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Structural and Functional Modularity of the U2 snRNP in pre-mRNA Splicing

Clarisse van der Feltz[1], **Aaron A. Hoskins**[1,*]

[1]Department of Biochemistry, 433 Babcock Dr., University of Wisconsin-Madison, Madison, WI 53706 USA

Abstract

The U2 small nuclear ribonucleoprotein (snRNP) is an essential component of the spliceosome, the cellular machine responsible for removing introns from precursor mRNAs (pre-mRNAs) in all eukaryotes. U2 is an extraordinarily dynamic splicing factor and the most frequently mutated in cancers. Cryo-electron microscopy (cryo-EM) has transformed our structural and functional understanding of the role of U2 in splicing. In this review, we synthesize these and other data with respect to a view of U2 as an assembly of interconnected functional modules. These modules are organized by the U2 small nueclear RNA (snRNA) for roles in spliceosome assembly, intron substrate recognition, and protein scaffolding. We describe new discoveries regarding the structure of U2 components and how the snRNP undergoes numerous conformational and compositional changes during splicing. We specifically highlight large scale movements of U2 modules as the spliceosome creates and rearranges its active site. U2 serves as a compelling example for how cellular machines can exploit the modular organization and structural plasticity of an RNP.

Keywords

pre-mRNA; RNA; SF3B1; snRNP; splicing; spliceosome

Introduction

The spliceosome is a prime example of how proteins and RNAs can function harmoniously together as RNPs. This mega-Dalton complex catalyzes the removal of introns and joining of exons in eukaryotic pre-mRNA. It contains five non-coding snRNAs (U1, U2, U4, U5, and U6) that recognize intronic splice sites by base pairing, organize the assembly of protein splicing factors, and catalyze the cleavage and ligation reactions. Each snRNA associates with a number of proteins and form snRNP complexes that comprise the spliceosome. Decades of study have revealed that the snRNPs are exceedingly dynamic and undergo dramatic changes in structure, base pairing, and protein composition during splicing. The cryo-EM revolution has led to an avalanche of structural information on the spliceosome in

[*]**Corresponding Author:** Aaron A. Hoskins, ahoskins@wisc.edu, 608-890-3101.

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which these snRNP dynamics can now be visualized (Kastner et al. 2019; Plaschka et al. 2019; Yan C et al. 2019).

Several excellent reviews have recently been published on the overall structures of spliceosomes, the molecular mechanism of splicing, and on the U6 and protein-only Nineteen Complex (NTC) spliceosome subunits (Wahl et al. 2009; de Almeida and O'Keefe 2015; Scheres and Nagai 2017; Didychuk et al. 2018; Kastner et al. 2019; Plaschka et al. 2019; Yan C et al. 2019). Here, we focus on the U2 snRNA and snRNP. The U2 snRNA has a remarkably modular design with distinct domains for interaction with the catalytic U6 snRNA, identification of the intronic branch site (BS) sequence, and scaffolding of large protein assemblies. These U2 modules work in concert for accurate identification of the BS, to regulate BS association with the spliceosome active site, and to link distant spliceosomal subunits together during structural transitions. We begin by providing an overview of the splicing reaction and identification of the key factors involved. Since we focus primarily on *Saccharomyces cerevisiae* (yeast) spliceosome, we most often employ the nomenclature commonly used for yeast splicing factors. Human homologs are identified in Table 1. We then describe U2 components in detail, highlight the role of each U2 snRNA module, and end with a description of the large-scale rearrangements of U2 during splicing as revealed by cryo-EM.

Overview of pre-mRNA Splicing by the Spliceosome

A spliceosome is a single turnover enzyme, responsible for recognizing and removing a single intron while joining the flanking exons. Spliceosomes process through a cycle of four distinct phases: assembly, activation, catalysis, and disassembly (Figure 1). During this procession, spliceosomes undergo dramatic compositional and structural reconfigurations. These result in formation of at least ten distinct, isolable complexes which have been characterized by biochemical, analytical, and very recently structural methods (Figure 1). Assembly begins by ATP-independent formation of the E (or "Early") complex in which the U1 snRNP recognizes the 5′ SS (Lerner et al. 1980; Ruby and Abelson 1988). During this stage, RNA-binding proteins also associate with the BS region: BBP (Msl5) and Mud2 in yeast or SF1 and U2AF1/U2AF2 ($U2AF^{35}$/$U2AF^{65}$) in humans (Ruskin et al. 1988; Abovich et al. 1994; Berglund et al. 1997). The A complex spliceosome is formed by ATP-dependent exchange of these RNA-binding proteins for the U2 snRNP (Konarska and Sharp 1987).

The A complex represents the earliest spliceosomal complex in which both sites used for 5′SS cleavage are identified by base pairing interactions between the intron and snRNAs: U1 snRNA/5′SS and U2 snRNA/BS duplexes. Biochemical and structural data from a number of organisms suggest that U1 and U2 can contact one another in A complex to form cross-intron bridging interactions that may be important for subsequent spliceosome assembly. In humans, biochemical data support direct interaction between U1 snRNA stem loop IV with the U2 protein SF3A1 (Donmez et al. 2007; Sharma et al. 2014). In *S. pombe*, the N-terminal serine/arginine (SR)-rich domain of the transiently-associated U2 protein Prp5 (discussed in detail later in this review) can associate with the U1 the snRNP and facilitate A complex formation (Shao et al. 2012). *S. cerevisiae* homologs lack both U1

snRNA stem loop IV and the SR domain of Prp5. However, recent cryo-EM structures of A complex spliceosomes show an interaction between the yeast U1 protein Prp39 and the U2 protein Lea1 (Plaschka et al. 2018). This interaction has not yet been functionally tested.

The tri-snRNP (a preformed complex of the U4, U5, and U6 snRNPs) joins the A complex to form the pre-B complex spliceosome. Initially, the tri-snRNP joins through protein/protein and snRNA/snRNA interactions with the U2 snRNP (Bai et al. 2018; Plaschka et al. 2018). At this point, the activation process begins by release of the U1 snRNP and transfer of the 5′ SS to the U6 snRNA to form B complex (Konarska and Sharp 1987; Staley and Guthrie 1999; Charenton et al. 2019). Activation next involves release of the U4 snRNA and many tri-snRNP proteins followed by recruitment of the Nineteen Complex (NTC) (Lamond et al. 1988; Yean and Lin 1991; Chan et al. 2003; Fabrizio et al. 2009; Hoskins et al. 2016). Activation ends by release of most U2 snRNP proteins and entry of the U2 snRNA/BS duplex into the active site to form the catalytically competent B* complex (Lardelli et al. 2010; Crawford et al. 2013; Rauhut et al. 2016; Yan C et al. 2016; Wan et al. 2019).

Catalysis occurs through two trans-esterification steps. In B*, the branch site has entered the catalytic center with the 2′ OH of the branch point adenosine positioned next to the catalytic magnesium ions and the scissile 5′ SS phosphodiester bond (Wan et al. 2019). During the first catalytic step, the nucleophilic 2′ OH of the branch point adenosine frees the 5′ exon and forms an intron lariat structure with three phosphodiester bonds attached to the branch point. Spliceosomes containing these products are described as C complexes.

For the second catalytic step, the spliceosome active site must be rearranged into the C* complex in which factors that promote 5′SS cleavage have been released, the U2/BS duplex reoriented, and the 3′SS has been allowed to dock into the active site (Fica et al. 2017; Yan C et al. 2017). The free 3′ OH of the 5′ exon then attacks the phosphodiester bond at the intron-3′ exon junction. This results in ligated exons and excision of the lariat intron in P (or “Product”) complex. Finally, release of the ligated exon product forms the intron lariat spliceosome (ILS) complex which is subsequently disassembled and recycled. While many intron lariats are degraded, some introns contain non-coding RNAs that serve various functions in the cell (Morgan et al. 2019; Parenteau et al. 2019; Zaffagni and Kadener 2019). A minor spliceosome with different snRNAs instead of U1, U2, U4 and U6 targets a small subset of introns with different consensus splice sequences (reviewed in (Turunen et al. 2013)). While some variations to the pathway do exist (Crispino and Sharp 1994; Tarn and Steitz 1994; Maroney et al. 2000; Shcherbakova et al. 2013), this fundamental reaction cycle for splicing is conserved in spliceosomes from yeast to humans.

The splicing cycle is driven forwards by eight RNA-dependent ATPases that are associated with transitions between complexes and release of splicing factors (Figure 1) (Staley and Guthrie 1998; Semlow and Staley 2012). The DEAD-box ATPases Sub2 and Prp5 are necessary for U2 association with the BS, although the mechanisms for this are not yet clear. Sub2 has been proposed to facilitate release of Mud2 from the pre-mRNA as well as participate in other early steps in spliceosome assembly (Kistler and Guthrie 2001; Libri et al. 2001; Zhang M and Green 2001). Prp5 interacts with U2 snRNP and may be involved in remodeling of the U2 snRNA during BS recognition (Ruby et al. 1993; Perriman R and Ares

2010). In the pre-B complex spliceosome, the DEAD-box ATPase Prp28 initiates the transfer of the 5′ SS from base pairing with U1 snRNA to pairing with U6 snRNA during B-complex formation (Staley and Guthrie 1999). This is followed by unwinding of the U4/U6 duplex during activation by the DEIH-box ATPase Brr2 (Raghunathan and Guthrie 1998). The last ATPase to act during activation is the DEAH-box Prp2, which initiates release of most U2 proteins and allows the U2/BS duplex to enter the spliceosome active site (Lardelli et al. 2010; Krishnan et al. 2013; Rauhut et al. 2016; Yan C et al. 2016; Wan et al. 2019). During catalysis and disassembly, the DEAH-box ATPases Prp16 and Prp22 promote the C to C* and P to ILS complex transitions, respectively (Semlow and Staley 2012; Semlow et al. 2016). The DEAH-box protein Prp43 facilitates disassembly of the ILS as well as other stalled spliceosome complexes (Arenas and Abelson 1997; Mayas et al. 2010). Prp2, Prp5, Prp16, and Prp43 have all been proposed to either directly or indirectly change U2 conformation, underscoring the close connection between U2 structure and the splicing process (Perriman R et al. 2003; Lardelli et al. 2010; Perriman R and Ares 2010; Wlodaver and Staley 2014; Liang and Cheng 2015; Fourmann et al. 2016; Galej et al. 2016; Wan et al. 2016; Fica et al. 2017; Yan C et al. 2017).

Modular Composition of the U2 snRNA

The U2 snRNA was identified nearly 50 years ago as a highly abundant, low molecular weight RNA that could be fractionated from hepatoma ascites cell nuclear extracts (Reddy et al. 1972; Shibata H. et al. 1975). Lerner and Steitz soon thereafter showed that U2 formed complexes with proteins and identified these complexes snRNPs (Lerner and Steitz 1979). The Steitz laboratory went on then prove U2's role in the splicing reaction by targeting the snRNA for degradation with RNaseH in HeLa nuclear extract and observing splicing inhibition (Black et al. 1985). The U2 snRNA is linearly organized into four modules spanning ~100 nucleotides (nt) that are well conserved between yeast and humans: the 5′ stem I region, the branch point recognition sequence (BPRS), the stem II region, and the Sm binding site/3′ stem loops (Figure 2)(Ares 1986). The first three modules and the Sm binding site are extremely well conserved, while the 3′ stem loops are more variable. In humans the 3′ end of U2 contains stems III and IV, but in yeast only a potential stem IV homolog is present. Yeast and many other fungal species contain a large fungal domain insertion in stem IV (~940 nt) that is nonessential for splicing (Igel and Ares 1988; Shuster and Guthrie 1988). Highlighting U2's high degree of evolutionary conservation, the shorter human U2 snRNA can replace yeast U2 snRNA *in vivo* (Shuster and Guthrie 1990).

The U2 snRNA contains a number of post-transcriptional modifications (Figure 2). Several of these modifications are consitutively included in the snRNA, such as the conserved pseudouridine modifications found in the BPRS (Reddy et al. 1972; Massenet et al. 1999). The human U2 snRNA also contains a number of other pseudouridine and 2′-*O*-methyl modifications (Reddy et al. 1972; Shibata Hirotoshi et al. 1974). These modifications are essential for U2 snRNP assembly and snRNA function (Yu et al. 1998; Donmez et al. 2004; Wu G, Adachi, et al. 2016). Recently, inducible pseudouridine modifications have been found in the yeast U2 snRNA (Wu G et al. 2011). Several U2 pseudouridine modifications have been implicated in regulating BS usage by the yeast spliceosome under certain growth conditions (Wu G, Adachi, et al. 2016).

Protein Composition of the U2 snRNP

The U2 snRNA interacts with a large cohort of proteins which can be grouped into subcomplexes related to their ability to be isolated after dissociation from the fully assembled 17S U2 snRNP (Behrens et al. 1993; Brosi, Groning, et al. 1993) (Table 1). These major subcomplexes and their combined molecular weights in yeast are SF3a (125 kDa), SF3b (360 kDa), and the snRNA 3′ binding proteins (130 kDa). Like U2 snRNA, most of the protein snRNP components are conserved between yeast and human (Table 1). The U2 proteins act in concert to support large U2 snRNA movements, guide pre-mRNA/U2 interactions, and make connections with other splicing factors.

SF3—The splicing factor 3 (SF3) complex spans the BPRS, stem II, and Sm binding modules of U2 (Figure 3). It was first isolated by chromatographic fractionation of the RNA splicing activity in HeLa nuclear extract (Kramer and Utans 1991). Further ion exchange chromatography of SF3 revealed the presence of two stable subcomplexes: SF3a and SF3b (Brosi, Hauri, et al. 1993). These complexes were subsequently shown to be components of the 17S U2 snRNP and to be required for spliceosome assembly (Behrens et al. 1993; Brosi, Groning, et al. 1993). Unlike the 3′ binding proteins, the SF3 complex does not remain bound to U2 throughout the splicing cycle (Lardelli et al. 2010). As described below, it is destabilized during activation to permit entry of the U2/BS duplex into the spliceosome active site.

SF3a—SF3a is a hetero-trimer composed of the yeast proteins Prp9, Prp11 and Prp21 (human SF3a60, SF3a66, and SF3a120, respectively) (Figure 3 and Table 1). Cryo-EM structural data show that Prp9 interacts directly with the Sm protein ring of U2 and Prp21. Prp21 in turn interacts with Prp11, which contacts the SF3b proteins Cus1 and Hsh155 as well as the U2/BS duplex (Bai et al. 2018; Plaschka et al. 2018). Combined, these proteins link the 5′ and 3′ regions of the U2 snRNA to one another by bridging the 3′ binding proteins to the SF3b subcomplex.

SF3b—SF3b is the largest U2 snRNP subcomplex and is comprised of the yeast proteins Hsh155, Rse1, Cus1, Hsh49, Rds3, and Ysf3 (human SF3b155/SF3B1, SF3b130, SF3b145, SF3b49, SF3b14b, and SF3b10, respectively) (Figure 3 and Table 1) (Dziembowski et al. 2004). SF3b surrounds the U2 BPRS and interacts with the U2/BS duplex throughout spliceosome assembly. It connects U2 to other spliceosome components through protein-protein interactions. Hsh155 serves as a scaffold for SF3b and interacts with most other SF3b proteins, the SF3a proteins Prp9 and Prp11, the transiently associated U2 proteins Cus2 and Prp5, and the U2/BS duplex (Tang et al. 2016; Carrocci et al. 2017; Loerch et al. 2019; Talkish et al. 2019). Hsh155 is largely composed of 20 HEAT repeats arranged in a ring conformation similar to a lock washer. The human homolog of Hsh155 (SF3B1) contains other protein interaction domains outside of the HEAT repeats, including five tandem U2AF ligand motifs (ULMs) that bind a number of other splicing factors including SF1 and U2AF1/2 (Loerch and Kielkopf 2016; Loerch et al. 2019). Since U2AF1 directly interacts with the conserved AG dinucleotide present at the 3′ SS, the multitude of interactions between SF3B1, SF1, and U2AF1/2 allow formation of complexes bridging the BS to the 3′ SS (Loerch and Kielkopf 2016; Mollet et al. 2006). Thus, U2 snRNP

positioning in humans is a highly interconnected process impacting both BS and 3′ SS selection.

Rds3 is located in the center of Hsh155's ring, and two of its three Zn-fingers directly contact the U2/BS duplex (van Roon AMM et al. 2008; Plaschka et al. 2018). A mutation in the first finger disrupts U2 snRNP stability and weakens interactions to the RES complex (the RES complex is described below) (Wang Q and Rymond 2003). In humans, one additional small protein, SF3b14a, interacts with SF3B1 as well as the U2 BPRS (MacMillan et al. 1994; Will et al. 2001; Dybkov et al. 2006; Schellenberg MJ, Dul, E. L., and A. M. MacMillan 2011). SF3b has been proposed to play roles in nuclear mRNA export and in tethering the U2 snRNP to histone pre-mRNAs to facilitate splicing-independent 3' end formation (Friend et al. 2007; Wang K et al. 2019).

3′ Binding Proteins—The Sm binding site/3′ stem loop module of U2 contains the most tightly associated U2 proteins (Figure 3; Lea1/U2A′, Msl1/U2B", and the Sm ring) which remain bound even under high salt conditions as part of the 12S U2 snRNP complex (Behrens et al. 1993). Unlike the other U2 subcomplexes, the 3′ binding proteins remain associated with the snRNA throughout the splicing cycle. The Sm ring is a seven-member heteromeric ring, comprised of Sm B, D1, D2, D3, E, F, and G. This protein complex is found on all of the spliceosomal U snRNPs with the exception of U6 which is bound by the homologous LSm complex (Hinterberger et al. 1983; Bringmann and Luhrmann 1986; Achsel et al. 1999; Mayes et al. 1999). Each of the seven Sm proteins shares a similar Sm-fold, and the snRNA passes through the Sm ring in a wheel and spoke-type arrangement (Leung et al. 2011). Even though they are present on multiple snRNPs, the seven Sm proteins have unique N and C terminal tails that enable distinct interactions with different snRNAs (Pomeranz Krummel et al. 2009; Leung et al. 2011; Montemayor et al. 2014).

Stem IV of U2 is bound by two other 3′ binding proteins: Lea1 (human U2A′) and Msl1 (human U2B"). Neither Lea1 or Msl1 are essential for yeast growth, although strains lacking these proteins grow slowly and exhibit impaired splicing (Caspary and Seraphin 1998). These proteins interact with one another and with the SmB and SmD3 components of the U2 Sm ring (Price et al. 1998; Schwer and Shuman 2015; Galej et al. 2016; Wan et al. 2016). Msl1/U2B" is highly homologous to the U1 snRNP protein Mud1/U1A. Specificity of Msl1/U2B" for U2 over U1 is due in part to cooperative binding with Lea1/U2A′ to stem IV (Scherly et al. 1990; Bentley and Keene 1991; Williams and Hall 2014a). In *C. elegans*, the U1A homolog can functionally replace U2B", and *Drosophila* only contain a single, dual-function protein (SNF) with specificity for both the U1 and U2 snRNAs (Polycarpou-Schwarz et al. 1996; Saldi et al. 2007; Williams and Hall 2014b; Weber et al. 2018).

Cus2 and Prp5—Additional factors associate with the U2 snRNA but are not stable components of the snRNP. Prior to U2 incorporation into the spliceosome A complex, Cus2 and Prp5 function to promote U2/BS duplex formation. Cus2 likely acts as an RNA chaperone to facilitate formation of the U2 stem IIa structure (Figure 2) and associates with U2 via protein interactions with Cus1, Prp11, and a U2AF homology motif (UHM) interaction with a conserved Hsh155 ULM (Ares and Igel 1990; Zavanelli and Ares 1991;

Wells and Ares 1994; Zavanelli et al. 1994; Yan D et al. 1998; Perriman R and Ares 2000; Rodgers et al. 2016; Loerch et al. 2019; Talkish et al. 2019).

Prp5 is a conformationally dynamic DEAD-box RNA-dependent ATPase which is required for A complex formation in the presence of Cus2 (Dalbadie-McFarland and Abelson 1990; Perriman R et al. 2003; Beier et al. 2019; Talkish et al. 2019). The exact function of Prp5 is not yet clear; however, it has been proposed to release Cus2 from U2 and facilitate annealing of the BPRS with the intron BS as discussed in greater detail below (Perriman R et al. 2003; Perriman R and Ares 2010). Prp5 is presumably recruited to U2 by interactions with the HEAT repeats of Hsh155 and cross-links to multiple nucleotides in U2's BPRS region (Liang and Cheng 2015; Tang et al. 2016; Carrocci et al. 2018). These interactions place Prp5 near the SF3b subcomplex, adjacent to the U2/BS duplex (Figure 3). Mutations in Prp5 can influence BS usage in yeast presumably by changing U2/Prp5 interactions to facilitate or inhibit recruitment of the tri-snRNP (Xu YZ and Query 2007; Liang and Cheng 2015). Recent structural and single molecule FRET data suggest that these U2/Prp5 interactions could be dependent on Prp5 conformation which are in turn correlated with Prp5's ATPase activity (Zhang ZM et al. 2013; Beier et al. 2019).

RES—Affinity purification of yeast Snu17-containing complexes led to the identification of the REtention and Splicing (RES) complex composed of yeast Ist3/Snu17, Bud13, and Pml1. While these proteins are not essential in yeast, deletion of the RES complex decreases splicing efficiency and results in leakage of unspliced pre-mRNAs from the nucleus and into the cytosol (Dziembowski et al. 2004). Additionally, the RES complex is necessary for activating the splicing of a subset of yeast pre-mRNAs expressed during meiosis (Scherrer and Spingola 2006). The RES complex binds HEAT repeats 8–10 of Hsh155 and has extensive contacts with the NTC-associated proteins Prp45, Cwc22, and the reverse transcriptase domain of U5 snRNP protein Prp8 (Rauhut et al. 2016; Yan C et al. 2016). Recently, Bao et al showed that the absence of the RES complex results in inefficient transition between the spliceosome B and B^{act} complexes (Bao et al. 2017). This inefficiency was probably due to premature association of the Prp2 ATPase with the spliceosome leading to its disassembly. The role of the RES complex in metazoans is less clear, and Bud13 is present in sub-stoichiometric amounts in human spliceosomes (Frankiw et al. 2019). It is possible that metazoan RES is only necessary to facilitate splicing of a subset of introns. This role for the RES is supported by recent observations that the RES complex regulates retention of small, GC-rich introns in zebrafish and during viral infection (Fernandez et al. 2018; Frankiw et al. 2019).

The 5′ Stem I Module

Prior to interacting with the pre-mRNA, the U2 5′ stem I module likely forms a 20-nucleotide hairpin structure near the 5′ end of the snRNA. Sashital *et al* showed that this structure can form in the absence of interaction partners and contains two stable sub-regions separated by wobble pairs (Sashital et al. 2007). In the context of the U2 snRNP, protein components of SF3b (Hsh49) and SF3a (Prp9) specifically recognize this region of U2 snRNA (Dybkov et al. 2006; van Roon AM et al. 2017). Human U2 stem I contains several post-transcriptional modifications that increase stem stability *in vitro* (Sashital et al. 2007).

The U2 5′ stem I module is conformationally dynamic, and the stem I hairpin must unwind for pairing with the U6 snRNA during spliceosome assembly (Figure 4A). Consistent with this prediction is the observation that hyperstabilizing mutations in stem I reduce splicing in human cells likely due to inhibition of unwinding and U6 pairing (Wu J and Manley 1992). Once paired with U6, the 5′ half of the stem I module forms U2/U6 helix II, while the 3′ half forms U2/U6 helices Ia and Ib. It is not known how the stem I hairpin is unwound by the splicing machinery; however, pairing with U6 at nucleotides flanking the 5′ portion of stem I may nucleate helix II formation and lead to stem I disruption.

In both yeast and human cryo EM structures of pre-B and B complex spliceosomes, U2/U6 helix II is the predominant feature connecting U2 to the tri-snRNP (Plaschka et al. 2017; Bai et al. 2018; Plaschka et al. 2018; Zhan et al. 2018; Charenton et al. 2019). This di-snRNA helix is conserved and disruption impacts viability (Wu J and Manley 1992; Field and Friesen 1996). In yeast, mutations in splicing factors involved in activation and catalysis were shown to be synthetic lethal with U2/U6 helix II disruption (Xu D et al. 1996; Xu D et al. 1998). This is consistent with a role for U2/U6 helix II in stabilization of the spliceosome during structural rearrangements such as those that take place during activation. In activated spliceosomes, several NTC proteins interact directly with U2/U6 helix II (Figure 4B) (Yan C et al. 2016). The functional consequences of these interactions are not yet clear. It is possible that these proteins are necessary to prevent helix II unwinding and/or that the spliceosome uses helix II as an anchor for other splicing factors.

In B^{act} spliceosomes the U2 stem I module becomes fully paired to U6 by formation of U2/U6 helix I. Concurrent formation of U2/U6 helix I and U6's internal stem-loop positions U6 bases to coordinate magnesium ions necessary for catalysis (Figure 4A). U2/U6 helix I can be further divided into helix Ia and Ib each with their own impact on splicing.

U2/U6 helix Ib contains the U6 catalytic triad, an AGC motif that together with a bulged nucleotide in the U6 internal stem loop (U80, Figure 4A) coordinates the magnesium ions used for splicing chemistry (Yean et al. 2000; Fica et al. 2014; Galej et al. 2018). The catalytic triad is directly paired to U2 bases 21–23 to form helix 1b, and this helix is surrounded by splicing proteins (Figure 4B) (Galej et al. 2016; Wan et al. 2016; Bao et al. 2018). Evidence from modeling, structural, and single molecule studies suggest that formation of U2/U6 helix Ib and U2 stem I are competitive with one another (Sun and Manley 1995; Sashital et al. 2004; Sashital et al. 2007; Karunatilaka and Rueda 2014). How this competition plays out during spliceosome activation is not yet known, but the protein shell surrounding U2/U6 helix I may function in part to direct the RNAs towards conformations productive for splicing.

U2/U6 helix Ia is separated from helix Ib by a two-nucleotide bulge in U2 (U24 and A25, yeast numbering). Shortening helix Ia by deletion of U2 nucleotides at position 26 and 27 causes splicing to stall during activation (Ryan and Abelson 2002). On the other hand, mutations at these positions can give rise to defects in the 2^{nd} catalytic step indicating an additional role for helix Ia during or just prior to exon ligation (McPheeters and Abelson 1992; Chang and McPheeters 2000). Consistent with these 2^{nd} step defects are observations that mutations in U2/U6 helix I have genetic interactions with Prp16, the RNA-dependent

ATPase that initiates rearrangements between catalytic steps (Madhani and Guthrie 1994; Hogg et al. 2014; Mefford and Staley 2009). Recently, strong biochemical evidence has been obtained for a conformational change within the U6 internal stem loop during catalysis consistent with the spliceosome switching from catalytic to noncatalytic conformations and back again (Eysmont et al. 2019). While the catalytic conformation predicted by Eysmont et al. has not yet been captured by cryo-EM, it could involve a conformational change in U2/U6 helix Ia, Ib, or both given helix Ib's role in structuring the spliceosome active site.

The BPRS Module

The U2 snRNA BPRS module contains the highly conserved 5′-GΨAGUA-3′ sequence which pairs with the intron BS to specify the nucleophile used during the 1st step of splicing and, consequently, the site of the branched nucleotide responsible for the lariat structure of excised introns. Intronic BS are almost always located near the 3' ends of introns and have a consensus sequence of UACUA**A**C in yeast and yUn**A**y in humans (Pikielny et al. 1983; Keller and Noon 1984; Gao et al. 2008). Human BS sequences can be highly divergent from one another and the vast majority of human BS remain unannotated. Compounding this problem is a recent observation obtained by meta-analysis of ~1.3 trillion sequencing reads from over 17,000 RNA-Seq data sets that many human introns can use multiple BS locations (Pineda and Bradley 2018). Even though this produces mRNA products of identical sequence, it is apparent that BS usage is being regulated by tissue-specific and development-specific programs.

BS recognitions occurs by pairing between the U2 snRNA 5′-GΨAGUA-3′ and the intron BS, resulting in a bulged RNA duplex in which the adenosine branch point nucleophile is flipped out from the duplex (Figure 5A) (Query et al. 1994). How the U2/BS duplex is formed is most clear with yeast BS, which nearly always have high complementarity with U2. It is less clear how mismatches between human BS and U2 impact the bulged duplex structure. Disruption of this U2/BS duplex in the case of non-consensus branch-site sequences reduces the efficiency or blocks splicing (Parker et al. 1987; Wu J and Manley 1989). As we describe below, cryo-EM studies have shown that the U2/BS duplex position changes dramatically during splicing. These structures have also revealed extended (and unexpected) base pairing between U2 and the intron both up and downstream of the BS (Galej et al. 2016; Rauhut et al. 2016; Wan et al. 2016; Yan C et al. 2016; Bertram et al. 2017; Zhang X et al. 2017). The functional importance of extended pairing outside the BS consensus region is an open question.

Mutational studies of the yeast U2 and intronic BS have significantly contributed to our functional understanding of the spliceosome. Surprisingly, the BS consensus and pairing sequence with U2 can be covaried with one another to an astounding degree and still function in splicing. For example, the WT U2/BS duplex of UACUAAC/GUAGUA (branch point underlined) can be exchanged (AUGAUAG/CAUCAU) or be composed entirely of GC base pairs (GGGCCAC/GGGCCC) (Smith et al. 2009). Instead, the position and identify of the bulged nucleotide are much more critical with A branch points functioning optimally in splicing, G branch points less well, and pyrimidine branch points having very poor activity (Smith et al. 2009).

Possible explanations for the high selectivity for adenosine branch points have come from cryo-EM structures of catalytic spliceosomes. During the 1st step of catalysis, the branch point adenosine N1 and 6-amino groups hydrogen bond to a BS uridine nucleotide (UAC**U**AAC, bold) (Figure 5B) (Galej et al. 2016). This establishes an unusual conformation of the U2/BS duplex that includes a base triple interaction between the U2/BS duplex and the 5′ SS. In sum, these interactions position the BS nucleophile towards the scissile phosphate. This conformation is potentially disrupted by pyrimidines at the branch positions.

A BS guanosine actually permits the 1st step of splicing but inhibits the 2nd step. This is surprising since exon ligation does not involve a reaction occurring at the BS. A possible explanation for this comes from cryo-EM structures of P complex spliceosomes, which have revealed that recognition of the 3′ SS sequence (YAG) comes, in part, from Hoogsteen pairing between the branch and 3′ SS (Figure 5C) (Bai et al. 2017; Liu et al. 2017; Wilkinson et al. 2017). Branch points other than adenosine can potentially distort the BS/3′ SS interaction, which in turn may misalign the 3′ SS in the active site and prevent exon ligation (Wilkinson et al. 2017). In humans, the impact of BS mutation is hard to predict *a priori* due to long intron lengths and cis-regulatory elements in pre-mRNAs (reviewed in (Ohno et al. 2018)). While mutations at human BS have been shown to be disruptive, these can also result in activation of cryptic BS not normally used by the spliceosome. Complicating this issue is that results from genome-wide approaches to identify human BS suggest that some non-consensus BS are recognized by unusual pairing interactions between U2 and the BS. These alternative pairing schemes may include looping out of mis-matched intron nucleotides (Gao et al. 2008; Taggart et al. 2017). In effect, this means that these introns contain BS which are non-contiguous.

Finally, we note that the BPRS module itself may adopt alternate structures prior to pairing with the intron BS. Disruption of the base of U2 stem I permits formation of an alternate stem loop structure with the BPRS module, called the branch point interacting stem loop (BSL, Figure 2A) (Perriman R and Ares 2010). This structure positions the U2 GUAGUA sequence within the loop and at the top of the BSL. This potentially facilitates formation of the U2/BS duplex by formation of a loop-loop ‘kissing’ complex intermediate (Paillart et al. 1996). Mutations that disrupt or stabilize the BSL structure interact genetically with mutations in the DEAD-box ATPase Prp5 which in turn can be cross-linked to the base of the BSL (Perriman R and Ares 2010; Liang and Cheng 2015). Current data suggest that Prp5 plays a role in BSL unwinding during U2/BS duplex formation.

BPRS Module Dynamics and Interactions with Splicing Factors—Once paired, the U2/BS duplex forms a functional unit that is sequentially recognized by many different splicing factors. Starting in the A complex and lasting until B^{act} (Figure 1), the U2/BS duplex is surrounded by the U2 snRNP proteins Hsh155, Rds3, and Ysf3 (Gozani et al. 1998; Rauhut et al. 2016; Yan C et al. 2016; Plaschka et al. 2017; Bai et al. 2018; Plaschka et al. 2018). Intronic RNA flanking the BS on the 5′ end travels through a protein tunnel formed by Prp11, Prp9, Cus1, and Hsh49 (Plaschka et al. 2017; Bai et al. 2018; Plaschka et al. 2018). Interactions with Hsh155 are particularly noteworthy as the protein’s HEAT repeats grasp the U2/BS duplex as well as provide a pocket for binding the bulged branch

point nucleotide (Figure 6A, B). Several of the contacts between Hsh155 and the phosphodiester backbone of the U2/BS duplex are essential for yeast viability (Carrocci et al. 2018). Moreover, it has been shown that mutation of the branch point adenosine pocket can change BS usage in yeast, presumably by altering how the protein accommodates duplexes containing mismatches (Carrocci et al. 2018). Different structural techniques using model RNAs have provided evidence for structural flexibility and dynamics of the U2/BS duplex including presence or absence of a bulged adenosine at the branch point position (Berglund et al. 2001; Newby and Greenbaum 2002; Lin and Kielkopf 2008; Kennedy et al. 2019). Given its extensive interactions with the U2/BS duplex, Hsh155 could help to induce and stabilize formation of the bulged duplex.

Hsh155 provides binding sites for a large number of other splicing factors including Cus2 and Prp5 (Wang Q et al. 2005; Tang et al. 2016; Carrocci et al. 2017). The protein may help to localize these factors near the U2 BPRS to facilitate U2/BS duplex formation. For reasons that are not yet clear, yeast Prp5 can remain associated with the U2 snRNP when mismatches are present between the U2 snRNA and intron BS (Liang and Cheng 2015). This fidelity checkpoint prevents tri-snRNP binding and subsequent spliceosome assembly and splicing of the pre-mRNA. These stalled complexes are disassembled by the DEAH-box ATPase Prp43 based on observations made using a chimera of Prp43 and a G-patch protein necessary for activation of its helicase activity (Fourmann et al. 2016). The Prp43 chimera physically interacted with the U2 proteins Hsh155 and Cus1 and was able to unwind U2/BS complexes. It was recently discovered that the poorly studied human splicing factor SUGP1 interacts with human Hsh155 (SF3B1) and contains a functionally important G-patch domain (Zhang J et al. 2019). It is possible that SUGP1 could provide the G-patch domain necessary for stimulation of Prp43 helicase activity for early spliceosomal disassembly.

The U2/BS duplex remains encased by SF3 proteins until B^{act} complex formation when the Prp2 ATPase releases the SF3 complex from the spliceosome (Lardelli et al. 2010). Single molecule FRET experiments provided initial evidence that the U2/BS duplex and 5′ SS are not juxtaposed for catalysis until after SF3 release (Crawford et al. 2013; Krishnan et al. 2013). This surprising finding was subsequently confirmed by cryo-EM analysis of assembled spliceosomes, which revealed the position of the U2/BS duplex changes by more than 160 Å between B and C complexes (Figure 7A) (Galej et al. 2016; Rauhut et al. 2016; Wan et al. 2016; Yan C et al. 2016; Plaschka et al. 2017; Wan et al. 2019). Prp2 docks onto Hsh155 and associates with the pre-mRNA substrate downstream of the U2/BS duplex. How Prp2 releases SF3 is unknown but involves triggering Hsh155 opening to free the U2/BS duplex. The duplex then integrates with the spliceosome active site in stages (Galej et al. 2016; Rauhut et al. 2016; Wan et al. 2016; Yan C et al. 2016; Wan et al. 2019). Interactions between the duplex and 1^{st} step splicing factors Yju2 and Cwc25 aid in final positioning of BS for catalysis.

The U2/BS duplex is again reoriented by the spliceosome after completion of the 1^{st} catalytic step. Between the C and C* complexes, the U2/BS duplex shifts by 60 Å, rotating in relation to the active site (Figure 7B). This movement is strikingly similar to a rearrangement observed in the active site of the evolutionarily related group II intron ribozyme during reverse splicing into DNA (Haack et al. 2019). In the spliceosome, this

movement provides space for the 3′ exon while reorienting the branched adenosine and 5′ SS to coordinate the 3′ SS bases as described above (Figure 5C) (Bai et al. 2017; Liu et al. 2017; Wilkinson et al. 2017). The reoriented U2/BS duplex is held in place through interactions with Prp8's endonuclease domain and Prp17 (Wilkinson et al. 2017). No further major structural rearrangements of the U2/BS duplex have yet been observed in P or ILS complex spliceosomes (Bai et al. 2017; Liu et al. 2017; Wan et al. 2017; Wilkinson et al. 2017).

BPRS Module-Binding Proteins are Mutated in Cancers and Targets for Chemotherapy—SF3B1 is the most commonly mutated splicing factor in a host of diseases including myelodysplastic syndromes, chronic lymphocytic leukemia, uveal melanoma, breast cancer, bladder cancer, and pancreatic cancer (Chen et al. 2011; Yoshida et al. 2011; Biankin et al. 2012; Ellis et al. 2012; Harbour et al. 2013; Landau et al. 2013). The highest mutational frequency is found in hematologic cancers (Dvinge et al. 2016). SF3B1 mutation causes selection of erroneous BS in a subset of introns, which in turn alters selection of the 3′ SS. This most often leads to use of a cryptic, upstream 3′ SS (Darman et al. 2015; Alsafadi et al. 2016). Consequently, these cells produce a number of incorrect mRNA isoforms, a large fraction of which may be degraded by nonsense-mediated mRNA decay (Darman et al. 2015; DeBoever et al. 2015; Alsafadi et al. 2016). It is unclear how SF3B1 mutation directly promotes cancer; however, recent work has demonstrated that these splicing perturbations can alter key cellular signaling pathways (Joshi et al. 2017; Lee et al. 2018; Xu JJ et al. 2019).

Hotspots for SF3B1 mutation cluster in HEAT repeats 4–7 (Figure 6A, B). Work in yeast has revealed that mutation of the homologous amino acids in Hsh155 can both alter BS selection (either increasing or decreasing fidelity for the UACUAAC sequence) and perturb interactions between Hsh155 and Prp5 (Tang et al. 2016; Carrocci et al. 2017). This suggests that the underlying mechanism for changes in BS usage observed in humans is conserved in yeast. This mechanism could involve alteration of an open-to-closed conformational change Hsh155/SF3B1 undergoes upon binding of the U2/BS duplex (Figure 6B) and/or changing the interaction with Prp5 (Cretu et al. 2016; Finci et al. 2018; Tang et al. 2016; Carrocci et al. 2017). Additionally, hotspot mutations in SF3B1 occur in a segment of the protein which contacts the intron immediately 3′ of the BS. In humans, this intron region usually contains a number of pyrimidine nucleotides (the polypyrimidine tract). Alteration of interactions between SF3B1 and the polypyrimidine tract by hotspot mutations could also play a role in BS selection. As mentioned above, the recent identification of SUGP1 in humans as a SF3B1 interacting protein indicates that other protein factors contribute to the splicing phenotype in cancers.

A meta-analysis of a large number of mutations in 33 different tumor types has identified a second, less prevalent SF3B1 mutational hot spot located in HEAT repeats 9–12 (Seiler et al. 2018). These mutations are most often associated with acute myeloid leukemia and endometrial and bladder cancers. Transcriptome analysis indicates that these mutations are associated with selection downstream of 3′ SS, rather than the upstream sites commonly found associated with mutations in HEAT repeats 4–6. Whether or not this is driven by altering BS usage is not known.

In addition to being a hot spot for cancer mutations, several anti-tumor agents target SF3B1. Molecules such as spliceostatin A, herboxidiene, and pladienolide B strongly inhibit spliceosome assembly and block cell proliferation (Figure 6C, D) (Kaida et al. 2007; Kotake et al. 2007; Roybal and Jurica 2010; Hasegawa et al. 2011). Biochemical and structural data indicate that these inhibitors have overlapping binding sites and prevent splicing by occupying the branch point adenosine binding pocket to occlude U2/BS duplex association and prevent SF3B1 conformational change (Corrionero et al. 2011; Folco et al. 2011; Effenberger et al. 2016; Teng et al. 2017; Cretu et al. 2018; Finci et al. 2018). This results in a blockage in spliceosome assembly and splicing inhibition (Folco et al. 2011). The *in vivo* mechanism of tumor growth inhibition by these compounds is complex as the excision of different introns is impacted in various ways: the removal of some introns can be strongly inhibited while others can be unaffected (Corrionero et al. 2011; Vigevani et al. 2017). Tumor cells, particularly those containing splicing factor mutations, are extremely sensitive to SF3B1 inhibitors, and an orally-available pladienolide B derivative is currently in clinical trials for cancer treatment (Agrawal et al. 2018; Seiler et al. 2018).

The Stem II Module

As we have described for the stem I and BPRS modules, the U2 snRNA stem II module can also adopt multiple structures and be influenced by protein binding partners. Stem II forms two mutually exclusive structures: stem IIa and IIc (Figure 8A). Biochemical and structural work support formation of stem IIa during spliceosome assembly followed by a structural rearrangement to stem IIc prior to catalysis (Ares and Igel 1990; Hilliker et al. 2007; Perriman RJ and Ares 2007; Galej et al. 2016; Wan et al. 2016; Plaschka et al. 2017; Wan et al. 2019). Stem IIb remains present in both conformations. Formation of stem IIa is essential for viability in yeast; however, neither stem IIb or stem IIc formation are required (Ares and Igel 1990).

During assembly of the spliceosome A complex, the formation of stem IIa is chaperoned by the RNA-binding protein Cus2 (TAT-SF1 in humans). Genetic and single molecule FRET data support a role for Cus2 in stabilizing the stem IIa conformation (Yan D et al. 1998; Rodgers et al. 2016). Cryo-EM data of spliceosome A and B complexes show that stem IIa associates with the SF3 components Prp9 and Cus1 in an arrangement which also places stem IIb in contact with RRM2 of Hsh49 (Plaschka et al. 2017; Plaschka et al. 2018). Genetic data agree with such an arrangement since SF3A protein alleles can compensate for weakening of stem IIa by mutation (Champion-Arnaud and Reed 1994; Wells and Ares 1994).

Despite the importance of stem IIa formation to spliceosome assembly (Zavanelli and Ares 1991), Cus2 is not essential in yeast (Yan D et al. 1998). Stem II can reversibly toggle between stem IIa and IIc in a RNA-only model system (Rodgers et al. 2016). In the absence of Cus2, stem IIa formation may happen spontaneously, and SF3 proteins may either capture stem IIa or promote its formation. Without Cus2, spliceosome A complex formation is ATP-independent and the ATPase activity of Prp5 is no longer required, although Prp5 itself remains essential (Perriman R and Ares 2000; Perriman R et al. 2003). Recent work suggests that the primary role for Cus2 is to enforce the ATP-dependence of spliceosome

assembly (Talkish et al. 2019). Defects in Prp5 activity result in Pol II accumulation on introns in yeast, and this is repressed by deletion of Cus2 (Chathoth et al. 2014). It may be that this particular ATP-dependent step in splicing has evolved to provide a checkpoint for proper co-transcriptional coupling.

Stem IIc formation occurs during the transition from the spliceosome B^{act} to B* complex and is coordinated with release of SF3A and SF3B proteins by Prp2 (Rauhut et al. 2016; Yan C et al. 2016; Wan et al. 2019). This suggests that stem IIa to IIc remodeling and docking of the U2/BS duplex into the spliceosome active site are coupled to one another. In the catalytic spliceosome, the U5 snRNP protein Prp8 contacts both U2 nucleotides 46–55 and the U2 Sm ring (discussed below), occluding the IIa conformation (Galej et al. 2016; Wan et al. 2016; Wan et al. 2019). Stem IIc forms a stacked duplex structure with stem IIb separated by a bulged region, and this stacked duplex appears to move as a single structural element for the remainder of the splicing reaction.

After its formation, stem IIb/IIc changes both its orientation and interacting partners as splicing progresses. In both B* and C complexes, stem IIb contacts the NTC-associated proteins Ecm2 and Cwc2 (Figures 8A, B) (Galej et al. 2016; Wan et al. 2016; Wan et al. 2019). Favorable docking of the stem IIb/c structure into the Cwc2/Ecm2 binding site is consistent with IIc formation supporting 5′ SS cleavage (Hilliker et al. 2007; Perriman RJ and Ares 2007). Spliceosome remodeling to form the C* complex results in release of stem IIb/IIc by Cwc2/Ecm2 and its movement by ~50 Å (Fica et al. 2017; Yan C et al. 2017). This shifts stem IIb/c towards Prp17's WD40 domain, where it remains through the end of the splicing cycle (Bai et al. 2017; Liu et al. 2017; Wan et al. 2017; Wilkinson et al. 2017). It has been proposed that stem IIb/c converts transiently to the IIa form between the 1^{st} and 2^{nd} steps of splicing as the active site is remodeled (Hilliker et al. 2007; Perriman RJ and Ares 2007). Evidence for this model comes, in part, from genetic suppression of mutants of the Prp16 ATPase responsible for active site remodeling. These Prp16 mutants are suppressed by U2 snRNA mutants that destabilize stem IIc or stabilize IIa. At the time of this review, a structure of a C complex spliceosome containing the stem IIa conformer has not been obtained, and it is unclear how C complex can accommodate such a structure. It is possible that U2 mutations also disrupt stem IIb/IIc interactions with Cwc2/Ecm2. This may suppress Prp16 mutation by facilitating exit from the 1^{st} step active site conformation.

In yeast, the stem II region is a target for stress-induced pseudouridylation. Pseudouridylation of U56 occurs during heat stress and favors IIc formation *in vitro*, while pseudouridylation of U93 occurs during nutrient depletion and favors formation of stem IIa (Wu G et al. 2011; Wu G, Radwan, et al. 2016; van der Feltz et al. 2018). Pseudouridylation of U93 is regulated by the TOR signaling pathway under starvation conditions (Wu G, Radwan, et al. 2016). This potentially links a fundamental cellular response to the core splicing machinery via stem II structure and function. Whether or not this results in splicing regulation of specific transcripts under stress conditions remains to be seen.

The Sm Binding Site/3′ Stem Loop Module

In comparison with the previous three U2 snRNA modules we have discussed, the Sm binding site/3′ stem loop module at first glance appears to be rather static. While there is no

evidence for the U2 snRNA in this region exchanging between alternate conformations or base pairing schemes during splicing, this module is nonetheless a dynamic component of the snRNP. As we will see in the following section, the Sm binding site/3′ stem loop module is reoriented several times during splicing with respect to the spliceosome active site. Along with these movements come changes in contacts between module proteins and other splicing factors.

As previously mentioned, the U2 SF3a protein Prp9 makes direct contact between the U2 Sm ring (SmD1 and SmD2) effectively linking the SF3 complex to this module (Figures 3, 9A). This interaction is observed in A and B complex spliceosomes but is disrupted by spliceosome activation and SF3 release (Plaschka et al. 2017; Bai et al. 2018; Plaschka et al. 2018). The functional consequence of this interaction is unclear. A genetic interaction has been reported between Prp9 and SmD2 in a high-throughput genetic screen (Costanzo et al. 2016); however, interpreting this result is complicated since Sm proteins are found on snRNPs other than U2, changes in snRNP stability can confound synthetic interactions, and Sm proteins exhibit some degree of functional redundancy (Wells and Ares 1994; Schwer et al. 2016).

After activation, the Sm binding site/3′ stem loop module engages in a number of other putative interactions with splicing factors based on cryo-EM data. These interactions can be difficult to interpret due to the low resolution of density in this region in many structures. Nonetheless, it is clear that the Sm ring, Lea1, and Msl1 all closely approach various splicing factors including the NTC component Syf1 and the WD40 domain of the 2nd step splicing factor Prp17 (Galej et al. 2016; Wan et al. 2016; Bai et al. 2017; Fica et al. 2017; Wan et al. 2017; Wilkinson et al. 2017; Yan C et al. 2017; Wan et al. 2019). As previously mentioned, Lea1 also is involved in a cross-intron bridging interaction with the U1 protein Prp39 in A complex (Plaschka et al. 2018). The combined structural data have led to a model in which Lea1 may specifically and sequentially interact with multiple splicing factors to facilitate positioning of the U2 Sm binding site/3′ stem loop module (Plaschka et al. 2017; Bai et al. 2018; Plaschka et al. 2018).

One of the most intriguing interactions that occurs in this module is that between the U2 Sm ring and the RNase H domain of Prp8 in B* and C complex spliceosomes (Figure 9B) (Galej et al. 2016; Wan et al. 2016; Wan et al. 2019). Prp8 is the largest spliceosome protein and provides a scaffold for much of the spliceosome including the RNA active site. The RNase H domain is an extremely dynamic subunit of Prp8 and is found in a different conformation in nearly every spliceosome complex (Plaschka et al. 2019). Importantly, mutations in the RNase H domain impact toggling of the spliceosome between 1st and 2nd step catalytic conformations (Liu L et al. 2007; Schellenberg MJ et al. 2013; Mayerle et al. 2017). In B* and C complexes, the RNase H domain is found abutted against the U2 Sm ring, contacting SmD1 and SmD2—the same Sm proteins which contact Prp9 in unactivated spliceosomes. It may be possible that Sm proteins bound to U2 could influence the catalytic steps in splicing by directly perturbing Prp8's RNase H domain conformation or function.

Molecular Choreography of the U2 snRNP During Splicing

As we have described in this review for each individual module, the U2 snRNP is an extremely dynamic (if not the *most* dynamic) component of the spliceosome. We finish by zooming out to illustrate and summarize how splicing results in a highly choreographed, 180° rotation of the BPRS, stem II, and Sm binding site/3′ stem loop modules around the spliceosome (Figure 10).

Beginning with the spliceosome pre-B complex, U2/U6 helix II (Figure 4) provides the main anchor point between U2 and the tri-snRNP (Bai et al. 2018; Plaschka et al. 2018). In our description of U2 movements, this helix serves as a useful axis around which U2 rotations can be described (Figure 10A). In the pre-B structure, every module of U2 is engaged in interactions with non-U2 splicing factors. The stem I module is paired with the U6 snRNA to form U2/U6 helix II; the BPRS and stem II modules bind the U2/BS duplex and interactions form between SF3 and tri-snRNP proteins; and the Sm binding site/3′ stem loop module proteins and snRNA interact with the U1 snRNP. The numerous contacts between tri-snRNP and SF3 proteins (Hsh155 contacts the U4/U6 snRNP protein Prp3, Cus1 and Rse1 contact the U4/U6 snRNA helicase Brr2) suggest that communication between U2 and the tri-snRNP is critical for proceeding through the next steps in splicing.

Activation begins with release of the U1 snRNP and transfer of the 5′ SS from the U1 to the U6 snRNA to form B complex. Only minor changes in U2 position occur at this stage. U2 repositioning begins in earnest during the next transition, from the B to B^{act} spliceosome (Figures 10A, B). Release of the U4 snRNA permits formation of U2/U6 helix I, its ensnarement by NTC proteins, and tugging of U2 modules towards the nascent spliceosome active site. At this point, the branch point nucleophile remains ~50Å away from the catalytic core, blocked from approaching the active site by a protein wall formed in part by the SF3 proteins Hsh155, Cus1 and Prp11 (Rauhut et al. 2016; Yan C et al. 2016). In B^{act}, the N-terminus of the SF3 protein Prp11 reaches down to the active site and contacts the 5′ SS while the remainder of the protein stays associated with the U2/BS duplex (Yan C et al. 2016). The functional significance of Prp11 linking the 5′ SS and BS prior to formation of the spliceosome active site is another open question.

The active site becomes fully assembled and functional during the B^{act} to B* transition by release of the SF3 proteins by Prp2. The specific order of SF3 protein release is not yet known; however, biophysical data support retention of Prp11 and Cus1 until late in the activation process (Ohrt et al. 2012). The U2/BS duplex enters the active site in stages with the help of the 1^{st} step splicing factors Yju2 and Cwc25 (Figure 10A) (Liu YC et al. 2007; Chiu et al. 2009). Yju2 binds the U2/BS duplex first, while Cwc25 binds subsequently to nudge the BS into the active site (Wan et al. 2019). Eventually, this results in another large rotation of U2 around the U2/U6 helix II axis prior to 5′ SS cleavage and C complex formation.

Prp16 initiates spliceosome remodeling between the C and C* complexes. This represents the last major rearrangement of U2. U2 is repositioned to extricate the U2/BS duplex from the active site and orient the duplex for interactions with the 3′ SS (Figure 10A, B). After this step, U2 remains in a relatively fixed position relative to U2/U6 helix II in C*, P, and

ILS complexes. It should be noted that C* structures obtained thus far do not have the 3′ SS resolved in the active site, and subtle U2 movements may be necessary for successful exon ligation. The final repositioning of U2 is accompanied by coordinated changes in the NTC, and the NTC protein Syf1 travels alongside its U2 partners (Galej et al. 2016; Wan et al. 2016; Fica et al. 2017; Yan C et al. 2017).

Conclusion

Each module of the U2 snRNA plays multiple roles during splicing; often changing configuration, orientation, interacting partners, or all three as splicing progresses. Cryo-EM structures coupled with data from genetic, biochemical, and biophysical experiments have provided a detailed look into how these modules contribute to spliceosome assembly and catalysis. Nonetheless, many outstanding questions remain concerning basic features of U2 structure and function.

While we have described several structures of U2 in various spliceosomes, structures of U2 alone and in the absence of U2/BS pairing have not yet been obtained. These will be vital for illuminating how U2 conformational changes are coupled with BS recognition. Current U2 structures provide little insight into how the snRNP would accommodate a BSL structure (Figure 2) or how the BSL and intron are oriented prior to pairing. Future structural work will need to be complemented by molecular studies in cells to understand U2 snRNP composition, interacting partners, and dynamics *in vivo*—all areas in which our knowledge is currently lacking.

In addition, little is known concerning how U2 association differs between strong BS (those with high complementarity between the intron BS and U2 BPRS) and weak BS (those containing mismatches between the RNAs). The vast majority of structural information to date has been obtained using model splicing substrates with very strong BS. Where weak BS have been used, limited resolution in the U2/BS duplex region limits interpretation of how the mismatched duplexes are accommodated (Plaschka et al. 2019). In humans, auxiliary splicing factors are often critical for promoting spliceosome assembly at these sites (Matlin et al. 2005). Higher resolution structures of human U2 bound to weak BS and spliceosomes in complex with their regulators are needed for understanding how splicing at these sites takes place.

Additional structural data will prove useful for explaining the function of the spliceosome's ATPases. Little is known about exactly how Prp2 manages to release the SF3 complex from U2 or how Prp5 functions in an assembly checkpoint. It is possible that the Prp5 checkpoint involves coordinated conformational changes in Prp5, Hsh155, or both (Beier et al. 2019). Clarification of these steps in spliceosome assembly is important for understanding both fundamental steps in BS recognition as well as how they can be coupled with transcription (Herzel et al. 2017). Since splicing and transcription do not occur at equilibrium in the cell, detailed kinetic analysis of this checkpoint *in vitro* and *in vivo* is needed for understanding how RNAs partition between pathways leading to productive or unproductive co-transcriptional processing. Molecular understanding of these steps is particularly desirable given that BS recognition appears to malfunction in several human diseases and that a

number of clinically important splicing inhibitors target the U2 snRNP protein Hsh155/SF3B1 (Agrawal et al. 2018).

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Abbreviations

snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoprotein
BS	branch site
BSL	branch point interacting stem loop
BPRS	branch point recognition sequence
ILS	Intron Lariat Spliceosome
NTC	Nineteen Complex
RES	Retention and Splicing complex

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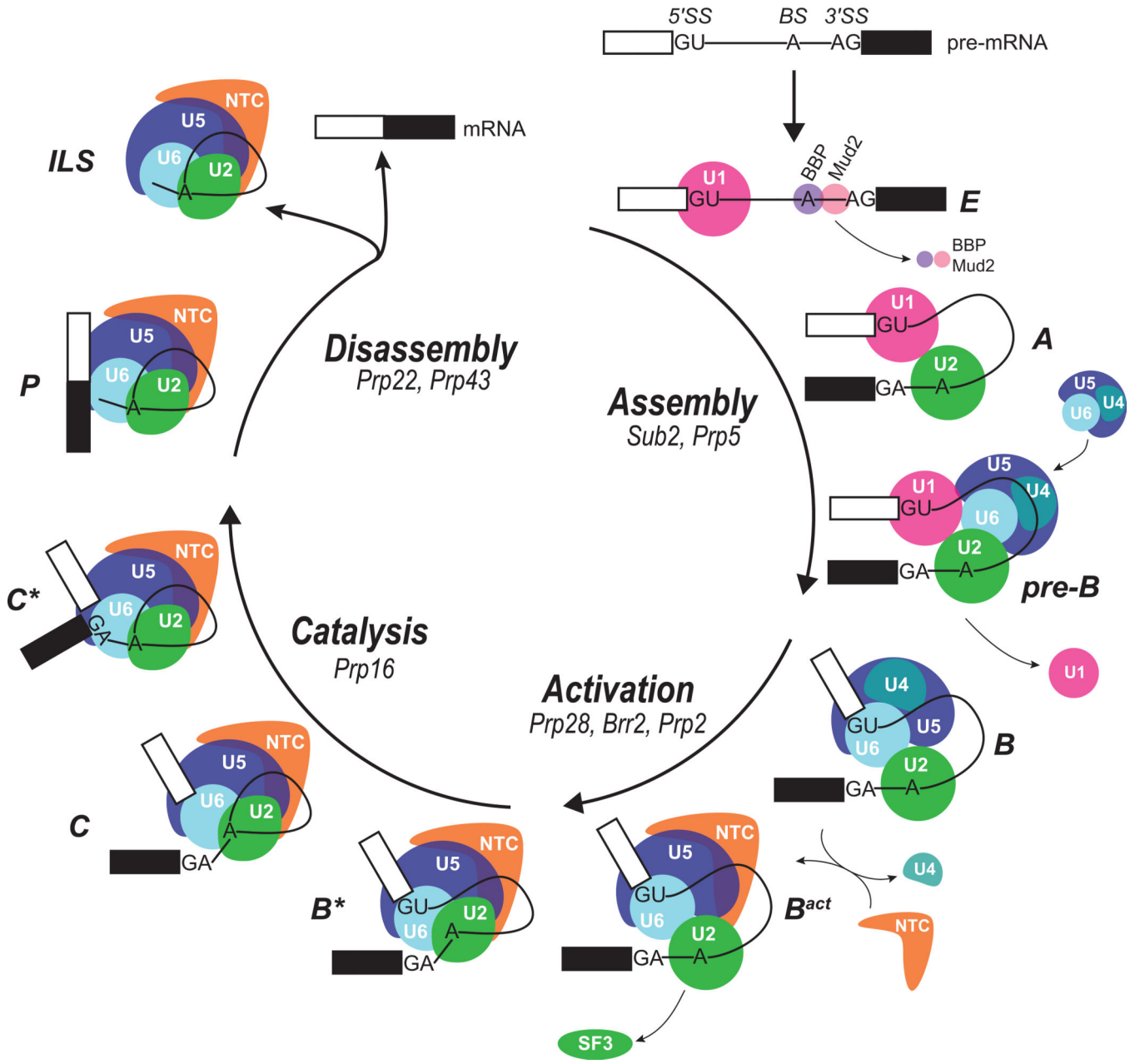

Figure 1.

Overview of the stages of pre-mRNA splicing. The spliceosome catalyzes splicing of the pre-mRNA by recognition of the 5′ SS, BS, and the 3′ SS. Many steps are required for excision of the intron and concomitant ligation of the 5′ (white box) and 3′ (black box) exons. The five U snRNPs and the NTC are labeled. BBP and Mud2 are splicing factors that recognize the BS in the E complex. The spliceosome complexes shown are the best characterized, and their respective names (***E***, ***A***, etc…) are indicated in bold italics. DExD/H-box ATPases necessary for splicing are named in italics below the corresponding stage during which they function (*Sub2*, *Prp5*, etc…). A color version of this figure is available online.

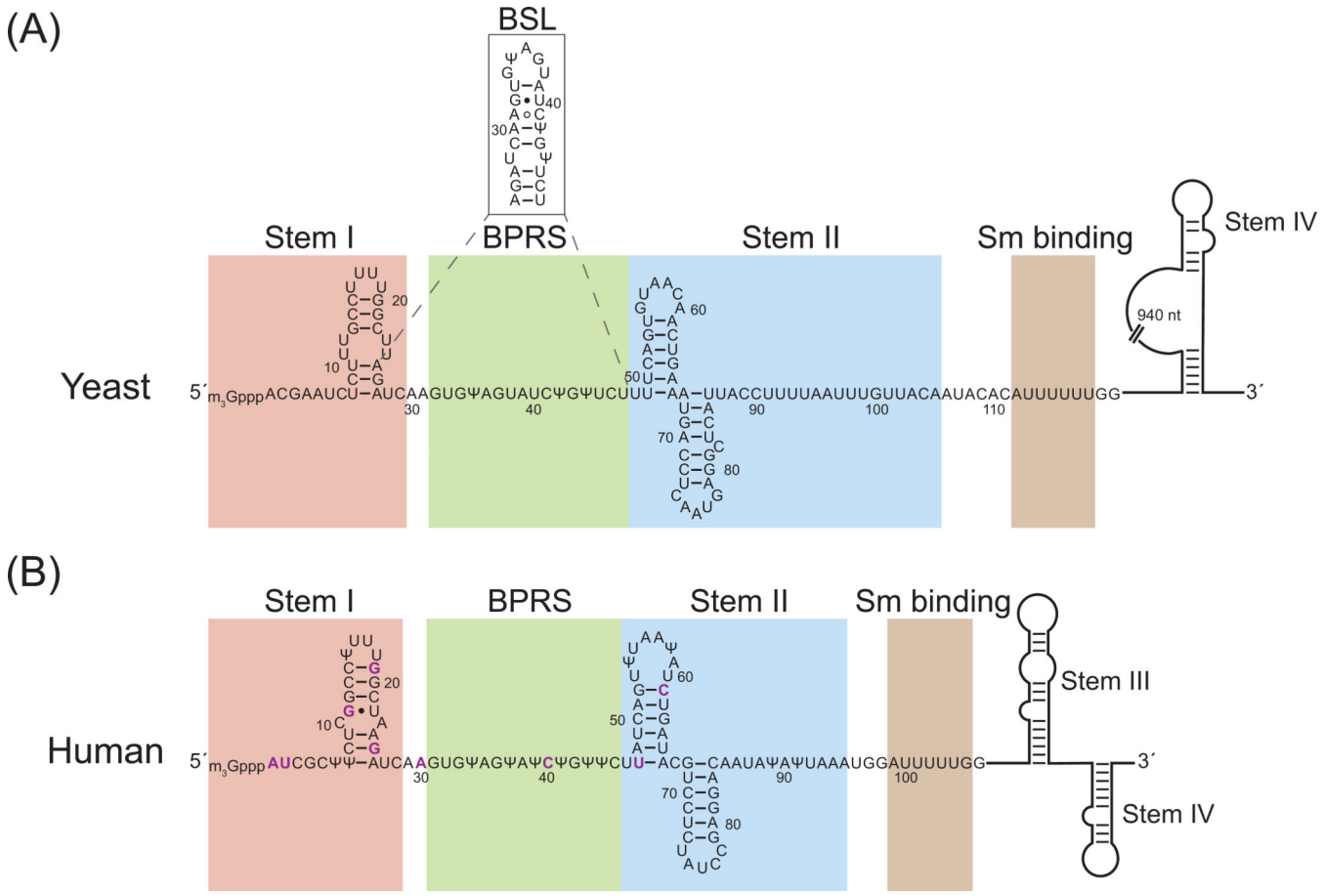

Figure 2.

Sequences and secondary structures of the conserved modules of the yeast (**A**) and human (**B**) U2 snRNAs. The modules consist of the Stem I (pink), branch point recognition sequence (BPRS, green), Stem II (blue), and Sm binding site/3′ stem loop (tan) regions. Also depicted are constitutively incorporated pseudouridines (Ψ) and positions of 2′ *O*-methylation (bold purple). An alternate structure for the yeast stem I and BPRS module called the branch point interacting stem loop (BSL) is shown in the inset. Human U2 snRNA includes a m^6Am post-transcriptional modification at position 30. A color version of this figure is available online.

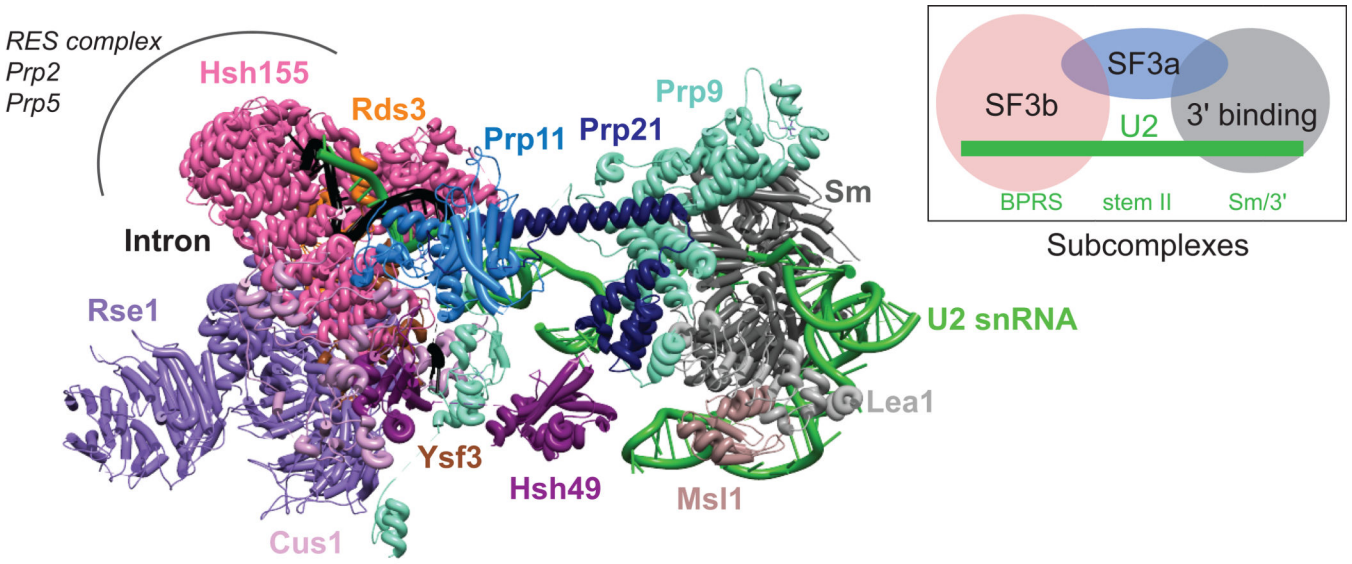

Figure 3.

Organization of the protein components of the U2 snRNP in the spliceosome B complex (PDB ID:5NRL). The BPRS, Stem II, and Sm binding site/3′ stem loop modules of U2 snRNA (green) scaffold an arrangement of snRNP proteins that anchors the SF3b subcomplex near the BPRS. The region of Hsh155 which interacts with Prp5, Prp2 and the RES complex is noted. A schematic representation of this organization is shown in the inset. A color version of this figure is available online.

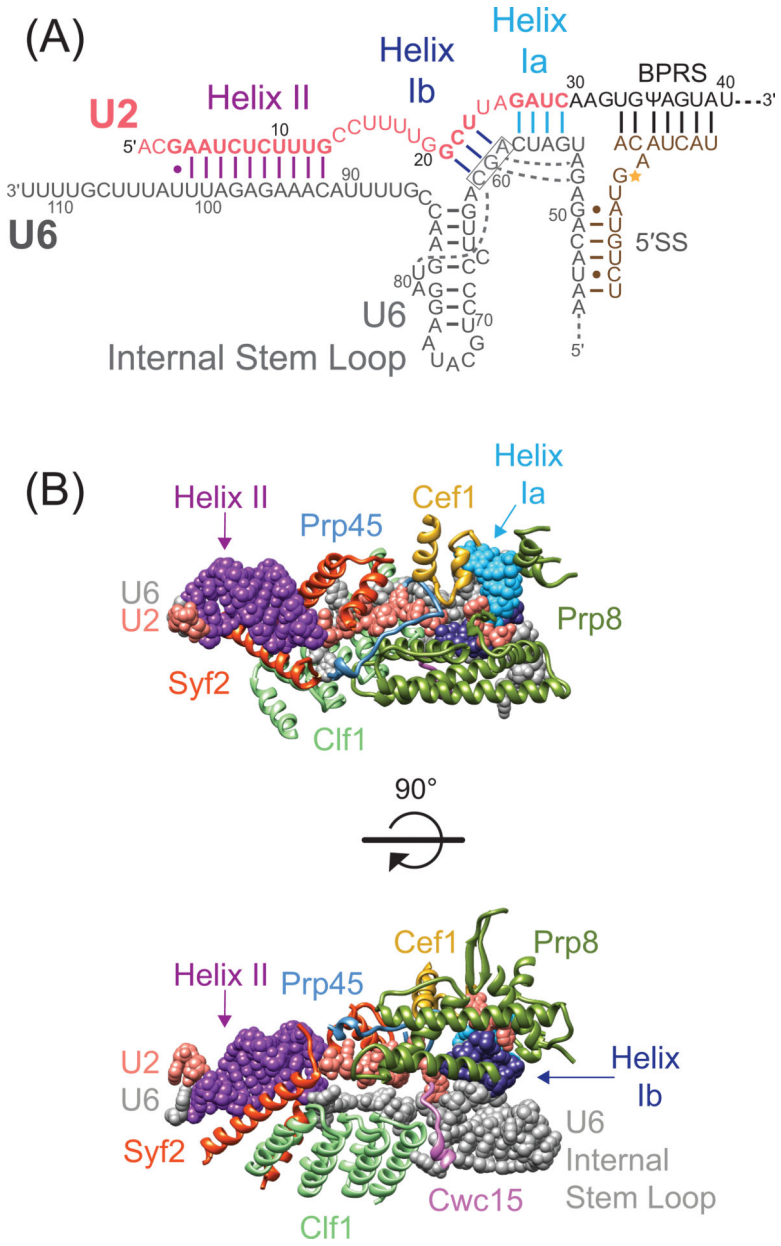

Figure 4.

Pairing between the U2 stem I module and the U6 snRNA in the spliceosome. (**A**) 2D schematic of U2/U6 helices Ia (light blue), Ib (navy) and II (purple) in the spliceosome prior to 5′ SS cleavage. The U2 bases that participate in base pairing are bolded. Pink nucleotides represent those found in U2 stem I (Figure 2). The internal stem loop of U6 coordinates magnesium ions essential for catalysis. U6's catalytic triplex is boxed and interactions with triplex bases are shown by dashed lines. The site of intron lariat formation is denoted by a gold star. (**B**) Cryo-EM structure of U2/U6 helices Ia, Ib, and II in the yeast B^{act} spliceosome. These RNA helices are encased by a number of proteins including Prp8 and a number of components of the NTC (Cef1, Clf1, Cwc15, Prp45, and Syf2; PDB ID: 5GM6). A color version of this figure is available online.

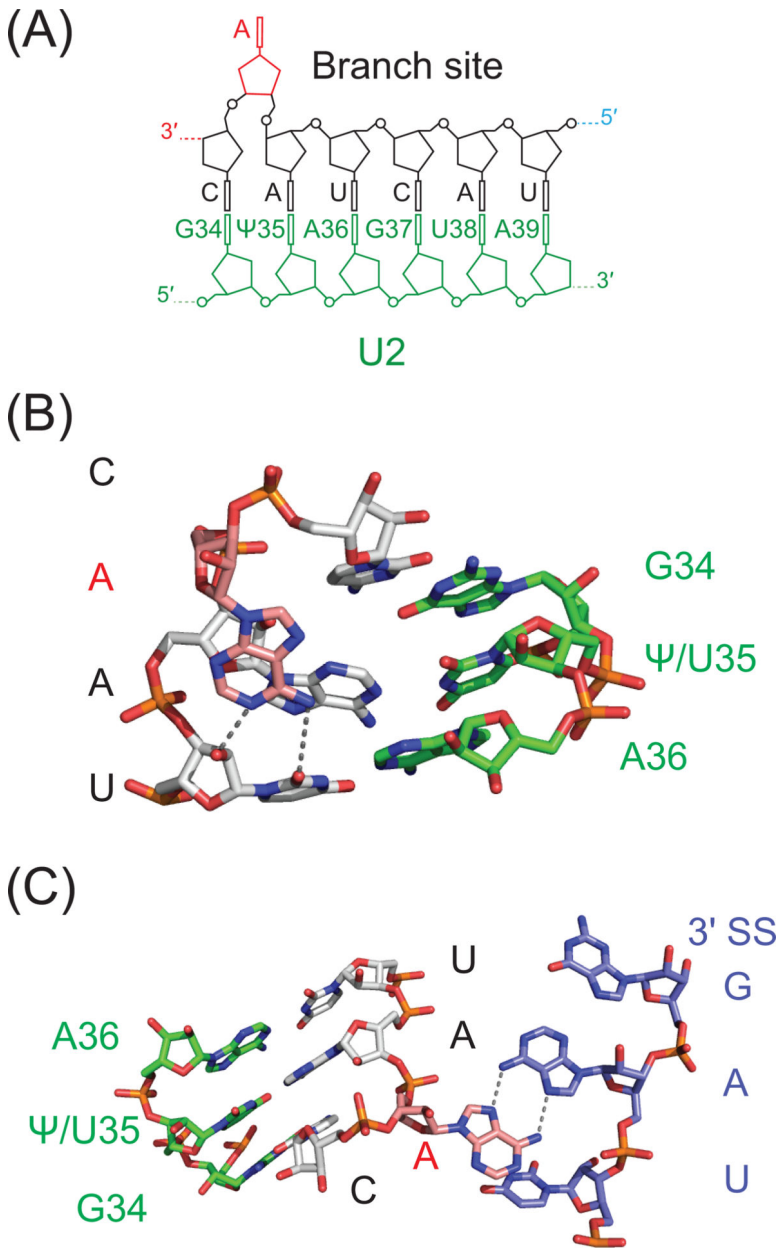

Figure 5.

Structure of the U2/BS duplex and its RNA interactions during splicing. (**A**) Schematic of the U2/BS RNA duplex. U2 snRNA (green) pairs to the branch site (black) resulting in expulsion of the branch site adenosine from the duplex. (**B**) Positioning of the 2' OH group of the branch point adenosine into the spliceosome active site for 5′ SS cleavage involves hydrogen bonding (black dashes) between the adenosine and a conserved uridine in the BS sequence (PDB ID:5LJ5). (**C**) The branch point adenosine participates in 3′ SS recognition during exon ligation by Hoogsteen pairing with the conserved adenosine of the 3′ SS (black dashes, PDB ID:6EXN). In panels (B) and (C), U2 snRNA position 35 was modeled as a uridine based on the cryo-EM data although U2 contains a highly conserved pseudouridine at this position. A color version of this figure is available online.

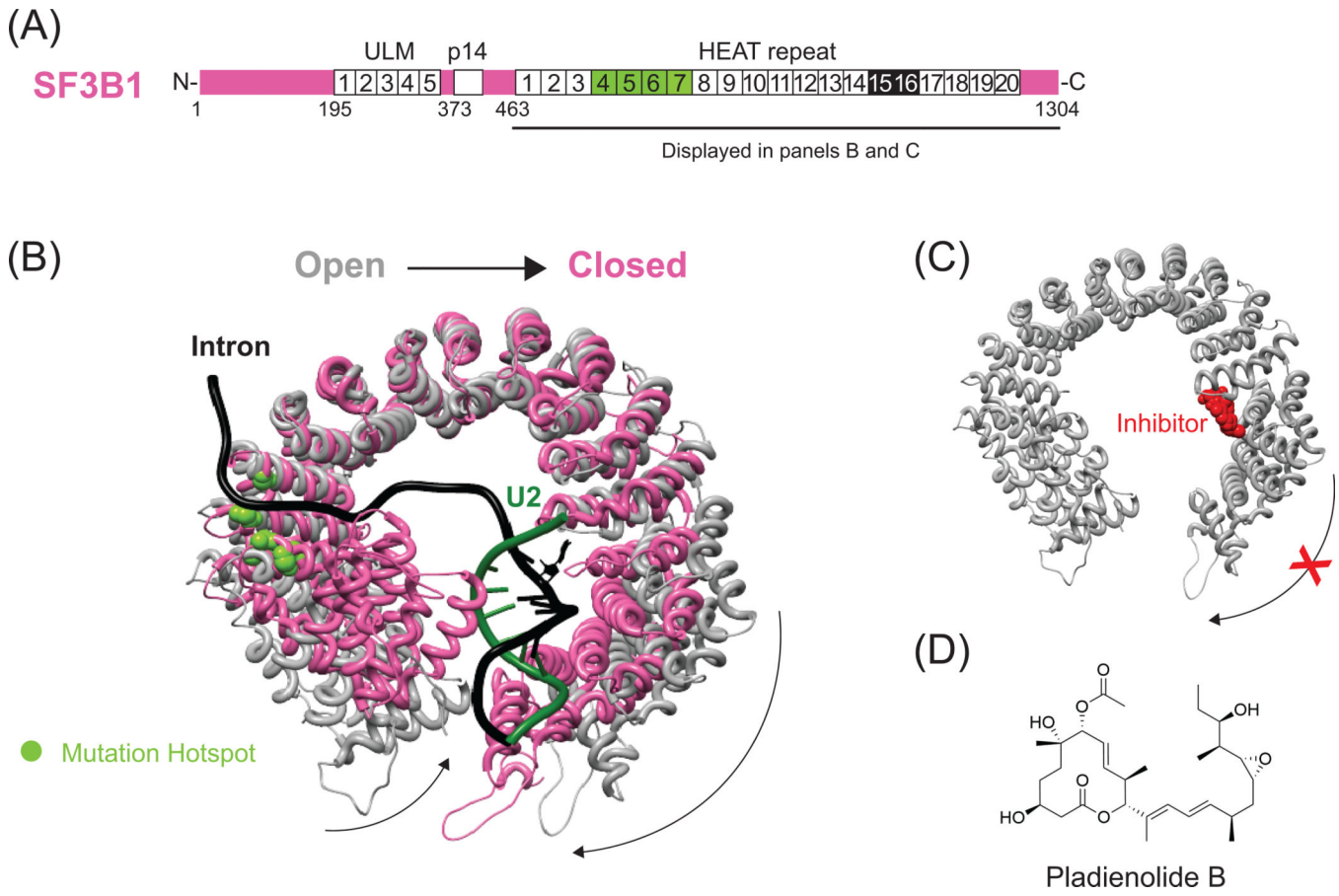

Figure 6.

Structure of the human U2 protein SF3B1. (**A**) Domain organization of human SF3B1. HEAT repeats 4–7 (green) is hot spot for mutations associated a variety of cancers. HEAT repeats 15 and 16 (black) form a binding pocket for the bulged branch site adenosine in the U2/BS RNA duplex. (**B**) Open (gray, PDB ID: 5IFE) and closed (pink, PDB ID: 6AHD) structures of SF3B1. The U2 snRNA BPRS (green) and intron (black) are shown as ribbons. Frequently mutated positions in SF3B1 are shown as green spheres (E622, R625, H662, K666, K700, and G742). (**C**) Structure of SF3B1 bound to the splicing inhibitor pladienolide B (red, PDB ID: 6EXN). (**D**) Chemical structure of pladienolide B. A color version of this figure is available online.

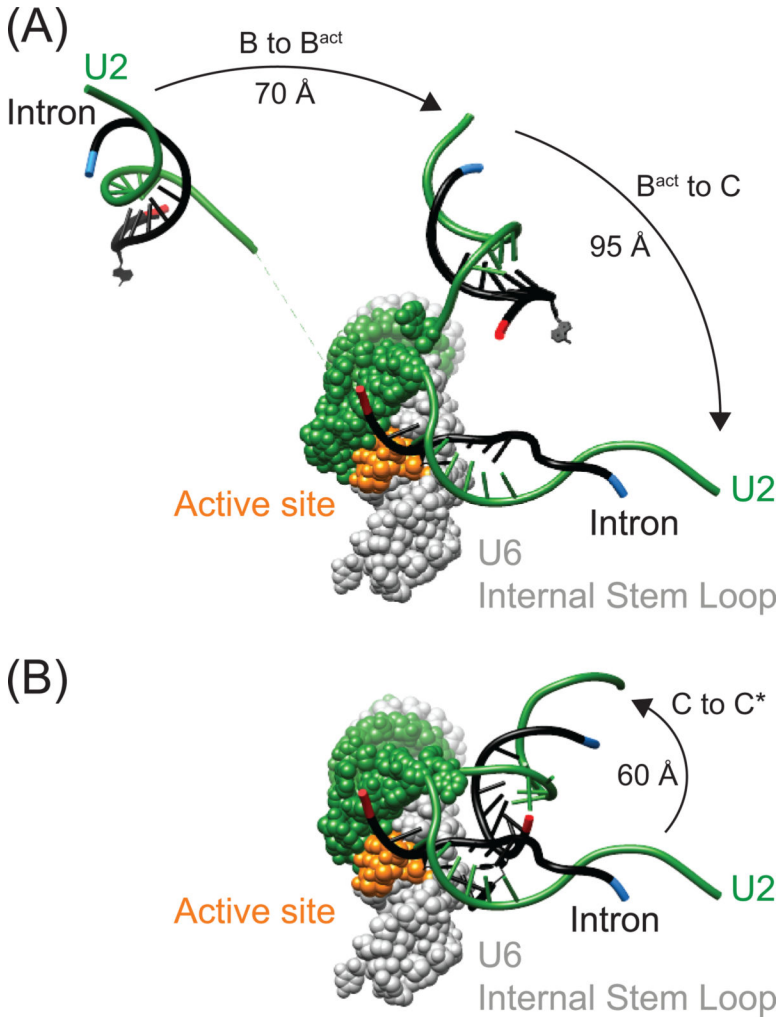

Figure 7.

Movements of the U2/BS duplex during splicing. (**A**) Movements of the duplex during B to B^{act} to C complex transitions. Movements are shown in relation to the active site and U6 catalytic triplex (orange). The U2 snRNA (green) in U2/U6 helices is shown space-filled and the U2 BPRS region shown as a ribbon. Base pairing between the BPRS and the intron (black; 5′ end, blue; 3′ end, red) varies between complexes (PDB IDs: 5NRL, 5GM6, and 5LJ3). (**B**) Movements occurring during the C to C* transition (PDB IDs: 5LJ3 and 5MQ0). Distances shown are measured from the U2 U47 backbone between structures. A color version of this figure is available online.

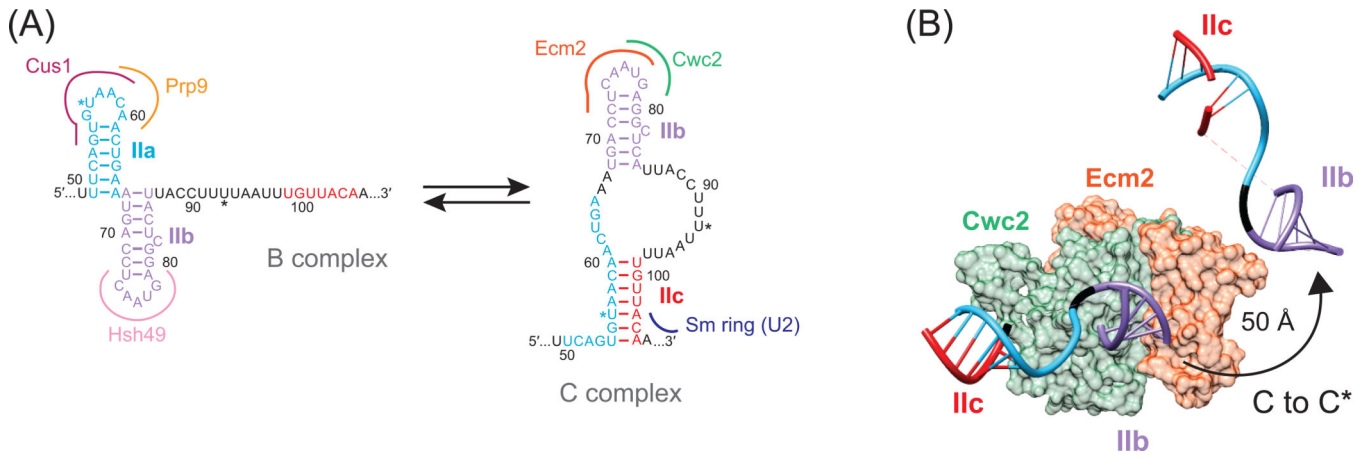

Figure 8.

U2 Stem II dynamics during splicing. (**A**) Stem IIa (blue) is a mutually exclusive structure to stem IIc (blue/red). Stem IIb (lavender) can be accommodated by both stem IIa and IIb pairing. In the spliceosome B complex, the stem loop of IIa interacts with Cus1 and Prp9 and the stem loop of IIb interacts with Hsh49 (PDB ID: 5NRL). In the spliceosome C complex, stem IIc has formed and interacts with U2's Sm ring. Also in this complex, stem loop IIb interacts with Ecm2 and Cwc2 (PDB ID: 5LJ3). (**B**) View of stem II's movement between the spliceosome C and C* complexes. As the active site of the spliceosome is remodeled, stem loop IIb undocks from Ecm2 and Cwc2 and moves ~50 Å (PDB ID: 5LJ3 and 5MQ0). A color version of this figure is available online.

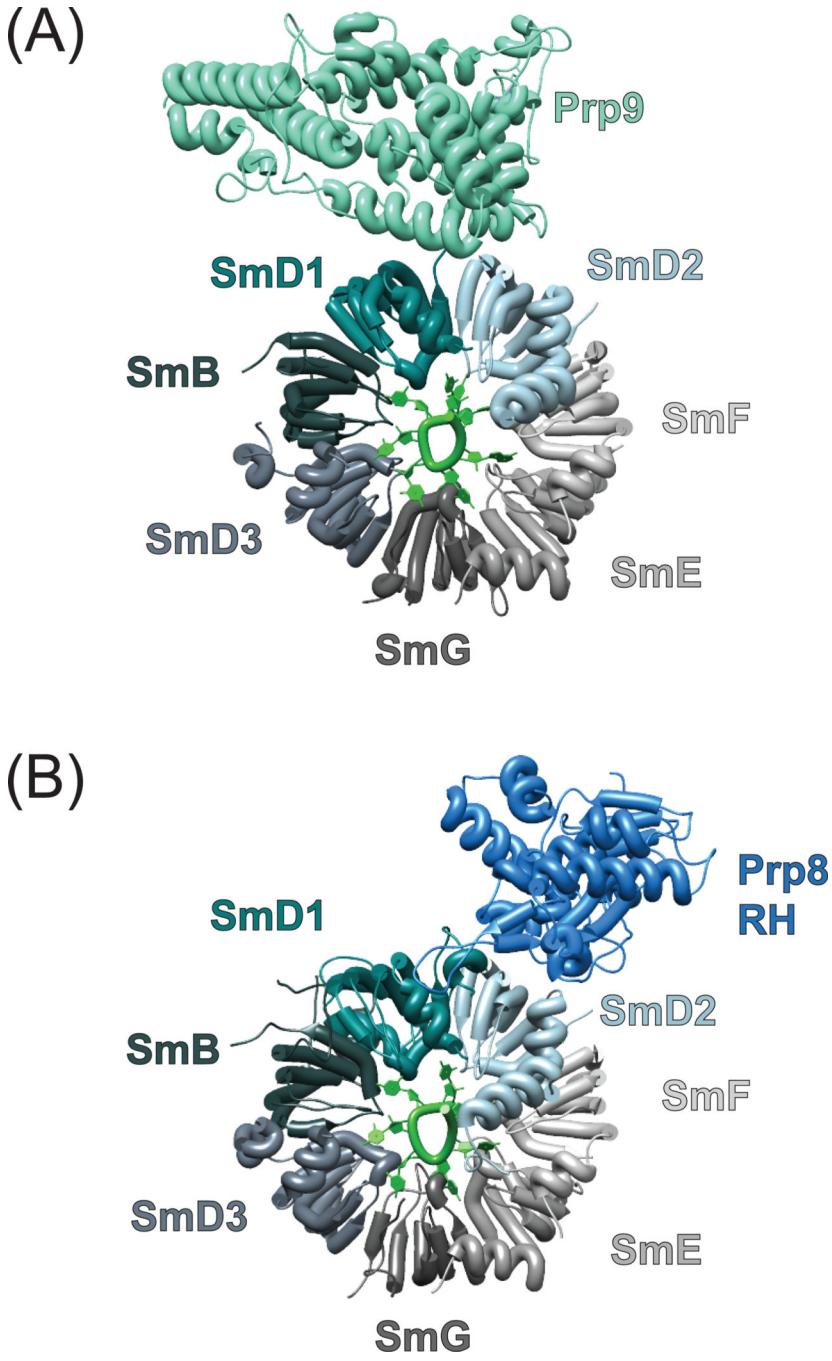

Figure 9.

Interactions between the U2 Sm ring and splicing factors in spliceosomes. The Sm binding site in U2 snRNA is shown in green. (**A**) Interaction between the SmD1 and SmD2 proteins and the SF3a protein Prp9 in B complex (PDB ID: 5NRL). (B) Interaction between SmD1 and SmD2 and the Prp8 RNase H (RH) domain in B* complex (PDB ID: 6J6Q). A color version of this figure is available online.

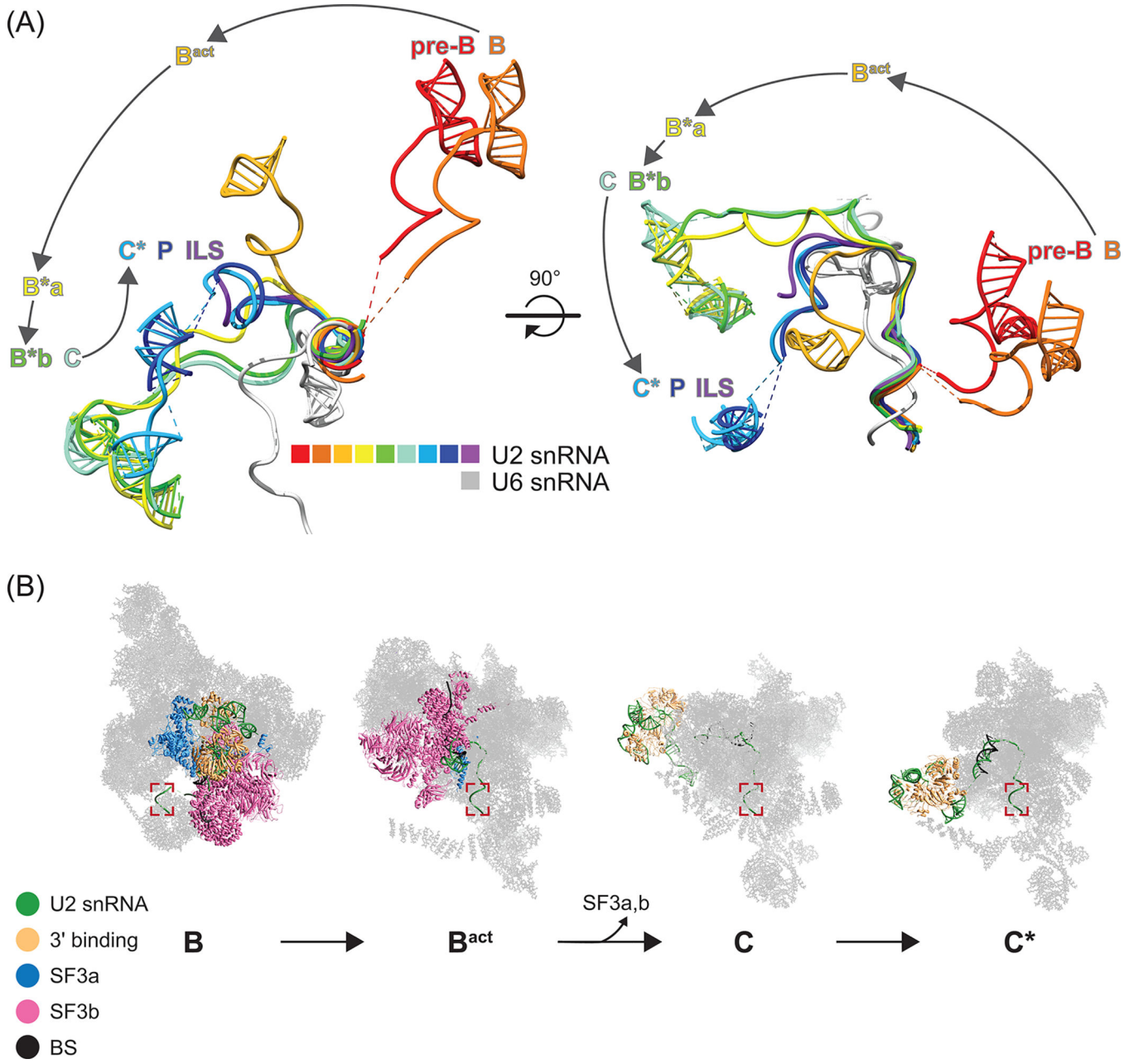

Figure 10.

Long-distance movements of the U2 BPRS, stem II, and 3′ modules during splicing. (**A**) Yeast U2 snRNA (nt 1–105) movement as the spliceosome progresses from pre-B to ILS complex formation in two orientations (PDB IDs: 5NRL, 5GM6, 6J6H, 6J6Q, 5LJ5, 5MQ0, 6EXN and 5Y88). For B* complex where multiple structures have been solved, B*a is PDB ID: 6J6H and B*b is PDB ID:6J6Q. Complexes were aligned to U2/U6 helix II (pre-B and B) or the U6 snRNA (B^{act} to ILS). The U6 snRNA from C complex is shown in grey for reference. (**B**) Motions of the U2 snRNP in context of the entire spliceosome. The U2 snRNP makes large movements accompanying U2 snRNA. Shown is a view of the spliceosome in the same orientation as the right panel in part (A). U2 snRNA, U2 snRNP

subcomplexes, and the branch site region of the intron (black) are highlighted. U2/U6 helix II is boxed in red. The remaining spliceosome components in each complex are shown in grey. A color version of this figure is available online.

Table 1:

Components of the U2 snRNP and associated factors

Subcomplex	Yeast Component	MW (kDa)	Human homologue	MW (kDa)
RNA	U2 snRNA	1,175 (nt)	U2 snRNA	188 (nt)
	Sm ring	91	Sm ring	94
3' binding	Lea1	27	U2A'/SNRPA1	28
	Msl1	13	U2B"/SNRPB2	25
	Prp9	63	SF3a60/SF3A3	59
SF3a	Prp11	30	SF3a66/SF3A2	49
	Prp21	33	SF3a120/SF3A1	89
	Hsh155	110	SF3b155/SF3B1	146
	Rse1	154	SF3b130/SF3B3	136
	Cus1	50	SF3b145/SF3B2	100
SF3b	Hsh49	25	SF3b49/SF3B4	44
	Rds3	12	SF3b14b/PHF5A	12
	Ysf3	10	SF3b10/SF3B5	10
	-	-	SF3b14a/p14	14
	Ist3	17	RBMX2/Snu17	37
RES	Bud13	30	Bud13/Cwc26	71/56
	Pml1	24	SNIP1	46
	Cus2	32	TAT-SF1	86
	Prp5	96	DDX46	117

Components that associate with U2 snRNP but are not part of the core 17S U2 snRNP are shaded in grey.