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# PSF Suppresses Tau Exon 10 Inclusion by Interacting with a Stem-Loop Structure Downstream of Exon 10

**Payal Ray · Amar Kar · Kazuo Fushimi · Necat Havlioglu · Xiaoping Chen · Jane Y. Wu**

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**Abstract** Microtubule binding protein Tau has been implicated in a wide range of neurodegenerative disorders collectively classified as tauopathies. Exon 10 of the human *tau* gene, which codes for a microtubule binding repeat region, is alternatively spliced to form Tau protein isoforms containing either four or three microtubule binding repeats, Tau4R and Tau3R, respectively. The levels of different Tau splicing isoforms are fine-tuned by alternative splicing with the ratio of Tau4R/Tau3R maintained approximately at one in adult neurons. Mutations that disrupt *tau* exon 10 splicing regulation cause an imbalance of different tau splicing isoforms and have been associated with tauopathy. To search for factors interacting with *tau* pre-messenger RNA (pre-mRNA) and regulating *tau* exon 10 alternative splicing, we performed a yeast RNA–protein interaction screen and identified polypyrimidine tract binding protein associated splicing factor (PSF) as a candidate *tau* exon 10 splicing regulator. UV crosslinking experiments show that PSF binds to the stem-loop structure at the 5′ splice site downstream of *tau* exon 10. This PSF-interacting RNA element is distinct from known PSF binding sites previously identified in other genes. Overexpression of PSF promotes *tau* exon 10 exclusion, whereas down-regulation of the endogenous PSF facilitates exon 10 inclusion. Immunostaining shows that PSF is expressed in the human brain regions affected by tauopathy. Our data reveal a new player in *tau* exon 10 alternative splicing regulation and uncover a previously unknown mechanism of PSF in regulating *tau* pre-mRNA splicing.

**Keywords** Tau · Alternative splicing regulation · Tauopathy · RNA stem-loop secondary structure · Polypyrimidine tract binding protein associated splicing factor (PSF)

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## Introduction

Alternative splicing is one of the most powerful mechanisms for gene regulation and increasing proteomic diversity in metazoans (Black 2000; Matlin et al. 2005; Nilsen and Graveley 2010). The majority of mammalian genes undergo alternative pre-messenger RNA (pre-mRNA) splicing to generate distinct splicing isoforms. Alternative splicing gives rise to isoforms that differ in their biochemical/biophysical properties, subcellular localization, and posttranslational modifications. Numerous genes utilize alternative splicing to achieve spatial and/or temporal regulation of their expression. The fundamental mechanisms regulating alternative splicing consist of complex and dynamic interactions between trans-acting splicing regulators and cis-elements in the intronic or exonic regions of individual pre-mRNAs (Wu et al. 2004; Wu and Postashkin 2009; Nilsen and Graveley 2010). The critical importance of alternative splicing regulation is demonstrated by large numbers of disease-causing mutations that affect alternative pre-mRNA splicing (Krawczak et al. 1992; Wu et al. 2004; Cooper et al. 2009; Ward and Cooper 2010). Splicing mutations can occur either in *cis* altering sequence elements within the splicing substrate genes or in *trans* affecting splicing accessory factors (Wang and Cooper 2007; Solis et al. 2008).

Fronto-temporal lobar degeneration (FTLD) refers to a group of neurological disorders that share the common pathological features of frontal and temporal lobe degeneration, accompanied by the presence of abnormal protein inclusions. There are several subgroups of FTLDs, which are classified on the basis of the predominant protein species found in the neuronal inclusion bodies. FTLD-tau is the condition wherein aggregated tau is the primary component of the neuronal inclusions, forming neurofibrillary tangles (Neumann et al. 2009; MacKenzie et al. 2010). The diseases that share this pathological feature are also known as tauopathies. Apart from FTLD-tau, tauopathies include Alzheimer's disease, Pick's disease, corticobasal degeneration, and progressive supranuclear palsy (Lee et al. 2001; Goedert and Jakes 2005; Mackenzie and Rademakers 2007; Boeve and Hutton 2008; Iqbal et al. 2010; Medeiros et al. 2010). Tau is a neuronally expressed microtubule binding protein that promotes microtubule assembly. It plays an important role in neuronal integrity and axonal transport by regulating microtubule stability and dynamics (Mercken et al. 1995; Panda et al. 1995; Iqbal et al. 2009; Morfini et al. 2009). Genetic studies have identified more than 30 mutations in the human *tau* gene among FTLD-tau patients (Hutton et al. 1998; Spillantini et al. 1998; D'Souza and Schellenberg 2000; Grover et al. 1999; Jiang et al. 2000; Pickering-Brown et al. 2000; Spillantini et al. 2000; Goedert and Spillantini 2001; Pickering-Brown et al. 2002; Jiang et al. 2003; Andreadis 2005, 2006; Goedert and Jakes 2005; Kar et al. 2005; Liu and Gong 2008; Wang et al. 2004). In addition to the mutations that alter the peptide sequence of Tau protein, many mutations have been found that affect alternative splicing of *tau* pre-mRNA, including both intronic and exonic ones (Lee et al. 2001; Goedert and Jakes 2005; Liu and Gong 2008; Colombo et al. 2009; Wolfe 2009).

The human *tau* gene contains 16 exons, of which exons 2, 3, and 10 are alternatively spliced to generate six isoforms, three containing exon 10 and three lacking exon 10 (Neve et al. 1986; Goedert et al. 1989a; Himmler 1989; Andreadis et al. 1992; Montejo de Garcini et al. 1992; Collet et al. 1997; Wei and Andreadis 1998; Andreadis 2005; Kar et al. 2005). Tau exons 9, 10, 11, and 12 encode four tandem microtubule binding repeat sequences (Goedert et al. 1989b; Goedert and Jakes 1990; Andreadis et al. 1992). Alternative splicing of exon 10 gives rise to two classes of tau isoforms: Tau4R (containing exon 10), which contains four microtubule binding repeats, and Tau3R (lacking exon 10), which contains three microtubule binding repeats. Tau4R and Tau3R isoforms differ in their binding affinity to microtubules (Lee et al. 1989; Goedert and Jakes 1990; Goode et al. 2000; Utton et al. 2002; Tomoo et al. 2005; Konzack et al. 2007; LeBoeuf et al. 2008). In the adult human brain, alternative splicing of *tau* exon 10 is tightly regulated to maintain the ratio of Tau4R to Tau3R at approximately one, whereas tau is expressed as almost exclusively Tau3R isoform in the fetal brain (Neve et al. 1986; Lichtenberg-Kraag and Mandelkow 1990; Andreadis et al. 1992; Gao et al. 2000). Disruption of this delicate balance in the Tau4R/Tau3R ratio is associated with tauopathies (Hutton et al. 1998; Hasegawa et al. 1998; Grover et al. 1999; Hasegawa et al. 1999; Spillantini et al. 2000; Connell et al. 2001; Cairns et al. 2007; Mackenzie et al. 2010). Studies over the last decade have revealed both *cis*-acting elements and *trans*-acting regulatory proteins that regulate *tau* exon 10 alternative splicing (Hartmann et al. 2001; Broderick et al. 2004; Kondo et al. 2004; Glatz et al. 2006; Kar et al. 2006; Wu et al. 2006; Dawson et al. 2007; Gao et al. 2007; Zhou et al. 2008; Wang et al. 2010). The RNA sequence at the exon 10–intron 10 boundary of *tau* pre-mRNA is predicted to form a stem-loop structure with 6 bp stem and six nucleotide loop (Hutton et al. 1998; Grover et al. 1999; Varani et al. 1999; Jiang et al. 2000; Varani et al. 2000; Kar et al. 2005; Donahue et al. 2006). Previous studies show that this stem-loop structure may influence the binding of U1snRNP (U1 small nuclear ribonucleoprotein) to the 5′ splice site (5′ss) of exon 10 (Varani et al. 1999; Jiang et al. 2000; Varani et al. 2000; Kalbfuss et al. 2001; Jiang et al. 2003; Donahue et al. 2006). A number of FTLD-tau-associated mutations are clustered in this exon 10–intron 10 boundary region. Interestingly, most of the mutations in the stem-loop region are associated with increased formation of Tau4R isoforms, suggesting that these mutations lead to inclusion of exon 10 by disrupting the stem-loop structure. However, splicing factors that interact with this region and regulate *tau* exon 10 alternative splicing have not been fully characterized.

In this study, we carried out a yeast RNA–protein interaction screen and identified PSF as one of factors interacting with the *tau* pre-mRNA in this stem-loop region. Using molecular and biochemical assays, we characterized the interaction between PSF and *tau* pre-mRNA and examined the role of PSF in regulation of *tau* exon 10 splicing. Our experiments demonstrate that PSF interacts with the stem-loop structure and acts to promote *tau* exon 10 skipping. Finally, the expression of PSF in the human brain is consistent with its involvement in tauopathy.

## Materials and Methods

*Yeast Three-Hybrid Screening for RNA–Protein Interaction* We used the RNA–protein Hybrid Hunter system (Invitrogen) to screen for proteins interacting with the *tau* exon 10 5′ splice site stem-loop structure. The bait plasmid was constructed by fusing the 5′ splice site stem-loop region of 96 nucleotides to the MS2 binding site in the pRH5′ plasmid. The bait plasmid was transformed into L40uraMS2 yeast strain that carried the reporters HIS3 and LacZ, downstream of four or eight LexA binding sites,

respectively. L40uraMS2 yeast strain carries the gene encoding LexA DNA binding domain–MS2 coat protein fusion. We confirmed that the bait we constructed did not activate reporter genes by itself in the L40 strain. The bait expressing yeast strain was transformed with a human fetal brain complementary DNA (cDNA) library that expresses cDNAs as proteins fused to B42 transcription activation domain. cDNA clones that activated the reporter genes were isolated and sequenced.

*Plasmids* The wild-type and DDPAC *tau* Ex9–11 and Ex10–11 minigene constructs have been described previously (Jiang et al. 2000, 2003). Mammalian expression plasmid for PSF-GFP and bacterial expression construct for PSF-GST were a kind gift of Dr. James Patton, Vanderbilt University.

*Transfection and RT-PCR Assay* HEK293 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (Gemini) and 1% penicillin-streptomycin (Gibco). For overexpression experiments, $2\times10^5$ cells/well were seeded onto six-well plates 24 h before transfection. Cells were transfected with 1 μg wild-type or DDPAC minigene and 3 μg expression vector using a modified calcium phosphate precipitation method (Jiang et al. 1998, PNAS). For small-interfering (siRNA) experiments, 250 pmol of either control or PSF specific siRNA (Ambion) were transfected using Lipofectamine 2000 following the manufacturer's instructions. For RT-PCR, cells were harvested 48 h after transfection, and RNA was extracted using the RNAspin Mini RNA isolation kit (GE). After RT-PCR amplification, cDNA was used for PCR using *tau*-specific primers (Jiang et al. 1998) in the presence of [$\alpha$-$^{32}$P]dCTP. The levels of Bcl-xl/xs, GAPDH, and β-actin were detected using corresponding specific primers. PCR products were separated on a 6% polyacrylamide gel, and the bands were quantified using a BAS 5000 Phosphorimager (Fuji).

*Antibodies and Western Blots* For detecting PSF expression, HEK293 cells expressing the corresponding minigenes with PSF protein or control GFP were lysed in lysis buffer [50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM DTT, 1× protease inhibitor (Roche)]. Equal amount of cell lysates were run on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred onto 0.22 μm Nitrocellulose membrane (Biorad) using a semi-dry transfer apparatus (Biorad). Membranes were blocked with 10% non-fat dry milk in PBST (phosphate-buffered saline, pH 7.4, 0.1% Tween-20), incubated overnight with corresponding primary antibody diluted in PBST containing 3% bovine serum albumin, washed, incubated with appropriate secondary antibody, and developed with ECL Plus Reagent (GE-Amersham). Antibodies used are as follows: anti-PSF (B92, Sigma), anti-GFP (B-34, Covance), and anti-beta actin (C-11, Santa Cruz).

*RNA–Protein Interaction Assay* Uniformly labeled *tau* wild-type or DDPAC pre-mRNA transcripts were prepared by in vitro transcription in presence of [$\alpha$-$^{32}$P]UTP using T7 polymerase from linearized DNA templates as described previously (Jiang et al. 2000). For preparing radioactively labeled oligomer, a labeling reaction was done with [$\gamma$-$^{32}$P]ATP by PNK. The RNA probes were used for UV crosslinking using cell lysates. Briefly, 50 μl HEK293 cell lysates overexpressing PSF or control protein were incubated with ~20 fmol of RNA probe for 2 h on ice. The samples were irradiated on ice under a 254-nm UV light for 10 min using a Stratalinker 2000 UV crosslinker (Stratagene) and subjected to RNase A (5 mg/ml, Sigma) digestion at 30°C for 30 min. End-labeled oligomer transcripts were not subjected to RNase digestion. For immunoprecipitation, anti-GFP antibody bound to protein A/G beads were added to the samples and incubated at 4°C for 2 h. Following precipitation, the beads were washed with cold buffer (20 mM Tris, 150 mM NaCl, 0.1% NP-40, pH 7.4), the RNA bound proteins eluted in sample buffer, and resolved on SDS-PAGE followed by autoradiography.

*Oligonucleotide Directed RNase H Cleavage Assay* [$\alpha$-$^{32}$P]UTP-labeled wild-type and mutant RNA transcripts were incubated at 37°C for 60 min in standard splicing buffer in the presence of 0.5 U of RNase H (USB) in 25-μl reaction mixtures with 200 fmol of an oligonucleotide (5′-GAAGGTACTCACACTGCC-3′) complementary to the exon 10 splice donor site. The reaction also contained either purified PSF or GST protein (for details, see Jiang et al. 2000). The recombinant PSF-GST protein was purified from *Escherichia coli* using standard purification protocol (Patton et al. 1993). Cleaved RNA products were then separated on a 6% polyacrylamide–8 M urea gel. The cleavage ratios were measured with a PhosphorImager (Fuji).

*Immunostaining* Human brain samples from autopsy material were fixed in formalin and paraffin-embedded. Six-micrometre-thick sections from the frontal and hippocampal regions were used for immunohistochemical staining. The Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) staining kit for mouse monoclonal antibody was used to perform immunostaining with the mouse anti-PSF antibody (B 92, Sigma-Aldrich). Antigen retrieval was carried out in the presence of 1 mM EDTA, pH 9.0. Primary antibody was used at a 1:1,000 dilution. Immunostaining was carried out following the instruction manual, color development was

performed with 3,3′-diaminobenzidine, and counterstaining was with hematoxylene.

## Results

### Identification of PSF as a Protein Binding to Tau Pre-mRNA in a Yeast RNA–Protein Interaction Screen

To understand molecular mechanisms regulating alternative splicing of *tau* exon 10, we employed a yeast RNA–protein interaction screen approach to search for proteins interacting with *tau* pre-mRNA in the region flanking the 5′ splice site, where a number of FTLD-tau associated mutations are located. A 96-nucleotide RNA corresponding to the last 40 nucleotides of *tau* exon 10 followed by 56 nucleotide of the downstream intronic sequence was used as bait. This *tau* RNA fragment was fused with the RNA fragment containing phage MS2 binding site to make the plasmid expressing MS2-tau-pre-mRNA bait. The bait *tau* RNA expressing plasmid was transformed into yeast strain L40MS2 expressing transcription activator protein containing LexA DNA binding domain–MS2 coat protein. We confirmed that expression of MS2-tau-pre-mRNA bait by itself does not activate reporter genes. A human fetal brain cDNA library expressing cDNAs as proteins fused to B42 transcription activation domain was transformed into the yeast expressing MS2-tau-pre-mRNA bait together with reporter genes *His3* and *LacZ* under the control of the promoter containing LexA binding sites (Fig. 1). A number of cDNA clones were isolated encoding proteins that activated the reporter genes in a manner dependent on the expression of the *tau* pre-mRNA. In this study, we focus on one of the candidate *tau* pre-mRNA interacting proteins identified in this screen (PSF). The remaining cDNA clones are being further characterized, and their detailed characterization will be described in separate reports.

### PSF Binds to Tau Pre-mRNA at the Stem-Loop Region at the Exon 10–Intron 10 Junction

We tested whether PSF interacted with *tau* pre-mRNA under splicing conditions. As illustrated in Fig. 2, a series of *tau* pre-mRNA transcripts were prepared containing either the intact *tau* exon 9–exon10–exon 11 (tau Ex9–11) or truncated sequences with wild-type sequence (tau Ex9–11wt) or mutant sequence, which contained a single nucleotide mutation (C to T) named disinhibition–dementia–parkinsonism–amyotrophy complex (DDPAC) at +14 position of *tau* intron 10 (Hutton et al. 1998; Jiang et al. 2000) (tau Ex9–11DDPAC). These *tau* pre-mRNA transcripts were radiolabeled by in vitro transcription reaction as described in our previous studies (Jiang et al. 2000, 2003) and used in UV crosslinking experiments. As shown in Fig. 2A, different *tau* pre-mRNA transcripts were UV-irradiated following incubation under splicing conditions with cell lysates prepared from HEK293 cells overexpressing PSF tagged with GFP. RNAs cross-linked to PSF were immunoprecipitated using a specific anti-GFP antibody and separated on SDS-PAGE following RNase digestion. UV crosslinking signals were detected by autoradiography. PSF interaction with *tau* pre-mRNA was detected when *tau* Ex9–11 wt (Fig. 2A, transcript 1, lane1) or *tau* exon9–10 together with the downstream intronic sequence (tau Ex9–10wt; Fig. 2A, transcript 2, lane 2) were used. However, when exon 10 with the intron 10 sequence was deleted (tau Ex9 wt; Fig. 2A, transcript 3), the UV crosslinking signal of PSF was no longer detectable (Fig. 2A, lane3). We then compared the wild-type and DDPAC mutant *tau* splicing substrates containing only exon 10–11 region. Interestingly, the wild-type (tau Ex10–11wt) transcript showed a strong UV crosslinking signal, but *tau* DDPAC (tau Ex10–11DDPAC) transcript had a significantly reduced signal (Fig. 2B, compare lanes 1 and 2). The crosslinking signal was specific because no signal was detected when the reactions were carried out using the control cell lysates expressing GFP vector alone (Fig. 2B, lanes 3 and 4). To further define the region of PSF binding on the *tau* pre-mRNA, a 48-nucleotide *tau* RNA oligomer containing the stem-loop region with either wild-type sequence or DDPAC mutation was used in the UV crosslinking experiments. Consistent with above results, the wild-type *tau* RNA oligomer showed a stronger UV crosslinking signal to PSF than the mutant DDPAC oligomer (Fig. 2C, compare lanes 4 and 3), suggesting that PSF preferentially binds to the stem-loop region. To rule out the possibility that PSF binds non-specifically to any RNAs with stem-loop secondary structures, we performed UV crosslinking experiments with Poly I/C and transfer RNA (tRNA), two molecules that have been predicted to form double stranded secondary structures in solution (Grant et al. 1968). PSF did not show any detectable binding to either tRNA or Poly I/C (Fig. 2C, lanes 3 and 4), indicating that PSF protein does not interact non-specifically with RNA containing double-stranded stem-loop structures. These results show that PSF specifically binds to the stem-loop region of *tau* pre-mRNA.

### PSF Regulates *tau* Exon 10 Splicing

To investigate the significance of PSF binding to the stem-loop region of *tau* pre-mRNA exon 10, we examined the effect on exon 10 splicing. HEK293 cells were co-transfected with *tau* exon 9–10–11 minigene (tau Ex9–11wt, Jiang et al. 2000) and either vector control or PSF expression plasmid. RNA was extracted 48 h after transfec-

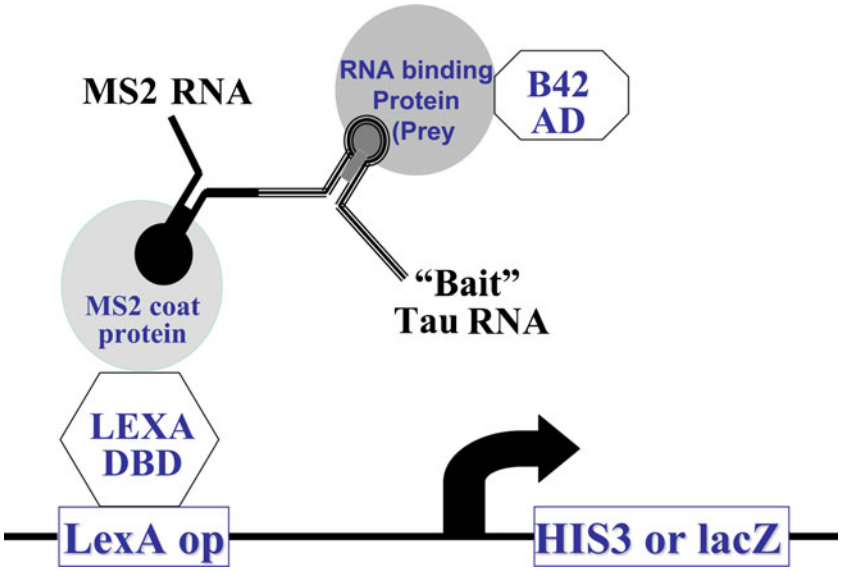


**Fig. 1** Schematic representation of the yeast RNA–protein interaction screen. As described in "Materials and Methods," the bait plasmid was constructed by fusing the 5′ splice site stem-loop region of exon 10 nucleotides to the MS2 binding site in the pRH5′ plasmid. The bait plasmid was transformed into L40uraMS2 yeast strain that carried the reporters HIS3 and LacZ and the LexA DNA binding domain-MS2 coat protein fusion. The cDNA library consists of proteins fused to the B42 transcription activation domain. When there is an interaction between RNA binding protein and the bait RNA, transcription is activated, then the LacZ and His3 reporters are expressed

tion and levels of exon 10 alternative splicing products were analyzed by RT-PCR (Fig. 3A), using specific primers, as reported previously (Jiang et al. 2000). Overexpression of PSF led to a significant increase in exclusion of *tau* exon 10 as compared to the vector control, resulting in increased levels of exon 10-lacking *tau* splicing products (E10−; Fig. 3A, top left panel, compare lanes 1 and 2).

Next, we tested whether PSF expression had a similar effect on the DDPAC mutant splicing as observed with *tau* wild-type minigene. After co-transfection of either vector control or PSF expression plasmid and *tau* exon 9–10–11 DDPAC minigene (tau Ex9–11 DDPAC, Jiang et al. 2000) in HEK293 cells, RNA was extracted, and cDNA levels were analyzed to determine the levels of *tau* transcripts containing or lacking exon 10. PSF overexpression resulted in a decrease in splicing products containing exon10 (E10+) in the DDPAC mutant background (Fig. 3C, top right panel, compare lanes 1 and 2).

To investigate the effect of down-regulating PSF on *tau* exon 10 alternative splicing, we used siRNA to decrease the endogenous levels of PSF in HEK293 cells. PSF-specific siRNA or a non-specific control siRNA were co-transfected together with the *tau* Ex9–11wt minigene into HEK293 cells. After two rounds of siRNA transfection, levels of alternatively spliced *tau* mRNA products containing or lacking exon 10 was examined by RT-PCR. Western blotting using anti-PSF-specific antibody confirmed that specific siRNA transfection led to a significant reduction in the endogenous PSF (see Fig. 3G, top panel). Specific down-regulation of the endogenous PSF in HEK293 cells led to increased levels of products containing exon 10 (E10+), accompanied by a comparable decrease in the levels of product lacking exon 10 (E10−) (Fig. 3E, top panel, lane 2). The effect of PSF knockdown was specific for *tau* pre-mRNA splicing because alternative splicing of *Bcl-x* was not affected (Fig. 3E, bottom panel). Together with the PSF overexpression data, these results show that PSF regulates the inclusion of exon10 of *tau* pre-mRNA.

## Oligonucleotide-Directed RNase H Cleavage Experiments Suggest that PSF Stabilizes the 5′ Splice Site Stem-Loop Structure

In our screen, PSF was identified as a protein interacting with the stem-loop region at exon 10 of *tau* pre-mRNA. To understand its mechanism of action, we examined whether PSF binding affected this secondary structure. Our group had previously reported an oligonucleotide directed RNase H cleavage assay to probe the secondary structure of this region using a DNA oligomer that is complementary to the 5′ splice site of the tau exon 10 (Jiang et al. 2000; Kar et al. 2011). This RNase H cleavage assay was carried out using either *tau* Ex10–11wt or *tau* Ex10–11DDPAC transcripts (as in Fig. 2B), and the splicing efficiency of these transcripts was measured following co-transfection with the PSF plasmid as described above for the *tau* Ex9–11wt minigene.

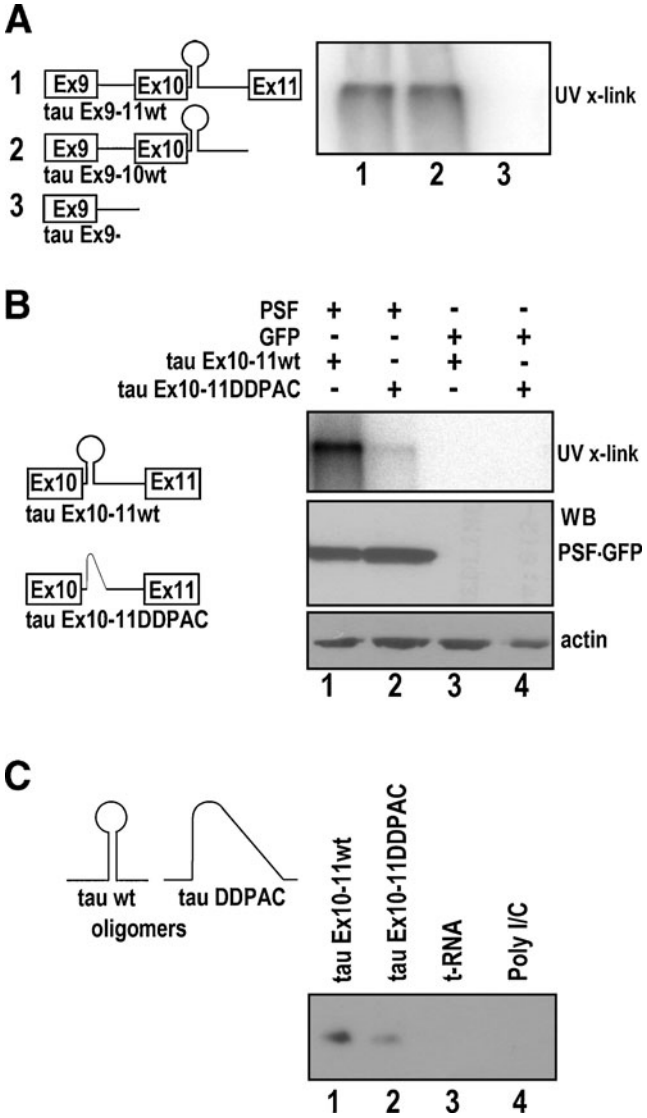


**Fig. 2** PSF interacts with *tau* Exon 10 stem-loop region. **A** HEK293 cells were transfected with PSF-GFP plasmid, and the lysates were used for UV crosslinking experiment with different *tau* wild-type (wt) transcripts (transcripts 1, 2, and 3). Following UV crosslinking and RNase treatment, the reaction products were analyzed by SDS-PAGE (*lanes 1–3*) and autoradiography. **B** Radiolabeled *tau*Ex10–11 wt or DDPAC transcripts (illustrated on *left*) were incubated with HEK293 cell lysates overexpressing PSF-GFP or control GFP and UV cross-linked. After RNase treatment, anti-GFP antibody was used for immunoprecipitation and the products subjected to SDS-PAGE (*lanes 1–4*) followed by autoradiography. *Middle and lower panels* show levels of PSF-GFP and actin, respectively, as detected by immunoblotting. **C** UV crosslinking experiments using cell lysates (similar to **B** above) were carried out with a radiolabeled 48 nucleotide oligomer (illustrated on *left*). Binding of wild type (*lane 1*) was compared with DDPAC (*lane 2*) oligomers as well as to transfer RNA (tRNA, *lane 3*) and Poly I/C (*lane 4*)

The ratio of spliced products and unspliced pre-mRNA was calculated to determine whether the effect of PSF on *tau* Ex10–11 transcript was comparable to the effect seen on *tau* Ex9–11 minigenes. PSF overexpression led to decreased levels of *tau* Ex10–11 mRNA, hence suppressing the splicing of exon 10 (see Fig. 4A and B). In the RNaseH cleavage assay, radiolabeled *tau* wt or *tau* DDPAC transcripts were incubated with control GST or purified PSF protein and then subjected to RNaseH treatment. Our results showed a decrease in the RNAase H cleavage products (indicative of the formation of DNA-RNA hybrid and thus the openness in the conformation of the stem-loop structure) in the reactions containing the purified PSF protein (Fig. 5A, left panel, compare lanes 1 and 3) as compared to the control reaction. This suggests that in the presence of PSF the stem-loop structure is less accessible for binding to the DNA oligonucleotide. To further identify the region responsible for PSF binding, we used a shorter oligomer RNA transcript corresponding to the stem-loop structure (as used in Fig. 2C). Consistent with our results using the longer *tau* pre-mRNA transcript, a reduction of cleavage of the *tau* wild-type oligomer RNA transcript was observed in the presence of PSF protein, suggesting that PSF interacts with *tau* pre-mRNA stem-loop structure in the region spanning the intron10–exon10 junction to ~48nucleotides downstream of exon 10. We also detected a concentration-dependent suppression of RNaseH cleavage in the presence of increasing amounts of PSF protein (data not shown, quantification shown in Fig. 5). This observation further supports the involvement of PSF protein in regulating the conformation of the *tau* stem-loop structure. We predicted *tau* DDPAC mutant to be more susceptible to RNaseH cleavage because of the destabilization of the stem-loop structure and the increased accessibility of the 5′ splice site to U1snRNP by the point mutation inside the stem structure. Consistent with our prediction, the DDPAC mutant RNA transcript showed an increased RNase H cleavage (Fig. 5A, right panel), suggesting that reduced PSF binding correlated with reduced protection from RNaseH cleavage. These results suggest that PSF may interact with the 5′ splice site stem-loop structure to prevent it from switching to a more open conformation that would allow an increased U1snRNP binding to the 5′ splice site.

## Expression of PSF in Mammalian Brain

PSF expression has been reported in the mammalian nervous system (Chanas-Sacre et al. 1999). In the mouse brain, PSF is expressed widely across all regions. PSF expression is under developmental control in the neural tissue, and PSF immunoreactivity has been detected in both neurons and oligodendrocytes (Chanas-Sacre et al. 1999). To determine whether PSF is expressed in neurons where *tau* exon 10 alternative splicing is important, we examined PSF expression in the human brains by immunohistochemical staining. Our results show that PSF is expressed at a high level in the hippocampus and the cortex (Fig. 6A, B)

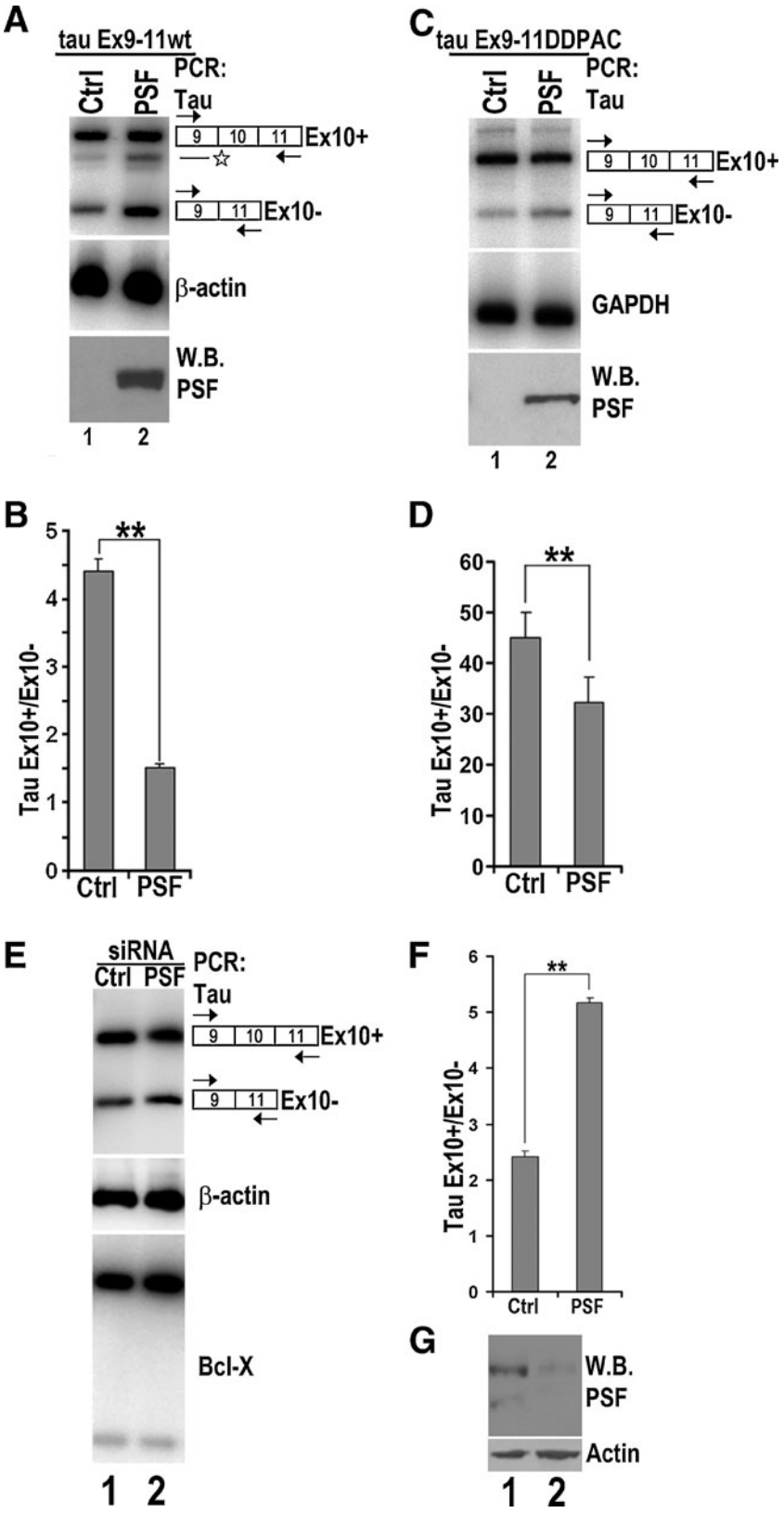


**Fig. 3** PSF regulates *tau* exon 10 exclusion. **A**–**D** The corresponding plasmids expressing either the vector control (*lane 1*) or PSF (*lane 2*) were co-transfected with the *tau*9–11 wild-type (tau Ex9–11wt) or *tau* Ex9–11 DDPAC minigene into HEK 293 cells. **A**, **C** *tau* exon 10 (tau Ex10) containing products were detected using RT-PCR, with β-actin or GAPDH transcripts in the corresponding groups showing comparable amounts of RNA used in each reaction. The levels of PSF protein in the corresponding reactions as detected by immunoblotting using anti-GFP antibody is shown in the *bottom panels*. *Asterisk* indicates background band in **A**. **B**, **D** Quantification of *tau* exon 10 splicing (ratio of *tau*Ex10+/Ex10− levels) as shown in **A** and **C**. The graph is average of five independent experiments. **E** The *tau* Ex9–11wt minigene was co-transfected into HEK293 cells together with either non-specific control RNAi (*lane 1*) or PSF-specific RNAi (*lane 2*). Alternative splicing products were detected by specific primers using RT-PCR. β-Actin levels were used as the internal control. Alternative splicing of the endogenous *Bcl-x* was not affected by decrease in PSF levels. **F** Quantification of *tau* exon 10 splicing isoforms as shown in **E**. The graph shows the average of six independent experiments. **G** Endogenous levels of PSF detected by anti-PSF antibody with actin levels used as loading control

and that PSF expression appears higher in the fetal than in the adult hippocampus (Fig. 6C). Therefore, PSF is expressed in the human brain regions affected by tauopathy and developmental changes in PSF expression appear to be consistent with the role of PSF in regulating *tau* exon 10 alternative splicing in humans.

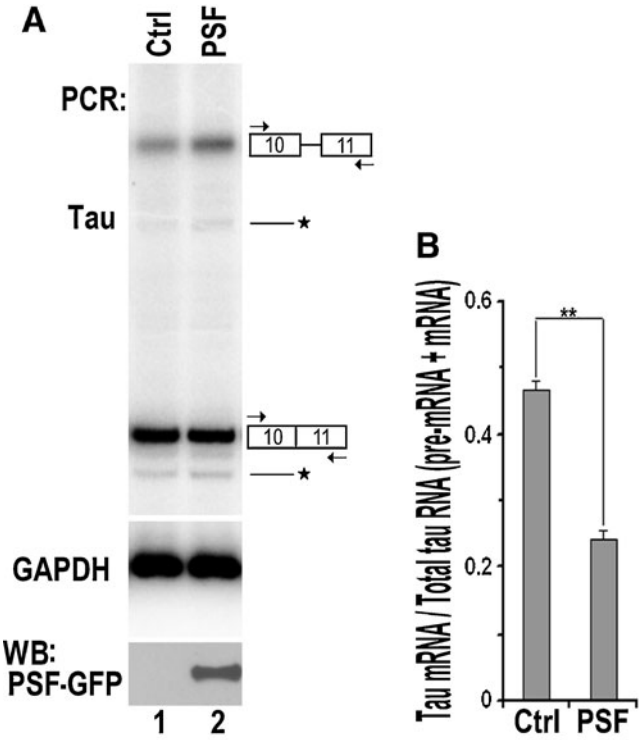


**Fig. 4** PSF overexpression affects exon 10 splicing. **A** *tau* exon 10–11 minigene plasmid was co-transfected along with vector control (*lane 1*) or PSF (*lane 2*) overexpression plasmids into HEK293 cells, cDNA extracted 48 h post-transfection and levels of mRNA analyzed by PCR with levels of GAPDH used as internal control. Western blot analysis of corresponding lysates using anti-GFP antibody show levels of PSF in the lysates used for the experiments. **B** Quantification of ratio of *tau* mRNA/pre-mRNA. *Graph* shows the average of three independent experiments

## Discussion

Genetic analyses of FTLD-tau patients have revealed a number of mutations near the 5′ splice site of exon 10 and the downstream intronic region in the human *tau* gene. Several studies have been conducted to characterize this region to elucidate molecular mechanisms of these splicing mutations and their effects on *tau* exon 10 alternative splicing (Wei et al. 2000; Hartmann et al. 2001; Broderick et al. 2004; Kondo et al. 2004; Glatz et al. 2006; Kar et al. 2006; Wu et al. 2006; Dawson et al. 2007; Gao et al. 2007; Zhou et al. 2008; Wang et al. 2010; Kar et al. 2011). These studies have uncovered several trans-acting proteins that interact with exonic or intronic cis-elements to regulate *tau* exon 10 alternative splicing. Interestingly, a significant fraction of tauopathy cases with increased Tau4R levels show no detectable mutation in the *tau* gene. It is possible that in such cases, increased expression or function of trans-acting factors that promote *tau* exon 10 inclusion play a role in the development of tauopathy.

Using a yeast RNA–protein interaction screen and biochemical assays, we identified PSF as a candidate regulator of *tau* exon 10 alternative splicing. PSF is a multifunctional nuclear protein that has been implicated in several cellular processes, including DNA recombination, transcription, pre-mRNA splicing (Patton et al. 1993), 3′ end processing (Kaneko et al. 2007), translation (Straub et al. 1998; Akhmedov and Lopez 2000; Urban et al. 2000; Mathur et al. 2001; Zhang and Carmichael 2001; Sewer et al. 2002; Shav-Tal and Zipori 2002; Cobbold et al. 2008), and control of circadian rhythm in mice (Duong et al. 2011). It has been proposed that PSF plays a role in tumor suppression (Wang et al. 2009). PSF has also been shown to be essential for cell survival and neuronal development in zebra fish (Lowery et al. 2007).

Our data show that PSF binds to the stem-loop structure at the exon 10–intron10 boundary of the *tau* pre-mRNA transcript. PSF was initially identified as a spliceosome associated factor (Patton et al. 1993). PSF is associated with the pre-spliceosome complex as well as spliceosomal snRNPs (Gozani et al. 1994; Peng et al. 2002). PSF also binds to U5snRNP and the 5′ splice site (Peng et al. 2002; Kameoka et al. 2004). Previous studies have reported the binding of PSF to different RNA sequences including AU-rich elements (Buxade et al. 2008), a stem-loop structure in hepatitis delta virus (Greco-Stewart et al. 2006), an exonic splicing suppressor sequence element in CD45 exon 4 (Melton et al. 2007), as well as other sequence elements in transcription promoter region (Yang et al. 2007). Furthermore, the binding of PSF to the stem 1b of U5snRNA has been shown to be dependent on both a specific sequence and a stem structure (Peng et al. 2002). In our studies, the wild-type *tau* RNA showed stronger PSF UV-crosslinking signal than the *tau* DDPAC mutant, suggesting that a more stable stem-loop structure may be necessary for efficient binding of PSF (Fig. 2). This reduced PSF binding to the DDPAC mutant *tau* RNA was accompanied by a reduction in the ability of PSF in suppressing exon 10 inclusion (Fig. 3). On the other hand, no UV-crosslinking signal was detected between PSF and Poly I/C or tRNA (Fig. 2C), indicating that stem-loop structures alone are not sufficient to confer PSF binding.

To better understand the mechanism of PSF in regulating *tau* exon 10 splicing, we investigated the effect of PSF binding on the stem-loop structure using oligonucleotide directed RNaseH cleavage assay. Our experiments show that the binding of PSF to the stem-loop structure is correlated with reduction of the association of U1snRNP to the 5′ splicing and with the exclusion of *tau* exon 10. Destabilization of this stem structure by the DDPAC mutation allows more U1snRNP binding to the 5′ splice site, leading to increased inclusion of exon 10 in *tau*

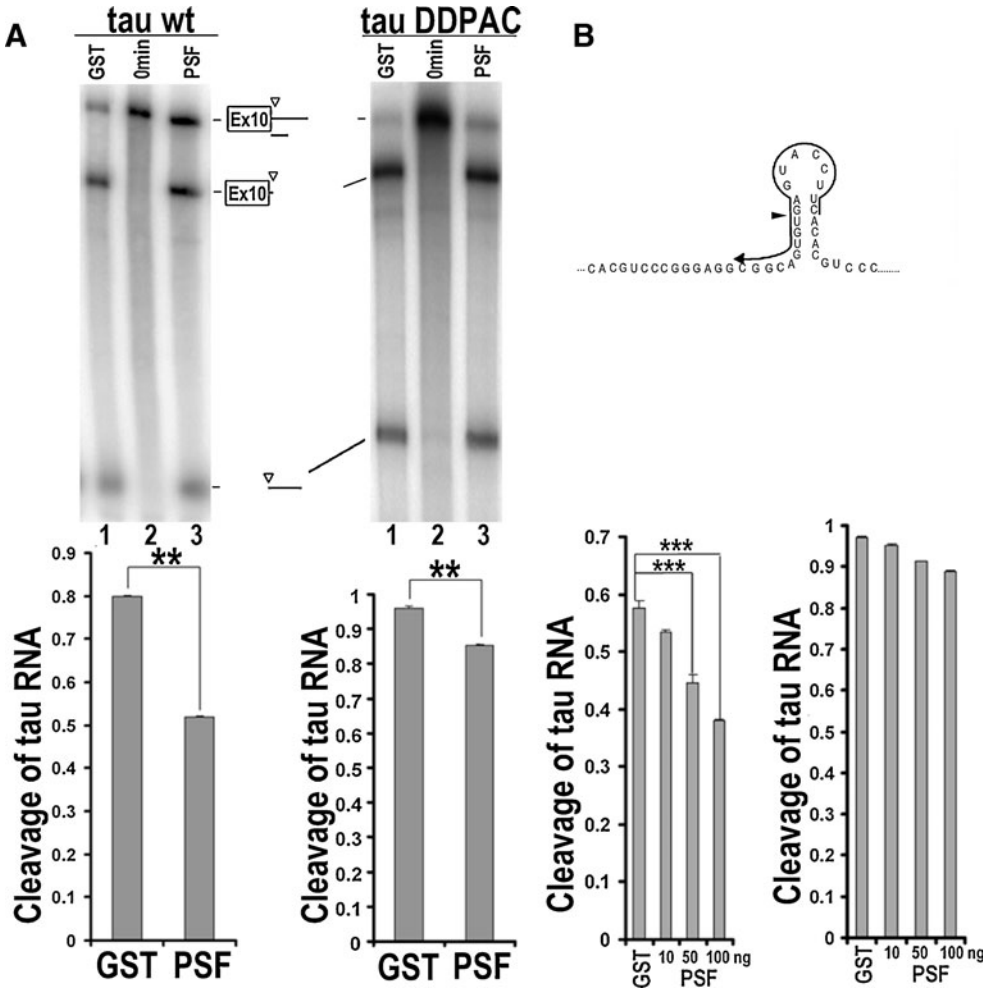


**Fig. 5** Oligonucleotide directed RNaseH cleavage assay shows that PSF protects the 5′ splice site of *tau* exon 10. **A** Radiolabeled *tau* wild-type (*left panel*) or DDPAC mutant (*right panel*) pre-mRNA transcripts containing exon 10 and intron 10 were incubated with RNase H and the oligonucleotide complementary to the 5′ splice site at 37°C for 20 min under splicing conditions in the presence of purified PSF (*lanes 2 and 3*) or control GST protein (*lanes 1*). After incubation, RNA was isolated, separated on denaturing gel and detected by autoradiography. *Lane 2* (0 min) in both the panels contain corresponding input RNA transcripts. *Tau* RNA transcript and its RNase H cleavage products are illustrated in the diagram between the two panels in **A**, with the *arrowhead* indicating the cleavage site and the *horizontal bar* below exon10–intron 10 boundary representing the DNA oligomer that is complementary to the 5′ splice site. *Bottom panel* represents the quantification of data from *lanes 1 and 3* in each panel, of cleavage of *tau* RNA as detected by autoradiography. Data are an average of four independent experiments. **B** RNase H cleavage assay performed with the 48 nucleotide oligomer under same conditions as described in **A** but with increasing amounts of purified PSF protein. Cleavage products were analyzed on denaturing gel followed by autoradiography, and efficiency of cleavage was determined by calculating the ratio of total cleavage products to the corresponding total transcript input. *Top panel* represents a schematic representation of the stem-loop structure with the *arrowhead* showing the cleavage site

transcripts. Previous studies have shown that different RNA secondary structures may influence interaction of the 5′ splice site with U1snRNP and play a role in regulating alternative splicing (Blanchette and Chabot 1997; Buratti and Baralle 2004; Eperon et al. 1988; Zychlinski et al. 2009). A stem-loop secondary structure has been reported in the 5′ splice site of *SMN2* exon 7 that regulates *SMN2* exon 7 alternative splicing (Singh et al. 2007). Other genes in which specific RNA secondary structures are associated with regulation of alternative exon inclusion include *dystrophin* (Matsuo et al. 1992) and the *β-tropomyosin* gene (Clouet d'Orval et al. 1991; Libri et al. 1991; Sirand-Pugnet et al. 1995).

PSF expression in the mouse brain is the highest around embryonic stage 16 (E16) and gradually decreases in the adulthood. In the developing mouse brain, PSF expression is high in the post-mitotic differentiating neurons in the cortex and cerebellum, whereas in the adult brain, PSF expression appears highest in the olfactory bulb and the hippocampus (Chanas-Sacre et al.

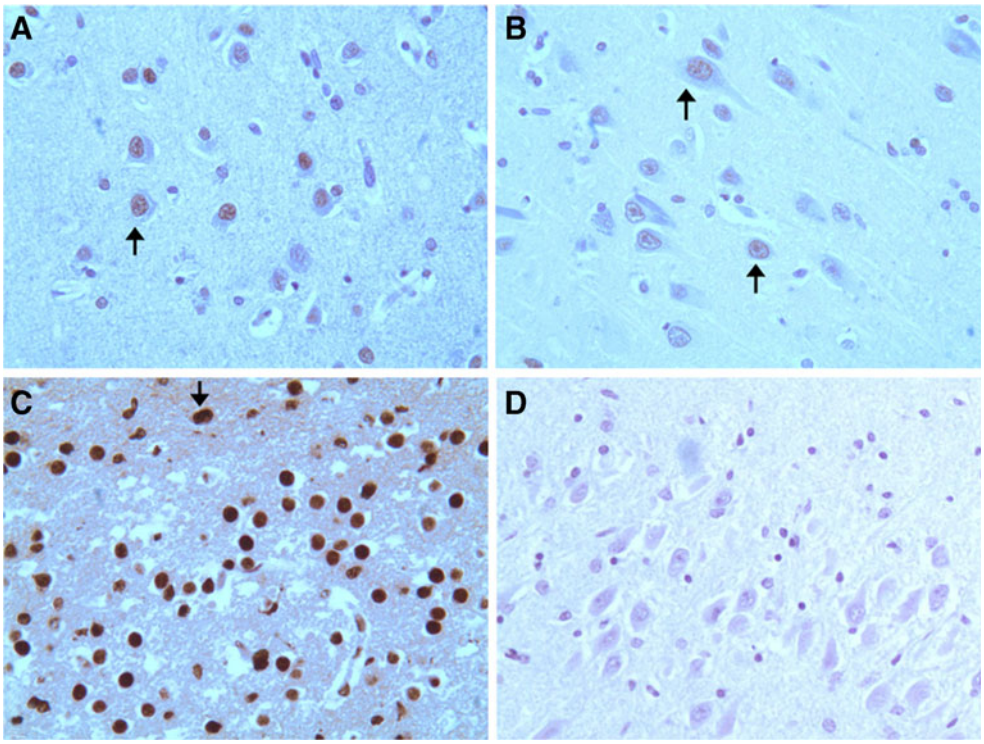


**Fig. 6** PSF is expressed in the brain regions affected by FTLD-tau. Human brain sections were stained using a specific anti-PSF antibody. **A**–**C** Staining of the adult frontal cortex (**A**), adult hippocampus (**B**), and fetal brain (**C**). **D** Negative control, staining of the hippocampal section in the absence of the primary antibody. PSF immunostaining was detected in the frontal cortex neurons (**A**) and hippocampal neurons (**B**, **C**). The *arrows* in **A** and **B** mark the pyramidal neurons. The pattern of staining is strong nuclear with fetal neurons displaying stronger signal. Stronger signals were detected in the fetal hippocampal neurons than that in the adult hippocampal neurons under the same staining conditions. All sections shown are of high magnification (×600)

1999). Interestingly, these are the brain regions that are frequently affected in tauopathy patients. Our immunohistochemical staining of the human fetal and adult brain sections showed higher levels of PSF in fetal hippocampus than that in the adult brain. This PSF expression pattern is consistent with the potential role of PSF in suppressing *tau* exon 10 inclusion in vivo. Previous work by our group shows that another regulatory factor, SRp54, is expressed at a high level in the fetal brain (Wu et al. 2006). These observations suggest that *tau* alternative splicing pattern may be a result of the complex interplay of different splicing factors whose expressions are controlled in a temporal and spatial manner. Although PSF expression is well documented in nervous system, neuronal specific splicing substrates of PSF had not been reported previously. Our study identifies *tau* pre-mRNA as a neuronally expressed alternative splicing substrate for PSF. A recent study reported an upregulation of PSF and the splicing factor SRp20 in mice during hippocampal memory formation (Antunes-Martins et al. 2007). Interestingly, SRp20 overexpression leads to increased exclusion of exon 10 not only in wild-type *tau* minigene but also in FTDP-17 mutants (Yu et al. 2004). It will be interesting to study how these splicing regulators act to affect learning and memory and whether their expression is altered in tauopathy patients.

In summary, we report the identification of PSF as a previously unknown alternative-splicing regulator of *tau* gene expression. This is the first report of PSF in regulating alternative splicing of a neuron-specific gene. PSF acts to repress the inclusion of *tau* exon 10 and gives rise to 3R isoform (Fig. 7). PSF functions by stabilizing the stem-loop structure at the 5′ splice site and blocking the accessibility of U1snRNP to the splice site. We also show that PSF is expressed in the brain regions that are affected in FTDP patients. The observation that PSF has a moderate effect on the splicing of *tau* DDPAC mutant that lacks a regular stem-

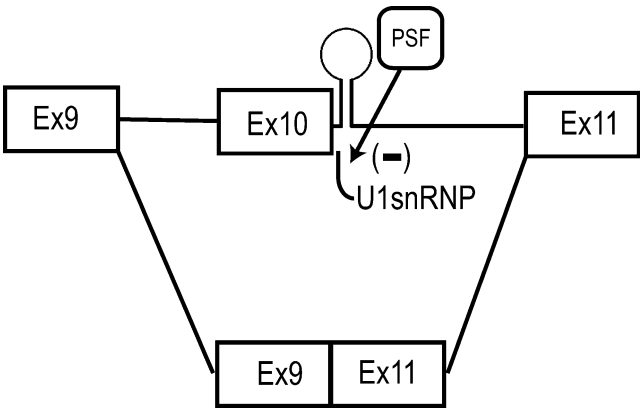


**Fig. 7** A model for the role of PSF in regulating *tau* exon 10 splicing. A schematic representation of *tau* exon 10 splicing regulation by PSF. Under normal conditions the closed stem-loop configuration is in a dynamic flux with the open configuration. PSF binds to and stabilizes the stem-loop structure at the 5′ splice site of exon 10 thereby facilitating the closed configuration. This prevents the association of U1snRNP with the 5′ splice site leading to exclusion of exon 10 from *tau* transcripts

loop secondary structure can be exploited to develop novel therapeutic approaches for FTDP patients.

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