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RNA helicases in splicing

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Abbreviations: 3'SS, 3' splice site; 5'SS, 5' splice site; aa, amino acid; BS, branch site; CBC, cap binding complex; DUF, domain of unknown function; NTC, NineTeen Complex; OB-fold, oligosaccharide binding fold; pre-mRNA, precursor messenger RNA; pre-rRNA, pre-ribosomal RNA; RNP, ribonucleoprotein complex; SF2, superfamily 2; snRNP, small nuclear RNP; snoRNA small nucleolar RNA; SR, serine-arginine; WH, winged helix

In eukaryotic cells, introns are spliced from pre-mRNAs by the spliceosome. Both the composition and the structure of the spliceosome are highly dynamic, and eight DExD/H RNA helicases play essential roles in controlling conformational rearrangements. There is evidence that the various helicases are functionally and physically connected with each other and with many other factors in the spliceosome. Understanding the dynamics of those interactions is essential to comprehend the mechanism and regulation of normal as well as of pathological splicing. This review focuses on recent advances in the characterization of the splicing helicases and their interactions, and highlights the deep integration of splicing helicases in global mRNP biogenesis pathways.

Pre-mRNA Splicing

Most eukaryotic precursor mRNAs (pre-mRNAs) contain introns that must be removed by RNA splicing to produce mature mRNAs. This is achieved in the spliceosome, a large and extremely dynamic ribonucleoprotein (RNP) complex (for recent reviews, see refs. 1 and 2). The spliceosome is highly conserved from yeast to human, with 85% of yeast splicing factors having an identified human ortholog. However, the human spliceosome contains about twice as many splicing factors as does the spliceosome of the budding yeast *Saccharomyces cerevisiae* (approximately 170 and 90 respectively),[3-5] likely reflecting the prevalence of alternative splicing mechanisms in higher eukaryotes. Thus, *S. cerevisiae* might be considered to have a minimal spliceosome.[3] Nevertheless, splicing in budding yeast is subject to regulation.[6]

Introns are identified by short sequences at the 5' splice site (5'SS), the branch site (BS) and the 3' splice site (3'SS). In budding yeast, these adhere quite closely to consensus sequences but are more varied in metazoans, where additional *cis*-acting elements and *trans*-acting factors affect splice site choice. RNA splicing must be highly accurate in order to join the coding exons correctly, and mechanisms exist to check the fidelity of splicing and to promote the discard and degradation of aberrant intermediates and products of splicing (proofreading see below). Among the many splicing factors, RNA helicases have been identified as important regulators of splicing, implicated in promoting conformational rearrangements as well as ensuring that only appropriate substrates proceed through the splicing reactions. These roles likely involve checking the configuration of the catalytic center of the spliceosome at each stage.

Spliceosome assembly is an ordered process in which the U1, U2, U4/U6 and U5 small nuclear RNPs (snRNPs) and non-snRNP splicing factors interact with the substrate pre-mRNA and with each other, defining the intron splice sites and the BS (reviewed in refs. 1, 2 and 7). In the commonly accepted step-wise model of spliceosome assembly that was defined mainly from in vitro studies (**Fig. 1**), the 5'SS is first recognized by the U1 snRNP and the BS by the SF1/BBP and U2AF proteins (Msl5 and Mud2 in yeast) that form the commitment complex (complex E). The U2 snRNP then associates with the BS, leading to formation of the pre-spliceosome, or complex A. Complex A is converted to complex B by addition of the U4/U6 and U5 snRNPs in the form of a pre-assembled tri-snRNP particle. Within the tri-snRNP the U4 and U6 snRNAs are base-paired via two regions of sequence complementarity, but are unwound in the spliceosome during a major reorganization that displaces the U1 and U4 snRNPs. At this point, the multi-protein 19-complex (NTC) joins the spliceosome to form the almost complete, but still inactive, complex B^{act}. The ATP dependent activation of the spliceosome (complex B*) precedes the first catalytic step of splicing. As a consequence of the first step, complex C is formed. This is reorganized again to perform the second reaction. Finally the spliceosome is dissociated and the products of splicing, i.e., the spliced mRNA and the excised intron, are released and either processed further and exported to the cytoplasm, or degraded.

Although splicing has long been known to involve two transesterification reactions, the precise chemistry and the structural changes that occur within the spliceosome before, during and after catalysis are still the subject of intense study. During the first reaction, the 2'hydroxyl moiety of a conserved adenosine (the BS adenosine), located toward the 3' end of the intron, attacks the 5'SS, cleaving the phosphodiester bond. This produces two

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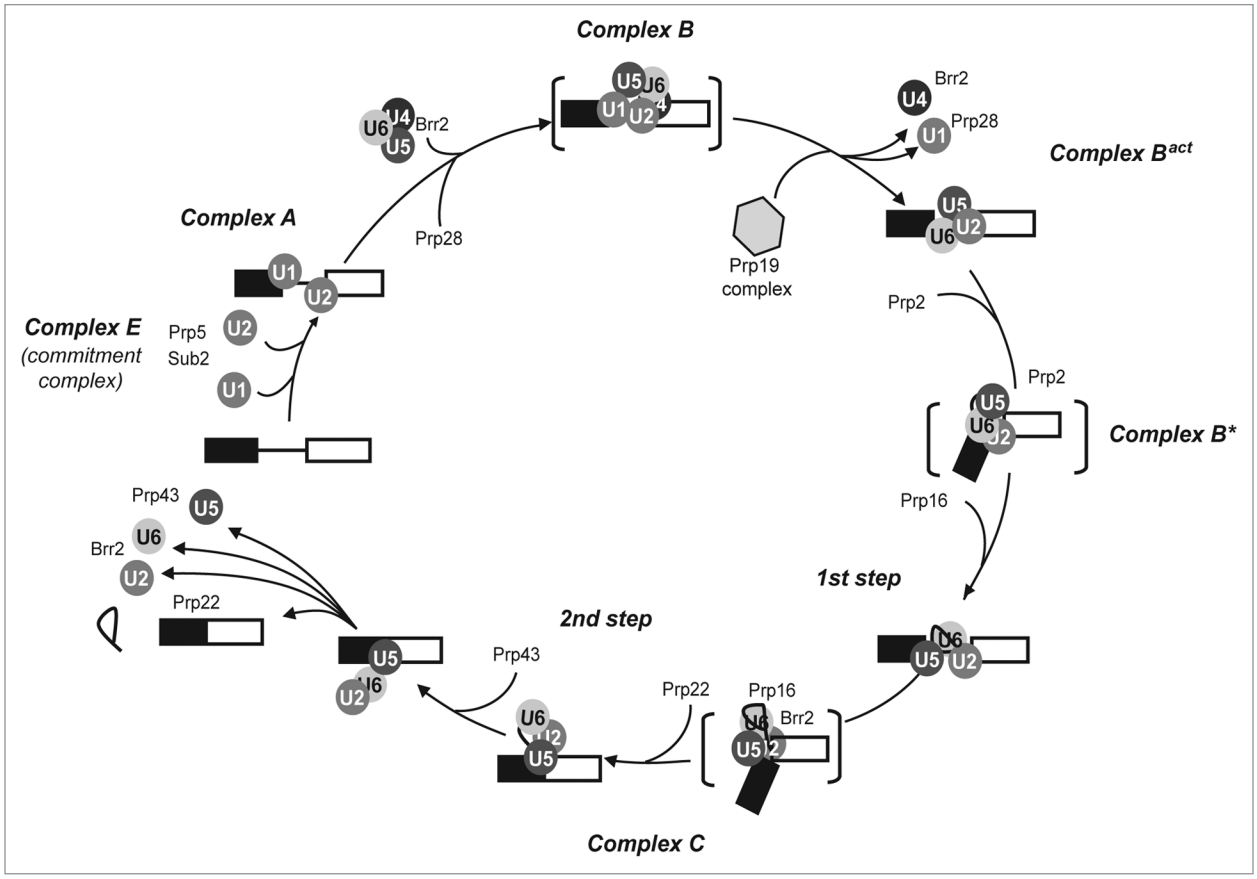


Figure 1. RNA helicases in the spliceosome assembly/disassembly pathway. The order of assembly of U snRNPs and the steps of association and activity of helicases are shown on a transcript containing a single intron. Names of complexes refer to the human nomenclature.

intermediates, the 5' exon and the intron-3' exon, in which the guanosine at the 5' end of the intron is covalently attached via a 2'-5' phosphodiester bond to the BS adenosine, forming a branched or lariat configuration. In the second catalytic step the 3' hydroxyl group of the 5' exon attacks the 3'SS, joining the exons and excising the intron in lariat form. The spliceosome has only one active site for both trans-esterification reactions. Thus reorganization of the catalytic center must occur between the two reactions, such that the products of the first reaction are repositioned as substrates for the second reaction.

Most early studies of the mechanism of splicing were performed with a few model substrates in vitro or using a small number of reporter constructs in vivo,[8-10] and the influence of transcription and other cellular processes on splicing was largely ignored. However, it is apparent that splicing factors can have distinct effects with different pre-mRNAs,[11] and that other pathways of RNA metabolism can affect splicing.[12-14] Therefore we conclude this review with a summary of links between splicing helicases and other RNA metabolic processes.

RNA Helicase Families and Mechanisms

Eight RNA helicases are required for pre-mRNA splicing in all eukaryotes (**Fig. 1**).[15] They all belong to the superfamily 2 (SF2) of helicases[16] that are characterized by the presence of two RecA-like domains and variable amino and/or carboxy terminal extensions. SF2 helicases generally function as monomers but some act as homo-dimers.[17,18] They share conserved motifs (**Fig. 2**) involved in NTP (usually ATP) binding and hydrolysis, and nucleic acid interaction. Motif III is involved in communicating between the motifs for nucleotide and nucleic acid binding. Other motifs are responsible for differences in activity observed between families. For example, among the eight spliceosomal RNA helicases, three (Prp5, Sub2 and Prp28) belong to the DEAD-box family, four (Prp2, Prp16, Prp22 and Prp43) to the DEAH-box family and one (Brr2) to the Ski-2 like family (**Fig. 2**) (reviewed in ref. 15).

DEAD-box helicases. DEAD-box RNA helicases are found in nearly every organism.[19] They are exclusively ATP specific with ATP hydrolysis usually being stimulated by RNA. Although commonly referred to as helicases, DEAD-box proteins are poor unwindases and might appropriately be considered ATP-dependent RNA binding proteins. DEAD-box proteins can bind a single strand of RNA, regardless of whether it is engaged in a duplex or not. Upon ATP binding, the helicase undergoes a conformational change, resulting in local physical constraint that destabilizes the structure of the bound RNA. In some cases the substrate can be a protein bound to the RNA. Additionally, some DEAD-box helicases possess bona fide RNA annealing activity.[20] These properties suggest that DEAD-box proteins could be efficient ATP-dependent switches. The three DEAD-box helicases

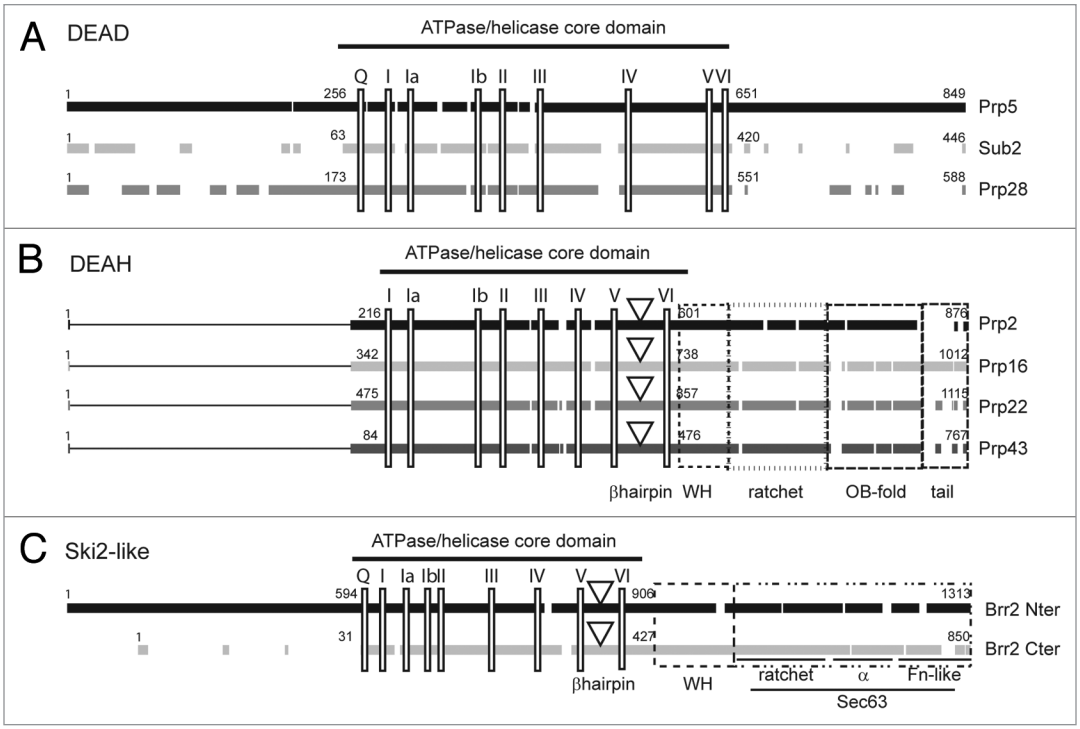


Figure 2. Splicing helicases belong to three distinct families. Primary sequence alignment of *S. cerevisiae* helicases from the DEAD-box (**A**), DEAH-box (**B**) and Ski2-like (**C**) families involved in pre-mRNA splicing. Black and gray blocks represent the conserved regions within each family. The lines in the amino-termini of DEAH-box helicases indicate the lack of conserved sequences in this region. The positions of the conserved motifs are indicated by vertical rectangles. For DEAH-box and Ski2-like helicases, a downward triangle indicates the position of the β-hairpin proposed to act as a strand separator. Dashed boxes indicate the conserved domains in the carboxy-termini of DEAH-box proteins and in the two helicase modules of the Ski2-like Brr2 helicase. For clarity of the figure we refer to the old nomenclature for conserved motifs[19] although a new nomenclature has been proposed.[17]

involved in splicing share a high degree of conservation in their core domains, whereas their amino- and carboxy-termini are poorly conserved (**Fig. 2A**). In higher eukaryotes, the amino termini of Prp5 and Prp28 contain serine-arginine (SR) repeats. SR repeats are commonly found in RNA splicing factors involved in alternative splicing[21-23] where they participate in protein or RNA binding.

DEAH-box helicases. Yeast DEAH-box helicases possess an extremely well conserved core domain that contains the common SF2 motifs, except for the Q-motif that confers ATP specificity (**Fig. 2B**), and conservation extends to the carboxy terminus. All possess a similar organization, that includes a conserved β-hairpin (5'HP) in their core domain, a winged helix (WH) domain, a ratchet domain involved in RNA binding and RNA translocation during duplex unwinding, and a DUF1605 domain (Domain of Unknown Function) that adopts the Oligosaccharide Binding fold (OB-fold)[24-27] (**Fig. 2B**). Unlike DEAD-box and Ski2-like helicases, DEAH-box helicases can bind and hydrolyse any NTP (or dNTP) in vitro,[15] although such substrate promiscuity may not be relevant in vivo. The conserved 5'HP and the DUF1605 domain participate in the control of the RNA binding and unwinding activities. The presence of these structures implies that DEAH-box helicases require a single-stranded region in the substrate on which to load. These domains also confer polarity and a certain degree of processivity to those helicases. Of six DEAH-box helicases in *S. cerevisiae*, three, Prp2, Prp16 and Prp22, participate specifically in pre-mRNA splicing, while Prp43 is necessary for both pre-mRNA splicing and rRNA processing.

Their extensive sequence conservation suggests a common mechanism of action and similar mode of regulation. In the spliceosome, the ATPase activity of both Prp2 and Prp43 is activated by G-patch proteins, Spp2 and Spp382, respectively.[5,26,28,29] For its role in rRNA processing, Prp43 is stimulated by another G-patch protein, Pfa1.[26] The G-patch proteins mediate regulation through interaction with the OB-fold domain of DEAH-box helicases.[25] Although very similar, the primary sequences of OB-fold domains of splicing helicases show clear differences that might account for partner-specific binding. In *S. cerevisiae*, no G-patch protein has been found associated with Prp16 or Prp22, although human Prp16 could be a target of GPNOW[30] (the ortholog of Spp2, that also interacts with hPrp2). The N-terminal domains of DEAH-box splicing helicases differ greatly in both primary sequence and length (from 84 amino acids for Prp43 to 475 amino acids for Prp22) (**Fig. 2B**) and little is known about their function. A PWI domain has been predicted in the N-terminus of human Prp2, but is absent in the *S. cerevisiae* ortholog.[31] In all splicing DEAH-box helicases a large portion of the N-terminus can be deleted without altering the function of the protein in vivo.[31-37]

Ski2-like helicases. Brr2 is the only Ski2-like helicase involved in pre-mRNA splicing (reviewed in ref. 18). Ski2-like helicases share structural features with both DEAD- and DEAH-box

helicases (reviewed in ref. 15). They possess a version of the Q-motif,[15] which is also present in DEAD-box RNA helicases, and a putative strand separator, the 5'HP located between motifs V and VI, also found in DEAH-box helicases.

Brr2 is unusual, in that it possesses two Ski2-like helicase modules, each of which comprises a Ski2-like helicase domain connected to a Sec63 domain through a structurally versatile WH domain (**Fig. 2C**). Only the N-terminal module has ATP hydrolysis and RNA unwinding activities in vitro,[38] and it alone interacts with RNA in vivo.[39] The sequence of the second module is divergent and appears to have a protein interaction function rather than the canonical RNA helicase function.[40,,41] The N-terminal module starts with a domain of unknown function (aa. 1–474 in budding yeast) that is essential in vivo (Turner, I.A. and Newman, A., personal communication). Interestingly it includes a PWI domain (aa. 258–338) that could participate in RNA binding.[31] Determination of the structure of the C-terminal WH-Sec63 domains[42,43] highlighted the presence of three conserved sub-domains[40] that strongly resemble the C-terminal domain of the Ski2-like DNA helicase Hel308.[44-46] The Sec63 domain itself includes a ratchet domain found in all Ski2-like and DEAH-box helicases,[15] followed by a short α-helical domain, which may provide flexibility to the Sec63 domain. Finally a fibronectin-like domain, rich in β-strands, interacts strongly with the other two domains. Altogether the Sec63 domain of Brr2 is likely to function in regulation of substrate binding by the helicase domain. A crystal structure of nearly full-length human Brr2 (residues 395–2129) was recently reported.[38] The structure confirmed the modular organization of Brr2 and, notably, the contribution of the first Sec63 domain to the formation of a tunnel in which RNA can bind and be translocated during unwinding. The second helicase domain and the second Sec63 domain form a similar tunnel, although negatively charged residues likely prevent RNA binding. It is proposed that the second helicase module has retained its capacity to bind ATP but not to hydrolyse it. The crystal structure reveals physical contacts between the RecA-1 domain of the N-terminal helicase module and the RecA-2 domain of the C-terminal helicase module, and a mutation within the carboxy terminal ATP-binding motif I reduced U4/U6 unwinding by the N-terminal helicase domain in vitro. Furthermore, the N-terminal ATPase activity was found to be enhanced in the presence of the C-terminal cassette.

Helicases in the Spliceosome

Sub2. Two RNA helicases, Sub2 (yeast)/UAP56 (human) and Prp5 participate in the recognition of the BS sequence by U2 snRNP during the formation of the pre-spliceosome (**Fig. 1**). UAP56 (**Fig. 3A**) was originally identified in humans as an interactor of U2AF65.[47] In vivo, Mud2 and Msl5 form a heterodimer and physically interact with U1 snRNP proteins, but not with U2 snRNP proteins, linking the recognition of the 5'SS by the U1 snRNP with recognition of the branch-site by Msl5.[48] The heterodimer Mud2/Msl5 is proposed to recruit ATP-bound Sub2/UAP56 at, or close to, the BS. Subsequently ATP hydrolysis by Sub2 triggers release of Msl5, leaving Mud2 associated with Sub2/UAP56, and allowing access for U2 snRNP factors and U2 snRNA.[49] The mechanism by which Sub2/UAP56 exerts its function is unclear; whether displacement of protein from an RNA or modulation of the Mud2/Msl5 protein interaction. The effects of mutations in motifs I and II of Sub2/UAP56 highlighted the need for ATP binding/hydrolysis but not unwinding for pre-spliceosome formation.[49] Although Sub2/UAP56 is essential for yeast viability in normal conditions, it is dispensable when *MUD2* or *MSL5* is deleted.[50] Therefore biochemical and genetic results are in good agreement.[49]

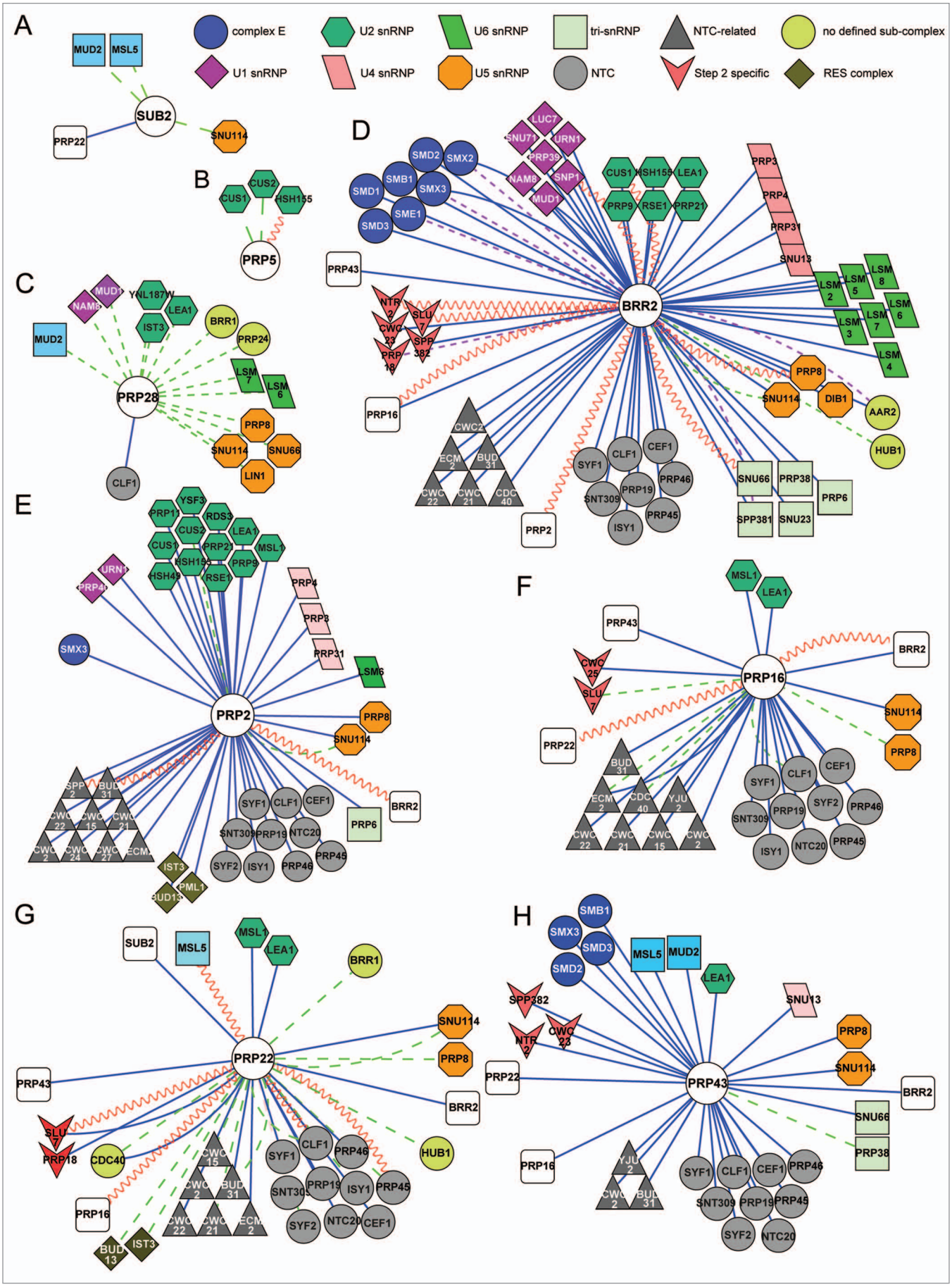
Recombinant Sub2/UAP56 can interact with U4 and U6 snRNAs in vivo and in vitro, and was proposed to play a role in unwinding the U4/U6 duplex in HeLa cell nuclear extracts.[49] However, the significance of this is unclear, as Brr2 was shown to perform this function (see below, and refs. 40, 51, 52). In addition to a role in pre-mRNA splicing, the Sub2/UAP56 DEAD-box RNA helicase is implicated in Pol II transcription regulation, mRNA transport and localization, and the control of cancer and virus expression.[49,53-58]

Prp5. The ATPase activity of Prp5 is necessary to facilitate and proof-read the interaction of U2 snRNP with the BS. Prp5 interacts genetically with several U2 snRNP factors, including Hsh155, Cus1 and Cus2 (**Fig. 3B**)[59-61] and several U2 snRNA mutations are suppressed by mutations in *PRP5*.[62] The U2 snRNP-associated factor Cus2 was proposed to promote or stabilize a conformation in the U2 snRNA that is favorable for association of the SF3a and SF3b proteins prior to interaction of the U2 snRNA with pre-mRNA.[62] Interestingly, when Cus2 is deleted, pre-spliceosome formation can proceed in the absence of ATP in vitro, although the presence of Prp5 is still required. Conversely, when recombinant Cus2 is added back to the extract, the ATP dependence is restored.[59] Prp5 could promote the displacement of Cus2 from the U2 snRNA, while helping to stabilize U2 snRNA in the stem IIa conformation.[59,63]

In *Schizosaccharomyces pombe*, SpPrp5 associates with the U1 snRNP by directly interacting with Rsd1, mediated by the SR-like domains of each protein.[64] The U1 snRNP proteins Snu71 and U1A, and the SF3b subunits of the U2 snRNP also co-purified with SpPrp5.[64] In contrast, the budding yeast Prp5 does not possess SR repeats and no Rsd1 ortholog is known. Thus, although the splicing machinery is highly conserved, organism-specific interactions are also found.

Prp28. Following the selection and recognition of the 5'SS and the BS, the tri-snRNP joins the pre-spliceosome to form the

Figure 3 (See opposite page). Cytoscape (www.cytoscape.org) representation of the interactome of *S. cerevisiae* splicing helicases. Splicing interactomes of (**A**) Sub2; (**B**) Prp5; (**C**) Prp28; (**D**) Brr2; (**E**) Prp2; (**F**) Prp16; (**G**) Prp22; (**H**) Prp43. The list of interactors was obtained from biogrid (www.thebiogrid.org). Only splicing factors are shown, grouped as in reference 1. Colored shapes indicate sub-complex associations of splicing factors. Connectors are colored according to the experimental system used: blue lines represent affinity-capture followed by identification of the prey by mass spectrometry or western blotting, dashed purple lines represent co-fractionation or co-purification experiments, dashed green lines represent genetic interactions and sinusoidal red lines represent yeast two-hybrid interactions.



transient complex B.[1] Unwinding of U4/U6 leads to a major reorganization of the spliceosome, with the displacement of the U1 and U4 snRNPs and addition of spliceosome activation factors converting it to the B^{act} complex (**Fig. 1**). Displacing U4 allows U6 snRNA to base pair with the 5'SS (following U1 snRNP displacement) and with the U2 snRNA, contributing to the formation of the catalytic center.

Two RNA helicases, Prp28p (**Fig. 3C**) and Brr2 (**Fig. 3D**), play crucial roles in these rearrangements.[1] Prp28, the third consecutive DEAD-box helicase to participate in spliceosome formation, was identified in a screen for cold-sensitive splicing mutants. Prp28 was initially proposed to be necessary for U4/U6 unwinding,[65,66] but was later shown to be required for dissociation of the U1 snRNA/5'SS base-pairing interaction.[67] The requirement for Prp28 can be bypassed by mutations in the U1 snRNP proteins U1C, Prp42 or Snu71, the cap-binding protein Cbp80 or Ynl187 that weakens the U1/5'SS interaction,[68,69] suggesting that Prp28 may destabilize the U1 snRNA/5'SS interaction indirectly, by displacing proteins that stabilize it.[68] Although Prp28 interacts genetically with two other U1 snRNP proteins, Nam8 and Mud1, mutation or deletion of these factors does not bypass Prp28.[69] Therefore, the destabilizing effect of Prp28 may be limited to a fraction of the U1 snRNP that contacts the 5'SS.[69]

The replacement of U1 snRNA by U6 snRNA at the 5'SS is tightly coupled. Mutations that strengthen the U6:5'SS interaction relieve the defect caused by mutations that hyper-stabilize U1:5'SS,[67] indicating an equilibrium between a "pre-spliceosome-5'SS" and a "complex B-5'SS." Compatible with this, Prp28 interacted in large scale genetic screens with components of the U6 snRNP as well as with U5 snRNP (**Fig. 3C**).[70-72] Thus Prp28 may proof-read the 5'SS based on the relative stability of its interactions with the U1 and U6 snRNAs.[67]

The N-terminal extension of the human ortholog of Prp28 has SR repeats that are targets of the SRPK2 kinase,[73] and the phosphorylation status of Prp28 impacts the stable recruitment of the tri-snRNP and complex B formation, suggesting a potential regulatory mechanism.[73]

Brr2. Brr2 is generally accepted to be responsible for U4/U6 unwinding,[40] although this activity of Brr2 appears to be functionally linked to Prp28 and Sub2/UAP56[49] in vivo (see above). Brr2 is a component of the U5 snRNP, within which it contacts Prp8 and the GTPase Snu114, as visualized by cryo-electronmicroscopy.[74] Brr2 associates with the U5 snRNP in a late maturation event of this particle[75] that is coupled with the release of the chaperone-like protein, Aar2.[75] Two recent studies showed that Brr2 loads onto the single stranded region of U4 located upstream of U4/U6 helix I and it was proposed to translocate 3' to 5' along the single stranded RNA to reach its duplexed target.[39,76] Interestingly the RNase H domain of Prp8 binds the same region of U4 and prevents Brr2 loading there.[76] Unwinding of the U4/U6 duplex by Brr2 in vitro is stimulated by a C-terminal region of Prp8 that contains the conserved RNase H and the ubiquitin-binding Jab1/MPN domains.[42,43,77] The ubiquitination status of Prp8 and the GDP/GTP bound state of Snu114 can also regulate the unwinding of U4/U6 by Brr2 in vivo.[71,78-80] Mutations that alter the interaction between Brr2 and Prp8 also reduce U4/U6 unwinding in vivo and in vitro.[71] Interestingly, in humans some of these mutations cause retinal degeneration and blindness.[81,82] It is unclear how essential and ubiquitous splicing factors can be responsible for a tissue-specific disorder.

A large body of results recently shed light on Brr2 structure and function. In addition to its association with U5 snRNP, Brr2 interacts genetically and physically with most spliceosomal subcomplexes (**Fig. 3D**), including components of U4/U6 snRNP, U1 snRNP and U2 snRNP.[71,83-88] Brr2 was also proposed to be responsible for U2:U6 dissociation during spliceosome disassembly.[79] In two-hybrid experiments the C-terminal domains of Brr2 interact with Prp2 and Prp16, and Brr2 was proposed to act as a receptor for these helicases at the catalytic center of the spliceosome.[41] Being present at the heart of the spliceosome, Brr2 could exert the dual functions of a helicase and a protein-protein interaction platform, thereby participating in the control of the progression of the splicing reaction through the sequential interaction of its partners with its C-terminal domain.[41-43,77] Furthermore, the use of a *brr2* mutant, *brr2-G858R*, in combination with UV cross-linking and sequencing has recently revealed a new function for Brr2, driving conformational rearrangements at the catalytic center of the spliceosome that lead to competence for the second step of splicing.[39]

Prp2. Prp2 (**Fig. 3E**) joins the B^{act} complex along with Spp2 and is required for activation of the spliceosome prior to the first transesterification reaction. Spp2 is a member of the G-patch protein family that contains a glycine-rich domain. Spp2 is necessary for the recruitment and the function of Prp2,[28,29] although it is not known how Spp2 affects Prp2 activity. The exact role of Prp2 is also unclear and its direct target is unknown, although an ATP-dependant conformational rearrangement promoted by Prp2 leads to destabilization of SF3a and SF3b proteins from the BS.[5,89] Prp2 activity may therefore contribute to positioning the branch-site adenosine for nucleophilic attack on the 5'SS.

Cwc22, which is loosely associated with the NTC, contributes to destabilization of the SF3a/b proteins.[90] In absence of Cwc22, Prp2 binds the spliceosome, hydrolyzes ATP and dissociates from the spliceosome, but fails to trigger SF3a/b release. It was suggested that Cwc22 could assist in positioning Prp2 close to the SF3-bound BS, thereby making use of the ATPase dependant 5' to 3' translocation of Prp2 to dissociate (directly or not) SF3a/b proteins from the BS. Indeed Prp2 cross-links beside the BS.[91] Another target of Prp2 activity could be the connection between Cwc24 and the NTC.[92,93]

Using a dual-color fluorescence cross-correlation spectroscopy approach to measure the affinity of several splicing factors in the activated spliceosome, Ohrt et al.[92] showed that Prp2 initiates a cascade of rearrangements, including displacement of the NTC-associated proteins Cwc24, Cwc27 and the RES complex protein Bud13 and reduced association of the SF3a/b proteins. Additionally, the two essential 1st step factors, Yju2 and Cwc25, become more strongly bound to the spliceosome. It was suggested that Cwc25 shifts the equilibrium from an inactive, step 1 incompetent catalytic center to an active, step 1 competent catalytic center.[5] In this way, Prp2 may not only expose the BS

adenosine but also promote the correct alignment of the 5'SS and the BS prior to the first trans-esterification reaction.

A large-scale two-hybrid screen of human splicing factors highlighted the profound integration of the various sub-complexes that form the spliceosome.[94] This screen illustrated the dynamics of interactions and confirmed the conservation in human of the interaction between Prp2 and Spp2 (GPNOW in human). Moreover, the authors suggested that two molecules of Prp2 could interact with Spp2 prior to the first step, with only one copy remaining after the first step. More strikingly the post-first step Prp2-Spp2 dimer might recruit Prp16 to the catalytic center of the spliceosome. It is still unclear whether these Prp2 interactions are direct or whether the interactions between Prp2, Spp2 and Prp16 impact the enzymatic activity of the helicases.

Prp16. Prp16 (**Fig. 3F**) was originally identified in a screen for suppressors of the C259 branch site mutation of an *ACT1-HIS4* gene fusion.[95] For a long time, Prp16 was believed to associate with the spliceosome only transiently, for the duration of the second catalytic step.[96] However, Prp16 was subsequently shown to be recruited to a suboptimal pre-mRNA after Cwc25 but before the first step of splicing.[97,98] At this stage, Prp16 could stabilize the interaction of Cwc25 with the BS sequence in an ATP independent fashion. Thus, during the first step of splicing, Prp16 may play a facilitator role (see below). During splicing of optimum pre-mRNAs, the ATPase activity of Prp16 is required for the transition between the first and second step of splicing, and is proposed to trigger the release of Yju2 and Cwc25.[97] Mutations in *ISY1, PRP8* or the U6 snRNA were shown to suppress *prp16* mutations, suggesting that those factors are also possible targets for Prp16.[99-101] The activity of Prp16 could "terminate" the first step of splicing and "initiate" the formation of a step 2 competent spliceosome. In the step 2 spliceosome, the 3'exon becomes resistant to RNase H cleavage, suggesting that it may be bound within the active site of the spliceosome.[102] Several factors (Cwc23, Slu7, Prp18, Prp22, Prp43, Ntr1 and Ntr2)[3] join the complex C spliceosome before the second catalytic step. However, besides Prp16, only Slu7, Prp18 and Prp22 are necessary for second step catalysis in vitro.[5,103]

Prp22. The precise mechanisms that govern disassembly of the spliceosome are not well understood. Prp22 is the first helicase to participate in spliceosome disassembly, triggering release of the spliced mRNA. The ATPase activity of Prp22 is dispensable for the second step itself, but is necessary for release of U5 snRNP proteins and the spliced mRNA after the second step.[104-107]

Based on yeast two-hybrid interactions and co-precipitation assays, Prp16 was proposed to serve as a receptor for Prp22 (**Fig. 3G**) in the spliceosome.[41] Moreover, both these proteins were observed to interact with the substrate near the 3'SS; Prp16 was found to crosslink from -4 to +13 relative to the 3'SS prior the second step, whereas Prp22 bound in a Prp16 dependent manner at positions -8 to -4 upstream of the 3'SS at this stage.[108,109] Prp22 also binds downstream of the splice junction in the spliced mRNA when the 3' exon is longer than 13 nucleotides.[110] Prp8 has a footprint of at least 13 nucleotides in the 3' exon,[111,112] and is thought to stabilize the duplex formed between the U5 snRNA and the first bases of the 3' exon during the second step. In vitro, Prp22 can unwind RNA duplex with a 3' to 5' polarity.[32] It is therefore tempting to speculate that Prp22 could bind the 3' exon just behind Prp8 and track along in a 3' to 5' direction, stripping away Prp8 and U5 snRNA loop I from the spliced RNA.[110]

Prp43. Release of the excised intron lariat and disassembly of the associated post-splicing complex necessitate ATP hydrolysis by Prp43.[37,113-116] Prp43 co-precipitates U2, U5 and U6 snRNAs,[117-119] although in a CRAC (UV crosslinking and cloning) analysis Prp43 was only crosslinked to the U6 snRNA (positions 18–43 and 76–83). In vivo, Prp43 (**Fig. 3H**) associates with Spp382[120-122] (also called Ntr1) and Ntr2.[113,121] Spp382 is a G-patch protein that functions as a receptor for Prp43 and also stimulates the otherwise weak RNA unwinding activity of Prp43 in vitro.[113] Prp43 was implicated not only in the disassembly of spliceosomes following the splicing of optimal pre-mRNA but also in the dissociation of spliceosomes that become stalled with sub-optimal substrates.[98] Curiously, Prp43 also participates in ribosome biogenesis (see below). Thus, Prp43 could be a general disassembly factor.

Proofreading in splicing. An increasing body of evidence links several helicases with proofreading and discard of defective substrates (reviewed in refs. 124–126). Prp5 is proposed to proofread the BS during pre-spliceosome formation,[127,128] Prp16 proofreads the 5'SS and the BS for the first step of splicing[98,129,130] and Prp22[131] appears to proofread the 3'SS and the BS for the second step. As previously mentioned, Prp28 was suggested to proofread the U1 snRNA:5'SS and/or U6 snRNA:5'SS interaction.[67]

The mechanism of proofreading is incompletely understood. Burgess and Guthrie[129] proposed a version of kinetic proofreading in which the rate of ATP hydrolysis by helicases determines the fate of pre-mRNAs and splicing reactions. The kinetic proofreading model is based on the equilibrium between rejection and acceptance of a substrate for the next step of splicing. In the case of a suboptimal pre-mRNA, the rate of rejection is normally higher than the rate of acceptance and the defective pre-mRNA is discarded. In a possible mechanism ("timer model"[124]), the activation of a helicase ATPase activity would restrict the time allotted for a given splicing event to occur. For example, in the case of a pre-mRNA with a normal BS, the recognition and the association of U2 snRNP would happen quickly. Activation of Prp5 ATPase activity would "validate" the U2:BS interaction and promote pre-spliceosome formation. In the case of a mutated or suboptimal BS, the establishment of the U2:BS interaction would be slow and Prp5 activation would "reject" the defective spliceosome and promote its dissociation. Thus the helicase could play a dual function of stabilizer of correct interactions and destabilizer of impaired interactions. Studies performed on Prp5, Prp16 and Prp22 showed that mutations that reduce the level of ATPase activity (not necessarily the rate of the ATP hydrolysis) allow the splicing of reporter constructs in which the 5'SS, BS or 3'SS is not optimal.[127-131]

Prp43 also plays a crucial role in the quality control of splicing. Prp43 is responsible for entry into the non-reversible discard pathway (reviewed in refs. 2, 124, 125, 132). Whereas pre-mRNAs rejected by Prp16 or Prp22 can re-enter the splicing cycle, activation of Prp43 seals the fate of discarded RNAs.[2,98,126,129,130,133]

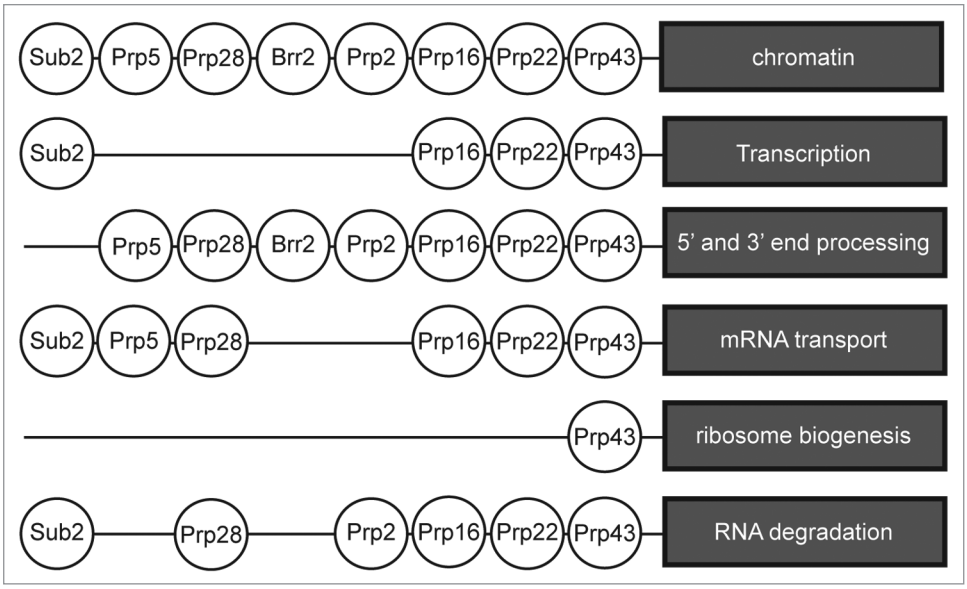


Figure 4. Splicing helicases are connected with other pathways of RNP metabolism. Based on large scale physical and genetic screens (see text), most splicing helicases are connected with several RNP biogenesis events. Only in few cases (see text) has the biological relevance of the proteome data been validated.

Possible Links Between Splicing Helicases and Other RNA Maturation Processes

The biogenesis of mRNPs involves a complex and highly integrated series of events that begins with initiation of transcription, quickly followed by addition of a monomethyl guanosine cap at the 5' end of the nascent transcript, co-transcriptional spliceosome assembly (if an intron exists), 3' end formation (cleavage and polyadenylation), association with nuclear pores and export to the cytoplasm,[134] and these processing events can influence each other. There is evidence that Sub2/UAP56 participates in several mRNA processing pathways in the nucleus.[53,134] The roles of other helicases have not been addressed so far. However, below we review physical and genetic interactions of splicing helicases that may suggest their participation in the regulation and/or integration of various processing events.

Links to transcription. There is considerable evidence for the coupling of transcription and pre-mRNA splicing.[135-140] Intriguingly, Chanarat et al.[141] showed that the yeast Prp19 complex is necessary for the recruitment of THO /TREX (transcription and export) complex during transcription of both intron-containing and intronless genes. They also showed that a mutant of *SYF1* (*syf1-37*), that encodes an NTC factor, slows transcription by Pol II while pre-mRNA splicing is unaffected. However, although Sub2, Prp16 and Prp22 associate with the THO (transcription elongation) complex in large scale proteomic and genetic screens,[70,89] to date there is no information regarding the direct participation of splicing RNA helicases in the coupling process. Most splicing helicases show functional and/or physical interaction with chromatin and chromatin remodelling complexes (**Fig. 4**) such as the Swi2/Snf2 complex (Prp5, Prp16, Prp22, Prp43), the Swr1 complex (Prp22, Prp28) or with the RNA polymerase machinery. The Swi/Snf complex is an ATP-dependent chromatin remodelling complex that can displace/remodel/modify nucleosomes.[142,143] The Swr1 complex catalyzes the replacement of histone H2A by histone H2Az at or around transcription start sites, thereby shifting the position of the reprogrammed nucleosome. This action can regulate transcription positively or negatively depending on the new position of the modified nucleosome. Somewhat confusingly, in addition to its role in splicing, U1 snRNA has been implicated in a splicing independent role in transcriptional activation.[144]

Among splicing helicases, Brr2 shows the least connections with proteins involved in chromatin remodelling or transcription (only Spt2 and Yta7[41]). This may underline a splicing-only role for Brr2 and suggest that *trans*-acting helicases are more likely candidates for co-regulating splicing and transcription.

Links to 5' and 3' end processing. Links between pre-mRNA splicing and 5' end capping or 3' end cleavage/polyadenylation of transcripts have long been known.[12,145,146] For example, in higher eukaryotes the 3' end processing machinery plays an important role in the definition of the last exon and, conversely, splicing influences the choice of cleavage/polyadenylation site (reviewed in ref. 12). In particular, U1 snRNPs inhibit premature cleavage and polyadenylation.[147]

In yeast, where the spliceosome appears to recognize introns rather than exons, a direct effect of the 3' end processing machinery on splicing is not clear. Nevertheless, most splicing helicases interact genetically and/or physically with factors involved in 3'end-processing such as Pab1 (Brr2, Prp43),[83,148] or Nab2 (Prp5, Prp28).[149]

Capping at the 5' end of transcripts occurs shortly (20 to 30 nucleotides) after initiation, as the nascent transcript emerges from the Pol II complex.[150] The cap binding proteins Cbp80 and Cbp20 (Sto1 and Cbc2/Mud13 in yeast) promote splicing of cap-proximal introns by stabilizing the 5'SS:U1 snRNA interaction[151-155] and deletion of the Cap Binding Complex (CBC) abolishes the co-transcriptional recruitment of the U1 snRNP to intron-containing transcripts.[156] In *Arabidopsis thaliana*, mutations in AtCbp80 or AtCbp20 affect alternative splicing and 5'SS selection preferentially in the first intron.[14] The cap binding protein Cbp20 co-purifies only with Brr2, Prp2, Prp16 and Prp22[89,157] while the regulatory subunit Cbp80 co-purifies with every splicing helicase[69,83,89,158] except Prp43. The biological significance of this difference is not clear. However, it may suggest that Cbp80 participates in the control of the early splicing helicases and formation of the commitment complex while the fully assembled cap binding complex would associate with the

spliceosome from complex B onwards. The absence of interaction between the CBC and Prp43 could imply that the dissociation of the CBC from the spliceosome precedes Prp43 recruitment and Prp43-dependent spliceosome disassembly.

Links to mRNA transport. Splicing and mRNA transport are tightly coupled in eukaryotes,[159] with spliced mRNAs being more efficiently exported to the cytoplasm than intronless RNAs.[160] Several splicing helicases (Sub2, Prp16, Prp22, Prp43) display both genetic and physical links to the THO/TREX complex that connects transcription and mRNA transport.[161-163] However, Sub2/UAP56 is the only splicing RNA helicase whose function in RNA transport is clearly established.[53,164] In yeast, a *sub2* mutation leads to nuclear accumulation of polyadenylated, spliced mRNA.[56] In higher eukaryotes, UAP56 is part of the Exon Junction Complex that is deposited on spliced mRNA 24 to 26 bases upstream of exon-exon junctions and plays a role in mRNP transport.[165-168] UAP56 also appears to bind intronless mRNAs prior to export.[167,169] The signals for binding to intronless RNAs are not currently known, but they may depend on the presence of non-splicing factors such as the CBC.[170] Sub2/UAP56 was shown to recruit the mRNA export factor Aly co-transcriptionally to both spliced and intronless mRNAs,[160,171] and was proposed to act as an ATP-dependent chaperone of the Aly-RNA interaction.[169] In yeast, ATP binding to Sub2 is necessary for mRNA transport[172] and in higher eukaryotes the association of UAP56, Aly and CIP29 with the TREX complex is also dependent on the presence of ATP.[173] CIP29 stimulates the helicase activity of UAP56,[174] while Aly stimulates ATP hydrolysis by UAP56.[169] UAP56/Sub2 is released prior to the export of mRNA to the cytoplasm.[175]

Links to ribosome biogenesis. During ribosome biogenesis two G-patch proteins, Gno1 and Pfa1, associate with Prp43.[26,117,176] Pfa1 stimulates Prp43 activity in vitro, and in vivo their interaction seems to be required for maturation of the 20S pre-rRNA (pre-rRNA).[117,177] Several Prp43 binding sites that were identified in 18S and 25S rRNA precursors mapped close to cleavage sites in the pre-rRNA, or close to snoRNA-rRNA base-pairing sites.[178] Those data support the likely involvement of Prp43 in remodelling snoRNAs and/or in displacing snoRNAs from pre-rRNA complexes. Thus, it appears that Prp43 functions as a disassembly factor during ribosome biogenesis and during pre-mRNA splicing, subject to control by different G-patch proteins.

Links to RNA degradation. Several RNA helicases co-purify or interact genetically with proteins involved in RNA degradation (**Fig. 4**) but the functional significance is currently not known. The mutant *sub2-201*[55] is synthetic sick when combined with depletion of the nuclear degradation factor Rrp6, but not with loss of the cytoplasmic degradation factor Xrn1.[179] Additionally, overexpression of *SUB2* leads to a synthetic growth defect when combined with mutations affecting the nuclear 5' to 3' exonuclease Rat1 or the TRAMP complex factor Mtr4.[180] Furthermore, Egecioglu et al.[125] found that the nuclear exosome and Rat1 participate in a quality control pathway that allows discard of aberrantly spliced mRNAs or incompletely spliced transcripts. For example the level of pre-mRNAs in a *prp2–1* mutant increased when components of the nuclear exosome or Rat1 were depleted or mutated.[181] The interaction of Prp43 with Xrn1 is likely linked to its role during pre-rRNA processing rather than its role in splicing.[117]

Conclusions

The fact that splicing helicases function within large and highly dynamic RNP complexes has greatly complicated the identification of their targets and characterization of their modes of action and regulation. Genetic studies, mainly in budding yeast, have been a rich source of information about interacting partners and potential targets. However, genetics may not distinguish functional interactions (which may be indirect) from direct physical interactions. In vitro analyses of splicing helicase activities using purified components have yielded limited information, and because pre-mRNA splicing is intricately intertwined with transcription and other RNA maturation events, in vitro studies of splicing factors will lack their influences unless coupled in vitro systems are developed. As discussed above, splicing RNA helicases are not only necessary for the progression of the spliceosome through the splicing cycle, they also function as proofreaders of the splicing process. Proofreading by helicases is only one level of quality control, which also involves the degradation of aberrant RNA molecules in the nucleus or in the cytoplasm.[124-126] Therefore, a full understanding of the contribution of helicases to this process will require detailed kinetic studies in vivo.

As RNA helicases make highly transient interactions with their targets, small molecule inhibitors, including substrate or cofactor analogs, may prove useful to capture transient complexes for structural studies. Similarly, mutant helicases in combination with cross-linking approaches may permit global "snapshots" to be obtained of normally transient protein-protein and protein-RNA interactions,[15] as mentioned above for Brr2. At the other end of the scale, combining chemical biology methodologies with single molecule fluorescence techniques now allows kinetic studies of spliceosome assembly and the splicing reactions to be performed on single transcripts in real time (reviewed in ref. 132).

Clearly, much remains to be unravelled about the precise role(s) played by splicing helicases, and how their activities are modulated and timed by their multiple partners, whether proteins, nucleic acids or small molecules. Deciphering the splicing helicase code remains an exciting challenge with profound repercussions in the understanding of normal and pathogenic pre-mRNA splicing.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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