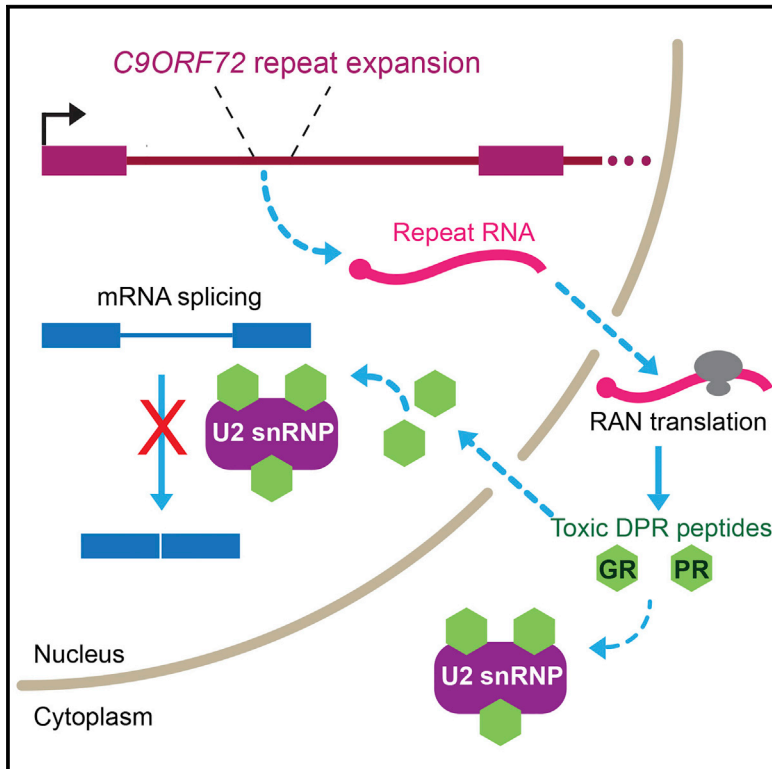


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Evidence that *C9ORF72* Di-peptide Repeat Proteins Associate with U2 snRNP to Cause Mis-splicing in ALS/FTD Patients

Graphical Abstract



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In Brief

Yin et al. report that GR and PR DPR peptides associate with U2 snRNP and block spliceosome assembly and splicing in vitro. U2 snRNP mis-localizes to the cytoplasm in *C9ORF72*-patient-iPSC-derived motor neurons. DPR-mediated dysfunction of U2 snRNP may explain a large portion of the mis-splicing in *C9ORF72* patients.

Highlights

- GR and PR toxic DPR peptides block spliceosome assembly and splicing in vitro
- GR and PR toxic DPR peptides specifically associate with U2 snRNP in nuclear extracts
- U2 snRNP mislocalizes to the cytoplasm in *C9ORF72*-patient-iPSC-derived motor neurons
- DPR-mediated dysfunction of U2 snRNP may explain mis-splicing in ALS/FTD patients



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Evidence that *C9ORF72* Dipeptide Repeat Proteins Associate with U2 snRNP to Cause Mis-splicing in ALS/FTD Patients

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SUMMARY

Hexanucleotide repeat expansion in the *C9ORF72* gene results in production of dipeptide repeat (DPR) proteins that may disrupt pre-mRNA splicing in amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) patients. At present, the mechanisms underlying this mis-splicing are not understood. Here, we show that addition of proline-arginine (PR) and glycine-arginine (GR) toxic DPR peptides to nuclear extracts blocks spliceosome assembly and splicing, but not other types of RNA processing. Proteomic and biochemical analyses identified the U2 small nuclear ribonucleoprotein particle (snRNP) as a major interactor of PR and GR peptides. In addition, U2 snRNP, but not other splicing factors, mislocalizes from the nucleus to the cytoplasm both in *C9ORF72* patient induced pluripotent stem cell (iPSC)-derived motor neurons and in HeLa cells treated with the toxic peptides. Bioinformatic studies support a specific role for U2-snRNP-dependent mis-splicing in *C9ORF72* patient brains. Together, our data indicate that DPR-mediated dysfunction of U2 snRNP could account for as much as ~44% of the mis-spliced cassette exons in *C9ORF72* patient brains.

INTRODUCTION

Expansion of a hexanucleotide repeat (G₄C₂) in the first intron of the *C9ORF72* gene is the most frequent cause of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Rutherford et al., 2012). The expansion generates both sense and anti-sense repeat RNAs, which form aggregates in patient brains (Cooper-Knock et al., 2014; DeJesus-Hernandez et al., 2011; Renton et al., 2011). The repeat RNAs also generate five dipeptide repeat

(DPR) proteins via translation of both the sense and anti-sense transcripts (Taylor et al., 2016). These DPR proteins (glycine-arginine [GR], proline-arginine [PR], glycine alanine [GA], glycine proline [GP], and proline alanine [PA]) are found in both the nucleus and the cytoplasm of *C9ORF72* patient tissues, including brain and spinal cord, as well as in patient induced pluripotent stem cell (iPSC)-derived motor neurons (Ash et al., 2013; Mori et al., 2013; Wen et al., 2014). Of the DPR proteins, GR and PR are toxic in human, yeast, and *Drosophila* model systems (Freibaum et al., 2015; Jovičić et al., 2015; Mizielinska et al., 2014; Tran et al., 2015; Wen et al., 2014; Yang et al., 2015; Zhang et al., 2015). Understanding the mechanisms by which the repeat RNAs and/or the DPRs cause neurodegeneration is a focus of intense investigation. Among the possibilities being examined are that the repeat RNAs or DPR proteins interfere with essential cellular processes (Taylor et al., 2016), such as nucleocytoplasmic trafficking (Freibaum et al., 2015; Jovičić et al., 2015; Zhang et al., 2015), protein synthesis (Kanekura et al., 2016), mitochondria function (Lopez-Gonzalez et al., 2016), and pre-mRNA splicing (Conlon et al., 2016; Cooper-Knock et al., 2014; Kwon et al., 2014; Prudencio et al., 2015). In addition, pathogenesis may involve DPR-protein-mediated disruption of the dynamics and assembly of membrane-less organelles such as nucleoli, stress granules, and nuclear speckle domains (Lee et al., 2016).

The focus of our study is to understand the mechanisms by which the *C9ORF72* repeat expansion causes mis-splicing. Several mechanisms have been proposed. For example, the repeat RNAs may sequester essential splicing factors and thereby block splicing (Conlon et al., 2016; Cooper-Knock et al., 2014, 2015; Haeusler et al., 2014). In addition, ~5,000 mis-splicing events were observed when cultured astrocytes were treated with PR toxic peptides, indicating that DPR proteins may play a role in mis-splicing (Kwon et al., 2014). It was also reported that synthetic DPR proteins expressed in tissue culture cells interact with RNA-binding proteins and, in particular, with those containing low-complexity domains (Lee et al., 2016; Lin et al., 2016). The low complexity domain proteins are thought to mediate the assembly of membrane-less cellular compartments, including nuclear speckle domains, which are where splicing components normally localize (Li et al., 2013;

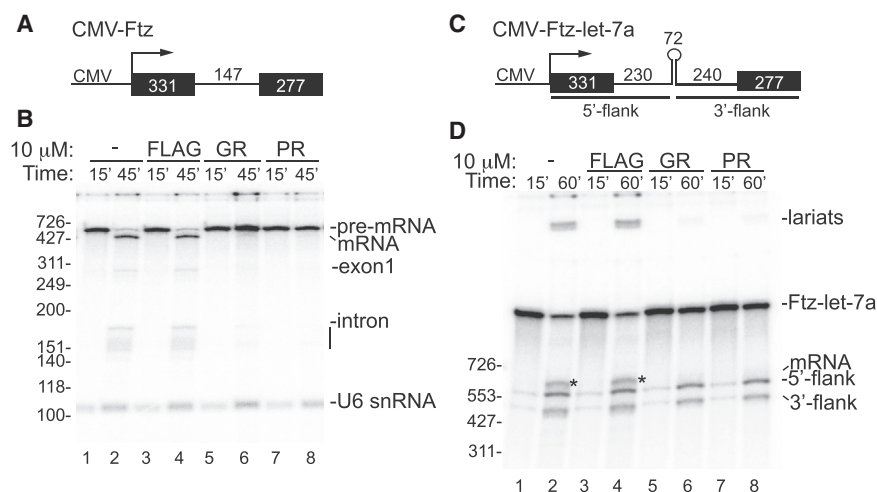


Figure 1. GR and PR Toxic Peptides Inhibit Splicing In Vitro

(A) Schematic of CMV-Ftz DNA template used for transcription-coupled splicing showing the CMV promoter and sizes of the exons and intron.

(B) CMV-Ftz DNA was incubated in transcription-coupled splicing reaction mixtures for 15 min with no peptide or in the presence of 10 μ M GR, PR, or FLAG peptides. α -Amanitin was then added to stop further transcription, and incubation was continued for 30 min to allow splicing. RNA species are indicated. The line below the intron marks intron breakdown products.

(C) Schematic of CMV-Ftz-let-7a DNA template which contains a pri-let-7a sequence in the Ftz intron.

(D) CMV-Ftz DNA was incubated in transcription/splicing/pri-miRNA processing reaction mixtures for 15 min with 10 μ M of the indicated peptides. After adding α -amanitin, incubation was continued for 45 min. The asterisks on the gel indicate the spliced mRNA. Markers in nucleotides (nts) are shown.

See also Figure S1.

Ramaswami et al., 2013; Taylor et al., 2016). Thus, mis-splicing in *C9ORF72* patient cells could be caused by disruption of nuclear speckles and/or to loss of RNA-binding proteins essential for splicing due to their low complexity domain-mediated aggregation.

Here, we report that both GR and PR toxic peptides block spliceosome assembly and splicing in nuclear extracts, and both peptides specifically associate with the U2 small nuclear ribonucleoprotein particle (snRNP). In *C9ORF72* patient iPSC-motor neurons, U2 snRNP is depleted from nuclear speckle domains and is mislocalized to the cytoplasm. This mislocalization may be directly caused by the DPR proteins, as we observe U2 snRNP mislocalization to the cytoplasm in HeLa cells treated with PR peptide. In addition, bioinformatic analyses indicate that the toxic peptides interfere with U2 snRNP to cause mis-splicing in *C9ORF72* patient brains. Together, these results provide evidence that DPR protein-mediated dysfunction of a major spliceosomal snRNP is a central mechanism underlying mis-splicing in *C9ORF72* patients. The genes disrupted by this mechanism include those with mitochondrial, neuronal, and gene expression functions and are candidate genes for contributing to ALS/FTD pathogenesis.

RESULTS

GR and PR Toxic Dipeptides Inhibit Splicing In Vitro

To investigate the mechanisms by which the DPR proteins and/or repeat RNAs encoded by the *C9ORF72* expansion inhibit splicing, we used an RNAP II transcription-coupled splicing system (Das et al., 2006, 2007; Folco et al., 2011). For this system, we used a DNA template driven by the cytomegalovirus (CMV) promoter and encoding the well-characterized Ftz splicing substrate (Das et al., 2006, 2007; Folco et al., 2011) (Figure 1A). This CMV-Ftz DNA template was incubated in HeLa cell nuclear extracts for 15 min to generate an RNAP II transcript, which is then spliced by the 45 min time point (Figure 1B, lanes 1 and

2). As shown in Figure S1A, increasing amounts of an RNA containing ~ 60 copies of the *C9ORF72* repeats does not affect transcription-coupled splicing. In contrast, a positive control RNA containing exonic splicing enhancers (Das et al., 2007) efficiently blocked splicing in this assay (Figure S1A).

We next tested the effects of the toxic repeat dipeptides on splicing in our system using synthetic FLAG-tagged peptides containing 20 repeats of GR or PR. FLAG peptide alone was used as a negative control. When these peptides were added to nuclear extracts, transcription occurred as efficiently as adding no peptide at all (Figure 1B, lanes 1, 3, 5, and 7). Strikingly, however, splicing was potently blocked by GR and PR toxic peptides, but not by FLAG peptide (Figure 1B, lanes 2, 4, 6, and 8). Moreover, splicing intermediates (exon 1 and lariat-exon 2) were not detected, indicating that the block occurs prior to or at the first catalytic step of splicing. We obtained the same results with another splicing substrate (CMV-AdML; Figures S1B and S1C), suggesting that the inhibitory effect of the toxic peptides on splicing is general.

The observation that the GR and PR peptides block splicing is consistent with previous data showing that these peptides cause extensive mis-splicing in cultured cells (Kwon et al., 2014). The inhibitory effect of GR and PR on splicing in vitro is dose dependent, as we observed no effect with 0.1 or 1 μ M of either GR or PR, $\sim 50\%$ inhibition with 5 μ M of either peptide, and complete inhibition with 10 μ M of the peptides (Figure S1D). Although high levels of the peptides are required to block splicing in the in vitro system, whereas only low levels of DPR proteins are present in *C9ORF72* patient cells, there are reasonable explanations for how these low levels might interfere with splicing. Specifically, mis-splicing in *C9ORF72* patient cells occurs in alternatively spliced exons (Prudencio et al., 2015). Such exons typically contain weak splicing signals, which are known to be sensitive to minor dysfunction in splicing factors (Fu and Ares, 2014). Thus, low levels of DPR proteins in patient cells might be sufficient to cause disruptions of alternative splicing. In contrast to patient

cells, in vitro systems require the use of highly efficient splicing substrates in order for splicing to occur, and splicing of these efficient substrates is not affected by small alterations in the functions/levels of splicing factors. Thus, it is not unexpected that low levels of GR/PR peptides have no effect on splicing the efficient substrates used for in vitro studies. Another notable difference between the patient cells and our in vitro model system is that patient cells typically harbor DPR proteins containing hundreds to thousands of repeats whereas the peptides that we use contain only 20 repeats. Longer peptides could not be tested because of the technical difficulties in synthesizing them (Kwon et al., 2014; Lin et al., 2016; Shi et al., 2017).

To determine whether other types of RNA processing are affected by GR or PR peptides, we added them to our transcription-coupled primary-microRNA (pri-miRNA) processing system (Yin et al., 2015). As shown in Figures S1E and S1F, no effect on pri-miRNA processing was observed when the peptides were present at the same levels that block splicing. We also developed an in vitro transcription/splicing/pri-miRNA processing system in which the pri-let-7a is located in the Ftz intron (Figure 1C). In the absence of peptides or in the presence of the FLAG peptide, both splicing and pri-miRNA processing products were observed (Figure 1D, lanes 2 and 4). However, when the toxic peptides were added to the reaction, splicing was blocked, whereas no apparent effect on pri-miRNA processing was observed (Figure 1D, lanes 6 and 8). The peptides also did not affect other gene expression steps, including transcription, U6 snRNA processing, or tRNA processing. (We note that processing of U6 and tRNAs results in their labeling by the ^{32}P -UTP added to the nuclear extract [Das et al., 2007], and we do not detect any effect on the levels of either labeled RNA species when the toxic peptides were added to the nuclear extract [Figures 1B and S1].) We conclude that GR and PR specifically block splicing in vitro.

GR and PR Associate with U2 snRNP

To investigate the mechanisms by which the toxic peptides block splicing, we identified their interacting proteins in HeLa nuclear extract. As a first purification step, we added GR, PR, or FLAG peptides to the extract and then size-separated these samples by gel filtration. This was followed by western blotting (Figure 2A) or ethidium bromide staining (Figure 2B) to detect the proteins and RNAs, respectively. Both GR and PR eluted in fractions containing high-molecular-weight RNP complexes, such as U1 and U2 snRNPs (Figures 2A and 2B). The toxic peptides also co-eluted with the ALS-causative protein FUS, but not with TDP-43 (Figure 2A). In contrast, FLAG peptide eluted in fractions containing free proteins. To identify proteins that interact with GR or PR, we individually pooled the fractions containing these peptides and used them for immunoprecipitations (IPs) with an antibody against FLAG. We found that these FLAG IPs were inefficient and thus tested pull-downs using avidin affinity selection of biotinylated peptides. As shown in Figure S1G, the biotinylated peptides inhibited splicing with similar efficiency as the non-biotinylated peptides. We then used the biotinylated peptides for pull-downs from the gel filtration fractions, and the set of proteins bound to them is shown on a silver-stained gel (Figure 2C). To identify the GR- or PR-interacting proteins, we

carried out quantitative mass spectrometry of the total proteins in each pull-down (Table S1). Gene Ontology (GO) and gene set enrichment analysis (GSEA) analyses of the proteins in both pull-downs revealed RNA splicing as the most enriched category (Figure S2; Tables S2 and S3). Strikingly, further examination of the proteomic data revealed that six U2 snRNP components were among the highest hits shared between the GR and PR pull-downs (Figure 2D, red dots). The U2 snRNP component SF3A1 was the highest specific hit in the GR pull-down, and five out of the top seven hits in this pull-down were U2 snRNP proteins (Table S1). Moreover, all of the other known U2 snRNP proteins, including the full sets of SF3a and SF3b sub-complexes, were present in both pull-downs (Figure 2E; Table S1). In contrast to U2 snRNP, components of other snRNPs were not enriched in the pull-downs, and their complete sets of cognate proteins were not detected (Table S1). For example, U5 snRNP is known to contain eight specific components. We only detected three of them, two of which are ~200 kDa and are found as common contaminants in many non-related pull-downs. These results suggest that U2 snRNP specifically associates with GR and PR. In addition to U2 snRNP, an enrichment of the barrier-to-autointegration factor (BAF) chromatin remodeling complex was seen in both pull-downs (Figure 2D, green dots; Table S1). We also detected a group of aminoacyl-tRNA synthetases that was specifically enriched in the PR, but not in the GR, pull-down (Figure 2D, purple dots; Table S1). We note that our silver-stained gel of the pull-downs revealed different patterns of proteins associated with the two peptides (Figure 2C). These differences can be ascribed to different levels of each protein pulled down by GR and PR (e.g., see GR and PR ranks in Table S1).

Importantly, only a few components of other multi-component complexes, such as the transcription export (TREX) or survival of motor neuron (SMN) complexes, were detected, indicating that GR and PR are not generally associated with all cellular complexes. Previous studies found that the translation machinery was the most abundant interactor of GR and PR expressed in HEK293 cells as GFP fusions from C9ORF72-repeat-containing RNA (Kanekura et al., 2016; Lee et al., 2016; Lopez-Gonzalez et al., 2016). We also detected most of the components of the large and small ribosomal subunits in our GR and PR pull-downs, but they were not top hits. One of the possible reasons for this is that we used nuclear extract whereas the other studies used whole-cell lysates, which are rich in ribosomes (Kanekura et al., 2016; Lee et al., 2016; Lopez-Gonzalez et al., 2016).

GR and PR Bind to U2 snRNP and Disrupt Spliceosome Assembly

We next analyzed the RNAs present in the pull-downs on an ethidium-bromide-stained gel (Figure 3A). Consistent with the proteomics results, U2 snRNA, but not the other spliceosomal snRNAs, was enriched in both GR and PR pull-downs, supporting a specific interaction between GR/PR and U2 snRNP. This was further confirmed by western blotting, which showed that the SF3a subunits (SF3A1–3) and SNRPB2 were enriched in the GR and PR pull-downs (Figure 3B). In contrast, the pri-miRNA processing factor DROSHA was not detected by western in the pull-downs, consistent with the observation that GR/PR

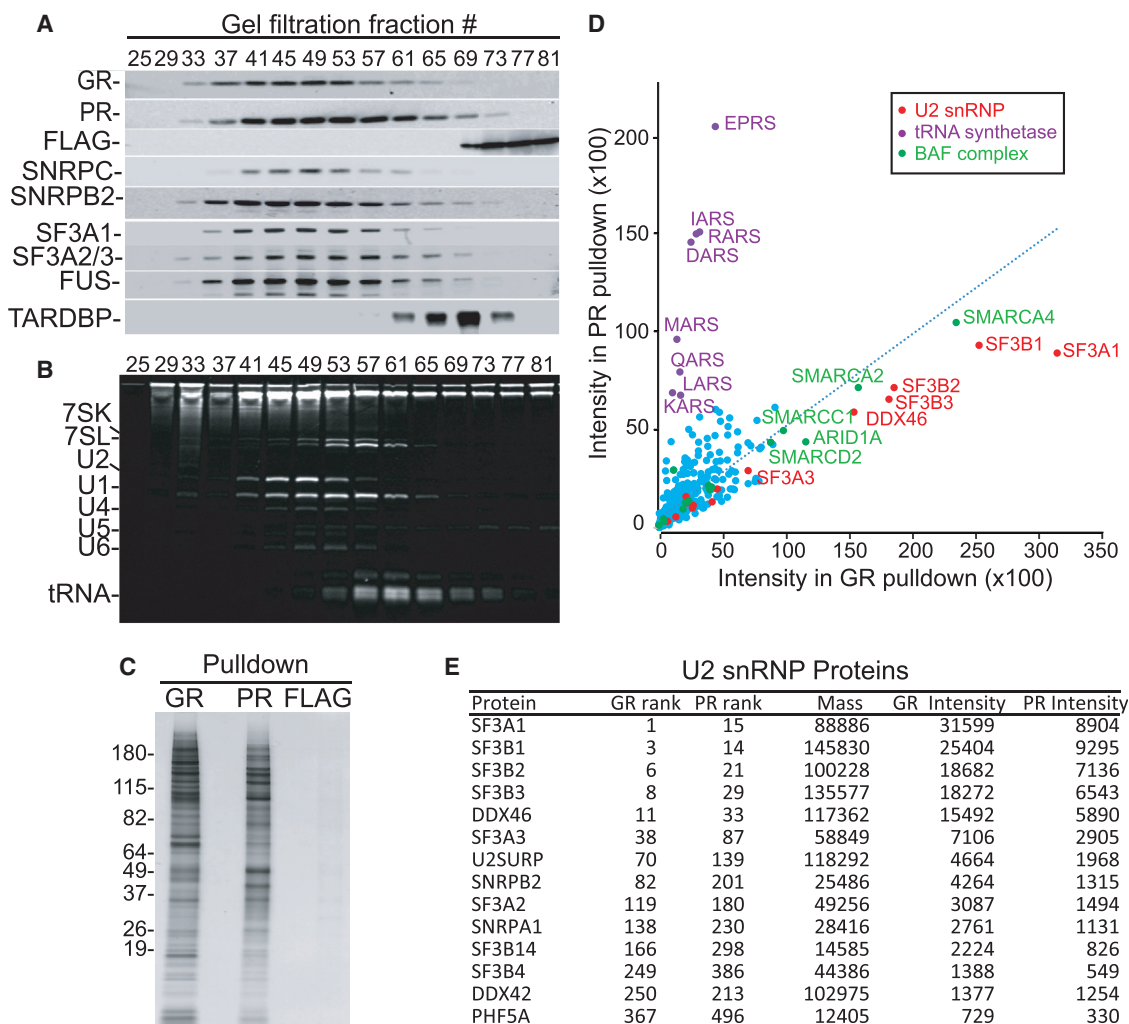


Figure 2. GR and PR Associate with U2 snRNP

(A) Nuclear extract reaction mixtures were incubated with 10 μ M of GR, PR, or FLAG peptide followed by separation on Sephacryl-S500 columns. The indicated fractions from each column were used for western blots with an antibody against FLAG (rows GR, PR, and FLAG). The GR gel filtration fractions were then used for western blotting analysis with antibodies against the U1 snRNP component SNRPC, the U2 snRNP components SNRPB2, SF3A1, SF3A2, and SF3A3 (the latter two proteins co-migrate on the SDS gel), FUS, and TARDBP. We obtained similar results when the PR or FLAG gel filtration fractions were used for the western blotting (data not shown). Fraction 25 is the void volume, and low-molecular-weight factors, such as free proteins, elute in fractions ~70–80.

(B) Same as (A), except that total RNA from the GR fractions was run on an 8% denaturing gel and stained with ethidium bromide. RNA species are indicated.

(C) Silver stained gel showing total proteins in GR, PR, or FLAG pull-downs from gel filtration fractions 40–60.

(D) Scatterplot depicting the protein intensity identified in GR or PR pull-downs. Red, purple, and green dots indicate U2 snRNP components, tRNA synthetases, and BAF complex components, respectively.

(E) Table showing quantitative mass spectrometry data for the U2 snRNP components in GR or PR pull-downs. The rank of each protein in the total pull-downs sorted by mass spectrometry intensity, the calculated mass, and the mass spectrometry intensity are shown.

See also Figure S2 and Tables S1, S2, and S3.

does not affect pri-miRNA processing (Figures 1C, 1D, S1E, and S1F). We also performed reverse IPs using antibodies to SF3a or SNRPB2, which confirmed that GR/PR associate with U2 snRNP (Figure 3C).

Previous studies showed that the functional form of U2 snRNP in nuclear extracts is a 17S particle consisting of the SF3a and SF3b sub-complexes and a 12S U2 snRNP core particle, which contains U2 snRNA, the Sm proteins, SNRPB2, and SNRPA1 (Wahl et al., 2009). Both the 12S and 17S U2 snRNP can be de-

tected on a native agarose gel using a 32 P-labeled 2' O-methyl oligonucleotide (U2 oligo) that base pairs to the 5' portion of U2 snRNA (Figures 3D and 3E, lane 1) (Folco et al., 2011; Ruby et al., 1993). We next used this assay to examine the effect of GR and PR on U2 snRNP. This analysis revealed that addition of either toxic peptide resulted in a quantitative mobility shift of the 12S and 17S U2 snRNP particles (Figure 3E, lanes 2 and 3), consistent with the observation that both peptides associated with this snRNP in the pull-down assay. These results raise the

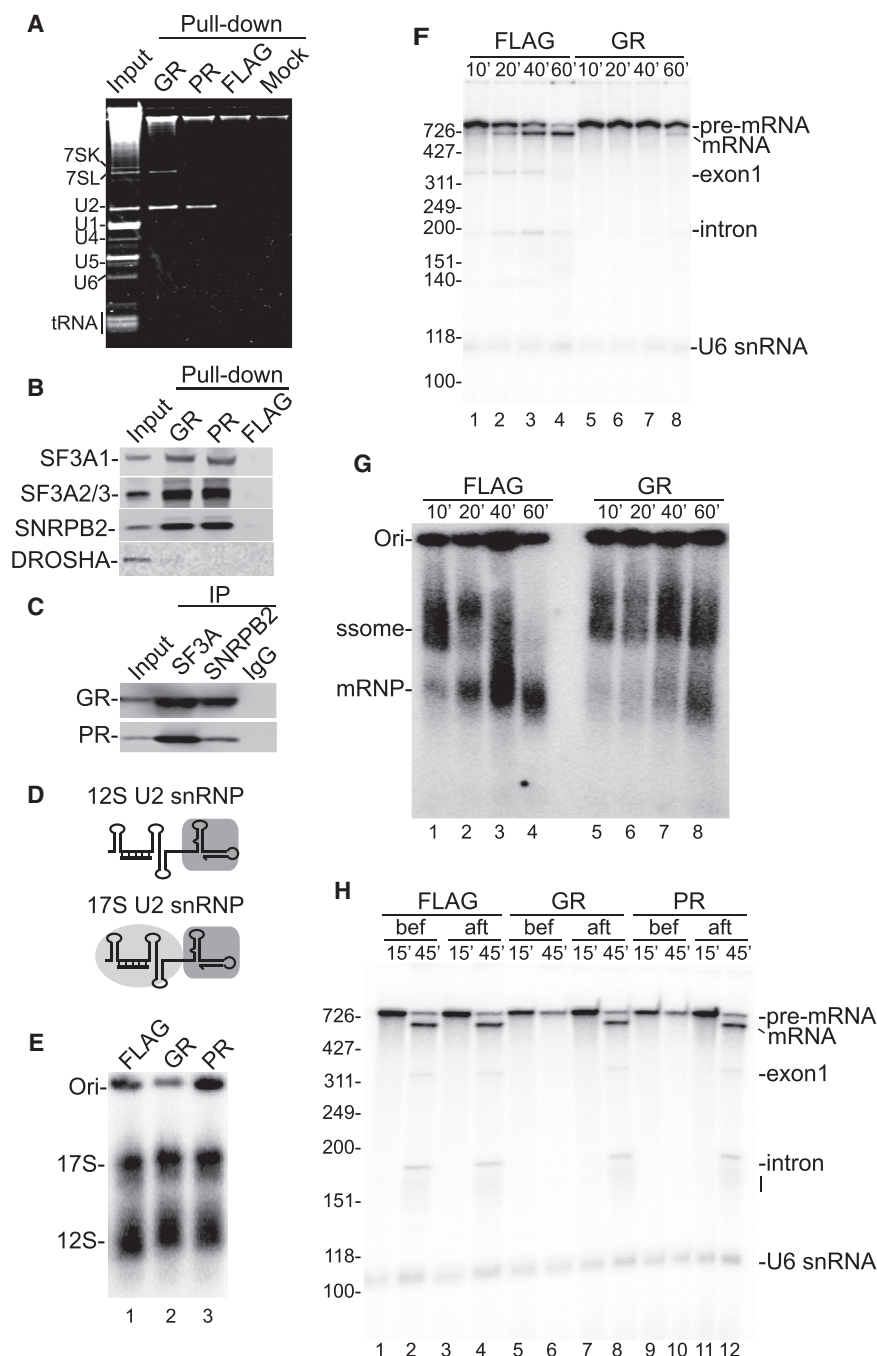


Figure 3. GR and PR Bind to U2 snRNP and Inhibit Spliceosome Assembly

(A) Ethidium-bromide-stained gel showing total RNA isolated from GR, PR, FLAG, or beads-alone (mock) pull-down from gel filtration fractions. A low level of the RNA component of signal recognition particle, 7SL RNA, was seen in the GR pull-down. However, the protein components of this complex were either low hits or not detected (Table S1). Thus, SRP is unlikely to be a genuine GR interactor.

(B) Pull-downs were analyzed by western blotting using the indicated antibodies.

(C) Antibodies against SF3A1-3, SNRNPB2, or IgG were used for IPs from nuclear extract followed by western blotting with antibodies against GR or PR.

(D) Schematic of 12S and 17S U2 snRNP showing the U2 2' O-methyl oligo that base pairs to the 5' end of U2 snRNA. The gray boxes indicate the Sm core, SNRNPB2 and SNRPA1, and the gray circle indicates the SF3a and SF3b complexes.

(E) Nuclear extracts were incubated with 10 μ M of the indicated peptides under splicing conditions for 5 min followed by a 5-min incubation with 32 P-labeled U2 2' O-methyl oligo. Samples were fractionated on a native agarose gel and detected by PhosphorImager. The 17S and 12S U2 snRNPs are indicated. Ori marks the gel origin.

(F and G) CMV-Ftz DNA template was incubated in transcription-coupled splicing mixtures in the presence of FLAG or GR for the indicated times. An aliquot from each time point was analyzed on an 8% denaturing polyacrylamide gel (F) or on a native agarose gel (G) to detect total RNA or RNP complexes, respectively. The spliceosome (ssome) and spliced mRNP complex are indicated, and Ori marks the gel origin.

(H) CMV-Ftz DNA template was incubated for 15 min in transcription-coupled splicing mixtures followed by addition of α -amanitin and continued incubation for 30 min. The indicated peptides were added either prior to (bef) or after (aft) transcription.

gel (Figure 3G). In the presence of FLAG peptide, the kinetics of splicing and spliceosome assembly were the same as with no peptide at all. Specifically, after 10 min of transcription, nascent transcripts were detected (Figure 3F, lane 1) and efficiently assembled into the spliceosome (Figure 3G, lane 1). Over time, the spliced mRNA and the spliced

possibility that GR and PR disrupt splicing by affecting normal U2 snRNP function.

As 17S U2 snRNP is essential for spliceosome assembly, we next examined the effects of the toxic peptides on this process. To do this, either FLAG or GR was added to nuclear extract followed by a transcription-coupled splicing reaction using the CMV-Ftz DNA substrate. The RNAs within the spliceosomal complexes were analyzed on a denaturing gel (Figure 3F), and the complexes were analyzed on a native

messenger ribonucleoprotein (mRNP) complex continued to accumulate (Figure 3F and 3G, lanes 2–4). In contrast, when GR was included in the reaction, splicing was inhibited (Figure 3F, lanes 5–8), and a complex with slower mobility than the spliceosome was detected by 10 min of incubation and was not significantly changed over time (Figure 3G, lanes 5–8; note that a low level of splicing [Figure 3F, lane 8] and spliced mRNP [Figure 3G, lane 8] are detected by 60 min, because the reaction is leaky by this late time point). We

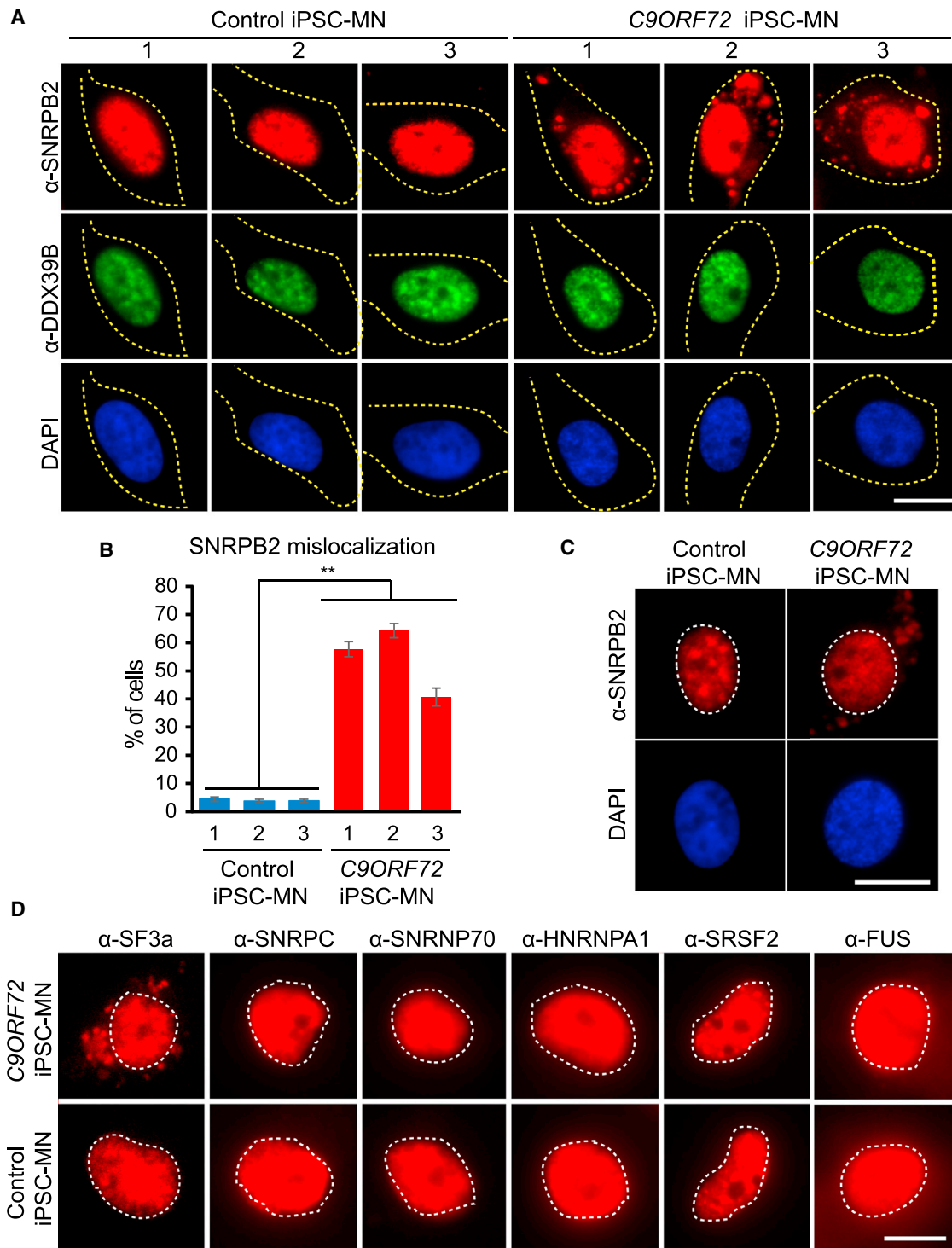


Figure 4. U2 snRNP Is Mislocalized to the Cytoplasm in C9ORF72 iPSC-Motor Neurons

(A) Immunofluorescent staining of SNRNPB2 (red), DDX39B (green), and DAPI (blue) in iPSC-motor neurons from control subjects (N = 3) or patients with C9ORF72 repeat expansion (N = 3). Dotted line outlines the cell body. See Figure S3 for fields.

(B) The percentage of cells with cytoplasmic SNRNPB2 signals in each cell line. Mean \pm SEM of three biological replicates is shown. Approximately 300 cells were counted for each line. **p < 0.001, two-way ANOVA.

(legend continued on next page)

conclude that GR inhibits spliceosome assembly, thereby resulting in defective splicing. Previously, we showed that the spliceosome assembles on nascent transcripts as rapidly as they are synthesized by RNAP II (Das et al., 2006). Consistent with these data, we observed that addition of GR or PR only blocked splicing when added before, but not after, transcription (Figure 3H). This order of addition experiment further demonstrates that the effect of the toxic peptides is specific, as they only cause a block to splicing at a specific step in the splicing pathway and do not interfere with splicing after transcription and spliceosome assembly occur.

U2 snRNP Mislocalizes to the Cytoplasm in *C9ORF72* Patient iPSC-Motor Neurons

A critical unanswered question is whether U2 snRNP is affected in *C9ORF72* patient cells. To investigate this possibility, we examined high-yield motor neuron cultures differentiated from 3 *C9ORF72* patient-derived iPSC lines (Lopez-Gonzalez et al., 2016). These iPSC-motor neurons are known to contain the *C9ORF72* repeat expansion as well as DPRs generated by translation of the repeat RNAs (Almeida et al., 2013; Ash et al., 2013; Donnelly et al., 2013; Lopez-Gonzalez et al., 2016; Mori et al., 2013; Su et al., 2014). iPSC-motor neurons derived from three non-*C9ORF72* subjects were used as controls. Immunofluorescent staining showed that, in the controls, SNRNPB2 and DDX39B were properly localized to the nucleus (Figure 4A). In contrast, we found a striking mislocalization of SNRNPB2 to the cytoplasm in the *C9ORF72* iPSC-motor neurons. This was not the case for DDX39B, which was properly localized to the nucleus (Figure 4A). Notably, mislocalization of SNRNPB2 in patient iPSC-motor neurons occurs in the same cells in which DDX39B is properly localized, indicating that the effect is on SNRNPB2 rather than a general disruption of the nucleus. Moreover, quantitation revealed mislocalization of SNRNPB2 in a large fraction of the patient iPSC-motor neurons (~40%–60%) (Figure 4B), and this was not seen in controls ($p < 0.001$, two-way ANOVA; see Figure S3 for fields). Notably, lighter exposures of our iPSC-motor neuron immunofluorescent staining data indicate that in *C9ORF72* patient cells in which SNRNPB2 was mislocalized to the cytoplasm, the level of SNRNPB2 in nuclear speckle domains was lower than in controls, and the nucleoplasmic level of SNRNPB2 was likewise reduced (Figure 4C; see Figure S4 for fields). Together, these data raise the possibility that U2 snRNP is mislocalized from its normal cellular location in *C9ORF72* patient cells.

To further investigate the significance of SNRNPB2 mislocalization, we carried out additional immunofluorescent staining in the iPSC-motor neurons. As shown in Figure 4D, SF3a is mislocalized to the cytoplasm only in patient iPSC-motor neurons and not in control iPSC-motor neurons. In contrast, the U1 snRNP components, SNRPC and SNRNP70, are properly localized to the nucleus in both patient and control iPSC-motor neurons. In addition, HNRNPA1, SRSF2, and FUS remain properly localized

to the nucleus in the patient and control cells. Together, these data support the conclusion that U2 snRNP, a major component of the splicing machinery, is mislocalized to the cytoplasm in *C9ORF72* iPSC-motor neurons, and this mislocalization may play a role in the mis-splicing observed in these patient cells.

U2 snRNP Mislocalizes to the Cytoplasm in HeLa Cells Treated with PR

To determine whether the toxic peptides could cause the mislocalization of U2 snRNP, we used immunofluorescent staining to examine HeLa cells treated with PR or FLAG peptides (GR peptide is unstable in cell culture media [Kwon et al., 2014] and was not tested in our study). As shown in Figure S5, both PR and FLAG peptides were distributed in both the nucleus and cytoplasm. Significantly, U2 snRNP components SNRNPB2 and SF3a were mislocalized to the cytoplasm in the PR-treated, but not control-treated, cells (Figures S5A and S5B; see Figure S5E for fields). In contrast, DDX39B and SRSF2 were properly localized to the nucleus in both the PR-treated and control-treated cells (Figures S5C and S5D; see Figure S5E for fields). These data are consistent with our results showing that U2 snRNP is mislocalized in *C9ORF72* patient iPSC-motor neurons and suggest a direct role of *C9ORF72* toxic peptides in causing U2 snRNP mislocalization.

U2-Dependent Exons Are Preferentially Mis-spliced in *C9ORF72* Patient Brains

Previous RNA-sequencing (RNA-seq) analyses found that 23% of cassette exons out of the whole genome (9,878 out of 42,216) were skipped when the U2 snRNP component SF3B1 was knocked down in HeLa cells (Kfir et al., 2015). If U2 snRNP dysfunction plays an important role in mis-splicing in *C9ORF72* patients, these “U2-dependent” exons might be preferentially mis-spliced in patients. To investigate this question, we reanalyzed RNA-seq datasets for cassette exons in *C9ORF72*, sporadic ALS (sALS), and control brains (Prudencio et al., 2015). In their study, they examined eight cerebellums and eight frontal cortices, and we used these data for our analysis. In conjunction, we performed a neutral simulation to estimate the extent of U2 dependency expected by chance in the whole genome. This analysis revealed that in the random sampling, the highest frequency of the percentage of U2-dependent exons in the genome was 23% (marked by red dot in Figures 5A and 5B). In contrast, in *C9ORF72* cerebellums, U2-dependent exons account for 35% (540/1,578) of the total skipped cassette exons, significantly higher than expected by chance ($\chi^2 = 98.3$, $p < 10^{-10}$, χ^2 test; Figure 5A). Notably, as shown in Figure 5A, an even greater enrichment of U2-dependent exons (42%, 83/200) was observed in the top 200 most significantly mis-spliced exons in *C9ORF72* cerebellum ($\chi^2 = 36.2$, $p < 10^{-10}$, χ^2 test). This was not the case for mis-spliced exons in sALS cerebellums, of which only 27% were U2 dependent (46 out of 170; $\chi^2 = 1$.

(C) Light exposure of immunofluorescent staining using SNRNPB2 (red) and DAPI (blue) in *C9ORF72* iPSC-motor neurons. Dotted line outlines the cell nucleus. See Figure S4 for fields.

(D) Immunofluorescent staining in patient or control iPSC-motor neurons using antibodies against SF3a, SNRPC, SNRNP70, HNRNPA1, SRSF2, and FUS. Dotted line outlines the cell nucleus.

MN, motor neuron. Scale bars, 20 μ m.

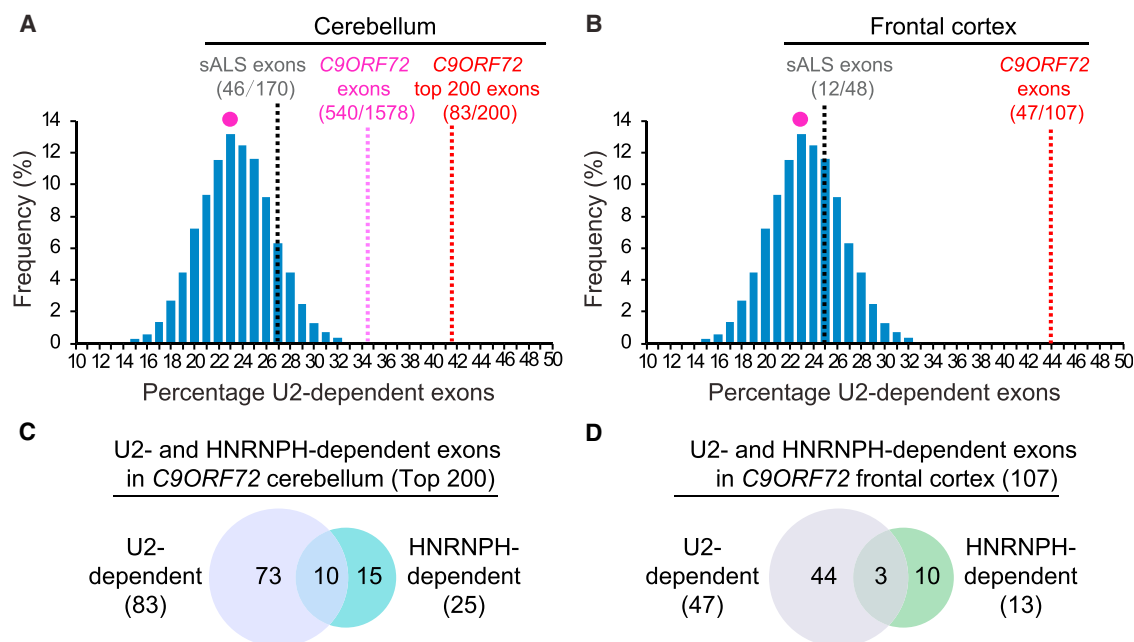


Figure 5. U2-Dependent Exons Are Preferentially Mis-spliced in C9ORF72 Patient Brain

(A and B) Histogram showing U2-dependent exons present in 10,000 random samplings. The red dot indicates the percentage (23%) of the U2-dependent exons in the genome that occur with the highest frequency. Lines indicate the percentage of U2-dependent exons that are mis-spliced in C9ORF72 or sALS patient cerebellums (A) or frontal cortex (B). For C9ORF72, U2-dependent exons for total mis-spliced (1,578) and top 200 mis-spliced cassette exons (FDR ranging from 7E-124 to 2E-12) are shown.

(C and D) Venn diagrams showing U2- and HNRNPH-dependent mis-spliced exons in C9ORF72 cerebellums (top 200 exons) (C) and frontal cortex (107 exons) (D).

See also Tables S4, S5, and S6.

3, $p = 0.26$, χ^2 test; Figure 5A). We obtained similar results when we reanalyzed the RNA-seq data from the frontal cortex in which a total of 107 cassette exons were skipped (Prudencio et al., 2015). In this case, our analysis revealed that U2-dependent exons were significantly enriched in C9ORF72 (44%, 47/107, $\chi^2 = 25.1$, $p = 1.0E-6$, χ^2 test; Figure 5B), but not in sALS (25%, 12/48, $\chi^2 = 0.07$, $p = 0.8$, χ^2 test; Figure 5B). Together, our results lend further support to our proteomic, biochemical, and U2 snRNP cellular localization studies, indicating that U2 snRNP plays an important role in the mis-splicing in C9ORF72 patients.

To further investigate whether mis-splicing in C9ORF72 was specific to U2 snRNP, we repeated the same analysis for “U1-dependent exons” using data from knockdown of the U1 snRNP component SNRNPC in HeLa cells (Rösel-Hillgärtner et al., 2013). Our results showed that 15% (232/1,578) of all cassette exons or 17% of the top 200 cassette exons altered in C9ORF72 patient cerebellums were U1 dependent, which was not significantly different from that of the global average of total exons (13%, $p = 0.11$ and $p = 0.12$, χ^2 test). Similarly, no enrichment of U1-dependent exons was observed for C9ORF72 patient frontal cortex (14%, 15/107, $\chi^2 = 0.05$, $p = 0.8$, χ^2 test).

In a recent study, it was reported that HNRNPH plays a role in mis-splicing in C9ORF72 patient brains (Conlon et al., 2016). Accordingly, we next examined the contribution of U2 snRNP versus HNRNPH to mis-splicing using the top 200 mis-spliced

exons from C9ORF72 patient cerebellum and the 107 exons from frontal cortex (Figures 5C and 5D). This analysis revealed that mis-splicing of 83 exons was U2 dependent, 25 exons were HNRNPH dependent, and 10 exons were both U2 and HNRNPH dependent in cerebellum (Figure 5C). Similar results were obtained in the frontal cortex (Figure 5D). These data indicate that U2 snRNP dysfunction has a greater contribution than HNRNPH to mis-splicing in C9ORF72 patient brain.

U2-Dependent Mis-splicing Occurs in PR-Treated Cells

In light of our multiple lines of evidence showing that GR/PR peptides associate with and affect U2 snRNP, we next investigated whether the U2-dependent mis-splicing observed in the bioinformatics analyses could be caused by the toxic peptides. A previous RNA-seq analysis showed that treatment of cultured astrocytes with PR resulted in splicing dysfunction, involving mis-splicing events in 4,298 genes (Kwon et al., 2014). We used these data to compare PR-dependent mis-spliced genes with the U2-dependent genes that we identified in C9ORF72 patient cerebellum and frontal cortex. This analysis revealed that 39% and 40% of the U2-dependent genes altered in the C9ORF72 cerebellum and frontal cortex, respectively, were also PR dependent, significantly more than expected by chance (20%, $p < 10^{-10}$ and $p = 1.0E-6$, χ^2 test). These data support the model that the toxic peptides directly lead to dysfunction of U2 snRNP and mis-splicing in C9ORF72 patient cells. Further

evidence for the importance of the toxic peptides in causing U2-dependent mis-splicing came from a genome-wide comparison of U2-dependent genes with PR-dependent genes. We found that 42% of the genes affected by PR are U2 dependent (1,797 out of 4,298), significantly higher than expected by chance (24%, $\chi^2 = 292.8$, $p < 10^{-10}$, χ^2 test). Thus, there is a high level of correlation between U2-dependent mis-splicing and PR-dependent mis-splicing events in the whole genome. Together, these analyses provide evidence that DPR proteins disrupt normal U2 snRNP function, and this could be an important mechanism underlying mis-splicing in *C9ORF72* patient cells.

DISCUSSION

Previous studies reported that mis-splicing occurs in *C9ORF72* patient brains, but the underlying mechanisms are not understood. Here, we obtained multiple lines of evidence that dysfunction of the general splicing factor U2 snRNP plays an important role in this mis-splicing. Our in vitro studies showed that *C9ORF72* toxic dipeptides (GR and PR) block spliceosome assembly and splicing and specifically associate and interfere with U2 snRNP. To investigate whether the effects on U2 snRNP are relevant to the mis-splicing observed in *C9ORF72* patient cells, we examined iPSC-motor neurons generated from these patients. We observed a dramatic mislocalization of U2 snRNP components, but not other snRNPs or splicing factors, to the cytoplasm in *C9ORF72* patient iPSC-motor neurons. Our data indicate that this mislocalization is due to the toxic peptides because we observed specific mislocalization of U2 snRNP components to the cytoplasm in HeLa cells treated with PR peptide. We also carried out bioinformatic analyses, which revealed that U2-dependent exons are preferentially mis-spliced in *C9ORF72* patient brain tissues, accounting for as much as ~44% of the most significantly mis-spliced exons. Of these, PR-dependent mis-splicing accounts for ~40% of them. Together, these data are consistent with the possibility that the mis-splicing observed in patients with *C9ORF72* repeat expansion is due, in part, to DPRs interfering with U2 snRNP. An important question for future work is to determine whether and how these U2-dependent mis-splicing events are involved in the pathogenesis of *C9ORF72* repeat expansion in ALS/FTD patients.

One obvious mechanism by which mis-splicing of U2-dependent exons could contribute to disease is by affecting genes that are essential for normal motor function. We listed the 81 U2-dependent genes that are affected by mis-splicing in *C9ORF72* cerebellum in Table S4 and the 46 U2-dependent genes affected by mis-splicing in the frontal cortex in Table S5. (Note that there are fewer genes than exons with mis-splicing, because some of the genes have more than one mis-splicing event.) We extracted potentially relevant information about the genes from the literature, and this information is shown in the tables. One of the largest categories of mis-spliced U2-dependent genes has mitochondrial functions (labeled red in Tables S4 and S5), an especially interesting finding in light of the known links between mitochondrial dysfunction and ALS resulting from multiple different causes (Cozzolino and Carri, 2012; Lopez-Gonzalez

et al., 2016). It was also recently reported that *C9ORF72* patient DPRs compromise mitochondrial function and cause oxidative stress (Lopez-Gonzalez et al., 2016). The U2-dependent genes related to mitochondria include *HIF1A*, *UQCRRH*, *DNM1L*, *COX16*, *TMEM126*, *NDUFAF5*, *PDHA1*, and *TIMM9*, and two mitochondrial ribosomal protein genes, *MRPL52* and *MRPS31* (Tables S4 and S5). *HIF1A* is interesting as it is a transcription factor that has a role in the distribution of mitochondria in axons. Genes with some relationship to neurons or neuronal disease were another large category in the U2-dependent mis-spliced genes (designated in blue in Tables S4 and S5). Notable among these are *PARD3* and *MARK2*, which modulate neuronal polarity and neurite outgrowth, respectively. Finally, we found that several U2-dependent genes have functions in different steps of gene expression. These include transcription and splicing factors (marked in gray and yellow, respectively, in Tables S4 and S5). Three of the splicing factors are members of the U2AF family (RBM39, U2AF1, and RBM23). These proteins are essential for recruiting U2 snRNP to the pre-mRNA, and it is possible that their mis-splicing further exacerbates the mis-splicing observed in *C9ORF72* patient brains. We also found that *GEMIN7* was mis-spliced (Table S5). This protein is a component of the SMN complex, which functions in snRNP biogenesis (Battle et al., 2006). Thus, defective snRNP biogenesis could also contribute to the mis-splicing observed in *C9ORF72* patients. Notably, mutation of the SMN protein causes the childhood motor neuron disease spinal muscular atrophy, and previous work revealed that this disease shares a biochemical pathway with ALS (Gama-Carvalho et al., 2017; Shan et al., 2010; Yamazaki et al., 2012). Our bioinformatic study also revealed that 40% of the U2-dependent genes in *C9ORF72* patient brains are also PR dependent. These genes are listed in Table S6, which reveals that they are enriched in genes with mitochondrial and pre-mRNA splicing functions. Together, our results raise the possibility that DPR-mediated dysfunction of U2 snRNP, a major component of the splicing machinery, causes mis-splicing of genes involved in cellular functions that have previously been associated with ALS/FTD pathogenesis.

Several mechanisms may underlie DPR-mediated U2-dependent mis-splicing in *C9ORF72* patients. For example, our in vitro data showing that U2 snRNP binds to GR/PR toxic peptides and disrupts splicing and spliceosome assembly raise the possibility that interactions between the DPRs and U2 snRNP directly disrupt the function of this essential splicing factor. Another possibility is that the loss of U2 snRNP to the cytoplasm that we observed in *C9ORF72* patient-iPSC derived motor neurons may decrease the levels of nuclear U2 snRNP required for proper splicing. In addition, we observed loss of U2 snRNP from nuclear speckle domains, and this may disrupt normal U2 snRNP function. In previous studies, overexpression of tagged GR/PR showed that it is primarily in the cytoplasm (Kwon et al., 2014; Lee et al., 2016), and we found that PR peptide localizes to the cytoplasm in HeLa cells. In addition, immunohistochemistry of *C9ORF72* patient brains showed that DPRs are present in both the nucleus and cytoplasm (Mori et al., 2013; Wen et al., 2014). These observations may explain how U2 snRNP is mislocalized to the cytoplasm in *C9ORF72* patient cells. Specifically, it is known that snRNP biogenesis takes place in the cytoplasm

(Battle et al., 2006). Thus, it is possible that interactions between the DPRs and U2 snRNP may sequester U2 snRNP in the cytoplasm.

In recent studies, GR or PR peptides overexpressed in cultured cells were found to associate with numerous cellular proteins (Lee et al., 2016; Lin et al., 2016; Lopez-Gonzalez et al., 2016). Among these were RNA-binding proteins that contain low complexity domains, and of these, several are known ALS-causative proteins (e.g., FUS, TARDBP, HNRNPA1, and MATR3). Interactions between GR/PR and the low complexity domain proteins disrupt the assembly and dynamics of membrane-less organelles, which may contribute to *C9ORF72* pathogenesis (Lee et al., 2016). Consistent with these studies, our proteomic analysis of GR/PR-interacting proteins revealed a large overlap with numerous ALS-causative proteins, including those known to contain low complexity domains (Figure 6A). In addition, the GR/PR interactors identified in our study overlap significantly with those identified in previous studies (Lee et al., 2016; Lopez-Gonzalez et al., 2016) (Figure 6B). Several observations suggest that at least some of the GR/PR interactors are physiologically relevant. Specifically, multiple U2 snRNP proteins were identified as GR/PR interactors not only in our work but also in the recent studies (Lee et al., 2016; Lopez-Gonzalez et al., 2016) (Figure 6B). In addition, these common interactors were identified using a variety of cell types and pull-down strategies and are independent of repeat protein lengths (Figure 6C). Many of these U2 snRNP proteins contain low complexity domains (Figures 6D and S6), and the interaction between GR/PR and these low complexity domain-containing U2 snRNP proteins may explain why U2 snRNP is lost from its normal location in the membrane-less organelle (nuclear speckle domains) and is mis-localized to the cytoplasm.

Our data indicate that U2-dependent mis-splicing accounts for a large fraction of the mis-splicing observed in patients with *C9ORF72* repeat expansion, which is the most frequent mutation in familial ALS/FTD. In addition, mis-splicing is present in other forms of familial ALS (e.g., those due to FUS or TDP-43 mutation). Sporadic ALS, which is the most common form of all types of ALS, also exhibits mis-splicing. Thus, correction of mis-splicing is a potential therapeutic approach for multiple forms of ALS. In this regard, splicing modulator compounds that can correct mis-splicing are emerging as potential therapies for other diseases, and these or related compounds may have efficacy for the treatment of ALS/FTD.

EXPERIMENTAL PROCEDURES

Plasmids

CMV-Ftz plasmid was described previously (Das et al., 2006). CMV-Ftz-let-7a plasmid was constructed by inserting a 395-nt pri-let-7a fragment into the BamHI site in the Ftz intron. CMV-DNA templates were amplified by PCR using forward (5'-TGGAGGTCGCTGAGTAGTGC-3') and reverse (5'-TAGAAGGCA CAGTCGAG GCT-3') primers.

Transcription-Coupled RNA Processing

For in vitro studies, we used HeLa cell nuclear extracts prepared according to Krainer et al. (1984). Transcription-coupled splicing reactions were performed as described elsewhere (Das et al., 2007; Folco et al., 2011), except that pre-initiation complexes (PICs) were assembled on the CMV-DNA templates prior to transcription (Yu et al., 2010). Briefly, CMV-DNA templates were incubated

in 15 μ L nuclear extract containing 3.2 mM $MgCl_2$ and 5 μ L polyvinyl alcohol for 20 min at 30°. The indicated peptides (10 μ M) were then added in a final reaction mixture volume of 25 μ L containing 0.5 mM ATP, 20 mM creatine phosphate (di-Tris salt), and 1 μ L 32 P-UTP (250 Ci/mmol; Perkin Elmer Life Sciences). Reaction mixtures were incubated for 15 min at 30°C to allow transcription followed by addition of α -amanitin (200 ng). Incubation was continued at 30°C for times indicated. For transcription/splicing/pri-miRNA processing, PICs were assembled using the same conditions as for transcription-coupled splicing. No peptide or 10 μ M of the indicated peptides was then added and incubation was continued for 15 min to allow transcription in the presence of 0.5 mM ATP, 20 mM creatine phosphate (di-Tris salt), and 1 μ L 32 P-UTP, and an additional 3.2 mM $MgCl_2$ was added to bring the final concentration to 6.4 mM in a final reaction mixture volume of 25 μ L. α -Amanitin (200 ng) was added and incubation was continued for 45 min to allow processing. Total RNA was fractionated on 8% denaturing polyacrylamide gels and detected by PhosphorImager.

Proteomic Analysis of GR and PR Interacting Proteins

GR, PR, or FLAG peptides were biotinylated using the EZ-Link TFPA-PEG3-Biotin kit (Thermo Fisher). Splicing reaction mixtures containing these peptides (10 μ M) were separated on Sephacryl-S500 gel filtration columns in a buffer containing 20 mM Tris (pH 7.8), 60 mM KCl, 0.1% Triton X-100, and 0.2 mM PMSF. Proteins in the gel filtration fractions were analyzed on NuPAGE Novex 4%–12% Bis-Tris Gels (Thermo Fisher) followed by western blotting. Total RNAs from the fractions were analyzed on an 8% denaturing polyacrylamide gel stained with ethidium bromide. The data for rows FLAG, SNRPC, SNRPB2, SF3A1, SF3A2/3, FUS, and TARDBP shown in Figure 2A and the RNA gel shown in Figure 2B were from the gel filtration column containing the GR peptide. Similar results were obtained with gel filtration fractions containing PR and FLAG peptides. Pull-downs were carried out from fractions 33–69 in the gel filtration buffer using streptavidin magnetic particles (Roche) at 4°C for 2 hr and washed five times with 1 \times PBS/0.1% Triton X-100. Proteins associated with biotinylated peptides were labeled using tandem mass tag (TMT) (McAlister et al., 2012) and analyzed with an Orbitrap Fusion mass spectrometer coupled to a Proxeon EASY-nLC 1000 liquid chromatography (LC) pump (Thermo Fisher Scientific).

Immunoprecipitations

A rabbit polyclonal antibody that recognizes the three SF3a complex subunits or a mouse monoclonal antibody against SNRPB2 were described previously (Das et al., 2000). Rabbit polyclonal antibodies against DROSHA and a control immunoglobulin G were from Abcam. For protein IPs, antibodies were cross-linked to protein Sepharose beads with dimethylpymelimidate (Sigma). Prior to IPs, 250- μ L reaction mixtures containing 75 μ L nuclear extract, 3.2 mM $MgCl_2$, 0.5 mM ATP, and 20 mM creatine phosphate (di-Tris salt) were incubated for 20 min at 30° and then spun at 14,000 rpm in a microfuge for 5 min at 4°C. The supernatant was mixed with 150 μ L PBS/0.1% Triton X-100/0.2 mM PMSF, protease inhibitor EDTA-free (Roche), and spun in a microfuge at 14,000 rpm for 5 min at 4°C. IPs were carried out at 4°C for 2 hr and washed five times with 1 \times PBS/0.1% Triton X-100. IPs were mixed with protein gel loading buffer without DTT at room temperature to elute the proteins. DTT (2 μ M final) was then added, and samples were boiled for 2 min and run on 4%–12% SDS-PAGE gels.

U2 snRNP and Spliceosome Assembly Assays

A U2 2'OMethyl oligo that base pairs to U2 snRNA (5'-mCmAmGmAmUmAmCmUmAmCmAmCmUmUmGmA-3') was 32 P-labeled with γ -ATP (250 Ci/mmol; Perkin Elmer Life Sciences) and T4 polynucleotide kinase. Reaction mixtures (25 μ L) containing 15 μ L nuclear extract were incubated in the presence of 0.5 mM ATP, 3.2 mM $MgCl_2$, 20 mM creatine phosphate (di-Tris salt), 10 μ M peptides, and 0.4 μ M U2 oligo for 5 min. For spliceosome assembly, transcription-coupled splicing reactions with or without the peptides were incubated for the times indicated and then run on G-50 micro columns (Amersham Biosciences) to remove unincorporated 32 P-UTP. Heparin (0.65 mg/mL final concentration) was added to the G-50 column-purified reactions before loading on a 1.2% low-melting-point agarose gel.

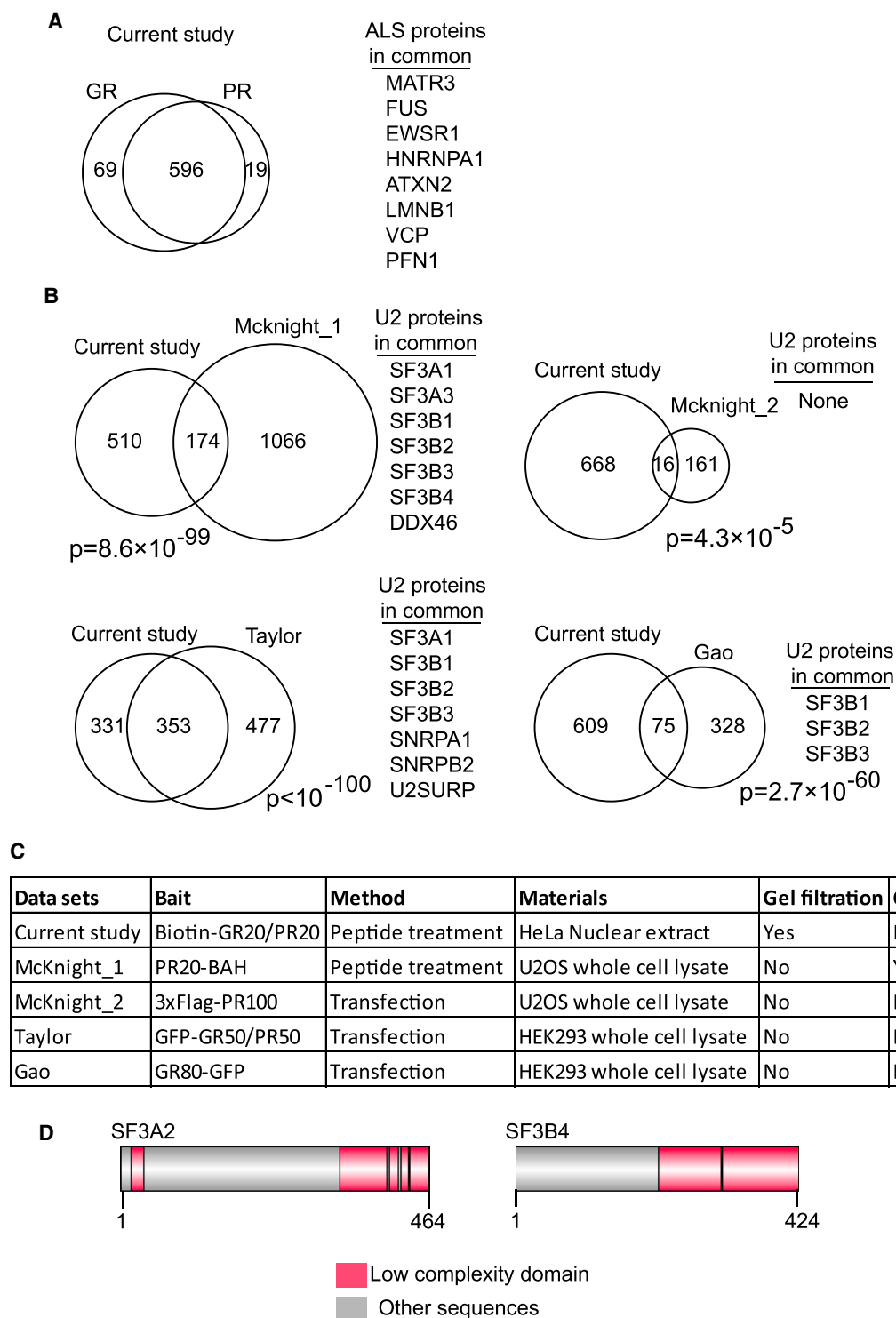


Figure 6. Comparison of Toxic Peptide Interactomes in Different Datasets

(A) Venn diagram showing overlap between GR- and PR-interacting proteins identified in this study. Known ALS proteins identified in both datasets are shown.

(B) Venn diagrams showing overlap between interacting proteins identified in this study and in four published datasets (the McKnight_1 and McKnight_2 datasets are described in [Lin et al., 2016](#); the Taylor dataset is described in [Lee et al., 2016](#); and the Gao dataset is described in [Lopez-Gonzalez et al., 2016](#)).

(C) Table showing methods used to generate the DPR interactome datasets.

(D) Examples of U2 snRNP proteins showing their low complexity domains.

See [Figure S6](#) for this analysis with all of the U2 snRNP components.

Generation of iPSC-Derived Motor Neurons

Generation of iPSC-derived neurons was described previously (Lopez-Gonzalez et al., 2016). Briefly, iPSCs were expanded in Matrigel-coated wells and at 60% confluency were split with Accutase into Matrigel-coated wells. 24 hr after plating, culture medium was replaced with neuroepithelial progenitor (NEP) medium, DMEM/F12, Neurobasal medium at 1:1, 0.5× N2, 0.5× B27, 0.1 mM ascorbic acid (Santa Cruz Biotechnology), 1X Glutamax (Invitrogen), 3 μ M CHIR99021 (Tocris Bioscience), 2 μ M DMH1 (Tocris Bioscience), and 2 μ M SB431542 (Stemgent) for 6 days. NEPs were dissociated with dispase and split 1:6 into Matrigel-coated wells. NEPs were cultured in motor neuron progenitor (MNP) induction medium (the same medium as described above with 0.1 μ M RA [Stemgent] and 0.5 μ M purmorphamine [Stemgent]) for 6 days. MNPs were dissociated with dispase to generate suspension cultures. After 6 days in culture, cells were dissociated into single cells, plated on laminin-coated plates/cover-slips in motor neuron differentiation medium (containing 0.5 μ M RA, 0.1 μ M purmorphamine, and 0.1 μ M compound E; Calbiochem), and cultured for 1 month.

Immunofluorescent Staining

For immunofluorescent staining, iPSC-motor neurons were fixed with 4% paraformaldehyde in PBS for 15 min and permeabilized with 0.1% Triton X-100 in PBS for 15 min. After incubation in 5% fetal bovine serum (FBS) for 1 hr at room temperature, cells were incubated overnight at 4°C in custom primary antibodies SNRPB2 (1:100), DDX39B (1:1,000), SF3a (1:1,000), and FUS (1:1,000) or in commercial antibodies SNRPC (Santa Cruz Biotechnology, 1:200), SNRPN70 (Millipore, 1:200), HNRNPA1 (Santa Cruz Biotechnology, 1:200), and SRSF2 (1:1000, Invitrogen). After three washes in PBS, cells were incubated with mouse Alexa Fluor 647 (for SNRPB2, SNRPN70, and SRSF2) or rabbit Alexa 488 (for HNRNPA1, DDX39B, SNRPC, SF3a, and FUS) secondary antibodies (Invitrogen, 1:1000) for 1 hr at room temperature, followed by three washes in PBS. Images were captured with a Nikon TE2000U inverted microscope.

Bioinformatic Analysis and Statistics

Brain transcriptome profiles of C9ORF72 ALS and sALS are described in GEO: GSE67196 (Prudencio et al., 2015). Processed RNA-seq data were provided by the authors, and a cutoff of Bonferroni-corrected false discovery rate (FDR) < 0.05 was used to specify significantly altered exons. RNA-seq data of SF3B1 knockdown was from GEO: GSE65644 (Kfir et al., 2015). RNA-seq data of HNRNPH or control knockdown in 293T cells was from GEO: GSE16642 (Xiao et al., 2009). RNA-seq data of SNRNPC or control knockdown in HeLa cells was from GEO: GSE42485 (Rösel-Hillgärtner et al., 2013). Misspliced genes in cultured astrocytes treated with PR peptide were from (Kwon et al., 2014). Low-complexity domain prediction was done using SMART online tool (Letunic et al., 2015). Statistical analyses were done with GraphPad Prism (GraphPad Software). Differences between groups were analyzed with ANOVA, Student's t test, or χ^2 test. $p < 0.05$ was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and six tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2017.05.056>.

AUTHOR CONTRIBUTIONS

S.Y. and R.R. conceived the project, and S.Y. carried out most of the experiments. F.-B.G. directed iPSC-motor neuron generation, which was carried out by R.L.-G. Imaging analyses were carried out by S.Y. and J.G. Splicing and U2 snRNP native gel analysis were done C.B. Mass spectrometry was performed by R.K. The data were analyzed by R.K., S.P.G., S.Y., and R.R. The manuscript was written by S.Y. and R.R. with input from F.-B.G. and R.L.-G. and assistance from all authors.

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