PERSPECTIVE

Spliceosome activation: U4 is the path, stem I is the goal, and Prp8 is the keeper. Let's cheer for the ATPase Brr2!

Klaus H. Nielsen and Jonathan P. Staley¹

Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, Illinois 60637, USA

During pre-mRNA splicing, the spliceosome is activated for catalysis by unwinding base-paired U4/U6 small nuclear RNAs, a step that must be precisely timed. We know that unwinding requires the ATPase Brr2, but the mechanism and regulation of unwinding have been understood poorly. In the November 1, 2012, issue of *Genes & Development*, Hahn and colleagues (pp. 2408–2421) and Mozaffari-Jovin and colleagues (pp. 2422–2434) defined a pathway for U4/U6 unwinding. Moreover, Mozaffari-Jovin and colleagues suggested a mechanism for regulating Brr2.

In pre-mRNA splicing, introns are removed and exons are joined to generate mature mRNA. In the first of two transesterification reactions required for splicing, the 2' OH group of an adenosine within a conserved intronic branch site attacks the 5' splice site (SS), generating a free 5' exon and a lariat intermediate. In the second reaction, the newly liberated 3' OH of the 5' exon attacks the 3' SS, generating mature mRNA and an excised intron lariat. These reactions are facilitated by five small nuclear RNAs (snRNAs) and 80 conserved proteins that together compose the spliceosome (Will and Lührmann 2011). Throughout the splicing cycle, the spliceosome undergoes extraordinarily intricate and dynamic transformations involving exchanges of protein–protein, protein–RNA, and RNA–RNA interactions. These rearrangements are in many cases catalyzed by one of the eight spliceosomal members of the DExD/H-box ATPase family (Cordin et al. 2012) and must occur in a regulated and timely manner (Will and Lührmann 2011).

After de novo assembly on a pre-mRNA, spliceosomes are inactive and require a dramatic rearrangement of catalytic components for catalysis. The catalytic core of the spliceosome is composed of base-paired U2/U6 snRNAs (Madhani and Guthrie 1992). This interaction juxtaposes

the reactants within the substrates, and the adjacent U6 intramolecular stem-loop (ISL) coordinates a divalent metal that functions at the catalytic stage (Yean et al. 2000). Importantly, neither base-paired U2/U6 nor the U6 ISL is preformed in spliceosomes. Instead U6 is base-paired with U4 within the U4/U6•U5 tri-snRNP in a mutually exclusive fashion. Thus, a key step in the activation of the spliceosome involves unwinding of U4/U6. This unwinding has been shown to depend on Brr2 and ATP in vivo and in vitro (Laggerbauer et al. 1998; Raghunathan and Guthrie 1998; Kim and Rossi 1999). This activity is compromised by mutations in human Brr2 that confer blindness in some cases of retinitis pigmentosa (Zhao et al. 2009). Because Brr2 is an integral factor of the U5 snRNP (Laggerbauer et al. 1996), the association of this snRNP with the di-snRNP U4/U6, a prerequisite for spliceosome assembly, brings Brr2 close to its substrate, U4/U6. Indeed, this U4/U6•U5 tri-snRNP can promote U4/U6 unwinding in an ATP-dependent manner in the absence of pre-mRNA (Raghunathan and Guthrie 1998). This premature activity is, however, antagonistic for spliceosome assembly, so U4/U6 unwinding must be timed appropriately (Small et al. 2006; Bellare et al. 2008; see below).

While we know that Brr2 is required for U4/U6 unwinding, precisely how Brr2 promotes U4/U6 unwinding is unknown. The U4/U6 duplex consists of two adjacent stems (I and II) separated by an ISL in U4, named the 5' SL. Based on structural homology with the DNA helicase Hel308, Brr2 is believed to function processively in a 3'-to-5' direction (Büttner et al. 2007; Pena et al. 2009; Zhang et al. 2009; Santos et al. 2012). Thus, if it unwinds U4/U6 directly, Brr2 could initiate at the 3' end of U6 and translocate along U6, unwinding stem II first and stem I second. Alternatively, Brr2 could initiate at the 3' end of U4 and translocate along U4, unwinding stem I (Fig. 1). In this case, before Brr2 could unwind stem II, Brr2 would encounter the 5' SL in U4, an element that is bound by proteins (Nottrott et al. 2002). In the November 1, 2012, issue of *Genes & Development*, two studies distinguished between these possibilities. Beggs and coworkers (Hahn et al. 2012), using in vivo cross-linking, and Lührmann and coworkers (Mozaffari-Jovin et al. 2012), using in vitro functional

[**Keywords:** splicing; UV cross-linking; conformational state; pre-mRNA splicing; RNA–protein complex; RNA–protein cross-linking; spliceosome catalytic activation]

¹Corresponding author

E-mail jstaley@uchicago.edu

Article is online at <http://www.genesdev.org/cgi/doi/10.1101/gad.207514.112>.

Figure 1. Prp8-regulated and Brr2-mediated unwinding of U4/U6. New results suggest a model for U4/U6 unwinding. (i) Hahn et al. (2012) showed that Brr2 (green oval) associates with the 3' side of the 3' SL of U4, a likely site of initial recruitment to U4/U6. It has been known that Brr2 activity is repressed by the GTPase Snu114 when in the GDP state and by ubiquitin when it modifies the U4/U6•U5 tri-snRNP, but it has been unclear how inhibition is mediated. Mozaffari-Jovin et al. (2012) showed that the RNase H domain of Prp8 (red oval) binds to U4/U6 adjacent to U4/U6 stem I, especially to the central domain of U4, and prevents direct binding of Brr2 to the central domain and unwinding of adjacent U4/U6 stem I. (ii) By some unknown mechanism, perhaps involving exchange of GDP for GTP in Snu114 and/or involving deubiquitylation, the RNase H domain of Prp8 is presumably displaced from the central domain of U4. Brr2 would then interact with the central domain, as established by both Hahn et al. (2012) and Mozaffari-Jovin et al. (2012). It is not yet clear whether Brr2 would at the same time dissociate from the 3' SL of U4 at this stage. (iii) Poised to translocate in the 3'-to-5' direction along U4, Brr2 translocates from the central domain to unwind U4/U6 stem I and, most likely, also a portion of the U4 5' SL, where it may stall due to bound proteins. It is currently unclear whether Brr2 continues to unwind stem II and, if it does, how (see the text for possibilities). The base-pairing of U4/U6 is depicted with U4/U6 stem II coaxially stacked on the 5' SL, as suggested by Lescote and Westhof (2006).

assays, provided evidence that Brr2 translocates along U4 and initiates unwinding at U4/U6 stem I. This surprising result raises the question of how stem II is unwound and sets the stage for understanding how Brr2 is regulated.

Brr2 binds the central domain of U4 and unwinds U4/U6 stem I

To identify candidate RNA targets of Brr2 *in vivo*, Hahn et al. (2012) performed cross-linking and analysis of cDNAs (CRAC). In this approach, analogous to HITS-CLIP, Brr2 is cross-linked *in vivo* by UV to RNA that is within a distance of one covalent bond length, the cross-linked RNA is then selected by affinity purification of Brr2, the RNA is cloned as a cDNA library, and finally the library is deep-sequenced to identify potential Brr2-binding sites in the cell. Mutations in sequence reads, particularly deletions, can be very informative because they likely reflect the actual site of the cross-link between the RNA and Brr2. However, because UV preferentially cross-links proteins to ssRNA, this approach biases against interactions with dsRNA (Kudla et al. 2011; Bohnsack et al. 2012). For the same reason, cross-linking to an RNA strand that is complementary to another strand may imply an unwound state of the duplex, especially if cross-linking is specific to one strand.

In applying CRAC to Brr2, Hahn et al. (2012) added a clever twist. Brr2 is an unusual DExD/H-box ATPase containing two DExD/H-box modules, each consisting of two RecA-like domains, a winged helix domain, and a Sec63 domain. The investigators found that the two DExD/H-box

modules of Brr2 can be expressed separately *in vivo* without compromising growth, allowing them to apply CRAC individually to the N-terminal or C-terminal DExD/H-box modules.

Based on the CRAC results, only the N-terminal DExD/H-box module interacts with RNA, in agreement with Kim and Rossi (1999), who demonstrated that only the N-terminal DExD/H-box module functions as an RNA-dependent ATPase. Not surprisingly, given that Brr2 is a component of the U4/U6•U5 tri-snRNP, the majority of interacting RNAs derive from U4, U5, and U6 snRNA. However, based on the number of sequence hits, Brr2 interacts very differently with each snRNA. The largest number of sequence reads is derived from U4 with two specific peaks. In stark contrast and despite the requirement for Brr2 in U4/U6 unwinding, ~100-fold fewer sequence reads derive from U6, and these were distributed evenly over the majority of its sequence. Even U5 and U2 yield more sequence reads than U6. Only U1 appears to cross-link less efficiently. These data demonstrate that the major interacting partner of Brr2 is U4. Importantly, one of the regions of U4 that Brr2 interacts with includes U4/U6 stem I and sequences immediately 3' to this structure. Therefore, in accordance with anticipated translocation in a 3'-to-5' direction, Hahn et al. (2012) proposed that Brr2 interacts with the central domain of U4 adjacent to U4/U6 stem I and translocates along U4, unwinding stem I before stem II is unwound. Proposing a mechanism solely from CRAC data could be misleading, given a potential bias against interactions with dsRNA or with transient ssRNA and uncertainty

about where in the protein the cross-links derive from or whether the cross-links are functionally relevant. Nevertheless, Mozaffari-Jovin et al. (2012) independently drew the same conclusion.

In their study of Brr2, Mozaffari-Jovin et al. (2012) performed both U4/U6 binding and unwinding assays *in vitro* to identify the requirements for each activity. In these experiments, Brr2 can bind naked U4/U6 in the absence of other proteins. They found that the central domain of U4, just 3' of U4/U6 stem I, is critical for binding of Brr2 to U4/U6. Most importantly, deletion of this single-stranded region severely compromises U4/U6 unwinding. Remarkably, the single-stranded region of U6 3' of U4/U6 stem II was not sufficient to support binding or unwinding. Thus, the investigators similarly proposed that Brr2 binds the central domain of U4 adjacent to U4/U6 stem I, translocates along U4 in the 3'-to-5' direction, and unwinds U4/U6 stem I first (Fig. 1). While one can question the relevance of a reductionist study, these *in vitro* studies by Mozaffari-Jovin et al. (2012) nicely complement the *in vivo* studies by Hahn et al. (2012). Furthermore, mechanistic studies of the activation pathway for the minor spliceosome provide support for their collective view that Brr2 unwinds U4/U6 stem I first.

In metazoans, introns can be divided into two distinct classes, with one being spliced by the major U2-dependent spliceosome, conserved throughout eukaryotes, and the other being spliced by the minor spliceosome, termed the U12-dependent spliceosome (Tarn and Steitz 1996). Although this minor spliceosome shares only one of its five snRNAs with the U2-dependent spliceosome, the U5 snRNA, the proteins are largely conserved, and the other four snRNAs each correspond directly to one of the four other snRNAs of the U2-dependent spliceosome in terms of both secondary structure and function. Moreover, the snRNA rearrangements of the U2-dependent spliceosome are conserved in the U12-dependent spliceosome. In particular, U4/U6 unwinding is recapitulated by U4_{atac}/U6_{atac} unwinding in the minor spliceosome—unwinding that is also expected to be catalyzed by Brr2, especially given that Brr2 is an integral component of the U5 snRNP that is conserved between the two spliceosomes. Importantly, RNA–RNA cross-linking analysis of rearrangements during spliceosome activation in the minor spliceosome revealed an intermediate in which U4_{atac}/U6_{atac} stem I was unwound and the alternative U12/U6_{atac} helix Ia was formed but U4_{atac}/U6_{atac} stem II remained intact (Frilander and Steitz 2001). These observations provide important mechanistic support for the conclusion that Brr2 translocates along U4, and not U6, in a 3'-to-5' direction, unwinding U4/U6 stem I before stem II is unwound—a finding that has important implications for the unwinding of U4/U6 stem II and the regulation of Brr2 activity (see below).

How is U4/U6 stem II unwound?

If Brr2 had been implicated in translocating along U6 in a 3'-to-5' direction, then one could easily imagine how Brr2 could unwind stem II and then stem I in one

continuous process. However, with the implication that Brr2 translocates along U4 in a 3'-to-5' direction, it follows that Brr2 unwinds stem I but then, before reaching stem II, encounters the U4 5' SL, which is bound by proteins. How, then, is stem II unwound? There are several possibilities. First, Brr2 could continue translocating, unwinding the U4 5' SL, and dissociating bound proteins and then continue to unwind stem II directly. In support of this model, *in vitro* unwinding experiments have demonstrated that Brr2 can unwind naked U4/U6 in an ATP-dependent manner in the absence of other proteins, indicating that Brr2 has the ability to unwind both stems (Laggerbauer et al. 1998; Mozaffari-Jovin et al. 2012). Challenging this view, however, Hahn et al. (2012) did not detect substantial numbers of CRAC hits between Brr2 and either strand of U4/U6 stem II. In a second possibility, Brr2 could unwind stem I as well as the 5' SL, both of which interact with Brr2 by CRAC, and trigger the unwinding of stem II indirectly by dissociating proteins bound to the 5' SL of U4. Indeed, a trimeric protein complex interacts with and likely stabilizes stem II, but recruitment of this complex to stem II requires proteins that bind a kink turn in the 5' SL (Nottrott et al. 2002). In support of a model wherein Brr2 displaces proteins bound to the 5' SL, the CRAC data suggest extensive interactions between Brr2 and the 3' side of the U4 5' SL but not the 5' side. Furthermore, it is known that DExD/H-box ATPases can disrupt protein–RNA interactions (Jankowsky et al. 2001). Nevertheless, it is also known that the stability of a protein–RNA interaction can present a challenge to a DExD/H-box ATPase (Jankowsky et al. 2001). In one view, the specific cross-linking of Brr2 to stem I and the 5' SL could reflect a relatively stable unwinding intermediate in which stem I and the upper portion of the 5' SL are unwound but Brr2 is stalled by proteins bound to the lower portion of the 5' SL; after displacing these proteins, Brr2 might translocate rapidly through stem II, resulting in transient interactions between Brr2 and U4 that may not be easily captured by cross-linking. In a third possibility, Brr2 could jump from stem I to stem II, continuing to unwind U4/U6 without unwinding the U4 5' SL (Diges and Uhlenbeck 2005). Finally, Brr2 could unwind stem I only, blocked by the proteins bound to the 5' SL, and other factors could unwind stem II. This possibility would be surprising, however, because stem I is short and, in principle, a suitable substrate for a nonprocessive DEAD-box ATPase, while stem II is longer and more suited as a substrate for a processive DExD/H-box ATPase, such as Brr2. Further studies will be required to distinguish these possibilities.

Does the 3' region of U4 position Brr2 for unwinding?

In light of the evidence that Brr2 translocates 3' to 5' along U4 rather than U6, we need to understand the determinants underlying this specificity. A strong CRAC signal between Brr2 and the 3' side of the U4 3' SL suggests that Brr2 interacts with this terminal feature of U4. Indeed, using an electrophoretic mobility shift assay (EMSA) to measure binding of Brr2 to U4/U6, Hahn

et al. (2012) found that the U4 3' SL is required but not sufficient for binding. The importance of this interaction may be reflected by their finding that the 3' SL is essential for growth in vivo. Consistent with these findings, Hayduk et al. (2012), using a U4 reconstitution assay, have previously found evidence for a protein-binding site at the 3' side of the U4 3' SL. Their data also suggest that features of the 3' SL are involved in tri-snRNP formation. Along with the results from Hahn et al. (2012), these observations suggest that the 3' strand of the 3' SL could be an initial binding platform for Brr2 that also promotes tri-snRNP formation (Fig. 1).

Despite this evidence for a role for the 3' strand of the 3' SL, Mozaffari-Jovin et al. (2012), also using EMSA assays, found that the 3' SL did not contribute significantly to binding of Brr2 to U4/U6. Furthermore, the 3' SL was not required for unwinding of U4/U6. While these differing views are difficult to reconcile, it may be significant that Hahn et al. (2012) used full-length U6 in these experiments, while Mozaffari-Jovin et al. (2012) used a truncated variant of U6 lacking the first 54 nucleotides. It is conceivable that the 3' SL of U4 is required only in the presence of the 5' end of U6. Regardless, Hahn et al. (2012) found that the 3' SL alone does not bind Brr2, implying that other RNA parts are required, and Mozaffari-Jovin et al. (2012) showed that the central domain of U4 plays a key role in Brr2 binding as well as in U4/U6 unwinding. Consistent with an important role for the central domain, bases in this domain adjacent to stem I have been shown to be important for splicing (Wersig and Bindereif 1992). Notably, however, the central domain of U4 can be endonucleolytically cleaved after formation of U4/U6 with little consequence for splicing (Hayduk et al. 2012). So, even if the 3' SL does recruit Brr2, it is unlikely that Brr2 would translocate from that point along U4 all the way to stem I.

Regulating Brr2-mediated U4/U6 unwinding

As noted above, premature U4/U6 unwinding compromises spliceosome assembly (Bellare et al. 2008). In addition, Brr2 mutations block not only U4/U6 unwinding, but also spliceosome disassembly (Small et al. 2006), and premature disassembly would also compromise splicing. Therefore, it was anticipated that Brr2 is regulated. Furthermore, given that Brr2 is a constitutive component of the spliceosome, some mechanism other than regulated recruitment would be required to control its activity. Indeed, evidence indicates that Brr2 is regulated by at least two splicing factors, Snu114 and Prp8, which, like Brr2, are constitutive components of the U5 snRNP. Snu114, an EF2-like GTPase (Bartels et al. 2002; Brenner and Guthrie 2005), regulates both U4/U6 unwinding and spliceosome disassembly, stimulating these rearrangements when bound to GTP and inhibiting these rearrangements when bound by GDP (Small et al. 2006). Regulation of Brr2 by Prp8 was first implicated by genetic experiments that revealed that *prp8* mutations suppress *brr2-1* (Kuhn et al. 2002). Subsequently, a C-terminal fragment (CTF) of Prp8 consisting of an RNase H domain and a Jab1/MPN

domain was shown to activate Brr2 unwinding activity in vitro (Maeder et al. 2009). The RNase H domain shows homology with a family of nucleases that cleave RNA when paired to DNA, just as the related argonaute proteins cleave RNA when paired to RNA, but in Prp8, this domain does not include the full complement of catalytic residues (see below). The Jab1/MPN domain shows homology with deubiquitylating enzymes. Although in Prp8 this domain also lacks catalytic residues, Prp8 does bind ubiquitin (Bellare et al. 2006), suggesting that ubiquitin may repress the activating function of Prp8. Indeed, deubiquitylation of U4/U6•U5 tri-snRNPs in vitro derepresses U4/U6 unwinding (Bellare et al. 2008). Curiously, Prp8 itself is ubiquitylated, suggesting that an intramolecular interaction may inhibit its stimulatory role in U4/U6 unwinding (Bellare et al. 2008). The U4 snRNP component Prp3 is another factor that, in its ubiquitylated state, binds the Jab1/MPN domain of Prp8 and stabilizes the tri-snRNP (Song et al. 2010). Despite the definition of these regulatory activities, in the case of neither Snu114, Prp8, nor Prp3 is it known how the regulation of Brr2 takes place at the molecular level. Lüthmann and coworkers (Mozaffari-Jovin et al. 2012) have now provided insight into this mechanism.

Specifically, Mozaffari-Jovin et al. (2012) have now provided evidence that the RNase H domain of Prp8 can repress Brr2 activity. Using in vitro binding assays, they showed that the RNase H domain can bind to U4/U6 and that the RNase H domain requires the single-stranded regions of U4 and U6 adjacent to U4/U6 stem I, with the U4 central domain being the main target. Interestingly, they mapped a cross-link between U4/U6 and the RNase H domain to a region of Prp8 that is sensitive to mutations. The binding is not sequence-specific because the central domain of U4 could be inverted without affecting the binding affinity, suggesting that the RNase H domain recognizes structure instead. Consistent with this view, the RNase H domain was previously shown to bind a number of different RNA complexes, with the strongest binders sharing structural features with a four-helix-junction RNA (Ritchie et al. 2008), although the binding between the RNase H domain and U4/U6 di-snRNA is even stronger. Since duplex nucleic acid represents the fundamental substrate for the RNase H family, it is tempting to propose U4/U6 stem I as the dsRNA substrate for this domain of Prp8, although U4/U6 stem I is not sufficient for binding. It remains to be determined whether stem I contributes to binding and/or specificity.

Importantly, the U4 region to which the RNase H domain binds is the same region that cross-links to Brr2 and promotes both U4/U6 binding and unwinding. This overlap suggests a novel regulatory function for the RNase H domain of Prp8 in which it prevents Brr2 from accessing U4/U6 stem I, its substrate. In support of this view, Mozaffari-Jovin et al. (2012) showed that the RNase H domain competed with Brr2 for binding to U4. Furthermore, by an in vitro U4/U6 unwinding assay, the RNase H domain reduced Brr2-mediated U4/U6 unwinding. While this regulation was observed in a minimalist system, there is evidence consistent with this regulatory

mechanism functioning in vivo. Specifically, mutations that reside in the RNase H domain of *prp8* suppress a U4 mutation (U4-cs1) that hyperstabilizes the U4/U6 stem I (Kuhn and Brow 2000). U4-cs1 is impaired in U4/U6 unwinding and activation of the spliceosome (Kuhn and Brow 2000). The data from Mozaffari-Jovin et al. (2012) suggest that the *prp8* mutations suppress U4-cs1 by relieving repression of Brr2. Supporting this view, they demonstrated that one of these *prp8* suppressor mutations, V1860D, compromises binding of Prp8 to U4/U6. Still, a rigorous test of this compelling model will require testing of specific predictions. For example, if repression is only mediated indirectly through competition resulting from specific binding of U4/U6 by Prp8, then the unwinding of a control duplex substrate should not be affected by the addition of the RNase H domain. Furthermore, if the RNase H domain acts as an inhibitor of Brr2 in the context of the spliceosome, then this domain, added in *trans*, should repress splicing in extracts.

If the activity of Brr2 is repressed by binding of the RNase H domain of Prp8 to U4, then what is regulating the binding of the RNase H domain to RNA? Prp8, one of the most conserved splicing factors, has been cross-linked to all key features of an intron—the 5' SS, the branch site, and the 3' SS—and a cross-link to the 5' SS has been mapped to residues within the RNase H domain (Reyes et al. 1996). Because spliceosome assembly and activation is driven by intron recognition, an attractive model is that the RNase H domain is ultimately regulated by the substrate. Nevertheless, how could the RNase H domain be regulated at the molecular level? The adjoining Jab1/MPN domain of Prp8 has been implicated in activating Brr2 through direct association with Brr2 (Maeder et al. 2009). Furthermore, as noted above, ubiquitin represses Brr2-mediated unwinding of U4/U6 (Bellare et al. 2008) and may repress the activating function of Jab1/MPN by binding directly to this domain. In this sense, ubiquitin and the RNase H domain would perform parallel functions in repressing Brr2. Given the adjoining nature of the RNase H and Jab1/MPN domains, an attractive model for the regulation of the RNase H domain is that binding of the RNase H domain to U4 requires ubiquitin binding to the Jab1/MPN domain. Deubiquitylation could then lead to stimulation of Brr2 both by direct activation by the Jab1/MPN domain and through conformational changes compromising binding of the RNase H domain to U4/U6. This model could resolve the apparent discrepancy between the stimulation of Brr2 activity by the C-terminal domain of Prp8, containing both the RNase H and Jab1/MPN domains (Maeder et al. 2009), and the inhibition by the RNase H domain alone (Mozaffari-Jovin et al. 2012). Taken together, these data suggest that Prp8 functions as a regulatory switch, repressing Brr2 in one state but activating Brr2 in another state and thereby promoting the proper timing of Brr2-mediated unwinding.

While we know that pre-mRNA splicing is catalyzed by divalent metal ions, it has been unclear what positions these critical metals. The RNase H domain of Prp8 was revealed simultaneously by three different groups through solution of its three-dimensional structure by X-ray crys-

tallography (Pena et al. 2008; Ritchie et al. 2008; Yang et al. 2008). An unusual β -hairpin (β HP) had prevented the discovery of the RNase H domain by sequence alignment. Consequently, the discovery of the RNase H domain was exciting, given that the domain belonged to a class of enzymes that positions divalent metals to cleave phosphodiester bonds. However, the RNase H domain does not contain a complete set of residues required for catalytic activity. The domain does contain one or two conserved acidic residues in the metal-binding pocket, and mutations in these residues confer a strong temperature-sensitive phenotype in budding yeast (Pena et al. 2008; Ritchie et al. 2008). This observation has sustained the possibility that Prp8 could be directly involved in spliceosomal catalysis (Abelson 2008). Indeed, the structures of the RNase H domain in the absence of RNA may not tell the whole story: A recent structure of an RNase H paralog, Argonaute from *Kluyveromyces polysporus*, revealed that binding of a guide RNA was necessary to arrange a catalytic core with a full complement of catalytic residues (Nakanishi et al. 2012). Furthermore, the RNase H domain of Prp8 cross-links to the 5' SS, although it is unclear whether this interaction persists during catalysis (Reyes et al. 1996). Whether the RNase H domain of Prp8 does in fact contribute to the catalysis of splicing will require further studies, but the evidence is now strong that the domain plays a regulatory role in controlling Brr2 activity.

Does Brr2 play a role during the catalytic stage of splicing?

Through their CRAC analysis, Hahn et al. (2012) identified not only abundant cross-links to U4, but also a significant number of cross-links to U5—in particular, the stem-loop I of U5. This loop has the capacity to interact with the termini of the exons, suggesting that the loop plays a role in aligning the exons for exon ligation. In support of this model, the loop I is required specifically for the exon ligation step in splicing (O'Keefe et al. 1996). Thus, the association of Brr2 with this loop suggests a function for Brr2 within the catalytic center of the spliceosome during the exon ligation step of splicing. Indeed, the CRAC data demonstrate that Brr2 preferentially interacts with the reactive sites of pre-mRNA substrates. To test for a function for Brr2 at the catalytic stage of splicing, the investigators first looked for genetic interactions between Brr2 and loop I of U5. They found that small deletions or insertions in the loop, which have no phenotypes on their own, are synthetic-lethal with the *brr2-G858R* mutation. Furthermore, Hahn et al. (2012) showed that the *brr2-G858R* mutation exacerbates a range of mutations defective in the exon ligation step of splicing, including mutations in *PRP16*, *SLU7*, *PRP18*, and *SNR6* (U6 gene). In addition, they showed that after a shift to the nonpermissive temperature, the *brr2-G858R* mutant is preferentially compromised for the exon ligation step in splicing, in contrast to another *brr2* mutant that shows a conventional defect in 5' SS cleavage. Consistent with the role of Slu7 and Prp18 in promoting splicing of substrates with long branch point-to-3' SS

distances (Zhang and Schwer 1997), the *brr2-G858R* mutation compromises splicing of substrates with branch point-to-3' SS distances that are long and structured. The *brr2-G858R* mutation is unusual in that it was originally identified as a suppressor of an exon ligation defect manifested by a secondary structure inserted in the 3' exon, near the 3' SS (Lin and Rossi 1996). Currently, it is unclear whether the *brr2-G858R* mutation modulates the unwinding activity of Brr2 and thereby suggests a role for Brr2 in unwinding during the exon ligation stage. The residue G858 is part of a β HP located between motifs V and VI in the RecA-like domain within the first helicase module. Based on the structure of the paralogous DNA helicase Hel308 in complex with nucleic acid, the β HP is expected to function in separating the strands of a duplex during unwinding (Büttner et al. 2007). However, in the structure of the paralogous DExD/H-box ATPase Prp43p, the β HP functions in concert with other domains and ADP to occlude the RNA-binding site (He et al. 2010). More work is necessary to determine the consequences of the G858R mutation in Brr2.

Whether or not Brr2 functions as an ATPase at the catalytic stage, the profile of snRNA cross-links that increase in the mutant provide clues to the conformation of the snRNAs at this stage. Genetic data have suggested that after 5' SS cleavage, the U6–5' SS interaction, which defines the 5' SS cleavage site, is unwound, and U2/U6 helix I, which juxtaposes the substrate for 5' SS cleavage, transiently opens (Konarska et al. 2006; Mefford and Staley 2009), both of which could facilitate substrate repositioning for exon ligation. The CRAC data provide biochemical support for these rearrangements as well as unwinding of U2/U6 helix II. Specifically, the CRAC analysis shows an increase in cross-linking to U6 snRNA but not to U2 snRNA in the *brr2-G858R* mutant. Furthermore, the U6 sequences flanking the U6 ISL cross-link more robustly than the SL itself, providing further support for a transient opening, given the expectation that CRAC will preferentially cross-link to RNA that is single-stranded. One caveat to this interpretation is that we do not know whether these cross-links occur in an on-pathway intermediate or in an off-pathway intermediate necessary for discard of stalled intermediates (Mayas et al. 2010). Regardless, these data demonstrate that CRAC can provide not only insight into RNA–protein interactions, but also RNA conformation. Both of these capabilities will be most powerful when combined with specific affinity purification strategies that select unique snRNPs or spliceosomal intermediates, thereby allowing unambiguous assignment of interactions to a specific state.

Summary

We have known for some time that Brr2 is required for unwinding base-paired U4/U6 in a key step in the activation of the spliceosome, but we have not understood how Brr2 unwinds U4/U6 or how Brr2 is regulated to correctly time its activity. Now, with state-of-the art in vivo cross-linking approaches from the Beggs laboratory

(Hahn et al. 2012) complemented by classical biochemical approaches from the Lührmann laboratory (Mozaffari-Jovin et al. 2012), we have strong evidence that Brr2 translocates along U4, unwinding U4/U6 stem I before stem II is unwound. These complementary approaches promise to reveal RNA targets for other DExD/H-box ATPases in splicing and beyond. Importantly, we also learned from the Lührmann laboratory (Mozaffari-Jovin et al. 2012) that the RNase H domain of Prp8 can inhibit the activity of Brr2 by binding to U4 precisely where Brr2 initiates unwinding of U4/U6 stem I. As is frequently the case in science, these findings have raised many new questions. In particular, how is U4/U6 stem II unwound, and how is the regulatory activity of Prp8 itself regulated? Answers to these and other questions will likely require further experimental innovations, but such answers will be necessary before we can appreciate how the game is played.

Acknowledgments

We apologize to those whose work was not discussed or cited due to space constraints. We thank the Staley laboratory for discussion. Research in our laboratory on spliceosome rearrangements has been funded by the National Institutes of Health (GM062264 to J.P.S.).

References

- Abelson J. 2008. Is the spliceosome a ribonucleoprotein enzyme? *Nat Struct Mol Biol* **15**: 1235–1237.
- Bartels C, Klatt C, Lührmann R, Fabrizio P. 2002. The ribosomal translocase homologue Snu114p is involved in unwinding U4/U6 RNA during activation of the spliceosome. *EMBO Rep* **3**: 875–880.
- Bellare P, Kutach AK, Rines AK, Guthrie C, Sontheimer EJ. 2006. Ubiquitin binding by a variant Jab1/MPN domain in the essential pre-mRNA splicing factor Prp8p. *RNA* **12**: 292–302.
- Bellare P, Small EC, Huang X, Wohlschlegel JA, Staley JP, Sontheimer EJ. 2008. A role for ubiquitin in the spliceosome assembly pathway. *Nat Struct Mol Biol* **15**: 444–451.
- Bohnsack MT, Tollervey D, Granneman S. 2012. Identification of RNA helicase target sites by UV cross-linking and analysis of cDNA. *Methods Enzymol* **511**: 275–288.
- Brenner TJ, Guthrie C. 2005. Genetic analysis reveals a role for the C terminus of the *Saccharomyces cerevisiae* GTPase Snu114 during spliceosome activation. *Genetics* **170**: 1063–1080.
- Büttner K, Nehring S, Hopfner KP. 2007. Structural basis for DNA duplex separation by a superfamily-2 helicase. *Nat Struct Mol Biol* **14**: 647–652.
- Cordin O, Hahn D, Beggs JD. 2012. Structure, function and regulation of spliceosomal RNA helicases. *Curr Opin Cell Biol* **24**: 431–438.
- Diges CM, Uhlenbeck OC. 2005. *Escherichia coli* DbpA is a 3' → 5' RNA helicase. *Biochemistry* **44**: 7903–7911.
- Frilander MJ, Steitz JA. 2001. Dynamic exchanges of RNA interactions leading to catalytic core formation in the U12-dependent spliceosome. *Mol Cell* **7**: 217–226.
- Hahn D, Kudla G, Tollervey D, Beggs JD. 2012. Brr2p-mediated conformational rearrangements in the spliceosome during activation and substrate repositioning. *Genes Dev* **26**: 2408–2421.
- Hayduk AJ, Stark MR, Rader SD. 2012. In vitro reconstitution of yeast splicing with U4 snRNA reveals multiple roles for the 3' stem-loop. *RNA* **18**: 1075–1090.

- He Y, Andersen GR, Nielsen KH. 2010. Structural basis for the function of DEAH helicases. *EMBO Rep* **11**: 180–186.
- Jankowsky E, Gross CH, Shuman S, Pyle AM. 2001. Active disruption of an RNA-protein interaction by a DEXH/D RNA helicase. *Science* **291**: 121–125.
- Kim D, Rossi J. 1999. The first ATPase domain of the yeast 246-kDa protein is required for in vivo unwinding of the U4/U6 duplex. *RNA* **5**: 959–971.
- Konarska MM, Vilardell J, Query CC. 2006. Repositioning of the reaction intermediate within the catalytic center of the spliceosome. *Mol Cell* **21**: 543–553.
- Kudla G, Granneman S, Hahn D, Beggs JD, Tollervey D. 2011. Cross-linking, ligation, and sequencing of hybrids reveals RNA–RNA interactions in yeast. *Proc Natl Acad Sci* **108**: 10010–10015.
- Kuhn AN, Brow DA. 2000. Suppressors of a cold-sensitive mutation in yeast U4 RNA define five domains in the splicing factor Prp8 that influence spliceosome activation. *Genetics* **155**: 1667–1682.
- Kuhn AN, Reichl EM, Brow DA. 2002. Distinct domains of splicing factor Prp8 mediate different aspects of spliceosome activation. *Proc Natl Acad Sci* **99**: 9145–9149.
- Laggerbauer B, Lauber J, Lührmann R. 1996. Identification of an RNA-dependent ATPase activity in mammalian U5 snRNPs. *Nucleic Acids Res* **24**: 868–875.
- Laggerbauer B, Achsel T, Lührmann R. 1998. The human U5-200kD DEXH-box protein unwinds U4/U6 RNA duplexes in vitro. *Proc Natl Acad Sci* **95**: 4188–4192.
- Lescoute A, Westhof E. 2006. Topology of three-way junctions in folded RNAs. *RNA* **12**: 83–93.
- Lin J, Rossi J. 1996. Identification and characterization of yeast mutants that overcome an experimentally introduced block to splicing at the 3' splice site. *RNA* **2**: 835–848.
- Madhani HD, Guthrie C. 1992. A novel base-pairing interaction between U2 and U6 snRNAs suggests a mechanism for the catalytic activation of the spliceosome. *Cell* **71**: 803–817.
- Maeder C, Kutach AK, Guthrie C. 2009. ATP-dependent unwinding of U4/U6 snRNAs by the Brr2 helicase requires the C terminus of Prp8. *Nat Struct Mol Biol* **16**: 42–48.
- Mayas RM, Maita H, Semlow DR, Staley JP. 2010. Spliceosome discards intermediates via the DEAH box ATPase Prp43p. *Proc Natl Acad Sci* **107**: 10020–10025.
- Mefford MA, Staley JP. 2009. Evidence that U2/U6 helix I promotes both catalytic steps of pre-mRNA splicing and rearranges in between these steps. *RNA* **15**: 1386–1397.
- Mozaffari-Jovin S, Santos KE, Hsiao HH, Will CL, Urlaub H, Wahl MC, Lührmann R. 2012. The Prp8 RNase H-like domain inhibits Brr2-mediated U4/U6 snRNA unwinding by blocking Brr2 loading onto the U4 snRNA. *Genes Dev* **26**: 2422–2434.
- Nakanishi K, Weinberg DE, Bartel DP, Patel DJ. 2012. Structure of yeast Argonaute with guide RNA. *Nature* **486**: 368–374.
- Nottrott S, Urlaub H, Lührmann R. 2002. Hierarchical, clustered protein interactions with U4/U6 snRNA: A biochemical role for U4/U6 proteins. *EMBO J* **21**: 5527–5538.
- O'Keefe RT, Norman C, Newman AJ. 1996. The invariant U5 snRNA loop 1 sequence is dispensable for the first catalytic step of pre-mRNA splicing in yeast. *Cell* **86**: 679–689.
- Pena V, Rozov A, Fabrizio P, Lührmann R, Wahl MC. 2008. Structure and function of an RNase H domain at the heart of the spliceosome. *EMBO J* **27**: 2929–2940.
- Pena V, Jovin SM, Fabrizio P, Orłowski J, Bujnicki JM, Lührmann R, Wahl MC. 2009. Common design principles in the spliceosomal RNA helicase Brr2 and in the Hel308 DNA helicase. *Mol Cell* **35**: 454–466.
- Raghubathan PL, Guthrie C. 1998. RNA unwinding in U4/U6 snRNPs requires ATP hydrolysis and the DEIH-box splicing factor Brr2. *Curr Biol* **8**: 847–855.
- Reyes J, Kois P, Konforti B, Konarska M. 1996. The canonical GU dinucleotide at the 5' splice site is recognized by p220 of the U5 snRNP within the spliceosome. *RNA* **2**: 213–225.
- Ritchie DB, Schellenberg MJ, Gesner EM, Raithatha SA, Stuart DT, Macmillan AM. 2008. Structural elucidation of a PRP8 core domain from the heart of the spliceosome. *Nat Struct Mol Biol* **15**: 1199–1205.
- Santos KE, Jovin SM, Weber G, Pena V, Lührmann R, Wahl MC. 2012. Structural basis for functional cooperation between tandem helicase cassettes in Brr2-mediated remodeling of the spliceosome. *Proc Natl Acad Sci* doi: 10.1073/pnas.1208098109.
- Small EC, Leggett SR, Winans AA, Staley JP. 2006. The EF-G-like GTPase Snul14p regulates spliceosome dynamics mediated by Brr2p, a DEXD/H box ATPase. *Mol Cell* **23**: 389–399.
- Song EJ, Werner SL, Neubauer J, Stegmeier F, Aspdén J, Rio D, Harper JW, Elledge SJ, Kirschner MW, Rape M. 2010. The Prp19 complex and the Usp4Sart3 deubiquitinating enzyme control reversible ubiquitination at the spliceosome. *Genes Dev* **24**: 1434–1447.
- Tarn WY, Steitz JA. 1996. A novel spliceosome containing U11, U12, and U5 snRNPs excises a minor class (AT-AC) intron in vitro. *Cell* **84**: 801–811.
- Wersig C, Bindereif A. 1992. Reconstitution of functional mammalian U4 small nuclear ribonucleoprotein: Sm protein binding is not essential for splicing in vitro. *Mol Cell Biol* **12**: 1460–1468.
- Will CL, Lührmann R. 2011. Spliceosome structure and function. *Cold Spring Harb Perspect Biol* **3**: 1–23.
- Yang K, Zhang L, Xu T, Heroux A, Zhao R. 2008. Crystal structure of the β -finger domain of Prp8 reveals analogy to ribosomal proteins. *Proc Natl Acad Sci* **105**: 13817–13822.
- Yean SL, Wuenschell G, Termini J, Lin RJ. 2000. Metal-ion coordination by U6 small nuclear RNA contributes to catalysis in the spliceosome. *Nature* **408**: 881–884.
- Zhang X, Schwer B. 1997. Functional and physical interaction between the yeast splicing factors Slu7 and Prp18. *Nucleic Acids Res* **25**: 2146–2152.
- Zhang L, Xu T, Maeder C, Bud LO, Shanks J, Nix J, Guthrie C, Pleiss JA, Zhao R. 2009. Structural evidence for consecutive Hel308-like modules in the spliceosomal ATPase Brr2. *Nat Struct Mol Biol* **16**: 731–739.
- Zhao C, Bellur DL, Lu S, Zhao F, Grassi MA, Bowne SJ, Sullivan LS, Daiger SP, Chen LJ, Pang CP, et al. 2009. Autosomal-dominant retinitis pigmentosa caused by a mutation in SNRNP200, a gene required for unwinding of U4/U6 snRNAs. *Am J Hum Genet* **85**: 617–627.

Spliceosome activation: U4 is the path, stem I is the goal, and Prp8 is the keeper. Let's cheer for the ATPase Brr2!

Klaus H. Nielsen and Jonathan P. Staley

Genes Dev. 2012, **26**:

Access the most recent version at doi:[10.1101/gad.207514.112](https://doi.org/10.1101/gad.207514.112)

Related Content

Brr2p-mediated conformational rearrangements in the spliceosome during activation and substrate repositioning

Daniela Hahn, Grzegorz Kudla, David Tollervey, et al.

[Genes Dev. November , 2012 26: 2408-2421](#) **The Prp8 RNase H-like domain inhibits Brr2-mediated U4/U6 snRNA unwinding by blocking Brr2 loading onto the U4 snRNA**

Sina Mozaffari-Jovin, Karine F. Santos, He-Hsuan Hsiao, et al.

[Genes Dev. November , 2012 26: 2422-2434](#)

References

This article cites 46 articles, 18 of which can be accessed free at:

<http://genesdev.cshlp.org/content/26/22/2461.full.html#ref-list-1>

Articles cited in:

<http://genesdev.cshlp.org/content/26/22/2461.full.html#related-urls>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).