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Dual-specificity Tyrosine Phosphorylation-regulated Kinase 1A (Dyrk1A) Modulates Serine/Arginine-rich Protein 55 (SRp55)-promoted Tau Exon 10 Inclusion*

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Background: Dysregulation of the alternative splicing of Tau exon 10 causes several types of neurodegenerative diseases.
Results: SRp55 promotes Tau exon 10 inclusion. Dyrk1A interacts with SRp55, mainly phosphorylates its proline-rich domain and inhibits its ability to promote Tau exon 10 inclusion.
Conclusion: Dyrk1A suppresses SRp55-promoted Tau exon 10 inclusion.
Significance: Up-regulation of Dyrk1A disrupts the alternative splicing of Tau exon 10.

***Tau* exon 10, which encodes the second microtubule-binding repeat, is regulated by alternative splicing. Its alternative splicing generates Tau isoforms with three- or four-microtubule-binding repeats, named 3R-tau and 4R-tau. Adult human brain expresses equal levels of 3R-tau and 4R-tau. Imbalance of 3R-tau and 4R-tau causes Tau aggregation and neurofibrillary degeneration. In the present study, we found that splicing factor SRp55 (serine/arginine-rich protein 55) promoted Tau exon 10 inclusion. Knockdown of SRp55 significantly promoted Tau exon 10 exclusion. The promotion of Tau exon 10 inclusion by SRp55 required the arginine/serine-rich region, which was responsible for the subnucleic speckle localization. Dyrk1A (dual specificity tyrosine-phosphorylated and regulated kinase 1A) interacted with SRp55 and mainly phosphorylated its proline-rich domain. Phosphorylation of SRp55 by Dyrk1A suppressed its ability to promote Tau exon 10 inclusion. Up-regulation of Dyrk1A as in Down syndrome could lead to neurofibrillary degeneration by shifting the alternative splicing of Tau exon 10 to an increase in the ratio of 3R-tau/4R-tau.**

The neuronal microtubule-associated protein Tau plays important roles in morphogenesis and axonal extension, as well as axonal vesicle and protein transport in neurons. Hyperphosphorylated Tau aggregates and deposits into neurofibrillary tangles in brains of individuals with Alzheimer disease and related tauopathies (1, 2). Tau is expressed in different isoforms generated by alternative splicing of its pre-mRNA encoded by a single gene. Alternative splicing of Tau exon 10 generates Tau isoforms with three- or four-microtubule-binding repeats, named 3R-tau and 4R-tau, respectively. Approximately equal levels of 3R-tau and 4R-tau exist in adult human brain (3, 4). Imbalance of 3R-tau and 4R-tau causes several types of neurodegenerative diseases, such as progressive supranuclear palsy, corticobasal degeneration, frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), Pick's disease, Down syndrome (DS),[3] postencephalitic Parkinsonism, and Niemann-Pick disease, suggesting that normal alternative splicing of Tau exon 10 is essential for maintaining neuronal physiology (5).

Alternative splicing is tightly regulated by the action of *trans*-acting factors (splicing factors) on the *cis*-elements (splicing enhancers or splicing silencers). Serine- and arginine-rich proteins (SR proteins) are a group of splicing factors and play important roles in alternative splicing of Tau exon 10. SRp55 (serine/arginine-rich protein 55) is a SR protein with an apparent molecular mass of 55 kDa and participates in constitutive and alternative pre-mRNA splicing (6). SRp55 was reported to regulate the alternative splicing of Bim (7), epidermal growth factor (8, 9), HIV-1 (10), and Tau (11).

The arginine/serine-rich (RS) domain of SR proteins is extensively phosphorylated on serine residues, and phosphorylation plays a critical role in their functions (12). We previously reported that Dyrk1A (dual-specificity tyrosine phosphorylation-regulated kinase 1A) phosphorylates SR proteins SF2/ASF (splicing factor 2/alternative splicing factor) and SC35 and inhibits their promotion of Tau exon 10 inclusion (13, 14). Dyrk1A is a proline- and arginine-directed Ser/Thr kinase that is localized in the DS critical region of chromosome 21 and contributes to several phenotypes of this disease in transgenic

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[3] The abbreviations used are: DS, Down syndrome; FITC, fluorescein isothiocyanate; SR proteins, serine- and arginine-rich proteins; RS, arginine/serine-rich; RRM, RNA recognition motifs; TRITC, tetramethylrhodamine isothiocyanate.

mice (15, 16). Overexpression of Dyrk1A caused by an extra copy of chromosome 21 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 (13).

In the present study, we investigated the role of SRp55 and its phosphorylation by Dyrk1A in Tau exon 10 splicing. We found that SRp55 promoted the inclusion of Tau exon 10, and phosphorylation of SRp55 by Dyrk1A inhibited this activity.

EXPERIMENTAL PROCEDURES

Plasmids, Proteins, and Antibodies—Recombinant rat Dyrk1A and mammalian expression vector pcDNA3 containing either rat Dyrk1A or dominant negative $Dyrk1A_{K188R}$ were kindly provided by Dr. Y.-W. Hwang of the New York State Institute for Basic Research and prepared as described previously (17). pCEP4/SRp55-HA was a gift from Dr. Tarn of the Institute of Biomedical Sciences, Academia Sinica, Taiwan. pCI/SI9-LI10, containing a Tau mini-gene SI9-LI10, comprising Tau exons 9–11 and part of intron 9 and the full length of intron 10, was described previously (11). Monoclonal antibody against Dyrk1A (8D9) was raised against a histidine-tagged protein containing the first 160 amino acid residues of rat Dyrk1A (18). Monoclonal anti-Dyrk1A (7D10) and anti-SRp55 were purchased from EMD Millipore (Billerica, MA). The monoclonal anti-HA, polyclonal anti-HA, anti-α-tubulin, and anti-β-actin were bought from Sigma. Peroxidase-conjugated anti-mouse and anti-rabbit IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). TRITC-conjugated goat anti-rabbit IgG, FITC-conjugated goat anti-mouse IgG, and siRNA of human SRp55 were from Santa Cruz Biotechnology (Santa Cruz, CA). Cy3-conjugated goat anti-rabbit IgG, Alexa 488-conjugated goat anti-mouse IgG, and TO-PRO-3 iodide (642/661) were from Invitrogen. The ECL kit was from Pierce. [γ-^{32}P]ATP was from MP Biomedicals (Irvine, CA).

Cell Culture and Transfection—COS-7, HEK-293T, HEK-293FT, HepG2, HeLa, and SH-SY5Y cells were maintained in DMEM supplemented with 10% fetal bovine serum (Invitrogen) at 37 °C. All transfections were performed in triplicate with Lipofectamine 2000 (Invitrogen) or FuGENE (Roche Applied Science) according to the manufacturer's instructions.

Plasmid Construction and DNA Mutagenesis—pGEX-2T/SRp55 was constructed by PCR amplification from pCEP4/SRp55 and subcloned into pGEX-2T to express GST-SRp55 protein. The deletion mutations of SRp55 were generated by amplifying an individual fragment, which contains part of the SRp55 coding regions into pCEP4 or pGEX-2T. Site mutations of SRp55 were performed with KOD PLUS (Toyobo, Japan) according to the manufacturer's instructions.

GST Pulldown—GST, GST-SRp55, and GST-SRp55 deletion mutants were purified by affinity purification with glutathione-Sepharose but without elution from the beads. The GST or GST-SRp55 beads was 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, 50 nM okadaic acid, 0.1% Triton X-100, 2 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin). After 4 h of incubation at 4 °C, the beads were washed with washing buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 1 mM DTT) six times, and then the bound proteins were eluted by boiling in Laemmli sample buffer, and the samples were subjected to Western blot analysis.

In Vitro Phosphorylation of SRp55 by Dyrk1A—For *in vitro* phosphorylation of SRp55 by Dyrk1A, GST-SRp55 or GST-SRp55 deletion mutants 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 a phosphorimaging system.

Phosphorylation of SRp55 in Cultured Cells—HEK-293FT cells were transfected with pCEP4/SRp55-HA and cultured in DMEM supplemented with 10% fetal bovine serum. After 45 h of transfection, the medium was replaced with [^{32}P]monosodium phosphate (10 mCi) in DMEM (without phosphate) supplemented with 10% fetal bovine serum. After a 3-h incubation, the cells were harvested in lysate buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, 50 nM okadaic acid, 0.1% Triton X-100, 0.1% Nonidet P-40, 0.25% sodium deoxycholate, 2 mM EDTA, 1 mM PMSF, and 10 μg/ml of aprotinin, leupeptin, and pepstatin). Insoluble materials were removed by centrifugation, and the supernatant was incubated with anti-HA precoupled to protein G-conjugated agarose for 4 h at 4 °C. After washing with TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl), the SRp55-HA immunoprecipitated by anti-HA was analyzed by immunoblotting and autoradiography.

Co-immunoprecipitation—HEK-293FT cells were co-transfected with pCEP4/SRp55-HA or its deletion mutants and pcDNA3/Dyrk1A for 48 h as described above. The cells were washed twice with PBS and lysed by sonication in lysate buffer (50 mM Tris-HCl, pH7.4, 8.5% sucrose, 50 mM NaF, 2 mM EDTA, 1 mM PMSF, 50 nM okadaic acid, and 10 μg/ml of aprotinin, leupeptin and pepstatin). Insoluble materials were removed by centrifugation; the supernatants were preabsorbed with protein G-conjugated agarose beads and incubated with anti-HA or anti-SRp55 overnight at 4 °C, and then protein G beads were added. After 4 h of incubation at 4 °C, the beads were washed twice each with lysate buffer and with TBS, 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 or HepG2 cells were plated onto coverslips 1 day prior to transfection at 50–60% confluence and were singly transfected or co-transfected with HA-tagged SRp55 or its deletion mutants and Dyrk1A or $Dyrk1A_{k188R}$ 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 polyclonal anti-HA antibody (1:200) and monoclonal anti-Dyrk1A (8D9, 1:10000) overnight at 4 °C. After washing and incubation with TRITC-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG, 1:200), the cells were

washed extensively in PBS and incubated with 5 μg/ml Hoechst 33342 for 15 min at room temperature. The cells were washed with PBS, mounted with Fluoromount-G, and revealed with a Leica TCS-SP2 laser scanning confocal microscope (in the Nantong laboratory). In some experiments, the cells were then washed and incubated for 1 h with secondary antibody (Alexa 488-conjugated goat anti-mouse IgG, 1:1000) plus TO-PRO-3 iodide at room temperature. The cells were washed with PBS, mounted with Fluoromount-G, and observed with a Nikon TCS-SP2 laser scanning confocal microscope (in the New York laboratory).

Quantitation of Tau Exon 10 Splicing by RT-PCR—Total cellular RNA was isolated from cultured cells by using RNeasy mini kit (Qiagen). One microgram of total RNA was used for first strand cDNA synthesis with oligo(dT)$_{15-18}$ by using an Omniscript reverse transcription kit (Qiagen). PCR was performed by using PrimeSTAR^{TM} HS DNA polymerase (Takara Bio Inc., Otsu, Shiga, Japan) with primers (forward, 5′-GGTGTCCACTCCCAGTTCAA-3′; and reverse, 5′-CCCTGGTTTATGATGGATGTTG CCTAATGAG-3′) to measure alternative splicing of Tau exon 10 under conditions: 98 °C for 3 min, 98 °C for 10 s, and 68 °C for 40 s for 30 cycles, followed by 68 °C for 10 min for extension. The PCR products were resolved on 1.5% agarose gels and quantitated using the Molecular Imager system (Bio-Rad).

Statistical Analysis—The data are presented as the means ± S.D. The data points were compared by the unpaired two-tailed Student's *t* test, and the calculated *p* values are indicated in the figures.

RESULTS

SRp55 Promotes Tau Exon 10 Inclusion—To learn the role of SRp55 in the alternative splicing of Tau exon 10, we used a mini-Tau gene pCI/SI9-LI10 containing exons 9–11 and partial intron 9 and full-length intron 10 to perform this study. We co-transfected pCEP4/SRp55 with Tau mini-gene pCI/SI9-LI10 into different types of cells and measured the splicing products of Tau exon 10 by RT-PCR 48 h after transfection. We observed different splicing patterns of Tau exon 10 in different types of cells (Fig. 1*A*). However, overexpression of SRp55 increased Tau exon 10 inclusion in all tested cell lines (Fig. 1*A*), suggesting that SRp55 promotes Tau exon 10 inclusion.

To confirm the participation of SRp55 in Tau exon 10 splicing, we knocked down SRp55 with siRNA in pCI/SI9-LI10 transfected HEK-293FT cells and then measured the splicing product of Tau exon 10 by RT-PCR. We found that expression of SRp55 siRNA (*siSRp55*), but not scrambled siRNA (mock), significantly inhibited exon 10 inclusion (Fig. 1*B*), supporting the promotion of SRp55 in Tau exon 10 inclusion.

To determine the role of SRp55 in endogenous Tau exon 10 splicing, we transfected siSRp55 into retinoid acid-treated SH-SY5Y cells and then measured the splicing products of Tau exon 10 by RT-PCR after 72 h of transfection. We observed that siSRp55 suppressed Tau exon 10 inclusion, resulting in decreased 4R-tau expression (Fig. 1*C*). These data suggest that SRp55 also works on endogenous Tau splicing and promotes Tau exon 10 inclusion.

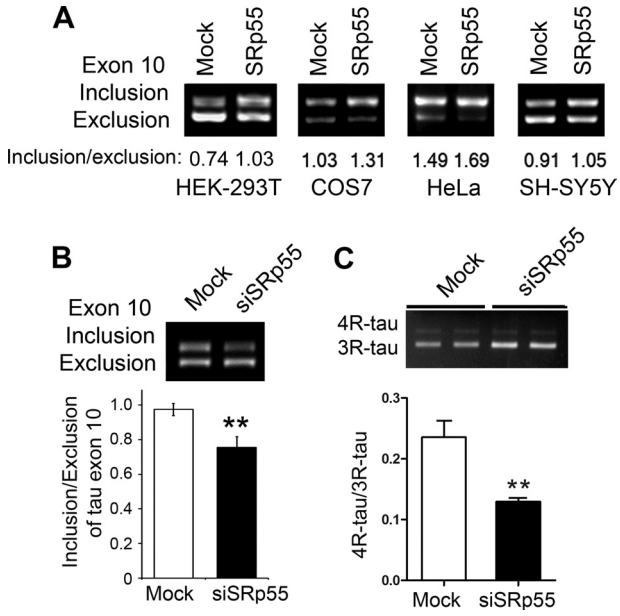

FIGURE 1. **SRp55 promotes Tau exon 10 inclusion.** *A*, SRp55 promoted Tau exon 10 inclusion in various cell lines. Cultured cells were co-transfected with pCEP4/SRp55 and Tau mini-gene pCI/SI9-LI10 for 48 h. The alternative splicing products of Tau exon 10 were measured by RT-PCR. The ratio of inclusion and exclusion of Tau exon 10 is presented under each lane; the ratio varies not only from cell line to cell line but also in different passages of the same cell line. *B*, knockdown of SRp55 suppressed Tau exon 10 inclusion. HEK-293FT cells were co-transfected with siRNA of human SRp55 and pCI/SI9-LI10 for 48 h, and then the splicing products of Tau exon 10 were measured by RT-PCR. The ratio of inclusion and exclusion of Tau exon 10 is presented as the mean ± S.D. **, $p < 0.01$. *C*, knockdown of SRp55 suppressed endogenous Tau exon 10 inclusion. SH-SY5Y cells were transfected with siRNA of human SRp55 and differentiated with 10 μM retinoid acid for 72 h, and then the splicing products of endogenous Tau exon 10 were measured by RT-PCR. The ratio of inclusion and exclusion of Tau exon 10 is presented as the mean ± S.D. **, $p < 0.01$.

RS Domains of SRp55 Are Required for Promoting Tau Exon 10 Inclusion—SRp55 contains two RNA recognition motifs (RRM) at the N terminus and two RS domains; separated by a proline-rich motif at the C terminus (Fig. 2*A*). To study the domain-specific role of SRp55, we generated various deletion mutants of this protein (Fig. 2*A*) and overexpressed them together with pCI/SI9-LI10 in HEK-293FT cells. We found that overexpression of SRp55 significantly increased Tau exon 10 inclusion, but expression of the deletion mutants failed to promote Tau exon 10 inclusion (Fig. 2*B*). Deletion of RS1, proline-rich, and RS2 domains suppressed exon 10 inclusion (Fig. 2*B*). These data suggest that SRp55 requires RS1and RS2 domains to promote Tau exon 10 inclusion.

To learn the effect of deletion mutation on subcellular localization of SRp55, we transfected HeLa cells with the HA-tagged mutants for 48 h and then studied them by immunocytochemistry. We found that SRp55 and its deletion mutants were localized in the nucleus (Fig. 2*C*). Enrichment of HA staining in speckles was seen with SRp55 and its deletion mutants SRp55$_{1-320}$ and SRp55$_{1-280}$. However, SRp55$_{1-183}$, which lacked both RS domains, was found to be diffused in nucleoplasm (Fig. 2*C*). When RS2 was added onto the C terminus to generate SRp55$_{\Delta 183-320}$, it translocated into speckles again (Fig. 2*C*). These results suggest that speckle localization of SRp55 requires at least one RS domain.

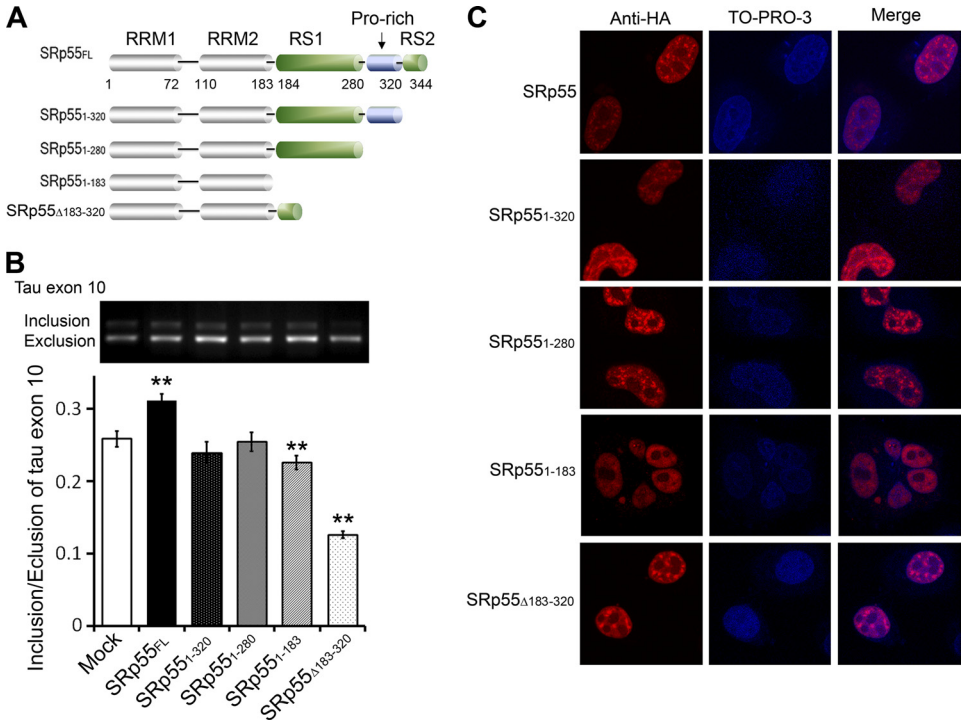

FIGURE 2. **SRp55 requires RS domain to promote Tau exon 10 inclusion.** *A*, schematic presentation of SRp55 deletion mutants. *B*, deletion mutants of SRp55 did not promote Tau exon 10 inclusion. HEK-293FT cells were co-transfected with pCEP4/SRp55 and its deletion mutants and pCI/SI9-LI10 for 48 h. The splicing products of Tau exon 10 were measured by RT-PCR. The ratio of inclusion/exclusion of Tau exon 10 is presented as the mean ± S.D. **, $p < 0.01$ as compared with mock transfection. *C*, SRp55 and its deletion mutants were localized in the nucleus. SRp55 and its deletion mutants tagged with HA were overexpressed in HeLa cells for 48 h. The cells were fixed and immunostained by anti-HA, followed by Cy3 anti-rabbit IgG. TO-PRO-3 was used for nuclear staining.

Dyrk1A Interacts with SRp55—Dyrk1A was previously shown to regulate the pre-mRNA alternative splicing of Tau through splicing factors (13, 14). To understand whether Dyrk1A interacts with SRp55 and modulates its function, we performed a GST pulldown assay. We expressed recombinant GST-SRp55 fusion protein and bound it onto glutathione-conjugated Sepharose (GSH beads). After incubation of the GST-SRp55-GSH beads with rat brain extract overnight at 4 °C, we detected Dyrk1A in the pulldown proteins by using Western blots. The results revealed that Dyrk1A was pulled down by GST-SRp55, but not GST itself (Fig. 3*A*). These results suggest that SRp55 may interact with Dyrk1A.

We further carried out the co-immunoprecipitation to confirm the interaction between SRp55 and Dyrk1A. Dyrk1A and SRp55 were co-transfected into HEK-293FT cells for 48 h, and SRp55 was immunoprecipitated with anti-HA. We found that Dyrk1A was co-immunoprecipitated by SRp55 (Fig. 3*B*), supporting the interaction of SRp55 with Dyrk1A.

To examine the interaction between endogenous of SRp55 and Dyrk1A, we immunoprecipitated endogenous SRp55 with anti-SRp55 from HEK-293FT cells. We found that SRp55 has the same apparent molecular mass as heavy chain of IgG (Fig. 3*C*, *lower panel*), and Dyrk1A was co-immunoprecipitated by anti-SRp55 (Fig. 3*C*, *upper panel*), indicating the existence of interaction between endogenous SRp55 and Dyrk1A.

To learn whether Dyrk1A is co-localized with SRp55 in intact cells, we expressed SRp55 tagged with HA and Dyrk1A in HepG2 cells and then immunostained the cells. We observed that Dyrk1A and SRp55 were co-localized in the nucleus with speckle enrichment (Fig. 3*D*). These results further support that Dyrk1A interacts with SRp55 in live cells.

SRp55 Interacts with Dyrk1A through Its RRM Domain—To look into the interaction of SRp55 molecule with Dyrk1A, we overexpressed SRp55 deletion mutants tagged with HA in HEK-293FT cells and then immunoprecipitated them with anti-HA. Western blots were conducted to analyze Dyrk1A in the anti-HA immunoprecipitated complex. We found that Dyrk1A was co-immunoprecipitated by $SRp55_{FL}$ and its deletion mutants at RS domain, but not by its deletion mutants at RRM (Fig. 4*A*), suggesting that RRM is required for the interaction of SRp55 with Dyrk1A.

To determine the subcellular localization of SRp55 deletion mutants and Dyrk1A, we co-transfected Dyrk1A with SRp55 deletion mutants tagged with HA in HeLa cells and then immunostained these cells. We observed that SRp55 deletion mutants detected by anti-HA located in nucleus and co-localized with Dyrk1A (Fig. 4*B*).

Dyrk1A Phosphorylates SRp55—To learn whether Dyrk1A phosphorylates SRp55, we performed *in vitro* phosphorylation of GST-SRp55 with various concentrations of Dyrk1A at 30 °C for 30 min. The reaction products were analyzed by SDS-PAGE, followed by autoradiography (Fig. 5*A*, *upper panel*). The incorporation of ^{32}P into SRp55 was determined by phosphorimaging (Fig. 5*A*, *lower panel*). We observed that GST-SRp55, but not GST, was phosphorylated by Dyrk1A dose-dependently (Fig. 5*A*).

To investigate the phosphorylation of SRp55 by Dyrk1A in cultured cells, we overexpressed SRp55 tagged with HA in

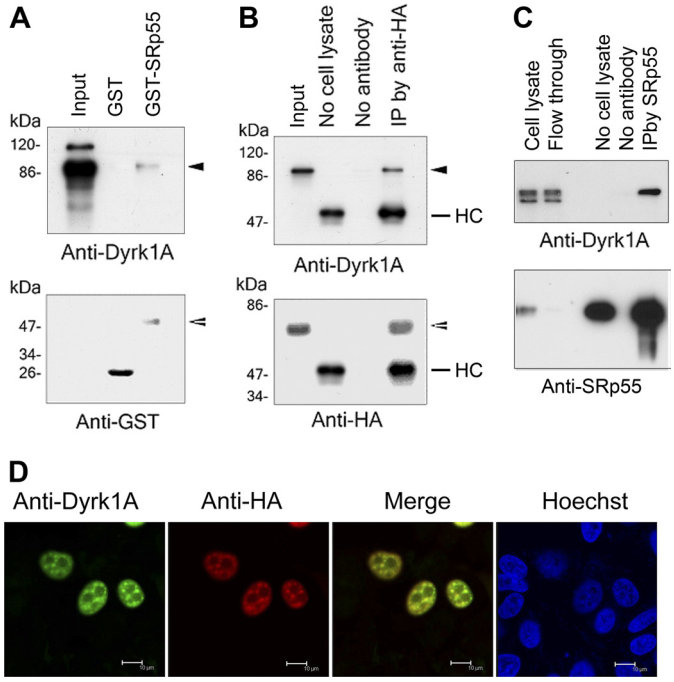

FIGURE 3. **SRp55 interacts with Dyrk1A.** *A*, Dyrk1A was pulled down from rat brain extracts by GST-SRp55. GST-SRp55 or GST coupled onto glutathione-Sepharose was incubated with rat brain extracts. After washing, the bound proteins were subjected to Western blots using anti-GST and anti-Dyrk1A. *B*, Dyrk1A was co-immunoprecipitated by HA-SRp55 using anti-HA. SRp55 tagged with HA and Dyrk1A was co-expressed in HEK-293T cells for 48 h. The cell extracts were incubated with anti-HA prebound onto protein G beads. The bound proteins were subjected to Western blots using antibodies indicated under each blot. *HC*, heavy chain of IgG. *C*, Dyrk1A was co-immunoprecipitated by anti-SRp55. HEK-293FT cell extracts were incubated with anti-SRp55 prebound onto protein G beads. The bound proteins were subjected to Western blots using antibodies indicated under each blot. *D*, co-localization of SRp55 with Dyrk1A in the nucleus. HA-SRp55 and Dyrk1A were co-transfected into HepG2 cells. After a 48-h transfection, the cells were fixed and immunostained by anti-HA and anti-Dyrk1A and followed by TRITC anti-rabbit IgG and FITC anti-mouse IgG. Hoechst 33342 was used for nuclear staining.

HEK-293FT cells and labeled the cells with [^{32}P]phosphate. We immunoprecipitated HA-SRp55 with anti-HA, and the immunoprecipitated protein was analyzed by SDS-PAGE, followed by autoradiography. The level of SRp55 was determined by Western blots developed with anti-HA. The results showed that the immunoprecipitated SRp55 was phosphorylated significantly. The protein band with higher ^{32}P incorporation moved fast in SDS-PAGE (Fig. 5*B*, *upper panel*), suggesting that phosphorylation of SRp55 increases its mobility.

In an attempt to learn the role of Dyrk1A in SRp55 phosphorylation, we treated SRp55-transfected cells with harmine, a selective Dyrk1A inhibitor, and/or Tg003, an inhibitor of the protein kinase Clk/Sty that is known to phosphorylate SRp55, and then measured the ^{32}P incorporation into SRp55 as described above. We found that Tg003 and harmine both significantly decreased ^{32}P incorporation into SRp55. Treatment with both Tg003 and harmine inhibited the ^{32}P incorporation more dramatically (Fig. 5*B*). In addition, the lower protein band with high ^{32}P incorporation markedly decreased after treatment with Clk/Sty or Dyrk1A inhibitors (Fig. 5*B*). These results indicate that SRp55 is phosphorylated by Dyrk1A as well as by Clk/Sty in cultured cells.

To confirm the phosphorylation of SRp55 by Dyrk1A in cultured cells, we co-expressed SRp55 and Dyrk1A or Dyrk1A$_{K188R}$ and then immunoprecipitated SRp55 with anti-HA. Incorporated ^{32}P into SRp55 was measured by autoradiography (Fig. 5*C*, *top panel*), and the level of immunoprecipitated SRp55 was measured by Western blot (Fig. 5*C*, *middle panel*). We found that co-expression of Dyrk1A increased SRp55 expression (Fig. 5*C*, *middle panel*), which is consistent with the previous finding (19). Phosphorylation of SRp55 was significantly increased by co-expression of Dyrk1A but decreased by co-expression of Dyrk1A$_{K188R}$ markedly (Fig. 5*C*), confirming the phosphorylation of SRp55 by Dyrk1A.

To identify which domains of SRp55 molecule are phosphorylated by Dyrk1A, we expressed and purified GST-SRp55 and its deletion mutants, GST-SRp55$_{1-320}$, GST-SRp55$_{1-280}$, and GST-SRp55$_{1-183}$ (Fig. 5*D*), and then phosphorylated them with Dyrk1A *in vitro*. The phosphorylated products were separated by SDS-PAGE, and the ^{32}P incorporation was determined by autoradiography. We found that deletion of RS2 domain did not affect ^{32}P incorporation, but deletion of proline-rich domain reduced it to 40% (Fig. 5*E*). Deletion of both the proline-rich domain and the RS1 domain decreased phosphorylation of SRp55 by Dyrk1A to 20%. These results suggest that the majority of the phosphorylation sites of SRp55 by Dyrk1A are located at the RS1 and proline-rich domains, especially the latter. Dyrk1A is a proline- and arginine-directed serine/threonine kinase and phosphorylates consensus motif, R*X*(*X*)(T/S)P. There are three putative Dyrk1A phosphorylation sites at these regions, including Ser-280, Ser-303, and Ser-316. Therefore, we speculate Dyrk1A mainly phosphorylates these three serine residues.

Dyrk1A modulates SRp55-mediated Tau Exon 10 Splicing—To determine the role of Dyrk1A in SRp55-mediated Tau exon 10 splicing, we co-expressed Dyrk1A with SRp55 in pCI/SI9-LI10 transfected HEK-293FT cells for 48 h and then measured the alternative splicing products of Tau exon 10 by RT-PCR. We found that the overexpression of Dyrk1A did not affect Tau exon 10 inclusion, whereas the expression of dominant negative Dyrk1A, Dyrk1A$_{K188R}$, which results in the loss of its kinase activity, significantly promoted Tau exon10 inclusion (Fig. 6, *A* and *B*). Furthermore, overexpression of SRp55 in the cells did not promote Tau exon 10 inclusion, but co-expression with Dyrk1A significantly decreased Tau exon 10 inclusion, and co-expression with Dyrk1A$_{K188R}$ dramatically increased Tau exon 10 inclusion (Fig. 6*A*). These findings suggested that phosphorylation of SRp55 by Dyrk1A inhibits the exon10 inclusion.

Next, we knocked down SRp55 in HEK-293FT cells by siRNA and found that it significantly decreased the expression of exon 10 (Fig. 6*B*). Also, overexpression of Dyrk1A suppressed Tau exon 10 inclusion, whereas dominant negative Dyrk1A promoted the inclusion of Tau exon 10 (Fig. 6*B*). Co-transfection of siSRp55 and Dyrk1A further suppressed Tau exon 10 inclusion (Fig. 6*B*). However, the suppression of Tau exon 10 inclusion by knockdown of SRp55 was reversed by Dyrk1A$_{K188R}$ (Fig. 6*B*). Taken together, the results from dominant negative Dyrk1A and from SRp55 knockdown studies suggest that Dyrk1A suppresses SRp55-promoted Tau exon 10 inclusion. Dyrk1A$_{K188R}$ could interact with, rather than phosphorylate target proteins,

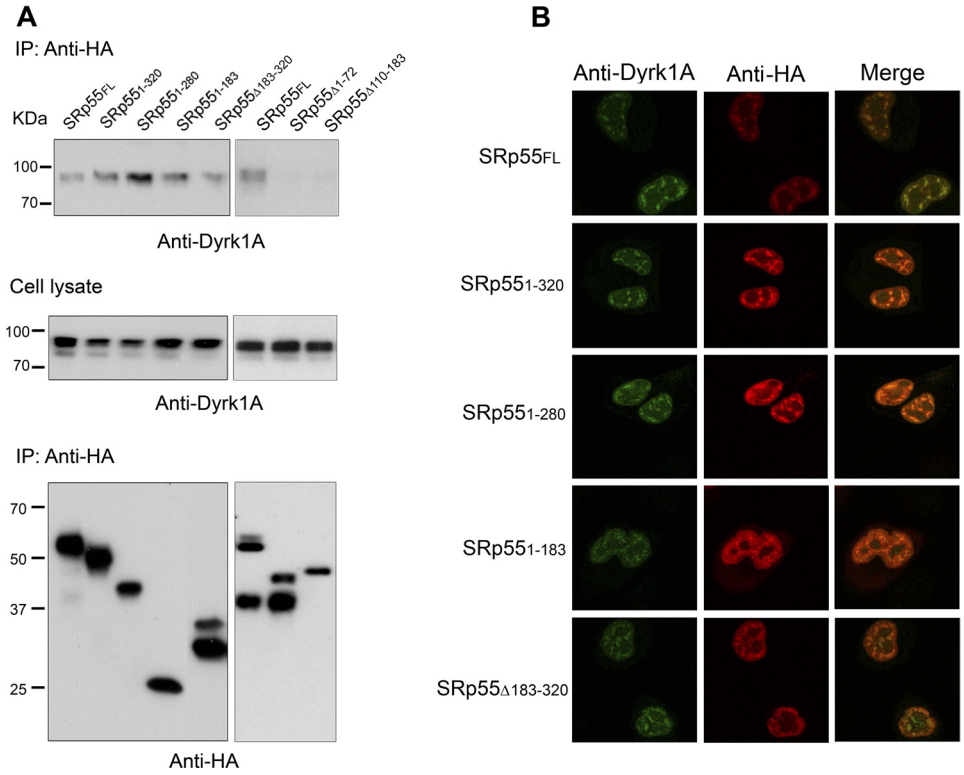

FIGURE 4. **SRp55 interacts with Dyrk1A through RRM domain.** *A*, co-immunoprecipitation of Dyrk1A by SRp55 required RRM. HA-tagged $SRp55_{FL}$ or its deletion mutants and Dyrk1A were co-expressed in HEK-293FT cells for 48 h. The cell extracts were incubated with anti-HA prebound onto protein G beads. The bound proteins were subjected to Western blots using antibodies indicated under each blot. *B*, co-localization of $SRp55_{FL}$ and its deletion mutants with Dyrk1A in the nucleus. HA-SRp55 and Dyrk1A were co-transfected into HeLa cells. After 48 h of transfection, the cells were fixed and immunostained by anti-HA or anti-Dyrk1A, followed by Cy3 anti-rabbit IgG or Alexa 488 anti-mouse IgG. *IP*, immunoprecipitation.

indicating that Dyrk1A modulates SRp55 function in Tau exon10 splicing via phosphorylation.

To know whether three putative Dyrk1A three sites, Ser-280, Ser-303, and Ser-316, may mediate the inhibitory role of Dyrk1A in SRp55-promoted Tau exon 10 inclusion, we mutated these three Ser sites to Ala and determined their effects on Tau exon 10 splicing. We found that $SRp55_{S303A}$ had stronger promotion in Tau exon 10 inclusion than wild type SRp55 ($SRp55_{WT}$) (Fig. 6*C*). However, other two mutations, S280A and S316A, did not alter SRp55 function in Tau exon 10 splicing (Fig. 6*C*), suggesting that phosphorylation of SRp55 at Ser-303 inhibits it to promote Tau exon 10 inclusion.

To learn whether Dyrk1A affects SRp55 subcellular localization, we co-expressed HA-SRp55 and Dyrk1A or $Dyrk1A_{K188R}$ in HeLa cells and studied them immunocytochemically. We observed that in Dyrk1A co-expressed cells, SRp55 was located in the nuclei with speckle enrichment (Fig. 6*D*). However, no clear speckle enrichment of HA-SRp55 was seen in $Dyrk1A_{K188R}$ co-expressed cells (Fig. 6*D*). These data suggest that Dyrk1A may phosphorylate SRp55, which affects its subcellular localization.

DISCUSSION

3R-tau and 4R-tau generated by alternative splicing of Tau exon 10 are different in modulation of microtubule dynamics and in their cellular distributions (20–24). Adult human brain expresses equal amounts of 3R-tau and 4R-tau, but fetal brain, which requires different microtubule dynamics, only expresses 3R-tau (4). During development where axonal extension and synaptogenesis occur, neurons express predominantly 3R-tau for more dynamic microtubules (20, 25–28). In contrast to developing neurons, more stable microtubules in the mature brain are required corresponding to an increase in 4R-tau expression.

Alternative splicing is controlled by multiple exonic and intronic *cis*-elements and *trans*-acting splicing factors. Somatic cells share some DNA sequences spatiotemporally, suggesting that the developmental and tissue specific alternative splicing is regulated by *trans*-acting splicing factors only. SR proteins are a family of splicing factors that are involved in alternative splicing (29, 30). Generally, SR proteins contain one or two RNA recognition motifs at the N terminus, which determine RNA binding specificity, and an RS domain at the C terminus, which promotes protein-protein interaction within the splicing complex (31, 32). SR proteins are essential for both constitutive splicing and alternative splicing. For constitutive splicing, SR proteins are required for the formation of the early prespliceosomal complex to stabilize U1 snRNP article and U2AF (33, 34). In alternative splicing, SR proteins function in modulating the 5′ splice site in a concentration-dependent manner. SRp55, one of the SR proteins, contains two RRMs and two RS domains separated by a proline-rich domain. In the present study, we found that the RS domains appear to be responsible for localization of SRp55 in speckles. Deletion of RS domain resulted in diffused nuclear localization.

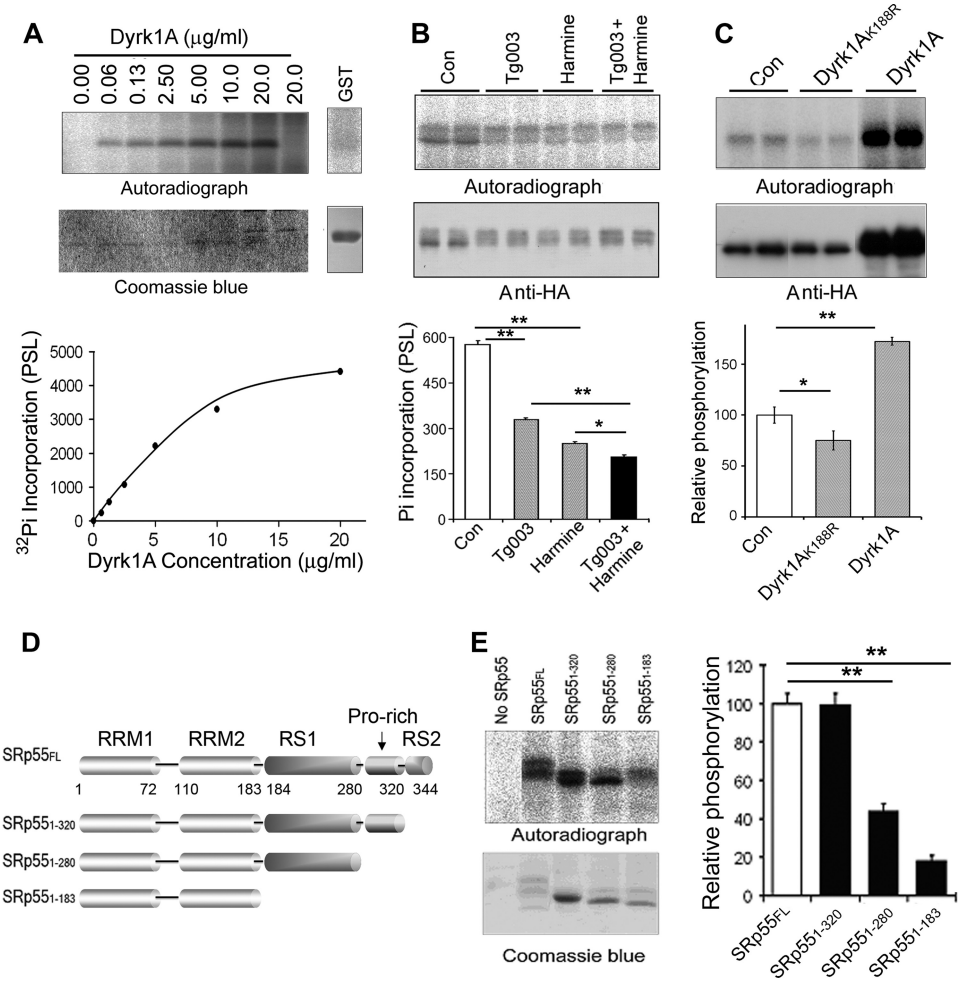

FIGURE 5. **Dyrk1A phosphorylates SRp55 *in vitro* and in cultured cells.** *A*, SRp55 was phosphorylated by Dyrk1A *in vitro*. Recombinant GST-SRp55 or GST was incubated with various concentrations of Dyrk1A or Dyrk1A alone (*last lane*) in the presence of [^{32}P]ATP at 30 °C for 30 min, and the reaction mixture was then separated by SDS-PAGE and visualized with Coomassie Blue staining or autoradiography (*upper panel*). Quantitation of ^{32}P incorporation is shown in the *lower panel*. *PLS*, photostimulated luminescence. *B* and *C*, SRp55 was phosphorylated by Dyrk1A in cultured cells. HEK-293FT cells were transfected with pCEP4/SRp55-HA for 45 h and then treated with 10 μM Tg003 and/or 20 μM harmine (*B*) or co-transfected with pCEP4/SRp55-HA and pcDNA3/Dyrk1A or pcDNA/Dyrk1A$_{K188R}$ for 48 h (*C*). After 45 h of transfection, [^{32}P]phosphate was added to label the phosphoproteins for 3 h, and then the cell lysates were subjected to immunoprecipitation with anti-HA. The immunoprecipitated HA-SRp55 was analyzed by autoradiography. Quantitation of ^{32}P incorporation is shown in the *lower panels* of *B* and *C* after normalization by SRp55 level detected by Western blots developed with anti-HA. *D*, schematic of SRp55 deletion mutants. *E*, GST fused deletion mutants of SRp55 were incubated with Dyrk1A *in vitro* for 30 min at 30 °C. ^{32}P incorporation into GST-SRp55 mutants was determined by autoradiography after the separation of the phosphorylation products by SDS-PAGE. Quantitation of the ^{32}P incorporation after normalization by the protein level is shown in the *right panel*. The results represent the means ± S.D. *, $p < 0.05$; **, $p < 0.01$. *Con*, control.

The 5′ end of Tau exon 10 contains three exonic splicing enhancers: a SC35-like enhancer, a polypurine enhancer, and an A/C-rich enhancer (35). Following the exonic splicing enhancer region, there is an exon splicing silencer. In addition, the 3′ end of exon 10 contains another exonic splicing enhancer sequence between the exon splicing silencer and the 5′ splice site. In intron 10, there are bipartite elements composed of the intronic splicing silencer (E10+11 to E10+18) and the intronic splicing modulator (E10+19 to E10+26). By bioinformatics analysis using an ESEfinder program, we found that Tau exon 10 has four potential SRp55 binding sites. They are sequences 2–7, 34–39, 49–55, and 73–78 of Tau exon 10. Among them, three acting sites are within the SC35-like enhancer, A/C-rich enhancer and C-terminal exonic splicing enhancer, respectively. In the present study, we found that SRp55 promotes Tau exon 10 inclusion. Expression of its deletion mutants fails to promote Tau exon 10 inclusion or even works as antagonist to inhibit exon 10 inclusion. However, the nature of the splicing enhancer(s) that SRp55 acts on to promote Tau exon 10 inclusion remains elusive.

SR proteins are extensively phosphorylated on serine residues, and phosphorylation plays an important role in regulating their nuclear activities. To date, multiple kinases, including SR protein kinase 1 (SRPK1) (36), SRPK2 (37), Cdc-like kinase (Clk/Sty) (38), DNA topoisomerase I (39), cAMP-dependent protein kinase (PKA), and AKT (40, 41), have been shown to phosphorylate the RS domain of SF2/ASF. We recently found that Dyrk1A phosphorylates SF2/ASF, SC35, and 9G8 and regulates both subcellular localization and activity (13, 14, 19). In the present study, we determined the phosphorylation of SRp55 by Dyrk1A and found that Dyrk1A phosphorylates SRp55 mainly at the proline-rich domain. Dyrk1A is a proline- and arginine-directed serine/threonine kinase and phosphorylates consensus motif, R*X*(*X*)(T/S)P. SRp55 has three such consensus

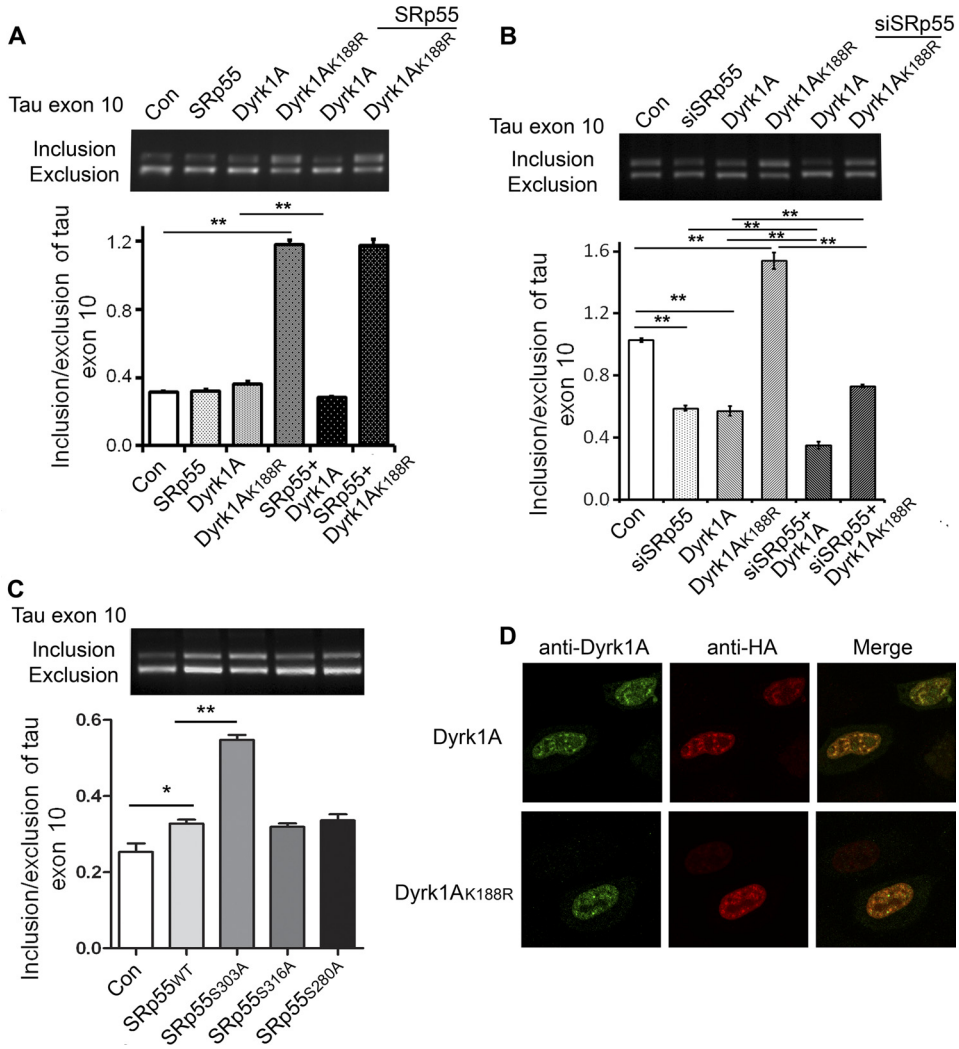

FIGURE 6. **Dyrk1A suppresses SRp55-promoted Tau exon 10 inclusion.** *A*, effects of Dyrk1A or dominant negative Dyrk1A, Dyrk1A$_{K188R}$, on SRp55-mediated Tau exon 10 splicing. Dyrk1A or Dyrk1A$_{K188R}$ was co-expressed with SRp55 in pCI/SI9-LI10 transfected HEK-293FT cells for 48 h. The alternative splicing products of Tau exon 10 were measured by RT-PCR. The ratio of inclusion and exclusion of Tau exon 10 is presented in the *lower panel*. *B*, syngeneic effect of Dyrk1A and siRNA of SRp55 (*siSRp55*) on Tau exon 10 splicing. Dyrk1A or Dyrk1A$_{K188R}$ was co-expressed with siRNA of human SRp55 in pCI/SI9-LI10 transfected HEK-293FT cells for 48 h. The alternative splicing products of Tau exon were measured with RT-PCR. The ratio of inclusion and exclusion of Tau exon 10 is presented in the *lower panel*. *C*, mutation of SRp55 at Ser-303 to Ala enhanced its promotion in Tau exon 10 inclusion. Wild type SRp55 (SRp55$_{WT}$) or its mutants (SRp55$_{S303A}$, SRp55$_{S316A}$, and SRp55$_{S280A}$) were expressed in pCI/SI9-LI10-transfected HEK-293FT cells for 48 h. The alternative splicing products of Tau exon 10 were measured by RT-PCR. The ratio of inclusion and exclusion of Tau exon 10 is presented in the *lower panel*. *D*, Dyrk1A or Dyrk1A$_{K188R}$ differently affected the subcellular localization of SRp55. HA-SRp55 and Dyrk1A or Dyrk1A$_{K188R}$ were co-transfected into HeLa cells. After 48 h transfection, the cells were fixed and double-immunostained by anti-HA and anti-Dyrk1A, followed by Cy3 anti-rabbit IgG and Alexa 488 anti-mouse IgG. The results represent the means ± S.D. *, $p < 0.05$; **, $p < 0.01$. *Con*, control.

motifs in the proline-rich domain: Ser-280, Ser-303, and Ser-316. Therefore, deletion of this domain reduced SRp55 phosphorylation by Dyrk1A dramatically. Mutation of Ser-303 to Ala, but not Ser-280 and Ser-316, increased the promotion of SRp55 in Tau exon 10 inclusion. In addition, it is known that in addition to the consensus sites, Dyrk1A also phosphorylates Ser/Thr within non-consensus site (42–45). The RS domain of SRp55 has many Ser/Thr resides that probably are phosphorylated by Dyrk1A with low efficiency.

Unlike in most proteins, phosphorylation of SRp55 increased its mobility shift in SDS-PAGE, and inhibition of Dyrk1A by harmine slowed its gel mobility. Overexpression of Dyrk1A inhibited SRp55-promoted Tau exon 10 inclusion, but dominant negative Dyrk1A (Dyrk1A$_{K188R}$) did not inhibit SRp55-mediated Tau exon 10 splicing (Fig. 6*A*). Thus, our data suggest that Dyrk1A modulates splicing activity of SRp55 by phosphorylating it at the proline-rich domain. When SRp55 had been knocked down, overexpression of Dyrk1A further suppressed Tau exon 10 inclusion, suggesting a possible involvement of other splicing factor(s). Our previous studies have demonstrated that Dyrk1A phosphorylates SF2/ASF and SC35 and inhibits Tau exon 10 inclusion (13).

Normal alternative splicing of Tau exon 10 is required for maintaining normal function of the central nervous system. Imbalance of 3R-tau and 4R-tau is a cause of several tauopathies (5). Overexpression of Dyrk1A in Down syndrome brain caused by an extra gene copy may dysregulate Tau exon10 splicing through phosphorylating and suppressing SR proteins, such as SF2/ASF, SC35, and SRp55, and contributes to early onset of Tau pathology in individuals with DS.

In summary, we found that SRp55 promoted Tau exon 10 inclusion and its RS domains were required for this function. SRp55 interacted with Dyrk1A through the RRM domain. Dyrk1A phosphorylated SRp55 mainly at the proline-rich domain. Phosphorylation of SRp55 by Dyrk1A inhibited its activity in promotion of Tau exon 10 inclusion. Overactivation of Dyrk1A as seen in DS may increase phosphorylation of SRp55 and thus inhibit its activity in Tau exon 10 splicing, resulting in an increase of 3R-tau expression.

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