, *C9ORF72* variant 3 forward- 5' -GGGTCTAGCAAGAGCAGGTG and reverse – 5'-AGCCCAAATGTGCCTTACTC, *PPIA* (cyclophilin A) forward-5' -TGCCATCGCCAAGGAGTAG and reverse -5'-TGCACAGACGGTCACTCAAA. Ct values for each sample and gene were normalized to *PPIA* gene. The 2exp (-ΔΔCt) method was used to determine the relative mRNA expression of each gene.

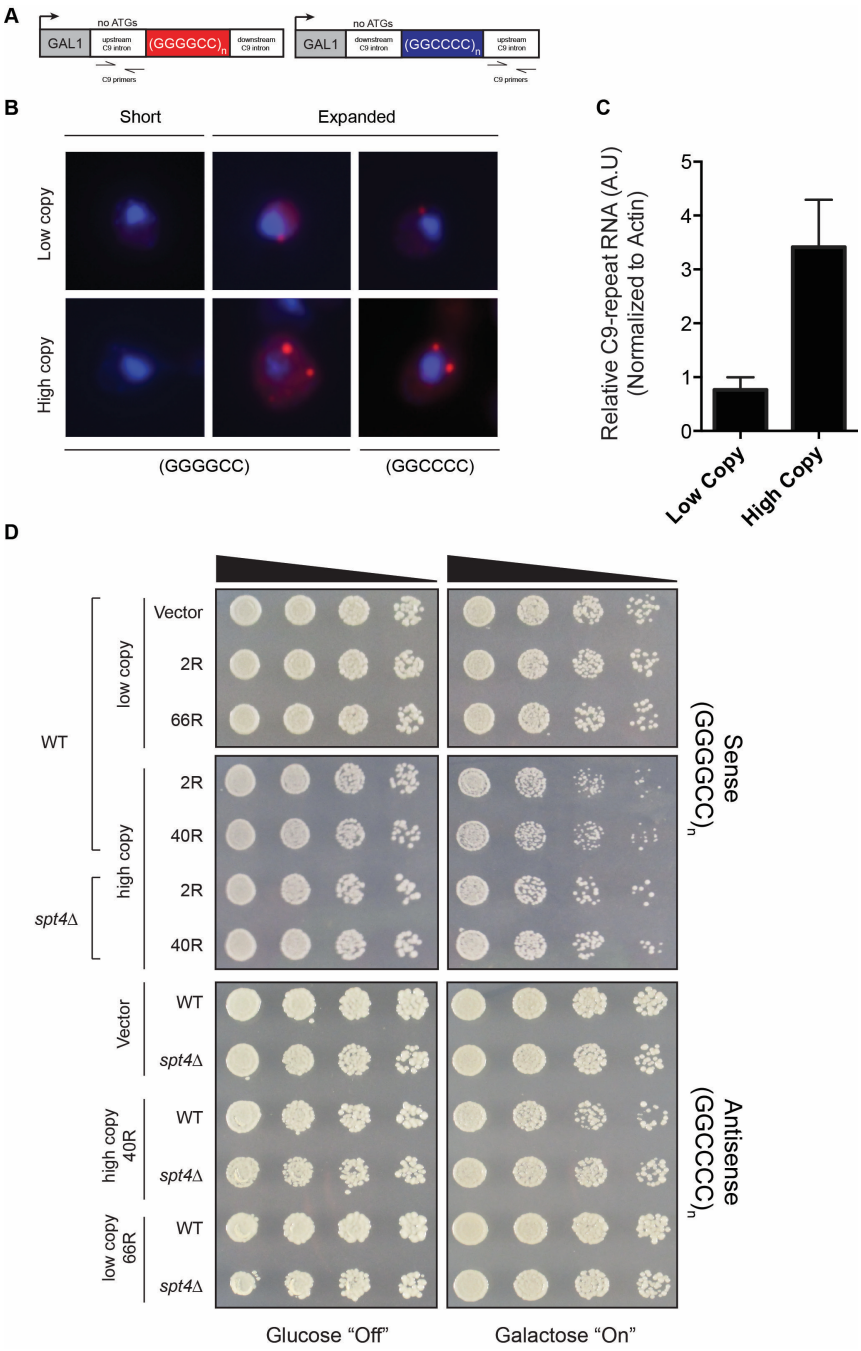


Fig. S1.

(A) Constructs used for expression of *C9orf72* sense GGGGCC and antisense GGCCCC repeats in yeast. (B) RNA foci observed by RNA FISH analysis in yeast expressing expanded (40R or 66R) but not short (2R) GGGGCC or GGCCCC repeats from either low-copy (CEN) or high-copy (2-micron) plasmids. (C) qRT-PCR comparison of C9-repeat RNA levels in yeast transformed with low-copy and high-copy plasmids expressing GGGGCC repeats. (D) Serial dilution growth analysis of yeast strains [wild type (WT) or *spt4Δ*] transformed with galactose inducible $(GGGGCC)_n$ or $(GGCCCC)_n$ expression constructs (n = 2, 40 or 66 repeats in either low-copy or high-copy plasmids). Glucose containing media='plasmid off', galatose containing media='plasmid on'.

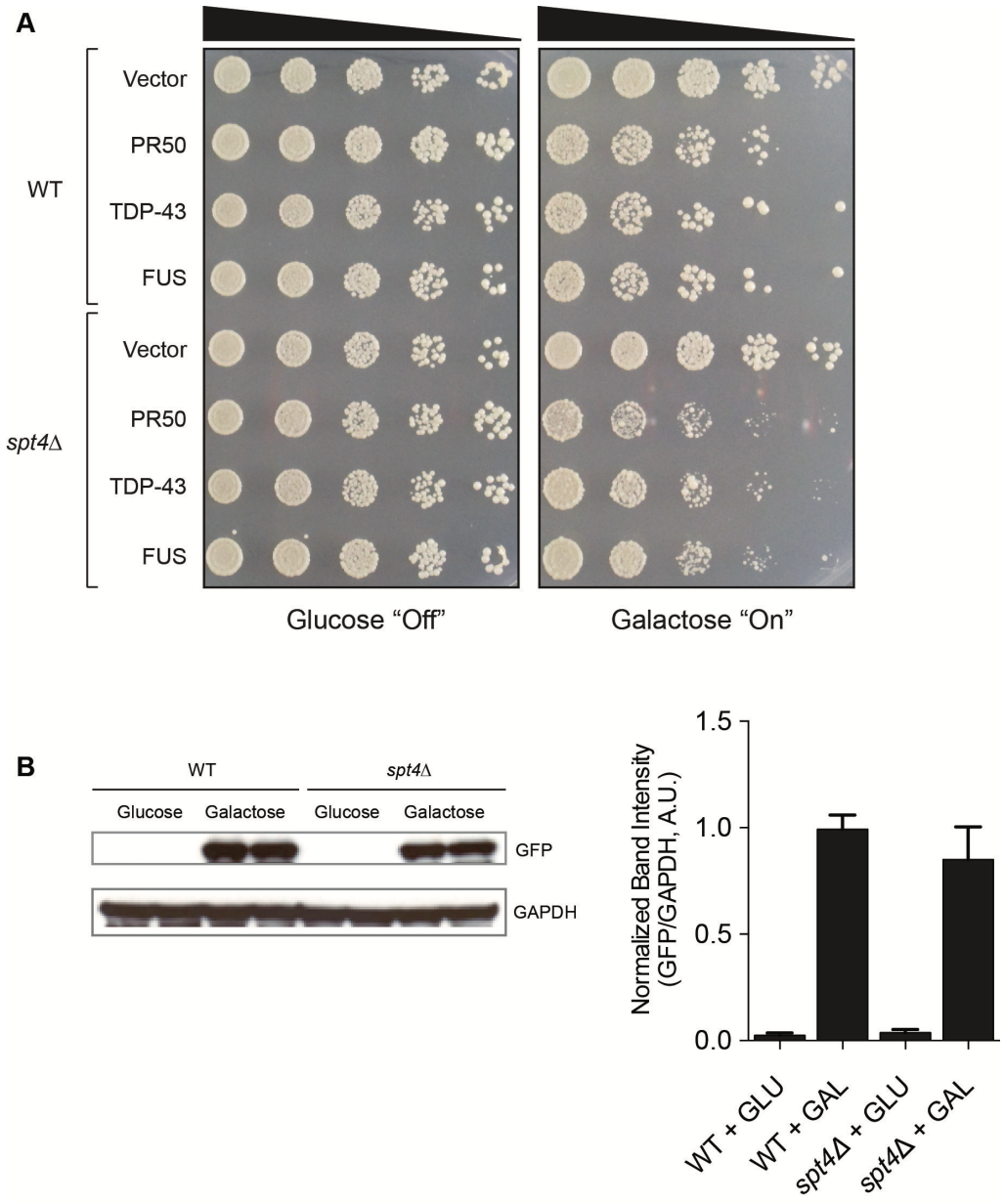
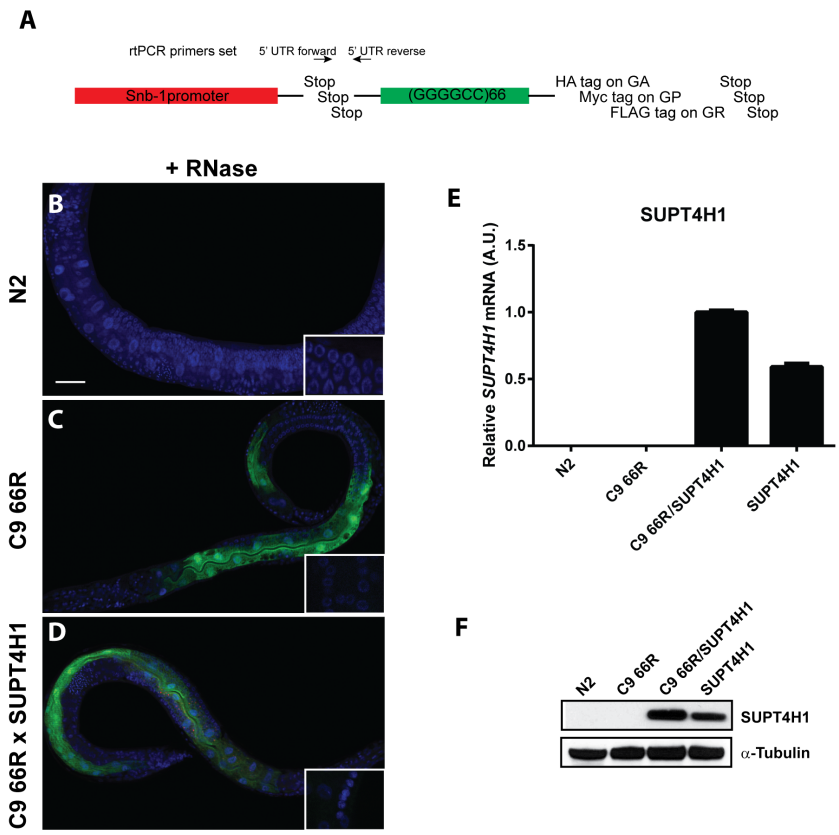


Fig. S2.

Specificity of Spt4 deletion in yeast to *C9orf72* repeats. (A) Serial dilution growth analysis of yeast transformed with codon optimized, interrupted *C9orf72* polyProline-Arginine (PR50, no-repeat context), TDP-43, and FUS expressing plasmids (galactose-inducible). *spt4Δ* does not modify these other disease-associated toxicity models in yeast. (B) *spt4Δ* does not significantly affect the expression of a control galactose-inducible plasmid expressing GFP. Western immunoblot detection and densitometric quantification of GFP and endogenous yeast GAPDH proteins in yeast cultures grown in glucose (plasmid repressed) or galactose (plasmid induced) for 24hr.



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Strain		Median Survival	Log-rank (Mantel-Cox) test P value
N2		16	p<0.0001§
SUPT4H1		17	n.s. # ; p<0.0001§
C966R		7	p<0.0001#
C9 66R/SUPT4H1		5	p<0.0001§,#
Len66.1R	OP50	9	p<0.0001¶
	RNAi *Spt4*	15	

Compared with N2 wild type animals
§ Compared with C9 66rp animals
¶ Compared with Len66.1rp animals subjected to control RNAi.

Fig. S3.

(A) Schematic diagram of the construct utilized to generate $(GGGGCC)_{66}$-expressing worms, which includes a stop codon in each frame following the promoter sequence, and a different tag to measure DPR protein production from each frame [i.e., HA tag for poly(GA), myc tag for poly(GP), and FLAG tag for poly(GR)]. (B-D) Worms were treated with RNase to confirm specificity of the FISH probe for GGGGCC repeat containing RNA. (E-F) Human SUPTH41 mRNA levels were measured by qPCR (E) and SUPTH41 protein levels were measured by immunoblot (F). (G) Lifespan comparison among different worm strains (median survival reported in days). n = 145 (N2 worms), n = 160 (C9 66R), n = 144 (SUPT4H1), n = 122 (C9 66R/SUPT4H1), n = 119 (OP50), n = 120 (RNAi Spt4). p-values were calculated using the log-rank test, as described in Methods.

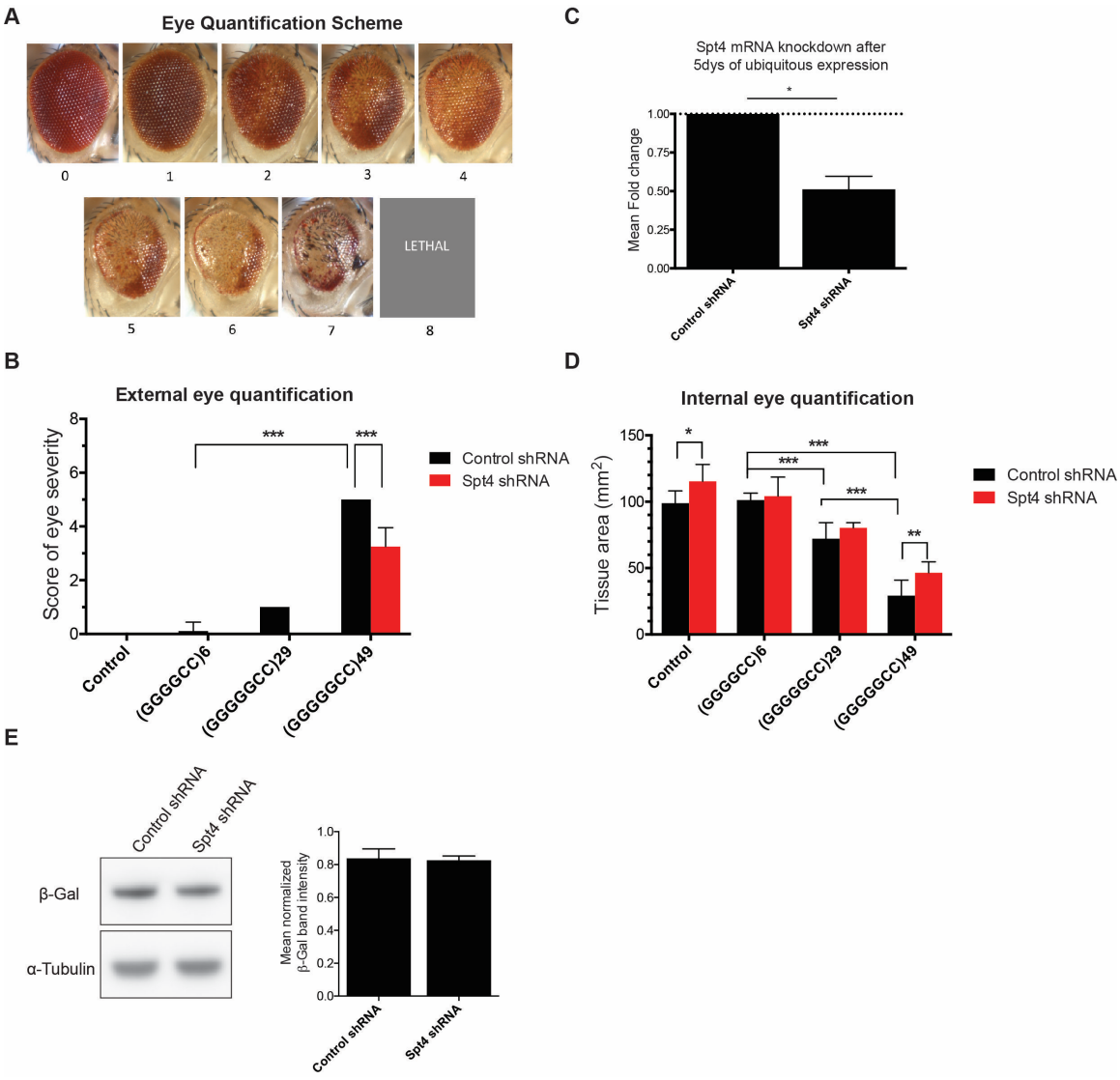

Fig. S4.

Quantification of the suppressive effects of reduced Spt4 expression in the eye of $(GGGGCC)_{49}$ *Drosophila*. (A) Analysis scheme for the level of neurodegeneration of the external fly eye, which was performed on imaged samples blinded to the experimenter. A score of 0 is given to animals with a normal eye, while a score of 8 is given to animals with extreme phenotypes (ie. lethality). (B) $(GGGGCC)_{49}$ control samples tend to get a score of 4-5 on this scale, and the phenotypes are summarized across genotypes. (C) Knockdown of Spt4 mRNA from fly heads was confirmed by qPCR. (D) By assessing the tissue are of the internal eye at the optic chiasm, the suppressive effects of reducing Spt4 can be seen in $(GGGGCC)_{49}$ animals. (E) The *gmr-GAL4* eye specific driver was used to express a control β-galactosidase transgene. Western immunoblot analysis of head tissue shows that expression of spt4 shRNA has no effect on the level of β-galactosidase compared to animals expressing a control luciferase shRNA. The mean band densities for triplicate samples from three independent assays were averaged after normalization to α–tubulin loading control. Error bars denote standard error of the mean.

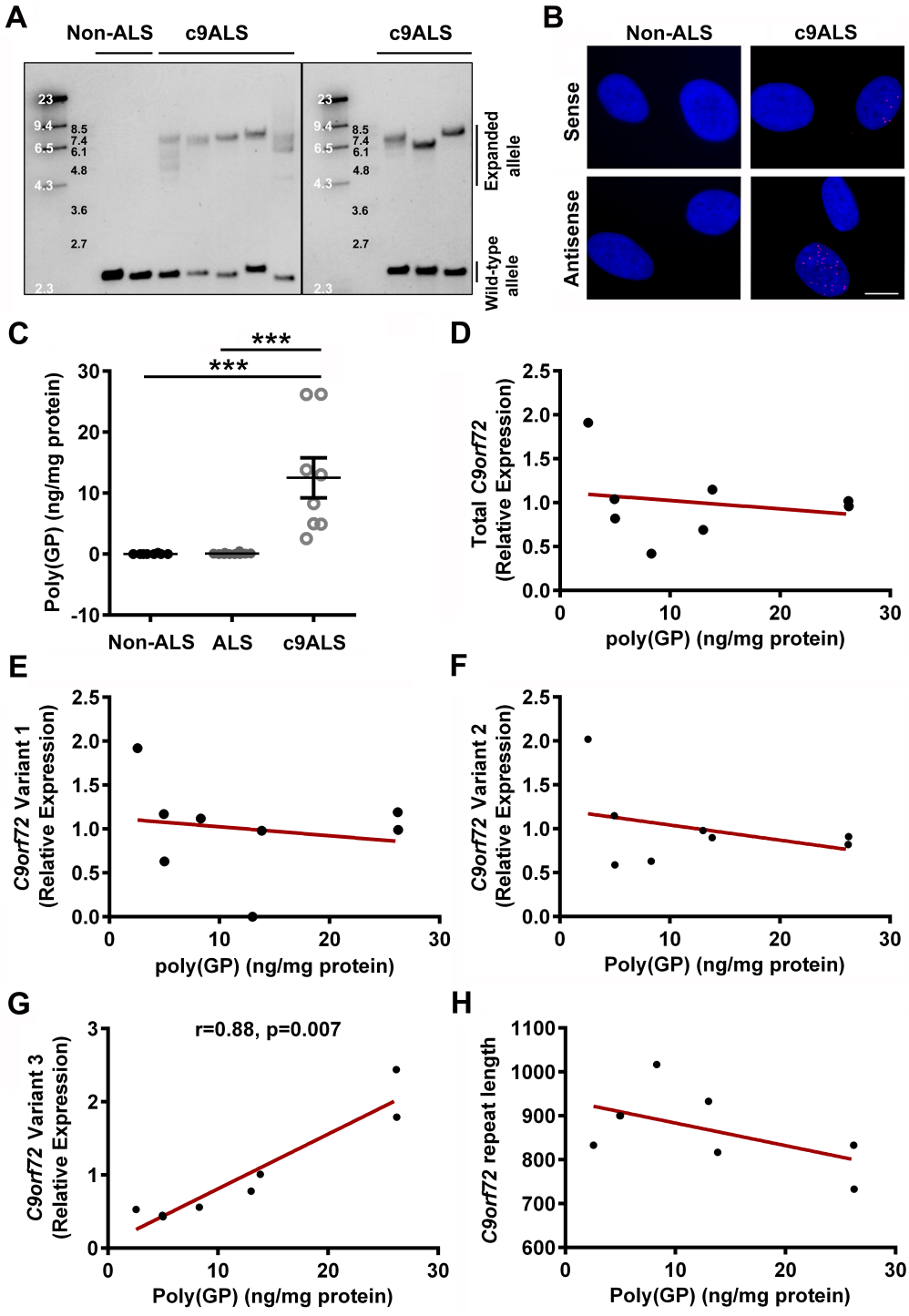

Fig. S5

Fibroblasts from c9ALS patients recapitulate pathological hallmarks of disease. (A) Southern blot analysis shows expanded GGGGCC repeat alleles in cultured fibroblasts from patients with c9ALS. (B) RNA FISH using probes for GGGGCC or GGCCCC RNA detected sense and antisense repeat-containing foci (red) in the nucleus (blue, Hoechst 33258) of cultured fibroblasts from patients with *C9orf72* repeat expansions but

not in individuals without an expansion. (C) Poly(GP) DPR proteins are detected in fibroblasts from eight c9ALS patients but not from ten *C9orf72*-negative ALS patients or eight individuals without ALS. *** $p < 0.001$, as assessed by one way ANOVA followed by Tukey's post-hoc analysis. (D-H) The concentration of poly(GP) in c9ALS fibroblasts significantly associates with *C9orf72* variant 3 mRNA levels measured by qPCR (G) but not with total *C9orf72* (D), *C9orf72* variant 1 (E) or *C9orf72* variant 2 (F), nor with repeat length (H), as assessed by Spearman's test of correlation.

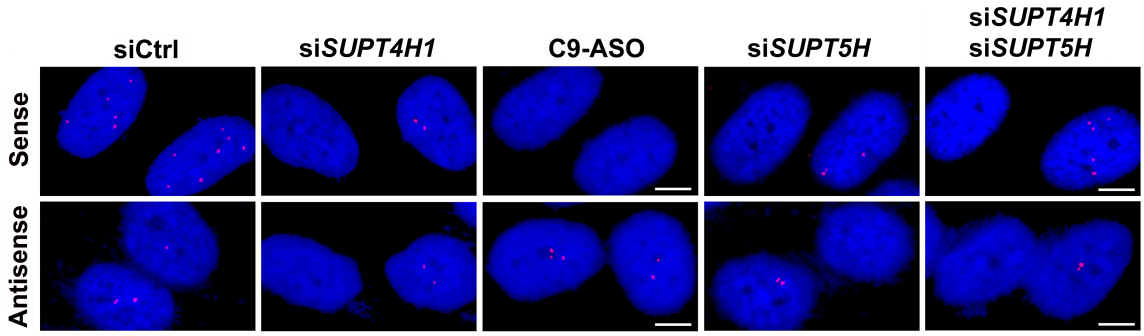

Fig. S6

Decreased sense and antisense RNA foci in c9ALS fibroblast cells following SUPT4H1 or SUPT5H depletion alone or in combination. Cultured fibroblasts from c9ALS patients were treated with a control siRNA (siCtrl) or siRNA towards *SUPT4H1*, *SUPT5H*, or both *SUPT4H1* and *SUPT5H*, or treated with a *C9orf72*-repeat targeting ASO (C9-ASO) for 10 days. Post-treatment, RNA fluorescence in situ hybridization with probes for GGGGCC or GGCCCC RNA was performed to detect foci containing sense or antisense repeats (red) in the nucleus of cells (blue, Hoechst 33258). Shown are representative images. This figure is related to Figure 3.

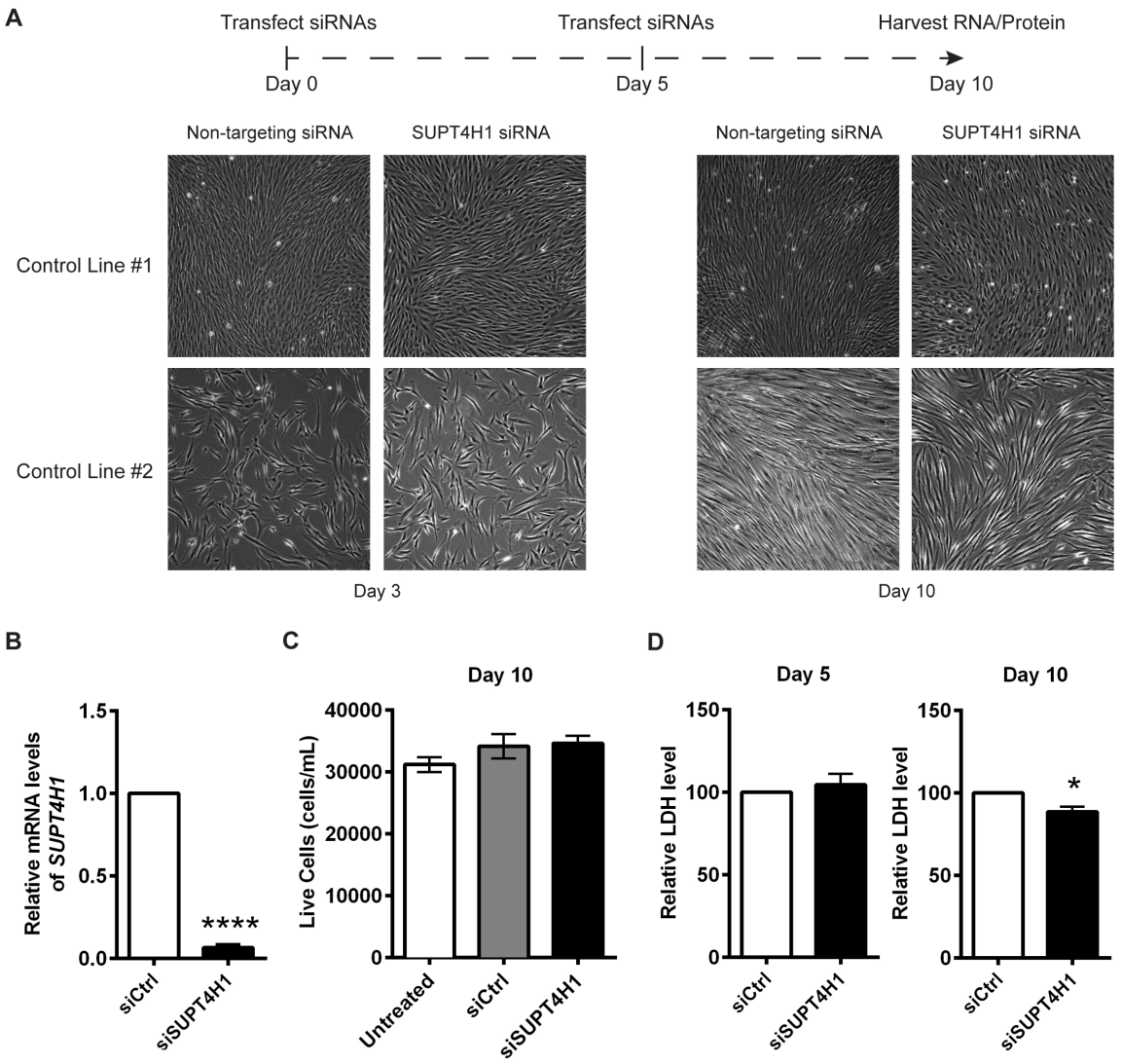

Fig. S7

Assessment of toxicity associated with SUPT4H1 siRNA treatment in human fibroblasts. (A) Schematic timeline of siRNA treatments in fibroblasts, two siRNA transfections were performed over the course of 10days in culture, and representative micrographs were taken throughout the experiment to monitor cell health and viability. (B) Knockdown of SUPT4H1 mRNA was confirmed by qRT-PCR in four fibroblast lines, and (C) live cells were counted on day 10 using a handheld automated cell counter. (D) A lactose dehydrogenase (LDH) assay was used as an independent readout of cell death. Reduction of SUPT4H1 mRNA levels by ~90% had little to no effect on fibroblast viability.

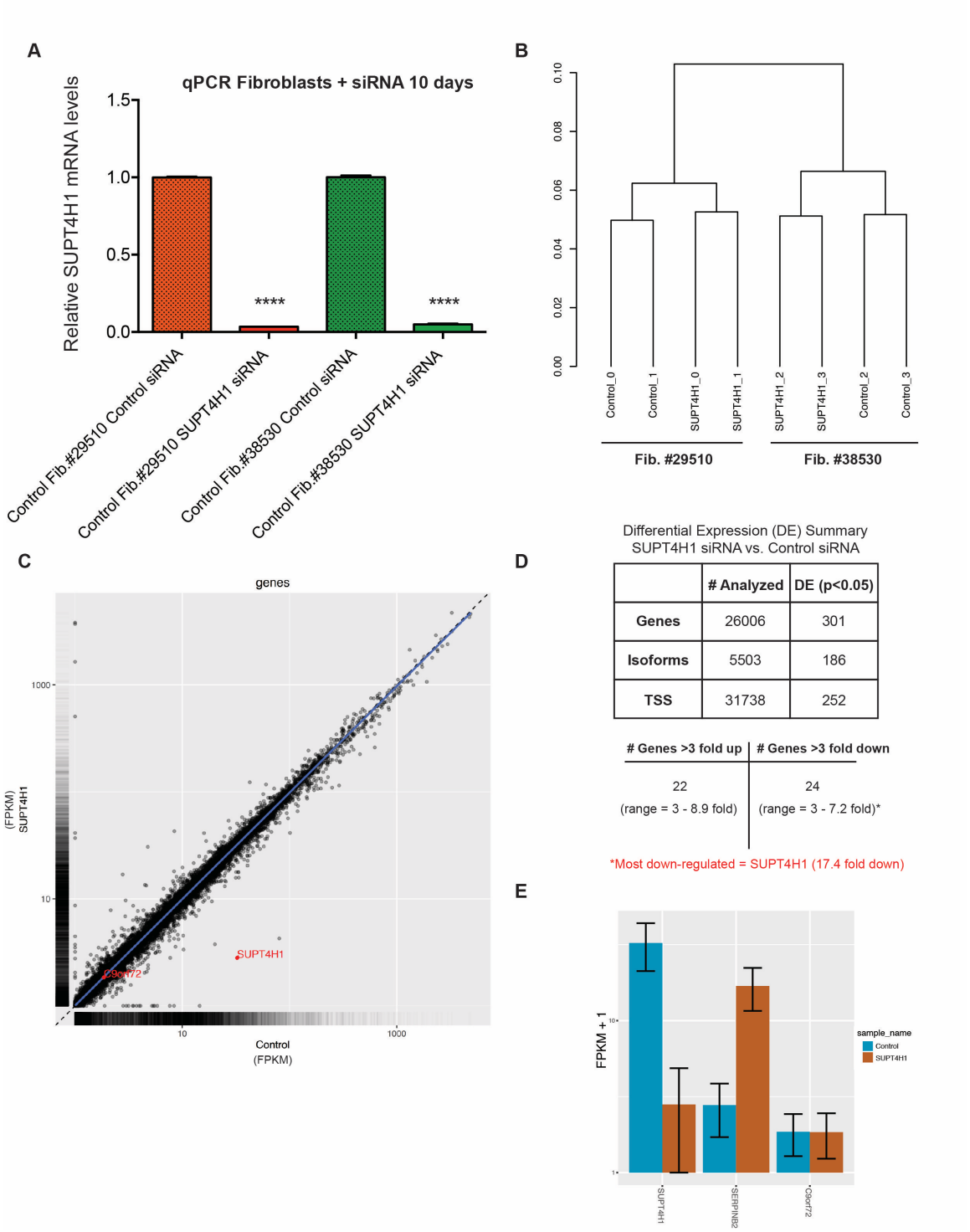

Fig. S8

RNA-sequencing of human fibroblasts treated with *SUPT4H1* siRNA (as in Fig. S7). (A) Knockdown of *SUPT4H1* was confirmed by qRT-PCR prior to RNA sequencing. (B) RNA-sequencing data illustrated as a dendrogram depicting the Jensen-Shannon distances between samples. Samples from each fibroblast line cluster together, with sub-clusters of replicate control siRNA treatments, and *SUPT4H1* siRNA treatments. Note,

cell lines are still clustered more closely than treatment, indicating little global transcriptional changes by *SUPT4H1* treatments. (C) Scatterplot of FPKM values per gene for groupings of Control siRNA treatments (combined two replicate transfections across two fibroblast lines = four samples per group) and *SUPT4H1* siRNA treatments (combined two replicate transfections across two fibroblast lines = four samples per group). (D) Summary data of significantly differentially expressed genes in *SUPT4H1* siRNA treated samples vs. Control siRNA treated samples, and (E) the most up- and down-regulated genes (SERPINB2 and SUPT4H1), along with C9orf72, plotted as FPKM values across treatments. *C9orf72* is highlighted as an unchanging gene, reinforcing the idea that SUPT4H1 reduction does not impact normal C9orf72 coding transcript levels.

Table S1.

Summary of human fibroblast lines

Cell line #	Clinical phenotype	Gender	*C9orf72* mutation
1	Asymptomatic	Female	Yes
2	ALS	Female	Yes
3	ALS	Female	Yes
4	Asymptomatic	Female	Yes
5	FTLD/ALS	Female	Yes
6	ALS	Female	Yes
7	ALS	Male	Yes
8	ALS	Male	Yes
9	FTLD/ALS	Male	No
10	ALS	Female	No
11	ALS	Female	No
12	ALS	Female	No
13	ALS	Male	No
14	ALS	Male	No
15	ALS	Female	No
16	ALS	Female	No
17	ALS	Female	No
18	ALS	Female	No
19	Healthy	Female	No
20	Healthy	Female	No
21	Healthy	Female	No
22	Healthy	Male	No
23	Sixth nerve palsy	Female	No
24	Healthy	Female	No
25	Healthy	Female	No
26	Healthy	NA	No