Nuclear Inhibitor of Protein Phosphatase-1 (NIPP1) Directs Protein Phosphatase-1 (PP1) to Dephosphorylate the U2 Small Nuclear Ribonucleoprotein Particle (snRNP) Component, Spliceosome-associated Protein 155 (Sap155)*

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Pre-mRNA splicing entails reversible phosphorylation of spliceosomal proteins. Recent work has revealed essential roles for Ser/Thr phosphatases, such as protein phosphatase-1 (PP1), in splicing, but how these phosphatases are regulated is largely unknown. We show that nuclear inhibitor of PP1 (NIPP1), a major PP1 interactor in the vertebrate nucleus, recruits PP1 to Sap155 (spliceosome-associated protein 155), an essential component of U2 small nuclear ribonucleoprotein particles, and promotes Sap155 dephosphorylation. C-terminally truncated NIPP1 (NIPP1-D/C) formed a hyper-active holoenzyme with PP1, rendering PP1 minimally phosphorylated on an inhibitory site. Forced expression of NIPP1-WT and -D/C resulted in slight and severe decreases in Sap155 hyperphosphorylation, respectively, and the latter was accompanied with inhibition of splicing. PP1 overexpression produced similar effects, whereas small interfering RNA-mediated NIPP1 knockdown enhanced Sap155 hyperphosphorylation upon okadaic acid treatment. NIPP1 did not inhibit but rather stimulated Sap155 dephosphorylation by PP1 in vitro through facilitating Sap155/PP1 interaction. Further analysis revealed that NIPP1 specifically recognizes hyperphosphorylated Sap155 throughout its Forkhead-associated domain and dissociates from Sap155 after dephosphorylation by associated PP1. Thus NIPP1 works as a molecular sensor for PP1 to recognize phosphorylated Sap155.

Pre-mRNA splicing is an essential step for expression of most genes in metazoans. Intron excision from a nascent transcript is achieved by pre-mRNA splicing catalyzed by the spliceosome, a macromolecular complex consisting of five small nuclear ribonucleoprotein particles (snRNPs) and a large number of non-snRNP proteins. Spliceosome assembly is an ordered process that includes stepwise recruitment of U1, U2, U5, and U4/6 snRNPs on a pre-mRNA and sequential formation of complex E → A/B → B* → C. The activated B* spliceosome catalyzes step I of splicing, whereas the C complex catalyzes step II. During and after splicing, spliceosome components dissociate and are recycled for further rounds of splicing. Spliceosome assembly/disassembly and splicing catalysis are thought to be regulated in part by reversible phosphorylation of spliceosomal proteins (1–3).

U2 snRNP includes U2 snRNA and two heteromeric protein complexes, Sf3a and Sf3b. Sap155, also known as Sf3b1 or Sf3b155, is a component of the Sf3b and becomes hyperphosphorylated concomitantly with or just after the first catalytic step of splicing in vitro (4). A recent study reveals that Sf3a/b proteins are destabilized and dissociate from the RNP core of the activated spliceosome during the transition from the B to C complex (5). Although Sf3a and Sf3b are essential early in the splicing reaction, they are apparently not required for the second catalytic step. Currently, it is not known what triggers exchange of proteins during spliceosome transitions.

Shi et al. (6) reported that the protein Ser/Thr phosphatase (PPase) type 1 (PP1) and/or type 2A (PP2A) are essential for splicing in vitro, in particular, at the second catalytic step. They also observed that Sap155 and U5-116k are potential substrates of these two PPases, suggesting the importance of dephosphorylation of spliceosomal proteins in spliceosome structural regulation.

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‡‡ The abbreviations used are: snRNP, small nuclear ribonucleoprotein particle; RNP, ribonucleoprotein; PP1, protein phosphatase-1; NIPP1, nuclear inhibitor of PP1; siRNA, small interfering RNA; PBS, phosphate-buffered saline; Dox, doxycycline; RNAi, RNA interference; OA, okadaic acid; PPase, phosphatase; HTO, HeLa-Tet-Off; RPA, RNase protection assay; FHA, Forkhead-associated; RT, reverse transcription; FHA, Forkhead-associated.

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rearrangement. However, how PP1 and PP2A regulate phosphorylation of spliceosomal proteins is not well understood.

Both PP1 and PP2A are often targeted to specific substrates by binding partners. For example, PP1 is regulated in vivo primarily by interaction with various regulatory subunits that dictate subcellular localization, substrate specificity, or enzymatic activity (7). In some cases, regulatory subunits are phosphorylated/dephosphorylated, resulting in activity changes that modulate PP1 activity in response to environmental cues such as hormones and second messengers (8–10). In addition to interacting with regulatory subunits, PP1 is regulated by phosphorylation. Phosphorylation by cyclin-dependent kinases lowers PP1 activity in vitro and vivo in Schizosaccharomyces pombe and in human cells (11, 12). The PP1 phosphorylation site is highly conserved among species and has been mapped to the C terminus. Studies in yeast and human cells suggest that PP1 phosphorylation is essential for proper cell cycle progression (13, 14).

A major nuclear interaction partner of PP1 in vertebrate cells is the nuclear inhibitor of PP1 (NIPP1) (7). Targeted disruption of the Nipp1 gene in mice results in embryonic lethality, indicating an essential role in vivo (15). NIPP1 has two PP1 binding regions as follows: a central domain containing a so-called RVXF-motif, which represents a high affinity binding motif and a low affinity binding region mapped to the NIPP1 C terminus (16, 17). The precise function of the latter interaction is not yet understood. Previous studies suggest the importance of NIPP1 in regulating pre-mRNA splicing. NIPP1 localizes in nuclear speckles, domains rich in splicing machinery components. Several splicing-related proteins such as Cdc5L, Sap155, and MELK have been identified as potential ligands of the Forkhead-associated (FHA) domain of NIPP1 (18–20). FHA is a modular phospho-Thr/Ser recognition motif (21, 22). Beullens and Bollen (23) reported that NIPP1 physically associates with spliceosomes through the FHA domain and that a dominant negative form of NIPP1 blocks the B to C transition of spliceosome assembly in the HeLa cell nuclear extracts by an unknown mechanism. Although there is a report that NIPP1 protein lacking the RVXF motif partially compromises splicing in vitro, data from splicing assays using HeLa nuclear extracts are contradictory (23, 24). Thus both the mode of NIPP1 action and the potential role of PP1/NIPP1 interaction in pre-mRNA splicing remain obscure.

To analyze function of NIPP1 in pre-mRNA splicing, we re-evaluated the physical and functional association among pre-mRNA/RNA, NIPP1, and PP1 in vivo using cell-based assays. We show that NIPP1 works as a targeting subunit that directs PP1 to dephosphorylate Sap155, not as an inhibitor of PP1. Our results suggest a regulatory mechanism ensuring proper Sap155 phosphorylation-dephosphorylation cycles during pre-mRNA splicing.

MATERIALS AND METHODS

Antibodies—Anti-U5-116k antibody was kindly provided by Dr. H. Brahms and Dr. R. Lührmann (25). Anti-Sap155 (26), anti-Myc 9e10, anti-Cdc5L, anti-FLAG M2, anti-phospho-Thr-320-PP1, anti-MELK, and anti-pan-PP1 E9 antibody were purchased from MBL (Nagoya, Japan), Roche Applied Science, BD Transduction Laboratories, Sigma, Cell Signaling Technology Inc. (Danvers, MA), Abcam (Cambridge, UK), and Santa Cruz Biotechnology Inc. (Santa Cruz, CA), respectively. Antibodies specific to each PP1 isoform have been described (27). Polyclonal or monoclonal anti-NIPP1 antibodies were generated against a peptide corresponding to the C-terminal 20 amino acids in our laboratory or purchased from BD Transduction Laboratories.

Plasmid Constructs—The pTRE-β-globin reporter has been described (28). The BsrRI-BstXI region of pTRE-β-globin, encompassing the β-globin gene from the 3′-half of exon 1 to the 5′-half of exon 3, was replaced with a corresponding BsrRI-BstXI fragment of β-globin cDNA derived from pTRE-β-globin; the sequence verified, and the resulting plasmid was designated pTRE-intronless-β-globin. Its nucleotide sequence is identical to that of pTRE-β-globin except intron 1 and 2 sequences are excluded. NIPP1 expression plasmids were constructed by subcloning cDNAs encoding wild-type or mutant forms of rat NIPP1 (29) into pCMV-FLAG2 (Sigma). Myc-PP1 constructs are described elsewhere (30).

Cell Culture and Transfection—HeLa-Tet-Off (HTO) cells (Clontech) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 250 μg/ml geneticin. Transfections were done using FuGENE 6 reagent (Roche Applied Science) according to the manufacturer’s recommendation. For stable transfection, cDNAs encoding FLAG-tagged wild-type or mutant NIPP1 were subcloned into the pTRE vector (Clontech). HTO cells in 10-cm dishes were transfected with 10 μg of pTRE-NIPP1-WT or -ΔC with 0.5 μg of pTK-Hyg (Clontech). Drug-resistant clones were selected in 200 μg/ml hygromycin, 250 μg/ml geneticin, and 1 μg/ml Dox. Two weeks later, ~150 independent clones for each construct were screened for expression of exogenous NIPP1 by immunoblotting with anti-FLAG antibody. Several clones for each construct (four for WT NIPP1 and three for NIPP1-ΔC) exhibited low and high levels of FLAG-NIPP1 proteins in the presence and absence of Dox, respectively, and were selected for further experiments. After establishment, stable clones were maintained in medium containing 5–10 ng/ml Dox.

In Vitro and in Vivo Splicing Assays—In vitro splicing was performed as described (23). RNP immunoprecipitation assays were performed essentially as described (31) with slight modification. Cells were fixed in 0.75% formaldehyde/PBS at room temperature for 10 min, washed twice in PBS, and harvested. Cells were lysed in 1 ml of RIPA buffer (20 mM Tris-Cl, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 0.1% SDS, 1% Triton X-100, and 0.1% deoxycholate) by sonication (Sonifier 450, Branson, Danbury, CT). 50 μl each of lysate was stored on ice as “Input,” and the rest was immunoprecipitated with anti-FLAG-agarose beads at room temperature for 2 h. Beads were collected by centrifugation and washed four times with gentle rotation for 5 min, followed by elution of RNPs with FLAG peptide (Sigma). “Inputs” and eluates were heated to 75 °C for 90 min to reverse cross-links and phenol-extracted twice. RNAs were ethanol-precipitated using yeast tRNA and Glyco-blue co-precipitants (Ambion) as carrier and treated with RNase-free DNase (DNA-free turbo, Ambion). RNA was reverse-transcribed using SuperScript III RTase (Invitrogen) using the RT primer 5′-
GTGCAGCTTGTACAGTGACG-3’. PCR was undertaken using standard protocols with Ex Taq HS polymerase (Takara Bio Inc., Otsu, Japan) and the following primers for β-globin sense, 5′-ATGGTGCACTGACTCCTGAG-3’; antisense, 5′-ACTAAAGGACCGACAGCTTTGTTG-3’. To analyze effects of NIPP1 on splicing rates, HTO cells in 10-cm dishes were transfected with 7.5 μg of NIPP1 expression plasmids with 2.5 μg of pTRE-β-globin. Four hours later, cells were split and further cultured without Dox for 20 h. At indicated time points after Dox addition, cells were harvested, and total RNA was isolated for Northern blot analysis with a 32P-labeled β-globin probe. For RPAs, antisense riboprobes complementary to exon 1/intron 1 or exon 2/intron 2 boundaries of probe. For RPAs, antisense riboprobes complementary to exon 1/intron 1 or exon 2/intron 2 boundaries of β-globin gene were synthesized by in vitro transcription using MAXI script (Ambion, Austin, TX) and T7 polymerase. Hybridization and RNase digestions were performed using the RPA III (Ambion) kit according to the manufacturer’s instructions. Protected RNAs were subjected to denaturing PAGE and visualized using the FLA system (Fuji Film Co., Tokyo, Japan).

**Immunoprecipitation and Western Blot Analysis**—Cells were lysed in RIPA or SDS buffer (125 mM Tris-Cl, pH 6.8, 1% SDS) by sonication using a “Bio-ruptor” sonicator (CosmoBio, Tokyo, Japan). For co-immunoprecipitation, cells were lysed in buffer (50 mM Tris-Cl, pH 7.4, 125 mM NaCl, 0.2% Triton X-100, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, leupeptin, and aprotinin) by sonication. Lysates were incubated with anti-FLAG-agarose or anti-Myc-agarose (Sigma) for 3–4 h at 4 °C. Immunocomplexes were collected by centrifugation, washed three times with lysis buffer without proteinase inhibitors, and immunoblotted.

**Immunohistochemistry**—Cells grown on collagen-coated coverslips were fixed in PBS containing 3.7% formaldehyde for 10 min. After permeabilization with 0.2% Triton X-100, cells were incubated in PBS containing 5% (w/v) bovine serum albumin and 0.5 μg/ml anti-FLAG M2 monoclonal antibody overnight at 4 °C. Immunoreactants were further visualized using Alexa Fluor488-conjugated anti-mouse IgG secondary antibody (Invitrogen), and images were acquired using a Pascal confocal laser-scanning microscope (Zeiss).

**32P-Orthophosphate Labeling**—Stable clones were cultured in the presence or absence of 10 ng/ml Dox for 24 h and then in phosphate-free medium (Invitrogen) containing 10% fetal calf serum (dialyzed three times against 100 volumes of Heps buffer) and 32P-orthophosphate (PerkinElmer Life Sciences) at 1 mCi/ml for an additional 4 h. Cells were harvested, lysed by sonication using the Bio-ruptor sonication machine, and subjected to immunoprecipitation. Immunoprecipitates were separated by SDS-PAGE, followed by immunoblot analysis and autoradiography using the FLA system.

**In Vitro De-phosphorylation Experiments**—HTO cells were lysed in buffer (50 mM Tris-Cl, 100 mM NaCl, 2 mM EDTA, 0.1% Triton X-100) by sonication. Lysates were incubated with or without either PP1 (a recombinant PP1α isoform; Calbiochem) or NIPP1-PP1 holoenzyme at 30 °C for 10–60 min. The NIPP1-PP1 holoenzyme was constituted by preincubation of recombinant PP1α with His-NIPP1 at several ratios on ice for 20 min in Tris-buffered saline. His-NIPP1 was expressed in Escherichia coli using the pET system (Novagen, San Diego, CA) and purified using TALON beads (Clontech) following the manufacturer’s recommendations. Sap155 was immunopurified from lysates of HeLa cells treated with 100 nM okadaic acid for 6–8 h using anti-Sap155 monoclonal antibody-conjugated agarose.

**Phosphatase Assay**—Immunoprecipitates with anti-FLAG-agarose were eluted twice by incubating beads with 200 μg/ml FLAG peptide. Eluates were subjected to a phosphatase assay and Western blotting. PP1 activity measurement was as described (26).

**RNAi Experiments**—siRNA duplexes against human NIPP1 (Stealth RNAi) were purchased from Invitrogen (HS513426 and HS143427). Stealth RNAi Negative control Medium GC duplex (Invitrogen) served as control. siRNA transfection was undertaken using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions at final siRNA concentrations of 5 nM in culture.

**Overlay Assay**—Sap155 was immunoprecipitated from HeLa cell lysates treated with 100 nM okadaic acid (OA) for 6–8 h, separated by SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was blocked with 5% bovine serum albumin in PBS and reacted with solutions supplemented with phosphatase inhibitors (Roche Applied Science) at 4 °C for 1 h. Membranes were washed with PBS, 0.1% Tween plus phosphatase inhibitors at 4 °C for 5 min twice and then UV-irradiated to fix complexes.

**Statistical Analysis**—One-way analysis of variance combined with Tukey’s test was used to analyze data with unequal variance between each group. A probability level of 0.05 was considered significant.

## RESULTS

**Physical and Functional Interaction between NIPP1 and Pre-mRNA Splicing in Intact Cells**—To elucidate interaction between NIPP1 and the spliceosome in vivo, we performed an RNP immunoprecipitation assay (31). Cells transfected with FLAG-NIPP1 (Fig. 1A) together with the β-globin reporter plasmid pTRE-β-globin were treated with formaldehyde, recovered, and lysed. RNPs containing FLAG-NIPP1 were immunoprecipitated using an anti-FLAG antibody. The cross-links were reversed, and the immunoprecipitated RNAs were detected by RT-PCR (“Minus RT” controls are presented as supplemental Fig. 1). As shown in Fig. 1B, both unspliced and spliced β-globin RNAs co-immunoprecipitated with FLAG-NIPP1-WT. We next examined interaction between NIPP1 mutants (Fig. 1A) and β-globin pre-mRNA/mRNAs. Similar results were obtained with NIPP1-R53A, which has a nonfunctional FHA domain (Fig. 1C), NIPP1-V201A/F203A (NIPP1-RATA), which is deficient in PPI binding (Fig. 1D), reproducibly co-immunoprecipitated pre-mRNA more efficiently than did NIPP1-WT. NIPP1-ΔC, which lacks the C-terminal 22 amino acids constituting a second PP1-binding region, interacted almost exclusively with unspliced pre-mRNA. This observation correlated with accumulation of unspliced reporter pre-mRNA in cell lysates, as detected by RT-PCR (“total RNA” in Fig. 1B), suggesting inhibition of splicing by that mutant form of NIPP1. Because transcription and “post-transcriptional” RNA processing are functionally coupled (32), the
effects of NIPP1-ΔC on splicing might represent an indirect transcriptional effect. To distinguish between these possibilities, we repeated the assays using a corresponding intron-less reporter gene. As shown in Fig. 1E, NIPP1-ΔC and other NIPP1 constructs did not significantly affect mRNA levels of the intron-less reporter, suggesting that the effect of NIPP1-ΔC on splicing is specific for a splicing-related event(s) or for splicing itself. Intriguingly, all NIPP1 variants co-precipitated mRNA derived from the intron-less reporter, consistent with a recent report that the U2 snRNP is recruited to both intron-containing transcripts and transcripts of intron-less genes, such as histone genes, to facilitate 3′-processing (33).

To further delineate effects of mutant NIPP1, we took advantage of the pTRE-β-globin reporter, in which the transcription is regulated by a TRE sequence upstream of the cytomegalovirus promoter (Fig. 1B). When introduced into cells expressing the Tet-repressor (Tet-Off cell lines), de novo transcription from the reporter is specifically and rapidly suppressed by the stable tetracycline analogue Dox (28). Northern blot analysis showed that expression of NIPP1-ΔC decreased steady-state levels of β-globin mRNA, whereas expression of NIPP1-WT had produced little if no effect (Fig. 2A). A band likely representing β-globin pre-mRNA (indicated by asterisk in Fig. 2A) migrated more slowly than β-globin mRNA and was more abundant in cells transfected with NIPP1-ΔC compared with mock- or NIPP1-WT-transfected cells. To confirm this observation, we performed an RPA (Fig. 2C; see supplemental Fig. 2 for assay design) and found that in mock-transfected cells levels of unspliced β-globin pre-mRNA were rapidly decreased after Dox-mediated block of de novo transcription of the reporter gene, as seen in Fig. 2C. This decrease is likely due to splicing of the reporter pre-mRNA. Strikingly, unspliced pre-mRNA was more abundant in cells transfected with NIPP1-ΔC, whereas mature spliced mRNA levels were decreased (Fig. 2C). Additionally, the half-life of unspliced pre-mRNA in NIPP1-ΔC-transfected cells appeared much longer than that seen in mock- and NIPP1-WT-transfected cells.
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As described, NIPP1-RATA associated with unspliced pre-mRNA more efficiently than did NIPP1-WT, whereas NIPP1-RATA did not accumulate unspliced pre-mRNA, in contrast to NIPP1-ΔC (Fig. 1B). It is noteworthy that decay of unspliced pre-mRNA of NIPP1-RATA-transfected cells was slightly slower than that seen in mock-transfected cells (Fig. 2, C and D). Migration of protected fragments derived from unspliced and spliced mRNAs is shown. Band intensities derived from the unspliced transcript were evaluated and are shown as values relative to those at 0 h (D). Data represent mean values of 3–4 independent RPAs with S.D. *, p < 0.001; **, p < 0.01 (ΔC versus WT).

Characterization of Splicing Inhibition by NIPP1-ΔC—To understand functional differences between NIPP1-ΔC and wild-type protein, we first investigated NIPP1-ΔC subcellular localization and found it indistinguishable from that of NIPP1-WT (Fig. 3A). We next prepared NIPP1 constructs containing point mutations in addition to the ΔC deletion to investigate domain requirements for splicing inhibition (Fig. 3B). The effects of these mutations on splicing of the β-globin construct were determined by analyzing accumulation of unspliced pre-mRNA. An RPA analysis revealed that NIPP1-ΔC, but not NIPP1-R53A/ΔC or NIPP1-RATA/ΔC, inhibited pre-mRNA splicing of the reporter gene (Fig. 3C). These results indicate that inhibition of splicing by NIPP1-ΔC requires both a functional FHA domain and interaction with PP1. Neither the R53A nor RATA mutation alone promoted accumulation of unspliced pre-mRNA (Fig. 1B and data not shown).

Because splicing inhibition by NIPP1-ΔC requires interaction with PP1, we compared NIPP1-WT and -ΔC mutant in terms of association with PP1. NIPP1-WT and NIPP1-ΔC were immunoprecipitated, and PP1 activities were evaluated using phosphorylase α as a substrate. PP1 activity complexed with NIPP1-ΔC was reproducibly higher than that seen with NIPP1-WT (Fig. 4A). Similar amounts of PP1 in immunoprecipitates of NIPP1-WT or -ΔC were confirmed by immunoblotting with anti-PP1 antibody (Fig. 4B). We reasoned that differences seen in Fig. 4A could be due to potential modification of the catalytic subunit PP1. It is well established that PP1 is phosphorylated by cyclin-dependent kinase(s) on a critical Thr residue in the C terminus (Thr-320 of PP1α) and inactivated (11, 12). NIPP1-WT or NIPP1-ΔC was immunoprecipitated from transfected cells, and the inhibitory phosphorylation of endogenous PP1 associated with NIPP1 was examined and compared. Strikingly, PP1 co-immunoprecipitated with NIPP1-WT was highly phosphorylated on the inhibitory site, whereas PP1 in association with NIPP1-ΔC was minimally phosphorylated (Fig. 4B). Thus, the specific activity of PP1 associated with NIPP1-ΔC could be due to lower levels of inhibitory phosphorylation of PP1, rendering it constitutively active. At present, we cannot differentiate the levels of inhibitory phosphorylation in each PP1 isoform because the phospho-Thr-320-PP1α antibody may also cross-react with other isoforms phosphorylated on the corresponding residue. Nevertheless, given that NIPP1 preferentially associates with PP1α (see below), it is likely that phospho-PP1 co-immunoprecipitating with NIPP1 is primarily PP1α.

NIPP1-associated PP1 Regulates Sap155 Phosphorylation—To further analyze NIPP1 actions in pre-mRNA splicing, stable cell lines conditionally expressing WT and mutant forms of NIPP1 (HeLa-TetOff (HTO)-NIPP1 clones) were developed. In these clones, FLAG-NIPP1 proteins were detected 8 h after removal of Dox, and levels were maximal at about 24 h (Fig. 5A). Previous studies had identified several potential effectors of NIPP1 and/or PP1 such as Cdc5L, Sap155, and U5-116k (6, 18, 19, 34). Levels of phosphorylation of these proteins in HTO-NIPP1 clones were investigated by metabolically labeling cells with 32P-orthophosphate. We found that Sap155 hyperphosphorylation was slightly decreased in cells expressing WT-NIPP1 (Fig. 5B). Importantly, this decrease was more apparent in cells expressing NIPP1-ΔC. Induction of NIPP1-ΔC expression by Dox removal caused virtual loss of Sap155 hyperphosphorylation. The slight decrease in Sap155 hyperphosphorylation of HTO-NIPP1-ΔC cells in the presence of Dox was probably due to leaky expression of the construct in this system. In contrast, neither Cdc5L nor U5-116k phosphorylation levels were affected by NIPP1-WT or
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-D.C. Taken together, these results demonstrate that Sap155 is a major dephosphorylation target of PP1, which is regulated by NIPP1 in vivo.

To test the effects of NIPP1 depletion on Sap155 phosphorylation, parental HTO cells were transfected with NIPP1 siRNA to knockdown NIPP1 (Fig. 5C). Little effect of NIPP1 knockdown on Sap155 phosphorylation was observed, as judged by assaying Sap155 mobility shifts on Western blotting. We next treated NIPP1-knockdown cells with the cell-permeable toxin OA, which could preferentially inhibit PP2A class PPases (PP2A, PP4, and PP6) at the adopted concentration of 100 nM. OA treatment of mock- or control siRNA-transfected cells slightly increased levels of hyperphosphorylated Sap155 compared with vehicle-treated cells (Fig. 5C). In NIPP1-knockdown cells, OA-induced hyperphosphorylation of Sap155 was more robust than that seen in mock- or control siRNA-transfected cells (Fig. 5C). These results indicate the importance of endogenous NIPP1 in regulating Sap155 phosphorylation.

The results in Fig. 5, B and C, strongly suggest that NIPP1 promotes dephosphorylation of hyperphosphorylated Sap155 by associated PP1. We further tested this hypothesis by in vitro dephosphorylation experiments. As shown in Fig. 6A, OA treatment of cells greatly induced Thr phosphorylation of numerous cellular proteins (lower panel) and also hyperphosphorylation of Sap155 (upper panel). Incubation of cell lysates under dephosphorylation conditions (i.e. without PPase inhibitors) resulted in time-dependent loss of most phospho-Thr, likely because of the activities of endogenous PPase(s) other than PP1, but did not affect Sap155 phosphorylation. However, addition of recombinant PP1α led to complete loss of hyperphosphorylated Sap155 within 60 min, indicating that Sap155 is a PP1 substrate. Remarkably, further addition of NIPP1 enhanced dephosphorylation of hyperphosphorylated Sap155 (Fig. 6B). The stimulating effect of NIPP1 on Sap155 dephosphorylation by PP1 was also observed with immunopurified Sap155, minimizing the possibility that scaffolding proteins other than NIPP1 mediate this effect (Fig. 6C and supplemental Fig. 3B). Furthermore, Sap155/PP1 interaction was detectable by far-Western analysis only when PP1 was pre-complexed with NIPP1 (Fig. 6D). Thus, we conclude that NIPP1 functions as a Sap155-targeting subunit for PP1.

Aberrent Sap155 Dephosphorylation Correlates with Inhibition of Pre-mRNA Splicing by Mutant NIPP1—How NIPP1 interacts with Sap155 was further investigated by immunoprecipitation assays. Sap155 was effectively co-immunoprecipitated with NIPP1-WT and -RATA but not with NIPP1-ΔC and -R53A (Fig. 7A). Importantly, Sap155 co-immunoprecipitated...
with NIPP1-WT was highly phosphorylated. These results are consistent with previous reports that NIPP1/Sap155 interaction requires both the NIPP1 FHA domain and Sap155 phosphorylation (19). Furthermore, interaction between NIPP1 FHA and Sap155 appears to require that Sap155 be “hyper”-phosphorylated because a faster migrating Sap155 species seen on SDS-PAGE was likely phosphorylated at relatively low levels (autoradiography in Fig. 5B) and was not significantly co-immunoprecipitated with NIPP1-WT. Notably, NIPP1-RATA, which binds PP1 less efficiently than NIPP1-WT, co-immunoprecipitated hyperphosphorylated Sap155 more efficiently than did NIPP1-WT. In contrast, NIPP1-ΔC again decreased hyperphosphorylation of Sap155 based on its mobility shift (“lysate” in Fig. 7A), and hence did not co-immunoprecipitate Sap155 (“IP” in Fig. 7A). Collectively, these results indicate that PP1, which is recruited to hyperphosphorylated Sap155 by NIPP1 through the FHA domain, dephosphorylates Sap155 and suggest that its de-regulation by a mutant form of NIPP1, NIPP1-ΔC, results in defects in pre-mRNA splicing. Strikingly, the NIPP1 mutants NIPP1-RATA/ΔC and -R53A/ΔC, which harbor second mutations that no longer inhibit splicing (Fig. 3), also failed to reduce levels of Sap155 hyperphosphorylation (Fig. 7B).

**PP1 Overexpression Compromises Pre-mRNA Splicing—** Three PP1 isoforms exhibiting differing N- and C-terminal sequences (PP1α, PP1γ, and PP1β) are expressed in mammalian somatic cells. To determine which isoforms bound to NIPP1, co-immunoprecipitation experiments were performed. Significant amounts of PP1α, the most abundant isoform expressed in HeLa cells, but not PP1γ and PP1β, co-immunoprecipitated with endogenous NIPP1, in the order PP1α > PP1γ > PP1β (Fig. 8A). Because NIPP1 recruited PP1 to dephosphorylate Sap155 and NIPP1 dysregulation resulted in splicing inhibition, one might predict that excess nuclear PP1 might also inhibit splicing. To test this possibility, the effects of PP1 overexpression on Sap155 phosphorylation and splicing were investigated. Hyperphosphorylated forms of Sap155 were reduced in cells transfected with PP1α-WT and PP1α-T320A but not in cells transfected with the inactive PP1α mutant, PP1α-H125A (Fig. 8C and D). As expected, either overexpression of wild-type or the active mutant of PP1α, PP1c-T320A, compromised splicing of the reporter gene (Fig. 8E). In contrast, the inactive PP1α mutant, PP1α-H125A had no effect.

**DISCUSSION**

In this study, we investigated physical and functional association between NIPP1, PP1, and pre-mRNA splicing in vivo. Recently, it was reported that PP1 and/or PP2A are required for a catalytic step in splicing, specifically the second step, in vitro and that Sap155 and U5-116k are potential targets of these two PPases (6). But how these PPases are recruited to spliceosomal substrates has not been elucidated. Our results demonstrate that NIPP1 targets PP1 to Sap155. Because NIPP1 has been considered a PP1 inhibitor, the stimulating effect of NIPP1 on Sap155 dephosphorylation by PP1 is unanticipated. These observations are reminiscent of Myp1, another PP1 regulatory protein. Myp1 inhibits PP1 activity against nonphysiological substrates such as phosphorylase a but stimulates it against

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**FIGURE 5. Regulation of Sap155 phosphorylation by NIPP1 in intact cells.** A, establishment of HTO clones conditionally expressing exogenous NIPP1. HTO-NIPP1 clones maintained in 5 ng/ml Dox were washed and cultured in the absence of Dox for the times indicated. Cell lysates were subjected to immunoblotting with the anti-FLAG antibody (upper) or the anti-NIPP1 antibody, which recognizes the Lys-rich region of NIPP1 (lower). The positions of exogenous and endogenous NIPP1 are shown at the left as exo. and endo., respectively. B, parental HTO and stable clones were cultured in the presence or absence of 10 ng/ml Dox. After 24 h, cells were labeled with 32P-orthophosphate for 4 h. Sap155, U5-116k, and Cdc5L were simultaneously immunoprecipitated by the corresponding antibodies, fractionated by SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was first immunoblotted with the mixture of mouse monoclonal anti-Sap155 and anti-Cdc5L antibodies, and then with rabbit anti-sera against U5-116k (WB). Subsequently, the membrane was subjected to autoradiography (32P). A portion of lysate was also loaded onto the gel to reveal positions of the three proteins shown at the left. C, HTO cells were transfected with control siRNA or siRNAs against human NIPP1. Forty eight hours later, transfected cells were incubated an additional 4.5 h with 100 mM okadaic acid (OA) or vehicle (DMSO). Immunoblots were performed using anti-Sap155, anti-NIPP1, or anti-vimentin antibodies.
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FIGURE 6. NIPP1 bridges Sap155 and PP1. A, dephosphorylation of Sap155 by PP1 in vitro. HTO cells were transfected with siRNA against NIPP1, treated with OA, and lysed. Lysates were incubated with 50 ng of recombinant PP1α at 30 °C for the times indicated. Immunoblots were done with anti-Sap155 or anti-pThr antibody. B, NIPP1 enhances Sap155 dephosphorylation by PP1 in vitro. Lysates of OA-treated HTO cells were incubated with increasing amounts of exogenous NIPP1 (upper) or PP1-NIPP1 holoenzyme (lower) at 30 °C for 10 min. Immunoblots were done with anti-Sap155 antibody. The holoenzyme was reconstituted using recombinant PP1α and His-NIPP1 expressed in and purified from E. coli. Lower panel, NIPP1 amounts are shown as a ratio relative to recombinant PP1α. C, dephosphorylation experiments using purified Sap155. In vitro dephosphorylation experiments were performed, as in B, using Sap155 immunopurified from HeLa cells treated with OA. Reactions were done using 50 ng of PP1α and/or His-NIPP1 per reaction and analyzed by immunoblot using anti-Sap155, anti-PP1α, and anti-NIPP1 antibodies. Asterisk marks residual signal of anti-PP1α blot. D, reconstitution of Sap155-NIPP1-PP1 ternary complex in vitro. Physical association between Sap155 and PP1 was analyzed by far-Western. Sap155 was immunopurified, size-fractionated by SDS-PAGE, and transferred to a membrane. Membrane pieces were reacted with 100 ng/ml of PP1α in the presence or absence of recombinant His-NIPP1 (+ + and + indicate 300 and 100 ng/ml His-NIPP1, respectively). PP1α overlaid on Sap155 was detected by immunoblot using anti-PP1α antibody. Similar amounts of Sap155 in each lane were confirmed by anti-Sap155 blot (lower).

FIGURE 7. Physical and functional associations between NIPP1 mutant proteins and Sap155. A, ΔC mutation of NIPP1 decreases Sap155 phosphorylation and results in dissociation of the NIPP1-Sap155 complex. HTO cells were transfected with FLAG-NIPP1 plasmids. Twenty eight hours later, cells were lysed and subjected to immunoprecipitation (IP) with an anti-FLAG antibody. Immunoprecipitates were analyzed by immunoblot using anti-Sap155 or anti-FLAG antibodies. Hyper-P indicates positions of hyperphosphorylated forms of Sap155. B, reduction of hyperphosphorylated Sap155 by PP1α-ΔC requires both a functional FHA domain and interaction with PP1. Cells were transiently transfected with FLAG-NIPP1 plasmids depicted in Fig. 1A and Fig. 3A, lysed, and immunoblotted with anti-Sap155 and anti-FLAG antibodies.

myosin light chain, a physiological substrate of the Mypt1/PP1 holoenzyme (35). We propose that the inhibitory activity of NIPP1 is also substrate-dependent.

Whereas overexpression of NIPP1-WT slightly reduced levels of hyperphosphorylated Sap155, expression of NIPP1-ΔC, which constitutes a hyper-active PP1 holoenzyme, produced more severe phenotypes, such as virtual loss of Sap155 hyperphosphorylation and defects in pre-mRNA splicing. The robust effect of NIPP1-ΔC is, at least in part, because of defects in regulation of the NIPP1-PP1 holoenzyme by inhibitory phosphorylation of the catalytic subunit. In contrast, PP1 bound to NIPP1-WT is highly phosphorylated at Thr-320. Although Thr-320 phosphorylation is implicated in regulating the cell cycle, our results suggest that regulation of PP1 by inhibitory phosphorylation is important in more general cellular functions, including regulation of pre-mRNA splicing.

Recently, it was reported that S3a and S3b are dispensable for the second splicing step while essential in early steps. Given that PP1/PP2A proteins, including Sap155, are destabilized and dissociate from the RNP core of the activated spliceosome during the transition from the B to C complex in vitro (5). Thus, it is likely that S3a and S3b are indispensable for the second splicing step while essential in early step(s). Given that PP1/PP2A are required for the second step, dephosphorylation of Sap155 may be critical for dissociation of S3a/b from the RNP core, facilitating structural rearrangement of the spliceosome required for further splicing steps. In this scenario, one could imagine that such regulation occurs by modulation of PPase activity through inhibitory phosphorylation of PP1 bound to NIPP1. Currently, why PP1 bound to NIPP1-ΔC is minimally phosphorylated on Thr-320 is not known and is under investigation.

Although our results show that NIPP1 enhances recruitment of PP1 to Sap155 and promotes Sap155 dephosphorylation, detailed mechanisms whereby NIPP1 stimulates Sap155 dephosphorylation remain unknown. It is important to determine how the PP1/NIPP1 holoenzyme can effectively and specifically dephosphorylate Sap155. PP1/NIPP1 interactions may be competed and transiently interrupted by element(s) within Sap155. Interestingly, Sap155 contains an RICF sequence (Arg-1057 to Phe-1060) resembling a consensus PP1-binding motif, although it has not been shown to be functional. Alternatively, NIPP1 may block substrates other than Sap155 from accessing the PP1 active pocket via steric hindrance. Future structural analysis of the PP1/NIPP1 holoenzyme should address these issues. In context, it is still possible that NIPP1 simultaneously
suppresses PP1 activity in a way that balances Sap155 phosphorylation activity to ensure proper phosphorylation/dephosphorylation cycle. Our results also do not exclude the possibility that higher activity of PP1-NIPP1ΔC against Sap155 in vivo is because of less inhibitory activity of the mutant NIPP1 itself. Currently, we cannot determine whether NIPP1-WT and -ΔC have similar inhibitory activity on hyperphosphorylated Sap155.

In cells expressing NIPP1-WT and -ΔC, Sap155 phosphorylation levels were specifically reduced, although normal phosphorylation levels of U5-116k were seen, suggesting des-regulation of Sap155 phosphorylation alone is sufficient to perturb splicing. But these results do not exclude the possibility that phosphorylation/dephosphorylation of U5-116k is also essential for splicing. Sap155 is the first example of a protein that phosphorylated exclusively at the time of the catalytic step of the splicing reaction (4). Indirect evidence has indicated the importance of Sap155 phosphorylation in splicing, although there has not yet been a direct demonstration. Our results provide additional evidence suggesting an essential role of Sap155 phosphorylation, namely NIPP1-ΔC constituted a hyper-active PP1 holoenzyme, decreasing hyperphosphorylated Sap155 and thereby inhibiting splicing. Sap155 has many potential phosphorylation sites, and several are phosphorylated in in vitro splicing reactions and also in vivo (36, 37). Identification of Sap155 phosphorylation sites and their functions during splicing is critically important for understanding how Sap155 is regulated by phosphorylation/dephosphorylation. Furthermore, recent reports revealed that Sap155 functions not only in constitutive splicing but also in alternative splicing and in epigenetic gene silencing (26, 38). Roles for Sap155 phosphorylation in these processes are the next questions to be analyzed.

NIPP1 knockdown sensitized Sap155 to hyperphosphorylation upon further stimulation of cells by OA. Because OA preferentially inhibits PP2A family PPases, our results are consistent with a previous report that PP1 and/or PP2A is essential for splicing in vitro (6). It is likely that PP1 and PP2A family PPase(s) indeed have overlapping roles in regulating Sap155 phosphorylation in intact cells. According to this notion, the relatively weak effects of mutant NIPP1 lacking the canonical PP1-binding motif (NIPP1-RATA) on splicing seem plausible. NIPP1-RATA was less efficient in binding PP1 and hence co-immunoprecipitated greater levels of hyperphosphorylated Sap155 compared with NIPP1-WT (Fig. 7), indicating that it functions as a dominant negative in some contexts, although its inhibition of splicing was partial (Figs. 1 and 2). One explanation for this observation would be that NIPP1-associated PP1 plays a kinetic role in splicing, affecting only splicing rate. Alternatively, we propose that lower levels of PP1 recruited by NIPP1-RATA could be compensated, for example, by PP2A class PPase(s). The relatively small increase in hyperphosphorylated Sap155 seen in mock- or control siRNA-treated cells with OA (Fig. 5C) strengthens this hypothesis, although the precise mechanism by which OA enhances Sap155 hyperphosphorylation is not clear. As seen in Fig. 5D, treating cells with OA globally affected phosphorylation levels of several cellular proteins, possibly because of a broad spectrum of targets of PP2A class PPases or to indirect effects. Thus, we cannot exclude the possibility that OA enhances Sap155 hyperphosphorylation by stimulating Sap155 kinase(s). We tried co-transfection of siRNAs targeting PP2A in addition to NIPP, but massive cell death resulting from that treatment prevented analysis of these cells.5 It is still possible that PP1 and PP2A dephosphorylate Sap155 differentially in terms of target residue or timing during splicing. Furthermore, PP1 and PP2A may be regulated differentially by intra- or extracellular signals through modification of the phosphatases themselves and/or inhibitory activity.

FIGURE 8. PP1 overexpression affects Sap155 phosphorylation and splicing. A, PP1 isoforms associating with NIPP1. Lysates of HTO cells were incubated with anti-NIPP1 antibody or control normal rabbit IgG. Immunoprecipitates (IP) were immunoblotted using antibodies specific to each PP1 isoform. B, diagram of wild-type and mutant forms of PP1α. Each is tagged with a Myc epitope at the N terminus. C, phosphatase activities of PP1α proteins expressed in HTO cells. Myc-PP1α were transiently expressed in HTO cells and immunoprecipitated with an anti-Myc antibody. Phosphatase activities were determined using phospho-phosphorylase peptide as substrate. Data represent means of three independent experiments with S.D. D, effects of PP1α overexpression on Sap155 phosphorylation. HTO cells were transiently transfected with myc-PP1α, lysed, and analyzed by Western blotting using anti-Sap155 and anti-Myc antibodies. E, cells were transiently transfected with the β-globin reporter together with myc-PP1α constructs. Total RNA was isolated and analyzed by RPA as in Fig. 2.

5 N. Tanuma and M. Nomura, unpublished observations.
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their regulatory protein. In addition to inhibitory phosphorylation of PP1, it should be noted that NIPP1 is also subject to regulation by phosphorylation, decreasing its association with PP1 (39, 40).

Interestingly, NIPP1ΔC promoted severe splicing defects in vivo but not in vitro. Although in vitro splicing assays are reliable and widely used, they may not always reflect in vivo conditions, at least in terms of PP1. In fact, some investigators have reported differences between in vivo and in vitro splicing. For example, a sub-domain of U2AF65, an essential splicing factor, plays important role in vivo but not in vitro (41).

In summary, our results provide evidence that NIPP1 directs PP1 to dephosphorylate Sap155. Further elucidation of the role of the NIPP1 C terminus in regulating PP1 and identification of specific Sap155 sites dephosphorylated by PP1 will be required to fully understand how pre-mRNA splicing is regulated by protein phosphorylation/dephosphorylation cycles. Also, cell lines conditionally expressing NIPP1−ΔC, which mediates a decrease in Sap155 hyperphosphorylation, will be useful to analyze the roles of Sap155 phosphorylation.

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REFERENCES

1. Misteli, T. (1999) Curr. Biol. 9, R198–R200
2. Hastings, M. L., and Krainer, A. R. (2001) Curr. Opin. Cell Biol. 13, 302–309
3. Stamm, S. (2008) J. Biol. Chem. 283, 1223–1227
4. Wang, C., Chua, K., Seghezzi, W., Lees, E., Gozani, O., and Reed, R. (1998) Genes Dev. 12, 1409–1414
5. Bessonov, S., Anokhina, M., Will, C. L., Urlaub, H., and Lührmann, R. (2008) Nature 452, 746–750
6. Shi, Y., Reddy, B., and Manley, J. L. (2006). Mol. Cell. Biol. 26, 191–192
7. Ceulemans, H., and Bollen, M. (2004) Physiol. Rev. 84, 39–58
8. Eto, M., Senba, S., Morita, F., and Yazawa, M. (1997) FEBS Lett. 410, 356–360
9. Greengard, P. (2001). Science 294, 1024–1030
10. Weiser, D. C., Sikes, S., Li, S., and Shenolikar, S. (2004) J. Biol. Chem. 279, 48904–48914
11. Dohadwala, M., da Cruz e Silva, E. F., Hall, F. L., Williams, R. T., Carbonaro-Hall, D. A., Nairn, A. C., Greengard, P., and Berndt, N. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6408–6412
12. Yamano, H., Ishii, K., and Yanagida, M. (1994) EMBO J. 13, 5310–5318
13. Berndt, N., Dohadwala, M., and Liu, C. W. (1997) Curr. Biol. 7, 375–386
14. Liu, C. W., Wang, B. H., Dohadwala, M., Schönthal, A. H., Villa-Moruzzi, E., and Berndt, N. (1999) J. Biol. Chem. 274, 29470–29475
15. Van Eynde, A., Nuyttens, M., Dewerchin, M., Schoonjans, L., Keppens, S., Beullens, M., Moons, L., Carmeliet, P., Stalmans, W., and Bollen, M. (2004) Mol. Cell. Biol. 24, 5863–5874
16. Eloff, M. P. F., Johnson, D., Moorhead, G., Cohen, P. T., Cohen, P., and Barford, D. (1997) EMBO J. 16, 1876–1887
17. Beullens, M., Vulteke, V., Van Eynde, A., Jagiello, I., Stalmans, W., and Bollen, M. (2000) Biochem. J. 352, 651–658
18. Boudrez, A., Beullens, M., Groenen, P., Van Eynde, A., Vulteke, V., Jagiello, I., Murray, M., Krainer, A. R., Stalmans, W., and Bollen, M. (2000) J. Biol. Chem. 275, 25411–25417
19. Boudrez, A., Beullens, M., Waekens, E., Stalmans, W., and Bollen, M. (2002) J. Biol. Chem. 277, 31834–31841
20. Vulteke, V., Beullens, M., Boudrez, A., Keppens, S., Van Eynde, A., Rider, M. H., Stalmans, W., and Bollen, M. (2004) J. Biol. Chem. 279, 8642–8647
21. Durocher, D., Taylor, I. A., Sarbassova, D., Haire, L. F., Westcott, S. L., Jackson, S. P., Smerdon, S. J., and Yaffe, M. B. (2000) Mol. Cell 6, 1169–1182
22. Kumeta, H., Ogura, K., Adachi, S., Fujioka, Y., Tanuma, N., Kikuchi, K., and Inagaki, F. (2008) J. Biol. Chem. 283, 5863–5874
23. Eto, M., Senba, S., Morita, F., and Yazawa, M. (1997) FEBS Lett. 410, 356–360
24. Achsel, T., Ahrens, K., Brahms, H., Teigelkamp, S., and Lührmann, R. (1998) Mol. Cell. Biol. 18, 6756–6766
25. Isono, K., Mizutani-Koseki, Y., Komori, T., Schmidt-Zachmann, M. S., and Koseki, H. (2005) Genes Dev. 19, 536–541
26. Takizawa, N., Mizuno, Y., Ito, Y., and Kikuchi, K. (1994) J. Biochem. (Tokyo) 116, 411–415
27. Yamashita, A., Ohashi, T., Kashiwa, I., Taya, Y., and Ohno, S. (2001) Genes Dev. 15, 2215–2228
28. Kim, S. E., Ishita, A., Shima, H., Nakamura, K., Yamada, Y., Ogawa, K., and Kikuchi, K. (2000) Int. J. Oncol. 16, 751–755
29. Mitsuhashi, S., Shima, H., Tanuma, N., Matsuura, N., Takekawa, M., Urano, T., Kataoka, T., Ubukata, M., and Kikuchi, K. (2003) J. Biol. Chem. 278, 82–88
30. Niranjanakumari, S., Lasda, E., Brazas, R., and Garcia-Blanco, M. A. (2002) Methods (San Diego) 26, 182–190
31. Maniatis, T., and Reed, R. (2002) Nature 416, 499–506
32. Friend, K., Lovejoy, A. F., and Steitz, J. A. (2007) Mol. Cell. Biol. 28, 240–252
33. Tran, H. T., Ulke, A., Morrice, N., Johannes, C. J., and Moorhead, G. B. (2004) Mol. Cell. Proteomics 3, 257–265
34. Johnson, D., Cohen, P., Chen, M. X., Chen, Y. H., and Cohen, P. T. W. (1997) Eur. J. Biochem. 244, 931–939
35. Seghezzi, W., Chua, K., Shanahan, F., Gozani, O., Reed, R., and Lees, E. (1998) Mol. Cell. Biol. 18, 4526–4536
36. de Graaf, K., Czajkowska, H., Rottmann, S., Packman, L. C., Lilischkis, R., Ruster, B., and Becker, W. (2006) BMC Biochem. 7, 7
37. Massiello, A., Roesser, J. R., and Chalfant, C. E. (2006) FASEB J. 20, 1680–1682
38. Beullens, M., Van Eynde, A., Bollen, M., and Stalmans, W. (1993) J. Biol. Chem. 268, 13172–13177
39. Van Eynde, A., Beullens, M., Stalmans, W., and Bollen, M. (1994) Biochem. J. 297, 447–449
40. Banerjee, H., Rahn, A., Gawebe, B., Guth, S., Valcarcel, J., and Singh, R. (2004) RNA (N. Y.) 10, 240–253

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