Functional mapping of the interaction between TDP-43 and hnRNP A2 *in vivo*

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ABSTRACT

Nuclear factor TDP-43 has been reported to play multiple roles in transcription, pre-mRNA splicing, mRNA stability and mRNA transport. From a structural point of view, TDP-43 is a member of the hnRNP protein family whose structure includes two RRM domains flanked by the N-terminus and C-terminal regions. Like many members of this family, the C-terminal region can interact with cellular factors and thus serve to modulate its function. Previously, we have described that TDP-43 binds to several members of the hnRNP A/B family through this region. In this work, we set up a coupled minigene/siRNA cellular system that allows us to obtain *in vivo* data to address the functional significance of TDP-43-recruited hnRNP complex formation. Using this method, we have finely mapped the interaction between TDP-43 and the hnRNP A2 protein to the region comprised between amino acid residues 321 and 366. Our results provide novel details of protein–protein interactions in splicing regulation. In addition, we provide further insight on TDP-43 functional properties, particularly the lack of effects, as seen with our assays, of the disease-associated mutations that fall within the TDP-43 321-366 region: Q331K, M337V and G348C.

INTRODUCTION

Nuclear factor TDP-43 is a multifunctional RNA binding protein that has been described to play a role in transcription, pre-mRNA splicing, mRNA stability and mRNA transport (1–4). Recently, it has also been described to participate in pathological processes such as cystic fibrosis (5) and a series of neurodegenerative diseases that include Fronto Temporal Lobar Degeneration (FTLD-U) and Amyotrophic Lateral Sclerosis (ALS) (6,7), as reviewed in several publications (8–12). For these reasons, the detailed characterization of the properties of this protein may well be crucial for future diagnostic, prognostic and therapeutic applications. TDP-43 is a member of the hnRNP protein family (13) that comprises several proteins with numerous functions (14,15) including some of the best well-known splicing modulators, such as PTB (hnRNP I), hnRNP A/B and hnRNP H (16). Structurally, the TDP-43 protein is divided in four well-defined regions, an N-terminal sequence that contains a Nuclear Localization Signal (17), two RNA Recognition Motifs of which only the first has been described to be involved in RNA binding (18) and a Gly-rich C-terminal region that in other hnRNPs normally mediates protein–protein interactions. Consistent with a role of the C-terminal tail in TDP-43 activity, we have previously demonstrated that this region is essential for TDP-43 to function as splicing silencer in the CFTR exon 9 and Apo AII exon 3 systems (19,20). We proposed that inhibition of splicing by TDP-43 depends on the interaction of the C-tail with members of the hnRNP A/B family and especially hnRNP A2, as determined by pull-down analysis (20). Interestingly, the C-terminal region is also required for the ability of TDP-43 to act as a transcriptional insulator for the mouse SP-10 gene, as reported by Abhyankar *et al.* (21). Taken together, the functional evidence gathered so far has identified the C-terminal region of TDP-43 as a crucial domain governing the protein's functional properties. The importance of the C-terminus has also been highlighted by the observation that ~2–3% of patients affected by sporadic and familial forms of ALS carry specific missense mutations in the C-terminus of TDP-43 (22–29), as recently reviewed by Banks *et al.* (30). Presently, there is no information with regards to which activity of the protein may be affected by these mutations although some have been associated with neurotoxicity (22) or increased degradation (23). From a molecular point of view, a likely possibility is that they may interfere with some essential protein–protein interactions such as the association with hnRNP A/B proteins.

In this work, we have further expanded our initial studies regarding the interaction of TDP-43 with hnRNP proteins by setting up an *in vivo* functional system that couples siRNA knock-down of endogenous TDP-43 and add-back of siRNA resistant proteins in HeLa cells. We have been able to finely map the region of interaction between TDP-43 and hnRNP proteins and to assess the importance of disease-related missense mutations in this region. Finally, we show that the hnRNP binding properties are evolutionarily conserved among TDP-43 orthologs even in conditions of limited sequence homology.

MATERIALS AND METHODS

Plasmid preparation

All GST tagged TDP-43 mutants were generated using as template the pGEX3X-TDP-43 (5) plasmid using primers TDP BamFW (5′-ggggatcctctgaatatattcgggtaac-3′), and TDP315EcoREV (5′-gggaattctcacgcaccaaagttcatcccaccac c-3′) or TDP 366EcoREV (5′-gggaattcggcctggtttggctccctc tg-3′) and cloned in pGEX3X between BamHI and EcoRI. The deletion mutants were generated with primers TDP BamFW, TDPEcoREV (5′-gggaattctcacattccccagccagaa gac-3′), TDPdelta321-366FW (5′-gcgttcagcattaatccattcggt tctggaaataac-3′) andTDPdelta321-366REV (5′-gttatttccag aaccgaatggattaatgctgaacgc-3′) whilst the Q331K, M337V and G348C carrying TDP-43 sequences were amplified with suitable primers carrying each point mutation (sequence available upon request). The GST-tagged TBPH, TBPH ΔC, have already been described by Ayala *et al.* (19) whilst GST-tagged hnRNP A2 proteins has been described in Buratti *et al.* (20).

The FLAG-tagged mutants generation has already been described by Ayala *et al.* (31) apart from the pFLAG Δ321-366 mutant that was amplified from pGEX3X Δ321-366 with primers TDPHindFW (5′-cccaagctttctgaat atattcgggtaaccg-3′) and TDPKpnREV (5′-ggggtacctcacatt ccccagccagaagac-3′). The FLAG-tagged TBPH was amplified from pGEX TBPH with primers DROHindFW (5′-cc caagcttgatttcgttcaagtgtcgga-3′) and DROKpnREV (5′-ggg gtaccaagaaagtttgacttctccg-3′). The FLAG-tagged TDP-43 mutants Q331K, M337V and G348C were amplified from the relative pGEX3X vectors with the primers TDPHindFW and TDPKpnREV. All the pFLAG TDP mutants were cloned HindIII/KpnI in pFLAG-CMV2. In addition, a silent mutation was introduced in all the pFLAG TDP-43 mutants using standard PCR procedure in order to make them resistant to the anti-TDP-43 siRNA. Primers were the following: siTDP_KOFW (5′-t aattctaagcagtcccaggatga-3′) and siTDP_KOREV (5′-tcatc ctgggactgcttagaatta-3′).

The reporter minigene used in all splicing assays, pTB CFTR C155T, has already been described by Pagani *et al.* (32).

Peptide synthesis

Peptide 321-366 was synthesized on solid phase (Fmoc/ *t*-Bu chemistry) using a home-built automatic synthesizer based on a Gilson Aspec XL SPE system. The peptide-resin (preloaded NovaSyn TGT, Novabiochem) was cleaved/deprotected using a modified Reagent H mixture (trifluoroacetic acid 80%, phenol 3%, thioanisole 3%, 3,6-dioxa-1,8-octanedithiol 8%, water 2.5%, methylethyl-sulfide 2%, hydroiodic acid 1.5% w/w) for 3 h. The peptide was then precipitated by diethylether, washed and freeze dried. The peptide was purified by preparative RP-HPLC on a 25 × 300 mm column (Load&Lock system, Varian) packed with VariTide RPC resin (Polymer Laboratories—Varian) using a gradient from 0.1% TFA in water to 0.1% TFA in acetonitrile. The purified fractions were checked by ESI-MS, pooled and freeze dried.

Tissue culture and add-back assay

HeLa cell line was grown in DMEM-Glutamax-I (GIBCO) supplemented with 10% fetal bovine serum (Euroclone) and Antibiotic–Antimycotic stabilized suspension (Sigma). Cells were grown overnight and transfected with Effectene Transfection reagent (Qiagen) according to manufacturer's instructions.

For the add-back experiment, HeLa cells were plated at 30% of confluence (Day 0) and two rounds of TDP-43 siRNA transfections were carried out according to the procedure already described (33) on Days 1 and 2 in order to maximize TDP-43 silencing efficiency. Transfection of 0.5 μg of the reporter minigene together with 1 μg of pFLAG-expressed proteins was performed on Day 3. Cells were harvested on Day 4 and total RNA was collected with Trizol Reagent (Invitrogen). The siRNA target sequences used to silence the different hnRNP proteins are the following: 5′-cagctgaggaagctcttca-3′ (hnRNP A1), 5′-ggaacagttccgtaagctc-3′ (hnRNP A2) and 5′-gcaaac aagcagtagagat-3′ (hnRNP C1/C2). Reverse transcription was performed using M-MLV Reverse Transcriptase (Invitrogen), according to the manufacturer's protocol. PCR with DNA Polymerase (Roche) was carried out for 35 amplification cycles (95°C for 30 s, 55°C for 30 s and 72°C for 30 s). PCR products were analyzed on 1.5% agarose gels.

Western blot and GST-overlay/Far Western analyses

Western blotting was performed according to standard protocols using rabbit polyclonal antibody specific for TDP-43 previously described by Buratti *et al.* (5), a commercially available mouse monoclonal antibody specific for the FLAG peptide (Sigma, F1804) and an in-house made mouse polyclonal antibody for tubulin. The GST-overlay/Far Western method has been described in detail elsewhere (20).

Protein expression and EMSA analyses

All pGEX-3X vectors were transformed in BL21-DE3 bacterial cells and recombinant protein expression and purification was performed according to the procedure already described by Buratti *et al.* (5). Electro-mobility shift assays (EMSAs) were conducted according to procedures already described (18) with minor modifications. The binding buffer contained 10 mM NaCl, 10 mM Tris pH 8.0, 2 mM MgCl$_2$, 5% glycerol and 1 mM DTT. The RNA oligonucleotide (UG)$_6$ 5′-uguguguguguga-3′

was made by Integrated DNA Technologies. The 5′-end labeling of the oligonucleotide was carried out with PNK according to standard protocols. In all experiments, 100 ng of each GST fusion protein and 0.5 ng of labeled oligonucleotide were incubated at room temperature for 10 min before loading in a final 20 µl volume. In supershift analysis, 1 µg of hnRNP A2 were added to this mix. Native 5% gels were run at 100–120 V at 4°C. Gels were dried before X-OMAT film or Cyclone (Packard) exposure.

RESULTS

Setting up a coupled minigene/siRNA system to assay TDP-43 splicing inhibitory activity

The aim of our work was to set up a cell-based system to provide an accurate *in vivo* testing system to characterize the structural and functional determinants of TDP-43 splicing activities. This assay was composed of three phases: RNAi mediated knock-down of endogenous TDP-43 in HeLa cells as previosuly described (34), add-back of wild-type (WT) or mutant TDP-43 proteins whose mRNA sequence was modified to be resistant to the siRNA used and finally the assessment of their splicing inhibitory activity using minigene analysis. Added-back TDP-43 proteins were FLAG tagged so the expression levels could be easily monitored through western blots. Concerning the minigene, we used a CFTR exon 9 minigene as a substrate (Figure 1A) containing a previously described disrupting mutation in a splicing regulatory element (C155T) (32). TDP-43 inhibition of CFTR exon 9 recognition depends on the presence of a specific target sequence composed of UG repeats at the 3′ splice site of the exon. In normal conditions, this resulted in ~50% of exon inclusion when transfected in HeLa cells (Figure 1C, lane 1). In order to test the performance of the system, we initially used two add-back plasmids coding for WT TDP-43 and for its *Drosophila* homolog (TBPH) previously shown to possess the same inhibitory effect of human TDP-43 in an *in vitro* splicing system (19). As negative control, we used a TDP-43 mutant that is unable to bind UG repeats due to the F147L and F149L mutations (18) in its first RRM-1 (Figure 1B). As shown in Figure 1C, transfection of the CFTR minigene in cells depleted of endogenous TDP-43 resulted in a substantial improvement of CFTR exon 9 inclusion (>80%) (Figure 1C, lane 2). As expected, inclusion levels could be successfully reduced following the addition of siRNA-resistant WT TDP-43 and of TBPH (Figure 1C, lanes 3 and 4), but were not affected by expression of the TDP-43 carrying the mutations in RNA binding domain 1 (Figure 1C, lane 5). The western blot against the Flag-tagged of the different proteins shows that all three were
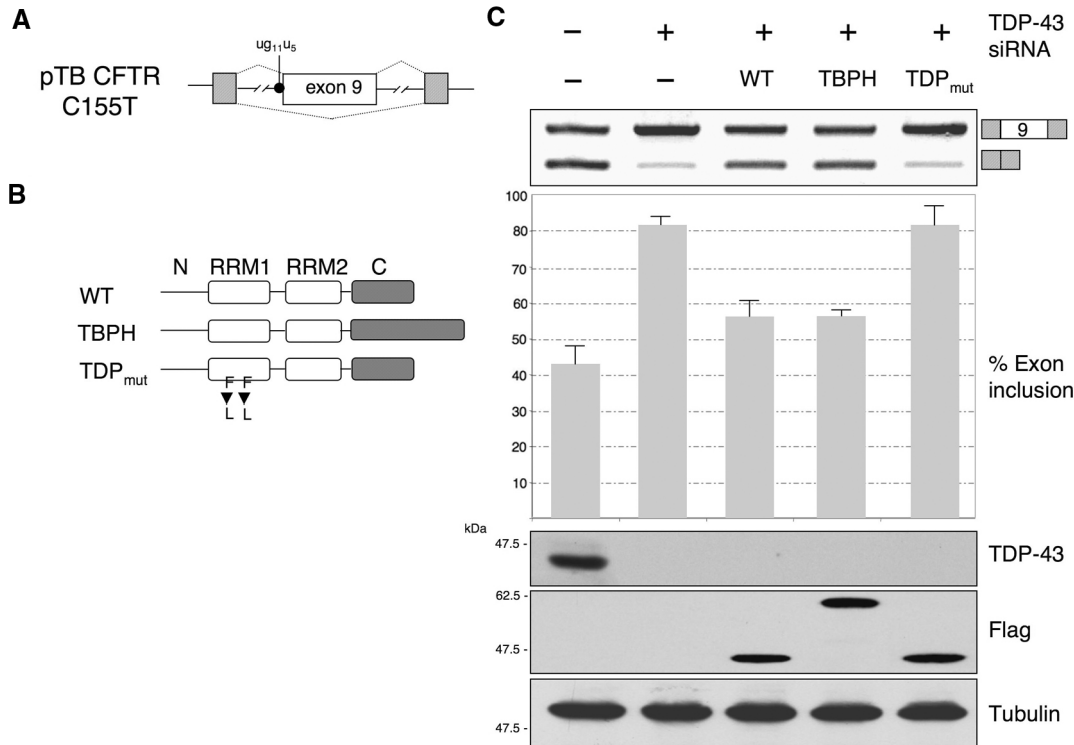
Figure 1. (A) A schematic diagram of the CFTR C155T minigene transfected in our add-back assay (dotted lines represent possible splicing outcomes). (B) Three schematic diagrams of TDP-43 WT, Drosophila TDP-43 (TBPH) and TDP-MUT proteins. The two crosses in the TDP MUT diagram represent the F147L/F149L mutations that make this mutant unable to bind UG repeats. (C) The effect on CFTR exon 9 splicing of adding back these three proteins following knock-down of the endogenous TDP-43. Standard deviations obtained in three independent transfection experiments are shown. The western blots against the endogenous TDP-43, tubulin and FLAG peptide are shown in the lower boxes to show silencing efficiency, equal loading and proper transgene expression.

expressed in similar quantities in the transfected cells. As shown in Figure 1C, however, the antibody against TDP-43 does not detect the transfected si-resistant proteins. The most plausible explanation for this is that the efficiency of the siRNA transfection is greater than the add-back efficiency. This has been confirmed by western blot experiments which show that the added-back protein becomes visible using the anti-TDP-43 antibody only after prolonged exposure of the autoradiographic film (data not shown). Although both the minigene and the Flag-expressing plasmid are presumably transfected together this difference in expression can also explain why recovery of the 'baseline' inhibitory activity by TDP WT and TBPH is not complete.

Testing the importance of hnRNP A/B proteins in CFTR exon 9 splicing

In previous studies, using *in vitro* splicing assays, we demonstrated that the ability of TDP-43 to repress a UG-containing substrate, closely correlated with the ability of its C-terminal tail to bind several hnRNP proteins (20). Currently, the only experimental evidence available on the impact of hnRNP A/B proteins in CFTR splicing *in vivo* is the observation from our lab that overexpression of hnRNP A1 can downregulate its inclusion (35). We have therefore performed a more extensive analysis of the functional implicatons of hnRNP A/B protein levels on CFTR exon 9 recognition through the knockdown of hnRNP A1/A2 and C, either alone or in combination. The results of this analysis are reported in Figure 2A. This figure shows that knocking down both hnRNP A1 and A2, but not hnRNP C, results in considerable increased levels of CFTR exon 9 inclusion with respect to normal inclusion levels, as detected using our minigene construct. Taken together, these results demonstrate that hnRNP A/B proteins are powerful negative modulators of CFTR exon 9 splicing. It could be argued, however, that hnRNP A/B proteins exert their action through their binding to other RNA sequence elements in our minigene. Therefore, to rule out this possibility, we have also performed overexpression studies using hnRNP A1 and A2 on the CFTR 155T reporter minigene and a minigene which carried a deletion of the TG sequence (ΔTG) and was thus unable to bind TDP-43. The results show that overexpression of A1 and A2 in a TDP-43-dependent CFTR context can successfully inhibit exon 9 recognition (Figure 2B). On the other hand, this effect cannot be observed in a TDP-43 independent context (Figure 2C). It should also noted that none of the hnRNP proteins tested can bind UG repeats in a band-shift, ruling out the possibility that hnRNP inhibitory action is mediated by direct binding to the UG tract (20). An additional consideration in this regard can also be made by the fact that CFTR exon 9 inclusion is almost complete following endogenous TDP-43 knockdown using siRNA (Figure 1). This observation argues against an independent role of hnRNP A1/A2 in CFTR exon 9 repression as this would have been unaffected by just the removal of TDP-43. Finally, Figure 2D shows that hnRNP A1 and A2 over-expression in the absence of endogenous TDP-43 have
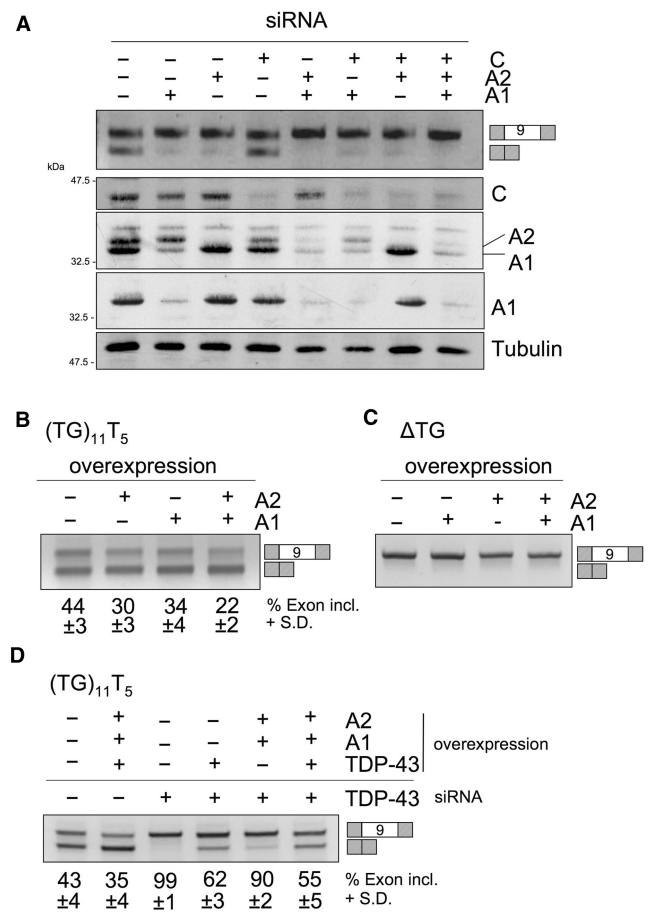
Figure 2. (**A**) The effect of siRNA knockdown of hnRNP A1, A2 and C on CFTR exon 9 splicing (either singly or in combination). The level of knockdown of each of these hnRNP proteins was assayed by western blot and is reported in the lower panels. The effects of overexpressing the hnRNP A1 and A2 proteins (either alone or in combination) in the presence of TG11T5- and ΔTG-containing CFTR minigenes are shown in (**B**) and (**C**), respectively. (**D**) The effect of overexpressing hnRNP A1, A2 and si-resistant TDP-43 on a CFTR $UG_{11}U_5$ background in the absence of endogenous TDP-43. Standard deviations obtained in three independent transfection experiments are shown for Figure S1B and S1D.

some minor independent activity on inhibiting exon inclusion ($\sim$10%), a phenomena that is found in a rather non-specific fashion in many exons whenever hnRNP A1 or A2 are overexpressed (36). This could be due to low-affinity RNA binding sequences for A1 and A2 to the CFTR RNA or to generalized protein–protein interactions.

Mapping the hnRNPA2–TDP-43 interaction using truncation and deletion mutants

To map the hnRNP binding region more precisely, we progressively shortened the TDP-43 C-terminus at residues 315 and 366, respectively (Figure 3A). The resulting mutants were then assayed both in our splicing system and in an *in vitro* super-shift binding assay with recombinant hnRNP A2. As shown in Figure 2B, the add-back of TDP-43 lacking the last 51 residues of the C-terminus (mutant 1-366) resulted in a recovery of inhibition (Figure 3B,
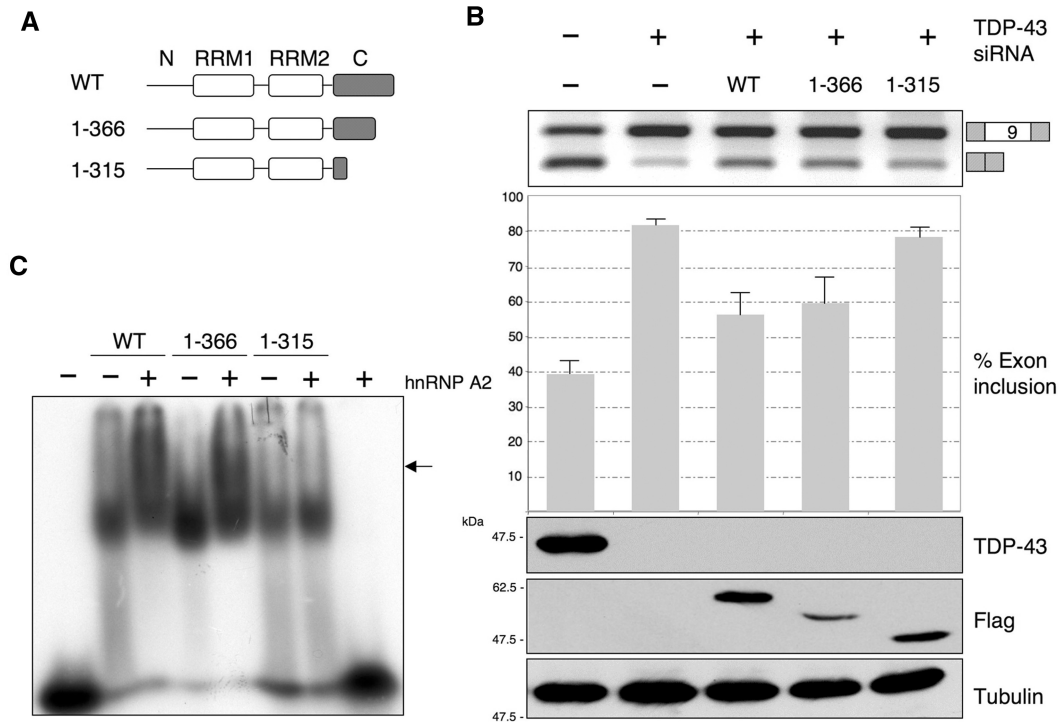
Figure 3. (**A**) A diagram of WT TDP-43 and of two mutants truncated at residues 315 and 366, respectively. The RT–PCRs in (**B**) show the splicing inhibitory activity of TDP-43 1-366 and 1-315 with respect to TD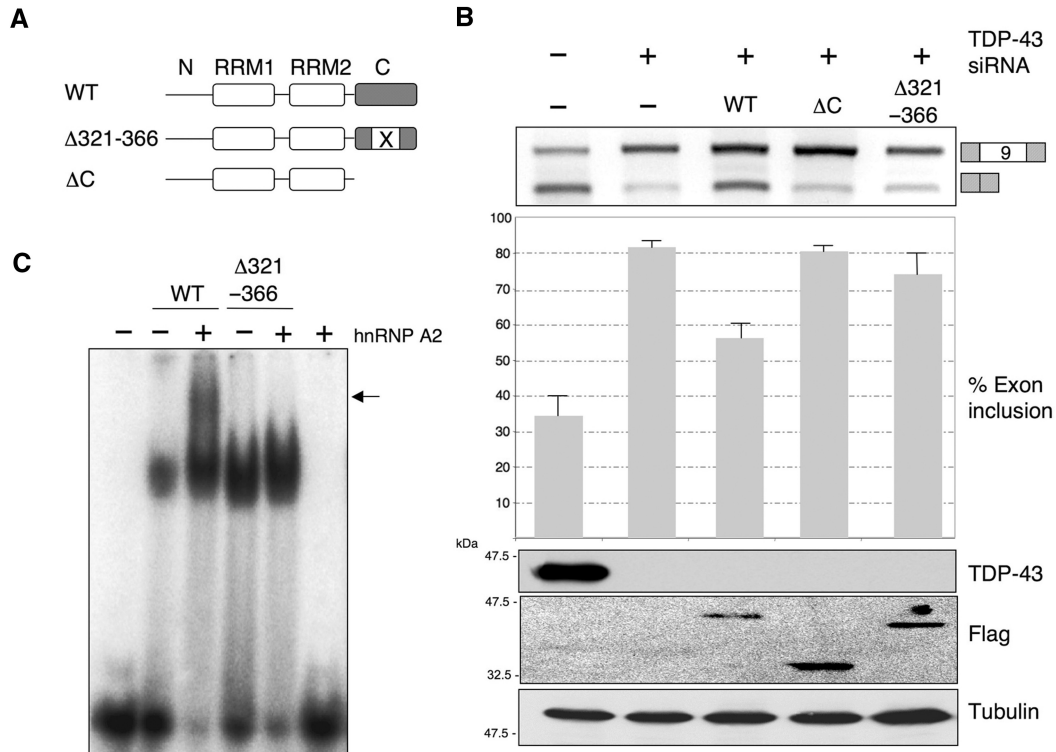P WT. Standard deviations obtained in three independent transfection experiments are shown. Western blots against the TDP-43, tubulin and FLAG peptide are shown below to show silencing efficiency, equal expression and proper transgene expression. (**C**) An EMSA super-shift analysis of the binding between the WT TDP-43, 1-366 and 1-315 TDP-43 mutants with hnRNP A2. Super-shifted complexes are indicated by an arrow.

lane 4) similar to that obtained with the WT protein (Figure 3B, lane 3). On the other hand, further shortening of the C-terminus, down to residue 315, resulted in a substantial reduction of inhibitory activity (Figure 3B, lane 5). Consistently, shortening of the C-terminus to residue 315 also decreased the ability to bind hnRNP A2 as seen by the lack of a super-shifted top band in EMSA (Figure 3C). Taken together, these results suggest that removing residues 366-414 has no effect both on A/B protein binding and splicing inhibition whilst removing residues 315-414 can abrogate both functional properties. Therefore, as a further control we removed the region between amino acid residues 321 and 366 (Δ321-366) (Figure 4A) and assayed this mutant for both minigene splicing and hnRNP A2 binding in EMSA analysis. As additional control for the minigene experiment, we used a previously described mutant that lacks the entire C-terminus region (ΔC) and is unable to restore CFTR exon 9 inhibition in an *in vitro* splicing system (19). The results obtained in the analyses with the Δ321-366 mutant are reported in Figure 4B and C. While the lack of interaction of Δ321-366 TDP-43 with hnRNP A2 is a clear and definitive result (Figure 4B), the recovery of splicing inhibition is not as clear as observed with the ΔC protein (compare inhibition levels of Figure 4B, lanes 4 and 5). Furthermore, when we tried to narrow down this region even further by introducing smaller deletions (residues 321 to 346 and 346 to 366, for a schematic diagram see

Figure S1A) both mutants were able to inhibit CFTR exon 9 inclusion in add-back assays, like the WT TDP-43 molecule (Figure S1B). These results suggest that, although the 321-366 region represents the minimal region required for binding hnRNP A2 and is important for splicing inhibition functionality, the total splicing inhibition effect of TDP-43 is not confined to just this specific punctual sequence but may be a function of a slightly wider region of the C-terminus.

A synthetic peptide spanning residues 321-366 can disrupt the TDP-43–hnRNP A2 interaction

In order to further establish whether residues 321-366 were the only requirement for interaction with hnRNP A2, a peptide containing these residues was synthesized (p321-366). This reagent was then used in a super-shift assay to test whether it was capable of competing the TDP-43–hnRNP A2 interaction. The results of this analysis are reported in Figure 5. They show that increasing concentrations up to a 240-fold molar excess of p321-366 can efficiently compete for the interaction between TDP-43 and hnRNP A2. A control peptide, pcont, added in equal amounts, had no effect on the TDP-43–hnRNP A2 interaction. Finally, in keeping with the results obtained in Figure S1B, the two peptides containing the smaller 321-346 and 346-366 deletions were unable to compete for hnRNPA2 binding to TDP-43 WT (Figure S1, panel C).

Figure 4. (**A**) A schematic diagram of the TDP-43 mutants lacking residues 321-366 and the entire C-terminal sequence from residue 216 (ΔC). (**B**) The splicing inhibitory activity of these two mutants compared to TDP-43 WT. The standard deviations obtained in three independent transfection experiments are reported. Western blots against the TDP-43, tubulin and FLAG peptide are shown below to show silencing efficiency, equal expression and proper transgene expression. (**C**) An EMSA analysis of the binding between TDP-43 WT, the 321-366 truncation mutant (Δ321-366) and hnRNP A2. Super-shifted complexes are indicated by an arrow.

Figure 5. Super-shift EMSA analysis of the binding between TDP-43 WT and hnRNP A2 in the presence of increasing quantities of a peptide (0.5, 1 and 2 μg, respectively, to obtain 60/120/240 molar excess, respectively) spanning residues 321-366 of TDP-43 (p321-366). Equal quantities of a control peptide (pcont) were used as control. Super-shifted complexes are indicated by an arrow.

Effect of ALS-associated missense mutations in the 321-366 C-terminus region

Interestingly, some of the familial and sporadic ALS patients bear mutations in TDP-43 and practically all with just one exception reside in the C-terminus (30). In particular, three mutations have been described to occur in the 321-366 region where we mapped the hnRNPA2 interaction: Q331K (22), M337V (22) and G348C (23) (Figure 6A). This observation raised the possibility that some of these mutations might interfere with hnRNP binding to TDP-43 and thus disrupt its splicing function. However, as shown in Figure 6B and C none of the mutants was able to disrupt either binding to hnRNP A2 in the EMSA assay or the splicing inhibitory activity on the CFTR exon minigene. Collectively, these results suggest that the effect of these mutants does not interfere with the RNA splicing function of TDP-43. We further investigated the effects of Q331K, M337V and G348C on their potential ability to disrupt the interactions with additional hnRNPs (A1, C, B1, A3), previously described for WT TDP-43 protein (20). The GST-overlay assays, shown in Figure 7, demonstrate that the entire cluster of hnRNP proteins recognized by TDP-43 is conserved for the mutants carrying the Q331K, M337V and G348C disease-related substitutions. The quantitative differences observed in the different samples are due to the fact that all filters have been developed using the ECL detection
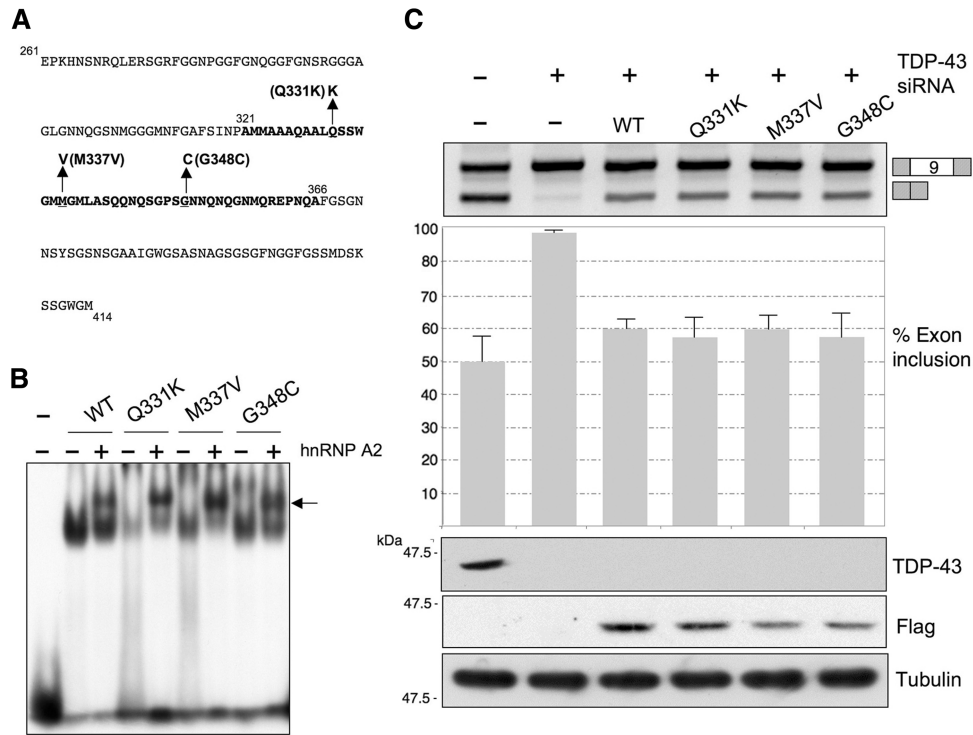
Figure 6. (**A**) The location of the disease associated missense substitutions found in ALS patients (Q331K, M337V and G348C). Their effect on the ability to bind hnRNP A2 in an EMSA super-shift analysis with hnRNP A2 (indicated by an arrow) and to inhibit splicing compared to TDP-43 WT are reported in (**B**) and (**C**), respectively. For the transfection experiments, standard deviations obtained in three independent experiments are shown. Western blots against the TDP-43, tubulin and FLAG peptide are shown below to show silencing efficiency, equal expression and proper transgene expression.

Figure 7. The left panel shows the Ponceau protein profile of HeLa cell nuclear extract (NE) and cytoplasmic extract (S100) run on a standard 10% SDS–PAGE gel. Molecular weights (kDa) are shown on the left. The remaining panels show the result of GST-overlay assays performed on a western blot containing the s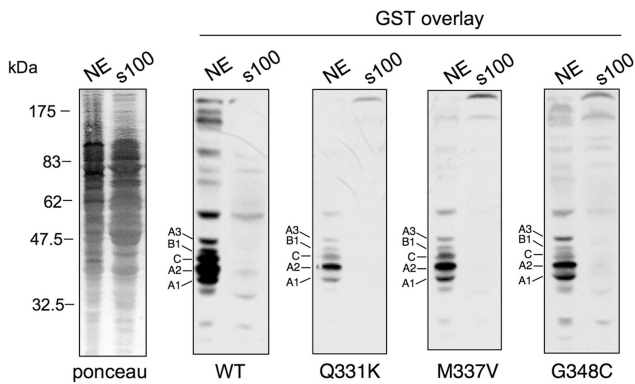ame amount of NE and S100 protein using TDP-43 WT and with TDP-43 carrying the following mutations: Q331K, M337V and G348C. Nuclear and cytoplasmic proteins specifically recognized by the GST-recombinant protein were revealed by ECL following incubation with an anti-GST antibody. The hnRNP cluster recognized by these proteins is indicated.

system for exactly the same amount of time. These results do not necessarily reflect a difference in binding affinity as it is very difficult to set up a quantitative GST-overlay assay when comparing different proteins obtained from different expression batches. What this experiment shows is that no new interactions have been created by the mutations introduced in the protein sequence and that all previous hnRNP interactions have been maintained within experimental limitations.

Comparing the sequence similarities and hnRNP binding abilities of TBPH and TDP-43

The alignment in Figure 8A shows the amino acid homology between the human TDP-43 C-terminal region and that of its homologous found in species from different phyla: marsupials (Opossum, *Monodelphis domestica*), birds (Chicken, *Gallus gallus*), amphibians (Frog, *Xenopus laevis*) and fish (Zebrafish, *Danio rerio*). This alignment indicates that the C-terminus is particularly conserved in the region between residues 321 and 350, suggesting that the ability of TDP-43 to bind hnRNP A2 may also be highly conserved as well. Intriguingly, however, the comparison between the sequences of TBPH and human TDP-43 sequences (Figure 8B) shows that the two proteins are highly similar in the N-terminal and the RRM-containing regions but are considerably divergent in the C-terminal tail (both with regards to the length and primary sequence). However, in Figure 1C we show that add-back of TBPH has the same inhibitory effects of TDP-43, a result that is consistent with previous experiments showing that the *in vitro* inhibitory ability of TBPH is comparable to that of TDP-43. The ability to block exon recognition by TBPH requires the presence of its C-terminal tail (19). We therefore wanted to see

A

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Hu  EPKHNSNRQLERSGRFGGNPGGFGNQGGFGNSRGGGAGLGNNQGSNMG------GGMNFG
Op  EPKHNSNRQLERSGRFGGNPGGFGNQGGFGNSRGGGAGLGNNQGSNMG------GGMNFG
Ch  EPKHNSNRQLERSGRFGGNPGGFGNQGGFGNSRGGGAGLGNNQGSNMG------GGMNFG
Fr  EPKHNNNRQLEREGRFPGPSFGSQ---GYPSNRPSGGTLGNNQGGNMGSGGGGGGGMNFG
Ze  EPKHNNTRQMMERAGRFGNGFGGQ---GFAGSRSNMGGGGGGGSSSSLG---------NFG
    *****..**: . .   *   *   *:..* . . *......:*        ***
Hu  AFSINPAMMAAAQAALQSSWGMMGMLASQQNQSGPSGNNQNQGNMQREPNQAFGSGNNSY
Op  AFSINPAMMAAAQAALQSSWGMMGMLASQQNQSGPSGNNQSQGNMQREPNQAFGSGNNSY
Ch  AFSINPAMMAAAQAALQSSWGMMGMLASQQNQSGPSGNNQPQGNMQREQNQGFSSGNNSY
Fr  AFSINPAMMAAAQAALQSSWGMMGMLASQQNQSGPQGSNPGQGNQQREQPQSFGSNNSYG
Ze  NFNLNPAMMAAAQAALQSSWGMMGMLAQQ-NQSGTSGTSTSGTSSSRDQAQTYSSANSNY
    *.:*********************.* **** ..*..   . .*: * :.* *.
Hu  SGSNSGAAIGWGSASNAGSG-SGFNGGFGSSMDSKSSGWGM
Op  SGSNSGAAIGWGSASNAGSG-SGFNGGFGSSMDSKSSGWGM
Ch  GGSNSGAAIGWGSASNAGSS-SGFNGGFGSSMDSKSSGWGM
Fr  SNSG---AIGWGSP-NAGSG-SGFNGGFSSSMESKSSGWGM
Ze  GSS--SAALGWGTGSNSGAASAGFNSSFGSSMESKSSGWGM
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B

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TDP-43  MSEYIRVTEDENDEPIEIPSEDDGTVLLSTVTAQFPGACGLRYRNPVSQCMRGVRLVEGI
TBPH    M-DFVQVSEEEGDEPIELPAEEDGTLLLSTLQAQFPGSCGLKYRNLDTKAVRGVRSNEGR
        * :::::*:*:*.*****:*:*:***:****: *****:***:*** ::.:****  **
TDP-43  LHAP--DAGWGNLVYVVVNYPKDNKRKMDETDASSAVKVKR--AVQKTSDLIVLGLPWKTT
TBPH    LFPPSVESGWGEYAYFCVFPKENKRKSDDNLENSTAKTKRIETRLRCTDLIVLGLPWKTT
        *..*  ::***: .*. :**:**** *:. .*:.*.** : : :*************
TDP-43  EQDLKEYFSTFGEVLMVQVKKDLKTGHSKGFGFVRFTEYETQVKVMSQRHMIDGRWCDCK
TBPH    EESLREYFETYGEVLMAQIKKDTKSGQSKGFGFVRFGSYDAQMRVLTNRHLIDGRWCEVK
        *:.*:***.*:*****.*:*** *:*:********* .*::*:::**::****: *
TDP-43  LPNSK-QSQDEPLRSRKVFVGRCTEDMTEDELREFFSQYGDVMDVFIPKPFRAFAFVTFA
TBPH    VPNSKGMGHQVPC---KVFVGRCTEDINSDDLREYFSKFGEVTDVFIPRPFRAFSFVTFL
        :**** .:: *    **********::.:***:**::*:* ***** :*****:****
TDP-43  DDQIAQSLCGEDLIIKGISVHISNAEPKHNSNRQLERSGRFGGNPGGFG-----NQGGFG
TBPH    DPDVAQSLCGEDHIIKGVSVHVSNAAPKAEQNRNQQVQSYNYNSANSFGMHSYHPQGNHM
        * ::********* ****:***:*** ** :.**: :  .. ....** ***..
TDP-43  NSRGGGAGLGNNQGSNMGGGMNFGAFSINPAMMAAAQAALQSSWGM----------MGML
TBPH    NPGRNGHHRGNNQHNAHGG---------ENAIVPNNHNIGTAGYGMGGNNYGGNSGGGYH
        *. .*  **** . **     : *::. :  :.:**           *
TDP-43  ASQQNQSGPSGNNQNQGNMQREPNQAFGSGNNSYSGSNSGAAIGWGSASNAGSGSGSFNGG
TBPH    NNGGNHSSGGNTNRQDGGSQYNSRQSNFHGMNQPHNGNVGGSNGWMNRGHLDMPNLQALG
        . *:*. ...*:::*. * :..*:  * *. ..* *.: ** ..: . *
TDP-43  FGSSMDSKSS------------------------------------------------
TBPH    INSQGSSSSNQGQNMSNQSMLNLNSLPINPALVAAALNQWSLVGNQLQNQNQDQQGGNFL
        :.*. .*.*.
TDP-43  ----------------------------------------------GWG-----------
TBPH    SWMAQNGGHNNANNFGGRKGPNNPNNNPAANGIKTDNSEPQNGNTGWSNQSSGSQNAAEK
                                                      **.
TDP-43  ---M
TBPH    SNFL
        :
```

C

D

Figure 8. The alignment in (**A**) shows the sequence homology between the human TDP-43 C-terminal region (hu) and its homologous sequences in Op, Opossum (*Monodelphis domestica*); Ch, Chicken (*Gallus gallus*); Fr, Frog (*Xenopus laevis*); and Ze, Zebrafish (*Danio rerio*). The 321–366 region is highlighted in bold. (**B**) The alignment between the full length human TDP-43 and full length Drosophila TBPH. The conserved RRM1 and RRM2 regions are highlighted in bold. (**C**) An EMSA super-shift analysis with hnRNP A2 of the binding between full length TBPH and a C-terminal truncated TBPH mutant (TBPH ΔC). Super-shifted complexes are indicated by an arrow. (**D**) A super-shift EMSA analysis of the binding between TBPH and hnRNP A2 in the presence of increasing quantities of the 321–366 TDP-43 peptide (p321-366) and of a control peptide (pcont) in the same quantities described for Figure 5. Super-shifted complexes are indicated by arrows.

whether the C-terminal tail of TBPH was also capable of binding to human hnRNP A2. The results in Figure 8C show that recombinant hnRNP A2 could efficiently bind to TBPH and that binding was abolished upon deletion of the C-terminal tail (TBPH ΔC mutant). In order to definitively confirm this, we have performed the same competition experiment described in Figure 5 using the 321-366 peptide as a competitor for TBPH-hnRNP A2 binding. The results shown in Figure 8D establish that the human 321-366 residues can be an effective competitor for this interaction. These results are consistent with the add-back assays reported in Figure 1 and suggest that hnRNP binding is conserved in the Drosophila homolog despite limited amino acid conservation. To address whether the ability to bind the other members of the

hnRNP family (20), in addition to hnRNP A2, is conserved in the case of TBPH we performed a GST-overlay experiment using TBPH and TBPH ΔC (as control). Figure 9 shows that the cluster of hnRNP proteins recognized by TDP-43 is also basically conserved for TBPH. Moreover, it shows that recognition of all these proteins is also completely abolished after the removal of the C-terminal tail (TBPH ΔC mutant).

DISCUSSION

TDP-43 is a member of the hnRNP protein family and its 414 amino acid sequence consists of an N-terminal region, two RRM domains and a C-terminal region. Like many members of this family, the C-terminal region contains

Figure 9. The left panel shows the Ponceau protein profile of HeLa cell nuclear extract (NE) and cytoplasmic extract (S100) run on a standard 10% SDS–PAGE gel. Molecular weights (kDa) are shown on the left. The remaining panels show the result of three GST-overlay assays using the following proteins: human TDP-43 WT, Drosophila TBPH and Drosophila TBPH ΔC as a control. Nuclear and cytoplasmic proteins specifically recognized by the GST-recombinant protein were revealed by ECL following incubation with an anti-GST antibody. The hnRNP cluster recognized by these proteins is indicated.

Gly-rich sequences that may be essential to recruit cellular factors that can modulate TDP-43 function. Currently, the only protein factors whose association to TDP-43 has been verified are several members of the hnRNP family (20) and the survival of motor neuron (SMN) protein (37). The C-terminal tail does not seem to play a role in the interaction with SMN. Other additional factors have also been suggested to interact with this protein on the basis of protein–protein association studies that use high-throughput methodologies (38). In particular, two proteomics studies (39,40) involving yeast two hybrid systems found some other potential TDP-43 binding partners, namely XRN2 and PM/Scl100, involved in mRNA decay, ZHX1, a transcriptional repressor, SETDB1, a chromatin remodeling regulator, and NSFL1C and ARF6, both involved in membrane trafficking. In this respect, it is important to note that TDP-43 has been described to be part of RNA granules responsible for trafficking, sequestering and degrading RNA species (41) and has been observed to colocalize strongly with Staufen, moderately with TIA-1 and weakly with XRN1, an exoribonuclease involved in mRNA decay (42). Finally, TDP-43 has also been found associated with both human and mouse microprocessor complexes (43,44), suggesting that it may also be involved in the biosynthesis of microRNAs. However, many of these interactions are difficult to interpret in terms of the functional role of the complexes due to the lack of more stringent biochemical evidence. In this work, we have presented a new siRNA/minigene coupled system to assess the *in vivo* splicing inhibitory activity of TDP-43 mutants and homolog proteins. Our results have fully confirmed previous *in vitro* data regarding the functional interaction of TDP-43 with hnRNP proteins. In particular, we have focused on hnRNP A2, the major hnRNP

protein recognized by TDP-43 according to pull-down assays (19,20). We have now identified residues 321 to 366 of the TDP-43 C-terminal tail as the minimal binding region required to bind hnRNP A2. In addition, we also find that this region is necessary for TDP-43 splicing inhibitory activity in our *in vivo* system. Taken together, these experiments strongly support the requirement for TDP-43 to form an hnRNP complex through its C-terminus to inhibit exon splicing. Interestingly, the size of the 321-366 region (although not its sequence) is strikingly similar to the 39 amino acid-long M9 regions described in the hnRNP A/B proteins that enables the bidirectional transport of these proteins across the nuclear envelope through binding to transportin, also known as Kapβ2 (45,46). It has been recently shown that the M9 region of hnRNP A1 protein binds a concave surface of a C-terminal arch in Kapβ2 in an extended conformation (residues 263 to 289 of this protein) making an extensive network of polar and hydrophobic contacts (45). A similar situation may occur in the TDP-43–hnRNPA2 interaction and could explain the fact that three missense mutations in the 321-366 region associated with neurodegeneration do not substantially affect binding efficiency and splicing function. Altogether, these data suggest that the mutations in TDP-43 found in patients do not contribute to a drastic protein loss of function. More likely, they may contribute to a predisposition to develop the disease (i.e. more readily form aggregates) through some still unidentified mechanisms. Given the late age of onset of both ALS and FTLD, another possibility is that the mutations affect TDP-43 function to a small degree (not detectable in our assays), but the slight disruption of its activity perpetuated over a long time might be the cause for the neurodegeneration. The same concept has been proposed to be the cause of neurodegeneration in Spinal Muscular Atrophy patients with a slight decrease in SMN protein (47,48). Further work is currently in progress to better define the TDP-43–hnRNP A2 interaction through the identification of the hnRNP A2 residues involved. The TDP-43 interacting domain should be localized in the C-terminal tail of hnRNP A2 as previously reported (20). These experiments will provide a better indication of the TDP-43 residues involved in the interaction and thus in a better position to judge the effect of eventual disease causing mutations.

From a functional point of view, characterization of the TDP-43–hnRNP interaction is essential to understand its splicing regulatory properties, especially in light of recent observations regarding the potential existence of human alternatively spliced variants that lack the C-terminal tail (49,50). Although at the moment there is scant biochemical evidence regarding the relative abundance or distribution pattern of these isoforms in humans, it is clear that their existence and or production would carry distinct biological properties with respect to the WT protein but would still be able to compete for the same binding sites (UG repeats). Therefore, in addition to variations in the relative hnRNP A/B proteins present in different tissues or developmental stages, expression of these truncated isoforms may also prove to be a good way to modulate

TDP-43 function(s) without necessarily altering *TARDBP* basal expression levels.

More in general, our results represent a clear indication that interactions between splicing regulatory proteins belonging to distinct classes can be a powerful modifier of their functional properties. In fact, it was well known that functional biochemical interactions between hnRNPs of the same type can explain their effect on splicing. For example, it has been proposed that a variety of proteins, such as PTB, hnRNP H and hnRNP A/B can potentially multimerize to create 'zones of silencing' across exons or modulate the conformation of the pre-mRNA and thereby influence exon recognition (51–54). Less often, protein networking has been described to occur between different factors. One such interaction has been described for the PTB–Raver1 interaction in the control of the tropomyosin gene (55,56), a case where both proteins can synergistically repress exon recognition. More recently, the presence of a functionally relevant biochemical interaction between the hnRNP H/F proteins and Fox2 has been reported (57). In this case, the interaction between hnRNP H and Fox2 is capable of altering the binding ability of the complex and thereby to influence the splicing inhibitory effect of the H/F proteins on FGFR2 exon IIIc.

Finally, all these observations of intricate biochemical connections between splicing factors of different classes will certainly add a layer of complexity to the well-established concept of combinatorial and context-dependent control in splicing (58–60). In order to understand splicing outcomes in the future, it will not be enough to simply identify all the trans-acting factors that bind to the RNA sequence in the vicinity of an exon. More probably, it will also be necessary to consider the relative expression levels and regulation of cellular factors that do not directly contact the RNA under study, but which can modify the functional properties of the factors that do.

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