Splicing proofreading at 5' splice sites by ATPase Prp28p

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ABSTRACT

Fidelity and efficiency of pre-mRNA splicing are critical for generating functional mRNAs, but how such accuracy in 5' splice site (SS) selection is attained is not fully clear. Through a series of yeast genetic screens, we isolated alleles of prp28 that improve splicing of suboptimal 5' SS substrates, demonstrating that WT-Prp28p proofreads, and consequently rejects, poor 5' SS. Prp28p is thought to facilitate the disruption of 5'SS–U1 snRNA pairing to allow for 5' SS–U6 snRNA pairing in the catalytic spliceosome; unexpectedly, 5' SS proofreading by Prp28p is dependent on competition with the stability of the 5' SS:U6 duplex, but not the 5' SS:U1 duplex. E404K, the strongest prp28 allele containing a mutation located in the linker region between adenosine triphosphatase (ATPase) subdomains, exhibited lower RNA-binding activity and enhanced splicing of suboptimal substrates before first-step catalysis, suggesting that decreased Prp28p activity allows longer time for suboptimal 5' SS substrates to pair with U6 snRNA and thereby reduces splicing fidelity. Residue E404 is critical for providing high splicing activity, demonstrated here in both yeast and Drosophila cells. Thus, the subdomain linker in Prp28p plays important roles both in splicing efficiency across species and in proofreading of 5' SS.

INTRODUCTION

Pre-mRNA splicing, catalyzed by the spliceosome, a large dynamic RNA–protein complex composed of five small nuclear ribonucleoproteins (snRNPs) and many protein factors, excises introns and ligates exons to generate mature mRNAs (1). Three conserved cis-elements in the intron, the 5' splice site (5'SS), branch site (BS) region and 3' splice site (3'SS), are critical for intron recognition, spliceosome assembly and splicing catalysis. The 5' SS is recognized by U1 snRNP, the 3' SS is bound by U2AFs and U2 snRNP binds to the BS region to form a stable pre-spliceosome. Subsequently, U4/U6–U5 tri-snRNP joins, and the active spliceosome forms after release of U1 and U4 snRNPs (2,3). Many RNA–RNA and RNA–protein interactions occur transiently and are disrupted during progression of the splicing pathway, most of which are facilitated by eight adenosine triphosphatases (ATPases) (4–6). For example, the early 5' SS–U1 snRNA interaction is exchanged for a 5' SS–U6 snRNA interaction before formation of spliceosomal complex B, facilitated by Prp28p (7,8). Likewise, U4–U6 snRNA interaction in the U4/U6–U5 tri-snRNP is replaced by U2–U6 snRNA interaction during formation of the 'activated spliceosome', facilitated by Brp2p (9–11).

Splicing fidelity is important for generating accurate mRNAs, and thereby functional proteins. The dynamic nature of spliceosome assembly and catalysis provides many opportunities for proofreading. Two non-mutually exclusive mechanisms have been proposed for altering fidelity, a two-state model and a kinetic proofreading model (12–14). The former was based on evidence of competition between spliceosomal conformations; such as, alleles of PRP8, U6 or CEF1 that inhibited first-step catalysis also improved second-step catalysis, and vice versa (15–18). The latter was based on the idea that ATPases function as timers to reject slowly progressing suboptimal substrates or as sensors of substrate identity to reject suboptimal substrates (3,14). For example, pep16 alleles with reduced ATPase activity improve splicing of poor...
first-step substrates, and prp22 alleles improve second-step catalysis on suboptimal substrates, presumably by allowing longer dwell time in the first and second-step conformations, respectively (15,19–24). Prp5p modulates BS fidelity by competing with the stability of the BS:U2 snRNA duplex (25,26); Prp43p promotes discard of intermediates and establishes a pathway for turnover of stalled intermediates at multiple stages (27). In addition, the specificity of the U2AF/3'sS interaction during early assembly can be increased by competition with DEK and hnRNP A1 binding in mammals (28,29).

Prp28p is a DEAD-box protein with nine conserved motifs (30,31). Genetic studies have demonstrated that Prp28p facilitates a destabilization of the 5'SS–U1 snRNP interaction, resulting in a switch of 5'SS:U1 pairing to 5'SS:U6 pairing (8,32). Mutations in U1 snRNP components, including U1C, Prp42p, Snu71p and U1 snRNA, can bypass the requirement of Prp28p (33–35). Mammalian Prp28 is a component of U5 snRNP (36), and its phosphorylation by SRPK2 is necessary for U4/U6–U5 tri-snRNP integration into the spliceosome (37). hPrp28 interacts directly with the 5'SS, detected by cross-links between motif III and positions +7 and +8 in the 5'SS (38). However, whether Prp28p is involved in substrate proofreading at the 5'SS is not yet known.

Here, we isolated prp28 alleles that improve splicing of suboptimal 5'SS substrates using yeast genetic screens. Alteration of 5'SS–U1 and 5'SS–U6 snRNA interactions demonstrated that 5'SS proofreading was sensitive to the stability of the 5'SS:U6 duplex, but not the 5'SS:U1 duplex. The strongest allele prp28-E404K has a reduced RNA-binding activity and improves splicing of 5'SS mutants at a stage before the first step of splicing. Furthermore, the ATPase subdomain linker sequence in Prp28p is phylogenetically variable; its replacements in both yeast and fly systems with heterologous linker sequences indicate that the yeast linker sequence and E404 are critical for cell growth and splicing efficiency. Thus, the Prp28p subdomain linker plays an important role in maintaining splicing activity across species, and mutations in this region have revealed a role in proofreading of 5'SS by Prp28p.

MATERIALS AND METHODS

Yeast strains and plasmids

Saccharomyces cerevisiae strains used in this study are listed in Supplementary Table S1. Plasmid-borne alleles of prp28, snr19 (U1 snRNA), snr6 (U6 snRNA), U1C and ACT1-CUP1 reporters were prepared by either in vivo gap repair cloning or traditional cloning using Escherichia coli.

Screening of prp28 alleles in S. cerevisiae

WT-PRP28 on pRS313(HIS) was digested by MfeI and transformed with a pool of error-prone polymerase chain reaction (PCR) products, covering nucleotides +525 to +1680 of PRP28, for gap repair in yYZX19 strain that contained various ACT1-CUP1 reporters. Transformants were replicated to 5-Fluoroorotic Acid (FOA) plates to lose the URA marked WT-PRP28 plasmid; then replicated to select for higher resistance to copper. Subsequent steps for identification and confirmation of prp28 alleles were carried out as described previously (26). Error-prone PCR was performed using buffer containing 0.5 mM MnCl₂, 0.05 U/μl of Taq DNA polymerase and 400 pg/μl templates for eight cycles to introduce 7–10 mutants per 1000 bp (39). Products from error-prone PCR were gel purified and amplified further under standard conditions.

Copper assay and primer extension

Copper assays were carried out as described previously (26,40). Plates were scored by the maximum copper concentrations of strain growth and photographed after 4 days at 30°C or 10 days at 20°C. For simplicity, growth of each strain with a reporter on two or three representative copper concentrations was chosen to illustrate altered splicing activities by prp28 alleles. Primer extensions were performed as described previously (41), using primer YAC6 5'-GGCACTCATGACCTTC, complementary to exon 2 of the ACT1-CUP1 reporter for detection of splicing products, and primer y5S 5'-ACGTTGATCGGACGGG, complementary to 5S rRNA, for a loading control. Extension products were quantified by phosphorimaging and Multi Gauge V3.0.

Purification of recombinant ScPrp28 proteins and UV cross-linking

His6-tagged Prp28p and mutant proteins were expressed in E. coli, then subsequently purified by Ni-NTA agarose (Qiagen), CHT-1 column (Bio-rad) and Superdex D75 10/60 (GE). Proteins were stored in buffer D [20 mM HEPES-KOH (pH 7.9), 0.2 mM ethylenediaminetetraacetic acid, 100 mM KCl, 0.5 mM dithiothreitol, 1 mM Phenylmethylsulfonyl fluoride and 20% glycerol]. In vitro ATP-binding assays were performed as described previously (42). Gel-purified 32P-labeled [U]10 (RiboBio Co., Ltd) was used for RNA-binding assays. Purified Prp28p was incubated with 0.1 pmol of 32P-[U]10 (5 x 10⁴ cpm) in 20 μl with 0.05 mM of ATP. Then UV cross-linking was performed as described previously (43) using 254-nm light for 20 min on ice, separated on 10% sodium dodecyl sulfate, visualized by autoradiography and quantified by Multi Gauge V3.0.

Construction of splicing reporter system in Drosophila S2 cell line

To generate expression plasmids of DmPrp28, fragments of hygromycin resistance gene with P copia promoter and simian vacuolating virus 40 poly(A) were cloned into pMT/V5-His B vector (Invitrogen), followed by insertion of DmPrp28 open reading frame with N-terminal FLAG tag. Then, the plasmids were transfected into S2 cell line and selected by 100 μg/ml of hygromycin to obtain stable cell lines that express similar levels of DmPrp28 proteins after induction with 0.5 mM CuSO₄. Sk2-EGFP reporters were constructed by insertion of DNA fragments with Kozak sequence (CGAAATGGGC), truncated Drosophila Sk2 gene (21 nt of exon 1; 479 nt of intron 1...
and 54 nt of exon 2) into pEGFP-N1 vector (CLONTECH), which is driven by the immediate early promoter of cytomegalovirus. Detection of EGFP fluorescence by fluorescence-activated cell sorting (FACS) (BD LSR II Flow Cytometer) was performed after transfection of Sk2-EGFP reporter and 2.5 days CuSO4 induction. To visualize the expressed proteins, western blots were probed using monoclonal M2– Peroxidase (Sigma) against FLAG, monoclonal antibody against GAPDH (ImB) and mAb 7G9 (Abmart) against GFP.

**Prp28 linker region**

The linker region of DEAD-box proteins was defined as the loop between the subdomain 1 and subdomain 2 of ATPase/helicase domains. Linkers of Dhh1 and eIF4A were extracted from published crystal structures, and the linker of Prp5 from our unpublished structure data. The linker sequences of Prp28 and Ded1 were acquired via comparison of their protein sequences with Prp5, Dhh1 and eIF4A using Basic Local Alignment Search Tool and confirmed by secondary structure predictions using software NetSurfIP (ver. 1.1).

**RESULTS**

**Genetic screens for prp28 alleles that improve splicing at suboptimal 5′SS**

Based on the idea that most of the spliceosomal ATPases contribute to kinetic proofreading (44), we used a series of genetic screens to identify alleles of prp28 that could improve splicing of suboptimal substrates. To identify substrates sensitive to altered Prp28p activity, we tested two mutants in motif III (TAT), which is thought to be required for coupling of ATP hydrolysis to conformational change: AAT and TAA. ACT1-CUP1 reporters, which confer copper tolerance to yeast lacking the chromosomal CUP1 genes, were used to monitor splicing efficiency in vivo (40). Sixteen reporters (Figure 1A), representing mutations at the 5′SS, BS and 3′SS, were tested for growth on copper in the presence of these two prp28 alleles. Subtle improvement by the two prp28 alleles was observed; however, subsequent screens using randomized motif III residues did not reveal stronger mutants. To identify alleles exhibiting more potent effects, we next mutated the entire ATPase domain (175–560 amino acids) using error-prone PCR; selection for improved growth on copper for 5′SS-A3G or A3U reporters identified several prp28 alleles (Figure 1B and C). The strongest allele, which improved A3U and A3G reporters >2-fold, contained four mutations: N178Y, M346V, L359F and E404K. To isolate the responsible residue(s) and obtain multiple mutations at the U1–C4 or –U6 position (33,46). If the 5′SS:U1 duplex was important for altered substrate use because of prp28 alleles, then changing the stability of the duplex would be expected to alter the potency of prp28 effects. To test this, we increased duplex stability using U1 snRNA mutants and decreased stability using a U1C mutant.

We first generated strains carrying U1 snRNA mutants along with a second plasmid carrying WT U1 snRNA because of the lethality of most U1 snRNA mutants as the sole copy (33,45,46). Compensatory mutations in U1 snRNA that restore pairing with 5′SS mutants improved their splicing (Figure 2A, col. 7, 10, 16, 19 and 22), whereas non-cognate U1s did not. For example, splicing of the A3G reporter was improved in the presence of U1–U6C, but not in the presence of U1–U6A or –U6G alleles. However, compensatory changes in U1 snRNA did not alter the level of improved splicing of suboptimal 5′SS substrates by prp28-E404K allele (Figure 2A). Same assay was also performed in the presence of a single copy of U1–C4U, the only viable U1 snRNA allele with mutation at the U1–C4 or –U6 position (33,46). Consistent with the aforementioned results, improved splicing of the 5′SS–G5A reporter by prp28-E404K was not changed when U1 snRNA was mutated to U1–C4U, which restored the pairing between 5′SS–pos.5 and U1 snRNA-pos.4 (Figure 2A, bottom). Second, we replaced the yeast chromosomal U1C gene with plasmid-borne WT-U1C or U1C-L13F allele that reduces the stability of the 5′SS–U1 snRNA interaction, resulting in a bypass of the requirement for Prp28p (33). In comparison with WT–U1C, splicing of most suboptimal 5′SS mutants was relative to WT-PRP28 and comparably increased A3G and A3U mRNA levels (Figure 1D).

**Alteration of 5′SS–U1 snRNA interaction does not change the effect of prp28-E404K**

Prp28p has been proposed to dissociate the duplex formed between U1 snRNA and the 5′SS (Figure 2A), resulting in release of U1 snRNAP and formation of 5′SS:U6 snRNA pairing (8,33). If the 5′SS:U1 duplex was important for altered substrate use because of prp28 alleles, then changing the stability of the duplex would be expected to alter the potency of prp28 effects. To test this, we increased duplex stability using U1 snRNA mutants and decreased stability using a U1C mutant.

Several E404 mutants were isolated and compared. prp28-E404K and -E404R alleles behaved similarly: both improved A3G and A3U growth on copper by 2- to 3-fold relative to WT-PRP28 and comparably increased A3G and A3U mRNA levels (Figure 1D). prp28-E404L, -E404V and -E404A alleles mildly improved A3G and A3U, whereas prp28-E404D mutant behaved exactly as WT (selected alleles are shown in Figure 1D). This pattern of effects suggests that disruption of the negative charge at position 404 is the key feature of this class of prp28 mutants. We chose the prp28-E404K allele for further analysis.

To investigate specificity of altered intron selection exhibited by prp28 alleles, we tested all possible 5′SS mutations at positions 1–6. The prp28-E404K allele strongly improved splicing of A3G, A3U, G5A and G5U substrates and slightly improved splicing of U4A and U6G substrates, but not of WT or other 5′SS mutant reporters nor of tested reporters having mutations within the BS region or 3′SS (Figure 1E). We conclude that our identified prp28 alleles alter substrate selectivity most strongly at +3 and +5 positions, although other 5′SS positions are also affected. As screens were performed on 5′SS mutants, the existence of prp28 alleles that improve splicing of BS or 3′SS mutants cannot be ruled out here.
decreased in the presence of the U1C–L13F allele. However, the splicing enhancement because of prp28-E404K did not change when WT–U1C was replaced with U1C–L13F (Figure 2B). For example, improvement of the A3G reporter was 2-fold in the presence of the prp28-E404K allele, which remained 2-fold with either WT–U1C or the U1C–L13F allele (Figure 2B, col. 6 to 5).

Thus, either restoration of 5'SS:U1 snRNA pairing or destabilization of 5'SS–U1 snRNP interaction does not alter the improvement of splicing of suboptimal 5'SS substrates by the prp28-E404K allele, suggesting that modulation of 5'SS selectivity by Prp28p is not dependent on 5'SS:U1 snRNA duplex stability.

Stabilization of the 5'SS:U6 snRNA duplex results in loss of prp28-E404K effects

We next asked whether the stability of the 5'SS:U6 snRNA duplex is important for altered substrate use because of prp28 alleles. As shown earlier in the text, effects of prp28-E404K are most strongly at 5'SS-pos.A3 and G5, which are matched with U6-pos.50 and 48, respectively. Because of the inviability of U6-pos.48 mutant alleles (47), we focused on U6-pos.50 and tested all possible combinations of 5'SS-pos.3 and U6-pos.50. Previous observed insensitivity of the A3C reporter to prp28-E404K might be due to its additional CG pair with WT U6–G50 (Figure 3A). Consistent with this hypothesis, improved splicing of 5'SS-A3G and A3U by
prp28-E404K was either cancelled or significantly decreased when U6-G50 was mutated to C or A, which also form an additional base pair (Figure 3A). In contrast, prp28-E404K improved the splicing of A3C reporter when U6–G50 was mutated to either A or U, disrupting the CG base pair (Figure 3A, col. 10 and 14). In all the 16 cases, improved splicing by prp28-E404K occurred when the nucleotide at 5’SS-pos.3 was mismatched with the nucleotide at U6-pos.50, except for the WT reporter that always exhibited high copper tolerance and the 5’SS-A3C reporter in the presence of U6–G50C. The inconsistency for the cases of WT reporter (Figure 3A, col. 1, 5, 9 and 13) would be due to an unknown reason. However, an early cross-linking assay demonstrated that U6-pos.51 interacts with various nucleotides in the 5’SS region, resulting in a shifted pairing between the 5’SS and U6 snRNA (48).

Therefore, the inability of prp28-E404K to improve splicing of the A3C reporter in the presence of U6–G50C might be due to a shifted and stable four base pairs interaction between the 5’SS and U6 snRNA (Figure 3A left), or another unknown mechanism that we cannot address here.

The extent of cancellation of the prp28-E404K effects by additional pairing is variable. GC base pairs fully cancelled the improvement; UG wobble or AU pairs only partly or did not cancel the improvement (Figure 3A). This might be due to either the identity or stability of CG pairs. To distinguish, we used 5’SS-A3C or A3G mutant reporters with additional mutations at the 5’SS-pos.4 and tested their splicing activities in the presence of U1C–L13F. RNA mutations are indicated in red; base pairs at mutation sites are shown by blue lines. The level of improved splicing by prp28-E404K allele is calculated as E404K/WT.
presence of U6 alleles (Figure 3B). First, in the presence of WT U6 snRNA, prp28-E404K allele did not improve splicing of the single mutant, A3C, as shown earlier in the text (for clarity, referred to here as C3U4), in which the duplex has four base pairs. However, splicing of reporters having additional mutations at 5'SS-pos.4 to disrupt the second base pair with U6–A49 was improved by prp28-E404K allele even if the first CG base pair was present (Figure 3B, col. 2–4 to 1). Likewise, improved splicing of A3G reporter was cancelled in the presence of U6–C50A49 that forms four base pairs; analysis was not available for reporters of A3G with additional pos.4 mutations because of their low splicing activity. Second, splicing of mutant reporters that disrupted two base pairs was even more strongly improved by prp28-E404K. For example, the improved splicing level of C3A4 reporter in the presence of U6–C50A49 is higher than the presence of WT U6 (Figure 3B, col. 6 to 2). These results strongly suggest that the base identity of CG pairs between 5' SS-pos.3 and U6-pos.50 is not important for the 5' SS selection by Prp28p, but instead that the 5'SS:U6 duplex stability is critical for the altered substrate use.

We then analyzed in vivo mRNA and lariat-intermediate products by primer extension. DBR1, the gene for debranching enzyme, was deleted to prevent degradation of lariat intermediates for accurate visualization (16,49). mRNA levels of all tested alleles by primer extension are consistent with the copper reporter assays described earlier in the text (Figure 4A, top graph). Further analysis revealed that the improved levels of mRNA by prp28-E404K allele were due to enhanced efficiency of the first step of splicing, with a level of 30–50% improvement when the mutant substrates formed only three base pairs with U6 snRNA (Figure 4A, middle graph), consistent with the observation from the gel (Figure 4A, upper). The small differences that we detected in the second step are insignificant, or alternatively might reflect an effect of prp28 on the second step that was not selected for in our screen (Figure 4A, bottom graph), suggesting that the prp28-E404K effects are before the first step of splicing.

**prp28-E404K alters RNA-binding activity**

Prp28p is an RNA-dependent ATPase; therefore, the E404K mutation may alter ATPase activity of Prp28p. However, ATP hydrolysis in vitro was undetectable when purified recombinant Prp28p was incubated with ATP, consistent with a previous report (7). Similarly, ATP binding by Prp28p was low; only faint bands of cross-linked 32P-ATP with Prp28p were observed by UV cross-linking (Figure 4B). To date, the direct RNA target...
E404 of Prp28p is critical for efficient splicing in yeast

DEAD-box proteins contain an ATPase/helicase domain and various flanking domains (30), in which the core ATPase/helicase domain comprises two RecA subdomains separated by a short peptide linker (Figure 5A). Glutamic acid 404 is the second residue in a linker region between the two Prp28p ATPase subdomains. Phylogenetic conservation of this region in Prp28p is much lower than in other DEAD-box proteins, such as eIF4Ap, Ded1p and Dhh1p; in particular, E404 is unique to budding yeast (Figure 5A).

The variation in Prp28p linkers might contribute to the maintenance of splicing activity or fidelity in yeast. To test this possibility, we constructed mosaic prp28 mutants in S. cerevisiae, in which the linker region was replaced by linkers from other species. Swapping the linker with metazoan Prp28ps, including linkers from Drosophila melanogaster; Branchiostoma floridae and Homo sapiens, resulted in growth defects of budding yeast, exhibited by a cs phenotype at 16°C, whereas linkers from fission yeast and plant showed no detectable growth defects. However, the cs phenotypes were rescued by a single mutation at position 404 back to Glu (Figure 5B).

To address whether these cs growth defects were due to decreased splicing activity, we measured copper resistance of ACT1-CUP1 reporters and mRNA levels of endogenous genes in yeast strains. First, prp28 alleles with replaced linkers significantly inhibited splicing of 5’SS-pos.4 mutant reporters, but not WT reporter, or slightly inhibited other mutant reporters at 30°C (Figure 5C). These mosaic prp28 alleles inhibited splicing of all tested reporters at lower temperature (20°C), including the WT reporter and 5’SS-pos.4 mutants that are the most inhibited substrates. Importantly, the splicing defects were partly rescued when residue 404 was mutated back to Glu (Figure 5C).

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Yeast Prp28p linker enhances splicing efficiency in Drosophila cells

To address whether the Prp28p linker is important for maintaining splicing efficiency in higher eukaryotes, we chose the Drosophila S2 cell line. Stable S2 cell lines carrying FLAG-tagged DmPrp28p or mutants, driven by an MT promoter, were transfected with Sk2-EGFP splicing reporters to monitor splicing activity. This reporter contains a truncated Drosophila Sk2 exon 2 and fused with EGFP (Figure 6B). Analyzed by FACS, the DmPrp28-Sc allele that contained the budding yeast linker sequence showed improved EGFP fluorescence for all three tested reporters, including 5′SS-GUAA4 (fly consensus), -GUAU4 (yeast consensus) and mutant -GUGU4 reporters, suggesting that the yeast Prp28p linker sequence provides higher splicing activity (Figure 6C). A single mutation at residue V634 in DmPrp28p to Glu also improved expression of EGFP, consistent with the previous findings in yeast. The pattern of EGFP expression analyzed by western blotting was consistent with the analysis by FACS, except that less improvement was seen (Figure 6C). In addition, splicing of the 5′SS-GUAA4 reporter was more efficient than the -GUAU4 reporter, consistent with adenosine being the favored nucleotide at 5′SS-pos.4 in higher eukaryotes. Taken together, these data demonstrate that the budding yeast linker sequence of Prp28p provides a higher splicing efficiency than the corresponding linker sequence from Drosophila Prp28p.

DISCUSSION

We have obtained prp28 alleles that specifically alter splicing of suboptimal 5′SS substrates, indicating proof-reading of 5′SS by Prp28p. The mutation in the strongest prp28 allele is located in the linker region between ATPase subdomains, close to the site of a previously observed cross-link between Prp28p and the 5′SS (38), and this mutation impaired RNA-binding ability. We propose that movement of the 5′SS across U6 snRNA facilitated by Prp28p competes with the stability of the 5′SS:U6 duplex, resulting in rejection of weak 5′SS:U6 pairs.

Supported both by genetic studies and in vitro assays, the common feature of proofreading by ATPases is that
WT ATPases discriminate suboptimal substrates, whereas slower ATPase mutants allow progression of splicing by providing a longer temporal opportunity for formation of productive complexes on suboptimal substrates (20,22–27). We provide several lines of evidence that Prp28p also modulates splicing fidelity, proofreading 5'SS at the stage of 5'SS–U6 snRNA association as follows: (i) isolated prp28 alleles improved splicing of suboptimal 5'SS substrates, but not BS or 3'SS mutants; (ii) either stabilization of the 5'SS:U1 snRNA duplex, attained by complementary changes of U1 snRNA, or destabilization of the 5'SS–U1 interaction, using a mutated U1C protein, resulted in neither cancellation nor exacerbation of the improved splicing activity by prp28-E404K allele; (iii) hyper-stabilization of the 5'SS:U6 snRNA duplex, attained by either mutations in the 5'SS or in U6 snRNA, cancelled the enhanced splicing activity conferred by the prp28 allele, and this cancellation was not because of nucleobase identity in the 5'SS. Furthermore, the prp28-E404K allele that improved splicing of 5'SS mutants exhibited reduced RNA-binding activity, which presumably results in a reduced ability to promote conformational change. The simplest explanation is a model in which these alleles allow longer time for an inefficient 5'SS:U6 snRNA duplex formation. In such a scenario, WT introns and U6 snRNA would pair efficiently, and subsequent activity by either WT-Prp28p or mutant Prp28p would facilitate a conformational change that stabilizes the association of U6 snRNA to introns (Figure 7A). By contrast, a suboptimal 5'SS would pair inefficiently and WT-Prp28p would function before duplex formation, resulting in a similar, but abortive, conformational change in the 5'SS–U6 snRNA complex. The slowed activity of mutant prp28 alleles would allow more time for the association of a weakened 5'SS:U6 snRNA duplex, thereafter

![Figure 6](image1.png) Linker switches of DmPrp28 in *Drosophila* S2 cell line. (A) The 5'SS region is more diverse in higher eukaryotes; see Supplementary Table S2 for details and additional species. The base pairing regions of U1 and U6 snRNAs to the 5'SS are invariant across species. (B) Strategy of constructing splicing reporter system in *Drosophila* S2 cell line. Stable cell lines were established by transfection and drug selection. (C) (Left) Western blot analysis indicated that FLAG-tagged DmPrp28s were expressed at similar levels in the three stable cell lines after induction with CuSO4. Switching yeast Prp28 link to DmPrp28 or single mutation of DmPrp28-V634E increases splicing efficiency of the three tested splicing reporters, indicated both by FACS detection of EGFP fluorescence (middle) and by western analysis of fusion Sk2-EGFP protein (right). Linker region sequences of WT and mutant DmPrp28s are indicated.

![Figure 7](image2.png) Prp28p proofreads splicing at 5'SS during the association of 5'SS with U6 snRNA. By either an unwinding activity or an RNPase activity, Prp28p disrupts the 5'SS–U1 interaction, then facilitates the 5'SS–U6 interaction and proofreads 5'SS before the first step of splicing. It is unclear whether this is a concerted one-step U1 release/U6 association or two distinct steps; therefore, disruption of 5'SS–U1 snRNA interaction by Prp28p is shown in dash. (A) Productive splicing pathway facilitated by Prp28p. Duplex formation between optimal 5'SS substrates and U6 snRNA is faster than the rate of conformational change by either WT-Prp28p or selected mutant Prp28p. (B) Conformational change by WT-Prp28p is faster than the rate of duplex formation between suboptimal 5'SS substrates and U6 snRNA, resulting in Prp28p-mediated transition prior to 5'SS:U6 pairing and thus rejecting suboptimal substrates. However, mutant Prp28p with a slower activity reduces the rate of conformational change, allowing longer temporal opportunity to achieve stable 5'SS:U6 pairing and thus progression of splicing.
progressing to allow an improved first step of splicing (Figure 7B).

We previously reported that the stability of the BS–U2 snRNA structure is in competition with the activity of Prp5p, linking an RNA duplex with the modulation of splicing fidelity by an ATPase (26). In this study, the stability of the 5′SS:U6 RNA duplex is proofread by Prp28p, providing another example of ATPase-mediated modulation of splicing fidelity being coupled to the stability of an RNA duplex. Prp28p has been proposed to mediate a switch of 5′SS:U1 pairing to 5′SS:U6 pairing, disrupting the 5′SS–U1 interaction by either its unwinding activity to separate the 5′SS:U1 duplex (8) or its RNase activity to remove U1 proteins (33,35); Prp28p was also proposed to translocate the 5′SS into a complex to interact with U6 snRNA by base pairing (38). Our results demonstrated that the level of prp28 allele’s effect was highly dependent on the stability of 5′SS:U6 duplex, but not 5′SS:U1 duplex, suggesting that ATP hydrolysis by Prp28p destabilizes the 5′SS–U1 interaction, resulting in release of U1 snRNPs; then, the 5′SS is translocated to U6 snRNA for formation of a second duplex, providing an opportunity for splicing proofreading. However, it is uncertain whether this is one coordinated step of U1 release/U6 association or two distinct steps that are facilitated by Prp28p and ATP hydrolysis, although we favor a coordinated one-step process (Figure 7).

The 5′SS sequence is more diverse in higher eukaryotes than in budding yeast, as are the BS and 3′SS (51). The base pairing partners for the 5′SS in U1 snRNA (4′ACUUACG) and U6 snRNA (5′ACAGAG5′) are invariant in all eukaryotes (52,53). Therefore, the 5′SS:U1 and 5′SS:U6 duplexes are generally uniform in budding yeast, but more variable and less stable in higher eukaryotes. In this study, we found that the linker region between Prp28p ATPase subdomains is variable between species and is particularly divergent in budding yeast ScPrp28p. A glutamic acid residue in the linker region is critical for maintaining high splicing efficiency, and swapping the entire linker sequence across budding yeast is the most sensitive position for mutant prp28 allele’s effect. Mutant assays in yeast is the most sensitive position for mutant prp28 alleles that contain replaced linker sequences. Mutant assays in Drosophila cells provided similar results. It has been demonstrated that linker regions are critical for maintenance of functional protein conformation from many studies of ATPases. For example, mutations in the linker region between the protease and helicase domains of dengue virus NS3 protein resulted in significantly lower ATPase and helicase activities and a reduction of viral RNA synthesis (54). Therefore, we propose that the linker region between Prp28p subdomains is a key element for maintenance of the active conformation of Prp28p, and its second residue Glu—the important feature being negative charge—is critical for high splicing efficiency in budding yeast. Our results also showed a strong correlation between the variation of Prp28p linkers and the fourth nucleotide in the 5′SS consensus sequence.

In addition, the more variable 5′SS sequences in higher eukaryotes, especially the fourth nucleotide, would form much different stabilities of RNA duplexes with the invariant U6 snRNA, thus, providing more opportunities for discrimination and alternative splicing by Prp28p in higher eukaryotes.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online: Supplementary Tables 1 and 2.

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**REFERENCES**

1. Will,C.L. and Lührmann,R. (2011) Spliceosome structure and function. Cold Spring Harb. Perspect. Biol., 3, a003707.
2. Wahl,M.C., Will,C.L. and Lührmann,R. (2009) The spliceosome: design principles of a dynamic RNP machine. Cell, 136, 701–718.
3. Hoskins,A.A. and Moore,M.J. (2012) The spliceosome: a flexible, reversible macromolecular machine. Trends Biochem. Sci., 37, 179–188.
4. Staley,J.P. and Guthrie,C. (1998) Mechanical devices of the spliceosome: motors, clocks, springs, and things. Cell, 92, 315–326.
5. Schwer,B. (2001) A new twist on RNA helicases: DExH/D box proteins as RNases. Nat. Struct. Biol., 8, 113–116.
6. Hahn,D. and Beggs,J.D. (2010) Brr2p RNA helicase with a split C terminus of Prp8. Nat. Struct. Mol. Biol., 17, 42–48.
7. Hahn,D., Kudla,G., Tollervey,D. and Beggs,J.D. (2012) Prp28p-mediated conformational rearrangements in the spliceosome during activation and substrate repositioning. Genes Dev., 26, 2408–2421.
8. Staley,J.P. and Guthrie,C. (1999) An RNA switch at the 5′ splice site requires ATP and the DEAD box protein Prp28p. Mol. Cell, 3, 55–64.
9. Maeder,C., Kutach,A.K. and Guthrie,C. (2009) ATP-dependent unwinding of U4/U6 snRNAs by the Br2 helicase requires the C terminus of Prp8. Nat. Struct. Mol. Biol., 16, 42–48.
10. Schwer,B. (2001) A new twist on RNA helicases: DExH/D box proteins as RNases. Nat. Struct. Biol., 8, 113–116.
11. Mozaffari-Jovin,S., Santos,K.F., Hsiao,H.H., Will,C.L., Urlaub,H., Wahl,M.C. and Lührmann,R. (2012) The Prp8 RNase H-like domain inhibits Br2-mediated U4/U6 snRNA unwinding by blocking Br2 loading onto the U4 snRNA. Genes Dev., 26, 2422–2434.
12. Egecioglu,D.E. and Chanfreau,G. (2011) Proofreading and spellchecking: a two-tier strategy for pre-mRNA splicing quality control. RNA, 17, 383–389.
13. Smith,D.J., Query,C.C. and Konarska,M.M. (2008) “Nought may endure but mutability”: spliceosome dynamics and the regulation of splicing. Mol. Cell, 30, 657–666.
14. Semlow,D.R. and Staley,J.P. (2012) Staying on message: ensuring fidelity in pre-mRNA splicing. Trends Biochem. Sci., 37, 263–273.
15. Query,C.C. and Konarska,M.M. (2004) Suppression of multiple substrate mutations by spliceosomal prp8 alleles suggests functional correlations with ribosomal ambiguity mutants. Mol. Cell., 14, 343–354.
16. Konarska,M.M., Vilardell,J. and Query,C.C. (2006) Repositioning of the reaction intermediate within the catalytic center of the spliceosome. Mol. Cell., 21, 543–553.
17. Liu,L., Query,C.C. and Konarska,M.M. (2007) Opposing classes of prp8 alleles modulate the transition between the catalytic steps of pre-mRNA splicing. Nat. Structure Mol. Biol., 14, 519–526.
18. Query,C.C. and Konarska,M.M. (2012) CEF1/CDC5 alleles modulate transitions between catalytic conformations of the spliceosome. RNA, 18, 1001–1013.
19. Schwer,B. and Guthrie,C. (1991) PRP16 is an RNA-dependent ATPase that interacts transiently with the spliceosome. Nature, 349, 494–499.
20. Burgess,S., Couto,J.R. and Guthrie,C. (1990) A putative ATP binding protein influences the fidelity of branchpoint recognition in yeast splicing. Genes Dev., 4, 705–717.
21. Burgess,S.M. and Guthrie,C. (1993) A mechanism to enhance mRNA splicing fidelity: the RNA-dependent ATPase Prp16 governs usage of a discard pathway for aberrant lariat intermediates. Cell, 73, 1377–1391.
22. Koodathingal,P., Novak,T., Piccirilli,J.A. and Staley,J.P. (2010) The DEAH box ATPases Prp16 and Prp43 cooperate to proofread 5’ splice site cleavage during pre-mRNA splicing. Mol. Cell., 39, 385–395.
23. Tseng,C.K., Liu,H.L. and Cheng,S.C. (2011) DEAH-box ATPase Prp5 and branch region-U2 snRNA pairing modulates the fidelity of the reaction intermediate within the catalytic center of the spliceosome. Mol. Cell, 30, 416–427.
24. Xu,Y.Z. and Query,C.C. (2007) Competition between the ATPase site of Prp5 and branch region-U2 snRNA pairing mediates the fidelity of spliceosome assembly. Mol. Cell, 28, 838–849.
25. Mayas,R.M., Maita,H., Semlow,D.R. and Staley,J.P. (2010) Spliceosome discards intermediates via the DEAH box ATPase Prp43. Proc. Natl Acad. Sci. USA, 107, 10020–10025.
26. Tavanez,J.P., Madl,T., Kooshapur,H., Sattler,M. and Valcarcel,J. (2000) The U1 snRNP protein U1C interacts with the Prp8p C domain of the spliceosome. Mol. Cell Biol., 20, 381–384.
27. Mathew,R., Hartmuth,M., Mohllmann,S., Urlaub,H., Fiener,R. and Luhrmann,R. (2008) Phosphorylation of human PRP28 by SRPK2 is required for integration of the U4/U6-U5 tri-snRNP into the spliceosome. Nat. Structure Mol. Biol., 15, 435–443.
28. Ismaili,N., Sha,M., Gustafson,E.H. and Konarska,M.M. (2001) The 100-kda U5 snRNP protein (hPrp28p) contacts the 5’ splice site through its ATPase site. RNA, 7, 182–193.
29. Wilson,D.S. and Keefe,A.D. (2001) Random mutagenesis by PCR. Curr. Protoc. Mol. Biol., Chapter 8, Unit 8.3.
30. Lessner,C.F. and Guthrie,C. (1993) Mutational analysis of pre-mRNA splicing in Saccharomyces cerevisiae using a sensitive new reporter gene, CUP1. Genetics, 133, 851–863.
31. Statecka,M., Reyes,J.L. and Konarska,M.M. (1999) Functional interactions of Prp8 with both splice sites at the spliceosomal catalytic center. Genes Dev., 13, 1983–1993.
32. Xu,Y.Z., Newham,C., Knight,N., Huang,T., Konarska,M.M. and Query,C.C. (2004) Prp5 bridges U1 and U2 snRNPs and enables stable U2 snRNP association with intron RNA. EMBO J., 23, 376–385.
33. Trier,J.R. and Guthrie,C. (1990) Multiple roles of the mammalian U6 small nuclear RNA: conserved functional implications. Trends Biochem. Sci., 15, 381–384.
34. Alvarey,C.J., Romfo,C.M., Vanhoey,R.W., Porter,G.L. and Wise,J.A. (1996) Mutational analysis of U1 function in Schizosaccharomyces pombe: pre-mRNAs differ in the extent and nature of their requirements for this snRNA in vivo. RNA, 2, 404–418.
35. Schwer,B. and Guthrie,C. (1993) Beat the clock: paradigms for NTPases in the maintenance of biological fidelity. Trends Biochem. Sci., 18, 381–384.
36. Madhani,H.D., Bordonne,R. and Guthrie,C. (1990) Multiple roles for U6 snRNA in the splicing pathway. Genes Dev., 4, 2264–2277.
37. Kim,C.H. and Abelson,J. (1996) Site-specific crosslinks of yeast U6 snRNA to the pre-mRNA near the 5’ splice site. RNA, 2, 995–1010.
38. Chapman,K.B. and Boeke,J.D. (1991) Isolation and characterization of the gene encoding yeast debbranching enzyme. Cell, 65, 483–492.
39. Schwer,B. (2008) A conformational rearrangement in the spliceosome sets the stage for Prp22-dependent mRNA release. Mol. Cell, 30, 743–754.
40. Burge,C.B., Tuscht,T. and Sharpe,P.A. (1999) Splicing of pre-mRNA splicing by the spliceosomes. In: Gesteland,R.F., Cech,T.R. and Atkins,J.F. (eds), The RNA World, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 525–560.
41. Wolf,T., Menssen,R., Hammel,J. and Bindereif,A. (1994) Splicing function of mammalian U6 small nuclear RNA: conserved positions in central domain and helix I are essential during the first and second step of pre-mRNA splicing. Proc. Natl Acad. Sci. USA, 91, 903–907.
42. Lee,C., Jaladat,Y., Mohammadi,A., Sharifi,A., Geisler,S. and Valadkhan,S. (2010) Metal binding and substrate positioning by evolutionarily invariant U6 sequences in catalytically active protein-free snRNAs. RNA, 16, 2226–2238.
43. Luo,D., Wei,N., Doan,D.N., Paradkar,P.N., Chong,Y., Davidson,A.D., Kotaka,M., Lescar,J. and Vasudevan,S.G. (2010) Flexibility between the protease and helicase domains of the dengue virus NS3 protein conferred by the linker region and its functional implications. J. Biol. Chem., 285, 18817–18827.