Title
Genetics and biochemistry remain essential in the structural era of the spliceosome

Authors
Mayerle, Megan
Guthrie, Christine

Publication Date
2017-08-01

DOI
10.1016/j.ymeth.2017.01.006

Peer reviewed

HHS Public Access

Author manuscript

Methods. Author manuscript; available in PMC 2018 August 01.

Published in final edited form as:

Methods. 2017 August 01; 125: 3–9. doi:10.1016/j.ymeth.2017.01.006.

Genetics and Biochemistry Remain Essential in the Structural Era of the Spliceosome

Megan Mayerle and **Christine Guthrie***

Department of Biochemistry and Biophysics, University of California San Francisco, San Francisco, California 94143, USA

Abstract

The spliceosome is not a single macromolecular machine. Rather it is a collection of dynamic heterogeneous subcomplexes that rapidly interconvert throughout the course of a typical splicing cycle. Because of this, for many years the only high resolution structures of the spliceosome available were of smaller, isolated protein or RNA components. Consequently much of our current understanding of the spliceosome derives from biochemical and genetic techniques. Now with the publication of multiple, high resolution structures of the spliceosome, some question the relevance of traditional biochemical and genetic techniques to the splicing field. We argue such techniques are not only relevant, but vital for an in depth mechanistic understanding of pre-mRNA splicing.

1.0 Introduction

Eukaryotic genes contain introns that must be accurately and efficiently removed to ensure fidelity in gene expression. Our understanding of the mechanisms and regulation of pre-mRNA splicing predominantly results from the clever combination of genetics and biochemistry. Recently the splicing field has been treated to high-resolution structures of human and *S. cerevesiae* tri-snRNP (PDB: 5GAN, 3JCM, 3JCR) [1–3], *S. cerevesiae* B^{act} (PDB: 5GM6, 5LQW) [4, 5], C (PDB: 5LJ3, 5GMK) [6, 7], and C*[8] complexes, as well as human C*[9], and the *S. pombe* Intron-Lariat Spliceosome (ILS) (PDB: 3JB9) [10]. These structures provide invaluable insights into spliceosome architecture, assembly, and function, and more spliceosome structures should be pursued. However, structural data are not without limitations. Here we review how genetic and biochemical approaches have traditionally informed our understanding of the spliceosome and discuss how these techniques remain essential in the structural era of the spliceosome.

The spliceosome is unique among cellular machines. Unlike the ribosome, which is assembled and then translates many cellular mRNAs, the spliceosome must assemble anew on each intron to be spliced. This elaborate assembly pathway allows for multiple points of regulation throughout the splicing cycle. Many assembly steps correspond to

*Corresponding author: christineguthrie@gmail.com.

interconversions between mutually exclusive RNA:RNA interactions, and are driven by DExD/H box ATPases (reviewed in [11]). These same DExD/H box ATPases also serve to regulate splicing fidelity, selectively promoting the advancement of "correct" substrates through consecutive splicing steps (reviewed in [12]).

The spliceosome (reviewed in [13]) catalyzes the removal of introns from nascent pre-mRNA transcripts via two transesterification reactions. The first transesterification reaction links the pre-mRNA 5' splice site (5'SS) and the branch site (BS) to form lariat-3'exon intermediate. The second reaction between the 5' and 3' splice sites forms spliced exon product. The spliceosome consists of five individual snRNAs and over one hundred unique proteins. These snRNAs and proteins are associated into a set of functional subcomplexes: the U1, U2, U4, U5, and U6 snRNPs, and the Nineteen Complex (NTC). These subcomplexes, in conjunction with associated proteins, proceed through the ordered series of compositional and conformational rearrangements required to splice each intron (Figure 1). Therefore, the spliceosome should not be thought of as a single entity, but rather as a dynamic, heterogeneous ensemble of subcomplexes that must interconvert throughout the splicing cycle.

2.0 Before the Structures

Here we highlight the genetic and biochemical approaches that have informed our understanding of spliceosome assembly and function.

2.1 In vitro Splicing and the Assembly of the Spliceosome

The spliceosome was first identified over 30 years ago[14]. Since then, *in vitro* splicing reactions have been used to define the canonical and alternative assembly pathways of the spliceosome and the components of the spliceosome present for each individual assembly step. In a typical *in vitro* splicing reaction, a labeled, *In vitro* transcribed pre-mRNA is incubated with ATP and yeast or human cell extract. This is followed by denaturing gel electrophoresis to visualize lariat-3' exon intermediate, free 5' exon, spliced mRNA product, and the excised lariat [15–17]. Nondenaturing gel electrophoresis of such splicing reactions allows detection of larger spliceosomal subcomplexes. In addition to the pre-mRNA, at least four distinct subcomplexes that differ in their snRNP complement and order of appearance can be resolved: the commitment complex (U1 and the Branchpoint Binding Protein (BBP), Complex E), pre-spliceosome (U1 and U2, Complex A), complete spliceosome (U1, U2, U4, U5, U6, Complex B), and active spliceosome (U2, U5, U6 and NTC, Complexes B^{act}, B*, and C), define the canonical assembly pathway (Figure 1) [18–20], which has been verified *in vivo*[21, 22].

Both steps of splicing can be reconstituted *in vitro* using a mixture of compositionally defined subcomplexes and recombinant proteins[23, 24]. Such assays have served as a basis for other more complex *in vitro* assays monitoring conformational rearrangements within the spliceosome[25], splicing fidelity and discard (release of the pre-mRNA from the spliceosome) [26], pre-mRNA conformation at the single-molecule level[27], and reversibility of the splicing reaction[28] (discussed in section 2.5).

In vitro splicing reactions have also been exploited to understand the function of individual components of the spliceosome. Endogenous U1, U2, U4, U5, and U6 snRNA can be depleted from yeast or mammalian cell extracts by DNA oligonucleotide-directed RNaseH cleavage, or by affinity selection with 2'-O-methyl RNA oligos complementary to the snRNA. Extracts treated in this way will, when supplied with an *in vitro* transcribed snRNA, process and then incorporate it into snRNPs and form functional spliceosomes[29–33]. This technique has been used to study the effects of otherwise lethal snRNA mutants, as well as to incorporate snRNAs containing fluorophores or other nucleotide modifications into the spliceosome for further study[32, 34, 35]. For example, metal ion rescue experiments, which required sulfur substitution at specific nucleotides in U6 snRNA, proved that RNA alone was the catalytic component of the spliceosome[35].

Conceptually similar immunodepletion-reconstitution assays have been exploited to study the protein components of the spliceosome(see [36–38] for examples). Such techniques can be adapted to isolate spliceosome subcomplexes through the immunoprecipitation of protein components, which have been studied in depth by mass spectrometry, crosslinking, and other biochemical techniques[39]. Studies of this type have produced an extensive list of "parts" and have been compiled into a searchable spliceosome database (http://spliceosomedb.ucsc.edu/)[40].

Decades of research into *in vitro* splicing and spliceosome purification techniques have yielded detailed purification protocols for the isolation of spliceosome A, B, B^{act}, B*, C, and ILS subcomplexes (Figure 1), as well as the individual snRNPs, the tri-snRNP, and the NTC (reviewed in [41, 42]). These provide an important starting point for structural studies of the spliceosome, as spliceosome subcomplexes isolated in this manner represent particularly stable intermediate forms of the spliceosome, and are thus excellent targets for structural determination.

2.2 Mutually Exclusive RNA Interactions in the Spliceosome

At the molecular level, many spliceosome assembly steps correspond to changes in mutually exclusive basepairing interactions between RNAs and between RNA and protein (reviewed in [43]). Three main techniques have contributed to our understanding of these structural switches in the spliceosome: phylogenetics, genetics, and photochemical crosslinking. These disparate approaches have yielded highly complementary data, establishing that direct physical interactions correspond to functional interactions and vice-versa.

The experiments that led to our understanding of how the 5'SS is recognized by the spliceosome provide an elegant example of how structural switches in the spliceosome are identified and characterized (Figure 2A). Basepairing between U1 snRNA and the pre-mRNA 5'SS was initially proposed based on sequences complementarity between the 5' end of U1 snRNA and the 5'SS consensus sequence[44, 45]. Subsequently it was shown that this interaction was functional; splicing of an intron with a mutated 5'SS could be rescued by expression of U1 snRNA containing a compensatory mutation in its 5'SS recognition sequence[46, 47]. This clever compensatory mutagenesis technique was further exploited to show that cleavage at the 5'SS was not simply a matter of maximizing Watson-Crick basepairing between U1 snRNA and the 5'SS. Instead, the 5'SS likely interacts with another

component of the spliceosome to direct 5'SS cleavage[48]. From crosslinking experiments it was known that, in addition to U1, the U6 snRNA interacts directly with the 5'SS[49, 50], and that the U1 and U6 snRNA interactions with the 5'SS are mutually exclusive (Figure 2A)[51]. Subsequent genetic experiments showed that the 5'SS:U6 snRNA interaction was required for selection of a correct 5'SS cleavage site[52].

This same general strategy was used repeatedly to discover and confirm other switches in the splicing cycle. Recognition of the pre-mRNA branch point requires sequential, mutually exclusive interactions with the branch point binding protein (BBP) and the U2 snRNA[53–56]. The stem II region of the U2 snRNA toggles between alternative conformations each required at distinct steps of the splicing cycle (Figure 2B)[57, 58]. U6 snRNA, extensively base-paired to the U4 snRNA while in the tri-snRNP[18, 59], base pairs with U2 snRNA once unwound [60] and forms an internal stem loop structure required for splicing catalysis[35, 61]. All of these mutually exclusive interactions were validated physically through crosslinking studies and functionally via phylogenetic and genetic approaches years before they could be visualized in high resolution structures of the spliceosome.

2.3 Control of Splicing Fidelity

Splicing must proceed with high fidelity to avoid introducing errors during gene expression. Estimates of the splicing error rate range from 1 in 100 to 1 in 100,000[62, 63], and errors in splicing have been shown to contribute to cancer[64] and other diseases[65]. Numerous core spliceosome components contribute to splicing fidelity. The best characterized are the DExD/H box ATPases Prp16, Prp22, and Prp5 (reviewed in [12]). These DExD/H box ATPases act via a kinetic proofreading mechanism (Figure 3) [66, 67]. In kinetic proofreading, the DExD/H box ATPases function as timers, restricting the window of time allotted for a particular splicing event before hydrolyzing ATP to promote a conformational change in the spliceosome. If a splicing substrate is proofread before the ATPase hydrolyzes ATP, ATPase activity promotes a productive conformational change in the spliceosome, driving the splicing reaction forward. If the substrate is still in the proofreading state when the ATPase hydrolyzes ATP, the ATPase instead promotes rejection of the substrate from the spliceosome (Figure 3) (reviewed in [12]).

Genetic screens for mutants in splicing factors, along with *in vivo* splicing reporter constructs that provide a means to link splicing efficiency to an easily measurable phenotype, have been key to dissecting splicing mechanics, including those that pertain to fidelity. For example, ACT-CUP splicing reporters link relative growth in copper-containing media to splicing efficiency[68]. Mutation of the core splicing sequences of these reporters allows screening for factors involved in splicing fidelity. Prp16 was the first spliceosome associated kinetic proofreader discovered by this method (Figure 3). Cold sensitive *prp16* alleles were identified in a screen designed to isolate alleles that could suppress a branch site A to C mutation[69,70]. Techniques to express and purify Prp16 *in vitro* followed by enzymatic assays revealed that these *prp16* mutants exhibit reduced ATPase and helicase activities *in vitro*. Most importantly, inhibition of ATPase activity correlated directly with how readily a given *prp16* allele would accommodate an aberrant splicing substrate, linking irreversible ATP hydrolysis to fidelity, a necessary condition for kinetic proofreading[70–

73]. Later screens identified suppressors of *prp16* cold sensitivity that also suppressed defects in Prp16 ATPase and helicase activities, demonstrating that fidelity could be regulated by the spliceosome[74]. Modified *in vitro* splicing assays have since shown that Prp16 proofreads by competing directly with 5'SS cleavage *in vitro* splicing reactions[37, 38].

In the currently available high resolution C complex structure, Prp16 is found on the spliceosome's periphery, distant from the spliceosome's active site, a position that does not immediately suggest that Prp16 would play a role in proofreading[7]. It is only because of the wealth of biochemical and genetic data available that Prp16's impact on splicing fidelity can be rationalized from this position[7]. Specifically, Prp16's position is consistent with crosslinking data between Prp16 and the pre-mRNA[75]. Furthermore, it is compatible with the current leading model for Prp16 action, wherein Prp16 functions at a distance by translocating towards, but not through, the intron branch point to disengage candidate splice sites from the active site of the spliceosome[76]. With only a structure, Prp16's role in fidelity would not have been apparent.

2.4 Reversibility in Splicing

Shrewd application of biochemical and genetic techniques have shown that spliceosome assembly, 5'SS cleavage (1st step), exon ligation (2nd step), and the discard of pre-mRNAs and splicing intermediates from the spliceosome are all reversible. Both single molecule FRET and *in vitro* splicing assays coupled with immunoprecipitation techniques have been essential for studies of splicing reversibility.

Previous bulk biochemical data had indicated that the spliceosome committed an intron to splicing very early in the splicing cycle, during the formation of the so-called commitment complex[77]. However, single molecule FRET experiments performed in yeast whole cell extracts show that each step of spliceosome assembly is reversible; the chances of an individual intron being spliced increase as the spliceosome successfully completes each assembly step[78]. Because of the heterogeneity inherent in *In vitro* splicing reactions, single molecule techniques are essential to our understanding of reversibility as well as other dynamic changes in the spliceosome.

Other aspects of pre-mRNA splicing are also reversible. DExD/H box ATPases function to promote forward progress through the splicing cycle (reviewed in [12]). When these ATPases are inhibited, or when splicing is slowed by other means, the spliceosome exhibits reversibility. By careful manipulation of potassium chloride levels and the use of a Prp22 variant defective in mRNA release, both catalytic steps of splicing can be reversed in the absence of ATP in vitro[28].

Together these biochemical and genetic studies demonstrate that essentially all aspects of pre-mRNA splicing are reversible, a conclusion that would be hard to support with structural data alone. It would be interesting to see the conformation of the spliceosome's catalytic core in a structure determined in conditions when the spliceosome exhibits reversibility.

3.0 After the Structures

High resolution structures of human and *S. cerevesiae* tri-snRNP [1–3], *S. cerevesiae* B^{act} [4, 5], C [6, 7], and C* complexes [8], human C* complex [9], and the *S. pombe* Intron-Lariat Spliceosome (ILS)[10] have recently been published and more will undoubtedly be forthcoming. Thus, we are now in the structural era of the spliceosome, allowing reinterpretation of previous genetic and biochemical data from a structural perspective. At the same time, spliceosome structures suggest new avenues for genetic and biochemical investigation. We anticipate that further elucidation of splicing mechanism and regulation will come from combining these traditional approaches with the detailed structural information. Here we focus on the spliceosome protein Prp8 as a case study. We discuss how structural data have advanced our understanding of Prp8 as well as key questions that remain best answered by a combination of genetics, biochemistry and other non-structural approaches.

Prp8 is the largest and most highly conserved protein in the spliceosome [79, 80]. Prp8 is present in multiple spliceosome subcomplexes including the U5 snRNP[81], the U4/U6-U5 tri-snRNP, and the catalytically active spliceosome (Figure 1). Long considered the “master regulator of splicing” Prp8 impacts both splicing efficiency and fidelity in a variety of contexts (reviewed in [82]). As Prp8 crosslinks to and genetically interacts with splicing sequences in the pre-mRNA[75, 83–94] as well as the U2, U4, U6 and U5 snRNAs [95–97], it has long been assumed to form part of the catalytic core of the spliceosome.

Sometimes a structure can validate a hypothesis, as in the case of Prp8 and the evolutionary origin of the spliceosome. The group II intron is thought to be the evolutionary precursor of the spliceosome as the catalytic cores of both the group II intron and the spliceosome are highly similar and catalysis occurs through a similar two-step mechanism in both systems [60, 98–101]. This hypothesis was strongly strengthened in 2013 when the crystal structure of Prp8 in complex with U5 biogenesis factor Aar2 was solved[102]. This structure revealed that Prp8 consisted of a Reverse Transcriptase domain followed by thumb/X, linker, endonuclease, RNaseH-like, and Jab1-MPN domains. Both the domain organization and fold of Prp8 are highly similar to that of group II intron maturase proteins[102–105]. In fact the overall fold of the maturase RT domain is more like the Prp8 RT domain than that of any other polymerase[105] confirming that Prp8 is likely the evolutionary descendent of a group II intron maturase protein.

Structural data can also clearly and directly rationalize seemingly conflicting or inexplicable genetic and/or biochemical data. Crystal structures of the Prp8 Jab1/MPN domain provided such a rationalization for control of Brr2 unwinding of the U4/U6 snRNAs during spliceosome activation ($B \rightarrow B^{act}$ transition), a key step in the splicing cycle (Figure 4) [106, 107]. This is a highly regulated process, as U4/U6 duplex unwinding allows the U6 snRNA to assume a catalytically competent form and premature catalytic activity could lead to errors in gene expression [60]. Genetics data originally hinted that the C-terminal Jab1/MPN domain of Prp8 negatively regulated Brr2 activity[108]. Later biochemical studies seemed to conflict with these data, showing that the Jab1/MPN domain promoted U4/U6 unwinding by stimulating Brr2 helicase activity and repressing ATPase activity (Figure 4A) [109, 110].

These data were resolved by a co-crystal of human Brr2 and the Prp8-Jab1/MPN domain, trapping a form of the complex wherein a long extension from the Jab1/MPN domain blocks the Brr2 RNA-binding channel (Figure 4B) [111]. Biochemistry confirmed that this extension inhibited Brr2 activity, however its truncation stimulates U4/U6 unwinding by Brr2[111]. Thus, structures rationalized how the Prp8 Jab1/MPN could both stimulate and repress Brr2 (Figure 4).

Now, a combination of genetics, biochemistry and structural data are what is most needed to understand the spliceosome. For example, the structures of the tri-snRNP, B^{act}, C, and ILS complexes revealed the existence of a prominent Prp8 surface loop. Dubbed the switch loop (amino acids 1402–1439 in *S. cerevisiae*), this stretch of amino acids adopts strikingly different conformations depending upon the structure examined [1, 4, 10]. In B^{act} and C complex the switch loop associates with the Prp8 NTD, RT domain, Thumb/X domains as well as the Nineteen Complex (NTC) protein Cwc21, GTPase Snu114, and the pre-mRNA 5’ exon, stabilizing the interaction between the pre-mRNA and stem-loop1 of the U5 snRNA (Figure 5A) [4]. This is consistent with previous genetic and physical interaction data that indicate that Cwc21 interacts with Prp8, the U5 stem-loop 1, Snu114 [87, 112, 113], and with NTC component Isy1 [114]. Furthermore, *cwc21Δ, isy1Δ*, and specific *prp8* point mutants all show defects in 3’SS positioning during exon ligation[74, 113–116]. These data suggest that the Prp8 switch loop positions the 5’ exon for branching and exon ligation during the 1^{st} and 2^{nd} catalytic steps, respectively[9]. Intriguingly, in the preand post-catalytic tri-snRNP and ILS, the switch loop has flipped approximately 180 degrees (Figure 5B), suggesting that the switch loop contributes to maintenance of a catalytically inactive conformation of the spliceosome. This hypothesis is far from proven, and the available structural data do not inform what drives this switch. If the Prp8 switch loop is critical for the positioning of the exons, one prediction is that mutagenesis of the Prp8 switch loop would result in temperature sensitive *prp8* alleles showing defects in 3’SS positioning. Such *prp8* alleles might also show genetic interactions with *cwc21* or other NTC alleles, as well as with various *snu114* alleles. Together these data could provide critical mechanistic details of how the spliceosome positions pre-mRNA exons for catalysis.

4.0 Moving Forward

The new spliceosome structures provide a much needed structural framework and are likely to transform the splicing field in the years to come, as high resolution ribosome structures did the translation field in the early 2000s. However, genetic and biochemical techniques remain essential to understanding the significance of these spliceosome structures, and to our understanding of splicing fidelity and mechanism.

Acknowledgments

The authors acknowledge Anne de Bruyn Kops for key discussions and commentary on this manuscript. This work was supported by grants RO1GM21119-42 (CG) and F32GM109512 (MM) from the National Institutes of Health National Institute of General Medical Science. CG is an American Cancer Society Research Professor of Molecular Genetics.

Bibliography

1. Wan R, Yan C, Bai R, Wang L, Huang M, Wong CCL, Shi Y. The 3.8 A structure of the U4/U6.U5 tri-snRNP: Insights into spliceosome assembly and catalysis. Science. 2016; 351:466–475. [PubMed: 26743623]
2. Nguyen THD, Galej WP, Bai X-C, Savva CG, Newman AJ, Scheres SHW, Nagai K. The architecture of the spliceosomal U4/U6.U5 tri-snRNP. Nature. 2015
3. Agafonov DE, Kastner B, Dybkov O, Hofele RV, Liu W-T, Urlaub H, Luhrmann R, Stark H. Molecular architecture of the human U4/U6.U5 tri-snRNP. Science. 2016
4. Yan C, Wan R, Bai R, Huang G, Shi Y. Structure of a yeast catalytically activated spliceosome at 3.5 A resolution. Science. 2016
5. Rauhut R, Fabrizio P, Dybkov O, Hartmuth K, Pena V, Chari A, Kumar V, Lee C-T, Urlaub H, Kastner B, Stark H, Luhrmann R. Molecular architecture of the Saccharomyces cerevisiae activated spliceosome. Science. 2016
6. Wan R, Yan C, Bai R, Huang G, Shi Y. Structure of a yeast catalytic step I spliceosome at 3.4 A resolution. Science. 2016
7. Galej WP, Wilkinson ME, Fica SM, Oubridge C, Newman AJ, Nagai K. Cryo-EM structure of the spliceosome immediately after branching. Nature. 2016
8. Fica SM, Oubridge C, Galej WP, Wilkinson ME, Bai X-C, Newman AJ, Nagai K. Structure of a spliceosome remodelled for exon ligation. Nature. 2017
9. Bertram K, Agafonov DE, Liu W-T, Dybkov O, Will CL, Hartmuth K, Urlaub H, Kastner B, Stark H, Lu Hrmann R. Cryo-EM structure of a human spliceosome activated for step 2 of splicing. Nature. 2017
10. Yan C, Hang J, Wan R, Huang M, Wong CCL, Shi Y. Structure of a yeast spliceosome at 3.6-angstrom resolution. Science. 2015; 349:1182–1191. [PubMed: 26292707]
11. Wahl MC, Will CL, Luhrmann R. The spliceosome: design principles of a dynamic RNP machine. Cell. 2009; 136:701–718. [PubMed: 19239890]
12. Semlow DR, Staley JP. Staying on message: ensuring fidelity in pre-mRNA splicing. Trends Biochem. Sci. 2012; 37:263–273. [PubMed: 22564363]
13. Will CL, Luhrmann R. Spliceosome structure and function. Cold Spring Harb. Perspect. Biol. 2011; 3
14. Brody E, Abelson J. The “spliceosome”: yeast pre-messenger RNA associates with a 40S complex in a splicing-dependent reaction. Science. 1985; 228:963–967. [PubMed: 3890181]
15. Hernandez N, Keller W. Splicing of in vitro synthesized messenger RNA precursors in HeLa cell extracts. Cell. 1983; 35:89–99. [PubMed: 6194902]
16. Lin RJ, Newman AJ, Cheng SC, Abelson J. Yeast mRNA splicing in vitro. J. Biol. Chem. 1985; 260:14780–14792. [PubMed: 2997224]
17. Padgett RA, Hardy SF, Sharp PA. Splicing of adenovirus RNA in a cell-free transcription system. Proc. Natl. Acad. Sci. U. S. A. 1983; 80:5230–5234. [PubMed: 6577417]
18. Cheng SC, Abelson J. Spliceosome assembly in yeast. Genes Dev. 1987; 1:1014–1027. [PubMed: 2962902]
19. Konarska MM, Sharp PA. Electrophoretic separation of complexes involved in the splicing of precursors to mRNAs. Cell. 1986; 46:845–855. [PubMed: 2944598]
20. Pikielny CW, Rymond BC, Rosbash M. Electrophoresis of ribonucleoproteins reveals an ordered assembly pathway of yeast splicing complexes. Nature. 1986; 324:341–345.
21. Huranova M, Ivani I, Benda A, Poser I, Brody Y, Hof M, Shav-Tal Y, Neugebauer KM, Stanek D. The differential interaction of snRNPs with pre-mRNA reveals splicing kinetics in living cells. J. Cell Biol. 2010; 191:75–86. [PubMed: 20921136]
22. Tardiff DF, Rosbash M. Arrested yeast splicing complexes indicate stepwise snRNP recruitment during in vivo spliceosome assembly. RNA N. Y. N. 2006; 12:968–979.
23. Warkocki Z, Odenwalder P, Schmitzova J, Platzmann F, Stark H, Urlaub H, Ficner R, Fabrizio P, Luhrmann R. Reconstitution of both steps of Saccharomyces cerevisiae splicing with purified spliceosomal components. Nat. Struct. Mol. Biol. 2009; 16:1237–1243. [PubMed: 19935684]

24. Ohrt T, Odenwalder P, Dannenberg J, Prior M, Warkocki Z, Schmitzova J, Karaduman R, Gregor I, Enderlein J, Fabrizio P, Luhrmann R. Molecular dissection of step 2 catalysis of yeast premRNA splicing investigated in a purified system. RNA N. Y. N. 2013; 19:902–915.
25. Schneider C, Agafonov DE, Schmitzova J, Hartmuth K, Fabrizio P, Luhrmann R. Dynamic Contacts of U2, RES, Cwc25, Prp8 and Prp45 Proteins with the Pre-mRNA Branch-Site and 3' Splice Site during Catalytic Activation and Step 1 Catalysis in Yeast Spliceosomes. PLoS Genet. 2015; 11:e1005539. [PubMed: 26393790]
26. Mayas RM, Maita H, Staley JP. Exon ligation is proofread by the DExD/H-box ATPase Prp22p. Nat. Struct. Mol. Biol. 2006; 13:482–490. [PubMed: 16680161]
27. Krishnan R, Blanco MR, Kahlscheuer ML, Abelson J, Guthrie C, Walter NG. Biased Brownian ratcheting leads to pre-mRNA remodeling and capture prior to first-step splicing. Nat. Struct. Mol. Biol. 2013
28. Tseng C-K, Cheng S-C. Both catalytic steps of nuclear pre-mRNA splicing are reversible. Science. 2008; 320:1782–1784. [PubMed: 18583613]
29. O'Keefe RT, Norman C, Newman AJ. The Invariant U5 snRNA Loop 1 Sequence Is Dispensable for the First Catalytic Step of pre-mRNA Splicing in Yeast. Cell. 1996; 86:679–689. [PubMed: 8752221]
30. Stark MR, Rader SD. Complementation of U4 snRNA in S. cerevisiae splicing extracts for biochemical studies of snRNP assembly and function. Methods Mol. Biol. Clifton NJ. 2014; 1126:193–204.
31. Hamm J, Dathan NA, Scherly D, Mattaj IW. Multiple domains of U1 snRNA, including U1 specific protein binding sites, are required for splicing. EMBO J. 1990; 9:1237–1244. [PubMed: 2138978]
32. McPheeters DS, Fabrizio P, Abelson J. In vitro reconstitution of functional yeast U2 snRNPs. Genes Dev. 1989; 3:2124–2136. [PubMed: 2560754]
33. Pan ZQ, Ge H, Fu XY, Manley JL, Prives C. Oligonucleotide-targeted degradation of U1 and U2 snRNAs reveals differential interactions of simian virus 40 pre-mRNAs with snRNPs. Nucleic Acids Res. 1989; 17:6553–6568. [PubMed: 2550896]
34. Abelson J, Hadjivassiliou H, Guthrie C. Preparation of fluorescent pre-mRNA substrates for an smFRET study of pre-mRNA splicing in yeast. Methods Enzymol. 2010; 472:31–40. [PubMed: 20580958]
35. Fica SM, Tuttle N, Novak T, Li N-S, Lu J, Koodathingal P, Dai Q, Staley JP, Piccirilli JA. RNA catalyses nuclear pre-mRNA splicing. Nature. 2013
36. Schneider S, Hotz H-R, Schwer B. Characterization of dominant-negative mutants of the DEAHbox splicing factors Prp22 and Prp16. J. Biol. Chem. 2002; 277:15452–15458. [PubMed: 11856747]
37. Koodathingal P, Novak T, Piccirilli JA, Staley JP. The DEAH box ATPases Prp16 and Prp43 cooperate to proofread 5' splice site cleavage during pre-mRNA splicing. Mol. Cell. 2010; 39:385–395. [PubMed: 20705241]
38. Tseng C-K, Liu H-L, Cheng S-C. DEAH-box ATPase Prp16 has dual roles in remodeling of the spliceosome in catalytic steps. RNA N. Y. N. 2011; 17:145–154.
39. Jurica MS, Moore MJ. Capturing splicing complexes to study structure and mechanism. Methods San Diego Calif. 2002; 28:336–345.
40. Cvitkovic I, Jurica MS. Spliceosome database: a tool for tracking components of the spliceosome. Nucleic Acids Res. 2013; 41:D132–D141. [PubMed: 23118483]
41. Luhrmann R, Stark H. Structural mapping of spliceosomes by electron microscopy. Curr. Opin. Struct. Biol. 2009; 19:96–102. [PubMed: 19211241]
42. Ilagan JO, Jurica MS. Isolation and accumulation of spliceosomal assembly intermediates. Methods Mol. Biol. Clifton NJ. 2014; 1126:179–192.
43. Staley JP, Guthrie C. Mechanical devices of the spliceosome: motors, clocks, springs, and things. Cell. 1998; 92:315–326. [PubMed: 9476892]
44. Rogers J, Wall R. A mechanism for RNA splicing. Proc. Natl. Acad. Sci. U. S. A. 1980; 77:1877–1879. [PubMed: 6246511]

45. Lerner MR, Boyle JA, Mount SM, Wolin SL, Steitz JA. Are snRNPs involved in splicing? Nature. 1980; 283:220–224. [PubMed: 7350545]
46. Zhuang Y, Weiner AM. A compensatory base change in U1 snRNA suppresses a 5’ splice site mutation. Cell. 1986; 46:827–835. [PubMed: 3757028]
47. Seraphin B, Rosbash M. Identification of functional U1 snRNA-pre-mRNA complexes committed to spliceosome assembly and splicing. Cell. 1989; 59:349–358. [PubMed: 2529976]
48. Siliciano PG, Guthrie C. 5’ splice site selection in yeast: genetic alterations in base-pairing with U1 reveal additional requirements. Genes Dev. 1988; 2:1258–1267. [PubMed: 3060402]
49. Sawa H, Shimura Y. Association of U6 snRNA with the 5’-splice site region of pre-mRNA in the spliceosome. Genes Dev. 1992; 6:244–254. [PubMed: 1310665]
50. Wassarman DA, Steitz JA. Interactions of small nuclear RNA’s with precursor messenger RNA during in vitro splicing. Science. 1992; 257:1918–1925. [PubMed: 1411506]
51. Konforti BB, Koziolkiewicz MJ, Konarska MM. Disruption of base pairing between the 5’ splice site and the 5’ end of U1 snRNA is required for spliceosome assembly. Cell. 1993; 75:863–873. [PubMed: 8252623]
52. Lesser CF, Guthrie C. Mutations in U6 snRNA that alter splice site specificity: implications for the active site. Science. 1993; 262:1982–1988. [PubMed: 8266093]
53. Ares M. U2 RNA from yeast is unexpectedly large and contains homology to vertebrate U4, U5, and U6 small nuclear RNAs. Cell. 1986; 47:49–59. [PubMed: 3530502]
54. Parker R, Siliciano PG, Guthrie C. Recognition of the TACTAAC box during mRNA splicing in yeast involves base pairing to the U2-like snRNA. Cell. 1987; 49:229–239. [PubMed: 3552247]
55. Query CC, Moore MJ, Sharp PA. Branch nucleophile selection in pre-mRNA splicing: evidence for the bulged duplex model. Genes Dev. 1994; 8:587–597. [PubMed: 7926752]
56. Berglund JA, Chua K, Abovich N, Reed R, Rosbash M. The splicing factor BBP interacts specifically with the pre-mRNA branchpoint sequence UACUAAC. Cell. 1997; 89:781–787. [PubMed: 9182766]
57. Zavanelli MI, Britton JS, Igel AH, Ares M. Mutations in an essential U2 small nuclear RNA structure cause cold-sensitive U2 small nuclear ribonucleoprotein function by favoring competing alternative U2 RNA structures. Mol. Cell. Biol. 1994; 14:1689–1697. [PubMed: 8114704]
58. Hilliker AK, Mefford MA, Staley JP. U2 toggles iteratively between the stem IIa and stem IIc conformations to promote pre-mRNA splicing. Genes Dev. 2007; 21:821–834. [PubMed: 17403782]
59. Konarska MM, Sharp PA. Interactions between small nuclear ribonucleoprotein particles in formation of spliceosomes. Cell. 1987; 49:763–774. [PubMed: 2953438]
60. Madhani HD, Guthrie C. A novel base-pairing interaction between U2 and U6 snRNAs suggests a mechanism for the catalytic activation of the spliceosome. Cell. 1992; 71:803–817. [PubMed: 1423631]
61. Burke JE, Sashital DG, Zuo X, Wang Y-X, Butcher SE. Structure of the yeast U2/U6 snRNA complex. RNA N. Y. N. 2012; 18:673–683.
62. Fox-Walsh KL, Hertel KJ. Splice-site pairing is an intrinsically high fidelity process. Proc. Natl. Acad. Sci. U. S. A. 2009; 106:1766–1771. [PubMed: 19179398]
63. Pickrell JK, Pai AA, Gilad Y, Pritchard JK. Noisy splicing drives mRNA isoform diversity in human cells. PLoS Genet. 2010; 6:e1001236. [PubMed: 21151575]
64. Bejar R. Splicing Factor Mutations in Cancer. Adv. Exp. Med. Biol. 2016; 907:215–228. [PubMed: 27256388]
65. Faustino NA, Cooper TA. Pre-mRNA splicing and human disease. Genes Dev. 2003; 17:419–437. [PubMed: 12600935]
66. Ninio J. Kinetic Amplification of Enzyme Discrimination. Biochimie. 1975; 57:587–595. [PubMed: 1182215]
67. Hopfield JJ. Kinetic Proofreading: A New Mechanism for Reducing Errors in Biosynthetic Processes Requiring High Specificity. Proc. Natl. Acad. Sci. 1974; 71:4135–4139. [PubMed: 4530290]

68. Lesser CF, Guthrie C. Mutational analysis of pre-mRNA splicing in Saccharomyces cerevisiae using a sensitive new reporter gene, CUP1. Genetics. 1993; 133:851–863. [PubMed: 8462846]
69. Madhani HD, Guthrie C. Genetic interactions between the yeast RNA helicase homolog Prp16 and spliceosomal snRNAs identify candidate ligands for the Prp16 RNA-dependent ATPase. Genetics. 1994; 137:677–687. [PubMed: 8088513]
70. Burgess SM, Guthrie C. A mechanism to enhance mRNA splicing fidelity: the RNA-dependent ATPase Prp16 governs usage of a discard pathway for aberrant lariat intermediates. Cell. 1993; 73:1377–1391. [PubMed: 8324826]
71. Schwer B, Guthrie C. A conformational rearrangement in the spliceosome is dependent on PRP16 and ATP hydrolysis. EMBO J. 1992; 11:5033–5039. [PubMed: 1464325]
72. Burgess S, Couto JR, Guthrie C. A putative ATP binding protein influences the fidelity of branchpoint recognition in yeast splicing. Cell. 1990; 60:705–717. [PubMed: 2138057]
73. Wang Y, Wagner JD, Guthrie C. The DEAH-box splicing factor Prp16 unwinds RNA duplexes in vitro. Curr. Biol. CB. 1998; 8:441–451. [PubMed: 9550699]
74. Villa T, Guthrie C. The Isy1p component of the NineTeen complex interacts with the ATPase Prp16p to regulate the fidelity of pre-mRNA splicing. Genes Dev. 2005; 19:1894–1904. [PubMed: 16103217]
75. McPheeters DS, Muhlenkamp P. Spatial organization of protein-RNA interactions in the branch site-3' splice site region during pre-mRNA splicing in yeast. Mol. Cell. Biol. 2003; 23:4174–4186. [PubMed: 12773561]
76. Semlow DR, Blanco MR, Walter NG, Staley JP. Spliceosomal DEAH-Box ATPases Remodel PremRNA to Activate Alternative Splice Sites. Cell. 2016; 164:985–998. [PubMed: 26919433]
77. Legrain P, Seraphin B, Rosbash M. Early commitment of yeast pre-mRNA to the spliceosome pathway. Mol. Cell. Biol. 1988; 8:3755–3760. [PubMed: 3065622]
78. Hoskins AA, Rodgers ML, Friedman LJ, Gelles J, Moore MJ. Single molecule analysis reveals reversible and irreversible steps during spliceosome activation. eLife. 2016; 5
79. Lossky M, Anderson GJ, Jackson SP, Beggs J. Identification of a yeast snRNP protein and detection of snRNP-snRNP interactions. Cell. 1987; 51:1019–1026. [PubMed: 2961458]
80. Hodges PE, Jackson SP, Brown JD, Beggs JD. Extraordinary sequence conservation of the PRP8 splicing factor. Yeast Chichester Engl. 1995; 11:337–342.
81. Stevens SW, Barta I, Ge HY, Moore RE, Young MK, Lee TD, Abelson J. Biochemical and genetic analyses of the U5, U6, and U4/U6 × U5 small nuclear ribonucleoproteins from Saccharomyces cerevisiae. RNA N. Y. N. 2001; 7:1543–1553.
82. Grainger RJ, Beggs JD. Prp8 protein: at the heart of the spliceosome. RNA N. Y. N. 2005; 11:533–557.
83. Vijayraghavan U, Parker R, Tamm J, Iimura Y, Rossi J, Abelson J, Guthrie C. Mutations in conserved intron sequences affect multiple steps in the yeast splicing pathway, particularly assembly of the spliceosome. EMBO J. 1986; 5:1683–1695. [PubMed: 3017708]
84. Wyatt JR, Sontheimer EJ, Steitz JA. Site-specific cross-linking of mammalian U5 snRNP to the 5' splice site before the first step of pre-mRNA splicing. Genes Dev. 1992; 6:2542–2553. [PubMed: 1340469]
85. Konforti BB, Konarska MM. U4/U5/U6 snRNP recognizes the 5' splice site in the absence of U2 snRNP. Genes Dev. 1994; 8:1962–1973. [PubMed: 7958870]
86. MacMillan AM, Query CC, Allerson CR, Chen S, Verdine GL, Sharp PA. Dynamic association of proteins with the pre-mRNA branch region. Genes Dev. 1994; 8:3008–3020. [PubMed: 8001820]
87. Teigelkamp S, Whittaker E, Beggs JD. Interaction of the yeast splicing factor PRP8 with substrate RNA during both steps of splicing. Nucleic Acids Res. 1995; 23:320–326. [PubMed: 7885825]
88. Umen JG, Guthrie C. A novel role for a U5 snRNP protein in 3' splice site selection. Genes Dev. 1995; 9:855–868. [PubMed: 7535718]
89. Reyes JL, Gustafson EH, Luo HR, Moore MJ, Konarska MM. The C-terminal region of hPrp8 interacts with the conserved GU dinucleotide at the 5' splice site. RNA N. Y. N. 1999; 5:167–179.

90. Maroney PA, Romfo CM, Nilsen TW. Functional Recognition of the 5′ Splice Site by U4/U6.U5 tri-snRNP Defines a Novel ATP-Dependent Step in Early Spliceosome Assembly. Mol. Cell. 2000; 6:317–328. [PubMed: 10983979]
91. Collins CA, Guthrie C. Genetic interactions between the 5' and 3' splice site consensus sequences and U6 snRNA during the second catalytic step of pre-mRNA splicing. RNA N. Y. N. 2001; 7:1845–1854.
92. Kuhn AN, Reichl EM, Brow DA. Distinct domains of splicing factor Prp8 mediate different aspects of spliceosome activation. Proc. Natl. Acad. Sci. U. S. A. 2002; 99:9145–9149. [PubMed: 12087126]
93. Query CC, Konarska MM. Suppression of multiple substrate mutations by spliceosomal prp8 alleles suggests functional correlations with ribosomal ambiguity mutants. Mol. Cell. 2004; 14:343–354. [PubMed: 15125837]
94. Liu L, Query CC, Konarska MM. Opposing classes of prp8 alleles modulate the transition between the catalytic steps of pre-mRNA splicing. Nat. Struct. Mol. Biol. 2007; 14:519–526. [PubMed: 17486100]
95. Dix I, Russell CS, O'Keefe RT, Newman AJ, Beggs JD. Protein-RNA interactions in the U5 snRNP of Saccharomyces cerevisiae. RNA. 1998; 4:1675–1686. [PubMed: 9848662]
96. Vidal VP, Verdone L, Mayes AE, Beggs JD. Characterization of U6 snRNA-protein interactions. RNA. 1999; 5:1470–1481. [PubMed: 10580475]
97. Li X, Zhang W, Xu T, Ramsey J, Zhang L, Hill R, Hansen KC, Hesselberth JR, Zhao R. Comprehensive in vivo RNA-binding site analyses reveal a role of Prp8 in spliceosomal assembly. Nucleic Acids Res. 2013; 41:3805–3818. [PubMed: 23393194]
98. Shukla GC, Padgett RA. A catalytically active group II intron domain 5 can function in the U12-dependent spliceosome. Mol. Cell. 2002; 9:1145–1150. [PubMed: 12049749]
99. Pyle AM. The tertiary structure of group II introns: implications for biological function and evolution. Crit. Rev. Biochem. Mol. Biol. 2010; 45:215–232. [PubMed: 20446804]
100. Fica SM, Mefford MA, Piccirilli JA, Staley JP. Evidence for a group II intron-like catalytic triplex in the spliceosome. Nat. Struct. Mol. Biol. 2014; 21:464–471. [PubMed: 24747940]
101. Robart AR, Chan RT, Peters JK, Rajashankar KR, Toor N. Crystal structure of a eukaryotic group II intron lariat. Nature. 2014; 514:193–197. [PubMed: 25252982]
102. Galej WP, Oubridge C, Newman AJ, Nagai K. Crystal structure of Prp8 reveals active site cavity of the spliceosome. Nature. 2013; 493:638–643. [PubMed: 23354046]
103. Dlakić M, Mushegian A. Prp8, the pivotal protein of the spliceosomal catalytic center, evolved from a retroelement-encoded reverse transcriptase. RNA N. Y. N. 2011; 17:799–808.
104. Query CC, Konarska MM. Structural biology: Spliceosome's core exposed. Nature. 2013; 493:615–616. [PubMed: 23354053]
105. Zhao C, Pyle AM. Crystal structures of a group II intron maturase reveal a missing link in spliceosome evolution. Nat. Struct. Mol. Biol. 2016; 23:558–565. [PubMed: 27136328]
106. Raghunathan PL, Guthrie C. RNA unwinding in U4/U6 snRNPs requires ATP hydrolysis and the DEIH-box splicing factor Brr2. Curr. Biol. CB. 1998; 8:847–855. [PubMed: 9705931]
107. Laggerbauer B, Achsel T, Luhrmann R. The human U5-200kD DEXH-box protein unwinds U4/U6 RNA duplices in vitro. Proc. Natl. Acad. Sci. U. S. A. 1998; 95:4188–4192. [PubMed: 9539711]
108. Kuhn AN, Brow DA. Suppressors of a cold-sensitive mutation in yeast U4 RNA define five domains in the splicing factor Prp8 that influence spliceosome activation. Genetics. 2000; 155:1667–1682. [PubMed: 10924465]
109. Pena V, Liu S, Bujnicki JM, Luhrmann R, Wahl MC. Structure of a multipartite protein-protein interaction domain in splicing factor prp8 and its link to retinitis pigmentosa. Mol. Cell. 2007; 25:615–624. [PubMed: 17317632]
110. Maeder C, Kutach AK, Guthrie C. ATP-dependent unwinding of U4/U6 snRNAs by the Brr2 helicase requires the C-terminus of Prp8. Nat. Struct. Mol. Biol. 2009; 16:42–48. [PubMed: 19098916]

111. Mozaffari-Jovin S, Wandersleben T, Santos KF, Will CL, Luhrmann R, Wahl MC. Inhibition of RNA helicase Brr2 by the C-terminal tail of the spliceosomal protein Prp8. Science. 2013; 341:80–84. [PubMed: 23704370]

112. Grainger RJ, Barrass JD, Jacquier A, Rain J-C, Beggs JD. Physical and genetic interactions of yeast Cwc21p, an ortholog of human SRm300/SRRM2, suggest a role at the catalytic center of the spliceosome. RNA N. Y. N. 2009; 15:2161–2173.

113. Gautam A, Grainger RJ, Vilardell J, Barrass JD, Beggs JD. Cwc21p promotes the second step conformation of the spliceosome and modulates 3' splice site selection. Nucleic Acids Res. 2015; 43:3309–3317. [PubMed: 25740649]

114. Khanna M, Van Bakel H, Tang X, Calarco JA, Babak T, Guo G, Emili A, Greenblatt JF, Hughes TR, Krogan NJ, Blencowe BJ. A systematic characterization of Cwc21, the yeast ortholog of the human spliceosomal protein SRm300. RNA N. Y. N. 2009; 15:2174–2185.

115. Collins CA, Guthrie C. Allele-specific genetic interactions between Prp8 and RNA active site residues suggest a function for Prp8 at the catalytic core of the spliceosome. Genes Dev. 1999; 13:1970–1982. [PubMed: 10444595]

116. Siatecka M, Reyes JL, Konarska MM. Functional interactions of Prp8 with both splice sites at the spliceosomal catalytic center. Genes Dev. 1999; 13:1983–1993. [PubMed: 10444596]

117. Staley JP, Guthrie C. An RNA switch at the 5' splice site requires ATP and the DEAD box protein Prp28p. Mol. Cell. 1999; 3:55–64. [PubMed: 10024879]

Highlights

- The spliceosome is a complex, heterogeneous, dynamic machine
- Our understanding of pre-mRNA splicing results from years of genetic and biochemical analyses, as little high-resolution structural data were available until very recently
- While structural data are critical for the advancement of the splicing field, genetic and biochemical techniques remain essential

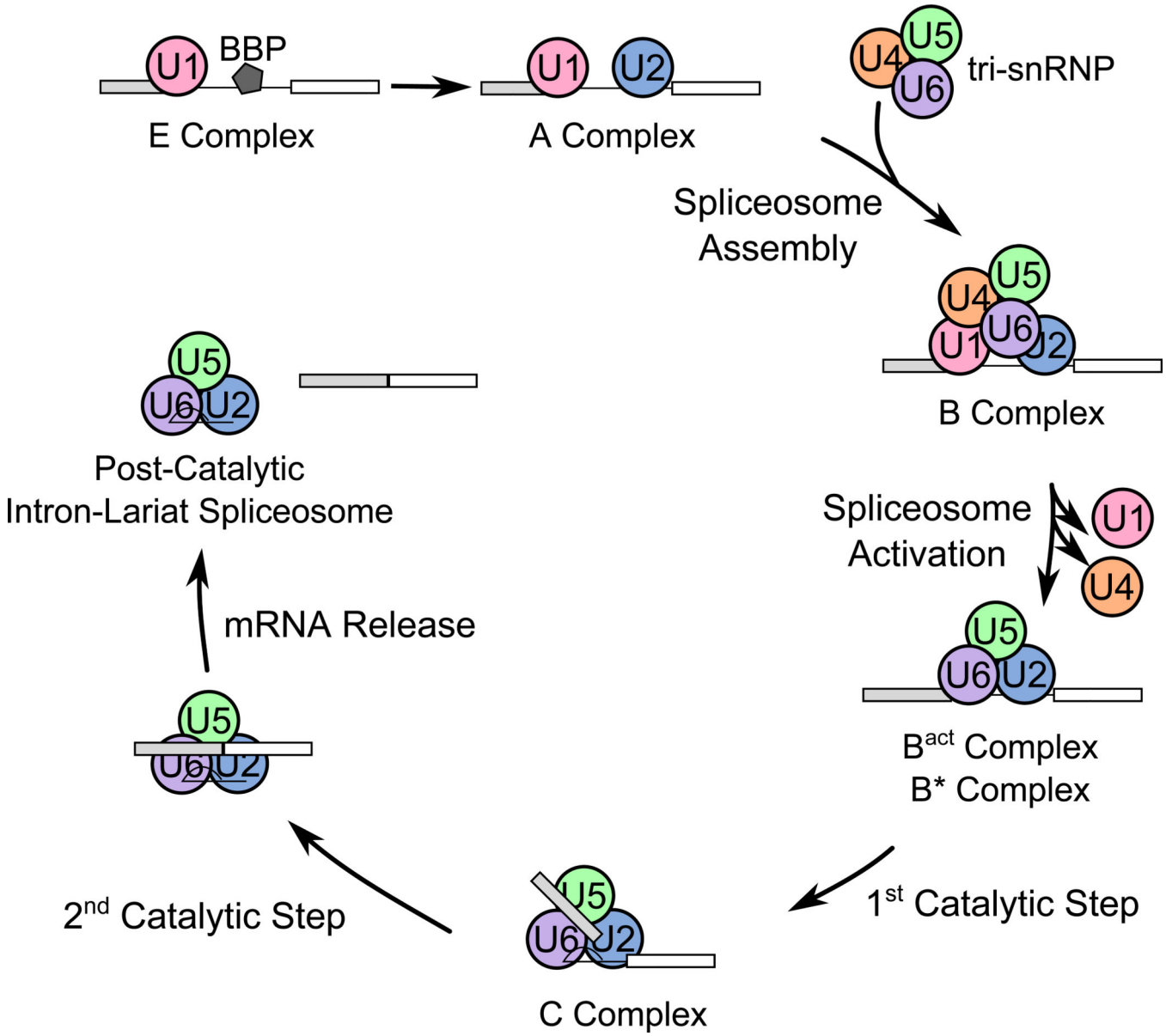

Figure 1.

The splicing cycle. SnRNPs are indicated as colored circles, the pre-mRNA exons are gray and white boxes connected by a line representing the intron. The transitions between key spliceosomal subcomplexes (E, A, B, B^{act}, C, ILS) are illustrated, and the two catalytic steps of splicing are labeled.

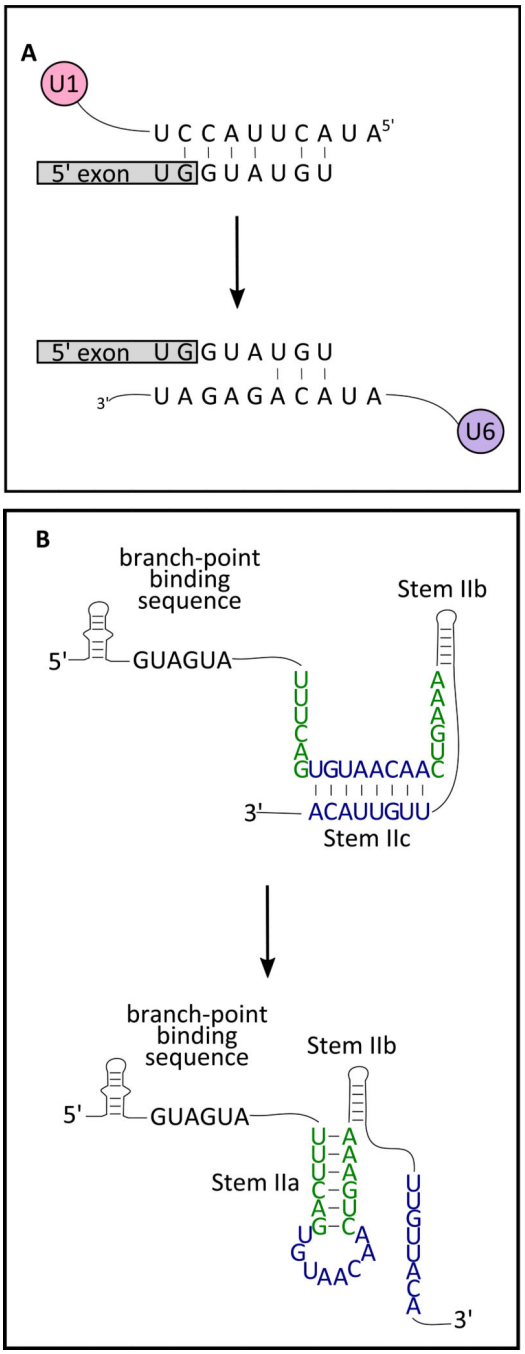

Figure 2.

Mutually exclusive RNA:RNA rearrangements during the splicing cycle. A. The 5'SS interacts sequentially with the U1 and U6 snRNAs[117]. B. U2 snRNA exhibits two conformations during the splicing cycle, stem IIc and stem IIa[58].

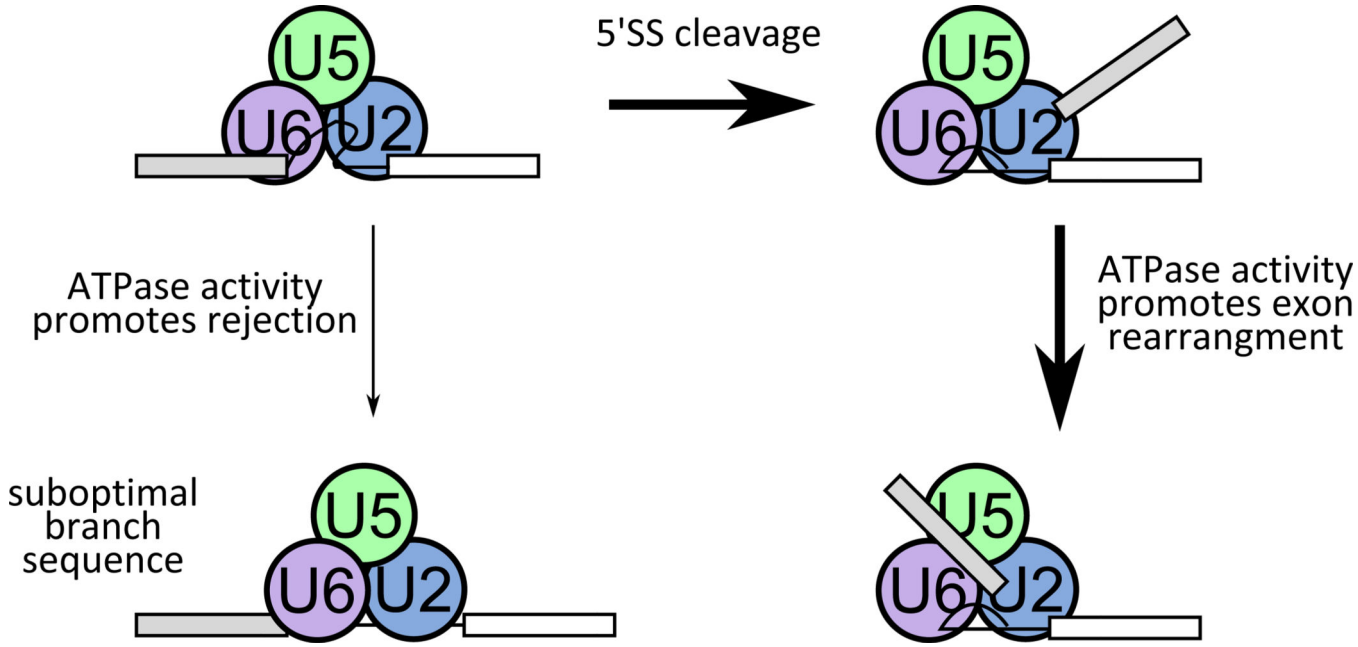

Figure 3.

Branch-site proofreading by DEAH-box helicase Prp16. For substrates with optimal, consensus branch-site sequences, after 5'SS cleavage (dark arrow), Prp16 promotes pre-mRNA rearrangements (dark arrow) required for exon ligation during the 2nd step. For substrates with a suboptimal (nonconsensus) branch-site sequence, Prp16-mediated rejection (light arrow) competes with 5'SS cleavage.

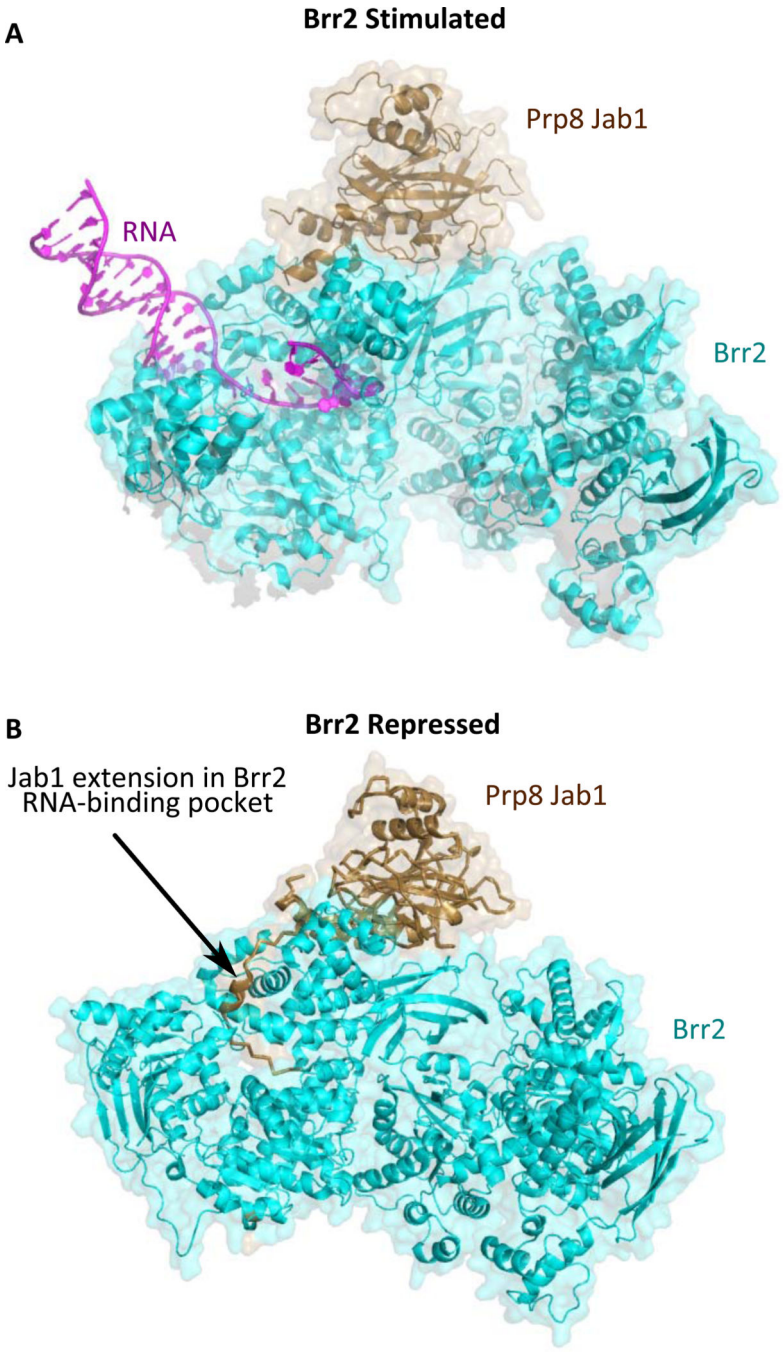

Figure 4.

Regulation of Brr2 activity by the Prp8 Jab1/MPN domain. A. Activated Brr2. When Jab1 is bound, and its extension is not in the Brr2 RNA-binding pocket, Brr2 can unwind dsRNA helices. B. Repressed Brr2. When Jab1 is bound, but its extension is inserted into the Brr2 RNA-binding pocket, Brr2 can no longer interact with RNA. Figures made using pdb files 4BGD and 5DCA.

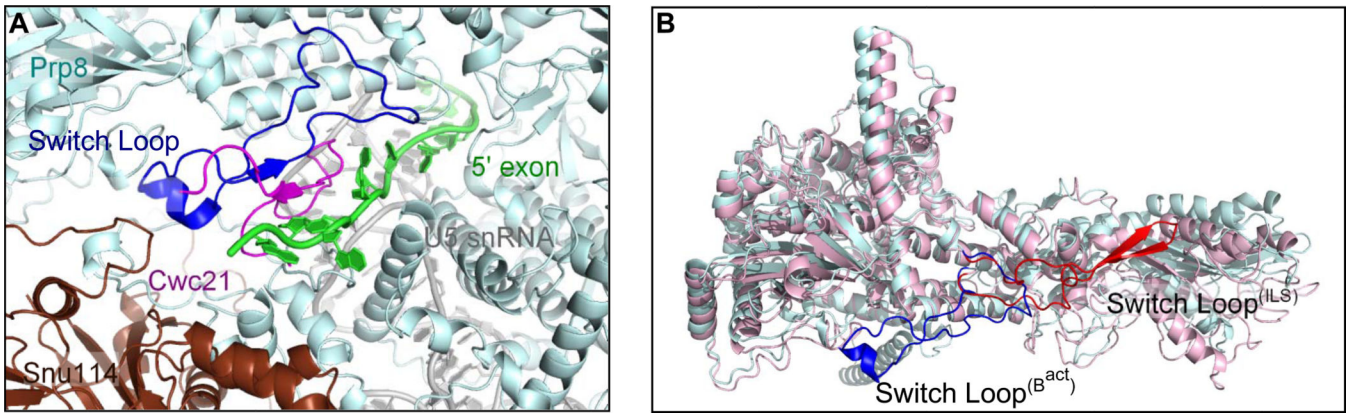

Figure 5.

The Prp8 switch loop. A. The switch loop (blue) of Prp8 (cyan) is shown in the context of an activated spliceosome[4]. Cwc21 is colored in magenta, the U5 snRNA in gray, the pre-mRNA 5' exon is shown in green, and Snu114 is shown in brown. B. Positional heterogeneity of the switch loop. The Prp8 large domains from B^{act} (cyan) and the ILS (pink) are aligned. The relative positions of the switch loop from B^{act} (blue) and the ILS (red) are indicated.