Release of SF3 from the intron branchpoint activates the first step of pre-mRNA splicing

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ABSTRACT

Eukaryotic pre-mRNA splicing is a complex process requiring the precise timing and action of >100 *trans*-acting factors. It has been known for some time that the two steps of splicing chemistry require three DEAH-box RNA helicase-like proteins; however, their mechanism of action at these steps has remained elusive. Spliceosomes arrested *in vivo* at the three helicase checkpoints were purified, and first step-arrested spliceosomes were functionally characterized. We show that the first step of splicing requires a novel ATP-independent conformational change. Prp2p then catalyzes an ATP-dependent rearrangement displacing the SF3a and SF3b complexes from the branchpoint within the spliceosome. We propose a model in which SF3 prevents premature nucleophilic attack of the chemically reactive hydroxyl of the branchpoint adenosine prior to the first transesterification. When the spliceosome attains the proper conformation and upon the function of Prp2p, SF3 is displaced from the branchpoint allowing first step chemistry to occur.

Keywords: ribonucleoprotein; spliceosome; RNA helicase; splicing mechanisms

INTRODUCTION

Eukaryotic protein-coding RNAs often contain one or more noncoding, intervening sequences (introns), which are removed in a two-step process within a massive ribonucleoprotein (RNP) termed the spliceosome (Brody and Abelson 1985). Inaccurate splicing leads to the production of mRNAs that do not authentically represent the intended gene product, thus, introns must be precisely removed to ensure the proper outcome. Indeed, pre-mRNA splicing defects have been correlated to a substantial percentage of human diseases including cancer (for review, see Faustino and Cooper 2003). Defining the mechanisms underlying the assembly of the spliceosome, the authorization for initiating the two chemical steps and proofreading of the splice sites are critical to a full understanding of pre-mRNA splicing.

Spliceosomal introns vary in size by a factor of almost 500 (from tens to thousands of nucleotides) and possess only three common, degenerate motifs at the 5' splice site, 3' splice site, and the branchpoint. In addition, the mech-

anism of pre-mRNA splicing is chemically indistinguishable from that of group II intron splicing, which can occur in the absence of *trans*-acting proteins and RNAs (for review, see Bonen and Vogel 2001). Although both intron types possess a 5' splice site, a 3' splice site, and a branchpoint sequence, spliceosomal introns lack the sequences and structures that group II introns possess for their removal. Thus accurate intron removal requires the regulated efforts of >100 snRNP- and non-snRNP-associated factors for the proper assembly of the substrate in the spliceosome and the precise formation of and rearrangements within the catalytic core of the spliceosome. Among these factors are eight highly conserved RNA helicase-like proteins of the DExH/D family (for review, see Staley and Guthrie 1998). Prp2p is required for the first chemical step (Chen and Lin 1990; King and Beggs 1990), and Prp16p (Schwer and Guthrie 1991) and Prp22p (Company et al. 1991) are required at the second step of the reaction, although the role of Prp22p at this step is ATP-independent (Schwer and Gross 1998). Although these factors have been studied for some time, the specific molecular rearrangements they initiate at each of the chemical steps have remained enigmatic for the last 20 years.

The first chemical step of splicing occurs when the 2'-OH of the branchpoint adenosine performs a nucleophilic attack

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at the phosphodiester bond of the 5' splice site, releasing the 5' exon and forming a branched lariat intermediate RNA (Domdey et al. 1984; Padgett et al. 1984; Ruskin et al. 1984). Prior to first step chemistry, the branchpoint sequence of the pre-mRNA is associated with the U2 snRNA by base-pairing and bound by a number of U2 snRNP-associated proteins contained within two sub-complexes termed SF3a (Brosi et al. 1993) and SF3b (Will et al. 2002). The second step reaction occurs when the 3'-OH of the 5' exon attacks the phosphodiester bond at the 3' splice site, joining the exons and liberating the intron lariat RNA. Both steps of splicing require significant structural rearrangements in the spliceosome to properly place the reactive sites at its catalytic center (Konarska et al. 2006). Defining these rearrangements and the mechanisms by which they occur has been challenging due in part to the complexity of spliceosome assembly and the dynamic nature of the spliceosome.

In this work, we have purified functional, synchronized spliceosomes genetically arrested at three critical checkpoints in the splicing reaction *in vivo* to enrich for on-pathway, functional complexes. Employing a biochemical approach, we analyzed spliceosomes stalled (1) prior to the first step (Fig. 1A, left panel), (2) prior to the second step (Fig. 1A, middle panel), and (3) prior to mRNA release (Fig. 1A, right panel) from the spliceosome. Compositional differences within these arrested spliceosomes were analyzed. Surprisingly, the multisubunit SF3a and SF3b complexes, which reside at and around the intron branchpoint (Macmillan et al. 1994; Gozani et al. 1996, 1998), are present prior to the first step and completely absent prior to the second step. We hypothesized that this release may coincide with the function of Prp2p and that once the spliceosome is properly organized, SF3 removal may initiate the activation of first step chemistry.

To test this, we functionally characterized the first-step-stalled spliceosome assembled *in vivo* and showed that prior to catalysis, a novel ATP-independent conformational rearrangement is followed by an ATP-dependent displacement of SF3 from the spliceosome. Removal of SF3 from the chemically reactive 2'-OH of the branchpoint adenosine at this time allows the proper nucleophilic attack at the 5' splice site. Thus, in addition to its role in branchpoint identification and binding, we propose that the SF3 complex functions to restrain the branchpoint hydroxyl from chemistry until the catalytic core of the spliceosome is properly assembled.

RESULTS

Characterization of first step-blocked spliceosomes

Prp2p acts almost immediately upstream of the first step of pre-mRNA splicing; therefore, we isolated spliceosomes from *Saccharomyces cerevisiae* arrested prior to Prp2p function. Prp2p-associated material was affinity purified from

FIGURE 1. Purification of the first step arrested spliceosome. (A) Graphical representation of the Prp2p-, Prp16p- and Prp22p-arrested and -associated spliceosomes purified in this work. RNA from glycerol sedimentation gradients corresponding to affinity purified (B) Prp2p-TAP and (C) prp2G551Np-TAP-associated material were Northern blotted for spliceosomal snRNAs. Splicing complexes are present only in the temperature-shifted mutant corresponding to the U2, U5, and U6 snRNAs sedimenting at ~40S. Ribosomal subunits were used to determine the peak area of 40S subunit sedimentation. The arrow indicates the direction of sedimentation. Positions of U1, U2, U4, U5, and U6 snRNAs are shown.

cells containing a TAP-tag (Puig et al. 2001) at the *PRP2* locus in a wild-type strain. Prp2p-associated complexes were layered onto a glycerol velocity gradient suitable for spliceosome sedimentation (Stevens and Abelson 2002). Northern blot analysis of snRNAs in the even fractions from this gradient shows that Prp2p does not stably interact with significant levels of endogenous spliceosomes or spliceosomal snRNAs (Fig. 1B). Prp2p is a transiently interacting factor (King and Beggs 1990); thus, we did not expect this strategy to yield spliceosomes, but instead to serve as a control.

To accumulate spliceosomes with which Prp2p is associated, but reversibly arrested prior to its ATPase-dependent function, we used a cold sensitive (cs) *prp2* mutant that interacts with the spliceosome, but is unable to function at nonpermissive temperatures (NPTs). A yeast strain containing the severely cs G551N mutation of Prp2p (Edwalds-Gilbert et al. 2000; also see Materials and Methods) was C-terminally TAP-tagged at the *prp2* locus. Cells were shifted to the NPT of 25°C prior to harvesting, trapping endogenous pre-mRNAs in a first step-arrested spliceosome. ATP-depleted, affinity-purified prp2G551Np-associated material was layered onto a glycerol velocity gradient for spliceosome isolation. A significant peak of U2, U5, and U6 snRNAs at ~40S is seen in these gradients (Fig. 1C), corresponding to the size of the yeast spliceosome (Brody and Abelson 1985).

To compositionally define the first step-arrested spliceosome, we performed MudPIT mass spectrometry analysis of the associated polypeptides (Washburn et al. 2001). In Figure 2, we outline the protein components present in this and downstream spliceosomal RNPs. We note the complete absence of all but three tri-snRNP components in the *prp2*-arrested spliceosome. Additionally, the first step-arrested spliceosome contains all of the known yeast SF3 components (Dziembowski et al. 2004). Also present in the first

step blocked spliceosome were the Sm core proteins of the U2 and U5 snRNPs (Séraphin 1995) and the U2 core proteins Lea1p and Msl1p (U2A' and U2B'', respectively in human) (Caspary and Séraphin 1998). We did not detect any U6-specific LSm proteins (Achse et al. 1999; Stevens et al. 2001) consistent with their reported destabilization during spliceosome activation (Chan et al. 2003). The entire Prp19p complex (NTC) was identified and remains bound throughout the splicing cycle during the window of time analyzed in these experiments, just prior to the first step until the splicing reaction has completed (Fig. 2). The second step factor Prp17p was detected prior to the first step and remains bound throughout splicing, consistent with a recent report (Sapra et al. 2008). Two pre-mRNA binding proteins (Sto1p and Cbc2p) comprising the cap-binding complex (Colot et al. 1996; Lewis et al. 1996; Fortes et al. 1999) were consistently detected in all mass spectrometry results. A number of other spliceosome-associated proteins were detected as well (Fig. 2).

Functional analysis of *prp2*-arrested spliceosomes

Although Prp2p function initiates the first step of splicing, the recently described factor Yju2p was reported to be required after Prp2p, but prior to chemistry (Liu et al.

		<i>prp2</i> 1st step block	<i>prp16</i> 2nd step block	<i>prp22</i> post-splicing
U2 snRNP core	Lea1p	7/27.7%	16/53.4%	15/59.2%
	Msl1p	4/29.7%	3/36.9%	3/36.9%
	Rse1p	20/13.7		
	Hsh155p	20/18.4%		
	Cus1p	27/39.7%		
SF3b	Hsh49p	4/24.4%		
	Ysf3p	2/25.9%		
	Rds3p	2/21.3%		
SF3a	Prp21p	13/29.3%		
	Prp11p	9/30.1%		
	Prp9p	38/47.7%		
U4/U5-U6 snRNP	Prp8p	60/20.3%	45/20.8%	87/37.8%
	Brr2p	56/21.7%	55/24.6%	26/16%
	Snu114p	27/26%	26/31.8%	44/44.1%
U2, U5 snRNPs	Sm core	✓	✓	✓
	Prp19p	28/40.8%	43/66.8%	44/62.4%
	Prp46p	16/28.6%	25/47.7%	33/60.5%
	Snt309p	13/41.7%	5/42.3%	10/68%
	Cef1p	36/53.7%	14/26.4%	32/48.1%
	Syf1p	13/15.9%	16/19.8%	36/38.8%
	Clf1p	14/17.6%	15/27.5%	27/44.7%
	Ntc20p	5/24.3%	6/20.7%	2/35.7%
	Cwc2p	14/35.4%	20/44%	19/48.1%
	Isy1p	6/31.5%	6/30.6%	7/31.5%
	Syf2p	11/49.3%	11/35.3%	11/40%
	Prp17p	15/28.4%	7/20.4%	15/35.6%
	Prp18p			7/32.7%
	Slu7			13/28.3%
Helicase/related	Prp2p	59/41.9%		
	Spp2p	13/50.3%		
	Prp16p		74/47%	
	Prp22p		6/7.1%	69/50.4%
	Prp43p		2/3.1%	22/33.3%
RES complex	Bud13p	14/34.6%		
	Pml1p	3/19.6%		
	Ist3p	3/26.4%		
mRNA binding	Sto1p	8/7.8%	7/7.3%	16/22.6%
	Cbc2p	5/19.7%	9/27.9%	4/31.2%
	Yra1p		7/27.4%	7/31%
THO/TREX	Tex1p			5/16.4%
	Hpr1p		2/4%	6/13.2%
	Sub2p			3/8.5%
	Thp2p		2/10.3%	2/13.4%
	Mft1p		3/6.9%	3/10.5%
Spliceosome associated	Yju2p		8/31.3%	
	Bud31p	7/17.2%	4/28%	5/22.3%
	Prp45p	21/33.5%	21/43%	21/55.7%
	Cwc22p	10/13.2%	12/24.4%	21/42.6%
	Cwc24p	15/44.8%		
	Cwc27p	12/33.6%		
	Cwc25p		12/50.3%	
	Cwc15p	6/40.6%	8/41.7%	9/45.1%
	Cwc21p	5/28.9%	6/31.1%	4/20%
	Ecm2p	7/22.8%	17/46.4%	15/37.6%
	Ubp3p		4/6.1%	16/30.6%
	Bre5p		8/25.8%	13/36.7%
	Novel			

FIGURE 2. Mass spectrometry analysis of the associated proteins from first step-arrested, second step-arrested, and post-second step spliceosomes. Polypeptides corresponding to peak fractions from Figure 1C (*prp2*-arrested first step), Figure 5B (*prp16*-arrested second step), from Figure 5E (*prp22*-arrested spliceosomes) were analyzed by MudPIT. Proteins are categorized by group or functional association. Data for the number of peptides identified and percentage polypeptide sequenced are shown. Polypeptide contaminants are listed in Supplemental Table S1.

2007). Yju2p is not detectable by mass spectrometry in this particle (Fig. 2), however to ensure that we could confidently distinguish between the functions of Prp2p and Yju2p in the arrested spliceosomes, we metabolically depleted Yju2p from *prp2G551Np*-TAP cells by repressing a *GAL*-driven, HA-tagged Yju2p (Fig. 3A; Longtine et al. 1998). We isolated first step-blocked spliceosomes by *prp2G551Np*-TAP chromatography from Yju2p-depleted cells that additionally contain a myc tag on Prp9p (Abovich et al. 1990; Legrain and Choulika 1990). Prp9p is the yeast homolog of mammalian SF3a60, which is a component of the U2 snRNP-associated trimeric SF3a complex. Purified, arrested spliceosomes were incubated under splicing conditions (Stevens and Abelson 2002) at NPT with no ATP treatment (Fig. 3B), under splicing conditions at permissive temperature (PT) with no ATP treatment (Fig. 3C), under splicing conditions at PT with AMP-PNP treatment (Fig. 3D), or under splicing conditions at PT with ATP (Fig. 3E). After incubation, the reactions were centrifuged through

spliceosome-resolving glycerol velocity gradients. Proteins from alternating fractions were subjected to Western blot analysis to detect Prp9p-myc and nucleic acids subjected to Northern blot analysis to detect spliceosomal snRNAs. Spliceosomes incubated at NPT sedimented identically to those resulting from an untreated sample in which Yju2p was not depleted (cf. Fig. 1C and Fig. 3B). In samples treated at PT without ATP, the peak of the signal was shifted three fractions higher in the gradient (Fig. 3C), indicating a substantial change in shape or in composition. This indicates that the novel ATP-independent event may be catalyzed by Prp2p itself and represents a conformational change rather than a compositional change and is corroborated by the AMP-PNP experiment in Figure 3D (see Discussion). Upon incubation at PT in the presence of ATP, Prp9p-myc is displaced from the spliceosome and sediments near the top of the gradient (Fig. 3E). A quantitative analysis of the sedimentation behavior of SF3 and the spliceosomes from Figure 3, B–E, is shown in

FIGURE 3. Functional characterization of the first step-arrested spliceosome. (A) Metabolic depletion of Yju2p from *prp2G551N* cells. Western blot analysis of GAL-HA-Yju2p through a time-course in galactose (left) or after shift to dextrose (right). Affinity-purified spliceosomes depleted for Yju2p and arrested at the first step of splicing using the *prp2G551N* mutation were treated at NPT with no added nucleotide (B), treated at PT with no added nucleotide (C), treated at PT with added ATP (D), or treated at PT with AMP-PNP (E). Proteins in the gradient fractions were subjected to Western blot analysis for Prp9-myc. (F) Prp9-myc signal was quantitated in gradient fractions from B–E, normalized and graphed (top panel). RNAs from the corresponding fractions were Northern blotted for snRNAs, which were quantitated, normalized, and graphed (bottom panel). (G) RT-PCR analysis of unspliced and partially spliced pre-mRNAs contained in the peak gradient fractions from B, C, and E. Lane 1 of each set is designed to amplify *RPP1B* pre-mRNA and mRNA. Lane 2 of each set amplifies the *RPP1B* 3' splice site junction. Lane 3 of each set specifically amplifies the *ACT1* lariar structure using a splint oligonucleotide that bridges the intron sequences of the 5' splice site and upstream of the branchpoint adenosine and another oligonucleotide that allows for specific amplification within the lariar structure.

Figure 3F. The displacement of SF3 (Fig. 3F, top panel) does not affect the sedimentation of the spliceosome as defined by the U2, U5, and U6 snRNAs at the $\sim 40S$ region of this gradient (Fig. 3F, bottom panel).

To further demonstrate that the shifts in glycerol gradient sedimentation correspond to authentic changes in shape for these complexes, we also performed native gel electrophoresis on the purified complexes after TEV elution (Supplemental Fig. S1). The shape change noted by the shift in sedimentation upon incubation at PT with no ATP (Fig. 3, cf. B and C), the migration of comparable spliceosomes in a native gel are measurably different (Supplemental Fig. S1, cf. lanes 1 and 2). Further migration differences were noted in the presence of ATP at PT (Supplemental Fig. S1, lane 3) or in the presence of AMP-PNP (Supplemental Fig. S1, lane 4).

To demonstrate the functionality of these arrested spliceosomes, we performed RT-PCR analysis on RNAs contained in representative fractions from affinity-purified, gradient-separated material. Using a strategy to detect pre-mRNA, lariat intermediate, and lariat, we show that pre-mRNA was exclusively found in arrested spliceosomes from peak fractions of both of the reactions lacking ATP, as expected (Fig. 3G). This also indicates that the ATP-independent conformational change is not coincident with or sufficient to promote the first step. In the presence of ATP at PT and in the absence of Yju2p, the vast majority of pre-mRNA was chased to lariat intermediate (Fig. 3G). These data demonstrate that even if Yju2p is required for the first step of splicing a subset of introns, it is not required for the displacement of SF3 under these conditions, as most of SF3 is not spliceosome associated after Prp2p function.

SF3b displacement is independent of Yju2p

The SF3a heterotrimer is a subfraction of the SF3 activity characterized by Krämer and colleagues (Brosi et al. 1993). The other portion of SF3 activity, the U2 snRNP-associated SF3b, contains seven polypeptides in humans (Will et al. 2002) and six orthologous polypeptides in yeast (Dziembowski et al. 2004). SF3a and SF3b are both present in the assembled, *prp2*-arrested spliceosome (Fig. 2); however since each subcomplex can be isolated independently, they may behave very differently in terms of their release from the spliceosome.

To test for the displacement of both SF3a and SF3b, we employed a glycerol-cushion pelleting assay to test for their release. In this assay, we used conditions designed to pellet intact spliceosomes, but incapable of pelleting displaced SF3a or SF3b. First step-arrested spliceosomes containing either Prp9p-myc (an SF3a component) or Hsh155p-myc (an SF3b component) were affinity-purified. To enrich for a homogeneous population, spliceosomes were further purified by glycerol velocity gradient and the peak fraction was selected. Spliceosomes were then incubated under one of

three conditions: (1) splicing conditions with no ATP at NPT (Fig. 4A, lanes 3,4); (2) splicing conditions with ATP at PT (Fig. 4A, lanes 5,6), or (3) treated in 1 M NaCl (Fig. 4A, lanes 7,8). The reactions were sedimented through a glycerol cushion to separate the spliceosome from released factors. Proteins extracted from the supernatant and the pellet were analyzed for the presence of the myc tag by Western blotting. The SF3a and SF3b complexes are known to be sensitive to elevated salt levels (Behrens et al. 1993; Bessonov et al. 2008); thus, the salt-treated samples serve as a control for displacement of SF3 from the purified spliceosome. Both SF3a and SF3b are displaced from the spliceosome upon Prp2p function only under splicing conditions (Fig. 4A lanes 5,6) as shown by the presence of Prp9p-myc and Hsh155p-myc in the supernatant. At NPT, they are found exclusively in the pellet.

We also affinity purified a first step-blocked, $\Delta Yju2p$ spliceosome containing Hsh155p-myc to ensure that Yju2p was not required for SF3b displacement. The above strategy was used for analysis of the $\Delta Yju2p$ spliceosome containing Hsh155p-myc (Fig. 4B). Yju2p, which has been proposed to act downstream from Prp2p in the splicing cycle, is not required for the displacement of SF3b as SF3b is released upon the action of Prp2p in the absence of Yju2p (Fig. 4B, lanes 5,6) (see Discussion).

Second step-arrested spliceosome characterization

After the first step of splicing, two additional DEAH-box proteins are required to proceed through the second step. The second transesterification reaction requires an ATPase-catalyzed reaction carried out by Prp16p (Schwer and Guthrie 1991), followed by an ATP-independent event requiring Prp22p (in conjunction with several other second step factors (Schwer and Gross 1998)). Spliceosomes arrested at the second step of splicing were affinity purified from *cs prp16* mutants. Prp16p was TAP-tagged in three strains:

FIGURE 4. SF3a and SF3b are released from the spliceosome concurrent with Prp2p action. (A) Affinity-purified spliceosomes were pelleted through glycerol cushions without splicing (lanes 3,4), with splicing (lanes 5,6), or after disruption with salt (lanes 7,8). Total proteins from supernatant and pellet fractions were Western blotted for the myc tag. (B) Same experiment performed for Hsh155p-myc in $\Delta Yju2p$ spliceosomes. Lane 1 contains 2% of the TEV eluates and lane 2 contains 10% of the 18th fraction from the reactive experiments.

BY4741 (wild-type *PRP16*) (Baker-Brachmann et al. 1998), *prp16-302* (Madhani and Guthrie 1994), and the allele of *prp16* in strain DBY4406 (*prp16R456K*; also see Materials and Methods; Noble and Guthrie 1996). Consistent with its transient interaction with the spliceosome and similar to wild-type Prp2p-TAP (Fig. 1B), affinity-purified, wild-type Prp16p-TAP is not associated with significant levels of spliceosomal snRNAs in the 40S region of the gradient (Fig. 5A). Spliceosomes purified at NPT from *prp16* cells are associated with U2, U5, and U6 snRNAs in the ~40S region (Fig. 5B).

We then analyzed the proteins in these second step-arrested spliceosomes by MudPIT mass spectrometry analysis. Unlike the first step-arrested spliceosome (Fig. 2), the second step-arrested spliceosome is devoid of all SF3 components. This independently supports that both SF3a and SF3b are displaced from the spliceosome between these steps. Other differences include the dissociation of the RES complex (Dziembowski et al. 2004), as well as the addition of the spliceosome disassembly factor Prp43p (Martin et al. 2002).

We measured the difference in sedimentation behavior between the first step-arrested and second step-arrested

FIGURE 5. Purification of the *prp16*- and *prp22*-arrested spliceosomes. RNA from glycerol sedimentation gradients corresponding to affinity purified (A) Prp16p-TAP and (B) *prp16-302p*-TAP-associated material were Northern blotted for spliceosomal snRNAs. Splicing complexes are present only in the temperature-shifted mutant corresponding to the U2, U5, and U6 snRNAs sedimenting at ~40S. (C) Sedimentation differences between first and second step-arrested spliceosomes were calculated using normalized snRNA signals present in the respective gradient fractions. (D) Prp22p-TAP and (E) *prp22-G613Q*p-TAP-associated material were Northern blotted for snRNAs as in A and B. (F) Graphical representation of sedimentation of *prp22*-arrested spliceosomes compared to those presented in C. (G) RT-PCR analysis of splicing intermediates and products from affinity purified, gradient isolated *prp16*- and *prp22*-arrested spliceosomes. First-step products are seen in *prp16*-arrested spliceosomes. Spliced mRNA and only the lariat intron are present in *prp22*-arrested spliceosomes. Lanes are as in Figure 3G.

spliceosomes. Despite the loss of >500 kDa of SF3 polypeptides, the *prp16*-arrested spliceosomes do not sediment measurably differently than spliceosomes arrested in the first step after the ATP-independent conformational rearrangement (cf. Fig. 3F and Fig. 5C). This is likely due to the added polypeptide mass of Prp16p, Prp22p, Prp43p, and others, which in total are similar in mass of the displaced SF3 and other first step components (Fig. 2). The similarity in mass and co-sedimentation suggest that there is not a dramatic conformational change after the first step and prior to the action of Prp16p.

Post-splicing *prp22*-arrested spliceosomes

After the second step, the spliced mRNA and the intron are retained within the spliceosome until the ATP-dependent function of the DEAH-box protein Prp22p releases the mRNA (Company et al. 1991; Schwer 2008). We completed the DEAH-box-arrested pre-mRNA- or mRNA-containing spliceosome purifications by isolating spliceosomes arrested after the second step, but prior to the release of mRNA using the same TAP-tagging strategy with two *cs* alleles of *prp22* (DBY4172—*prp22D613Q* and DBY4423—*prp22G394R,D613Q*) (Noble and Guthrie 1996). Similar to that for wild-type Prp2p (Fig. 1B) and Prp16p (Fig. 5A), Prp22p was not associated with significant levels of snRNAs (Fig. 5D). Spliceosomes arrested at the *prp22* step were affinity purified and sedimented through a glycerol velocity gradient. RNA extracted from gradient fractions was Northern blotted for snRNAs (Fig. 5E), and the post-splicing *prp22*-arrested spliceosomes sedimented three fractions higher in the gradient than both the *prp2*- and *prp16*-arrested spliceosomes (Fig. 5F). There are very few compositional differences between *prp16*- and *prp22*-arrested particles, however we note that Yju2p and Cwc25p are absent after Prp16p action and prior to dissociation of the spliceosome.

RT-PCR analysis of the mRNA/pre-mRNA/lariat contained in the peak fractions for *prp16*- and *prp22*-arrested spliceosomes was performed (Fig. 5G). As expected, the *prp16* spliceosomes contained no pre-mRNA, but did contain the 3' splice site junction and lariat RNA, indicative of the first step products. The *prp22* spliceosomes contained only mRNA and lariat intron, indicative of the post-splicing, second step products. This *in vivo* evidence is supported by *in vitro* experiments performed with these mutants (Edwards-Gilbert et al. 2004; Villa and Guthrie 2005).

Similar to the *prp2*- and *prp16*-arrested particles, *prp22*-arrested spliceosomes contain several known spliceosome-associated proteins present as well as the second step factors Prp18p and Slu7p. These act together after Prp16p is released but prior to Prp22p binding (James et al. 2002). A more complete set of THO/TREX complex components is present in the *prp22*-arrested spliceosome although

Tho2p is again undetectable. Interestingly, the presence of a deubiquitinase Ubp3p and its interaction partner Bre5p were present in both the *prp16*- and *prp22*-arrested spliceosomes. Although no published work has demonstrated a function for these proteins in pre-mRNA splicing, we have also detected these factors in post-splicing lariat RNP complexes (J Combs and SW Stevens, unpubl.).

DISCUSSION

The pre-mRNA splicing machinery and the mechanism of splicing have been extensively studied by both genetic and biochemical means for many years. Although the basic chemical mechanism of the two transesterification reactions of pre-mRNA splicing was proposed 25 yr ago (Domdey et al. 1984; Padgett et al. 1984; Ruskin et al. 1984), the specific molecular means by which the chemical steps of splicing are authorized and promoted remain unknown. A general mechanism for the function of the DEAH-box protein Prp16p was first proposed by Guthrie and colleagues (Burgess et al. 1990). Work by Query and Konarska (2004) has further shaped this model; however, specific functions for both Prp2p and Prp16p in the first and second step, respectively, have best been described as enigmatic rearrangements.

In this work we show that the mechanism for initiating the attack of the branchpoint adenosine on the 5' splice site involves the removal of SF3 from the spliceosome at the time of Prp2p action, thus unsheathing the pre-mRNA branchpoint/U2 snRNA duplex. Our data support a model in which the U2-associated SF3a and SF3b protein complexes sequester this region and prevent its premature reactivity (Fig. 6; see below).

prp2-, *prp16*-, and *prp22*-arrested spliceosomes

Many important discoveries in the field of pre-mRNA splicing have resulted from compositional comparisons of (1) constituent parts of the spliceosome (Neubauer et al. 1997; Caspary et al. 1999; Stevens and Abelson 1999; Gottschalk et al. 2001; Stevens et al. 2001, 2002), (2) assembled or partially assembled functional complexes (Hartmuth et al. 2002; Jurica et al. 2002; Zhou et al. 2002; Chen et al. 2007; Bessonov et al. 2008), and (3) products of the reaction (Makarov et al. 2002). In this work, we compositionally define spliceosomes arrested at three helicase checkpoints *in vivo*, each purified by gentle, but stringent affinity chromatography techniques. Due to the differences likely to be encountered through the assembly of the spliceosome *in vitro* upon a single transcript versus the co-transcriptional assembly of the spliceosome *in vivo*, we believe that the data presented here accurately represent the composition and functionality of these complexes.

Our mass spectrometry data (Fig. 2) reveal that one striking feature among the helicase-arrested spliceosomes is

FIGURE 6. Model for the activation of the first chemical step of pre-mRNA splicing. (A) The SF3b complex (purple oval) resides on the branchpoint/U2 RNA duplex, while SF3a (orange oval) resides just upstream of SF3b during spliceosome assembly and until the ATP-dependent action of Prp2p. The pre-mRNA is denoted by the green and blue exon boxes and the bottom RNA strand. snRNPs are shown as the red ovals with U2 and U6 snRNAs forming duplexes with each other and the pre-mRNA. U5 snRNP contacts the 5' splice site. (B) After addition of Prp2p, an ATP-independent rearrangement concomitant with a structural rearrangement occurs. (C) Upon ATP hydrolysis by Prp2p, the SF3a and SF3b components are displaced from the pre-mRNA, uncovering the reactive branchpoint adenosine hydroxyl, which is now properly positioned for in-line attack of the 5' splice site. (D) Transesterification releases exon1 and the lariat intermediate, formed by the 2'–5' linkage between the branchpoint and the 5' splice site.

the absence of most of the U4/U6•U5-associated factors, with the exceptions of the Sm core, Prp8p, Brr2p, and Snu114p, which are present throughout assembly and splicing. Our data show that the majority of U4/U6•U5-associated factors are dispensable during the catalytic steps of splicing. This result differs slightly from that of activated human spliceosomes (Makarov et al. 2002) in which other tri-snRNP components were detected. This discrepancy may be due to the operational differences in purifying synchronized splicing complexes formed in vivo versus those formed in vitro, or less likely, might reflect functional differences in the two systems.

More striking is that the SF3a and SF3b components are completely absent after the first step. Other differences between *prp2*- and *prp16*-arrested spliceosomes include the addition of factors known to participate in the second step of splicing (e.g., Prp16p, Prp22p), and others that have been implicated in pre-mRNA splicing by association with spliceosomes (e.g., several Cwc proteins), but for which there are not yet data demonstrating a function in splicing. Interestingly, THO/TREX complex components appear only after the first step of splicing and persist through the second step. This temporal difference is consistent with data showing that the THO/TREX complex loads late during transcription on most intron-containing transcripts, after spliceosome assembly (Abruzzi et al. 2004).

We also note that the entire RES complex is absent after the first step of splicing. This heterotrimer has been implicated in the retention of unspliced pre-mRNA in the nucleus and indeed, tagged RES components only co-purify radiolabeled pre-mRNA and no other splicing intermediates (Dziembowski et al. 2004), consistent with our mass spectrometry results. How the RES complex might enforce a nuclear retention phenotype when it is only associated prior to the first step is not currently understood. Other differences between the *prp2*- and *prp16*-arrested spliceosomes include the addition of the post-splicing Prp43p helicase (Arenas and Abelson 1997). While Prp43p is not known to be required for the second step or for mRNA release, its presence indicates that it may either have an unknown role at these steps, or that its presence is required or tolerated without playing a catalytic role.

Compositional differences between the *prp16*- and *prp22*-arrested spliceosomes are relatively minimal; however, they are consistent with previous reports. These include the addition of Prp18p and Slu7p in the *prp22*-arrested spliceosome; both have been shown to bind and function after the ATPase-dependent function of Prp16p (Jones et al. 1995), but prior to the second step of chemistry. Several THO/TREX complex components are stably bound after the second step. However, in none of the isolated spliceosomes do we find the largest THO/TREX component Tho2p.

Other differences include the removal of Yju2p and Cwc25p after the second step. Previous work from Cheng and colleagues has suggested that these two factors act prior to the first step (Liu et al. 2007; Chiu et al. 2009). Our data demonstrate that in vivo-assembled spliceosomes do not require Yju2p for the first step, at least for the pre-mRNA tested (Fig. 3G), and that Prp2p-initiated removal of SF3a and SF3b also occurs in its absence (Fig. 4B). However, our data cannot rule out that Yju2p joins after Prp2p and prior to the first step. One possibility for this difference is that Yju2p may be required for the splicing of only a subset of introns in yeast. This seems to be the most likely possibility, and indeed, intron microarray analysis has revealed vast differences in splicing efficiency between different splicing

factor mutants (Pleiss et al. 2007). Another possibility is that the difference between our in vivo-assembled spliceosomes and those formed in vitro using Yju2p-depleted extracts reflect pathway-specific differences resulting from transcription-coupled pre-mRNA processing versus those assembled in vitro. We note that SF3a and SF3b displacement is quantitative under these conditions, independent of the presence of Yju2p in the spliceosomes.

We have not presented data regarding the mechanism of Prp16p in authorizing the second step, however previous work has revealed that a significant change in the conformation of the spliceosome occurs at this stage, inducing protection of the 3' splice site (Schwer and Guthrie 1992). Our data show a limited number of polypeptides are released at this stage (Fig. 2). If the Prp16p-catalyzed structural rearrangement positions the 5' exon for attack at the 3' splice site, it is possible that one or more of the factors that depart at this stage (e.g., Yju2p and/or Cwc25) are protecting the 3'-OH of the free 5' exon and/or sequences around the phosphodiester bond of the 3' splice site until this point. Candidates for 3' splice site protection include Prp8p or Prp16p and Prp22p (Umen and Guthrie 1995; McPheeters et al. 2000).

Spliceosome remodeling at the first step of splicing

Our data show that two rearrangements are affected to initiate the first chemical step of splicing. First, a novel ATP-independent conformational change occurs that is significant enough to shift the sedimentation value of the spliceosome (Fig. 3, cf. B and C and D). This change in the spliceosome may result from a repositioning of a snRNP, the pre-mRNA, or both to favor the arrangement of the reactive sites for the first step (Konarska et al. 2006). Upon ATP treatment, SF3a and SF3b are quantitatively removed from spliceosomes (Figs. 3E, 4A) and pre-mRNA is chased into first step products (Fig. 3G) demonstrating that the *prp2* arrested spliceosomes are functional and are not defective, off-pathway intermediates. The spliceosomes purified in this work were prepared under moderately stringent conditions. Purification of spliceosomes using conditions of reduced salt allows pre-mRNA to be chased to fully spliced mRNA (data not shown), likely due to the presence of contaminating amounts of second step factors resulting from less stringent purification.

ATP-independent and ATP-dependent roles for helicases

Several DExH/D-box proteins have been reported to have both ATP-dependent and ATP-independent functions, including Prp5p during the assembly of the spliceosome (Newnham and Query 2001; Perriman et al. 2003) and Prp22p, which has an ATP-independent role in the second step of splicing (Schwer and Gross 1998) prior to its ATP-

dependent role in displacing the spliceosome from the spliced mRNA (Company et al. 1991; Schwer 2008). Similarly, we have shown a parallel ATP-independent rearrangement at the time of Prp2p function, and defined an ATP-dependent role for Prp2p in initiating the first step of pre-mRNA splicing. Although we cannot yet say definitively that Prp2p is required for the ATP-independent step, the inability to proceed through this step using a *prp2* mutant at NPT strongly suggests that Prp2p plays a role. The nature of this event is currently under investigation.

Model for first step activation and catalysis

Structural analysis of the human SF3b complex demonstrated that the SF3b14a protein, which crosslinks to the branchpoint adenosine (Macmillan et al. 1994) and U2 snRNA (Dybkov et al. 2006), resides in a cleft through which the pre-mRNA has been predicted to thread (Golas et al. 2005). Although yeast SF3b does not contain an ortholog of this protein, the high degree of conservation of the other SF3b subunits suggests that the U2 snRNA/intron branchpoint duplex is sequestered within SF3b in a protective manner.

Others have shown that early in spliceosome assembly, the branchpoint adenosine is arranged in such a way as to expose the reactive 2'-OH upon base-pairing to the U2 snRNA (Newby and Greenbaum 2002). The importance of restraining this highly reactive nucleophile was highlighted in work showing that, in the presence of a defective 5' splice site, the branchpoint can attack the U2 snRNA itself (Smith et al. 2007). In our model, SF3b provides a temporary steric barrier prior to the activation of the first step of splicing (Fig. 6). Physically obscuring this region at the time of base pairing with the U2-associated SF3b complex allows the numerous rearrangements required to proceed through spliceosome assembly to occur without premature branchpoint reactivity. When the spliceosome achieves the proper conformation (Fig. 6A), an ATP-independent rearrangement occurs (Fig. 6B). Although we do not have direct evidence for the participation of Prp2p in the ATP-independent event, the block induced by *prp2G551Np* at NPT suggests that Prp2p plays a role in this process. Prp2p then induces an ATP-dependent change that destabilizes both SF3b from the branchpoint region and SF3a from the region upstream of the branchpoint (Fig. 6C). This unleashes the branchpoint 2'-OH, which is now positioned for proper in-line attack at the 5' splice site to form the lariat intermediate and free 5' exon (Fig. 6D).

In this work, we have defined the mechanism of activation of the first step of splicing. Future work should reveal how Prp2p displaces SF3a and SF3b, whether by translocating along the pre-mRNA, as Prp22p does in spliceosome disassembly (Schwer 2008), or by affecting local pre-mRNA or snRNA structure to initiate SF3 release without translocation.

MATERIALS AND METHODS

Yeast strains

The yeast strains used were: SS1347 (MATa, *ura3 leu2, trp1, his3, lys2, prp2::TRP1* pRS415-PRP2-TAP::URA3), SS1348 (MATa, *ura3, leu2, trp1, his3, lys2, prp2::TRP1* pRS415-*prp2G551N*-TAP::URA3); SS1353 (MATa *ura3, leu2, trp1, his3, lys2, prp2::TRP1* pRS415-*prp2G551NTAP::URA3* PRP9MYC::HIS3); SS1354 (MATa *ura3, leu2, trp1, his3, lys2, prp2::TRP1*, pRS415-*prp2G551NTAP::URA3*, HSH155MYC::HIS3); SS1357 (MATa *ura3, leu2, trp1, his3, lys2, prp2::TRP1*, pRS415-*prp2G551NTAP::URA3*, PRP9MYC::HIS3, KAN::GAL-HA-YJU2); SS1358 (MATa *ura3, leu2, trp1, his3, lys2, prp2::TRP1*, pRS415-*prp2G551NTAP::URA3*, HSH155MYC::HIS3 KAN::GAL-HA-YJU2); SS492 (MAT a *his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, PRP16-TAP::TRP1*); SS1316 (MAT a *ade2, his3, leu2, lys2, trp1, ura3, prp16::LYS2* pSE358-*prp16-302-TAP::URA3*); SS1311 (parent DBY4406) (MAT α *gal-, mal-, his4-619, prp16R456K-TAP::KAN*); SS1330 (parent DBY4172) (MAT α *gal-, mal-, his4-619, prp22D613Q-TAP::KAN*); and SS1332 (parent DBY4423) (*gal-, mal-, his4-619, prp22-G394R,D613Q-TAP::KAN*).

Mapping helicase mutations

To map the mutations in the *prp16* and *prp22* genes in the DBY strains, the entire open reading frame was PCR-amplified from the respective strains. The *prp16* mutation contained in DBY4406 is *prp16R456K*, located just downstream from motif Ib; the mutation in *prp22* contained in DBY4172 is D613Q, a charge reversal located just after the DEAH-box (motif II); the mutation in *prp22* contained in DBY4423 is G394R, as well as the same D613Q mutation in DBY4172. We note that in addition to the *prp2G551N* mutation, the *prp2* cs mutation used in this work also contains a D547E mutation, which suppresses the dominant negative phenotype reported by Lin and colleagues (Edwalds-Gilbert et al. 2000).

Cell growth and temperature shifts

Wild-type strains were grown in YPD at 31°C to OD (A_{600}) = 1.5 before harvesting. Mutant strains were grown at PT (*prp2G551* strains at 37°C; *prp16-302* and *prp22* strains at 31°C) to OD (A_{600}) = 1.0 and then shifted to NPT (*prp2G551N* strains to 25°C; *prp16-302* and *prp22* to 16°C) for 45 min. Cells were harvested by centrifugation, washed in cold water, washed in buffer A (10 mM HEPES at pH 7.9, 200 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, and 10% glycerol), and resuspended in one volume (w:v) buffer A before freezing dropwise in liquid nitrogen.

Isolation of arrested spliceosomes

Frozen cells were milled using a Retch ball mill (Mayas et al. 2006). ATP was depleted by addition of glucose to 2 mM. Whole cell extract was prepared as described (Stevens and Abelson 2002) except dialysis buffer contained 150 mM KCl. Extract (40 mL) was incubated with IgG sepharose (400 μL) at 4°C for 1 h with rotation. The resin was washed thoroughly and resuspended in wash buffer (10 mM Tris at pH 8.0, 150 mM NaCl, 0.1% NP-40, 1.5 mM MgCl₂, 8% glycerol, 1 mM DTT, 0.2 mM PMSF). Bound material was incubated for 1 h at 16°C with TEV protease and RNase inhibitor (Invitrogen). The eluted material was layered

onto 10%–30% glycerol gradients (made in 20 mM HEPES at pH 8.0, 150 mM KCl, 1.5 mM MgCl₂, 0.1% NP-40), and were centrifuged for 10 h at 29,000 RPM in an SW41 rotor. Fractions were phenol/chloroform extracted, and protein and RNA were precipitated (Stevens and Abelson 2002). RNA was subjected to Northern blot analysis probing for U1, U2, U4, U5, and U6 snRNAs, and corresponding protein fractions were subjected to mass spectrometry analysis or Western blot analysis.

Metabolic depletion of YJU2

Strains containing a metabolically depletable *YJU2* gene were grown in YP + 2% galactose at PT to OD (A_{600}) = 0.5, washed in prewarmed YPD, resuspended in fresh YPD, and allowed to grow for 3 h. Cells were then shifted to 25°C for 45 min before harvesting.

Functional analysis of arrested spliceosomes

Arrested spliceosomes were purified as described above. Following TEV elution, purified material was treated (1) under splicing conditions (Stevens and Abelson 2002) in the presence of ATP, (2) under splicing conditions in the absence of ATP, or (3) incubated on ice in the absence of ATP. Splicing reactions were layered onto 10%–30% glycerol gradients and centrifuged as described above. Extracted proteins from even fractions were analyzed by Western blotting for the myc tag. Extracted RNA was precipitated and analyzed by Northern blotting for spliceosomal snRNAs.

Native gel electrophoresis of splicing reactions using affinity purified spliceosomes was performed using 20 cm × 20 cm × 0.75 mm gels (0.5X TAE and 4% [80:1]). Gels were electrophoresed at 4°C at 250 V for 11.75 h in 0.5X TAE, and analyzed by Northern blotting for U6 snRNA.

For the spliceosome pelleting experiments, TEV elutions were layered on 11 mL glycerol gradients (10%–30%) under conditions described above. The peak spliceosome fraction from the glycerol gradient was split into three equal samples. One sample was treated with 1 M NaCl, another adjusted to splicing conditions in the absence of ATP and incubated in ice, and another treated at 37°C under splicing conditions with ATP. After a 20 min incubation, each of the samples were layered onto a 30% glycerol cushion containing 20 mM HEPES at pH 7.9, 150 mM KCl, 1.5 mM MgCl₂, 0.1% NP-40, 1 mM DTT, 0.2 mM PMSF, and centrifuged at 80,000 RPM for 80 min in a TLA 100.3 rotor. The supernatant was removed and the pellet was resuspended. Extracted protein was analyzed by Western blotting for the myc tag.

Protein and RNA quantitation and normalization

Quantitation of proteins in Western blots was performed using a NightOWL II LB 983 (Berthold Technologies) by ultracold-CCD camera detection. Signal intensities were integrated over the lanes of the Western blot and values for each glycerol gradient fraction were normalized against the lane with the most intense signal, which gave a value of 1 for the most abundant fraction. Automatic average background subtraction was performed for each Western blot independently.

Quantitation of snRNA was performed by phosphorimage analysis using the Quantity One suite of software (Bio-Rad). Values for each band within a Northern blot were quantitated using identically sized analysis frames. Background levels were

subtracted from each of these values using an identically sized analysis frame in a region of the Northern blot that contained no signal. Background subtracted RNA signals from each glycerol gradient fraction were normalized against the lane with the most intense signal for that Northern blot, which gave a value of one (1) for the most abundant fraction.

RT-PCR

Peak fractions from the glycerol gradients were treated with DNase I and RNase inhibitor for 15 min at 37°C. RNA was phenol extracted, precipitated, and reverse transcribed with AMV reverse transcriptase (Promega) using an oligonucleotide primer specific to the *RPP1B* pre-mRNA/mRNA or the *ACT1* lariat/lariat intermediate (J Beggs and D Barrass, pers. comm.). PCR on cDNA was performed using Pfu DNA polymerase using the above primers in pairs designed to detect the species of interest.

Mass spectrometry analysis

To each sample, 60 μ L of solubilization buffer (8 M Urea, 100 mM Tris-HCl at pH 8.5) were added. The subsequent mixture was then reduced by adding TCEP to 5 mM. Iodoacetamide (10 mM final concentration) was added to alkylate cysteine residues and the samples were subsequently incubated at room temperature in the dark for 15 min. Reactions were diluted to 2 M urea by addition of 180 μ L of 100 mM Tris at pH 8.5. Calcium chloride was added to a final concentration of 1 mM. Two micrograms of Trypsin were added and the resulting mixtures were shaken for 18 h in the dark at 37°C. Formic acid (90%) was added to a final concentration of 5% formic acid. The tubes were centrifuged for 30 min at 2°C on a table top centrifuge.

The protein digest was pressure-loaded onto a fused silica capillary desalting column containing 5 cm of 5 μ m Polaris C18-A material (Metachem) packed into a 250 μ m i.d. capillary with a 2 μ m filtered union (UpChurch Scientific). The desalting column was washed with buffer containing 95% water, 5% acetonitrile, and 0.1% formic acid. After desalting, a 100 μ m i.d. capillary with a 5 μ m pulled tip packed with 10 cm 3- μ m Aqua C18 material (Phenomenex) followed by 3 cm of 5 μ m Partisphere strong cation exchanger (Whatman) was attached to the filter union and the entire split-column (desalting column–filter union–analytical column) was placed inline with an Agilent 1100 quaternary HPLC and analyzed using a modified six-step separation described previously (Washburn et al. 2001). The buffer solutions used were 5% acetonitrile/0.1% formic acid (buffer A), 80% acetonitrile/0.1% formic acid (buffer B), and 500 mM ammonium acetate/5% acetonitrile/0.1% formic acid (buffer C). Step 1 consisted of a 90-min gradient from 0%–100% buffer B. Steps 2–5 had the following profile: 3 min of 100% buffer A, 2 min of X% buffer C, a 10-min gradient from 0%–15% buffer B, and a 97-min gradient from 15%–45% buffer B. The 2-min buffer C percentages (X) were 20%, 40%, 60%, and 80%, respectively, for the six-step analysis. For the final step, the elution consisted of: 3 min of 100% buffer A, 20 min of 100% buffer C, a 10-min gradient from 0%–15% buffer B, and a 107-min gradient from 15%–70% buffer B.

As peptides eluted from the microcapillary column, they were electrosprayed directly into an LTQ two-dimensional ion trap mass spectrometer (ThermoFinnigan) with the application of

a distal 2.4-kV spray voltage. A cycle of one full-scan mass spectrum (400–1400 m/z) followed by eight data-dependent MS/MS spectra at a 35% normalized collision energy was repeated continuously throughout each step of the multidimensional separation. Application of mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcalibur data system.

MS/MS spectra were analyzed using the following software analysis protocol. Poor quality spectra were removed from the data set using an automated spectral quality assessment algorithm (Bern et al. 2004). MS/MS spectra remaining after filtering were searched with the SEQUEST algorithm (Eng et al. 1994) against the SGD *S. cerevisiae* (December 16, 2005) protein database (created on December 17, 2006) concatenated to a decoy database in which the sequence for each entry in the original database was reversed (Peng et al. 2003). All searches were parallelized and performed on a Beowulf computer cluster consisting of 100 1.2 GHz Athlon CPUs (Sadygov et al. 2002). No enzyme specificity was considered for any search. SEQUEST results were assembled and filtered using the DTASelect (version 2.0) program (Tabb et al. 2002; Cociorva et al. 2006). DTASelect 2.0 uses a linear discriminant analysis to dynamically set XCorr and DeltaCN thresholds for the entire data set to achieve a user-specified false-positive rate. The false-positive rates are estimated by the program from the number and quality of spectral matches to the decoy database.

Parameters for inclusion in the data presented in Figure 2 include: sequence confirmation of >5% of the protein and absence from the mock purification MS/MS data. Mock purification was performed identically to that of the experimental samples from nontagged strain of yeast. Polypeptides identified in this mock analysis as well as contaminants identified in the *prp2*, *prp16*, and *prp22* particles are presented in Supplemental Table S1.

SUPPLEMENTAL MATERIAL

Supplemental material can be found at <http://www.rnajournal.org>.

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