# Figure S1



**A** Rb. IgG hnRNP A2/B1
In. Sup. IP In. Sup. IP
A2/B1

**B** Rabbit IgG High RNAse Low RNAse
A2/B1

**C** p=3.6x10^-7 p=9.6x10^-29
CLIP Cluster 6-mer Enrichment vs RBNS R Z-score
Contains UAGG
Other

**D**

| Gene Name | iCLIP Binding |
|-----------|---------------|
| MT-ND2 | CDS |
| Fus | Prox. Intron |
| Hnrnpa2b1 | 3'UTR |
| Slc1a2 | 3'UTR |
| Nefh | 3'UTR |
| Ubqln2 | 3'UTR |
| Pcp4 | 3'UTR |

**E** hnRNP A2/B1 Rb. IgG
Input IP In. IP
5x 2x
RNAse I

**F** Cluster Distribution
527 genes bound
(866 clusters)
Genomic Distribution

5' UTR CDS 3' UTR
Proximal Intron 2kb 2kb Distal Intron

**G** Distance to UAGG Motif
Real Shuffled
Fold Enrichment vs Distance to Center (bp)

**H** Mouse Targets (iCLIP) 252 | 35 | 492 Human Targets (eCLIP)

**I**

| KIF1A |
|-------|
| DDX5 | KIF1B |
| PNISR | DYNC1I2 |

| TIA1 | GLUL | ARNT2 |
|------|------|-------|
| DDX17 | NKTR | NDRG4 |
| SF3B1 | EPB41L1 | ATP8A1 |
| SFPQ | SON | MAT2A |
| SRSF6 | MYCBP2 | ZNF462 |
| HNRNPU | MAPRE2 | STXBP1 |
| EIF4G2 | TMBIM6 | PTPRG |
| HNRNPA2B1 | TTC3 | FNBP1 |
| RBM5 | DPYSL2 | LENG8 |
| HNRNPH1 | RTN3 | OGT |

**J** Pnisr (mouse)
iCLIP 4.7 0
PNISR (human)
eCLIP 2.7 0
1100 bp

**K** Leng8 (mouse)
iCLIP 8.4 0
LENG8 (human)
eCLIP 2.4 0
2500 bp
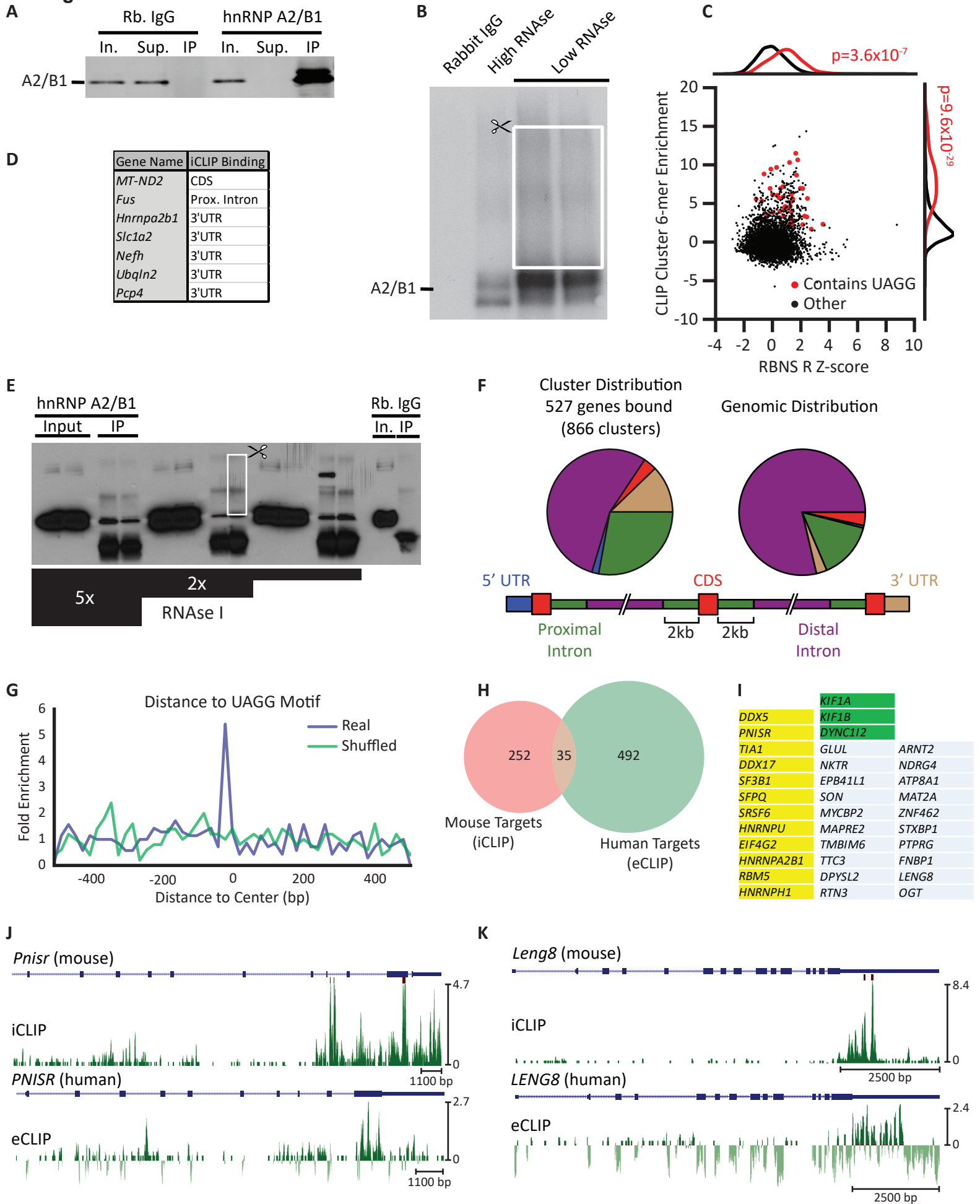
**Figure S1. Supplemental data related to iCLIP, eCLIP and RNA Bind-N-Seq (Figure 1).**

**(A)** Efficient immunoprecipitation of hnRNP A2/B1 complexes used for iCLIP. Western blot of mouse spinal cord lysate immunoprecipitated with either hnRNP A2/B1 specific antibody or rabbit IgG (Rb. IgG) and used for iCLIP. Input (In.), Supernatant (Sup.), Immunoprecipitate (IP).

**(B)** Autoradiogram of $^{32}$P-labeled protein-RNA complexes fractionated by PAGE. White box indicates the area cut and used for iCLIP library preparation.

**(C)** RNA sequences found in 3'UTRs by iCLIP contain k-mers that are significantly enriched for UAGG (red dots) in RBNS data compared to all other k-mers (black dots). *p*-values were calculated by HOMER.

**(D)** Table of significantly bound iCLIP target genes that have been previously implicated in ALS.

**(E)** Efficient immunoprecipitation of hnRNP A2/B1 complexes used for eCLIP. Western blot of iPSC-MN lysate immunoprecipitated with either hnRNP A2/B1 specific antibody or rabbit IgG (Rb. IgG) and used for eCLIP. Input (In.), Supernatant (Sup.), Immunoprecipitate (IP). Black bars denote serial dilution of RNase I used for RNA fragmentation. White box indicates the area cut and used for eCLIP library preparation.

**(F)** hnRNP A2/B1 eCLIP-derived clusters are enriched in 3'UTRs of protein coding genes (left) when compared to the expected distribution of gene regions (5' and 3'UTRs, exons, and exon-proximal and -distal portions of introns; right). Proximal intron regions are defined as extending up to 2 kb from an exon-intron junction (bottom).
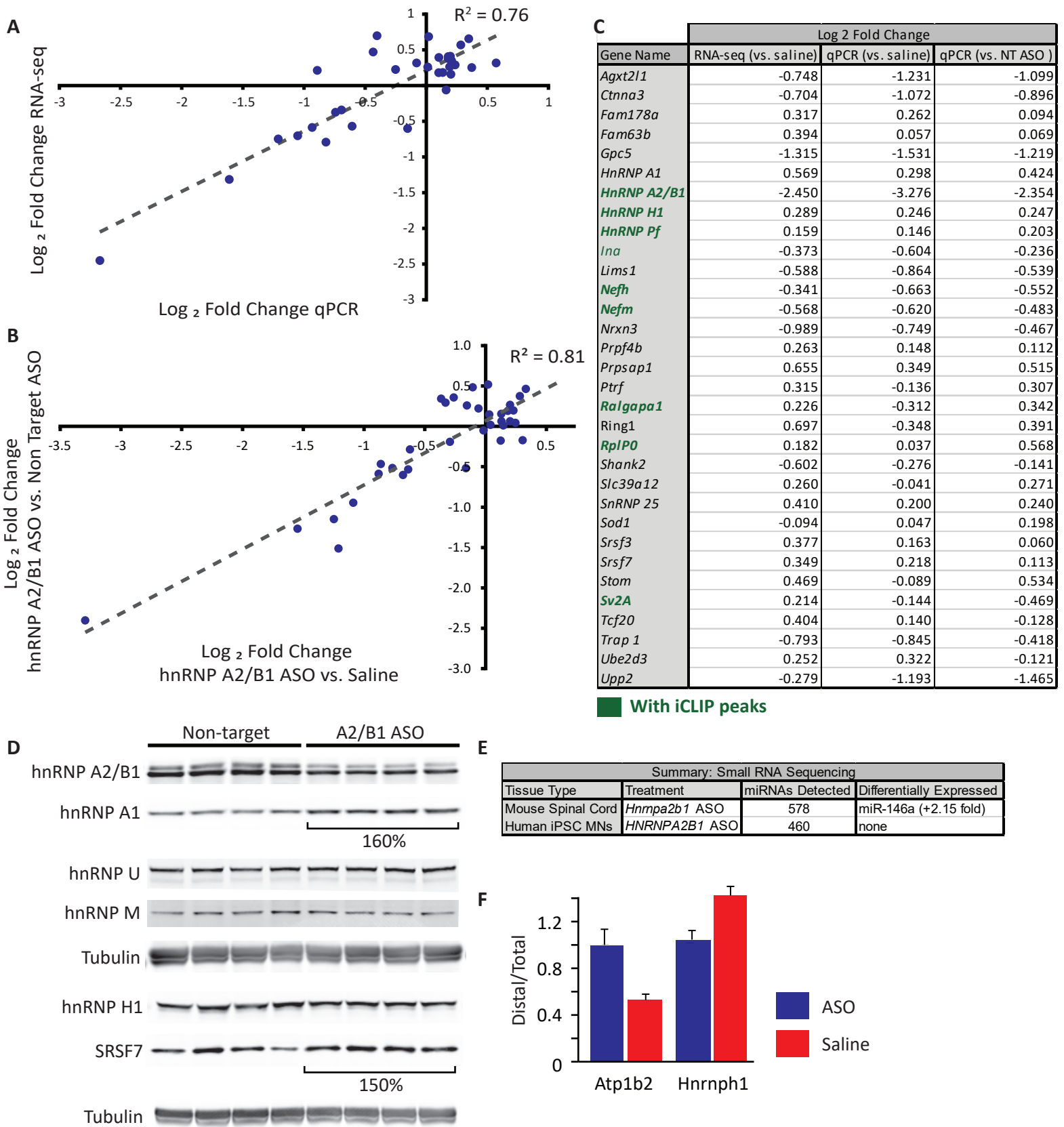
**(G)** Fold-enrichment of UAGG motifs in the genome plotted as a function of their distance to the center of the actual eCLIP peaks vs a shuffled control set.

**(H)** Venn diagram showing the overlap between the sets of hnRNP A2/B1-bound target genes from eCLIP and iCLIP in human motor neurons and mouse spinal cord, respectively.

**(I)** Table of the 35 hnRNP A2/B1-bound genes from (G) overlapping in both CLIP datasets. The 12 genes in yellow are RBPs. The 3 genes in green are motor proteins (2 kinesins and 1 dynein).

**(J-K)** Genome browser tracks of hnRNP A2/B1 iCLIP (mouse spinal cord) and eCLIP (human iPSC-MNs) targets.

# Figure S2

**A**

Log $_2$ Fold Change RNA-seq (y-axis) vs. Log $_2$ Fold Change qPCR (x-axis), $R^2 = 0.76$

**B**

Log $_2$ Fold Change hnRNP A2/B1 ASO vs. Non Target ASO (y-axis) vs. Log $_2$ Fold Change hnRNP A2/B1 ASO vs. Saline (x-axis), $R^2 = 0.81$

**C**

| Gene Name | Log 2 Fold Change | | |
|---|---|---|---|
| | RNA-seq (vs. saline) | qPCR (vs. saline) | qPCR (vs. NT ASO) |
| *Agxt2l1* | -0.748 | -1.231 | -1.099 |
| *Ctnna3* | -0.704 | -1.072 | -0.896 |
| *Fam178a* | 0.317 | 0.262 | 0.094 |
| *Fam63b* | 0.394 | 0.057 | 0.069 |
| *Gpc5* | -1.315 | -1.531 | -1.219 |
| *HnRNP A1* | 0.569 | 0.298 | 0.424 |
| *HnRNP A2/B1* | -2.450 | -3.276 | -2.354 |
| *HnRNP H1* | 0.289 | 0.246 | 0.247 |
| *HnRNP Pf* | 0.159 | 0.146 | 0.203 |
| *Ina* | -0.373 | -0.604 | -0.236 |
| *Lims1* | -0.588 | -0.864 | -0.539 |
| *Nefh* | -0.341 | -0.663 | -0.552 |
| *Nefm* | -0.568 | -0.620 | -0.483 |
| *Nrxn3* | -0.989 | -0.749 | -0.467 |
| *Prpf4b* | 0.263 | 0.148 | 0.112 |
| *Prpsap1* | 0.655 | 0.349 | 0.515 |
| *Ptrf* | 0.315 | -0.136 | 0.307 |
| *Ralgapa1* | 0.226 | -0.312 | 0.342 |
| *Ring1* | 0.697 | -0.348 | 0.391 |
| *RplP0* | 0.182 | 0.037 | 0.568 |
| *Shank2* | -0.602 | -0.276 | -0.141 |
| *Slc39a12* | 0.260 | -0.041 | 0.271 |
| *SnRNP 25* | 0.410 | 0.200 | 0.240 |
| *Sod1* | -0.094 | 0.047 | 0.198 |
| *Srsf3* | 0.377 | 0.163 | 0.060 |
| *Srsf7* | 0.349 | 0.218 | 0.113 |
| *Stom* | 0.469 | -0.089 | 0.534 |
| *Sv2A* | 0.214 | -0.144 | -0.469 |
| *Tcf20* | 0.404 | 0.140 | -0.128 |
| *Trap 1* | -0.793 | -0.845 | -0.418 |
| *Ube2d3* | 0.252 | 0.322 | -0.121 |
| *Upp2* | -0.279 | -1.193 | -1.465 |

■ **With iCLIP peaks**

**D**

Non-target | A2/B1 ASO

hnRNP A2/B1
hnRNP A1
160%
hnRNP U
hnRNP M
Tubulin
hnRNP H1
SRSF7
150%
Tubulin

**E**

| Summary: Small RNA Sequencing | | | |
|---|---|---|---|
| Tissue Type | Treatment | miRNAs Detected | Differentially Expressed |
| Mouse Spinal Cord | *Hnrnpa2b1* ASO | 578 | miR-146a (+2.15 fold) |
| Human iPSC MNs | *HNRNPA2B1* ASO | 460 | none |

**F**

Distal/Total (y-axis): Atp1b2, Hnrnph1

■ ASO  ■ Saline

**Figure S2. Supplemental data related to gene expression changes upon hnRNP A2/B1 depletion (Figure 2).**

**(A)** Correlation of log$_2$ fold change in hnRNP A2/B1 ASO treated mouse spinal cord compared to saline treated spinal cord for 32 genes with the highest fold-change as judged by RNA-seq or microarray. Fold change is determined by qPCR (x-axis) and RNA-seq (y-axis). Dotted line is the least-squares linear regression.

**(B)** Correlation of log$_2$ fold change in hnRNP A2/B1 ASO treated mouse spinal cord vs. saline treated spinal cord (X-axis) and hnRNP A2/B1 ASO treated spinal cord, vs. NTC ASO (y-axis), for the same 32 genes. Dotted line is the least squares linear regression.

**(C)** Table of the 32 most highly differentially expressed upon hnRNP A2/B1 depletion in mouse spinal cord. Differential expression was determined by RNA-seq and microarray and then confirmed by qPCR. Genes marked in green contain iCLIP enrichment.

**(D)** Western blotting showing upregulation, upon hnRNP A2/B1 depletion, of the levels of hnRNP A1 and SRSF7, but not of the other splicing factors indicated. Tubulin served as a loading control. Each lane represents lysate prepared from one spinal cord.

**(E)** Summary of small RNA sequencing results from mouse spinal cords and human iPSC-MNs in which hnRNP A2/B1 was depleted by ASO treatment, compared to control treatment (n=3 replicates per treatment and cell/tissue type, for a total of 12 samples). The data show that among 578 and 460 microRNAs detected in mouse spinal cords and human iPSC-MNs, respectively, only one microRNA (miR-146a) was significantly differentially expressed in mouse spinal cords, while none were found to be affected by hnRNP A2/B1 depletion in human iPSC-MNs.

**(F)** Validation of alternative poly-A site usage upon knockdown of hnRNP A2/B1 of events in Figures 2I and 2J. qPCR was performed for each gene with primer sets detecting the proximal and distal cleavage sites. The ratio is displayed on the y-axis.

**Figure S3**
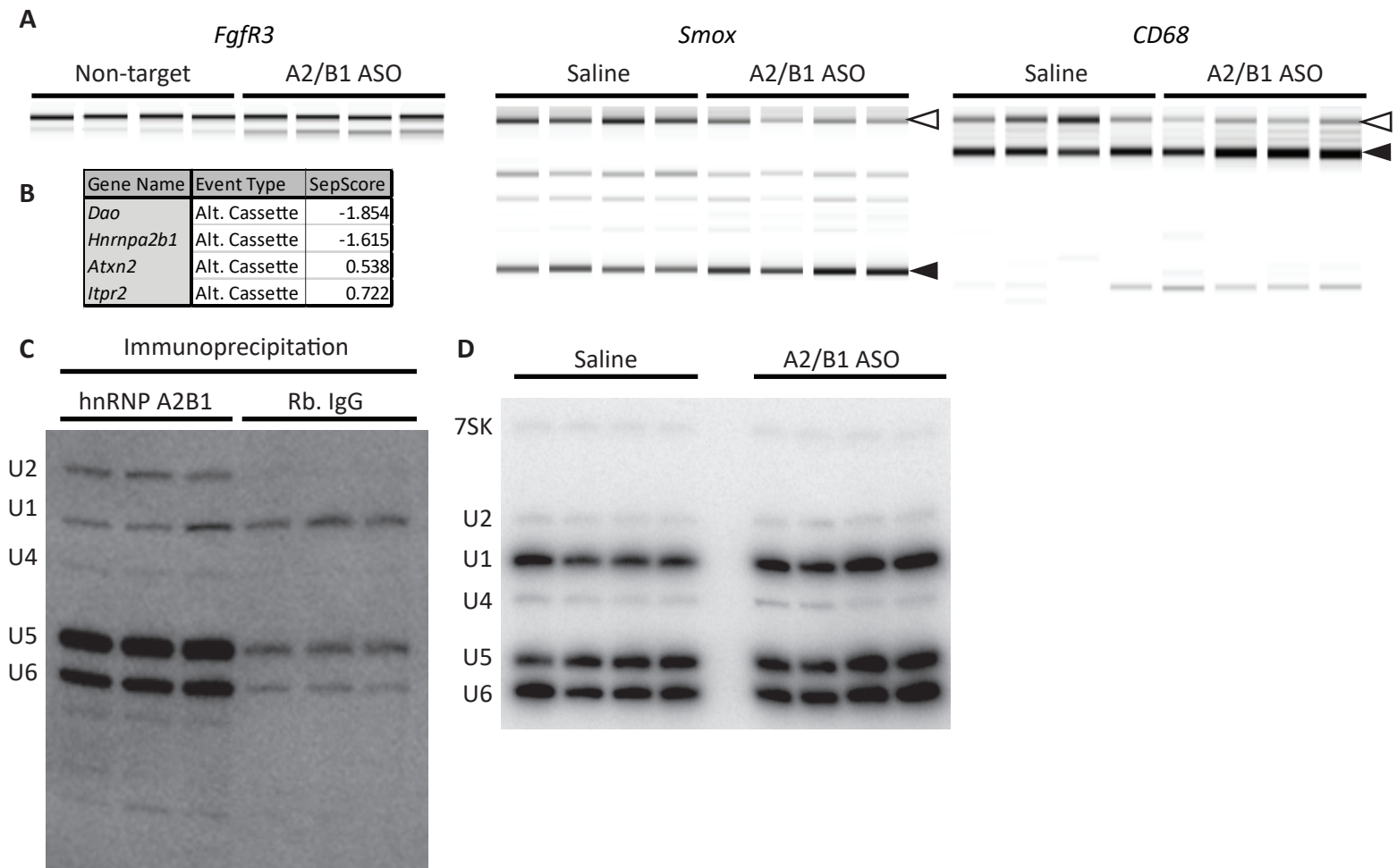


**Figure S3. Supplemental data related to AS changes induced upon hnRNP A2/B1 depletion (Figure 3).**

**(A)** Three additional splicing changes detected in ASO treated mouse spinal cords validated by RT-PCR and capillary electrophoresis. Arrows point to the transcripts produced by alternative cassette events.

**(B**) Table of significantly changed splicing events, altered upon depletion of hnRNP A2/B1 in mouse spinal cord, that overlap with previously published ALS related genes.

**(C)** Enrichment of hnRNP A2/B1 on RNA components of the spliceosome. hnRNP A2/B1 was immunoprecipitated from mouse spinal cord lysates. The bound RNA was recovered and detected by northern blot against five snRNAs.

**(D)** Northern blot against five snRNAs from mouse spinal cord total RNA. There is no consistent difference in snRNA levels after hnRNP A2/B1 depletion.

| Gene Name | Event Type | SepScore |
|---|---|---|
| Dao | Alt. Cassette | -1.854 |
| Hnrnpa2b1 | Alt. Cassette | -1.615 |
| Atxn2 | Alt. Cassette | 0.538 |
| Itpr2 | Alt. Cassette | 0.722 |
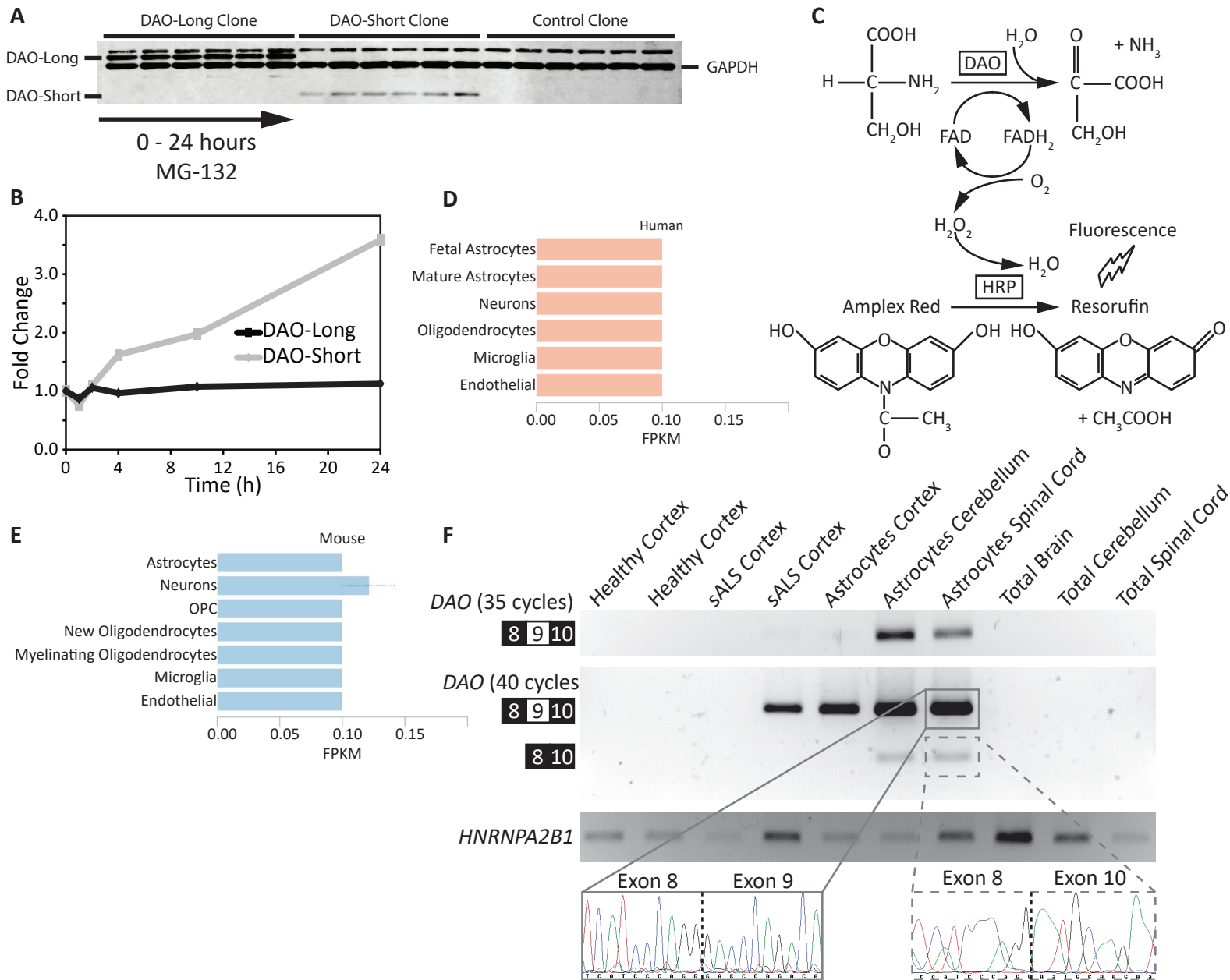
# Figure S4



**Figure S4. Supplemental data related to exon skipping in the _Dao_ gene after hnRNP A2/B1 depletion in mouse spinal cord (Figure 4).**

**(A)** Western blot of DAO in transgenic cell lines expressing the long or short isoforms of the protein. Cells were treated with MG-132 and harvested 1h, 2h, 4h, 10h and 24 later. GAPDH served as a loading control. These data were used to generate the graph in Figure 4H. The top band is an unspecific band.

**(B)** Relative fold change in DAO protein after treatment with MG-132 determined by Western blotting. The zero time point for each line is normalized to 1.

**(C)** A schematic representation showing how the Amplex Red assay is coupled to DAO and used to read out the activity level by fluorescence emission.

**(D-E)** Expression data from the Brain RNA-Seq project. FPKM values for _DAO/Dao_ are at the noise level for all samples in both human (C) and mouse (D).

**(F)** RT-PCR analysis of the long and short _DAO_ transcript isoforms in a panel of RNA from h isolated from healthy cortex and sALS primary motor cortex obtained from deceased individuals, primary human astrocytes (ScienCell) and total brain, cerebellum and spinal cord (Clontech). The isoforms detected and the PCR amplification cycle numbers used are depicted to the left of the agarose gel image. Sanger sequencing from the indicated gel-extracted PCR amplicons (Insets) confirmed the identity of the PCR products (bottom).

# Figure S5

**A**

| Designation | Cell Type | Gender | Disease Status | Genotype | Karyotype |
|---|---|---|---|---|---|
| Ctrl. JCV | hiPSC | Male | Unaffected | WT | 46 XY |
| H1 | hESC | Male | N/A | WT | 46 XY |
| H7 | hESC | Fermale | N/A | WT | 46 XX |
| H9 | hESC | Fermale | N/A | WT | 46 XX |
| VCP R155H-1.1 | hiPSC | Male | MSP | VCP R155H | 46 XY |
| VCP R155H-1.2 | hiPSC | Male | MSP | VCP R155H | 46 XY |
| A2B1 D290V-1 | hiPSC | Fermale | MSP | hnRNP A2/B1 D290V | 46 XX |
| A2B1 D290V-2 | hiPSC | Fermale | MSP | hnRNP A2/B1 D290V | 46 XX |
| A2B1 D290V-3 | hiPSC | Male | MSP | hnRNP A2/B1 D290V | 46 XY |

**B**
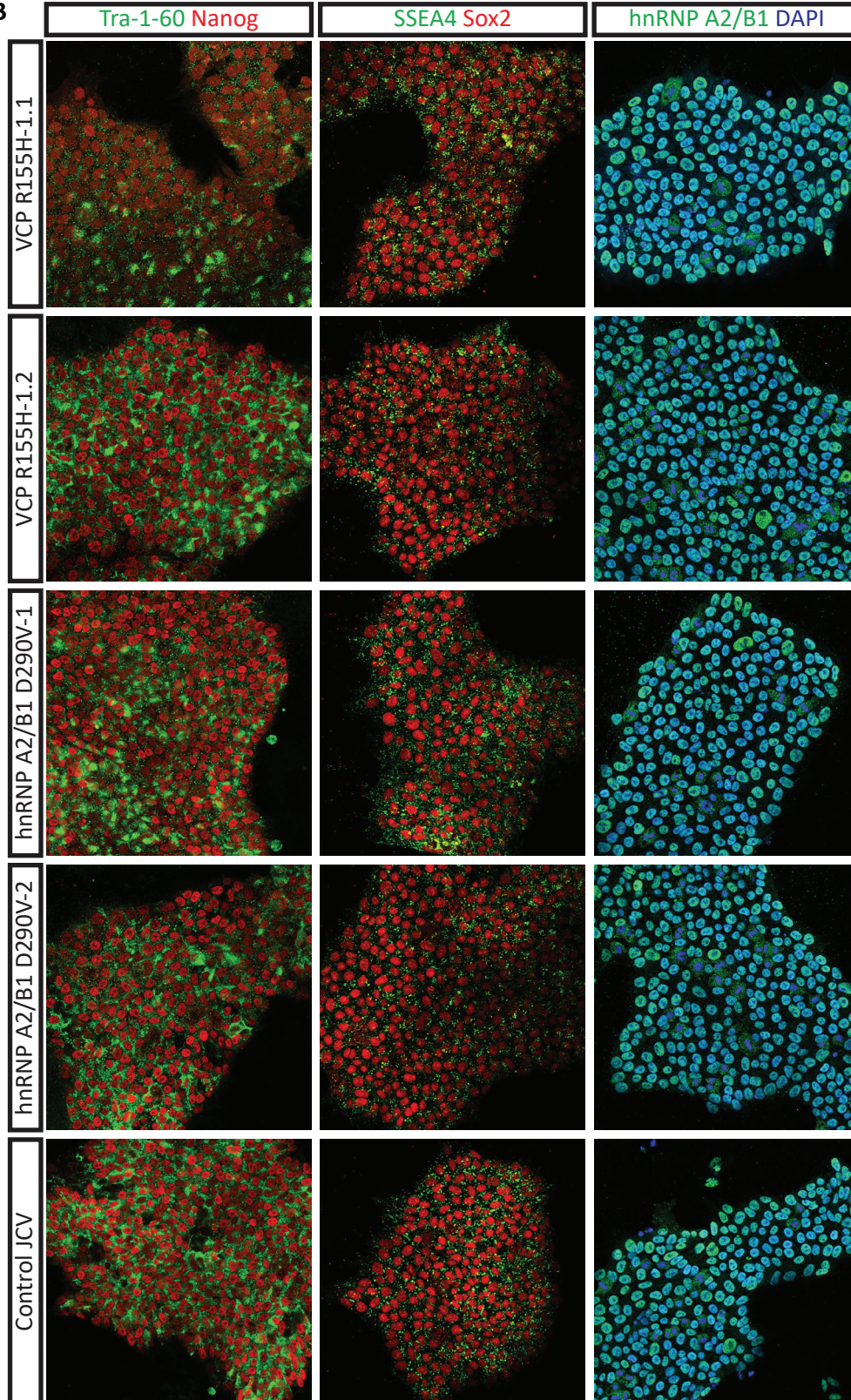
**Figure S5. Supplemental data related to the characterization of human iPSC and ESC lines used in Figures 6 and 7.**

**(A)** Summary of PSCs used in Figures 6 and 7 and associated patient information where applicable.

**(B)** Immunofluorescence staining of selected iPSC lines demonstrating the expression of pluripotency markers.

**Figure S6**

**A** Time (days) Harvest

1  7  9  18  22  25  53

KOSR → N2

N2 + B27 + Ascorbic Acid + BDNF + GDNF + CNTF

Dorsomorphin + SB431542

Aphidicolin

Retinoic Acid + SAG

DAPT

Antisense Oligonucleotide Treatment

Matrigel

PDL + Laminin

**B** Day 28 | Day 53

HB9 Islet1

Nestin HuC

PAX6 OLIG2

SMI31 MAP2

NKX6.1 DCX

**C** Day 32 | Day 46

Activity heatmap

**D** Waveforms

**E** Raster plot

Time (s)

**F** Mean Firing Rate

mHz

Day 32  Day 46

**G** Mean Number of Bursts

Day 32  Day 46

**Figure S6. Supplemental data related to the characterization of human PSC derived motor neurons used in Figures 6 and 7.**

**(A)** A schematic of the differentiation and treatment of human PSC derived motor neurons. For stress granule experiments (Figure 7; Figure S7) cells were treated at day 28 and were not treated with aphidicolin. For ASO depletion experiments (Figure 6) cells were harvested at day 53 after aphidicolin treatment. For MEA experiments (Figure S6C-S6F), cells were recorded on the indicated day and were not treated with aphidicolin.

**(B)** Immunofluorescence staining of the indicated protein markers in iPSC-MNs derived from an unaffected individual. Cells were fixed at day 28 without aphidicolin treatment, and day 53 after aphidicolin treatment. Scale bare = 100 μm.

**(C)** Heat map of spontaneous electrophysiological activity on an multielectrode array (MEA) in four wells (64 electrodes per well) recorded at the indicated time points. MNs were derived from unaffected control hiPSCs.

**(D)** Six examples of spikes recorded by the MEA at the indicated time points.

**(E)** Raster plot of MEA activity across 64 electrodes (rows) in a single well over 30 seconds. Tick marks indicate single spikes. Blue ticks indicate bursting events closely spaced in time (5 spikes in less than 100 msec). Pink boxes denote spontaneous synchronous activity distributed over many electrodes (10 spikes in less than 100 msec).

**(F–G)** Quantification of firing rate (F) and mean number of bursts (G) over a 5 minute recording session in unaffected iPSC-MNs. Error bars are SEM calculated using four wells per condition.

**A**

Detergent Insoluble

| | Nuclear Fraction | | | | Cytoplasmic Fraction | | | |
|---|---|---|---|---|---|---|---|---|
| | Affected VCP Q155H | Affected A2/B1 D290V | Affected A2/B1 D290V | Unaffected A2/B1 WT | Affected VCP Q155H | Affected A2/B1 D290V | Affected A2/B1 D290V | Unaffected A2/B1 WT |

A2/B1

1.73    2.15    0.93    1.00

Histon H3

β-Actin

Detergent Soluble

| | Nuclear Fraction | | | | Cytoplasmic Fraction | | | |
|---|---|---|---|---|---|---|---|---|
| | Affected VCP Q155H | Affected A2/B1 D290V | Affected A2/B1 D290V | Unaffected A2/B1 WT | Affected VCP Q155H | Affected A2/B1 D290V | Affected A2/B1 D290V | Unaffected A2/B1 WT |

A2/B1

Histon H3

β-Actin

**Figure S7. Supplemental data related to stress granule formation and nuclear aggregation in iPSC-MNs (Figure 7).**

**(A)** Western blots showing results of nuclear / cytoplasmic (left / right) and detergent insoluble / soluble fractionation (top / bottom) in unaffected and MSP iPSC-MN. Histone H3 and β-Actin were used as nuclear and cytoplasmic loading controls, respectively. Quantification is shown for the hnRNP A2/B1 bands in the nuclear insoluble fraction. Quantification determined relative to the unaffected sample.

**(B)** Immunofluorescence staining for hnRNP A2/B1 (green) and PABP (red) (top row; see Figure S7C for unmerged channels), as well as the indicated additional stress granule markers (red) in iPSC-MNs from an unaffected individual and an hnRNP A2/B1 D290V patient (middle and bottom rows). The images of TIA1 staining in controls also serve as controls in Figure S7D. Scale bar = 10 µm. Magenta arrows indicate hnRNP A2/B1 localized to stress granules. White arrows indicate stress granules negative for hnRNP A2/B1 staining.

**(C)** Immunofluorescence staining for PABP and hnRNP A2/B1 in iPSC-MNs from an unaffected individual (control) and an hnRNP A2/B1 D290V patient, treated with puromycin or PBS (vehicle). Arrows indicate hnRNP A2/B1 located to stress granules. Scale bar = 10 µm.

**(D)** Immunofluorescence staining showing the localization of hnRNP A2/B1 (green) and TIA-1 (red) in control and VCP R155H iPSC-MNs. Nuclei are DAPI-stained (blue). Scale bar = 10 µm.

**Figure S8**

A — Disease Status flowchart: Unaffected (Wildtype, n=3), Affected (A2B1 D290V n=3, VCP R155H n=1). hiPSCs / hESCs → Reprogramming → Motor neurons → Differentiation. Unaffected/Affected split into Saline and Puromycin. CTRL 1, CTRL 2, CTRL 3 (Changed in 3 healthy); D290V 1, D290V 2, D290V 3, VCP (Changed in 4 affected). Changed in all 7 comparisons.

B — PBS vs. Puromycin clustering heatmap.

C — Genes Changed in All. Row Z-Score (Fold Change), +3 to −3. Affected: VCP, D290V-1, D290V-2, D290V-3; Unaffected: CTRL-1, CTRL-2, CTRL-3.

D — Splicing Events Changed in All. Row Z-Score (Sepscore), +3 to −3. Affected: D290V-3, VCP, D290V-1, D290V-2; Unaffected: CTRL-2, CTRL-3, CTRL-1.

**Figure S8. Supplemental data related to splicing and expression changes resulting from puromycin-induced stress in iPSC-MNs from hnRNP A2/B1 D290V patients and the VCP R155H patient (Figure 8).**

**(A)** Schematic of the experimental design.

**(B)** Heat map of the correlation of the raw intensities of the 42 splicing-sensitive microarrays used in this experiment. Hierarchical clustering shows clear separation of puromycin- and vehicle (PBS)-treated samples.

**(C-D)** Heat maps of fold-changes in gene expression (C) and AS (SepScore; D) in those gene that are changed upon stress in all samples. Hierarchical clustering shows clear separation of affected and unaffected control samples. Color mapping is by Z-score computed for each row.

**Supplemental Data Legends**

**Data S1. Supplemental data and inventory of oligonucleotides, antibodies and other materials related to Figures 1-8, Figures S1-S8 and supplemental experimental procedures.**

**(A)** iCLIP clusters in mouse spinal cord.

**(B)** Cell lines used. hFibs = human fibroblasts. GBM = glioblastoma multiforme.

**(C)** eCLIP peaks in human iPSC-MNs. Fold change refers to IP sample over the matching input sample.

**(D)** RNA-seq data from mouse spinal cord treated with ASO against hnRNP A2/B1.

**(E)** Gene expression data from microarray analysis of mouse spinal cord treated with ASO against hnRNP A2/B1. Fold change refers to ASO depleted over control.

**(F)** microRNA expression data from small RNA-seq in mouse spinal cord. Fold change refers to ASO depleted over control.

**(G)** microRNA expression data from small RNA-seq in human iPSC-MNs. Fold change refers to ASO depleted over control.

**(H)** Alternative polyadenylation events in mouse spinal cord.

**(I)** AS data from microarray analysis of mouse spinal cord treated with ASO against hnRNP A2/B1.

**(J)** AS data from microarray analysis of human fibroblasts from hnRNP A2/B1 D290V patients and controls. Columns are sepscore for pairwise comparison of the various samples.

**(K)** AS data from microarray analysis of iPSC-MNs from CTRL.JCV (unaffected individual) treated with ASO against hnRNP A2/B1 vs. nontargetting control ASO.

**(L)** AS data from microarray analysis of iPSC-MNs from D290V-2.1 (MSP individual, hnRNP A2/B1 D290V) treated with ASO against hnRNP A2/B1 vs. nontargetting control ASO.

**(M)** AS data from microarray analysis of iPSC-MNs from CTRL.JCV (unaffected individual) vs. D290V-2.1 (MSP individual, hnRNP A2/B1 D290V).

**(N)** Gene expression data from microarray analysis of puromycin-treated PSC-MNs. Columns indicate fold change calculated for each of the various samples and it's matching unstressed control.

**(O)** AS data from microarray analysis of puromycin-treated PSC-MNs. Columns indicate sepscore calculated for each of the various samples and it's matching unstressed control.

**(P)** Antibodies used. The lot number is mentioned for antibodies that were lot controlled. The applications column lists the application and the dilution used. WB = western blot; IF = immunofluorescence; IP = immunoprecipitation.

**(Q)** Oligonucleotide sequences.

**Data S2. Sequences for 3xFLAG tagged *Dao* open reading frames related to Figure 4 and Figure S4 and supplemental experimental procedures.**

**(A)** mDao_Long_3xFLAG:

GGTACCGCCACCATGCGCGTGGCCGTGATCGGAGCAGGAGTCATTGGGCTCTCCACAGCCCTCTGCAT-
TCATGAGCGTTACCACCCAACACAGCCACTGCACATGAAGATCTATGCAGATCGATTCACCCCGTTCAC-
CACGAGCGATGTGGCCGCCGGCCTCTGGCAGCCTTATCTCTCTGACCCCAGCAACCCTCAGGAGG-
CGGAGTGGAGCCAGCAAACGTTTGATTACCTGCTGAGCTGCCTCCATTCTCCAAACGCTGAAAAAAT-
GGGCCTGGCCCTAATCTCAGGCTACAACCTCTTCCGAGATGAAGTTCCGGACCCTTTCTGGAAAAAC-
GCAGTTCTGGGATTCCGGAAGCTGACCCCCAGTGAGATGGACCTGTTCCCTGATTATGGCTACGGCT-
GGTTCAATACAAGCCTCCTTCTAGAGGGGAAGAGCTACCTGCCATGGCTAACTGAGAGGTTAACTGA-
GAGGGGAGTGAAGCTTATCCATCGGAAGGTGGAGTCTCTCGAAGAGGTGGCAAGAGGAGTGGATGT-
GATTATCAACTGCACCGGGGTGTGGGCCGGGGCCCTGCAAGCAGATGCCTCCCTGCAGCCAGGCCG-
GGGCCAGATCATCCAGGTGGAGGCCCCTTGGATTAAACACTTCATCCTCACCCATGATCCTAGCCTTGG-
TATCTACAACTCTCCGTACATCATCCCAGGTTCCAAGACAGTTACGCTCGGGGGTATATTCCAGCTGGG-
GAACTGGAGCGGGTTAAACAGCGTCCGTGACCACAATACCATTTGGAAGAGCTGCTGTAAACTGGAGC-
CCACCCTGAAGAATGCAAGAATTGTGGGTGAACTCACTGGCTTCCGGCCAGTCCGGCCTCAGGTCCG-
GCTAGAAAGAGAATGGCTTCATTTTGGATCTTCAAGTGCAGAGGTCATCCACAACTATGGTCATGGAG-
GTTACGGGCTCACAATCCACTGGGGTTGTGCAATGGAGGCGGCCAACCTCTTCGGGAAAATTCTAGAG-
GAAAAGAAGTTGTCCAGGTTGCCTCCCTCCCACCTCGACTACAAAGACCATGACGGTGATTATAAAGAT-
CATGACATCGATTACAAGGATGACGATGACAAGTGAGCGGCCGC

**(B)** mDao_Short_3xFLAG:

GGTACCGCCACCATGCGCGTGGCCGTGATCGGAGCAGGAGTCATTGGGCTCTCCACAGCCCTCTGCAT-
TCATGAGCGTTACCACCCAACACAGCCACTGCACATGAAGATCTATGCAGATCGATTCACCCCGTTCAC-
CACGAGCGATGTGGCCGCCGGCCTCTGGCAGCCTTATCTCTCTGACCCCAGCAACCCTCAGGAGGC-
GGAGTGGAGCCAGCAAACGTTTGATTACCTGCTGAGCTGCCTCCATTCTCCAAACGCTGAAAAAATG-
GGCCTGGCCCTAATCTCAGGCTACAACCTCTTCCGAGATGAAGTTCCGGACCCTTTCTGGAAAAACG-
CAGTTCTGGGATTCCGGAAGCTGACCCCCAGTGAGATGGACCTGTTCCCTGATTATGGCTACGGCTG-
GTTCAATACAAGCCTCCTTCTAGAGGGGAAGAGCTACCTGCCATGGCTAACTGAGAGGTTAACTGAGAG-
GGGAGTGAAGCTTATCCATCGGAAGGTGGAGTCTCTCGAAGAGGTGGCAAGAGGAGTGGATGTGAT-
TATCAACTGCACCGGGGTGTGGGCCGGGGCCCTGCAAGCAGATGCCTCCCTGCAGCCAGGCCGGGGC-
CAGATCATCCAGGTGGAGGCCCCTTGGATTAAACACTTCATCCTCACCCATGATCCTAGCCTTGGTATCTA-
CAACTCTCCGTACATCATCCCAGGAATGCAAGAATTGTGGGTGAACTCACTGGCTTCCGGCCAGTCCGG-
CCTCAGGTCCGGCGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGAC-
GATGACAAGTAGGCGGCCGC

## Supplemental Experimental Procedures

### RNA Bind-n-Seq (RBNS)

*RBNS experimental procedure*

RBNS was performed as previously described (Lambert et al., 2014). Briefly, a truncated reading frame of *HNRNPA2B1* (amino acids 1-197), which contains both RRMs, was cloned downstream of a tandem GST-SBP tag into a modified pGex6p-1 vector (GE). The truncated protein was recombinantly expressed, purified via the GST tag, and used for RBNS performed at 5 concentrations (5 nM, 20 nM, 80 nM, 320 nM, and 1300 nM) with a pool of randomized 20-mer RNAs flanked by short primers. Preparation of the randomized RNA pool and experimental conditions were described in (Lambert et al., 2014), with the modification that RBNS was performed at 4 degrees C rather than 37 degrees C.

*RBNS Computational Analysis*

Motif enrichment (R) values were calculated for 6-mers as the motif frequency in the RBP-selected pool over the frequency in the input RNA library. R values were considered significant if they had a Z-score $\geq 2$ (mean and standard deviation calculated over all 6-mers). Values in Fig. 1D are for the protein concentration library with the highest overall enrichment (80 nM). For comparison with CLIP-seq data, RBNS enrichments were determined from the concentration with the largest enrichment. For enrichment in CLIP-seq 6-mers, FASTQ sequences were extracted from all clusters, and a matched number of random clusters from the same genomic region (5′UTR, CDS, 3′UTR, proximal introns, and distal introns. EMBOSS compseq was performed on the true and background set, and a delta between real and background k-mers was calculated with the equation:

$$\Delta \text{kmer} = \frac{\frac{f_{CLIP}}{N_{CLIP}} - \frac{f_{background}}{N_{background}}}{\sqrt{\left(\left(\frac{1}{N_{CLIP}} + \frac{1}{N_{background}}\right) * g * (1-g)\right)}}, \text{ for } g = \frac{f_{CLIP} + f_{background}}{N_{CLIP} + N_{background}}$$

where N is the number of times the motif occurs in the set and f is observed frequency of the motif. To plot enrichment, all 6-mers with the 4-mer of interest were highlighted and a KDE plot was created for all 6-mers. Statistical significance in differences between distributions was determined by the Kolmogorov–Smirnov 2-tailed test.

### CLIP analysis of mouse and human samples

*iCLIP experimental procedure*

8-week old, female, C57/BL6J mice were obtained from Jackson Labs (Bar Harbor, ME). Mice were sacrificed and the spinal cords were removed, flash frozen in liquid nitrogen, and ground finely with a mortar and pestle. The frozen cords were UV crosslinked at $\lambda$=254nm with 400 mJ/cm$^2$ of energy. iCLIP libraries were prepared as previously described (Huppertz et al., 2014) using rabbit polyclonal antibody GTX127928 (Genetex) (10 mg per sample) (Data S1P) (RRID: AB_2616069). Libraries were sequenced on the HISEQ 2500 platform in single end mode with a read length of 50 base pairs.

*eCLIP experimental procedure*

eCLIP was performed according to (Van Nostrand et al., 2016). Human iPSC-MNs were differentiated in culture for 28 days. Approximately 20 million cells were UV crosslinked at $\lambda$=254nm with 400 mJ/cm$^2$ of energy. Crosslinked cell pellets were lysed and treated with RNAse I (Ambion). Lysate was immunoprecipitated using rabbit polyclonal antibody GTX127928 (Genetex) (10 µg per sample) and Dynabeads Protein G (Thermo Fisher Scientific). Immunoprecipitated samples were washed and dephosphorylated with Fast AP (Thermo Fisher Scientific) and T4 PNK (NEB). A 3' RNA adapter was ligated using T4 RNA Ligase (NEB). Samples were run on PAGE gels and transferred to nitrocellulose membranes. A region of membrane spanning 75kDa above the molecular weight of hnRNP A2/B1 was cut and RNA was recovered using Proteinase K and phenol-chloroform. RNA was reverse transcribed using Affinityscript (Agilent Technologies) and treated with ExoSAP-IT (Affymetrix). A DNA adapter containing a 5nt randomer was then ligated to the 3' end (T4 RNA Ligase, NEB). Samples were purified using Dynabeads MyOne Silane (Thermo Fisher Scientific), quantified by qPCR, and then PCR amplified

to the appropriate number of cycles. Amplified libraries were size selected with an agarose gel, quantified using the Agilent Tapestation, and sequenced on the Illumina HiSeq 2500 platform using paired end 50bp reads.

## iCLIP computational analysis

Raw CLIP-seq reads were trimmed of polyA tails, adapters and low quality ends using Cutadapt with parameters:

> --match-read-wildcards --times 2 -e 0 -O 5 --quality-cutoff' 6 -m 18 -b TCGTATGCCGTCTTCTGCTTG -b ATCTCGTATGCCGTCTTCTGCTTG -b CGACAGGTTCAGAGTTCTACAGTCCGACGATC -b TGGAATTCTCGGGTGCCAAGG -b AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAA -b TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT

Trimmed reads were mapped against a database of repetitive elements derived from RepBase (version 18.05) using Bowtie (version 1.0.0) with parameters (Langmead et al., 2009):

> -S -q -p 16 -e 100 -l 20

Reads not mapped to repetitive elements were mapped to the mm9 mouse genome (UCSC assembly) using STAR (version 2.3.03) with parameters (Dobin et al., 2013):

> --outSAMunmapped Within –outFilterMultimapNmax 1 –outFilterMultimapScoreRange 1

Reads having the same 5' mapping position were collapsed to a single read to eliminate PCR duplication. CLIP-seq peaks were identified as previously described (Zisoulis et al., 2010).

## eCLIP computational analysis

Reads were demultiplexed using custom scripts and the randomer was appended to the read name. Reads were trimmed, filtered for repetitive elements, and mapped to human genome assembly hg19 as described in iCLIP computational analysis. PCR duplicated reads were removed based on the start positions of read1, read2, and the sequence of the randomer. eCLIP peaks were identified using CLIPPER with parameters (Lovci et al., 2013):

> –s hg19 –o –bonferroni –superlocal --threshold-method binomial --save-pickle

Peak strength was then normalized against a size matched input by calculating fold enrichment of number of reads in IP versus number of reads in size matched input.  Peaks were called significant if the number of reads in IP was greater than the number of reads in input and the peaks a Bonferroni corrected Fisher exact $p$-value of less than .05.

## Distance away from motif

All significant peaks were used to compute distance of motifs away from peaks.  Distance was calculated using the HOMER command:

> annotatePeaks.pl <peaks> hg19 -m <motif file> -hist 20 -size 1000 -noann.

 Peaks were then shuffled with the number of peaks binding in each region (5'UTR, 3'UTR, CDS, proximal and distal introns) in the transcriptome being preserved and a background distribution was computed.

## De novo motif analysis

Motif analysis was performed using the findMotifs program in the HOMER software package with parameters (Lovci et al., 2013):

> -p 4 -rna -S 10 -len 5,6,7,8,9 (REF)

A background set was obtained using random clusters in the same genic regions as CLIPPER-identified hnRNP A2/B1 clusters.

**Cell culture**

All cell cultures were kept humidified at 37°C and 5% $CO_2$.

*Human fibroblasts*

Human fibroblasts were grown on Poly-L-Lysine coated plates in DMEM (High Glucose) supplemented with 10% FBS, 1% NEAA, and 1% L-glutamine (Thermo Fisher Scientific). Cells were passaged using TrypLE select (Thermo Fisher Scientific). Cell line information is detailed in Data S1B.

*Human iPSC generation*

Human fibroblasts were reprogrammed using the CytoTune iPS Sendai Reprogramming kit, according to the manufacturer's instructions (Thermo Fisher Scientific). Nascent colonies were manually passaged onto Matrigel coated dishes (Becton Dickenson) for several passages and then expanded using Accutase (Innovative Cell Technologies). In some cases, previously generated iPSC lines from individual J. Craig Venter were used (Gore et al., 2011). Cell line information is detailed in Data S1B.

*iPSC and ESC culture*

All human pluripotent cells (PSCs) were grown on Matrigel coated dishes (Becton Dickenson), in mTesR1 media (Stem Cell Technologies) and passaged manually or with Accutase (Innovative Cell Technologies). During all passaging or thawing steps, the ROCK inhibitor Y27632 (Tocris) was used at a concentration of 10µM for 24 hours.

*Differentiation of human motor neurons from PSCs*

Human iPSCs and ESCs were differentiated according to a modified version of (Burkhardt et al., 2013; Chambers et al., 2009). Confluent dishes of feeder free PSCs were placed in hEB Media: Knockout D-MEM + 10% Knockout Serum Replacement (Thermo Fisher Scientific) + 10% Plasmanate (Biocare) + GLUTAMAX + NEAA (Thermo Fisher Scientific) and supplemented with 10 uM SB431542, 10 uM Dorsomorphin dihydrochloride, and 4 µM CHIR99021 (Tocris). SB431542 and Dorsomorphin were maintained in all cultures until day 18 of differentiation CHIR99021 was maintained until day 6 of differentiation. On days 4, 5 and 6 of differentiation, hEB media was mixed with N2 Base media: D-MEM/F12 + GLUTAMAX, 1% N-2 Supplement + 4.5 mM D-Glucose, 0.05 mM Ascorbic Acid (Sigma-Aldrich) at a ratio of 70:30, 50:50, and 50:50, respectively. On days 7 and 8 of differentiation, cells were maintained in a 50:50 combination of hEB media and Maturation Media: D-MEM/F12 + GLUTAMAX, 2% N-2 Supplement, 4% B-27 Serum-Free Supplement (Thermo Fisher Scientific), 9.0 mM D-Glucose, 0.1 mM Ascorbic Acid (Sigma-Aldrich) in addition to 2 ng/mL each of Ciliary Neurotrophic Factor (CNTF), Brain-Derived Neurotrophic Factor (BDNF), and Glial Cell-Derived Neurotrophic Factor (GDNF) (Peprotech). Beginning on day 7, the neurons were patterned with 200 nM Smoothened Agonist (EMD Biosciences) and 1.5uM Retinoic Acid (Sigma-Aldrich). These compounds were maintained until day 22. After 18 days of differentiation, cells were dissociated using Accutase, and transferred to Poly-D-Lysine (Sigma-Aldrich) + Laminin (Thermo Fisher Scientific) coated dishes and maintained in Maturation Media with Retinoic Acid and Smoothened Agonist. On day 22, RA and SAG were withdrawn and the cells were maintained in Maturation Media containing 2 µM DAPT (Tocris) for a period of 3 days. After this time, cells were maintained in Maturation Media only. Throughout the protocol media was changed daily. We analyzed PSC-MNs for neuronal markers at two or more time points including 25 and 32 days after neural induction. Cells in PSC-MN cultures had processes that stained positive for axonal protein neurofilament and expressed lineage markers of motor neurons that innervate the limb: ISLET1 and HB9.

*Multi-electrode array recordings*

On day 18 of differentiation, PSC-MNs were dissociated into plates containing multi-electrode arrays (Axion Biosystems). PSC-MNs were matured according to our standard protocol until day 46. On day 32 and day 46 neurons were recorded for 5 minutes in standard media using Axion Biosystems Maestro amplifier.

_FLP-In-293 (HEK293-FRT) stable cell line generation_

FLP-In-293 cells were obtained from Thermo Fisher Scientific. Cells were grown on Poly-L-Lysine coated plates in DMEM (High Glucose) supplemented with 10% FBS, 1% NEAA, and 1% L-glutamine (Thermo Fisher Scientific). Cells were passaged using TrypLE select (Thermo Fisher Scientific). For stable cell line generation, $1x10^6$ cells were plated into each well of a 6 well plate. After 24 hours, wells were transfected with Lipofectamine 2000 and 3µg of pOG44 + 1µg of plasmid containing the gene of interest (pcdna5/FRT backbone). 48 hours after transfection, cells were split into 10cm dishes and supplemented with 100µg/ml hygromycin. Treatment with hygromycin continued until colonies appeared. Drug resistant colonies were manually passaged and then expanded. Expression of the gene of interest was confirmed by western blotting.

_U-251 MG cell culture and shRNA transfection_

The U-251 MG human glioblastoma cell line was obtained as a gift from Frank Funari. Cells were grown in conditions identical to what is described above for fibroblasts. For shRNA mediated hnRNP A2/B1 depletion, U-251 cells were seeded at a density of 100,000 cells per well in 12 well plates. 24 hours after plating, cells were transfected with the pLKO.1 vector containing shRNA constructs targeting hnRNP A2/B1 or GFP (Sigma-Aldrich). Transfection was achieved using Lipofectamine 3000 (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. 2µg of DNA and 3µL of reagent was used per well. Cells were harvested with Trizol (Thermo Fisher Scientific) 48 hours after transfection. hnRNP A2/B1 depletion was verified by western blotting. The sequences and catalog numbers for the shRNA constructs used are as follows. All shRNA constructs were acquired as glycerol stocks (Sigma-Aldrich).

shRNA 59 (CAT#: TRCN0000001059):

CCGGCAGAAATACCATACCATCAATCTCGAGATTGATGGTATGGTATTTCTGTTTTT

shRNA 61 (CAT#: TRCN0000001061):

CCGGTGACAACTATGGAGGAGGAAACTCGAGTTTCCTCCTCCATAGTTGTCATTTTT

shRNA 82 (CAT#: TRCN0000010582):

CCGGAGAAGCTGTTTGTTGGCGGAACTCGAGTTCCGCCAACAAACAGCTTCTTTTTT

shRNA GFP:

GCAAGCTGACCCTGAAGTTCAT3

**Antisense oligonucleotide (ASO) based depletion of hnRNP A2/B1**

_Stereotactic injections of ASOs in mouse_

8-week old, female, C57/BL6J mice were anesthetized with isoflurane. Saline containing 500 µg of antisense oligonucleotide targeting Hnrnpa2b1 was delivered by intracerebroventricular injection using a Hamilton syringe. Control animals were injected with saline alone or with an ASO that does not target any known region of the mouse genome. The sequences for the ASOs are as follows: GT$_o$A$_o$C$_o$AacagcttcttA$_o$A$_o$C$_o$TC (_Hnrnpa2b1_), CC$_o$T$_o$A$_o$TaggactatccA$_o$G$_o$G$_o$AA (Control). ASOs were mixed backbone "gapmers" where capitalized nucleotides contain 2'-O-(2-methoxy)ethyl modifications. The backbone was phosphorothioate DNA except for nucleotides flagged with $_o$ which were phosphodiester DNA. Mice were routinely monitored and sacrificed 28 days post injection. The spinal cords were harvested and flash frozen. RNA and protein were extracted using Trizol (Thermo Fisher Scientific) according to the manufacturer's instructions. Successful knockdown was validated by western blot using sc-32316 (RRID: AB_2279639), sc-53531 (RRID: AB_2248245) (Santa Cruz), or GTX127928 (GeneTex). All procedures were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of Isis Pharmaceuticals and the University of California at San Diego.

<u>ASO depletion of hnRNP A2/B1 in human fibroblasts</u>

Patient fibroblasts from control and affected individuals were grown to confluence in triplicate wells. For knockdown experiments 20ng of ASO targeting HNRNPA2B1 or control ASO were transfected using Lipofectamine 2000, in accordance with the manufacturer's instructions (Thermo Fisher Scientific). The sequences for the ASOs are as follows: TTCACatgtcacctgAAACT (*HNRNPA2B1-1*), GCTTCttaactctacACACG (*HNRNPA2B1-2*), CTCAGTAACATTGACACCAC (Control). ASOs were phosphorothioate "gapmers" where capitalized nucleotides contain 2'-O-(2-methoxy)ethyl modifications. Successful knockdown was verified by western blot and qRT-PCR.

<u>ASO depletion of hnRNP A2/B1 in human PSC derived motor neurons</u>

PSC-MNs were differentiated from control individuals and D290V patients as described above. After 25 days 5um ASO against hnRNP A2/B1 or non-targeting ASO (detailed above) was added to the media. For the first 5 days of ASO treatment, cells were also treated with 4μm aphidicolin (Sigma-Aldrich) to eliminate any remaining undifferentiated cells. The media and ASO was replaced every 5 days until 53 days post differentiation. RNA and protein were extracted using TRIzol (Thermo Fisher Scientific) according to the manufacturer's instructions. Successful knockdown was determined by western blotting.

**Western blotting**

Unless otherwise noted, protein samples were extracted and solubilized from TRIzol lysates according to the manufacturer's instructions (Thermo Fisher Scientific). Protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). For samples solubilized in urea containing buffer (Figure S7A) the Pierce 660nm Protein Assay was used instead (Thermo Fisher Scientific). Proteins were separated on Novex Bis-Tris 4-12% PAGE gels (Thermo Fisher Scientific) and transferred onto nitrocellulose or PVDF membranes. Membranes were blocked for one hour in the Odyssey Blocking Buffer (LiCor) and probed with the appropriate primary antibody (Data S1P). Detection was achieved using species specific LiCor IRDye secondary antibodies and an Odyssey infrared imaging system (LiCor).

**Expression analysis by RNA-seq**

<u>Library preparation, sequencing, and analysis</u>

RNA was extracted using TRIzol in accordance with the manufacturer's instructions. RNA quality was determined using the Agilent TapeStation according to the manufacturer's instructions. 1μg of total RNA was used to prepare each library. Library preparation was achieved using a TruSeq stranded total RNA with Ribozero Gold kit (Illumina RS-122-2301). Sequencing was performed on a HISEQ 2500 platform in single end mode with a read length of 100bp. Reads were trimmed to remove adapters and mapped to the genome using STAR with parameters identical to those used for iCLIP analysis. Counts were calculated with featureCounts (Liao et al., 2014) and RPKMs were then computed. Genes with RPKM values lower than 0.1 were not used. Differential expression was measured by calculating two sample T-statistics between ASO treated and control mice. P-values were calculated and then adjusted using a Benjamini-Hochberg FDR <0.01.

<u>Small RNA Sequencing</u>

Mouse spinal cords and human iPSC-MNs were generated and harvested as described in the corresponding sections. Samples were treated with ASOs targeting HNRNPA2B1 or non-target controls in biological triplicate. Libraries were generated using the Illumina TruSeq Small RNA Library Prep Kit using 500ng of total RNA. Libraries were sequenced on the Illumina MiSeq with 50 cycles.

<u>miRNA expression analysis</u>

First, 3' adapters were trimmed from sequencing reads using cutadapt. The data was then mapped to either the human or mouse (hg19 and mm10 respectively; University of California, Santa Cruz [UCSC] [http://genome.ucsc.edu]) genomes using Bowtie software (version 1.1.1); the following parameters were used:

-k [valid alignments per read], 1; -m [number of possible alignments], 10; -l [seed length], 25; --best [optimal alignments]; --chunkmbs 128

featureCounts was used with the mirBase (http://mirbase.org/) miRNA annotations for human (v20; hg19) and mouse (v21; mm10). Differential miRNA expression was determined using the DESeq2 software (https://bioconductor.org/packages/release/bioc/html/DESeq2.html) (Data S1F and S1G).

## Quantitative (qPCR) analysis

500 ng-1 µg of total RNA was reverse transcribed using Superscript III (Thermo Fisher Scientific) with random hexamer primers. cDNA was diluted 10-fold in water and quantified with Power SYBR Green Master Mix (Thermo Fisher Scientific). Detection was carried out on an iQ5 real-time PCR detection system (Bio-Rad). Biological samples were prepared in technical triplicate. Analysis was performed using iQ5 optical system software. Median cT values were calculated from each triplicate set. Genes of interest were normalized by appropriate reference genes (Data S1Q). Relative transcript levels were estimated using the $2^{-\Delta cT}$ method. Biological replicates were averaged to generate mean fold changes. Primer sequences were generated from Primer3 (Untergasser et al., 2012) software or obtained from Integrated DNA Technologies PrimeTime qPCR assays. Wherever possible, primers were designed such that the primer pair flanked an exon-exon junction. Sequences are detailed in Data S1Q.

## Alternative splicing analysis by microarray

### Splicing analysis in mouse spinal cord samples and human PSC derived motor neurons (Figure 8)

500 ng of total RNA was used to prepare each microarray sample. Mouse splicing junction mJay or HTA 2.0 (Affymetrix) arrays were prepared and hybridized by the UCSD VA hospital microarray facility according to the manufacturer's instructions. Data analysis was performed as previously described (Huelga et al., 2012). Significant and high quality events were selected using a q-value < 0.05 and an absolute sepscore > 0.5, where sepscore = $\log_2$[hnRNP A2/B1 depleted or puromycin stress (Skip/Include)/Control treated (Skip/Include)]. For each set of replicates, the log2 ratio of skipping to inclusion was calculated using robust least squares regression. Differential expression data was also calculated from array data as previously described. Significant events were selected for p-value < 0.05 and log2 fold change > 0.5.

### Splicing analysis in human fibroblasts

Human splicing junction HJAY (Affymetrix) microarrays were prepared and hybridized by the UCSD VA hospital microarray facility according to the manufacturer's instructions in technical triplicates. Analysis of AS and differential expression was performed identically to mJay arrays described above. For D290V and VCP mutant patient fibroblast samples, sepscores and q-values were calculated relative to two different fibroblast samples from unaffected individuals. Sepscores and q-values were also calculated between the two control fibroblast samples used. High confidence differential splicing events were filtered by requiring samples to pass the sepscore and q-value cutoff relative to both control samples. Splicing events were further filtered by removing events that were flagged as differentially spliced between the two control samples used.

### Splicing analysis in human PSC derived motor neurons (Figure 6)

500 µg of total RNA was used to perform microarray preparation and hybridization. Samples were hybridized in technical triplicates. RNA samples for microarray profiling were prepared using WT Expression Kit (Ambion), hybridized to Affymetrix HTA 2.0 microarrays, and imaged. Signal for all probes was RMA-normalized (Affymetrix Expression Console). Probes quantifying cassette exons (comprising exclusion junction, upstream and downstream inclusion junction, and inclusion exonic probes) were normalized against the average signal for the entire gene (to remove gene expression changes), and Student's *t*-Test was performed on residuals comparing control versus knockdown in WT and D290V mutant lines separately. Inclusion probes and exclusion probes were analyzed separately, with significant events defined as those with either inclusion or exclusion probe p-value ≤ 0.001. To reduce the confounding effects of genetic variation withing samples, we considered only AS events that were sensitive to hnRNP A2/B1 depletion. Loss of function events were those that were changed in WT samples only. Gain of function events were those that were changed in D290V mutant samples only. *Normal* function events were those that were changed in all samples.

_RT-PCR analysis of splicing changes_

1ug of total RNA was reverse transcribed using superscript III (Thermo Fisher Scientific) and random hexamer primers. For RT-PCR experiments, primers were designed in constitutive exons flanking the alternative exon of interest. Primer sequences are detailed in Data S1Q. cDNA was column purified (Qiagen) and quantified. 4 ng of cDNA was subjected to 35 cycles of PCR. The products were analyzed using the Agilent TapeStation. The peaks from the electropherogram were integrated and the normalized included fraction was calculated as:

$$[(included)/(included+skipped)]_{ASO}/[(included)/(included+skipped)]_{control}.$$

In some cases, PCR products were run on agarose gels, gel extracted and Sanger sequenced to confirm the identity of the observed isoforms. Primer sequences were generated from Primer3 (Untergasser et al., 2012) software.

_Alternative Polyadenylation_

Alternative polyadenylation (APA) sites were identified from RNA-seq data using the bioinformatics algorithm DaPars (Xia et al., 2014), which uses a regression model to locate endpoints of alternative polyadenylation sites, was used to identify differences in APA events between hnRNP A2/B1 depleted samples and controls. To identify significant APA events, we used the following cutoffs:

$$FDR < 0.05, |\Delta PDUI| \geq 0.2, \text{ and } |dPDUI| \geq 0.2.$$

APA scatter plots were generated using the R-Studio program with the ggplot2 library. Selected APA changes were validated using qPCR. The primer sequences are detailed in Data S1Q.

**Northern blotting and RNA immunoprecipitation (RIP) northern blotting**

For northern blots, RNA from ASO injected mouse spinal cord was used (see above for details). For RIP northern blots, 8 weeks old female C57/BL6J mice were sacrificed and the spinal cords extracted as above. Cords were flash frozen in liquid nitrogen and ground into a fine powder with a mortar and pestle. Ground spinal cords were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% NP-40; 0.1% SDS; 0.5% sodium deoxycholate) supplemented with protease Inhibitor (Roche) and murine RNase inhibitor (New England Biolabs). Protein G Dynabeads (Thermo Fisher Scientific) were washed and conjugated with 10µg of antibody against hnRNP A2/B1 (Genetex) (GTX127928) or normal rabbit IgG (Thermo Fisher Scientific). Lysates were rotated overnight with conjugated beads at 4°C. The supernatant was removed and beads were washed 8 times with wash buffer (10 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1% Triton-X 100). The bound RNA was extracted from the beads with Trizol (Thermo Fisher Scientific), quantified and mixed with TBE-Urea (8M) loading buffer. In subsequent steps, RIP northern and standard northern samples were treated identically. RNA was separated by electrophoresis on 9% 19:1 polyacrylamide, 0.6x TBE, 8M Urea at 20 mA/gel for 2 hrs. Gels were transferred in 0.5x TBE to nylon membrane at 25V for 16 hours. Membranes were UV crosslinked, blocked with Ultrahyb Oligo (Life Technologies), and hybridized with 5' end-radiolabeled DNA oligos (see Data S1Q).

**Measurements of alternative isoforms of Dao**

_RT-PCR of human DAO transcript_

Healthy and sporadic ALS (sALS) RNA samples were obtained from the motor cortex of deceased individuals (Courtesy J. Ravits). Astrocyte RNA from cortex, spinal cord, and cerebellum was purchased from Sciencell Research Laboratory. RNA from total brain, total spinal cord, and total cerebellum was purchased from Agilent Technologies. Samples were reverse transcribed and subject to PCR as above. The PCR products were gel extracted and Sanger sequenced with the same primers used to generate the products (Data S1Q).

## Constructs

Open reading frames for the mouse *Dao* long and short isoforms were determined from the UCSC genome browser. A 3xFLAG tag was appended to the 3' end of the sequence. Constructs containing the tagged ORFs were synthesized by Genscript. The 3xFLAG tagged ORFs were cut out with restriction enzymes and subcloned into the multiple cloning site of the pcDNA5/FRT vector. See Data S2A and S2B for sequences.

## Tertiary structure prediction

Predicted amino acid sequences for DAO long and short isoforms were obtained from UCSC genome browser. The sequences were submitted to:

Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/) and

SWISS-MODEL (http://swissmodel.expasy.org/interactive.

DAO Isoform 1 (Long Isoform):

MRVAVIGAGVIGLSTALCIHERYHPTQPLHMKIYADRFTPFTTSDVAAGLWQPYLSDPSNPQEAEWSQQTF-
DYLLSCLHSPNAEKMGLALISGYNLFRDEVPDPFWKNAVLGFRKLTPSEMDLFPDYGYGWFNTSLL-
LEGKSYLPWLTERLTERGVKLIHRKVESLEEVARGVDVIINCTGVWAGALQADASLQPGRGQIIQVEAP-
WIKHFILTHDPSLGIYNSPYIIPGSKTVTLGGIFQLGNWSGLNSVRDHNTIWKSCCKLEPTLKNARIVGELTG-
FRPVRPQVRLEREWLHFGSSSAEVIHNYGHGGYGLTIHWGCAMEAANLFGKILEEKKLSRLPPSHLDYKDH-
DGDYKDHDIDYKDDDD

DAO Isoform 2 (Short Isoform):

MRVAVIGAGVIGLSTALCIHERYHPTQPLHMKIYADRFTPFTTSDVAAGLWQPYLSDPSNPQEAEWSQQTF-
DYLLSCLHSPNAEKMGLALISGYNLFRDEVPDPFWKNAVLGFRKLTPSEMDLFPDYGYGWFNTSLL-
LEGKSYLPWLTERLTERGVKLIHRKVESLEEVARGVDVIINCTGVWAGALQADASLQPGRGQIIQVEAPWIKH-
FILTHDPSLGIYNSPYIIPGMQELWVNSLASGQSGLRSGDYKDHDGDYKDHDIDYKDDDDK

## Proteasome assay

Stable cell lines expressing either the long or short isoform of DAO or control cells expressing no transgene, were plated at $2.5 \times 10^5$ cells/well in a 6-well plate. 72 hours after plating, cells were treated with 4 μM Mg132 proteasome inhibitor (Sigma-Aldrich) for 0, 1, 2, 4, 10, and 24 hours. Cells were harvested in RIPA buffer (50 mM Tris-HCl pH. 7.4, 150 mM NaCl, 0.1% SDS, 0.5% Sodium deoxycholate, 1% NP-40, 1x protease inhibitor (Roche)), incubated at 4°C on a shaker for 30 min, and shredded with a QIAShredder (Qiagen). Harvested cells were stored at -20°C until western blotting.

## Cell based activity assay

FLP-In-293 cells expressing either DAO Isoform 1 (long) or DAO Isoform 2 (short) or control cells expressing no transgene were plated at 100,000 cells/well in a 96-well plate overnight. Clones were plated in technical triplicate. The Amplex Red kit (Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit, cat. A-22188, Molecular Probes/Invitrogen) was used to measure $H_2O_2$ released into the medium during the oxidation of DAO. Prior to the start of the assay, the media in each well was replaced with 100 μL of HANKS buffer solution (Invitrogen/GIBCO) with 20 mM HEPES for 5-10 min. In the wells containing control cells, $H_2O_2$ was added to final concentrations of 0 μM, 1.25 μM, 2.5 μM, 5 μM, 7.5 μM, 10 μM, 15 μM, and 20 μM to create a standard curve. The media in each well was then mixed with 25 μL of a 5x mixture of either assay buffer (50 mM D-Serine, 0.125 units HRP, 50 μM Amplex Red stock solution in HANKS with 20 mM HEPES) or control buffer (assay buffer without D-Serine) and incubated at 37°C for 30 min. The fluorescence signal was measured on an Infinite 200PRO (Tecan) with excitation at 544 nm and emission at 590 nm at 30 min, 60 min, 100 min, and 200 min after the addition of assay or control buffer.

3XFLAG tagged versions of the long and short isoforms of the mouse Dao protein were expressed in rabbit reticulocyte lysates using the TNT Quick Coupled Reticulocyte Lysate kit (Promega). In a 50-μL reaction, 1 μL of pcDNA5/FRT_mDAO_FLAG (1000 ng/μL) DNA was incubated with 1 μL methionine (1 mM), 41 μL of reticulocyte lysate, and 9 μL water at 30°C for 60 min. Translation product was flash-frozen in dry ice and methanol and then immediately dissolved in 2.5 mM FAD in HANKS buffer (HBSS, calcium, magnesium, no phenol red; Thermo Fisher Scientific) + 10.5 mM HEPES at 4°C for 30 min. The final 100-μL reaction contained the following components: 1 μL reticulocyte lysate (mDAO1_FLAG, mDAO2_FLAG, or T7 Luciferase Control), 4 μM Amplex Red (Thermo Fisher Scientific), 0.6 μL DMSO, 0.1 U/mL HRP (Thermo Fisher Scientific), 50 mM D-serine (Sigma-Aldrich), 500 nM FAD (Sigma-Aldrich), and $H_2O_2$ standards, all in HANKS buffer + 10.5 mM HEPES. The $H_2O_2$ standards were incubated in a dark room with Amplex Red and HRP in HANKS buffer prior to plating. Resorufin, caused by the oxidation of Amplex Red during mDAO-catalyzed turnover of D-serine, was measured on an Infinite 200PRO (Tecan) during 4-hour kinetic run (5 min time points with agitation) with excitation and emission wavelengths of 563 and 594 nm respectively. The $H_2O_2$ concentration was calculated using linear regression on the fluorescence.

## Stress granule assays

*Treatment of cells*

Motor neurons from control and affected individuals were differentiated and matured as described above. On day 18 the cells were re-plated into glass bottom chamber slides or glass bottom 96 well plates. On day 28 cells were treated with 36 μM puromycin or saline for 24 hours. After 24 hours the cells were fixed and processed for immunofluorescence as described below.

*Immunofluorescence and imaging*

Cells were fixed for 15 minutes in 4% PFA then washed 3 times with PBS + 0.01% Triton-X. Cells were permeablized with PBS+0.1% Triton-X for 45 minutes then blocked for 1 hour in PBS+0.01% Triton-X + 5% normal goat serum. Primary antibodies were incubated overnight at 4°C in PBS+0.01% Triton-X + 5% normal goat serum according to dilutions listed in Data S1P. After incubation the primary antibody was removed and the cells were washed 5 times for 10 minutes in PBS+0.01% Triton-X. Alexa fluor conjugated secondary antibodies were then incubated for 90 minutes at room temperature in PBS+0.01% Triton-X + 5% normal at a dilution of 1:1000 (Thermo Fisher Scientific). Next cells were washed 5 times for 10 minutes in PBS+0.01% Triton-X with the fourth wash containing 10 mg/mL DAPI. Images were acquired on a Zeiss 780 inverted laser scanning confocal microscope using a 20X Plan Apo air objective (Zeiss) or a 63X Plan Apo oil immersion objective (Zeiss). Primary antibodies used are detailed in Data S1P.

*Image analysis*

Four images were taken for each condition in Figure 7 and Figure S7. In Figures 7D and Figures S7B, images were blinded and then nuclei and G3BP1 and hnRNP A2/B1 foci were counted manually. Bar graphs are expressed as foci / nucleus.

*Nuclear / cytoplasmic and Detergent soluble / insoluble fractionation*

Nuclear and cytoplasmic fractions were extracted according to (Rio et al., 2010). Cells were rinsed in cold PBS and detached by gentle scraping. Cells were pelleted and resuspended in hypotonic lysis buffer (20 mM Tris HCl pH7.5, 10 mM KCl, 1.5 mM MgCl, 5 mM EGTA, 1 mM EDTA, 1 mM DTT, Protease Inhibitor Cocktail III (EMD Millipore)). The suspension was homogenized with 10 strokes of a dounce homogenizer. The nuclei were pelleted by centrifugation at 1200g and the cytoplasmic fraction was cleared of nuclei and debris by further spins at 1200g. The supernatant containing the cytoplasmic fraction was solubilized by adding 1% NP-40, 0.5 % Na-Deoxycholate, and 0.1% SDS.  The nuclei were resuspended in a sucrose buffer (0.32 M sucrose, 3 mM $CaCl_2$, 2 mM MgOAc, 0.1 mM EDTA, 10 mM Tris HCl pH 8, 1 mM DTT, 0.5% NP-40, Protease Inhibitor Cocktail III) and homogenized with 3 strokes of a dounce homogenizer. The nuclear suspension was layered on top of a 2.0 M sucrose cushion (2 M sucrose, 5 mM MgOAc, 0.1 mM EDTA, 10 mM Tris pH 8, 1 mM DTT, Protease Inhibitor Cocktail III) and centrifuged at 20,000g for one hour. Pelleted nuclei were solubilized in RIPA buffer (50

mM Tris HCl pH7.4, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, Protease Inhibitor Cocktail III). Cytoplasmic and nuclear fractions solubilized in RIPA were treated with Turbo DNAse (Thermo-Fisher) for 1 hour at 37°C and then sonicated in the Bioruptor (Diagenode) for 10 minutes on high power. The detergent insoluble material was pelleted by centrifugation at 20,000g for one hour and the supernatant was removed (soluble fraction). The detergent insoluble pellets were solubilized in buffer containing (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris HCl pH 8.5, Protease Inhibitor Cocktail III) and sonicated in the Bioruptor (Diagenode) for 10 minutes on high power. All fractions were filtered using a 0.22 μM cellulose acetate spin filter (Costar). Western blotting was performed as above. Antibodies against histone H3 and β-Actin were used to verify the nuclear and cytoplasmic fractions, respectively. See data S1P.

## Long Term Imaging Experiments

### Differentiation and Cell Culture

iPSC lines from two ALS patients with hnRNP A2/B1 D290V mutations and two healthy individuals (Miyaoka et al., 2014) were confirmed for normal karyotype.  The iPSCs were differentiated into motor neurons using a dual SMAD inhibition protocol as previously described (Burkhardt et al., 2013; Chambers et al., 2009) with modifications from Du et al 2015 (Du et al., 2015).  Briefly, the iPSCs were neuralized in IMDM/F12 medium (Thermo Fisher) with SMAD inhibitors: 0.2 μM LDN-193189 (Stemgent) and 10 μM SB431542 (Stemgent). Cultures were split 1:2 after 1 week in medium containing 0.1 μM RA, (Sigma-Aldrich) and 1 μM SAG (Fisher Scientific) and 3 μM CHIR99021, 0.2 μM LDN193189 and 10 μM SB431542. After 1 more week, the cultures were banked frozen in Synthafreeze (Thermo Fisher).  All cell lines were then thawed simultaneously to synchronize neuron maturation for imaging experiments. The MN cultures were fed with medium containing Compound E (0.1 μM, Fisher), 2.5 μM DAPT (Fisher), 0.1 μM db-cAMP (Sigma-Aldrich), 200 ng/mL ascorbic acid (Sigma-Aldrich), 10 ng/mL each BDNF and GDNF (Fisher Scientific), together with 0.5 μM all-trans RA and 0.1 μM SAG for an additional 6 days.  MN cultures were trypisizined and plated into 96-well plates, lipofected with a plasmid expressing the red fluorescent protein mApple under the neuronal specific promoter Synapsin.

### Imaging and analysis.

The iPSC-neurons were imaged starting 2 days after transfection, once every six hours on a custom-built robotic microscopy system comprised of a Nikon T2i widefield fluorescence microscope and 888 Andor camera, and kept in a robotic incubator (Liconic).  Individual neurons were tracked using custom algorithms and neuronal survival was analyzed in time-lapse images.  Kaplan-Meier survival analysis was used to measure survival of individual neurons over time.  Cumulative risk of death curves were derived from the Kaplan Meier survival curves.  Cox proportional hazard analysis was used to measure cumulative hazard ratio of iPSC-MNs from ALS patients compared with healthy individuals and derive P values as previously described (Skibinski and Finkbeiner, 2013).

## Supplemental References

Burkhardt, M.F., Martinez, F.J., Wright, S., Ramos, C., Volfson, D., Mason, M., Garnes, J., Dang, V., Lievers, J., Shoukat-Mumtaz, U., et al. (2013). A cellular model for sporadic ALS using patient-derived induced pluripotent stem cells. Molecular and Cellular Neurosciences 56, 355-364.

Chambers, S.M., Fasano, C.A., Papapetrou, E.P., Tomishima, M., Sadelain, M., and Studer, L. (2009). Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nature Biotechnology 27, 275-280.

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. Bioinformatics (Oxford, England) 29, 15-21.

Du, Z.W., Chen, H., Liu, H., Lu, J., Qian, K., Huang, C.L., Zhong, X., Fan, F., and Zhang, S.C. (2015). Generation and expansion of highly pure motor neuron progenitors from human pluripotent stem cells. Nature Communications 6, 6626.

Gore, A., Li, Z., Fung, H.L., Young, J.E., Agarwal, S., Antosiewicz-Bourget, J., Canto, I., Giorgetti, A., Israel, M.A., Kiskinis, E., et al. (2011). Somatic coding mutations in human induced pluripotent stem cells. Nature 471, 63-67.

Huelga, S.C., Vu, A.Q., Arnold, J.D., Liang, T.Y., Liu, P.P., Yan, B.Y., Donohue, J.P., Shiue, L., Hoon, S., Brenner, S., et al. (2012). Integrative genome-wide analysis reveals cooperative regulation of alternative splicing by hnRNP proteins. Cell Reports 1, 167-178.

Huppertz, I., Attig, J., D'Ambrogio, A., Easton, L.E., Sibley, C.R., Sugimoto, Y., Tajnik, M., Konig, J., and Ule, J. (2014). iCLIP: protein-RNA interactions at nucleotide resolution. Methods 65, 274-287.

Lambert, N., Robertson, A., Jangi, M., McGeary, S., Sharp, P.A., and Burge, C.B. (2014). RNA Bind-n-Seq: quantitative assessment of the sequence and structural binding specificity of RNA binding proteins. Molecular cell 54, 887-900.

Langmead, B., Trapnell, C., Pop, M., and Salzberg, S.L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome biology 10, R25.

Liao, Y., Smyth, G.K., and Shi, W. (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics (Oxford, England) 30, 923-930.

Lovci, M.T., Ghanem, D., Marr, H., Arnold, J., Gee, S., Parra, M., Liang, T.Y., Stark, T.J., Gehman, L.T., Hoon, S., et al. (2013). Rbfox proteins regulate alternative mRNA splicing through evolutionarily conserved RNA bridges. Nature structural & Molecular Biology 20, 1434-1442.

Miyaoka, Y., Chan, A.H., Judge, L.M., Yoo, J., Huang, M., Nguyen, T.D., Lizarraga, P.P., So, P.L., and Conklin, B.R. (2014). Isolation of single-base genome-edited human iPS cells without antibiotic selection. Nature Methods 11, 291-293.

Rio, D.C., Ares, M., Jr., Hannon, G.J., and Nilsen, T.W. (2010). Preparation of cytoplasmic and nuclear RNA from tissue culture cells. Cold Spring Harbor Protocols 2010, pdb.prot5441.

Skibinski, G., and Finkbeiner, S. (2013). Longitudinal measures of proteostasis in live neurons: features that determine fate in models of neurodegenerative disease. FEBS Letters 587, 1139-1146.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., and Rozen, S.G. (2012). Primer3--new capabilities and interfaces. Nucleic Acids Research 40, e115.

Van Nostrand, E.L., Pratt, G.A., Shishkin, A.A., Gelboin-Burkhart, C., Fang, M.Y., Sundararaman, B., Blue, S.M., Nguyen, T.B., Surka, C., Elkins, K., et al. (2016). Robust transcriptome-wide discovery of RNA-binding protein binding sites with enhanced CLIP (eCLIP). Nature Methods 13, 508-514.

Xia, Z., Donehower, L.A., Cooper, T.A., Neilson, J.R., Wheeler, D.A., Wagner, E.J., and Li, W. (2014). Dynamic analyses of alternative polyadenylation from RNA-seq reveal a 3'-UTR landscape across seven tumour types. Nature Communications 5, 5274.

Zisoulis, D.G., Lovci, M.T., Wilbert, M.L., Hutt, K.R., Liang, T.Y., Pasquinelli, A.E., and Yeo, G.W. (2010). Comprehensive discovery of endogenous Argonaute binding sites in Caenorhabditis elegans. Nature Structural & Molecular BWiology 17, 173-179.