POST-TRANSCRIPTIONAL CONTROL

Mechanisms of alternative splicing regulation: insights from molecular and genomics approaches

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Abstract | Alternative splicing of mRNA precursors provides an important means of genetic control and is a crucial step in the expression of most genes. Alternative splicing markedly affects human development, and its misregulation underlies many human diseases. Although the mechanisms of alternative splicing have been studied extensively, until the past few years we had not begun to realize fully the diversity and complexity of alternative splicing regulation by an intricate protein–RNA network. Great progress has been made by studying individual transcripts and through genome-wide approaches, which together provide a better picture of the mechanistic regulation of alternative pre-mRNA splicing.

Small nuclear ribonucleoprotein particle (snRNP). A protein, including U1, U2, U4, U5 and U6, which contains U-rich small nuclear RNAs (snRNAs) and both small nuclear ribonucleoprotein (snRNP)-specific and common proteins, and is a core component of the spliceosome.

Branch point
A nucleotide, usually an adenosine, within a variably conserved branch point sequence upstream of the 3′ splice site, the 2′ hydroxyl group of which attacks the 5′ splice site in the first step of splicing.

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Alternative splicing is a crucial mechanism for gene regulation and for generating proteomic diversity. Recent estimates indicate that the expression of nearly 95% of human multi-exon genes involves alternative splicing[1,2]. In metazoans, alternative splicing plays an important part in generating different protein products that function in diverse cellular processes, including cell growth, differentiation and death.

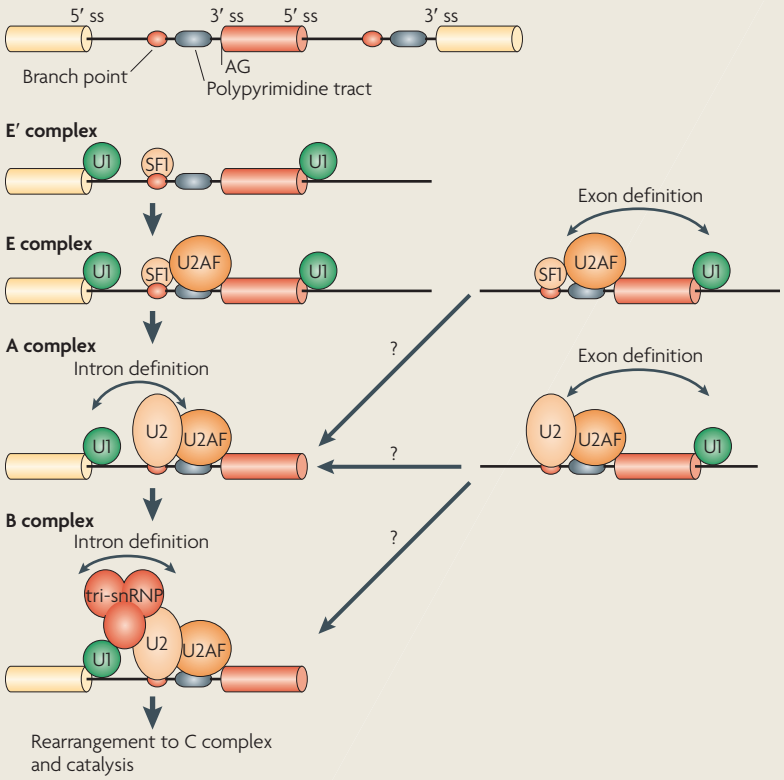
Splicing is carried out by the spliceosome, a massive structure in which five small nuclear ribonucleoprotein particles (snRNPs) and a large number of auxiliary proteins cooperate to accurately recognize the splice sites and catalyse the two steps of the splicing reaction[1,2] (BOX 1). Spliceosome assembly (BOX 1) begins with the recognition of the 5′ splice site by the snRNP U1 and the binding of splicing factor 1 (SF1) to the branch point[3] and of the U2 auxiliary factor (U2AF) heterodimer to the polypyrimidine tract and 3′ terminal AG[4,5]. This assembly is ATP independent and results in the formation of the E complex, which is converted into the ATP-dependent, pre-spliceosomal A complex after the replacement of SF1 by the U2 snRNP at the branch point. Further recruitment of the U4/U6–U5 tri-snRNP complex leads to the formation of the B complex, which is converted into to the catalytically active C complex after extensive conformational changes and remodelling.

The decision as to which exon is removed and which exon is included involves RNA sequence elements and protein regulators. Depending on the position and function of the *cis*-regulatory elements, they are divided into four categories: exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs) and intronic splicing silencers (ISSs). ESEs are usually bound by members of the SR (Ser–Arg) protein family[6–8] (BOX 2). ISSs and ESSs are commonly bound by heterogeneous nuclear RNPs (hnRNPs; TABLE 1), which have one or more RNA-binding domains and protein–protein interaction domains[9,10]. ISEs are not as well characterized as the other three types of element, although recently several proteins, such as hnRNP F, hnRNP H, neuro-oncological ventral antigen 1 (NOVA1), NOVA2, FOX1 and FOX2 (also known as RBM9), have been shown to bind ISEs and to stimulate splicing[11–14].

Choices of alternative splicing have long been thought to be made at the stages of splice site recognition and early spliceosome assembly, and indeed this is frequently the case[1]. However, several recent studies have shown that the decision can be made at different stages of spliceosome assembly, and even during conformational changes between the two transesterification steps[15–17]. In addition, there has been accumulating evidence showing the coupling of RNA transcription to splicing regulation[18–22].

Alternative splicing contributes to genomic diversity and tissue specificity[23]. Comprehending tissue-specific alternative splicing requires an understanding of the regulatory network of protein–protein, protein–RNA and RNA–RNA interactions that are involved in this process[1,24]. Tissue-specific alternative splicing is thought to be controlled by differentially expressed splicing regulators[1,25] and/or ubiquitously expressed splicing factors of different concentrations and/or activity[26,27]. In addition, tremendous progress has been made using high-throughput

Box 1 | **Splicing and spliceosome assembly**



Pre-mRNA splicing is a process in which intervening sequences (introns) are removed from an mRNA precursor. Splicing consists of two transesterification steps, each involving a nucleophilic attack on terminal phosphodiester bonds of the intron. In the first step this is carried out by the 2′ hydroxyl of the branch point (usually adenosine) and in the second step by the 3′ hydroxyl of the upstream (5′) exon[1,2]. This process is carried out in the spliceosome, a dynamic molecular machine the assembly of which involves sequential binding and release of small nuclear ribonucleoprotein particles (snRNPs) and numerous protein factors as well as the formation and disruption of RNA–RNA, protein–RNA and protein–protein interactions.

The basic mechanics of spliceosome assembly are well known. Briefly, the process begins with the base pairing of U1 snRNA to the 5′ splice site (ss) and the binding of splicing factor 1 (SF1) to the branch point[3] in an ATP-independent manner to form the E′ complex (see the figure; double-headed arrows indicate an interaction). The E′ complex can be converted into the E complex by the recruitment of U2 auxiliary factor (U2AF) heterodimer (comprising U2AF65 and U2AF35) to the polypyrimidine tract and 3′ terminal AG[158]. The ATP-independent E complex is converted into the ATP-dependent pre-spliceosome A complex by the replacement of SF1 by U2 snRNP at the branch point. Further recruitment of the U4/U6–U5 tri-snRNP leads to the formation of the B complex, which contains all spliceosomal subunits that carry out pre-mRNA splicing. This is followed by extensive conformational changes and remodelling, including the loss of U1 and U4 snRNPs, ultimately resulting in the formation of the C complex, which is the catalytically active spliceosome.

methods, which has not only revealed many more new alternative splicing events[28–30], but has also accelerated the process of understanding its regulation in different tissues by examining the expression levels of protein regulators and helping to define *cis*-regulatory elements[31,32]. In this Review, we discuss mechanisms of alternative splicing control from different perspectives. At which stage of spliceosome assembly is the decision of whether to include an alternative exon made? What protein factors are involved? How does RNA polymerase II (RNAP II) function in alternative splicing regulation? Are the kinetics of spliceosome assembly important for this process? Which mechanisms control tissue-specific alternative splicing? We address these questions and conclude by summarizing the diversity of the mechanisms of alternative splicing regulation and speculate on the future directions of alternative splicing research.

SR (Ser–Arg) protein family
A family of nuclear factors that have many important roles in splicing mRNA precursors in metazoan organisms, functioning in both constitutive and alternative splicing.

Splice site recognition and selection

Most human genes contain multiple exons, and the average length of exons (50–250 bp) is much shorter than that of intervening sequences (frequently thousands of bps). Early stages of spliceosome assembly occur around the exons owing to the large size of introns[33]. This type of exon-centred splice site recognition is referred to as 'exon definition' (REF. 34) (BOX 1). Exon definition must eventually be converted to intron definition, which occurs by cross-intron interactions between the U1 and U2 snRNPs[35,36]. The best-studied mechanisms of alternative splicing regulation involve controlling splice site recognition by facilitating or interfering with the binding of the U1 or U2 snRNP to the splice sites.

Facilitating splice site recognition. SR proteins have important roles in facilitating splice site recognition. For example, they recruit the U1 snRNP to the 5′ splice site and the U2AF complex and U2 snRNP to the 3′ splice site by binding to an ESE and directly interacting with protein targets[37–40] (FIG. 1a). These interactions are mediated by their RS (Arg–Ser repeat-containing) domains[41,42], which have to be properly phosphorylated and dephosphorylated[1,43,44]. SR proteins also cooperate with other positive regulatory factors to form larger splicing enhancing complexes by interacting with other RS domain-containing proteins, such as transformer 2 (TRA2) and the SR-related nuclear matrix proteins SRm160 (also known as SRRM1) and SRm300 (also known as SRRM2)[45–47] (FIG. 1a). Binding and recruiting can also be achieved by intronic binding proteins: for example, T cell-restricted intracellular antigen 1 (TIA1) binds a U-rich sequence downstream of weak 5′ splice sites to recruit the U1 snRNP[48,49]; and Src-associated in mitosis 68 kDa protein (SAM68; also known as KHDRBS1) binds and recruits U2AF to the 3′splice site of exon V5 of the transmembrane glycoprotein *CD44* pre-mRNA[50].

The SR protein SRp38 (also known as TASR[51], NSSR[52], FUSIP1 and SRrp40 (REF. 53)) has been characterized as a general splicing repressor that is activated by dephosphorylation[54,55] (see below). However, recent studies indicate that it also functions as a sequence-dependent splicing activator when phosphorylated[39]. It was found that *in vitro* SRp38 activates the formation and splicing of the A complex by facilitating the recruitment of the U1 and U2 snRNPs to the pre-mRNA and stabilizing the 5′ splice site and branch site recognition (FIG. 2a). Notably, SRp38, unlike other SR proteins, cannot complement cytoplasmic S100 extracts (which contain all factors required for splicing except SR proteins) to activate splicing[56]. Although an A-like complex is formed in the presence of S100 and SRp38, it is stalled or inactive and requires a specific (and currently unknown) coactivator

Box 2 | **SR proteins**

The SR (Ser–Arg) proteins are a family of nuclear factors that have many important roles in the splicing of mRNA precursors in metazoan organisms, functioning in both constitutive and alternative RNA splicing[7]. They are involved in many steps of splicing regulation, by binding exonic splicing enhancers (ESEs) through their RNA recognition motifs (RRMs) and mediating protein–protein[41,42], and perhaps protein–RNA[159], interactions through their RS (Arg–Ser repeat-containing) domains. All canonical SR proteins have common characteristics (see the table). They have a similar structure, with one or two ribonucleoprotein particle (RNP)-type RNA-binding domains at their amino termini and a variable-length domain enriched in Arg–Ser dipeptides at their carboxyl termini (the RS domain). RS domains are extensively phosphorylated and they function in splicing, usually as activators. Most SR proteins function as pivotal regulators in multiple aspects of mRNA metabolism, such as mRNA nuclear export[160], nonsense-mediated mRNA decay[161] and translation[162]. Numerous additional RS domain-containing proteins have been identified; proteins known to be involved in alternative splicing are listed in the table.

Name*	Domains	Binding sequence	Target genes
Canonical SR proteins			
SRp20 (SFRS3)	RRM and RS	GCUCCUCUUC	*SRP20*, *CALCA* and *INSR*
SC35 (SFRS2)	RRM and RS	UGCUGUU	*ACHE* and *GRIA1–GRIA4*
ASF/SF2 (SFRS1)	RRM, RRMH and RS	RGAAGAAC	*HIPK3*, *CAMK2D*, HIV RNAs and *GRIA1–GRIA4*
SRp40 (SFRS5)	RRM, RRMH and RS	AGGAGAAGGGA	*HIPK3*, *PRKCB* and *FN1*
SRp55 (SFRS6)	RRM, RRMH and RS	GGCAGCACCUG	*TNNT2* and *CD44*
SRp75 (SFRS4)	RRM, RRMH and RS	GAAGGA	*FN1*, *E1A* and *CD45*
9G8 (SFRS7)	RRM, zinc finger and RS	(GAC)n	*TAU*, *GNRH* and *SFRS7*
SRp30c (SFRS9)	RRM, RRMH and RS	CUGGAUU	*BCL2L1*, *TAU* and *HNRNPA1*
SRp38 (FUSIP1)	RRM and RS	AAAGACAAA	*GRIA2* and *TRD*
Other SR proteins			
SRp54	RRM and RS	ND	*TAU*
SRp46 (SFRS2B)	RRM and RS	ND	NA
RNPS1	RRM and Ser-rich	ND	*TRA2B*
SRrp35	RRM and RS	ND	NA
SRrp86 (SRrp508 and SFRS12)	RRM and RS	ND	NA
TRA2α	RRM and two Arg-rich	GAAARGARR	dsx
TRA2β	RRM and two RS	(GAA)n	*SMN1*, *CD44* and *TAU*
RBM5	RRM and RS	ND	*CD95*
CAPER (RBM39)	RRM and RS	ND	*VEGF*

*Alternative names are provided in brackets. *ACHE*, acetylcholine; *BCL2L1*, BCL-2-like 1; *CAMK2D*, calcium/calmodulin-dependent protein kinase II-δ; *CALCA*, calcitonin-related polypeptide-α; CAPER, coactivator of activating protein 1 and oestrogen receptors; *FN1*, fibronectin 1; FUSIP, FUS-interacting serine-arginine-rich protein 1; *GNRH*, gonadotropin-releasing hormone; *GRIA*, glutamate receptor, ionotropic, AMPA; *HIPK3*, homeodomain-interacting protein kinase 3; *HNRNPA1*, human nuclear RNP A1; *INSR*, insulin receptor; *PRKCB*; protein kinase Cβ; RBM, RNA-binding protein; RNPS1, RNA-binding protein with Ser-rich domain 1; RRMH, RRM homology; NA, not applicable; ND, not determined; SFRS, splicing factor, Arg- and Ser-rich; *SMN1*, survival of motor neuron 1; *TNTT2*, troponin T type 2; *TRA2*, transformer 2; *TRD*, tradin; *VEGF*, vascular endothelial growth factor.

to proceed[39] (FIG. 2a). *In vivo*, SRp38 was shown to favour inclusion of the Flip exon of the *GRIA2* (glutamate receptor, ionotropic, AMPA 2; also known as *GLURB*) pre-mRNA[52], whereas the mutually exclusive Flop exon is included when SRp38 is absent[39]. Interestingly, both exons contain SRp38-binding sites, and it was proposed that the intracellular concentrations of SRp38 as well as differential binding to the exons (that is, stronger binding to the Flip exon) influence the decision to include either the Flip or Flop exon[39].

Inhibiting splice site recognition. Inhibition of splice site recognition can be achieved in many ways. First, when splicing silencers are located close to splice sites or to splicing enhancers, inhibition can occur by sterically blocking the access of snRNPs or of positive regulatory factors. For example, polypyrimidine-tract binding protein (PTB; also known as PTB1 and hnRNP I), binds the polypyrimidine tract and blocks the binding of U2AF to regulated exons[57–59]. In addition, hnRNP A1 binds ISSs that are located upstream of exon 3 in HIV *Tat* pre-mRNA and prevents binding of the U2 snRNP[60]. Finally, tissue-specific splicing factors FOX1 and FOX2 inhibit the formation of the E′ complex by binding to an intronic sequence to prevent SF1 from binding to the branch site of *CALCA* (calcitonin-related polypeptide-α) pre-mRNA[61] (FIG. 1b).

Splicing inhibitors also sterically block the binding of activators to enhancers. Hu/ELAV family proteins inhibit U1 snRNP binding by competing with the binding of TIA1 to an AU-rich sequence downstream of the 5′ splice site of exon 23a of neurofibromatosis type 1 pre-mRNA[62]. FOX1 and FOX2 also inhibit E complex formation by binding to an exonic sequence in the *CALCA* pre-mRNA close to the ESE that TRA2 and SRp55 bind to, preventing the recruitment of U2AF by the activators[61] (FIG. 1b). Finally, hnRNP A1 binds an ESS upstream of the TRA2-dependent ESE in exon 7 in the *SMN2* (survival of motor neuron 2) pre-mRNA, possibly inhibiting the formation or stabilization of the U2 snRNP complex[63,64] (FIG. 1c).

Some silencers can be over 100–200 bp away from enhancers, and a simple 'bind and block' model thus cannot explain their inhibitory effect. One explanation for the activity of such splicing inhibitors is that they function by masking splice site recognition through multimerization along the RNA[58]. Another model proposes that the alternative exon might be 'looped out' in a process involving protein–protein interactions between RNA-binding proteins bound at sites spanning the alternative exon[58,65–67], and that this loop formation may sterically interfere with further spliceosome assembly, even though splice site recognition may not be inhibited[67]. For example, hnRNP A1 binds to elements upstream and downstream of exon 7B in its own pre-mRNA to promote skipping of exon 7B[68]. hnRNP A1 has also been shown to bind to exonic and intronic silencers in *SMN2* exon 7 and intron 7, and it is proposed that an interaction between hnRNP A1 molecules is required to fully suppress the inclusion of *SMN2* exon 7 (REFS 64,69,70). In addition, PTB has been shown to bind sites flanking the *SRC* N1 exon, and mutating one of the PTB sites affects the binding of PTB to the other site[58,71].

Table 1 | **Ribonucleoproteins that are involved in pre-mRNA splicing**

Name	Other names	Domains*	Binding sequences	Target genes
hnRNP A1	NA	RRM, RGG and G	UAGGGA/U	*SMN2* and *RAS*
hnRNP A2 hnRNP B1	NA	RRM, RGG and G	(UUAGGG)n	HIV *tat* and *IKBKAP*
hnRNP C1 hnRNP C2	AUF1	RRM	U rich	*APP*
hnRNP F	NA	RRM, RGG and GY	GGGA and G rich	*PLP*, *SRC* and *BCL2L2*
hnRNP G	NA	RRM and SRGY	CC(A/C) and AAGU	*SMN2* and *TMP1*
hnRNP H hnRNP H′	DSEF1	RRM, RGG, GYR and GY	GGGA and G rich	*PLP*, HIV *tat* and *BCL2L1*
hnRNP I	PTB	RRM	UCUU and CUCUCU	*PTB*, *nPTB*, *SRC*, *CD95*, *TNTT2*, *CALCA* and *GRIN3B*
hnRNP L	NA	RRM	C and A rich	*NOS* and *CD45*
hnRNP LL	SRRF	RRM	C and A rich	*CD45*
hnRNP M	NA	RRM and GY	ND	*FGFR2*
hnRNP Q	NA	RRM and RGG	ND	*SMN2*

*Domains are enriched in particular amino acids, as indicated by their names. *APP*, amyloid-β precursor protein; *BCL2L1*, BCL-2-like 2; *CALCA*, calcitonin-related polypeptide-α; *GRIN3B*, glutamate receptor, ionotropic, NMDA 3B; *FGFR2*, fibroblast growth factor receptor 2; hnRNP, heterogeneous nuclear ribonucleoprotein particle; hnRNP LL, hnRNP L-like; *IKBKAP*, inhibitor of κ-light polypeptide gene enhancer in B cells, kinase complex-associated protein; NA, not applicable; ND, not determined; *NOS*, nitric oxide synthase; *SMN2*, survival motor neuron protein 2; *PLP*, proteolipid protein; *PTB*, polypyrimidine-tract binding protein; RRM, RNA recognition motif; *TNTT2*, troponin T type 2; TPM1, α-tropomyosin.

Heterologous nuclear RNP (hnRNP). A pre-mRNA- or mRNA-binding protein that associates with transcripts during or after transcription and influences their function and fate. Some hnRNPs shuttle in and out of nuclei, whereas others are constitutively nuclear.

Alternative exon
An exon that is included in mature mRNA in certain cellular contexts but excluded in others.

RS (Arg–Ser repeat-containing) domain
A protein domain that is variable in length and enriched in Arg–Ser dipeptides and seems to be involved in protein–protein and protein–RNA interactions.

Hu/ELAV family protein
A protein belonging to a family of nervous system-specific RNA-binding proteins that specifically bind to AU-rich sequences.

CLIP
A method that combines cross-linking and immunoprecipitation to identify *in vivo* targets of RNA-binding proteins.

Combinatorial effects of activators and inhibitors. Splicing of individual pre-mRNAs is frequently controlled by combinatorial or competitive effects of both activators and inhibitors. The final decision of whether an alternative exon is included is determined by the concentration or activity of each type of regulator, often by SR proteins and hnRNPs[72–74]. For example, the SR protein 9G8 (also known as SFRS7) and hnRNP F and hnRNP H regulate the splicing of α-tropomyosin exon 2 by competing for binding to the same element[75]; hnRNP A1 and the SR proteins ASF/SF2 (also known as SFRS1) and SC35 (also known as SFRS2) have antagonistic functions in splicing of β-tropomyosin exon 6B[76]; and CELF (CUGBP- and ETR3-like factor)-family proteins ETR3 (also known as CELF2) and CUGBP1 activate the splicing of exon 5 of *TNTT2* (troponin T type 2; also known as *cTNT*) by displacing PTB[77]. A recent study showed that at least some *Drosophila melanogaster* SR proteins and hnRNPs do not have as many common targets as had been thought[78]. Using small interfering RNAs (siRNAs) to deplete individual splicing factors followed by splicing-sensitive microarray analysis, the authors compared genes regulated in opposite directions by hnRNPs and two SR proteins (dASF and BC52). Surprisingly, less than 5% of the genes overlap. However, a systematic analysis of more SR and hnRNPs will be necessary to decide whether antagonizing effects between these proteins is a main mode of alternative splicing regulation.

Position-dependent splicing regulation. The nature of the activity of *cis*-acting elements and their cognate binding proteins in some cases depends on their position relative to regulated exons. Several proteins, such as NOVA1, NOVA2, FOX1, FOX2, hnRNP L, hnRNP L-like, hnRNP F and hnRNP H, have been shown to act as either repressors or activators depending on the location of their binding site[11–14,79–83]. For example, NOVA1 binds to an ISE in *GABRG2* (GABA A receptor, γ2) pre-mRNA and promotes inclusion of exon 9 (REF. 84), but it binds to the ESS in the alternative exon 4 of its own pre-mRNA and prevents exon 4 from being included[81]. Similarly to NOVA1, hnRNP L can activate or repress upstream alternative exons, and this probably depends on the location of its binding site relative to the regulated 5′ splice site[12]. hnRNP H promotes the formation of ATP-dependent spliceosomal complexes when it binds to G-rich sequences (G runs) downstream of the 5′ splice site[85], but it inhibits splicing when the G-rich sequences are located in exons[86]. By using information from mRNA transcripts that are know to be targeted by NOVA1 and NOVA2 and by searching for YCAY clusters (which are NOVA1 and NOVA2 binding sequences) around regulated exons, one group[11] drew an 'mRNA map' that includes the location of NOVA1 and NOVA2 binding sites and the consequence of each binding event. This map provides insight into the mechanisms underlying the effects of NOVA1 and NOVA2 on splicing. For example, binding of NOVA1 and NOVA2 to an ESS inhibits the formation of the pre-spliceosomal E complex by altering its composition before the binding of hnRNPs and inhibits the binding of U1 snRNP[11]. By contrast, NOVA1 and NOVA2 binding to an ISE downstream of the alternative exon promotes the formation of spliceosomal complexes A, B and C[11]. A new technique that combines CLIP and high-throughput sequencing, known as HITS-CLIP[80] (CLIP-seq)[13,87,88], not only verified the reproducibility of this mRNA map, but

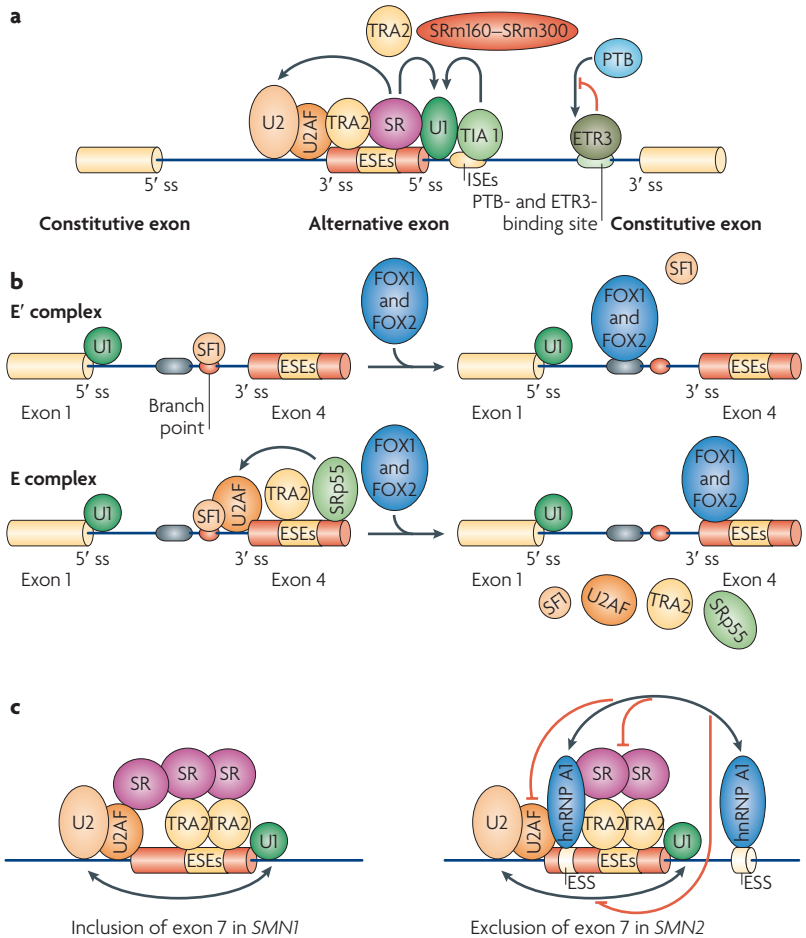


Figure 1 | **Mechanisms of alternative splicing by splice site selection.** Schematic depicting mechanisms of splicing activation. **a** | SR (Ser–Arg) proteins bind to exonic splicing enhancers (ESEs) to stimulate the binding of U2AF to the upstream 3′ splice site (ss) or the binding of the U1 small nuclear ribonucleoprotein (snRNP) to the downstream 5′ ss. SR proteins function with other splicing co-activators, such as transformer 2 (TRA2) and the SR-related nuclear matrix proteins SRm160–SRm300. T cell-restricted intracellular antigen 1 (TIA1) binds to U-rich sequences (intronic splicing enhancers (ISEs)) immediately downstream of 5′ splice sites to facilitate U1 binding. CELF (CUGBP- and ETR3-like factor) proteins, such as ETR3, bind to similar sequences as polypyrimidine-tract binding protein (PTB), thereby activating splicing by competing with PTB. **b** | FOX1 and FOX2 inhibit the inclusion of *CALCA* (calcitonin-related polypeptide-α) exon 4 by blocking the binding of splicing factor 1 (SF1) to the branch point (top panel) and of TRA2 and SRp55 to ESEs (bottom panel), thereby inhibiting spliceosome assembly at two stages, the E′ and E complexes. The arrow indicates that SRp55 and TRA2 promote binding of the U2AF complex. **c** | Single-nucleotide differences in *SMN2* (survival of motor neuron 2) compared with *SMN1* create binding sites for heterogeneous nuclear ribonucleoprotein particle A1 (hnRNP A1) (or hnRNP A2) in exon 7 and in the downstream intron in *SMN2* pre-mRNA. hnRNP A1 (or hnRNP A2) may then inhibit the formation or stabilization of the U2 snRNP complex, either directly or indirectly by blocking the activity of the downstream TRA2-dependent ESE. Note that it has also been suggested that the base change in exon 7 destroys an ASF/SF2-dependent ESE[163,164] (but see also REF. 69).

also provided genome-wide information on the target genes of NOVA1, NOVA2 and FOX2 as well as possibly mechanisms by which these proteins are regulated[80].

Why does the position of splicing regulatory elements determine the action of cognate splicing factors? It is possible that enhancers are positioned so that when the splicing factors bind to them, the splice sites of the alternative exon are better presented to the splicing machinery by changing the local mRNA structure. By contrast, silencing elements function in the opposite manner, by competing with components of the splicing machinery or by changing the structure of the mRNA to impede splice site recognition.

Roles for RNA in alternative splicing regulation. The selection of the splice site can also be influenced by secondary structures in the pre-mRNA. Perhaps the most striking example of this is the complex alternative splicing that is observed in *D. melanogaster Dscam* pre-mRNA. The exon 6 cluster of *Dscam* consists of 48 mutually exclusive exons. Pairing between a conserved sequence located downstream of constitutive exon 5 (the docking site) and another conserved sequence, a variant of which is located upstream of each exon 6 variant (the selector sequence), allows the inclusion of only one exon 6 variant[89]; the other variants are excluded by the binding of hrp36, a *D. melanogaster* hnRNP A homologue, to the selector sequence[90].

Secondary structures can affect alternative splicing by masking splice sites[91] or by binding sites for splicing factors[92,93]. For example, a stem and loop secondary structure was shown to sequester alternative exon 6B of the chicken β-tropomyosin pre-mRNA, leading to its exclusion[94]. The IDX exon of *RAS* can form a secondary structure with an ISS (RASISS1), preventing the binding of hnRNP H to RASISS1. Unwinding this secondary structure by the RNA helicase P68 exposes the binding site, partially explaining the exclusion of IDX[93]. Riboswitches, which control gene expression in prokaryotes[95], also have the potential to modulate alternative splicing. Splicing of the *NMT1* (*N*-tetradecanoyltransferase 1) pre-mRNA in *Neurospora crassa* was found to respond to the coenzyme thiamine pyrophosphate through a riboswitch-like structure[96]. It remains to be seen, however, whether this intriguing mechanism operates in higher eukaryotes. In mammals, small nucleolar RNAs (snoRNAs) have also been implicated in alternative splicing regulation[97]. For example, the snoRNA HBII 52 regulates the alternative splicing of *HTR2C* pre-mRNA by binding to a silencing element in exon Vb to promote its inclusion[98].

Regulation by U1 and U2 snRNP pairing

After the 5′ and 3′ splice sites are recognized and exons are defined, exon definition must be converted to intron definition, which involves cross-intron interaction between U1 and U2 snRNPs, to form a functional spliceosome. Precisely when this occurs and when the commitment to splice site pairing happens have been intensively investigated over the past few years. Several studies have shown that the commitment to splicing of at least some alternative exons occurs during splice site pairing in the A complex[35,36]. For example, ATP hydrolysis is required for splice site pairing, which locks splice sites into a splicing pattern after U2 snRNP binding to the branch site[36]. Additional studies, discussed below, also provided evidence that binding of U1 and U2 snRNPs to splice sites to define an exon does not necessarily commit the exon to splicing[15,16,99].

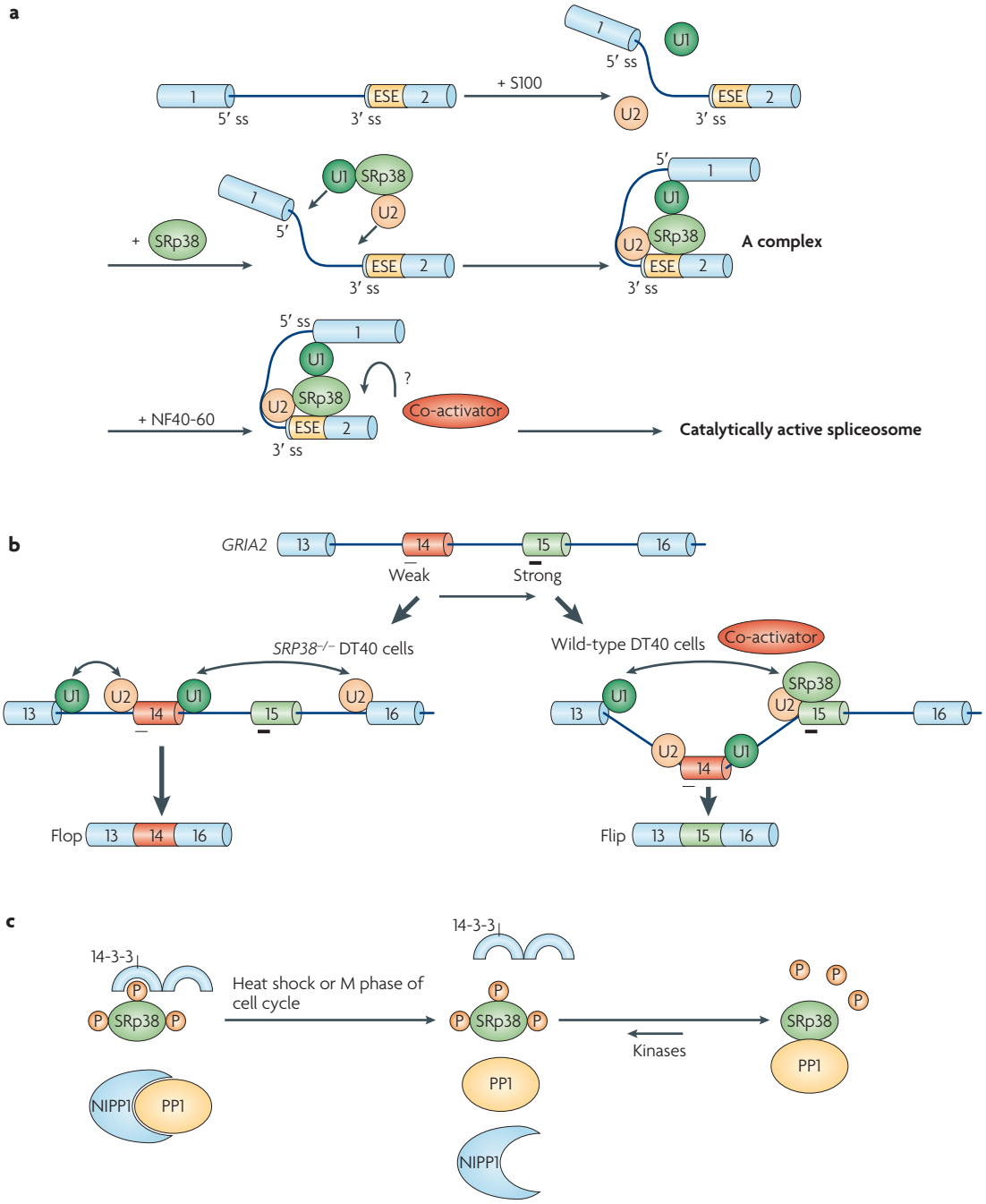


Figure 2 | **Phosphorylation switches the general splicing repressor SRp38 into a sequence-specific activator.** **a** | Phosphorylated SRp38 (Ser–Arg protein 38) activates splicing by recruiting the U1 and U2 small nuclear ribonucleoprotein particles (snRNPs) to splice sites (ss). SRp38 binds SRp38-dependent exonic splicing enhancers (ESEs) in target transcripts and facilitates the association of U1 and U2 snRNPs with the pre-mRNA to stabilize 5′ ss and branch site recognition by interacting with U1 and U2 snRNPs, respectively. However, the spliceosomal A complex formed is stalled in S100 extract, in which an SRp38-specific cofactor from NF40-60 is absent, which is required to proceed through the splicing pathway. **b** | SRp38 enhances the inclusion of the Flip exon of *GRIA2* (glutamate receptor, ionotropic, AMPA 2) pre-mRNA relative to the mutually exclusive Flop exon. Both exons contain SRp38-binding sites (indicated by black bars under exon 14 (Flop) and exon 15 (Flip)), but the site in Flip is stronger (indicated by the thicker bar), and Flip inclusion is therefore favoured in the presence of SRp38. **c** | Protein phosphatase 1 (PP1) dephosphorylates SRp38 on heat shock. Under normal conditions, phosphorylated SRp38 is associated with 14-3-3 proteins, which help to protect SRp38 from dephosphorylation, and PP1 activity is inhibited by PP1-associated proteins, including nuclear inhibitor of PP1 (NIPP1). During heat shock, PP1 dissociates from NIPP1 and directly binds to and dephosphorylates SRp38, which has dissociated from 14-3-3 proteins. Part **a** of the figure modified, with permission, from *Nature Struct. Mol. Biol.* REF. 39 © (2008) Macmillan Publishers Ltd. All rights reserved. Part **c** of the figure modified, with permission, from REF. 151 © (2007) Elsevier.

Regulation by protein factors. A new splicing inhibition mechanism was demonstrated recently by a study showing that binding of hnRNP L to an ESS can inhibit the pairing of U1 and U2 snRNPs[15]. An ATP-dependent spliceosome-like complex, known as A-like exon-definition complex (AEC), was found to form across alternative exon 4 of the *CD45* pre-mRNA, even when its inclusion was inhibited. The AEC contains U1 and U2 snRNPs and displays the same gel mobility as the A complex, but progression into the B complex is inhibited. They proposed a model in which an A-like complex forms across exons when hnRNP L is not present, after which the U4/U6–U5 tri-snRNP complex is recruited to the intron-defined A complex to form the B complex. However, when hnRNP L is present, an hnRNP L-containing AEC prevents the U1 or U2 snRNPs bound to the splice sites of exon 4 from cross-intron pairing with the adjacent U2 or U1, resulting in exon 4 skipping. There are two possible ways in which the binding of hnRNP L might interfere with snRNP pairing. One is that binding of hnRNP L physically shields the interaction between the snRNPs. Another possibility is that hnRNP L induces a change in the conformation of the pre-mRNA that prevents cross-intron pairing of the snRNP-bound alternative exon. It is intriguing that the AEC, at least superficially, resembles the stalled A complex formed by SRp38 in S100 extract (see above). Although the importance of this is unknown, the following example suggests that such complexes may be more widespread than realized.

PTB is another inhibitory splicing factor that has been shown to function, in some cases, by blocking the transition from exon definition to intron definition[16,65] (FIG. 3a). One group[16] studied the mechanism of PTB inhibition by comparing the active and inactive spliceosomal complexes from neuronal WERI-1 cell nuclear extracts, in which the *SRC* N1 exon is included, and from HeLa cell nuclear extracts, in which the *SRC* N1 exon is excluded. PTB is highly expressed by HeLa cells, whereas a less repressive brain paralogue, nPTB (also known as PTB2 and brPTB), is expressed by WERI-1 cells[100–103] (see below). Similarly to the example provided by hnRNP L, ATP-dependent exon definition complexes form in nuclear extracts from both cell lines. The protein compositions of the exon definition E and A complexes (EDE and EDA, respectively) that formed on constitutive exon 4 on nuclear extracts from both cell lines were similar, and PTB was found only in complexes formed in HeLa nuclear extracts. By contrast, the EDE and EDA complexes that formed on the substrate containing both exon N1 and exon 4 in WERI-1 nuclear extracts and in HeLa nuclear extracts differed in their properties and protein compositions. The WERI-1 EDE can move on to functional A, B and C complexes following ATP addition, whereas the EDE formed in HeLa nuclear extracts can only localize to a 'dead end' A complex. Several proteins only exist in the functional A complex formed in WERI-1 nuclear extracts, such as the PRP19 complex[104,105] and SRm160–SRm300 complex[46,106], which are might be important for 3′ and 5′ splice site bridging and exon N1 inclusion, and are excluded from the spliceosome by PTB. This study provides a new mechanism for how different protein compositions in different tissues can help to determine the alternative splicing pattern through a silencing factor and its interactions with other splicing factors to prevent intron definition. This study also reveals for the first time that the protein composition of different exon and intron definition complexes can vary, and thus begins to decipher a new mechanism for alternative splicing regulation and tissue specificity.

RBM5 (RNA-binding protein 5; also known as LUCA15 and H37) is a putative tumour suppressor protein[107,108] that promotes the exclusion of exon 6 of *CD95* (also known as *FAS*) pre-mRNA[109]. RBM5 was found to interact with sequences in exon 6 but to not affect the association of U1 and U2 snRNPs to the adjacent splice sites. Instead, RBM5 inhibited the incorporation of the U4/U6–U5 tri-snRNP complex on the introns flanking exon 6, thereby blocking the maturation of pre-spliceosomes. RBM5 also promoted the pairing of U1 and U2 at the distal splice sites, contributing further to the exclusion of exon 6 (REF. 109) (FIG. 3b).

In addition to alternative splicing regulation by the inhibition of intron definition, it is also possible that alternative splicing is stimulated by the activation of intron definition. *In vitro* experiments with substrates containing expanded introns have shown that the presence of binding sites for hnRNPs near intron boundaries can facilitate splicing[82]. This presumably involves cross-intron interactions between hnRNPs that help to bring together the ends of the introns, indicating a possible positive regulatory role for hnRNPs in splicing.

Cis-acting elements that affect U1 and U2 snRNP pairing by modulating 5′ splice site competition have recently been identified. In one study an *in vitro* screen was carried out for splicing silencers (ESSs and ISSs) that alter the selection of the 5′ splice site by choosing a distal, weak 5′ splice site over a proximal, strong 5′ splice site[99]. The isolated silencers did not affect whether U1 snRNP bound to the 5′ splice site, but instead somehow altered the conformation of the proximal U1 snRNP–5′ splice site complex so that it lost its advantage to compete with the distal U1 snRNP–5′ splice site complex for the pairing with the U2 snRNP–3′ splice site complex. This study suggests a new mechanism by which silencers can subtly affect splice site selection that does not involve sequestering splice sites, but instead entails changing the conformation of the snRNP–pre-mRNA complex. Moreover, the silencers do not affect the rate-limiting step of splicing but instead affect how well U1 and U2 snRNPs pair, which in turn can influence splice site choice.

Transcription-coupled alternative splicing

Two models have been proposed to explain the role of RNAP II in the regulation of alternative splicing[110]. The first model is known as the recruitment model, in which RNAP II and transcription factors interact, directly or indirectly, with splicing factors[22,111,112], thereby increasing or decreasing the efficiency of splicing. One study has shown that the structure of the promoter can affect the splicing pattern of the *FN1* (fibronectin 1) pre-mRNA by facilitating the differential recruitment of ASF/SF2 (REF. 113). It is conceivable that this reflects the ability of different

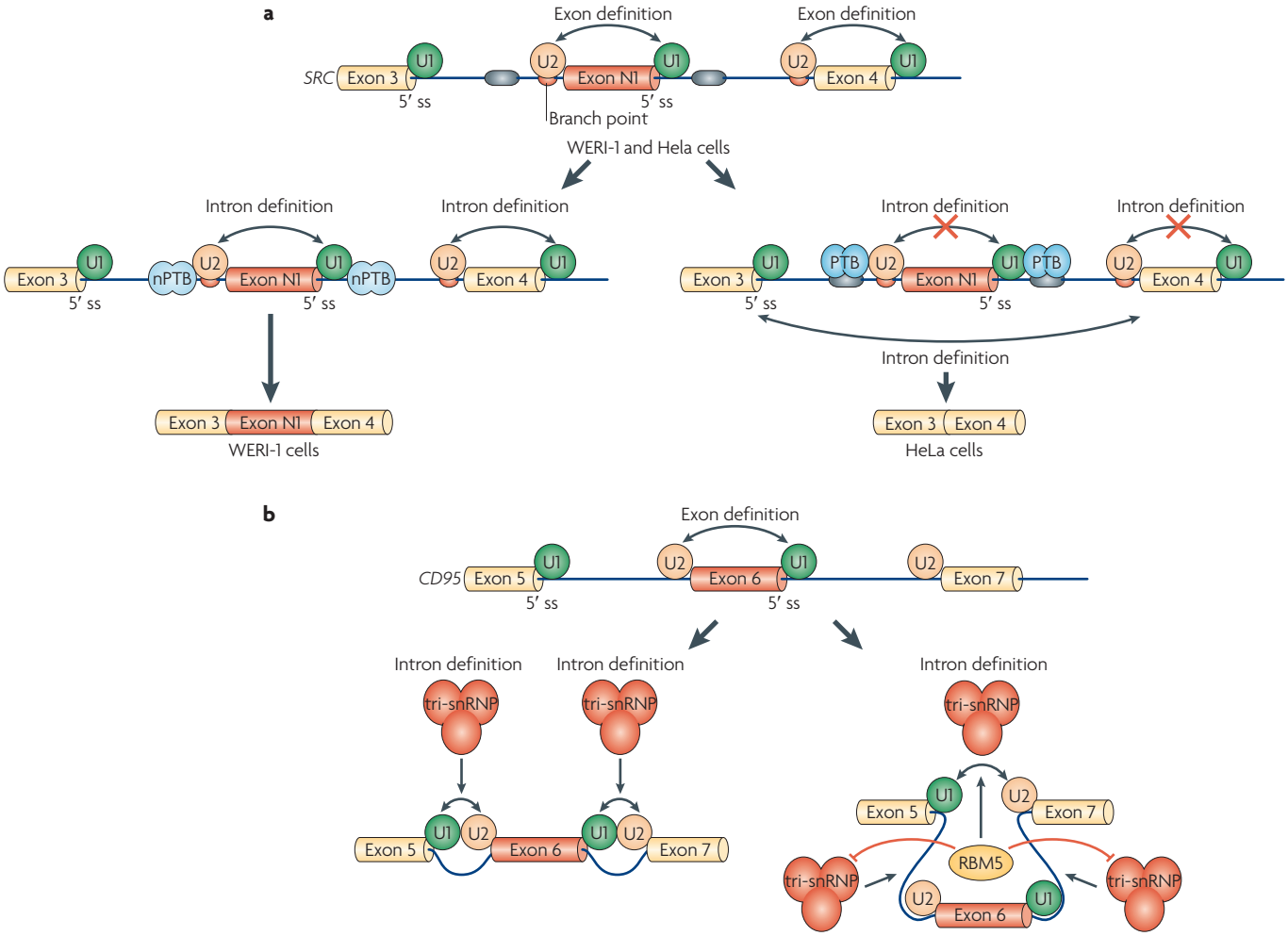


Figure 3 | **Mechanisms of alternative splicing regulation at the transition from exon definition to intron definition.** **a** | Polypyrimidine-tract binding protein (PTB) inhibits the inclusion of *SRC* exon N1 by inhibiting the interactions of the U1 and U2 small nuclear ribonucleoprotein particles (snRNPs) and intron definition. In both WERI-1 and HeLa cells, the N1 exon is defined by the binding of U1 snRNP to the 5′ splice site (ss) and of U2 snRNP to the branch point. In WERI-1 cells, in the absence of PTB, U1 and U2 snRNPs bound to the N1 exon interact with the U2 and U1 snRNP on adjacent constitutive exons, respectively, thereby allowing efficient spliceosome assembly on introns flanking exon N1. In HeLa cells, PTB binds to sequences flanking exon N1 and prevents the cross-intron interactions that occur in WERI- cells, thereby excluding exon N1. **b** | RBM5 (RNA-binding protein 5) regulates alternative splicing of *CD95* by inhibiting the inclusion of exon 6. It does so not at the stage of U1 and U2 snRNP binding, but instead by promoting tri-snRNP assembly on the intron-defined spliceosomal complex between exon 5 and exon 7, while blocking tri-snRNP recruitment to complexes that would result in inclusion of exon 6. Double-headed arrows indicate intron and exon definition.

transcription factors to influence the recruitment of distinct splicing factors to the nascent pre-mRNA, resulting in the inclusion or exclusion of the alternative exon. For example, PGC1 (peroxisome proliferator-activated receptor-γ coactivator 1), a transcription co-activator that is recruited to target genes by specific transcription factors, can modulate the alternative splicing of nascent RNA transcripts by interacting with other splicing factors through its RS domain[114].

Another study showed that differential recruitment of transcription co-activators to progesterone- and oestrogen-responsive elements upstream of reporter genes, such as *CD44*, alters the alternative splicing of the resultant mRNA transcripts[112]. Specifically, recruitment of activating signal cointegrator 1(ASC1) and ASC2 and their associated proteins to these elements was found to both activate the transcription of the reporter genes and affect splicing, but in opposite ways. The ASC1-associated protein CAPER (coactivator of activating protein 1 and oestrogen receptors; also known as RBM39) contains an RS domain and two RRM domains, similarly to SR proteins, whereas the ASC2-associated protein COAA (also known as RBM14) is structurally related to hnRNP A1. It was suggested that the antagonistic effects of the ASC1 and ASC2 complexes are mediated by these factors.

A second, kinetic model proposes that the rate of transcription elongation influences the inclusion of alternative exons by affecting whether the splicing machinery is recruited sufficiently quickly for spliceosome

RRM domain
(RNA recognition motif domain). A protein domain that is frequently involved in sequence-specific single-stranded RNA binding. Also known as an RNP-type RNA-binding domain.

assembly and splicing to occur. In support of this model, an RNAP II with a reduced elongation rate caused by a point mutation was found to greatly stimulate the inclusion of an alternative exon that has weak splice sites[115]. Specifically, it was found that slow transcription favours the inclusion of *FN1* EDI exon, which was excluded when transcription was more rapid. Consistent with this, a recent study showed that changes in the RNAP II elongation rate following ultraviolet irradiation could lead to changes in alternative splicing that occur in response to DNA damage[116].

One way of changing the transcription rate is through changing the phosphorylation status of RNAP II. The carboxy-terminal domain of the largest subunit of RNAP II consists of up to 52 tandem repeats of the heptapeptide consensus sequence YSPTSPS[117]. Excess phosphorylation on Ser5 of the C-terminal domain is associated with RNAP II stalling downstream of the promoter region, whereas phosphorylation on Ser2 is associated with elongation through the gene[117,118]. One group[18] showed that a subunit of the human SWI–SNF (switching-defective–sucrose non-fermenting) complex, BRM, regulates changes in the alternative splicing of *CD44* pre-mRNA that are stimulated following T cell activation. On T cell stimulation, RNAP II phosphorylated on Ser5 pauses at the variant exon region of *CD44* by a mechanism requiring its association with BRM. Interestingly, this also results in increased association of BRM with components of the splicing machinery and splicing factor SAM68, leading to inclusion of the V5 exon. This study not only provides direct support of the kinetic model, but also shows that the mechanism for alternative splicing regulation by transcription can result from a combination of transcription elongation-related effects and differential recruitment of splicing factors.

Alternative splicing and tissue specificity

Alternative splicing regulation by tissue-specific splicing factors. Alternative splicing has an important role in defining tissue specificity. Recent high-throughput studies have shown that, of the human tissues examined, 50% or more of alternative splicing isoforms are differently expressed among tissues[29], indicating that most alternative splicing is subject to tissue-specific regulation.

Tissue-specific alternative splicing events can be explained in part by tissue-specific expression of splicing factors, and the corresponding regulation of their target mRNA transcripts[31,119,120]. In keeping with this, numerous tissue-specific alternative splicing regulators have now been identified (TABLE 2). Among all human tissues, brain is the most functionally diverse tissue, as it has the highest occurrence of tissue-specific alternative splicing isoforms. Accordingly, several brain-specific factors have been identified, such as nPTB[101,121], NOVA1, NOVA2 (REFS 81,84,122) and Hu/Elav proteins[123–125]. In addition, region- and cell type-specific expression of most of the >300 RNA-binding proteins examined was observed in proliferating and post-mitotic mouse brain cells[126]. PTB is expressed in neural progenitor cells, but its expression levels are greatly downregulated in differentiated neurons, where nPTB is upregulated[100,101]. Recent experiments provided evidence that the PTB-to-nPTB switch provides a post-transcriptional mechanism that is important for programming neuronal differentiation[101]. Using microarray analysis, it was shown that siRNA-mediated PTB depletion in N2A neuroblastoma cells caused the upregulation of nPTB and an altered alternative splicing pattern. Most of the observed changes were also detected when P19 cells (derived from an embryonal carcinoma) were differentiated into neuronal cells. In neuronal cells, expression of nPTB and downregulation of PTB explains ~25% of nervous system-specific alternative splicing[101]. However, the molecular mechanism that allows nervous system-specific alternative exons to be included in neuronal cells even when bound by nPTB is still unclear. It may reflect differences in the ability of nPTB and PTB to interact with other splicing factors and/or in the presence or absence of other splicing factors (for example, in the presence of Nova proteins) in neuronal and non-neuronal cells.

Other brain-specific factors, including Nova proteins, may be involved in fine tuning the programming of different types of neuronal cell. NOVA1 and NOVA2 are differentially expressed in post-natal mouse brain, with NOVA2 being highly expressed in the neocortex and hippocampus, and NOVA1 being expressed primarily in the hindbrain and spinal cord[127]. NOVA1-null mice die shortly after birth from a motor defect that is associated with apoptotic cell death of spinal and brainstem neurons. Furthermore, studies in mice in which NOVA2 has been conditionally knocked out showed that NOVA2 regulates ~7% of brain-specific splicing in the neocortex and that NOVA2-dependent alternative splicing regulates the expression of mRNA transcripts that encode synaptic functions[122]. These findings indicate that Nova proteins regulate alternative splicing events that result in transcripts with specific functions in the brain. Similarly to the roles of PTB and nPTB in helping to define non-neuronal and neuronal tissues, the expression of NOVA1 and NOVA2 may contribute to different functions of different brain regions. However, owing to the limited number of exons analysed and the overlap between PTB or nPTB and Nova targets, more detailed studies are needed to obtain a complete understanding of tissue-specific alternative splicing regulation by these factors.

Tissue-specific alternative splicing factors have recently been shown to be important in controlling the expression of epithelial cell-specific exons. One study[128] identified two paralogues, RBM35a (also known as ESRP1) and RBM35b (also known as ESRP2), that are important for the inclusion of epithelial cell-specific exons in several mRNA transcripts[128]. In addition, downregulation of RBM35a was found to coincide with the loss of epithelial splicing during the epithelial-to-mesenchymal cell transition, and ectopic expression of RBM35a in mesenchymal cells restored epithelial splicing. These data show that RBM35a and RBM35b contribute to defining the distinguishing characteristics of epithelial cells.

Table 2 | **Tissue-specific alternative splicing factors**

Name	Other names	Binding domain	Binding motif	Tissue expression	Target genes
nPTB	brPTB and PTBP2	RRM	CUCUCU	Neurons, myoblasts and testes	*BIN1*, *GLYRA2*, *ATP2B1*, *MEF2*, *NASP*, *SPAG9* and *SRC*
NOVA1	NA	KH	YCAY	Neurons of the hindbrain and spinal cord	*GABRG2*, *GLYRA2* and *NOVA1*
NOVA2	NA	KH	YCAY	Neurons of the cortex, hippocampus and dorsal spinal cord	*KCNJ*, *APLP2*, *GPHN*, *JNK2*, *NEO*, *GRIN1* and *PLCB4*
FOX1	A2BP1	RRM	(U)GCAUG	Muscle, heart and neurons	*ACTN*, *EWSR1* , *FGFR2*, *FN1* and *SRC*
FOX2	RBM9	RRM	(U)GCAUG	Muscle, heart and neurons	*EWS*, *FGFR2*, *FN1* and *SRC*
RBM35a	ESRP1	RRM	GU rich	Epithelial cells	*FGFR2*, *CD44*, *CTNND1* and *ENAH*
RBM35b	ESRP2	RRM	GU rich	Epithelial cells	*FGFR2*, *CD44*, *CTNND1* and *ENAH*
TIA1	mTIA1	RRM	U rich	Brain, spleen and testes	*MYPT1*, *CD95*, *CALCA*, *FGFR2*, *TIAR*, *IL8*, *VEGF*, *NF1* and *COL2A1*
TIAR	TIAL1 and mTIAR	RRM	U rich	Brain, spleen, lung, liver and testes	*TIA1*, *CALCA* , *TIAR*, *NF1* and *CD95*
SLM2	KHDRBS3 and TSTAR	KH	UAAA	Brain, tests and heart	*CD44* and *VEGFA*
Quaking	QK and QKL	KH	ACUAAY[…]UAAY	Brain	*MAG* and *PLP*
HUB	HUC, HUD and ELAV2	RRM	AU rich	Neurons	*CALCA*, *CD95* and *NF1*
MBNL	NA	CCCH zinc finger domain	YGCU(U/ G)Y	Muscles, uterus and ovaries	*TNTT2*, *INSR*, *CLCN1* and *TNNT3*
CELF1	BRUNOL2	RRM	U and G rich	Brain	*TNTT2* and *INSR*
ETR3	CELF2 and BRUNOL3	RRM	U and G rich	Heart, skeletal muscle and brain	*TNTT2*, *TAU* and *COX2*
CELF4	BRUNOL4	RRM	U and G rich	Muscle	*MTMR1* and *TNTT2*
CELF5	BRUNOL5 and NAPOR	RRM	U and G rich	Heart, skeletal muscle and brain	*ACTN*, *TNTT2* and *GRIN1*
CELF6	BRUNOL6	RRM	U and G rich	Kidney, brain and testes	*TNTT2*

A2BP1, ataxin 2-binding protein 1; *ACTN*, α-actinin; *APLP2*, amyloid-β precursor-like protein 2; *ATP2B1*, ATPase, Ca^{2+} transporting, plasma membrane 1; *BIN1*, bridging integrator 1; *CALCA*, calcitonin-related polypeptide-α; CELF, CUGBP- and ETR3-like factor; *CLCN1*, chloride channel 1; *COL2A1*, collagen, type II, α1; *COX2*, cytochrome *c* oxidase II; *CTNND1*, catenin δ1, *EWSR1*, Ewing sarcoma breakpoint region 1; *FGFR2*, fibroblast growth factor receptor 2; *FN1*, fibronectin 1; *GABRG2*, GABA A receptor, γ2; *GLYRA2*, glycine receptor, α2 subunit; *GPHN*, gephyrin; *GRIN1*, glutamate receptor, ionotropic, NMDA 3B; *IL8*, interleukin-8; *INSR*, insulin receptor; *JNK2*, Jun N-terminal kinase 2; *KCNJ*, potassium inwardly-rectifying channel, subfamily; KHDRBS3, KH domain-containing, RNA-binding, signal transduction-associated protein 3; *MAG*, myelin associated glycoprotein; MBNL, muscleblind; *MEF2*, myocyte enhancing factor 2; *MTMR1*, myotubularin-related protein 1; *NASP*, nuclear autoantigenic sperm protein; *NEO*, neogenin; *NF1*, neurofibromin 1; *NOVA*, neuro-oncological ventral antigen; *PLCB4*, phospholipase C β4; *PLP*, proteolipid protein; PTB, polypyrimidine-tract binding protein; RBM, RNA-binding protein; RRM, RNA recognition motif; *SLM2*, SAM68-like mammalian protein 2; *SPAG9*, sperm associated antigen 9; TIA1, T cell-restricted intracellular antigen 1; TIAR, TIA1-related protein; *TNTT2*, troponin T type 2; *VEGF*, vascular endothelial growth factor.

Alternative splicing regulation by constitutive splicing factors. SR proteins were originally discovered by biochemical methods as general, or non-sequence-specific, splicing activators[7]. However, more recent findings indicate that individual SR proteins can act as specific alternative splicing regulators in different cell types and tissues. Disruption of the genes that encode ASF/SF2 and SC35 specifically in the heart have shown that they have important but distinct roles in tissue development[129,130]. In addition, a recent study showed that mice with complete ablation of SRp38 survived through early embryogenesis and, strikingly, displayed only cardiac defects; these mice showed differences in alternative splicing[131].

Core spliceosomal proteins (CSPs) are also involved in alternative splicing regulation. Analysis of microarray-based expression profiles from mouse, chimpanzee and human tissues revealed that snRNPs are differentially expressed in particular tissues[132]. This is consistent with results from an RNA interference (RNAi) screen in *D. melanogaster*, which showed that changing levels of CSPs leads to changes in alternative splicing[26]. These CSPs include components of U1, U2 and U4/U6 snRNPs, as well as the U2AF heterodimer. Further evidence was provided by RNAi knockdown of the isoforms of U2AF35, U2AF35a and/or U2AF35b, and a subunit of splicing factor SF3B, SAP155 (also known as SF3B1), in human cells[44,133,134]. Knockdown of these CSPs was found to affect only alternative splicing of a subset of transcripts: for example, mRNAs encoding cell cycle phosphatases in the case of U2AF35 and 5′ splice site selection of *BCL2L1* (BCL-2-like 1; also known as *BCLX*) pre-mRNA in response to ceramide in the case of SAP155. In addition, evidence from budding yeast that showed differences in splicing patterns in response to different kinds of stress also suggests that CSPs are involved in alternative splicing regulation[135].

SMN, which is part of the SMN complex, has recently been shown to regulate alternative splicing in many mouse tissues[27]. The SMN complex is important for efficient assembly of snRNPs, and depletion of SMN in HeLa cells leads to a decrease in snRNP levels. SMN-deficient mice showed tissue-specific alterations in snRNAs, and different snRNPs were affected in different tissues, leading to an altered stoichiometry of snRNPs[27]. In addition, microarray analysis of total RNA samples from different tissues of SMN-deficient mice revealed changes in several alternative splicing events in various tissues[27]. The mechanism of alternative splicing alteration by SMN deficiency is unknown, but it is probable that the resulting changes in snRNP levels directly affected alternative splicing of specific pre-mRNAs. It will be important to understand how, and whether, these changes in alternative splicing contribute to spinal muscular atrophy, which is caused by SMN deficiency[136,137].

Alternative splicing regulation by post-translational modifications of splicing factors. Differences in protein expression levels of either tissue-specific splicing regulators or CSPs may not fully explain how cells can change alternative splicing patterns rapidly, for example in response to cellular stress. Mounting evidence has shown that post-translational modification of splicing factors can affect alternative splicing. The best studied modification is phosphorylation, and consistent with this, several well-studied cell signalling pathways have been shown to be involved in alternative splicing regulation (reviewed in REFS 138,139). Phosphorylation has been shown to affect the local concentration of splicing factors that are adjacent to pre-mRNA substrates, by altering their intracellular localization[140–144], protein–protein[43] and protein–RNA interactions[50,145] and even intrinsic splicing activity[39,54].

Phosphorylation can change the ability of splicing factors to interact with other proteins or mRNA substrates, leading to changes in splice site selection. Phosphorylation of RS domains in SR proteins affects their interaction with CSPs[43] and is necessary for sequence-specific mRNA binding *in vitro*[146]. Phosphorylation of TIA1 and TIA1-related protein (TIAR) by Fas-activated serine/threonine kinase (FASTK) enhances TIA1 and TIAR-mediated recruitment of U1 snRNP to a sub-optimal 5′ splice site, leading to the inclusion of *CD95* exon 6 (REF. 147). Tyrosine phosphorylation of SAM68 by Fyn protein kinase favours the formation of the anti-apoptotic *BCLXL* (B cell lymphoma XL) mRNA by interfering with the interaction between SAM68 and hnRNP A1, and with the interaction of both proteins with the pre-mRNA[148]. Phosphorylation of RS motifs of PTB-associated splicing factor PSF (also known as SPFQ) by SR kinases inhibits the binding of PSF to mRNA[145].

Phosphorylation can also change the intracellular localization of splicing factors. Osmotic shock stresses cells and activates the signalling pathway involving MEK3 (also known as MAPKK3), MEK6 (also known as MAPKK6) and p38, which leads to the relocalization of hnRNP A1 to the cytoplasm as a result of hyperphosphorylation. This change in hnRNP A1 localization can alter the alternative splicing pattern of an adenovirus *E1A* reporter transcript[141,144]. Furthermore, ischaemia triggers changes in Ca^{2+} concentration, leading to hyperphosphorylation of a TRA2 isoform, TRA2β1, and to its localization to the cytoplasm[143]. In addition, protein kinase A phosphorylates PTB on Ser16, which leads to its translocation to the cytoplasm[149,150].

Phosphorylation status can also, in one case, determine whether a splicing factor functions as a splicing repressor or activator. SRp38 acts as a global splicing repressor when dephosphorylated in the M phase of the cell cycle and following heat shock[54,55]. However, it becomes a sequence-specific activator when phosphorylated[39]. Shi and Manley[151] elucidated the detailed regulatory mechanism of SRp38 phosphorylation in response to heat shock. Specifically, at normal temperatures two mechanisms ensure that SRp38 remains phosphorylated (FIG. 2c): first, SRp38 is bound and protected by 14-3-3 proteins, and second, protein phosphatase 1 (PP1), which has been shown to target SRp38, is masked by associated proteins, including nuclear inhibitor of PP1 (NIPP1), which had previously been implicated in splicing control[152,153]. At increased temperatures 14-3-3 proteins dissociate from SRp38, and PP1 is released from NIPP1, thereby freeing PP1 and allowing it to dephosphorylate SRp38. Unlike other SR proteins, SRp38 is a poor substrate for the SR protein kinases CLK1 (CDC-like kinase; also known as STY) and SRPK1. Therefore, after dephosphorylation SRp38 remains dephosphorylated and can thus repress splicing events, unlike other SR proteins, which are rapidly rephosphorylated.

Conclusions and perspectives

The studies described here reveal the complexity of alternative splicing regulation. Alternative splicing can be regulated at different steps of spliceosome assembly by different splicing factors, both general and specific, and by many mechanisms that rely on *cis*-acting elements. Although alternative exons are shorter than constitutive exons and are flanked by longer introns, alternative exons are more conserved than constitutive exons, especially the exon–intron junctions, and these conserved regions often extend into flanking introns for 80–100 nucleotides[154], where *cis*-regulatory elements are embedded. Correct alternative splicing also depends on the stoichiometry and interactions of positive and negative regulatory proteins, including CSPs. Each cell type has a unique repertoire of SR proteins and hnRNPs, and moderate changes in their relative stoichiometry can have great effects on the pattern of alternative splicing[1,63]. It is possible that changes in the stoichiometry of snRNPs perturb the complex network of splicing factors and the interactions between splicing factors and CSPs. Therefore, alternative splicing regulatory networks have such an exquisite architecture that perturbation of any single step can lead to alternative splicing misregulation.

Diverse mechanisms are used to ensure tissue and cell type-specific splicing regulation. Accumulating evidence has shown that alternative splicing plays an important part in defining tissue specificity. The action of sequence-specific transcription factors has been thought to be the most robust way of defining tissue specificity[155].

14-3-3 protein
A protein belonging to a family of conserved proteins that bind to phosphorylated serine and threonine residues and that are encoded by seven genes in most mammals. They bind diverse regulatory proteins, including kinases, phosphatases and transmembrane receptors.

SELEX
A technique to determine the DNA or RNA sequence that is specifically recognized by a protein. The method involves multiple rounds of binding to an initially random sequence until a high-affinity consensus sequence emerges.

Importantly, >2,500 transcription factors have been identified in humans[156], whereas the reported number of sequence-specific alternative splicing factors is <50. Given that it now seems that alternative splicing is as prevalent and perhaps as important a mechanism as transcriptional control, what might be the explanation for this? One possibility is that many more splicing regulators remain to be discovered. However, the total number of putative RBPs in mice has been estimated to be less than 400 (REF. 126), and some fraction of these proteins will not be involved in splicing. Another possibility is that there are fundamental differences in how splicing and transcription are regulated. For example, individual splicing regulators control much larger groups of genes than specific transcription factors. This is consistent with the large numbers of neuronal transcripts that are thought to be controlled by PTB and Nova proteins[80,101,103,122]. It is also probable that considerable regulation is achieved by combinations of abundant regulators with limited sequence-specificity (that is, the SR proteins and hnRNPs), which act in concert to regulate different mRNA transcripts in different tissues depending on their relative concentrations.

Important goals of future studies of alternative splicing regulation include understanding how regulators switch key splicing events during development and in response to environmental stimuli, and how misregulation of alternative splicing leads to disease. Complete characterization of tissue-specific patterns of expression is of great importance to defining mechanisms of alternative splicing regulation in different cell types. *De novo* identification of regulatory motifs[31] and HITS-CLIP[80] are two complementary approaches to achieve this goal. However, until now only a limited number of alternative splicing regulators and tissues have been studied by these methods. In addition to Nova proteins and FOX2, CLIP-seq also provided a landscape of potential ASF/SF2 target mRNA transcripts and characterized a purine-rich consensus motif[87,88] that is nearly identical to a consensus sequence obtained previously by an *in vitro* SELEX (systematic evolution of ligands by exponential enrichment) approach[157]. A database that includes more comprehensive information on the expression patterns of proteins that regulate alternative splicing, definition of potential target mRNA transcripts and positions of binding motifs on these transcripts will facilitate searches for regulatory proteins that control specific splicing events, such as of genes that are involved in disease, and possibly provide insights into underlying mechanisms.

Owing to the dynamic nature of spliceosome assembly and the potential for regulation at multiple points, detailed proteomic analysis will be important in completely elucidating the molecular mechanisms of alternative splicing regulation. For example, how do RNA-binding proteins interact with other factors and core splicing factors? When and where do splicing factors function to regulate the spliceosome? How do post-translational modifications influence these events? Obtaining a full understanding of the mechanisms underlying alternative splicing and its role in defining tissue specificity will require multiple approaches and methods.

1. Black, D. L. Mechanisms of alternative pre-messenger RNA splicing. *Annu. Rev. Biochem.* **72**, 291–336 (2003).
2. Wahl, M. C., Will, C. L. & Luhrmann, R. The spliceosome: design principles of a dynamic RNP machine. *Cell* **136**, 701–718 (2009).
3. Berglund, J. A., Chua, K., Abovich, N., Reed, R. & Rosbash, M. The splicing factor BBP interacts specifically with the pre-mRNA branchpoint sequence UACUAAC. *Cell* **89**, 781–787 (1997).
4. Zamore, P. D. & Green, M. R. Identification, purification, and biochemical characterization of U2 small nuclear ribonucleoprotein auxiliary factor. *Proc. Natl Acad. Sci. USA* **86**, 9243–9247 (1989).
5. Nelson, K. K. & Green, M. R. Mammalian U2 snRNP has a sequence-specific RNA-binding activity. *Genes Dev.* **3**, 1562–1571 (1989).
6. Graveley, B. R. Sorting out the complexity of SR protein functions. *RNA* **6**, 1197–1211 (2000).
7. Tacke, R. & Manley, J. L. Determinants of SR protein specificity. *Curr. Opin. Cell Biol.* **11**, 358–362 (1999).
8. Long, J. C. & Caceres, J. F. The SR protein family of splicing factors: master regulators of gene expression. *Biochem. J.* **417**, 15–27 (2009).
9. Smith, C. W. & Valcarcel, J. Alternative pre-mRNA splicing: the logic of combinatorial control. *Trends Biochem. Sci.* **25**, 381–388 (2000).
10. Dreyfuss, G., Kim, V. N. & Kataoka, N. Messenger-RNA-binding proteins and the messages they carry. *Nature Rev. Mol. Cell Biol.* **3**, 195–205 (2002).
11. Ule, J. *et al.* An RNA map predicting Nova-dependent splicing regulation. *Nature* **444**, 580–586 (2006).
12. Hui, J. *et al.* Intronic CA-repeat and CA-rich elements: a new class of regulators of mammalian alternative splicing. *EMBO J.* **24**, 1988–1998 (2005).
13. Yeo, G. W. *et al.* An RNA code for the FOX2 splicing regulator revealed by mapping RNA-protein interactions in stem cells. *Nature Struct. Mol. Biol.* **16**, 130–137 (2009).
14. Mauger, D. M., Lin, C. & Garcia-Blanco, M. A. hnRNP H and hnRNP F complex with Fox2 to silence fibroblast growth factor receptor 2 exon IIIc. *Mol. Cell. Biol.* **28**, 5403–5419 (2008).
15. House, A. E. & Lynch, K. W. An exonic splicing silencer represses spliceosome assembly after ATP-dependent exon recognition. *Nature Struct. Mol. Biol.* **13**, 937–944 (2006).
16. Sharma, S., Kohlstaedt, L. A., Damianov, A., Rio, D. C. & Black, D. L. Polypyrimidine tract binding protein controls the transition from exon definition to an intron defined spliceosome. *Nature Struct. Mol. Biol.* **15**, 183–191 (2008).
This study shows that PTB inhibits *SRC* exon N1 inclusion by preventing the transition from an exon-definition to an intron-definition complex and analyses the protein composition of different complexes.
17. Lallena, M. J., Chalmers, K. J., Llamazares, S., Lamond, A. I. & Valcarcel, J. Splicing regulation at the second catalytic step by Sex-lethal involves 3′ splice site recognition by SPF45. *Cell* **109**, 285–296 (2002).
18. Batsche, E., Yaniv, M. & Muchardt, C. The human SWI/SNF subunit Brm is a regulator of alternative splicing. *Nature Struct. Mol. Biol.* **13**, 22–29 (2006).
This study shows that BRM promotes the inclusion of variable exons of *CD44* pre-mRNA by stalling RNAP II at the variable exon-containing region of the *CD44* gene. It also shows that BRM interacts with splicing factor SAM68.
19. de la Mata, M. & Kornblihtt, A. R. RNA polymerase II C-terminal domain mediates regulation of alternative splicing by SRp20. *Nature Struct. Mol. Biol.* **13**, 973–980 (2006).
20. Sims, R. J. 3rd *et al.* Recognition of trimethylated histone H3 lysine 4 facilitates the recruitment of transcription postinitiation factors and pre-mRNA splicing. *Mol. Cell* **28**, 665–676 (2007).
21. Lin, S., Coutinho-Mansfield, G., Wang, D., Pandit, S. & Fu, X. D. The splicing factor SC35 has an active role in transcriptional elongation. *Nature Struct. Mol. Biol.* **15**, 819–826 (2008).
22. Moldon, A. *et al.* Promoter-driven splicing regulation in fission yeast. *Nature* **455**, 997–1000 (2008).
23. Graveley, B. R. Alternative splicing: increasing diversity in the proteomic world. *Trends Genet.* **17**, 100–107 (2001).
24. Blencowe, B. J. & Graveley, B. R. (eds) *Alternative Splicing in the Postgenomic Era*. (Springer, the Netherlands, 2007).
25. Grabowski, P. J. & Black, D. L. Alternative RNA splicing in the nervous system. *Prog. Neurobiol.* **65**, 289–308 (2001).
26. Park, J. W., Parisky, K., Celotto, A. M., Reenan, R. A. & Graveley, B. R. Identification of alternative splicing regulators by RNA interference in *Drosophila*. *Proc. Natl Acad. Sci. USA* **101**, 15974–15979 (2004).
27. Zhang, Z. *et al.* SMN deficiency causes tissue-specific perturbations in the repertoire of snRNAs and widespread defects in splicing. *Cell* **133**, 585–600 (2008).
This paper shows that SMN deficiency regulates snRNP levels in a tissue-specific manner, and this was reflected in altered alternative splicing patterns in different mouse tissues.
28. Blencowe, B. J. Alternative splicing: new insights from global analyses. *Cell* **126**, 37–47 (2006).
29. Wang, E. T. *et al.* Alternative isoform regulation in human tissue transcriptomes. *Nature* **456**, 470–476 (2008).
30. Pan, Q., Shai, O., Lee, L. J., Frey, B. J. & Blencowe, B. J. Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nature Genet.* **40**, 1413–1415 (2008).
31. Castle, J. C. *et al.* Expression of 24,426 human alternative splicing events and predicted *cis* regulation in 48 tissues and cell lines. *Nature Genet.* **40**, 1416–1425 (2008).
A transcriptome study that analyses alternative splicing events from 48 tissues and identifies tissue-specific regulatory motifs and cognate binding proteins.
32. Sultan, M. *et al.* A global view of gene activity and alternative splicing by deep sequencing of the human transcriptome. *Science* **321**, 956–960 (2008).
33. Sterner, D. A., Carlo, T. & Berget, S. M. Architectural limits on split genes. *Proc. Natl Acad. Sci. USA* **93**, 15081–15085 (1996).

34. Berget, S. M. Exon recognition in vertebrate splicing. *J. Biol. Chem.* **270**, 2411–2414 (1995).
35. Lim, S. R. & Hertel, K. J. Commitment to splice site pairing coincides with A complex formation. *Mol. Cell* **15**, 477–483 (2004).
36. Kotlajich, M. V., Crabb, T. L. & Hertel, K. J. Spliceosome assembly pathways for different types of alternative splicing converge during commitment to splice site pairing in the A complex. *Mol. Cell. Biol.* **29**, 1072–1082 (2009).
This study shows that the commitment to splicing of some alternative exons occurs during splice site pairing in the A complex and that ATP hydrolysis is required for splice site paring, thereby locking splice sites into a splicing pattern after U2 snRNP binding to the branch site.
37. Bourgeois, C. F., Popielarz, M., Hildwein, G. & Stevenin, J. Identification of a bidirectional splicing enhancer: differential involvement of SR proteins in 5′ or 3′ splice site activation. *Mol. Cell. Biol.* **19**, 7347–7356 (1999).
38. Zuo, P. & Maniatis, T. The splicing factor U2AF35 mediates critical protein–protein interactions in constitutive and enhancer-dependent splicing. *Genes Dev.* **10**, 1356–1368 (1996).
39. Feng, Y., Chen, M. & Manley, J. L. Phosphorylation switches the general splicing repressor SRp38 to a sequence-specific activator. *Nature Struct. Mol. Biol.* **15**, 1040–1048 (2008).
This paper shows that phosphorylation switches SRp38 from a general repressor to a sequence-specific activator that functions by recruiting and stabilizing U1 and U2 snRNP at splice sites.
40. Graveley, B. R., Hertel, K. J. & Maniatis, T. The role of U2AF35 and U2AF65 in enhancer-dependent splicing. *RNA* **7**, 806–818 (2001).
41. Kohtz, J. D. *et al.* Protein–protein interactions and 5′-splice-site recognition in mammalian mRNA precursors. *Nature* **368**, 119–124 (1994).
42. Wu, J. Y. & Maniatis, T. Specific interactions between proteins implicated in splice site selection and regulated alternative splicing. *Cell* **75**, 1061–1070 (1993).
43. Xiao, S. H. & Manley, J. L. Phosphorylation of the ASF/SF2 RS domain affects both protein-protein and protein-RNA interactions and is necessary for splicing. *Genes Dev.* **11**, 334–344 (1997).
44. Pacheco, T. R., Coelho, M. B., Desterro, J. M., Mollet, I. & Carmo-Fonseca, M. *In vivo* requirement of the small subunit of U2AF for recognition of a weak 3′ splice site. *Mol. Cell. Biol.* **26**, 8183–90 (2006).
45. Longman, D. *et al.* Multiple interactions between SRm160 and SR family proteins in enhancer-dependent splicing and development of *C. elegans*. *Curr. Biol.* **11**, 1923–1933 (2001).
46. Blencowe, B. J. *et al.* The SRm160/300 splicing coactivator subunits. *RNA* **6**, 111–120 (2000).
47. Tacke, R. & Manley, J. L. Functions of SR and Tra2 proteins in pre-mRNA splicing regulation. *Proc. Soc. Exp. Biol. Med.* **220**, 59–63 (1999).
48. Izquierdo, J. M. *et al.* Regulation of Fas alternative splicing by antagonistic effects of TIA-1 and PTB on exon definition. *Mol. Cell* **19**, 475–484 (2005).
49. Forch, P., Puig, O., Martinez, C., Seraphin, B. & Valcarcel, J. The splicing regulator TIA-1 interacts with U1-C to promote U1 snRNP recruitment to 5′ splice sites. *EMBO J.* **21**, 6882–6892 (2002).
50. Tisserant, A. & Konig, H. Signal-regulated pre-mRNA occupancy by the general splicing factor U2AF. *PLoS ONE* **3**, e1418 (2008).
51. Yang, L., Embree, L. J., Tsai, S. & Hickstein, D. D. Oncoprotein TLS interacts with serine-arginine proteins involved in RNA splicing. *J. Biol. Chem.* **273**, 27761–27764 (1998).
52. Komatsu, M., Kominami, E., Arahata, K. & Tsukahara, T. Cloning and characterization of two neural-salient serine/arginine-rich (NSSR) proteins involved in the regulation of alternative splicing in neurones. *Genes Cells* **4**, 593–606 (1999).
53. Cowper, A. E., Caceres, J. F., Mayeda, A. & Screaton, G. R. Serine-arginine (SR) protein-like factors that antagonize authentic SR proteins and regulate alternative splicing. *J. Biol. Chem.* **276**, 48908–48914 (2001).
54. Shin, C. & Manley, J. L. The SR protein SRp38 represses splicing in M phase cells. *Cell* **111**, 407–417 (2002).
55. Shin, C., Feng, Y. & Manley, J. L. Dephosphorylated SRp38 acts as a splicing repressor in response to heat shock. *Nature* **427**, 553–558 (2004).
56. Krainer, A. R., Conway, G. C. & Kozak, D. Purification and characterization of pre-mRNA splicing factor SF2 from HeLa cells. *Genes Dev.* **4**, 1158–1171 (1990).
57. Singh, R., Valcarcel, J. & Green, M. R. Distinct binding specificities and functions of higher eukaryotic polypyrimidine tract-binding proteins. *Science* **268**, 1173–1176 (1995).
58. Spellman, R. & Smith, C. W. Novel modes of splicing repression by PTB. *Trends Biochem. Sci.* **31**, 73–76 (2006).
59. Sauliere, J., Sureau, A., Expert-Bezancon, A. & Marie, J. The polypyrimidine tract binding protein (PTB) represses splicing of exon 6B from the β-tropomyosin pre-mRNA by directly interfering with the binding of the U2AF65 subunit. *Mol. Cell. Biol.* **26**, 8755–8769 (2006).
60. Tange, T. O., Damgaard, C. K., Guth, S., Valcarcel, J. & Kjems, J. The hnRNP A1 protein regulates HIV-1 *tat* splicing via a novel intron silencer element. *EMBO J.* **20**, 5748–5758 (2001).
61. Zhou, H. L. & Lou, H. Repression of prespliceosome complex formation at two distinct steps by Fox-1/Fox-2 proteins. *Mol. Cell. Biol.* **28**, 5507–5516 (2008).
62. Zhu, H., Hinman, M. N., Hasman, R. A., Mehta, P. & Lou, H. Regulation of neuron-specific alternative splicing of neurofibromatosis type 1 pre-mRNA. *Mol. Cell. Biol.* **28**, 1240–1251 (2008).
63. Kashima, T. & Manley, J. L. A negative element in SMN2 exon 7 inhibits splicing in spinal muscular atrophy. *Nature Genet.* **34**, 460–463 (2003).
64. Martins de Araujo, M., Bonnal, S., Hastings, M. L., Krainer, A. R. & Valcarcel, J. Differential 3′ splice site recognition of *SMN1* and *SMN2* transcripts by U2AF and U2 snRNP. *RNA* **15**, 515–523 (2009).
65. Sharma, S., Falick, A. M. & Black, D. L. Polypyrimidine tract binding protein blocks the 5′ splice site-dependent assembly of U2AF and the prespliceosomal E complex. *Mol. Cell* **19**, 485–496 (2005).
66. Damgaard, C. K., Tange, T. O. & Kjems, J. hnRNP A1 controls HIV-1 mRNA splicing through cooperative binding to intron and exon splicing silencers in the context of a conserved secondary structure. *RNA* **8**, 1401–1415 (2002).
67. Nasim, F. U., Hutchison, S., Cordeau, M. & Chabot, B. High-affinity hnRNP A1 binding sites and duplex-forming inverted repeats have similar effects on 5′ splice site selection in support of a common looping out and repression mechanism. *RNA* **8**, 1078–1089 (2002).
68. Hutchison, S., LeBel, C., Blanchette, M. & Chabot, B. Distinct sets of adjacent heterogeneous nuclear ribonucleoprotein (hnRNP) A1/A2 binding sites control 5′ splice site selection in the hnRNP A1 mRNA precursor. *J. Biol. Chem.* **277**, 29745–29752 (2002).
69. Kashima, T., Rao, N., David, C. J. & Manley, J. L. hnRNP A1 functions with specificity in repression of *SMN2* exon 7 splicing. *Hum. Mol. Genet.* **16**, 3149–3159 (2007).
70. Kashima, T., Rao, N. & Manley, J. L. An intronic element contributes to splicing repression in spinal muscular atrophy. *Proc. Natl Acad. Sci. USA* **104**, 3426–3431 (2007).
71. Chou, M. Y., Underwood, J. G., Nikolic, J., Luu, M. H. & Black, D. L. Multisite RNA binding and release of polypyrimidine tract binding protein during the regulation of c-src neural-specific splicing. *Mol. Cell* **5**, 949–957 (2000).
72. Mayeda, A., Helfman, D. M. & Krainer, A. R. Modulation of exon skipping and inclusion by heterogeneous nuclear ribonucleoprotein A1 and pre-mRNA splicing factor SF2/ASF. *Mol. Cell. Biol.* **13**, 2993–3001 (1993).
73. Zahler, A. M., Damgaard, C. K., Kjems, J. & Caputi, M. SC35 and heterogeneous nuclear ribonucleoprotein *A/B* proteins bind to a juxtaposed exonic splicing enhancer/exonic splicing silencer element to regulate HIV-1 *tat* exon 2 splicing. *J. Biol. Chem.* **279**, 10077–10084 (2004).
74. Zhu, J. & Krainer, A. R. Pre-mRNA splicing in the absence of an SR protein RS domain. *Genes Dev.* **14**, 3166–3178 (2000).
75. Crawford, J. B. & Patton, J. G. Activation of α-tropomyosin exon 2 is regulated by the SR protein 9G8 and heterogeneous nuclear ribonucleoproteins H and F. *Mol. Cell. Biol.* **26**, 8791–802 (2006).
76. Expert-Bezancon, A. *et al.* hnRNP A1 and the SR proteins ASF/SF2 and SC35 have antagonistic functions in splicing of β-tropomyosin exon 6B. *J. Biol. Chem.* **279**, 38249–59 (2004).
77. Charlet, B. N., Logan, P., Singh, G. & Cooper, T. A. Dynamic antagonism between ETR-3 and PTB regulates cell type-specific alternative splicing. *Mol. Cell* **9**, 649–658 (2002).
78. Blanchette, M. *et al.* Genome-wide analysis of alternative pre-mRNA splicing and RNA-binding specificities of the *Drosophila* hnRNP *A/B* family members. *Mol. Cell* **33**, 438–449 (2009).
79. Hung, L. H. *et al.* Diverse roles of hnRNP L in mammalian mRNA processing: a combined microarray and RNAi analysis. *RNA* **14**, 284–296 (2008).
80. Licatalosi, D. D. *et al.* HITS-CLIP yields genome-wide insights into brain alternative RNA processing. *Nature* **456**, 464–469 (2008).
This paper introduces a method that combines CLIP and high-throughput sequencing to identify targets of Nova proteins.
81. Dredge, B. K., Stefani, G., Engelhard, C. C. & Darnell, R. B. Nova autoregulation reveals dual functions in neuronal splicing. *EMBO J.* **24**, 1608–1620 (2005).
82. Martinez-Contreras, R. *et al.* Intronic binding sites for hnRNP A/B and hnRNP F/H proteins stimulate pre-mRNA splicing. *PLoS Biol.* **4**, e21 (2006).
83. Wang, Z. & Burge, C. B. Splicing regulation: from a parts list of regulatory elements to an integrated splicing code. *RNA* **14**, 802–813 (2008).
84. Dredge, B. K. & Darnell, R. B. Nova regulates $GABA_A$ receptor γ2 alternative splicing via a distal downstream UCAU-rich intronic splicing enhancer. *Mol. Cell. Biol.* **23**, 4687–4700 (2003).
85. Schaub, M. C., Lopez, S. R. & Caputi, M. Members of the heterogeneous nuclear ribonucleoprotein H family activate splicing of an HIV-1 splicing substrate by promoting formation of ATP-dependent spliceosomal complexes. *J. Biol. Chem.* **282**, 13617–13626 (2007).
86. Caputi, M. & Zahler, A. M. Determination of the RNA binding specificity of the heterogeneous nuclear ribonucleoprotein (hnRNP) H/H′/F/2H9 family. *J. Biol. Chem.* **276**, 43850–43859 (2001).
87. Sanford, J. R. *et al.* Identification of nuclear and cytoplasmic mRNA targets for the shuttling protein SF2/ASF. *PLoS ONE* **3**, e3369 (2008).
88. Sanford, J. R. *et al.* Splicing factor SFRS1 recognizes a functionally diverse landscape of RNA transcripts. *Genome Res.* **19**, 381–394 (2009).
89. Graveley, B. R. Mutually exclusive splicing of the insect *Dscam* pre-mRNA directed by competing intronic RNA secondary structures. *Cell* **123**, 65–73 (2005).
90. Olson, S. *et al.* A regulator of Dscam mutually exclusive splicing fidelity. *Nature Struct. Mol. Biol.* **14**, 1134–1140 (2007).
91. Grover, A. *et al.* 5′ splice site mutations in *tau* associated with the inherited dementia FTDP-17 affect a stem-loop structure that regulates alternative splicing of exon 10. *J. Biol. Chem.* **274**, 15134–15143 (1999).
92. Hiller, M., Zhang, Z., Backofen, R. & Stamm, S. Pre-mRNA secondary structures influence exon recognition. *PLoS Genet.* **3**, e204 (2007).
93. Camats, M., Guil, S., Kokolo, M. & Bach-Elias, M. P68 RNA helicase (DDX5) alters activity of *cis*- and *trans*-acting factors of the alternative splicing of H-Ras. *PLoS ONE* **3**, e2926 (2008).
94. Libri, D., Balvay, L. & Fiszman, M. Y. *In vivo* splicing of the beta tropomyosin pre-mRNA: a role for branch point and donor site competition. *Mol. Cell. Biol.* **12**, 3204–3215 (1992).
95. Henkin, T. M. Riboswitch RNAs: using RNA to sense cellular metabolism. *Genes Dev.* **22**, 3383–3390 (2008).
96. Cheah, M. T., Wachter, A., Sudarsan, N. & Breaker, R. R. Control of alternative RNA splicing and gene expression by eukaryotic riboswitches. *Nature* **447**, 497–500 (2007).
97. Kishore, S. & Stamm, S. Regulation of alternative splicing by snoRNAs. *Cold Spring Harb. Symp. Quant. Biol.* **71**, 329–334 (2006).
98. Kishore, S. & Stamm, S. The snoRNA HBII-52 regulates alternative splicing of the serotonin receptor 2C. *Science* **311**, 230–232 (2006).
99. Yu, Y. *et al.* Dynamic regulation of alternative splicing by silencers that modulate 5′ splice site competition. *Cell* **135**, 1224–1236 (2008).
The authors screen for splicing silencers that favour the inclusion of a distal 5′ splice site and provide evidence that the silencers work by changing the conformation of the pre-mRNA–U1 snRNP complex and promoting pairing of the distal U1 snRNP with U2 snRNP.

100. Makeyev, E. V., Zhang, J., Carrasco, M. A. & Maniatis, T. The microRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. *Mol. Cell* **27**, 435–448 (2007).
101. Boutz, P. L. *et al.* A post-transcriptional regulatory switch in polypyrimidine tract-binding proteins reprograms alternative splicing in developing neurons. *Genes Dev.* **21**, 1636–1652 (2007).
Using siRNA and microarray analysis, the authors show that the PTB-to-nPTB switch provides a post-transcriptional mechanism that is important for programming neuronal differentiation.
102. Coutinho-Mansfield, G. C., Xue, Y., Zhang, Y. & Fu, X. D. PTB/nPTB switch: a post-transcriptional mechanism for programming neuronal differentiation. *Genes Dev.* **21**, 1573–1577 (2007).
103. Spellman, R., Llorian, M. & Smith, C. W. Crossregulation and functional redundancy between the splicing regulator PTB and its paralogs nPTB and ROD1. *Mol. Cell* **27**, 420–434 (2007).
104. Ohi, M. D. *et al.* Structural and functional analysis of essential pre-mRNA splicing factor Prp19p. *Mol. Cell. Biol.* **25**, 451–460 (2005).
105. Bessonov, S., Anokhina, M., Will, C. L., Urlaub, H. & Luhrmann, R. Isolation of an active step I spliceosome and composition of its RNP core. *Nature* **452**, 846–850 (2008).
106. Eldridge, A. G., Li, Y., Sharp, P. A. & Blencowe, B. J. The SRm160/300 splicing coactivator is required for exon-enhancer function. *Proc. Natl Acad. Sci. USA* **96**, 6125–6130 (1999).
107. Edamatsu, H., Kaziro, Y. & Itoh, H. *LUCA15*, a putative tumour suppressor gene encoding an RNA-binding nuclear protein, is down-regulated in ras-transformed Rat-1 cells. *Genes Cells* **5**, 849–858 (2000).
108. Mourtada-Maarabouni, M., Sutherland, L. C. & Williams, G. T. Candidate tumour suppressor LUCA-15 can regulate multiple apoptotic pathways. *Apoptosis* **7**, 421–432 (2002).
109. Bonnal, S. *et al.* RBM5/Luca-15/H37 regulates Fas alternative splice site pairing after exon definition. *Mol. Cell* **32**, 81–95 (2008).
110. Kornblihtt, A. R. Chromatin, transcript elongation and alternative splicing. *Nature Struct. Mol. Biol.* **13**, 5–7 (2006).
111. Das, R. *et al.* SR proteins function in coupling RNAP II transcription to pre-mRNA splicing. *Mol. Cell* **26**, 867–881 (2007).
112. Auboeuf, D. *et al.* Differential recruitment of nuclear receptor coactivators may determine alternative RNA splice site choice in target genes. *Proc. Natl Acad. Sci. USA* **101**, 2270–2274 (2004).
113. Cramer, P. *et al.* Coupling of transcription with alternative splicing: RNA pol II promoters modulate SF2/ASF and 9G8 effects on an exonic splicing enhancer. *Mol. Cell* **4**, 251–258 (1999).
114. Monsalve, M. *et al.* Direct coupling of transcription and mRNA processing through the thermogenic coactivator PGC-1. *Mol. Cell* **6**, 307–316 (2000).
115. de la Mata, M. *et al.* A slow RNA polymerase II affects alternative splicing *in vivo*. *Mol. Cell* **12**, 525–532 (2003).
116. Munoz, M. J. *et al.* DNA damage regulates alternative splicing through inhibition of RNA polymerase II elongation. *Cell* **137**, 708–720 (2009).
117. Phatnani, H. P. & Greenleaf, A. L. Phosphorylation and functions of the RNA polymerase II CTD. *Genes Dev.* **20**, 2922–2936 (2006).
118. Lin, P. S., Marshall, N. F. & Dahmus, M. E. CTD phosphatase: role in RNA polymerase II cycling and the regulation of transcript elongation. *Prog. Nucleic Acid Res. Mol. Biol.* **72**, 333–365 (2002).
119. David, C. J. & Manley, J. L. The search for alternative splicing regulators: new approaches offer a path to a splicing code. *Genes Dev.* **22**, 279–285 (2008).
120. Ohno, G., Hagiwara, M. & Kuroyanagi, H. STAR family RNA-binding protein ASD-2 regulates developmental switching of mutually exclusive alternative splicing *in vivo*. *Genes Dev.* **22**, 360–374 (2008).
121. Li, Q., Lee, J. A. & Black, D. L. Neuronal regulation of alternative pre-mRNA splicing. *Nature Rev. Neurosci.* **8**, 819–831 (2007).
122. Ule, J. *et al.* Nova regulates brain-specific splicing to shape the synapse. *Nature Genet.* **37**, 844–852 (2005).
123. Perrone-Bizzozero, N. & Bolognani, F. Role of HuD and other RNA-binding proteins in neural development and plasticity. *J. Neurosci. Res.* **68**, 121–126 (2002).
124. Zhu, H., Hasman, R. A., Barron, V. A., Luo, G. & Lou, H. A nuclear function of Hu proteins as neuron-specific alternative RNA processing regulators. *Mol. Biol. Cell* **17**, 5105–5114 (2006).
125. Soller, M., Li, M. & Haussmann, I. U. Regulation of the ELAV target *ewg*: insights from an evolutionary perspective. *Biochem. Soc. Trans.* **36**, 502–504 (2008).
126. McKee, A. E. *et al.* A genome-wide *in situ* hybridization map of RNA-binding proteins reveals anatomically restricted expression in the developing mouse brain. *BMC Dev. Biol.* **5**, 14 (2005).
127. Yang, Y. Y., Yin, G. L. & Darnell, R. B. The neuronal RNA-binding protein Nova-2 is implicated as the autoantigen targeted in POMA patients with dementia. *Proc. Natl Acad. Sci. USA* **95**, 13254–13259 (1998).
128. Warzecha, C. C., Sato, T. K., Nabet, B., Hogenesch, J. B. & Carstens, R. P. ESRP1 and ESRP2 are epithelial cell-type-specific regulators of FGFR2 splicing. *Mol. Cell* **33**, 591–601 (2009).
The authors identify ESRP1 and ESRP2 as epithelial cell-specific alternative splicing factors and find that they regulate several epithelial cell-specific exons. They also show that ESRP1 and ESRP2 expression levels correlate with the splicing pattern change that is observed during the epithelial-to-mesenchymal cell transition.
129. Ding, J. H. *et al.* Dilated cardiomyopathy caused by tissue-specific ablation of SC35 in the heart. *EMBO J.* **23**, 885–896 (2004).
130. Xu, X. *et al.* ASF/SF2-regulated CaMKIIδ alternative splicing temporally reprograms excitation-contraction coupling in cardiac muscle. *Cell* **120**, 59–72 (2005).
131. Feng, Y. *et al.* SRp38 regulates alternative splicing and is required for Ca^{2+} handling in the embryonic heart. *Dev. Cell* **16**, 528–538 (2009).
This study shows that mice lacking SRp38 die perinatally and have multiple cardiac defects, and that the mRNA encoding cardiac triadin is a direct target of SRp38.
132. Grosso, A. R. *et al.* Tissue-specific splicing factor gene expression signatures. *Nucleic Acids Res.* **36**, 4823–4832 (2008).
133. Massiello, A., Roesser, J. R. & Chalfant, C. E. SAP155 binds to ceramide-responsive RNA *cis*-element 1 and regulates the alternative 5′ splice site selection of Bcl-x pre-mRNA. *FASEB J.* **20**, 1680–1682 (2006).
134. Pacheco, T. R., Moita, L. F., Gomes, A. Q., Hacohen, N. & Carmo-Fonseca, M. RNA interference knockdown of hU2AF^{35} impairs cell cycle progression and modulates alternative splicing of Cdc25 transcripts. *Mol. Biol. Cell* **17**, 4187–4199 (2006).
135. Pleiss, J. A., Whitworth, G. B., Bergkessel, M. & Guthrie, C. Rapid, transcript-specific changes in splicing in response to environmental stress. *Mol. Cell* **27**, 928–937 (2007).
136. Monani, U. R. Spinal muscular atrophy: a deficiency in a ubiquitous protein; a motor neuron-specific disease. *Neuron* **48**, 885–896 (2005).
137. Gabanella, F. *et al.* Ribonucleoprotein assembly defects correlate with spinal muscular atrophy severity and preferentially affect a subset of spliceosomal snRNPs. *PLoS ONE* **2**, e921 (2007).
138. Shin, C. & Manley, J. L. Cell signalling and the control of pre-mRNA splicing. *Nature Rev. Mol. Cell Biol.* **5**, 727–738 (2004).
139. Tarn, W. Y. Cellular signals modulate alternative splicing. *J. Biomed. Sci.* **14**, 517–522 (2007).
140. Huang, Y., Yario, T. A. & Steitz, J. A. A molecular link between SR protein dephosphorylation and mRNA export. *Proc. Natl Acad. Sci. USA* **101**, 9666–9670 (2004).
141. van der Houven van Oordt, W. *et al.* The MKK(3/6)-p38-signaling cascade alters the subcellular distribution of hnRNP A1 and modulates alternative splicing regulation. *J. Cell Biol.* **149**, 307–316 (2000).
142. Habelhah, H. *et al.* ERK phosphorylation drives cytoplasmic accumulation of hnRNP-K and inhibition of mRNA translation. *Nature Cell Biol.* **3**, 325–330 (2001).
143. Daoud, R. *et al.* Ischemia induces a translocation of the splicing factor tra2-beta 1 and changes alternative splicing patterns in the brain. *J. Neurosci.* **22**, 5889–5899 (2002).
144. Guil, S., Long, J. C. & Caceres, J. F. hnRNP A1 relocalization to the stress granules reflects a role in the stress response. *Mol. Cell. Biol.* **26**, 5744–5758 (2006).
145. Huang, C. J., Tang, Z., Lin, R. J. & Tucker, P. W. Phosphorylation by SR kinases regulates the binding of PTB-associated splicing factor (PSF) to the pre-mRNA polypyrimidine tract. *FEBS Lett.* **581**, 223–232 (2007).
146. Tacke, R., Chen, Y. & Manley, J. L. Sequence-specific RNA binding by an SR protein requires RS domain phosphorylation: creation of an SRp40-specific splicing enhancer. *Proc. Natl Acad. Sci. USA* **94**, 1148–1153 (1997).
147. Izquierdo, J. M. & Valcarcel, J. Fas-activated serine/threonine kinase (FAST K) synergizes with TIA-1/TIAR proteins to regulate Fas alternative splicing. *J. Biol. Chem.* **282**, 1539–1543 (2007).
148. Paronetto, M. P., Achsel, T., Massiello, A., Chalfant, C. E. & Sette, C. The RNA-binding protein Sam68 modulates the alternative splicing of Bcl-x. *J. Cell Biol.* **176**, 929–939 (2007).
149. Ma, S., Liu, G., Sun, Y. & Xie, J. Relocalization of the polypyrimidine tract-binding protein during PKA-induced neurite growth. *Biochim. Biophys. Acta* **1773**, 912–923 (2007).
150. Xie, J., Lee, J. A., Kress, T. L., Mowry, K. L. & Black, D. L. Protein kinase A phosphorylation modulates transport of the polypyrimidine tract-binding protein. *Proc. Natl Acad. Sci. USA* **100**, 8776–8781 (2003).
151. Shi, Y. & Manley, J. L. A complex signaling pathway regulates SRp38 phosphorylation and pre-mRNA splicing in response to heat shock. *Mol. Cell* **28**, 79–90 (2007).
152. Van Eynde, A. *et al.* Molecular cloning of NIPP-1, a nuclear inhibitor of protein phosphatase-1, reveals homology with polypeptides involved in RNA processing. *J. Biol. Chem.* **270**, 28068–28074 (1995).
153. Cohen, P. T. Protein phosphatase 1— targeted in many directions. *J. Cell Sci.* **115**, 241–256 (2002).
154. Kim, E., Goren, A. & Ast, G. Alternative splicing: current perspectives. *Bioessays* **30**, 38–47 (2008).
155. Hallikas, O. *et al.* Genome-wide prediction of mammalian enhancers based on analysis of transcription-factor binding affinity. *Cell* **124**, 47–59 (2006).
156. Babu, M. M., Luscombe, N. M., Aravind, L., Gerstein, M. & Teichmann, S. A. Structure and evolution of transcriptional regulatory networks. *Curr. Opin. Struct. Biol.* **14**, 283–291 (2004).
157. Tacke, R. & Manley, J. L. The human splicing factors ASF/SF2 and SC35 possess distinct, functionally significant RNA binding specificities. *EMBO J.* **14**, 3540–3551 (1995).
158. Kent, O. A., Ritchie, D. B. & Macmillan, A. M. Characterization of a U2AF-independent commitment complex (E′) in the mammalian spliceosome assembly pathway. *Mol. Cell. Biol.* **25**, 233–40 (2005).
159. Shen, H. & Green, M. R. RS domains contact splicing signals and promote splicing by a common mechanism in yeast through humans. *Genes Dev.* **20**, 1755–1765 (2006).
160. Huang, Y., Gattoni, R., Stevenin, J. & Steitz, J. A. SR splicing factors serve as adapter proteins for TAP-dependent mRNA export. *Mol. Cell* **11**, 837–843 (2003).
161. Zhang, Z. & Krainer, A. R. Involvement of SR proteins in mRNA surveillance. *Mol. Cell* **16**, 597–607 (2004).
162. Sanford, J. R., Gray, N. K., Beckmann, K. & Caceres, J. F. A novel role for shuttling SR proteins in mRNA translation. *Genes Dev.* **18**, 755–768 (2004).
163. Cartegni, L. & Krainer, A. R. Disruption of an SF2/ASF-dependent exonic splicing enhancer in SMN2 causes spinal muscular atrophy in the absence of SMN1. *Nature Genet.* **30**, 377–384 (2002).
164. Cartegni, L., Hastings, M. L., Calarco, J. A., de Stanchina, E. & Krainer, A. R. Determinants of exon 7 splicing in the spinal muscular atrophy genes, SMN1 and SMN2. *Am. J. Hum. Genet.* **78**, 63–77 (2006).

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DATABASES

UniProtKB: http://www.uniprot.org
FOX1 | FOX2 | hnRNP A1 | hnRNP F | hnRNP H | hnRNP L | NOVA1 | NOVA2 | nPTB | PTB | RBM5 | SAM68 | SRp38

FURTHER INFORMATION

James L. Manley's homepage: http://www.columbia.edu/cu/biology/faculty/manley

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