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## Review

Combinatorial regulation of alternative splicing<sup>☆</sup>Hossein Shenasa, Klemens J. Hertel<sup>\*</sup>

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## ABSTRACT

The generation of protein coding mRNAs from pre-mRNA is a fundamental biological process that is required for gene expression. Alternative pre-mRNA splicing is responsible for much of the transcriptomic and proteomic diversity observed in higher order eukaryotes. Aberrations that disrupt regular alternative splicing patterns are known to cause human diseases, including various cancers. Alternative splicing is a combinatorial process, meaning many factors affect which two splice sites are ligated together. The features that dictate exon inclusion are comprised of splice site strength, intron-exon architecture, RNA secondary structure, splicing regulatory elements, promoter use and transcription speed by RNA polymerase and the presence of post-transcriptional nucleotide modifications. A comprehensive view of all of the factors that influence alternative splicing decisions is necessary to predict splicing outcomes and to understand the molecular basis of disease. This article is part of a Special Issue entitled: RNA structure and splicing regulation edited by Francisco Baralle, Ravindra Singh and Stefan Stamm.

## 1. Introduction

Pre-mRNA splicing entails the simultaneous excision of introns and ligation of exons to form a contiguous stretch of mRNA [1,2]. Alternative pre-mRNA splicing is a process in which various exons are included, sometimes in different forms, while other exons are excluded. This process is responsible for much of the transcriptomic and proteomic diversity in higher order eukaryotes [3]. Because alternative splicing generates so many different mRNA isoforms, there must be sufficient flexibility in the splicing code to allow for different isoforms to be generated, while maintaining an astonishingly high level of fidelity [4]. Thus, the regulation of alternative splicing is a highly combinatorial process, where many inputs dictate the splicing outcome of each exon [5]. The selection of alternative splice sites is influenced by a multitude of cis- and trans-acting features. These can be categorized by the strength of splice sites flanking exons, the presence or absence of splicing regulatory elements that recruit splicing activators or repressors, the propensity for RNA secondary structure formation, the influence of transcription kinetics by RNA polymerase, the length of introns and exons and the presence of modified RNA nucleotides, which in turn can modulate protein binding or RNA secondary structure formation [6–22]. Given the sequence and context differences between annotated exons, it is expected that the splicing outcome of most exons is regulated through a unique set of parameters. This review will focus

on the alternative splicing mechanisms of metazoans and in particular humans, which have a greater degree of splicing and a more complex splicing machinery.

## 2. The role of splice sites in mediating alternative splicing

The fundamental sequences that direct the spliceosome to the pre-mRNA are the splice site sequence elements. These cis-elements in the pre-mRNA distinguish intron/exon boundaries and recruit components of the spliceosome. The 5' splice site is a nine-nucleotide consensus sequence that demarcates the 5' intron/exon boundary and recruits the U1 snRNP (Fig. 1). The 5' end of U1 snRNA base pairs with the 5' splice site consensus sequence and this complementarity is the basis of efficient U1 snRNP recruitment [2,23,24]. The greater the complementarity, the more efficient U1 snRNP binding is to a 5' splice site. By analogy, lower complementarity between the 5' splice site sequence and the 5' end of U1 snRNA leads to weaker binding of U1 snRNP to the pre-mRNA [25]. The 3' splice site is composed of an AG dinucleotide that delineates the downstream exon from the intron (Fig. 1). The 3' splice site is preceded by a polypyrimidine tract, another essential sequence element, which recruits the heterodimer U2AF. While U2AF65 preferentially binds stretches of pyrimidines, the U2AF35 subunit has been demonstrated to recognize the AG dinucleotide at the intron/exon junction [26]. U2AF bound to the intron/exon junction assists in the

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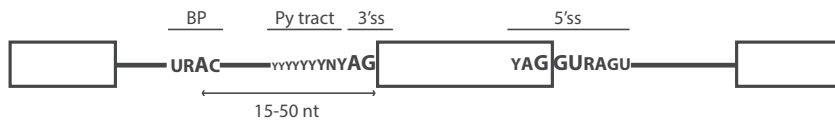
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the branch point sequence (BP), the polypyrimidine tract (Py) and the AG dinucleotide at the intron/exon junction (3'ss). The Py tract is a binding site for U2AF, which assists in the recruitment of U2 snRNP to the branch point sequence. The distance between the branch point and the exon/intron junction is indicated below. The size of the letters indicates their importance with smaller letters representing nucleotide positions with a higher degree of degeneracy. The letter Y symbolizes pyrimidine nucleotides C and U. The letter R symbolizes purine nucleotides A and G. The letter N represents any nucleotide: A, C, G or U.

subsequent recruitment of U2 snRNP, which recognizes the intronic branch point sequence through base pairing interactions [1,2,24,27]. In the human genome, the distance between the intron/exon junction and the branch point ranges between 15 and 50 nucleotides [28]. It is well appreciated that the strength of these splice junction signals dictates the rate and efficiency at which they recruit spliceosomal components. As such, the splice sites are essential pre-mRNA elements that direct splicing through base pairing and RNA-protein interactions and modulate alternative splicing through their affinity for U1 and U2 snRNP. Given their central role in directing the spliceosome to the pre-mRNA several approaches have been implemented to derive numerical scores that describe the strength of a given splice site. The most frequently used approach uses a maximum entropy principle to calculate the likelihood of a sequence to act as a 5' or 3' intron/exon junction [29]. Using this scoring scheme, it has been shown that the 5' and 3' splice site strengths play a near equal role in the promotion of cassette exon inclusion [6]. Thus, neither the 5' nor the 3' splice site exhibit a dominant influence on exon inclusion. Furthermore, the sum of the splice site scores is a much better predictor of exon inclusion compared to the 5' or the 3' splice site score alone [6]. Interestingly, the splice site score analysis also demonstrated that the difference between preferential exon inclusion and exon exclusion is strikingly narrow suggesting cooperative mechanisms of exon recognition. As expected from such splice site score analysis the use of both splice sites in splicing prediction algorithms significantly improves the accuracy of splicing predictions.

### 3. The influence of RNA secondary structure in mediating exon recognition

RNA secondary structure has been shown to play an important role in pre-mRNA splicing [11–13]. While pre-mRNAs are often depicted in a linear fashion, local RNA secondary structures can form and influence pre-mRNA splicing. On a theoretical level it is easy to envision how RNA secondary structure modulates alternative splicing. This is based on the verified notion that spliceosomal components and splicing regulators interact with single-stranded RNA [30]. For example, the formation of an RNA helix could mask splice sites or the binding sites for splicing regulators (Fig. 2A). As a consequence, pre-mRNA binding by spliceosomal components or regulators would be hindered, inhibiting the splicing of certain isoforms. This has been observed in exon 7 of the *SMN* genes, where the weak 5' splice site is partially sequestered by an RNA helix that interferes with U1 snRNA base pairing [31]. Using similar logic, RNA secondary structure can theoretically promote splicing by masking repressive splicing regulatory elements or by decreasing the distance between two splice sites. An *in silico* analysis of RNA secondary structure potential around intron/exon junctions showed that alternative splicing correlated with the ability to form RNA secondary structures at the junction. Furthermore, up to 4% of phylogenetically conserved alternative splicing events were shown to be correlated with conserved RNA secondary structure formation *in silico*. These results point to a generic role for RNA secondary structure in alternative splicing [10]. There are also examples of RNA secondary structure being the primary driver of alternative splicing. For example, in the *Drosophila Dscam* exon cluster 6, alternative splicing is driven by RNA duplexes forming between a RNA docking sequence and various RNA

selector sequences [32]. Furthermore, it was shown that the strength of the RNA-RNA duplex formed between the docking site and the selector sequence plays a role in the level of exon inclusion [33]. The *Dscam* exon 6 cluster is one example of RNA secondary structure modulating alternative splicing in a mutually exclusive way.

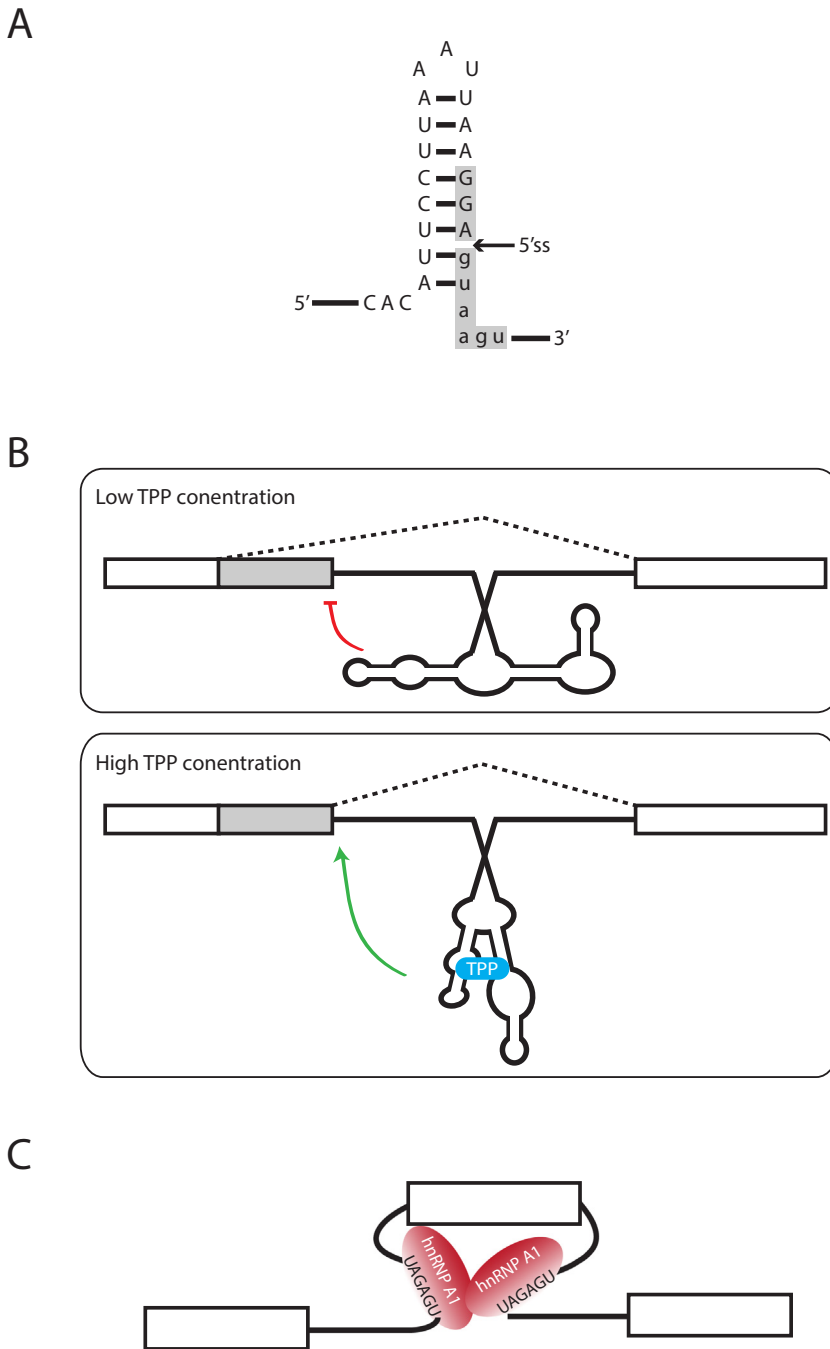
An additional elegant example of RNA secondary structure modulating alternative splicing can be seen in the *NMT1* gene of *N. crassa* (Fig. 2B). The intron of the *NMT1* gene contains a thiamine pyrophosphate (TPP) binding riboswitch, which senses levels of TPP. In the absence of TPP the intronic RNA secondary structure masks an alternative 5' splice site [34]. However, in presence of TPP the aptamer domain of the riboswitch binds TPP and causes structural rearrangements in the intron, which promote usage of the alternative 5' splice site. This structural switch results in mRNA transcripts that have competing upstream open reading frames and repress *NMT1* expression [34].

Another way in which RNA secondary structure affects alternative splicing is through protein-mediated RNA structural changes. For example, the protein hnRNPA1 has been shown to promote distal (upstream) 5' splice site activation by looping out an internal exon (Fig. 2C). Importantly, these splicing effects were replicated when hnRNPA1 binding sites were replaced with inverted repeats that could form RNA duplexes and were predicated to loop out the internal exon [35]. A similar looping mechanism has been proposed for the polypyrimidine tract binding protein (PTB), which is often associated with exon repression. It has been shown that the third and fourth RNA binding domains of PTB can simultaneously bind separate pyrimidine tracts separated by a linker and bring their 3' and 5' ends together, presumably verifying PTB's ability to loop exons out [36]. While some clear-cut examples of RNA secondary structure driving alternative splicing decisions have been described here, RNA secondary structure often has a more peripheral albeit understated effect on alternative pre-mRNA splicing.

4. The influence of splicing regulatory elements on alternative splicing

### 4. The influence of splicing regulatory elements on alternative splicing

Splicing regulatory elements (SREs) are cis-acting sequence elements that are often located adjacent to the splice sites. They represent binding sites that recruit trans-acting splicing regulatory proteins to the pre-mRNA, which then modulate spliceosomal assembly (Fig. 3A). The two major classes of splicing regulatory proteins include SR proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs) [2,5,8,23,24]. SR proteins are multidomain proteins characterized by the presence of serine arginine repeats and at least one RNA recognition motif (RRM). While the RRM is responsible for RNA interactions, which can be specific as demonstrated for *dsx* alternative exon 4 inclusion (Fig. 3B), the SR domain is thought to mediate protein/protein interactions [37–39]. Interestingly, U1 snRNP and the heterodimer U2AF also contain RS domains [40,41]. It has been proposed that SR proteins interact directly with these core spliceosomal particles through RS-RS domain contacts to mediate splice site recognition [42]. Aside from assisting pre-mRNA splicing, SR proteins are known to contribute to other gene expression steps, such as mRNA export, transcription and translation. The phosphorylation status of the RS domain is likely key to transitioning



**Fig. 2.** The influence of RNA secondary structure on alternative splicing. (A) RNA secondary structure can repress splicing by masking splice sites or the binding sites for splicing activators in double stranded helical regions. This is illustrated for exon 7 of *SMN2*, where a stem loop structure sequesters a weak 5'ss. This structure is termed terminal stem-loop 2 (TSL2) and mutations that weaken it promote exon 7 inclusion while mutations that strengthen it promote exon 7 exclusion. The 5' splice site is highlighted in grey. (B) The *NMT1* gene of *N. crassa* contains a TPP binding riboswitch, shown here as a structured intronic region. When TPP concentrations are low the riboswitch in the intron adopts a conformation that masks the proximal 5' splice site. However, when TPP concentrations are high the aptamer domain of the TPP riboswitch binds TPP, causing allosteric changes that unmask the proximal 5' splice site. TPP binding results in increased proximal 5' splice site usage. Preferred splice patterns are indicated by dotted lines. (C) Protein mediated RNA structural rearrangements can modulate alternative splicing, as demonstrated by hnRNP A1-mediated exon repression. In this mechanism, hnRNP A1 proteins bind flanking sites near an exon and loop it out, which inhibits its selection.

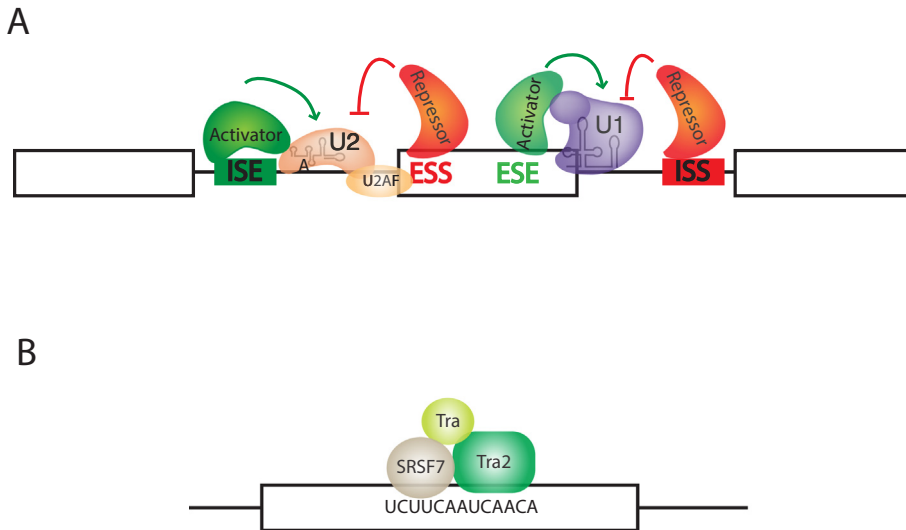
between the multiple roles SR proteins play in gene expression [42].

HnRNPs also contain RRM domains, however they are not characterized by a unique amino acid residue or motif. HnRNPs often contain RGG motifs known as RGG boxes, glycine rich, proline rich or acidic residues. Like SR proteins, hnRNPs are known to interact with spliceosomal core particles to enhance or repress splicing [43].

Based on their influence on pre-mRNA splicing and their location relative to the regulated exon, splicing regulatory elements can be categorized into four categories: exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs) and intronic splicing silencers (ISSs) [5,8,23]. ESEs are best characterized as recruiting SR proteins to exonic positions. SR proteins are known to localize spliceosomal components to adjacent introns through protein-protein contacts. Detailed *in vitro* studies have shown that unlike transcription factors, splicing activation by SR proteins leads to additive

increases in intron removal efficiency [9]. In addition, there is a distance relationship between the activated splice site and pre-mRNA-bound SR proteins and the activity of SR proteins is correlated to the number RS repeats they contain [44]. Interestingly, *in vitro* assays have also demonstrated that SR proteins have an important function in pre-mRNA splicing that does not entail binding exons [45]. Thus, it is possible that SR proteins have an active role in mediating the many rearrangements that ultimately lead to the activated spliceosome.

ESS sequences have traditionally been shown to recruit hnRNP proteins [2,23,24]. For example, hnRNP L has been shown to bind the ESS on *CD45* exon 4 and recruit hnRNP A1 to repress exon inclusion [46]. Many other examples of SRE functions and mechanisms have been detailed in the literature. Genome-wide interaction and function studies suggest that some splicing regulatory proteins may exhibit position-dependent activities [47]. A comprehensive study demonstrated that



**Fig. 3.** Splicing regulatory elements are involved in modulating alternative splicing. (A) Splicing regulatory elements recruit splicing activators or repressors, which in turn modulate spliceosomal assembly by recruiting or inhibiting individual components of the spliceosome such as U1 snRNP, U2AF and U2 snRNP. ISE represents intronic splicing enhancer elements, ESS represents exonic splicing silencer elements, ESE represents exonic splicing enhancer elements and ISS represents intronic splicing silencer elements. (B) The *dsx* splicing enhancer complex is illustrated. Tra, Tra2 and SRSF7 bind cooperatively to the 13 nt repeat element.

this is indeed the case for almost all SR and hnRNP proteins tested [7]. SR proteins were shown to activate splicing when bound exonicly upstream of the regulated 5' splice site but repress splicing when bound intronically, downstream of the 5' splice site. The opposite effect was demonstrated for hnRNPs, which were shown to repress splicing when bound exonicly, upstream of the 5' splice site, but activate splicing when bound intronically downstream of the 5' splice site. This study demonstrates that splicing regulators can exert opposite effects on splice site recognition in a position-dependent manner and highlights the flexibility of splicing regulatory elements in modulating splicing [7]. It is accepted that changes in alternative splicing are often mediated through the differential expression of SR proteins and hnRNPs as seen when analyzing various tissues or cells exposed to different external conditions [48,49].

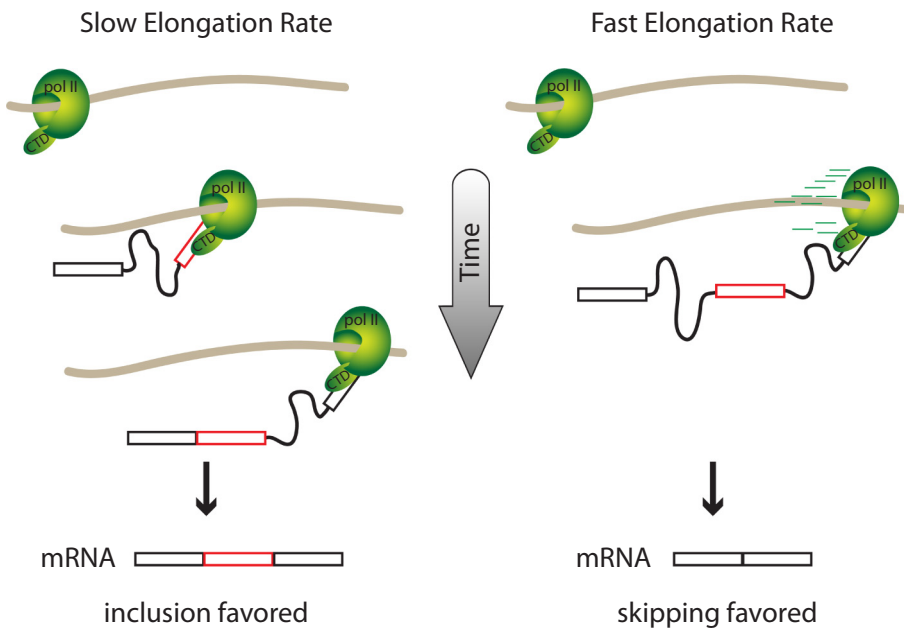
### 5. The influence of transcription by RNA polymerase II on splice site selection

Pre-mRNA splicing has been shown to be coupled to 5' end capping, transcription by RNA polymerase II and 3' end processing. The process of synthesizing pre-mRNAs suggests multiple forms of possible regulation [50]. For example, a faster polymerase that reaches downstream constitutive splice sites more quickly may promote alternative exon skipping (Fig. 4). Experimentally, it has been shown that the promoter architecture is important in alternative pre-mRNA splicing. Different promoters affect the ratio of the *fibronectin* EDI exon inclusion, and this differential exon usage is independent of promoter strength [15]. Additionally, it has been demonstrated that the elongation speed of RNA polymerase influences alternative splicing. A mutation (R749H) in the large subunit of RNA polymerase II causes a slower elongation rate, which modulates the alternative splicing of the EDI exon or the adenovirus *E1a* pre-mRNA under conditions where endogenous RNA polymerase II is inactivated by  $\alpha$ -amanitin [14]. However, the splicing of other transcripts, such as the *hnRNP A1* pre-mRNA, were unaffected by the elongation rate of RNA polymerase II. In *Drosophila* embryos the slow polymerase promotes re-splicing in the *Ultrabithorax* gene [14]. The C-terminal domain of RNA polymerase II (CTD) is known to have both conserved and degenerate heptad repeats that become phosphorylated during the transcription cycle. The deletion of RNA polymerase II's CTD can also lead to changes in alternative splicing that are independent of the elongation rate. As an example, deletion of RNA polymerase II's C-terminal domain leads to increased levels of *fibronectin* EDI cassette exon inclusion. This deletion of the CTD causes a loss of SRSF3 (SRp20) function, which inhibits EDI exclusion [51]. Two different models that are not mutually exclusive have been proposed for

transcriptional control of alternative pre-mRNA splicing. In the first model the primary driver of alternative splicing is the elongation rate, where slower polymerases allow recognition of suboptimal splice sites or the formation of RNA secondary structures that modulate alternative splicing. In the second model unique promoters or post-transcriptional states of RNA polymerase II's CTD are proposed to recruit different trans-acting protein factors that interact with the splicing machinery [51]. The coordinated assembly of the spliceosome is then believed to modulate splice site recognition. Recent genome-wide approaches have demonstrated that the majority of splice sites in eukaryotes are defined co-transcriptionally, meaning that spliceosomal components such as U1 or U2 snRNP assemble onto the nascent pre-mRNA while it is being transcribed [52–55]. The co-transcriptional assembly of spliceosomal factors increases the efficiency of the splicing reaction and ensures that most pre-mRNAs are destined for nuclear export and translation [50]. It is important to note that the majority of *in vitro* splicing assays uncouple splicing from transcription, which may heavily influence results and interpretations. To address this potential limitation, methods have been described to analyze co-transcriptional splicing in the test tube [56,57]. These approaches show that RNA polymerase II couples transcription to spliceosomal assembly *in vitro*, thereby increasing the overall splicing efficiency.

### 6. The influence of the intron-exon architecture on alternative splicing

The intron-exon architecture refers to the length of introns and exons within particular genomes and genes. Two models for splice site recognition have been suggested entailing recognition across the intron, which would be favored for genes with short introns (intron definition) (Fig. 5, left panel), and recognition across the exon, which would be favored in genes with long introns (exon definition) (Fig. 5, right panel) [58]. The influence of the intron-exon architecture on exon skipping has been demonstrated *in vivo*. For example, large-exons (> 500 nucleotides) are skipped when flanked by long introns (> 500 nucleotides), but the same large exons are efficiently recognized when flanked by short introns (< 500 nucleotides). Short exons can be efficiently recognized by the splicing machinery, even when flanked by large introns [16]. *In vitro* analyses have revealed that the intron recognition mechanism employed by the spliceosome is more efficient and leads to higher levels of exon inclusion. Furthermore, it has been shown that intron recognition is employed for introns that are 200–250 nucleotides (nts) or shorter [17]. Interestingly, the intron-exon architecture of multiple model organisms has been studied. A weak, but significant correlation has been shown between increasing genome size and intron

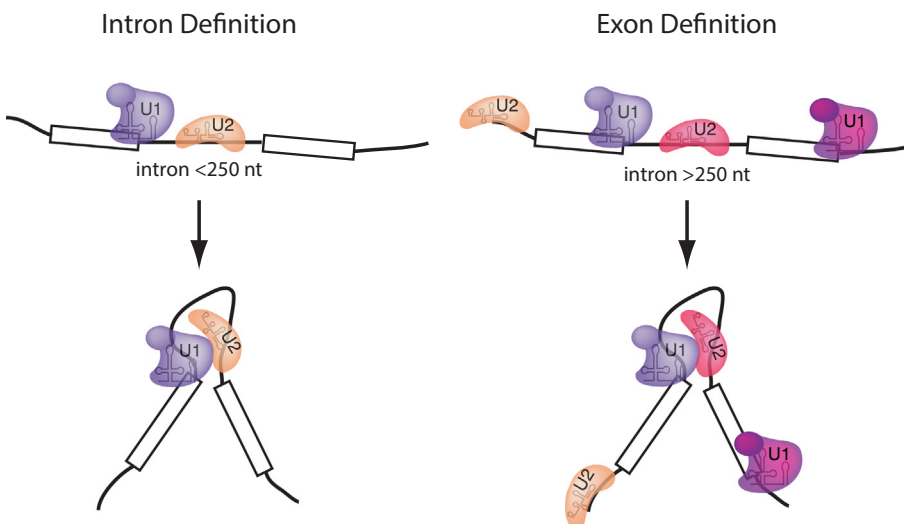


**Fig. 4.** The kinetics of transcription influence alternative splicing. Slow transcription elongation (left panel) results in the preferential inclusion of the alternative exon (red box) because competing downstream exons are not generated fast enough. Fast elongation kinetics (right panel) results in more frequent exon skipping because competing downstream exons are synthesized more quickly. Pre-mRNA exons and introns are depicted by open boxes and black lines. The DNA template is represented by a thick light brown line. Pol II and its CTD domain are shown in green.

length per kilobase of coding sequence. There is also a global shift in the intron-exon architecture of model organisms, with yeast, fungi and *Drosophila* having on average shorter introns and longer exons relative to vertebrates; this is in contrast to humans and other vertebrates having shorter exons and longer introns [59]. Intriguingly, in species where gene architecture is mainly defined by intron definition (*Drosophila*), the length of flanking introns is a significant predictor of alternative splicing. In agreement with model investigations, *Drosophila* exons flanked by large introns (> 1000 nucleotides) are much more likely to undergo alternative splicing than exons that are flanked by short introns (< 250) [17]. Surprisingly, this alternative splicing correlation is almost nonexistent in human, where the vast majority of the intron-exon architecture is defined by exon definition. Presumably, intron expansion was accompanied by additional regulatory sequences to ensure efficient intron removal. These observations demonstrate that the intron-exon architecture of genomes and genes can play a fundamental role in alternative splicing.

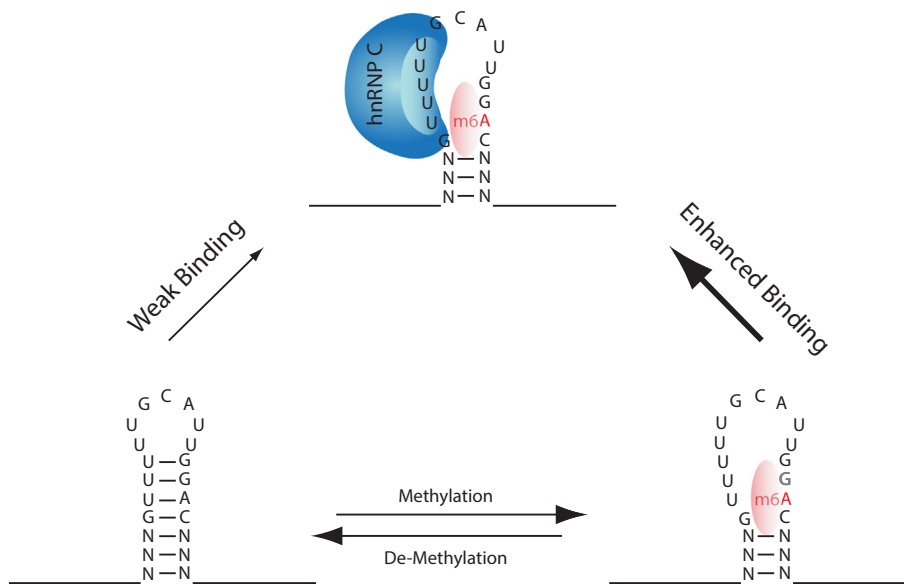
## 7. The influence of modified nucleotides and RNA editing on alternative splicing

Modified nucleotides and the emergence of a functional epitranscriptome is a rapidly developing area of RNA biology. Post-transcriptionally modified nucleotides have been identified in U-snRNPs, ribosomal RNA, mRNA, long non-coding RNA (lncRNA) and transfer RNAs (tRNA) [19]. While some modified nucleotides have well defined functions, such as N6-methyladenosine (m6A) and its critical role in *XIST* mediated X-chromosome transcriptional repression, the function of other modified nucleotides remains unclear [60]. Within the context of pre-mRNA splicing, modified nucleotides can be divided into two categories: nucleotide modifications that are located within the snRNA components of the spliceosome and those that are located within the pre-mRNA. It has long been known that snRNAs contain a modified trimethylated guanosine cap (m3G) (except for U6 snRNA), 2'-O-methyl residues and pseudouridine residues [19,61,62]. Pseudouridine and 2'-O-methyl residues within U2 snRNA have been shown to be critical for E-complex formation and the splicing reaction, while the m3G cap of U-snRNPs has been shown to be critical for nuclear import [61,63]. The other category of emerging modified nucleotides includes those within



**Fig. 5.** Intron length influences the mechanisms of splice site recognition. When introns are short (< 250 nts in length) spliceosomal assembly occurs across the intron. This mode of splice site recognition is referred to as intron definition (left panel). Splice sites of exons that are flanked by larger introns (> 250 nts) are recognized through exon definition (right panel). It is not known how spliceosomal components assembled across exons are combined to define the intron that will be excised.





**Fig. 6.** The influence of m6A modifications on alternative splicing. m6A modifications destabilize double stranded regions of RNA. Such RNA secondary structure disruptions can promote binding of RBPs, which typically prefer single stranded RNAs. Shown here is the m6A-mediated recruitment of hnRNP C to an RNA binding site that is usually less accessible due to secondary structure.

the pre-mRNA that can modulate pre-mRNA splicing [19]. For example, it has been shown that the Fat Mass and Obesity associated protein (FTO) decreases m6A levels. Increases in m6A mRNA levels due to FTO knockdown increase SRSF2 recruitment to the pre-mRNA and promote exon inclusion on a genome-wide level [20]. This study highlights how the epitranscriptome can also act as a cis-regulatory element. Interestingly, hnRNP C and hnRNP G were demonstrated to be recruited by a m6A switch mechanism in which m6A nucleotides disrupt local RNA secondary structures and allow for the binding of these two RNA binding proteins (RBPs) (Fig. 6). In general, m6A modifications destabilize RNA structure and their presence induces single-strandedness, which could make unique RNA binding motifs more accessible. Both hnRNP C and hnRNP G modulate the alternative splicing of a unique, non-overlapping set of genes [21,22]. Finally, hnRNP G was shown to bind m6A modified RNA using its low complexity domain rather than its canonical globular RNA recognition motif, highlighting an interesting interaction between m6A sites and hnRNP G. These results demonstrate that m6A modifications expand hnRNP G's RNA target specificity [22].

Another nucleotide modification that has been verified to occur in pre-mRNA and influence alternative splicing is the conversion of adenosine to inosine through ADAR (adenosine deaminase acting on RNA) mediated RNA editing [64,65]. This process, which requires a double stranded RNA substrate containing the adenosine nucleotide to be edited, was first discovered to regulate alternative splicing in rat mRNAs. Rat ADAR2 (rADAR2)-mediated RNA editing of rADAR2 pre-mRNA creates a proximal 3' splice site by converting an AA dinucleotide to an AI dinucleotide. The resulting AI dinucleotide is recognized by the spliceosome as a functional 3' splice site, thereby activating alternative splicing. The use of the proximal alternative splice site adds 47 nucleotides to rADAR2 mRNA, and this sequence addition is predicted to change the reading frame. Interestingly, leaky ribosome scanning allows for use of a downstream initiation codon and the production of functional protein, albeit at lower efficiency. Given the activity of rADAR2 on its own pre-mRNA, it has been proposed that the editing of rADAR2 pre-mRNA is an auto-regulatory mechanism that tunes the level of rADAR2 expression [64,65]. While editing adenosine to inosine can create new splice sites, this type of conversion has not been observed as a broad mechanism. A global study of alternative splicing changes due to ADAR-mediated RNA editing showed that this is rarely the case [66]. *In silico* analyses suggest that the effects of ADAR-mediated RNA editing are partially modulated through the creation or destruction of SREs. Knockdown RNA-seq and exon specific microarrays

were used to show trans-acting pre-mRNA processing factors are enriched among transcripts that undergo alternative splicing upon ADAR knockdown. This observation suggests a mechanism where the alternative splicing of a trans-acting factor due to RNA editing affects multiple downstream alternative splicing events [66]. It remains to be seen whether other nucleotide modifications play such significant roles in mediating alternative splicing either in gene-specific instances or on a global scale as demonstrated for m6A and ADAR-mediated A to I editing.

## 8. Combinatorial exon recognition and alternative splicing predictors

Alternative splicing is an extremely complex process that usually cannot be explained by the influence of any single mechanism or class of splicing regulators. While the presence of splicing regulatory elements is a strong determinant in the final outcome of spliced products, other factors such as intron-exon architecture, splice site strength, RNA secondary structure, RNA modification and transcription significantly influence the final splice pattern and, in many specific examples, are the primary drivers of alternative splicing. In the face of the remarkable complexity of splicing regulation, the chances of predicting splicing outcomes in different cellular or experimental contexts appear to be low. However, recent computational approaches have demonstrated that the type and direction of alternative splicing can be anticipated. These splicing code algorithms rely on defining hundreds of RNA sequence features that are tested alone or in combination using machine learning approaches to define sets of RNA elements that guide predictive success. One approach intended to determine whether a particular exon is prone to undergo one or multiple types of alternative splicing. The results suggest that exons have an innate ability to undergo alternative splicing that is primarily due to the strength of present and competing splice sites and the intron-exon architecture [18]. The presence of cis-acting sequence elements that often serve as binding sites for splicing regulators such as SR or hnRNP proteins are responsible for most tissue- and species-specific alternative splicing patterns [67–69]. Presumably, variable expression of trans-acting splicing factors in different cell types or at different external conditions mediates differential recognition of exons, thus generating alternative mRNA isoforms. Interestingly, the predictive success of many splicing code approaches hinges on a feature that defines the conservation of nucleotide sequences surrounding the splice junctions. The increased level of conservation strongly suggests that additional cis-

acting elements are positioned within the area of analyzed conservation. However, the identity and mechanism of action for these hidden RNA elements have yet to be determined. In summary, the splicing code results suggest that type of alternative splicing is controlled primarily by the core splicing signals and intron-exon architecture, but the magnitude of alternative splicing is modulated by trans-acting splicing regulatory proteins in a tissue and species dependent manner [18,67–70].

## 9. Perspectives and novel technologies

Alternative pre-mRNA splicing is an extremely complicated process that historically has been mostly studied at the single transcript level. While experiments that study the mechanisms of splicing for a particular transcript have been highly informative, they are often performed using *in vitro* approaches, such as the nuclear extract splicing system, and are limited in the number of transcripts investigated. Recent advances have led to enhanced crosslinking and immunoprecipitation (eCLIP) methodologies, which allow RBP binding site discovery at single nucleotide resolution [71]. eCLIP will allow scientists to create RBP binding maps that show where RBPs bind specific transcripts across the entire transcriptome. In combination with knock-down RNA-seq approaches, this technology will allow scientists to discover where RBPs bind and what effect they have on splicing [47,72]. A wonderful resource to create such binding and activity maps is the RNA binding protein interaction and function arm of the ENCODE project (<https://www.encodeproject.org>), an open source undertaking, which offers complete RNA-seq and eCLIP datasets for hundreds RBPs.

There has also been a renaissance in RNA structure probing technologies [73]. Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) allows for the interrogation of RNA flexibility and dynamics at every residue [74,75]. SHAPE reagents are amenable to use inside cells and can be used to interrogate transcriptome structure [76–78]. Dimethyl sulfate, which reacts with the Watson-Crick side adenosine and cytosine and the unpaired face of guanosine residues, has also been used to probe RNA base-pairing interactions *in vivo* [79,80]. Lastly, nicotinoyl azide (NAz) is a reagent that can be used to probe the solvent accessibility of guanosine and adenosine residues inside cells in order to “footprint” RNA-protein binding interactions [81]. All of these reagents have been combined with next generation sequencing and will enable scientists to study changes in pre-mRNA flexibility, base-pairing and solvent accessibility *in vitro* and *in vivo* at the single transcript level or at a transcriptome-wide level [80,82–86]. These tools will help elucidate and validate additional structural mechanisms that govern pre-mRNA splicing *in vivo*. For example, *in vivo* click selective 2'-hydroxyl acylation analyzed by primer extension (icSHAPE) was used to compare the *in vivo* profile vs the *in vitro* profile of RBfox2 binding motifs as determined by iCLIP [77]. The unique icSHAPE profiles of RBfox2 binding sites were deemed indicative of RBP binding and used to further increase the accuracy of machine learning algorithms. icSHAPE profiles were also utilized for *de novo* prediction of HuR binding sites with a high rate of accuracy [77]. Given the technical improvements in monitoring RNA structures *in vivo* it is likely that RNA secondary structure will emerge as a more significant contributor to alternative splicing, perhaps even explaining some of the as of yet uncharacterized splicing code contributions that are currently categorized by high levels of sequence conservation.

The tremendous improvement in resolution obtained by cryo-electron microscopy has also led to a revolution in structural biology and the field of RNA splicing [87]. High resolution structures of the human spliceosomal B and C complex have been solved at previously unimaginable resolutions [88,89]. The inevitable solving of human spliceosomal complex E and A will further expand our understanding of the mechanisms of splice site selection and pairing. Furthermore, high resolution structures of proteins that have historically been difficult to solve, such as SR proteins (which have eluded scientists for many years)

may now become solvable when in complex with the splicing machinery. It is certain that with the recent technological advances the next decade will be an exciting time in the alternative splicing field.

## Transparency document

The Transparency document associated with this article can be found, in online version.

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