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Cryo-EM snapshots of the human spliceosome reveal structural adaptions for splicing regulation☆

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Introns are excised from pre-messenger RNAs by the spliceosome, which produces mRNAs with continuous protein-coding information. In humans, most pre-mRNAs undergo alternative splicing to expand proteomic diversity. Cryo-electron microscopy (cryo-EM) structures of the yeast spliceosome elucidated how proteins stabilize and remodel an RNA-based active site to effect splicing catalysis. More recent cryo-EM snapshots of the human spliceosome reveal a complex protein scaffold and provide insights into the role of specific human proteins in modulating spliceosome activation, splice site positioning, and the ATPase-mediated dynamics of the active site. The emerging molecular picture highlights how, compared to its yeast counterpart, the human spliceosome has coopted additional protein factors to allow increased plasticity of splice site recognition and remodeling, and potentially to regulate alternative splicing.

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Introduction

Transcripts from most mammalian genes are synthesized as precursor mRNAs (pre-mRNAs), from which non-coding introns are spliced out [1,2]. Introns allow a single gene to encode multiple protein isoforms with distinct activities, thus expanding proteomic diversity through alternative splicing [3]. Indeed, splicing modulates transcription, export, localization, translation, and the stability of mammalian transcripts [4].

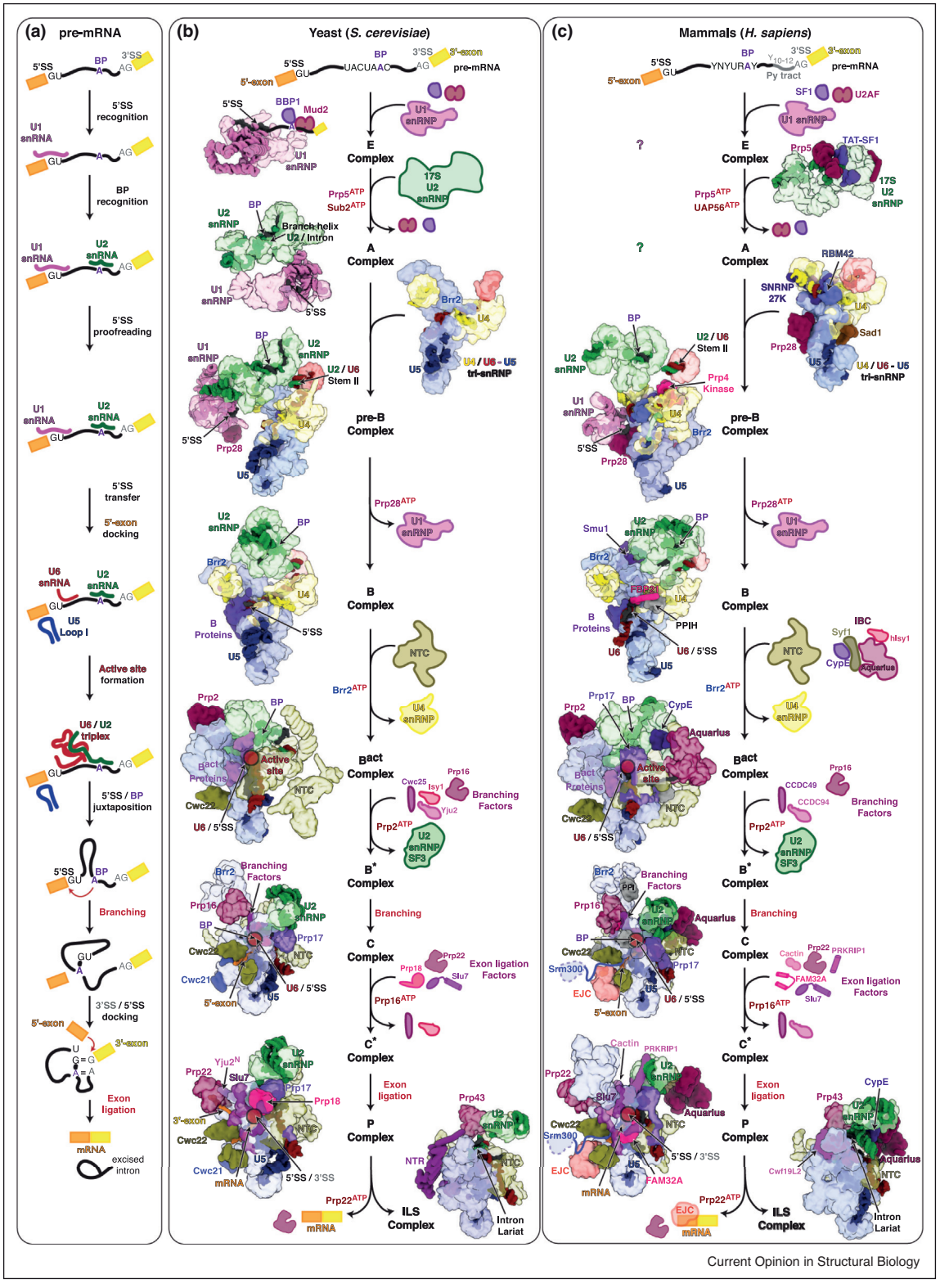
To excise introns, the spliceosome assembles *de novo* on each pre-mRNA through protein and RNA interactions that recognize the splice sites. Following assembly, the splice sites dock at an RNA-based active site that produces mRNA by catalyzing two sequential transesterifications – branching and exon ligation (Figure 1a) [5••]. In the last five years structures of the spliceosome from the budding yeast *Saccharomyces cerevisiae* have rationalized decades of biochemistry and genetics and provided a molecular view of the basic mechanism of splicing, showing how specific factors promote splice site recognition and catalysis [5••,6–8]. Whereas in yeast very few pre-mRNAs contain introns and only a handful are alternatively spliced [9], in humans alternative splicing occurs in 94% of genes [2] and the spliceosome has adapted to act on pre-mRNAs with multiple introns. Although the core of the spliceosome is conserved in higher eukaryotes, cryo-EM structures have also shed light on the increased complexity of the mammalian spliceosome [1]. This review focuses on how additional *trans*-acting factors that associate with human spliceosomes modulate splice site recognition and dynamics of the conserved spliceosome core.

A regulated assembly pathway

During initial assembly, the U1 snRNP pairs with the 5′-splice site (5′SS) to form the E complex (Figure 1a–c), in which the branch point adenosine (BP) and the 3′ end of the intron are also recognized cooperatively by binding of the trans-acting factors SF1 and U2AF [1,10]. In mammals this assembly step is regulated by the strength of U2AF interactions with a stretch of pyrimidines preceding the 3′splice site (3′SS), termed the polypyrimidine (Py) tract. Indeed, U2AF binding determines the efficiency of subsequent A complex formation [11], during which the U2 snRNP pairs with the intron around the BP adenosine to form the branch helix (Figure 1). The transition from the E to the A complex is mediated by Prp5 and UAP56, two ATPases with potential RNA helicase activity, whose mechanism of action remains largely obscure in mammals but which likely remodel the U2 snRNP and proofread BP recognition, as is the case of Prp5 in yeast [12,13]. A recent structure of the free human 17S U2 snRNP provides the molecular basis for understanding how Prp5 and the transcription elongation factor TAT-SF1, which binds the U2 snRNP, can modulate initial BP recognition [14]. The structures of the yeast

☆ I dedicate this review to the memory of Kiyoshi Nagai (1949–2019)

Figure 1



Splicing pathway. The names of specific spliceosome complexes were initially derived from their order of observation on native assembly gels. **(a)** Conceptual cartoons of key steps in spliceosome assembly and splicing catalysis. **(b)** Structures of key spliceosome complexes in yeast: E complex, PDB 6N7P [15]; A complex, PDB 6G90 [16]; U4/U6-U5 tri-snRNP, PDB 5GAN [26]; pre-B complex, PDB 5ZWM [27]; B complex, PDB 5NRL, [56]; B^{act} complex, PDB 5GM6 [57]; C complex, PDB 5LJ5 [50], P complex PDB 6EXN [44]; ILS complex PDB 5Y88 [48]. **(c)** Structures of

E and A complexes provide molecular clues about initial splice site recognition, suggesting how dynamic interactions between the U1 and U2 snRNPs govern A complex assembly and how additional trans-acting factors may regulate this process [15,16]. However, in mammals, the U2 and U1 snRNPs from adjacent introns are thought to interact across an exon in a process called exon definition [17], which has been proposed to control alternative splicing decisions [18], though some genome-wide studies suggest exon definition may only affect a subpopulation of introns [19,20]. Early recognition events and transition from E to A complex are further regulated in mammals by many RNA-binding proteins, which control alternative splicing [21]. Understanding how such factors regulate assembly of the A complex in molecular detail remains one of the foremost future challenges in the structural study of splicing.

By contrast, the molecular events following A complex formation are becoming increasingly clear. The A complex recruits a pre-formed U4/U6-U5 tri-snRNP, in which the Prp8 protein constitutes the core scaffold of the spliceosome (Figure 2). In the tri-snRNP, U6 snRNA, which will form the active site [22], is held in an inactive conformation through base-pairing with U4 snRNA. The helicase Brr2 unwinds these U4/ U6 interactions at a later stage. In humans, the tri-snRNP contains the DEAD-box helicase Prp28, which engages the U1 snRNP and is required for formation of the fully assembled spliceosome pre-B complex (Figure 1) [23,24••]. Indeed tri-snRNP association in humans is regulated by phosphorylation of Prp28 [25]. As in yeast, association of the U2 snRNP is stabilized at this stage by the newly formed U2/U6 stem II (Figure 1b,c). In yeast, the helicase Brr2 has already engaged its U4 snRNA substrate in the tri-snRNP and is thus poised for unwinding in the pre-B complex [26,27]. In contrast, human Brr2 is held in an unengaged position in the tri-snRNP by Sad1, which may repress premature Brr2 unwinding of U4/U6 interactions [5••] (Figure 1). Brr2 remains unengaged in the pre-B complex, resulting in a more mobile U2 snRNP that does not appear to stably contact the U1 snRNP. Such plasticity may promote U1 snRNP engagement with the tri-snRNP body thus priming the spliceosome for transition into the pre-catalytic B complex. In yeast, by contrast, Prp28 is not stably bound to the tri-snRNP and in pre-B may only interact with the U1 snRNP, though it remains unclear whether the absence of a stably engaged Prp28 in the yeast pre-B complex reflects a genuinely different assembly pathway [5••,24••]. Regardless, ATP-driven Prp28 action causes dissociation of U1 snRNP and 5′SS transfer from U1 to U6 snRNA (Figure 1), allowing Prp28 to proofread 5′SS recognition in humans, as in yeast [28]. In humans, this transition is further regulated by the Prp4 kinase, which phosphorylates core components of the tri-snRNP [29]. In the resulting B complex, Brr2 is now relocated and engaged on U4 snRNA, as in yeast. Importantly, the B complex is a key intermediate before formation of the active site, as even complexes that may initially assemble across an exon at the A complex stage proceed through a B complex conformation across the intron [30].

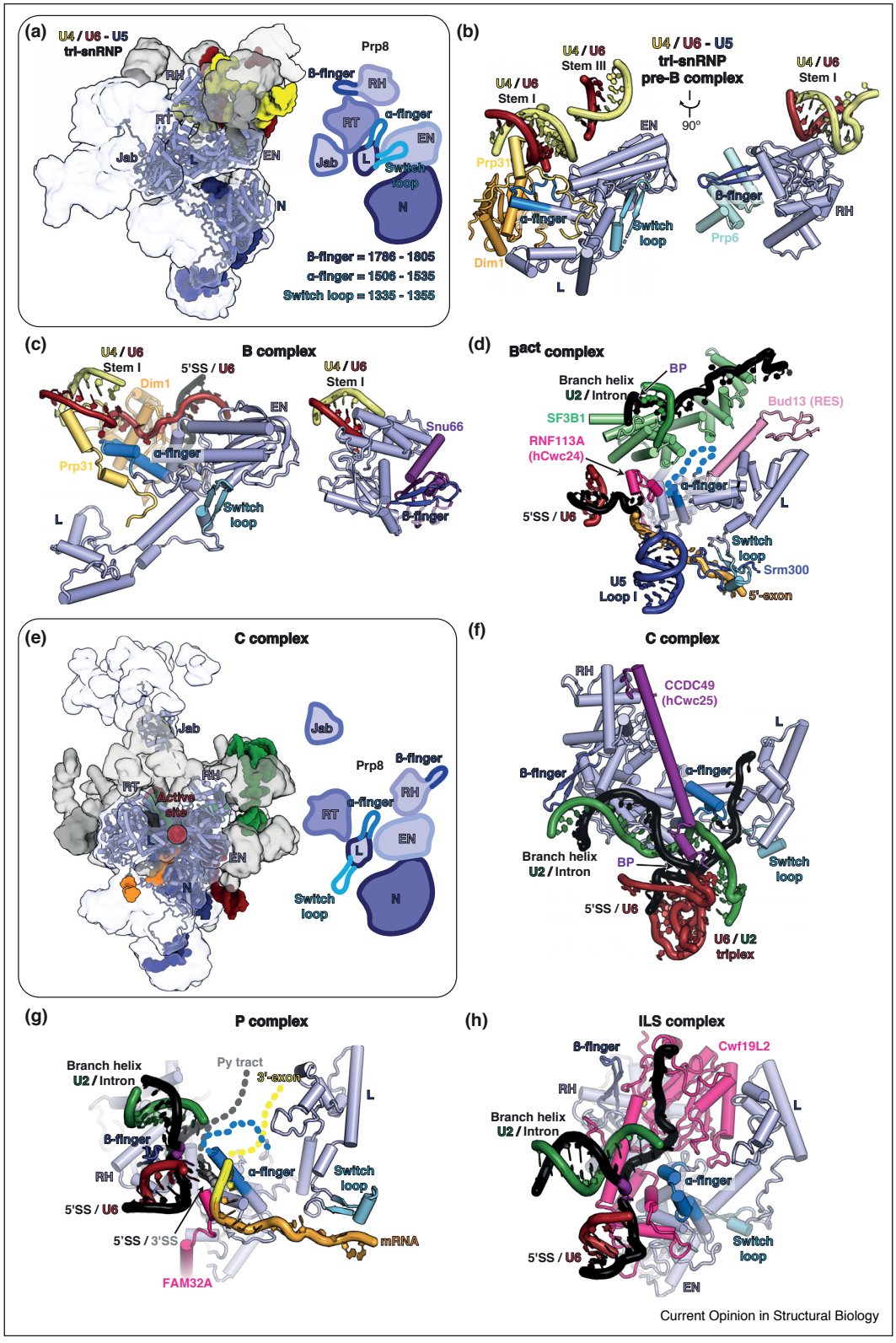
Brr2 remodels the B complex by unwinding U4/U6 interactions, thus inducing dissociation of the U4 snRNP. In humans, premature Brr2-mediated unwinding may be prevented by FBP21, which bridges Brr2 to the U4 snRNP and may regulate alternative splicing [31,32••,33]. Remodeling of the B complex assembled on short introns is further modulated by Smu1 and RED, two additional factors which bridge Brr2 and the U2 snRNP (Figure 1) [31,32••,34].

Brr2 action allows formation of the B^{act} complex, in which the freed U6 snRNA pairs with U2 snRNA to fold into a triple helix that constitutes the active site of the spliceosome and positions two catalytic Mg^{2+} ions (Figure 1) [5••,22,35]. During this transition, a large complex of Prp19 and associated factors (termed NTC) engages the spliceosome in a step-wise manner, followed by further remodeling mediated by the association of the intron-binding complex (IBC) comprising the mammalian helicase Aquarius, which is required for spliceosome activation [36,37••,38••]. Cryo-EM snapshots of different B^{act} conformations suggest that the NTC and IBC undergo extensive dynamic rearrangements [36,37••], likely to promote productive docking of the 5′SS at the newly formed active site, which occurs in B^{act} (Figure 1). Indeed, stable integration of Prp17, which remains bound throughout catalysis, appears coupled to binding of IBC in the mature B^{act} complex [36,37••]. The docked 5′SS is stabilized, as in yeast, by specific protein factors and by binding of the 5′-exon onto Loop I of U5 snRNA [1,5••]. In mammals the path of the 5′-exon is further guided by the SR protein Srm300, which is the largest single polypeptide in the spliceosome [39]; though mostly disordered Srm300 remains associated throughout the catalytic stage (Figure 1c) and may regulate splicing of specific transcripts [40].

During the final steps of spliceosome activation, Prp2 activity extensively remodels the B^{act} complex to promote exchange of factors and juxtaposition of the BP

(Figure 1 Legend Continued) key spliceosome complexes in humans: 17S U2 snRNP, PDB 6Y5Q [14]; U4/U6-U5 tri-snRNP, PDB 6QW6 [24••]; pre-B complex, PDB 6QX9 [24••]; B complex, PDB 6AHD [31]; B^{act} complex, PDB 5Z56 [36]; C complex, PDB 5YZG [42]; P complex, PDB 6QDV [45••]; ILS complex, PDB 6ID1 [46]. Note that no structures of the human E and A complexes have been reported to date. The B^* and C^* complexes adopt the same conformation as the post-catalytic C and P complexes, respectively, and were omitted for clarity. ATP indicates the activity of a specific DEAH/D-box helicase.

Figure 2



Rearrangements of Prp8 mobile elements mediate association of step-specific factors during spliceosome assembly and catalysis. **(a)** Relative arrangement of Prp8 domains in the U4/U6-U5 tri-snRNP: N, N-terminal domain; EN, endonuclease domain; RH, RNaseH-like domain; RT, reverse-transcriptase-like domain; Jab, Jab1/Mpn-like domain; L, linker domain[24••,49]. Boundaries for the three key loop regions are indicated.

adenosine to the 5′SS, which is then stabilized by branching factors (Figure 1) [1,5••]. The resulting B* complex effects branching by catalyzing attack of the 5′SS by the BP adenosine, thus cleaving the 5′exon (Figure 1) [5••]. Following branching, a complex specific to higher eukaryotes termed the Exon-Junction Complex (EJC) is stably recruited to the 5′-exon through interactions with Cwc22 in the C complex [1,5••], although its components may already loosely bind the B^{act} complex [37••,41]. The EJC is important for mRNA stability and for translation quality control [4]. Several peptidyl-prolyl isomerases also associate with the C complex [42] (e.g. PPI, Figure 1), though their functions remain unclear. Following branching, the ATPase Prp16 rearranges the C complex into a new C* conformation (Figure 1), which allows docking of the 3′-splice site (3′SS) [43,44]. In mammals several additional factors not observed in yeast promote the C* conformation [45••], as detailed below. The C* complex catalyzes attack of the cleaved 5′-exon on the 3′SS to effect exon ligation and is converted to the post-catalytic P complex, from which the ATPase Prp22 releases the mRNA bound to the EJC (Figure 1c). In the resulting intron-lariat spliceosome (ILS) the excised intron is bound by Cwf19L2 [46], the human homolog of yeast Drn1, an auxiliary factor that in yeast recruits the Dbr1 debranchase to initiate degradation of the intron [47]. The ATPase Prp43 binds the ILS and dissociates the spliceosome to allow snRNP recycling. Although the yeast ILS structure reveals how Prp43 may be recruited by auxiliary factors such as Ntr1 [48], the published human ILS structure lacks such factors [46], suggesting human spliceosome disassembly may be more complex.

Prp8 dynamics mediate association of *trans*-acting factors

Prp8 forms the core scaffold of the spliceosome from the pre-B complex onwards and the active site is cradled between its endonuclease (EN), linker (L), and reverse-transcriptase-like (RT) domains (Figure 2a,e) [5••,49,50]. Cryo-EM structures of the human spliceosome reveal how two mobile regions — the Switch loop and the α-finger — that protrude from the Linker domain cooperate with the β-finger of the RNaseH-like domain (Figure 2a) to mediate association of step-specific factors during assembly, activation, and catalysis.

In the tri-snRNP and pre-B complex, the Switch loop abuts the Prp8 EN domain, thus stabilizing binding of Prp31, a U4 snRNP protein, which rigidifies the tri-snRNP assembly together with Prp6, whose N-terminus latches onto the Prp8 β-finger (Figure 2b). The α-finger interacts with Prp31 and Dim1 to hold in place U4/U6 stem I, thus priming the U6 snRNA to engage the 5′SS in the B complex (Figure 2c). Indeed, only the position of the Prp8 β-finger changes from the pre-B to the B complex, where it interacts with Snu66 (Figure 2c), a factor that also helps to stabilize U4/U6 stems I and III in pre-B and may thus couple 5′SS transfer to U6 with spliceosome activation [5••,24••] (see also below). Reflecting dynamics of the B^{act} complex, the Prp8 α-finger becomes partly disordered following activation but is likely positioned to serve as a nexus of interactions, binding RNF113A (hCwc24) to promote docking of the 5′SS near the active site but also potentially linking SF3B1 of the repositioned U2 snRNP to Bud13 of the RES complex (Figure 2d). Since both SF3b and RES complexes dissociate following Prp2 activity [1], the Prp8 α-finger may act as a relay to promote Prp2-induced remodeling. The Switch loop also swings almost 180° from the B to the B^{act} complex (Figure 2c,d), where it remains throughout final activation and catalysis and clamps onto Srm300 to stabilize binding of the 5′-exon onto U5 snRNA Loop I between the Prp8 L and EN domains.

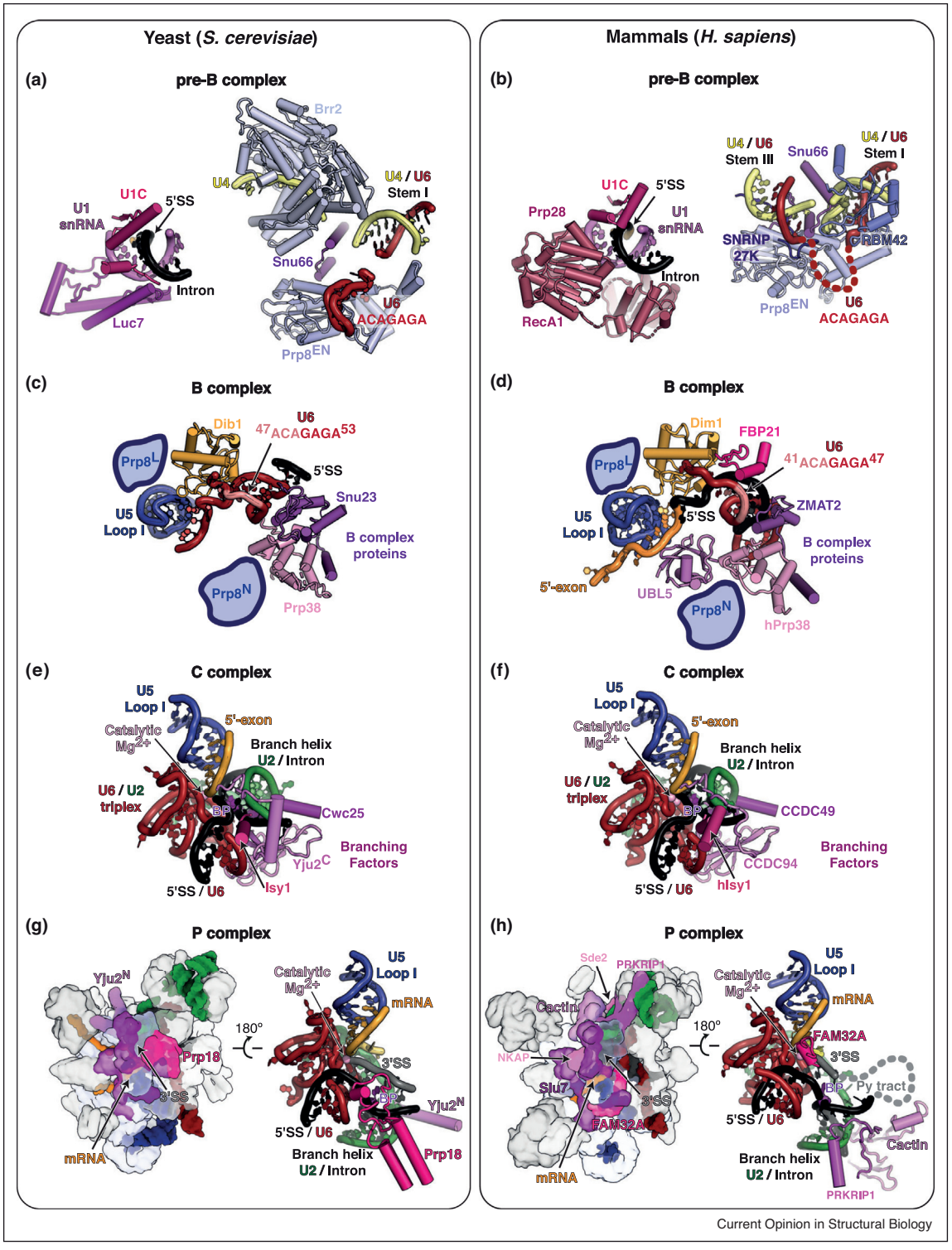
The relative orientation of Prp8 domains changes during final activation, as the RH domain engages the EN domain throughout the catalytic stage (Figure 2e–g). In the C complex, the Prp8 α-finger and β-finger stabilize the branch helix in its branching conformation, with the α-finger promoting binding of CCDC49 (hCwc25) to clamp on the branch helix (Figure 2f). Then, in the C* and P complex, the α-finger and β-finger act as pincers to rigidify the repositioned branch helix and clamp the docked 3′SS in the active site during exon ligation (Figure 2g). Finally, in the post-catalytic ILS complex, the α-finger and β-finger clamp onto Cwf19L2, a factor that is also observed in the *Schizosaccharomyces pombe* ILS and promotes spliceosome disassembly and intron degradation (Figure 2h) [46]. Notably, in the ILS complex, the Prp8 Switch loop returns to its original position abutting the EN domain, ready to re-form the tri-snRNP after spliceosome disassembly.

Specific human proteins modulate splice site recognition

Although splice site recognition and selection is generally believed to occur mainly in the E and A complexes [3,21], the human spliceosome structures suggest that selection of splice sites can also occur later during assembly, and

(Figure 2 Legend Continued) (b) Three Prp8 regions stabilize U4/U6 helices in the pre-B complex. **(c)** Prp8 interactions in the B complex. Note engagement of the Prp8 β-finger by Snu66, which may regulate spliceosome activation [24••]. **(d)** The Switch loop of Prp8 engages Srm300 to stabilize binding of the 5′-exon at the active site in the mature B^{act} complex [36]. **(e)** Relative orientation of Prp8 domains during the catalytic stage. **(f)** The Prp8 α-finger and β-finger stabilize the branch helix during catalysis of branching. **(g)** The Prp8 α-finger and β-finger act as pincers to rigidify the docked 3′SS in the active site during exon ligation. **(h)** The Prp8 α-finger and β-finger stabilize Cwf19L2 binding to the excised intron. Note that in the ILS complex the Prp8 Switch loop returns to its original position abutting the Prp8 EN domain, ready to re-form the tri-snRNP after spliceosome disassembly.

Figure 3



Specific human protein factors modulate splice site recognition during assembly and catalysis. **(a)** Different conformations of the U6 snRNA ACAGAGA in yeast and human pre-B complexes. Note that the ACAGAGA box forms a stem loop in the yeast pre-B complex and is not ready to stably engage the 5′SS. **(b)** Human proteins prime the U6 snRNA in the pre-B complex for engagement with the 5′SS. Similarly, Prp28 is engaged in the human pre-B complex and primed to transfer the 5′SS to U6 snRNA. **(c)** The U6 ACAGAGA does not engage the 5′SS productively in the yeast B complex. **(d)** FBP21 stabilizes the productive helix between the U6 ACAGAGA and the 5′SS, while UBL5 clamps the 5′-exon onto U5 snRNA Loop I. **(e–f)** The conformation of the active site during branching is conserved from yeast to humans. Note that similar human branching

during catalysis. Indeed, most of the ATPases that act on the pre-B, B^{act}, C, and P complexes have been implicated in proofreading splice site choice [5••,6].

While Prp8 rearrangements are conserved, human and yeast spliceosomes differ in their initial recognition of the 5′SS in the pre-B complex and its transfer to the U6 snRNA ACAGAGA box in the B complex, a process mediated by specific human proteins. In yeast the 5′SS/ U1 snRNA pairing is still stabilized by Luc7 in pre-B [27], whereas in humans Prp28 has already engaged the 5′SS/ U1 helix in at least a subset of pre-B complexes (Figure 3a,b) [24••]. More importantly, the U6 snRNA ACAGAGA forms a stem-loop in the yeast pre-B and B complexes, whereas in humans the ACAGAGA is flexible at the pre-B stage but has already paired with the 5′SS in the B complex, an interaction stabilized by FBP21 (Figure 3c,d). Indeed, by stabilizing the human-specific U4/ U6 stem III in pre-B, RBM42 and SNRNP-27K prime the ACAGAGA in a flexible conformation to receive the 5′SS [5••,24••]. These differences allow the human spliceosome to uncouple 5′SS transfer to U6 snRNA, which occurs early, in the B complex, from unwinding of U4/ U6 and formation of the active site in B^{act}. In contrast, in yeast, pairing of the 5′SS to the ACAGAGA box occurs later and is coupled to formation of the active site during transition to B^{act} [5••,24••]. This mechanism may allow usage of alternative 5′SS in mammals, which accounts for 8% of in-frame alternative splicing events in humans [51]. Consistently, SNRNP-27K was shown to regulate 5′SS usage in *Caenorhabditis elegans* [52]. In humans, 5′SS transfer is instead accompanied by binding of the 5′-exon onto U5 snRNA Loop I (Figure 1a) [5••,24••]. Indeed, in the B complex the 5′-exon is stabilized onto Loop I by the ubiquitin-like UBL5 (Figure 3d), which may further modulate splice site choice similarly to its yeast homolog Hub1 [9].

While human factors promote plasticity of 5′SS recognition, after final activation, the yeast and human spliceosomes have a strikingly similar structure of the active site as well as the same complement of branching factors to stabilize the juxtaposed BP and 5′SS in the B* and C complexes (Figure 3e,f). By contrast, subsequent remodeling to the C* conformation allows increased flexibility during docking of the 3′SS in humans. The human C* complex is stabilized by several additional factors including PRKRIP1, which rigidifies the branch helix [45••,53], and Cactin and Sde2, which have been implicated in alternative splicing in *S. pombe* [45••]. Cactin partly substitutes for the N-terminal domain of Yju2, which stabilizes the yeast C* and P complexes (Figure 3g,h) [44,45••]. Cactin is also positioned to modulate docking of the 3′SS through interactions with the polypyrimidine tract (Figure 3h) and a recent genome-wide study implicates the polypyrimidine tract in controlling exon ligation of a subset of human introns [20]. Finally, whereas in yeast 3′SS docking is promoted by Prp18, in humans Prp18 is partly substituted by FAM32A (Figure 3h), which binds the 5′-exon in C* to promote exon ligation and has been implicated in splicing of a subset of pre-mRNAs [45••]. Thus several human factors have the potential to modulate 3′SS choice during exon ligation, which may emerge as a major regulatory step since 20% of in-frame alternative splicing events involve a choice between alternative 3′SS situated only a few nucleotides apart and which are unlikely to be discriminated by U2AF during initial assembly [51].

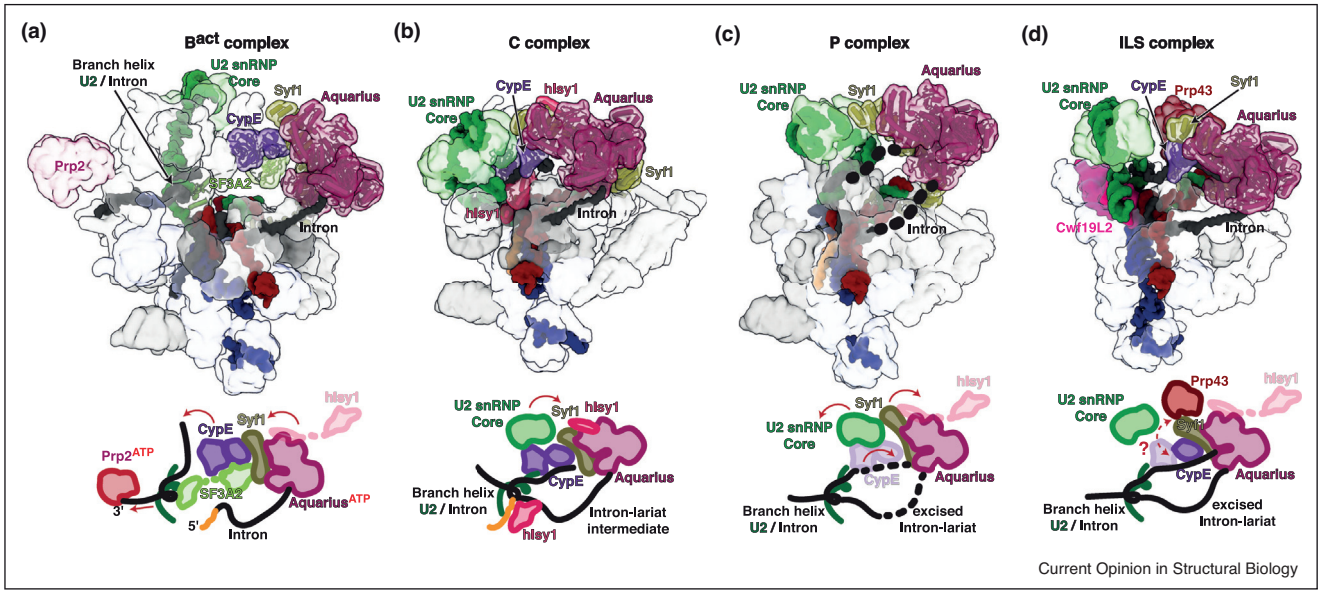
Specific human remodeling chaperones

In addition to conserved ATPases that mediate spliceosome remodeling in both yeast and humans, several additional ATPases associate with the human spliceosome [10,54]. Of these, only Aquarius — a large SF1 family helicase — has been observed in spliceosome structures thus far. Aquarius associates in the IBC with the cyclophilin CypE, the NTC-interacting Syf1, and with human Isy1 (hIsy1) [38••] and may cooperate with these factors as a general chaperone for human spliceosome remodeling.

The IBC joins the spliceosome at the B^{act} stage following formation of the active site triplex (Figure 1) [38••,55]. ATP hydrolysis by Aquarius, but not RNA unwinding, is required during the B^{act} to C transition, suggesting Aquarius may remodel RNA-protein interactions instead of acting as an RNA helicase [38••]. Consistently, repositioning of the branch helix during remodeling of B^{act} by Prp2 dissociates SF3a and SF3b complexes (Figure 1b,c) and Aquarius may assist in this process through interactions mediated by CypE with SF3A2, which binds the branch helix (Figure 4a). In the resulting C complex, Aquarius binds the intron downstream of the U6/ 5′SS helix, while CypE engages the intron upstream of the branch helix, which is clamped into the active site by the IBC component hIsy1 (Figure 4b). Thus Aquarius may serve as a ribonucleoprotein chaperone by threading the intron to promote juxtaposition of the 5′SS and BP in the B* and C complex (Figure 4a,b), consistent with Aquarius being necessary for branching [38••]. Subsequently, hIsy1 disengages the spliceosome core and becomes disordered in the C* and P complex, where stable density was only observed for the repositioned Syf1 and Aquarius (Figure 4c) [45••,53]. Indeed, Aquarius activity is not required during exon ligation [38••]. Intriguingly, CypE re-engages the intron upstream of the branch helix in the

(Figure 3 Legend Continued) factors stabilize the branch helix near the catalytic Mg^{2+}. **(g–h)** Additional exon ligation factors stabilize the P complex conformation in humans. Note how in humans FAM32A partially substitutes for Prp18 near the docked 3′SS.

Figure 4



The ATPase Aquarius and its associated intron-binding complex (IBC) modulate spliceosome rearrangements in humans. **(a)** Aquarius and CypE may chaperone B^{act} remodeling by the Prp2 ATPase. Note that Prp2 action dissociates the SF3a and SF3b complexes and Aquarius may assist in this process through interactions mediated by CypE with SF3A2. **(b)** Interactions with CypE and Aquarius may drive the intron and branch helix into the C complex active site. **(c)** Aquarius may promote engagement of Syf1 with the repositioned U2 snRNP core during the C to P transition. **(d)** Aquarius and CypE may assist recruitment of Prp43 to mediate disassembly of the post-catalytic ILS complex and release of the excised intron.

post-catalytic ILS and may modulate recruitment of Prp43 for spliceosome disassembly (Figure 4d) [46], suggesting that the IBC may also act as chaperone during human spliceosome disassembly.

Future perspectives

Cryo-EM snapshots have provided unprecedented molecular insights into splice site recognition and catalysis by the human spliceosome. However, many human factors detected by mass spectrometry have not yet been identified in any spliceosome structure and their roles remain largely obscure, raising the possibility that such factors may engage the spliceosome in a transcript-specific manner. The challenge for the next decade of spliceosome structural biology will be to understand how these specific human proteins mediate remodeling, proofreading of splice site choice, and alternative splicing for individual human transcripts.

Conflict of interest statement

Nothing declared.

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