ALS-implicated protein TDP-43 sustains levels of STMN2, a mediator of motor neuron growth and repair

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The findings that amyotrophic lateral sclerosis (ALS) patients almost universally display pathological mislocalization of the RNA-binding protein TDP-43 and that mutations in its gene cause familial ALS have nominated altered RNA metabolism as a disease mechanism. However, the RNAs regulated by TDP-43 in motor neurons and their connection to neuropathy remain to be identified. Here we report transcripts whose abundances in human motor neurons are sensitive to TDP-43 depletion. Notably, expression of STMN2, which encodes a microtubule regulator, declined after TDP-43 knockdown and TDP-43 mislocalization as well as in patient-specific motor neurons and postmortem patient spinal cord. STMN2 loss upon reduced TDP-43 function was due to altered splicing, which is functionally important, as we show STMN2 is necessary for normal axonal outgrowth and regeneration. Notably, post-translational stabilization of STMN2 rescued neurite outgrowth and axon regeneration deficits induced by TDP-43 depletion. We propose that restoring STMN2 expression warrants examination as a therapeutic strategy for ALS.

ALS is a neurodegenerative disease characterized by the loss of motor neurons 1. Patients with ALS experience progressive paralysis, usually succumbing to disease 1–5 years after diagnosis 2. Aside from two Federal Drug Administration approved drugs, which only modestly slow disease, treatment for ALS is limited to supportive care 3. ALS is recognized to be on the same pathological spectrum as frontotemporal dementia (FTD), the most common cause of pre-senile dementia 4. Although the causes of most ALS and FTD cases remains unknown, pathological findings and family-based linkage studies have demonstrated overlap in molecular pathways involved in both diseases 4. In a landmark finding, TDP-43 was discovered to be a major constituent of inclusions in many sporadic cases of ALS and FTD 5. TDP-43 is a predominantly nuclear DNA/RNA-binding protein with functional roles in transcriptional regulation, splicing, pre-microRNA processing, stress granule formation and messenger RNA transport and stability 6,7. Subsequently, autosomal-dominant mutations in the gene TARDBP were identified in both ALS and FTD families, linking genetics and pathology with neurodegeneration 8. Thus, elucidating the role that perturbations to TDP-43 play in disease is essential to understanding both sporadic and familial ALS.

Whether neurodegeneration associated with TDP-43 pathology is the result of loss-of-function mechanisms, toxic gain-of-function mechanisms or both remains unclear 8. Early studies showed that overexpression of wildtype and mutant TDP-43 led to its aggregation and nuclear loss 9. While these studies along with the autosomal-dominant inheritance pattern of TARDBP mutations support a gain-of-function view, the loss of nuclear TDP-43 and its aggregation suggests its normal functions might also become impaired. Subsequent findings revealed that TDP-43 depletion in the developing embryo or motor neurons has profound consequences 9.

Given the myriad roles TDP-43 plays, a key question is: what RNA substrates are perturbed on alterations of TDP-43 localization or by TDP-43 mutation and how do they contribute to neurodegeneration? Initial efforts to answer this question used cross-linking and immunoprecipitation with RNA-sequencing of whole brain homogenates 10. These studies led to an understanding that many transcripts are regulated by TDP-43 with a preference towards RNAs containing UG repeats and long introns; however, the prominence of glial RNAs in the samples limited insights into the neuronal targets of TDP-43. Consequently, few clear connections between the TDP-43 target RNAs and neurodegenerative mechanisms have been forged.

To identify substrates potentially contributing to neurodegeneration, we sought to identify RNAs regulated by TDP-43 in human...
motor neurons. Because the vulnerable neurons in living ALS patients are fundamentally inaccessible for isolation, we and others have developed approaches for directing the differentiation of human pluripotent stem cells into human motor neurons (hMNs)\textsuperscript{12–14}. Here, we performed RNA-sequencing (RNA-Seq) after TDP-43 knockdown in hMNs and identified TDP-43-regulated transcripts in this cellular context. In total, we found 885 transcripts for which TDP-43 was needed to maintain normal RNA levels. Although any number of these targets may play subtle roles in neurodegeneration, we noted that one of the most abundant transcripts in hMNs encoding STMN2, also known as SCG10 a regulator of microtubule stability, was particularly sensitive to a decline in TDP-43. Additionally, we determined that STMN2 levels were also decreased following pharmacological induction of TDP-43 relocalization from the nucleus and in induced pluripotent stem cell- (iPSC-) derived hMNs generated from patients with TDP-43 mutations\textsuperscript{15}. We further show that STMN2 encodes a protein necessary for normal hMN outgrowth and repair. Importantly, we established that post-translational stabilization of STMN2 through inhibition of c-Jun N-terminal kinases (JNK) can rescue deficits in motor neurite outgrowth and axon regeneration induced by TDP-43 knockdown. Finally, STMN2 depletion resulting from loss of TDP-43 activity is probably relevant to people with ALS, as we found that STMN2 expression is decreased in the spinal cord of ALS patients postmortem.

**Results**

**Motor neuron RNAs regulated by TDP-43.** To produce hMNs for studying RNAs regulated by TDP-43, we differentiated the human embryonic stem cell (hESC) line HUES3 Hb9::GFP\textsuperscript{+} into GFP\textsuperscript{+} hMNs under adherent culture conditions. On day 14 of differentiation, we routinely obtained cultures comprised of approximately 18–20%GFP\textsuperscript{+} cells. Subsequent analysis of these GFP\textsuperscript{+} hMNs following purification by flow cytometry demonstrated that they possessed expected functional and molecular properties (Supplementary Fig. 1a–i).

The reduced levels of nuclear TDP-43 observed in ALS has been hypothesized to contribute to downstream neurodegenerative events through alterations in the metabolism of the RNAs it binds. We, therefore, sought to identify the RNAs regulated by TDP-43 in purified hMNs through a combination of knockdown and RNA-Seq approaches. Using a fluorescently conjugated siRNA (siRED), we validated transfection conditions to achieve high-levels of siRNA Seq approaches. Using a fluorescently conjugated siRNA (siRED), purified hMNs through a combination of knockdown and RNA-seq. Using this method, we isolated neurons from five control iPSC lines and four iPSC lines with distinct TDP-43 mutations (Fig. 2a and Supplementary Fig. 5a). As anticipated, each cell line exhibited its own differentiation propensity into hMNs (Supplementary Fig. 4f–g), but after sorting we could readily obtain homogenous neurons and stem cells (Supplementary Fig. 4a). These studies revealed that by isolating NCAM\textsuperscript{+}/EpCAM\textsuperscript{−} cells, we could obtain neuronal cultures with reproducible properties (Supplementary Fig. 4h–i).

Using this method, we isolated neurons from five control iPSC lines and four iPSC lines with distinct TDP-43 mutations (Fig. 2a and Supplementary Fig. 5a). As anticipated, each cell line exhibited its own differentiation propensity into hMNs (Supplementary Fig. 4f–g), but after sorting we could readily obtain homogenous neuronal cultures from each line (Fig. 2b). We used these hMNs to explore how mutations in TARDBP influence TDP-43 localization, solubility and ability to regulate select RNA targets. The majority of mutations in TDP-43 are dominant missense mutations in the C-terminal glycine-rich domain, but how mutations trigger disease or if all mutations are equivalent remains unclear\textsuperscript{7}. Previous studies have reported that iPSC-derived neuronal cultures expressing mutant TDP-43 recapitulate some aspects of TDP-43 pathology including its accumulation in both soluble and insoluble cell protein extracts, as well as some cytoplasmic mislocalization\textsuperscript{24–26}. We first tested whether expression of mutant TDP-43 was sufficient to induce cytoplasmic mislocalization using immunofluorescence (Fig. 2b and Supplementary Fig. 5b). However, we did not find this to be the case. In both control and mutant neurons, we observed primarily nuclear TDP-43 staining using two different metrics. Pearson’s coefficient analysis revealed a strong correlation between TDP-43 immunostaining and the DNA counterstain for both groups (Fig. 2c). We also calculated the nuclear to cytoplasmic ratio of TDP-43 staining with no significant difference observed between
Increased abundance (392) in the TDP43-knockdown experiment were selected for validation by qRT-PCR. Data are displayed as mean with s.d. of technical replicates.

Differential transcript abundance scatter plot comparing TPM values for all transcripts expressed in hMNs treated with control siRNAs vs. the fold change in expression for those transcripts in cells treated with siTDP43.

Fig. 5d–e). These results are consistent with some TDP-43 iPSC disease modeling studies26, yet inconsistent with others24 and suggested that additional perturbations could be required to induce TDP-43 mislocalization27.

Although we did not detect gross changes to TDP-43, we hypothesized that TARDBP mutations might impact the metabolism of a subset of TDP-43 target RNAs. Thus, we collected RNA from hMNs and performed qRT-PCR to investigate levels of the transcripts most reproducibly impacted by TDP-43 depletion (ALOX5AP, STMN2, ELAVL3, PFKP and RCAN1). For three of the genes (STMN2, PFKP and ELAVL3), we observed a significant decrease in abundance.
(Fig. 2d). Consistent with the TDP-43 depletion experiments, we did not observe significant changes to the abundance of the closely related STMN1 RNA suggesting a specific relationship between TDP-43 and STMN2 (Fig. 2d and Supplementary Fig. 6e). Additionally, we did not observe significant differences in TARDBP transcript levels between mutant and control neurons (Fig. 2d).

Fig. 2 | A subset of transcripts with altered abundance after TDP-43 depletion also displayed altered abundance in hMNs expressing mutant TDP-43.

a, Strategy for assessing candidate TDP-43 target transcripts in ALS patient iPSC-derived hMNs expressing mutant TDP-43. b, Representative micrographs of control and patient neurons immunostained for TDP-43 (red), β-III tubulin (green) and counterstained with DAPI (blue). Scale bar, 100 μm. n = 4 control and three patient lines with similar results in two independent experiments. c, Pearson’s correlation analysis for TDP-43 immunostaining and DAPI fluorescence comparing control neurons to those with TDP-43 mutations. Dots represent individual cells and are displayed as mean with s.d. for 60 cells from n = 4 control and three patient lines (unpaired t test, two-sided, P < 0.05). Two independent experiments were performed. d, qRT-PCR analysis of the transcripts with altered abundance after TDP-43 knockdown in neurons differentiated from controls or TDP-43 patients. Data are displayed as mean with s.d. from two independent experiments with n = 4 control and three patient lines in experiment 1 and n = 5 control and four patient lines in experiment 2 (unpaired t test, two-sided, P < 0.05).
TDP-43 regulates STMN2 levels. Due to their broad expression pattern, previous studies have detected links between both PKP and as well as ELAVL3,28,29 and TDP-43. We, therefore, focused our efforts on exploring how STMN2, which shows a more neuroanatomically restricted expression pattern, might be regulated by TDP-43. STMN2 is one of four proteins belonging to the Stathmin family of microtubule-binding proteins, with functional roles in neuronal cytoskeletal regulation and axonal regeneration pathways.30,31,32 In humans, STMN1 and STMN3 transcripts exhibit ubiquitous expression, whereas STMN2 and STMN4 are enriched in central nervous system tissues.33 Considering the growing evidence for cytoskeletal defects in ALS22–24 and the importance of cytoskeletal function in projection neurons, characterizing a putative relationship between STMN2 and TDP-43 seemed to be of potential relevance to ALS.

To this end, we first examined whether the decline in the STMN2 transcript abundance after TDP-43 knockdown was reflected at the protein level. Following independent RNAi experiments, we performed qRT-PCR with two different sets of primer pairs and found STMN2 (~50–60%) transcript abundance significantly reduced in hMNs relative to controls (Fig. 3a). Immunoblot assays on protein lysates from these same hMN cultures demonstrated STMN2 protein levels were also significantly reduced following siTDP-43-treatment (Fig. 3b).

To probe how selective the changes in STMN2 levels were to TDP-43 knockdown, we knocked down two additional ALS-linked genes, FUS and C9ORF72.41 FUS protein is structurally similar to TDP-43 and is also involved in RNA metabolism. The function of C9ORF72 is under investigation, but a large intronic GGGGCC repeat expansion is responsible for a substantial number ALS and FTD cases through three proposed mechanisms: the creation of long repetitive RNAs, the translation of these repeats into toxic, repetitive dipeptides or haploinsufficiency. Following induction of RNAs targeting TDP-43, FUS or C9ORF72, we found significant downregulation of the respective siRNA-targeted genes by qRT-PCR (Supplementary Fig. 6a–c). Although knockdown of TARDBP reduced levels of STMN2, STMN2 levels were unaltered following knockdown of FUS and C9ORF72 (Fig. 3c). These results confirm that STMN2 downregulation was not a trivial consequence of RNAi induction. Furthermore, these data suggest that reduced function of FUS and C9ORF72 were not sufficient to induce a reduction in STMN2 abundance.

TDP-43 can bind to and regulate several aspects of RNA metabolism. To determine whether TDP-43 associated directly with STMN2 RNA, which contains many TDP-43 binding motifs (Supplementary Fig. 6a,g), we developed conditions for TDP-43 immunoprecipitation and subsequent formaldehyde RNA immunoprecipitation ( RIP) (Fig. 3d). After reversal of cross-linking, we performed qRT-PCR to detect RNA molecules bound to TDP-43. We first examined whether transcripts encoding TDP-43 itself were enriched as this autoregulation is well-established.34 Indeed, we observed enrichment of TARDBP RNA following TDP-43 pull-down, but not in an IgG control or when we pulled down a distinct ALS-associated protein, SOD-1 (Fig. 3e). We next asked whether additional transcripts influenced by TDP-43 knockdown, housekeeping transcripts (PGKI, UBC, GAPDH), or a motor neuron-specific transcript (MNX1) were enriched. Of these, we observed strong and significant enrichment for STMN2 transcripts (Fig. 3e).

TDP-43 suppresses cryptic exon inclusion. Emerging evidence highlights the importance of nuclear TDP-43 in suppressing non-conserved or cryptic exons to maintain splicing integrity.35 When cryptic exons are included in RNA transcripts, their inclusion can affect transcript abundance by disrupting translation and promoting nonsense-mediated decay.36 Interestingly, little or no overlap in the genes regulated by TDP-43 through cryptic exon suppression has been observed between mouse and man.35 We, therefore, examined our motor neuron sequencing data for evidence of cryptic exons in the 95 human genes reported to contain TDP-43-regulated cryptic exons.36 We found reads mapping to cryptic exons in nine of these genes including PKP (Supplementary Fig. 7a). Encouraged, we used our data to search for neuronal transcripts that might be regulated by this mechanism. We identified cryptic exons in both ELAVL3 and STMN2 (Supplementary Fig. 7b,c). In the case of STMN2, we observed splicing tracks emanating from exon 1 and entering a cryptic exon, but no splice ribbons from the cryptic exon to either exon 2 or other exons downstream (Fig. 3f,g). Moreover, the inclusion of this cryptic exon would lead to a very early stop codon (Supplementary Fig. 10h). As a result, STMN2 transcripts that include this cryptic exon cannot yield functional STMN2 protein.

STMN2 levels tuned by TDP-43 levels and localization. TARDBP mutations and overexpression have been proposed to perturb autoregulation of its transcript leading to altered TDP-43 protein levels.37 Thus, we asked whether expression of additional wildtype or mutant TDP-43 could act dominantly to influence expression of STMN2. To answer this question in a controlled manner, we knocked-in a single copy of either wildtype TDP-43 or mutant TDP-43 M337V into the AAVS1 safe harbor locus of the HUES3 H9:GFP cell line (Supplementary Fig. 8a). Genotyping of the AAAS1 locus confirmed single copy integration of each construct (Supplementary Fig. 8b). Following directed differentiation, GFP+ hMN purification and RNA isolation, we found that additional expression of either wildtype or mutant TDP-43 significantly depressed the abundance of STMN2 (Supplementary Fig. 8c). These data together with our siRNA data indicate that both depleting and increasing TDP-43 levels can alter STMN2 expression.

Aggregation and loss of nuclear TDP-43 are pathological hallmarks of ALS. To investigate a potential connection between these postmortem findings and STMN2 abundance, we explored whether MG-132-mediated proteasome inhibition could influence TDP-43 localization in hMNs and serve as a potential model of acute TDP-43 dysfunction. After establishing the range and timing of tolerable proteasome inhibition (Supplementary Fig. 9a–c), we performed a pulse-chase experiment to determine the consequences of proteasome inhibition on TDP-43 localization (Supplementary Fig. 10a). Strikingly, using the Pearson’s coefficient analysis and nuclear to cytoplasmic ratio, we observed that TDP-43 staining in the nucleus was greatly diminished after 24h (Supplementary Fig. 10b–d). Following MG-132 washout, we found that TDP-43 staining became indistinguishable from that in unchallenged neurons after 4days (Supplementary Fig. 10b–d). Thus, proteasome inhibition in hMNs induced reversible TDP-43 mislocalization.

In ALS patients, TDP-43 mislocalization is associated with accumulation of insoluble TDP-43 protein. After proteasome inhibition in hMNs, we examined TDP-43 levels by immunoblot analysis in both the detergent-soluble and detergent-insoluble fractions. In lysates from neurons treated with MG-132 (Supplementary Fig. 10a), we observed a significant decline in soluble TDP-43 (Supplementary Fig. 10e), which could be readily explained by an increase of insoluble TDP-43 found after urea exposure (Supplementary Fig. 10e). After establishing that proteasome inhibition induced TDP-43 mislocalization and insolubility, we asked if this change in TDP-43 also influenced STMN2 transcript levels and again found a significant decline in its abundance (Supplementary Fig. 10f). We next used RT-PCR with primers spanning into the STMN2 cryptic exon observed after TDP-43 depletion to ask if proteasome inhibition also induced its inclusion in hMNs (Supplementary Fig. 10g). Although hMNs growing under normal conditions showed no detectable evidence for this cryptic event, following proteasome inhibition, it could be readily observed (Supplementary Fig. 10g) Sequencing of the PCR product confirmed the splice junction and...
premature stop codon within the cryptic splice-form predicted by our RNA-seq analysis (Supplementary Fig. 10h). These data connect impaired protein homeostasis to TDP-43 localization and solubility as well as STMN2 levels. We found it notable that an identical cryptic splicing event was induced in STMN2 following either RNAi-mediated TDP-43 depletion or proteasome inhibition induced TDP-43 insolubility.

**STMN2 localization in human neurons.** Given the importance of TDP-43 in ALS and its tight regulation of STMN2, we wondered...
if alterations in STMN2 might influence motor axon biology. Supporting previous expression studies, we found that STMN2 protein was selectively expressed in differentiated neurons and could not be as readily detected in stem cells or neuronal progenitors (Supplementary Fig. 6d). We used immunocytochemistry to probe the subcellular localization of STMN2 and found it both at neurite tips and in peri-nuclear regions of the cell bodies (Fig. 4a). Further co-staining for the Golgi-associated protein GOLGIN97, indicated STMN2 was localizing to this subcellular compartment (Fig. 4b). On the basis of rodent studies, STMN2 would be predicted to function at the human growth cone during neurite extension and repair. When we stained hMNs after differentiation and sorting, we observed strong staining of STMN2 at the interface between microtubules and F-actin bundles; cytoskeletal components defining the growth cone (Fig. 4c).

**TDP-43 depletion inhibits neurite and axon growth.** On the basis of predicted STMN2 function, we investigated whether TDP-43 knockdown compromised neurite outgrowth and axonal regeneration in hMNs. Three days after siTDP43 transfection, hMNs were fixed and stained for β-III-tubulin to label neuronal processes. Sholl analysis, which quantifies the number of neurite branches at a given interval from the center of the soma, revealed significantly reduced neurite complexity in neurons treated with siTDP-43 (Fig. 5a–c).

To test if reduced TDP-43 levels inhibited axonal regrowth after injury, we plated hMNs in microfluidic devices that permitted axon growth into a chamber distinct from the neuronal cell bodies. Neurons cultured for 7 days in the soma compartment of the device extended axons through the microchannels into the axon chamber. We severed axons without disturbing cell bodies in the soma compartment and then measured axon extension from the microchannel across a time course to assess regrowth after injury. Our analysis revealed significantly reduced regrowth in the siTDP-43-treated neurons for all time points measured (Fig. 5d–f).

**STMN2 is necessary for normal neurite growth.** Because TDP-43 regulates many transcripts, we confirmed that loss of STMN2 was sufficient to induce phenotypes similar those observed after
TDP-43 knockdown. We used CRISPR/Cas9 to produce a large deletion within STMN2 in two independent hES cell lines (WA01 and HUES3 Hb9::GFP). We confirmed loss of protein expression in differentiated hMNs by both immunoblotting and immunocytochemistry (Fig. 6a–d). We then characterized neurite outgrowth in STMN2−/− mutant hMNs (Fig. 6e). After 7 days in culture, Sholl analysis revealed significantly reduced neurite extension in STMN2−/− mutant neurons relative to controls (Fig. 6f,g). Separately, we cultured neurons in the presence of a ROCK inhibitor, Y-27632, which has been shown to increase neurite extension. The difference in neurite outgrowth was even more striking in these experiments with the molecule enhancing the outgrowth of the STMN+/+ line but not the STMN−/− line (Fig. 6h). Inactivation of STMN2 in the WA01 background resulted in hMNs with similar outgrowth phenotypes (Supplementary Fig. 11). We also queried whether STMN2 functions in neuronal repair after injury (Fig. 6i). To this end, hMNs were cultured for 7 days in microfluidic devices before performing axotomy. Our experiments revealed
Fig. 6 | STMN2 mutant neurons have neurite outgrowth and regrowth deficits similar to neurons treated with siTDP43. a, Knockout strategy targeting two constitutive exons of the human STMN2 gene. Ex., exon. b–d, STMN2 elimination was confirmed in the HUES3 Hb9::GFP line by RT-PCR analysis of genomic DNA (b), by immunoblot analysis (c) and by immunofluorescence (d). Similar results were obtained from n = 2 biologically independent experiments. e, Experimental strategy used to assess the cellular effect of STMN2 elimination in hMNs. f–h, Sholl analysis of hMNs with and without STMN2 and in the absence (g) or presence (h) of an ROCK inhibitor (Y-27632, 10 μM). Lines represent sample means and shading represent the s.e.m. with unpaired t test between siTDP43 and siSCR, two-sided, \( P < 0.05 \), with all values in Supplementary Table 1. Similar results were obtained in n = 2 biologically independent experiments. i, Experimental strategy used to assess the cellular effect of STMN2 elimination in hMNs after axonal injury. j,k, Axonal regrowth after injury: representative micrographs of hMNs in the microfluidics device prior to and after axotomy (j). Measurements of axonal regeneration after axotomy. Individual neurites are displayed as dots along with the mean and s.d. (unpaired t test, two-sided, \( P < 0.05 \), \( 18\ h = 0.0005, 24\ h = 0.0001, 48\ h \leq 0.0001 \)) (k). Similar results were obtained in n = 4 devices from two independent experiments. Similar results were obtained in n = 2 biologically independent experiments.
significantly reduced regrowth in the STMN2−/− mutant hMNs when compared to STMN2+/+ controls for all time points measured (Fig. 6j,k and Supplementary Fig. 11).

**STMN2 is altered in postmortem ALS spinal cord.** We next asked if our findings were relevant to the motor neurons of ALS patients in vivo. To this end, we used immunohistochemistry to investigate

![Image](https://via.placeholder.com/150)

**Fig. 7 | ALS patient spinal cord motor neurons have decreased expression of STMN2 and express transcripts containing the cryptic exon.** a–c, Histologic analysis of human adult lumbar spinal cord from postmortem samples collected from a subject with no evidence of spinal cord disease (control) (a) or two patients diagnosed with sporadic ALS (b,c). The experiment was performed with n = 3 controls and three ALS cases. STMN2 immunoreactivity in lumbar spinal motor neurons from control and ALS cases was scored as ‘strong’ or ‘absent’. Scale bars, 50 μm. d, The percentage of lumbar spinal motor neurons with strong STMN2 immunoreactivity was significantly lower in ALS tissue samples. Data are displayed as the mean with s.d. for n = 3 controls and three ALS cases; approximately 40 motor neurons were scored for each subject (unpaired t test, two-sided, P < 0.05). e–g, Meta-analysis of STMN2 transcript abundance in previously published datasets for laser-captured lumbar motor neurons analyzed by microarray, n = 10 controls and 12 ALS cases displayed (Rabin et al., ref. 38 e) and laser-captured lower motor neurons analyzed by microarray, n = 6 controls and six ALS cases (Highley et al., ref. 39 f), with individuals displayed as dots with mean and s.d. (moderated t test, P < 0.05, e,f); and spinal cord ventral horns analyzed by RNA-Seq for n = 8 controls and nine ALS cases, with individuals displayed as dots with mean (D’Erchia et al., ref. 40, Wald test and a cutoff of 0.1 for Benjamini–Hochberg adjusted P values with no log, fold-change ratio cutoff, g). h–i, Visualization of the cryptic exon for STMN2 from the NINDS datasets for the ventral horns of controls (h) and sporadic ALS patient (i) spinal cords. Read coverage and splice junctions are shown for alignment of the samples to the human hg19 genome. Splice ribbons from exon 1 to the cryptic exon are highlighted in orange.
STMN2 expression in control and ALS patient postmortem spinal cord. Similar to our observations in stem cell-derived hMNs, strong STMN2 immunoreactivity was present in the cytoplasmic region of lumbar spinal hMNs, but absent in the surrounding glial cells (Fig. 7a–c). We determined the percentage of hMNs exhibiting STMN2 immunoreactivity in lumbar spinal cord tissue sections in three controls and in three ALS cases. Consistent with our hypothesis, we found that the percentage of lumbar hMNs with immunoreactivity to STMN2-specific antibody was significantly reduced in sporadic ALS cases (Fig. 7d). Our results are further supported by several independent expression analyses of ALS postmortem spinal cord samples\(^{38-40}\). We independently interrogated these data and...
Finally, we demonstrated JNK inhibition could rescue STMN2 levels after TDP-43 depletion (Fig. 8c). Interestingly, partial depletion of FUS and C9ORF72 did not alter STMN2 abundance. It is well known that patients with C9ORF72 display TDP-43 pathology and consistent with this we found reduced STMN2 levels in postmortem material from such patients. These findings suggest that either the gain of function effects of the C9ORF72 mutation or further events subsequent to knockdown are required to induce TDP-43 pathology and reduced STMN2 levels. In the instance of patients with mutations in FUS who do not display TDP-43 pathology, it seems plausible that distinct defects in RNA metabolism underlie neuropathy. While they are beyond the scope of this study, further studies with iPSC-derived neurons from FUS and C9ORF72 patients could shed light on these hypotheses.

The Stathmin family of proteins are regulators of microtubule stability46. Using gene editing we demonstrated STMN2 has important functions in both motor axon outgrowth and regeneration, phenocopying results in neurons with diminished TDP-43 levels. Although hMNs generated in vitro share many molecular and functional properties with bona fide motor neurons13, the in vivo validation of stem cell-based ALS model discoveries is a critical test of their relevance46. Using spinal cord tissues and published expression studies, we provide in vivo evidence corroborating the finding that STMN2 levels are altered in ALS. Furthermore, we found that the probable mechanism for this decline, as in our cell models, was through splicing into a cryptic exon. Finally, we demonstrate JNK inhibition increases STMN2 levels, which was sufficient to rescue axonal phenotypes induced by TDP-43 depletion. While we suggest that restoring STMN2 levels in ALS patients to normal levels is an attractive target, it is important this be done with care as overexpression of Stathmins can cause Golgi fragmentation as shown in the transgenic SOD1 mouse model of ALS47.

Discussion
An outstanding question has been: what are the mechanistic consequences of TDP-43 mutations and how do their effects relate to the events that occur when TDP-43 becomes pathologically re-localized in patients? Identifying transcripts regulated by TDP-43 provided us the opportunity to explore the potential impact of different types of TDP-43 manipulations relevant to both familial and sporadic disease. First, we addressed whether a subset of the target RNAs identified after TDP-43 depletion change in hMNs produced from patients with TDP-43 mutations. Interestingly, we found modest but significant changes in the expression of a number of interesting genes41 including the microtubule regulator STMN2. Thus, cells expressing patient mutations displayed partial loss of function. But if so, how can their dominant nature be explained?

Upon overexpression, TDP-43 is prone to aggregation, and some studies suggest mutant TDP-43 is prone to aggregation when expressed at native levels in patient-specific motor neurons42-45. To determine whether aggregation or loss of nuclear mutant TDP-43 could be contributing to decreased expression, we carefully monitored TDP-43 in these patient hMNs, but could not detect such a defect. We are, however, confident that if larger scale aggregation, or nuclear loss of mutant TDP-43 were occurring in iPSC hMNs that we would have detected it, as we found that proteasome inhibition induced dramatic nuclear loss of TDP-43 along with its insoluble accumulation. These results are akin to our previous results with mutant SOD-1 being selectively insoluble after proteasome inhibition44. We also found these data captivating because disrupted proteostasis induced by any number of ALS implicated mutations or events could be upstream of the most common histopathological finding in sALS, TDP-43 pathology and our finding that TDP-43 targets are depleted following proteasome inhibition is consistent with that model. This model is attractive because it reconciles how overexpression of an additional transgenic copy of TDP-43 might lead to a decrease in STMN2 expression. Two recent publications describing mice with endogenous TDP-43 mutations also provide insight into the potential gain and loss of TDP-43 functions. Both studies suggest splicing abnormalities are a major consequence of TDP-43 mutations45,46. Our study also spotlights splicing defects downstream of TDP-43 perturbations, and, because of the lack of cryptic exon conservation among species45, showcases the advantage of human cellular disease models in identifying STMN2 RNA as a TDP-43 target.

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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/s41593-018-0300-4.

Received: 25 May 2018; Accepted: 13 November 2018; Published online: 14 January 2019
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Methods
Cell culture and differentiation of hESCs and human induced pluripotent stem cells into motor neurons. Pluripotent stem cells were grown with mTeSR1 medium (Stem Cell Technologies) on tissue culture dishes coated with Matrigel (BD Biosciences), and maintained in 5% CO2 incubators at 37 °C. Stem cells were passaged as small aggregates of cells after 1 mM EDTA treatment. 10 μM ROCK inhibitor (Sigma, Y-27632) was added to the cultures for 16–24 h after dissociation to prevent cell death. The HUES3 H9::GFP cell line has been previously described (1–3). Motor neuron differentiation was achieved using a modified 14-day strategy. This approach relies on neural induction through small molecule inhibition of SMAD signaling, accelerated neural differentiation through FGF and NOTCH signaling inhibition and motor neuron patterning through the activation of retinoic acid and Sonic Hedgehog signaling pathways. In brief, embryonic stem cells were dissociated to single cells using accutase (Stem Cell Technologies) and plated at a density of 80,000 cells per cm² on Matrigel-coated culture plates with mTeSR1 medium (Stem Cell Technologies) supplemented with ROCK inhibitor (10 μM Y-27632, Sigma). When cells reached 100% confluency, medium was changed to differentiation medium (½ Neurobasal (Life Technologies) ½ DMEM-F12 (Life Technologies) supplemented with b-27 supplement (Gibco), x1 N-2 supplement (Gibco), x1 Gibco GlutaMAX (Life Technologies) and 100 μM non-essential amino acids (NEAA)). This time point was defined as day 0 (d0) of motor neuron differentiation. Treatment with small molecules was carried out as follows: 10 μM SB431542 (Custom Synthesis), 100 nM LDN-193189 (Custom Synthesis), 1 μM retinoic acid (Sigma) and 1 μM Smoethond agonist (Custom Synthesis) on d0–d5; 5 μM DAPT (Custom Synthesis), 4 μM SU-5402 (Custom Synthesis), 1 μM retinoic acid (Sigma) and 1 μM Smoethond agonist (Custom Synthesis) on d6–d14.

Cell acquisition and authentication. The hESCs and iPSCs used in this study were previously approved by the institutional review boards of Harvard University, Massachusetts General Hospital and Columbia University. Specific point mutations were confirmed by Sanger sequencing. Cell line authentication was performed using a lab checks for mycoplasma contamination using the MycoAlert kit (Lonza) with no positive cultures identified. Cells were confirmed by PCR amplification followed by Sanger sequencing. Weekly, our lab checks for mycoplasma contamination using the MycoAlert kit (Lonza) with no positive cultures identified. Cells were confirmed by PCR amplification followed by Sanger sequencing.

FACS of hMNs. After 14 days, differentiated cultures were dissociated to single cells using accutase treatment for 1 h inside a 5% CO2 per 37 °C incubator. Repeated (10–20 times) but gentle pipetting with a 1,000 μl pipette tip was used to achieve a single cell preparation. Cells were spun down, washed x1 with PBS and resuspended in sorting buffer (x1 cation-free PBS 15 mM HEPES at pH 7.5 (Gibco), 1% BSA (Gibco), x1 penicillin-streptomycin (Gibco), 1 mM EDTA and DAPI (1 μg/ml)). Cells were passed through a 45 μm filter immediately before FACS analysis. The BD FACs Aria II cell sorter was routinely utilized to purify H9-derived hMNs into cell collection tubes containing motor neuron medium (Neurobasal (Life Technologies), x1 N-2 supplement (Gibco), B-27 supplement (Gibco), GlutaMAX and NEAA) with 10 μM ROCK inhibitor (Sigma, Y-27632) and 10 μM CHIR99021 (Sigma) in conditioned media. Cells were then incubated overnight at 4 °C with primary antibody (diluted in blocking buffer) with 20 μM DAPT (Sigma, 4 μM SU-5402) (Custom Synthesis), 1 μM retinoic acid (Sigma) and 1 μM Smoethond agonist (Custom Synthesis) on d6–d14.

RNA preparations. RNA was isolated from cells using TRIzol LS (Invitrogen) according to manufacturer’s instructions. 200 μl of TRIzol buffer was added per well of the 24-well culture. A total of 300–1,000 ng of total RNA was used to perform complementary DNA (cDNA) synthesis. Reaction conditions for the iscript kit (Bio-Rad). qPCR–PCRamplification was performed using SYBR green (Bio-Rad) and the iCycler (Bio-Rad). Quantitative levels for all genes assayed were normalized using GAPDH expression. Normalized expression was displayed relative to the control sample. For comparison between patient lines, normalized expression was calculated relative to the pooled data of all samples. A two-tailed t-test or a Mann-Whitney U test comparing control and ALS cell lines was performed with five control lines and four patient lines with three technical replicates. All primer sequences are available on request. For next-generation RNA-Seq, two independent experiments were performed (Exp. 1 and Exp. 2) with multiple unique siRNAs used for the two groups control (siRED, siSCR1 and siSCR2) versus siTDP43 (stTDP1 and stTDP2) with a total of n = 15 samples. After RNA extraction, samples with RNA integrity numbers above 7.3, determined by a BioAnalyzer, were used for library preparation. In brief, RNA sequencing libraries were generated from ~250 ng of total RNA using the Illumina TrueSeq RNA kit V2, according to the manufacturer’s directions. Libraries were sequenced at the Harvard Bauer Core Sequencing facility on a HiSeq 2000 platform. All FASTQ files were analyzed using the hcsvialSeq workflow and toolchain (4). The FASTQ files were aligned to the GRCh37/hg19 reference genome. Differential expression testing was performed using DESeq2 suite of bioinformatics tools (5). The DEXSeq module of Bioconductor was used to identify differential splicing (6). We used salmon to generate the counts and trimmomatic to load them at gene level (7). All P-values are corrected for multiple comparisons using the method of Benjamini and Hochberg (8). We used an adjusted P value cutoff of 0.05 with no log fold-change ratio cutoff for differential gene expression analysis and a 0.1 cutoff with no log fold-change ratio cutoff for differential exon usage.

TDP-43 localization analysis. For analysis of TDP-43 nuclear localization analysis, hESCs were co-transfected with TDP-43 (ProteinTech), β-III tubulin (R&D Systems) and counterstained with DAPI. Images were acquired using a Nikon Eclipse Ti microscope. β-III tubulin staining was used to determine the cell body as the...
region of interest and then the Pearson's correlation coefficient was calculated for TDP-43 and DAPI staining using NIS-Elements (Nikon) with a minimum of 30 neurons analyzed. NIS-Elements was used to segment cells into the nuclear (DAPI positive) and cytoplasmic (p-III tubulin positive DAPI negative) regions. The mean TDP-43 intensity in these two regions were used to calculate the nuclear to cytoplasmic ratio for TDP-43 immunofluorescence.

Electrophysiology recordings. GFP+ motor neurons were plated at a density of 5,000 cells per cm² on poly-D-lysine/laminin-coated coverslips and cultured for 10 days in MN condition media (containing serum and replaced with mouse glial cells and supplemented with 10 ng/ml of each GDNF, BDNF and CNTF (R&D Systems). Electrophysiology recordings were carried out as previously reported. Briefly, whole-cell voltage-clamp or current-clamp recordings were made using a Multiclamp 700B (Molecular Devices) at room temperature (21–23°C). Data were digitized with a Digidata 1440A A/D interface and recorded using pCLAMP 10 software (Molecular Devices). Data were sampled at 20 kHz and low-pass filtered at 2 kHz. Patch pipettes were pulled from borosilicate glass capillaries on a Sutter Instruments P-97 puller and had resistances of 2–4 MΩ. The pipette capacitance was reduced by wrapping the shank with Parafilm and compensated for using the amplifier circuitry. Series resistance was typically 5–10 MΩ, always less than 15 MΩ, and compensated by at least 80%. Linear leak currents were digitally subtracted using a P/4 protocol. Voltages were elicited from a holding potential of −80 mV to test potentials ranging from −80 to 30 mV in 10 mV increments. The intracellular solution was a potassium-based solution and contained KCl, 150; MgCl₂, 2; HEPES, 10; Mg ATP, 4; EGTA, 1 (pH 7.4 with KOH). NaCl, 135; KCl, 5; HEPES, 10; pH 7.4 with NaOH). Kainate was purchased from Sigma. For multielectrode array analysis, hMNs were plated onto the multielectrode array plate and co-cultured with mouse p0–2 astrocytes (Axion Biosystems M768-KAP-96). The neuronal activities including spike and bursting number for individual wells were measured and analyzed by Axion NeuroMetric tool software.

Formaldehyde RNA immunoprecipitation. One well of a six-well plate of hMNs (2 million cells) were crosslinked and processed according to the MagnaRIP instructions (Millipore). The following antibodies were used in this study: SOD1 (1/50, Cell Signaling Technologies), TDP-43 (1/500, gift of D. Cleveland) and motor neuron (1/500, Cell Signaling Technology). We generated short-tagged RIP RNA 5' fractions' Ct value to the Input RNA fraction Ct value for the same quantitative PCR assay to account for RNA sample preparation differences. To calculate the dCt[normalized RIP], we determined Ct[Input] = Ct[Input] − log2 (Input Dilution Factor), where the dilution factor was 100 or 1%. To determine the fold enrichment, we calculated the dCt[normalized IgG] then fold enrichment = 2^−dCt.

STMN2 knockout generation. STMN2 guide RNAs were designed using the following web resources: CHOPCHOP (https://chopchop.ucsf.edu) from the Schier Laboratory. Guides were cloned into a vector containing the human STMN2 transcript (synthetic transcript) followed by the cloning site available by cleavage with BslII, as well as ampicillin resistance. To perform the cloning, all the gRNAs were modified before ordering. The following modifications were used to generate overlaps compatible with a BslII sticky end: if the 5' nucleotide of the sense strand was not a G, this nucleotide was removed and substituted with a G; for the reverse complement strand, the most 3' nucleotide was removed and substituted with a G, while AAc was added to the 5' end. The resulting modified STMN2 gRNA sequences were used for Cas9 nucleosome genome editing: guide 1: 5'- CACCGTTATAGTATGTAGTGGCC 3' (Exon 2), guide 2: 5'- CACCTGAAACAATTGGCAAGAG 3' (Exon 3), guide 3: 5'- CACAGCTCTCCAGAAGGCTTTGG 3' (Exon 4). Cloning was performed by first annealing and phosphorylating both the gRNAs in PCR tubes. 1 µl of both the strands at a concentration of 100 µM was added to 1 µl of T4 PNK (New England Biolabs), 1 µl of T4 ligation buffer and 6 µl of H₂O. The tubes were placed in the thermocycler and incubated at 37°C for 30 min, followed by 5 min at 95°C and a slow ramp down to 25°C at a rate of 5°C per min. The annealed oligos were subsequently digested and phosphorylated with the gRNAs in PCR tubes. 1 µl of both the strands at a concentration of 100 µM was added to 1 µl of T4 PNK (New England Biolabs), 1 µl of T4 ligation buffer and 6 µl of H₂O. The tubes were placed in the thermocycler and incubated at 37°C for 30 min, followed by 5 min at 95°C and a slow ramp down to 25°C at a rate of 5°C per min. The annealed oligos were subsequently digested and phosphorylated with the gRNAs in PCR tubes. The PCR products were analyzed on a 1% Agarose Gel. In brief, the targeted sequence was PCR amplified by a pair of primers external to the deletion, designed to produce a 1,100 base pair deletion-band to detect deleted clones. Sequences of the primers used are as follows: OUT_FWD, 5'- GCAAGAGGCTCTAAGTGCGC 3' and OUT_REV, 5'- GGAAGGTGACCTGACTGCTGC 3'. Knockout lines were further confirmed using immunoblot analysis.

Neurite outgrowth assay. Individual TuJ1-positive neurons used for Sholl analyses were randomly selected and imaged using a Nikon Eclipse TE300 with a XPlan 20 objective (40×). The neurite length and surface area were traced using the ImageJ (NIH) plugin NeuroJ, and Sholl analysis was carried out using the Sholl tool of Fiji, quantifying the number of intersections at 10-µm intervals from the cell body. The Sholl analysis was carried out on a minimum of n = 50 neurons. Statistical analysis was carried out by comparing the number of intersections of knockout clones with the parental wildtype line for each 10-µm interval using Prism 7 (GraphPad). Significance was assessed by a standard Student's t-test, with a P value of P < 0.05 considered as significant.

Aneurysm. Sorted motor neurons were cultured in standard neuron microfluidic devices (SND150, XONIA Microfluidics) mounted on glass coverslips coated with 0.1 mg/ml poly-L-ornithine (Sigma-Aldrich) diluted in 50 mM Borate buffer, pH 8.5 and 3 µg/ml laminin (Invitrogen) at a concentration of around 250,000 neurons per device. Aneurysm was performed at day 7 of culture by repeated vacuum aspiration and reperfusion with x1 PBS in the axon chamber until axons were cut effectively without distorting cell bodies in the soma compartment. For HUES3 experiments, two independent replicates were performed with the first using one device and 20–30 neurites measured, and for the second experiment three devices were used with 70–90 neurites measured. For the WA01 cell lines, one replicate was performed with two devices and 50–60 neurites measured.

TDP-43 and STMN2 immunohistochemical analyses. Postmortem samples from three sporadic ALS cases (1 male, 2 female) and three controls (2 male, 1 female) (no evidence of spinal cord disease) were gathered from the Massachusetts Alzheimer’s Disease Research Center in accordance with Partners and Harvard International Review Board protocols. Histologic analysis of TDP-43 immunoreactivity (rabbit polyclonal, ProteinTech Group) was performed to confirm the diagnosis. For STMN2 analyses, sections of normal fixed formalin fixed lumbar spinal cord were stained using standard immunohistochemical procedure with the exception that citrate buffer antigen retrieval was performed before blocking. Briefly, samples were rehydrated, rinsed with water, blocked in 3% hydrogen peroxide then normal serum, incubated with primary STMN2 rabbit-derived antibody (1/100 dilution, Novus), followed by incubation with the appropriate secondary antibody (anti-rabbit IgG conjugated to horsesradish peroxidase 1/200), and exposure to ABC Vectastain kit and DAB peroxidase substrate, and briefly counterstained with hematoxylin before mounting. Multiple levels were examined for each sample.

STMN2 splicing analysis. Total RNA was isolated from neurons using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. A total of 300–1,000 ng of total RNA was used to synthesize complimentary DNA by reverse transcription according to the iscript kit (Bio-Rad). RT-PCR was then performed using one cryptic exon specific primer and then analyzed using the Agilent 2200 Tapestation.

Data presentation and statistical analysis. In the figure elements, bars and lines represent the median with error bars representing standard deviation. The box and whiskers plots display the minimum to maximum. Data distribution was assumed to be normal but this was not formally tested. Unless stated otherwise, the statistical analyses were performed using a two-tailed unpaired Student’s t-test, with a P value of *P < 0.05 considered as significant using Prism 7 (GraphPad).

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.
Data availability
The authors will make all data available to readers upon reasonable request. The RNA-seq data discussed in this publication have been deposited in the Gene Expression Omnibus and are accessible through GEO series accession number GSE121569. The patient spinal cord RNA-seq data are available through dbGaP (phs000747.v2.p1).

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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).

- [ ] 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
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- [ ] 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 | No custom software was used to collect data. Other programs used to collect and analyze data include: Licor (Image Studios version 2.1), Flow Jo (Version 8.7), GraphPad Prism (Version 7), IGV (Version 6), 4Peaks (version 1.8), Geneious (version 10), ImageJ with NeuronJ (1.4.2 by Erik Meijering), Nikon NIS Elements (Version 4.0). |
| Data analysis | RNA-seq data was processed with the bcbio pipeline and analyzed with the bcbioRNASeq R package. Detailed information on the source code and program versions (update version 06) used for analysis, including installation instructions, are available at our Github repo: https://github.com/mjsteinbaugh/eggan-es-derived_motor_neuron_knockdown-rnaseq-human. All methods used in the RNA-seq analysis are published in peer-reviewed journals. bcbio and bcbioRNASeq source code are provided under an MIT license (https://github.com/hbc/bcbioRNASeq/blob/master/LICENSE). Software URLs: bcbio http://bcbio-nextgen.readthedocs.io https://github.com/hbc/bcbio-bcbio-nextgen bcbioRNASeq http://bioinformatics.sph.harvard.edu/bcbioRNASeq https://github.com/hbc/bcbioRNASeq https://dx.doi.org/10.12688/f1000research.12093.1 salmon |
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Data

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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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The authors will make all data available to readers upon reasonable request. The RNA-seq data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) Series accession number GSE121569 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE121569). The patient spinal cord RNA-Seq is available through dbGaP (phs000747.v2.p1).

Field-specific reporting

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Life sciences

Life sciences study design

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

| Sample size | No statistical methods were used to pre-determine sample sizes but our sample sizes are similar to those reported in previous publications including Egawa et al 2012 Sci Transl Med, Bilican et al 2012 PNAS, and Fujimori et al 2018 Nature Medicine |
| Data exclusions | No data were excluded from the analyses. |
| Replication | For the RNAi experiments, replication was achieved through multiple independent differentiations and through using two unique siRNAs for the control and siTDP-43 groups. For the iPSC cell studies, we used multiple control cell lines and all available mutant TDP-43 lines for replication. For the STMN2 knockout studies, we generated knockout in two distinct hES cell lines for replication. Technical replication was used throughout for molecular studies. All attempts at replication were successful. |
| Randomization | No randomization was used. We compared specifically designated control samples and test samples through either treatment or genetic information. |
| Blinding | Blinding was used for the Sholl analysis for the researcher tracing the neurites. For other experiments, 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            | Source and Catalog Numbers |
|----------------------------|----------------------------|
| NCAM, BD Biosciences Alexa Fluor® 700 Mouse Anti-Human CD56, Cat # 557919, Clone 8159, Lot 5020744 |                              |
| EpCAM, BD Biosciences PE Mouse Anti-Human EpCAM, Cat # 347198, Clone EBA-1, Lot 717457 |                              |
| TDP-43, ProteinTech TDP-43 Antibody, Cat# 10782-2-AP, Polyclonal, Lot 00052103 |                              |
| TUJ1, R&D Systems Neuron-specific beta-III Tubulin NL493 Mab, Cat# NL11956, Clone Tuj-1, Lot HGQ0116111 |                              |
| STMN2 (F) Novus Biologicals Stathmin-2/STMN2 Antibody, Cat# NBP1-49461, Polyclonal, Lots C7 and D1 |                              |
| STMN2 (WB) R&D Systems Human/Mouse Stathmin-2/STMN2 Antibody, Cat# MAB6930, Clone 684433, Lot CFIL0114101 |                              |
| GAPDH, Millipore Anti-Glyceraldehyde-3-Phosphate Dehydrogenase Antibody, Cat# MAB374, Clone 6C5, Lot 2792998 |                              |
| MAP2, Abcam Anti-MAP2 antibody, Cat# ab5392, Polyclonal, Lot GR286806-6 |                              |
| GFP, Invitrogen Anti-GFP antibody, Cat# A10262, Polyclonal |                              |
| Golgin97, Invitrogen Golgin-97 Monoclonal Antibody, Catalog # A-21270, Clone CDF4, GR230216-3 |                              |
| Hb9 DSHB 81.5C10, Cat# 81.5C10, Clone AB_2145209, Supernatant |                              |
| Isl1, Abcam Anti-Isl1 antibody, Cat# ab109517, Clone EP4182, Lot GR24182, Lot GR224528-5 |                              |
| TDP-43, gift from D Cleveland, Clone FL9 |                              |
| SOD1, Cell Signaling Technologies Anti-SOD1 antibody, Cat# 4266, Clone 71G8 |                              |
| Mouse IgG, Cell Signaling Technologies isotype control antibody, Cat# 5415, Clone G3A1 |                              |

Secondary antibodies were purchased from Invitrogen conjugated to Alexa Fluor 488, 555, 594, and 647

Validation

All antibodies have their respective source company and clone number and are validated for the applications used within this manuscript. This information is available on the manufacturers publicly available datasheets, which we have provided links for in here:

- http://www.bdbiosciences.com/us/applications/research/stem-cell-research/hematopoietic-stem-cell-markers/human/negative-markers/alexia-fluor-700-mouse-anti-human-cd56-b159/p/557919
- http://www.bdbiosciences.com/us/applications/research/stem-cell-research/cancer-research/human/pe-mouse-anti-human-epcam-eba-1/p/347198
- https://ptgb.com/products/TARDBP-Antibody-10782-2-AP.htm?gclid=EAIaIQobChMIIt4j8671gIVT8DICh1vdweSEAAAYASAEgjcBvD_BwE#datasheet
- https://resources.rndsystems.com/pdfs/datasheets/nl1195g.pdf
- https://www.novusbio.com/products/stathmin-2-stmn2-antibody-nbp1-49461
- https://resources.rndsystems.com/pdfs/datasheets/mab6930.pdf
- http://www.emdmillipore.com/US/en/product/Anti-Glyceraldehyde-3-Phosphate-Dehydrogenase-Antibody-clone-6C5.MM_NF-MAB374
- https://www.abcam.com/anti-mouse-antibody-5392.html
- https://www.thermofisher.com/antibody/product/GFP-Tag-Antibody-Polyclonal/A10262
- https://www.thermofisher.com/antibody/product/Golgin-97-Antibody-clone-CDF4-Monoclonal/A-21270
- http://dbsh.biology.uiowa.edu/B1-SC10_2
- https://www.abcam.com/islet-1-antibody-ep4182-ab109517.html
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- https://www.thermofisher.com/us/en/home/life-sciences/antibodies/secondary-antibodies/fluorescent-secondary-antibodies/alexia-fluor-secondary-antibodies.html

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

- HUES9 Hb9::GFP is a human embryonic stem cell line derived at Harvard University study.
- WA01 is a human embryonic stem cell line derived at the University of Wisconsin Madison

The control iPS cell lines (11a, 15b, 17a, 18a, 20b) and two TDP-43 lines (36a Q343R and 47d) were generated in our lab with fibroblasts under IRB approved protocols of collaborative study with Dr. Chris Henderson at the ALS Clinic at Columbia University.

The TDP-43 lines (RB20a) was generated in our lab with fibroblasts from a collaborative study with Dr. Robert Brown through ongoing IRB approved protocol in collaboration with Dr. Merit Cudkowicz and Dr. James Berry at the MGH Neurological Research Institute.

The TDP-43 line with the M337V mutation was a generous gift from Dr. Christopher Shaw

Authentication

Specific point mutations were confirmed by PCR amplification followed by Sanger sequencing.

Mycoplasma contamination

Weekly, we both specifically and randomly check cultures within the lab for mycoplasma contamination using the MycoAlert kit (Lonza). All cell lines used in this study were tested and none tested positive.

Commonly misidentified lines

(See ICLAC register)

No commonly misidentified cell lines were used.
## Flow Cytometry

### Plots

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

### Methodology

#### Sample preparation

Differentiated cultures were dissociated to single cells using accutase™ treatment for 1 hour inside a 5% CO2 / 37°C incubator. Repeated (10-20 times) but gentle pipetting with a 1000 µL Pipetman® was used to achieve a single cell preparation. Cells were spun down, washed 1x with PBS and resuspended in sorting buffer (1x cation-free PBS 15mM HEPES at pH 7 (Gibco®), 1% BSA (Gibco®), 1x penicillin-streptomycin (Gibco®), 1 mM EDTA, and DAPI (1 µg/mL). Cells were passed through a 45 µm filter immediately before FACS analysis and purification.

#### Instrument

The BD FACS Aria II cell sorter (SORP) was used.

#### Software

FlowJo was used to analyze the data.

#### Cell population abundance

The purity of samples was determined post sort using microscopy.

#### Gating strategy

Forward and side scatter was used to resolve cells from debris with doublet discrimination by forward scatter area vs forward scatter width. DAPI signal was then used to determine cell viability, and differentiated cells not exposed to MN patterning molecules (RA and SAG) were used as negative controls to gate for green fluorescence. For lines not containing the Hb9::GFP reporter, single cell suspensions were incubated with antibodies against NCAM (BD Bioscience, BD8557919, 1:200) and EpCAM (BD Bioscience, BD8347198, 1:50) for 25 minutes in sorting buffer, then washed once with PBS 1x and resuspended in sorting buffer. The population that was negative for EpCAM but positive for NCAM was sorted. We can provide additional figures exemplifying all the gating strategies if the ones provided are insufficient.

- Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.