Context-dependent control of alternative splicing by RNA-binding proteins

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Abstract | Sequence-specific RNA-binding proteins (RBPs) bind to pre-mRNA to control alternative splicing, but it is not yet possible to read the 'splicing code' that dictates splicing regulation on the basis of genome sequence. Each alternative splicing event is controlled by multiple RBPs, the combined action of which creates a distribution of alternatively spliced products in a given cell type. As each cell type expresses a distinct array of RBPs, the interpretation of regulatory information on a given RNA target is exceedingly dependent on the cell type. RBPs also control each other's functions at many levels, including by mutual modulation of their binding activities on specific regulatory RNA elements. In this Review, we describe some of the emerging rules that govern the highly context-dependent and combinatorial nature of alternative splicing regulation.

Spliceosome
A macromolecular RNA–protein complex that is responsible for intron removal and that consists of U1, U2, U4, U5 and U6 small nuclear ribonucleoproteins (snRNPs) and many auxiliary protein factors.

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doi:10.1038/nrg3778
Published online 12 August 2014

The split arrangement of protein-coding information in DNA and its reconstitution by RNA splicing after transcription remain one of the most intriguing features of eukaryotic genome structure and evolution. The seemingly inefficient strategy of keeping introns in a gene only to transcribe and remove them using a large, complex macromolecular machine (that is, the spliceosome) must have provided evolutionary advantages[1,2], particularly during the rise of metazoan organisms. Such advantages may have included protection from mutation and transposons, expanded and flexible genomic coding capacity, protein diversity and opportunities for regulation[3]. Large-scale surveys of alternative splicing reveal its pervasive impact on the transcriptomes of *Drosophila melanogaster*[4] and other metazoans[5,6]. In present-day mammalian genomes, RNA splicing has evolved to serve complex regulatory functions that are coupled with other gene expression steps[7–9].

The splicing reactions are carried out in the spliceosome — a dynamic RNA–protein complex with a highly regulated functional cycle found in all eukaryotes[10]. This sophisticated RNA–protein enzyme has remarkable plasticity in substrate recognition and can incorporate different parts of a pre-mRNA into a mature mRNA under the influence of a large number of regulatory proteins, many of which have the ability to directly bind to the pre-mRNA. Through alternative splicing, pre-mRNA transcripts can be processed to produce mRNA isoforms with different stability or coding potential, thus allowing quantitative control of protein production and synthesis of related proteins with qualitatively distinct functions[11].

The large number of splicing regulatory proteins that recognize distinct RNA sequences across the transcriptome suggests the existence of a 'splicing code' (REF. 12), which could be used to predict splicing responses in different cell types under different experimental conditions. However, the depths of the complexity of this code have yet to be understood and measured. Deciphering the splicing code will require a comprehensive list of splicing regulatory RNA-binding proteins (RBPs) and their *cis*-acting binding sites. A major challenge is that the same genomic sequences are recognized differently by a given RBP in different cell types, partly depending on which other RBPs are expressed in that cell type (see below). Many proteins may function in complex with other proteins, thus influencing the binding specificity of one another. Complicating the problem, the same short degenerate RNA sequence motif can be recognized by multiple different proteins or protein isoforms. Furthermore, there is no simple division of positive and negative splicing regulators, as this behaviour frequently depends on the location of the binding site relative to the regulated exon. Attempts to predict splicing regulation from sequence features and tissue splicing data through computer learning approaches[13,14] have so far been

limited by the fact that tissues are composed of multiple cell types with superimposed splicing responses, as well as by the inability to assign a unique protein to a unique RNA sequence motif.

As a complete understanding of alternative splicing and its regulation will be necessary for comprehending its biological impact on developmental and disease processes[15–18], our goal in this Review is to outline the emerging regulatory rules with attention to their limitations. Future experimentation will be necessary both for understanding the mechanisms of splicing regulation and for identifying the genomic location at which each regulator functions in different cell types.

Defining the regulatory landscape

When considering splicing regulation, it is helpful to distinguish between two classes of splicing events: first, constitutive splicing events that are recognized efficiently by the spliceosome and that are spliced the same way in each pre-mRNA from a given gene; second, alternative splicing events, in which recognition and joining of a 5′ and 3′ splice site pair are in competition with at least one other 5′ or 3′ splice site (FIG. 1a). Accordingly, *cis*-acting RNA elements involved in splicing reaction and regulation may also be divided into two classes. The first comprises the pre-mRNA reactive sites known as splicing signals, including the 5′ splice site, the branchpoint and the 3′ splice site, each of which is strictly required by the spliceosome for substrate recognition and catalysis[10], whereas the second class comprises other RNA elements — collectively known as splicing regulatory elements (SREs) — which are often target sites for *trans*-acting factors.

The context and positional relationship of these signals are paramount to their function. For example, a weak 3′ splice site is more efficiently recognized when there is a strong functional 5′ splice site within 100–150 nucleotides downstream through a phenomenon called exon definition[19], whereby splice sites influence each other's recognition by the U1 small nuclear ribonucleoprotein (snRNP) at the 5′ splice site and the U2 snRNP at the 3′ splice site[20] (FIG. 1b). Exon definition for genes with complex architecture is followed by splice site pairing, which is another key regulatory step in alternative splicing (FIG. 1c). Importantly, the accessibility of any sequence-based RNA signal will depend on the folding landscape of the sequence environment in which it is embedded[21]. This has been exemplified by long-distance RNA–RNA interactions that facilitate pairing of a common exon to different alternative exons[22]. Recent fine-mapping of RNA secondary structure in yeast, plant and mammalian genomes[23–25] provides rich information for associating local RNA structure with function of specific RBP-binding sites in an effort to refine the prediction of functional sites from genomic context.

In principle, regulatory elements may act from any location in a pre-mRNA, but most studies focus on 200–300 nucleotides adjacent to observed splice sites, which seem to have the most identifiable sequence features[26]. However, a recent study showed that remote regulatory sequences are just as important as those proximal to a functional splice site[27], which suggests a major limitation associated with current experimental and computational approaches in defining functional RNA elements in mammalian genomes. In fact, many long introns in mammals may carry more regulatory information than we currently appreciate. It is now more feasible to dissect these distant regulatory regions by genome engineering using CRISPR–Cas (clustered regularly interspaced short palindromic repeat–CRISPR-associated) technology[28,29]. Mutations of SREs in the proximal intron are known to contribute to many human diseases, which indicates that more such mutations might be found in distal regions. This highlights a major limitation of exome sequencing efforts, which will not detect crucial disease-associated mutations that may be found by complete sequencing of patient genomes. Below, we focus on the class of SREs that act by capturing specific RBPs to achieve splicing regulation.

Dissecting SREs on model genes. Two approaches have traditionally been used to identify and dissect *cis*-acting SREs, which can be divided into exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs) and intronic splicing silencers (ISSs) (FIG. 1d). As extensively reviewed previously[30], serial deletion constructs can be analysed to expose positive and negative SREs or to characterize putative SREs in a heterozygous exon. Mutational analyses of model alternative splicing substrates can be informative for single cases but suffer from limited generalizability owing to undefined contributions of sequence context. Furthermore, it is often unclear how results from minigene constructs relate to sequence elements and functions in the endogenous gene in its natural genomic context.

The second approach is *in vitro* selection of RNAs that bind to a splicing regulatory protein of interest to identify sequences that resemble those found in natural pre-mRNAs. These studies often use stringent *in vitro* binding conditions that result in overspecification of a sequence that does not represent the more diverse set of natural sites that function *in vivo*. A new method that selects sequences with high rates of RNA binding in a kinetic capture experiment has revealed sequence specificity that is not found using thermodynamic end-point binding studies[31]. Kinetic sequence preferences are beyond the reach of widely used methods, and yet many of these have important regulatory roles. Even so, the general limitation with single model reporters is that most regulatory contexts arise from multiple overlapping sets of RNA elements and that one reporter only samples the activity of the regulator under study in only one such context. More inclusive global approaches that are now widely used in the field (see below) have the potential to offset and deconvolute some of these limitations in defining the regulatory landscape in a genome.

In silico capture of regulatory RNA elements. Computational approaches have been used to filter out some regulatory sequence features. One study attempted

Alternative splicing
Differential inclusion of exons in the final processed RNA product by splicing of a precursor RNA segment.

Transcriptomes
Complete sets of RNA transcripts in a cell.

mRNA isoforms
Different mRNAs produced from the same precursor mRNA.

Cis-acting RNA elements
RNA sequences in precursor mRNA that are important for both constitutive and regulated splicing.

Splicing signals
Essential sequences in the pre-mRNA for recognition by the core splicing machinery.

Branchpoint
A sequence as part of the 3′ splice site that is recognized by the spliceosome and that reacts with the 5′ splice site in the first step of the splicing reaction to form the lariat.

Splicing regulatory elements
(SREs). RNA motifs in precursor RNA that have regulatory roles in splice site selection.

Trans-acting factors
Proteins that interact with *cis*-acting regulatory RNA elements.

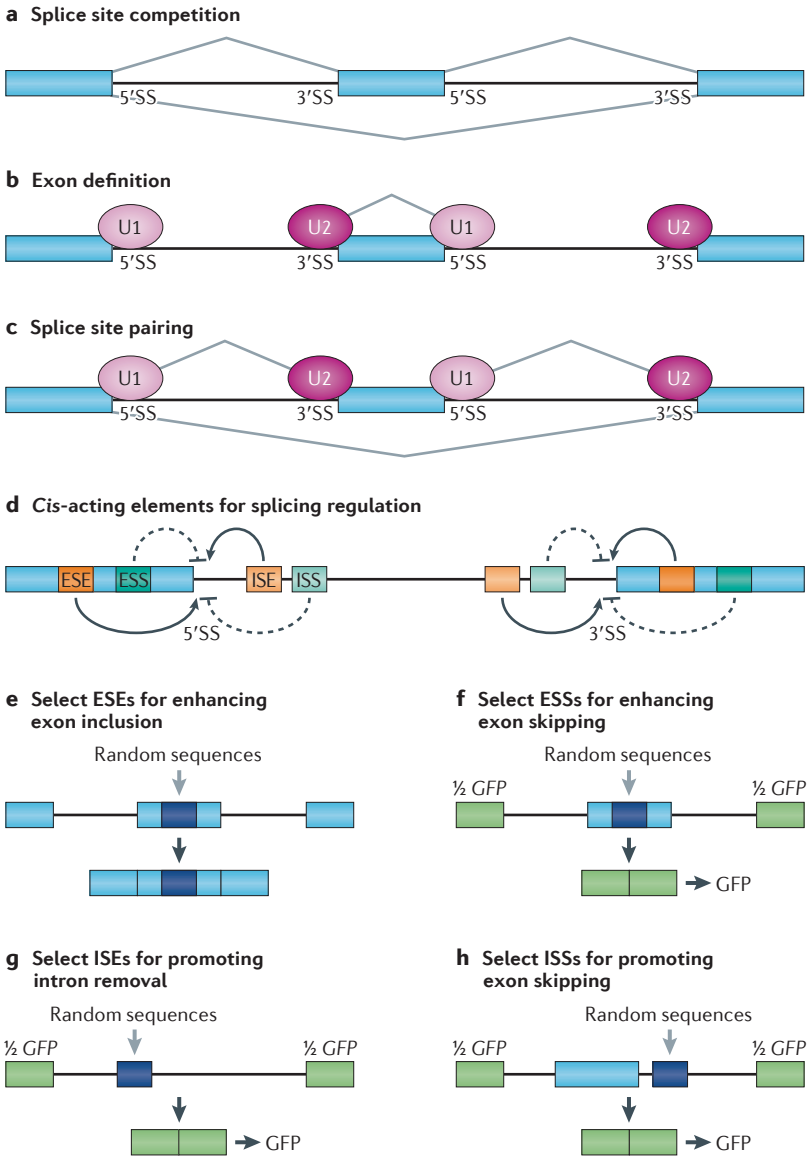

Figure 1 | **Regulation of alternative splicing by *cis*-acting enhancers and silencers.** **a** | A pre-mRNA substrate has two 5′ splice sites (5′SSs) and two 3′SSs that are engaged in competition with each other for assembly of splicing complexes, which causes the alternative exon in the middle to be either included or skipped in the final mRNA products. In a given cell type, the ratio of included and skipped isoforms varies, and such variation is regulated by a large array of splicing regulators. **b** | Most exons in mammals, including both constitutive and alternative ones, are recognized by U1 small nuclear ribonucleoprotein (snRNP) binding at the 5′SS and U2 snRNP binding at the 3′SS that enhance one another. This exon definition process determines the selection of functional splice sites in the genome, which is subjected to modulation by various RNA-binding splicing regulators. **c** | Upon initial splice site selection, functional splice sites are paired to allow subsequent steps of spliceosome assembly to occur. Such a pairing process can also be regulated to generate alternatively spliced mRNA isoforms. **d** | *Cis*-acting RNA elements that positively or negatively influence splice site selection are shown. Depending on their locations and functions, they are referred to as exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs) and intronic splicing silencers (ISSs). **e** | A selection of ESEs from a random sequence library are inserted into an alternative exon in a splicing reporter. In this setting, the middle exon is weakly included. From a random pool of sequences that have been individually inserted in the alternative exon, specific ESEs can be isolated, amplified and reselected from the mRNA product pool generated by *in vitro* splicing or from transfected cells that contain the alternative exon. **f–h** | Selection of ESSs (part **f**), ISEs (part **g**) and ISSs (part **h**) are shown. In each case, a sequence from a random sequence library is inserted into different locations in a splicing reporter that splits the *GFP* gene (green) into two halves. Upon transfection into an experimental cell line, GFP-positive cells are selected, and the pool of inserted sequences are then amplified by PCR and sequenced to identify sequence motifs that can modulate splicing.

to identify sequence motifs representing elements that promote exon inclusion (that is, ESEs) by postulating that they would be needed to compensate for exons with weak matches to the consensus sequences at the 5′ and 3′ splice sites. A set of motifs were enriched in exons with weak splice sites[32], some of which were further linked to specific features in the splice sites[33]. Related studies compared internal non-coding exons to pseudo-exons and 5′ untranslated regions (UTRs) of intronless genes[34,35]; alternatively, sequence conservation at wobble positions can be taken into consideration to minimize the confounding effect of protein-coding information[36]. Except in cases of alternative 5′ or 3′ splice site usage in which an intronic regulatory sequence may overlap with an extension of an exon, the superposition of splicing regulatory information and protein-coding information does not interfere with identification of ISEs and ISSs.

Motif collections deduced by different computational methods are not identical, which suggests that not all features associated with regulatory RNA sequences are represented by any one approach. In addition, many computationally identified sequences cannot be easily matched to a known RBP. The same motifs are also sometimes found to function as either an enhancer or a silencer in different locations in the same exon[36]. The poor reliability of any single prediction, together with the challenge in experimentally connecting a motif to a particular regulator, limits the usefulness of computational approaches for dissecting biological functions.

Reporter-based sequence library screens. Diverse sequence populations can be screened for function as SREs using reporters that have been engineered to accept randomized oligonucleotides at positions within or near an alternative exon. For example, to identify ESEs, each sequence from a library can be inserted in the internal alternative exon of a three-exon splicing reporter to

select specific sequences that can enhance the inclusion of the alternative exon in an *in vitro* splicing reaction[37] or in transfected cells[38] (FIG. 1e). Common motifs can then be deduced from these sequences and further characterized as ESEs, which are largely purine-rich, consistent with their functions as SR protein-binding sites. However, there are other non-purine-rich sequences, which suggests the existence of other types of ESEs. Again, these results are constrained by the context of both the chosen reporter and the cell type in which the selection is carried out, as well as by the uncertainty in pinpointing the specific *trans*-acting regulatory RBPs involved.

More elaborate strategies have used GFP-based vectors in transfected HEK293T cells, in which a short randomized sequence from a library was inserted into an efficiently included internal exon that prevents *GFP* expression, and sorting of GFP-positive cells allowed recovery of ESSs[39] (FIG. 1f). More recently, this approach was adapted to screen for ISEs or ISSs by inserting a random sequence from a library into a weak intron or a location downstream of an internal exon that splits the *GFP* gene[40,41] (FIG. 1g,h). These approaches yielded a large collection of sequences that function as putative SREs, many of which resemble binding sites for some known splicing regulators. A major caveat to these approaches is that the motifs might function only in the specific contexts (reporter gene and cell type) in which the selection was carried out. This was underscored in a recent screen in which library sequences were inserted into two different positions in a three-exon reporter with different outcomes[42]. Thus, although these screens provide valuable information on SREs, they offer little insight into the problem of context.

With each new SRE sequence there is the question of which RBP binds to it. However, affinity purification of RBPs using SREs shows that a one-to-one relationship between an SRE and an RBP is not always the case[40,41]. As association under native conditions used in these studies does not distinguish between direct and indirect interactions, some of the identified proteins may interact indirectly with RNA through protein–protein interactions. In any case, these possibilities create ample mechanistic scenarios to positively or negatively modulate splice site selection in the cell.

High-throughput approaches. It is crucial to answer the following questions: how many proteins encoded in mammalian genomes have the capacity to bind to RNA, and how many of them are involved in splicing control? Recent proteomic studies indicate that up to 1,000 proteins can be directly crosslinked to mRNA in HeLa cells or mouse embryonic stem cells[43,44]. Interestingly, many of these proteins do not contain known RNA-binding domains that are similar to those in the known repertoire of RBPs[45]. As the first step to characterize their roles in splicing regulation or RNA metabolism in general, we need to know the RNA-binding site sequences for known RBPs. A high-throughput methodology was recently applied to this question[46]. In this study, individual purified RBPs (although in most cases only known RNA-binding domains were expressed instead of whole proteins) were incubated with a pool of RNA oligonucleotides. After affinity selection, enriched sequences were identified by microarray, and the consensus motifs were deduced. This information enables inference of potentially functional RNA elements in the genome and lends itself to experimental tests. This approach can be coupled with system-wide RNA interference (RNAi) and splicing profiling to deduce a full complement of splicing regulators in a genome[47,48].

As RBPs exert their function partly by binding to pre-mRNA, the knowledge of where and when they bind could help to define the context in which splicing regulation takes place. Transcriptome-wide identification of binding sites can be sampled for a given RBP using the crosslinking and immunoprecipitation (CLIP) method, which detects direct RNA–protein interactions because ultraviolet (UV) radiation-induced crosslinking requires the RNA and the protein to be within ~1 Å of each other[49,50]. After UV crosslinking in live cells or extracts, immunoprecipitation and denaturing gel electrophoresis can be used to purify covalently linked protein–RNA adducts. RNA is then recovered, sequenced and mapped back to the genome. Since the initial development of the CLIP technology, several refinements have been made to increase the crosslinking efficiency (such as photoactivatable ribonucleoside-enhanced CLIP (PAR-CLIP))[51] or the mapping precision (such as individual-nucleotide-resolution CLIP (iCLIP))[52]. Although each method has intrinsic caveats, such as variable crosslinking efficiencies and recovery biases at different RNA sites, the CLIP approach greatly enables qualitative identification of specific binding sites and deduction of consensus binding sequences, and it also allows specific binding events (or patterns) to be linked to function (TABLE 1). This approach is particularly powerful if the derived information is cross-analysed with high-throughput analyses of binding specificity by individual purified RBPs[46,53]. Owing to its power to identify *cis*-acting regulatory RNA elements in mammalian genomes, multiple regulatory principles have emerged from the use of the CLIP technology.

Trans-*acting splicing regulators.* Our understanding of splicing regulation has benefitted tremendously from biochemical studies on *in vitro* splicing systems. Classic *trans*-acting splicing regulators identified are SR proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs)[54]. The SR protein family can be further divided into core members and RS domain-containing polypeptides. SR proteins are widely viewed as positive splicing regulators that promote exon inclusion both by binding to exons and by recruiting U1 snRNP to the 5′ splice site and U2 auxiliary factor (U2AF) to the 3′ splice site through protein–protein interactions in early steps of spliceosome assembly[55–57] (FIG. 2a,b). In general, SR proteins have a binding preference for purine-rich exonic sequences, and this observation is now largely confirmed by global mapping of protein–RNA interactions[58–61].

Table 1 | **Genomic landscape of splicing regulators as revealed by CLIP experiments**

Splicing factors	Binding profile and functions	Motifs*	Refs
Splicing regulators			
CUGBP1	Binds to intronic regions and antagonizes MBNL1	UGUU	166
MBNL1 and MBNL2	Bind to intronic regions with positional effects	YGCY	83,167
NOVA	Binds to both exons and introns with positional effects	YCAY	79
PTB and nPTB	Bind to intronic regions to mediate splicing repression	CU-rich	68,123
RBFOX1 and RBFOX2	Bind to intronic regions with positional effects	GCAUG	82,168
TIA-1 and TIAL1	Bind to intronic regions with positional effects	U-rich	169
QKI	Binds to intronic regions with positional effects	UAA	51
ESRPs	Bind to intronic regions with positional effects	GU-rich	87
RBM4	Binds to intronic regions with positional effects	CGG or CTAACG	40,170
RBM5	Binds to intronic regions with positional effects	UCAUC or UGUAA	118
RBM6 and RBM10	Bind to intronic regions with positional effects	CUCUGAA	118
ELAV1	Binds to intronic and 3′ untranslated regions	U-rich or AU-rich	171–173
3′splice site factors			
U2AF65	Defines functional 3′ splice sites in most introns	Pyrimidine-rich	139
SF1	Binds to branchpoint sequences and other intronic regions	ACUNAC	174
SR proteins			
SRSF1	Enhances splicing on ESEs in a context-sensitive manner	GGAGGA	58,61
SRSF2	Enhances splicing on ESEs in a context-sensitive manner	SSNG	59
SRSF3	Has a slight preference for exonic over intronic regions	Pyrimidine-rich	58
SRSF4	Has a slight preference for exonic over Intronic regions	Purine-rich	58
hnRNPs			
hnRNP A1	Binds to intronic regions to repress splicing on ESSs	GNNAGN	78
hnRNP A2 and hnRNP B1	Bind to intronic regions to repress splicing on ESSs	GGUAGU	78
hnRNP C	Binds to intronic regions and competes with U2AF65 at Alu sequences	Pyrimidine-rich	52
hnRNP F	Binds to intronic regions to enhance or repress splicing	GU-rich	78
hnRNP H1	Binds to intronic regions to enhance or repress splicing	G-run	78
hnRNP L	Binds to intronic regions to enhance or repress splicing	CA-rich	175,176
hnRNP M	Binds to intronic regions to enhance or repress splicing	GGUGG	78
hnRNP U	Binds to intronic regions to regulate U2 snRNP maturation	GU-rich	78,92

CLIP, crosslinking and immunoprecipitation; CUGBP1; CUG-binding protein 1; ELAV1, Elav-like protein 1 (also known as HuR); ESE, exonic splicing enhancer; ESRP, epithelial splicing regulatory protein; ESS; exonic splicing silencer; hnRNP, heterogeneous nuclear ribonucleoprotein; MBNL1, muscleblind-like protein 1; nPTB, neural polypyrimidine tract-binding protein (PTB); QKI, protein quaking; RBFOX1, RNA-binding protein fox-1 homologue 1; RBM4, RNA-binding protein 4; SF1, splicing factor 1; snRNP, small nuclear ribonucleoprotein; SRSF1, serine/arginine-rich splicing factor 1; TIA-1, nucleolysin TIA-1 isoform p40; TIAL1, nucleolysin TIAR; U2AF65, U2 auxiliary factor 65 kDa subunit. *S represents C or G; R represents G or A; Y represents C or U; and N is any nucleotide.

The best-characterized hnRNPs involved in splicing control are negative regulators hnRNP A/B and polypyrimidine tract-binding protein (PTB; also known as hnRNP I). hnRNP A/B seems to antagonize the function of SR proteins[62,63]. The mechanism by which this is accomplished is unclear, as high-affinity hnRNP A/B-binding sites do not often overlap with SR protein-binding sites on exons. A potential mechanism involves cooperative binding of hnRNP oligomers that spreads along a transcript to prevent the binding of SR proteins over an extended region of RNA[64,65]. PTB has a binding preference for polypyrimidine sequences[66], mostly in intronic regions, and this finding has also been confirmed by genome-wide mapping[67,68]. The binding specificity of PTB is similar but not identical to that of U2AF65, which promotes U2 snRNP binding to the intron, suggesting that PTB may interfere with functional recognition of the 3′ splice site at least for certain genes. PTB prevents onward spliceosome assembly after initial exon definition[69,70] (FIG. 2c), and a more recent study indicates that PTB might additionally bind to U1 snRNA to cause an extended interaction between U1 and the 5′ splice site[71]. Extended U1–5′ splice site interaction has also been attributed to the splicing repressor activities of hnRNP L and hnRNP A1 (REF. 72). Although this interaction is subtle and non-rate limiting in most cases, under other conditions this extended base-pairing may be kinetically manifested to shift net splice site choice markedly[73] (FIG. 2d).

These findings underscore the fact that there is no substitute for biochemistry to understand the mechanism of splicing regulation and that much remains to be done to illuminate the mechanisms of action of SRE-recognizing RBPs. A wide range of functional screens and differential expression profiling strategies have successfully identified tissue-specific *trans*-acting splicing regulators and have demonstrated the roles of epithelial splicing regulatory proteins (ESRPs)[74], hnRNP LL in the control of T cell signalling[75,76] and nSR100 in the development of the nervous system[77].

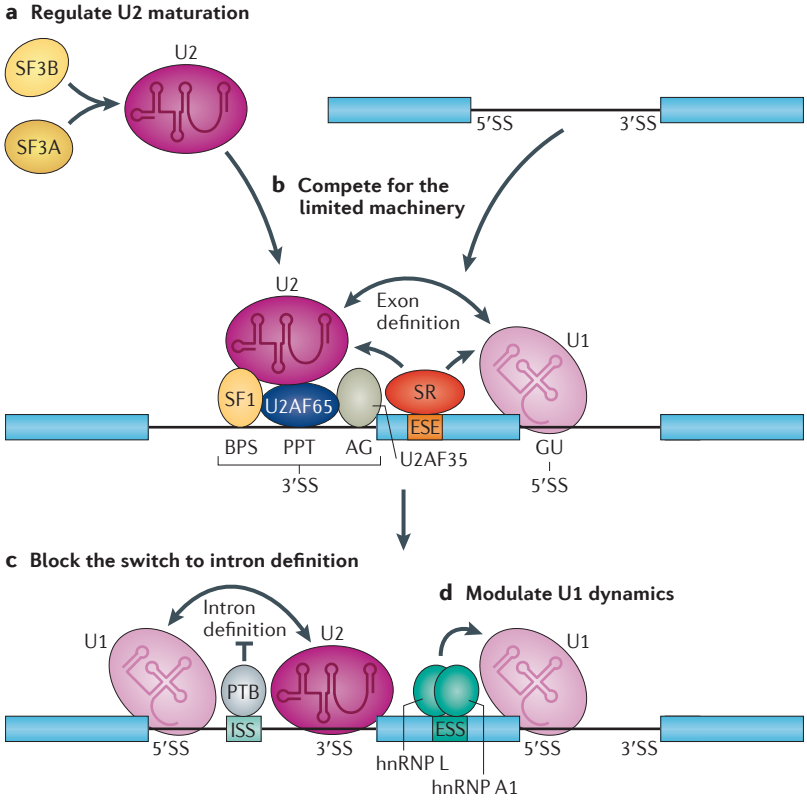

Figure 2 | **Regulated splicing through controlling the assembly of the core splicing machinery.** All splicing regulators must exert their activities, either directly or indirectly, through the core splicing machinery. **a** | Modulation of small nuclear ribonucleoprotein (snRNP) assembly can change the pool of active splicing components in the cell, thereby influencing alternative splice sites that are particularly sensitive to the levels of spliceosome components. **b** | The available functional splice sites in different pre-mRNAs — including the branchpoint sequence (BPS), polypyrimidine tract (PPT) and AG sequence in the 3′ splice site (3′SS), and the GU sequence in the 5′SS — are all competing for the splicing machinery. As a result, strong splice sites may titrate core components of the splicing machinery away from weak splice sites, thus indirectly influencing global splicing patterns in the cell. **c** | After initial recognition of splicing signals, the functional sites have to be efficiently paired for next steps of spliceosome assembly to take place, which also represents key steps for regulation. The binding of polypyrimidine-tract binding protein (PTB) to an intronic splicing silencer (ISS) inhibits intron definition. **d** | The U1 snRNP is about tenfold higher in abundance than other splicing snRNPs in mammalian cells. This may allow efficient recognition of most 5′SSs in both constitutive and alternative exons, as well as protection of inappropriate polyadenylation sites. However, the U1 snRNP has to be released later in the spliceosome cycle, which is essential for the formation of the catalytic core in the spliceosome. Interference of U1 release by heterogeneous nuclear ribonucleoprotein L (hnRNP L) and hnRNP A1 can thus also inhibit the selection of a regulated splice site, which acts as a repressive complex. ESE, exonic splicing enhancer; ESS, exonic splicing silencer; SF3A, splicing factor 3A; U2AF65, U2 auxiliary factor 65 kDa subunit.

Although the simple 'division of labour' between SR proteins as positive splicing factors and hnRNPs as negative ones is generally useful, it does not hold true in every case. Splicing profiling experiments have now shown roughly equal numbers of induced exon inclusion and skipping events in cells treated with small interfering RNA (siRNA) against SR proteins[59] or hnRNPs[67,68,78]. This is also true for several well-studied tissue-specific splicing regulators, such as NOVA[79,80], RBFOX[81,82], muscleblind-like proteins (MBNLs)[83,84], protein quaking (QKI)[85] and ESRPs[86,87]. Although some of the documented events may be indirectly affected by depletion of a splicing factor, biochemical characterization, motif enrichment analyses and CLIP all reveal binding sites that are indicative of direct action. Despite these advances, it is clear that we are still far from deciphering all the regulatory rules and mechanisms for both known and new splicing regulators.

Binding and context: rules

Numerous splicing factors have now been characterized, and they seem to share sets of behaviours. Below, we summarize these behaviours in the form of rules. The general message is that all regulatory activities of RBPs are coordinated with the function of the core splicing machinery to first define functional splice sites and then to modulate selective pairing of differentially defined splice sites.

Core machinery threshold for splice site recognition. A common feature of alternative splice sites is their divergence from the consensus derived from 'strong' constitutive splice sites, which has been considered to render them 'weaker' than typical constitutive splice sites. Consistent with an important regulatory role preserved during evolution, weak alternative splice sites tend to be more conserved than constitutive splice sites[88]. This suggests that the sequence context sets idiosyncratic constraints for the core splicing machinery to recognize alternative splice sites, which would then be influenced by regulatory factors. This implies that the level or functionality of the core splicing machinery also contributes to splicing control (FIG. 3a), and this possibility may explain why mutations in the core splicing machinery lead to highly specific phenotypes and diseases. Accordingly, depletion of some core components of the spliceosome in RNAi-treated cells shows selective effects on certain alternative splicing events[48,89], which could reflect the fact that some RBPs interact with specific core components of the spliceosome to achieve regulation. For example, during 5′ splice site selection, the RBP nucleolysin TIA-1 isoform p40 (TIA-1) functions through the U1 snRNP protein U1-C[90], and the RBP P-element somatic inhibitor (PSI) exerts its effect through U1-70K[91]. In cassette exons where the proximal splice sites are regulated, this rule predicts that a compromised core splicing machinery would induce exon skipping to a large extent, which seems to be the case in a study that broadly examined the splicing responses upon reducing the level of a specific U2 snRNP component[92].

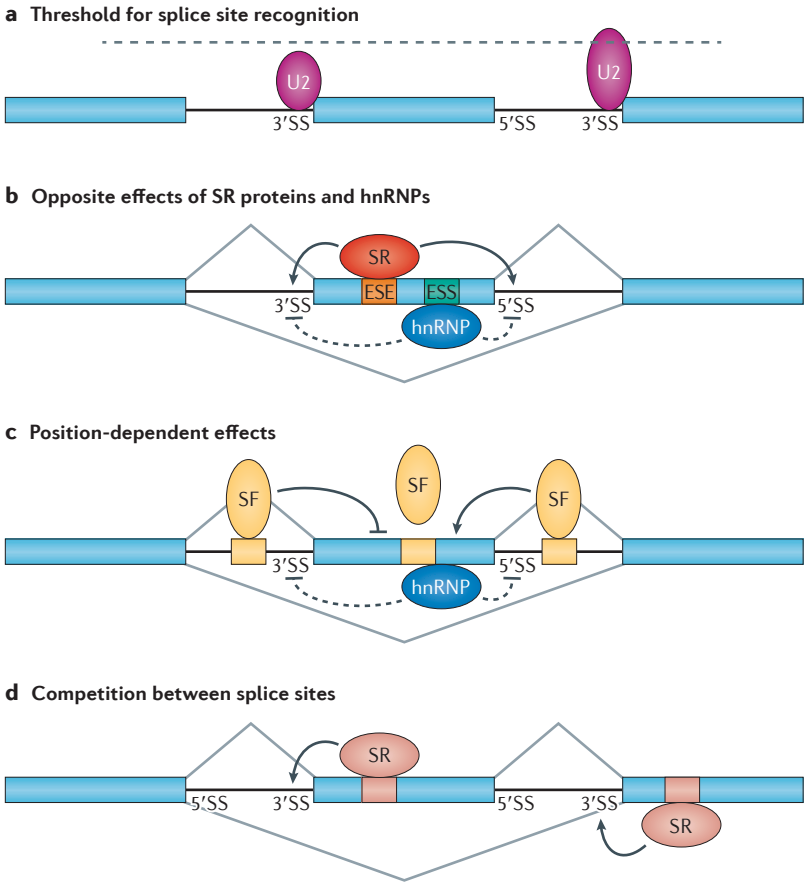

Figure 3 | **Rules for context-dependent and position-sensitive regulation of alternative splicing.** **a** | In general, the alternative exon (in the middle) is associated with weak splice site signals, which render partial selection by core components of the splicing machinery. A small U2 small nuclear ribonucleoprotein (snRNP) is illustrated to show its inefficient interaction with the alternative splice site. The flanking sites, which are strongly recognized by the splicing machinery, are engaging in competition with the alternative splice sites. A large U2 snRNP is illustrated to show its strong interaction with the flanking 3′ splice site (3′SS). According to the threshold rule, a partially compromised splicing machinery would selectively affect the alternative splice sites relative to the competing sites, thus leading to induced exon skipping. **b** | Exon-bound SR proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs) have opposite effects on the recognition of the adjacent splice sites by the core splicing machinery. **c** | For many sequence-specific splicing factors (SFs) — for example, NOVA — their binding on exons generally inhibits the selection of the alternative exon. They are inhibitory when they are bound to an upstream intronic region but enhance the selection of the alternative exon when bound to a downstream intronic region. The mechanism for the polarity effect remains to be fully understood. **d** | Enhanced recognition of the alternative splice site induces exon inclusion, whereas increased recognition of the flanking competing splice site causes exon skipping. ESE, exonic splicing enhancer; ESS, exonic splicing silencer.

The differential response of splicing substrates to depletion of core components of the splicing machinery suggests that competition between pre-mRNAs for the available splicing machinery may influence splicing across the whole transcriptome. Consistent with this, extensive changes in pre-mRNA pools in budding yeast that reduce demand for the splicing machinery increase splicing efficiency for poorly spliced transcripts[93]. The phenomenon whereby changes in the activity of core splicing components alter the entire balance between competing splicing events may underlie numerous specific diseases, such as retinitis pigmentosa[94], spinal muscular atrophy[95] and myelodysplasia[96].

Opposite effects from exon versus intron binding. In general, hnRNPs inhibit splicing when binding to exons but can have either positive or negative effects on splicing when bound to intronic sites. This is best illustrated with hnRNP L and hnRNP H/F. On several model substrates, hnRNP L represses or enhances splicing by binding to CA-rich motifs in exons[97,98] or introns[99,100], respectively. hnRNP H binds to intronic G-rich elements to promote the selection of nearby 5′ splice sites[101,102] but represses splicing when bound to exons[103–105]. In many cases, hnRNP H seems to function together with the related hnRNP F[105–108].

This rule has been generalized by tethering several well-studied SR proteins and hnRNPs to either side of a regulated 5′ splice site in a three-exon reporter[109] (FIG. 3b). An SR protein tethered to an intronic region upstream of the functional 5′ splice site interferes with exon definition, which mimics the activity of natural decoys or pseudoexons in regulated splicing[14,110,111]. By contrast, an hnRNP tethered to an exon represses productive splicing in a similar manner to PTB[69,70], and the mechanism involved formation of non-productive complexes that prevent subsequent splice site pairing[109]. However, there are exceptions to this general rule because positioning of an SR protein-binding site in two different regions in an alternative exon elicited opposite splicing responses[36]; similarly, the inhibitory effect of exon-bound hnRNP L could be switched to a positive one when an adjacent sequence was mutated[112]. These observations suggest that the positional effect is also subject to modulation by sequence context that has yet to be defined.

Intron binding position and polarity. The positional effect of splicing regulators in different intronic locations was first illustrated with NOVA[79]. In this study, NOVA activated exon inclusion when binding downstream of the alternative exon but repressed its inclusion when binding upstream of the exon. Subsequently, these polarity effects were observed with multiple splicing regulators based on CLIP–seq maps and motif enrichment analyses that link their binding events to functional responses (FIG. 3c; TABLE 1).

The molecular mechanism for this phenomenon remains to be fully understood, and this represents a key area for future experimentation. A recent study using tethered RBFOX1 showed that the carboxy-terminal region of this protein was sufficient to promote exon inclusion when tethered downstream of the alternative exon, but both its RNA-binding domain and the carboxy-terminal region seemed to be essential for repression when tethered upstream of the alternative exon, which indicated distinct requirements for the activating and repressing functions of RBFOX1 in splicing regulation[113]. Although several other proteins — including hnRNP H, a newly identified splicing regulator TFG and the mRNA export factor RALY — have all been found to bind to RBFOX1, none of these factors

showed selectivity in RBFOX1-mediated exon inclusion or skipping in different intronic locations. Thus, both the mechanism and the auxiliary proteins that might be involved to account for the position–polarity effect remain elusive. In this regard, it is interesting to note that a dominant-negative RBFOX protein was found to interfere with exon activation but not repression, which indicates that repression might be mediated by other factors at or near some RBFOX-binding sites[114]. This may also provide an explanation for the negative regulation of apparent MBNL-regulated splicing before the induction of MBNL expression in embryonic stem cells[115]. It is also important to emphasize that this positional rule needs to be further generalized because some reporter-selected SREs seem to activate inclusion both when they are placed in the upstream intron and when they are in the downstream intron[40].

Effect of binding on distal competing sites. Splice site selection is almost never an isolated event that solely depends on the intrinsic properties of the splice site; more often, it occurs in cases in which two or more potential splice sites are engaged in competition. Using an alternative cassette exon as an example (FIG. 3d), the 5′ splice site of the upstream exon competes for the 3′ splice sites of both the internal exon and the downstream exon; similarly, the 3′ splice site of the downstream exon competes for the 5′ splice sites of both the upstream exon and the internal exon. In principle, inclusion of the internal exon could be enhanced by strengthening the recognition of either its 5′ splice site or its 3′ splice site, or by weakening the recognition of either the 5′ splice site on the upstream exon or the 3′ splice site of the downstream exon. By tethering an SR protein to the internal exon or to a flanking competing exon of model genes, the predicted opposite effects were achieved. The presence of the SR protein on the internal exon promoted its inclusion, but the opposite result (that is, repression of the internal exon) was obtained when the SR protein was tethered to either of the adjacent exons[116,117].

Similarly, when a repressive PTB-binding site was placed near the 5′ splice site of the upstream exon or the 3′ splice site of the downstream exon, the inclusion of the internal exon was enhanced in a PTB-dependent manner[68]. This is consistent with the positive effect of PTB when tethered in an intronic location in a related study[67]. Interestingly, opposite to the effect of PTB, RNA-binding protein 6 (RBM6) seems to promote exon skipping by enhancing the selection of the competing flanking splice site[118]. Therefore, an intronic splicing repressor or activator may be selectively directed to one of the competing sites to induce exon inclusion or skipping. This rule may apply to several other hnRNPs and could account for the roughly equal numbers of induced exon inclusion and skipping events in response to RNAi-mediated depletion of traditionally classified positive and negative splicing regulators in the cell[59,78].

In concluding this section, it is important to emphasize that regulatory RBPs must function in conjunction with specific components of the core splicing machinery to modulate splice site selection and pairing (FIG. 2). Therefore, regulated splicing is a highly orchestrated biochemical process in mammalian cells.

Strategies to play on the rules

It has become increasingly evident that splicing regulators themselves are also subjected to regulation. Below, we highlight the emerging mechanisms that underlie combinatorial splicing controls, which have previously been highlighted[119]. More recent studies further emphasize that the consequences observed in any loss-of-function study are likely to reflect not only the loss of function of the factor under investigation but also the effect of perturbing its cooperating factors, thus providing an opportunity for gain-of-function conditions for other splicing regulators. These mechanisms are likely to provide an explanation for the composite and complex phenotypes observed in splicing-factor-knockout animals[80,120–123].

Cooperativity between splicing regulators. An earlier study provided evidence for cooperativity between SUP-12 and the worm homologue of RBFOX1 in the regulation of tissue-specific splicing in *Caenorhabditis elegans*[124]. A systematic study of co-occurring regulatory motifs in mammals also identified a global relationship between NOVA and RBFOX family proteins[125]. One of the best-characterized cases is perhaps the cooperativity between two T-STAR family members in *C. elegans*, in which ASD-2 and SUP-12 act together on the *unc-60* gene to regulate its splicing in muscle[126]. These two related splicing factors each bind to a separate motif with low affinity, but one binding event enhances the other, thereby creating a synergy between the two factors. This proves to be required for muscle-specific processing of their target genes. Another recent study documented extensive overlap between the splicing regulatory networks of PTB and QKI in muscle cells[85], which indicates the broader regulatory potential of such interactions. These findings illustrate synergistic interactions both between the same family of RBPs and between different families of RBPs in regulating their target genes (FIG. 4a), and our knowledge of such partnerships is almost certain to be expanded in the future.

An important aspect of cooperative action of RBPs on their respective binding sites is protein–protein and protein–RNA interactions. Interestingly, many splicing factors, especially those involved in early spliceosome assembly steps, seem to have intrinsically disordered regions (that is, segments that lack a stable protein structure in solution) — a property that may allow these proteins to adopt functional confirmations after they are engaged in interactions with their binding partners[127]. Recent studies show that low-complexity domains that are characteristic of numerous RBPs can nucleate protein–protein networks in the form of hydrogels[128,129], which might underlie the organization of not only various types of RNA granules but also the splicing machinery, as well as the formation of pathological aggregates in neurological diseases, such as amyotrophic lateral sclerosis and Parkinson's disease[17,130,131].

Tissue specificity and regulation by family members. Different RBP family members bind to the same RNA target sequence but have distinct biochemical properties that might create distinct splicing regulatory outcomes in different tissues or developmental stages. The best-studied example of this is PTB and neural PTB (nPTB): PTB is expressed in most cell types and tissues, whereas nPTB expression is highly restricted to the nervous system[132]. Such tight tissue specificity is maintained by PTB autoregulation and by PTB-mediated repression of nPTB in non-neuronal cells[133–135]. Interestingly, the regulated expression of PTB seems to have a central role in neuronal induction[134,136], and the induced nPTB expression is eventually diminished in matured neurons[137]. These programmed switches are likely to create a sequentially regulated splicing programme during development of the nervous system[122,123].

Context-dependent cooperation and competition. As distinct splicing factors can have overlapping sequence recognition specificity, they may collaborate or compete in different contexts. Context-dependent splice site recognition has been extensively studied in a neuron-specific exon at which a splicing regulator selectively operates in combination with certain 3′ splice site features to achieve regulation[138]. A more recent study knocked down one SR protein and monitored binding of another SR protein in the mouse genome. On certain regulated exons, loss of one SR protein was found to cause reduced binding of another SR protein but, at other locations, loss of one SR protein allowed another SR protein to bind more efficiently[59]. These observations indicate that SR proteins act both cooperatively and competitively in the regulation of alternative splicing in mammalian cells (FIG. 4a,b). Such cooperativity and competition may also apply to various hnRNPs and other splicing regulators. For example, a recent study showed that hnRNP C competes with U2AF65 on some Alu-associated 3′ splice sites and, as a result of hnRNP C removal, U2AF65 gained the ability to activate a large set of exons from expressed Alu transcripts[139].

Cross-regulation among splicing regulators. Given such complex relationships in controlling splicing, it is not surprising that RBP expression is frequently autoregulated and cross-regulated, thus creating extensive regulatory networks in the cell[140–142]. SR proteins have long been known to regulate their own expression at the level of splicing, which seems to be a key mechanism for homeostatic expression of most SR proteins in mammalian cells and tissues[143–145]. A network of potential cross-regulatory interactions has also been observed in an integrated study of hnRNPs, in which different hnRNP-encoding genes were found to contain binding sites for other hnRNPs; in conjunction with protein–protein interactions, it is conceivable that it is the cross-regulation that gave rise to the complex functional response in the cell[78]. Interestingly, in many loss-of-function studies, regulatory RBPs seem to be among the most affected, which may underlie the function and regulation of many RBPs, such as disease-associated FUS (also known as TLS) and TAR DNA-binding protein 43 (TDP-43), the mutations of which may be manifested into broad alterations in gene expression[146,147].

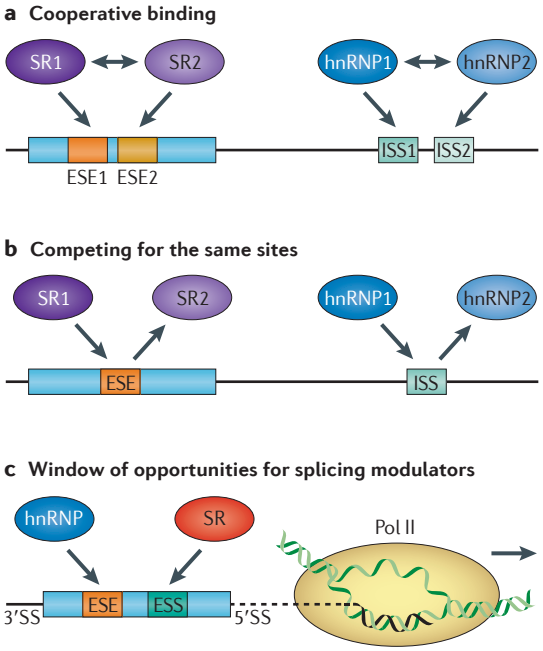

Figure 4 | **Cooperation and competition of splicing factors in regulated splicing.** **a** | Distinct splicing regulators may bind to adjacent splicing regulatory elements (SREs) in a cooperative manner, which may be mediated, at least partly, by their protein–protein interactions (indicated by two-headed arrows). **b** | The same *cis*-acting SREs, regardless of whether they are located in exons or introns, may be recognized by related RNA-binding splicing regulators. This results in competition between related splicing regulators. **c** | An increasing amount of evidence suggests that the speed of transcription elongation may influence splice site selection, which may create window of opportunities for both positive and negative splicing regulators to recognize their binding sites co-transcriptionally, thus modulating alternative splicing during these integrated events in gene expression. 3′SS, 3′ splice site; ESE, exonic splicing enhancer; ESS, exonic splicing silencer; hnRNP, heterogeneous nuclear ribonucleoprotein; ISS, intronic splicing silencer; Pol II, RNA polymerase II.

Besides the regulation of RBPs by RBPs at the splicing level, altered transcription elongation rates might create windows of opportunity for different RBPs to gain increased kinetic access to their regulatory sequences on nascent transcripts during co-transcriptional splicing[7] (FIG. 4c). Conversely, many RBPs that control splicing also seem to function at other steps in the gene expression pathway. Of particular interest is the finding that the SR protein SRSF2 seems to be directly involved in regulating RNA polymerase II pause release from promoters and elongation in the gene body[148,149], which further complicates the interpretation of the splicing responses upon depletion of this SR protein. Similarly, an increasing amount of evidence suggests that various hnRNPs

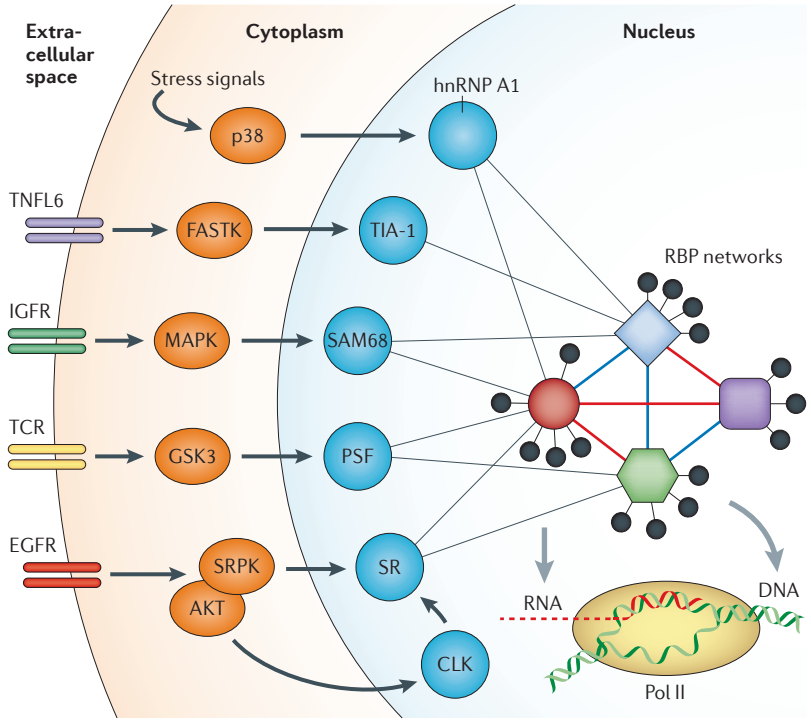

Figure 5 | **Signal transduction to regulate alternative splicing in the nucleus.** Both internal and external signals can influence alternative splicing through post-transcriptional modification of specific splicing regulators as illustrated, which may modulate both protein–protein and protein–RNA interactions to produce a network response to affect many regulated splicing events. In terms of signal transduction pathways, the SR protein system seems to have a dedicated pathway through SR protein kinases (SRPKs) and CDC-like kinases (CLKs) in the cytoplasm and the nucleus, respectively. These kinases are responsible for controlling the phosphorylation state of SR proteins to modulate their activities in splicing. Various phosphatases may be also involved to antagonize the activity of these splicing kinases. EGFR, epidermal growth factor receptor; FASTK, Fas-activated serine/threonine kinase; GSK3, glycogen synthase kinase 3; hnRNP A1, heterogeneous nuclear ribonucleoprotein A1; IGFR, insulin-like growth factor receptor; MAPK, mitogen-activated protein kinase; p38 is a MAPK involved in the DNA damage response; Pol II, RNA polymerase II; PSF, PTB (polypyrimidine-tract binding protein)-associated splicing factor; RBP, RNA-binding protein; TCR, T cell receptor; TIA-1, nucleolysin TIA-1 isoform p40; TNFL6, tumour necrosis factor ligand superfamily member 6 (also known as FasL).

are widely involved in the regulation of mRNA stability, subcellular localization and translational control through several mechanisms, including direct binding to mRNA 3′UTRs and regulating microRNA processing and function. It is therefore conceivable that splicing regulation *in vivo* represents a system-wide response to the composition of RBPs in different cell types or during development and disease processes. As transcription is thought to have various influences on mRNA splicing and other post-transcriptional regulatory events[7,9], dissection of gene networks with an aim to understand the splicing regulation involved has now become a systems biology problem and represents a major new challenge in the research field.

Modulating splicing regulators. Splicing regulatory networks are subject to signalling events that control alternative splicing. This has been best illustrated by the Ca^{2+} ion-mediated regulation of alternative splicing of a calcium-activated potassium channel by calcium/calmodulin-dependent protein kinase type IV (CaMK IV)[150]. In response to membrane depolarization, an influx of Ca^{2+} ions triggers a splicing response through an RNA element called the CaRRE motif near the 3′ splice site. The process not only involves CaMK IV phosphorylation-regulated binding of hnRNP L[151] but also requires some undefined transcription-induced splicing regulators. Most studies of signal-regulated splicing have so far been focused on the identification of specific signal-induced post-transcriptional modifications (mostly phosphorylation) on some known splicing regulators[152,153]. Key mechanistic insights have been gained through dissection of signal-induced splicing events on model genes (FIG. 5), including cellular sequestration of splicing regulators (for example, hnRNP A1 and EWS) in response to osmotic stress or DNA damage[154,155], enhanced RNA-binding activity of an RBP (for example, SAM68) upon activation of the mitogen-activated protein kinase (MAPK) pathway[156] or in response to depolarization[157], conversion from an activator to a repressor in response to heat shock[158], release from inhibitory protein complexes (for example, PTB-associated splicing factor (PSF) and hnRNP L)[97,159] and/or transcriptional induction of a cofactor during T cell signalling[75,76]. In general, however, we only have little understanding of how a specific signalling event might trigger system-wide changes in control of splicing regulators and how such changes might in turn induce global changes in alternative splicing.

SR proteins are well known to depend on phosphorylation to regulate their activities in different phases of spliceosome assembly and catalysis[57,160]. Two families of kinases are involved in the regulation of SR protein phosphorylation: SR protein kinases (SRPKs) in the cytoplasm and CDC-like kinases (CLKs) in the nucleus, both of which consist of multiple family members. Recent studies revealed that the SRPK family of kinases is regulated by molecular chaperones in the cytoplasm[81]. Upon activation by growth factors — such as epidermal growth factor (EGF), which activates AKT — these SRPKs translocate to the nucleus to cause widespread changes in alternative splicing[161,162] (FIG. 5). Remarkably, this AKT–SRPK–SR protein pathway seems to be responsible for EGF-induced splicing to a large extent, as removal of SRPK1 and/or SRPK2 by RNAi abolishes most EGF-induced events[162], and such functional interplay between SRPKs and AKT seems to be a key tumour-promoting event[163]. As inhibition of mammalian target of rapamycin (mTOR) has little effect, these findings suggest that SRPKs are major transducers of growth signals that impinge on the regulation of alternative splicing in the nucleus. Interestingly, during the recovery phase after cellular stress, CLKs seem to be responsible for restoring the proper phosphorylation state in SR proteins[164]. Therefore, the dual kinase systems for SR proteins might contribute in a coordinated manner to splicing regulation in response to different types of signals. In the future, large-scale splicing profiling coupled with systematic RNAi against splicing regulators will help to dissect signalling-induced splicing in different cell types.

Conclusions and perspectives

Many regulatory principles have been deduced by studies on model genes, but these principles require global analyses to establish their scope and general applicability. In this regard, the elegant studies on the sex determination pathway in *Drosophila melanogaster* have provided many guiding principles for understanding regulatory mechanisms in alternative splicing, including the negative effect of intronic binding of the Sex-lethal (Sxl) protein on spliceosome assembly, the positive enhancement of splice site selection by Transformer 2 (Tra2) on the *doublesex* (*dsx*) pre-mRNA to dictate sex-specific alternative splicing, as well as the requirement for Tra (which does not bind directly to RNA) to cooperate with Tra2 in *dsx* splicing[165]. Many of these regulatory principles have been extended in whole-genome studies.

Recent progress in understanding this area, particularly using global approaches, has revealed new sets of rules for context-sensitive and position-dependent splicing. Currently, some of these rules are still unclear, and the molecular mechanisms that underlie these phenomena await detailed biochemical dissection. Thus, we are left with a set of fundamental questions that will take some time to address. Given the diversity of *cis*-acting elements and the large array of *trans*-acting factors that recognize them in cooperative and antagonistic ways, what is the code for the control of alternative splicing? If an RBP has multiple tasks in the cell, then how are its different functions reconciled and controlled? How can we dissect and disentangle such complex and overlapping networks to understand their integrated function and regulation? Finally, how do specific alternative splicing events contribute to crucial phenotypes in development, behaviour and human disease? It is almost certain that the community will continue to rely on functional genomics to discover and generalize regulatory paradigms, and on biochemistry to tackle the direct effects and the mechanisms. This effort will be undoubtedly aided by systematic identification of RBPs that can be crosslinked to diverse RNA populations in the cell, large-scale decoding of binding specificities of RBPs using high-throughput methods; dissection of both RNA-independent and RNA-dependent protein–protein interactions involved in regulated splicing; computational analyses of the effect of RNA secondary structure on RBP access to RNA sequence information; genome engineering to study *cis*-acting elements in their native context; and deconvolution of gene networks through genome-wide loss- and gain-of-function studies.

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Acknowledgements

This Review has benefitted from numerous discussions with colleagues in the field. The work in the authors' laboratories were supported by grants GM040478 (to M.A.Jr), and GM049369 and GM052872 (to X.-D.F.) from the US National Institutes of Health.

Competing interests statement

The authors declare no competing interests.