RNA Helicase p68 (DDX5) Regulates tau Exon 10 Splicing by Modulating a Stem-Loop Structure at the 5′ Splice Site

Amar Kar, Kazuo Fushimi, Xiaohong Zhou, Payal Ray, Chen Shi, Xiaoping Chen, Zhiren Liu, She Chen, and Jane Y. Wu

Department of Neurology, Center for Genetic Medicine, Lurie Cancer Center, Northwestern University Feinberg School of Medicine, 303 E. Superior Street, Chicago, Illinois 60611; Department of Biology, Georgia State University, Atlanta, Georgia 30302; and National Institute of Biological Sciences, Beijing, China

Received 29 September 2010/Returned for modification 27 October 2010/Accepted 14 February 2011

Regulation of tau exon 10 splicing plays an important role in tauopathy. One of the cis elements regulating tau alternative splicing is a stem-loop structure at the 5′ splice site of tau exon 10. The RNA helicase(s) modulating this stem-loop structure was unknown. We searched for splicing regulators interacting with this stem-loop region using an RNA affinity pulldown-coupled mass spectrometry approach and identified DDX5/RNA helicase p68 as an activator of tau exon 10 splicing. The activity of p68 in stimulating tau exon 10 inclusion is dependent on RBM4, an intronic splicing activator. RNase H cleavage and U1 protection assays suggest that p68 promotes conformational change of the stem-loop structure, thereby increasing the access of U1snRNP to the 5′ splice site of tau exon 10. This study reports the first RNA helicase interacting with a stem-loop structure at the splice site and regulating alternative splicing in a helicase-dependent manner. Our work uncovers a previously unknown function of p68 in regulating tau exon 10 splicing. Furthermore, our experiments reveal functional interaction between two splicing activators for tau exon 10, p68 binding at the stem-loop region and RBM4 interacting with the intronic splicing enhancer region.

Alternative splicing is one of most powerful posttranscriptional mechanisms for generating multiple gene products from individual gene loci, thereby contributing to genetic and proteomic diversity (7, 84, 86, 89). Numerous studies have provided evidence for cell-type-specific and developmentally regulated alternative splicing events (6, 87). Alternative splicing plays critical roles in the nervous system, where production of distinct splicing isoforms regulates multiple cellular processes, including cell fate determination, axon guidance, synaptogenesis, and neural transmission (reviewed in references 8, 30, 73, and 80). Defects or disruption in regulation of alternative splicing contributes to the pathogenesis of a large number of neurodegenerative disorders, such as amyotrophic lateral sclerosis (ALS), familial Alzheimer’s disease (FAD), and frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17, or frontalotemporal lobar degeneration with tauopathy [FTLD-tau]) (reviewed in references 31, 40, 41, 42, 53, 71, and 96).

Tau, a microtubule-associated protein (MAP) enriched in axons, plays an important role in polymerization and stabilization of neuronal microtubules. Tau is critical for maintenance of neuronal cytoskeleton and axonal transport (reviewed in references 31, 40, 41, 42, 53, 71, and 96). Human Tau protein is encoded by a single gene on chromosome 17q21 containing 16 exons with microtubule-binding repeats in the human Tau protein, and they are encoded by exons 9, 10, 11, and 12, respectively (3, 51). tau exon 10 (E10), which encodes the second microtubule-binding domain, is alternatively spliced, resulting in Tau protein isoforms containing either three (E10−) or four (E10+) microtubule-binding domains referred to as three-repeat Tau (Tau3R) and four-repeat Tau (Tau4R), respectively (21, 42, 52). Tau4R and Tau3R show distinct activities in microtubule binding (43, 68, 69, 70, 100–103, 105). tau exon 10 alternative splicing undergoes developmental stage-specific regulation, with almost exclusive expression of Tau3R in the fetal brain and both isoforms being expressed equally (Tau4R/Tau3R ratio of 1:1) in the adult human brain (3, 4, 38, 60, 74, 88, 90).

FTLD-tau is a group of neurodegenerative diseases characterized by the presence of Tau-immunoreactive lesions in the frontotemporal regions and elsewhere, often with the formation of disease-specific tangles or other types of inclusion bodies containing hyperphosphorylated Tau protein (references 12 and 78 and references therein). FTLD-tau can be caused by either missense or splicing mutations in the tau gene (24, 49, 50, 54; reviewed in references 53 and 66). In some forms of familial FTLD-tau, splicing mutations disrupt tau exon 10 splicing regulation and alter the Tau4R/Tau3R ratio in the brain, leading to neurodegeneration (17, 19, 23, 24, 26, 43, 49, 55, 97, 110). Several studies have begun to reveal cis-acting regulatory elements and trans-acting factors that regulate tau exon 10 splicing (9, 26, 27, 28, 37, 45, 46, 60, 61, 67, 106). One of the cis-acting elements is a stem-loop structure downstream of the 5′ splice site of exon 10 (45, 54, 60, 103, 104). Biochemical and structural studies suggest that this stem-loop structure may promote exon 10 exclusion by preventing U1snRNP binding to the 5′ splice site (60, 64, 103, 104). However, RNA helicases that interact with the stem structure and regulate tau exon 10 splicing have not previously been reported.

* Corresponding author. Mailing address: Northwestern University Feinberg School of Medicine, 303 E. Superior St., Lurie 6-117, Chicago, IL 60611. Phone: (312) 503-0684. Fax: (312) 503-5603. E-mail: jane-wu@northwestern.edu.

1 Published ahead of print on 22 February 2011.
In this study, we have developed an RNA affinity pulldown-coupled mass spectrometry (MS) approach to identify regulatory factors interacting with the stem-loop structure at the 5′ splice site of tau exon 10. One of the proteins identified is DEAD/H box polypeptide 5 (DDX5), also known as RNA helicase p68. We show that p68 regulates tau exon 10 splicing by interacting with the stem-loop region, destabilizing the stem structure, and facilitating U1snRNP binding to this 5′ splice site. Our biochemical experiments demonstrate that p68 interacts with an intronic splicing activator, RNA binding motif protein 4 (RBM4), thereby stimulating tau exon 10 inclusion.

**MATERIALS AND METHODS**

**RNA affinity pulldown-coupled mass spectrometry.** Biotinylated RNA oligonucleotides corresponding to the tau gene containing the last 24 nucleotides of exon 10 and the first 24 nucleotides of intron 10 were synthesized together with a control RNA with random sequence (Dharmacon). In RNA affinity pulldown experiments, 0.5 pmol of biotinylated RNA oligonucleotide was incubated in a 500-μl reaction mixture containing 100 microliters of HeLa cell nuclear extract under splicing conditions (20 mM HEPES, 72 mM KCl, 1.5 mM MgCl2, 1.6 mM magnesium acetate [MgOAc], 0.5 mM dithiothreitol [DTT], 4 mM glycerol, 1 mM ATP, 200 units of RNasin; Promega) at 30°C for 30 min. After incubation, 100 μl of preequilibrated magnetic streptavidin beads (Dynal Corporation) was added to the reaction mixture and incubated for 1 h at room temperature. After three washes with reaction buffer (20 mM HEPES, 72 mM KCl, 1.5 mM MgCl2, 1.6 mM MgOAc, 0.5 mM DTT, 4 mM glycerol, 1 mM ATP, 200 units of RNasin; Promega), the resin-bound proteins were eluted with 100 μl of 7 M urea and were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Following elution, the samples were analyzed at the proteomics facility of the National Institute of Biological Sciences. After tryptic digestion, the peptide mixtures were separated by either a single C18 reversed-phase column or a two-dimensional high-performance liquid chromatography (2D-HPLC) setup (cation exchange column coupled to C18 reversed-phase column). The eluted peptides were ionized and directly sprayed into an LTQ tandem mass spectrometer (ThermoFisher Scientific). The MS data were processed on an in-house Sequest (29) cluster to generate a protein list for each sample. The protein lists were then compared to determine the proteins that specifically interact with the tau exon 10 stem-loop RNA transcript.

**Plasmids, antibodies, and purified proteins.** The wild-type (wt) tau10-11 and tau10-11 minigene constructs or corresponding mutant minigene constructs containing a C-to-T single-nucleotide mutation in intron 10 at the +14 position, named the DDPC mutation, were described previously (60). Mammalian expression plasmids for hemagglutinin (HA)-tagged wild-type or LGLD mutant p68 (76) and Flag-RBM4 and Flag-Raver (44, 76) were previously described. Backbone expression plasmids for His- or LGLD mutant p68 were previously described (52, 75, 107). Monoclonal anti-Flag antibody was purchased from Sigma. Monoclonal anti-HA antibody was purchased from Covance. Wild-type or LGLD mutant 6×His-tagged p68 protein was purified as previously described using HisTrap and AKTA purifier (GE Biomedical) using T7 RNA polymerase from corresponding linearized DNA templates as described previously (60, 61). Wild-type or DDPC mutant (containing a single-nucleotide C-to-T change at position +14 of intron 10) tau RNA oligonucleotides containing the last 24 residues of exon 10 and the first 24 residues of intron 10 of the human tau gene were end labeled using polynucleotide kinase (Roche) in the presence of [γ-32P]ATP (7,000 Ci/mmol; MP Biomedical). For UV cross-linking, ~20 fmol of RNA probe was incubated at 30°C with 100 μl of cell lysates in a 150-μl reaction mixture under in vitro splicing conditions (60, 61). The samples were irradiated on ice under a 254-nm UV lamp for 10 min (120 micromoles/cm2) using a Stratalinker 2000 UV cross-linker (Stratagene). The probes were digested in equal volumes of RNase A (5 mg/ml; Sigma) at 30°C for 20 min, except for experiments in which the end-labeled probes were used. For immunoprecipitation, following the RNase treatment, samples were incubated with monoclonal M2 anti-Flag antibody (Sigma) at 4°C for 2 h. Protein A/G agarose beads were then added with further incubation and gentle mixing. Following washing, the immunoprecipitated RNA-bound proteins were eluted and resolved on SDS-PAGE followed by autoradiography.

**Oligonucleotide-directed RNase H cleavage assay.** [α-32P]UTP-labeled wild-type and DDPC tau pre-mRNA transcripts containing exon 10-intron 10 sequence were used in this assay, as illustrated in Fig. 5 and 6. The RNA probes were incubated at 37°C for 60 min in standard splicing buffer in the presence of 0.5 μl of RNase H (Roche) in 25-μl reaction mixtures with 50 fmol of an oligonucleotide (5′-GAAGGTACTCACACTGCCC-3′) complimentary to the 5′ splice site of exon 10 together with the purified recombinant wild-type or LGLD mutant p68 protein as described earlier (60). Cleaved RNA products were separated on 6% polyacrylamide denaturing gels containing 8 M urea followed by autoradiography and quantification using a PhosphorImager as described previously (60). The efficiency of RNase H cleavage was calculated by dividing the band intensities of the cleaved fragments by the band intensities of the total RNA fragments in each lane.

**U1snRNP depletion and U1snRNP protection assay.** U1snRNP was depleted using oligonucleotide-targeted RNase H cleavage as described in our previous study (60). Briefly, HeLa cell nuclear extract was incubated at 30°C for 1 h in the presence of RNase H and the oligonucleotide 5′-CCAGGTACAAAT-3′ complementary to the 5′ end of U1snRNP (reference 60 and references within). As a control, U7-depleted nuclear extract was obtained by incubation with RNase H and a U7snRNA-specific oligonucleotide. The U1snRNP protection assay was carried out under splicing reaction conditions with 50,000 cpm of [32P]-labeled tau pre-mRNA transcripts with nontreated, U1-depleted, or U7-depleted nuclear extracts at 30°C for 0 or 20 min. RNase H (0.4 μl) and 20 pmol (molar excess) of the oligonucleotide complementary to the 5′ splice site of exon 10 (5′-GAAGGTACCACTGCCC-3′) were then added to 25-μl splicing reaction mixtures (60), and incubation was continued for 30 min at 37°C to completely cleave the pre-mRNA transcripts that were not protected. Each reaction mixture was then separated on 6% polyacrylamide denaturing gels containing 8 M urea followed by autoradiography and quantification using a PhosphorImager as described previously (60). The efficiency of RNase H cleavage was calculated by dividing the band intensities of the cleaved fragments by the total band intensities of the total RNA bands in each lane.

**Immunoprecipitation and Western blot analysis.** HEK293 cells transfected with plasmids expressing HA-tagged wild-type p68 or LGLD mutant p68 or Flag-tagged RBM4 or Raver were lysed with a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM DTT, 1× protease inhibitor mixture [Roche Molecular Biochemicals]) as described previously (65). Lysates were immunoprecipitated with specific antibodies and protein A/G agarose beads for 2 h at 4°C. Fifty percent of the immunoprecipitated proteins and 20% of the cell lysate used for the pulldown were separated using SDS-PAGE and detected by Western blotting using corresponding specific antibodies with an enhanced chemiluminescence kit (GE Healthcare).

**RESULTS**

DDX5/RNA helicase p68 interacts with the tau exon 10 stem-loop structure. Previous studies indicate that splicing mutations in the human tau gene that are predicted to destabilize the exon 10 stem-loop structure increase tau exon 10 inclusion, suggesting that this stem-loop region plays an important role in regulating tau exon 10 splicing (54, 60, 103, 104). This model also suggests that proteins influencing the stability of this stem-loop structure may regulate tau exon 10 splicing. To search for
splicing regulators that interact with the tau exon 10 stem-loop structure, we established an RNA affinity pulldown-coupled mass spectrometry approach. As illustrated in Fig. 1A, a biotinylated RNA oligomer corresponding to the tau exon 10 stem-loop region was incubated under splicing conditions with HeLa nuclear extract (HeLaNE). RNA-protein complexes were isolated by affinity pulldown using streptavidin beads. Protein components of the RNA-protein complex were identified using liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS). A random RNA oligonucleotide was used as an internal control to exclude proteins that nonspecifically bind RNA. Table 1 profiles the proteins identified by this RNA affinity pulldown approach. Candidate proteins of interest included alternative splicing regulators such as SAP62, TIA-1, Fox2, p68, and RBM25. We were particularly interested in p68, as it had been reported to be an alternative splicing regulator that functioned by influencing RNA second-order structures such as that in ras (13, 16).

To confirm that p68 interacted with tau pre-mRNA in the stem-loop region downstream of exon 10, UV cross-linking-coupled immunoprecipitation experiments were carried out. The wild-type (wt) tau stem-loop-containing RNA oligomer used in the RNA affinity pulldown experiment or a mutant tau stem-loop oligomer containing a C-to-T mutation at the intron 10 +14 position, named the DDPAC mutation (17, 54), was end labeled using [γ-32P]ATP and incubated with protein lysates prepared from HEK293 cells transfected with plasmids expressing either wild-type p68 or LGLD mutant p68. This p68 mutant lacks ATPase/helicase activity and contains an arginine (R)-to-leucine (L) change in the consensus sequence motif (V), RGLD, thus being named the LGLD mutant (75). As shown in Fig. 1B, both wild-type and mutant HA-p68 interacted with the wt tau stem-loop-containing RNA (lanes 3 and 4). However, no interaction was detected between the DDPAC mutant tau RNA and either wild-type or mutant p68 protein (Fig. 1B, lanes 1 and 2). These results suggest that p68 indeed interacts with wt tau RNA in the stem-loop region but not with the DDPAC mutant tau RNA containing a single-nucleotide change in the stem region that destabilizes the stem structure.

**RNA helicase p68 stimulates tau exon 10 inclusion.** To examine the functional significance of the interaction of p68 with tau pre-mRNA at the exon 10 stem-loop region, we tested the effect of p68 on tau exon 10 splicing. HEK293 cells were cotransfected using a wild-type tau minigene containing exons 9, 10, and 11 (tau9-11wt [60]) together with either the vector control or wild-type or LGLD mutant p68 expression plasmid (Fig. 2A and B). The tau exon 10 alternative splicing products were detected using RT-PCR with specific primers as described earlier (60). Overexpression of wild-type p68 increased tau exon 10 inclusion compared to that in the vector control (Fig. 2A and B, lanes 1 and 2). Interestingly, overexpression of LGLD mutant p68 suppressed tau exon 10 inclusion, leading to increased formation of the isoform lacking exon 10 (E10−) (Fig. 2A and B, compare lanes 1 and 3), indicating that the RNA helicase activity is involved in the p68 activity in regulating tau exon 10 splicing. Next, we performed RNA interference (RNAi)-mediated knockdown of p68 to test the effect of the endogenously expressed p68 on tau exon 10 splicing. HEK293 cells were cotransfected using the tau9-11wt minigene together with p68-specific interfering RNA or control interfering RNA or wild-type p68 expression plasmid (Fig. 2C). Consistent with the previous studies in which p68 knockdown led to increased cell death (59, 96), it was difficult to drastically downregulate p68 expression without causing significant cytotoxicity. Under our conditions, without eliciting significant cell death, we were able to achieve approximately a 50% decrease in p68 transcript levels, as detected by RT-PCR with p68-specific primers and Western blotting (Fig. 2C, compare lanes 2 and 3). With RNAi-mediated reduction in p68 expression, the tau exon 10-containing splicing product (E10+) was signific-

<table>
<thead>
<tr>
<th>Protein description and/or name</th>
<th>GI no.</th>
<th>Mass (Da)</th>
<th>x-corr</th>
<th>Sequence coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDX5/p68</td>
<td>GE:4758138</td>
<td>69,148</td>
<td>3.71</td>
<td>5</td>
</tr>
<tr>
<td>prp40</td>
<td>GE:7106840</td>
<td>105,270</td>
<td>3.65</td>
<td>5</td>
</tr>
<tr>
<td>hnRNPC</td>
<td>GE:13937888</td>
<td>35,598</td>
<td>3.35</td>
<td>18</td>
</tr>
<tr>
<td>TIA</td>
<td>GE:6094480</td>
<td>42,460</td>
<td>3.21</td>
<td>8.5</td>
</tr>
<tr>
<td>TIA-1-related protein, TIA</td>
<td>GE:4507499</td>
<td>41,250</td>
<td>2.27</td>
<td>6.5</td>
</tr>
<tr>
<td>RBM9/Fox2</td>
<td>GE:6572238</td>
<td>41,360</td>
<td>3.68</td>
<td>6.5</td>
</tr>
<tr>
<td>hnRNP M</td>
<td>GE:33874022</td>
<td>44,443</td>
<td>2.44</td>
<td>4</td>
</tr>
<tr>
<td>SF3A, subunit 1</td>
<td>GE:5032087</td>
<td>87,230</td>
<td>2.11</td>
<td>4</td>
</tr>
<tr>
<td>SF3B subunit 1/SAP155</td>
<td>GE:6912654</td>
<td>145,724</td>
<td>3.42</td>
<td>4</td>
</tr>
<tr>
<td>SAP62</td>
<td>GE:21361376</td>
<td>51,040</td>
<td>2.55</td>
<td>4</td>
</tr>
<tr>
<td>U1-70K</td>
<td>GE:29568103</td>
<td>48,070</td>
<td>2.84</td>
<td>8</td>
</tr>
<tr>
<td>RBM25</td>
<td>GE:33874022</td>
<td>84,560</td>
<td>2.43</td>
<td>6</td>
</tr>
</tbody>
</table>

*a x-corr, cross correlation score.*
standard errors, with data from six independent experiments (of the 5/H11032 intronic splicing enhancer located 100 nucleotides downstream (65). RBM4 increases tau activator.

The graph shows the average ratios of data derived from six independent experiments. The graph shows the antibody, are shown. (B) Quantification of reaction mixtures, as detected using Western blotting with anti-HA showing comparable amounts of RNA used in each reaction mixture. 

RBM4-mediated exon 10 splicing stimulation was unclear. Be-

cause p68 had a similar effect on tau exon 10 splicing, we investigated if RBM4 interacted with p68. We performed co-immunoprecipitation experiments using HEK293 cells cotransfected with epitope-tagged p68 and RBM4-HA-tagged p68 and Flag-tagged RBM4 (Fig. 3). RBM4 was coimmunoprecipitated with either wt or LGLD mutant p68 (Fig. 3A, lanes 4 and 5). This interaction with p68 was specific to RBM4, because another RNA binding motif-containing protein, Raver, was not coimmunoprecipitated with p68 (Fig. 3A, lane 6). To test if the interaction of RBM4 with p68 was dependent on RNA, we carried out coimmunoprecipitation experiments in the presence of RNase A. The specific association of both wt and LGLD mutant p68 with RBM4, but not with Raver, was detected in coimmunoprecipitation following treatment with RNase A, demonstrating that p68 specifically interacted with RBM4 in an RNA-independent manner (Fig. 3B).

RNA helicase p68-mediated stimulation of tau exon 10 inclusion is dependent on RBM4. To examine the functional significance of the interaction between p68 and RBM4, we tested the effects of RBM4 on p68-mediated tau exon 10 regulation by knocking down RBM4 (Fig. 4). In cells treated with the control interfering RNA, overexpression of wild-type p68 stimulated, whereas the LGLD mutant p68 lacking ATPase/helicase activity inhibited, tau exon 10 inclusion (Fig. 4A and B, compare lanes 5 and 6 with lanes 4), similar to our previous observation (Fig. 2). When RBM4 was downregulated by RBM4-specific RNAi, effects of either wt or LGLD mutant p68 on tau exon 10 splicing were no longer detectable (Fig. 4A, compare lanes 2 and 3 with lane 1). These data indicate that the activity of p68 in regulating tau exon 10 splicing is RBM4 dependent.

We further examined whether this activity of p68 was dependent on the interaction of RBM4 with the tau pre-mRNA. We made use of a mutant tau pre-mRNA minigene in which the RBM4 binding site was mutated, tau9-11mtRBM4/B65 (65). As shown in Fig. 4C and D, both wt p68-mediated stimulation of tau exon 10 inclusion and LGLD mutant p68-mediated
exon 10 alternative splicing was assayed using RT-PCR, with quantification of data from six independent experiments (Fig. 4A). The graphs show the average ratios of p68 wt or LGLD mutant p68 to E10+ transcript detected by RT-PCR or RNase H cleavage assay using either wt tau pre-mRNA or DDPAC mutant tau transcript containing the single-nucleotide change that reduced the base pair interaction in the stem region. The RNase H cleavage assay was performed using control bovine serum albumin (BSA) or purified p68 protein (Fig. 5). Incubation of wt p68 protein with wt tau RNA transcript led to a concentration-dependent increase in the cleavage of the wt tau RNA transcript (Fig. 5A, lanes 3 to 5 and lane 1; Fig. 5B, compare lanes 2 and 3), whereas LGLD mutant p68 decreased the cleavage of the wt tau RNA transcript (Fig. 5B, compare lanes 2 and 4). On the other hand, neither wt p68 nor LGLD mutant p68 changed the cleavage of DDPAC mutant tau transcript by RNase H (Fig. 5A, lanes 6 to 10; Fig. 5B, lanes 6 to 8). These results suggest that interaction of p68 with the tau stem structure may lead to a more open conformation in this region, allowing enhanced access of the DNA oligomer and increased RNase H cleavage. Once the stem structure is destabilized by the DDPAC mutation, the effect of p68 is no longer detectable. Also, the p68-mediated conformational change is dependent on the RNA helicase activity, because the helicase-dead LGLD mutant p68 decreased RNase H cleavage of the wt tau RNA.

RNA helicase p68 increases U1snRNP binding to the 5′ splice site of tau exon 10. Because the stem-loop structure overlaps with the site for U1snRNP binding to the tau exon 10 5′ splice site, U1snRNP access to this 5′ splice site may be affected by the stem-loop structure. Consistent with this model, FTLD-tau mutations that destabilize this stem structure increase exon 10 inclusion (17, 53, 54, 60). Our results show that overexpression of p68 increased tau exon 10 splicing (Fig. 2) and that p68 affected the conformation of the stem-loop structure at the 5′ splice site of tau exon 10 (Fig. 5); hence, we investigated whether p68 influenced U1snRNP interaction with this 5′ splice site. We had previously established a U1 protection assay to measure U1snRNP binding to tau pre-mRNA at this 5′ splice site (60). In this assay, U1snRNP binding at the 5′ splice site of tau exon 10 was assessed as a measure of the U1snRNP-mediated protection of the tau RNA from RNase H cleavage following incubation of the tau RNA transcripts with HeLa nuclear extract (HeLaNE) either containing or lacking U1snRNP under splicing conditions. The radiolabeled wt tau RNA transcript was first preincubated with HeLaNE under splicing conditions for 0 or 30 min to recruit U1snRNP to the 5′ splice site (Fig. 6). The DNA oligomer complementary to the 5′ splice site was then added together with RNase H. The RNase H-cleaved and -protected RNA fragments were resolved by denaturing polyacrylamide gel electrophoresis and quantified with a PhosphorImager. As suppression of tau exon 10 splicing were abolished when the interaction of RBM4 with tau pre-mRNA was disrupted by its binding site mutation (Fig. 4C and D, compare lanes 1 to 3 with lanes 4 to 6). These results indicate that p68-mediated exon 10 splicing activation is dependent on its interaction with RBM4 and on the ability of RBM4 to interact with the tau pre-mRNA.

**RNA helicase p68 promotes conformational changes in tau exon 10 stem-loop structure.** To further investigate the mechanism underlying p68-mediated tau exon 10 splicing activation, we examined the effect of p68 on the tau exon 10 stem-loop structure. We had developed an oligonucleotide-directed RNase H cleavage assay in which a DNA oligomer complementary to the 5′ splice site downstream of tau exon 10 was used to examine conformational changes in this stem-loop region (60). We performed the oligonucleotide-directed RNase H cleavage assay using either wt tau pre-mRNA or DDPAC mutant tau transcript containing the single-nucleotide change that reduced the base pair interaction in the stem region. The RNase H cleavage assay was performed using either wt or DDPAC mutant tau transcripts in the presence of control bovine serum albumin (BSA) or purified p68 protein (Fig. 5). Incubation of wt p68 protein with wt tau RNA transcript led to a concentration-dependent increase in the cleavage of the wt tau RNA transcript (Fig. 5A, lanes 3 to 5 and lane 1; Fig. 5B, compare lanes 2 and 3), whereas LGLD mutant p68 decreased the cleavage of the wt tau RNA transcript (Fig. 5B, compare lanes 2 and 4). On the other hand, neither wt p68 nor LGLD mutant p68 changed the cleavage of DDPAC mutant tau transcript by RNase H (Fig. 5A, lanes 6 to 10; Fig. 5B, lanes 6 to 8). These results suggest that interaction of p68 with the tau stem structure may lead to a more open conformation in this region, allowing enhanced access of the DNA oligomer and increased RNase H cleavage. Once the stem structure is destabilized by the DDPAC mutation, the effect of p68 is no longer detectable. Also, the p68-mediated conformational change is dependent on the RNA helicase activity, because the helicase-dead LGLD mutant p68 decreased RNase H cleavage of the wt tau RNA.
shown in Fig. 6A and B, without preincubation with HeLaNE (lanes 2 to 4), wt p68 protein enhanced RNase H cleavage (compare lane 3 with lane 2). This enhancement was significantly reduced upon preincubation of the tau RNA transcript with HeLaNE, suggesting that HeLaNE contained a factor(s) that protected this 5'H11032 splice site from the binding of the DNA oligomer and RNase H cleavage. Consistent with the finding that LGLD mutant p68 interacted with wt tau RNA (Fig. 1B), this helicase-dead mutant appeared to stabilize the stem structure, making the tau RNA less accessible to RNase H cleavage, and the preincubation with HeLaNE further decreased tau RNA cleavage (Fig. 6A, lanes 4 and 7). We next tested the role of U1snRNP in protecting this 5' splice site of tau pre-mRNA using the HeLaNE in which U1snRNP was depleted by RNase H with an oligonucleotide specific against U1snRNA, NE-U1 (60). Such a U1-depleted HeLaNE was splicing incompetent as confirmed using an in vitro splicing assay (60). As a control, HeLaNE depleted of U7snRNA was used. HeLaNE depleted in either U1snRNA or U7snRNA was used in the U1 protec-
tion together with the wt tau RNA transcript. Depletion of U1snRNA in the nuclear extract (NE-U1) led to significantly increased RNase H cleavage of the wt tau RNA compared with that in the reaction using the control U7-depleted nuclear extract (NE-U7) in the presence of wild-type p68 under the same preincubation conditions (Fig. 6C, compare lane 4 with lane 2; Fig. 6D). Interestingly, the LGLD mutant p68 seemed to interact with and protect the wt tau RNA from RNase H cleavage, and this protection was not affected by the depletion of U1snRNP from nuclear extract (Fig. 6C, compare lanes 3 and 5 with lanes 2 and 4; Fig. 6D). These results support our finding that p68 interacts with and destabilizes the stem structure, thereby enhancing U1snRNP recognition of the 5′ splice site downstream of tau exon 10.

**DISCUSSION**

Multiple lines of evidence suggest that disruption of tau exon 10 splicing regulation plays an important role in the pathogenesis of tauopathy. More than 30 mutations have been identified among FTD-P17 patients, many of which affect tau exon 10 splicing (26, 45, 54, 60, 61, 91, 92, 97; reviewed in references 2, 53, and 66). In addition, a number of studies have reported an altered Tau4R-to-Tau3R ratio or aberrant splicing in a range of sporadic tauopathies, including progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), multiple system atrophy with dementia (MSTD), and argyrophilic grain disease (AGD), and even in some Alzheimer’s patients, among others (20, 39, 101; reviewed in references 2, 12, 36, and 78 and references therein). It is interesting that a significant fraction of these patients show increased levels of Tau4R without detectable mutations in the tau gene. These studies underscore the significance of tau exon 10 splicing regulation in the maintenance and function of the nervous system and also suggest that factors other than tau mutation may play a role in tauopathy, including tau alternative splicing regulators.

Molecular studies from several groups, including ours, have begun to reveal cis elements and trans-acting factors regulating tau exon 10 alternative splicing. One of the cis elements involved is the stem-loop structure at the tau exon 10-intron 10 junction, which blocks the maximal access of U1snRNP to the 5′ splice site (45, 54, 60, 103, 104). Mutations that destabilize the base pairing of the stem structure lead to increased exon 10 inclusion (54, 60, 103, 104, 109). These observations suggest that modulation of the conformation or stability of this stem-loop structure may serve as a critical mechanism for tau exon 10 splicing regulation. Prior to this study, no protein factors had been identified to destabilize this stem structure and no RNA helicase had been found to modulate the function of this stem-loop region in tau splicing. RNA secondary structures have been implicated in regulation of alternative splicing in a number of genes (reviewed in references 10, 47, and 83). Understanding how RNA stem-loop structures are regulated by RNA helicases may have general implications for alternative splicing regulation.

In this study, we have developed an RNA affinity pulldown-coupled mass spectrometry approach to search for potential trans-acting factors that interact with the tau exon 10 stem-loop structure. This approach can be used for studying other important cellular processes involving regulatory RNA elements. With this approach, we have identified candidate trans-acting factors (Table 1), including p68, that interact with the tau pre-mRNA stem-loop region. ATP-dependent DEAD/H box-containing RNA helicase p68 is involved in a wide range of cellular processes from transcriptional to posttranscriptional gene regulation: transcription activation, pre-mRNA splicing, and rRNA, microRNA (miRNA), and ribosome biogenesis. Some of the p68-interacting proteins have been reported, including transcription factors (AF1, MyoD, and p53) and splicing regulatory proteins such as hnRNP A1, hnRNP H, and FUS-TLS, among others (11, 13, 14, 16, 22, 32, 33, 34, 35, 46, 57, 58, 75, 77, 93–95, 108; for a recent review, see reference 59).

Previous analyses of spliceosomal complexes have revealed p68 as a component of mammalian splicing machinery (15, 48, 63, 79). Detection of cross-linking of p68 to the U1snRNA-5′ splice site duplex led to the proposal of p68 as an essential pre-mRNA splicing factor involved in unwinding this RNA duplex (77). Our RNase H cleavage experiments using purified p68 protein show that p68 alone is sufficient for interacting with the wt tau pre-mRNA and also suggest that p68 may be recruited to the 5′ splice site prior to U1snRNP recruitment to change the secondary structure of tau pre-mRNA, thus enhancing U1snRNP binding to the 5′ splice site (Fig. 7). Our studies on the role of p68 in promoting tau exon 10 inclusion reveal that its ATPase/RNA helicase activity is essential for its splicing activity.

It has been suggested that p68 is responsible for ATP-dependent unwinding of the U1snRNA-5′ splice site duplex (75, 77). However, it was not clear whether p68 affects pre-mRNA splicing of all genes or certain specific targets. We have tested a number of alternative splicing substrates and found that not all splicing events are affected by p68, including Bel-x (Fig. 2D) and others. This suggests that p68 may affect only selected alternative splicing events. Biochemical studies on CD44 and H-ras splicing show that p68 suppresses inclusion of alternative exons (13, 16, 46). In the case of CD44 splicing, the role of p68 in suppressing CD44 variable exon inclusion is suggested to be cotranscriptional, wherein p68 enhances transcription of androgen-responsive genes by binding to the androgen receptor response element (ARE) in the promoter regions of these genes. It was proposed that p68 might affect splicing factor recruitment to RNA, thereby influencing exon splicing (16). In the H-ras gene, inclusion or skipping of its alternative intron D exon (IDX) leads to formation of p19 or p21 isoforms, and p68 suppresses IDX inclusion by interacting with an intronic element that may contain an extended stem-loop structure (13, 46). In this case, p68 blocks the binding of hnRNP H to the intronic region in ras pre-mRNA and acts as a splicing suppressor (13).

Overexpression of wild-type p68 increased tau exon 10 inclusion, whereas LGLD mutant p68 lacking ATPase/helicase activity decreased exon 10 splicing (Fig. 2A), suggesting that the ATPase/helicase activity of p68 is required for its function in regulating tau exon 10 splicing and that the mutant p68 may bind to and stabilize the stem structure, making the 5′ splice site less accessible to splicing machinery. Such ATPase/helicase-dependent regulatory activity of p68 in modulating alternative splicing has not been reported for other previously studied splicing target genes of p68.
Our results show, for the first time, that p68 promotes the recruitment of U1snRNP by binding to a stem-loop structure at the 5' splice site and activates tau exon 10 inclusion in an ATPase/helicase-dependent manner (Fig. 7). Therefore, the role of p68 in tau exon 10 splicing regulation and the underlying mechanism reported in this study are different from that of p68 in regulating alternative splicing of either H-ras or CD44 genes.

Many tau splicing mutations associated with FTD/p-tau, such as DDPAC, increase exon 10 splicing by destabilizing the stem-loop structure (60, 103, 104). Consistent with this, lengthening the stem region or binding of antibiotics that increase the stability of the stem structure decreases tau exon 10 inclusion (25, 64, 98, 109). Using RNase H cleavage and U1 protection assays, we probed potential conformational changes in the stem-loop region (Fig. 5 and 6). Our results suggest that the wild-type p68 may destabilize the stem structure, releasing the 5' splice site and making it more accessible for U1snRNP interaction, thereby increasing exon 10 inclusion. In contrast, the ATPase/helicase-dead LGLD mutant p68 decreased tau RNA cleavage, suggesting stabilization of the stem-loop structure (Fig. 5). These results are consistent with the splicing changes observed in transfected cells. On the other hand, DDPAC mutant tau RNA did not show any changes in RNase H cleavage or U1snRNP protection, consistent with the UV cross-linking data showing that DDPAC mutant tau RNA did not interact with either wild-type or mutant p68. This further supports the idea that the interaction with tau pre-mRNA and the splicing activation activity of p68 are dependent on the presence of a more stable stem structure at the exon 10-intron 10 junction of tau pre-mRNA. However, these results do not preclude other potential p68-mediated effects such as remodeling of RNA-protein and protein-protein interaction at the 5' splice site of tau exon 10 as possible mechanisms of action for p68.

RNA binding protein RBM4 is a multifunction protein containing an RNA recognition motif (82). RBM4 plays a role in regulating alternative splicing of alpha-tropomyosin, survival of motor neuron gene 2 (SMN2), SRp20 reporter genes, and tau exon 10 (65, 76, 81). An expression cloning screening of an adult human brain cDNA library together with biochemical studies identified RBM4 as a regulator of tau exon 10 splicing (65). RBM4 promotes tau exon 10 splicing by interacting with an intronic splicing enhancer element 100 nucleotides downstream of exon 10 (65). In the current study, we demonstrate that RBM4 interacts with RNA helicase p68 in an RNA-independent manner (Fig. 3). However, the splicing activation function of p68 is dependent on RBM4 binding to the intronic element downstream of the 5' splice site (Fig. 4). These results suggest that RBM4 might function to recruit p68 to a nearby splice site, thus explaining the necessity for RNA interaction. Alternatively, recruiting p68 to RBM4-containing protein complexes might allow p68 to function by remodeling protein-protein interactions as reported earlier for H-ras and CD44 splicing, the RNAi pathway, and cellular differentiation (13, 16, 22, 46, 99, 104).

Studies of RBM4 activity in regulating pre-mRNA splicing of other substrates such as alpha-tropomyosin suggested possible recruitment of U1snRNP (76). Our studies suggest that RBM4-mediated exon splicing enhancement may be mediated by its interaction with p68. Our data demonstrate functional interaction between two splicing activators of tau exon 10, p68 binding to the stem-loop region at the 5' splice site and RBM4 interacting with the intronic splicing enhancer in tau exon 10 splicing. This also suggests a potential selective mechanism by which RNA helicases such as p68 might influence only specific alternative splicing events. However, the details of the interaction and mechanism require further study. Taken together, our work uncovers a previously unknown mechanism in which trans-acting regulators p68 and RBM4 cooperate to promote inclusion of tau exon 10.

ACKNOWLEDGMENTS

We thank members of the Wu laboratory for helpful discussions and Eileen Bigio for critical reading and suggestions.
REFERENCES


VOL. 31, 2011 REGULATION OF
Montejo de Garcini, E., et al.


