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Current Opinion in Structural Biology

The spliceosome: disorder and dynamics defined

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Among the many macromolecular machines involved in eukaryotic gene expression, the spliceosome remains one of the most challenging for structural biologists. Defining features of this highly complex apparatus are its excessive number of individual parts, many of which have been evolutionarily selected for regions of structural disorder, and the remarkable compositional and conformation dynamics it must undertake to complete each round of splicing. Here we review recent advances in our understanding of spliceosome structural dynamics stemming from bioinformatics, deep sequencing, high throughput methods for determining protein–protein, protein–RNA and RNA–RNA interaction dynamics, single molecule microscopy and more traditional structural analyses. Together, these tools are rapidly changing our structural appreciation of this remarkably dynamic machine.

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Current Opinion in Structural Biology 2014, **24**:141–149

This review comes from a themed issue on **Nucleic acids and their protein complexes**

Edited by **Karolin Luger** and **Simon EV Phillips**

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http://dx.doi.org/10.1016/j.sbi.2014.01.009

Introduction

In all organisms, gene expression requires coordinate action of multiple macromolecular machines, many with multi-megadalton (MDa) molecular weights. Whereas high-resolution crystal structures have revealed the overall architecture and detailed inner workings of many such machines (e.g., ribosomes and RNA polymerases), one elusive 'structure of desire' [1] is the spliceosome. Weighing in at over 3 MDa, the spliceosome is the ribonucleoprotein (RNP) complex responsible for excision of intragenic regions (introns; Box 1) from eukaryotic RNA polymerase II transcripts (precursors to messenger RNAs; pre-mRNAs). The spliceosome must be at once highly accurate — a single nucleotide shift in the site of splicing (splice site; SS) within an open reading frame will result in a non-functional mRNA — and highly malleable to permit alternative splicing, the process by which expressed regions (exons) are spliced together in different arrangements enabling synthesis of many different protein isoforms from a single gene. The proliferation of alternative splicing is the primary reason why organismal complexity is not tightly linked to gene number in the eukaryotic lineage [2,3].

To achieve the right balance between precision and malleability, the spliceosome contains scores of individual parts, many of which are structurally disordered. Working in a highly orchestrated manner, these parts perform incredible feats of molecular gymnastics with each round of splicing. These extremes of complexity and dynamics are no doubt to blame for the spliceosome's recalcitrance to crystallize despite intense efforts by multiple labs over many years. Nonetheless, significant progress is now being made by combining crystal structures of smaller pieces with EM reconstructions of larger assemblages. As detailed in the upcoming review [4], solution of several high-resolution structures containing pieces of Prp8, the massive and highly conserved protein at heart of the spliceosome, is rapidly transforming our understanding of the catalytic core. In this review, we will focus instead on recent progress in understanding spliceosome evolutionary and structural dynamics.

Evolutionary dynamics

One of the defining features of pre-mRNA splicing is the sheer number of components that must come and go to accurately identify and excise each new intron (Figure 1a). In budding yeast, this includes five small nuclear RNAs (snRNAs) and ~100 different proteins, whereas mammals utilize nine unique snRNAs and over 300 different proteins [5,6]. Metazoans have more spliceosomal snRNAs because they contain not one, but two spliceosomes: the more abundant 'major spliceosome' responsible for removing 99.5% of introns and the 'minor spliceosome' excising the other 0.5% [7] (Figure 1b). A long-standing question regarding the function of these minor introns was recently addressed by Younis *et al.* [8•], who showed that under normal growth conditions, their splicing is limited by rapid decay of the key minor snRNA U6atac. In the presence of stress, however, U6atac is stabilized, allowing splicing of preexisting minor intron-containing transcripts, which can then be rapidly translated to help alleviate the stress.

The existence of two spliceosomes is thought to reflect a long ago merging of two eukaryotic genomes that had diverged and separately evolved for prior untold generations. By the time of the merge, so many mutations had

Box 1 Splicing nomenclature

Exons: Regions of pre-mRNA that are ligated together by the spliceosome.

Introns: Regions of pre-mRNA excised by the spliceosome.

5′SS: 5′ splice site; a.k.a. splice donor. The 3′–5′ phosphodiester bond at the 5’ boundary of an intron. This bond is exchanged for a 2′–5′ phosphodiester linking the 5′SS with the BP during the first chemical step of splicing.

BP: Branch point. An intronic adenosine near the 3′SS whose 2′-OH serves as the nucleophile for the first chemical step of splicing.

3′SS: 3′ splice site; a.k.a. splice acceptor. The 3′–5′ phosphodiester bond at the 3′ boundary of an intron. This bond is exchanged for a 3′–5′ phosphodiester linking the two exons during the second chemical step of splicing.

Consensus sequence: A region of sequence conservation that helps to define one of the three sites of chemistry.

snRNA: Small nuclear ribonucleic acid. Spliceosomal snRNAs vary in length from ~90 nts to ~1200 nts and are uridine-rich, so are named U1, U2, etc.

snRNP: Small nuclear ribonucleoprotein particle. A complex containing one or more snRNAs plus stably bound proteins.

NTC: Nineteen complex. A protein-only complex containing Prp19 and other proteins. Stable association of the NTC is the final step in spliceosome assembly, and mediates the transition from the pre-catalytic to the catalytic spliceosome.

First chemical step: Attack by the 2′-OH of the BP adenosine on the 5′SS to form a 2′–5′ branched lariat intron and liberate the 5′ exon.

Second chemical step: Attack by the 3′-OH of the 5′ exon on the 3′SS to join the exons and liberate the lariat intron.

Splicing factor: A protein involved in splicing that is not a stable snRNP component.

accumulated in the separate lineages that the two machineries were no longer fully compatible. Nonetheless, the major and minor spliceosomes do share some key components, and much can be learned about core spliceosome structure from comparing their commonalities and differences (Figure 1b). The largest common component is U5 snRNP, which contains U5 snRNA and 14–15 stably bound proteins [6]. Of all the snRNAs, U5 has the highest percentage of internal secondary structure and is the least accessible to RNase digestion or nucleotide modification reagents [9•], consistent with it being almost entirely coated with proteins. The only region of U5 snRNA making intermolecular RNA–RNA interactions is a U-rich loop that contacts exonic nucleotides to either side of the intron and is thought to help align the exons to facilitate both steps of splicing. Because exon sequences are subject to selective pressures dictated by the encoded protein, the nucleotides at exon ends are not highly conserved; therefore, the contacts made with U5 snRNA are perforce relatively non-specific.

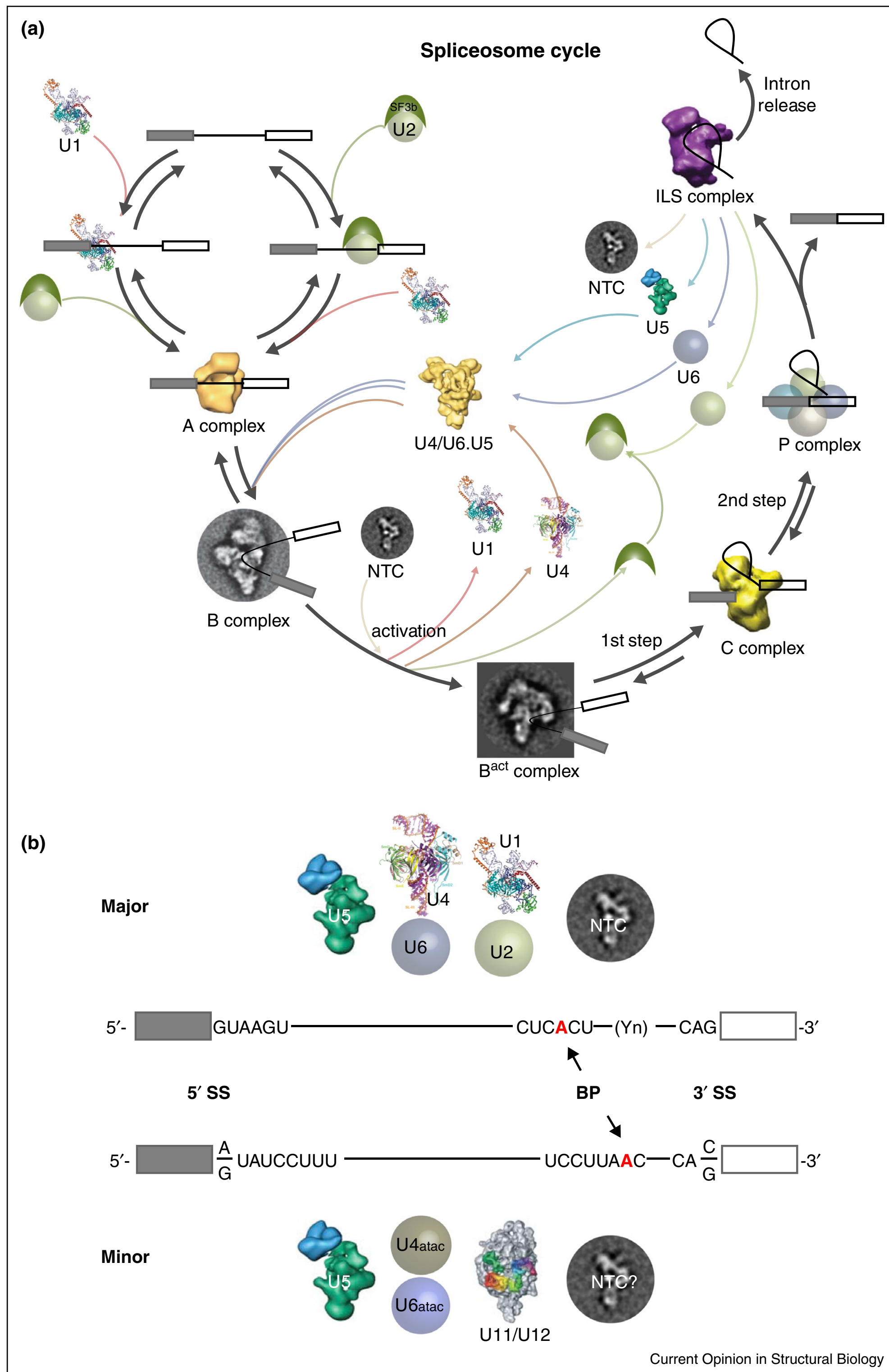
Among all spliceosomal proteins, those comprising the U5 snRNP are the most conserved across species. In Prp8, the largest spliceosomal protein, 61% of its >2300 amino acids are completely conserved from yeast to humans. Such a high level of conservation is indicative of strong structural constraints both internally (to maintain overall folding and activity) and externally (to maintain intermolecular interactions) [10]. Consistent with this, Prp8 is known to directly contact eight other spliceosomal proteins, all three splice site consensus sequences, and U1, U2, U5 and U6 snRNAs [11]. As discussed in the accompanying review [4], Prp8 both supplies amino acids necessary for catalysis and serves as the structural scaffold upon which the entire spliceosome is built. Two other highly conserved U5 snRNP proteins that interact with this scaffold are Brr2 and Snu114, two large NTPases necessary for key structural transitions during spliceosome assembly and disassembly [12].

Each of the other major spliceosomal snRNAs (U1, U2, U4 and U6) has a distinct ortholog in the minor spliceosome (U11, U12, U4atac and U6atac, respectively) (Figure 1b). These snRNAs make key intermolecular base pairs with other snRNAs and/or the splice site consensus sequences (Figure 1b). Because RNA–RNA interactions have simple substitution rules (e.g., A-U → G-C), compensatory mutations could easily accumulate over evolutionary time, resulting in incompatibilities when the two eukaryotic predecessor genomes merged. In comparison, many proteins were apparently less mutable and therefore remained sharable. Other shared proteins between the major and minor spliceosomes include the SF3B proteins required for early spliceosome assembly, the 110K and 65K tri-snRNP proteins [13] and possibly the NTC complex [7]. Among non-shared components, the 20 and 35 kDa proteins in 18S U11/U12 are likely orthologs of U1C and U1-70K [7]. U1C protein recognizes the 5′SS consensus of introns removed by the major spliceosome [14]; the different 5′SS consensus in minor spliceosomal introns may therefore explain the need for a different, but functionally related protein. Interactions between the N-terminal half of U1-70K and SR proteins modulate both exon definition and alternative splicing [15], and this homology region is retained in the U11/U12 35K protein. The unique C-terminal half of U1-70K interacts with polyA polymerase and suppresses pre-mRNA polyadenylation [16]. This additional activity of U1 snRNP was recently shown to be crucial for preventing premature polyadenylation at cryptic polyA sites within introns [17] and to control 3′-UTR length [18•]. Consistent with the lack of this domain in U11/U12 35K, no such activity has yet been ascribed to the U11/U12 snRNP.

Evolving toward disorder?

Many spliceosomal proteins contain intrinsically disordered regions (IDRs), polypeptide stretches that in isolation lack stable, well-defined 3D structures. IDRs have a variety of useful functions — they can serve as linkers between structured domains, as sites of post-translational

Figure 1



An updated spliceosome assembly cycle. **(a)** Structural overview of the yeast spliceosome assembly, activation, and disassembly cycle (adapted from [36•]). The crystal structures of human U1 and U4 snRNPs [51,52] and EM structures of larger complexes are shown [6,53–58]. **(b)** Shared and unique components of the major and minor spliceosomes. Structures of NTC, U1, U4, and U5 snRNPs are as in A; U11/U12 di-snRNP structure is from [59]. NTC complex association with the minor spliceosome has only been inferred to date, not structurally validated. Also shown are the conserved sequences of major and minor introns with exons (boxes), introns (lettering or solid line), the branch adenosine (bold red) indicated (adapted from [7]).

modification, and as sites of protein–protein and protein–RNA recognition [19]. A general feature of IDRs is their ability to transition to a more ordered state upon interaction with a specific binding partner. Many IDR-containing proteins are able to bind multiple targets simultaneously, thereby facilitating larger complex assembly (Figure 2a). Another important feature, however, is the capacity of some IDRs to adopt different conformations upon interaction with different binding partners. This capacity for multiple mutually exclusive specific interactions (multi-specificity) makes IDRs particularly adept at facilitating structural transitions within larger complexes.

Remarkably, one recent comprehensive bioinformatics analysis has predicted that IDRs occupy almost half of the combined sequences of abundant human spliceosomal proteins! [20••] In *Saccharomyces cerevisiae*, ~47% of spliceosomal proteins contain predicted IDRs in comparison to only ~13% of the entire yeast proteome [21•]. Within the spliceosome, the distribution of intrinsic order and disorder is highly uneven, with essential catalytic core proteins generally being more ordered than those involved in spliceosome assembly and dynamics. Further, proteins involved in later stages of assembly (e.g., spliceosome activation) tend to be less disordered than components acting at earlier stages (e.g., initial splice site recognition) (Figure 2b). This suggests that over evolutionary time, a preexisting ordered catalytic core gained functionality and flexibility through addition of peripheral and evolutionarily younger IDR-containing proteins [20••]. In higher eukaryotes, this increased flexibility likely enabled the spliceosome to integrate more diverse information than just the canonical 5′SS, branch site and 3′SS consensuses, thereby facilitating the proliferation of alternative splicing. Unfortunately for humans, however, the accompanying proliferation of IDR-containing splicing factors also has a cost. Because of their tendency toward disorder, IDR-containing proteins tend to form protein aggregates [22], and aggregates of splicing factors have been implicated in multiple human diseases, especially neurodegeneration [23].

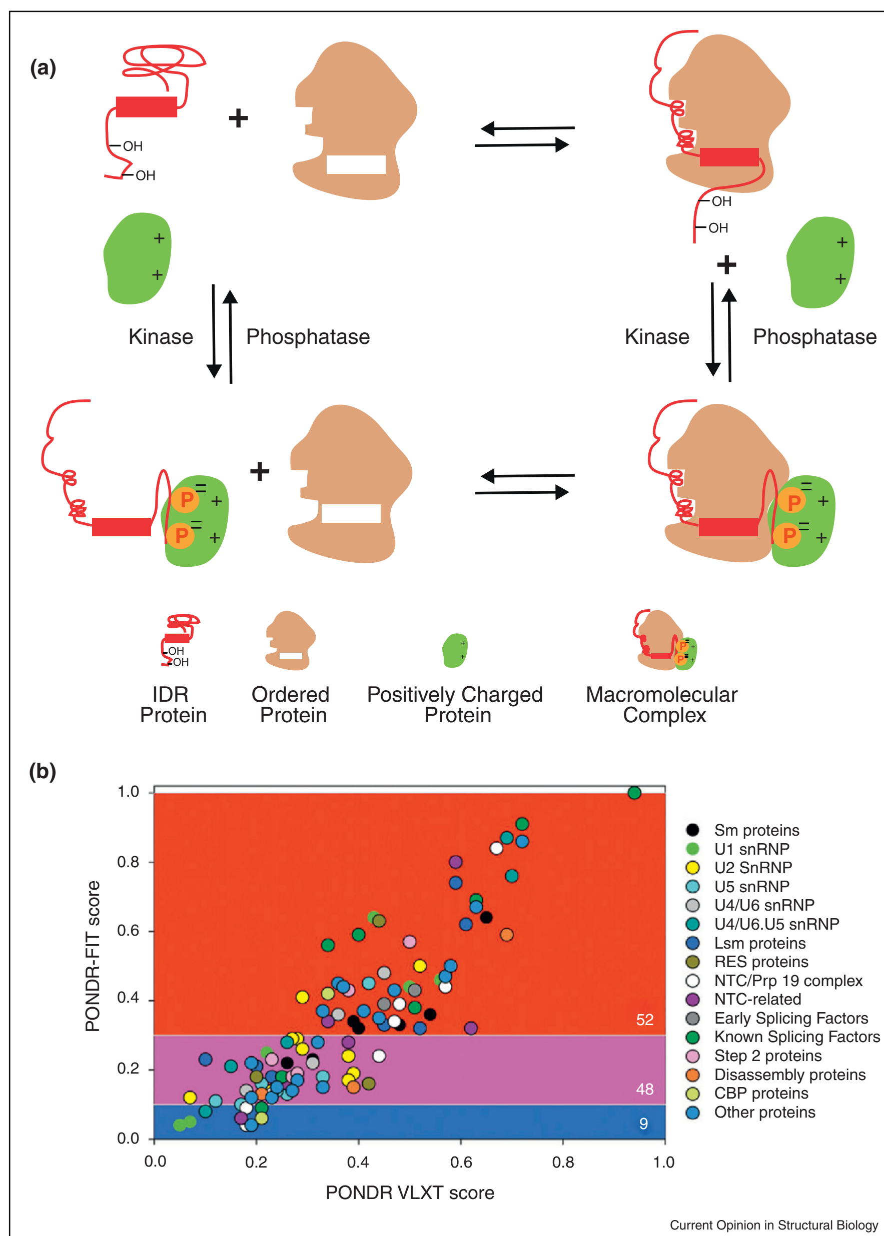
Reversibility is the rule

Another major theme emerging in recent years is that, rather than being the one-way pathway typically drawn in textbooks, almost every step in the spliceosome cycle is readily reversible (Figure 1a). Each required structural and chemical transition is nearly energy neutral, with the overall process being driven in the forward direction by coupling these reversible transitions to an energetically favorable reaction (e.g., ATP hydrolysis by an RNA helicase). One example of this inherent reversibility is the phosphodiester exchange reactions comprising the first and second chemical steps of splicing (lariat formation and exon ligation) [24]. Tseng and Cheng [25] recently showed that not only can the spliceosome catalyze both chemical steps in forward and reverse, it can even convert spliced products (lariat intron and ligated exons) back into unspliced pre-mRNA! Catalysis of the first and second step chemistries requires two different active site configurations between which the spliceosome toggles to favor one step or the other [26,27]. The equilibrium between these two configurations can be altered by mono and divalent metal ion concentrations [28•] and by mutations in the pre-mRNA, in U2 and U6 snRNAs, and in Prp8 and Brr2 [29,30], all of which together comprise the catalytic core. The overall process is driven in the forward direction by coupling these active site structural changes to ATP hydrolysis by the RNA helicases Brr2, Prp2, Prp16, and Prp22 [6].

One methodology rapidly rewriting our understanding of splicing dynamics and reversibility is single molecule microscopy. Supporting the idea that the splicing cycle can be thought of as a network of near energy neutral structural transitions separated by relatively low energy barriers, one single molecule fluorescence resonance energy transfer (smFRET) study suggested that pre-mRNA conformation is highly dynamic over the course of spliceosome assembly, with various states interchanging on 'seconds' timescales [31]. Using a protein-free system, Guo *et al.* [32] found evidence for at least three distinct conformations of U2 and U6 snRNA duplexes whose equilibrium and dynamics were functions of Mg^{2+} concentration and U6 snRNA sequence. These conformational changes bear strong resemblance to those proposed to occur in the transition between the first and second step catalytic states of the intact spliceosome [26,27,33].

Observation of single molecules in biochemically active cellular extracts has recently enabled researchers to directly measure the dynamic comings and goings of individual spliceosomal components, to distinguish on-pathway from off-pathway assembly events and to tie specific conformational changes to individual binding events [34]. Using Colocalization Single Molecule Spectroscopy (CoSMoS), Hoskins *et al.* [35••] demonstrated the reversibility of every major subcomplex addition step along the yeast spliceosome assembly pathway. More recently, Shcherbakova *et al.* [36•] showed that functional A complex formation can occur by either a U1-first or a U2-first pathway (Figure 1a). Intriguingly, if the pre-mRNA contains multiple 5′SS's, there appears to be an ATP-dependent mechanism to ensure that only one U1 snRNP is stably incorporated into complex A [37]. Together with data indicating the likelihood of even more diverse spliceosome assembly pathways in mammalian cells [38,39], the above studies have profound consequences for our understanding of alternative splicing regulation. That is, alternative splicing decisions *in vivo* likely result from kinetic modulation of competing spliceosome assembly pathways, and the inherent reversibility of these pathways means that such

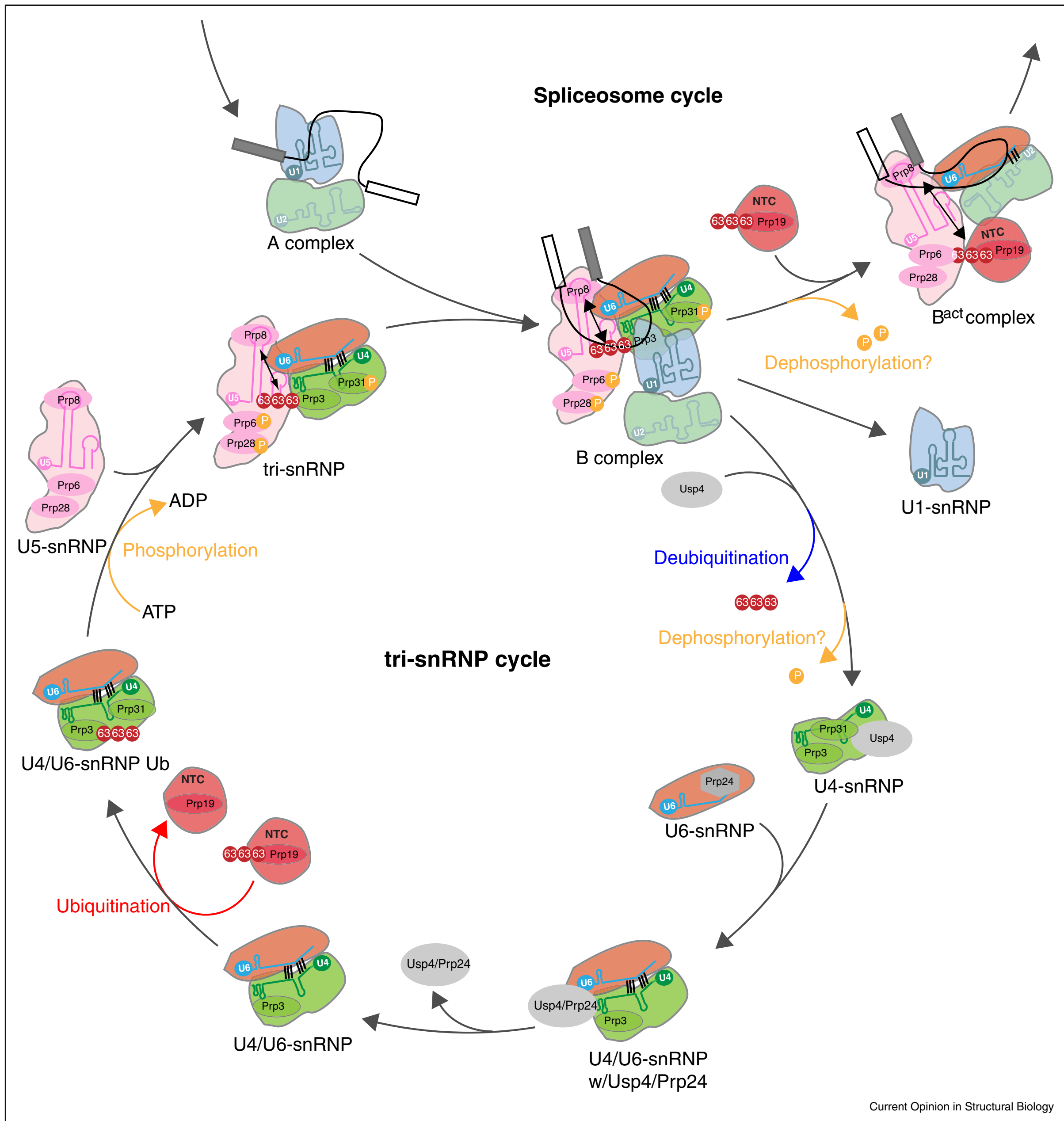
Figure 2



Intrinsic disorder among spliceosomal proteins. **(a)** Illustration of how IDRs can facilitate formation of larger macromolecular assemblies by adopting ordered structures upon binding to a specific partner and/or serving as sites of post-translational modification. **(b)** Plot showing the correlation between predicted protein disorder of various yeast spliceosomal proteins as calculated by PONDR-FIT (*y*-axis) and PONDR® VLXT (x-axis) (reproduced with permission from [21•]). These two programs use different algorithms to generate a score for each protein sequence that reflects the fraction of amino acids likely to be in IDRs. This plot shows that both algorithms generate highly correlated outputs with regard to spliceosomal proteins and splicing factors [21•]. Using arbitrary PONDR-FIT cutoffs, proteins were classified as highly ordered (blue field, 9 proteins), moderately disordered (pink field, 48 proteins) and highly disordered (red field, 52 proteins). Proteins are color coded as indicated to indicate their relationship to different components and complexes.

modulation could occur at late as well as early assembly steps. By combining CoSMoS with smFRET, Crawford *et al.* [40•] recently demonstrated that the 5′SS and BS regions remain physically separate until after spliceosome activation. This opens the possibility that the final decision of where to splice might occur much later in the spliceosome cycle than previously thought, further increasing the options for alternative splicing regulation.

Figure 3



Ubiquitination and phosphorylation cycles involved in U4/U6.U5 tri-snRNP dynamics (adapted from [48•]). See text for details. Question marks indicate steps that are yet to be elucidated.

Dynamic PPIs and post-translational modifications

In addition to the dynamic RNA–RNA and RNA–protein interactions discussed above, the spliceosome cycle also involves innumerable dynamic protein–protein interactions (PPIs). Recently, Hegele *et al.* elucidated the complete PPI 'wiring diagram' of the human spliceosome [41••]. Employing a combination of yeast two-hybrid (Y2H) and coimmunoprecipitation analyses, the authors systematically investigated 632 possible PPIs among core and noncore components. They then used link clustering to integrate 242 positively confirmed core factor interactions with spliceosome subcomplex purification data. The result was a highly expanded understanding of core PPI dynamics during splicing, particularly with regard to mutually exclusive binding partner interactions that help drive the splicing cycle forward. This systematic Y2H and comparative proteomics approach can also be used to

identify new splicing factors, as exemplified by the recent discovery of six novel splicing factors in *Schizosaccharomyces pombe* [42].

Also driving spliceosome assembly forward are dynamic post-translational modifications. Some spliceosomal proteins are acetylated, and dynamic acetylation/deacetylation is important for spliceosome assembly and rearrangement [6]. Numerous others are subject to reversible methylation and/or phosphorylation, with dynamic SR protein phosphorylation being particularly important for mammalian spliceosome assembly [6,43]. A recent bioinformatics study revealed that sites of dynamic phosphorylation tend to occur at intermolecular binding interfaces, where they can 'orthosterically' modulate the strength of protein–protein interactions [44]. Further, phosphorylation, disorder-to-order transitions and formation of new binding partner interactions are all highly coupled (Figure 2a), with about one quarter of all Ser/Thr/Tyr residues at interfaces being phosphorylated. Among these, phospho-Ser residues are the most likely to occur within intrinsically disordered regions (IDRs). Conversely, phospho-Tyr is more often observed at ordered interfaces (i.e., structures predicted to be ordered even in the unbound state) [45]. The prevalence of Ser phosphorylation on spliceosomal proteins is therefore consistent with the above discussion regarding the remarkable abundance of IDRs within the splicing machinery [20••,21•].

Another post-translational modification driving spliceosome dynamics is reversible ubiquitination. The best understood example is the polyubiquitination cycle involving the U4 snRNP protein Prp3, the NTC component PRP19, the deubiquitinating enzyme Usp4 and its binding partner in U6 snRNP Prp24/Sart3. Two early studies reported that Prp19 contains a U-box, allowing it to ubiquitinate itself via nonproteolytic K63-linked chains [46], and that dynamic ubiquitination/deubiquitination controls tri-snRNP levels, likely by regulating U4/U6 snRNA winding and unwinding [47]. These observations were recently integrated by Song *et al.* [48•], who found that Prp3 is the downstream target of both Prp19 ubiquitination and Usp4 deubiquitination (Figure 3). Through interaction of the NTC with the U4/U6 di-snRNP, Prp19 transfers its ubiquitin chains to Prp3; this facilitates U4/U6.U5 tri-snRNP reassembly by increasing Prp3's affinity for Prp8, likely via Prp8's ubiquitin-binding JAMM domain. Once the U4/U6.U5 tri-snRNP has joined the spliceosome, Usp4 deubiquitinates Prp3, decreasing its affinity for Prp8. This both facilitates U4 departure, enabling interaction of U6 snRNA with U2 snRNAs to form the catalytic core, and frees up Prp8 to interact with the ubiquitin chains on Prp19 to stabilize NTC addition. Once U4 snRNP has been ejected from the spliceosome, it can bind a molecule of free U6 snRNA facilitated by Prp24/Sart3. Through its own series of structural transitions [49,50], Prp24/Sart3 promotes U4/U6 snRNA reannealing to reform the U4/U6 snRNP within which Prp3 can once again be ubiquinated by Prp19 to favor U4/U6.U5 tri-snRNP formation [48•]. Intersecting with this ubiquitination–deubiquitination cycle is a phosphorylation–dephosphorylation cycle on tri-snRNP proteins Prp6, Prp28 and Prp31 [6]. This phosphorylation cycle is crucial for both tri-sRNP formation and B complex assembly.

Perspective

The ever-increasingly complicated and interconnected cycles of dynamic structural and post-translational changes shown in Figures 1 and 3 illustrate the incredible complexities facing structural biologists bold enough to even contemplate complete structural understanding of the splicing machinery within their lifetimes. Given its remarkable dynamics, a high-resolution crystal structure of any fully assembled spliceosome may yet be years away. Nonetheless, with new tools such as single molecule microscopy, bioinformatics, and high throughput methods for determining protein–protein, protein–RNA and RNA–RNA interaction dynamics increasingly being developed and applied, structural biologists do have much to celebrate. No doubt the splicing machinery has many structural surprises yet to be revealed.

Acknowledgements

We thank many members of our research group for critical reading of the manuscript, especially J Braun, I Shcherbakova and V Serebrov. This work was supported by funding from HHMI and NIH RO1-GM53007 (MJM). MJM is an HHMI Investigator.

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- •• of outstanding interest

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