Premature polyadenylation-mediated loss of stathmin-2 is a hallmark of TDP-43-dependent neurodegeneration

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Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) are associated with loss of nuclear transactive response DNA-binding protein 43 (TDP-43). Here we identify that TDP-43 regulates expression of the neuronal growth-associated factor stathmin-2. Lowered TDP-43 levels, which reduce its binding to sites within the first intron of stathmin-2 pre-messenger RNA, uncover a cryptic polyadenylation site whose utilization produces a truncated, non-functional mRNA. Reduced stathmin-2 expression is found in neurons trans-differentiated from patient fibroblasts expressing an ALS-causing TDP-43 mutation, in motor cortex and spinal motor neurons from patients with sporadic ALS and familial ALS with GGGGCC repeat expansion in the C9orf72 gene, and in induced pluripotent stem cell (iPSC)-derived motor neurons depleted of TDP-43. Remarkably, while reduction in TDP-43 is shown to inhibit axonal regeneration of iPSC-derived motor neurons, rescue of stathmin-2 expression restores axonal regenerative capacity. Thus, premature polyadenylation-mediated reduction in stathmin-2 is a hallmark of ALS-FTD that functionally links reduced nuclear TDP-43 function to enhanced neuronal vulnerability.
recruited to growth cones of regenerating axons. Moreover, stathmin-2 has been proposed as an axonal-maintenance factor whose loss accelerates a neuronal degeneration program. In Drosophila, expression of a mutant stathmin causes retraction of motor neurons from innervated neuromuscular junctions.

We now identify that the mRNA encoding stathmin-2 is significantly lost after TDP-43 depletion and in neurons trans-differentiated from multiple patient fibroblasts, each carrying an ALS-causing mutation in TDP-43. Mechanistically, TDP-43 disruption is shown to drive premature polyadenylation and aberrant splicing in intron 1 of stathmin-2 pre-mRNA, producing a non-functional mRNA. Aberrant polyadenylation/splicing of stathmin-2 pre-mRNA is also consistently found in spinal motor neurons and motor cortex of sporadic ALS and C9orf72 ALS patients, supporting stathmin-2 loss of function as a key driver of motor neuron degeneration. Indeed, suppression of TDP-43 or stathmin-2 in iPS-derived motor neurons leads to inhibition of axonal regeneration after induced damage. Importantly, restoration of stathmin-2 levels rescues axonal regeneration ability in the absence of TDP-43, evidence supporting rescue of stathmin-2 levels as a potential therapeutic approach in neurodegenerative diseases—especially ALS and FTD—affected by TDP-43 proteinopathy.

Results

TDP-43 depletion or disease-causing mutation suppresses stathmin-2. Two independent strategies were undertaken to manipulate TDP-43 function in a human neuronal cell line: (1) depletion of TDP-43 by short interfering RNA (siRNA) and (2) genome editing with clustered regularly interspaced short palindromic repeats (CRISPR) to create a conditional isogenic line (iNeurons) where TDP-43 was either eliminated or replaced with a familial ALS-causing mutant allele. The human neuronal cell line SH-SY5Y was chosen as a model as it has been shown to maintain a diploid karyotype and acquire substantial neuronal character when differentiated. siRNA treatment reduced TDP-43 mRNA levels by >80% when compared with cells treated with a control siRNA (Fig. 1a). The consequences of lowering TDP-43 were then examined on a genome-wide basis. Libraries of RNAs were prepared, sequenced, and mapped to the human genome. This approach confirmed reduction of TDP-43 to one-fourth of its initial level (Fig. 1b). Amid 299 and 219 mRNAs that were down- or upregulated, respectively (with fold changes >1.5, false discovery rate (FDR) <0.05) (Supplementary Table 1), the most affected mRNA (with >85% reduction; Fig. 1b and Supplementary Fig. 1a) was the one encoding the neuronal growth-associated protein stathmin-2. Reduction in stathmin-2 mRNA upon TDP-43 depletion was confirmed by quantitative PCR (qPCR) (Fig. 1c) and an 8-fold reduction of the 22 kDa stathmin-2 protein was identified by immunoblotting (Fig. 1d and Supplementary Fig. 1b).

Using a CRISPR–Cas9 site-selective nuclease, we next genetically engineered SH-SY5Y cells to express a familial ALS-causing mutation (asparagine substituted to serine at amino acid 352 (TDP-43N352S)) from both endogenous TDP-43 alleles (Supplementary Fig. 2a). Immunofluorescence imaging confirmed that mutant TDP-43 protein remained mostly nuclear, similar to wild-type TDP-43 expressed in the original SH-SY5Y line (Fig. 1e and Supplementary Fig. 2b). Transcriptional profiling by RNA sequencing (RNA-seq) of the wild-type and mutant lines revealed that introduction of the disease-causing TDP-43 mutation led to altered expression of 950 mRNAs (451 downregulated and 499 upregulated; fold change >1.5, FDR <0.05) (Supplementary Table 2), including moderate reduction in TDP-43 (Fig. 1f). In addition, previously described alternative splicing changes linked to TDP-43 loss of function were observed in TDP-43 mutant-expressing cells (Supplementary Fig. 2c), consistent with both loss of normal TDP-43 function and a gain of aberrant function, as seen previously in mice.

Analysis of RNAs from isogenic wild-type and mutant lines (using qPCR) confirmed a 1.7-fold reduction of stathmin-2 mRNA (Fig. 1g), while immunoblotting revealed more than 2-fold reduction of stathmin-2 protein in TDP-43N352S mutant cells (Supplementary Fig. 2d). A search for gene expression changes that overlapped between cells with TDP-43 loss of function (Fig. 1b) and TDP-43N352S mutant cells (Fig. 1f) identified stathmin-2 mRNA (and 18 additional RNAs) to be downregulated, together with another 23 that were upregulated (Fig. 1h).

Reduced stathmin-2 in TDP-43 mutant human neurons. We then tested whether stathmin-2 expression was reduced in human neurons directly converted from ALS patients' fibroblasts. To this end, we used eight fibroblast lines obtained from an extended family that included four carriers heterozygous for the ALS-linked mutant TDP-43N352S and four individuals without the mutation (Supplementary Fig. 3a and Supplementary Table 3). Overall, stathmin-2 mRNA levels were already reduced (by 50%) (Supplementary Fig. 3b) in the mutant fibroblast lines relative to controls despite primarily nuclear localization of TDP-43 protein both in control and ALS patient fibroblasts (Supplementary Fig. 3c,d). Fibroblasts from all eight lines were directly induced into neurons (herein referred to as iNeurons) by expression of the neuronal-specific transcription activator Brn2 and reduction of the polypyrimidine track binding protein (PTB) (Fig. 2a).

TDP-43 was almost exclusively nuclear in wild-type and mutant fibroblasts (Supplementary Fig. 3d). When converted into iNeurons, a higher proportion of wild-type TDP-43 accumulated in the cytoplasm and this relocalization was enhanced in iNeurons expressing the N352S mutation (Fig. 2b and Supplementary Fig. 3e). Importantly, stathmin-2 mRNA was significantly downregulated (more than threefold) in all four sets of TDP-43N352S iNeurons relative to the four iNeuron lines derived from fibroblasts of healthy family members (P <0.01, n=4; Fig. 2c).

We next tested iNeurons generated from familial ALS patient fibroblasts carrying three other mutations: glycine to serine at position 298 (TDP-43G298S), alanine to threonine at position 382 (TDP-43A382T), and asparagine to serine at position 390 (TDP-43N390S). Stathmin-2 mRNA was reduced (relative to iNeurons from non-ALS individuals) in all of these familial ALS iNeurons (Fig. 2c,d). These data demonstrate that, similar to reduction of TDP-43, ALS-linked mutations in TDP-43 suppress stathmin-2 expression level in human iNeurons.

TDP-43 represses premature polyadenylation in stathmin-2 pre-mRNA. The stathmin-2 gene is annotated to contain five constitutive exons (Refseq ID: NM_001199214.1) plus a proposed alternative exon between exons 4 and 5 whose use was undetectable (using RNA-seq) under any condition in our SH-SY5Y cells (Fig. 3a). Reduction or mutation in TDP-43, however, induced a new spliced exon, with RNA-seq reads mapping within intron 1 (Fig. 3a, red arrow). This new exon, herein called exon 2a, was absent in wild-type cells, but appeared either when TDP-43 was depleted or when endogenous TDP-43 was edited to carry the N352S mutation. Prominent utilization of this new exon was observed when TDP-43 was reduced, in line with more than six-fold suppression of RNAs containing exons 2 to 5 (Fig. 3a, upper panel). This was accompanied by the corresponding loss of stathmin-2 protein (Fig. 1d and Supplementary Fig. 1b).

Altered splicing and ligation of exon 1 to exon 2a in SH-SY5Y cells after TDP-43 depletion or in the presence of a TDP-43 mutation was then confirmed by reverse transcription followed by PCR (RT–PCR) and qPCR (Fig. 3b). However, while fusion between exon 1 and exon 2a was consistently observed (Fig. 3b), no RNAs containing exon 2a ligated to the downstream exon 2 were identified using primers targeting the flanking exons 1 and 2 (Supplementary Fig. 4a-c).
TDP-43 depletion or genome editing in a human neuronal cell line identifies a significant reduction in stathmin-2 expression levels. **Fig. 1 |** a, Quantitative real-time PCR analysis confirming siRNA-mediated reduction of TDP-43 mRNA levels in SH-SY5Y cells. Expression of TFRC and GAPDH mRNAs were used as endogenous controls. Cells were treated with siControl (white bar, black dots, mean $= 1$) or siTDP-43 (gray bar, red dots, mean $= 0.19$) for 96 h in three biologically independent experiments (two-tailed $t$ test, $P = 0.0012, n = 3$). Error bars represent s.e.m. ** $P < 0.01$. b, Volcano plot showing differentially expressed genes in SH-SY5Y cells depleted of TDP-43 by siRNA treatment. Genes with significant changes in mRNA levels are represented by red dots ($n = 3$ biologically independent experiments, fold change $> 1.5$ and FDR $< 0.05$ by DESeq2). Larger red dots represent increased statistical significance (measured by FDR $< 0.05$). RNA-seq analysis identified 518 misregulated genes and confirmed four-fold reduction in TDP-43 mRNA levels. Stathmin-2 mRNA showed the strongest reduction (6.5-fold) after TDP-43 suppression. Up- and downregulated genes’ counts are indicated. Expression values were calculated as TPM. c, Representative immunofluorescence of TDP-3 (green) and lamin-B (red) in SH-SY5Y lines expressing wild-type or mutant TDP-43 by genome editing. Genotypes are indicated; experiment was reproduced three times independently with similar results. d, Immunoblotting of TDP-43 and stathmin-2 in SH-SY5Y cells treated with siControl or siTDP-43 for 96 h. α-Tubulin served as a loading control. Three biological replicates are shown. For unprocessed blots, see Supplementary Fig. 10. e, Quantitative real-time PCR analysis confirming reduction of stathmin-2 mRNA expression levels ($mean = 0.13$) in SH-SY5Y cells treated with siRNA targeting TDP-43 compared with cells treated with siControl ($mean = 1$) for 96 h in three biologically independent experiments (two-tailed $t$ test, $P = 0.0005, n = 3$). Error bars represent s.e.m. ** $P < 0.001$. f, Gene expression changes linked to TDP-43 loss (1.7-fold) after TDP-43 suppression. Up- and downregulated genes’ counts are indicated. f, Gene expression changes linked to TDP-43 loss (1.7-fold) after TDP-43 suppression. Expression values were calculated as TPM. ** $P < 0.01$. h, Relative expression of 42 overlapping genes linked to TDP-43 N352S/N352S. Genotypes are indicated; experiment was reproduced three times independently with similar results. f, Volcano plot depicting 950 differentially expressed genes identified by genome-wide RNA-seq. Significant changes in mRNA levels between SH-SY5YWT/WT and SH-SY5YN352S/N352S lines are represented by red dots ($n = 2$ biologically independent experiments, fold change $> 1.5$, FDR $< 0.05$) by DESeq2. Larger red dots represent increased statistical significance (measured by FDR $< 0.05$). Expression values were calculated as TPM. g, Gene expression changes linked to TDP-43 loss (4-fold) after TDP-43 suppression. Expression values were calculated as TPM. ** $P < 0.01$. h, Expression changes of 42 overlapping genes from Fig. 1b,f are plotted.
Fig. 2 | Reduced stathmin-2 levels in human neurons produced by direct conversion from ALS patient fibroblasts expressing mutant TDP-43.

a. Summary of the direct conversion strategy adopted from Xue et al.28. 
b. iNeurons produced by direct conversion from fibroblasts of control and ALS patients. Immunofluorescence staining at day 20 of differentiation revealed partial cytoplasmic mislocalization of TDP-43 (green) in ALS iNeurons. Neuron-specific class III tubulin (Tuj1, red) was used as a neuronal marker; cell nuclei were visualized by DAPI staining (blue). The experiment was repeated independently with similar results from three control individuals and three familial ALS donor lines (with mutant TDP-43). c. qPCR analysis of stathmin-2 mRNA levels in iNeurons from control (gray bars, black dots) and familial ALS patients with different TDP-43 mutations (white bars, turquoise dots). Plotted are two biologically independent experiments per each individual from an extended family that included four carriers heterozygous for the familial ALS-linked mutant TDP-43N352S (Supplementary Fig. 4e), confirming physical interaction with TDP-43 protein. Altogether, these three biologically independent experiments of three additional ALS patients (nonfamily members) with the indicated mutations. a Asymptomatic. 

d. Summary of stathmin-2 expression level measured by qPCR in iNeurons from controls (n = 4, gray, mean = 1) and familial ALS patients (n = 7, white, mean = 0.36). P = 0.0005, two-tailed t test. Error bars represent s.e.m. **P < 0.01; ***P < 0.001.

To test the possibility that incorporation of exon 2a drives alternative polyadenylation within what is normally intron 1 (Fig. 3c), we used anchored oligo(dT) primers for reverse transcription followed by PCR and sequencing. This analysis confirmed polyadenylation of the stathmin-2 RNA containing exon 2a as the terminal exon (Fig. 3d,c).

A polyadenylation signal ‘AUUAAA’ was identified 24 nucleotides upstream to the polyadenylation site (Fig. 3d). This cryptic polyadenylation sequence in the human stathmin-2 gene is conserved among most primates, but is notably absent in mouse or rat (Supplementary Fig. 4d). qPCR analysis on nascent stathmin-2 pre-mRNAs identified reduction in full-length stathmin-2 transcripts in SH-SY5Y cells expressing mutant TDP-43 (Supplementary Fig. 4e), consistent with increased levels of truncated exon 2a-containing RNAs (Fig. 3b).

The prematurely polyadenylated RNA includes 227 nucleotides originating from exon 2a (hg38; chr8:79,616,822–79,617,048) with its predicted 16 amino acid translation product initiating at the normal AUG codon in exon 1 and ending 11 codons into exon 2a (Fig. 3c,d). Three ‘GUGUGU’ hexamers marking potential binding sites of TDP-43 (as we and others have previously described29) were identified in a region spanning 30 nucleotides downstream of the 3′ splice site that produces exon 2a (Fig. 3d). Indeed, analysis of ultraviolet cross-linking and immunoprecipitation (iCLIP) data for TDP-43 in human SH-SY5Y cells’ confirmed TDP-43 binding (shown by mapped reads) to the stathmin-2 pre-mRNA at a single region containing the three GUGUGU sequences (Fig. 3f), thus confirming physical interaction with TDP-43 protein. Altogether, we conclude that reduction in TDP-43 causes de-repression and efficient use of a cryptic polyadenylation site in what normally is intron 1, which results in truncation of the stathmin-2 pre-mRNA and suppression of functional stathmin-2 expression.

Stathmin-2 mRNA is prominently expressed in motor neurons. Comparison of published evidence of the abundance of stathmin-2 mRNA in ribosome-bound RNAs across specific cell types of the...
murine central nervous system revealed 25- and 15-fold enrichment of translated stathmin-2 mRNAs in motor neurons relative to astrocytes and oligodendrocytes, respectively (Supplementary Fig. 5a). Remarkably, further analysis of these data identified stathmin-2 to be among the 25 most abundant actively translated mRNAs isolated from adult motor neurons in mice. Similarly, our analysis of RNA sequencing from laser capture microdissected human lumbar spinal motor neurons from seven healthy controls determined that in human spinal motor neurons stathmin-2 is the 20th most abundant mRNA, only slightly less abundant than the mRNAs encoding the three neurofilament subunits NF-L, NF-M, and NF-H (Supplementary Fig. 5b). Further, of the four stathmin genes, only stathmin-2 was enriched within human motor neurons (Supplementary Fig. 5c).

**Fig. 3** | TDP-43 regulates stathmin-2 mRNA levels by repressing premature polyadenylation. a, RNA-seq reads mapped to the genomic region of stathmin-2 revealed incorporation of a new exon, originated from intron 1, into the mature stathmin-2 mRNA. Red arrows indicate the intronic region of aberrant splicing (exon 2a) in SH-SY5Y cells after TDP-43 depletion (top) or expression of mutant TDP-43 (bottom). The experiment was repeated independently three times (TDP-43 depletion) and twice (expression of mutant TDP-43) with similar results. b, Representative RT-PCR (left; experiment was repeated three times independently with similar results) and qPCR (right; n = 3 biologically independent experiments) analyses confirmed expression of the new spliced mRNA isoform containing exon 2a upon TDP-43 depletion (mean = 8.7, P = 0.00078) or mutation (mean = 3.8, P = 0.0001). The location of primers in exons 1 and 2a is shown. RT–PCR of the CENP-A transcript was used as loading control for RT–PCR, and TFRC and GAPDH were used as qPCR normalizers (**P < 0.001, two-tailed t test; error bars represent s.e.m.). For uncropped gel images, see Supplementary Fig. 10. c, Schematic of stathmin-2 pre-mRNA (top) and alternative RNA isoforms (bottom) in normal cells (1) or cells with TDP-43 deficiency (2). Constitutive exons are represented by black boxes; exon 2a is in red and the thin red box represents the newly acquired 3′ UTR. Light grey boxes represent 3′ or 5′ UTRs of normal stathmin-2 mRNA. d, Sequence of exon 2a is shown in red, including the embedded in-frame UAG codon generating a premature stop codon. Highlighted are potential TDP-43 binding sites located 127 nucleotides upstream of the alternative polyadenylation signal. e, 3′ end sequencing by reverse transcription using oligo(dT)-VN primers confirmed exon 2a as the terminal exon of short stathmin-2 mRNA. f, Genome browser track obtained from iCLIP for TDP-43 in human SH-SY5Y cells; revealing TDP-43 physical binding to exon 2a, located in intron 1 of stathmin-2 pre-mRNA.
**Fig. 4** | Abnormal stathmin-2 mRNA processing is a disease hallmark in affected spinal motor neurons of sporadic ALS patients. **a**, RNA sequencing of control and sporadic ALS laser-captured spinal motor neurons revealed a robust signature of exon 2a incorporation in stathmin-2 mRNA in sporadic ALS samples, but not in non-ALS aged controls. Lower diagram shows the genomic region of stathmin-2, and RNA reads mapped to exon 2a are indicated by a red arrow. Data were analyzed from from Krach et al. Stathmin-2 mRNA expression was suppressed in spinal motor neurons of sporadic ALS patients (gray bar, \( n = 13 \), mean = 423 TPM, \( P = 0.03 \)) relative to non-ALS aged controls (white bar, \( n = 7 \), mean = 971 TPM, \( P = 0.019 \)). Two-tailed t test, error bars represent s.e.m.. RNA sequencing data was analyzed from Krach et al. Stathmin-1 mRNA expression is shown to the right (control mean = 25, ALS patients mean = 44.5). TPM counts are represented, two-tailed t test. \( P = 0.02 \). *P < 0.05, error bars represent s.e.m.**. **c, d**, RT–PCR using primers targeted to exon 1 and exon 2a confirmed expression of truncated stathmin-2 isoform in (c) thoracic spinal cord or (d) motor cortex from sporadic and C9orf72 ALS patients, but not in spinal cords of mutant SOD1 carriers. For uncropped gel images, see Supplementary Fig. 10. **e, f**, Lumbar spinal cord and motor cortex sections isolated from control individuals and sporadic ALS patients were hybridized with locked nucleic acid probes targeting exon 2a (within intron one) of stathmin-2 pre-mRNA (for the truncated RNA) or exon 5 of stathmin-2 pre-mRNA. Signal is shown in blue, counterstain is nuclear fast red. **g**, Summary of human samples with expression of stathmin-2 truncated RNA, identified by RT–PCR or in situ hybridization. Data shown in Supplementary Fig. 7 are included in this table. NA, not applicable.
Importantly, just as we had determined in TDP-43 mutant expression experiments (Fig. 4a and Supplementary Fig. 6), overall stathmin-2 mRNA levels in sporadic ALS patients were significantly decreased relative to those in motor neurons from non-ALS individuals (Fig. 4b). By contrast, stathmin-1 mRNA expression was low in both healthy and ALS spinal motor neurons (Fig. 4b). Additionally, analysis of RNAs extracted from anterior horns of thoracic spinal cord from 13 sporadic ALS patients and three familial ALS patients carrying GGGGCC expansion in C9orf72 revealed aberrant processing of stathmin-2 mRNA in all, but not in RNAs from eight healthy individuals or three familial ALS patients with superoxide dismutase 1 (SOD1) mutations (Figs. 4c and Supplementary Fig. 7a). Consistent with the latter, mice developing fatal motor neuron disease from expression of an ALS-linked mutation in SOD1 retained normal stathmin-2 mRNA levels despite apparent motor neuron damage, demonstrating that stathmin-2 expression is not affected by motor neuron degeneration per se (Supplementary Fig. 5a). Recognizing that TDP-43 pathology is found in sporadic and familial ALS linked to C9orf72 expansion but not SOD1-mediated ALS (Supplementary Fig. 8)19, these results are consistent with premature polyadenylation of stathmin-2 mRNA being triggered by TDP-43 dysfunction and the corresponding suppression of stathmin-2 expression in both thoracic (Fig. 4c and Supplementary Fig. 7a) and lumbar (Fig. 4a) motor neurons.

Extension of similar analyses to the motor cortex identified altered processing of stathmin-2 mRNA to include exon 2a in two of two C9orf72 samples and six of nine sporadic ALS samples (Fig. 4d). For ALS cases that were also diagnosed with FTD, premature polyadenylation was seen in frontal cortex-derived RNAs in three of four sporadic ALS–FTD patients, and one of one C9orf72 expansion patient (Supplementary Fig. 7b).

Lastly, chromogenic in situ hybridization was used to test on a cell-by-cell basis for stable accumulation of exon 2a-containing stathmin-2 RNAs in spinal cord and motor cortex samples from five control individuals and four sporadic ALS patients (Fig. 4e–g and Supplementary Fig. 7c,d). In agreement with prior analyses of RNAs derived by laser microdissection (Fig. 4a) or from bulk tissues (Fig. 4c,d), RNA probes hybridizing to stathmin-2 exon 2a were increased in both nuclear and cytoplasmic compartments of spinal cord neurons (97% of sporadic ALS motor neurons versus 0% of control; n = 69 and 41, respectively) and in neurons from layers 3 and 5 of motor cortex from all ALS-derived samples, with 100% of scored sporadic ALS cortical neurons staining positive for exon 2a (n = 50) versus 0% of control cortical neurons (n = 50). Low basal levels of hybridization (presumably corresponding to the newly transcribed stathmin-2 pre-mRNA) were found in tissues from non-ALS individuals, with an intensity only slightly above that of a scrambled sequence control probe.

A probe hybridizing to exon 5 of the stathmin-2 mRNA revealed an apparent reduction in affected regions of sporadic ALS, with only 35% of sporadic ALS lumbar motor neurons showing weakly positive staining for STMN2 exon 5 versus 70% of control non-ALS motor neurons (n = 65 and 61, respectively). The reduction of exon 5 staining was even more substantial in the motor cortex where 0% of scored neurons showed a positive signal, compared with a strong signal in 100% of non-ALS cortical neurons (Fig. 4e,f and Supplementary Fig. 7c,d).

Impaired axonal regeneration on TDP-43 loss in human iPSC-derived motor neurons is alleviated by restoring stathmin-2. To determine the functional consequence of TDP-43-mediated reduction in stathmin-2, induced pluripotent stem cells (iPSCs) were generated using a non-integrating approach, validated to retain a normal karyotype (Supplementary Fig. 9a) and expression of pluripotent stem cell markers (Supplementary Fig. 9b–d), and differentiated into motor neurons (Supplementary Fig. 9e). Motor neuron precursors (expressing homeobox gene HB9) appeared within 21 days (Supplementary Fig. 9f) and by 28 days developed MAP2-positive dendrites and long axons accumulating neurofilaments (Supplementary Fig. 9g,h). At day 29, motor neurons were treated with antisense oligonucleotides (ASOs) that direct catalytic suppression of stathmin-2 by premature polyadenylation is a hallmark of ALS. Analysis of the RNA sequencing reads mapping to the stathmin-2 gene from lumbar motor neurons captured by laser microdissection revealed a clear segregation between controls and sporadic ALS cases. RNAs from all analyzed sporadic ALS patients (n = 13) contained inclusion of exon 2a (Fig. 4a and Supplementary Fig. 6). In contrast, RNAs including exon 2a were absent in mRNA from all tested (n = 7) non-ALS individuals. In an additional control, RNAs using exon 2a were significantly decreased relative to those in motor neurons from non-ALS individuals (Fig. 4b). By contrast, stathmin-1 mRNA expression was low in both healthy and ALS spinal motor neurons (Fig. 4b). Additionally, analysis of RNAs extracted from anterior horns of thoracic spinal cord from 13 sporadic ALS patients and three familial ALS patients carrying GGGGCC expansion in C9orf72 revealed aberrant processing of stathmin-2 mRNA in all, but not in RNAs from eight healthy individuals or three familial ALS patients with superoxide dismutase 1 (SOD1) mutations (Figs. 4c and Supplementary Fig. 7a). Consistent with the latter, mice developing fatal motor neuron disease from expression of an ALS-linked mutation in SOD1 retained normal stathmin-2 mRNA levels despite apparent motor neuron damage, demonstrating that stathmin-2 expression is not affected by motor neuron degeneration per se (Supplementary Fig. 5a). Recognizing that TDP-43 pathology is found in sporadic and familial ALS linked to C9orf72 expansion but not SOD1-mediated ALS (Supplementary Fig. 8)19, these results are consistent with premature polyadenylation of stathmin-2 mRNA being triggered by TDP-43 dysfunction and the corresponding suppression of stathmin-2 expression in both thoracic (Fig. 4c and Supplementary Fig. 7a) and lumbar (Fig. 4a) motor neurons.

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degradation of TDP-43 mRNAs through the action of endogenous RNase H. TDP-43 mRNA levels were reduced in a dose-dependent manner (Fig. 5a).

ASO-induced reduction in TDP-43 mRNA (up to 55%) was consistently accompanied by a greater (up to 12-fold) dose-dependent reduction in stathmin-2 mRNAs, coupled with a corresponding increase in RNAs containing exon 1 ligated to exon 2a (Fig. 5b). Dose-dependent suppression (up to 15-fold) of stathmin-2 expression in iPSC-derived motor neurons (without affecting TDP-43 levels) was also achieved with an ASO targeted to the 3’ UTR of full-length stathmin-2 mRNA (Fig. 5c). Immunoblotting confirmed that within 12 days of treatment with ASOs targeting either TDP-43 or stathmin-2 mRNAs there was nearly complete loss of the 22kD stathmin-2 protein (Fig. 5d). Motor neuronal viability was not affected by reduction in either TDP-43 or stathmin-2 (Supplementary Fig. 9i).
To assess the functional consequences of stathmin-2 depletion in axonal regeneration (Fig. 5e), motor neuron precursors were seeded into the proximal somatic compartment of a microfluidic device. Over a 9-day maturation period, axons extended through microgroove-embedded channels (830 μm in length) that exclude neuronal cell bodies but allow axonal extension into the distal compartment. Matured motor neurons were treated for 20 days with ASOs added to the somatic compartment to reduce synthesis and accumulation of either TDP-43 or stathmin-2 (Fig. 5f). Axons were maintained after loss of either protein. After axons in the distal compartment were mechanically axotomized, axonal regrowth of motor neurons treated with nontargeting control ASOs initiated within 24 h (visualized by NF-H immunostaining in red; Fig. 5g,h). Stathmin-2 appeared in a punctate pattern along these regenerating axons and accumulated in the growth cones (Fig. 5g), consistent with a role in promoting axonal regrowth. Indeed, following ASO-mediated reduction in stathmin-2 (Fig. 5i,j) or TDP-43 (Fig. 5k,l), axonal regeneration after axotomy was almost completely suppressed, with 10% (Fig. 5j) and 13% (Fig. 5i) recovery rates relative to those treated with control ASOs, respectively. Together, these data demonstrate increased vulnerability of motor neurons with reduced accumulation of the neuronal-growth factor stathmin-2.

We next tested whether the failure to regenerate after axotomy of iPSC-derived human motor neurons following depletion of either stathmin-2 or TDP-43 could be rescued by restoration of stathmin-2 expression. To do this, after a 16-day ASO-mediated suppression of stathmin-2 or TDP-43, iPSC-derived motor neurons were transduced with a lentivirus carrying a stathmin-2-encoding gene whose RNA did not contain the cryptic polyadenylation site found in the endogenously encoded stathmin-2 gene. Despite the prior sustained (16 days) reduction in stathmin-2, subsequent restoration of stathmin-2 expression almost completely rescued regeneration after axotomy (Fig. 5m,n). More remarkably, although TDP-43 affects the levels or splicing of many RNAs (Fig. 1b and refs. 8,9), restoration of stathmin-2 alone was sufficient to rescue regeneration after axotomy of TDP-43-depleted motor neurons (Fig. 5o,p).

Discussion

Using three independent approaches, we have identified that reduction or mutation in TDP-43 induces aberrant processing of stathmin-2 pre-mRNA, leading to efficient usage of a cryptic polyadenylation site that truncates mRNAs encoding stathmin-2, thereby suppressing stathmin-2 synthesis. Our data strongly support that under normal conditions TDP-43 acts to maintain stathmin-2 expression by repressing this premature polyadenylation within intron 1 of the stathmin-2 pre-mRNA. Loss of nuclear TDP-43 is a nearlyuniversal pathological hallmark in ALS, and notably, we observed efficient premature polyadenylation of stathmin-2 in motor neurons of all tested sporadic ALS patients and in patients with repeat expansion in C9orf72, the most common inherited cause of ALS and FTD. In contrast, altered processing of stathmin-2 was not found in SOD1 ALS patients who do not exhibit TDP-43 pathology. Thus, our data offer strong support that loss of stathmin-2 RNA by premature polyadenylation and subsequent reduced levels of stathmin-2 protein in affected neurons are hallmarks of sporadic and C9orf72-expansion-mediated ALS and FTD.

Thousands of mRNA targets affected by TDP-43 dysfunction have been identified in multiple cell lines and in vivo14,15,22. Notably, reduced TDP-43 has been linked to the abnormal inclusion of a set of cryptic exons that may lead to nonsense-mediated decay and reduced levels of transcripts in different cell types and tissues from mouse and human16,17. To that, we have established here that TDP-43 also acts in the normal situation to suppress polyadenylation within exon 2a in stathmin-2 pre-mRNA. Reduced TDP-43 in human motor neurons is accompanied by near complete loss of expression of functional stathmin-2. Recognizing that splicing typically occurs co-translationally23, it is likely that the premature polyadenylation in stathmin-2 mRNA is the driving event in stathmin-2 suppression, with premature polyadenylation inducing the splicing machinery to use a previously cryptic 3′ site to resolve splicing initiated at the normal 5′ splice site in intron 1.

We note that abnormal processing of stathmin-2 is not recapitulated in mice expressing TDP-43 transgenes or in TDP-43 deficient mice, as the cryptic polyadenylation signal and three GU-rich TDP-43 binding sites in intron 1 of the human stathmin-2 gene are not found in the corresponding mouse intron (Supplementary Fig. 4d). Consistently, stathmin-2 pre-mRNA is not bound by TDP-43 in the murine nervous system and stathmin-2 mRNA level is not altered after ASO-mediated reduction of TDP-43 in wild-type mice24. By mining TDP-43 iCLIP data25, we have identified physical interaction of TDP-43 with the GU-rich sequences in exon 2a of human stathmin-2 pre-mRNA. This strongly supports that in healthy neurons, TDP-43 binding represses premature polyadenylation of stathmin-2 pre-mRNA. TDP-43 loss of function during disease relieves this inhibition, producing exon 2a-containing, truncated stathmin-2 mRNAs as seen in all sporadic and C9orf72-mediated ALS examples tested here.

Our evidence supports a broader role for TDP-43 in modulating polyadenylation within the nervous system, similar to the role previously shown for the neuronal factor Nova26, as well as additional RNA-binding proteins27,28 including the ALS-associated protein FUS29,30. Since computational analysis has recently identified extensive alternative polyadenylation in cerebellum of C9orf72 ALS and sporadic ALS subjects31, as well as in HEK293 cells with TDP-43 suppression32, the global impact of TDP-43 on alternative polyadenylation in human motor neurons should now be determined.

Impaired microtubule dynamics has been widely implicated in neurodegeneration, and microtubule-stabilizing agents have been proposed as a therapeutic approach33. While disruption of TDP-43 nuclear function affects the processing of numerous RNA targets, our data provide a link between increased motor neuron vulnerability in ALS and suppression of stathmin-2, a tubulin binding protein previously established to affect microtubule dynamics34,35. The family of vertebrate stathmins includes stathmins 1–4 that are likely to serve both complementary and distinct functions in the nervous system36. Each stathmin possesses two conserved tubulin-binding domains that are capable of interacting with two α/β tubulin heterodimers37.

Individual stathmins may have unique functional properties. Stathmin-2 and -4 are highly expressed in neurons38, with only stathmin-2 mRNA enriched in motor neurons (Supplementary Fig. 5b, c). Increased expression of stathmin-1 and -2 has been reported in cellular and mouse models of spinal muscular atrophy46,47. Stathmin-2 has been proposed as a neuronal regeneration marker21 (with expression similar to the axonal growth-associated protein GAP-4348) and we have shown here that axonal regeneration after reduction in TDP-43 can be restored by maintaining stathmin-2 levels (Fig. 5). In contrast, reduction in stathmin-1, which we show to be expressed in motor neurons at 1/30th the level of stathmin-2 (Fig. 4b), has been reported to rescue axonal pathology in motor neurons in pmn mice that have mutation in the TBCE chaperone for folding tubulin49.

The stathmin-2 loss we have identified in motor neurons from ALS patients is strongly expected to alter microtubule dynamics and function, thereby contributing to denervation from neuromuscular junctions and/or axonal degeneration. This scenario is supported by evidence of destabilized neuromuscular junctions and impaired synapse stability in Drosophila expressing mutant stathmin2. Likewise, a paralytic phenotype was reported following loss of stathmin function in Drosophila50. The high level of stathmin-2
shown here to accumulate in normal human spinal motor neurons highlights the potentially deleterious impact from its profound reduction following reduced nuclear TDP-43 function in the neuronal population preferentially affected in ALS.

To this, our data provide evidence that reduction in stathmin-2, as the consequence of neuronal damage or stress that leads to diminished nuclear TDP-43 function, is itself sufficient to inhibit regeneration of motor axons. Restoring the regenerative capability to iPSC-derived motor neurons depleted of TDP-43 simply by restoring stathmin-2 levels offers a new insight into how TDP-43 dysfunction drives increased neuronal vulnerability. Perhaps most importantly, our evidence supports development of therapeutic strategies for ALS, FTD, and other neurodegenerative diseases affected by TDP-43 proteinopathy through restoration of stathmin-2 to increase sustainability, including regenerative capacity of affected neurons.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41593-018-0293-z.

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**Author contributions**

Z.M., J.L.-E., S.D.C, C.L.-T., and D.W.C. designed the research; Z.M., J.L.-E., M.W.B., K.D, O.Z., Y.S., S.D.C., C.L.-T., and D.W.C. analyzed the data; Z.M., J.L.-E., M.W.B., K.D, J.A., F.F., M.A.M., M.S.B., T.O, M.R., D.W., and N.L. performed the research; F.R., C.F.B., J.R., D.W., and N.L. contributed key reagents and methodology; Z.M., C.L.-T., and D.W.C. wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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Methods
RNA extraction and complementary DNA synthesis. For RNA extraction from cultured cells and neurons, direct lysis was applied using Trizol reagent (Invitrogen) and RNA was purified according to the manufacturer’s instructions. Human tissue samples were first homogenized in Trizol, using a mechanical tissue homogenizer, and RNA was extracted accordingly.

RT–PCR and real-time PCR. For cDNA synthesis, 1 µg total RNA was reverse transcribed using the high-capacity reverse transcription kit (ABI) or superscript III (Invitrogen) according to the manufacturers’ instructions. RT–PCR reactions were performed using Q5 High-Fidelity DNA polymerase (NEB) in a T100 thermocycler PCR machine (Bio-Rad). For splicing analyses, RT–PCR products were separated on 2% polyacrylamide gels and then incubated with SYBR gold (Invitrogen) for imaging and analysis. Quantification of alternative splicing band intensities were determined using ImageJ software and an average of three biological replicates was plotted. Quantitative real-time PCR was carried out in triplicates, using Taq Universal SYBR green (Bio-Rad) in a CFX384 real-time PCR machine. mRNA expression of transferrin receptor protein-1 (TFRC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as endogenous control genes, as indicated. All primers are listed in Supplementary Table 4.

RNA sequencing. Total RNA was extracted from SH-SY5Y cells as indicated above. Subsequently, 1 µg of total RNA was used for mRNA libraries preparation using the TruSeq RNA kit (Illumina). For the TDP-43 knockdown experiment, three biological replicates were sequenced per each condition (siControl, siTDP-43). For TDP-43 genome-editing experiments, two biological replicates were sequenced per each condition (TDP-43, TDP-43*). Real-time qPCR was performed in triplicates with at least three replicates. RNA sequencing was carried out on an Illumina HiSeq 4000 platform with a median of 46 M reads per sample. Fifty-base-pair single-end FASTQ files were obtained using the Illumina demultiplexing pipeline. STAR and RSEM were used to align the reads to the human reference sequence HG38 and to calculate the raw counts and transcripts per million values for genes, respectively. Genes differentially expressed between sample groups were identified by DESeq2.

Genome editing. Both TDP-43 alleles were genetically modified in SH-SY5Y cells. A single guided RNA targeting TDP-43 was designed (Benchling webtool) and cloned into pSpCas9–2A-green fluorescent protein (GFP) plasmid54 (px558, px555, px556, px557). Bamhi restriction site is required for guiding RNA targeting TDP-43 is: CCGGGTTAATAACCAAAACCA. To promote homologous recombination, pSpCas9–2A-GFP-gRNA plasmid was electroporated using the Amaxa Nucleofactor (assay A-023) along with a 180-nucleotide long single-stranded donor oligonucleotides (IDT), containing the desired ALS-causing mutation and four homologous single-nucleotide replacements to avoid DNA cleavage recurrence by Cas9. Forty-eight hours following electroporation, cell colonies were collected and associated using Accutase. GFP-positive cells were sorted and single-cell seeded into 96-well plates using the SH800S Sony cell sorter. Individual clones were expanded and DNA was extracted for PCR amplification of the TDP-43 genomic locus. Replacement of ATG to ATG (c.1055A>G) was confirmed by Sanger sequencing. For TDP-43 coding region, all exons and introns (>500-bp length of each intronic region) were then sequenced to verify the absence of any additional DNA alteration. qPCR analysis was performed with a wild-type isogenic SH-SY5Y cell line that had undergone the mutagenesis attempt but not acquired the TDP-43 mutation during the original screening process.

Immunoblotting. Total-cells extracts were collected in radioimmunoprecipitation lysis buffer (RIPA buffer). Proteins concentrations were determined by Bradford assay (Bio-Rad) and equal amounts of total protein were boiled in SDS sample buffer for 10 min before running in 10% acrylamide gel. Proteins were transferred to PVDF and blocked in 5% milk solution in tris-buffered saline and 0.1% Tween-20 (TBST) for 1 h, before overnight incubation with the following primary antibodies: anti-TDP-43 (1:1,000) (ProteinTech, 10782–2-AP), anti-stathmin-2 (1:2,000) (npb1–49461, NovusBiologicals), anti-tubulin (1:10,000) (DM1A, Abcam). Immunoblots were washed in TBST and probed with horseshad peroxidase-conjugated secondary antibodies diluted 1:5,000 for 1 h at room temperature (GE Healthcare), before being exposed to film.

Immunofluorescence and image acquisition. SH-SY5Y cells were grown on poly-D-lysine (Sigma)-coated four-well glass bottom chambers (Thermo). Neurons were grown in matrigel-coated (Corning, 356230) four-well glass bottom chambers (Thermo). At the end point, cells were washed once with phosphate buffer (PBS) and fixed by 4% paraformaldehyde in phosphate buffer (PBS) for 30 min at room temperature. Two washes with PBS were followed by cell membrane permeabilization using 0.1% Triton X-100 (Sigma) for 15 min at room temperature. After one wash with PBS, cells were incubated for 30 min with blocking solution containing 3% bovine serum albumin (BSA)+ 4% donkey serum (Jackson ImmunolResearch) in PBS. Blocking solution was removed and replaced with primary TDP-43 antibody (Proteintech no. 10782–2-AP) diluted 1:1,000 in fresh blocking solution for overnight incubation at 4 °C. Following overnight incubation and three washes with PBS, cells were incubated with fluorescein tagged secondary antibody conjugated to Alexa-488 (Thermo Fisher Scientific) diluted 1:500 in blocking solution. Finally, cells were washed three times in PBS, the second wash included 10 min incubation with DAPI solution (Thermo Fisher Scientific, 100 ng ml−1) for nuclear staining. The preparation was mounted with ProLong Gold antifade reagent (Thermo Fisher Scientific). Fluorescence images were acquired with Olympus FV1000 confocal microscope, at the microscopy core, the Ludwig Institute, UCSD.

Fluorescence quantification from micrographs. Fluorescence quantifications were performed using FIJI image processing software (http://imagej.net). Freehand tool of the software was used to select the subcellular regions such as nuclei or cytoplasm for quantification. Data were plotted in GraphPad Prism software for illustration.

In situ hybridization of STMN2 RNA isoforms. Short 5′-digoxigenin-labeled locked nucleic acid probes were designed by QIAGEN (Truncated Eosin2A STMN2 RNA isoform probe sequence: TCACACAGAGAGCCAAAATCTCTT; Normal STMN2 mature mRNA probe sequence to the 3′UTR of STMN2: ATTCGGATAGCGATGATCCAT). For each tissue, two serial tissue sections (7 µm) were cut onto charged glass slides. Each of the two sections was hybridized with one of the STMN2 probes using standard ISH protocols and revealed with a commercial anti-DIG antibody and NBT developer kit. Slides were inspected under brightfield microscopy and serial sections of individual neurons were identified and imaged at ×40 magnification on a Keyence BZ-XR fluorescence microscope. Specific hybridization signals were identified by their large cytoplasm and/or nucleus, the presence of lipofuscin, and their position within Rexed lamina IX of the spinal cord. Neurons of the motor cortex were identified by their shape and relative size and position within cortical layering, with special attention to layers three and five.

Immunohistochemistry for TDP-43 and phosphorylated TDP-43. Formalin-fixed paraffin-embedded sections with 6 µm thickness were deparaffinized with Citrovol (Decon Lab no. 1601H) and hydrated with different dilutions of alcohol. Endogenous peroxidase activity was quenched with 0.06% H2O2 in methanol for 15 min. Antigen retrieval was performed in a high pH solution (Vector no. H-3301) in a pressure cooker at 125 °C for 20 min. Sections were blocked with 2% FBS (Gibco no. 10438–026) and 0.2% Triton X-100 (Sigma no. T8778) in PBS for 60 min. Following antigen retrieval and blocking, the sections were incubated with primary antibodies at 4 °C overnight as follows: TDP-43 (anti-rabbit, Proteintech no. 10782–2-AP; 1:5,000), and phosphorylated TDP-43 (pTDP-43) (pS409/410, anti-rabbit, Cosmobi no. TIP-PTD-P02, 1:1,000). The secondary antibody was TRIFLEX HRP Reagent Kit, anti-rabbit (Millipore, no. MP-7401) was incubated at room temperature for 60 min, and signals were detected using NovaRED peroxidase substrate kit (Vector no. SK-4800) for 2 min. Counterstaining was performed with hematoxylin (Rica chemical no. 3537–32). TDP-43 and pTDP-43 immunohistochemistry was performed using consecutive sections for each patient.

Cell culture and transfection. SH-SY5Y. The neuroblastoma cells (ATCC) were cultured in DMEM/F12 (Gibco) supplemented with 10% fetal-bovine serum (Omega) and 1% penicillin-streptomycin (Gibco) at 37 °C with 5% CO2. For knockdown experiments, cells were transfected with SMARTpool ON-TARGETplus siRNA targeting TDP-43 (L-012394) or control siRNA pool (D001801–10) (GE Dharmacon) at a final concentration of 50 nM, for 72 h in two doses (0, 24 h), after complexing with Lipofectamin RNAiMAX (Invitrogen) in Opti-MEM (Gibco) for 20 min.

Fibroblasts. Fibroblasts were cultured in DMEM/F12 (Gibco) containing 20% tetracycline-free FBS (Omega) and 1% penicillin-streptomycin (Gibco).

Direct conversion of human fibroblasts into induced neurons. The transdifferentiation assay was performed according to a protocol previously described1 and is based on expression of neuronal-specific transcriptional factor Brn2 and knockdown of the splicing blocker PTB. Briefly, initial lentiviral transduction of fibroblasts by doxycycline-induced Brn2 expressing plasmid was performed. After 16 h, the medium was replaced and cells were allowed to recover for 24 h. Cells were then grown under 1 µg ml−1 puromycin selection (Gibco) for 4 days and remaining cells were transferred into a new culturing dish. After expansion to 40–50% confluence, cells were transfected with short hairpin RNA (shRNA) lentivirus designed to suppress PTB mRNA expression. Sixteen hours later, the medium was replaced for 24 h recovery. After selection with hygromycin B (100 µg ml−1) for 4 days, remaining cells were seeded for terminal differentiation in matrigel-covered (Corning, 356230) glass chambers (Thermo) for immunofluorescence or six-well dish for RNA extraction. Twenty-four hours later, the medium was switched to Neurobasal/F12/F (Gibco) and supplemented with 20 ng ml−1 of Dox (Sigma), FGfB 10 ng ml−1 (In vitrogen), and 0.4% B27 (Gibco). The N3 medium contains: 25 µM insulin (Sigma, I9278), 50 µg ml−1 apo-transferrin (T1147),
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30 nM sodium selenite, 20 nM progesterone (Sigma), 100 nM putrescine (Sigma), 0.4% B27 and penicillin/streptomycin (Gibco). Small molecules were added 48 h later: CHIR99021 1 μM (Stemgent, 04-0004-02), SB434524 10 μM (Stemgent, 04-0010), and DbcAMP 0.5 μM (Sigma, D6027). Forty-eight hours later, cells were switched to N3 medium containing neuronal growth factors: CNTF (20 ng ml\(^{-1}\)), BDNF (10 ng ml\(^{-1}\)), NT3 (10 ng ml\(^{-1}\)), and GDNF (20 ng ml\(^{-1}\)) purchased from R&D systems, plus 2% FBS, Dox (1 μg ml\(^{-1}\)), FGFβ (10 ng ml\(^{-1}\)), 0.4% B27, forskolin 10 μM (FSK) (Sigma F8886), and DM 1 μM (AMPK Inhibitor, Compound C; Millipore 17126). Medium was changed every other day for 2–4 wk.

**iPSC and motor neurons differentiation.** The iPSC line was derived from peripheral blood mononuclear cells donated by a 58-year-old healthy Caucasian male, by introducing the episomal DNAs expressing Oct4, Sox2, Klf4, L-Myc, Nanog, and shRNA against p53. The selected clone was fully characterized and demonstrated a normal karyotype with normal self-renewal and differentiation capacity comparable to H9 human embryonic stem cells. NextGen sequencing data shows that no modifications occurred on ALS-related genes. iPSCs were differentiated into motor neurons using the proprietary differentiation protocol patented by iXCell Biotechnologies (Provisional Application no. 14559–001–888), summarized in Supplementary Fig. 9.

**ASO treatment.** ASOs mediating RNase H-dependent degradation of the TDP-43 mRNA, AAGGCTTCATATTGTACTTT (Ionis Pharmaceuticals), stathmin-2 mRNA, GGTCTTATGCAAGCTCAGAG (Ionis Pharmaceuticals), or murine Malat-1 as control, GGTCAGCTGCCAATGCTAG (Ionis Pharmaceuticals) were added to the iPSC-derived motor neurons culture medium at day 29 of maturation. Medium was changed every 3–4 d without addition of ASOs, and RNA was collected after 12 d of treatment (day 41). The MOE-gapper ASOs are 20 nucleotides in length, wherein the central gap segment comprising ten 2′-deoxyribonucleotides that are flanked on the 5′- and 3′-linkages in the 5′-phosphorothioate, except for the stathmin-2 ASO, which has phosphodiester linkages and 3′-methoxyethyl-modified nucleotides. The internucleotide linkages are 2′-deoxyribonucleotides that are flanked on the 5′- and 3′-linkages in the 5′-methoxyethyl-modified nucleotides.

**Compartmentalized microfluidic devices and anatomy.** The master molds to prepare the microfluidic devices were fabricated by photolithography, as previously described\(^9\), by the Bioengineering Department of the University of California, San Diego, Nano3 Cleanroom Facility. Two compartment devices were molded by soft lithography using Sygild 182 (Ellsworth Adhesives) as previously described\(^9\). Each compartment of the device was 108 μm wide, and 830 μm long. The proximal compartment contained one hole in each side of the compartment 8 mm in diameter, while the distal compartment's holes were 4 mm in diameter. These characteristics are important to perform an effective vacuum-based axotomy. After cutting, the cur devices were bath-sonicated in water, washed in 70% ethanol and sterilized under ultraviolet light before mounting onto glass coverslips. The devices were coated with matrigel (Corning, 356230) for 1 h at 37°C and rinsed with DMEM before plating the cells. Half a million iPSC-derived motor neuron precursors were plated in the proximal somatic compartment, and axonal growth to distal compartment was achieved in 8 d of maturation. ASOs were then added into the somatic compartment for 20 d of treatment, half media was changed every 4 d and a second dose of ASOs was added after 12 d. For aspiration-induced axotomy, media was simultaneously aspirated from one side and added back to the other side of the distal (axonal) compartment, until complete removal of axons from the distal compartment was confirmed.

**Lentiviral transduction.** A stathmin-2 open reading frame was cloned into a PL-SIN18-lentivector under transcriptional control of the phosphoglycerate kinase (PGK) promoter. HEK293T cells were used for packaging lentiviruses. Briefly, 0.5 × 10\(^5\) per well of 293T cells were seeded in a six-well plate. For lentiviral transfection, 2.5 μg of the lentiviral plasmid (containing stathmin-2 cDNA), 1.25 μg of pMD2.G, and 0.625 μg of psPAX2 were co-transfected to each well using Mirus transIT-X2 transfection reagent (Mirus). Culture medium was changed to fresh medium at ~24 h post transfection. Virus containing supernatants were collected at 48 h and 72 h after transfection and concentrated using ultracentrifugation. Viral concentrations were stored at ~80°C. Human iPSC-derived motor neurons were cultured to maturation and then infected with lentiviral particles encoding stathmin-2 in the presence of polybrene (Millipore).

**Cell viability assay.** Human iPSC-derived motor neurons were matured as described in Supplementary Fig. 9e, and then treated with control ASOs or ASOs targeting TDP-43 or stathmin-2 for 20 d. Viability of iPSC-derived human motor neurons was examined using LIVE/DEAD Reduced Biohazard Viability/Cytotoxicity Kit (Molecular Probes), according to the manufacturer’s instructions. Quantification of living cells was performed using the Olympus FV1000 confocal microscope.

**Human post-mortem tissues.** Human tissues were obtained using a short post-mortem interval acquisition protocol that followed Health Insurance Portability And Accountability Act (HIPAA)-compliant informed consent procedures and were approved by institutional review boards (IRBs) (Benaroya Research Institute, Seattle, WA IRB no. 10058 and University of California San Diego, San Diego, CA IRB no. 120056).

Tissue samples were obtained from patients who met the modified El Escorial criteria for definite ALS\(^8\). Control nervous systems were obtained from non-neurological patients when life support was withdrawn, or from patients on hospice. Autopsies were performed within 10 h of death, with an average post-mortem interval of 5 h for the cohort of patients used in this study.

Information on human samples is provided in Supplementary Table 5.

**Statistics.** Statistical tests were performed using GraphPad Prism. Student’s t-tests (two-tailed or one-tailed) were used as indicated in the text. No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those reported in previous publications. Experiments were not randomized. Data distribution was assumed to be normal but this was not formally tested. Data collection and analysis were not performed blind to the conditions of the experiments.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

All RNA sequencing data generated and analyzed for this study have been deposited in the Gene Expression Omnibus database under accession number GSE122069. The data that support the findings of this study are readily available from the corresponding authors upon reasonable request.

**References**

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Reporting Summary

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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

☐ n/a Confirmed
☐ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
☐ An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐ The statistical test(s) used AND whether they are one- or two-sided
☐ Only common tests should be described solely by name; describe more complex techniques in the Methods section.
☐ A description of all covariates tested
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☐ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated
☐ Clearly defined error bars
☐ State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

Data collection was done using software provided with the relevant equipment: Illumina HiSeq 4000, Bio-Rad CFX384 Real time PCR machine running Bio-Rad CFX Manager 3.1 (version 3.1.1517.0823), and Olympus FV1000 confocal microscope running controller software FV10-ASW Version 03.01.03.03.

Data analysis

Fiji/Image J software (version 2.0.0-rc-68/1.52e) was used to analyze confocal raw files, GraphPad Prism (version 5.0a) was used for statistical analyses. Bioinformatics: RNA sequencing analysis was performed using STAR (version 2.5.3a with default setting) and RSEM (version 1.3.0 with default setting). Differentially expressed genes were determined using DESeq2 (version 1.14.1). For more information see Methods.

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
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- A list of figures that have associated raw data
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The data that support the findings of this study are readily available from the corresponding authors upon reasonable request.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size          No statistical methods were performed to pre-determine sample sizes. Sample sizes were determined based on existing studies in the field to enable statistical analyses and reproducibility.

Data exclusions      No data was excluded.

Replication          Experiments were independently repeated, the numbers of biological replicates are presented in the Figures and text. Our attempts for replicating experiments were reliably reproduced.

Randomization        Randomization was not relevant to the experiments performed in this study. Experiments were not randomized.

Blinding             Data collection and analysis were not performed blind to the conditions of the experiments.

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☒   | Unique biological materials |
| ☒   | Antibodies |
| ☒   | Eukaryotic cell lines |
| ☒   | Palaeontology |
| ☒   | Animals and other organisms |
| ☒   | Human research participants |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☒   | ChIP-seq |
| ☒   | Flow cytometry |
| ☒   | MRI-based neuroimaging |

Antibodies

| Antibodies used                           |
|-------------------------------------------|
| Rabbit anti TDP-43 (1:1000) (ProteinTech, 10782-2-AP). Lot not available. |
| Rabbit anti Stathmin-2 (1:2000) (Cat#: nbp1-49461, NovusBiologics; Lot: D2) |
| Mouse anti Tubulin (1:10,000) (Sigma, Catalog # T6199; Lot: 048M4751V) |
| Mouse anti Beta III-Tubulin (1:1000) (BioLegend, Catalog # 801202; Lot: B205807) |
| Goat anti Lamin-B M-20 (1:1000) (SantaCruz Biotechnology, Catalog # SC-6217; Lot: F2514) |
| Rabbit anti HSP-90 (1:2000) (Cell Signaling Catalog # 4877s; Lot: 4) |
| Mouse anti NF-H (1:1000) (Millipore, Catalog # MAB5262, clone RT97) |
| Chicken anti MAP2 (1:1000) (NOVUS, Catalog # NB300-213; Lot: 7225-7) |
| Rabbit anti Nanog (1:200) (4903S, Cell Signaling; Lot# 6) |
| Mouse anti SOX2 (1:100) (MAB2018-SP, R&D Systems; Lot# KGQ0316111) |
| Rabbit anti OCT4 (1:200) (2840, Cell Signaling) |
| Mouse anti SSEA4 (1:200) (330401, Biolegend; Lot# B134262) |
Validation

Knockdown experiments of TDP-43 or stathmin-2 confirmed specificity of related antibodies by immunoblotting. LaminB specificity was determined by immunofluorescence. NF-H and MAP2 demonstrated distinct immunofluorescence staining in iPSC-derived motor neuron cultures. All antibodies that were used have been either validated in literature or commonly used by our colleagues in the field for similar approaches.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

SH-SY5Y cell were obtained from ATCC. Cells were cultured in 1:1 v:v mix of F12 (Gibco Cat# 31765-035) and Minimum Essential Medium (Gibco Cat# 11095-080) supplemented with 10% v:v Fetal Bovine Serum (omega scientific Cat # FB-16 Lot 531342), maintained at 50-70% confluence in a 37ºC incubator at 5% CO2 and passaged with 0.05% Trypsin-EDTA (Gibco Cat# 25300-054).

Fibroblast lines obtained from Dr. John Ravits were obtained from patient or familial control skin biopsies after informed consent, cultured in high-glucose DMEM (Sigma Cat# D5796) supplemented with 10% fetal bovine serum for two weeks without disturbance before media was refreshed. At weeks two through six of culture, media was supplemented with 10ug/ml Ciprofloxacin (Hospira Cat# 0409-4765-86) as a precautionary prophylactic against possible mycoplasma contamination that could be residual from clinical isolation. Cells were sequenced by PCR and Sanger sequencing for TDP43 mutational status to confirm clinical sequencing results. Lines obtained from other sources were accepted as-is, but tested for mycoplasma and Sanger sequenced to confirm TDP43 mutational status.

The iPSC line was derived from peripheral blood mononuclear cells donated by a 58 year old healthy Caucasian male, by introducing the episomal DNAs expressing Oct4, Sox2, Klf4, L-Myc, Nanog, and shRNA against p53. The selected clone was fully characterized and demonstrated a normal karyotype with normal self-renewal and differentiation capacity comparable with H9 human embryonic stem cells. NextGen sequencing data showed that no modifications occurred on ALS-related genes. iPS cells were differentiated into motor neurons using the proprietary differentiation protocol patented by iXCells Biotechnologies (Provisional Application # 14359-001-888).

Authentication

Human iPS cells were characterized for maintaining normal karyotype, pluripotency, and capability to differentiate into neuronal stem cells. SH-SY5Y cells were obtained from ATCC with certificate.

Mycoplasma contamination

All lines were tested negative for mycoplasma.

Commonly misidentified lines (See ICLAC register)

No commonly misidentified cell lines were used. Aside from the TDP43 mutational status confirmation, karyotyping, and mycoplasma testing, cells from non-commercial sources were not further genetically characterized to authenticate their identity.