



Functional roles of protein splicing factors

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Synopsis

RNA splicing is one of the fundamental processes in gene expression in eukaryotes. Splicing of pre-mRNA is catalysed by a large ribonucleoprotein complex called the spliceosome, which consists of five small nuclear RNAs and numerous protein factors. The spliceosome is a highly dynamic structure, assembled by sequential binding and release of the small nuclear RNAs and protein factors. DEXD/H-box RNA helicases are required to mediate structural changes in the spliceosome at various steps in the assembly pathway and have also been implicated in the fidelity control of the splicing reaction. Other proteins also play key roles in mediating the progression of the spliceosome pathway. In this review, we discuss the functional roles of the protein factors involved in the spliceosome pathway primarily from studies in the yeast system.

Key words: protein splicing factor, RNA splicing, small nuclear ribonucleoprotein (snRNP), spliceosome, yeast

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INTRODUCTION

Most eukaryotic genes are disrupted by intervening sequences (introns or IVS) that are removed after transcription by RNA polymerases in a process called RNA splicing. Different classes of RNAs use different chemical strategies and different machineries to splice out their introns. Among them, the process of pre-mRNA splicing is the most complicated. Splicing of pre-mRNA takes place on a large ribonucleoprotein particle called the spliceosome, within which two transesterification reactions take place (Figure 1) [1–3]. The spliceosome comprises five snRNAs (small nuclear RNAs), U1, U2, U4, U5 and U6, and many other protein factors. It is assembled through ordered binding of these factors to the pre-mRNA for each round of splicing, and then disassembled after completion of the reaction to recycle the splicing factors (Figure 2) (reviewed in [4,5]). Since the splicing reactions are mechanistically identical with those of Group II self-splicing introns [6,7], it is widely believed that pre-mRNA splicing is also an RNA-catalysed reaction [8]. The spliceosome presumably plays a role in folding the pre-mRNA for proper alignment of the two splice sites so that the splicing reaction can take place. While the RNA–RNA interactions involved in spliceosome formation have been extensively studied [9,10], the functional roles of protein factors have only been better studied more recently.

OVERVIEW OF THE SPLICEOSOME

Most introns have common consensus sequences near their 5' and 3' ends that are recognized by the spliceosomal components (Figure 1). Metazoan genes, however, have been found to contain a different class of rare introns that have non-canonical consensus sequences and use a distinct set of snRNAs for their recognition [11]. These snRNAs, U11, U12, U4atac and U6atac, are functionally analogous to U1, U2, U4 and U6 used in the splicing of the major class of introns [12]. This review focuses on the U2-dependent spliceosome with emphasis on findings from yeast. The known spliceosomal protein components are listed in Table 1.

snRNPs (small nuclear ribonucleoprotein particles) and their protein components

Each of the snRNAs, except for U6, is in complex with Sm core proteins and several other proteins unique to the snRNA to form an snRNP [13,14]. U4 forms base-pairs with U6 to form a single RNP, the U4/U6 di-snRNP, which can further interact with U5 snRNP to form the U4/U6.U5 tri-snRNP [15–18]. The protein components of the snRNPs are evolutionarily conserved, but some mammalian proteins contain additional domains, presumably to increase their capacity for

Abbreviations used: BBP branchpoint-binding protein; CWC, complexed with Cef1; eIF4G, eukaryotic initiation factor 4G; NTC, NineTeen Complex; RRM, RNA-recognition motif; snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein particle.

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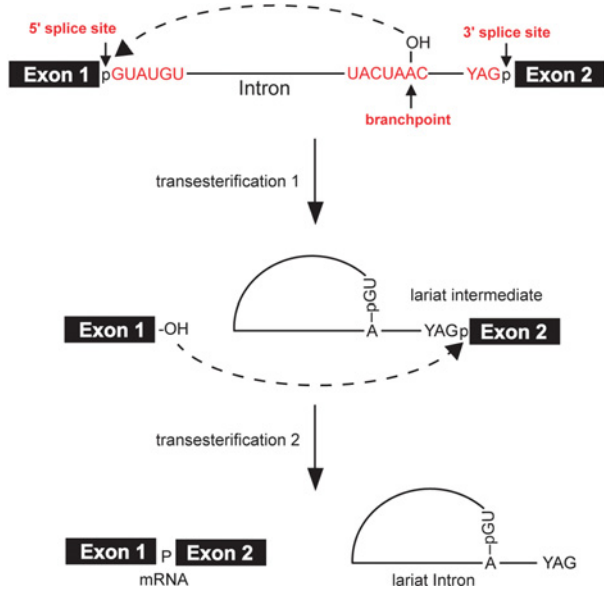


Figure 1 Two-step transesterification reaction of pre-mRNA splicing

Yeast splice site conserved sequences are shown in red. The splicing reaction takes place in two steps. The first step is cleavage of the 5' splice site and formation of lariat intron–exon 2 via a 2'–5' phosphodiester linkage. The second step is cleavage of the 3' splice site and ligation of the two exons.

protein–protein interactions and/or for regulation. The protein components of snRNPs not only function in maintaining the structure of the snRNPs but also in regulating the splicing activity by interacting with other spliceosomal components.

Prp8, Brr2 and Snu114 are integral components of the U5 snRNP. Prp8 is a large protein of more than 250 kDa in size and is one of the most highly conserved nuclear proteins [19,20]. Prp8 interacts with many spliceosomal proteins, with snRNAs and with both 5' and 3' splice sites [21–26]. Prp8 contains an RRM (RNA-recognition motif) domain in the middle of the protein (amino acid residues 1059–1151 in yeast), and a Jab1/MPN domain at its C-terminus (amino acid residues 2173–2310 in yeast) [25,27]. Several mutations linked to human RP (retinitis pigmentosa) are located at the extreme C-terminus of Prp8. They were found to affect splicing and U5 snRNP maturation [28]. Prp8 lacks the catalytic residues in its Jab1/MPN domain required for the activity of deubiquitination, but has been shown to bind ubiquitin *in vitro* [29]. Although ubiquitin has been implicated in the spliceosome pathway [30], the functional significance of ubiquitin binding or whether this function is related to Prp8 is not known. Upstream from the Jab1/MPN domain, a segment of ~250 amino acids has been demonstrated to form an RNase H-like structure (1833–1950 in yeast) [31–34]. Since the putative catalytic residues of the RNase H enzyme are displaced in the Prp8 domain, whether Prp8 retains its nucleolytic function is questionable. A C-terminal fragment of Prp8 comprising the RNase H, Jab1/MPN and RP domains has been shown to stimulate the unwinding activity of Brr2 [35–37].

Brr2 is a DExD/H-box RNA helicase that mediates the unwinding of U4/U6 helices to release U4 from the spliceosome [38,39]. It has also been implicated in the mediation of spliceosome disassembly [40]. Unlike the other spliceosomal DExD/H-box proteins, Brr2 has two helicase domains and remains associated with the spliceosome throughout the reaction after its association as a component of tri-snRNP [41]. The N-terminal helicase domain is responsible for the unwinding activity [42]. The C-terminal helicase domain is not catalytically active, but serves as a platform for interaction with many spliceosomal components [26,37].

Snu114 is a GTPase that is highly homologous with translation elongation factor G [43] and has been suggested to be involved in spliceosome activation [44,45]. Despite never having been shown to hydrolyse GTP *in vitro*, Snu114 has been demonstrated to bind GTP by UV-cross-linking [43,46], and its binding of GTP/GDP has been proposed to regulate spliceosome dynamics mediated by Brr2 during spliceosome activation and disassembly [40]. Furthermore, a functional GTPase domain is required for assembly of Snu114 into U5 snRNP [47].

Prp24 is an RNA-binding protein containing four RRM [48]. Prp24 has been shown to interact with U6 and U4/U6, and stimulate annealing of U4 and U6 for formation of the di-snRNP [49–52]. In extracts, while U4 always complexes with U6 as di-snRNP or tri-snRNP, U6 also exists as a distinct RNP particle, which contains Prp24. A role for Prp24 in recycling the spliceosome has been demonstrated [49].

DExD/H-box RNA helicases

DExD/H-box proteins are a ubiquitous class of enzymes that mediate rearrangement of RNA–RNA or RNA–protein interactions in an ATP-dependent manner [53,54]. Eight DExD/H-box proteins are required for the splicing process [55,56]. They function in mediating structural rearrangements of the spliceosome at different steps in the spliceosome pathway. Several of them have been shown to unwind RNA duplexes *in vitro* [38,39,57–60], but no direct evidence shows that they catalyse RNA unwinding in the spliceosome. Two of these proteins, Sub2 and Prp5, are involved in the early steps of spliceosome assembly. Sub2 has been proposed to displace Mud2 and BBP (branchpoint-binding protein), which bind to the 3' splice site and branch site respectively (see the section 'Spliceosome assembly' for details), to facilitate the association of U2 snRNP with the spliceosome [61]. Prp5 also functions in facilitating U2 binding to the spliceosome, in part by ATP-dependent displacement of Cus2 from U2 snRNP to convert U2 into a functional form [62,63]. Prp28 and Brr2 are required for spliceosome activation in mediating the release of U1 and U4 respectively [38,39,64]. The requirement of Prp28 can be bypassed if the interaction of U1C protein (Yhc1 in yeast) or U1 snRNA with the 5' splice site is destabilized by mutations [65]. Brr2 is required for unwinding of U4/U6 during spliceosome activation [38,39] and also for separation of U2/U6 interaction in disassembly of the spliceosome [40]. Prp2 and Prp16 are involved in catalytic steps [66,67]. The action of Prp2 is associated with the release of SF3a/b after the activation of the spliceosome

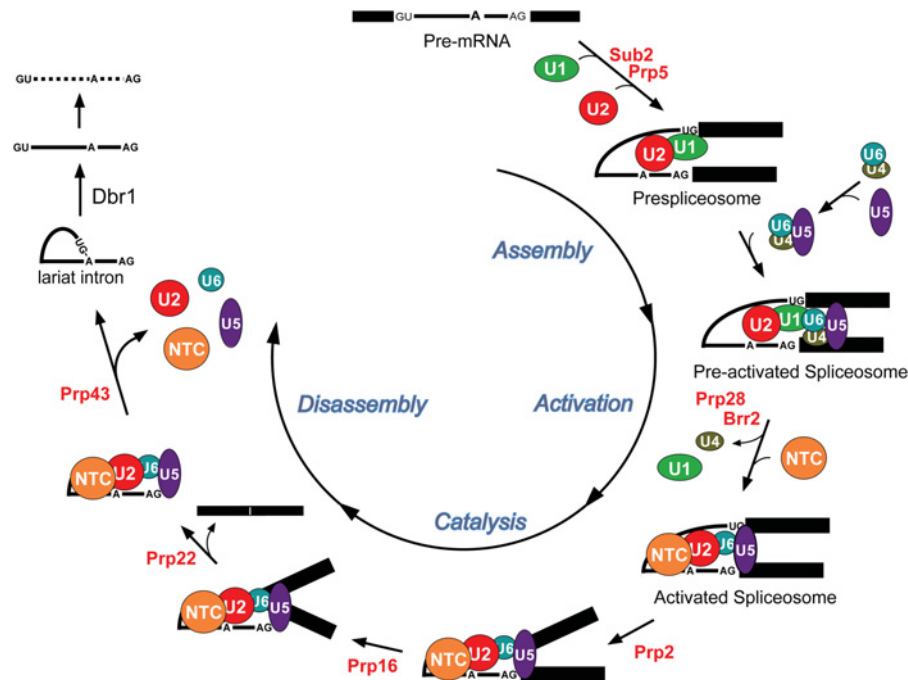


Figure 2 Schematic representation of the spliceosome pathway in yeast

The pathway can be divided into four stages: spliceosome assembly, spliceosome activation, catalysis and spliceosome disassembly. Spliceosome assembly involves ordered interactions of snRNPs with pre-mRNA. Spliceosome activation starts with the release of U1 and U4, followed by binding of the NTC. After the two catalytic reactions, the spliceosome is disassembled first by releasing the mature mRNA, and the spliceosome is then dismantled.

[68,69], and that of Prp16 is associated with the release of Yju2 and Cwc25 after lariat formation [70]. After completion of splicing, two DExD/H-box proteins are required to disassemble the spliceosome. Prp22 first mediates the release of mature mRNA from the spliceosome, and Prp43 then catalyses disassembly of the intron-containing spliceosome [71,72].

In addition to these ATP-dependent functions, several DExD/H-box splicing factors have been shown to have an ATP-independent role in splicing. Although Prp5 is required for displacement of Cus2 from U2 snRNP before the binding of U2 to the spliceosome, it is still needed for pre-spliceosome formation in the absence of Cus2 independent of its ATPase function [63]. Prp2 has been implicated in a novel ATP-independent conformational change [68]. Prp16 has been shown to stabilize the association of Cwc25 with the spliceosome formed on pre-mRNA carrying mutations at the branchpoint [70]. Prp22 also participates in the second transesterification reaction independent of ATP in addition to its role in mRNA release [59]. It will be interesting to see whether it is a general property for splicing DExD/H-box proteins to have both ATP-dependent and ATP-independent functions.

The Prp19 complex

The Prp19 complex [or NTC (NineTeen Complex)] was identified as a large RNA-free protein complex associated with essential

splicing factor Prp19 [73,74]. Eight proteins, Prp19, Syf1/Ntc90, Cef1/Ntc85, Clf1/Ntc77, Syf2/Ntc31, Isy1/Ntc30, Snt309/Ntc25 and Ntc20, have been shown to be components of the complex, and several others have also been suggested to be putative NTC components [74–80]. The NTC is recruited to the spliceosome after the release of U1 and U4 and remains associated with the spliceosome until completion of the catalytic reactions. The NTC is required for stabilizing the association of U5 and U6 with the spliceosome [81]. It promotes specific interactions of U5 and U6 with pre-mRNA within several bases, and also triggers the release of Lsm from binding to the 3'-end of U6 snRNA to allow further interaction of U6 with the intron sequence near the 5' splice site [81,82]. Proteomic analysis of proteins associated with Cef1/Ntc85 has also identified a complex, called CWC (complexed with Cef1), containing U2, U5 and U6 snRNAs and more than 25 proteins, including all the NTC components [83]. Cwc2 directly interacts with U6 snRNA, and has been suggested a role in linking the NTC to the spliceosome [84]. In contrast, Cwc22 and Cwc25 are required for the catalytic steps, and only bind to the spliceosome after its activation [85,86].

The functions of NTC and several of its putative components in splicing have been characterized. Prp19 contains an U-box motif for ubiquitin ligase at its N-terminus, a coiled-coil domain for tetramerization at central region and a WD40 repeat domain as a splicing scaffold at its C-terminus [87–90]. Prp19 has been demonstrated to promote ubiquitination of Prp3

**Table 1 Protein splicing factors**

SF3a and SF3b are subcomplexes of U2 snRNP. Asterisks mark proteins not found on the spliceosome. Non-snRNP proteins are sorted by their functions.

(a) snRNP proteins

U1	U2	U4	U5	U6	U4/U6	U4/U6.U5
SmB, D1, D2, D3, E, F and G	SmB, D1, D2, D3, E, F and G	SmB, D1, D2, D3, E, F and G	SmB, D1, D2, D3, E, F and G	Lsm2–8	SmB, D1, D2, D3, E, F and G	SmB, D1, D2, D3, E, F and G
Snp1	Lea1	Prp3	Dib1	*Prp24	Lsm2–8	Lsm2–8
Mud1	Msl1	Prp4	Prp8		Prp3	Prp3
Yhc1	*Cus2	Snu13	Prp28		Prp4	Prp4
Luc7	SF3a		Brr2		Snu13	Snu13
Nam8	Prp9		*Lin1		Prp31	Prp31
Prp39	Prp11		Snu114			Dib1
Prp40	Prp21		Prp6			Prp8
Prp42	SF3b		*Aar2			Prp28
Snu56	Cus1					Brr2
Snu71	Rse1					Snu114
	Hsh49					Prp6
	Hsh155					Prp38
	Rds3					Snu23
	Ysf3					Snu66
	RES					Spp381
	Ist3					*Sad1
	Bud13					
	Pml1					

(b) Non-snRNP proteins

Assembly	Activation	First reaction	Second reaction	Disassembly	Unknown
Msl5	Prp19	Cwc22	Prp17	Spp382	Bud31
Mud2	Snt309	Spp2	Prp16	Ntr2	Cwc15
Sub2	Cef1	Prp2	Slu7	Prp43	Cwc24
Prp5	Syf1	Cwc25	Prp18		Cwc27
	Clf1	Yju2	Prp22		Urn1
	Syf2				
	Isy1				
	Ntc20				
	Cwc2				
	Prp45				
	Prp46				
	Ecm2				
	Cwc21				

during the splicing reaction [91]. Snt309/Ntc25 is required for the integrity of the NTC. It interacts with Prp19 to regulate the interaction of Prp19 with Cef1/Ntc85, which further interacts with Syf1/Ntc90 and Clf1/Ntc77 in the formation of the NTC [75,92]. Although Clf1/Ntc77 has been shown to bind to the spliceosome as an integral component of the NTC, it has also been reported to be required for the addition of tri-snRNP to the spliceosome [75,93]. Syf1/Ntc90, Syf2/Ntc31, Isy1/Ntc30 and Ntc20 form a stable subcomplex, which is not required for spliceosome activ-

ation, but is required for the recruitment of step-one factor Yju2 [94]. Deletion of *ISY1/NTC30* gene was found to partially rescue the cold-sensitive growth phenotype of *prp16-302* mutant and restore fidelity of branchpoint usage in *prp16-302* cells to wild-type level, leading to the hypothesis that Isy1/Ntc30 can regulate the function of Prp16 in splicing fidelity control [95]. Ecm2 was shown to be a component of the CWC complex, and was also identified as Slt11 on the basis of synthetic lethality with a mutation in the 5'-end of U2 snRNA that disrupts U2/U6 helix

II [83,96]. Ecm2 contains two conserved zinc-finger motifs and two regions of the RRM, and has been shown to bind specific RNA structures reminiscent of U2/U6 helix II, suggesting a role for Ecm2 in the formation of helix II [97]. Cwc2 has been shown to interact with Prp19 directly by two-hybrid assays [79] and is likely to be the Ntc40 detected to interact with Prp19 by far Western blotting [74]. Cwc2 also contains an RRM and a zinc-finger motif, and can directly bind U6 snRNA [84], but how binding of Cwc2 to U6 mediates spliceosome activation remains to be investigated.

SPLICEOSOME ASSEMBLY

The spliceosome is assembled via sequential binding of snRNPs to the pre-mRNA in the order of U1, U2 and then U4/U6.U5 (as the preformed tri-snRNP) [17,98]. U1 snRNP binds to the 5' splice site through base-pairing of U1 with the 5' splice site to form the commitment complex [99–101]. Binding of U1 to the pre-mRNA is ATP-independent and can occur without incubation [98,99]. U2 binds to the branch site also through base-pairing to form the pre-spliceosome, which requires ATP [98,102–105]. The U4/U6.U5 tri-snRNP is recruited to the spliceosome following binding of U2 [17,98] and interacts with the 5' splice site, but such interaction can occur independent of prior binding of U2 to the branch site in HeLa or nematode cell extracts [106,107]. The BBP [SF1 (splicing factor 1) in humans] is thought to bind the branchpoint sequence early and can bridge the 5' splice site and the 3' splice site by interacting with U1 components Prp40 and Mud2 (U2AF65 in humans) [108–110]. BBP is displaced by U2 snRNP in an ATP-dependent step upon formation of the pre-spliceosome [111]. A large 45S particle containing all five snRNAs, called penta-snRNP, has been detected in yeast cell extracts and demonstrated to be functional for splicing upon addition of pre-mRNA substrate [112]. It has also long been known that large 200S complexes, containing all five snRNAs and protein splicing factors exist in HeLa nuclear extracts [113,114]. These observations raise the possibility that splicing occurs on preassembled spliceosomes *in vivo*. However, *in vivo* studies of spliceosome formation in yeast by chromatin immunoprecipitation analysis suggest spliceosome assembly to be co-transcriptional and occur in an ordered manner [115,116].

SPLICEOSOME ACTIVATION

After the binding of tri-snRNP, the spliceosome undergoes a major conformational rearrangement in which U1 and U4 are dissociated from the spliceosome [17,117]. The release of U1 and U4 requires the unwinding of U4/U6 duplexes and destabilization of U1–5' splice site base-pairing, both of which require ATP. DExD/H-box RNA helicases Prp28 and Brr2 have been

implicated in the release of U1 and U4 respectively [38,39,64]. Brr2 is a component of the U5 snRNP [41] and has been demonstrated to catalyse the unwinding of the U4/U6 RNA duplexes *in vitro* [38,39]. Human, but not yeast, Prp28 is associated with the tri-snRNP [118], which requires phosphorylation of Prp28 by SRPK2 (serine/arginine protein-specific kinase2) [119]. Genetic studies have revealed that Prp28 is required to destabilize U1–5' splice site base-pairing [64], but the mechanism underlying the action of Prp28 is not clear. Prp28 may act by directly unwinding U1–5' splice site duplex, although RNA unwinding activity has not been demonstrated *in vitro*. Alternatively, Prp28 may displace stabilizing proteins that bind to the 5' splice site. Supporting this notion, U1 component U1C has been shown to directly bind to the 5' splice site [120], and mutation in U1C was able to bypass the requirement of Prp28 [65].

The release of U1 and U4 allows new base-pair formation between U6 and the 5' splice site and between U6 and U2, as demonstrated by extensive genetic and UV-cross-linking analyses [9,10] (Figure 3). Interactions between U5 and the exon sequence at the splice junction have also been demonstrated [121–123]. Although such RNA base-pairings constitute the framework of the catalytic core of the spliceosome, they are usually in short stretches and require protein factors to stabilize their structure. Prp8 has been shown to cross-link to both 5' and 3' splice site regions, suggesting its binding to the pre-mRNA may stabilize the base-pairing of U5 with the exon sequences for splice site alignment [22,121].

The NTC was shown to be required for spliceosome activation after the release of U1 and U4 [81]. The binding of NTC does not require much of the sequence downstream of the branchpoint [124], but how it is recruited to the spliceosome remains unknown. The NTC is required for stabilizing the association of U5 and U6 with the spliceosome in formation of the active spliceosome [81]. In the absence of the NTC, both U5 and U6 interact with the pre-mRNA in a dynamic manner after U1 and U4 are released, as revealed by UV-cross-linking analysis. The presence of the NTC renders base-pairings of U5 and U6 with defined residues of the pre-mRNA [81,82]. The binding of the NTC also promotes the release of Lsm proteins from U6 to allow for the interaction of the Lsm-binding site near the 3'-end of U6 snRNA with the intron in a region approximately 30 bp downstream from the 5' splice site [81].

CATALYTIC STEPS

After pairing of U6 to the 5' splice site and to U2 snRNA, the catalytic core of the spliceosome is established and stabilized by the binding of the NTC and protein components of snRNPs. The splicing reaction is completed via two consecutive transesterification reactions on the activated spliceosome. The first step is cleavage at the 5' splice site and formation of the lariat intron–exon 2 via a 2'–5' phosphodiester linkage. The second step is cleavage at the 3' splice site and ligation of the two exons (Figure 1) [125–127].

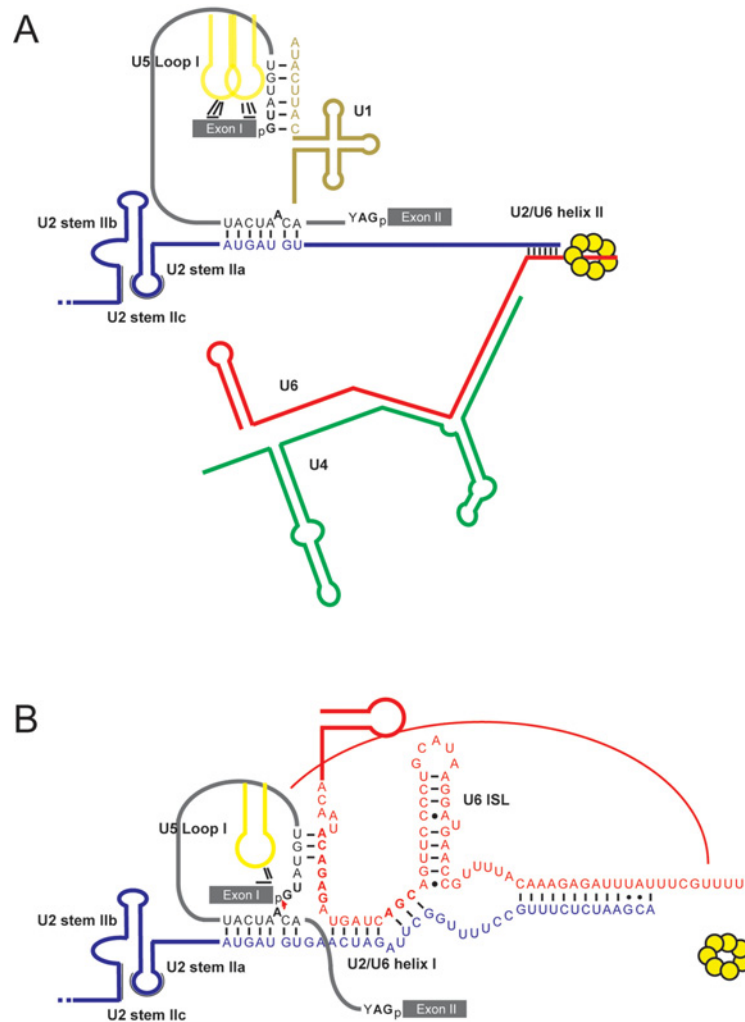


Figure 3 RNA-RNA interactions in pre-activated and activated spliceosomes

(A) The pre-activated spliceosome contains five snRNAs. U1 snRNA base-pairs with the 5' splice site and U2 snRNA base-pairs with the branch site with a stem Ila structure. U2/U6 helix II has been shown to be required for tri-snRNP recruitment in mammalian cells. Loop I of U5 snRNA interacts with two broad regions of exon 1. (B) On the activated spliceosome, base-pairings of the 5' splice site with U1 are displaced by U6. In addition, the Lsm complex is dissociated from U6 to allow interactions of its 3' end with the intron sequence in a region downstream from the 5' splice site.

Although transesterification reactions do not require energy, both catalytic steps require DExD/H-box ATPases, Prp2 for the first step and Prp16 for the second step, and several other proteins [56] (Figure 4). Much of the work concerning the mechanism of the catalytic steps has been conducted using the yeast *Saccharomyces cerevisiae*.

First catalytic step

It has been shown that the 3' splice site is not required for the first reaction [128], but the lack of most nucleotides between the branchpoint and the 3' splice site blocks the first reaction without affecting spliceosome activation, indicating that this region is important for the first reaction [81,124,129]. The first cata-

lytic step can be divided into two stages based on the ATP requirement, the Prp2-mediated ATP-dependent step with no chemical change to the RNA substrate, followed by the ATP-independent step, which requires additional protein factors to promote the chemical reaction [130].

Prp2 can associate with the spliceosome and directly interact with pre-mRNA in the absence of ATP prior to the first reaction [66,131,132]. Upon ATP hydrolysis, Prp2 is dissociated from the spliceosome [132]. The recruitment of Prp2 to the spliceosome requires a co-factor Spp2, originally identified as a high-copy suppressor of temperature-sensitive *prp2-1* mutation [133,134]. The C-terminal region of Prp2 is important for its interaction with Spp2 and for spliceosome binding [135,136]. Spp2 interacts with Prp2 through its G-patch domain, which has been

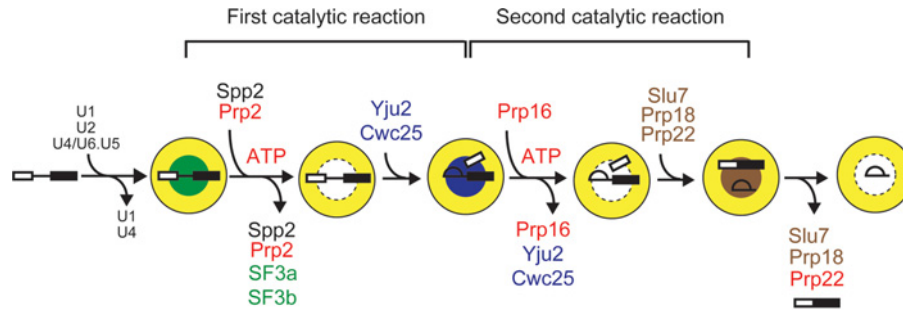


Figure 4 Spliceosome dynamics during the catalytic steps

Both catalytic steps can be divided into ATP-dependent and ATP-independent steps. Prp2 and Prp16 are required in ATP-dependent steps. Hydrolysis of ATP results in the release of SF3a/b and Yju2/Cwc25 in the first and second step respectively. This allows Yju2/Cwc25 and Slu7/Prp18/Prp22 to bind to the spliceosome and promote the first and second transesterification reactions respectively in an ATP-independent manner. Yellow circles with different colours in the centre represent spliceosomes with different sets of splicing factors binding at the catalytic centre.

proposed to mediate RNA binding [136,137]. Spp2 can associate with the spliceosome prior to the first reaction and is dissociated from the spliceosome with Prp2 upon ATP hydrolysis [134].

The function of Prp2 in the first catalytic step was obscure until recently, when it was demonstrated that the ATPase function of Prp2 is associated with the release of SF3a and SF3b from the spliceosome [68,69]. SF3a and SF3b are subunits of U2 snRNP, comprising three and six or seven proteins respectively. SF3b subunit SAP155 (Hsh155 in yeast) has been shown to cross-link to intron sequences flanking the branchpoint, suggesting a role for SF3b in stabilizing U2-branch site base-pairing during spliceosome assembly [138–140]. Conceivably, the removal of SF3b from binding to the branch site is necessary to expose the branchpoint to initiate the chemical reaction. Prp2 and Spp2 are dissociated from the spliceosome together with SF3a/b. More recently, another splicing factor, Cwc22, was shown to be required for the Prp2-dependent step [86]. Cwc22 is an eIF4G (eukaryotic initiation factor 4G)-like protein and contains both MIF4G (middle of eIF4G) and MA3 domains found in eIF4G. It binds to the spliceosome after the binding of NTC and is not required for spliceosome activation. Although Cwc22 is not required for the binding of Prp2 to the spliceosome, in the absence of Cwc22, Prp2 is released from the spliceosome upon ATP hydrolysis without promoting the release of SF3a/b [86].

The release of SF3a/b, however, is not sufficient for the first reaction to take place. A protein factor of unknown identity, named HP (heat-resistant protein) for its heat-resistant property, was previously shown to be required for the first catalytic reaction independent of ATP [130]. Two proteins, Yju2 and Cwc25, were later found to be required for the first reaction after the action of Prp2. Yju2 has been shown to interact with the NTC via its interaction with Ntc90 and Ntc77, and can bind to the spliceosome before or after the action of Prp2 [141]. In contrast, Cwc25 is recruited to the spliceosome exclusively after the action of Prp2 but only in the presence of Yju2 [85], and is the last protein factor recruited to the spliceosome before the first catalytic re-

action [69,85]. Both Yju2 and Cwc25 become stably associated with the spliceosome after the first reaction, but are released prior to the second reaction [70] (see below).

Second catalytic step

The second catalytic step concerns the identification of the 3' splice site to align with the 5' splice site for exon ligation. U5 snRNP plays an important role in the alignment of exons. Loop 1 of U5 snRNA contains nine evolutionarily invariant nucleotides that can interact with both exons at the splice junction [122,142–144]. In yeast, loop 1 of U5 is required only for the second reaction *in vitro* [145,146], but can play a role in the first step [144]. Surprisingly, loop 1 is totally dispensable for the human *in vitro* splicing reaction [147]. The U5 component Prp8 has been shown to cross-link to both exons at sites near the splice junction, and proposed to stabilize the interaction of U5 with the exon sequences during the second catalytic reaction [21,22,148]. Genetic interactions between Prp8 and the 5' splice site, 3' splice site, and almost all the other second-step factors (see below) have also been demonstrated [148–151]. In addition, the non-Watson–Crick interaction between the first and last intron nucleotides has been shown to be required for the second reaction [152,153], and the interactions between both splice site consensus sequences and U6 snRNA also play an important role to juxtapose the two exons in the second step [154]. Mutations that affect these interactions can be suppressed by allele-specific *prp8* mutants [149,150,155], suggesting that Prp8 may modulate the conformational transition between the two transesterification reactions [156,157].

Like the first step, the second catalytic step also involves an ATP-dependent step, which requires DEXD/H-box protein Prp16, followed by an ATP-independent step, in which transesterification occurs [158]. Prp16 was originally identified as a suppressor of branchpoint mutant A259C (or brC) of the actin intron [159,160], but was found to be only required for the second reaction *in vitro* [67]. It has been shown that Prp16 can associate with the spliceosome in the absence of ATP, and upon ATP hydrolysis,



Prp16 is released from the spliceosome [67]. The N-terminus of Prp16 is required and sufficient for its association with the spliceosome [161]. UV-cross-linking analysis has revealed that Prp16 interacts with pre-mRNA in the intron sequence near the 3' splice site [23,138]. Prp17 has also been demonstrated to function in the ATP-dependent step, but the binding of Prp16 to the spliceosome is not affected in the absence of Prp17 [23,162]. Prp16 has been proposed to mediate a conformational change in the spliceosome prior to the second reaction, since the 3' splice site becomes protected from RNase H cleavage after Prp16 action [163]. This protected region was later shown to cross-link with Prp22, suggesting that the binding of Prp22 may confer the nuclease protection phenotype [59,164].

Several cold-sensitive *prp16* alleles have been shown to suppress mutations in U2 and U6 snRNAs in the regions that are involved in the formation of RNA duplexes, suggesting a role of Prp16 in disruption of these helices [165–167]. Although Prp16 has been shown to unwind synthetic RNA duplexes, there is no direct evidence for such function on the spliceosome [57]. The recent demonstration of Prp16- and ATP-dependent release of the first step factors Yju2 and Cwc25 suggests that Prp16 may play a role in remodelling the spliceosome to prepare the spliceosome for the second catalytic reaction [70]. Whether destabilization of Yju2 and Cwc25 by Prp16 leads to destabilization of RNA structures, or vice versa, is unclear.

Following Prp16 action, Slu7, Prp18 and Prp22 join the spliceosome to promote the second reaction in an ATP-independent manner [59,158,168]. The fact that Yju2 and Cwc25 are removed from the spliceosome prior to the association of Slu7, Prp18 and Prp22 suggests that displacement of proteins binding to the catalytic centre is necessary for positioning of the 5' and 3' splice sites for exon ligation. These three proteins interact with the 3' splice site, and both Prp22 and Slu7 directly contact the intron, as revealed by cross-linking analysis [23,164]. Prp22 is a DEXD/H-box protein, but its role in the second catalytic step is ATP-independent [59,72]. Prp22 is further required for the release of mature mRNA after completion of splicing, which depends on its ATPase activity, and together with Slu7 and Prp18, it is released from the spliceosome along with mRNA [58,59,72,169]. Prp18 has been shown to stabilize the interaction between loop 1 of U5 snRNA and both exons during the second reaction [142,170]. The requirement of Slu7, Prp18 and Prp22 for the second reaction depends on the distance between the branch site and the 3' splice site of precursor mRNA [59,171,172]. None of the three proteins is needed if the distance is less than 7 nt [171]. The binding of these proteins follows an order of Slu7, Prp18 and then Prp22 [169]. Together, the second-step factors bind to the 3' splice site, and coordinate with Prp8 to stabilize interactions of U5 loop 1 and the exons for the second transesterification.

Similarities between the two steps

The two catalytic steps are mechanistically similar. Both steps can be divided into two stages, an ATP-dependent step, which requires a DEXD/H-box protein, followed by an ATP-independent catalytic reaction. Each step starts with displacement of protein

binding to the catalytic centre to allow binding of another set of proteins to promote the catalytic reaction. Prp2 is required to mediate the release of SF3a/b, which bind to the branch site, to allow binding of Yju2 and Cwc25 to promote the first transesterification. Prp16 is required to mediate the release of Yju2 and Cwc25, to allow binding of Slu7, Prp18 and Prp22 to promote the second transesterification. Cwc25 binds to the branch site as it can cross-link to substrate RNA near the branch site (H.-C. Chen and S.-C. Cheng, unpublished work), and mutations at the branchpoint prevent its binding to the spliceosome [70]. Conceivably, the removal of factors binding to the catalytic centre and functioning in the previous step converts the catalytic centre into an open state, allowing splice sites to interact in a dynamic manner. Subsequent binding of specific protein factors, Yju2/Cwc25 for the first step and Slu7/Prp18/Prp22 for the second step, facilitates and confines the interaction at specific sites. It is interesting that Cwc25 was shown to be dispensable for the first reaction if the purified spliceosome was incubated in the presence of Mn^{2+} , suggesting that increasing the dynamics of splice-site interaction can bypass the need for Cwc25 [85]. Similarly, Slu7/Prp18/Prp22 can be dispensable when the 3' splice site is very close to the branch site so that the 3' splice site is more susceptible to interaction with the 5' splice site [59,171,172]. Moreover, both Prp16 and Prp22 have two roles in the splicing pathway. Prp22 has a primary role in promoting mRNA release, which requires the ATPase function of the protein, and is conditionally required for the second reaction in positioning the 3' splice site in an ATP-independent manner. Prp16 also has an ATP-dependent role in promoting the release of Yju2/Cwc25, and has an ATP-independent role in the first catalytic step in stabilizing the binding of Cwc25 when pre-mRNA carries a mutation at the branchpoint.

DISASSEMBLY OF THE SPLICEOSOME

mRNA release

Following exon ligation, the mature mRNA is released before the spliceosome is disassembled. Prp22 was initially identified from a genetic screen for factors defective in pre-mRNA splicing [173]. The *prp22-1* mutant accumulates mRNA and excised lariat-intron on the spliceosome at the non-permissive temperature [72]. Prp22 has been demonstrated to unwind RNA duplexes *in vitro* [58,59], and the helicase activity is required for mRNA release [174]. Mutations in motif III (SAT) of the helicase domain are defective in unwinding RNA duplex and releasing mRNA with no effect on the ATPase activity or the binding of Prp22 to the spliceosome [174]. It has been shown that cold-sensitive growth defect elicited by Prp22 helicase-defective mutants can be suppressed by several mutant alleles of Prp8 at Arg¹⁷⁵³, suggesting that Prp22 may disrupt RNA–RNA or RNA–protein interaction stabilized by Prp8 during mRNA release [175]. In agreement with this notion, specific mutant alleles of U5 loop 1, which stabilizes the interaction of U5 loop 1 with splicing intermediate and suppresses *prp8-R1753* mutant, can aggravate the defect of Prp22 helicase-defective mutants [151]. Prp22 was demonstrated to bind to the

3' splice site during the second step [164] and then translocate to mRNA downstream of exon–exon junction after exon ligation [176]. It is proposed that Prp22 functions to liberate mRNA by moving along mRNA in a 3' to 5' directionality to disrupt U5–mRNA interaction [176]. More than 13 nt of 3' exon in length is necessary for mRNA release, presumably to allow stable binding of Prp22 after translocation [176].

Spliceosome disassembly

After mRNA is released, the spliceosome is disassembled to free the lariat-intron and all associated spliceosomal components for recycling of splicing factors. The free lariat-introns are debranched, catalysed by debranchase (Dbr1), to yield linear intron molecules, which are generally degraded [177,178]. Prp43, a DExD/H-box RNA helicase, is the key player in mediating disassembly of the spliceosome [71,178] and is also involved in the biogenesis of both large- and small-subunits of the ribosome [179–181]. Prp43 has been demonstrated to unwind RNA duplexes *in vitro* [60]. The ATPase activity of Prp43 is required for spliceosome disassembly, but the level of the helicase activity does not have any apparent correlation with the disassembly efficiency [60,178].

The function of Prp43 in mediating spliceosome disassembly requires two co-factors, Ntr1 and Ntr2 [182,183]. Ntr1 interacts with Ntr2 to form a stable complex, which further interacts with Prp43 to form a functional NTR complex [182]. Spliceosome disassembly can be assayed *in vitro* using purified NTR. The post-splicing spliceosome isolated from NTR-depleted extracts, containing the lariat-intron, is dissociated into U2, U5, U6 and NTC upon incubation with purified NTR and ATP [182]. Ntr1 interacts with Ntr2 via the middle region of the protein, and with Prp43 via the N-terminal G-patch domain [182]. The G-patch domain of Ntr1 has been shown to stimulate the helicase activity, but not the ATPase activity, of Prp43 [184]. Ntr2 interacts with U5 snRNP via its interaction with U5 component Brr2, and such interaction is suggested to mediate the recruitment of NTR to the spliceosome [185].

How NTR mediates spliceosome disassembly is not known. To disassemble the spliceosome, it is essential to disrupt base-pairings of U6/5' splice site, U2/U6 and U2/branch site. Prp43 has been shown to cross-link to U6, but the precise cross-link sites have not been determined [186]. Prp43 may destabilize base-pairings of U6/5' splice site or U2/U6 by unwinding RNA duplex or by displacing proteins binding to specific RNA structures, which then triggers disruption of other RNA duplexes. It has also been shown that mutations in Brr2 impede disassembly of the spliceosome isolated via its association with Prp43, and the ATPase activity of Brr2 is regulated by Snu114 through its binding of GTP versus GDP [40]. How Brr2 co-ordinates with Prp43 to mediate spliceosome disassembly remains unknown.

NTRI, previously identified as *SPP382*, was shown to function as a suppressor of *prp38-1* mutation [187]. Prp38 is a component of the yeast tri-snRNP [188,189]. Several mutations in *PRP43* affecting the ATPase activity have been shown to suppress the growth defect of *prp38-1* with efficiencies inversely proportionate

to the measured ATPase activities [187], suggesting that reducing the activity of Prp43 could partially compensate for impaired spliceosome assembly. The involvement of Prp43 in the discard of spliceosome intermediates is supported by the findings that the dissociation of pre-mRNA or the lariat intermediate from the impaired spliceosome depends on Prp43 [190,191].

SPLICING FIDELITY CONTROL

The mechanism of splicing fidelity control has been best studied in yeast. DExD/H-box protein Prp16 was first discovered to have a function in promoting the fidelity of branch site recognition [159,160]. Two other DExD/H-box proteins, Prp22 and Prp5, were later also implicated in the control of splicing fidelity [192,193]. Prp16 was identified in a genetic screen as a suppressor of branchpoint mutation brC (changing A to C). ATPase-defective mutants of Prp16 could suppress branchpoint mutation and accumulate lariat-intermediates with aberrant branch nucleotide, suggesting that the ATPase activity of Prp16 is required to discriminate mutations at the branchpoint [194]. Prp16 was further found to be essential *in vitro* for the second reaction but dispensable for the first reaction [67]. Based on these studies, a model for how splicing fidelity is controlled by a kinetic proofreading mechanism was formulated [194]. It was proposed that kinetic competition between a switch to the second-step conformation and the ATP-dependent rejection of substrate mediated by Prp16 serves as the basis for the control of fidelity [194]. The kinetic competition mechanism was further supported by a recent study using a mutant U6 snRNA with sulfur substitution for pro-*Sp* non-bridging oxygen at position U80 (U6–sU80) [190]. In the first reaction, splicing was impeded in the presence of Mg²⁺ using U6–sU80. Prp16 was able to bind to such spliceosomes and mediates rejection of the slow spliceosome in the way that requires ATP and the ATPase function of Prp16 [190].

An ATP-independent role for Prp16 in the first reaction has recently been demonstrated [70]. Cwc25 is known to bind to the spliceosome in the presence of Yju2 after the release of SF3a/b to promote the first reaction on wild-type pre-mRNA [69,85]. Mutations at the branchpoint prevent binding of Cwc25, and consequently inhibit the reaction [70]. It was found that the presence of Prp16, regardless of its ATPase activity, stabilizes binding of Cwc25 to the brC spliceosome and promotes the first reaction. This ATP-independent function of Prp16 counteracts its ATP-dependent function in mediating Cwc25 removal. The balance between the two activities determines the level of splicing of brC pre-mRNA. Reducing the ATPase activity of Prp16 promotes the reaction. In this view, although Prp16 is the key player in mediating fidelity control of the first reaction, Cwc25 plays an important role in enforcing specificity of branchpoint recognition [70,195].

Mutations at the 3' splice site block exon ligation, which requires Prp22. Prp22 was shown to repress exon ligation at the mutated 3' splice site in an ATP-dependent manner. Prp22 mutants that reduce ATPase activity compromise the fidelity of



exon ligation both *in vivo* and *in vitro* [192]. These results suggest that Prp22 may play a role in proofreading exon ligation. It has also been shown that mutations within Prp5 in motif III of the helicase domain with reduced ATPase activity also increase the splicing activity of suboptimal substrate with less stable branch region-U2 snRNA pairing, suggesting a link between fidelity control in branch recognition and Prp5 ATPase activity [193].

CONCLUSIONS AND PERSPECTIVES

Pre-mRNA splicing is a complex process requiring assembly of a large ribonucleoprotein particle that involves a large number of factors. Over the years, genetic and biochemical studies have provided comprehensive insights into the mechanism of the splicing reaction. Compositional and structural analyses of the spliceosome and its components by MS, electron microscopy and X-ray crystallography have facilitated further understanding of the function of the spliceosome and its components at the structural level [196–201]. Approaches at the single-molecule level are also expected to result in new mechanistic insights [202,203], shedding further light on the molecular mechanism of nuclear pre-mRNA splicing.

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