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Sad1 Counteracts Brr2-Mediated Dissociation of U4/U6.U5 in Tri-snRNP Homeostasis

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The yeast Sad1 protein was previously identified in a screen for factors involved in the assembly of the U4/U6 di-snRNP particle. Sad1 is required for pre-mRNA splicing both *in vivo* and *in vitro*, and its human orthologue has been shown to associate with U4/U6.U5 tri-snRNP. We show here that Sad1 plays a role in maintaining a functional form of the tri-snRNP by promoting the association of U5 snRNP with U4/U6 di-snRNP. In the absence of Sad1, the U4/U6.U5 tri-snRNP dissociates into U5 and U4/U6 upon ATP hydrolysis and cannot bind to the spliceosome. The separated U4/U6 and U5 can reassociate upon incubation more favorably in the absence of ATP and in the presence of Sad1. Brr2 is responsible for mediating ATP-dependent dissociation of the tri-snRNP. Our results demonstrate a role of Sad1 in maintaining the integrity of the tri-snRNP by counteracting Brr2-mediated dissociation of tri-snRNP and provide insights into homeostasis of the tri-snRNP.

Splicing of precursor mRNA takes place via two steps of transesterification reactions and is catalyzed by a large ribonucleoprotein complex called the spliceosome. The spliceosome consists of five small nuclear RNAs (snRNAs), U1, U2, U4, U5, and U6, and nearly a hundred proteins. These snRNAs are associated with specific protein components to form small nuclear ribonucleoprotein particles (snRNPs), among which U4 and U6 base pair with each other to form a U4/U6 di-snRNP, which can further associate with U5 snRNP to form a U4/U6.U5 tri-snRNP. The spliceosome is assembled via sequential binding of snRNPs to the pre-mRNA in the order U1, U2, and then the tri-snRNP. U1 binds to the 5′ splice site through base pairing of the U1 snRNA with the 5′ splice site sequence, and U2 recognizes the branch site through base pairing of U2 snRNA with the branch site sequence (1–5). A subsequent conformational rearrangement results in the release of U1 snRNP and U4 snRNP, accompanied by new base pairings between U6 and U2 and between U6 and the 5′ splice site (6–8). A protein complex associated with Prp19, called NTC (for *n*ine*t*een *c*omplex), is then added to the spliceosome to stabilize specific interactions of U5 and U6 with pre-mRNA to form the activated spliceosome (9–11), which can catalyze transesterification reactions.

The release of U1 and U4 from the spliceosome involves destabilization of base pair interactions between U1 and the 5′ splice site and the unwinding of the U4/U6 duplex, which are mediated by DEXD/H-box RNA helicases Prp28 and Brr2, respectively. Genetic studies have revealed a requirement for Prp28 to destabilize U1-5′ splice site base pairing, and this requirement can be eliminated by mutations in U1 snRNA or U1-C protein that destabilize the base pair interaction of U1 and the 5′ splice site (12, 13). Prp28 may act by directly unwinding the U1-5′ splice site duplex or by displacing U1C to destabilize U1-5′ splice site base pairing (12, 14, 15). Brr2 is an intrinsic component of the U5 snRNP and is associated with the spliceosome, along with the binding of the tri-snRNP. Brr2 has been demonstrated to unwind RNA duplexes *in vitro* (16, 17) and implicated in mediating U4/U6 unwinding during spliceosome activation (16–18). Unwinding of the U4/U6 duplex has also been shown to occur on the purified tri-snRNP (19). The unwinding activity of Brr2 is stimulated by a C-terminal segment of Prp8 containing the Jab1/MPN domain (18) but is inhibited by the C-terminal tail of Prp8 (20). It has been proposed that Brr2 is loaded onto U4 in a region preceding the U4/U6 stem I and translocates along U4 in the 3′-to-5′ direction to disrupt stem I in separating U4 and U6 (21, 22). The RNase H domain of Prp8 can also bind to this region of U4 to compete with binding of Brr2 and thereby inhibit U4/U6 unwinding (21).

The tri-snRNP contains at least 28 proteins in yeast, including components of U4/U6 di-snRNP and U5 snRNP and proteins specifically associated with the tri-snRNP (23–25). Prp8, Brr2, and Snu114 are three large proteins associated with U5 and form a molecular motor in spliceosome activation (26). Human orthologues of Snu66 and Sad1 have been shown to specifically associate with the tri-snRNP, and both are required for the recruitment of the tri-snRNP to the spliceosome but not for maintenance of tri-snRNP stability (27). In yeast, Snu66 was also found to associate with the tri-snRNP (23).

The yeast *SAD1* gene was identified from a screen for mutants defective in U4/U6 snRNP assembly (28). The temperature-sensitive *sad1-1* mutant is blocked for assembly of newly synthesized U4 snRNA into U4/U6 snRNP at nonpermissive temperatures and is also defective in splicing both *in vivo* and *in vitro* (28). Human Sad1 was shown to associate with the tri-snRNP, and its depletion prevents the recruitment of the tri-snRNP to the spliceosome (27). Sad1 exhibits significant homology to the zinc finger ubiquitin-binding protein (ZnF-UBP) domain and ubiquitin

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C-terminal hydrolase (UCH) domain, known as deubiquitin enzymes (29). However, a functional cysteine residue in the catalytic center of UCH is replaced by aspartate in Sad1 (D159), and some conserved residues in the ZnF-UBP domain of Sad1 involved in ubiquitin binding, as demonstrated for USP39, are also replaced (29). Consequently, Sad1 may not be a canonical ubiquitin-binding protein and also lacks hydrolase activity due to substitution in catalytic residues (29).

In this study, we analyzed the function of Sad1 in the *in vitro* splicing reaction. We found that Sad1 promotes the association of U5 with U4/U6 to form the tri-snRNP and is required for maintaining the integrity of the tri-snRNP upon ATP hydrolysis catalyzed by Brr2. In the absence of Sad1, the tri-snRNP likely fails to bind to the spliceosome under normal splicing conditions due to its dissociation into U4/U6 and U5. Our results uncover homeostasis of the tri-snRNP, with Sad1 promoting the association of U4/U6 and U5 to counteract Brr2-mediated dissociation of the tri-snRNP.

MATERIALS AND METHODS

Yeast strains. The yeast strains used were BJ2168 (*MAT***a** *prc1 prb1 pep4 leu2 trp1 ura3*), YSCC4 (*MAT***a** *prc1 prb1 pep4 leu2 trp1 ura3 PRP4-HA*), YSCC9 (*MAT***a** *prc1 prb1 pep4 leu2 trp1 ura3 ntc25*::*LEU2*), YSCC032 (*MAT***a** *prc1 prb1 pep4 leu2 trp1 ura3 GAL-BRR2*), YSCC033 (*MAT***a** *prc1 prb1 pep4 leu2 trp1 ura3 GAL-BRR2 PRP4-HA*), YSCC034 (*MAT***a** *prc1 prb1 pep4 leu2 trp1 ura3 sad1*::*LEU2* pRS416.SAD1), YSCC035 (*MAT***a** *prc1 prb1 pep4 leu2 trp1 ura3 SAD1-3HA*), YSCC036 (*MAT***a** *prc1 prb1 pep4 leu2 trp1 ura3 GAL-SAD1*), and EGY48 (*MAT*α *ura3 his3 trp1 LexAop-leu2 ade2*).

Antibodies and reagents. Antihemagglutinin (anti-HA) monoclonal antibody 8G5F was produced by immunizing mice with a keyhole limpet hemocyanin (KLH)-conjugated HA peptide (our unpublished data), and the anti-Sad1 polyclonal antibody was produced by immunizing rabbits with the full-length protein expressed in *Escherichia coli*. Protein A-Sepharose and streptavidin-Sepharose were from GE, Inc.

Splicing extracts and substrates. Yeast whole-cell extracts were prepared according to the method of Cheng et al. (30). Actin precursors were synthesized using SP6 RNA polymerase according to the method of Cheng and Abelson (31). Biotinylated pre-mRNA was synthesized according to a procedure described previously by Chan et al. (11).

Immunodepletion, immunoprecipitation, and precipitation of the spliceosome by streptavidin-agarose. Immunodepletion of Sad1 was performed by incubation of 100 μl of splicing extracts with 150 μl of anti-Sad1 antiserum conjugated to 50 μl of protein A-Sepharose, and the procedure was as described by Tarn et al. (10) using 10 μl of anti-HA for Prp4-HA or 1.5 μl of anti-Prp8 antibody for each 20-μl reaction mixture. Precipitation of the spliceosome with streptavidin-agarose was carried out according to methods described previously by Chan et al. (11).

Purification of Sad1 and Brr2. The full-length *SAD1* gene, the C63A point mutant, and the truncated mutants were cloned into a modified version of pET15b for the expression of His-tagged proteins in *E. coli*. Recombinant proteins were purified on Ni-nitrilotriacetic acid (NTA) agarose (Novagen) according to the manufacturer's instructions.

Yeast two-hybrid assays. The *SAD1*, *SNU114*, *SNU66*, *BRR2*, *PRP8*, *PRP6*, *PRP4*, and *PRP3* genes were fused to the LexA-DNA binding domain in plasmid pEG202 and the GAL4-activation domain in plasmid pACT2, and each pair of plasmids was transformed into *Saccharomyces cerevisiae* strain EGY48, together with the β-galactosidase reporter plasmid pSH18-34. Interactions were examined for expression of β-galactosidase on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase) plates.

Gradient sedimentation. Splicing reaction mixtures or cell extracts were sedimented on 10 to 30% glycerol gradients in a buffer containing 20 mM HEPES, pH 7.9, 50 mM NaCl, 0.5 mM dithiothreitol (DTT), and 0.2 mM EDTA. The gradients were centrifuged in an SW60Ti rotor at 50,000 rpm for 2.5 h at 4°C and collected in 0.2-ml fractions.

Northern blotting. RNA from cell extracts or from splicing reactions was fractionated by electrophoresis on 5% denaturing or nondenaturing polyacrylamide (29:1) gels and electroblotted onto GeneScreen membranes for Northern blotting probed with U4, U6, or five snRNAs.

RESULTS

Interaction of Sad1 with components of the tri-snRNP. The *sad1-1* mutant was isolated for its accumulation of *de novo*-synthesized U4 snRNA at the nonpermissive temperature (28). Comparative study revealed a phenotype similar to that of the *prp19-1* mutant (28). Although Prp19, or any other NTC component, is not tightly associated with snRNAs, accumulation of free U4 is a general phenotype for cells depleted of one or two NTC components (32). This raised a question of whether there is a functional relationship between Sad1 and NTC. However, immunoprecipitation analysis did not detect association of Sad1 with NTC or with the spliceosome during the splicing reaction (data not shown). Nevertheless, Sad1 is required for binding of NTC to the spliceosome (see Fig. S1 in the supplemental material), suggesting a role of Sad1 in the stage prior to or during spliceosome activation.

Human Sad1 (hSad1) has been shown to coprecipitate U4, U5, and U6 and is suggested to be a component of U4/U6.U5 tri-snRNP (33). Yeast two-hybrid assays have also revealed interactions of hSad1 with components of the tri-snRNP, hBrr2, hPrp3, and hSnu66 (34). We first examined whether yeast Sad1 is also associated with the tri-snRNP by immunoprecipitation. Extracts prepared from a strain in which Sad1 was tagged with 3 copies of HA were precipitated with anti-HA antibody, followed by Northern blotting (Fig. 1A). Only residual amounts of U4, U5, and U6 were coprecipitated (lane 2), and the total amounts of the three snRNAs in the supernatant fraction were not drastically changed (lane 6). Control experiments using Prp4-HA extracts showed coprecipitation of U4, U5, and U6 (lane 4), with the majority of U4 and U6 depleted from the extract in the supernatant fraction (lane 8), consistent with Prp4 being a component of U4 snRNP. Western blotting showed that both Sad1-HA and Prp4-HA were nearly completely precipitated from the extracts by anti-HA antibody (Fig. 1B, lane 4 versus lane 10, and lane 6 versus lane 12). These results indicate that yeast Sad1 may also be associated with the tri-snRNP, but only weakly. Two-hybrid analysis revealed strong interaction of Sad1 with Snu114 and weaker interaction with Snu66 among the tri-snRNP components tested (Fig. 1C). Unlike hSad1, yeast Sad1 did not show two-hybrid interaction with Brr2 or Prp3 in our assays. Although Snu114 and Sad1 showed strong two-hybrid interaction, they do not appear to interact in a stable manner, as immunoprecipitation of Sad1 did not significantly coprecipitate Snu114 (data not shown), consistent with its weak interaction with the tri-snRNP.

Sad1 domains required for the interaction with Snu114 and Snu66. To study the functional role of Sad1 in the splicing reaction, we set up an *in vitro* complementation assay by depleting Sad1 from splicing extracts using anti-Sad1 antibodies (Fig. 2A). The splicing activity was abolished when the extract was depleted of Sad1 (lane 3) but was restored upon the addition of recombinant Sad1 protein (lanes 4 to 6), indicating that depletion of Sad1 did not codeplete significant amounts of any other factor essential for the reaction. The Sad1 protein comprises a ZnF-UBP domain at the amino terminus (residues 47 to 96) and a UCH domain at

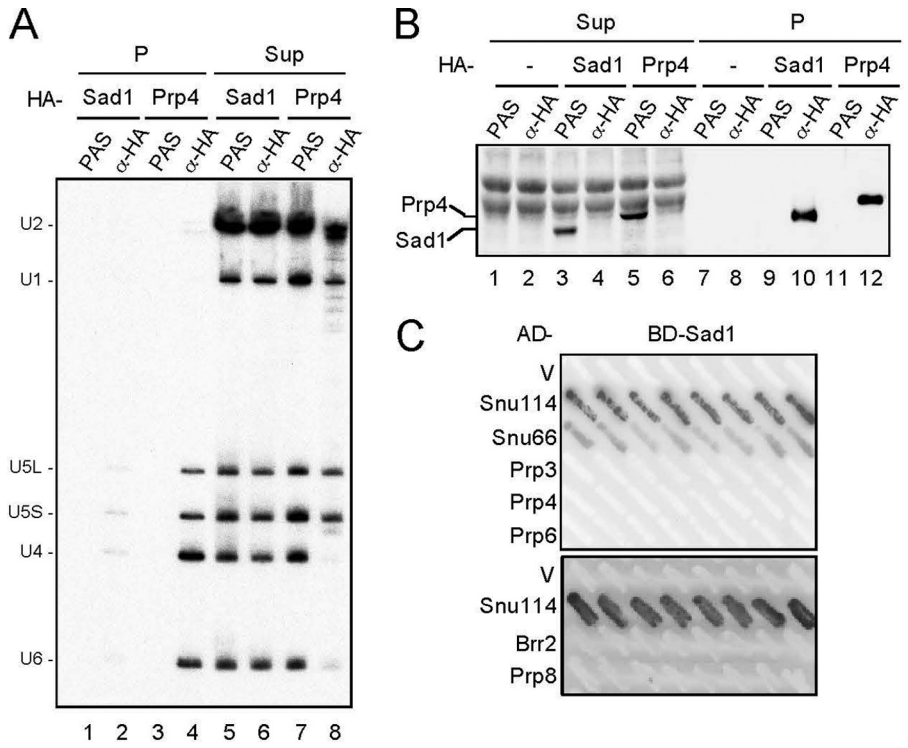

FIG 1 Interaction of Sad1 with components of tri-snRNP. (A) Extracts prepared from Sad1-HA (tagged with 3 copies of HA; lanes 1, 2, 5, and 6) and Prp4-HA (lanes 3, 4, 7, and 8) strains were immunoprecipitated with the anti-HA antibody, followed by Northern blotting using probes against U1, U2, U4, U5, and U6. (B) Western blot showing precipitation of Sad1 or Prp4 with anti-HA antibody from Sad1-HA (lanes 3, 4, 9, and 10) or Prp4-HA (lanes 5, 6, 11, and 12) extracts, respectively. (C) Sad1 was fused to the LexA-DNA binding domain (BD), and tri-snRNP protein (Snu114, Snu66, Brr2, Prp8, Prp6, Prp4, or Prp3) was fused to the GAL4 activation domain (AD). The interactions were assayed by measuring β-galactosidase activity. PAS, protein A-Sepharose; α-HA, anti-HA antibody; P, pellet; Sup, supernatant; V, vector.

the carboxy terminus (residues 150 to 448) (Fig. 2B). Both domains are essential for cellular viability (Fig. 2C). Mutations at conserved C_2H_2 Zn-binding residue cysteine 60 or 63 also resulted in lethality, and mutation at histidine 79 produced severe growth defects. However, mutation at histidine 85 showed no growth defect, raising a question of whether zinc binding is essential for its function. Nevertheless, the requirements for these motifs for cellular growth appear to reflect their requirements for splicing, as recombinant protein with a deletion in either domain (Fig. 2D, lanes 4 and 6) or with a C63A mutation (lane 5) did not rescue the splicing activity of Sad1-depleted extracts. The interaction of Sad1 with Snu114 involves only the C-terminal UCH domain, as deletion of the ZnF-UBP domain or C63A mutation retained the ability of Sad1 to interact with Snu114 (Fig. 2E). In contrast, both domains are required for the interaction with Snu66.

Sad1 is not required for binding of the tri-snRNP to the spliceosome. To see whether Sad1 is required for the association of the tri-snRNP with the spliceosome, spliceosomes were formed on biotinylated pre-mRNA in Sad1-depleted extracts and isolated by precipitation with streptavidin-Sepharose, followed by Northern blotting to analyze their snRNA contents. The 3′ splice site mutant pre-mRNA ACAC was used to prevent cycling of the spliceosome (35). We have previously shown that activation of the spliceosome requires higher concentrations of ATP, and splicing reactions carried out at lower ATP concentrations are blocked for spliceosome activation with U4 retained on the spliceosome (10). Splicing reactions were performed in the presence of 0, 0.2, and 2 mM ATP to arrest the spliceosome at different stages (Fig. 3A). In mock-treated extracts, U4, U5, and U6 were all retained on the spliceosome at 0.2 mM ATP (lane 2), indicating arrest of the spliceosome at the preactivation stage. At 2 mM ATP, when the spliceosome can be activated to proceed through the first catalytic step, U4 was absent from the spliceosome (lane 3). In Sad1-depleted extracts, the association of U4, U5, and U6 was not affected at 0.2 mM ATP (lane 5). Unexpectedly, none of the three snRNAs were retained on the spliceosome when the splicing reaction was carried out at 2 mM ATP (lane 6). These results indicate that Sad1 is not required for the recruitment of the tri-snRNP to the spliceosome but may be required at higher ATP concentrations for spliceosome activation, which involves unwinding of the U4/U6 duplex to release U4 from the spliceosome. We speculated that after unwinding of U4/U6 at 2 mM ATP, Sad1 might be required to stabilize the association of U5 and U6 with the spliceosome and anticipated that the absence of Sad1 would not affect U4/U6 unwinding.

It is known that U6 also exists in a free form, besides those paired with U4 to form U4/U6 di-snRNP and U4/U6.U5 tri-snRNP (36). The free U6 particle contains the recycling factor Prp24, which efficiently promotes the annealing of U4 and U6 to form U4/U6 di-snRNP upon the release of U4 during spliceosome activation (37–40). Consequently, free U4 is normally not detected in the extract or during the splicing reaction. We have previously shown that cells defective in NTC function contain smaller amounts of U6 snRNAs, and extracts prepared from an *NTC25*-null strain (ntc25Δ) contain only minor amounts of free U6 (32).

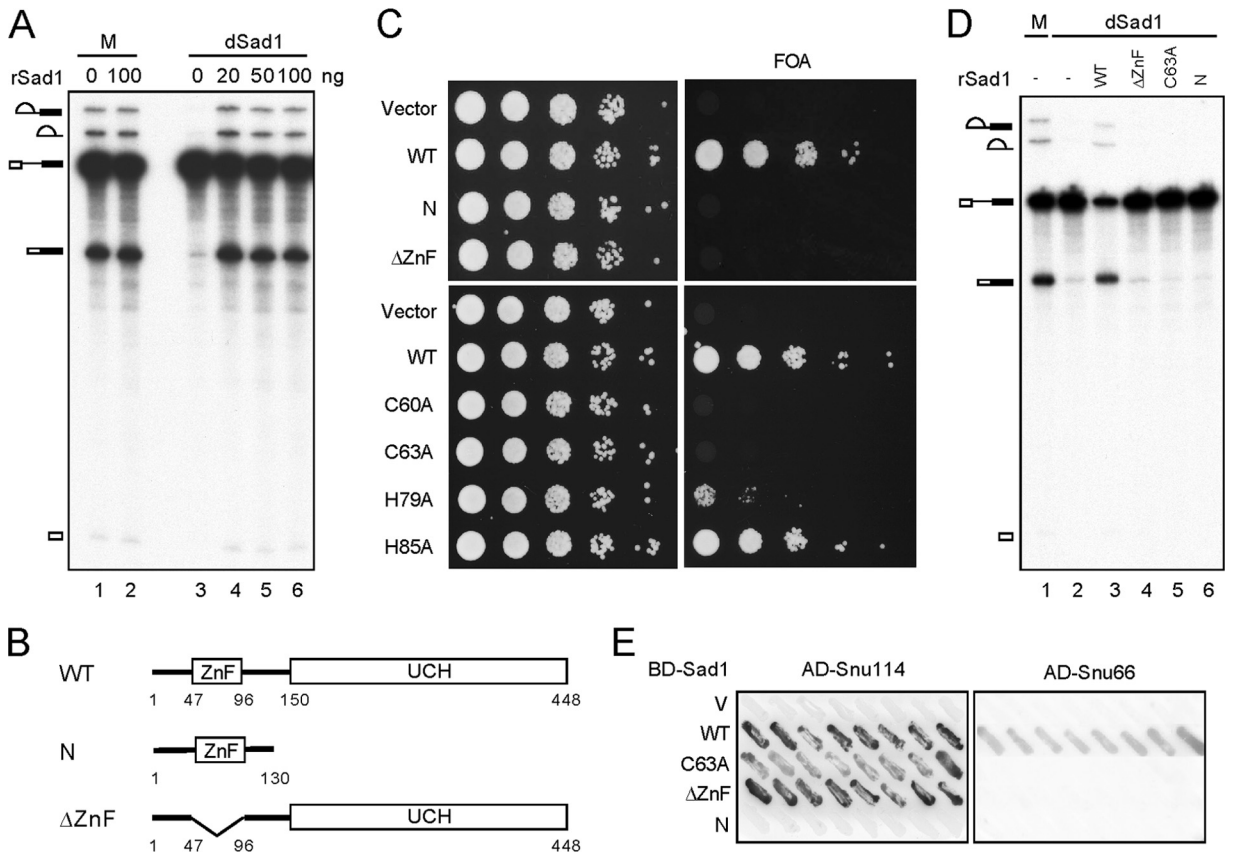

FIG 2 Requirement for ZnF-UBP and UCH domains in Sad1 function. (A) Splicing was carried out in mock-treated (lanes 1 and 2) or Sad1-depleted (lanes 3 to 6) extracts using actin pre-mRNA with (lanes 2, 4, 5, and 6) or without (lanes 1 and 3) the addition of recombinant Sad1 (rSad1). (B) Schematic of Sad1 domains and the structures of deletion mutants. (C) *SAD1* deletion or point-mutated clones in yeast strain YSCC034 grown in synthetic minimum medium were spotted onto 5-fluoroorotic acid (FOA)-containing synthetic minimum medium plates after serial dilutions and grown at 30°C for 3 days. (D) Recombinant wild-type Sad1 (lane 3) or Sad1-deleted or point-mutated protein (lanes 4 to 6) was added to Sad1-depleted extracts (lane 2) for splicing assays. rSad1, recombinant Sad1; M, mock; dSad1, Sad1 depletion. (E) Deleted or point-mutated mutants of Sad1 were fused to the LexA-DNA binding domain (BD), and Snu114 or Snu66 was fused to the GAL4 activation domain (AD). The interactions were assayed by measuring β-galactosidase activity. V, vector.

Prp24 is also present in insignificant amounts in ntc25Δ extracts (see Fig. S2 in the supplemental material). Northern blotting of total RNA fractionated on native gels revealed that only a residual amount of U6 was present in ntc25Δ extracts (Fig. 3B, lane 4) as opposed to that present in wild-type extracts (lane 1). After splicing, while no free U4 was detected in wild-type extracts (lanes 2 and 3), the free form of U4 accumulated in ntc25Δ extracts with or without the addition of NTC to restore the splicing activity (lanes 5 and 6). Free U6 also accumulated after splicing in ntc25Δ extracts, presumably in association with the spliceosome. In contrast to our expectation, depletion of Sad1 did not result in the release of U4 after incubation with pre-mRNA substrate under splicing conditions in the presence or absence of NTC (lanes 8 and 9), indicating either no unwinding of U4/U6 or that reannealing of U4 and U6 occurs immediately following unwinding during the reaction. We reasoned that since Prp24 or free U6 was not present in sufficient amounts to promote efficient reannealing of U4 and U6, it is less likely that U4/U6 helices unwound and then reannealed. This raised a question of whether the tri-snRNP would be destabilized from the spliceosome at high ATP concentrations when U4/U6 unwinding was blocked and, further, whether the tri-snRNP remained intact after being dissociated from the spliceosome in the nonproductive pathway.

Sad1 prevents ATP-dependent dissociation of the tri-snRNP into U5 and U4/U6 di-snRNP. We then examined whether the tri-snRNP remained intact after splicing in the absence of Sad1 by sedimentation analysis (Fig. 4A). Splicing was carried out at 0.2 mM or 2 mM ATP in Sad1-depleted or mock-treated extracts, and the reaction mixtures were fractionated on 10 to 30% glycerol gradients. Unreacted cell extracts were also analyzed for comparison (Fig. 4A, a and d). As shown in Fig. 4A, a, for mock-treated and unreacted extracts, while a small amount of U4/U6 sedimented in fractions 11 and 12, the majority of U4 and U6 cosedimented with U5 as the tri-snRNP in fractions 7 and 8. The identity of the tri-snRNP in these fractions was further confirmed by coprecipitation of U4, U5, and U6 with the tri-snRNP-specific protein Snu66 (see Fig. S3 in the supplemental material). Free U5 sedimented in fractions 9 to 11 and U2 in fractions 8 to 10. Depletion of Sad1 did not affect the distribution of snRNAs (Fig. 4A, d). When splicing was carried out at 0.2 mM ATP, a large fraction of U2, U4, U5, and U6 sedimented in fractions 4 to 6 in association with the spliceosome, regardless of the presence of Sad1 (Fig. 4A, b and e). At 2 mM ATP, while U6 remained in fractions 4 to 6, U4 sedimented in fractions 12 to 14 in mock-treated extracts (Fig. 4A, c), indicative of its release from the spliceosome. In Sad1-depleted extracts, U4 and U6 were found to sediment primarily as U4/U6

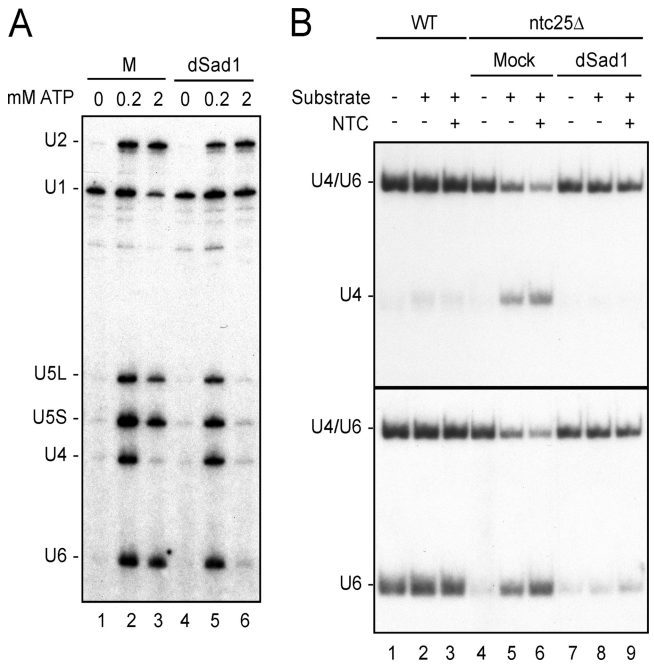

FIG 3 Sad1 is not required for binding of the tri-snRNP to the spliceosome. (A) Splicing reactions were carried out in mock-treated (lanes 1 to 3) or Sad1-depleted (lanes 4 to 6) extracts in the absence (lanes 1 and 4) or presence of 0.2 mM (lanes 2 and 5) or 2 mM (lanes 3 and 6) ATP with biotinylated ACAC pre-mRNA substrates. The spliceosome was precipitated with streptavidin-Sepharose, and RNA was extracted and analyzed by Northern blotting. Probes were mixed against U1, U2, U4, U5, and U6. (B) Splicing reactions were carried out in wild-type extracts (lanes 1 to 3) and mock-treated (lanes 4 to 6) or Sad1-depleted (lanes 7 to 9) *NTC25*-null extracts (ntc25Δ) in the absence (lanes 1, 4, and 7) or presence (lanes 2, 3, 5, 6, 8, and 9) of ACAC pre-mRNA substrates at 25°C for 30 min with (lanes 3, 6, and 9) or without (lanes 1, 2, 4, 5, 7, and 8) NTC. RNA was isolated and fractionated on a 5% nondenaturing polyacrylamide gel for Northern blotting and probed with U4 (top) or U6 (bottom).

di-snRNP in fractions 11 and 12 rather than as the tri-snRNP (Fig. 4A, f), indicating dissociation of the tri-snRNP into U5 and U4/U6 di-snRNP. These results suggest that in the absence of Sad1, the tri-snRNP can bind to the spliceosome when the ATP concentration is low but may dissociate into U5 and U4/U6 under normal splicing conditions, resulting in their release from the spliceosome. Alternatively, the tri-snRNP dissociates when incubated with higher concentrations of ATP in the absence of Sad1 and consequently is unable to bind to the spliceosome.

We then examined whether the tri-snRNP dissociates in the absence of Sad1 and substrate pre-mRNA (Fig. 4B) and found that U4/U6 accumulated irrespective of the presence of the substrate (Fig. 4A, b and c), indicating that the tri-snRNP dissociates in the absence of splicing. These results suggest that the tri-snRNP may dissociate into U4/U6 and U5 in the presence of 2 mM ATP and is therefore unable to bind to the spliceosome during the splicing reaction. Notably, the amount of U4/U6 di-snRNP also increased in mock-treated extracts upon incubation with ATP (compare Fig. 4A, a, and B, a), suggesting a dynamic change of the tri-snRNP structure in cell extracts during incubation.

ATP-dependent dissociation of the tri-snRNP in cell extracts was further confirmed by immunoprecipitation analysis using antibodies against the U5 component Prp8 and anti-HA antibody for the U4 component Prp4 tagged with the HA epitope (Fig. 5A). In mock-treated or Sad1-depleted extracts, U4, U5, and U6 were coprecipitated with Prp4 and Prp8 (lanes 1, 2, 5, and 6). Incubation with 2 mM ATP, while not affecting coprecipitation of U6 with U4, reduced the amount of U5 coprecipitated with U4/U6 in mock-treated extracts (compare lanes 3 and 4 with lanes 1 and 2). In Sad1-depleted extracts, U5 was entirely dissociated from U4/U6 (lanes 7 and 8). These results suggest that the tri-snRNP might undergo dynamic dissociation in the presence of higher concentrations of ATP and that Sad1 stabilizes the tri-snRNP either by preventing dissociation or by facilitating the interaction of U4/U6 and U5.

To see whether Sad1 facilitates the interaction of U5 with U4/U6, we separated U4/U6 and U5 by incubation of Sad1-depleted extracts in the presence of 2 mM ATP and added back recombinant Sad1 after depletion of ATP, followed by immunoprecipitation. Figure 5B shows that U4/U6 and U5 could reassociate upon incubation of dissociated tri-snRNP (lanes 1 and 6), even without the addition of Sad1 (lanes 2 and 7), suggesting dynamic interactions of U4/U6 and U5. Addition of Sad1 to the reaction mixture increased the amount of association by approximately 2-fold (lanes 4 and 9), indicating that Sad1 promotes the association of U5 with U4/U6. Furthermore, while addition of 4 mM ATP to the reaction mixture decreased the amount of tri-snRNP formed by approximately 80% (lanes 3 and 8), further addition of Sad1 reduced dissociation (lanes 5 and 10). Titration of Sad1 from 0.1 μg to 1 μg revealed a slight increase in the amount of tri-snRNP with increasing amounts of Sad1 added in the presence or absence of ATP (Fig. 5C). These results suggest that Sad1 promotes tri-snRNP formation to counteract ATP-dependent dissociation of the tri-snRNP.

The requirement for Sad1 in tri-snRNP formation was further confirmed by *in vivo* analysis. When extracts prepared from Sad1 metabolically depleted cells were fractionated on glycerol gradients, only a small amount of the tri-snRNP was detected. The majority of U5 and U4/U6 was separated in different fractions (Fig. 5D, top), but more tri-snRNP was observed when recombinant Sad1 was added to the extract (bottom).

Brr2 mediates ATP-dependent dissociation of U4/U6.U5. The ATP dependency of tri-snRNP dissociation suggests the involvement of an ATPase in the process. Two DEXD/H-box ATPases, Brr2 and Prp28, are associated with the tri-snRNP (41, 42), and both are required for spliceosome activation. Prp28 has been implicated in U1 displacement (12, 13) and Brr2 in unwinding of U4/U6 duplexes (19, 21, 22). Based on their functional targets, Brr2 is more likely to be responsible for the ATP-dependent dissociation of the tri-snRNP. We generated a *BRR2*-inducible Prp4-HA-tagged yeast strain using the *GAL1* promoter and prepared extracts from *BRR2*-repressed cells to analyze the association of U4/U6 with U5. Western blotting revealed Brr2 was not detected in significant amounts in the extract (see Fig. S4 in the supplemental material). The Brr2-depleted extract was precipitated with anti-Prp8 (Fig. 6A) or anti-HA (Fig. 6B) antibody, and the RNA content was analyzed by Northern blotting. U4/U6 and U5 were found to remain associated, since both U4/U6 and U5 were precipitated by either antibody (Fig. 6, lane 1). Addition of Brr2, purified from overexpressing yeast cells, resulted in less U4/U6 association upon incubation with ATP (lanes 4). Notably, depletion of Sad1 had no effect on tri-snRNP dissociation (lanes 6), and when Brr2 was added back to the extract, the tri-snRNP

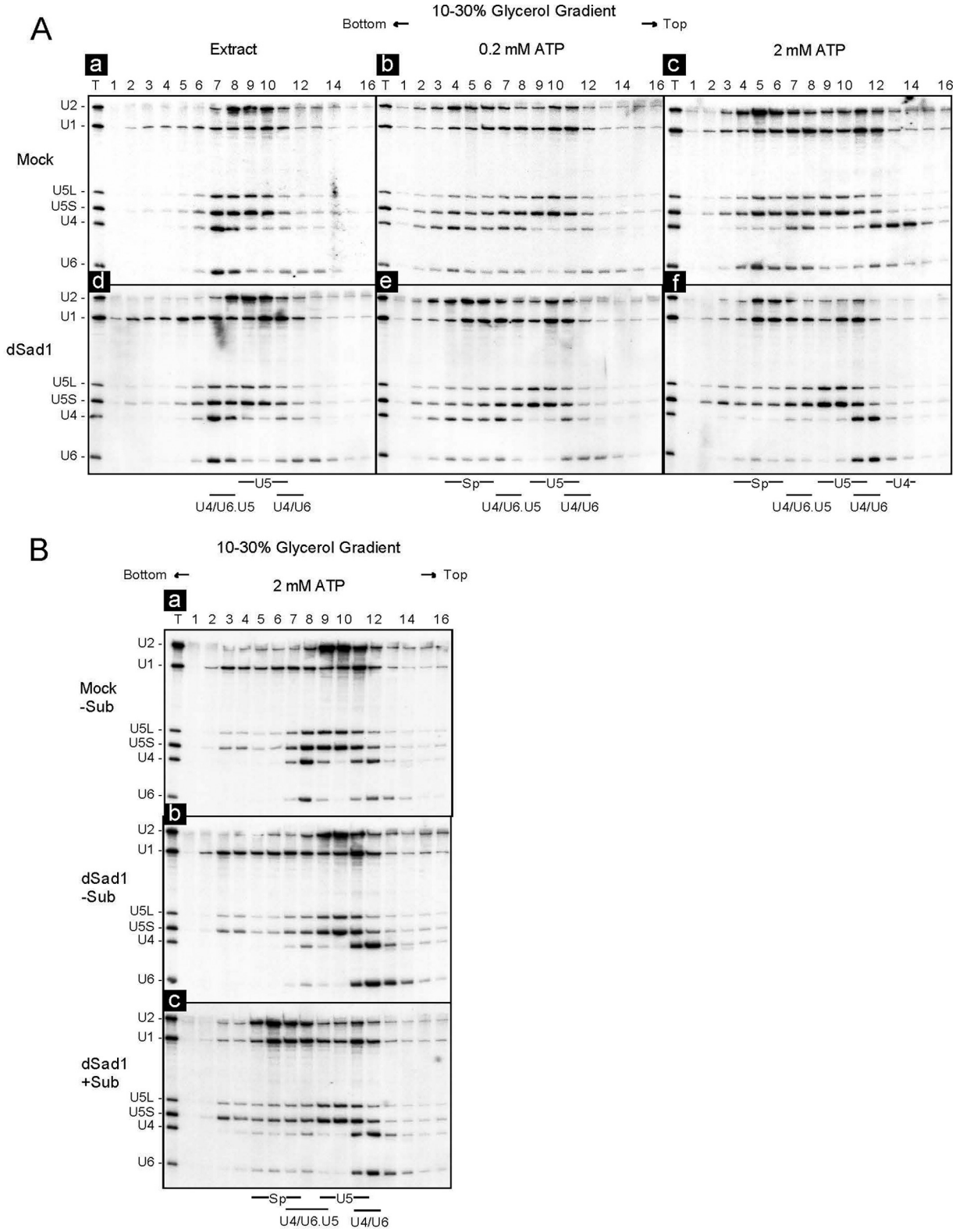

FIG 4 Sad1 prevents ATP-dependent dissociation of the tri-snRNP into U5 and U4/U6 di-snRNP. (A) Splicing was carried out in mock-treated (b and c) or Sad1-depleted (e and f) ntc25Δ extracts (a and d) at 0.2 mM (b and e) or 2 mM (c and f) ATP with actin RNA substrates. (a and d) RNAs from mock-treated or Sad1-depleted extracts. (B) Splicing was carried out in mock-treated (a) or Sad1-depleted (b and c) ntc25Δ extracts at 2 mM ATP in the presence (c) or absence (a and b) of actin RNA substrates, and the reaction mixtures were fractionated on 10 to 30% glycerol gradients. RNA from each tube was isolated and analyzed by Northern blotting, probed with U1, U2, U4, U5, and U6. The spliceosome is in fractions 4 to 6, tri-snRNP in fractions 7 and 8, U5 snRNP in fractions 9 to 11, U4/U6 di-snRNPs in fractions 11 and 12, and free U4 snRNP in fractions 12 to 14. Sp, spliceosome.

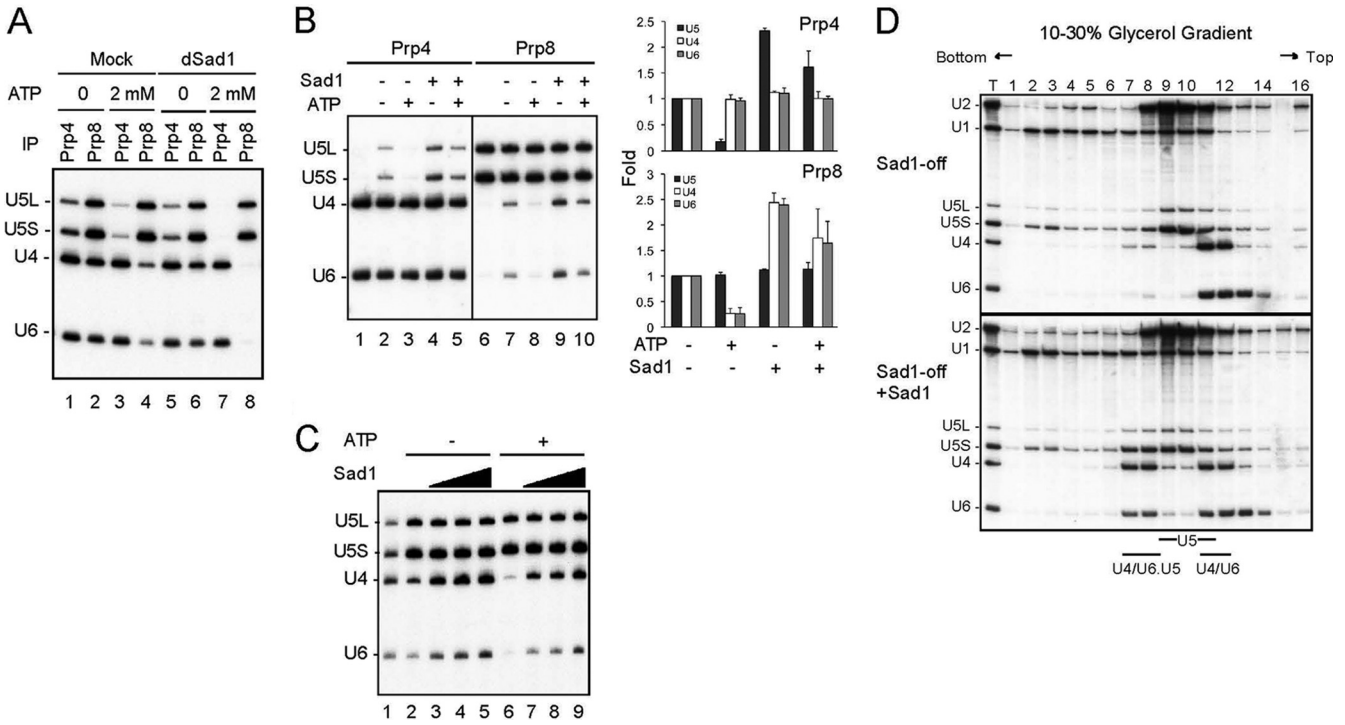

FIG 5 Sad1 promotes tri-snRNP formation. (A) Mock-treated (lanes 1 to 4) or Sad1-depleted (lanes 5 to 8) Prp4-HA extracts were incubated with (lanes 3, 4, 7, and 8) or without (lanes 1, 2, 5, and 6) 2 mM ATP and then precipitated with anti-HA or anti-Prp8 antibody. (B) Sad1-depleted Prp4-HA extracts were incubated with 2 mM ATP, followed by depletion of ATP upon incubation with 2 mM glucose. Following the addition of 0.4 μg of Sad1 (lanes 4, 5, 9, and 10) and/or 4 mM ATP (lanes 3, 5, 8, and 10), samples were incubated for 10 min at 25°C and then precipitated with anti-HA or anti-Prp8 antibody. The amounts of U4, U5, and U6 from three independent experiments were quantified with a phosphorimager, normalized to the control, and plotted as a bar graph. The error bars indicate standard deviations. (C) Sad1-depleted extracts were incubated without (lanes 2 to 5) or with (lanes 6 to 9) 2 mM ATP without (lanes 2 and 6) or with the addition of 0.1 μg (lanes 3 and 7), 0.4 μg (lanes 4 and 8), or 1 μg (lanes 5 and 9) of Sad1 and then precipitated with anti-Prp8 antibody. (D) Sad1 metabolically depleted cell extracts were incubated with (bottom) or without (top) the addition of 0.4 μg of Sad1 protein in the presence of 2 mM ATP. Samples were subjected to sedimentation on 10 to 30% glycerol gradients. RNA was isolated and analyzed by Northern blotting, probed with U1, U2, U4, U5, and U6.

was nearly completely dissociated upon incubation with ATP (lanes 8). These results indicate that Brr2 is the key player in mediating tri-snRNP dissociation.

DISCUSSION

In this study, we investigated the function of Sad1 in splicing using the *in vitro* reconstitution system. Sad1 has been implicated in the assembly of U4 into the U4/U6 di-snRNP and is also required for the splicing reaction. The human Sad1 protein was shown to associate with the tri-snRNP, and its depletion prevents the recruitment of the tri-snRNP to the spliceosome (27). We found that yeast Sad1 is not directly involved in the recruitment of the tri-snRNP to the spliceosome and instead plays a role in maintaining the intact tri-snRNP form. Yeast Sad1 also interacts with the tri-snRNP, but very weakly or not in a stable manner, so that only a residual amount of the tri-snRNP was coprecipitated with Sad1 and depletion of Sad1 with anti-Sad1 antibody did not significantly codeplete the tri-snRNP or other essential splicing factors.

Despite having weak interaction with the tri-snRNP, Sad1 promotes formation of the tri-snRNP. We show that the tri-snRNP is a dynamic complex that undergoes dynamic dissociation and reassociation. In Sad1-depleted extracts, the tri-snRNP was nearly completely dissociated into U5 snRNP and U4/U6 di-snRNP upon incubation with 2 mM ATP. As a consequence, splicing was likely blocked and arrested at the prespliceosome stage due to failure to recruit the tri-snRNP to the spliceosome. Titration of ATP revealed that nearly all tri-snRNP was dissociated at ATP concentrations greater than 1 mM (see Fig. S5 in the supplemental material). Incubation in the absence of ATP also resulted in decreased amounts of U4/U6.U5, but to a lesser extent. In the presence of Sad1, incubation with ATP also resulted in reduced levels of the tri-snRNP, suggesting that the ATP-dependent tri-snRNP dissociation still occurs in the presence of Sad1. On the other hand, incubation of the separated U4/U6 and U5 in the absence of ATP led to formation of the tri-snRNP even in the absence of Sad1, but more tri-snRNP formed when Sad1 was added during the incubation. Altogether, our results suggest that U4/U6 and U5 undergo dynamic association and dissociation, with ATP hydrolysis facilitating dissociation and Sad1 promoting association. Thus, Sad1 counteracts the ATP-stimulated dissociation of U4/U6.U5 in homeostasis of the tri-snRNP. Since ATP is required for normal splicing reactions and is maintained *in vivo* in millimolar concentrations, it is necessary to have Sad1 to maintain a sufficient level of the tri-snRNP. In the absence of Sad1, the tri-snRNP will likely be completely dissociated into U4/U6 and U5 and cannot bind to the spliceosome. It has previously been shown that depletion of U4 or U5 prevented the association of the other two snRNAs with the spliceosome (43, 44). Furthermore, mixing of extracts individually depleted of U4 or U6 did not reconstitute the splicing activity (45). These results suggest that intact U4/U6 di-

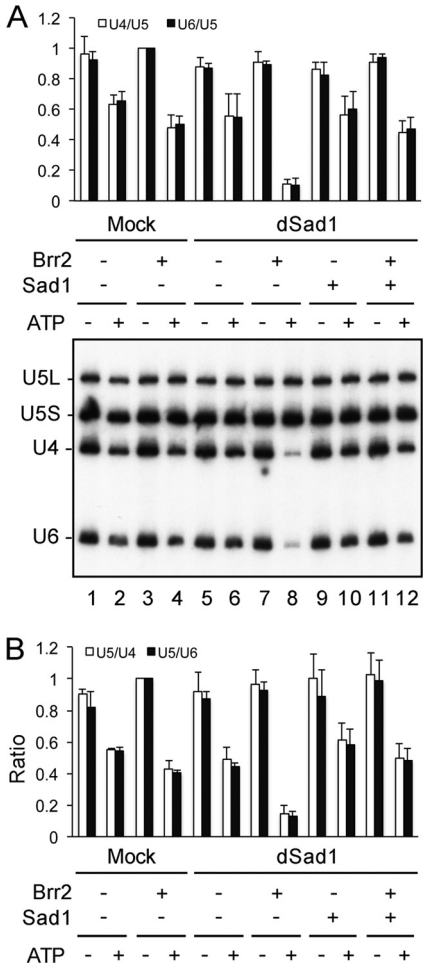

FIG 6 Brr2 mediates ATP-dependent dissociation of U4/U6.U5. Incubations were carried out in mock-treated or Sad1-depleted Brr2 metabolically depleted Prp4-HA cell extracts with the addition of Brr2 (lanes 3, 4, 7, 8, 11, and 12) and/or Sad1 (lanes 9 to 12) protein. Samples were incubated in the absence (lanes 1, 3, 5, 7, 9, and 11) or presence (lanes 2, 4, 6, 8, 10, and 12) of 2 mM ATP at 25°C for 10 min and then subjected to immunoprecipitation with anti-Prp8 (A) or anti-HA (B) antibody. RNA was isolated and analyzed by Northern blotting, probed with U1, U2, U4, U5, and U6. The amounts of U4, U5, and U6 from three independent experiments were quantified with a phosphorimager, and the ratios of U4 to U5 and U6 to U5 (A) or U5 to U4 and U5 to U6 (B) were plotted as a bar graph. The error bars indicate standard deviations.

snRNP may be required for splicing. Our study further demonstrates that intact U4/U6.U5 tri-snRNP is required for the recruitment of U4/U6 and U5 to the spliceosome under conditions where U4, U5, and U6 were all present in the extract. It is worth noting that although human Sad1 was shown to be essential for the recruitment of tri-snRNP to the spliceosome (27), the splicing assay using hSad1-depleted extracts was performed under conditions where the tri-snRNP might have been completely dissociated. It is thus likely that failure in recruiting tri-snRNP to the spliceosome was also ascribed to lack of intact tri-snRNP in human extracts under the assay conditions.

Using extracts from Brr2 *in vivo* depletion, we demonstrated that Brr2 is responsible for mediating dissociation of the tri-snRNP. Brr2 is known to mediate unwinding of the U4/U6 duplex on the purified tri-snRNP and remains associated with U5 after U4 and U6 are dissociated from each other (19). It has been proposed that Brr2 is loaded onto U4 in a region preceding the U4/U6 stem I and translocates along U4 in the 3′-to-5′ direction to disrupt stem I in separating U4 and U6 (21, 22). The function of Brr2 is regulated by two other U5 components, Prp8 and Snu114 (18, 21, 46). It is not known whether Brr2 directly mediates the dissociation of U5 from U4/U6 snRNP without duplex unwinding or if U5-U4/U6 dissociation is a consequence of Brr2-mediated U4/U6 unwinding followed by rapid reannealing to reform the U4/U6 di-snRNP. The RNA binding protein Prp24 is known to promote annealing of U6 with U4 to form the di-snRNP and is important for cycling of the tri-snRNP in the spliceosome pathway (37, 38, 40). Prp24 is associated with free U6, which can efficiently capture the U4 snRNP released from the spliceosome during spliceosome activation or released from the tri-snRNP upon ATP hydrolysis catalyzed by Brr2. Conceivably, reannealing of U4/U6 may be impeded in the absence of Prp24. We addressed whether U4/U6 unwinding occurs in the absence of Sad1 using ntc25Δ extracts, which were previously demonstrated to contain only residual amounts of free U6 (32) and were shown here to also be limited in Prp24 (see Fig. S2 in the supplemental material). We found that although splicing in ntc25Δ extracts resulted in accumulation of free U4, due to insufficient amounts of free U6 and Prp24 to reform the di-snRNP, prior depletion of Sad1 prevented U4 accumulation. Considering the role of Sad1 in promoting the association of U4/U6 and U5, Brr2 might simply disrupt the interaction of proteins that bridge the two snRNP particles with U4 or U6 snRNA upon ATP hydrolysis to weaken their association, or U4 and U6 might rapidly reanneal following unwinding to reform U4/U6 di-snRNP even in the absence of Prp24 so that free U4 or U6 was not seen. It is interesting that while U4/U6 unwinding was highly efficient with purified tri-snRNP, proceeding to near completion upon incubation with ATP, only a small amount of free U4 was detected upon incubation of the Prp24-depleted or ntc25Δ extracts with ATP (38) (see Fig. S6 in the supplemental material). It is possible that some factor(s) in the extract prevents complete separation of U4 and U6 during or after Brr2-mediated unwinding so that U4 and U6 can rapidly reassociate without needing Prp24. Consequently, only a little unwinding can be detected in the extract. Such factors may interact only weakly with the tri-snRNP and are not copurified with the tri-snRNP to prevent complete separation of U4 and U6. A scheme for the proposed tri-snRNP homeostasis pathways is shown in Fig. 7. We propose that the tri-snRNP can dissociate into U4/U6 and U5 by two pathways. One pathway, which predominates with purified tri-snRNP, leads to full dissociation of U4, U5, and U6 and requires Prp24 to facilitate formation of U4/U6 di-snRNP. The other pathway, which predominates in extracts, does not require Prp24 to reform U4/U6 di-snRNP due to incomplete separation of U4 and U6. Although Sad1 is not tightly associated with the tri-snRNP, it promotes the association of U4/U6 and U5 to form the tri-snRNP. Under the condition of Prp24 depletion, free U4 and U6 accumulate in small amounts. Codepletion of Sad1 and Prp24 results in reduced amounts of free U4 and U6 due to reduced amounts of the tri-snRNP.

Although immunoprecipitation did not reveal Sad1 interaction with the tri-snRNP in a stable manner, Sad1 shows strong interactions with Snu114 by two-hybrid assays. Supporting this, *sad1-1* has demonstrated strong synthetic interactions with several *snu114* alleles (47). Sad1 was also found to interact with

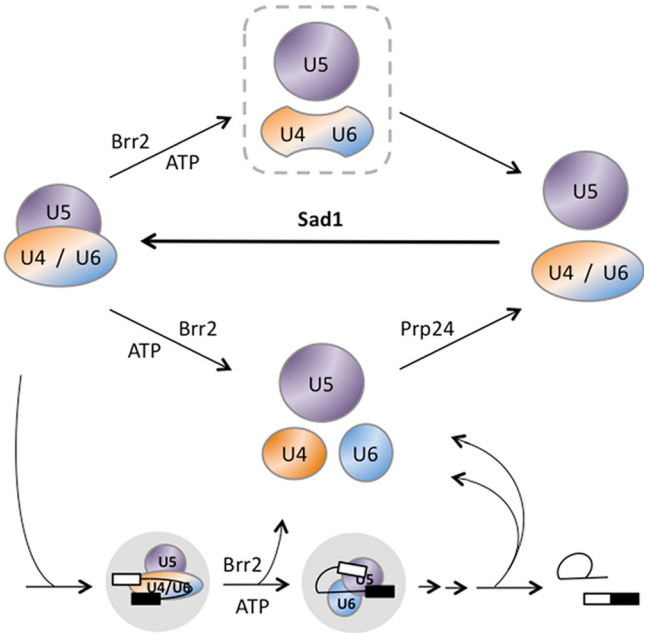

FIG 7 Schematic showing homeostasis of the tri-snRNP. While Sad1 promotes the association of U5 with U4/U6, Brr2 facilitates their dissociation by hydrolyzing ATP. Dissociation of U5 from U4/U6 may be a consequence of U4/U6 unwinding followed by Prp24-mediated reannealing (lower pathway) or simply disruption of the interaction between U5 and U4/U6 snRNP particles following unwinding and rapid reannealing independent of Prp24 (upper pathway).

Snu66, but less strongly, by two-hybrid assays. The interaction with Snu114 does not require the N-terminal ZnF-UBP domain, but that with Snu66 involves both the ZnF-UBP and UCH domains. Previous studies have suggested Prp6 to be a docker in establishing contacts between U5 and U4/U6 snRNPs (48–51). Snu66 has also been proposed to bridge the interaction between U4/U6 and U5 based on the results of two-hybrid assays (34). Nevertheless, *in vivo* depletion of Snu66 did not affect the integrity of the tri-snRNP, as determined by immunoprecipitation analysis (data not shown). Snu114 is associated with the tri-snRNP and with free U5 and is regarded as a component of U5 snRNP. Sad1 may interact with Snu114 and Snu66 to stabilize the association of U4/U6 and U5, but the interaction may be too weak to resist wash-off in immunoprecipitation assays. Since Snu114 has been shown to play a role in regulating Brr2 activity, whether the interaction of Sad1 with Snu114 negatively regulates Brr2 activity remains an open question. We show that Sad1 promotes the association of U4/U6 and U5 even in the absence of ATP (Fig. 5B), and the amount of association increases with increasing amounts of Sad1 (Fig. 5C), suggesting that the counteracting of Brr2-mediated tri-snRNP dissociation by Sad1 may be a consequence of shifting the equilibrium in favor of tri-snRNP formation. Nevertheless, we cannot exclude the possibility that Sad1 also inhibits Brr2-mediated tri-snRNP dissociation via interacting with Snu114.

Sad1 contains a ZnF-UBP domain at the N terminus and a UCH domain at the C terminus. Both domains are essential for cellular viability and for the function of Sad1 in the splicing reaction. The ZnF-UBP domain is predicted to coordinate zinc ions and to interact with ubiquitin (52). Although mutation in the zinc-coordinating residue C60 or C63 leads to cellular lethality, mutation in H85 has no effect on cellular growth and in H79 results in slow growth. In this context, it is questionable that zinc binding is essential for Sad1 function. A subclass of the ZnF-UBP proteins has been shown to bind ubiquitin (29, 52–55). Sad1 and its human homologue Usp39 harbor substitutions among the residues involved in ubiquitin binding (29). Accordingly, we have been unable to demonstrate ubiquitin binding of Sad1 despite intensive efforts using biochemical and yeast two-hybrid assays. It is thus possible that ubiquitin binding is unnecessary for the function of Sad1.

Sad1 was initially identified in a screen for factors involved in the biogenesis of U4 snRNP, and the *sad1-1* mutant accumulates the free form of *de novo*-synthesized U4 snRNA (28). Our results uncover how Sad1 affects the biogenesis of U4 by regulating the level of the tri-snRNP. Sad1 deficiency results in dissociation of the tri-snRNP, with U4 and U6 remaining associated under physiological conditions. Consequently, free U4 is unable to displace the existing U4 in the di-snRNP whether the U4/U6 duplex undergoes transient unwinding or not in the dissociation of the tri-snRNP. This explains why *sad1-1* accumulates *de novo*-synthesized U4 snRNA. Cells with defects in the NTC function have also been reported to accumulate free U4, accompanied by a lower level of U6 (28, 32). Distinct from Sad1, NTC functions in stabilization of U5 and U6 on the spliceosome after U4 is released during spliceosome activation (11). Accumulation of free U4 in NTC-deficient cells is likely a result of downregulation of U6 for efficient recycling of the U4/U6 di-snRNP. Our study elucidates the functional role of Sad1 in the splicing pathway, provides novel insights into homeostasis of the tri-snRNP, and also reveals distinct mechanisms for the accumulation of free U4 in *SAD1*-deficient and NTC-deficient cells.

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