The DExD/H-box ATPase Prp2p destabilizes and proofreads the catalytic RNA core of the spliceosome

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ABSTRACT

After undergoing massive RNA and protein rearrangements during assembly, the spliceosome undergoes a final, more subtle, ATP-dependent rearrangement that is essential for catalysis. This rearrangement requires the DEAH-box protein Prp2p, an RNA-dependent ATPase. Prp2p has been implicated in destabilizing interactions between the spliceosome and the protein complexes SF3 and RES, but a role for Prp2p in destabilizing RNA–RNA interactions has not been explored. Using directed molecular genetics in budding yeast, we have found that a cold-sensitive *prp2* mutation is suppressed not only by mutations in SF3 and RES components but also by a range of mutations that disrupt the spliceosomal catalytic core element U2/U6 helix I, which is implicated in juxtaposing the 5' splice site and branch site and in positioning metal ions for catalysis within the context of a putative catalytic triplex; indeed, mutations in this putative catalytic triplex also suppressed a *prp2* mutation. Remarkably, we also found that *prp2* mutations rescue lethal mutations in U2/U6 helix I. These data provide evidence that RNA elements that comprise the catalytic core are already formed at the Prp2p stage and that Prp2p destabilizes these elements, directly or indirectly, both to proofread spliceosome activation and to promote reconfiguration of the spliceosome to a fully competent, catalytic conformation.

Keywords: DExD/H-box ATPase; Prp2; U2/U6 helix I; spliceosome activation; spliceosome catalytic core

INTRODUCTION

Most eukaryotic genes are interrupted by introns, which must be excised from pre-mRNA for proper gene expression (Wahl et al. 2009). The chemistry of this pre-mRNA splicing reaction is a relatively simple sequence of two transesterification reactions, first between an adenosine in the branch point sequence and the 5' splice site (5' splice site cleavage) and then between the 5' splice site and the 3' splice site (exon ligation). These reactions are catalyzed by the spliceosome, an RNA–protein assembly of 80 conserved proteins and five small nuclear RNAs (snRNAs): U1, U2, U4, U5, and U6 (Wahl et al. 2009). The spliceosomal snRNAs recognize the consensus sequences of the intron at the 5' splice site and the branch site, and subsequently during spliceosome activation, the snRNAs form key structures that compose the catalytic core of the spliceosome. These structures include the U2/U6 helix I (Madhani and Guthrie 1992; Sun and Manley 1995; Mefford and Staley 2009), composed of U2/U6 helix Ia, which juxtaposes the reactants for the first step of splicing, and U2/U6 helix Ib (Madhani and Guthrie 1994b; Hilliker and Staley 2004; Mefford and Staley 2009),

which, together with residues in the U6 intramolecular stem-loop (ISL), has been shown to position catalytic metal ions, similar to the catalytic domain V of the self-splicing group II intron (Fabrizio and Abelson 1992; Yu et al. 1995; Yean et al. 2000; Toor et al. 2008; Koodathingal et al. 2010; Fica et al. 2013). Before the catalytic activation of the spliceosome, these key structures are precluded by mutually exclusive base-pairing interactions between U6 and U4 snRNAs (Brow and Guthrie 1988; Madhani and Guthrie 1992). Unwinding of the U4/U6 duplex during spliceosome activation provides the potential to form these catalytic components, but it is not yet known when these catalytic features form.

Spliceosome assembly and activation occur *de novo* on each pre-mRNA and follow an elaborate pathway involving dramatic RNA and protein rearrangements (Wahl et al. 2009). Numerous steps in this pathway require ATP hydrolysis by DExD/H-box ATPases; in several cases, these factors have been shown additionally to promote the fidelity of splicing through kinetic proofreading mechanisms (Semlow and

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Staley 2012). DExD/H-box ATPases make up a large protein family of about 50 members that participate in nearly all aspects of RNA metabolism throughout the cell, and they are found in all kingdoms of life (Putnam and Jankowsky 2013). The range of fundamental, biochemical activities of DExD/H-box ATPases includes the unwinding of RNA duplexes, the disruption of RNA–protein complexes, and the assembly of RNA–protein complexes (Linder and Jankowsky 2011). Indeed, several spliceosomal DExD/H-box ATPases have been implicated in unwinding specific RNA duplexes or to disrupt specific RNA–protein interactions (Schwer and Guthrie 1992; Fleckner et al. 1997; Kim and Rossi 1999; Staley and Guthrie 1999; Perriman et al. 2003; Hilliker et al. 2007; Perriman and Ares 2007, 2010; Schwer 2008; Mefford and Staley 2009). However, the biological roles of many DExD/H-box ATPases remain poorly understood.

The DEAH-box ATPase *Prp2p* functions at a late stage of spliceosome activation (Kim and Lin 1993, 1996). Indeed, *Prp2p* functions in the final ATP-dependent step necessary for 5' splice site cleavage (Fig. 1A). In this final step, *Prp2p* promotes conversion of the spliceosome from a pre-catalytic conformation into a catalytic conformation. Like most other spliceosomal DExD/H-box ATPases, the association of *Prp2p* with the spliceosome is transient (King and Beggs 1990). When *Prp2p* activity is required during spliceosome activation, *Prp2p* is recruited to the spliceosome through its interaction with the G-patch protein *Spp2* and then, after ATP hydrolysis by *Prp2p*, both factors dissociate (Roy et al. 1995; Kim and Lin 1996; Silverman et al. 2004; Fabrizio et al. 2009). Like many other DExD/H-box ATPases, the ATPase activity of *Prp2p* is stimulated by RNA binding (Kim et al. 1992). *Prp2p* cross-links to pre-mRNA downstream from the branch site, and this region of the substrate is required during spliceosome activation, so it has been proposed that *Prp2p* could destabilize interactions involving the pre-mRNA near the branch site (Teigelkamp et al. 1994; Liu and Cheng 2012). Indeed, recent studies have begun to elucidate the hallmarks of the role of *Prp2p* in activation as characterized by protein dynamics at the branch site. Proteomics studies have implicated *Prp2p* in destabilizing the spliceosomal association of the U2 snRNP complex SF3, the RES complex, and the NTC-related proteins *Cwc24p* and *Cwc27p*, although not necessarily in dissociation of these factors (Bessonov et al. 2008; Fabrizio et al. 2009; Warkocki et al. 2009; Lardelli et al. 2010; Agafonov et al. 2011; Ohrt et al. 2012), with the destabilization of SF3 requiring *Cwc22p* (Yeh et al. 2010). *Prp2p* activity results in high-affinity binding sites for *Cwc25p* and *Yju2p*, two factors required specifically for 5' splice site cleavage (Liu et al. 2007b; Chiu et al. 2009; Ohrt et al. 2012), and low-affinity binding sites for the second-step factors *Prp16p*, *Slu7p*, and *Prp18p*—factors required for docking of the 3' splice site to the active site (Ohrt et al. 2013). *Cwc25p*, like the SF3b subcomplex, binds the pre-mRNA at or near the branch site (Gozani et al. 1996, 1998; Query et al. 1996; Will et al. 2001; McPheeters

FIGURE 1. Mutations in motif VI of *PRP2* lead to cold-sensitive growth and splicing. (A) Diagram of the linear pathway of spliceosome assembly, spliceosome activation, and the first step in catalysis. After *Prp2p* promotes an RNA rearrangement in the catalytic core, *Cwc25p* binds the spliceosome, promoting 5' splice site cleavage. (B) Mutations in *PRP2* lead to cold-sensitive growth. Serial dilutions of wild-type (WT) *PRP2*, *prp2-Q548E*, and *prp2-Q548N* were grown on rich media at the permissive temperature (30°C, 2 d) or the restrictive temperature (15°C, 13 d). (C) The mutant *prp2-Q548N* compromises splicing. Strains carrying the indicated *PRP2* variant were grown at 30°C or shifted to 15°C and then harvested for whole-cell RNA, which was analyzed for splicing of pre-U3 snoRNA by primer extension. The quantitation below shows the splicing efficiency calculated as M/P , where M is the mature U3 snoRNA and P is the sum of pre-U3A and pre-U3B. The values were normalized to the splicing efficiency in *PRP2* at 30°C. Each bar shows the mean of three replicates, and the error bars show the range of replicates. U14 snoRNA was probed as an internal control. Growth and splicing phenotypes were tested in parental strain yJPS1393, after transformation and shuffle with *PRP2* variants and wild-type U2 and U6.

and Muhlenkamp 2003; Chiu et al. 2009; Tseng et al. 2010; Liu and Cheng 2012). Thus, it has been proposed that *Prp2p* destabilizes SF3 from the pre-mRNA so that *Cwc25p* can bind and promote catalysis (Lardelli et al. 2010; Yeh et al. 2010), perhaps by positioning the branch-site adenosine for attack at the 5' splice site. While our understanding of *Prp2p*-mediated protein dynamics has advanced, little is known about RNA dynamics at this final ATP-dependent stage of spliceosome assembly and activation.

To investigate the role of *Prp2p* in promoting RNA dynamics, we took a genetic approach used previously to successfully identify functional targets of other DExD/H-box ATPases (Madhani and Guthrie 1994a; Staley and Guthrie 1999; Kistler and Guthrie 2001; Perriman et al. 2003; Schneider et al. 2004; Hilliker et al. 2007; Perriman and Ares 2007; Xu and Query 2007; Mefford and Staley 2009). We generated a cold-sensitive allele of *PRP2* and screened for mutations in snRNAs, in addition to proteins, that suppressed the cold-sensitive growth defect. We found that the *prp2* mutation

was suppressed not only by mutations in the SF3a-, RES-, and NTC-related complexes, but also in U2/U6 helix I and in U6 residues implicated in a putative catalytic triplex (Toor et al. 2008), providing the first evidence that Prp2p destabilizes snRNA structure, directly or indirectly. Conversely, we found that the cold-sensitive allele of *prp2* suppressed dominant-negative and lethal phenotypes of mutations in U2/U6 helix Ia, implicating a role for Prp2p in the fidelity of spliceosome activation. Our data provide evidence that the snRNAs assume a conformation with catalytic features at the Prp2p stage and that this DExD/H-box ATPase promotes activation by proofreading and destabilizing the catalytic core of the spliceosome.

RESULTS

Mutations in motif VI of Prp2p compromise growth and splicing in the cold

In our molecular genetic strategy to investigate the role of Prp2p in spliceosome activation, we reasoned that alleles of *PRP2* that were cold-sensitive would fail to destabilize the pre-catalytic conformation and thereby prevent the spliceosome from rearranging into the catalytic conformation. Furthermore, mutations in proteins or snRNAs that suppressed a cold-sensitive *PRP2* allele would reveal the configuration and dynamics of the spliceosome at this stage. Of 18 alleles that we screened on the basis of cold sensitivity in other spliceosomal DEAH-box ATPases, we found two alleles, *prp2-Q548E* and *prp2-Q548N*, that were cold-sensitive (Fig. 1B). The *prp2-Q548N* mutation compromised splicing of an endogenous transcript, U3 snoRNA, as revealed by primer extension analysis (Fig. 1C). Because the mutations change a residue within the DExD/H-box motif VI, they likely compromise the catalytic functions of Prp2p in ATP binding and/or hydrolysis (Roy et al. 1995; Silverman et al. 2004; Linder and Jankowsky 2011).

PRP2 mutations are suppressed by mutations in the SF3 and RES complexes

If a role of Prp2p is to destabilize interactions between the spliceosome and the SF3 and RES complexes as well as the NTC-related proteins Cwc24p and Cwc27p (Warkocki et al. 2009; Lardelli et al. 2010; Ohrt et al. 2012), then mutations in these factors that weaken these interactions should suppress the *prp2* mutants. Thus, to test for a role in destabilizing the SF3 complex, we asked whether the cold-sensitive phenotypes of *prp2* mutants could be suppressed by the *prp9-1* mutation, a temperature-sensitive allele of the gene encoding the SF3a component Prp9p (Legrain and Choulika 1990). This allele disrupts a salt bridge within Prp9p (Lin and Xu 2012) and compromises binding of the U2 snRNP to pre-mRNA (Arenas and Abelson 1993; Ruby et al. 1993). Significantly, the cold-sensitive *prp2* mutants were both suppressed by the

FIGURE 2. Prp2p destabilizes SF3, RES, and NTC-related proteins in vivo. (A) The *prp9-1* mutation (Legrain and Choulika 1990) suppresses the cold-sensitive defect of *prp2-Q548N*. The double deletion strain *prp2Δprp9Δ* carrying plasmids encoding *PRP9* or *prp9-1* and *PRP2*, *prp2-Q548E*, or *prp2-Q548N* was spotted onto rich media and grown for 2 d at 37°C or for 14 d at 15°C. Images were from the same plate but cropped and aligned for clarity. (B) Deletion of *PML1*, encoding an RES complex component, and deletion of the NTC-related gene *CWC27* suppress the *prp2-Q548N* mutant. Strains deleted for *PRP2* and the indicated genes and carrying a plasmid encoding *PRP2* or *prp2-Q548N* were spotted onto rich media and grown for 6 d at 15°C. The components of the RES complex are indicated. The contrast for the entire image was adjusted for clarity. (C) A mutation in the FHA motif of *PML1* expected to compromise phosphothreonine recognition (Brooks et al. 2009; Trowitzsch et al. 2009) suppresses *prp2-Q548N*. The wild-type *PML1* or mutant *pml1Δ* strains carrying a plasmid encoding *PRP2* or *prp2-Q548N* and a plasmid encoding *PML1*, *pml1-R108A*, *pml1-S137A*, or an empty parent plasmid (pRS313) were grown for 10 d at 15°C on media lacking histidine to maintain the pRS313 plasmids. All spots were grown on the same plate, but cropped and grouped together for clarity.

prp9-1 mutation (Fig. 2A). This result provides evidence in vivo that Prp2p destabilizes the SF3 complex, weakening interactions between the U2 snRNP and the pre-mRNA.

To test for a role for Prp2p in rearranging the RES complex (Dziembowski et al. 2004), composed of Pml1p, Bud13p, and Snu17p, or in rearranging the NTC-related protein Cwc27p, we asked whether the cold-sensitive phenotype of a *prp2* mutant could be suppressed by deletion of any of the encoding genes, as compared with deletion of control genes, *PRP17* and *ISY1*; Prp17p and Isy1p bind the spliceosome before the Prp2p-dependent step (Fabrizio et al. 2009; Lardelli et al. 2010), but Prp17p functions afterward (Jones et al. 1995), and the *ISY1* deletion suppresses a *prp16* mutation that compromises an ATP-dependent step in splicing that follows Prp2p action (Villa and Guthrie 2005). We found that the *prp2-Q548N* mutant was suppressed specifically by deletion of *PML1* or *CWC27* (Fig. 2B). Interestingly, Pml1p has an FHA domain; such domains can bind phosphothreonine

peptides (Brooks et al. 2009; Trowitzsch et al. 2009), and in the crystal structure of Pml1p, the highly conserved residue R108, in the putative phosphopeptide-binding core, binds sulfate, strongly implicating this residue in binding phosphate as in other FHA family members (Brooks et al. 2009; Trowitzsch et al. 2009). Strikingly, mutation of this residue, but not another, was sufficient to suppress the *prp2* mutant; whereas wild-type *PML1* complemented the deletion of *PML1* and abolished suppression of *prp2*, *pml1-R108A* did not (Fig. 2C); an equivalent mutation in the FHA domain of Rad53 decreased binding of phosphothreonine peptides 10-fold (Durocher et al. 1999). Thus, the suppression of *prp2* by *pml1-R108A* suggests a role for recognition of a phosphothreonine at this stage of splicing (cf. Brooks et al. 2009) and destabilization of this interaction by Prp2p. Overall, these data provide evidence in vivo that Prp2p destabilizes the SF3 and RES complexes and Cwc27p.

Prp2p destabilizes U2/U6 helix Ia

To test whether Prp2p also rearranges RNA, we asked whether mutations in snRNAs suppressed *prp2-Q548N*. We screened mutations in residues involved in several structures, including U2/U6 helix I (Madhani and Guthrie 1992; Sun and Manley 1995; Mefford and Staley 2009), the extended U6 ISL (Sun and Manley 1995; Sashital et al. 2004), U2 stem I (Wu and Manley 1992), U2 stem II (Ares and Igel 1990; Zavanelli et al. 1994), and the U2 branch point stem-loop (BSL) (Perriman and Ares 2010). With only one exception (see below), we did not observe suppression of *prp2-Q548N* by any mutations in the U6 ISL, U2 stem I, U2 stem II, or the U2 BSL. In contrast, we observed broad and dramatic suppression in residues that participate in U2/U6 helix I. The *prp2-Q548N* mutant was suppressed by mutations in seven of the eight nucleotides (nt) of helix Ia (Fig. 3A–E), the structure implicated in juxtaposing the branch site and 5' splice site; mutations at the remaining residue, U2-G26, are lethal in a wild-type strain. Furthermore, a compensatory analysis of suppressors that would disrupt the U2/U6-C29/G55 base pair revealed that double mutations that restored Watson–Crick base-pairing specifically abolished suppression, providing evidence that this base pair is formed before Prp2p acts and is destabilized when Prp2p acts (Fig. 3E; Supplemental Fig. S1A). For example, the point mutations U2-C29A and U6-G55U each suppressed *prp2-Q548N*, but the compensatory mutation U2/U6-C29A/G55U abolished suppression; in contrast, noncompensatory mutations, such as U2/U6-C29A/G55A, did not abolish suppression. While a simple compensatory analysis of the U2/U6 base pair U28/A56 was not sufficient to reveal evidence for base-pairing at this position (Supplemental Fig. S1B), we found serendipitously that in the background of a third aphentotypic mutation (U2-Ψ44C), a compensatory analysis did provide evidence for base-pairing at this position (Fig. 3F). At the U2/U6 base pair A27/U57, the suppression of *prp2* by U6-U57A was

FIGURE 3. Prp2p destabilizes U2/U6 helix Ia. (A) Secondary structural model of U2/U6 helix I showing residues in helix Ia that were tested as boxed letters and residues that suppress *prp2-Q548N* when mutated as shaded boxes. (B–F) Mutations that disrupt U2/U6 helix Ia suppress *prp2-Q548N*. (B–D) Point mutations in U2/U6 base pairs G26/C58 (B), U28/A56 (C), and A27/U57 (D) illustrate the suppression of *prp2-Q548N* by mutations that disrupt helix Ia. (E, F) Compensatory analyses at the U2/U6 base pairs C29/G55 (E) and U28/A56 (F) show that suppression is specifically abolished by restoration of base-pairing. In B–F, *PRP2* or *prp2-Q548N* cells containing the indicated U2 and U6 alleles were grown for 10–16 d at 15°C. For B, C, E, and F, cells were grown on rich media. For D, in which U2 dominant phenotypes were assayed, cells were grown on selective media, after transformation with U2 plasmids, to retain both the wild-type and mutant U2 plasmids. Phenotypes were tested in parental strain yJPS1393. (*) This U2 variant included the mutation U2-Ψ44C, which shows no phenotype alone, yielding the double U2 mutation U2-U28C/Ψ44C.

also specifically compromised by a compensatory mutation (Supplemental Fig. S1C; Supplemental Note 1). While some *prp2* mutant suppressors in U2/U6 helix Ia were not abolished by compensatory mutations, as for some *prp16* mutant suppressors in U2/U6 helix Ia (Mefford and Staley 2009), such negative results may reflect, for example, a requirement for the purine–pyrimidine polarity of the base pair; constraints imposed by additional, tertiary interactions; or limitations of the approach resulting from disruption of alternative interactions that function at other stages. Taken together, these data provide evidence that helix Ia forms before Prp2p acts, and the data suggest further that U2/U6 helix Ia is destabilized by Prp2p.

Prp2p destabilizes U2/U6 helix Ib

Similar to mutations in helix Ia, *prp2-Q548N* was suppressed by several of the 11 aphentotypic point mutations in helix Ib, a

FIGURE 4. Prp2 destabilizes U2/U6 helix Ib. (A) Secondary structural model of U2/U6 helix I showing residues in helix Ib that were tested as boxed letters and residues that suppress *prp2-Q548N* when mutated as shaded boxes. (B,C) Mutations in U2/U6 helix Ib residues suppress *prp2-Q548N*. PRP2 (bottom) or *prp2-Q548N* (top) cells containing the indicated U2 and U6 alleles were grown on rich media. (B) Compensatory analysis of U2/U6 base pair U23/A59. Cells were grown at 16°C for 5 d (PRP2) or 10 d (*prp2-Q548N*). (C) Compensatory analysis of U2/U6 base pair C22/G60. Cells were grown at 16°C for 5 d (PRP2) and 10 d (*prp2-Q548N*). (D) Compensatory analysis of U2/U6 base pair G21/G61. Cells were grown for 6 d at 20°C to assay for exacerbation of the *prp2-Q548N* mutant. Phenotypes were tested in parental strain yJPS1393. (*) Secondary mutations at U2-U12 preserve bulge in U2 stem I. (**) Compensatory mutations at U2-G13 preserve Watson-Crick base-pairing in U2 stem I.

component of the structure that acts at the catalytic stage and positions catalytic metals (Hilliker and Staley 2004; Mefford and Staley 2009; Fica et al. 2013). Specifically, *prp2-Q548N* was suppressed by the three U2 mutations, C22U, U23G, and U23A (Fig. 4A–C). Suppression of *prp2-Q548N* by two of these mutations, U2-C22U and U2-U23G, was abolished by the U6 compensatory mutations that restored helix Ib (Fig. 4B,C), but not by compensatory mutations in the competing structure U2 stem I or the extended U6 ISL, and point mutations specific to these alternative base-pairing partners did not suppress *prp2-Q548N* on their own, with only one exception (Supplemental Fig. S2). These data provide evidence that helix Ib forms before Prp2p acts and suggest further that Prp2p destabilizes helix Ib.

In contrast with helix Ia mutations, we also found that six point mutations in U2/U6 helix Ib exacerbated the *prp2-Q548N* mutant. For example, in the helix Ib base pair U2/U6-G21/C61, the base substitutions U6-C61U and U6-C61A and all three base substitutions of U2-G21 exacerbated

prp2-Q548N or resulted in synthetic lethality with *prp2-Q548N* (Fig. 4D). Although in the one testable case, restoring base-pairing in helix Ib with a compensatory mutation did not rescue growth, compensatory mutations in alternative structures, including U2 stem I or the extended U6 intramolecular stem-loop (ISL), also failed to improve growth, and point mutations specific to these alternative base-pairing partners did not exacerbate *prp2-Q548N* on their own, with the exception of only one residue (Supplemental Fig. S2). Taken together, our observations are consistent with the exacerbation of *prp2* deriving from destabilization of helix Ib and suggest that Prp2p promotes a conformation particularly sensitive to base-pair identity in helix Ib. Indeed, repair of helix Ib with compensatory U6 mutations that restored base-pairing with U2-C22U or U23G not only abolished suppression of *prp2-Q548N* by these U2 mutations but also exacerbated the cold-sensitive phenotype of the *prp2* mutant (Fig. 4B,C). Overall, these data provide evidence that Prp2p destabilizes helix Ib and does so to promote a conformation of helix Ib within a new context that promotes catalysis (see Discussion).

Prp2p destabilizes putative base triple interactions

Because our data suggested that the catalytic core components U2/U6 helix Ia and helix Ib are both formed before Prp2p acts, we hypothesized that additional features of the catalytic core have also formed by this stage—in particular, a putative catalytic triplex that would result from base triple interactions between U2/U6 helix Ib and residues in the highly conserved U6 ACAGAGA and the U6 ISL (Fig. 5A). This putative triplex is predicted from a crystal structure of a self-splicing group II intron, in which the triplex plays a key role in positioning catalytic divalent metal ions (Fig. 5B; Toor et al. 2008). To test our hypothesis, we asked whether *prp2-Q548N* could be suppressed by mutations in the U6 residues predicted to interact with U2/U6 helix Ib: A53, G52, and U80. We also asked whether the *prp2-Q548N* mutant could be suppressed by mutations in U6-U54, since U54 could potentially extend the triplex through an interaction with the U2/U6 helix Ia pair G26/C58, as suggested by covariation among different species (Madhani and Guthrie 1992).

Consistent with base triple interactions already being formed at the Prp2p stage, *prp2-Q548N* was strongly suppressed by mutations in three of the four putative U6 base triple partners: the mutations U6-A53U, U6-A53G, U6-U80C, and all three mutations at U6-U54 (Fig. 5A,C,E,F; cf. 5D). In total, six of nine testable mutations suppressed *prp2-Q548N*. Consistent with the formation of an extended triplex, the suppression by mutations in U6-U54 was not abolished by either compensatory mutations in U2 that would restore a potential lengthening of U2/U6 helix Ia (Supplemental Fig. S3A,B) or by compensatory mutations in the U6 central stem (Supplemental Fig. S3C,D). Furthermore, at the putative triple partner, U2/U6 base pair G26/C58, two allele

FIGURE 5. Prp2p destabilizes putative base triple interactions. (A) Diagram of predicted base triple interactions in the catalytic core of the spliceosome. (Solid red arrows) Base triple interactions predicted by the group II intron structure (Toor et al. 2008); (dashed red arrow) a base triple implicated by covariation (Madhani and Guthrie 1992). (Boxed residues) Bases that were tested for genetic interactions with *prp2-Q548N*; (shaded boxes) positions or base pairs that showed suppression. Within the putative triplex, helix Ib residues involved in Watson–Crick base-pairing between U2 (green) and U6 (blue) are shown, as are U6 residues of the ACAGAGA sequence and U80 (orange) proposed to interact with helix Ib. (B) Base triple interactions of the catalytic triplex observed in the crystal structure of the *Oceanobacillus iheyensis* group II intron (Toor et al. 2008). The color coding of the equivalent triplex residues is the same as in panel A. The two putative catalytic metals are shown as spheres. (C–F) Mutations in the third base of three predicted base triples suppress *prp2-Q548N*. Cells were grown on rich media at 15°C for 5 d (*PRP2*) or 12 d (*prp2-Q548N*). (G) At the U2/U6 base pair G26/C58, non-wild-type, Watson–Crick base-pair combinations suppress *prp2-Q548N*. Cells were grown on rich media for 10 d at 15°C. (Black boxes) Allele combinations that were not included in the analysis because they have previously been reported as lethal (Mefford and Staley 2009). In each panel, all strains were grown on the same plate; images were cropped and grouped together for clarity. Phenotypes were tested in parental strain yJPS1393.

combinations that maintained base-pairing but altered base-pair identity also suppressed *prp2-Q548N* (Fig. 5G). These data are not only consistent with the presence of the putative base triple interactions at the Prp2p stage, but also suggest that Prp2p destabilizes tertiary RNA interactions, in addition to base-pairing and protein–RNA interactions (Warkocki et al. 2009; Lardelli et al. 2010). Still, proof that the putative triples form will require further genetic and biochemical investigations.

Prp2p proofreads catalytic core formation

Unexpectedly, we not only observed that snRNA mutations suppressed *prp2-Q548N* but also conversely that a *prp2* mutation suppressed select snRNA mutations—that *prp2* and snRNA mutations mutually suppressed one another. Specifically, we discovered that *prp2-Q548N* rescued the dominant-negative phenotype of two mutations, U2-A27U and -A27C (Fig. 3D). While this rescue could have formally indicated that the *prp2* mutation somehow prevented incorporation of U2 harboring these mutations into the spliceosome, thereby favoring incorporation of wild-type U2, the same U2 mutations suppressed the *prp2* mutation, arguing against this possibility and for a role for the *prp2* mutation in suppression by enabling the dominant-negative U2 mutations to function within the spliceosome. Confirming this view, *prp2-Q548N* also suppressed the lethal phenotypes of two U2 mutations in helix Ia: A27U and A27G (Fig. 6). These surprising observations indicate that the dominant-negative and lethal phenotypes of these mutations depend on the wild-type activity of Prp2p. That is, wild-type Prp2p activity is required to ensure that suboptimal spliceosomes compromised by these U2/U6 helix Ia mutations do not function. While a final test of the model will require biochemical tests, these data implicate Prp2p for the first time as a spliceosomal DExD/H-box fidelity factor (Semlow and Staley 2012) and one that proofreads formation of the catalytic core.

DISCUSSION

The role of Prp2p in spliceosome activation is essential to pre-mRNA splicing (Kim and Lin 1993, 1996). Several studies have implicated Prp2p in destabilizing protein complexes (Bessonov et al. 2008; Fabrizio et al. 2009; Warkocki et al. 2009; Lardelli et al. 2010; Ohrt et al. 2012), but to date, there has been little indication that Prp2p destabilizes RNA, despite its membership in the DExD/H-box family of ATPases. Furthermore, while features of the spliceosome's catalytic RNA core have been defined, such as U2/U6 helix I (Madhani and Guthrie 1992; Sun and Manley 1995; Hilliker

FIGURE 6. Prp2p proofreads the catalytic core. The *prp2-Q548N* mutant rescues the lethality of U2-A27U and U2-A27G. To assay recessive U2 phenotypes, the indicated strains carrying a copy of both wild-type U2 (on a *URA3*-marked plasmid) and the indicated U2 variant were streaked onto 5-FOA to counterselect against cells containing the wild-type U2 plasmid. Cells were grown for 4 d at 30°C. Phenotypes were tested in parental strain yJPS1393.

and Staley 2004; Mefford and Staley 2009), it has remained unclear when these features form during spliceosome activation. Here, using a cold-sensitive *PRP2* mutation as a tool to study the activation stage, we have provided evidence that key features of the catalytic core, including U2/U6 helix I and putative, catalytic base triples, are already formed *in vivo* at the Prp2p stage (Figs. 3–5; Supplemental Fig. S1). Since these features are not yet competent to promote catalysis without the role of Prp2p in the final ATP-dependent step of spliceosome activation, we infer that formation of U2/U6 helix I and the putative base triples is not sufficient to catalyze splicing. Indeed, providing the first evidence indicating that Prp2p destabilizes RNA structure, our data imply that Prp2p destabilizes the catalytic RNA core, directly or indirectly (Figs. 3–5). Strikingly, a *prp2* mutation rescues lethal helix Ia mutations, establishing evidence that Prp2p not only destabilizes the catalytic core but also proofreads this essential RNA element (Fig. 6).

A nascent catalytic core is formed before the final stages of spliceosome activation

U2/U6 helix Ia is thought to juxtapose the branch site and the 5' splice site (Madhani and Guthrie 1992; Sun and Manley 1995; Mefford and Staley 2009). Consistent with recent chemical probing data (Anokhina et al. 2013), our data indicate that U2/U6 helix Ia is already formed before Prp2p promotes the final stages of spliceosome activation (Fig. 3). Consistent with this conclusion, recent single-molecule FRET studies indicate that the branch site and 5' splice site move closer to one another at or near the Prp2p stage (Crawford et al. 2013; Krishnan et al. 2013). Since helix Ia is already formed at the Prp2p stage and the spliceosome is not yet competent to undergo the chemistry of splicing, our data imply that helix Ia-mediated juxtaposition of the 5' splice site and branch site is not sufficient to promote the first step of splicing. Other features of the catalytic core must be limiting for the complete activation of the spliceosome.

We also provide evidence that U2/U6 helix Ib is already formed before the final stages of activation (Fig. 4), and our data are consistent with formation of putative base triple interactions (Toor et al. 2008) by the Prp2p stage (Fig. 5), although further studies will be required to unequivocally establish formation of the triples. Residues that would engage in the triples include the AGC triad of helix Ib and the bulged U80 of the U6 ISL. These residues have recently been shown to coordinate metal ions that mediate the catalysis of both steps of pre-mRNA splicing (Fica et al. 2013). Since our data imply that helix Ib, and potentially the catalytic triplex, is already formed at the Prp2p stage, binding of metals by helix Ib may not be limiting at the final step of spliceosome activation, similar to helix Ia-mediated substrate juxtaposition. Thus, our findings support a view in which the final rearrangements in spliceosome activation involve fine-tuning of substrate conformation.

Differences in snRNA structure formed at the Prp2p stage and the catalytic stage

Mutations in U2/U6 helix I suppress not only a cold-sensitive mutation in *PRP2* but also a cold-sensitive mutation in *PRP16* (Madhani and Guthrie 1992, 1994a; Mefford and Staley 2009), a DExD/H-box ATPase that is required just after 5' splice site cleavage (Schwer and Guthrie 1992). This parallel establishes that helix I is formed and destabilized at both of these stages. Still, despite this parallel, more subtle differences in the genetic interactions between snRNA mutations and *prp2* and *prp16* mutants point to conformational differences between these stages that result from Prp2p action. First, two mutations in helix Ia, U6-U57A and U6-U57C, have opposite effects on the phenotype of a *prp16* mutation, leading to the interpretation that U57A is relatively destabilizing to the 5' splice site conformation while U57C is stabilizing; further genetics indicate that these mutations act inversely at the second step of splicing: U57A is relatively stabilizing for the exon ligation conformation, while U57C is relatively destabilizing (McPheeters 1996; Query and Konarska 2004; Konarska et al. 2006; Liu et al. 2007a; Mefford and Staley 2009; Query and Konarska 2012). In contrast, these U6-U57 alleles do not show this dichotomy of genetic interactions with *prp2*; *prp2-Q548N* is modestly suppressed by U6-U57A, but it is not exacerbated by U6-U57C (Fig. 3D). Second, while some mutations in the U2/U6 helix Ib pair C21-G61 suppress *prp16* (Mefford and Staley 2009), no mutations in this pair suppress *prp2*—indeed, all exacerbate (Fig. 4D). Third, while only one mutation in U6-U54 suppresses *prp16*, all base substitutions in U6-U54 suppress *prp2* (cf. Fig. 5D and Mefford and Staley 2009), perhaps by destabilizing a putative base triple between U6-U54 and U2/U6-G26/C58, suggested by co-variation (Madhani and Guthrie 1992). In this case, the genetic differences may reflect that the possible base triple is formed at the Prp2p stage but not at the Prp16p stage. Finally, while deletion of the gene encoding the NTC protein Isy1p suppresses the cold-sensitive defect of *prp16-302* (Villa and Guthrie 2005), deletion of this gene does not suppress *prp2-Q548N* (Fig. 2B). Together, these genetic differences indicate that unique features of the catalytically active core are not yet formed at the Prp2p stage and suggest a role for Prp2p in consolidating interactions around the catalytic core during the final stage of spliceosome activation.

The role for Prp2p in destabilizing snRNA structure in the catalytic core

Complementing the already established role that Prp2p plays in destabilizing proteins (Bessonov et al. 2008; Fabrizio et al. 2009; Warkocki et al. 2009; Lardelli et al. 2010; Ohrt et al. 2012), our investigation has provided evidence that Prp2p destabilizes U2/U6 helix I, in addition to putative base triples (Figs. 3–5). For several other DExD/H-box ATPases, it is clear from molecular genetic studies that destabilization of a

structure equates with disruption of the structure because the ATPases drive the formation of alternative, mutually exclusive structures (Fleckner et al. 1997; Staley and Guthrie 1999; Perriman et al. 2003; Hilliker et al. 2007; Perriman and Ares 2007, 2010). If by destabilizing U2/U6 helix I, Prp2p fully disrupts the catalytic core, directly or indirectly, then the catalytic core would need to be re-stabilized for catalysis. Establishing precedence for such a toggling rearrangement, Prp16p destabilizes the catalytic core transiently: After Prp16p destabilizes the 5' splice site cleavage conformation, promoting formation of an intermediate state, the spliceosome re-stabilizes the catalytic core for exon ligation (Mefford and Staley 2009). This transient disruption of the catalytic core is thought to allow repositioning of the reactants for exon ligation (Schwer and Guthrie 1992). Prp2p could similarly destabilize the catalytic core to allow positioning of the substrate for 5' splice site cleavage, a function that could couple with the role for Prp2p in destabilizing SF3 to exposes the branch site adenosine for attack (Warkocki et al. 2009; Lardelli et al. 2010), especially since helix I is immediately adjacent to the U2/branch site helix (Fig. 3A). Together, genetic investigations of helix I at the Prp2p and catalytic stages suggest the intriguing notion that the catalytic core is inherently stable, forming in ATP-independent steps, and that DExD/H-box ATPases function in part to counteract this stabilizing force to enable rearrangements of the substrate.

In addition to destabilizing SF3 to promote the binding and stabilization of Cwc25p and Yju2p (Liu et al. 2007b; Chiu et al. 2009; Ohrt et al. 2012), Prp2p may function, through reconfiguration of the nascent catalytic core, to enable binding of proteins to the catalytic core to induce a catalytically active configuration. Recent biochemical experiments indicate that Cwc2p, a protein with an RNA recognition motif (RRM), protects helix I from modification before Prp2p acts (McGrail et al. 2009; Rasche et al. 2012; Schmitzová et al. 2012). Although this protection could be indirect, destabilization of helix I may consequently remodel interactions between Cwc2p and the catalytic core. Indeed, Cwc2p cross-links to the U6 ISL, and this cross-linking changes as a consequence of Prp2p function in both yeast and mammals (Rasche et al. 2012). In addition to Cwc2p, Prp8p is thought to function as a scaffold for the catalytic RNA core (Galej et al. 2013), as implicated by cross-linking (Turner et al. 2006), genetics (Query and Konarska 2004), and crystallography (Pena et al. 2008; Ritchie et al. 2008; Yang et al. 2008; Galej et al. 2013). Thus, by destabilizing U2/U6 helix I, Prp2p may also promote this scaffolding function of these proteins.

The role of Prp2p in destabilizing proteins

Our results show that *prp9-1*, a temperature-sensitive mutation in the SF3a protein Prp9p (Legrain and Choulika 1990), suppresses *prp2* (Fig. 2A), consistent with the proteomic result that SF3 is destabilized by Prp2p (Warkocki et al. 2009; Lardelli et al. 2010). The human SF3 components

SAP155p (also known as SF3b1p or SF3b155p), SAP145p (also known as SF3b2p, Cus1p, or SF3b145p), and p14 cross-link near the branch site (Gozani et al. 1996, 1998; Query et al. 1996; Will et al. 2001), as does the yeast SF3b155p homolog Hsh155p (McPheeters and Muhlenkamp 2003; Liu and Cheng 2012). Thus, it has been hypothesized that destabilization of SF3 by Prp2p could remove steric constraints and allow Cwc25p binding to enable positioning of the branch site for 5' splice site cleavage (Chiu et al. 2009; Warkocki et al. 2009; Lardelli et al. 2010; Tseng et al. 2010; Yeh et al. 2010). Since Prp2p cross-links to the pre-mRNA just downstream from the branch site and this region is required for Prp2p function (Teigelkamp et al. 1994; Liu and Cheng 2012), Prp2p has been proposed to destabilize directly interactions between the pre-mRNA and SF3 near the branch site of the pre-mRNA (Warkocki et al. 2009; Lardelli et al. 2010). The suppression of *prp2* by *prp9-1* (Fig. 2A) is consistent with Prp2p-mediated repositioning of the branch site, because the *prp9-1* mutation destabilizes the interaction between the branch site and the U2 snRNP (Ruby et al. 1993). Furthermore, these phenotypes of *prp9-1* together indicate that alternative interactions must substitute for SF3 in stabilizing the U2/branch site interaction, which is required at the catalytic stage of splicing (Smith et al. 2009).

Interestingly, our data also implicate a role for the RES complex in phosphothreonine recognition at, and likely before, the Prp2p stage. Specifically, a *prp2* mutant is robustly suppressed by a point mutation predicted from homology (Durocher et al. 1999) and crystallography (Brooks et al. 2009; Trowitzsch et al. 2009) to directly disrupt phosphothreonine recognition by the FHA domain of Pml1p (Fig. 2C). This observation implies that phosphothreonine recognition by Pml1p stabilizes an interaction that Prp2p destabilizes. A number of splicing factors are phosphorylated, and two are phosphorylated in a dynamic manner (Wang et al. 1998; Agafonov et al. 2011). However, few phosphorylations are understood in terms of their importance or their recognition. Several SF3 factors are phosphorylated (Wang et al. 1998; Agafonov et al. 2011), suggesting the possibility that Pml1p binds to one of these phosphorylated residues to stabilize SF3 binding to the spliceosome. Future experiments will be required to identify the putative target of Pml1p.

A role for Prp2p in proofreading the catalytic core

It has been predicted that all spliceosomal DExD/H-box ATPases act as proofreading factors, in addition to driving the spliceosome forward along the splicing pathway (Burgess and Guthrie 1993b). However, it is not yet known if this is the case. Extending support for this hypothesis, we show here that Prp2p functions as a fidelity factor, in addition to five of the other seven DExD/H-box ATPases (Burgess and Guthrie 1993a; Mayas et al. 2006, 2010; Xu and Query 2007; Koodathingal et al. 2010; Perriman and Ares 2010; Yang et al. 2013). A role for Prp2p in fidelity was revealed through the

striking observation that *prp2-Q548N* rescues the dominant-negative or lethal phenotypes of mutations in U2-A27 (Figs. 3D, 6). Mutation of U2-A27 could in principle compromise growth by destabilizing any of various structures, including U2 stem I, the U2 BSL, or U2/U6 helix Ia, but given our evidence that U2/U6 helix Ia is formed before Prp2p acts and that Prp2p destabilizes helix Ia, we infer that the defect of U2-A27 mutations that is suppressed by *prp2* results from disruption of base-pairing in U2/U6 helix Ia. While the mechanism of this proofreading could be indirect, such as by premature destabilization of SF3, the function of this proofreading must be tied, minimally, to the role of U2-A27. Thus, our data provide the first evidence that a DExD/H-box ATPase proofreads formation of an snRNA/snRNA interaction within the spliceosome. In a wild-type spliceosome, we imagine that formation of the catalytic core is not a straightforward RNA folding problem and that folding “errors” occur that must be corrected in a manner parallel and mechanistically equivalent to the correction of substrate-binding “errors” (see below). Given the proximity of helix Ia to the U6/5′ splice site interaction and the U2/branch site interaction, a concomitant role for Prp2p in proofreading these interactions is an intriguing possibility. While preliminary tests have not revealed such a role, further experiments are required to test this possibility rigorously.

The prevailing model for how spliceosomal DExD/H-box ATPases act as fidelity factors (for review, see Semlow and Staley 2012) specifies that DExD/H-box ATPases can compete with a productive step in splicing, such as U2 snRNP binding (Xu and Query 2007; Perriman and Ares 2010), 5′ splice site cleavage (Burgess and Guthrie 1993a; Koodathingal et al. 2010), or exon ligation (Mayas et al. 2006), and thereby establish a rejection branch. By this kinetic proofreading model, Prp2p can compete (Fig. 7, step i), through premature destabilization of U2/U6 helix I and SF3, with a currently undefined step (see below) in the productive pathway of spliceosome activation (ii). If the spliceosome is suboptimal, for example, by not fully forming helix Ia or interactions with helix Ia, then Prp2p would act before the productive step (ii) to reject the suboptimal spliceosome (i); if the spliceosome is optimal, for example, by fully forming helix Ia, the spliceosomes would proceed along the productive pathway (ii), and Prp2p would act after the productive step (ii) to activate the spliceosome (iii).

In kinetic proofreading, the sorting of suboptimal and optimal spliceosomes between rejection and productive pathways can be achieved by one of two nonmutually exclusive mechanisms (Semlow and Staley 2012). First, these ATPases could generally enforce a time limit on the productive step in splicing. In support of this possibility, Prp16p has been shown to preferentially reject spliceosomes that catalyze 5′ splice site cleavage slowly (Koodathingal et al. 2010; Koodathingal and Staley 2013). Similarly, Prp2p could act as a proofreading timer by limiting the amount of time available for the productive step (ii). If the productive step (ii) does not occur

FIGURE 7. Model of the branched pathway for the proofreading activity of Prp2p. In this kinetic proofreading model (Semlow and Staley 2012), Prp2p-dependent rejection (i) competes with a yet undefined transition along the productive pathway of activation (ii) to antagonize activation of a suboptimally configured spliceosome. Prp2p-dependent rejection would yield an intermediate that could either undergo discard by Prp43p (v) (Pandit et al. 2006; Koodathingal et al. 2010; Mayas et al. 2010; Chen et al. 2012) or re-engage the productive pathway (vi). In contrast, an optimally configured spliceosome would proceed preferentially through the undefined stage in activation (ii), after which Prp2p would promote spliceosome activation (iii). The prevailing model for activation posits that Cwc25p replaces binding of SF3 near the branch site (iv) (Yeh et al. 2010; Ohrt et al. 2012). Our data indicate that, in addition, Prp2p promotes destabilization of snRNA structure in the catalytic core (iii), pictured by the loss of both U2/U6 helix I base-pairing and putative base triples. In the model, Prp2p drives these protein and RNA dynamics in both rejecting suboptimal spliceosomes and in promoting optimal spliceosomes. Thus, the consequence of Prp2p activity, productive or antagonistic, depends on its timing of action. (Thick arrows) The canonical activation pathway; (gray arrows in the secondary structure diagrams) putative base triple interactions.

in a given window of time, because U2-A27U or helix Ia mispairing, for example, delays the productive step (ii), then Prp2p would reject the spliceosome (i). One attractive candidate for the productive step is Yju2 binding, since Prp2p can function before or after Yju2 binds to the spliceosome (Liu et al. 2007b), just as Prp16p, for example, can function before or after 5′ splice site cleavage (Koodathingal et al. 2010; Tseng et al. 2010). Another attractive candidate is Cwc2p association with the catalytic core, given that Cwc2p protects helix I before Prp2p acts (Rasche et al. 2012); if, for example, a mutation or mispairing in helix I delays Cwc2p binding, Prp2p may act prematurely, rejecting the configuration.

In the second possible mechanism for sorting, the proofreading DExD/H-box ATPase could sense the authenticity of a substrate. In this case, the suboptimal U2-A27U catalytic core or suboptimal helix Ia pairing, for example, would stimulate the rate of Prp2p-mediated rejection (i), which would proceed faster than the productive step (ii), perhaps simply

because the catalytic core is less stable. Indeed, we expect that the U2-A27U mutation would destabilize U2/U6 helix I by disrupting the base pair with U6-U57, and we know that U2-A27U suppresses the *prp2-Q423N* mutation (Fig. 3D), consistent with the U2 mutation accelerating or facilitating Prp2p function.

Evidence suggests that Prp16p- and Prp22p-mediated rejection is reversible, enabling re-sampling of the substrate for optimal sites (Mayas et al. 2006; Koodathingal et al. 2010). Similarly, Prp2p-mediated rejection of the catalytic core may enable further attempts at assembly of a stable and/or productive core (vi) and thereby function akin to an RNP chaperone. Although Prp16p- and Prp22p-mediated rejection may be reversible, rejection can also lead to irreversible discard by the DExD/H-box ATPase Prp43p, which results in release of the substrate and disassembly of the spliceosome (Pandit et al. 2006; Koodathingal et al. 2010; Mayas et al. 2010). Interestingly, Prp43p can bind to the spliceosome not only after Prp16p and Prp22p function but also after Prp2p functions (Chen et al. 2012). Thus, Prp2p-mediated rejection (i) may similarly lead to discard by Prp43p (v). Regardless, our data provide evidence that Prp2p destabilizes U2/U6 helix I not only to advance the spliceosome along the productive pathway but also to proofread formation of the catalytic core.

MATERIALS AND METHODS

Yeast strains and plasmids

For the screen for cold-sensitive alleles of *PRP2*, for primer extension assays, and for growth assays testing U2 and U6 mutations, we used the shuffle strain yJPS1393, which was generated from the previously described strain yHM118 (Madhani and Guthrie 1994b) by transforming it with the plasmid pPRP2U2U6-URA3 (pJPS2503) and then deleting *PRP2* with KanMx4. The plasmid pJPS2503 was generated by cloning a *PRP2*-containing SmaI/SacI fragment into the SmaI site of the previously described pU2U6-URA3 (Madhani and Guthrie 1994b). Variants of the plasmid pJPS2500 (pPRP2-pASZ11) were used to screen for cold-sensitive alleles of *prp2*. pJPS2500 was generated by cloning the *PRP2*-containing BamHI fragment from pRS415-*PRP2* (Edwalds-Gilbert et al. 2000) into the BamHI site of pASZ11 (Stotz and Linder 1990). Variants were generated by QuikChange site-directed mutagenesis (Agilent) and confirmed by sequencing.

For growth assays testing mutations in Prp9p, RES factors, and NTC-related factors, isogenic strains were made from the previously described *prp2Δ* strain YTY1 (Edwalds-Gilbert et al. 2000) by deleting with KanMx4 the gene for either *PRP9* (yJPS1416 and yJPS1417), *PML1* (yJPS1421), *BUD13* (yJPS1418), *SNU17* (yJPS1419), *CWC27* (yJPS1422), *PRP17* (yJPS1423), or *ISY1* (yJPS1420). yJPS1416 contains *PRP9*-pRS313 (pJPS2504), while yJPS1417 contains *prp9-1*-pRS313 (pJPS2505); to generate pJPS2504 and pJPS2505, *PRP9*- or *prp9-1*-containing BamHI fragments were amplified, respectively, from the previously described strains SS330 and SRY9-1c (Ruby et al. 1993) and cloned into the BamHI site of pRS313 (Sikorski and Hieter 1989). A *PML1*-containing EcoRI fragment was amplified from ge-

nomic DNA from the strain BY4741 (Open Biosystems) and cloned into the EcoRI site of pRS313 to generate pJPS2530. The *PML1* variants *pml1-R108A* (pJPS2532) and *pml1-S137A* (pJPS2533) were generated by QuikChange mutagenesis and confirmed by sequencing. Variants of *PRP2*-pRS415 (Edwalds-Gilbert et al. 2000) that were used in these strains were generated by QuikChange mutagenesis and confirmed by sequencing. See the Supplemental Data for a complete list of yeast strains (Supplemental Table S1) and plasmids (Supplemental Table S2) used in this study.

Growth assays

To screen for interactions between *prp2-Q548N* and snRNA mutations, variants of pJPS2500 (*PRP2*-pASZ11), pJPS216 (Hilliker and Staley 2004), and pSX6 (Madhani and Guthrie 1992) were cotransformed into yJPS1393. Transformants were streaked in triplicate onto 5-FOA (5-fluoroorotic acid)-containing media and grown for 3–5 d at 30°C to counterselect against strains with the pU2U6PRP2-URA3 plasmid (Boeke et al. 1987). Colonies were streaked onto YPDA (yeast peptone dextrose adenine) media and grown for 3 d at 30°C. The purified colonies were grown in liquid YPDA to an OD₆₀₀ of ~0.8 and then spotted onto solid YPDA media. Cells were grown at the permissive temperature of 30°C for 2–3 d and the restrictive temperature of 15°C for 10–16 d for phenotypic analysis. Yeast cells were transformed by a standard lithium acetate method (Ito et al. 1983).

To screen for interactions between *prp2-Q548N* and *prp9-1*, *pml1Δ*, *bud13Δ*, *snu17Δ*, *cwc27Δ*, *prp17Δ*, and *isy1Δ*, the appropriate double deletion strain or YTY1 was transformed with variants of *PRP2*-pRS415. Transformants were streaked onto 5-FOA, colony-purified on YPDA, and spotted onto solid media as above. To test for interactions with *prp2-Q548N* and putative phosphate binding mutations in *PML1*, pJPS2530 variants were cotransformed with *PRP2*-pRS415, *prp2-Q548N*-pRS415, or *prp2-Q548E*-pRS415; plasmid shuffle and phenotypic analysis were performed on solid media lacking histidine to maintain the *PML1*-pRS313 plasmids.

The mutual suppression of *prp2-Q548N* and U2-A27U and -A27C, which was observed in a pseudo-diploid also expressing wild-type U2, and the rescue of lethality of U2-A27U and -A27G by *prp2-Q548N*, in a true haploid, were revealed using strains derived from yJPS1393 that carried either *PRP2*-pASZ11 or *prp2-Q548N*-pASZ11 and pU2U6-URA3 (Madhani and Guthrie 1994a). These strains were transformed with pJPS216 variants and pSX6 variants and selected on media lacking histidine, tryptophan, and uracil for the pseudo-diploid experiment and on media lacking histidine and tryptophan for the haploid experiment. For the haploid experiment, transformants were colony-purified on media lacking histidine and tryptophan to give them the opportunity to lose the pU2U6PRP2-URA3 plasmid and then streaked onto media containing 5-FOA and grown for 4 d at 30°C.

Primer extension analysis

Cells were grown to log phase at 30°C, whole-cell RNA was harvested from half the cell culture for the permissive temperature samples, and RNA was harvested from the remaining half after a temperature shift for 4 h to 15°C. Whole-cell RNA was isolated by hot phenol extraction, and primer extensions were performed as described (Stevens et al. 2002) using ³²P-end-labeled primers complementary

to the 3' exons of *SNR17A* and *SNR17B*, which encode U3 snoRNA (5'-CCAAGTTGGATTCACTGGCTC-3') (Dobbyn and O'Keefe 2004), and to *SNR128*, which encodes U14 snoRNA (5'-ACGATGGGTTTCGTAAGCGTACTCCTACCGTGG-3') (Hilliker and Staley 2004). Products were separated on a 6% denaturing polyacrylamide gel, developed by PhosphorImager, and quantitated by Image J (NIH).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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