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RNA splicing factors as oncoproteins and tumor suppressors

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Preface

The recent genomic characterization of cancers has revealed recurrent somatic point mutations and copy number changes affecting genes encoding RNA splicing factors. Initial studies of these ‘spliceosomal mutations’ suggest that the proteins bearing these mutations exhibit altered splice site and/or exon recognition preferences relative to their wild-type counterparts, resulting in cancer-specific mis-splicing. Such changes in the splicing machinery may create novel vulnerabilities in cancer cells that can be therapeutically exploited using compounds that can influence the splicing process. Further studies to dissect the biochemical, genomic, and biological effects of spliceosomal mutations are critical for the development of cancer therapies targeted to these mutations.

Splicing of mRNA precursors is required for the maturation of almost all human mRNAs, and furthermore is a key step in the regulation of expression of many genes. Alternative splicing, wherein a pre-mRNA can be processed into different mature mRNAs via splice site selection, allows multiple potential protein products to be generated from a single gene and thereby expands the cellular proteome. Although the functional roles of most isoforms generated by alternative splicing are unknown, specific isoforms have been identified that are selected in cancer due to their ability to promote neoplastic transformation, cancer progression, and/or therapeutic resistance (reviewed in[1,2]). In some cases, specific splicing changes are promoted or repressed by recurrent somatic point mutations near splice sites, which may promote cancer by inducing mis-splicing of genes encoding tumor suppressors[3,4].

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In addition to differential splicing of specific genes, cancers may also exhibit global splicing abnormalities. Genome-wide analyses of cancer transcriptomes[5–7] have revealed widespread splicing alterations such as inefficient intron removal in neoplastic tissues relative to their normal counterparts, but the functional consequences of these differences are unknown.

Finally, recurrent somatic mutations within genes encoding core spliceosomal proteins and associated RNA splicing factors are present at high frequencies in several cancers[8–12]. These mutations provide a direct genetic link between dysfunction of the splicing machinery and cancer. Both the genetic spectrum of mutations and functional studies of their consequences indicate that RNA splicing factors can act as proto-oncoproteins and tumor suppressors.

In this Review, we outline the current genetic and functional links between dysregulated and/or mutated RNA splicing factors and cancer. We discuss how recurrent mutations affecting splicing factors might promote the development and/or maintenance of cancer. We describe the challenges inherent in connecting mutations in spliceosomal proteins to specific downstream splicing changes, as well as the importance of testing whether mutated splicing factors dysregulate biological processes other than splicing itself. Finally, we discuss how determining the mechanistic consequences of mutated splicing factors may facilitate the identification of novel therapeutic opportunities to selectively target cancers with spliceosomal mutations.

RNA splicing catalysis and regulation

RNA splicing is essential for processing pre-mRNA transcribed from the >90% of human protein-coding genes that contain more than one exon into mature mRNAs prior to translation into proteins[13,14]. The primary function of splicing is the removal of non-coding introns, a process carried out by the large macromolecular machineries known as the major spliceosome and minor spliceosome (reviewed in[15]). The major spliceosome consists of five small nuclear ribonucleoprotein complexes (snRNPs, pronounced “snurps”), U1, U2, U4, U5 and U6, and it is responsible for the excision of >99% of human introns. The minor spliceosome contains the U5 snRNP, along with the U11, U12, U4atac and U6atac snRNPs, which are the functional analogues of the corresponding snRNPs in the major spliceosome (reviewed in[16]). Each constituent snRNP contains a different short non-coding RNA, an Sm or Sm-like protein complex that is required for the formation of the mature snRNP complex and proteins specific to each snRNP (reviewed in[15,17]).

Intron excision is facilitated by short sequence motifs in the pre-mRNA, in particular at boundaries between the upstream exon and intron (the 5′ splice site) and the intron and downstream exon (the 3′ splice site) (Fig. 1a). Although splicing itself is catalyzed by RNA[18], the proper recognition of splice sites relies upon RNA:RNA, RNA:protein and protein:protein interactions. U1 snRNP recognizes and binds to the 5′ splice site, whereas U2 snRNP interacts with the branch site region adjacent to the 3′ splice site, facilitated by interactions with U2 auxiliary factors (U2AFs, which form the U2AF complex) that bind the 3′ splice site. Following recruitment of the U4/U6.U5 tri-snRNP, the assembled spliceosome enters its active conformation and splicing proceeds via two sequential transesterification reactions (reviewed in[15,17]) (Fig. 1b).

Splice sites are typically categorized as constitutive splice sites or alternative splice sites, depending on whether they are always (constitutive) or only sometimes (alternative) recognized as splice sites and spliced into the mature mRNA. Splicing of both categories of splice sites is catalyzed by the same molecular machinery, although the efficient recruitment of spliceosomal proteins to alternative splice sites frequently depends on the binding of additional *trans*-acting factors. Although most splicing reactions in some eukaryotes such as *Saccharomyces cerevisiae* are constitutive — meaning that the same 5′ and 3′ splice sites are always ligated together — splicing is more complex in mammals.

Transcripts from almost all human multi-exon genes are alternatively spliced, where a particular 5′ splice site can be joined to different 3′ splice sites (or vice versa), frequently in a regulated fashion[13,14]. Alternative splicing events can be further classified based on how they affect the exonic structure of the mature mRNA (Fig. 1c). The distinction between constitutive and alternative splicing is empirical, and so can vary over time as additional transcript sequencing data becomes available. For example, high-throughput sequencing has transformed the study of RNA splicing by enabling the rapid and accurate quantification of genome-wide splicing patterns (Box 1). This has led to the realization that many splice sites and/or exons that were previously classified as constitutive are actually alternatively spliced (for example, in distinct cell types or in response to particular stimuli).

Alternative splicing is frequently regulated by *trans*-acting splicing factors, which bind to sequence motifs that are associated with the promotion (enhancers) or repression (silencers) of splicing. These motifs lie in both exons and introns, and frequently have the strongest effect when in close proximity to a splice site[19] (Fig. 1a; reviewed in[20,21]). Splicing enhancers and silencers are bound by diverse RNA-binding proteins, exemplified by the serine/arginine-rich proteins (SR proteins) and heterogeneous nuclear ribonucleoproteins (hnRNPs; reviewed in[22–25]). SR proteins contain one or two copies of an RNA recognition motif (RRM) domain at the N-terminus that provides RNA-binding specificity and a C-terminal arginine/serine-rich (RS) domain that contributes to protein-protein interactions[26,27]. hnRNPs similarly contain both RNA-binding domains and relatively unstructured domains that likely contribute to protein-protein interactions[28].

SR proteins and hnRNPs canonically promote and repress splicing, respectively, in a sequence-specific manner[22]. However, recent studies have revealed further nuances to these roles. The regulatory consequences of SR protein or hnRNP binding within pre-mRNA is frequently context-specific, giving rise to complex regulatory relationships (reviewed in[21]). For example, SR proteins can promote or repress splicing, depending on where they bind within a pre-mRNA[29,30]. Similarly, canonical splicing repressors such as the hnRNP polypyrimidine tract binding protein 1 (PTBP1; also known as PTB), can activate splicing in a context-dependent manner[31,32]. Some hnRNPs primarily act as splicing repressors, whereas others activate exon inclusion[33]. SR proteins and hnRNPs regulate splicing in diverse ways, including facilitating recruitment of the U1 or U2 snRNP, occluding a splice site, ‘looping out’ an exon and other mechanisms (reviewed in[20,21]).

Many other RNA-binding proteins regulate splicing in addition to the SR proteins and hnRNPs. These include the CUG-BP- and ETR-3-like (CELF) proteins (reviewed in[34]),

Muscleblind-like (MBNL) proteins (reviewed in[35]), RBFOX proteins, Signal transduction and activation of RNA (STAR) proteins, NOVA proteins[36], epithelial splicing regulatory protein (ESRP) families, T-cell-restricted intracellular antigen-1 (TIA1) and TIA1-like (TIAL1), and many others (reviewed in[21]). Furthermore, alterations in the levels of core spliceosomal components such as the snRNP proteins SmB/B′ (also known as snRNP-B)[37] can regulate splicing. Mass spectrometry studies indicate that the spliceosome is associated with >170 proteins[38], and computational studies of exon recognition suggest that hundreds of sequence motifs contribute to the regulation of splicing[39]. Although the total number of splicing factors is unknown, the above studies suggest that hundreds of proteins may have a role in splicing regulation.

Dysregulation of splicing in cancer

Pro- and anti-tumorigenic splicing factors

Just as regulation of alternative splicing has essential roles in cellular growth, differentiation and tissue development, dysregulated splicing can give rise to protein isoforms that contribute to tumor establishment, progression, and resistance to therapy. Many studies have found links between the altered expression and/or activity of splicing factors, cancer-associated splicing and transformation (reviewed in[1,2]) (Table 1). SR proteins, hnRNPs and other splicing factors can act as both oncoproteins and tumor suppressors.

Some SR proteins can act as oncoproteins when overexpressed in the correct cellular context. For example, SR splicing factor 1 (SRSF1; also known as ASF/SF2) is upregulated in cancers including lung, colon and breast cancer[40,41]. Modest overexpression of SRSF1 drove the immortalization of murine fibroblasts and human and mouse mammary epithelial cells[40,41]. SRSF1 promotes transformation in part by inducing mis-splicing of *MNK2* and *S6K1* and activating the mTOR pathway, which is required for SRSF1-mediated transformation[40,42], and promoting the expression of *BIM* (also known as *BCL2L11*) and bridging integrator 1 (*BIN1*) protein isoforms without pro-apoptotic functions[41]. A recent study proposed that SRSF3 is also an oncoprotein when overexpressed[43]. Consistent with this, SRSF3 downregulation promoted p53-mediated cellular senescence in part by promoting the expression of the p53β isoform[44]. *SRSF6* has also been characterized as a proto-oncogene that is frequently overexpressed in human skin cancer[45]. Transgenic overexpression of SRSF6 from the collagen type Iα1 (*Col1a1*) locus in mice induced hyperplasia of skin sensitized by shaving or wounding, partially through aberrant alternative splicing of tenascin C (*Tnc*)[45]. SRSF6 may act as an oncoprotein in lung and colon cancer as well[46].

hnRNPs have been implicated in cancer in both pro- and anti-tumorigenic capacities. For instance, two studies reported that MYC-mediated upregulation of specific hnRNPs (hnRNP A1, A2 and PTB) resulted in exclusion of exon 9 of pyruvate kinase muscle (*PKM*), thus promoting expression of the cancer-associated embryonic PKM2 isoform and aerobic glycolysis in glioma[47,48]. Expression of the constitutively active variant of epidermal growth factor receptor (EGFR), EGFRvIII, in gliomas was shown to upregulate hnRNP A1 which, in turn, contributed to alternative splicing of MYC associated factor X (*MAX*) to produce delta *MAX* and further promote glycolytic gene expression and proliferation[49]. The splicing

factor hnRNP A2/B1 also acts in a pro-tumorigenic capacity. hnRNP A2/B1 is overexpressed in gliomas, where it correlates with poor prognosis, and its overexpression transforms cells *in vitro*[50].

Conversely, other hnRNPs may act as tumor suppressors. Motivated by the observation that *HNRNPK* (among other genes) lies within a chromosomal locus that is recurrently deleted in acute myeloid leukemia (AML)[51], one recent mouse study[52] found that deletion of one allele of *Hnrnpk* resulted in myeloid hematologic transformation. However, it remains unknown whether the observed myeloid transformation phenotype is due to changes in splicing or other biological pathways. hnRNP K has been implicated in many biological processes in addition to RNA splicing, including cellular proliferation and cellular senescence through its effects on p53[53] and p21[52], as well as myeloid differentiation through its effects on CCAAT/enhancer-binding protein-α (C/EBPα) and C/EBPβ[52].

Many splicing factors other than SR proteins and hnRNPs have also been implicated in cancer in pro- as well as anti-tumorigenic capacities. For example, the STAR family protein quaking (QKI) may act as a tumor suppressor in lung cancer, where it is commonly downregulated[54]. QKI overexpression inhibits the proliferation of lung cancer cells *in vitro* and *in vivo*, in part by regulating the alternative splicing of *NUMB*[54]. Other splicing factors are also linked to cancer by their regulation of *NUMB*. RNA binding motif protein 5 (*RBM5*), *RBM6* and *RBM10*, which encode homologous RNA-binding proteins, are commonly deleted, mutated, and/or under- or overexpressed in lung and other cancers[55–59]. RBM5 or RBM6 depletion has an opposite effect to RBM10 depletion on *in vitro* colony formation. This is partially due to the antagonistic functions of these factors in regulating the alternative splicing of *NUMB*[60]. RBM4 is also downregulated in multiple cancers and has been characterized as a tumor suppressor that regulates *BCLX* (also known as *BCL2L1*) splicing and antagonizes the pro-tumor activity of SRSF1[61]. The epithelial to mesenchymal transition (EMT) is regulated by splicing factors including epithelial splicing regulatory protein 1 (ESRP1; also known as RBM35A) and ESRP2 (also known as RBM35B), which promote epithelial splicing programs in breast cancer, and RBFOX2, which promotes mesenchymal splicing programs in breast cancer[62,63]. MBNL and CELF proteins and hnRNPs have also been implicated in EMT[63], highlighting the complexity of cancer-associated splicing dysregulation.

As described above, both functional and prognostic data indicate that many splicing factors play pro- or anti-tumorigenic roles. In a few cases, such as for hnRNP K, RBM5, RBM6 and RBM10, genetic changes such as chromosomal deletions may alter the expression of splicing factors. However, in general, the splicing factors described above are not known to be subject to recurrent, high-frequency mutations in cancer. Although these data suggest that dysregulated expression of splicing factors plays important roles in tumor development or progression, thus far there is limited functional evidence that altered levels of splicing factors alone can drive cancer initiation or that altered levels of splicing factors are required for cancer maintenance.

Recurrent mutations in spliceosomal genes in cancer

The discovery of recurrent somatic mutations in genes encoding core spliceosomal proteins throughout diverse cancer types provided the first genetic evidence directly linking RNA splicing regulation to cancer. These spliceosomal mutations were initially discovered in hematological malignancies including myelodysplastic syndromes (MDS)[8–10] and other myeloid neoplasms as well as chronic lymphocytic leukemia (CLL)[11,12] (Fig. 2a and Table 2). More recently, recurrent spliceosomal mutations have also been found in several solid tumor types, including uveal melanoma (18.6%)[64,65], pancreatic ductal adenocarcinoma (4%)[66], lung adenocarcinoma (3%)[56] and breast cancers (1.8%)[67–70]. Most reported spliceosomal mutations are concentrated in four genes: splicing factor 3B, subunit 1 (*SF3B1*), serine/arginine-rich splicing factor 2 (*SRSF2*), U2 small nuclear RNA auxiliary factor 1 (*U2AF1*) and zinc finger, RNA-binding motif and serine/arginine-rich 2 (*ZRSR2*)[8–10].

Several features of spliceosomal gene mutations immediately suggested their potential functional consequences. First, with the exception of *ZRSR2* mutations, these mutations affect highly restricted amino acid residues in an exclusively heterozygous state with the wild-type allele (Fig. 2b). These data suggest that mutations in most spliceosomal genes likely confer gain or alteration of function, except *ZRSR2* mutations, which follow a pattern consistent with loss of function. Second, splicing factor mutations are mutually exclusive of one another, which may be due to either functional redundancy or synthetic lethality, possibilities that have not yet been explored in published studies (Fig. 2c).

These genetic observations suggest that *SF3B1*, *SRSF2* and *U2AF1* may be proto-oncogenes, as they are subject to highly specific missense mutations are suggestive of gain or alteration of function. In contrast, *ZRSR2* may play a tumor suppressor role, as *ZRSR2* mutations frequently introduce in-frame stop codons or disrupt the reading frame, likely inactivating the gene and/or protein. Functional evidence supporting pro-oncogenic versus tumor suppressor roles for these four proteins is described below.

Although each of these four proteins have some impact on 3′ splice site recognition, it is not clear why they are the targets of recurrent mutations in cancer. The >30 other proteins which comprise the U2 snRNP, the U2AF complex or the SR protein family are not recurrently mutated despite having similarly important roles in 3′ splice site and exon recognition. The highly specific nature of spliceosomal mutations suggests that these mutations, and presumably not others, alter RNA:protein and/or protein:protein interactions in a manner that promotes a specific set of downstream changes that are critical for oncogenesis.

Specific splicing factors are most frequently mutated in distinct cancer subtypes (Fig. 2a). For example, *SF3B1* is the only splicing factor that has been identified as a common mutational target in breast cancer[67–70] (based on cohorts sequenced to date), whereas *U2AF1* is the most commonly mutated factor in non-small cell lung cancer[56]. Moreover, in diseases such as MDS in which multiple splicing factors are commonly mutated, specific splicing factor mutations associate with distinct MDS subtypes. Mutations in *SF3B1* are highly enriched in refractory anemia with ringed sideroblasts (RARS), a form of MDS characterized by a typically indolent course, anemia, and the accumulation of erythroid

precursor cells with abnormally iron-loaded mitochondria[8,10]. In contrast, *SRSF2* is the most commonly mutated splicing factor in MDS with a more fulminant course[71] (Fig. 2a). Currently, the mechanistic basis for this association of different splicing factors with distinct histological subtypes of cancer is not known. Moreover, specific mutated residues in SF3B1 appear to be associated with distinct diseases (Fig. 2d). For instance, *SF3B1*R625 mutations represent the most common *SF3B1* mutation in uveal melanoma, yet are far less frequent in hematological malignancies[64,65]. Similarly, *U2AF1* mutations affect both the S34 and Q157 residues in hematopoietic malignancies[8,9], but only mutations affecting S34 have been identified in lung cancer[56].

Knock-in mouse models in which mutated spliceosomal genes are expressed from their endogenous loci have been created for *Srsf2* (two distinct models)[72,73] and *Sf3b1*[74,75]. A transgenic model of inducible *U2AF1*S34F expression has also been described[76] (Box 2). Phenotypic analyses of a *Srsf2*^{P95H} conditional knock-in model[72] and the transgenic *U2AF1*^{S34F} model[76] revealed that the expression of these mutated proteins in post-natal hematopoietic tissue recapitulated key features of MDS, including leukopenia and increased numbers of hematopoietic stem and myeloid progenitor cells. *Srsf2*^{P95H} knock-in mice developed morphological dysplasia whereas the transgenic *U2AF1*^{S34F} mice did not. However, the transcriptomes of hematopoietic stem or progenitor cells (HSPCs) from each model showed that these cells had the same genome-wide alterations in exonic splicing enhancer (*Srsf2*^{P95H}) and 3′ splice site (*U2AF1*^{S34F}) preferences as those that were observed in patients' cells with these mutations. Additionally, in both the *Srsf2*^{P95H} and *U2AF1*^{S34F} mouse models, there were some genes that were differentially spliced in mouse cells as well as patient cells, suggesting that the models recapitulate many molecular phenotypes of human disease.

The high frequencies with which *SF3B1*, *SRSF2*, *U2AF1* and *ZRSR2* are subject to specific mutations in cancer suggest that spliceosomal mutations drive tumorigenesis, at least in some cellular contexts, and are not merely passenger mutations. Spliceosomal mutations are likely to occur as both initiating and secondary genetic events, a distinction that has been best studied in liquid neoplasms. The clonal architecture of MDS indicates that *SF3B1* mutations are initiating genetic events[77]. Similar studies of secondary AML revealed that *SRSF2*, *U2AF1* and *ZRSR2* mutations also occurred early during the leukemogenic process[78]. In contrast, even though *SF3B1* is the second most commonly mutated gene in CLL, *SF3B1* mutations occur most frequently in advanced versus early disease, suggesting that they are secondary genetic events that facilitate progression[11,79].

Mutations affecting 3′ splice site recognition via U2

SF3B1 is mutated at significant rates in both hematological and solid cancers, including MDS, CLL, uveal melanoma and breast cancer, rendering it the most commonly mutated spliceosomal gene (Fig. 2a). It encodes a core spliceosomal protein that binds upstream of the pre-mRNA branch site in a manner that is likely to be largely sequence independent. SF3B1 is probably required for the recognition of most 3′ splice sites[17]. *SF3B1* mutations are concentrated in sequence encoding its HEAT repeat domains (Fig. 2b); however, the normal function of these domains is poorly characterized, rendering it difficult to predict the

mechanistic consequences of *SF3B1* mutations. DeBoever *et al.*[80] recently reported that *SF3B1* mutations were associated with enhanced recognition of cryptic 3′ splice sites between the branch point and normal 3′ splice site. While U2 snRNP binding to the branch point normally prevents recognition of AG dinucleotides immediately downstream of the branch point by steric occlusion, DeBoever *et al.*[80] hypothesized that *SF3B1* mutations prevent this normal steric occlusion, thereby enhancing recognition of cryptic splice sites (Fig. 3a). Darman *et al.*[81] similarly reported that mutant SF3B1 enhanced recognition of intron-proximal cryptic 3′ splice sites, which frequently involved normally unused upstream branch points. However, the exact mechanism(s) by which mutations might alter SF3B1 interactions with pre-mRNA, components of the U2 snRNP or other proteins remains unknown. A precise understanding of how mutations alter the role of SF3B1 in RNA splicing will likely require further studies of normal SF3B1 function.

The consequences of *SF3B1* mutations may be cell type-dependent and/or allele-specific, as different *SF3B1* mutations may not be phenotypically equivalent. *SF3B1* mutations in MDS versus CLL constitute initial versus secondary genetic insults and associate with favorable versus poor prognosis, respectively[10–12,79]. Different *SF3B1* mutations are more enriched in MDS compared with CLL and other cancers (Fig. 2d).

Despite the close association between *SF3B1* mutations and the presence of ring sideroblasts in MDS, no studies have clearly demonstrated that *SF3B1* mutations induce abnormal iron metabolism. However, the recent report of Darman *et al.*[81] that ATP-binding cassette subfamily B member 7 (*ABCB7*) is mis-spliced in *SF3B1*-mutant cells hints at possible connections between altered splicing and sideroblastic anemia. *ABCB7* encodes an iron transporter that is essential for hematopoiesis and that is mutated in X-linked sideroblastic anemia with ataxia, a genetic disease that is also characterized by the presence of ring sideroblasts[82,83].

U2AF1 is mutated in both liquid and solid tumors, with the highest reported rates in MDS and lung cancer (Fig. 2a). Unlike SF3B1, which is required for recognition of many or most 3′ splice sites, U2AF1 binds the 3′ splice site in a highly sequence-specific manner, and is only required for recognition of a subset of ‘AG-dependent’ 3′ splice sites[84,85]. *U2AF1* mutations are concentrated in sequence encoding the S34 and Q157 residues, which lie within the first and second C_3H zinc ‘knuckles’ of the protein (Fig. 2b). In hematological cancer, such mutations have been shown to induce sequence-specific alterations in the preferred RNA motif bound by U2AF1, which normally recognizes the motif yAG|r (y = pyrimidine; r = purine; lower-case nucleotides are preferred but not always required, while upper-case nucleotides are usually required; ‘|’ = intron-exon boundary)[86–89] (Fig. 3b). Interestingly, the molecular consequences of *U2AF1* mutations are allele-specific[88]. S34 and Q157 mutations respectively affect recognition of the −3 (pyrimidine normally preferred) and +1 (purine normally preferred) positions, where the coordinates are defined with respect to the intron-exon boundary to induce different changes in 3′ splice site recognition. At a global level, S34 mutations promote recognition of 3′ splice sites with cAG|r and aAG|r motifs over those with tAG|r, whereas Q157 mutations promote 3′ splice sites with yAG|g motifs over those with yAG|a motifs.

Many downstream targets of mutant U2AF1 have been identified using patient transcriptomes, a transgenic mouse model of *U2AF1*^{S34F} and transgenic human cells bearing each of the common *U2AF1* mutations affecting the S34 and Q157 residues[76,86–89]. These mis-spliced genes fall into biological pathways including the DNA damage response (ataxia telangiectasia- and Rad3-related (*ATR*) and Fanconi anemia complementation group A (*FANCA*)), epigenetic regulation (H2A histone family member Y (*H2AFY*)), and apoptosis (caspase 8 (*CASP8*))[76,88]. Intriguingly, several of the identified target genes are recurrently mutated in MDS (for example, BCL6 corepressor (*BCOR*)[90]) and other cancers (for example, *CASP8*[67,91]). However, functional studies are needed to determine whether these and other mis-spliced genes contribute to the disease process.

Mutations affecting 3′ splice site recognition via U12

ZRSR2 mutations found in MDS are distributed throughout the gene, which lies on the X chromosome (Xp22.1), and frequently interrupt the coding sequence by directly or indirectly introducing in-frame stop codons (Fig. 2a). Together with the common occurrence of *ZRSR2* mutations in male patients with cancer[8], this pattern is consistent with loss of function. *ZRSR2* mutations therefore contrast with the mutations observed in the spliceosomal genes *SF3B1, SRSF2* and *U2AF1*, which cause missense changes at specific residues and never introduce in-frame stop codons (Fig. 2b).

Whereas biochemical assays suggested that ZRSR2 promotes recognition of both U2- and U12-type introns, the phylogenetic observation that organisms with U12-type introns have ZRSR2 and those lacking U12-type introns also lack ZRSR2 suggested that ZRSR2 is particularly important for U12-type splicing[92]. Madan *et al.*[93] reported that MDS transcriptomes harboring mutations likely to inactivate *ZRSR2* are characterized by frequent retention of U12-type introns, consistent with a crucial role for ZRSR2 in the minor spliceosome (Fig. 3c). *ZRSR2* knockdown altered the *in vitro* differentiation potential of cord blood-derived CD34$^+$ cells by promoting myeloid differentiation and impairing erythroid differentiation[93], consistent with features of human MDS. However, *ZRSR2* knockdown also impaired the growth of K562 cells *in vitro* and following subcutaneous xenografting *in vivo*[93], indicating that *ZRSR2* loss does not convey a proliferative advantage in the K562 genetic background. *ZRSR2* mutations were associated with mis-splicing of genes relevant to the MAPK pathway and E2F transcription factor signaling[93], but functional experiments are needed to determine whether these or other splicing changes contribute to the hematopoietic phenotypes of ZRSR2-deficient cells.

Mutations affecting exon recognition

SRSF2 mutations appear most commonly (in 40–50% of patients[8]) in chronic myelomonocytic leukemia (CMML), and are also enriched in subtypes of high-risk MDS, where they portend an increased risk of transformation to acute leukemia[94] (Fig. 2a). *SRSF2* encodes a member of the SR protein family that contributes to both constitutive and alternative splicing. SRSF2 canonically promotes exon recognition by binding to exonic splicing enhancer (ESE) motifs within pre-mRNAs through its RRM domain[95–97]. All recurrent *SRSF2* mutations affect the P95 residue[8], which is immediately downstream of the RRM domain (Fig. 2b). RNA-seq analyses of hematopoietic stem and progenitor cells from

Srsf2^{P95H} knock-in mice[72] and transgenic[72] and knock-in[98] K562 cells expressing *SRSF2*$^{P95H/L/R}$, and human AML and CMML patients with or without *SRSF2* mutations[72], revealed that SRSF2 mutations alter its normal sequence-specific RNA-binding activity. Mutant SRSF2 preferentially recognizes a C-rich CCNG motif versus a G-rich GGNG motif, whereas wild-type SRSF2 binds both motifs with similar affinity[72,98,99] (Fig. 3d). These alterations in the RNA-binding activity of SRSF2 promote or repress recognition of exons containing C- or G-rich ESEs[72,98].

Altered ESE recognition causes widespread splicing changes in hundreds of genes[72,98]. Several of these downstream mis-spliced genes are themselves recurrent mutational targets in myeloid malignancies, including enhancer of zeste homolog 2 (*EZH2*)[100,101] and *BCOR*[90]. *SRSF2* mutations promote inclusion of a 'poison exon' of *EZH2* that introduces an in-frame stop codon to induce nonsense-mediated decay (NMD) of the *EZH2* transcript and consequent global downregulation of EZH2 protein and histone H3 lysine 27 trimethylation (H3K27me3) levels[72]. Loss-of-function mutations in *EZH2* occur in MDS[100,101], and *Ezh2* loss has been functionally linked to MDS development and aberrant hematopoietic stem cell self-renewal *in vivo*[102]. Therefore, decreased EZH2 levels may partially explain how *SRSF2* mutations drive MDS, and also explain the previously observed mutual exclusivity of *SRSF2* and *EZH2* mutations in MDS[71,103].

Mutations affecting other splicing factors

In addition to *SF3B1*, *SRSF2*, *U2AF1* and *ZRSR2*, other genes encoding splicing factors are recurrently mutated in cancer. Pre-mRNA processing factor 8 (*PRPF8*) is subjected to mutations or hemizygous deletions in 1–5% of patients with myeloid leukemias[104]. Biochemical studies in yeast suggest that PRPF8 mutations may affect recognition of suboptimal 3′ splice sites[104].

Genes encoding splicing factors have also been identified as recurrent targets of translocations in cancer. For example, splicing factor proline/glutamine-rich (*SFPQ*; also known as *PSF*) is reportedly recurrently fused to *ABL1* in B-cell acute lymphoblastic leukemia[105] and to transcription factor binding to IGHM enhancer 3 (*TFE3*) in papillary renal cell carcinomas[106]. *SRSF3* is also reportedly involved in rare fusions with *BCL6* in B-cell lymphomas[107]. Currently, it is not known if these fusions affect the function of the splicing factors involved in the chimeric protein product. In the case of *SFPQ* fusions, the sequence encoding the coiled-coil domain (which is important for protein dimerization) of SFPQ appears to be consistently included in the chimeric transcript[108], suggesting that *SFPQ* fusions may contribute to cancer by promoting aberrant dimerization of SFPQ's partner protein.

Inherited genetic variation affecting RNA splicing factors has also been implicated in cancer. A missense genetic variant in serine/arginine repetitive matrix 2 (*SRRM2*; also known as *SRm300*) was recently found to segregate with incidence of familial papillary thyroid carcinoma[109]. Patients carrying this *SRRM2* variant exhibited mis-splicing of specific cassette exons, suggesting that the variant altered the normal function of SRRM2 in splicing. Somatic mutations may also interact with genetic variants to promote cancer. Recent work identified both somatic mutations and genetic variants affecting DEAD-box helicase 41

(DDX41) that are associated with high-penetrance familial MDS and AML[110]. Although the normal molecular role of DDX41 is incompletely understood, mass spectrometry data indicated that DDX41 interacts with core spliceosome components and that the likely loss-of-function DDX41 mutations perturb these interactions. Therefore, DDX41 may play a role in RNA splicing that is disrupted by MDS and AML – associated mutations, although that hypothesis remains to be tested.

Connections to other cellular processes

Splicing factor dysregulation, including spliceosomal mutations, may directly or indirectly affect many cellular processes in addition to RNA splicing. These processes include the maintenance of genome integrity, epigenetic regulation, transcription, nuclear export, and translation-dependent mRNA decay.

Depletion of SRSF1 or SRSF2 gives rise to DNA damage and genomic instability via the formation of RNA:DNA hybrids ('R loops')[111,112], which expose the unhybridized DNA strand to DNA damage (Fig. 4a). Splicing factors are also linked to the DNA damage response. For example, BRCA1 is reportedly physically associated with SF3B1 and U2AF1 specifically in response to DNA damage[113]. A recent study proposed that the spliceosome is an effector of ataxia-telangiectasia mutated (ATM) signaling, wherein DNA lesions displace spliceosomes, resulting in R loop formation and ATM activation[114].

Splicing is also closely linked to epigenetic regulation. Nucleosomes are preferentially positioned over exons versus introns[115,116]. SF3B1 is preferentially associated with nucleosomes positioned over exons, facilitating recognition of these exons[116]. Histone H3 lysine 36 trimethylation (H3K36me3) is further enriched over exons[117,118], and modulation of H3K36me3 can influence splice site choice[119]. Conversely, modifying splice site recognition can influence H3K36me3 deposition[120,121]. The literature linking splicing and epigenetics is reviewed in more detail in Refs[122,123].

Connections between splicing and epigenetics may contribute to the oncogenic activity of spliceosomal mutations. As described above, mutant SRSF2 prevents hematopoiesis in part by promoting a non-functional isoform of EZH2, resulting in global decreases in H3K27me3 levels[72]. Mutant U2AF1 promotes a cancer-associated isoform of the histone variant macro-H2A.1[76,88]. Connections between *SF3B1* mutations and epigenetic dysregulation have not been identified, but are plausible given the published links between splice site recognition and chromatin described above. However, further studies are needed to determine whether potential epigenetic changes caused by *U2AF1* and/or *SF3B1* mutations are important for cancer initiation and progression.

Splicing factor dysregulation may also affect transcription independently of epigenetic regulation. For example, SRSF2 regulates transcriptional elongation in a sequence-specific manner via the 7SK complex that governs RNA polymerase II pause release[124]. Similarly, hnRNP A1, hnRNP A2 and hnRNP K are linked to cancer, and hnRNP A1 and A2 have reported roles in transcription elongation whereas hnRNP K is linked to transcription termination[125,126]. Many splicing factors co-transcriptionally associate with the C-terminal

domain of RNA polymerase II, thereby coupling transcription, splicing, cleavage and polyadenylation (reviewed in[127]).

Finally, molecular changes induced by splicing factor dysregulation may not be confined to the nucleus. Multiple SR proteins facilitate nuclear export of both unspliced and spliced mRNAs[128,129]. SRSF1 shuttles between the nucleus and cytoplasm and promotes cap-dependent translation of bound mRNAs in a eukaryotic translation initiation factor 4E (eIF4E)-dependent manner[130,131]. SRSF1 activates the mammalian target of rapamycin (mTOR) signaling pathway, which is required for SRSF1-mediated cell transformation[40,42]. Splicing itself promotes translation via the deposition of multiprotein exon-junction complexes (EJCs) near exon-exon junctions[132].

NMD provides a concrete example of a cytoplasmic process that is likely affected by cancer-associated mutations affecting SF3B1, SRSF2, U2AF1 and ZRSR2, even though those proteins localize to the nucleus. NMD is a translation-dependent RNA surveillance process that degrades mRNAs containing premature termination codons (Fig. 4b; reviewed in[133,134]). Splicing and NMD are closely linked in human cells for several reasons. First, many NMD substrates are generated by alternative splicing, wherein premature termination codons are introduced via inclusion of alternatively spliced sequence containing an in-frame premature stop codon or exclusion of sequence resulting in a frameshift. Second, stop codons are recognized as premature by the NMD machinery if they lie >50 nucleotides upstream of a splice junction[133,134]. The 50 nucleotide threshold arises because the translating ribosome dislodges EJCs and prevents EJC-facilitated activation of NMD. However, if the ribosome stalls sufficiently upstream of an EJC, then NMD factors are recruited and activated[133,134]. Third, specific splicing factors including SRSF1, regulator of differentiation 1 (ROD1; also known as PTBP3) and PTB can enhance or repress NMD[135–137].

Human cells express an abundance of mRNAs containing premature termination codons (one-third of all alternatively spliced isoforms by one estimate[138]), including the *EZH2* poison exon that is promoted by *SRSF2* mutations[72]. A subset of poison exons are among the most evolutionarily conserved elements in the human genome[139–141]. These poison exons enable splicing factors to post-transcriptionally down- or upregulate expression of specific genes, including the genes encoding many splicing factors themselves, likely explaining the extreme sequence conservation of many poison exons[139–141].

Interestingly, in the earliest report of splicing factor mutations, genes involved in NMD were upregulated following overexpression of mutant U2AF1[8], suggesting a potential link between spliceosomal mutations and overproduction of NMD substrates. However, such high levels of NMD substrates have not been observed in subsequent studies of mutations affecting U2AF1 or other splicing factors.

The recent discovery of recurrent mutations in *UPF1*, which encodes a RNA helicase that is central to NMD, in pancreatic adenosquamous carcinoma provided a genetic link between NMD and cancer[142]. The observed mutations induced abnormal *UPF1* splicing and skipping of sequence encoding core domains, potentially resulting in partial or complete loss of UPF1

function, although further work is required to determine how these mutations affect global RNA surveillance. Deficiencies in different NMD factors have been previously linked to disorders including intellectual disability[143], thrombocytopenia with absent radii syndrome[144] and muscular dystrophy[145].

Implications for therapy

Given the crucial roles of specific alternatively spliced isoforms in cancer biology[146,147], as well as the potentially increased sensitivity of cancers to global perturbation of splicing efficiency relative to normal cells[148,149], pharmacological modulation of splicing may represent an important therapeutic strategy. Spliceosomal gene mutations that cause alteration or gain of function are mutually exclusive with one another and are always co-expressed with a wild-type allele, suggesting that cells bearing spliceosomal mutations may be unable to tolerate further perturbations in splicing, and could therefore be preferentially sensitive to pharmacological splicing inhibition. A number of compounds and oligonucleotides that can disrupt or modulate normal splicing catalysis or alter splice site recognition through distinct pathways have been identified (Fig. 4c).

Several compounds with antitumor activity were identified through natural product screens prior to the discovery of spliceosomal gene mutations. Synthetic analogues with higher stability, solubility and activity were subsequently developed, including E7107, meayamycin, spliceostatin A, and sudemycins (reviewed in[150,151]). Biochemical studies identified SF3B1 as the likely target of these drugs, consistent with the observation that mutations affecting the R1074 residue of SF3B1 confer resistance to pladienolide and E7107[152,153]. Unfortunately, two separate Phase I clinical trials of E7107 revealed an unexpected and unexplained side effect of visual disturbances in 5% of subjects[154,155]. Further efforts are needed to determine whether this was an on- or off-target effect of U2 snRNP inhibition *in vivo*. In the meantime, several preclinical studies are evaluating the utility and safety of sudemycins[156,157] for cancer therapy.

Although the origin of splicing inhibitors' general antitumor activities is unknown, two studies provided evidence that MYC expression renders cells sensitive to compounds that inhibit 3′ splice site recognition[148,149]. More recently, Lee *et al.*[158] reported that the splicing inhibitor E7107 reduced the leukemic burden and prolonged survival of mice carrying oncogene-driven myeloid leukemias if the leukemias had *Srsf2* mutations, but not if the leukemias expressed only wild-type *Srsf2*. Lee *et al.* observed similarly specific targeting of patient-derived xenograft (PDX) models of leukemias with spliceosomal mutations. These data suggest that splicing inhibitors such as E7107 are synthetically lethal with genetic lesions affecting the spliceosome.

Interventions that target post-translational modification of splicing factors might also prove effective for therapy. For example, SR proteins are phosphorylated by kinases including topoisomerase I and members of the SR protein kinase (SRPK) and CDC2-like kinase (CLK) families[159–161]. These kinases affect SR protein subcellular localization and splicing activity[162,163], exhibit altered expression and/or activity in cancer[164,165], and can potentially act as oncogenes[165]. Small molecules that block activity of these kinases have been

identified, including TG003, an inhibitor of CLK1 and CLK4, and SRPIN340, an SRPK1 and SRPK2 inhibitor that inhibits angiogenesis[166,167]. In addition, indole derivatives, such as benzopyridoindoles and pyridocarbazoles, are a class of compounds that were recently discovered to modulate splicing by altering the ESE-dependent splicing activity of individual SR proteins[168]. Indole derivatives have been shown to modulate the splicing event that generates the cancer-associated, constitutively active ΔRon isoform of the recepteur d'origine nantais (*RON*, also known as *MST1R*) proto-oncogene and revert the invasive phenotype of cancer cells expressing ΔRon[169].

Summary and future perspectives

The recent discovery of recurrent spliceosomal mutations as likely cancer drivers has underscored the pressing need to identify connections between abnormal pre-mRNA processing and tumorigenesis. Emerging evidence supports a model in which many spliceosomal mutations induce specific changes in splice site or exon recognition, frequently via altered RNA binding, leading to genome-wide splicing changes that presumably promote cancer development.

Despite these mechanistic advances, efforts to link altered splice site or exon recognition to specific pathological splicing events are nascent. Challenges including identifying and prioritizing among hundreds of downstream mis-spliced isoforms, as well as determining the biological roles of specific isoforms. Furthermore, it is unknown whether the pro-tumorigenic effects of mutated spliceosomal proteins are mediated by just a handful of mis-spliced isoforms, or instead are due to many splicing changes, which may even be functionally interdependent. Although many mis-spliced isoforms have been identified in cells bearing spliceosomal mutations, very few of these isoforms have been functionally characterized to date.

Spliceosomal mutations likely both indirectly and directly dysregulate diverse cellular processes. In principle, spliceosomal mutations could affect almost any biological process by inducing mis-splicing of key regulators (for example, the connection between *SRSF2* and H3K27me3 deficiency via EZH2 mis-splicing). Spliceosomal mutations may also dysregulate processes including transcriptional elongation, the DNA damage response and NMD, in which splicing factors play key roles (Fig. 4a–b).

Although spliceosomal mutations provide the most direct link between splicing and cancer, it is also important to note that abnormal splicing is a feature of most cancers even in the absence of spliceosomal mutations[5–7]. Abnormal cancer-associated splicing may result from both specific and global perturbations to the splicing machinery. Specific perturbations may arise from dysregulation of single splicing factors that play pro- or anti-tumorigenic roles (Table 1), whereas global perturbations may arise from effects including potential transcriptional amplification driven by MYC[149] or mutations affecting epigenetic regulators such as isocitrate dehydrogenase (IDH) or SET domain containing 2 (SETD2)[5,7].

Although incomplete, our current understanding of spliceosomal mutations suggests that these mutations may create new therapeutic opportunities. Because splicing factors can act

as both oncoproteins and tumor suppressors, distinct therapeutic interventions may prove necessary for treating cancers harboring different spliceosomal mutations. Possible therapeutic interventions fall into several broad categories, including restoring normal splicing and exploiting vulnerabilities to specifically target mutant cells.

Normal splicing could potentially be restored by specifically inhibiting the mutant protein, manipulating downstream splicing events or other methods. These approaches are promising, yet each requires further investigation. For example, specific inhibition or sequestration of mutant SRSF2 and U2AF1 may be possible given their altered RNA-binding preferences. However, definitive evidence that cancer cells depend on these mutated proteins, or that inhibiting the mutant allele is sufficient to restore normal splicing, is currently absent. (Mutant SRSF2 and U2AF1 likely act as oncoproteins to promote tumor formation, yet may not be required for subsequent tumor maintenance or growth.) The same caveat applies to inhibition of downstream mis-splicing. Specific mis-splicing events could potentially be corrected with antisense oligonucleotides, which have shown promise in clinical trials of disorders such as Duchenne muscular dystrophy[170] and spinal muscular atrophy[171,172]. However, our current understanding of how spliceosomal mutations perturb cellular function is insufficient to determine which mis-splicing events to correct in cancer. Furthermore, because inhibiting a mutant oncoprotein is likely more feasible than restoring the function of a disabled wild-type protein, restoring normal splicing may not be possible in the context of spliceosomal mutations that disable tumor suppressors. For example, *ZRSR2* mutations cause loss of ZRSR2 expression or function, and it is unclear whether restoring U12-type intron recognition in the absence of ZRSR2 is possible.

Conversely, it may be feasible to selectively target cells expressing mutated splicing factors. Recent work suggested that inhibiting splicing catalysis itself may provide a therapeutic index in cells bearing spliceosomal mutations[158,173]. Non-cell autonomous approaches to target cells with spliceosomal mutations may also be possible. Just as increased somatic mutational burdens may generate neo-epitopes and render specific subsets of cancer sensitive to cancer immunotherapies[174–177], so may abnormal mRNAs generated by spliceosomal mutations result in neo-epitope production in cancers bearing these lesions. Notably, these two approaches—inhibition of splicing catalysis and immunotherapy—could potentially be efficacious in the context of spliceosomal mutations that generate oncoproteins as well as those that inactivate tumor suppressors. Recurrent mutations in *SF3B1*, *SRSF2*, *U2AF1*, and *ZRSR2* cause very different mechanistic alterations in splicing, yet each may render cells susceptible to further perturbation of splicing catalysis or result in the generation of neo-epitopes.

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Glossary

Major spliceosome
A ribonucleoprotein complex consisting of five small nuclear RNAs (termed U1, U2, U4, U5 and U6), each in complex with a set of proteins to form snRNPs, that are together responsible for excision of the majority of introns

Minor spliceosome
A ribonucleoprotein complex that catalyzes splicing of a small subset of 'U12-type' introns. The introns recognized by the minor spliceosome are typically defined by different sequence elements than those that define U2-type introns, which are recognized by the major spliceosome

Small nuclear ribonucleoprotein complexes
(snRNPs). These complexes assemble on pre-mRNA to catalyze splicing

U2AF complex
A heterodimeric protein complex consisting of U2AF1 and U2AF2. U2AF1 and U2AF2 bind the polypyrimidine tract and AG dinucleotide of the 3′ splice site to facilitate splice site recognition. Only a subset of 'AG-dependent' 3′ splice sites require U2AF1 binding for efficient splice site recognition

Constitutive splice sites

Splice sites that are always recognized and used by the spliceosome. Similarly, constitutive exons are always included in the mature mRNA

Alternative splice sites
Splice sites that are variably recognized and used by the spliceosome. Similarly, alternative exons (also known as cassette or skipped exons) are sometimes, but not always, included in the mature mRNA. Recognition of alternative splice sites is frequently cell type-specific and may rely upon the binding of additional *trans*-acting factors

Serine/arginine-rich proteins
(SR proteins). A family of splicing factors that frequently promote splicing, although their action is context-dependent. Many of these proteins bind pre-mRNA in a sequence-specific manner to activate splicing. Some of the family are implicated in other cellular processes, including mRNA export and translation

Heterogeneous nuclear ribonucleoproteins
(hnRNPs). Many members of this protein family are splicing factors, although they also participate in other diverse RNA metabolic processes. These proteins frequently repress splicing, although their action is context-dependent

Acute myeloid leukemia
A type of cancer characterized by the rapid growth of abnormal white blood cells that accumulate in the bone marrow and interfere with the production of normal blood cells

Myelodysplastic syndromes
(MDS). A heterogeneous group of clonal disorders of hematopoiesis characterized by an impaired ability to generate mature blood cells as well as aberrant cell morphologies (termed ‘dysplasia’).

Chronic lymphocytic leukemia
(CLL). A type of cancer characterized by accumulation of aberrant mature-appearing B lymphocytes.

SF3B1
This gene encodes a key component of the U2 snRNP that binds upstream of the branch point to facilitate 3′ splice site recognition. SF3B1 is likely required for the splicing of most introns and is the most commonly mutated splicing factor in cancer.

SRSF2
This gene encodes an SR protein that binds specific exonic splicing enhancer motifs to promote recognition and inclusion of exons containing these motifs

ZRSR2
A gene encoding a component of the minor spliceosome that contacts the 3′ splice site of specific U12-type introns to promote their excision

Synthetic lethality

The situation in which two cellular perturbations (for example, two distinct mutations, or a mutation and a particular drug) result in cell death when combined whereas each perturbation alone does not

Secondary AML
(sAML) Acute myeloid leukemia that develops following a previous chronic myeloid malignancy such as a myelodysplastic syndrome

Cryptic 3′ splice sites
Potential 3′ splice sites that are not normally recognized by the spliceosome. By chance, introns and exons contain many AG dinucleotides that are not used as splice sites. Perturbations such as spliceosomal mutations can cause such 'decoy splice sites' to be incorrectly recognized as authentic splice sites

Stop codon
UAA, UAG, or UGA codons, signaling the end of translation. Also known as a termination codons

Chronic myelomonocytic leukemia
A clonal disorder with features of both myelodysplastic and myeloproliferative syndromes in which there are too many monocytes in the blood

Exonic splicing enhancer
A typically short sequence motif in pre-mRNA that is bound by a splicing factor to promote exon recognition and subsequent inclusion of the exon in the mature mRNA. Many SR proteins bind exonic splicing enhancers to activate splicing

Poison exon
A cassette exon containing an in-frame premature stop codon. A premature stop codon lies upstream of the normal stop codon, resulting in premature termination of translation of the mRNA when included in a transcript. Poison exons can induce nonsense-mediated decay of the mRNA or production of a truncated protein

Nonsense-mediated decay
An RNA surveillance process that recognizes and degrades mRNAs containing premature stop codons, as well as other abnormal RNAs and a subset of normal coding transcripts. Splicing is closely linked to NMD, as exon-exon junctions are important components of NMD activation in human cells

RNA polymerase II pause release
The process by which RNA polymerase II that is paused (not actively transcribing) after the initiation of transcription is released, enabling transcriptional elongation

Frameshift
The disruption of an open reading frame by the insertion or deletion of nucleotide sequence whose length is not a multiple of three

Expressed sequence tag

(EST). Portions of cDNA sequences

Unannotated splicing

Splicing events that have not been previously reported by published studies or genomic databases such as Ensembl, UCSC, Vega and RefSeq

ψ value

The percentage of all mRNAs transcribed from a gene that correspond to a particular isoform or contain a particular alternatively spliced sequence relative to all transcripts of the parent gene. For example, the ψ value for a cassette exon is the fraction of all mRNAs that contain the cassette exon. The ψ value is independent of gene expression and falls within the range 0–100%

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Box 1

Methods to determine differential RNA splicing using RNA-seq data

Accurate detection and quantification of differential splicing remain bioinformatic challenges[178,179]. Important aspects of the methods used to measure splicing are described below and include: the quality and origins of the genomic annotation of splicing events, whether to consider full-length isoforms or single splice sites (or single exons) in isolation and the choice of statistical method to identify differences between samples.

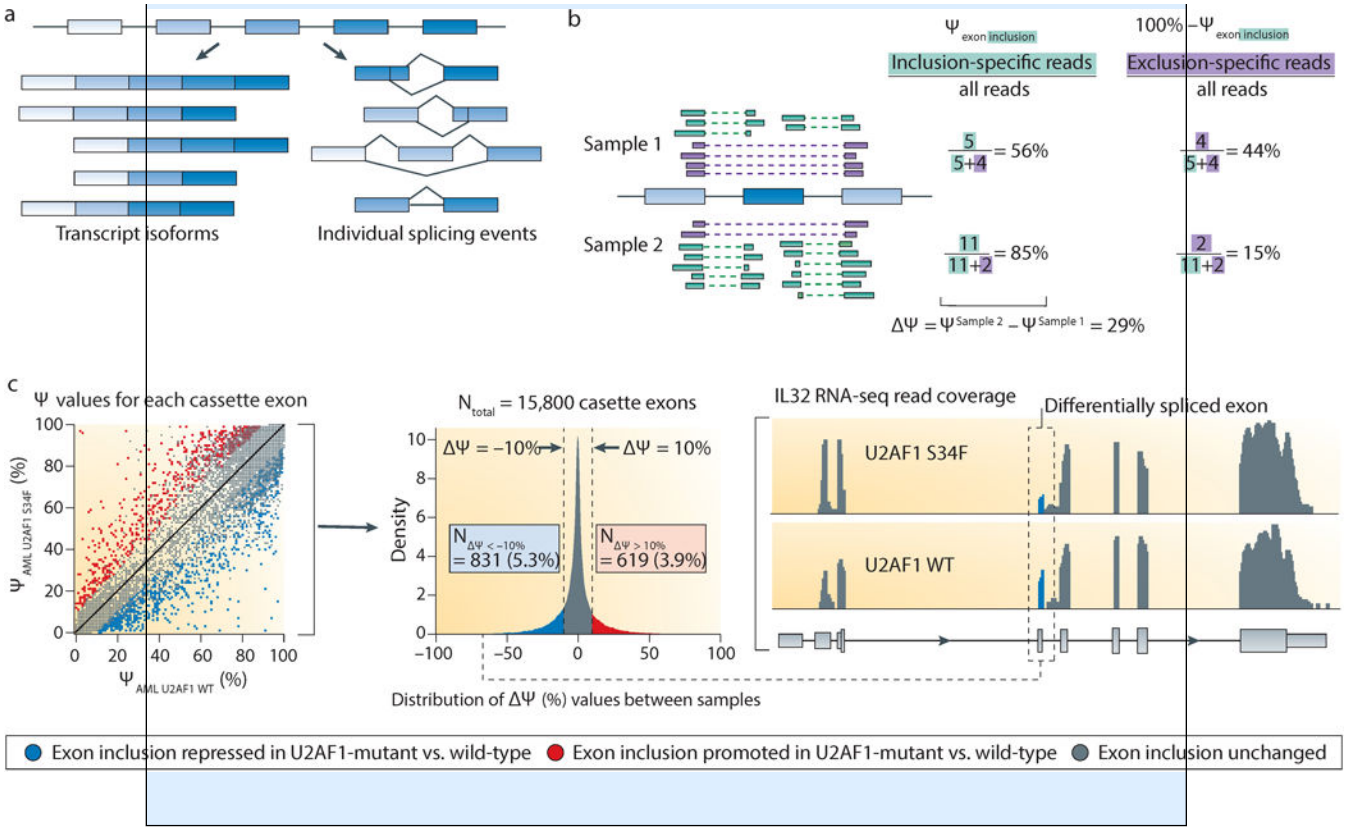
Genome-wide splicing annotations are available from both general-purpose genomic databases[180–182] and specialized software packages[183]. These annotations are frequently based on exon-exon junctions inferred from expressed sequence tags (ESTs) and full-length cDNAs, limiting the annotation to the extent that such sequences are available for a given cell type or organism. For example, there are >2.5-fold more alternative splicing events annotated in humans than in mice[183], which is a product of the more complete human genome annotation as well as increased rates of alternative splicing in human versus mouse tissues[184]. As comparatively few splicing events have been annotated in many model organisms, detecting unannotated splicing (or novel splicing) with RNA-seq is frequently necessary. Novel splicing detection can involve searching for reads overlapping new combinations of known 5′ and 3′ splice sites of a gene, detecting alternate 5′ or 3′ ends of exons that constitute novel splice junctions[185,186] or conducting *de novo* isoform reconstruction[187,188] (methods ordered by increasing complexity). These approaches are more computationally intensive than using existing annotations, and experimental validation of novel splicing events is crucial.

Differential splicing can be analyzed at the level of differentially expressed full-length isoforms or at the level of single splicing events (for example, the inclusion or exclusion of a particular cassette exon; see panel (**a**) of the figure). Full-length isoforms are the biologically relevant entities that encode the final protein products[189], but the exon composition of each isoform must be statistically inferred due to the short read lengths of most high-throughput sequencing platforms. Conversely, focusing on single splicing events may be beneficial when analyzing splicing mechanisms and regulation—for example, the altered motif preferences induced by *U2AF1* and *SRSF2* mutations[72,76,88,98]—or when a particular alternatively spliced region has an important impact on gene function. Many such studies focus on single events and then infer full-length isoforms for downstream functional studies. In either case, splicing is quantified using a ‘percent spliced in’ value (PSI, or ψ value) ranging from 0 to 100%[14,183], defined as the percentage of all mRNAs that correspond to the isoform or alternatively spliced sequence of interest relative to all transcripts of the parent gene (see panel (**b**) of the figure), independent of gene expression. (For simplicity, only junction-spanning reads are illustrated in the computation of ψ.)

Quantifying differences in splicing, whether of full-length isoforms or of single events, also involves statistical choices with biological implications. Fold-change in ψ values can be used to quantify differential splicing, but may not correlate well with biological

importance. For example, consider an isoform with ψ values of 0.1% and 1% in two samples. This corresponds to a fold-change of 10, yet the isoform is low-abundance relative to other isoforms of that gene in both samples. Therefore, many studies instead measure absolute changes in splicing as Δψ, the difference in ψ value between two samples, and apply thresholds on Δψ to identify potentially important changes in splicing[14,183] (for example, using *U2AF1*-mutant (*U2AF1* S34F, shown on the y axis) versus wild-type (*U2AF* WT, shown on the x axis) acute myeloid leukemia (AML) samples, with exons satisfying $\Delta\psi \geq 10\%$ (red) or $\Delta\psi \leq 10\%$ (blue) highlighted; see panel (**c**) of the figure). This measure is more statistically robust, but may ignore differential splicing of low-abundance yet important isoforms, such as a novel isoform that confers gain-of-function or dominant-negative activity.

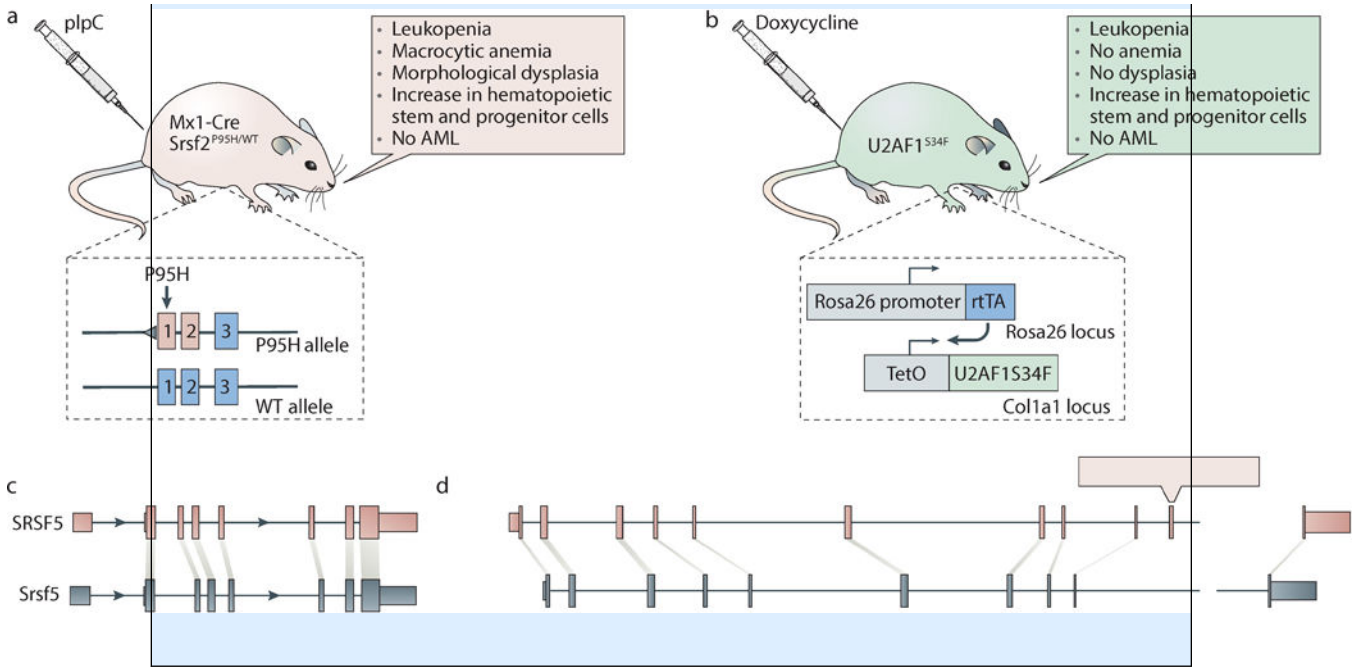
Ultimately, the optimal splicing analysis strategy depends upon the specific aims of a given study and the downstream data interpretation plan. Three common goals of splicing studies are described below. First, many studies seek to identify mechanistic changes in the splicing process itself, such as alterations caused by spliceosomal mutations or global differences between tumor and normal samples[5,7]. In such cases, meta-analyses created by averaging over many individual splicing events typically yield the highest statistical power. Second, a study may focus on specific isoforms of biological importance, such as ligand-independent isoforms of the androgen receptor[190,191], in which case quantifying entire isoforms is essential. Third, a study may seek to identify differentially spliced isoforms that contribute to a known biological phenotype, such as impaired hematopoiesis or enhanced cell migration. Although the biologically relevant entities are whole isoforms, combining single-event and whole-isoform analysis is frequently useful given the exploratory nature of such studies.



Box 2

Models of spliceosomal gene mutations in cancer

The genetic heterogeneity of primary patient samples render genetic models important tools when studying the roles of spliceosomal mutations in cancer pathogenesis. As the wild-type and mutant alleles of spliceosomal genes are consistently co-expressed at similar levels in patients, mimicking this expression pattern is likely important in order to create biologically realistic genetic models. Analyses of the hematopoietic transcriptomes of *Srsf2*^{P95H} conditional knock-in[72] (shown in figure panel **a**; light pink) and *U2AF1*^{S34F} transgenic[76] (shown in figure panel **b**; light green) murine models demonstrated that the mechanistic alterations in exon and splice site recognition induced by these mutations are conserved between human and mouse and validate these murine models for mechanistic studies of the role of splicing alterations in cancer pathogenesis. These observations are consistent with the deep conservation of *SRSF2* and *U2AF1* across eukaryotes. However, even though the functions of these spliceosomal proteins are conserved, the sets of downstream mis-spliced genes may differ between human and murine models due to species-specific differences in splicing. (**c**) Tissue-specific splicing events are more frequently conserved between human (pink) and mouse (blue) than their unregulated counterparts, as is the flanking intronic sequence[14,192]. This even extends to conservation between the human and fungal genomes for a splicing event that enables autoregulation of *SRSF5*[141]. (**d**) Nonetheless, the majority of alternative isoforms are species-specific[184,193,194], such as the illustrated example of *CD55* (pink, human exons; blue, mouse exons). This situation—where a subset of regulated isoforms is highly conserved, but most alternative splicing events are likely not to be—is analogous to that observed for transcription factor binding, in which detailed studies of transcription factors active in the liver revealed that the majority of binding sites are species-specific[195,196]. It remains to be determined what fraction of species-specific isoforms contribute to phenotypic differences between species compared with those that constitute non-functional products of noisy splicing. However, as only approximately one quarter of alternative exons are conserved between human and mouse[184], mis-spliced isoforms that are relevant to human disease pathogenesis yet absent from the mouse genome are likely to exist. Given these differences between species, the parallel use of genome-engineering techniques to generate isogenic human cell lines carrying spliceosomal gene mutations in endogenous loci may prove particularly useful for detailed transcriptional studies. For example, Zhang *et al.*[98] recently generated *SRSF2*P95H knock-in K562 cells to identify the changes in exon recognition and differential splicing induced by *SRSF2* mutations. Combined studies of murine models, isogenic human cells, and patient cohorts will likely prove essential to identify the direct targets of mutant spliceosomal proteins with cancer relevance.



Key points

- Genetic and functional data indicate that RNA splicing factors can act as oncoproteins as well as tumor suppressors.
- A subset of RNA splicing factors are recurrent targets of specific point mutations in cancer. Many other splicing factors exhibit dysregulated expression in cancer.
- In many cases, recurrent spliceosomal mutations alter splice site or exon recognition preferences to cause abnormal RNA splicing.
- Spliceosomal mutations are sufficient to impair myeloid differentiation in murine models. In the case of *SRSF2*, impaired differentiation has been linked to a specific splicing change in a downstream gene (*EZH2*).
- Spliceosomal mutations may affect cellular processes, including epigenetic regulation, the DNA damage response and nonsense-mediated decay, in addition to regulation of RNA splicing.
- Small molecules that disrupt splicing catalysis and/or targeted correction of specific splicing changes may provide novel therapeutic opportunities for cancers bearing spliceosomal mutations.

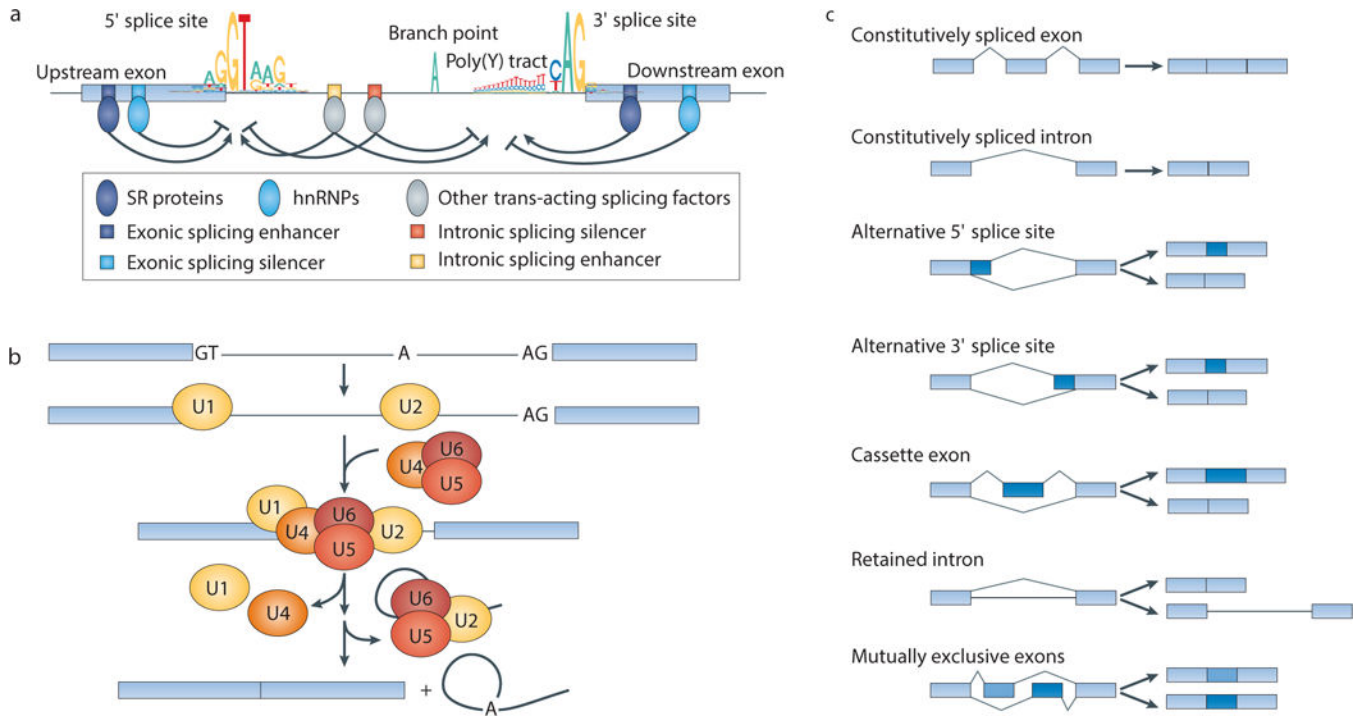


Figure 1. Simplified model of constitutive and alternative splicing

(**a**) The key sequence features that govern splicing are shown, including consensus sequences of the 5′ and 3′ splice sites and sequence motifs bound by *trans*-acting splicing factors (serine/arginine-rich (SR) proteins, heterogeneous nuclear ribonucleoproteins (hnRNPs) and others). Sequence elements required for assembly of the spliceosome onto the pre-mRNA, including the splice sites themselves, polypyrimidine (poly(Y)) tract and branch point, frequently follow the illustrated consensus motifs, whereas the sequences of enhancer or silencer elements depend upon the specific RNA-binding protein that recognizes them. Consensus motifs are illustrated as sequence logos, where the height of each nucleotide corresponds to its approximate genome-wide frequency in bits. Sequence motifs are illustrated as genomic DNA sequence rather than pre-mRNA sequence (“T” instead of “U”). Note that splicing factors such as SR proteins and hnRNPs frequently play context-dependent regulatory roles. (**b**) Simplified schematic of intron excision and ligation of two adjacent exons. The steps shown are: recognition of the 5′ and 3′ splice sites by the U1 and U2 small nuclear ribonucleoprotein complexes (snRNPs), assembly of the snRNPs into the active spliceosome, the excision of the intron lariat and the ligation of the two exons. (**c**) Schematic of constitutive and alternative splicing events. Light blue: constitutive sequence that always forms part of the mature mRNA; dark blue: alternative sequence that can be either included or excluded in the mature mRNA.

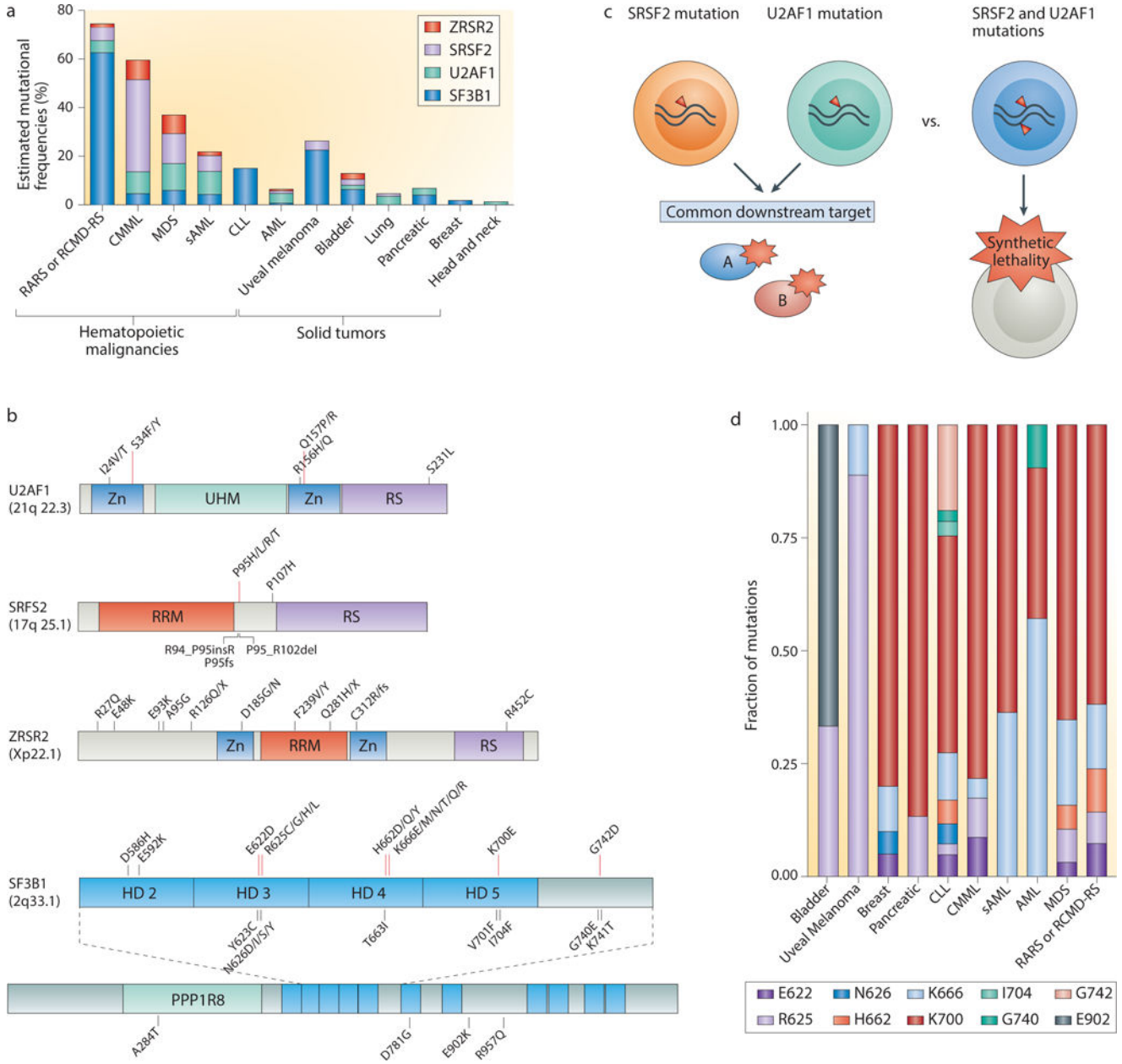


Figure 2. Commonly mutated spliceosomal proteins and their associations with specific cancer types

(**a**) The incidence and cancer distribution of the most frequently mutated spliceosomal genes are shown. (**b**) The spectrum of mutations that have been identified in *U2AF1* (U2 small nuclear RNA auxiliary factor 1), *SRSF2* (serine/arginine-rich splicing factor 2), *ZRSR2* (zinc finger, RNA-binding motif and serine/arginine-rich 2) and *SF3B1* (splicing factor 3B, subunit 1). Mutations shown in bold text occur at hotspots; other illustrated mutations are recurrent but rare. As *ZRSR2* mutations do not occur at hotspots, very rare or private mutations are shown as examples. (**c**) Schematic illustrating the potential reasons that spliceosomal gene mutations are mutually exclusive with one another: they either converge on a common downstream target or result in synthetic lethality. (**d**) The frequency of specific

mutations in *SF3B1* across various histological subtypes of cancer. AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CMML, chronic myelomonocytic leukemia; HD, HEAT domain; PPP1R8, binding site for protein phosphatase 1 regulatory subunit 8; RARS, refractory anemia with ringed sideroblasts; RCMD-RS, refractory cytopenia with multilineage dysplasia and ringed sideroblasts; RRM, RNA recognition motif; RS, arginine/serine-rich domain; sAML, secondary AML; UHM, U2AF homology motif; ZN, zinc finger domain.

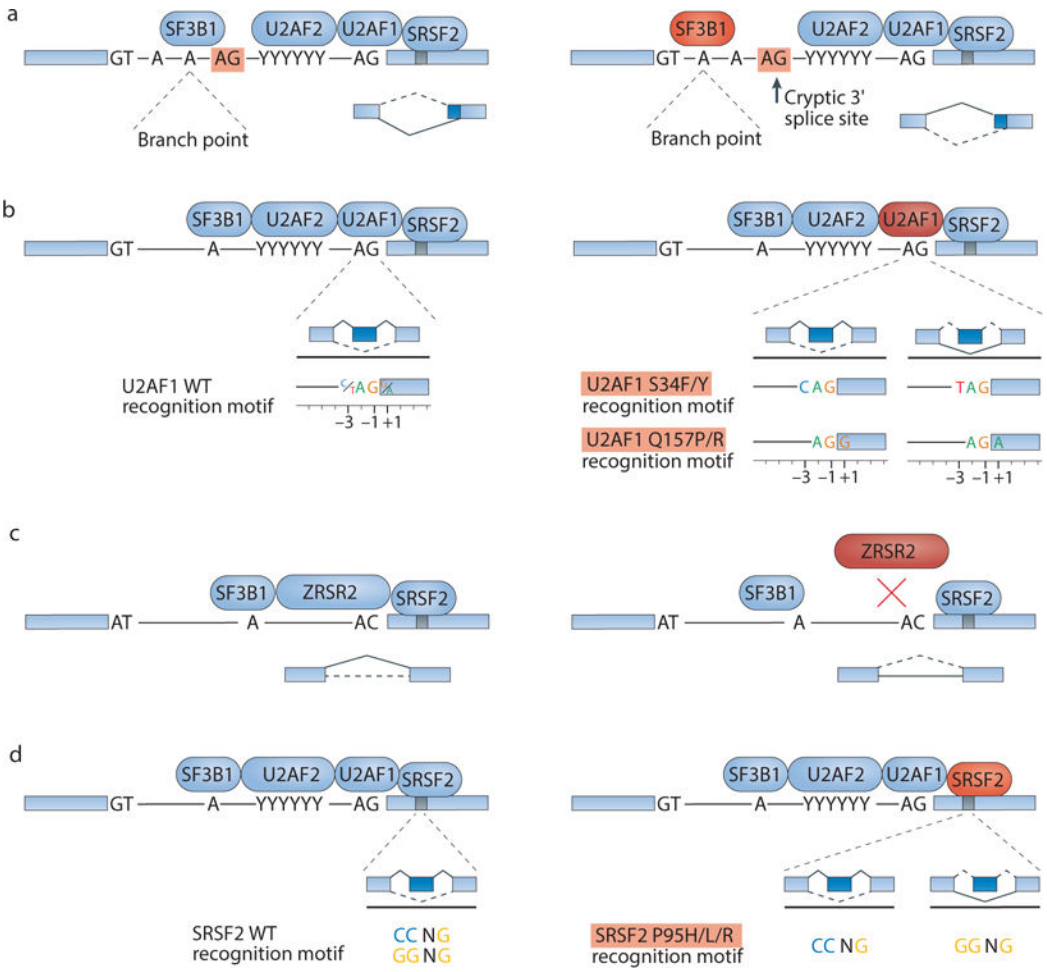


Figure 3. Current understanding of the mechanistic consequences of spliceosomal gene mutations for RNA splicing

(**a**) Splicing factor 3B, subunit 1 (*SF3B1*) mutations (mutant form shown in red) alter 3′ splice site (ss) selection by permitting or enhancing recognition of cryptic upstream 3′ splice sites. It is not yet known how mutations affecting SF3B1 alter its target protein:RNA and/or protein:protein interactions to drive this cryptic splice site recognition. Sequence motifs are illustrated as genomic DNA sequence rather than pre-mRNA sequence ("T" instead of "U"). (**b**) U2 small nuclear RNA auxiliary factor 1 (*U2AF1*) mutations alter 3′ splice site consensus sequences. Wild-type U2AF1 recognizes the consensus motif yAG|r at the intron|exon boundary (y = pyrimidine, r = purine, '|' = intron-exon boundary). S34F or S34Y mutations (shown in red) promote recognition of cAG|r over tAG|r, whereas Q157P or Q157R mutations (shown in red) promote recognition of yAG|g over yAG|a**.** (**c**) Zinc finger, RNA-binding motif and serine/arginine-rich 2 (*ZRSR2*) mutations (shown in red) cause loss of ZRSR2 function to induce splicing defects, primarily involving the aberrant retention of U12-type introns. (**d**) Serine/arginine-rich splicing factor 2 (*SRSF2*) mutations (shown in red) alter exonic splicing enhancer (ESE) preferences. Wild-type SRSF2 recognizes the consensus motif SSNG (S = C or G), whereas mutant SRSF2 preferentially recognizes the CCNG motif over the GGNG motif.

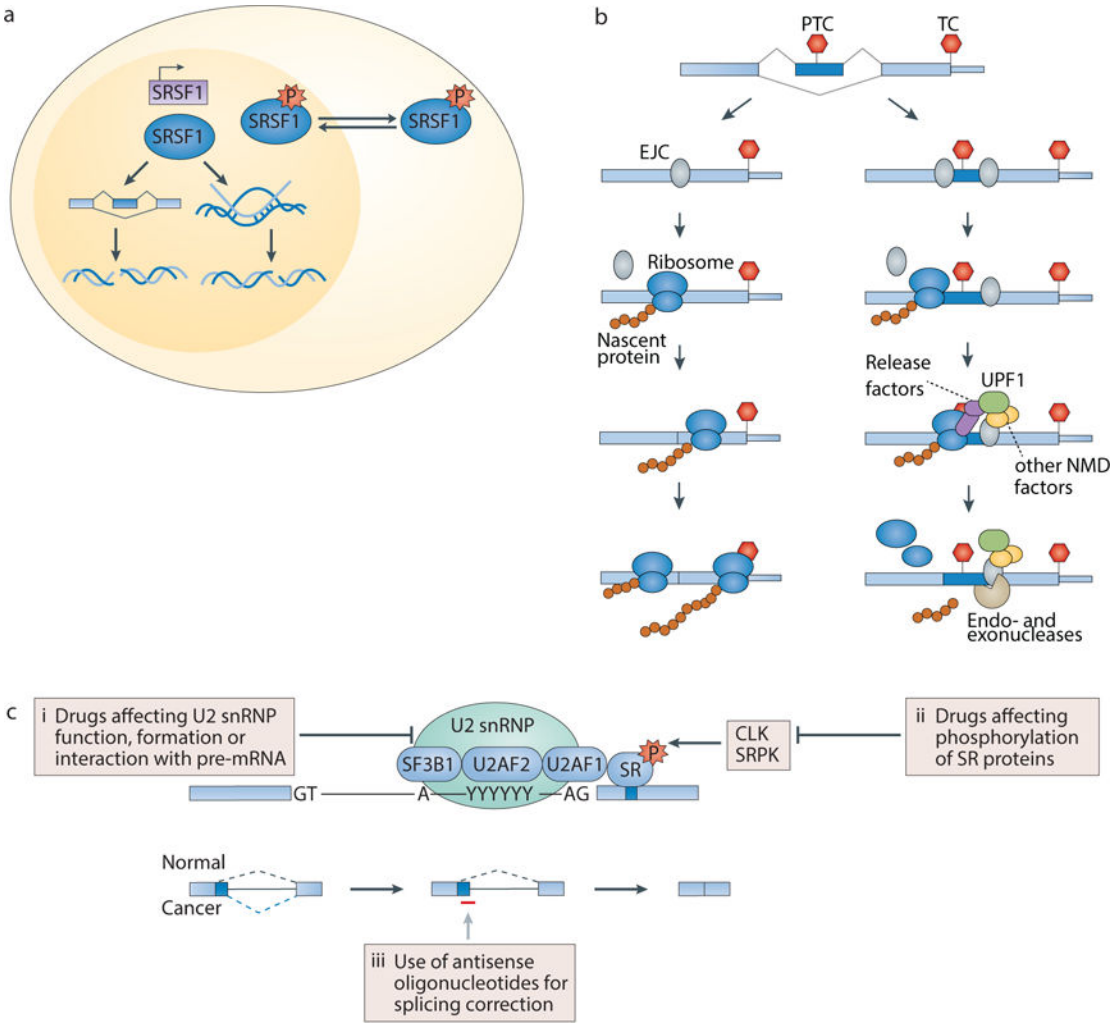


Figure 4. Links between splicing factors and diverse biological processes and potential methods for therapeutic manipulation of splicing

(**a**) Changes in the abundance, post-translational modifications and/or subcellular localization of splicing factors such as serine/arginine-rich splicing factor 1 (SRSF1) can cause DNA damage or influence the DNA damage response (DDR). Splicing factors have been linked to DNA damage and the DDR both directly (for example, insufficient levels of splicing factors can cause R loop formation and genomic instability) and indirectly (for example, through downstream changes in splicing). (**b**) The steps involved in nonsense-mediated decay (NMD) are shown. The exon junction complex (EJC; grey) is deposited upstream of exon-exon junctions on the processed mRNA, and is displaced by the ribosome (blue) during the pioneer (first) round of translation. Ribosome stalling at a premature termination codon (PTC) >50 nt upstream of an EJC promotes interactions between release factors (purple) and UPF1 (green), recruitment of other NMD components (orange), and RNA degradation by endo- and exonucleases (beige). In contrast, if only a normal termination codon (TC) is present, then all EJCs are displaced by the ribosome during the pioneer round of translation and NMD is not triggered. Red stop signs indicate stop codons. (**c**) Compounds and oligonucleotides that can disrupt or modulate normal splicing catalysis

or alter splice site recognition through distinct pathways include (**i**) drugs affecting U2 small nuclear ribonucleoprotein (snRNP) function, formation, and/or interaction with pre-mRNA, (**ii**) drugs affecting post-translational modifications of serine/arginine-rich (SR) proteins and potentially other splicing factors, and (**iii**) oligonucleotides to manipulate specific mRNA isoforms that may be important in tumor maintenance. CLK, CDC2-like kinase; SF3B1, splicing factor 3B, subunit 1; SRPK, SR protein kinase.

Table 1

Unmutated splicing factors that function as proto-oncogenes or tumour suppressors

Splicing factor	Downstream dysregulated isoforms that are functionally linked to cancer
ESRP1 and ESRP2	Promotes an epithelial splicing program to regulate EMT[62,63].
hnRNP A1	Contributes to aerobic glycolysis in cancer by promoting the expression of specific isoforms of pyruvate kinase (*PKM2* isoform)[47,48] and *MAX* (the Delta *MAX* isoform)[49].
hnRNP A2	Contributes to aerobic glycolysis in cancer by promoting the expression of a specific isoform of pyruvate kinase (*PKM2* isoform)[47,48]. Increases breast cancer cell invasion by promoting the expression of a specific isoform of *TP53INP2*[197].
hnRNP A2/B1	Acts as an oncogenic driver in glioblastoma by regulating splicing of several tumor suppressors and oncogenes, including *RON*[198].
hnRNP H	Contributes to survival of gliomas as well as invasion by promoting the expression of specific isoforms of *IG20* and *RON*, respectively[199].
hnRNP K	Serves as a tumor suppressor in leukemia. Deletion is associated with aberrant p21 and C/EBP expression (although mechanistic links to splicing and gene expression are unclear)[52].
hnRNP M	Contributes to EMT in breast cancer and increases metastasis in mice by promoting the expression of a specific isoform of *CD44* (CD44s)[200].
PRPF6	Promotes cell proliferation in colon cancer by altering splicing of genes associated with growth regulation, including the kinase gene *ZAK*[201].
PTB (PTBP1)	Contributes to aerobic glycolysis in cancer by promoting the expression of a specific isoform of pyruvate kinase (*PKM2* isoform)[47,48]. Also promotes the expression of an isoform of the deubiquitinating enzyme-encoding gene *USP5*[202] that has been shown to promote glioma cell growth and mobility (although the mechanism underlying this phenotypic association is not resolved).
QKI	Acts as a tumor suppressor by regulating alternative splicing of *NUMB* in lung cancer cells[54].
RBFOX2	Promotes a mesenchymal splicing program to regulate EMT[63].
RBM4	Acts as a tumor suppressor by promoting the pro-apoptotic isoform BCL-X_S of *BCL2L1* and opposing the pro-tumorigenic effects of SRSF1 on mTOR activation[61].
RBM5, RBM6 and RBM10	RBM5 modulates apoptosis by regulating alternative splicing of *CASP2*[203] and *FAS*[204]. RBM5 or RMB6 depletion has an opposite effect to RBM10 depletion, as these factors antagonistically regulate the alternative splicing of *NUMB*[59,60].
SRSF1	Promotes an isoform of the kinase MNK2 that promotes eIF4E phosphorylation independently of MAP kinase signaling[40]. In the context of breast cancer, SRSF1 overexpression promotes alternative splicing of *BIM* and *BIN1*[41] to promote the expression of isoforms that lack pro-apoptotic functions.
SRSF3	Regulates alternative splicing of *TP53*[44] such that SRSF3 loss promotes expression of p53β, an isoform of p53 that promotes p53-mediated senescence.
SRSF6	Promotes expression of isoforms of the extracellular matrix protein tenascin C that are characteristic of invasive and metastatic skin cancer[45], contributing to epithelial cell hyperplasia.
SRSF10	Promotes cell proliferation and colony formation *in vitro* and increases tumorigenic capacity of colon cancer cells in mice by inducing expression of a specific isoform of *BCLAF1* (BCLAF1-L)[205].

BCLAF1, Bcl-2-associated transcription factor 1; EMT, epithelial to mesenchymal transition; ESRP, epithelial splicing regulatory protein; hnRNP, heterogeneous nuclear ribonucleoprotein; NMD, nonsense-mediated decay; PRPF, pre-mRNA-processing factor; PTB, polypyrimidine tract-binding protein 1; QKI, quaking; RBM, RNA binding motif protein; SRSF, serine/arginine-rich splicing factor.

Table 2

Recurrent mutations affecting splicing factors in cancer and their disease associations.

Splicing factor	Diseases	Mutated residue*	Frequencies	Clinical associations
SF3B1	RARS	K700, K666, H662, R625, E622	48 – 75.3%	Increased event-free or overall survival, lower risk of transformation to AML[10]
	CMML	K700, K666, R625, E622	4.3 – 4.7%	
	MDS	K700, K666, H662, R625, E622	4.4 – 7%	
	sAML	K700, K666	3.7 – 4.8%	
	AML	K700, K666, G740	2.6 – 7.3%	
	CLL	K700, K666, H662, G742	15%	Reduced treatment-free or overall survival, faster disease progression[11,12,79]
	Pancreatic	K700, R625	4%	–
	Breast	K700, K666, E622, N626	1.80%	–
	Uveal melanoma	R625, K666	15 – 20%	Relatively favorable prognosis[64,65]
	Bladder	R625, E902	4%	–
U2AF1	RARS	S34, Q157	5%	Less favorable overall survival, higher risk of transformation to AML[9]
	CMML		8 – 11%	
	MDS		10.3 – 11.6%	
	sAML		9.7%	
	AML		1.3%	
	Lung	S34	3%	Reduced progression-free survival[56]
SRSF2	RARS	P95	5.5%	Unfavorable prognostic factor for AML transformation, reduced overall survival[206]
	CMML		28 – 47%	
	MDS		11.6%	
	sAML		6.5%	
	AML		0.7%	
ZRSR2	RARS	No evident mutational hotspot (presumed loss-of-function)	1.4–10%	Higher risk of transformation to AML[207]
	CMML		8.0%	
	MDS		7.7%	
	sAML		1.6%	

AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CMML, chronic myelomonocytic leukemia; MDS, myelodysplastic syndromes; RARS, refractory anemia with ring sideroblasts; sAML, secondary AML; SF3B1, splicing factor 3B, subunit 1; SRSF2, serine/arginine-rich splicing factor 2; U2AF1, U2 small nuclear RNA auxiliary factor 1.

*Only residues recurrently affected by somatic mutations are listed.