

# Functional interactions between Prp8, Prp18, Slu7, and U5 snRNA during the second step of pre-mRNA splicing

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## ABSTRACT

After the second transesterification step of pre-mRNA splicing, the Prp22 helicase catalyzes release of spliced mRNA by disrupting contacts in the spliceosome that likely involve Prp8. Mutations at Arg1753 in Prp8, which suppress helicase-defective *prp22* mutants, elicit temperature-sensitive growth phenotypes, indicating that interactions in the spliceosome involving Prp8-R1753 might be broken prematurely at 37°C. Here we report that mutations in loop 1 of the U5 snRNA or in Prp18 can suppress the temperature-sensitive *prp8-R1753* mutants. The same gain-of-function *PRP18* alleles can also alleviate the growth phenotypes of multiple *slu7-ts* mutants, indicating a functional link between Prp8 and the second step splicing factors Prp18 and Slu7. These findings, together with the demonstration that changes at Arg1753 in Prp8 impair step 2 of pre-mRNA splicing *in vitro*, are consistent with a model in which (1) Arg1753 plays a role in stabilizing U5/exon interactions prior to exon joining and (2) these contacts persist until they are broken by the helicase Prp22.

**Keywords:** Prp8; Prp18; Prp22 helicase; mRNA release; mRNA splicing

## INTRODUCTION

The spliceosome, an assembly of snRNAs (U1, U2, U4/U6, and U5) and proteins, catalyzes the excision of introns from pre-mRNAs in two successive transesterification reactions (Burge et al. 1999). The first step entails attack of the branch site 2'-OH on the 5' splice site (5'ss) with formation of a branched lariat intermediate. The second step is the attack of the 3'-OH of the 5' exon on the 3' splice site (3'ss) to join the exons and expel the lariat-intron. For the second transesterification reaction to occur, the 3'ss phosphodiester must be identified and positioned for attack by the 3' hydroxyl of exon 1.

Step 2 depends on integral spliceosome constituents such as U5 snRNA and Prp8 and nonspliceosomal proteins Prp16, Slu7, Prp18, and Prp22 (Umen and Guthrie 1995b; Grainger and Beggs 2005). ATP hydrolysis by the DEAH-box enzyme Prp16 promotes a conformational change in the spliceosome that leads to protection of the 3'ss from targeted RNase H cleavage (Schwer and Guthrie

1992). This change, which likely reflects binding of the 3'ss PyAG↓ in the catalytic center of the spliceosome, requires the ordered recruitment of Slu7, Prp18, and Prp22 to the spliceosome (James et al. 2002).

Interactions between the U5 snRNA and exon bases immediately adjacent to the splice sites assist in aligning the exons for step 2 catalysis (Newman 1997; Crotti et al. 2007). Whereas the nucleotides in the U5 snRNA "loop 1" that interact with exon sequences are phylogenetically invariant (Frank et al. 1994), the involved exon bases are not conserved in pre-mRNAs. Cross-linking data implicate the Prp8 protein, a component of the U5 snRNP, in stabilizing the inherently weak U5/exon interactions (Teigelkamp et al. 1995).

The Prp18 protein also plays a role in fortifying U5/exon contacts prior to exon joining (Baciková and Horowitz 2005; Crotti et al. 2007). Structural analysis of a functional Prp18 fragment revealed five tightly packed  $\alpha$  helices and an unstructured 36-amino acid loop between  $\alpha$  helices 4 and 5 (Jiang et al. 2000). Included in the 36-amino acid segment are 19 amino acids, the so-called conserved region (CR), which are deleted in the temperature-sensitive *prp18 $\Delta$ CR* mutant (Baciková and Horowitz 2002). The idea that Prp18, via its CR, stabilizes U5/exon interactions was suggested by the finding that mutations in the U5 snRNA loop 1 suppress the growth phenotypes and the

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second step splicing defects elicited by *prp18ΔCR* mutants (Bacíková and Horowitz 2005). Furthermore, the second step of splicing with Prp18ΔCR, but not wild-type Prp18, is sensitive to mutations in exon bases adjacent to the splice sites that interact with loop 1 of U5 (Crotti et al. 2007).

The DEAH-box helicase Prp22 enters the spliceosome prior to the second transesterification step, after which it catalyzes the release of mRNA from the spliceosome (Schwer and Gross 1998). The ATPase and helicase activities of Prp22 are required for product release. ATPase-deficient Prp22 mutants are lethal, and Prp22 mutants that retain ATPase activity, yet fail to unwind RNA duplexes in vitro, are lethal or elicit severe cold-sensitive growth defects (Schwer and Meszaros 2000; Campodonico and Schwer 2002; Schneider et al. 2002, 2004). Mutations at Arg1753 in the 2413-amino acid Prp8 protein suppress the cold-sensitive growth phenotypes of helicase-defective Prp22 mutants, suggesting that wild-type Prp22 effects mRNA release by disrupting contacts in the spliceosome that involve Arg1753 of Prp8. Whether Prp22 breaks a direct connection between Prp8 and the mRNA or whether Prp22 breaks U5/exon interactions that might be stabilized by Prp8 is not known. In *PRP22* wild-type cells, the *prp8-R1753* mutant alleles elicit temperature-sensitive growth defects, suggesting that contacts involving Arg1753 are not formed properly or are broken prematurely at 37°C.

Here we tested whether the contacts with spliced mRNA that are broken by the Prp22 helicase (1) involve the U5 snRNA in addition to the U5 snRNP component Prp8, and (2) are established prior to exon joining. We show that U5 snRNAs carrying specific mutations in loop 1 suppress the temperature-sensitive growth defects of *prp8-R1753* mutants and exacerbate the growth defects of *prp22-cs* cells. The temperature sensitivity of *prp8-R1753* mutants and of multiple *slu7-ts* alleles was also relieved by gain-of-function *PRP18* alleles *V191A* and *S162P*, indicating a close functional relationship between Prp8, Prp18, and Slu7. In vitro studies show that Prp8-R1753 mutants slow the second step of splicing certain pre-mRNAs containing mutations in the exons adjacent to the splice sites. Our findings are consistent with a model whereby Arg1753 of Prp8 collaborates with Prp18 to stabilize U5/exon contacts that are important for the second transesterification step, and that the Prp22 helicase disrupts interactions between the U5 snRNP and mRNA to elicit mRNA release.

## RESULTS

### Mutations in U5 snRNA loop 1 suppress the growth phenotype of Prp8-R1753 mutants

If Prp8-R1753 mutants are temperature sensitive because they fail to stabilize contacts of the U5 snRNP with exon sequences, then mutations in the U5 snRNA that increase pairing might rescue the growth defects. Because exon

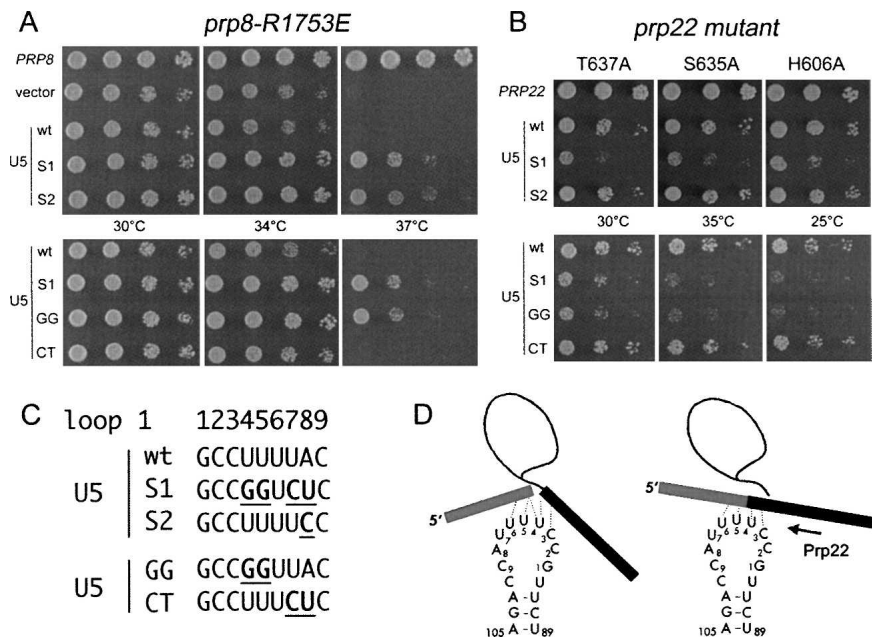
sequences are not conserved, Prp8-R1753 mutants likely influence splicing of a subset of precursor RNAs. To identify U5 alleles that rescue the splicing defects of affected RNAs, we screened for dominant suppressors of the temperature-sensitive phenotype of *prp8-R1753E* cells. We introduced into *prp8-R1753E* cells that contained a wild-type U5 gene (*SNR7*) a plasmid library of U5 mutants in which the nucleotides of loop 1 were randomized (Newman and Norman 1991). Of approximately 15,000 transformants analyzed, seven grew at 37°C, the nonpermissive temperature for *prp8-R1753E* cells. Plasmids were recovered from these strains and the U5 genes analyzed (Fig. 1C). U5-S1, which was found in six isolates, carried four mutations in loop 1 (U4G, U5G, U7C, A8U). The U5-S2 allele, which was isolated once, contained a single A8C change. Plasmids bearing *PRP8*, wild-type U5, U5-S1, and U5-S2 were reintroduced into *prp8-R1753E SNR7* cells and growth of the resulting strains was compared (Fig. 1A). Whereas *PRP8* restored growth to *prp8-R1753E* cells at 37°C, an extra copy of the gene for wild-type U5 snRNA did not. However, the U5-S alleles supported growth at nonpermissive temperature. U5-S1 and U5-S2 also suppressed the temperature sensitivity of *prp8-R1753K* and *prp8-R1753A* cells (not shown).

To determine which of the four base changes in U5-S1 were responsible for suppression, we generated two U5 double mutants, U5-GG (U4G U5G) and U5-CT (U7C A8U), and analyzed their effects on growth of *prp8-R1753E* cells. Whereas U5-CT had no beneficial effect, *prp8-R1753E* cells harboring U5-GG grew as well as cells carrying the U5-S1 allele (Fig. 1A).

Although the sequence or length of loop 1 does not appear to affect the assembly of the U5 snRNP particle in vitro (O'Keefe et al. 1996; O'Keefe and Newman 1998; Ségault et al. 1999; McGrail et al. 2006), the function of the U5 snRNP might be disrupted by mutations in the loop sequence (Frank et al. 1992; O'Keefe 2002). We therefore tested whether U5-S1, U5-S2, U5-GG, and U5-CT supported viability of a *U5Δ* strain. U5-S2 and U5-CT supported normal growth of *U5Δ* cells at all temperatures (not shown; Bacíková and Horowitz 2005). U5-GG cells grew poorly, forming only pinpoint colonies after more than 6 d (not shown). U5-S1 failed to complement a *U5Δ* strain at 18°C, 30°C, or 37°C (not shown). The finding that U5-S1 was lethal yet suppressed the growth defect of *prp8-R1753* mutants argues that (1) the altered U5 snRNA functions specifically with the mutated Prp8 to splice certain precursor RNAs, and (2) wild-type U5 snRNA is required for splicing of other essential RNAs.

### U5-S1 (U4G, U5G, U7C, A8U) exacerbates the growth defects of helicase-defective Prp22 mutants

Bases C3 to U6 of the U5 loop are thought to interact with bases in the exons adjacent to the splice sites (Newman



**FIGURE 1.** Genetic interactions between Prp8, Prp22, and the U5 snRNA. (A) Fivefold serial dilutions of *prp8-R1753E* cells bearing plasmids (*URA3 CEN*) for expression of wild-type Prp8, wild-type U5 snRNA, and the mutants U5-S1, U5-S2, U5-GG, and U5-CT were spotted to minimal agar medium lacking uracil. The plates were photographed after 3 d of incubation at 30°C, 34°C, and 37°C. (B) *prp22-T637A*, *prp22-S635A*, and *prp22-H606A* were transformed with plasmids expressing wild-type Prp22 and U5 snRNAs as indicated at the left. Serial dilutions (10-fold) were spotted to agar medium and photographed after 3 d of incubation at 30°C and 35°C, and 4 d at 25°C. (C) Alignment of the wild-type U5 loop 1 sequence with those of the suppressors U5-S1 and U5-S2. Mutated nucleotides in the suppressor U5's are in bold and underlined. The loop 1 sequences in U5-GG and U5-CT are U4G, U5G and U7C, A8U, respectively. (D) Model for interactions between the U5 loop and exon sequences prior to step 2 (left) and after exon joining (right). The dashed lines indicate putative base-pairing interactions between the U5 loop 1 and exon sequences (Newman 1997). Exons 1 and 2 are drawn as gray and black rectangles, respectively; the intron is indicated by a line. Loop 1 nucleotides, which are numbered 1–9 according to (Newman and Norman 1991), correspond to nucleotides 93–101 in the U5 snRNA (Frank et al. 1994).

1997; Crotti et al. 2007). The multiple mutations in U5-S1 (specifically U4G, U5G) might enhance the base-pairing of U5 with exon sequences in one or more pre-mRNAs. If Prp22 normally disrupts those contacts to release the mRNA, then U5-S1 and U5-GG might exacerbate the phenotype of helicase-defective *prp22-cs* mutants. To test this, *prp22-T637A*, *prp22-S635A*, and *prp22-H606A* cells (containing wild-type alleles for Prp8 and U5) were transformed with centromeric plasmids carrying either *PRP22*, wild-type U5, or the various U5 mutants and then plated at various temperatures (not shown; Fig. 1B). *PRP22* restored growth to each of the *prp22-cs* mutants. Whereas an extra copy of wild-type U5, U5-S2, or U5-CT did not affect growth of the *prp22-cs* mutants at any temperature, *T637A*, *S635A*, and *H606A* cells bearing U5-S1 or U5-GG grew more slowly at 30°C, 35°C and 25°C, their respective semipermissive temperatures (Fig. 1B).

The findings that the *snr7* alleles U5-S1 and U5-GG suppressed the temperature-sensitive growth phenotype of *prp8-R1753* mutants and enhanced cold sensitivity of heli-

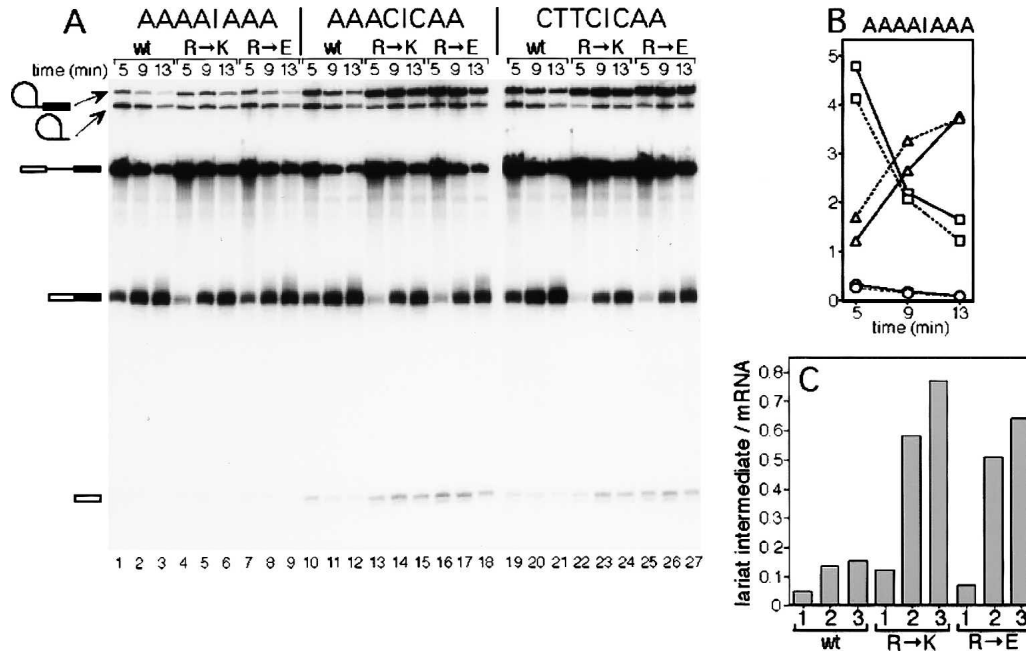
case-defective *prp22* mutants are consistent with a model wherein the Prp22 helicase disrupts interactions between the U5 snRNP and mRNA (Fig. 1D).

### Prp8-R1753 mutants are defective for the second step of splicing in vitro

To determine whether Prp8-R1753 mutants were defective for pre-mRNA splicing in vitro, we prepared extracts from isogenic *PRP8*, *prp8-R1753K*, and *prp8-R1753E* cells and assayed their splicing activities in vitro. *ACT1* pre-mRNA was spliced with comparable efficiency in all three extracts at 23°C and 32°C, and preincubation at 37°C did not result in selective inhibition of mRNA formation in Prp8-R1753K and Prp8-R1753E extracts (not shown). Thus, splicing of *ACT1* pre-mRNA in vitro appears to be insensitive to mutations at Arg1753 of Prp8 that cause temperature-sensitive growth defects in vivo. Although we cannot rule out the possibility that Prp8-R1753 mutant proteins cannot be heat inactivated in vitro, it seems more likely that the function of Arg1753 in Prp8 might not be rate limiting for splicing of *ACT1*.

If Prp8-R1753 stabilizes the interaction of U5 with the exons, then altering the exon sequences so that their interaction with U5 is weakened might affect

splicing in *prp8-R1753* mutant extracts. Modified *ACT1* precursors with mutations at exon sequences that affect the second step of *prp18ΔCR* splicing have been identified (Crotti et al. 2007). For example, AAAA|AAA (the last four bases of exon 1 and the first three bases of exon 2 are separated by "|") was spliced better in *prp18ΔCR* extract than *ACT1* (TCTG|AGG), while the mutants AAAC|CAA and CTTC|CAA were spliced worse (Crotti et al. 2007). We assayed splicing of these three mutant pre-mRNAs in *PRP8*, *prp8-R1753K*, and *prp8-R1753E* extracts at 32°C (Fig. 2). The AAAA|AAA pre-mRNA was spliced with similar rates and efficiencies in extracts from wild-type or Prp8 mutant cells (Fig. 2B). However, splicing of CTTC|CAA and AAAC|CAA pre-mRNAs was impaired, insofar as splicing intermediates accumulated to higher levels, especially in mutant extracts. The relative amounts of intermediates (expressed as the ratio of intermediates/mRNA) from the CTTC|CAA and AAAC|CAA pre-mRNAs were increased four- to sixfold compared to AAAA|AAA pre-mRNA at 5, 9, and 13 min (Fig. 2A,C). Splicing in wild-type extracts



**FIGURE 2.** (A) Splicing of three exon-mutant substrates in vitro using extracts from wild-type *PRP8* or mutant *prp8* cells. *ACT1* substrate with the exon mutations shown at the top of each panel was spliced in vitro in wild-type *PRP8* extract (wt), in *prp8-R1753K* extract (R→K), or in *prp8-R1753E* extract (R→E), as indicated above each panel. Aliquots were withdrawn at the times indicated along the top of each gel and analyzed by denaturing PAGE. Autoradiographs of the gels are shown. The positions of the pre-mRNA, splicing intermediates, and mRNA are depicted at the left. (B) Relative molar amounts of RNA species derived from the wild-type (lanes 1–3) and *prp8-R1753E* (lanes 7–9) time courses are graphed for the AAAA|AAA pre-mRNA. The pre-mRNA is represented by squares, lariat intermediate by circles, and mRNA by triangles; the dashed lines represent wild-type extracts and the solid lines *prp8-R1753E* extracts. (C) Graphical representation of the relative amounts of the intermediates/mRNA for the 9 min time points (wt extract: lanes 2,11,20; for R→K: lanes 5,14,23; for R→E: lanes 8,17,26). The number beneath each bar indicates the pre-mRNA: 1: AAAA|AAA; 2: AAAC|CAA; and 3: CTTC|CAA.

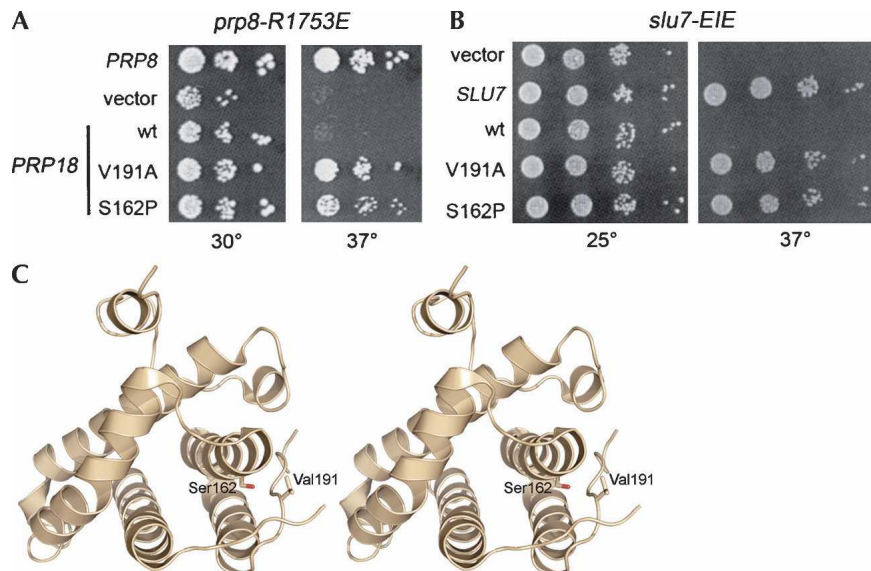
was less sensitive to mutations in the exons. Because different extracts were used, we cannot assess whether mutations at Arg1753 have an effect on the first step of splicing. The observed second step defects likely reflect the failure of Prp8-R1753 mutants to stabilize U5/exon contacts required to configure the active site for step 2 catalysis.

### Mutations in *PRP18* suppress the temperature sensitivity of *prp8-R1753* mutants

Two lines of evidence suggested that Prp8 and Prp18 have closely related functions: (1) *prp8-R1753E* and *prp18ΔCR* mutations were suppressed by mutations in the gene for U5 (Fig. 1; Bacíková and Horowitz 2005) and (2) the second step of splicing of pre-mRNAs with exon mutations was impaired in extracts with mutant Prp8-R1753 proteins or with Prp18ΔCR protein (Fig. 2; Crotti et al. 2007). If Prp18 and Prp8 cooperate in stabilizing U5/exon interactions, then mutations in Prp18 might suppress the phenotype of *prp8-R1753E* mutants at 37°C. To identify gain-of-function mutations in *PRP18*, a Prp18-mutant library was generated by error-prone PCR and the plasmid library (*URA3 CEN*) was transformed into *prp8-R1753E PRP18* cells. Of approx-

imately 3000 *Ura*<sup>+</sup> cells that were plated, 4 grew at 37°C. The plasmids were recovered from the suppressor strains and the *PRP18* genes were sequenced. One of the dominant *PRP18* suppressor alleles carried a single mutation, resulting in a Val-191-Ala change. The other three suppressors contained a Ser-162-Pro change and were judged to be independent isolates insofar as they bore other unique changes (Materials and Methods). The single V191A or S162P changes in Prp18 were sufficient to restore growth to *prp8-R1753E PRP18* cells at 37°C (Fig. 3A). V191A and S162P also suppressed the temperature sensitivity of *prp8-R1753K* and *prp8-R1753A* cells (not shown). The *PRP18-V191A* and *PRP18-S162P* alleles were fully functional in lieu of wild-type *PRP18*, insofar as they restored wild-type growth to *prp18Δ* cells (not shown).

The close functional relationship between Prp8 and Prp18 was underscored by the finding that *prp18* alleles with mutations in three functionally important regions (Bacíková and Horowitz 2002), including *prp18ΔCR*, were synthetic lethal with *prp8-R1753* mutants (not shown). Taken together, the genetic data support the idea that Prp8 (through Arg1753) and Prp18 (via its CR) cooperate to stabilize contacts between the U5 snRNA and exon sequences during the second step of splicing (Fig. 1D).



**FIGURE 3.** Gain-of-function mutations in Prp18. (A) Serial 10-fold dilutions of *prp8-R1753E* cells bearing plasmids that carry the *PRP8* gene or various *PRP18* alleles as indicated at the left were plated to agar medium lacking uracil. Cells containing the empty plasmid (vector) were analyzed in parallel. The plates were photographed after 3 d of incubation at 30°C and 37°C. (B) A photograph of *slu7-EIE* cells containing the indicated expression plasmids after 4 and 2 d of incubation at 25°C and 37°C, respectively, is shown. (C) Stereo representation of Prp18 (80–251) shown as a ribbon diagram (Jiang et al. 2000). Ser162 and Val191 are shown as sticks.

### Prp18-V191A and Prp18-S162P suppress the temperature sensitivity of *slu7* mutants

Prp18 interacts with Slu7, and together they recruit Prp22 to the spliceosome (Zhang and Schwer 1997; James et al. 2002). To explore the functional connection between Slu7 and Prp18 further, we screened the Prp18 mutant library for suppressors of the temperature-sensitive growth defect of *slu7-EIE*, an alanine cluster (E215A–I216A–E217A) mutant of Slu7. The EIE mutations abolished Slu7 binding to Prp18 in vitro and in a two-hybrid assay in vivo (James et al. 2002). We reasoned that Prp18 mutants selected by the screen might restore interaction with Slu7-EIE. The Prp18-mutant library was introduced into *slu7-EIE PRP18* cells, and candidate suppressors were selected at 37°C. Plasmids carrying the dominant *PRP18* alleles were isolated (seven from a total of approximately 5000 transformants) and sequenced. Five isolates contained a Ser-162-Pro change, and two carried Val-191-Ala mutations, the same *prp18* mutations that were isolated as suppressors of *prp8-R1753E*. The single amino acid changes S162P or V191A sufficed for suppression (Fig. 3B).

Mutations at Ser162 and Val191 in Prp18 did not restore interaction with Slu7-EIE in a 2-hybrid assay (not shown), arguing that suppression occurs independent of the physical interaction between Slu7 and Prp18. Suppression was not allele specific insofar as S162P and V191A suppressed the temperature sensitivity of eight *slu7* mutants containing lesions throughout the polypeptide (Table 1), and of two

previously described *slu7*(125–382) and *slu7*(163–382) alleles (not shown; Zhang and Schwer 1997). Yet, Prp18-V191A and Prp18-S162P did not bypass the requirement for Slu7 in vivo or in vitro (not shown).

We surmise that V191A and S162P suppress *slu7* mutants by improving the functionality of Prp18. Consistent with this idea, we found that introducing a Val-191-Ala change into mutant Prp18s (R151E-R152E, R151A-R152A, H118E-R151A) that were defective for Slu7 interaction partially suppressed the temperature sensitivity of *prp18-R151E-R152E*, *prp18-R151A-R152A*, and *prp18-H118E-R151A* cells (Baciková and Horowitz 2002), without restoring Slu7 interaction (not shown).

## DISCUSSION

The temperature-sensitive *PRP8* allele *R1753K* was isolated as a suppressor of the cold sensitivity of *prp22-S635A*, an allele encoding a helicase-defective

Prp22 protein that is unable to release mature mRNA from the spliceosome (Schwer and Meszaros 2000; Schneider et al. 2004). The present study of *prp8-R1753* mutants provides insights to the mechanism of the second step of splicing and the release of mature mRNA from the spliceosome. The major findings are (1) mutations in loop 1 of U5 snRNA suppress the temperature sensitivity of *prp8-R1753* mutants; (2) the second step of splicing certain pre-mRNAs is impaired in cell-free extracts from *prp8-R1753* mutant cells; (3) gain-of-function mutations in *PRP18* suppress *prp8-R1753* and multiple *slu7* mutants; and (4) mutations in loop 1 of U5 snRNA exacerbate the growth defects of *prp22-cs* mutants.

Multiple mutations at Arg1753 (R1753K, R1753A, R1753Q, R1753E) suppressed helicase-defective *prp22*

**TABLE 1.** Temperature-sensitive *slu7* mutants

Allele	Missense changes
<i>slu7-11</i>	H75R, R243G, D267G
<i>slu7-12</i>	K94E, K324E
<i>slu7-13</i>	S230P, L278P
<i>slu7-14</i>	R173G, W210R, L297Q
<i>slu7-15</i>	T246A, Y266C
<i>slu7-16</i>	K138E, S230P, L278P, R290G
<i>slu7-17</i>	G96D, N124S, N150K, W189R, K202R
<i>slu7-18</i>	Y36H, S178P, D235G, K284E

mutants and elicited temperature-sensitive growth defects in *PRP22* cells, leading to the proposal that Arg1753 makes contacts in the spliceosome that are loosened in the *prp8-R1753* mutants (Schneider et al. 2004). Our findings that (1) mutations in loop 1 of U5 snRNA alleviate the temperature sensitivity of *prp8-R1753* mutants, and (2) splicing of transcripts with mutations in exon sequences is impaired in *prp8-R1753* mutant extracts implicate the U5 snRNA and exon bases in contacts that are strengthened by Arg1753. A role for Prp8 in stabilizing U5/exon interactions has been suggested based on cross-linking studies, which placed Prp8 close to bases in loop 1 of U5 and to bases at the ends of both exons (Teigelkamp et al. 1995; Dix et al. 1998; Turner et al. 2006). Using site-specific photocross-linking and proteolytic analyses, Turner et al. (2006) have established a physical map of Prp8 contacts with the U5 and U6 snRNAs and the 5' splice site and branch point in the pre-mRNA. None of the analyzed RNA-protein cross-links mapped in the vicinity of Arg1753, which could indicate that (1) Arg1753 makes contact with the U5/3' exon RNA, (2) Arg1753 does not interact directly with the RNA, or (3) the interaction cannot be captured by photocross-linking. In any event, our results provide functional evidence for an involvement of Prp8 in stabilizing U5/exon interactions.

The gain-of-function *PRP18-V191A* and *PRP18-S162P* alleles suppress *prp8-R1753E* and also a variety of *slu7* mutants (Fig. 3). In the crystal structure of Prp18 $\Delta$ 79 (Jiang et al. 2000), Ser162 and Val191 are near each other (Fig. 3C). Val191 is located within Prp18's conserved region. Prp18 proteins from human, plant, fly, and worm contain an alanine at the equivalent position; thus, the V191A mutation increases the identity of the *Saccharomyces cerevisiae* protein with other Prp18s. Ser162 is situated in helix3, which contacts the conserved loop in the crystal structure. The spatial proximity of the two suppressor mutations suggests that they are mechanistically similar, perhaps altering the conformation and/or the flexibility of Prp18's conserved loop. The isolation of *S162P* and *V191A* in two independent screens for dominant suppressors and the finding that *prp18* mutations suppressed multiple *slu7* alleles support the idea that the mutations improve the functionality of Prp18, likely by enhancing the ability of Prp18 to stabilize contacts between U5 and the exons. The notion that Prp18's conserved region plays an important role in facilitating U5/exon interactions comes from genetic and biochemical studies of *prp18 $\Delta$ CR*, an allele that encodes a Prp18 protein lacking the conserved loop (Baciková and Horowitz 2002, 2005; Crotti et al. 2007).

*Prp8-R1753* mutants resemble the *prp18 $\Delta$ CR* allele in three aspects. First, the U5 allele *U5-S2* (A8C) suppresses the growth defect of *prp8-R1753E* and of *prp18 $\Delta$ CR*, and it improves the step 2 splicing defect in *prp18 $\Delta$ CR* cells (Baciková and Horowitz 2005). *U5-S2* (A8C) alleviates temperature sensitivity in the absence or presence of wild-type U5, indicating that A8C is a gain-of-function

mutation. Second, *Prp8-R1753E* and *prp18 $\Delta$ CR* are also suppressed by mutations in the bases of loop 1 of U5 that interact directly with the exons (Newman 1997; Crotti et al. 2007); *U5-GG* (U4G U5G) and *U5-U4A* suppress *prp8-R1753E* and *prp18 $\Delta$ CR*, respectively. These U5 alleles suppress only in the presence of wild-type U5, suggesting that they are necessary for splicing a subset of pre-mRNAs that rely on Prp8 or Prp18 to strengthen U5/exon interactions, whereas wild-type U5 is required for splicing other essential transcripts. Third, the *Prp8-R1753* mutants and *Prp18 $\Delta$ CR* impair the second step of splicing pre-mRNAs with exon mutations that, based on genetic and biochemical analyses, weaken interaction with U5 (Newman 1997; Crotti et al. 2007). Taken together, the findings are consistent with the idea that Prp8 and Prp18 cooperate to stabilize U5/exon interactions that are important for the second transesterification reaction.

The *PRP18* alleles *V191A* and *S162P* suppress the growth defects of several *slu7* mutants (Fig. 3). *SLU7* was originally isolated in a synthetic lethal screen with the *U5-U98A* mutant that carries a U to A change at position 6 in loop 1 (Frank et al. 1992). *Slu7* affects 3' splice site choice in yeast and in human cell extracts; when presented with competing 3' splice sites, yeast *Slu7* is necessary for selection of the distal PyAG $\downarrow$  (Frank and Guthrie 1992) and human *Slu7* is reported to discriminate against aberrant AGs (Chua and Reed 1999). In vitro studies suggested that human *Slu7* plays a role in properly holding exon 1 within the spliceosome (Chua and Reed 1999). The gain-of-function mutations in Prp18 identified here are not allele specific, but suppress multiple *slu7* mutants. We surmise that suppression occurs at the stage when the U5/exon contacts are formed during the second step of splicing. Whether *Slu7* plays a direct role in stabilizing contacts between U5 and exon bases is not known.

*Prp8-R1753* mutants affect the second step of splicing certain pre-mRNAs in vitro. Prp8 is essential early in splicing (Jackson et al. 1988; Brown and Beggs 1992), and a role for Prp8 during the second step of splicing is well documented. For example, *prp8-101* is defective for the second step of splicing precursor RNAs that have sub-optimal 3' splice sites (Umen and Guthrie 1995a), and several *prp8* alleles suppress intronic mutations including changes at the 3' splice site PyAG $\downarrow$  (Umen and Guthrie 1995a, 1996; Collins and Guthrie 1999; Siatecka et al. 1999; Konarska et al. 2006). Our findings indicate that Prp8 also plays a role in modulating the second step of splicing based on exonic sequences, extending earlier experiments that showed that Prp8 contacts the exons during the second step (Teigelkamp et al. 1995; Turner et al. 2006; McPheeters et al. 2000; McPheeters and Muhlenkamp 2003).

Unlike Prp8 and U5/exon pairing, other interactions that facilitate the formation of the active site for step 2 catalysis might not be maintained after exon ligation when Prp22 helicase acts to release mRNA. This is indicated by the findings that the gain-of-function mutants *U5-S2* (A8C)

and the *PRP18* alleles *V191A* and *S162P* do not exacerbate the phenotypes of *prp22-cs* mutants (data not shown; Fig. 1B). Our results are consistent with the idea that the Prp22 disrupts contacts between the U5 snRNP and mRNA. The biochemical characteristics of the Prp22 helicase, the 3 → 5' directionality, and the requirement for a single-stranded RNA segment of ≥20 nt (Tanaka and Schwer 2005) have implications for the positioning of Prp22 on the RNA target (Fig. 1D).

## MATERIALS AND METHODS

### Plasmids

The plasmid library for the U5-loop I mutants was generously provided by Andy Newman (MRC Laboratory of Molecular Biology, Cambridge, United Kingdom). Mutations in the *SNR7* gene to generate U5-GG and U5-CT were introduced using the two-stage overlap PCR method. PCR products were restricted and the 590 base pair (bp) *Cla*I–*Hind*III fragments were inserted into pSE360 (*URA3 CEN*) and pRS413 (*HIS3 CEN*) vectors. To generate a Prp18 mutant library, a genomic *Hinc*II/*Sph*I fragment spanning the *PRP18* coding sequence plus 1417 and 211 bp upstream and downstream of the ATG and stop codon, respectively, was inserted into pUC18. The plasmid pUC18-PRP18 was used as the template for PCR amplification of the *PRP18* ORF in vitro with *Taq* DNA polymerase. The standard PCR reaction mixture was modified to contain 0.2 mM  $MnCl_2$ , and the concentration of dATP was reduced to 1/5 compared to the other three dNTPs (Leung et al. 1989). The PCR products were digested with *Bgl*II (which cleaves immediately downstream of the ATG) and *Eco*RV (which cleaves 40 bp downstream of the stop codon), and the mutagenized Prp18 DNA fragment was ligated into pUC18-PRP18 to replace the wild-type segment. The ligation mixes were transformed into *Escherichia coli* dg98 and a pooled plasmid library was prepared from approximately 6000 ampicillin-resistant colonies harvested directly from agar plates. From the pUC18-prp18 mutant library DNA, a 2.3-kb DNA fragment was excised, ligated into pSE360 (*URA3 CEN*), and transformed. The p360-prp18 mutant library DNA was prepared from approximately 50,000 transformants. To gauge the mutation frequency, an aliquot of the plasmid pool was transformed into *E. coli*. Plasmid DNA was recovered from six individual ampicillin-resistant colonies and the *PRP18* gene was sequenced. Each of the plasmids contained one or more lesions, but none carried mutations at Ser162 or Val191, indicating that these changes were not overrepresented in the library.

### Strains

The *prp8-R1753E* strain [Mata *ura3-1 trp1-1 his3-11,15 leu2-3,112 ade2-1 can1-100 Δprp8::kanMX*] carries the *prp8-R1753E* allele on pRS413 (*HIS3 CEN*) (Schneider et al. 2004). *prp22-T637A*, *prp22-S635A*, and *prp22-H606A* strains were made by replacing p360-PRP22 (*URA3 CEN*) with the respective p358-prp22 mutant (*TRP1 CEN*) in a *prp22Δ* strain using the plasmid shuffle method (Schwer and Meszaros 2000). Strain *slu7-EIE* [Mata *leu2 his7 trp1 ura3 slu7::hisG*] harbors the *slu7-E215A-*

*I216A-E217A* allele on a *TRP1 CEN* plasmid (James et al. 2002). To generate strains for extract preparation, the *PRP8* gene was disrupted by insertion of the *kanMX* marker in BJ2168 cells harboring p360-PRP8 (*URA3 CEN*) (Schneider et al. 2004). Using the plasmid shuffle procedure, we then replaced the *URA3*-marked *PRP8* by the wild-type or the *R1753K* and *R1753E* mutant alleles on *TRP1 CEN* plasmids.

### Screen for suppressors of *prp8-R1753E*

The *prp8-R1753E* cells were transformed with the plasmid libraries of U5 loop I or of *prp18* mutants.  $Ura^+$  colonies were selected at 37°C. Plasmids were recovered from individual colonies, amplified in bacteria, and the genes for the U5 snRNA or for Prp18 were sequenced. The four independently isolated *prp18* suppressors carried the following mutations: (1) *S162P* and a silent change at Thr31, (2) *S162P*, (3) *V191A*, and (4) *K141E* and *S162P*.

### Screen for *prp18* suppressors of the temperature-sensitive growth defect of *slu7-EIE* cells

The *prp18* mutant library DNA was introduced into *slu7-EIE* cells, and  $Ura^+$  colonies were selected at 37°C. The plasmid DNAs were recovered and analyzed after amplification in *E. coli*. Three individual isolates carried a single *S162P* change, and one isolate contained a *V191A* mutation. Three other suppressors contained the following mutations: (1) *K125E*, *I176V*, *V191A*; (2) *K141E*, *S162P*; (3) *S162P*, and silent mutations at Leu109 and His203.

### Isolation of temperature-sensitive *slu7* mutants

A DNA fragment spanning the *Slu7* coding region was amplified by *Taq* DNA polymerase under mutagenic conditions (Leung et al. 1989). The PCR product was restricted and inserted into p358-*Slu7*-(B), thereby replacing the wild-type DNA fragment. Ligation mixes were transformed into *E. coli* dg98 and a pooled plasmid library was prepared from approximately 30,000 ampicillin-resistant colonies harvested directly from agar plates. The mutant library was transformed into a *slu7Δ* strain harboring *SLU7* on a *URA3*-marked plasmid.  $Trp^+$  transformants were selected at 25°C and replica plated to 5-FOA twice. One thousand 5-FOA survivors were patched to rich medium (YPD) and replica-plated to 25°C and 37°C. The *TRP1 slu7* plasmids were isolated from cells that grew at 25°C, but failed to grow at nonpermissive temperature, amplified in *E. coli*, and the *SLU7* genes were sequenced to determine the lesions.

### Extract preparation and in vitro splicing reactions

Yeast whole cell extract from BJ-Prp8, BJ-Prp8-R1753K, or BJ-Prp8-R1753E cells was prepared by grinding in liquid nitrogen as described (Ansari and Schwer 1995). Labeled precursor RNAs for in vitro splicing were synthesized using SP6 RNA polymerase and  $\alpha$ -<sup>32</sup>P-UTP from plasmids pDBN1a (AAAA|AAA), pDBN6a (AAAC|CAA) and pDBN7a (CTTC|CAA) that had been restricted with *Nde*I (Crotti et al. 2007). Splicing reactions were carried out at 32°C and analyzed as described (Lin et al. 1985). The RNA species were visualized by autoradiography and quantified using a phosphorimager. To determine the relative molar amounts, the values were normalized for the length of the respective RNA species (Crotti et al. 2007).

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