Title: Neuronal hyperexcitability drives TDP43 pathology by upregulating shortened TDP43 protein isoforms

Key Words: TDP43, ALS, hyperexcitability, alternative splicing, TDP43 pathology, iPSC, iNeuron

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Supplemental Materials and Methods

RNA-sequencing

We utilized previously described RNA sequencing(seq) data[1,2], and determined transcript abundance as described above.

Tissue preparation and immunohistochemistry in murine tissue

Vertebral columns were dissected from 5 month old C57Bl6 J mice, fixed in 4% paraformaldehyde (PFA) at 4°C for 48h, washed in phosphate buffered saline (PBS), and dissected to extract the spinal cords. The lumbar enlargement was sub-dissected, cryoprotected in 30% sucrose at 4°C, embedded in M1 matrix (Thermo Scientific #1310) in a silicone mold, frozen on dry ice, and sectioned at a thickness of 16µm onto charged slides (Thermo Scientific J1800AMNZ). Sections were then briefly air dried and stored at -80°C. For immunohistochemistry (IHC), sections were washed in distilled water, and blocked and permeabilized in blocking buffer (5% BSA, 0.5% Triton X-100, and 5% goat serum (Gibco 16210-064)) for 1h at room temperature. Slides were then incubated with primary antibody at 4°C overnight in blocking buffer diluted 2-fold with PBS (Supplemental Table 4). Sections were washed 3x for 5m in PBS, then incubated at room temperature for 1h with Alexa Fluor goat anti-mouse 488 (Life Technologies AB150113), Alexa Fluor donkey anti-goat 647 (Life Technologies AB150131), and Alexa Fluor goat anti-rabbit 568 (Life Technologies AB175470) secondary antibodies diluted 1:500 in blocking solution. Sections were then washed 3x for 5m, counterstained, and mounted with Vectashield Hardset with DAPI (Vector Labs H-1500). Images were acquired using Olympus Whole Slide Scanner (VS120) with a 40x objective.

Differentiation of iPSC-derived astrocytes

iPSC-derived astrocyte progenitors were derived, cultured, and expanded as described previously[3]. The glial progenitors were pre-differentiated in Neurobasal Medium containing 1% B27 Supplement, 1% NEAA, and 1% PS for 10d on 6-well plates before cryopreservation. Before the experiment, batches of pre-differentiated astrocytes were defrosted and plated at 50k per well in 8-well Ibidi imaging chambers (Ibidi 80841), and cultured for an additional 6 days in Neurobasal Medium containing 1% B27, 1% NEAA, 1% PS, and 20 ng/mL ciliary neurotrophic factor (CNTF, Thermo Fisher PHC7015). Astrocytes were then fixed in 4% PFA for 15m and immunostained as described above (Supplemental Table 4) using Alexa Fluor goat anti-rabbit 647 (Thermo Fisher A-21245) and Alexa Fluor goat anti-mouse IgG1 (Thermo Fisher A-21121) secondary antibodies diluted 1:1000 in 3% goat serum in PBS. Imaging was performed on a Leica DMi8 with CooLED light source at 63x and analyzed with ImageJ.

Supplemental Figure Legends

Supplemental Figure 1. TDP43 abundance is modulated by neuronal activity.

(**A**) Western blot demonstrating an increase in TDP43 abundance in iNeurons stimulated with TEA, and a reduction in TDP43 levels upon inhibition with TTX. (**B**) Quantification of TDP43 abundance normalized to a GAPDH loading control, relative to vehicle-treated (V) neurons (3 experimental replicates, *p<0.05, ***p<0.001, ****p<0.0001, one-way ANOVA with Dunnett's post-test). (**C**) Rodent primary mixed cortical neurons exhibited similar increases in TDP43 abundance with bicuculline, and reductions in TDP43 levels with TTX. (**D**) Quantification of TDP43 abundance normalized to GAPDH, relative to vehicle-treated (V) neurons (3 experimental replicates, *p<0.05, one-way ANOVA with Dunnett's post-test).

Supplemental Figure 2. sTDP43 is deficient in splicing activity.

(**A**) Schematic of the *CFTR* minigene reporter. TDP43-mediated splicing of the reporter results in exon 9 exclusion. Arrows indicate primers used to amplify the splice junction. (**B-C**) EGFP-flTDP43, but not EGFP-sTDP43, effectively excludes *CFTR* exon 9 in HEK293T cells overexpressing the reporter (3 replicates, *p<0.05, one-way ANOVA, Dunnett's post-test).

Supplemental Figure 3. sTDP43 autoregulatory function is impaired.

(**A**) Schematic of the TDP43 autoregulation reporter in which the fluorescent protein mCherry is fused to the *TARDBP* 3' UTR. (**B**) Rodent primary mixed cortical neurons overexpressing EGFP-flTDP43 show a significant reduction in reporter signal, consistent with autoregulation, while those overexpressing EGFP-sTDP43 do so to a lesser degree (EGFP n=2044, EGFP-flTDP43 n=2375, EGFP-sTDP43 n=2208, 3 replicates, ****p<0.0001, one-way ANOVA, Dunnett's post-test). (**C**) Fluorescence microscopy of rodent primary mixed cortical neurons demonstrating reduced reporter fluorescence in neurons co-expressing EGFP-flTDP43, in comparison to those co-expressing EGFP or EGFP-sTDP43. Scale bar in (**C**), 10 µm.

Supplemental Figure 4. sTDP43 colocalizes with components of stress granules.

(**A**) HEK293T cells were transfected with EGFP-tagged sTDP43 and immunostained using antibodies against the stress granule marker G3BP1 and endogenous TDP43. (**B**) Overexpressed sTDP43-EGFP colocalizes with endogenous TDP43 and stress granule markers G3BP1 and TIA1 in HEK293T cells treated with 0.4M sorbitol. Scale bar in (**A**) 20 µm, scale bar in (**B**), 10 µm.

Supplemental Figure 5. sTDP43 transcripts are present in a variety of tissue types and disease states.

(**A**) Summary of previously published RNA-seq studies analyzed for sTDP43. (**B**) sTDP43-1 represents 30% of *TARDBP* transcripts in spinal cord ventral horn homogenate isolated from both control and sALS patients, with the remainder corresponding to flTDP43. (**C**) Similarly, sTDP43-1 makes up 30% of *TARDBP* transcripts in cerebellum, and 55% of *TARDBP* transcripts in frontal cortex. In each case, sTDP43-2 transcripts were largely undetectable, and there was no significant change in transcript isoform abundance in C9ALS or sALS patients compared to controls.

Supplemental Figure 6. Endogenous sTDP43 is expressed by neurons and glia in murine lumbar spinal cord.

Immunohistochemistry in murine spinal cord, showing colocalization of sTDP43 immunoreactivity (green) with the neuronal marker NeuN (red) in the ventral horn (**A**) and with the astrocytic marker GFAP (**B**, purple). Scale bars (**A**) and (**B**), 200 µm.

Supplemental Figure 7. Endogenous sTDP43 is produced by human iPSC-derived astrocytes.

(**A**) Immunocytochemistry using antibodies against flTDP43 (red) and sTDP43 (green) in astrocytes differentiated from human iPSCs. (**B**) Reflecting their unique subcellular distributions, flTDP43 displays a significantly higher nuclear-cytoplasmic ratio (NCR) than sTDP43 (3 replicates, flTDP43 n=136, sTDP43 n=136, ****$p<0.0001$, two-tailed t-test). Scale bar in (**A**) 10 µm.

Supplemental Table 1. Amino acid sequence of the sTDP43 C-terminal tail is conserved in humans, non-human primates, and lesser mammals.

Supplemental Table 2. Nucleotide sequence of the sTDP43-1 and -2 splice junctions are conserved in humans, non-human primates, and lesser mammals.

Supplemental Table 3. Constructs and primer sequences used to generate iPSC lines

Supplemental Table 4. Primary Antibodies

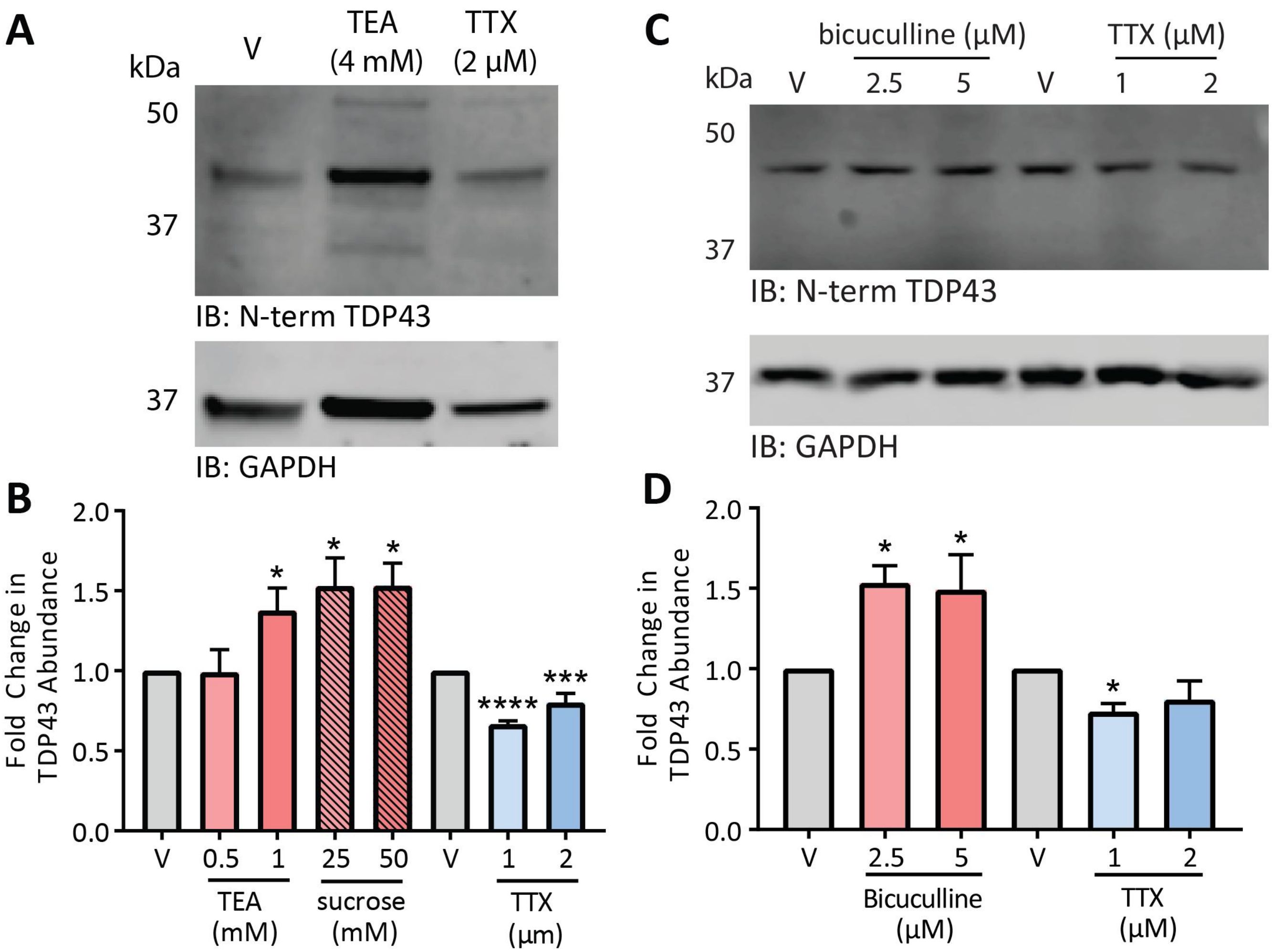
Supplemental Table 5. Primers used in RT PCR and CFTR Assays

Supplemental Table 6. Source and Construction of Plasmid Vectors

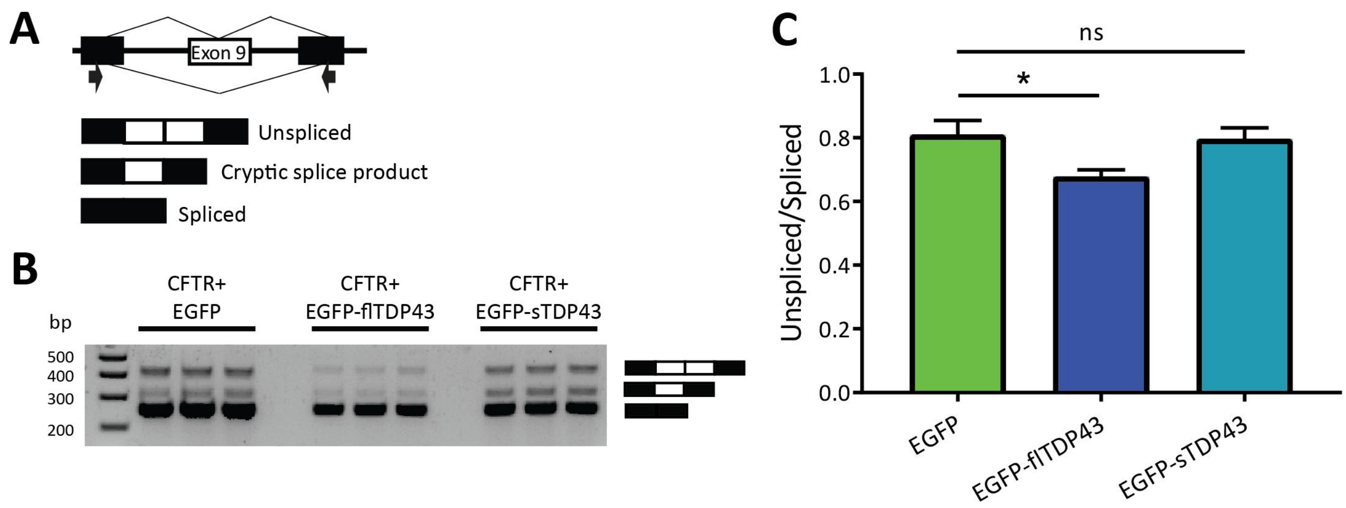
Supplemental Bibliography

1. Prudencio, M. *et al.* Distinct brain transcriptome profiles in C9orf72-associated and sporadic ALS. *Nat. Neurosci.* **18**, 1175–1182 (2015).
2. D'Erchia, A. M. *et al.* Massive transcriptome sequencing of human spinal cord tissues provides new insights into motor neuron degeneration in ALS. *Sci. Rep.* **7**, 10046 (2017).
3. Serio, A. *et al.* Astrocyte pathology and the absence of non-cell autonomy in an induced pluripotent stem cell model of TDP-43 proteinopathy. *Proceedings of the National Academy of Sciences* **110**, 4697–4702 (2013).

Supplemental Figure 1.



Supplemental Figure 2.



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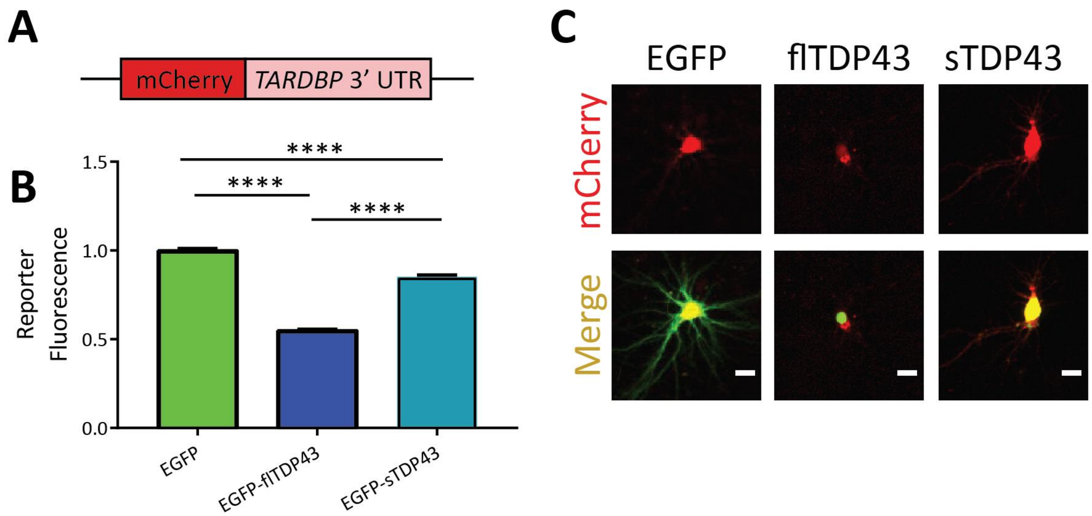

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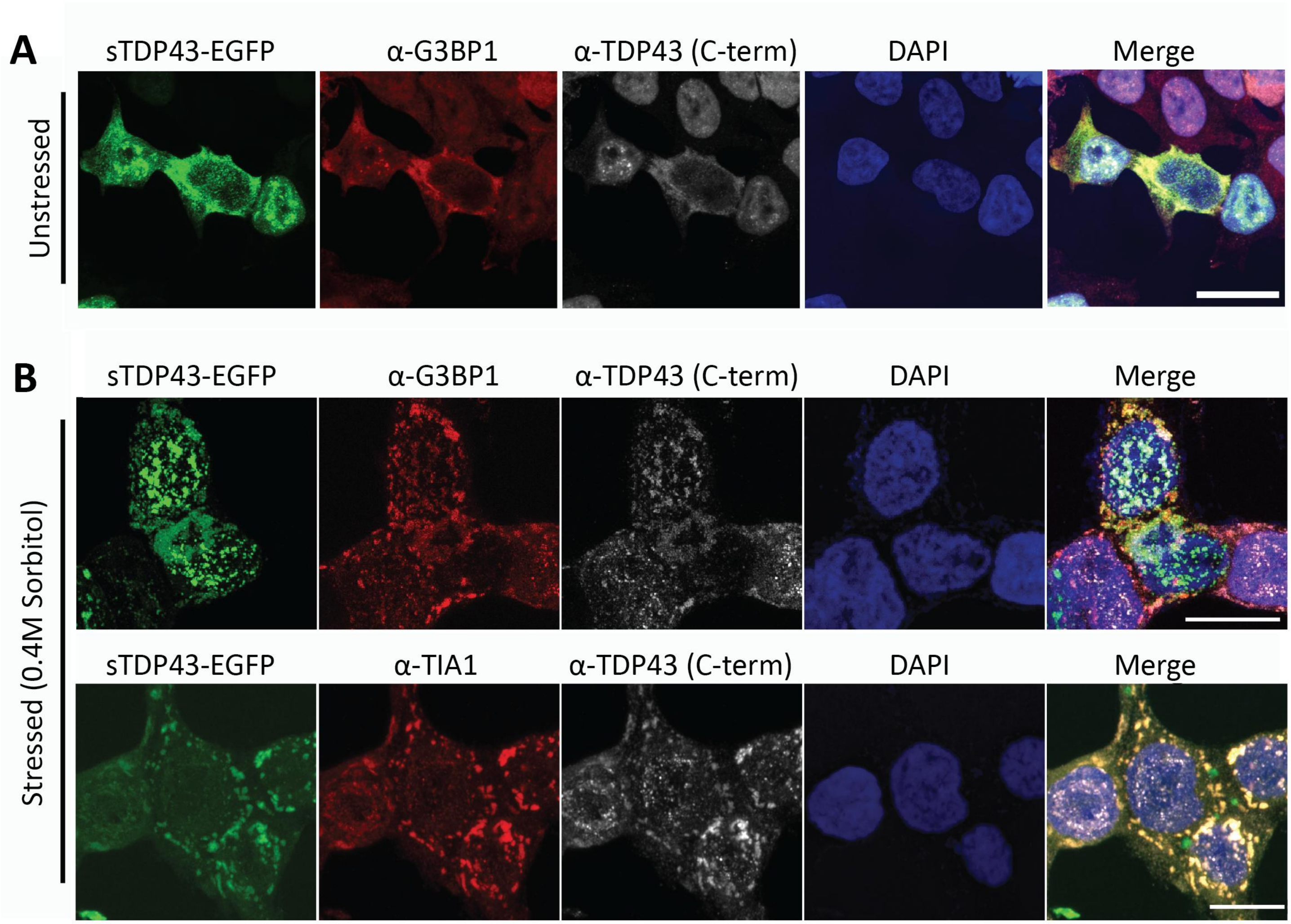
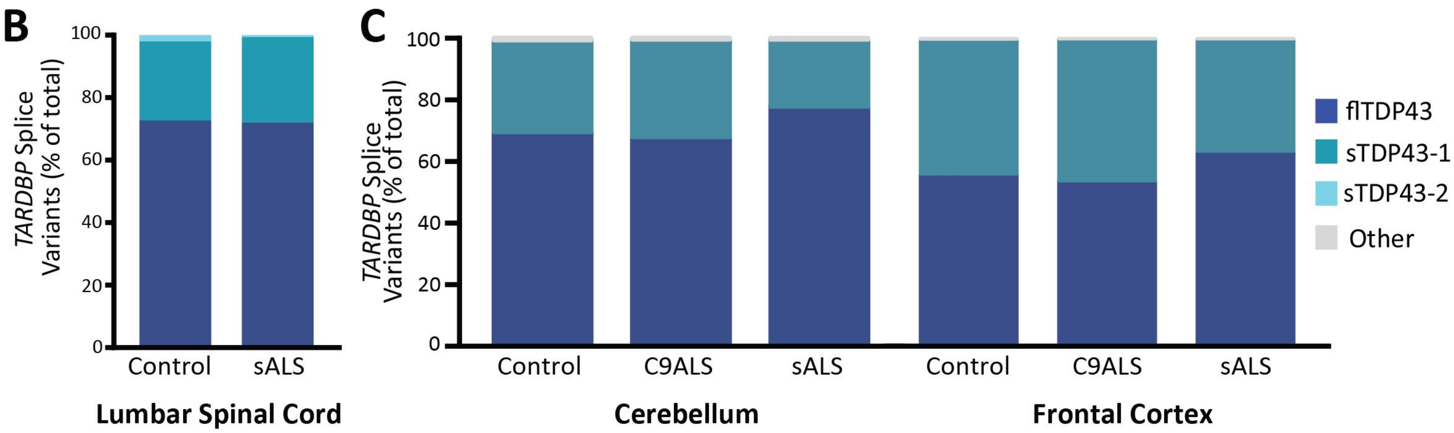


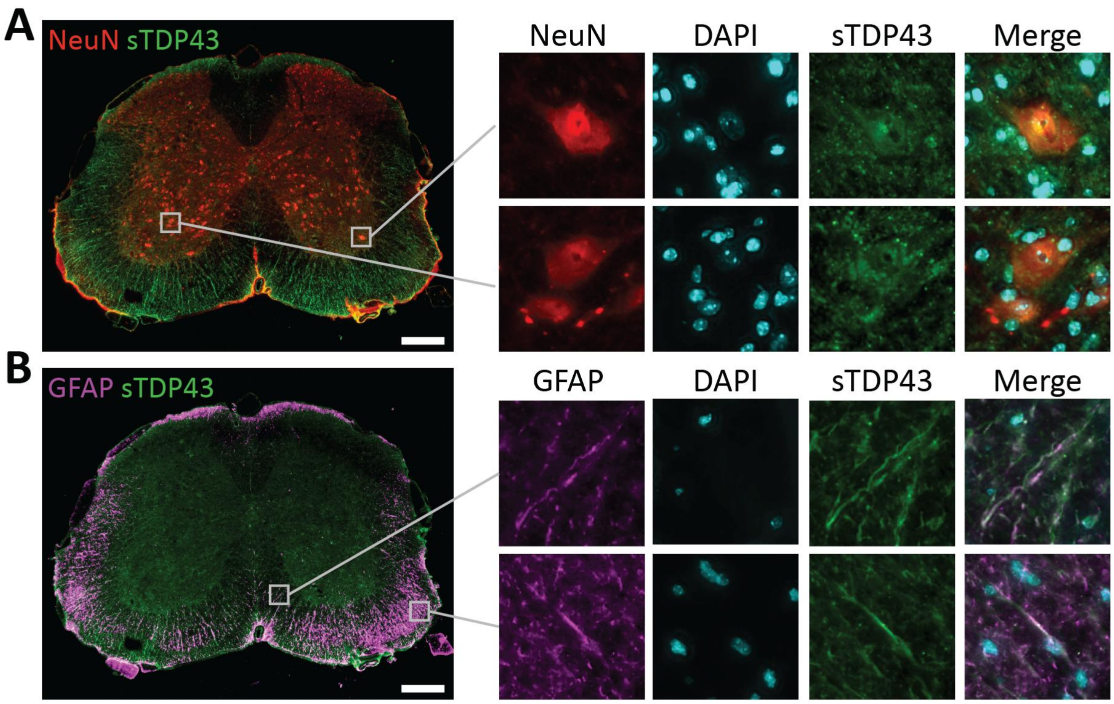
Supplemental Figure 5.

A

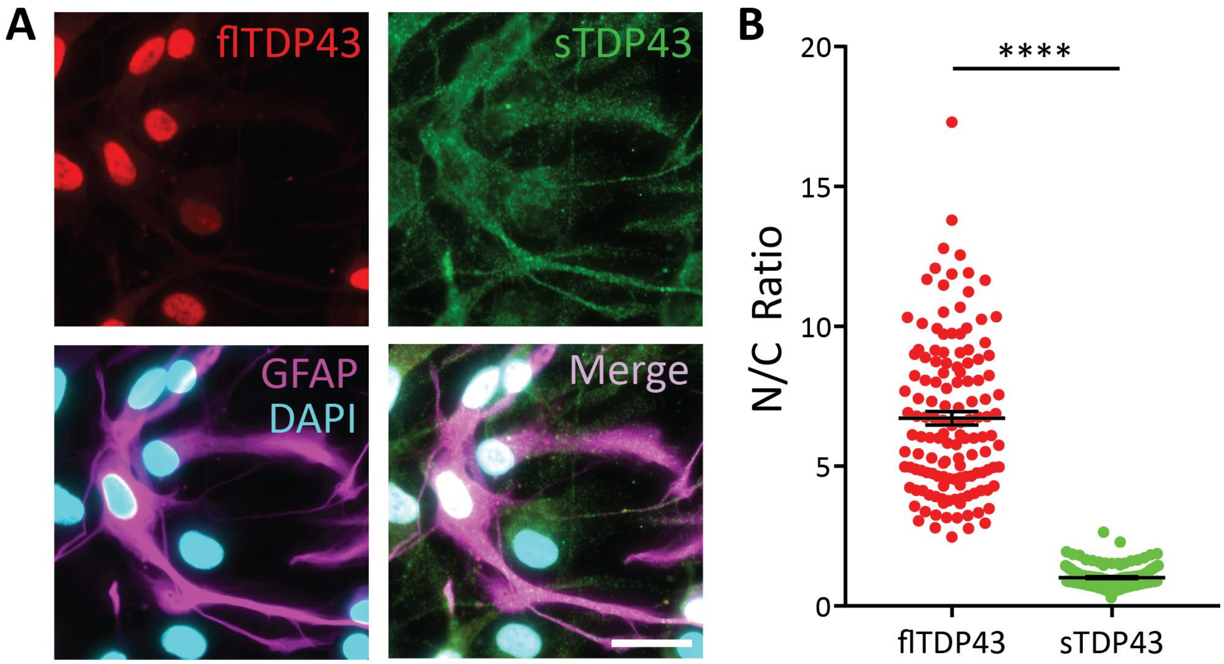
Original Study	Species	Library preparation	Read Length	Cell or Tissue Type	Disease Condition	n
White et al. 2018	Mouse	Clontech SMART-seq and Illumina Nextera XT	100 bp, paired	Laser captured spinal motor neuron	Wildtype	4
White et al. 2018	Mouse	Illumina TruSeq	100 bp, paired	Frontal cortex	Wildtype	6
Krach et al. 2018	Human	NuGEN Ovation	50 bp, single	Laser captured spinal motor neuron	Control and sALS	21
D'Erchia et al. 2017	Human	Illumina TruSeq	100 bp, paired	Ventral horn spinal cord	Control and sALS	11
Preduncio et al. 2015	Human	Illumina TruSeq	100 bp, paired	Cerebellum and Frontal cortex	Control, C9ALS, and sALS	53



Supplemental Figure 6.



Supplemental Figure 7.



Supplemental Table 1. Amino acid sequence of the sTDP43 C-terminal tail is conserved in humans, non-human primates, and lesser mammals.

Species	Amino Acid Sequence																	
Reference Sequence	V	H	L	I	S	N	V	Y	G	R	S	T	S	L	K	V	V	L
Chimpanzee (*Pan troglodytes*)	V	H	L	I	S	N	V	Y	G	R	S	T	S	L	K	V	V	L
Orangutan (*Pongo abelii*)	V	H	L	I	S	N	V	Y	G	R	S	T	S	L	K	V	V	L
Baboon (*Papio anubis*)	V	H	L	I	S	N	V	Y	G	R	S	T	S	L	K	V	V	L
Drill (*Mandrillus leucophaeus*)	V	H	L	I	S	N	V	Y	G	R	S	T	S	L	K	V	V	L
Black and white colobus (*Colobus angolensis palliatus*)	V	H	L	I	S	N	V	Y	G	R	S	T	S	L	K	V	V	L
Goat (*Capra hircus*)	V	H	L	I	S	N	V	Y	G	R	S	T	S	L	K	V	V	L
Cat (*Felis catus*)	V	H	L	I	S	N	V	Y	G	R	S	T	S	L	K	V	V	L
Alpine marmot *(Marmota marmota marmota*)	V	H	L	I	S	N	V	Y	G	R	S	T	S	L	K	V	V	L
Long-tailed chincilla (*Chinchilla lanigera*)	V	H	L	I	S	N	V	Y	G	R	S	T	S	L	K	V	V	L
Ground squirrel (*Ictidomys tridecemlineatus*)	V	H	L	I	S	N	V	Y	G	R	S	T	S	L	K	V	V	L
Kangaroo rat (*Dipodomys ordii*)	V	H	L	I	S	N	V	Y	G	R	S	T	S	L	K	V	V	L
Guinea pig (*Cavia porcellus*)	V	H	L	I	S	N	V	Y	G	R	S	T	S	L	K	V	V	L
Brown rat (*Rattus norvegicus*)	V	H	L	I	S	N	V	Y	G	R	S	T	S	L	K	V	V	L
Darama mole rat (*Fukomys damarensis*)	V	H	L	I	S	N	V	Y	G	R	S	T	S	L	K	V	V	L
Blind Mole Rat (*Nannospalax galili*)	V	H	L	I	S	N	V	Y	G	R	S	T	S	L	K	V	V	L
Golden hampster (*Mesocricetus auratus*)	V	H	L	I	S	N	V	Y	G	R	S	T	S	L	K	V	V	L
Deer mouse (*Peromyscus maniculatus bairdii*)	V	H	L	I	S	N	V	Y	G	R	S	T	S	L	K	V	V	L
House mouse (*Mus musculus*)	V	H	L	I	S	N	V	Y	G	R	S	T	S	L	K	V	V	L
Naked mole rat (*Heterocephalus glaber*)	V	H	L	I	S	N	V	F	G	R	S	T	S	L	K	V	V	L
Horse (*Equus calabus*)	V	H	L	M	S	N	V	Y	G	R	S	T	S	L	K	V	V	L
Tibetan antelope (*Pantholops hodgsonii*)	V	H	L	I	S	N	V	H	G	R	S	T	S	L	K	V	V	L
Chinese tree shrew (*Tupaia chinensis*)	V	H	L	I	S	N	V	S	G	R	S	T	S	L	K	V	V	L

Supplemental Table 2. Nucleotide sequence of the sTDP43-1 and -2 splice junctions are conserved in humans, non-human primates, and lesser mammals.

Species	Isoform 1 Identity	Isoform 2 Identity
Baboon (Papio anubis)	99%	99%
Drill (Mandrillus leucophaeus)	96%	96%
Angolan black and white colobus (Colobus angolensis palliatus)	96%	96%
Goats (Capra hircus)	96%	96%
Chinese tree shrew (Tupaia chinensis)	96%	96%
Horse (Equus caballus)	96%	96%
Cat (Felus catus)	96%	96%
Ground Squirrel (Ictidomys tridecemlineatus)	96%	96%
Shrew (Mus pahari)	96%	96%
Brown rat (Rattus novegicus)	96%	96%
Deer mouse (Peromyscus maniculatus bairdii)	96%	96%
Prairie vole (Microtus ochrogaster)	96%	96%
House mouse (Mus musculus)	96%	96%
Ryukyu mouse (Mus caroli)	96%	96%
Damaraland mole rat (Fukomys damarensis)	96%	96%
Tibetan antelope (Pantholops hogsonii)	96%	96%
Blind mole rat (nannospalax galili)	96%	96%
Chinese hamster (Cricetulus griseus)	95%	95%
Guinea pig (Cavia procellus)	95%	95%
Golden hampster (Mesocricetus auratus)	95%	92%
Lesser Egyptian jerboa (Jaculus jaculus)	95%	93%
Cow (Bos tauru)	95%	95%

Supplemental Table 3. Constructs used for generation of iPSC Lines

Construct	Source	Complementary Oligomers	Sequence (5' to 3')
pUCM-CLYBL-NGN1-2-RFP	Gift from M. Ward		
pLTC13-L1	Gift from M. Ward		
pLTC13-R1	Gift from M. Ward		
pX335-U6-Chimeric_BB-CBh-hSpCas9n(D10A)	Addgene (42335, donated by Feng Zhang)		
pX330S-4	Addgene (58780, donated by Feng Zhang)		
pUCM-N-term-TARDBP-D2-HDR	Synthesized by Blue Heron, LLC		
pX335-sgRNA-D2-TDP43-Upstream	This paper	Sense	GACCGTTCATATCTCTTTTCTCTTT
		Antisense	AAACAAAGAGAAAAGAGATATGAAC
pX335-sgRNA-D2-TDP43-Downstream	This paper	Sense	CACCGGGGCTCATCGTTCTCATCTT
		Antisense	AAACAAGATGAGAACGATGAGCCCC
pUCM-C-term-TARDBP-D2-HDR	Synthesized by Blue Heron, LLC		
pX335-sgRNA-TDP43-D2-Upstream	This paper	Sense	CACCGGTTGGTTGGTATAGAATGG
		Antisense	AAACCCATTCTATACCAACCAACC
pX335-sgRNA-TDP43-D2-Downstream	This paper	Sense	CACCGACCACTGCCCGACCCTGCAT
		Antisense	AAACATGCAGGGTCGGGCAGTGGTC

Supplemental Table 4. Primary antibodies

Antibody	Source	Catalog Number	Species	Dilution
Vglut1	Synaptic Systems	135303	rabbit	1:200 for ICC in iNeurons
Tuj1	BioLegend	801202	mouse	1:500 for ICC in iNeurons
N-term TDP43	Sephton et al. 2011; Barmada et al. 2014	NA	rabbit	1:5000 for ICC and western
C-term TDP43	Sephton et al. 2011; Barmada et al. 2014	NA	rabbit	1:5000 for ICC and western
sTDP43	Custom-made from Genscript	NA	rabbit	1:1000 for ICC and western
				1:500 for murine IHC
				1:250 for human IHC
Map2	Milipore	MAB3418	mouse	1:1000 for ICC in iNeurons
GAPDH	Milipore	MAB374	mouse	1:1000 for ICC in iNeurons
GFAP	Abcam	AB53554	goat	1:500 for murine IHC
NeuN	Abcam	AB104225	mouse	1:500 for murine IHC
GFAP	Milipore	AB5541	chicken	1:500 for human IHC
NeuN	Milipore	MAB377	mouse	1:250 for human IHC
TDP43	R&D Biosystems	MAB7778	mouse	1:1000 for western and ICC
				1:500 for ICC in iPSC-derived astrocytes
				1:250 for human IHC
GFAP	Sigma	C9205	mouse	1:500 for ICC in iPSC-derived astrocytes

Supplemental Table 5. Primers used in qRT-PCR and CFTR splicing assays

Target	Location	Primer	Sequence (5' to 3')
ARC	*ARC* exon 2	Forward	CCTGTACCAGACGCTCTACG
		Reverse	GCAGGAAACGCTTGAGCTTG
Total TARDBP	*TARDBP* exon 1 and 2	Forward	CTGCTTCGGTGTCCCTGTC
		Reverse	TGGGCTCATCGTTCTCATCT
full-length (fl) TARDBP	*TARDBP* exon 6 stop codon	Forward	GTGGCTCTAATTCTGGTGCAG
		Reverse	CACAACCCCACTGTCTACATT
sTDP43-1	*sTDP43-1* splice donor	Forward	AGAAGTGGAAGATTTGGTGTTCA
		Reverse	GCATGTAGACAGTATTCCTATGGC
sTDP43-2	*sTDP43-2* splice donor	Forward	AGATTTGGTGGTAATCCAGTTCA
		Reverse	GGCCTGTGATGCGTGATGA
CFTR minigene	*CFTR* exon 9 splice junction	Forward	CAACTTCAAGCTCCTAAGCCACTGC
		Reverse	TAGGATCCGGTCACCAGGAAGTTGGTTAAATCA

Supplemental Table 6. Source and construction of plasmid vectors

Plasmid	Source	Amplicon or Insert	Primer	Sequence (5' to 3')
pGW1-mApple	Barmada et al. 2014			
pGW1-EGFP(1)	Arrasate et al. 2004			
pGW1-TDP43-EGFP	Barmada et al. 2014			
pGW1-sTDP43-EGFP	This paper	sTDP43	NA	
pGW1-sTDP43(mNES)-EGFP	This paper	sTDP43(mNES)	Forward	CGCGGGCCCATGTCTGAATATATTCG
			Reverse	GCGACCGGTCGCAGCACTCCACCCCCTCCGCCGCTTCTTCCATAAAC
pGW1-EGFP(2)	This paper	EGFP	Forward	GCGAAGCTTGCCACCATGGTGAGCAAG
			Reverse	CGCGGTACCCTTGTACAGCTCGTCCAT
pGW1-EGFP-tail	This paper	tail	Forward	CGTTCATCTCATTTCAAATGTTTATGGAAGAAGCACTTCATTGAAAGTAGTGCTGTAAG
			Reverse	CTAGCTTACAGCACTACTTTCAATGAAGTGCTTCTTCCATAAACATTTGAAATGAGATGAACGGTA
pGW1-EGFP-tail(mNES)	This paper	tail(mNES)	Forward	CGTTCATCTCATTTCAAATGTTTATGGAAGAAGCGGCGGAGGGGGTGGAGTGCTGTAAG
			Reverse	CTAGCTTACAGCACTCCACCCCCTCCGCCGCTTCTTCCATAAACATTTGAAATGAGATGAACGGTA
pGW1-EGFP-flTDP43	This paper	TDP43	Forward	GCG GGT ACC ATG TCT GAA TAT ATT CGG
			Reverse	CGC GCT AGC TTA TCC CCA GCC AGA AG
pGW1-EGFP-sTDP43	This paper	sTDP43	Forward	GCGGGTACCATGTCTGAATATATTCGG
			Reverse	CGCGCTAGCTTACAGCACTACTTTCAATG
pGW1-Halo	This paper	Halo	Forward	AAAAAA TCTAGA GCCACCATGGCAGAAATCGG
			Reverse	AAAAAA CCTGCAGG CTA GGAAATCTCGAGCGTCGACA
pGW1-flTDP43-Halo	This paper	TDP43, Halo	Forward	AAAAAA TCTAGA ATGGCAGAAATCGGTACTGG
			Reverse	AAAAAA CCTGCAGG CTA GGAAATCTCGAGCGTCGACA
pGW1-sTDP43-Halo	This paper	sTDP43	Forward	GCG GCTAGC GCCACC ATGTCTGAATATATTCG
			Reverse	CGCACCGGTGGCAGCACTACTTTC
pCaggs-mCherry-TARDBP3'UTR	This paper	TARDBP Exon 6 and 3'UTR	Forward	ATATTGTACATTGCGCAGTCTCTTTGTGGA
(TDP43 autoregulation reporter)			Reverse	ATATGGCCGAGGCGGCCATCGTGTTTTTCCAGTAAGACTCCAGAC
pcDNA3.1-NLS-mCherry-NES (shuttle-RFP)	Addgene (72660, donated by B. Di Ventura and R. Eils)			
pFN21A HaloTag PUM2 RBD R6SYE	Gift from A. Goldstrohm			
pTB CFTR A455E TG13T5 (CFTR minigene)	Gift from Y. Ayala			
pGW1-CMV	Gift from S. Finkbeiner			
pCaggs-mCherry	Gift from S. Finkbeiner			
pSMART Lenti-shTARDBP (human) CAG-TurboRFP-VSVG	Dharmacon (V3SH11240-224779127)			
pSMART Lenti-NT shRNA CAG-TurboRFP-VSVG	Dharmacon (VSC11719)			