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Regulation of the alternative splicing of tau exon 10 by SC35 and Dyrk1A

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ABSTRACT

Abnormal alternative splicing of *tau* exon 10 results in imbalance of 3R-tau and 4R-tau expression, which is sufficient to cause neurofibrillary degeneration. Splicing factor SC35, a member of the superfamily of the serine/arginine-rich (SR) proteins, promotes tau exon 10 inclusion. The molecular mechanism by which SC35 participates in tau exon 10 splicing remains elusive. In the present study, we found that tau pre-mRNA was coprecipitated by SC35 tagged with HA. Mutation of the SC35-like exonic splicing enhancer located at exon 10 of tau affected both the binding of SC35 to tau pre-mRNA and promotion of tau exon 10 inclusion, suggesting that SC35 acts on the SC35-like exonic splicing enhancer to promote tau exon 10 inclusion. Dyrk1A (dual-specificity tyrosine-phosphorylated and regulated kinase 1A) phosphorylated SC35 *in vitro* and interacted with it in cultured cells. Overexpression of Dyrk1A suppressed SC35′s ability to promote tau exon 10 inclusion. Downregulation of Dyrk1A promoted 4R-tau expression. Therefore, upregulation of Dyrk1A in Down syndrome brain or Alzheimer's brain may cause dysregulation of tau exon 10 splicing through SC35, and probably together with other splicing factors, leading to the imbalance in 3R-tau and 4R-tau expression, which may initiate or accelerate tau pathology and cause neurofibrillary degeneration in the diseases.

INTRODUCTION

Tau is a neuronal microtubule-associated protein that promotes microtubule (MT) assembly and stabilizes MT network. Therefore, tau plays important roles in neuronal morphogenesis, axon polarity and axonal transport (1,2). Aggregation of hyperphosphorylated tau in the brain causes diverse set of sporadic and familial neurodegenerative diseases called tauopathies (3,4).

The human *tau* gene lies on the long arm of chromosome 17 and contains 16 exons from which 6 different tau isoforms are generated in the adult central nervous system by alternative splicing of exons 2, 3 and 10 (5). The alternative splicing of exon 10 of the *tau* gene results in the presence or absence of the second MT-binding repeats, leading to the expression of tau containing either four (4R-tau) or three MT-binding repeats (3R-tau) (6,7). Approximately equal amount of 3R-tau and 4R-tau are expressed in normal adult human brain (8,9).

At least 39 different mutations in the *tau* gene have been identified from patients with frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) (10–12). More than a half of these mutations only alters splicing of exon 10 and consequently disrupts 3R-tau/4R-tau balance but does not disrupt tau's primary sequence (13). Thus, alteration in the 3R-tau/4R-tau ratio is sufficient to trigger neurodegeneration and dementia. Dysregulation of alternative splicing of human tau exon 10 is one of the important etiologic mechanisms in the pathogenesis of tauopathies.

Alternative splicing is regulated by multiple exonic and intronic *cis*-element and *trans*-acting splicing factors. Serine/arginine-rich (SR) proteins are one group of the splicing factors involved in alternative splicing (14,15). All SR proteins are highly conserved in eukaryotes and have a modular organization. They contain an N-terminal RNA-recognition motif (RRM) that interacts with the pre-mRNA and a C-terminal arginine–serine-rich (RS) domain that promotes protein–protein interactions within the splicing complex (16,17).

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

SC35 is one of the SR proteins, which was identified by monoclonal antibodies directed against purified spliceosomes (18). Previous studies reported that the extent of SC35 recruitment to alternatively spliced transcripts of tau exon 10 is related to exon 10 inclusion (19). Tau exon 10 contains a SC35-like enhancer at 5′-end (20,21). However, there is no direct evidence that SC35 acts on SC35-like enhancer to promote tau exon 10 inclusion.

SR protein is a phosphoprotein. Its function is highly regulated by the phosphorylation. Several kinases could phosphorylate SR proteins and regulate their function (22–25). We recently reported that dual-specificity tyrosine-phosphorylated and regulated kinases 1A (Dyrk1A) phosphorylates SF2/ASF, a SR protein and inhibits its promotion of *tau* exon 10 inclusion (26). Dyrk1A is a proline- and arginine-directed Ser/Thr kinase. It lies at the Down syndrome (DS) critical region of chromosome 21 and contributes to several phenotypes of DS in transgenic mice (27,28). Individuals with DS develop Alzheimer-type neurofibrillary degeneration as early as the fourth decade of life (29). Overexpression of Dyrk1A due to an extra-copy of chromosome in DS brain leads to the dysregulation of tau exon 10, resulting in an increase in 3R-tau expression and causing early onset of tau pathology in DS brain (26). The early onset of tau pathology in DS could also be caused or promoted by over-production of amyloid-β peptide as a result of an extra copy of the gene for amyloid-β precursor protein, which is also located on chromosome 21. Overexpression of Dyrk1A changes the distribution of SC35 from speckles to more diffuse in nucleus (30), suggesting that Dyrk1A may also modulate SC35′s function.

In the present study, we investigated the molecular mechanism by which SC35 regulates tau exon 10 splicing and Dyrk1A regulates SC35-mediated tau exon 10 splicing. The findings of this study suggest that SC35 promotes exon 10 inclusion by acting on the SC35-like enhancer and that Dyrk1A phosphorylates SC35 and suppresses its function in promotion of tau exon 10 inclusion.

MATERIALS AND METHODS

Plasmids and antibodies

pCEP4/SC35-HA was a gift from Dr Tarn of the Institute of Biomedical Sciences, Academia Sinica, Taiwan. Mammalian expression vector pcDNA3.1 containing either rat Dyrk1A or kinase-dead $Dyrk1A_{K188R}$ were described previously (26). pCI/SI9–SI10 containing a tau minigene, SI9/SI10, comprising tau exons 9, 10 and 11, part of introns 9 and 10 was as described (31). Mouse monoclonal antibody 8D9 was raised against a histidine-tagged protein containing the first 160 residues of rat Dyrk1A (32). Rabbit polyclonal anti-HA, mouse monoclonal anti-HA and mouse monoclonal anti-β-actin were from Sigma (St Louis, MO, USA). Rabbit polyclonal anti-tau (R134d) was described previously (33). Peroxidase-conjugated anti-mouse and anti-rabbit IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA); tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG, and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG, siRNAs of human or mouse Dyrk1A, and siRNA of mouse SC35 were from Santa Cruz Biotechnology (Santa Cruz, CA). The ECL kit was from Thermo Scientific (Rockford, IL, USA), and [γ-^{32}P] ATP and [^{32}P] orthophosphate were from MP Biomedicals (Irvine, CA, USA). Alkaline phosphatase from bovine calf intestine was from Sigma (St Louis, MO, USA)

Plasmid construction and DNA mutagenesis

pGEX-2T/SC35 was constructed by PCR amplification from pCEP4/SC35 and subcloned into pGEX-2T to express GST-SC35 protein. The deletion mutations of SC35 were generated by amplifying an individual fragment, which contains part of the SC35 coding region into the HindIII–XhoI sites of pCEP4. Mutants of SI9/SI10 were created by site-directed mutagenesis using KOD-Plus-Mutagenesis kit (TOYOBO) with primers (forward, 5′gg ctaccaaaggtgcGgataattaataagaagctggatctta3′, and reverse, 5′taagatccagcttcttattaattatc**A**gcacctttggtagcc3′) for SI9/$SI10_{E10A5G}$, primers (forward, 5′ctaccaaaggtgca**T**ataattaa taagaagctggatcttag3′, and reverse, 5′ctaagatccagcttcttattaa ttatTtgcacctttggtag3′) for SI9/$SI10_{E10G6A}$ and primers (forward, 5′tggctaccaaaggtgattaataagaagctggatcttagcaac3′, and reverse, 5′gttgctaagatccagcttcttattaatcacctttggta gcca3′) for SI9/$SI10_{E10\Delta3\text{-}9}$.

Cell culture and transfection

COS-7, HEK-293T, N2a, 3T3, SH-SY5Y and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen, Carsbad, CA, USA) at 37°C (5% CO_2). Transfections were performed with Lipofectamine 2000 (Invitrogen, Carsbad, CA, USA) or FuGene 6 (Roche, Indianapolis, IN), according to the manufacture's instructions.

Expression and purification of recombinant SC35

For purification of GST-SC35, pGEX-2T/SC35 plasmid was transformed into BL21 (DE3) host strain for expression. A 1 l culture of *Escherichia coli* harboring pGEX-2T/SC35 plasmid was grown at 37°C to OD600 $\approx$0.6, and induced with 0.5 mM IPTG at 16°C overnight before harvesting by centrifugation. Cells were re-suspended in 30 ml of lysis buffer (50 mM Tris–HCl, pH 7.8, 150 mM NaCl, 1 mM DTT, 1 mM EDTA and protease inhibitor cocktail) and lysed with sonication. All purification procedures were carried out at 4°C. The total soluble fraction was recovered by centrifugation at 36 000*g* for 60 min, and loaded onto 10 ml of glutathione sepharose beads. The resin was washed with 2 M NaCl high salt buffer (50 mM Tris–HCl, pH 7.8, 1 mM DTT, 1 mM EDTA, 10% glycerol) and subsequently with 50 mM NaCl low salt buffer. The GST-fused SC35 bound to GST beads was eluted with 10 mM glutathione in 50 mM Tris–HCl, pH 8.0, 50 mM NaCl and dialyzed against 50 mM Tris–HCl, pH 7.4). The purified GST-SC35 was aliquoted and stored at −80°C.

For purification of HA-SC35 from cultured mammalian cells, pCEP4-SC35 was transfected into HEK-293T Cells with FuGENE 6 for 48 h, and then cells were lysed in 0.5 ml of IP lysis/wash buffer (Pierce Crosslink Immunoprecipitation Kit). One milligram of cell lysate protein was added to protein G-Sepharose beads cross-linked to anti-HA and incubated overnight at 4°C. After extensively washing, SC35 was eluted in three successive 100 µl fractions with elusion buffer provided in the kit and neutralized with 1 M Tris.

Phosphorylation of SC35 by Dyrk1A *in vitro*

For phosphorylation of SC35 by Dyrk1A *in vitro*, GST-SC35 or GST (0.2 mg/ml) was incubated with various concentrations of Dyrk1A in a reaction buffer consisting of 50 mM Tris–HCl, pH 7.4, 10 mM β-mercaptoethanol, 0.1 mM EGTA, 10 mM $MgCl_2$ and 0.2 mM [γ-^{32}P] ATP (500 cpm/pmol). After incubation at 30°C for 30 min, the reaction was stopped by adding an equal volume of 2× Laemmli sample buffer and boiling. The reaction products were separated by SDS–PAGE. Incorporation of ^{32}P was detected by exposure of the dried gel to phosphorimaging system (BAS-1500, Fuji film).

Dephosphorylation of SC35 by alkaline phosphatase *in vitro*

SC35 was overexpressed in HEK-293T cells and immnoprecipitated with anti-HA crosslinked onto protein G beads as described above. The immunocomplex on the beads was dephophorylated with alkaline phosphatae in reaction buffer (50 mM Tris–HCl, pH 8.5, 10 µg/ml aprotinin, 10 µg/ml leupeptine, 10 µg/ml pepstatin and 1 mM AEBSF) for 30 min at 37°C. The dephosphorylated product was eluted with 2× Laemmli buffer and boiling and subjected to western blot analysis.

GST pull down

GST or GST-SC35 was purified by affinity purification with glutathione Sepharose without elution from the beads. Beads coupled with GST or GST-SC35 were incubated with crude extract from rat brain homogenate in buffer (50 mM Tris–HCl, pH 7.4, 8.5% sucrose, 50 mM NaF, 1 mM Na_3VO_4, 0.1% Triton X-100, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 10 µg/ml pepstatin). After 4 h incubation at 4°C, the beads were washed with washing buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl and 1 mM dithiothreitol) six times and the bound proteins were eluted by boiling in Laemmli sample buffer, and the samples were subjected to western blot analysis.

CO-IMMUNOPRECIPITATION

HEK-293T cells were co-transfected with pCEP4-SC35-HA and pcDNA3.1-Dyrk1A for 48 h. The cells were washed twice with phosphate-buffered saline (PBS) and lyzed by sonication in lysate buffer containing phosphatase and protease inhibitors (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, 2 µg/ml leupeptin and 2 µg/ml pepstatin). Insoluble materials were removed by centrifugation; Protein G beads were incubated with anti-HA overnight at 4°C, and then the antibody bound beads were incubated with the cell lysate. After a 4 h incubation at 4°C, the beads were washed with lysate buffer twice and with Tris-buffered saline twice, and bound proteins were eluted by boiling in Laemmli sample buffer. The samples were subjected to western blot analysis with the indicated primary antibodies.

Co-localization study

HeLa cells were plated in 24-well plates onto coverslips 1 day prior to transfection at 30–40% confluence. These cells were transfected with HA-tagged SC35 constructs or co-transfected with Dyrk1A as described above. Two days after transfection, the cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. After washing with PBS, the cells were blocked with 10% goat serum in 0.2% Triton X-100/PBS for 2 h at 37°C and incubated with rabbit anti-HA (1:200) and mouse anti-Dyrk1A (8D9, 1:5000) overnight at 4°C. After washing and incubation with secondary antibodies (TRITC-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG, 1:200), the cells were washed extensively with PBS and incubated with 5 µg/ml Hoechst 33 342 for 5 min at room temperature. The cells were washed with PBS, mounted with Fluoromount-G and visualized with a Leica TCSSP2 laser-scanning confocal microscope.

Quantitation of tau exon 10 splicing by reverse transcription–PCR

Total cellular RNA was isolated from cultured cells by using an RNeasy mini kit (Qiagen GmbH). Six hundred nanograms of total RNA was used for first-strand cDNA synthesis with oligo (dT)18 by using an Omniscript reverse transcription kit (Qiagen GmbH). PCR was performed by using Prime-STARTM HS DNA Polymerase (Takara Bio Inc., Otsu, Shiga, Japan) with primers (forward 5′-GGTG TCCACTCCCAGTTCAA-3′ and reverse 5′-CCCTGGT TTATGATGGATGTTGCCTAATGAG-3′) for transfected pCI/SI9–SI10, and with primers (forward 5′-AAC ACCGCCCACCCGGGAG-3′ and reverse 5′-GTCTGTC TTGGCTTTGGCATTCTC-3′) for endogenous mouse tau to measure alternative splicing of tau exon 10 under conditions: denaturation for 5 min at 98°C was followed by 30 cycles with denaturation for 10 s at 98°C, annealing for 15 s at 55°C, polymerization for 30 s at 72°C and a final extension for 10 min at 72°C. The PCR products were resolved on 1.5% agarose gels and quantitated using the Molecular Imager system (Bio-Rad).

Electrophoretic mobility shift assay

Tau RNA primer of the wild-type SC35-like element 5′-G UGCAGAUAAUUAAUAAGAAGCUGGAUCUU-3′ (Tau-RNA) or RNA primer of SC35-like element deleted 5′-GUAUUAAUAAGAAGCUGGAUCUU-3′ (Tau-$RNA_{\Delta SC35\text{-like}}$) was labeled with [$\gamma$-^{32}P]ATP (4500 Ci/mM) using T4 polynucleotide kinase (New England Biolabs)

and subsequently purified with MicroSpin G-25 column (Amersham Biosciences). To perform electrophoretic mobility shift assay (EMSA), the immunopurified SC35 in 50 mM Tris–HCl buffer (pH 7.5) containing 50–mM NaCl, 1 mM EDTA and 1 mM dithiothreitol was mixed with ^{32}P-labeled wild-type SC35-like RNA primer or SC35-like enhancer deleted RNA primer in a total volume of 10 µl. The reaction mixture was incubated at 37°C for 40 min, and analyzed with a 6% non-denaturing polyacrylamide gel, which was pre-run at 100 V for 10 min. Electrophoresis was carried out in TBE buffer (89 mM Tris borate, 2 mM EDTA) at 100 V for 60 min. The gel was dried and visualized with a PhosphorImager (BAS-1500, Fujifilm). The RNA substrates used in all experiments were at 2.4 nM, and the amounts of proteins were indicated in the figure legends.

RNA immunoprecipitation

The RNA immunoprecipitation (RNA-IP) experiment was performed as described (34–36). Briefly, HEK-293T cells co-transfected with pCEP4/SC35 and pCI/SI9–SI10 were crosslinked with 1% formaldehyde for 10 min at room temperature. After quenching with 125 mM glycine, the cells were lyzed in lysis buffer (16.7 mM Tris–HCl, pH 8.1, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 1× Roche protease inhibitors cocktail and 50 U/ml RNasin® Plus RNase Inhibitor) on ice for 10 min, and centrifuged at 2000 *g* for 5 min to pellet nuclei. The nuclear fraction was sonicated in buffer B (50 mM Tris–HCl, pH 8.1, 1% SDS, 10 mM EDTA, 1× protease inhibitors cocktail and 50 U/ml RNasin® Plus RNase Inhibitor). After centrifugation at 16 000*g* for 10 min, the supernatant was subject to immunoprecipitation with anti-HA in IP buffer (16.7 mM Tris–HCl, pH 8.1, 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 1× protease inhibitors cocktail and 50 U/ml RNasin® Plus RNase Inhibitor) for 2 h. Immune-complex was washed sequentially with low-salt buffer (20 mM Tris–HCl, pH 8.1, 150 mM NaCl, 0.1% SDS, 1% Triton X-100 and 2 mM EDTA), with high-salt buffer (20 mM Tris–HCl, pH 8.1, 500 mM NaCl, 0.1% SDS, 1% Triton X-100 and 2 mM EDTA), with LiCl buffer (10 mM Tris–HCl, pH 8.1, 250 mM LiCl, 1% NP-40, 1% deoxycholate and 1 mM EDTA), and with TE buffer (10 mM Tris pH 8.0, 1 mM EDTA). Immune-complex was eluted with elution buffer (1% SDS, 0.1 M $NaHCO_3$ and 50 U/ml RNasin® Plus RNase Inhibitor). The crosslinking was reversed by incubation with 200 mM NaCl at 65°C for at least 2 h. After digestion with 0.4 mg/ml Proteinase K (Invitrogen) at 42°C for 45 min and 1 mg/ml of RQ1 Rnase-free Dnase (Promega) at 37°C for 15 min, respectively, RNA was extracted by RNeasy Mini Kit (Qiagen) and subjected to first-strand cDNA synthesis with random primer or Oligo-(dT)15–18 by using the Omniscript Reverse Transcription Kit (Qiagen). cDNA was amplified by PrimeSTARTM HS DNA Polymerase (Takara Bio Inc.) with two sets of primers against tau introns 9 and 10: one set primers: Forward 5′-AGGCGGGTCCAGGGTGGCGTGTCAC TCATCC-3′, Reverse 5′-CTAATAATTCAAGCCACAG CACGGCGCATGGGACG-3′; another set of primers: Forward 5′-AGGGTGGCGCATGTCACTCATCGAAA GTGGAGGCG-3′, Reverse 5′-GGATTTATTCTATG C AGTGTCTCGCAAGTGTACGC-3′. An initial denaturation for 5 min at 98°C was followed by 30 cycles with denaturation for 10 s at 98°C, annealing for 15 s at 55°C, polymerization for 30 s at 72°C and a final extension for 10 min at 72°C. PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining.

RESULTS

SC35 promotes tau exon 10 inclusion

To elucidate the role of SC35 on tau exon 10 splicing, we co-transfected mini-tau gene pCI/SI9–SI10, consisting of tau exons 9, 10 and 11, part of introns 9 (SI9) and 10 (SI10) (31) together with various amount of pCEP4/SC35 into HEK-293T cells. The transfected cells were harvested at 48 h and analyzed for the splicing product of tau exon 10 by reverse transcription (RT)–PCR. We found that overexpression of SC35 promotes the tau exon 10 inclusion concentration dependently. The ratio of inclusion/exclusion of tau exon 10 increased along with transfection concentration and the peak appeared at 0.8 µg/well pCEP4/SC35 transfection (Figure 1A). To examine whether the effect of SC35 on tau exon 10 splicing was cell type specific, we also co-transfected pCI/SI9–SI10 and

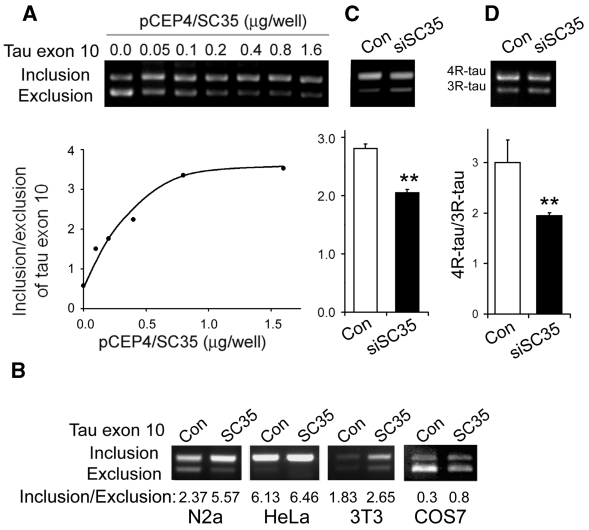

Figure 1. SC35 promotes tau exon 10 inclusion. (**A**) SC35 promoted tau exon 10 inclusion dose dependently. The pCI/SI9–SI10 mini-tau-gene was co-transfected with different amount of pCEP4-SC35 into HEK-239T cells. Total RNA was subjected to RT–PCR for measurement of tau exon 10 splicing after 36 h transfection. (**B**) SC35 promoted tau exon 10 inclusion cell-type independently. pCI/SI9–SI10 was co-transfected with pCEP4/SC35 into various cell lines indicated under each panel. Tau exon 10 splicing was measured by RT–PCR after 36 h transfection. (**C** and **D**) siRNA of SC35 suppressed tau exon 10 inclusion. pCI/SI9–SI10 was co-trasnfected with siRNA of SC35 into N2a cells for 48 h, and then the splicing products of tau exon 10 of mini gene (C) and endogenous mouse tau (D) were measured by RT–PCR. The same amount of scramble siRNA was used for controls. The data are presented as mean ± SD. $^{**}P < 0.01$.

pCEP4/SC35 into N2a, HeLa, 3T3 and COS7 cells. We found that SC35 promoted tau exon 10 inclusion in these four types of cells as well (Figure 1B). Thus, SC35 promotes tau exon 10 inclusion.

To confirm the effect of SC35 on the promotion of tau exon 10 inclusion, we transfected siRNA of SC35 or control siRNA with pCI/SI9–SI10 into N2a cells to knock down SC35 expression and then measured the splicing products by RT–PCR. We found that as compared with control siRNA, siRNA of SC35 suppressed exon 10 inclusion and decreased the ratio of tau exon 10 inclusion to exclusion significantly (Figure 1C).

To determine the role of SC35 in alternative splicing of endogenous tau exon 10, we knocked down the expression of SC35 by siRNA of SC35 in N2a cells and then measured the splicing products of mouse tau exon 10 by RT–PCR. We observed that siRNA of SC35 suppressed mouse 4R-tau expression and decreased the ratio of 3R-tau/4R-tau significantly (Figure 1D), which suggests that SC35 also works on endogenous tau and promotes tau exon 10 inclusion.

SC35 binds to the pre-mRNA of tau via SC35-like element of exon 10

To determine the molecular mechanism by which SC35 promotes the inclusion of tau exon 10, we co-transfected pCI/SI9–SI10 with pCEP4/SC35-HA into HEK-293T cells, and immunoprecipitated SC35 with anti-HA antibody from the cell lysates. The co-immunoprecipitated pre-mRNA of tau with SC35 by anti-HA was amplified with RT–PCR by using two kinds of primers, random primer and oligo dT primer, for reverse transcription and two sets of primers against introns 9 and 10 of tau to get 194 and 294 bp of PCR products, respectively (Figure 2A). We observed that pre-mRNA of the mini-tau-gene was co-immunoprecipitated with SC35 (Figure 2A and B), suggesting that SC35 could act on the pre-mRNA of tau.

Next, we determined which domain of SC35 was responsible for recruitment to exon 10 of pre-mRNA of the mini-tau-gene. SC35 contains one RNA-recognition motif (RRM) at its N-terminal half and a characteristic SR-rich protein–protein interaction domain at its C-terminus. We transfected a set of previously characterized domains of SC35 (Figure 2C) together with the pCI-SI9/SI10 mini-tau-gene into HEK-293T cells. After 48 h transfection, RNA-IP was also used to detect the binding ability between the fragments of SC35 and pre-mRNA of the mini-tau-gene. We found that HA-$SC35_{1–117}$ showed the strongest binding ability to the mini-tau-gene among the SC35 fragments (Figure 2D). However, only $SC35_{FL}$ and $SC35_{1–191}$ promoted tau exon 10 inclusion (Figure 2E). These results indicate that SC35 binds with pre-mRNA of mini-tau-gene through its RRM and that both RRM and RS domains are required to promote tau exon 10 inclusion.

SC35-dependent splicing enhancers are known to contain UGCNGYY sequence (37). A SC35-like enhancer located at the 5′-end of tau exon 10 contains the sequence TGCAGAT (38). Whether the binding between

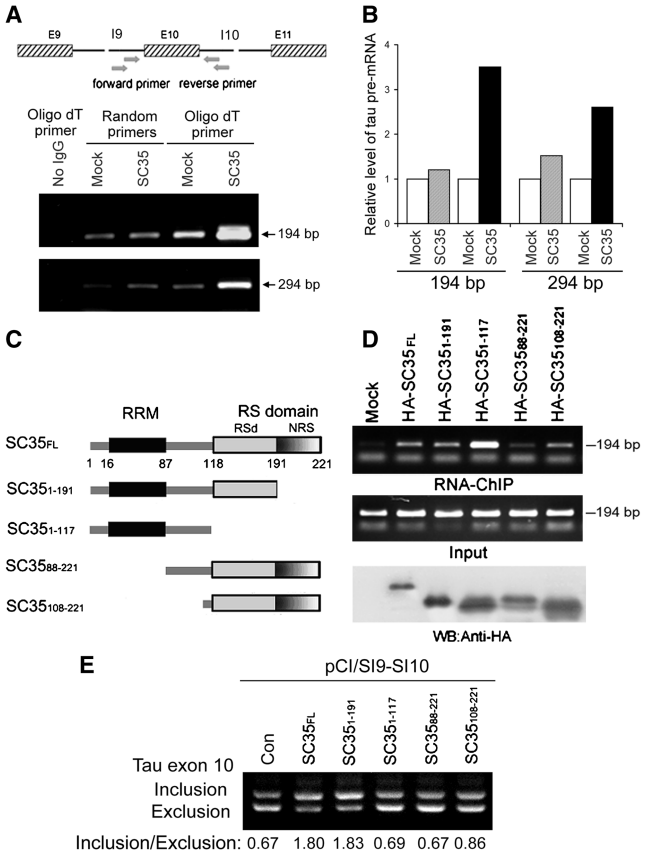

Figure 2. SC35 acts on exon 10 of tau pre-mRNA to promote tau exon 10 inclusion. (**A**) Tau pre-mRNA could be immunoprecipitated by SC35. pCI/SI9–SI10 was co-transfected with pCEP4/SC35-HA into HEK-293T cells. SC35 was immunoprecipitated with anti-HA antibody. Co-immunoprecipitated pre-mRNA of tau with SC35 was determined by RT–PCR with random primer or oligo-dT for generating cDNA and with two sets of primers specific to introns 9 and 10 as indicated for amplifying the cDNA derived from tau pre-mRNA. The RT–PCR product was separated by agarose electrophoresis and quantitated by densitometry and presented in **B** from two separated experiments. (**C**) Schematic of SC35 deletion mutants. (**D**) Tau pre-mRNA was immunoprecipitated by deletion mutants of SC35 differentially. Different deletion mutants of SC35 showed in panel C tagged with HA were overexpressed in pCI/SI9–SI10 transfected HEK-293T cells. RNA-IP was carried out with anti-HA antibody and co-immunoprecipitated pre-mRNA of tau was measured by RT–PCR as in panel A. Total pre-mRNA of tau, Input, was also measured by RT–PCR with same primers. The immunoprecipitated deletion mutations of SC35 were examined by western blot using anti-HA antibody (lower panel). (**E**) Deletion mutations of SC35 promoted tau exon 10 inclusion differentially. pCI/SI9–SI10 was co-transfected with different deletion mutants of SC35 into HEK-293T. Total RNA was extracted and subjected to RT–PCR for measurement of tau exon 10 splicing after 36 h transfection.

SC35 and pre-mRNA of tau exon 10 depends on SC35-like enhancer remains elusive. To answer this question, HA-tagged SC35 was overexpressed in HEK-293T cells and purified using Protein G beads crosslinked with anti-HA monoclonal antibody. Two major forms of SC35 were eluted in the first fraction, and only one form was found in fractions 2 and 3 (Figure 3A, left panel). It is well-known that phosphorylation affects the gel mobility

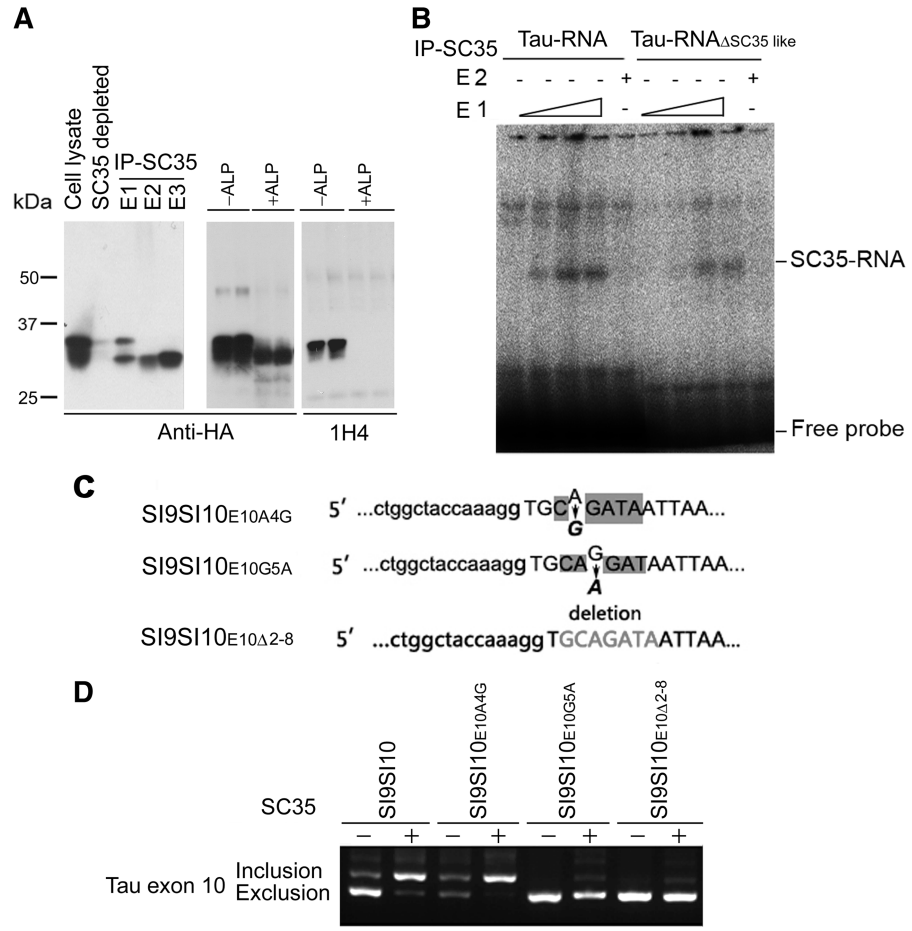

Figure 3. SC35 promotes tau exon 10 inclusion through SC35-like enhancer. (**A**) SC35 was immunopurified by anti-HA. SC35 tagged with HA was overexpressed in HEK-293T cells and immunopurified with anti-HA-crosslinked protein G beads. Different elution fractions (left panel) were subjected to western blot analysis with anti-HA. Immunoprecipitated SC35 without elution was dephosphorylated with alkaline phosphatase and determined by anti-HA and 1H4, an antibody to phosphorylated SR proteins. E1, elution fraction 1; E2, elution fraction 2; E3, elution fraction 3; ALP, alkaline phosphatase. (**B**) SC35 bound to RNA of tau exon 10. Immunopurified SC35 (E1 and E2) was incubated with tau pre-mRNA (tau-RNA) or SC35-like enhancer deleted tau pre-mRNA (tau-$RNA_{\Delta SC35\ like}$) pre-labeled with γ-^{32}P ATP. The incubation products were subjected to native-gel electrophoresis. After drying, the gel was analyzed with phosphoimaging device (BAS-1500, Fujifilm). (**C**) Schematic of mutations of mini-tau-gene on SC35-like enhancer of tau exon 10. (**D**) Mutations of SC35-like enhancer affected SC35 promoted tau exon 10 inclusion. Different mutants of mini-tau-gene, pCI/SI9–SI10, at SC35 like enhancer were transfected alone or together with pCEP4/SC35. RT–PCR was carried out to measure tau exon 10 splicing after 36 h transfection.

of proteins. To define whether phosphorylation slows down the mobility of SC35, we immunopurified SC35 with anti-HA crosslinked protein G beads, and then dephosphorylated it with alkaline phosphatase. The dephosphorylated product was subjected to western blots with anti-HA and 1H4, an antibody against phospho-SR proteins. We found that upon dephosphorylation with alkaline phosphatase, the upper band of SC35 disappeared and the lower SC35 band was not recognized by antibody 1H4 (Figure 3A, right two panels). These results indicated that the upper SC35 band was the phosphorylated SC35 and the lower band was the unphosphorylated one. To determine whether SC35 binds to tau RNA and whether its phosphorylation affects the binding, EMSA was carried out. We observed that the phosphorylated SC35 bound RNA oligomer containing SC35-like sequence in a dose-dependent manner (Figure 3B, lanes 2–4), whereas the unphosphorylated SC35 completely lost the binding ability. The binding activity of SC35 to the RNA oligomer was decreased when CAGATA was deleted in the SC35-like ESE. These data suggest that SC35 binds SC35-like enhancer specifically and the binding requires its phosphorylation.

To validate our hypothesis that SC35 promotes tau exon 10 inclusion by acting on the SC35-like enhancer of exon 10 of tau pre-mRNA, we transfected the wild-type and the mutant SI9/SI10 tau mini-genes alone or together with pCEP4/SC35 into HEK-293T cells. The level of the alternative splicing of exon 10 was detected by RT–PCR. The results showed that mutation of A to G at the fourth base pair of exon 10 increased the inclusion of exon 10 (Figure 3D, lane 3), while mutation of G to A at the fifth base pair of exon 10 dramatically inhibited the exon 10 inclusion (Figure 3D, lane 5). Deletion of

SC35-like enhancer, GCAGATA, also suppressed the exon 10 inclusion (Figure 3D, lane 7). Overexpression of SC35 increased tau exon 10 inclusion markedly in SI9–SI10 and in SI9–SI10$_{E10A4G}$, but much less in SI9–SI10$_{E10G5A}$ and SI9–SI10$_{E10\Delta2-8}$ (Figure 3D). These results verify that SC35-like enhancer acts as a splicing enhancer and that the promotion of tau exon 10 inclusion by SC35 depends on the SC35-like enhancer located on tau exon 10.

SC35 interacts with Dyrk1A

To address whether Dyrk1A regulates SC35 activity in splicing, we first studied the interaction between SC35 and Dyrk1A. GST-pull down was used to detect the protein–protein interaction *in vitro*. We found that Dyrk1A from rat brain extract was pulled down with GST-SC35, but not with GST itself (Figure 4A). To further validate the interaction, co-immunoprecipitation and confocal microscopy were employed in cultured cells. Dyrk1A could be co-immunoprecipitated with HA-SC35 by anti-HA antibody (Figure 4B), confirming the interaction between SC35 and Dyrk1A.

To study the interaction of SC35 with Dyrk1A in intact cells, we coexpressed HA-SC35 and Dyrk1A in HeLa cells and then immunostained the cells with anti-HA and anti-Dyrk1A. By employing confocal microscopy, we observed that both Dyrk1A and SC35 were mainly located in the nucleus, colocalized and enriched in the nuclear speckles, giving further evidence to their possible interaction in cultured cells (Figure 4C).

To map the domain of SC35 that interacts with Dyrk1A, HA-tagged full-length and various deletion mutations of SC35, HA-SC35$_{1–191}$, HA-SC35$_{1–117}$, HA-SC35$_{88–221}$ and HA-SC35$_{108–221}$, were coexpressed with Dyrk1A, respectively, in HEK-293T cells for co-immunoprecipitation assays or in HeLa cells for confocal microscopy. The results from co-IP showed that Dyrk1A was co-immunoprecipitated by SC35, SC35$_{1–191}$ or SC35$_{1–117}$, but not by SC35$_{88–221}$ or SC35$_{108–221}$ (Figure 5A), suggesting that the interaction between SC35 and Dyrk1A was through the N-terminal domain of SC35. HA-SC35$_{FL}$, HA-SC35$_{1–191}$ and HA-SC35$_{1–117}$ also showed subcellular colocalization with Dyrk1A, and they were enriched in the nuclear speckles (Figure 5B). However, HA-SC35$_{88–221}$ and HA-SC35$_{108–221}$ showed some what different distribution patterns from that of Dyrk1A, although they were both in the nucleus. These results further support that the N-terminus of SC35 interacts with Dyrk1A.

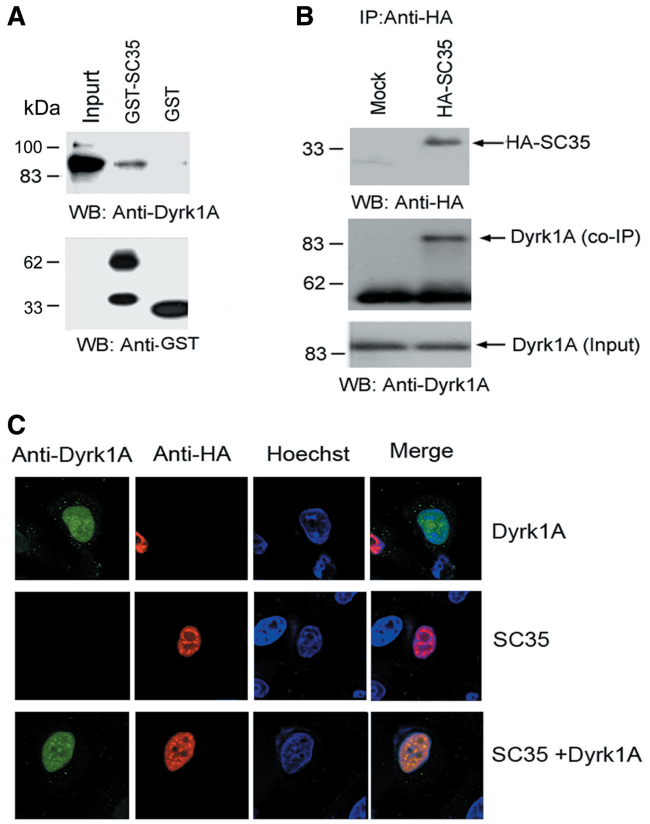

Figure 4. SC35 interacts with Dyrk1A. (**A**) Dyrk1A was pulled down from rat brain extract by GST-SC35. GST-SC35 or GST coupled onto glutathione Sepharose was incubated with rat brain extract. After washing, bound proteins were subjected to western blots using anti-GST and anti-Dyrk1A antibody. (**B**) Dyrk1A could be co-immunoprecipitated by HA-SC35 using anti-HA antibody. SC35 tagged with HA and Dyrk1A were coexpressed in HEK-293T cells for 48 h. The cell extract was incubated with anti-HA antibody coupled onto protein G beads. The bound proteins were subjected to western blots using antibodies indicated under each blot. (**C**) Colocalization of SC35 with Dyrk1A in nucleus. HA-SC35 and Dyrk1A were co-transfected into HeLa cells. After a 48 h transfection, the cells were fixed and immunostained by anti-HA or anti-Dyrk1A and followed by TRITC-anti-rabbit IgG or FITC-anti-mouse IgG. Hoechst was used for nuclear staining.

Dyrk1A inhibits SC35′s activity to promote tau exon 10 inclusion

Our observations of the physical interaction between SC35 and Dyrk1A led us to further investigate the functional relationship between them. We incubated GST-SC35 with Dyrk1A *in vitro* and found that GST-SC35, but not GST, was phosphorylated by Dyrk1A in an enzyme concentration-dependent manner (Figure 6A and B).

Then we studied the impact of Dyrk1A on the biological activity of SC35. pCI/SI9–SI10 was co-transfected with pcDNA3/Dyrk1A or pcDNA3/Dyrk1A$_{K188R}$ alone, or in combination with pCEP4/SC35, and the amounts of exon 10 inclusion and exclusion were measured by RT–PCR. We found that Dyrk1A, but not Dyrk1A$_{K188R}$, a dead enzyme, suppressed SC35′s activity to promote tau exon 10 inclusion (Figure 6C). These results suggest that phosphorylation of SC35 by Dyrk1A inhibits its activity to promote tau exon 10 inclusion.

To confirm that Dyrk1A suppresses tau exon 10 inclusion, we knocked down the expression of Dyrk1A by its siRNA (26) in pCI/SI9–SI10 transfected HEK-293FT cells and then measured splicing products by RT–PCR. We observed that transfection of Dyrk1A siRNA enhanced the SC35 promoted tau exon 10 inclusion dose dependently (Figure 6D). To determine the role of Dyrk1A in endogenous tau exon 10 splicing, we transfected siRNA of Dyrk1A into N2a or SH-SY5Y cells, both expressing tau, for 48 h, and then measured the products of the splicing by RT–PCR. We found that transfection with

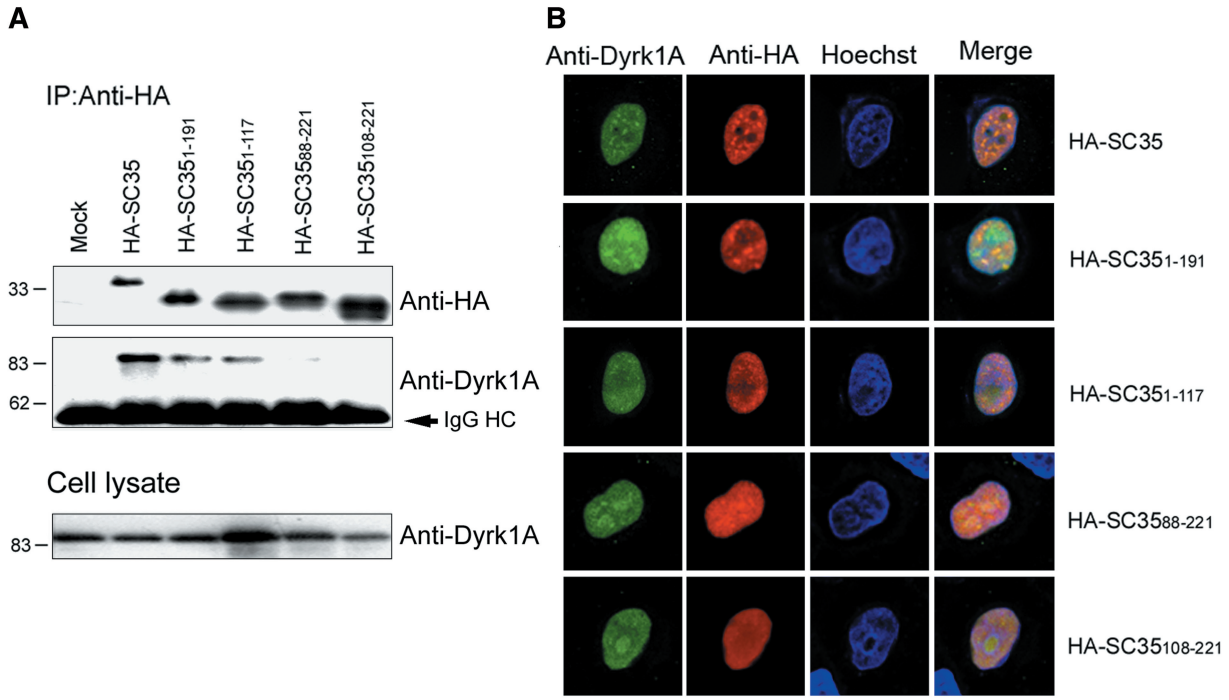

Figure 5. N-terminus of SC35 interacts with Dyrk1A. (**A**) Dyrk1A was co-immunoprecipitated by N-terminus of SC35 using anti-HA antibody. Dyrk1A was coexpressed with different deletion mutations of SC35 tagged with HA in HEK-293T cells for 48 h. The cell extract was incubated with anti-HA coupled to protein G beads. The bound proteins were subjected to western blots using antibodies indicated at the right of each blot. Dyrk1A in cell lysate was used as loading control (lower panel). (**B**) colocalization of N-terminus of SC35 with Dyrk1A in nucleus. HA-$SC35_{1–191}$, HA-$SC35_{1–117}$, HA-$SC35_{88–221}$ or HA-$SC35_{108–221}$ was co-transfected with Dyrk1A respectively into HeLa cells. After a 48-h transfection, the cells were fixed and immunostained by anti-HA or anti-Dyrk1A and followed by TRITC-anti-rabbit IgG or FITC-anti-mouse IgG. Hoechst was used for the staining of nuclei.

Dyrk1A siRNA significantly increased 4R-tau expression in the both cells (Figure 6E), suggesting that Dyrk1A also suppresses exon 10 inclusion of endogenous tau.

DISCUSSION

The present study provides the first directly experimental evidence that SC35 acts on the SC35-like enhancer located at the 5′-end of tau exon 10 and promotes tau exon 10 inclusion. Dyrk1A interacts with and phosphorylates SC35 and inhibits its activity to promote tau exon 10 inclusion. Taken together with our recent findings that Dyrk1A phosphorylates splicing factor SF2/ASF and suppresses SF2/ASF-promoted tau exon 10 inclusion (26,39), we conclude that up-regulation of Dyrk1A in DS individuals due to trisomy 21 may suppress the function of SC35 and SF2/ASF in promoting tau exon 10 inclusion and lead to increase in 3R-tau expression, which may initiate or accelerate tau pathology in DS brain. In addition, overproduction of amyloid-β peptide, as a result of an extra copy of the gene for amyloid-β precursor protein located on chromosome 21, in DS brain may also initiate or accelerate tau pathology. Therefore, the early onset of tau pathology in DS could result from the overexpression of both Dyrk1A and amyloid-β precursor protein.

Tau exon 10 is flanked by large introns 9 (13.6 kb) and 10 (3.8 kb). It has two weak splice sites, a weak 5′ splice site and a weak 3′ splice site (10,12,38). Several *cis*-elements in exon 10 and intron 10, which modulate the use of the weak 5′- and 3′ splice sites, have been identified and extensively characterized (20,21). The 5′ end of exon 10 contains three ESEs (exonic splicing enhancer), a SC35-like enhancer, a polypurine enhancer (PPE) and an A/C-rich enhancer (ACE). Splicing factors act on these elements and regulate the alternative splicing of tau exon 10. Mutations in these elements, including Δ280, N279K and L284L, cause FTDP17.

SC35-dependent splicing enhancers have the sequence of UGCNGYY (where Y = C or U, and N is any base) (37). The 5′-end of exon 10 of tau pre-mRNA contains the sequence, UGCAGAU, that matches with the above consensus sequence and was named as SC35-like enhancer (38). The present study showed that deletion of this element resulted in increased tau exon 10 exclusion, supporting that the SC35-like element acts as an enhancer. Mutation of guanosine to adenosine (UGCA**G**AU to UGCA**A**AU) led tau exon 10 exclusion, confirming that the G at base 5 is required for the enhancer. However, mutation of adenosine to guanosine at base 4 (UGC**A**AGAU to UGC**G**GAU) increased tau exon 10 inclusion, suggesting that the fourth base at SC35-dependent splicing enhancer, UGC**N**GYY, could modulate its splicing activity.

As implied by the name, SC35 acts on SC35-dependent splicing enhancer and promotes the exon inclusion. In the present study, by employing EMSA and RNA-IP, we

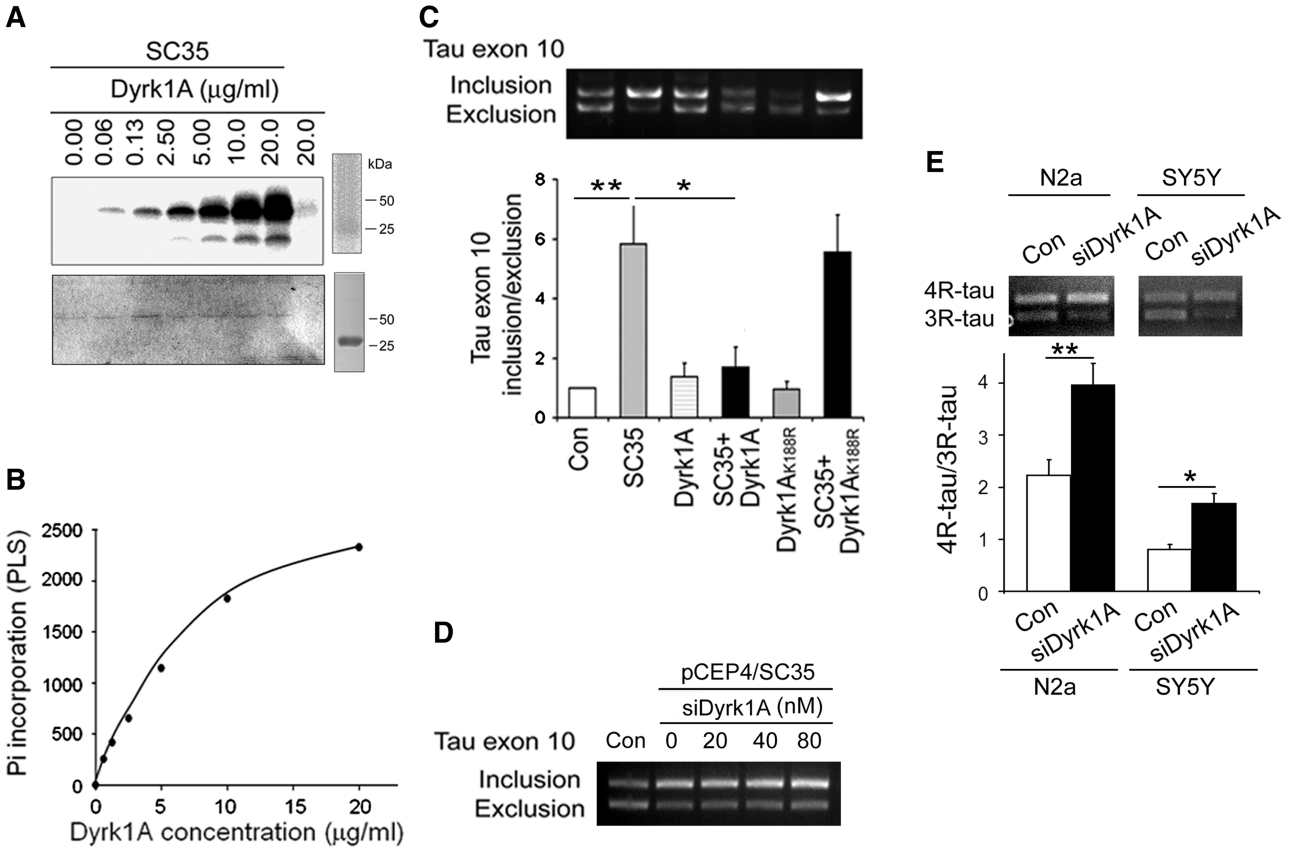

Figure 6. Dyrk1A phosphorylates SC35 and suppresses SC35 promoted tau exon 10 inclusion. **(A)** autoradiography of SC35 phosphorylation by Dyrk1A *in vitro*. Recombinant GST-SC35 was incubated with various concentrations of Dyrk1A indicated above each lane for 30 min at 30°C and separated by SDS–PAGE and visualized with Coomassie blue staining (lower panel). The last lane is Dyrk1A alone, without GST-SC35. After drying the gel, the ^{32}P incorporated into SC35 was measured by using a phosphorimaging device (BAS-1500, Fuji) (upper panel). **(B)** The incorporated ^{32}P into SC35 was by different concentration of Dyrk1A. **(C)** Dyrk1A, but not $Dyrk1A_{K188R}$, inhibited tau exon 10 inclusion promoted by SC35. pcDNA/Dyrk1A or pcDNA/$Dyrk1A_{K188R}$ was transfected only or together with SC35 into HEK-293T. Total RNA was subjected to RT–PCR for measurement of tau exon 10 splicing after 36 h transfection. **(D)** siRNA of Dyrk1A enhanced SC35-promoted tau exon 10 inclusion. pCEP4/SC35 was co-transfected with various concentration of siRNA of Dyrk1A into pCI/SI9–SI10 transfected HEK-293FT cells for 48 h, and the products of tau exon 10 splicing were measured by RT–PCR. **(E)** siRNA of Dyrk1A promoted 4R-tau expression. N2a or SH-SY5Y cells were transfected with Dyrk1A siRNA for 48 h, and then 3R-tau and 4R-tau were measured by RT–PCR. The same amount of scramble siRNA was used for controls. $^{*}P < 0.05$; $^{**}P < 0.01$.

demonstrated that SC35 mainly acts on SC35-like enhancer to promote the exon 10 inclusion and that the regulatory efficiency is dependent on SC35-like enhancer sequence. It is well known that in SR proteins, RRM interacts with the pre-mRNA and RS domain promotes protein–protein interactions within the splicing complex. SC35 has a RRM at its N-terminus and a RS domain at C-terminus. In this study, we observed that RRM bound to the pre-mRNA most strongly, and RS domain weakly interacts with the pre-mRNA. However, both RRM and RS domains are required for promoting tau exon 10 inclusion.

It has been reported that the serine residues within the RS domains of SR proteins are extensively phosphorylated. First, this phosphorylation appears to influence the subcellular localization of SR proteins (22,24). Second, phosphorylation affects protein interactions involving SR proteins (23,40). Both of them may change the ability of SR proteins in splicing function. To date, several kinases, including SR-protein kinases 1 and 2 (SRPK1 and SRPK2), the cell cycle-dependent dual specificity kinase (Clk/Sty), Akt/protein kinase B and DNA topoisomerase I (Topo I), have been reported to phosphorylate and regulate localization and function of SR proteins (22,24,25,41,42). SC35 is also phosphorylated by GSK-3β at N-terminal of SC35 primed by other kinases. Inhibition of GSK-3 increases 4R-tau expression (43). Amyloid-β peptide treatment reduces 4R-tau expression via the GSK-3β-SC35 pathway (44).

We previously found Dyrk1A phosphorylates SR proteins SF2/ASF and regulates their function in tau splicing (26). SF2/ASF plays a very important role in tau exon 10 inclusion (45). Dyrk1A phosphorylates and drives SF2/ASF into nuclear speckles, and prevents it from facilitating tau exon 10 inclusion (26). Overexpression of Dyrk1A due to trisomy 21 in DS brain leads to increased 3R-tau/4R-tau ratio, an imbalance that is known to associate with neurofibrillary

degeneration in Pick disease and some cases of FTDP-17 (26). Mutations of L266V, G272V, D280, E9+33, E10+19 and E10+29 result in an increase in tau exon 10 exclusion. The molecular mechanism of 3R-tau associated neurofibrillary degeneration in Pick's disease is unclear. It was reported that as compared with controls, the mRNA level of Dyrk1A in the hippocampus of patients with AD is increased (46). Our unpublished data also demonstrated that Dyrk1A was truncated and activated by calpain I in AD brain. Moreover, calpain I also proteolyzes GSK-3β and PKA-Cα, which promotes tau exon 10 exclusion and 3R-tau expression (47–49). Dyrk1A is a proline directed Ser/Thr kinase and phosphorylates Thr or Ser at the consensus motif of RX(X)T/SP of numerous proteins. SC35 harbors four Dyrk1A-consensus motifs. They are located at both RRM and RS domain. However, the exact phosphorylation sites of SC35 by Dyrk1A remain to be determined. In the present study, we demonstrated that Dyrk1A interacted with SC35 through the RRM of SC35, phosphorylated SC35 effectively and suppressed tau exon 10 inclusion, suggesting that an upregulation of Dyrk1A may cause an imbalance in the alternative splicing of tau exon 10, leading to or accelerating tau pathology in DS, AD and related disorders via ASF and SC35, as well as other splicing factors.

In summary, SC35 specifically binds to pre-mRNA product of the SC35-like enhancer on tau exon 10 both *in vitro* and *in vivo*. SC35 promotes tau exon 10 inclusion that is dependent on the SC35-like enhancer of SC35. Dyrk1A interacts and phosphorylates SC35, resulting in an inhibition of tau exon 10 inclusion promoted by SC35. These findings provide a new insight into mechanisms of the regulation of tau exon 10 splicing and shed new light into the dysregulation of tau exon 10 splicing in DS where there is an extra copy of Dyrk1A. These findings can help in development of novel therapeutic strategies to prevent or inhibit neurofibrillary degeneration in tauopathies.

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REFERENCES

1. Schoenfeld,T.A. and Obar,R.A. (1994) Diverse distribution and function of fibrous microtubule-associated proteins in the nervous system. *Int. Rev. Cytol.*, **151**, 67–137.
2. Mandelkow,E. and Mandelkow,E.M. (1995) Microtubules and microtubule-associated proteins. *Curr. Opin. Cell Biol.*, **7**, 72–81.
3. Ballatore,C., Lee,V.M. and Trojanowski,J.Q. (2007) Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. *Nat. Rev. Neurosci.*, **8**, 663–672.
4. Hernandez,F. and Avila,J. (2007) Tauopathies. *Cell Mol. Life Sci.*, **64**, 2219–2233.
5. Goedert,M. and Spillantini,M.G. (2001) Tau gene mutations and neurodegeneration. *Biochem. Soc. Symp.*, 59–71.
6. Goedert,M., Spillantini,M.G., Potier,M.C., Ulrich,J. and Crowther,R.A. (1989) Cloning and sequencing of the cDNA encoding an isoform of microtubule-associated protein tau containing four tandem repeats: differential expression of tau protein mRNAs in human brain. *EMBO J.*, **8**, 393–399.
7. Andreadis,A., Brown,W.M. and Kosik,K.S. (1992) Structure and novel exons of the human tau gene. *Biochemistry*, **31**, 10626–10633.
8. Kosik,K.S., Orecchio,L.D., Bakalis,S. and Neve,R.L. (1989) Developmentally regulated expression of specific tau sequences. *Neuron*, **2**, 1389–1397.
9. Goedert,M. and Jakes,R. (1990) Expression of separate isoforms of human tau protein: correlation with the tau pattern in brain and effects on tubulin polymerization. *EMBO J.*, **9**, 4225–4230.
10. Hutton,M., Lendon,C.L., Rizzu,P., Baker,M., Froelich,S., Houlden,H., Pickering-Brown,S., Chakraverty,S., Isaacs,A., Grover,A. *et al.* (1998) Association of missense and 5′-splice-site mutations in tau with the inherited dementia FTDP-17. *Nature*, **393**, 702–705.
11. Poorkaj,P., Bird,T.D., Wijsman,E., Nemens,E., Garruto,R.M., Anderson,L., Andreadis,A., Wiederholt,W.C., Raskind,M. and Schellenberg,G.D. (1998) Tau is a candidate gene for chromosome 17 frontotemporal dementia. *Ann. Neurol.*, **43**, 815–825.
12. Spillantini,M.G., Murrell,J.R., Goedert,M., Farlow,M.R., Klug,A. and Ghetti,B. (1998) Mutation in the tau gene in familial multiple system tauopathy with presenile dementia. *Proc. Natl Acad. Sci. USA*, **95**, 7737–7741.
13. Liu,F. and Gong,C.X. (2008) Tau exon 10 alternative splicing and tauopathies. *Mol. Neurodegener.*, **3**, 8.
14. Dreyfuss,G., Kim,V.N. and Kataoka,N. (2002) Messenger-RNA-binding proteins and the messages they carry. *Nat. Rev. Mol. Cell Biol.*, **3**, 195–205.
15. Graveley,B.R. (2000) Sorting out the complexity of SR protein functions. *RNA*, **6**, 1197–1211.
16. Caceres,J.F., Misteli,T., Screaton,G.R., Spector,D.L. and Krainer,A.R. (1997) Role of the modular domains of SR proteins in subnuclear localization and alternative splicing specificity. *J. Cell Biol.*, **138**, 225–238.
17. Zahler,A.M., Lane,W.S., Stolk,J.A. and Roth,M.B. (1992) SR proteins: a conserved family of pre-mRNA splicing factors. *Genes Dev.*, **6**, 837–847.
18. Fu,X.D. and Maniatis,T. (1990) Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. *Nature*, **343**, 437–441.
19. Mabon,S.A. and Misteli,T. (2005) Differential recruitment of pre-mRNA splicing factors to alternatively spliced transcripts in vivo. *PLoS Biol.*, **3**, e374.
20. D'Souza,I. and Schellenberg,G.D. (2005) Regulation of tau isoform expression and dementia. *Biochim. Biophys. Acta*, **1739**, 104–115.
21. Andreadis,A. (2005) Tau gene alternative splicing: expression patterns, regulation and modulation of function in normal brain and neurodegenerative diseases. *Biochim. Biophys. Acta*, **1739**, 91–103.
22. Gui,J.F., Lane,W.S. and Fu,X.D. (1994) A serine kinase regulates intracellular localization of splicing factors in the cell cycle. *Nature*, **369**, 678–682.
23. Wang,H.Y., Lin,W., Dyck,J.A., Yeakley,J.M., Songyang,Z., Cantley,L.C. and Fu,X.D. (1998) SRPK2: a differentially

expressed SR protein-specific kinase involved in mediating the interaction and localization of pre-mRNA splicing factors in mammalian cells. *J. Cell Biol.*, **140**, 737–750.
24. Colwill,K., Pawson,T., Andrews,B., Prasad,J., Manley,J.L., Bell,J.C. and Duncan,P.I. (1996) The Clk/Sty protein kinase phosphorylates SR splicing factors and regulates their intranuclear distribution. *EMBO J.*, **15**, 265–275.
25. Rossi,F., Labourier,E., Forne,T., Divita,G., Derancourt,J., Riou,J.F., Antoine,E., Cathala,G., Brunel,C. and Tazi,J. (1996) Specific phosphorylation of SR proteins by mammalian DNA topoisomerase I. *Nature*, **381**, 80–82.
26. Shi,J., Zhang,T., Zhou,C., Chohan,M.O., Gu,X., Wegiel,J., Zhou,J., Hwang,Y.W., Iqbal,K., Grundke-Iqbal,I. *et al.* (2008) Increased dosage of Dyrk1A alters alternative splicing factor (ASF)-regulated alternative splicing of tau in Down syndrome. *J. Biol. Chem.*, **283**, 28660–28669.
27. Arron,J.R., Winslow,M.M., Polleri,A., Chang,C.P., Wu,H., Gao,X., Neilson,J.R., Chen,L., Heit,J.J., Kim,S.K. *et al.* (2006) NFAT dysregulation by increased dosage of DSCR1 and DYRK1A on chromosome 21. *Nature*, **441**, 595–600.
28. Gwack,Y., Sharma,S., Nardone,J., Tanasa,B., Iuga,A., Srikanth,S., Okamura,H., Bolton,D., Feske,S., Hogan,P.G. *et al.* (2006) A genome-wide Drosophila RNAi screen identifies DYRK-family kinases as regulators of NFAT. *Nature*, **441**, 646–650.
29. Wisniewski,K.E., Wisniewski,H.M. and Wen,G.Y. (1985) Occurrence of neuropathological changes and dementia of Alzheimer's disease in Down's syndrome. *Ann. Neurol.*, **17**, 278–282.
30. Alvarez,M., Estivill,X. and de la Luna,S. (2003) DYRK1A accumulates in splicing speckles through a novel targeting signal and induces speckle disassembly. *J. Cell Sci.*, **116**, 3099–3107.
31. Yu,Q., Guo,J. and Zhou,J. (2004) A minimal length between tau exon 10 and 11 is required for correct splicing of exon 10. *J. Neurochem.*, **90**, 164–172.
32. Wegiel,J., Kuchna,I., Nowicki,K., Frackowiak,J., Dowjat,K., Silverman,W.P., Reisberg,B., DeLeon,M., Wisniewski,T., Adayev,T. *et al.* (2004) Cell type- and brain structure-specific patterns of distribution of minibrain kinase in human brain. *Brain Res.*, **1010**, 69–80.
33. Liu,F., Iqbal,K., Grundke-Iqbal,I., Hart,G.W. and Gong,C.X. (2004) O-GlcNAcylation regulates phosphorylation of tau: a mechanism involved in Alzheimer's disease. *Proc. Natl Acad. Sci. USA*, **101**, 10804–10809.
34. Niranjanakumari,S., Lasda,E., Brazas,R. and Garcia-Blanco,M.A. (2002) Reversible cross-linking combined with immunoprecipitation to study RNA-protein interactions in vivo. *Methods*, **26**, 182–190.
35. Gilbert,S.L., Pehrson,J.R. and Sharp,P.A. (2000) XIST RNA associates with specific regions of the inactive X chromatin. *J. Biol. Chem.*, **275**, 36491–36494.
36. Sun,B.K., Deaton,A.M. and Lee,J.T. (2006) A transient heterochromatic state in Xist preempts X inactivation choice without RNA stabilization. *Mol. Cell*, **21**, 617–628.
37. Schaal,T.D. and Maniatis,T. (1999) Selection and characterization of pre-mRNA splicing enhancers: identification of novel SR protein-specific enhancer sequences. *Mol. Cell. Biol.*, **19**, 1705–1719.
38. D'Souza,I. and Schellenberg,G.D. (2000) Determinants of 4-repeat tau expression. Coordination between enhancing and inhibitory splicing sequences for exon 10 inclusion. *J. Biol. Chem.*, **275**, 17700–17709.
39. Zhong,X.Y., Wang,P., Han,J., Rosenfeld,M.G. and Fu,X.D. (2009) SR proteins in vertical integration of gene expression from transcription to RNA processing to translation. *Mol. Cell*, **35**, 1–10.
40. Xiao,S.H. and Manley,J.L. (1998) Phosphorylation-dephosphorylation differentially affects activities of splicing factor ASF/SF2. *EMBO J.*, **17**, 6359–6367.
41. Colwill,K., Feng,L.L., Yeakley,J.M., Gish,G.D., Caceres,J.F., Pawson,T. and Fu,X.D. (1996) SRPK1 and Clk/Sty protein kinases show distinct substrate specificities for serine/arginine-rich splicing factors. *J. Biol. Chem.*, **271**, 24569–24575.
42. Blaustein,M., Pelisch,F., Tanos,T., Munoz,M.J., Wengier,D., Quadrana,L., Sanford,J.R., Muschietti,J.P., Kornblihtt,A.R., Caceres,J.F. *et al.* (2005) Concerted regulation of nuclear and cytoplasmic activities of SR proteins by AKT. *Nat. Struct. Mol. Biol.*, **12**, 1037–1044.
43. Hernandez,F., Perez,M., Lucas,J.J., Mata,A.M., Bhat,R. and Avila,J. (2004) Glycogen synthase kinase-3 plays a crucial role in tau exon 10 splicing and intranuclear distribution of SC35. Implications for Alzheimer's disease. *J. Biol. Chem.*, **279**, 3801–3806.
44. Chen,K.L., Yuan,R.Y., Hu,C.J. and Hsu,C.Y. (2010) Amyloid-beta peptide alteration of tau exon-10 splicing via the GSK3beta-SC35 pathway. *Neurobiol. Dis.*, **40**, 378–385.
45. D'Souza,I. and Schellenberg,G.D. (2006) Arginine/serine-rich protein interaction domain-dependent modulation of a tau exon 10 splicing enhancer: altered interactions and mechanisms for functionally antagonistic FTDP-17 mutations Delta280K AND N279K. *J. Biol. Chem.*, **281**, 2460–2469.
46. Kimura,R., Kamino,K., Yamamoto,M., Nuripa,A., Kida,T., Kazui,H., Hashimoto,R., Tanaka,T., Kudo,T., Yamagata,H. *et al.* (2007) The DYRK1A gene, encoded in chromosome 21 Down syndrome critical region, bridges between beta-amyloid production and tau phosphorylation in Alzheimer disease. *Hum. Mol. Genet.*, **16**, 15–23.
47. Goni-Oliver,P., Lucas,J.J., Avila,J. and Hernandez,F. (2007) N-terminal cleavage of GSK-3 by calpain: a new form of GSK-3 regulation. *J. Biol. Chem.*, **282**, 22406–22413.
48. Liang,Z., Liu,F., Grundke-Iqbal,I., Iqbal,K. and Gong,C.X. (2007) Down-regulation of cAMP-dependent protein kinase by over-activated calpain in Alzheimer disease brain. *J. Neurochem.*, **103**, 2462–2470.
49. Shi,J., Qian,W., Yin,X., Iqbal,K., Grundke-Iqbal,I., Gu,X., Ding,F., Gong,C.X. and Liu,F. (2011) Cyclic AMP-dependent protein kinase regulates the alternative splicing of tau exon 10: a mechanism involved in tau pathology of Alzheimer's disease. *J. Biol. Chem.*, doi:10.1074/jbc.M110.204453.