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Alternative-splicing defects in cancer: splicing regulators and their downstream targets, guiding the way to novel cancer therapeutics

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Abstract

Defects in alternative splicing are frequently found in human tumors and result either from mutations in splicing-regulatory elements of specific cancer genes or from changes in the regulatory splicing machinery. RNA splicing regulators have emerged as a new class of oncoproteins and tumor suppressors, and contribute to disease progression by modulating RNA isoforms involved in the hallmark cancer pathways. Thus dysregulation of alternative RNA splicing is fundamental to cancer and provides a potentially rich source of novel therapeutic targets. Here we review the alterations in splicing regulatory factors detected in human tumors, as well as the resulting alternatively spliced isoforms that impact cancer hallmarks, and discuss how they contribute to disease pathogenesis. RNA splicing is a highly regulated process and, as such, the regulators are themselves tightly regulated. Differential transcriptional and post-transcriptional regulation of splicing factors modulates their levels and activities in tumor cells. Furthermore, the composition of the tumor microenvironment can also influence which isoforms are expressed in a given cell type and impact drug responses. Finally, we summarize current efforts in targeting alternative splicing, including global splicing inhibition using small molecules blocking the spliceosome or splicing-factor-modifying enzymes, as well as splice-switching RNA-based therapeutics to modulate cancer-specific splicing isoforms.

Graphical Abstract

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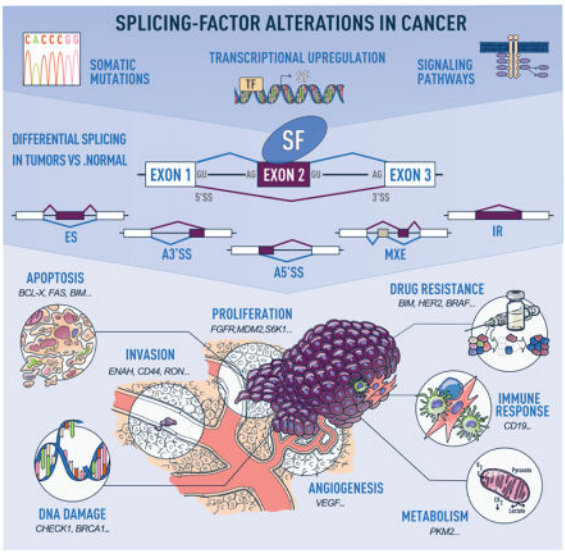

INTRODUCTION

Cancers arise as a consequence of the dysregulation of cellular homeostasis and of its multiple control mechanisms. Alternative RNA splicing is a key step of post-transcriptional gene expression regulation. It contributes to proteomic and functional diversity by enabling the production of distinct RNA isoforms from a single gene. Alternative splicing provides transcriptional plasticity by controlling which RNA isoforms are expressed at a given time point in a given cell type. Cancer cells subvert this process to produce isoforms that benefit cell proliferation or migration, or unable escape from cell death (Figure 1)[1].

RNA splicing is a highly controlled process that relies on cis-regulatory elements and trans-regulatory factors. The core splicing machinery, the spliceosome, removes introns and joins exons together to generate a mature mRNA molecule. This machinery assembles on the pre-mRNA molecule on specific sequences located at the exon-intron boundaries and that define the 3′ and 5′ splice sites (SSs) and the branch point site (BPS). The core human spliceosome, together with associated regulatory factors, comprise more than 300 proteins and five small nuclear RNAs (snRNAs), and catalyze both constitutive and regulated alternative splicing[2–5]. The architecture of the spliceosome undergoes dynamic remodeling in preparation for, during, and after the splicing reaction (Figure 2). In addition to the core spliceosome, regulatory proteins are involved in modulating the splicing reaction, and act as splicing activators or repressors by binding to exonic or intronic enhancer or silencer elements.

Defects in alternative splicing are frequently found in human tumors and result either from mutations in splicing-regulatory elements of specific cancer genes or from changes in the regulatory splicing machinery[6]. Alterations of the splicing machinery are particularly important in cancer because they affect a network of downstream splicing targets, whereas a mutation affecting splicing of a single gene often affects only one isoform. RNA splicing regulators have recently emerged as a new class of oncoproteins or tumor suppressors, and

contribute to disease progression by modulating RNA isoforms involved in the hallmarks cancer pathways. Dysregulation of alternative splicing is a fundamental process in cancer and provides a potentially rich source of novel therapeutic targets and biomarkers for disease progression. A better understanding of the regulators of the splicing machinery is a crucial step in understanding the role of RNA splicing in cancer. Here we review the alterations in splicing regulatory factors detected in human tumors, as well as the alternatively spliced isoforms that impact cancer hallmarks, and discuss how they contribute to disease pathogenesis. Finally, we summarize current efforts in targeting alternative splicing as cancer therapeutics.

ALTERATIONS IN SPLICING REGULATORY COMPONENTS

Splicing-factor mutations associated with malignancies

Recurrent somatic mutations in components of the human splicing machinery occur in human tumors, most frequently in hematological malignancies[7], suggesting that splicing-factor alterations are a hallmark of cancer. Interestingly, the two most frequently mutated splicing factors are SF3B1, a core component of U2 snRNP involved in BPS selection, and SRSF2, a serine/arginine-rich (SR) protein that acts both in alternative and constitutive splicing and interacts with U1 snRNP (Figure 2). Mutations in other splicing factors have been also been identified, and the list is growing every day as more human tumors are sequenced. However, the functional consequences of most of these mutations and their roles in tumor progression remain to be characterized.

SF3B1 (alias SF3B155)—SF3B1, the key protein component of U2 snRNP, is crucial for formation of the spliceosomal A complex. SF3B1 interacts directly with the RNA-recognition motif (RRM) of U2AF2 as well as with SF3B14a, thus creating a stable complex that directs the recognition of the BPS by U2 snRNA[5]. SF3B1 also interacts with nucleosomes suggesting that chromatin structure can modulate its splicing functions[8]. Recurrent somatic *SF3B1* mutations occur in myelodysplastic syndromes (MDS), including 83% of refractory anemia with ringed sideroblasts (RARS), an MDS variant with erythroid dysplasia and favorable outcomes, and 76% of refractory cytopenia with multilineage dysplasia and ringed sideroblasts (RCMD-RS) which carries a less favorable survival rate[9–12]. *SF3B1* mutations cluster in exons 12–15, which encode HEAT repeats, a region important for the association of SF3B1 with SF3B14a[13]. *SF3B1* missense mutations alter the recognition of alternative or cryptic 3′SS leading to differential splicing of transcripts, 70% of which are novel isoforms and 50% undergo nonsense-mediated decay (NMD)[14,15]. In mouse models, this differential exon usage disrupts key pathways in hematopoiesis and iron metabolism and blocks erythroid differentiation, thus providing a basis for the pathogenesis of RARS and RCMD-RS[16,17]. The K700E missense mutation, which accounts for more than half of *SF3B1* mutations in MDS patients and is associated with a better prognosis, promotes splicing of an isoform of the erythroid lineage transcription factor *TAL1* that reduces erythroid differentiation *in vitro*[10,13,18]. *SF3B1* mutations are also detected in other cancers including 15% of chronic lymphocytic leukemia (CLL), in which *SF3B1* mutations are associated with an anti-apoptotic role and correlate with poor overall prognosis[12,19,20]. Additionally, the K700E mutation is detected in 3% of pancreatic and

1.8% of breast cancers, both of which exhibit alterations in RNA splicing patterns[21,22]. *SF3B1* mutations also occur in 1% of cutaneous melanomas and 20% of uveal melanomas in which they are associated with aberrant splicing, chromosome 3 disomy, and intermediate prognosis[23–26].

SRSF2 (alias SC35)—The splicing factor SRSF2 belongs to the SR protein family and is involved in regulation of both alternative and constitutive splicing. SRSF2 coordinates recognition of the 5′ and 3′ SS by the U1 and U2 snRNPs, respectively. SR proteins recognize enhancer and silencer sequences in pre-mRNA exons and introns and thereby favor exon inclusion or skipping by recruiting or inhibiting spliceosome assembly[5]. Mutations in *SRSF2* are frequently observed in hematologic malignancies including 10% of MDS, 31–47% of chronic myelomonocytic leukemia (CMML), and 2% of acute myeloid leukemia (AML)[13]. *SRSF2* mutations in MDS are associated with decreased overall survival and increased progression rate from MDS to AML[27]. Interestingly, *SRSF2* missense mutations cluster at proline 95, in a region proximal to the RRM domain, which confers the RNA binding specificity[13,28]. In mouse models, blood lineage-specific *SRSF2* knockout (KO) or heterozygous expression of $Srsf2^{P95H}$ causes defective hematopoiesis[29]. The $SRSF2^{P95H}$ mutation induces splicing changes in mouse and human myeloid cell models, which likely result from alterations in pre-mRNA sequences recognized by the RRM of SRSF2[29–32]. Indeed, mutant SRSF2 exhibits increased binding specificity for the CCNG consensus sequence, whereas wild-type SRSF2 recognize both CCNG and GGNG sequences[29]. This alteration in sequence specificity leads to the inclusion of a premature termination codon (PTC)-containing exon in *EZH2*, a histone methyltransferase implicated in the pathogenesis of MDS[29]. Finally, deletion of *Ezh2* in mouse hematopoietic stem cells causes MDS, providing a causal link between the *SRSF2* mutation, *EZH2* loss of function, and MDS[33].

U2AF1, ZRSR2, RBM10 and other splicing-factor mutations—While *SF3B1* and *SRSF2* are the most frequently mutated splicing factors in hematologic malignancies, other factors also exhibit recurrent mutations in MDS.

U2AF1 (alias U2AF35) is involved in the formation of the spliceosomal E complex. As a heterodimer with U2AF2 (U2AF65), it is responsible for the recognition of the 3′SS and BPS as well as for stabilizing U2 snRNA binding to the BPS[5]. In addition to MDS, *U2AF1* is also mutated in 3% of lung adenocarcinomas[34]. Missense mutations in *U2AF1* occur almost exclusively at S34 and Q157, thus affecting the C-terminal zinc finger domain. Expression of $U2AF1^{S34F}$ in HeLa cells leads to an increase in PTC-containing transcripts, suggesting global splicing defects[13]. *U2AF1* mutants disrupt proliferation in HeLa cells and exhibit a decreased ability to reconstitute the hematopoietic system when introduced into mouse hematopoietic stem cells, thereby convoluting the link between these mutations and MDS[13]. However, a recent study described a gain-of-function role for mutant U2AF1[35]. When overexpressed in human hematopoietic progenitor cells, $U2AF1^{S34F}$ promotes lineage-specific splicing changes, most notably in *H2AFY* and *STRAP* isoforms, which are not rescued by co-expression of wild-type U2AF1. These splicing isoforms disrupt normal erythroid and granulomyelocytic differentiation in hematopoietic progenitors[35].

Interestingly, expression of the canonical isoforms is capable of rescuing the differentiation defect[35]. Taken together, these findings suggest that mutant *U2AF1* blocks terminal differentiation of hematopoietic cells, but does not grant a growth or survival advantage, and may therefore require further mutational hits to lead to MDS.

ZRSR2 is involved in the recognition of 3′SS in both major and minor introns, a class of intronic sequences recognized by the minor U12-dependent spliceosome[36]. In addition, ZRSR2 also promotes the removal of the intron lariat and stitching of the adjacent exons[37]. *ZRSR2* mutations in MDS lead to the retention of minor introns without affecting the major introns[38]. In contrast to the hotspot mutations in other factors, *ZRSR2* mutations are widely distributed and create loss-of-function mutants, thus suggesting that ZRSR2 functions as a tumor suppressor[13,38].

The RNA-binding protein RBM10 is a component of the pre-spliceosomal complex A. Mutations in *RBM10* are associated with the TARP syndrome, an X-linked recessive disorder with congenital heart malformation and developmental abnormalities, often associated with neonatal lethality[39]. Somatic mutations in *RBM10* are found in lung adenocarcinoma[34,40], including 21% of invasive lung adenocarcinoma [41], as well as less frequently in non-anaplastic thyroid cancers[42], colorectal carcinoma[43], pancreatic adenocarcinoma[44] and intraductal papillary mucinous neoplasm[45]. *RBM10* mutations are widely distributed and create loss-of-function mutants, indicating that RBM10 functions as a tumor suppressor[46]. Furthermore, the presence of *RBM10* mutations is associated with a significant reduction in *RBM10* expression in lung tumors, and is accompanied by changes in proliferation rates and in alternative splicing of RBM10 target genes[47]. For example, missense or truncating *RBM10* mutations found in lung cancer patients disrupts RBM10-mediated regulation of *NUMB* splicing, inducing a pro-proliferative isoform[46]. Conversely, in pancreatic cancer, *RBM10* mutations are associated with longer survival in spite of histological features of aggressive disease[44].

Mutations in other components of the spliceosome, e.g., *PRPF40B*, *U2AF2*, *SF3A1*, or *SF1,* occur sporadically in MDS patients. PRPF40B interacts with SF1 and U2AF2 to enhance the inclusion of exons with weak SSs, and regulates splicing of apoptotic isoforms of *FAS* and *BCL-x*[48]. U2AF2 is involved in 3′SS recognition, and in some cases can promote exon skipping[49,50]. SF3A1 interacts with both the U1 snRNA and U2 snRNP complex to mediate communication between the 5′SS and 3′SS complexes[51]. Additionally, PRPF40B and U2AF2 are also upregulated or downregulated in several solid tumors[52], including melanoma, where U2AF2 promotes metastasis by regulating splicing of *CD44*[53].

Alterations in splicing-factor levels

In solid tumors, splicing factors exhibit frequent changes at the copy number or expression levels but are rarely mutated[54]. Splicing factors bind directly to pre-mRNA and regulate their downstream targets in a concentration-dependent manner[55]; thus, changes in splicing-factor levels cause splicing deregulation in tumors even in the absence of mutations. Two major protein families play a critical role in the regulation of alternative splicing through recognition of intronic or exonic enhancer and silencer sequences. The serine/arginine-rich(SR) protein family is composed of 12 members (SRSF1-12) containing SR domains

that contribute to protein-protein interactions, and one or two RRMs that allow sequence-directed binding[55]. Heterogenous nuclear ribonucleoproteins (hnRNPs), which are a large and structurally diverse family of RNA-binding proteins with diverse roles in splicing, mRNA transport, and translation, often function as antagonists to SR-protein-regulated alternative splicing events[56]. Below, we discuss in more detail several RNA-binding proteins that exhibit expression changes in human tumors, and for which there is compelling *in vitro* or *in vivo* evidence that their alterations affect cellular processes involved in transformation (Figure 3).

SRSF1 (alias ASF/SF2)—SRSF1 is a proto-oncogene that controls alternative splicing but also regulates other steps of RNA metabolism[57]. SRSF1 is frequently upregulated in breast, lung, colon and bladder tumors, in part due to an amplification of Chr.17q23[58–60]. In breast cancer models, SRFS1 overexpression promotes transformation *in vivo* and *in vitro* by enhancing proliferation and decreasing apoptosis[58]. Additionally, SRSF1 acts synergistically with MYC, and their co-expression correlates with higher tumor grade and decreased survival in breast and lung cancer patients[58,60–62]. SRSF1 oncogenic activity relies on the regulation of splicing isoforms involved in apoptosis (e.g., *BCL2L1*, *BCL2L11*, *BIN1*), cell growth (e.g., *RPS6KB1*), cell survival (e.g., *MKNK2*), or motility (e.g., *RON*)[58–60,63,64]. In lung cancer, *SRSF1* upregulation is associated cisplatin and topotecan resistance[65].

SRSF3 (alias SRp20)—In addition to its role in splicing regulation, SRSF3 is also involved in transcription termination, IRES-dependent viral RNA translation, and homologous recombination-mediated DNA repair[66–68]. Additionally, SRSF3 together with SRSF1 associates with hypo-phosphorylated chromatin, and controls G0/G1 re-entry[69]. *SRSF3* is overexpressed in lung, breast, ovarian, stomach, bladder, colon, liver, and oral tumors, in part due to copy number changes on Chr.6p21[70–72], and shows variable expression in renal cancers[52,70]. SRSF3 acts as a proto-oncogene as its overexpression is capable of transforming human fibroblasts *in vitro*, while its depletion causes growth arrest of cancer cell lines[70]. SRSF3 regulates alternative splicing of genes involved in tumorigenesis, such as isoforms *PKM2* that alters cell metabolism or *TP53β* that induces cellular senescence[73,74].

SRSF6 (alias SRp55)—*SRSF6* is frequently upregulated in breast, lung, pancreatic and colon cancers, in part due to an amplification of its locus[60]. SRSF6-overexpression synergizes with MYC to promote transformation of lung epithelial cell lines[75], while, its knockdown in lung carcinoma cells decreases proliferation and prevents tumor formation in immunocompromised mice[75]. SRSF6 promotes pro-oncogenic splice variants of the insulin receptor *INSR*, the tumor suppressor *DLG1*, and the downstream effector of the MAPK pathway *MKNK2*[75]. SRSF6 is also upregulated in multiple subtypes of skin cancer, and its overexpression in murine skin promotes splicing of cassette exons, coordinates wound healing, and induces hyperplasia[76]. Conversely, PLX4720, a BRAF inhibitor, induces SRSF6 expression in $BRAF^{V600E}$ melanoma cell lines, which in turn promotes splicing of the pro-apoptotic isoform *BIM-S* leading to increased cell death[77]. While *SRSF6* upregulation also correlates with increased *BIM-S* expression post-treatment, continued

exposure to PLX4720 leads to drug resistance[78]. Finally, SRSF6 is downregulated in kidney tumors, which could indicate cell type-specific functions[52].

Other SR proteins—Other SR proteins are also altered in human tumors but have less well-defined roles in transformation. SRSF5 is upregulated in breast tumors with lymph node metastasis[79] and oral tumors[80]. SRSF5 or SRSF7 are upregulated in lung cancer, and their knockdown impacts cell proliferation[81]. Interestingly, *SRSF5* shows broad downregulation in breast, lung, liver, and kidney tumors[52]. SRSF4 regulates alternative splicing events leading to cell death in cisplatin treated breast cancer cells[82]. Renal tumors show a broad differential expression of various SR proteins[83]. Co-expression of these splicing factors may indicate that the robust network of splicing changes in cancer cells is due to an imbalance among multiple splicing factors rather than differential splicing regulated by a single splicing factor.

TRA2β—TRA2β is an SR-like protein that regulates alternative splicing and is essential for embryonic development[84]. Overexpression of *TRA2β* occurs in lung, breast, ovarian, cervical, prostate, colon, and central nervous system tumors, where it correlates with an aggressive phenotype, whereas downregulation is detected in thyroid and renal cancers[52,71,85–91]. *TRA2β*-overexpression promotes proliferation in human lung carcinoma cells, while its knockdown induces apoptosis[85]. *TRA2β* overexpression in human glioma cells promotes proliferation and migration[89], and *TRA2β* KO leads to defects in murine brain development, highlighting the importance of TRA2β homeostasis in neurogenesis[92]. TRA2β regulates the inclusion of *CD44* exons v4 and v5 in breast tumors[90], and inclusion of estrogen receptor alpha *ERα* exon 7, creating a dominant negative isoform in endometrial tumors[93]. Interestingly, lung tumors exhibit a rare fusion protein between *TRA2β* and *DNAH5* that preferentially localizes to the cytoplasm, activates ERK1/2 through inhibition of SIRT6, and promotes lung cancer[94].

hnRNPA1—hnRNPA1 regulates alternative splicing and translation, and is overexpressed in blood, lung, and colorectal malignancies[52,95–98]. hnRNPA1 upregulation in lung adenocarcinoma is associated with increased tumor staging; conversely, hnRNPA1 knockdown decreases cell proliferation and induces cell cycle arrest in lung cancer cell lines[97]. In response to ultraviolet radiation, hnRNPA1 expression is increased in skin cells, consequently modulating splicing of *HDM2* and promoting cell survival by activating the mTOR pathway[99,100]. Furthermore, hnRNPA1 is upregulated in AML, where it functions to prevent myeloid differentiation by binding to the 3′-UTR and thereby preventing translation of C/EBPα mRNA, a critical transcription factor for myelopoiesis[98].

hnRNPA2/B1—hnRNPA2/B1, a splicing regulator closely related to hnRNPA1, is frequently overexpressed in lung, breast, colorectal, and brain tumors[52,101–103]. Up-regulation of hnRNPA2/B1 in bronchial lavage specimens predicts the diagnosis of a lung neoplasm with high sensitivity and specificity[101], and its degree of overexpression correlates with microsatellite instability[104], increased tumor stage, and decreased overall survival[105]. hnRNPA2/B1 mediates its tumorigenic effect in glioblastoma through alternative splicing of key oncogenes and tumor suppressors. For example, hnRNPA2/B1 overexpression causes

skipping of *RON* exon 11, creating an oncogenic isoform involved in cell motility; skipping of exon 11 in the insulin receptor *INSR* leading to an isoform with altered substrate specificity that binds to broader range of mitogens; or inclusion of exon 12a in the tumor suppressor *BIN1m* creating an isoform that is unable to stimulate apoptosis[102].

hnRNPK—hnRNPK is a splicing factor that can act as a tumor suppressor but also exhibits oncogenic functions. Heterozygous deletion of 9q, where hnRNPK is located, is a characteristic of AML and results in hnRNPK decreased expression and haplo-insufficiency[106,107]. hnRNPK interacts directly with *C/EBPα* mRNA, and heterozygous hnRNPK KO mice express low levels of the C/EBPα p42 isoform and eventually develop abnormal myelopoiesis[98,106]. *hnRNPK* expression is also decreased in renal tumors[52]. Consistent with its role as a tumor suppressor, hnRNPK is an HDM2-regulated cofactor for p53, and its expression increases upon DNA damage[108]. Furthermore, hnRNPK knockdown leads to defects in DNA-repair and to increased DNA damage after gamma-irradiation[108,109]. However, hnRNPK also exhibits oncogenic functions and is upregulated in breast, colorectal, and pancreatic cancer tissues and cell lines [110–113]. For example, inhibition of hnRNPK in human cancer cells decreases cell motility, whereas its upregulation increases proliferation and migration[110,114]. In colorectal and pancreatic tumors and cell lines, oncogenic hnRNPK is translocated from the nucleus to the cytoplasm, thus suggesting a potential explanation for its ability to act as either an oncogene or a tumor suppressor[112–114].

Other hnRNPs—Upregulation of hnRNPM is detected in metastatic breast tumor[115]. hnRNPM regulates epithelial-mesenchymal transition (EMT) in breast epithelial cells, in part by promoting splicing of the *CD44s* isoform, and by altering TGF-β signaling[115]. hnRNPM upregulation is also a poor prognostic factor for Ewing's sarcoma, where inhibition of the PI3K/AKT/mTOR pathway causes broad transcriptome changes mediated by hnRNPM-regulated splicing events[116]. Additionally, hnRNPH1 contributes to the aggressiveness of glioblastoma via alternative splicing of *IG20/MADD* and *RON,* creating anti-apoptotic and pro-motility protein isoforms[117]. Moreover, hnRNPC is upregulated in lung and colorectal cancers, and downregulated in kidney cancers [52]. hnRNPC acts as a tumor suppressor and alters DNA damage repair by binding to *BRCA1*, *BRCA2*, *RAD51*, and *BRIP1* mRNA and modulating the inclusion of intronic Alu transposable elements[118]. hnRNPE1 upregulation in pancreatic cancer is associated with metastasis and promotes alternative splicing of integrin β1, a transmembrane protein involved in cell adhesion[119]. Finally, PTBP1, also known as hnRNPI is upregulated in breast, brain, colon, endometrial, and ovarian tumors and cell lines[120–124]. PTBP1-overexpression increases proliferation, anchorage-independent growth, and invasion in cancer cell lines, but does not transform murine fibroblasts[124,125].

Other splicing factors—The epithelial-specific splicing factors ESRP1 and ESRP2 affect splicing of target genes involved in EMT, including *CD44*, *ENAH*, *FGFR2*, and *RAC1*[126–131]. They are often upregulated in normal epithelium but downregulated in invasive fronts[132]. Paradoxically, they have been assigned both a tumor suppressor and an oncogenic function[133–135].

Similarly, the splicing factor RBFOX2 has been linked with EMT, and regulates splicing targets in breast, pancreatic and colon tumors[128,136–138].

Additionally, splicing factors RBM5 and 10 are found upregulated or downregulated in several solid tumors, and are implicated in the splicing of apoptotic proteins BAX and BCL-x, and the notch pathway regulator NUMB[34,139–143].

Finally, QKI downregulation is a common event in several solid tumors and is associated with poor prognosis[144–146]. Interestingly, MYB-QKI fusions have been identified as a driver event in glioma[147].

Defects in pathways regulating splicing factors

Alterations in splicing-factor levels can be explained by gene amplifications or deletions only in a fraction of the tumors that exhibit splicing-factor defects[52,54]. RNA splicing is a highly regulated process and hence the splicing regulators are themselves tightly regulated. Differential regulation of splicing factors can thus affect their levels and activities in tumors even in the absence of copy-number changes or mutations. Here we discuss examples of transcriptional and post-transcriptional regulation that could explain the defects in splicing-factor levels observed in tumors (Figure 4).

Transcriptional Regulation—The transcription factor MYC is a well-studied oncogene that is overactive in a variety of cancers. However, part of MYC's oncogenic potential may result from its ability to regulate splicing factors at the transcriptional level. The oncogenic factor SRSF1 is a direct transcriptional target of MYC, and synergizes with MYC to promote tumorigenesis in breast and lung tumors[58,62]. In gliomas, c-MYC drives the expression of PTBP1, hnRNPA1, and hnRNPA2/B1, all of which favor splicing of the PKM2 isoform used in aerobic glycolysis[148]. PTBP1 or hnRNPA1 are directly regulated by n-MYC in neuroblastomas, where they controls cell survival and correlate with a worse prognosis[149]. Conversely, knockdown of hnRNPA1 or hnRNPA2 reduces splicing of *PKM2* and alters cell metabolism[150]. Moreover, MYC-driven tumors exhibit differential expression of spliceosomal components or their regulators, e.g., BUD31 and PRMT5, as well as of their downstream targets[151,152]. In addition to MYC, other pathways control splicing-factor transcriptional activation. In colorectal tumors, the Wnt signaling pathway, which is frequently dysregulated through *APC* mutation, directly controls *SRSF3* level [153,154]. The transcription factors Ets1 and HSF1 mediate basal and oxidant-stress responses by inducing *TRA2β* expression in colorectal cancer[88]. Together, these pathways represent key points for potential targeted therapies that could be used to disrupt splicing regulators in tumors.

Alternative splicing and nonsense-mediated mRNA decay—The expression of many RNA-binding proteins is regulated through the splicing of their own pre-mRNA. SR proteins auto-regulate their levels by enhancing the inclusion of PTC-containing cassette exons, termed "poison exons", within their mRNA. These transcripts are degraded by NMD creating a negative feedback loop when SR-protein levels become elevated[155–159]. Although auto-regulation has not been experimentally demonstrated for all SR proteins, poison exons are highly conserved throughout evolution, and isoforms containing these ultraconserved regions are detected in human [160,161]. While SR proteins auto-regulate through inclusion of

poison exons, auto-regulation of hnRNPs involves both inclusion and skipping of PTC-containing regions[161–164].

Splicing factors can also cross-regulate the expression of other RNA-binding proteins, through splicing of their respective ultraconserved regions[155,162]. In murine cells, exogenous SRSF3 enhances inclusion of its own poison exon, while SRSF1 overexpression inhibits *SRSF3* exon inclusion[155]. Similarly, RBFOX2, which coordinates mesenchymal splicing networks in cancer tissues, regulates alternative splicing of a number of different RNA-binding proteins[137,165,166]. Alternative splicing of murine Quaking, *Qk,* generates three isoforms *Qk5*, *Qk6*, and *Qk7,* that exhibit both auto- and cross-regulation. Specifically, Qk5 enhances expression of total *Qk* mRNA while also binding to its own 3′-UTR and downregulates Qk5 protein expression. Qk6 negatively regulates protein expression of Qk5, while also stimulating translation of *Qk6* mRNA[167]. Human lung tumors express high levels of *QKI5* vs. *QKI6*[168], suggesting that this extensive network of auto and cross-regulation could exist in humans and that a similar mode of regulation may exist across other splicing factors.

Regulation by lncRNAs—Long non-coding RNA (lncRNAs) are involved in the regulation of alternative splicing, for example by facilitating splicing-factor binding to exonic splicing silencer or intronic splicing silencer elements. lncRNAs *PCGEM1* and *BC200* regulate alternative splicing of *AR* and *BCL-x*, respectively, through interaction with hnRNPA1, hnRNPA2/B1, or U2AF65[169,170]. Moreover, *MALAT1* modulates alternative splicing by influencing SR protein subnuclear localization[171]. Additionally, *LINC01133* sequesters SRSF6, and its knockdown allows SRSF6 to promote EMT and metastasis in colorectal cancer mouse models[172].

Regulation by miRNAs—MicroRNAs (miRNA) can act as tumor suppressors or as oncogenes and can play a role in the regulation of splicing-factor expression. Expression of SRSF7 is regulated by miR-30a-5p and miR-181a-5p in renal tumors, and this miRNA-mediated suppression of SRSF7 alters splicing patterns[83,173]. Conversely, SRSF7 regulates splicing and expression of these miRNAs, thus forming a negative feedback loop[173]. miR-30a-5p is upregulated in glioma cells by Wnt signaling and acts as an oncogene, perhaps superseding the pro-tumorigenic roles of either SRSF7 and miR-30a-5p in different cancers[174]. Additionally, SRSF1 is the target of miR-28, miR-505, miR-10a, and miR-10b[175,176]. The oncogenic lymphoma/leukemia-related factor LRF represses miR-28 and miR-505 expression and potentially leads to increased SRSF1 expression in tumors[175]. Upregulation of miR-10a and miR-10b in response to retinoic acid causes terminal differentiation of neuroblastoma cells, possibly through repression of SRSF1 levels[176]. Additionally, miR-10a and miR-10b also target TRA2β, which promotes proliferation in glioblastoma cells[89,176]. Finally, miR-451 targets hnRNPA1 in human leukemia cells, potentially acting as a tumor suppressor by repressing hnRNPA1 expression[98].

Post-translational regulation—SR proteins undergo extensive post-translational modifications which impacts their subcellular localization and thus activity. For example, phosphorylation of the C-terminal RS domain by SR-specific protein kinases (SRPKs) allows nuclear import via interactions with transportin-SR2[177–179]. Once in the nucleus,

Cdc-like kinases (CLK) control the nuclear distribution of SR proteins[180–182]. Additionally, SRPK and CLK kinases can alter the functionality of SR proteins independently of their effect on splicing-factor localization. For example, CLK2-mediated phosphorylation prevents the auto-regulation of TRA2β[157]. CLK2 acts as an oncogene in breast cancer where it alters splicing, possibly linking the regulation of splicing-factor phosphorylation and splicing dysregulation in cancer[183]. Moreover, the oncogenic kinase AKT directly phosphorylates SRSF1, SRSF7, and SRSF5[184,185]. AKT promotes phosphorylation and subsequent activation of SRPKs, thereby indirectly regulating SR proteins[186]. Finally, SRPK1 is overexpressed in various cancer types including breast, colon, pancreatic, prostate, and ovarian[187–189].

TUMOR-ASSOCIATED ALTERNATIVELY SPLICED ISOFORMS

The hallmarks of cancer described by Hanahan and Weinberg can be used to understand the capabilities acquired by cells during tumor development and progression[190]. These ten hallmarks include a cancer cell's ability to sustain proliferation, avoid cell death, invade and metastasize, and even deregulate cellular energetics. Alternative splicing leads to the production of tumor-associated isoforms that function within these hallmarks to promote tumorigenesis. Here we describe several alternative splicing events, providing compelling evidence for their role in tumorigenesis and discuss how these isoforms relate to the cancer hallmarks (Figure 5).

Isoforms sustaining proliferation

RPS6KB1—The gene *RPS6KB1* encodes the protein S6K1, a substrate of mTOR, which controls translation and cell growth. The full-length protein is produced from the *RPS6KB1*-isoform 1 (*RPS6KB1-1*), whereas inclusion of three cassette exons 6a, 6b, and 6c generates the shorter isoform 2 (*RPS6KB1-2*)[60]. A PTC in exon 6c causes the shorter isoform to lack a portion of the kinase domain[60,191]. Alternative splicing of *RPS6KB1-2* is regulated by SRSF1, an oncogenic factor overexpressed in human breast tumors[60]. High levels of *RPS6KB1-2* are detected in breast and lung cancer cell lines and primary tissues[191,192]. Expression of *RPS6KB1-2* in non-transformed cell lines promotes transformation, whereas knockdown in breast, prostate, and lung cancer cells decreases proliferation and tumor growth. Conversely, knockdown of *RPS6KB1-1* in cancer cell lines induced transformation[191,192]. These data suggest that *RPS6KB1-1* plays a role as a tumor suppressor whereas *RPS6KB1-2* contributes to cell proliferation and tumor growth via mTORC1 and 4E-BP1 phosphorylation.

FGFR—*FGFR1, FGFR2 and FGFR3* belong to the fibroblast growth factor receptor (*FGFR*) family, members of which are involved in cell proliferation and migration during embryologic development, but also during tissue repair and wound healing of adult tissue. These receptors contain a cytoplasmic kinase domain, a transmembrane domain, and an extracellular ligand binding portion that consists of three immunoglobulin domains, Ig-I to III[193]. The second half of Ig-II is generated by alternative splicing of one of two mutually exclusive exons -exon 8 or 9- to generate isoform *FGFR-IIIb* or *FGFR-IIIc* respectively. This differential splicing, regulated by splicing factors hnRNPH1, hnRNPF, ESRP1, and ESRP2,

changes the binding specificity of the FGF ligand[130,193,194]. Increased levels of *FGFR-IIIc* isoforms are detected in a variety of tumors, and correlate with tumor progression, increased grading, and invasiveness[195–197]. For example, increased expression of *FGFR2-IIIc* is detected in renal, endometrial, pancreatic and colorectal carcinoma compared to normal tissues[195,198–200]. *FGFR2-IIIb*, but not *FGFR2-IIIc*, is expressed in non-transformed and non-invasive breast cancer cells, whereas *FGFR2-IIIc* is detected in invasive cell lines[201]. Most renal tumor samples have high levels of *FGFR2-IIIc,* but those tumors with high *FGFR2-IIIb* expression have a lower grade and stage, and are associated with longer survival[198]. Furthermore, expression of *FGFR-IIIb* isoforms is tumor-suppressive *in vitro* and *in vivo* in bladder cancer cells, whereas *FGFR-IIIc* isoforms promote tumor growth, invasion, and metastasis in colorectal, pancreatic, and cervical cancer cells[196,197,200,202,203]. However, a high *FGFR-IIIc/IIIb* ratio is not always correlated with poor prognosis[199], suggesting that *FGFR* isoforms may exhibit different functions in specific tissues or tumors.

In addition to their role in cell proliferation, *FGFR* isoforms also impact EMT, a key step in tumor dissemination and metastasis. The expression of *FGFR-IIIb* isoforms correlates with epithelial markers and *FGFR-IIIc* with mesenchymal markers, in prostate, bladder, and renal carcinoma primary tumors and cell lines[198,204–206]. Finally, expression of *FGFR2-IIIc* induces human epithelial keratinocyte cells to acquire mesenchymal characteristics[207].

MKNK2—Mnk2, a kinase in the MAPK pathway encoded by the *MKNK2* gene, has two main spliced isoforms, Mnk2-a and Mnk2–b, which differ in their C terminal domain. Inclusion of exon 13a produces Mnk2-a, the full-length protein isoform, whereas skipping of exon 13a and inclusion of exon 13b encodes Mnk2-b, an isoform lacking the MAPK domain[208]. Both isoforms are capable of phosphorylating the translation initiation factor eIF4E, which promotes cell growth; however, Mnk2-a is also capable of phosphorylating p38 in response to stress leading to cell death[209]. SRSF1 regulates *MNKN2* alternative splicing, promoting Mnk2-b and decreasing Mnk2-a expression[60,62]. Normal breast, lung, and colon tissues express higher levels of *Mnk2-a* than *Mnk2-b*, whereas the corresponding tumors exhibit a shift towards the *Mnk2-b* isoform[208,209]. Since Mnk2-b lacks the MAPK domain, it is unable to activate the p38 stress response, tipping the balance to promote cell growth[208,209]. Isoform-specific overexpression or knockdown experiments demonstrate that Mnk2-a overexpression inhibits soft-agar colony formation, whereas Mnk2-b expression or Mnk2-a knockdown increases transformation[209].

HRAS—H-ras belongs to the *Ras* GTPase family, a class of proto-oncogenes regulating proliferation, survival, and differentiation. These proteins exert their stimulatory effects when bound to GTP but become inactive when GTP is hydrolyzed to GDP. Alternative splicing of *HRAS* produces two distinct proteins, p19 and p21. The full-length isoform, p21, contains exons 0 through 4B, whereas isoform p19 includes the alternative exon IDX between exons 3 and 4a. This exon contains a stop codon, and therefore IDX-containing transcripts produce a truncated protein[210,211]. p19 is unable to bind GTP and therefore cannot function like other Ras proteins; however, the unique C-terminus of p19 allows binding to the scaffolding protein RACK1[212], There are limited data regarding the relative

expression of p19 and p21 in tumors; however, evidence suggests that p19 may serve as a tumor suppressor, and that its ectopic expression delays G1/S transition[213].

CCND1—*CCND1* undergoes alternative splicing to generate two isoforms: cyclin D1a, the conventional isoform, and cyclin D1b, which lacks the C-terminal protein domains. Usage of an alternative 5′SS in exon 4 introduces a PTC, and the resulting D1b isoform lacks the GSK-3β phosphorylation site encoded by exon 5. This causes the protein to remain in the nucleus[214,215]. Increased expression of *CCND1b* is observed in breast, lung and prostate tumors[216–219]. However, both *CCND1a* and *CCND1b* are expressed in lymphoma, bladder, cervical, esophageal and breast cancer[215,220–223]. *CCND1b* expression, but not *CCND1a*, correlates with tumor grade, metastasis, and patient survival in lung and breast cancer[218,222,224]. Interestingly, both isoforms enhance tumor formation, although through different mechanisms. Cyclin D1a promotes cell proliferation and G1/S transition, while cyclin D1b impacts invasion and metastasis[215,217,220,222,225,226]. Cyclin D1b is unable to phosphorylate RB, which is required for cell cycle progression, thus the D1b isoform lacks the proliferative effects of D1a[214,216,220,221]. However, the role of cyclin D1b in promoting tumor growth remains controversial with several studies claiming it activates proliferation[222,224] while others stating it inhibits proliferation[221]. Therefore, tumors that express both cyclin isoforms may have advantages in proliferation as well as invasion, and the two isoforms are likely to play distinct roles in different cell types.

Isoforms preventing cell death

BCL2L1—*BCL2L1*, a member of the Bcl-2 family, generates two isoforms, *BCL-xL* and *BCL-xS*, which have opposing functions in apoptosis; the first prevents apoptosis while the latter promotes it [227]. *BCL-xS* is generated via an alternative 5′SS in exon 2 (Figure 4), and lacks the exons encoding the Bcl-2 homology domains, BH1 and BH2, but still includes BH3 and BH4[228]. Sam68 modulated by FBI-1, RBM4, PTBP1, or RBM25 upregulates *BCL-xS* expression[229–233], whereas SRSF1 promotes *BCL-xL* splicing [234]. Increased expression of *BCL-xL* and decreased expression of *BCL-xS* are detected in lymphoma, glioma, myeloma, and neuroblastoma cell lines and primary tumors[235–238]. Expression of the pro-apoptotic BCL-xS isoform in cancer cell lines decreases cell viability and sensitizes cells to chemotherapy and radiation[236,239,240]. Conversely, expression of BCL-xL promotes cell survival and increases resistance to apoptosis following chemotherapy[238,241–243]. Therefore BCL-xS can antagonize the protective effects of BCL-xL.

FAS—*FAS* is a member of the TNF-receptor superfamily known for promoting the extrinsic pathway of apoptosis. An alternatively spliced isoform, soluble Fas (*sFAS*), is produced by the skipping of exon 6, which encodes the transmembrane domain, and therefore the protein cannot localize to the plasma membrane[244]. Alternative splicing of *FAS* is controlled by multiple regulators, including EWS, hnRNPA1, and TIA1, all of which promote exon 6 inclusion[245–247], whereas RBM5 and PTBP1 favor exon 6 skipping[248]. A genome-wide siRNA screen identified close to 200 additional genes that may be implicated in regulating *FAS* alternative splicing[249]. sFas is expressed in leukemias and lymphomas[250–252], as well as in solid tumors including renal, cervical, endometrial, ovarian, and bladder cancer[253–256].

Expression of sFas is inversely correlated with patient survival and tumor progression in leukemia, gynecological, and bladder tumors[252,254–257].

BIN1—*BIN1* is tumor suppressor that functions by interacting with and inhibiting c-MYC. Inclusion of exon 12A of *BIN1* generates a protein isoform that no longer binds Myc and therefore eliminates BIN1 tumor-suppressive function[258]. SRSF1 overexpression promotes inclusion of exon 12A, and this isoform plays a role in escaping cell death in SRSF1-dependent breast tumors[58,60]. Expression of the *BIN1+12A* isoform is detected in melanoma and breast cancer cell lines[60,259].

CASP2—Caspase 2 is an initiator of apoptosis and functions as a tumor suppressor. Splicing of *CASP-2* generates two main isoforms; skipping of exon 9 produces the pro-apoptotic Casp-2L protein isoform, whereas exon 9 inclusion results in a PTC, leading to the anti-apoptotic Casp-2S isoform[262,263]. Splicing of *CASP2* is regulated by RBM5, which promotes exon 9 inclusion[260], and SRSF3, which promotes exon 9 skipping[261]. The major isoform in adult tissue is Casp-2L, whereas Casp-2S is found in brain and muscle tissues[262,263]. Expression of *CASP2* isoforms is detected in various cancers and immortalized cell lines[261,264,265].

MCL1—The BH3-containing member of the Bcl-2 family, *MCL1*, has three major isoforms which differ in their apoptotic potential[266,267]. Full length *MCL1-L* includes exons 1 to 3 and displays anti-apoptotic activity. Skipping of exon 2 introduces a PTC, leading to the *MCL1-S* isoform, which contains the BH3 domain but lacks the BH1, BH2, and transmembrane domains. Finally, truncation of exon 1 produces the *MCL1-ES* isoform which maintains the BH1-BH3 and transmembrane domains but lacks the PEST domain, a site of cleavage and phosphorylation for caspases[268]. SRSF1 is one of the regulators of *MCL1* splicing in cancer cell lines[269]. Increased expression of Mcl-1L is observed in oral cancers and basal cell carcinoma compared to normal tissues[270,271]. High MCL1-L expression correlates with increased tumor size and decreased survival in oral cancers[272], as well as with resistance to treatment in oral squamous cell carcinoma[273]. Interestingly, melanocytes upregulate MCL-1L in response to UVB radiation, which protects them against apoptosis; whereas melanoma cell lines that have elevated MCL1-L expression without UV exposure are resistant to apoptosis[274]. Finally, MCL1-ES differs from other Bcl-2 family members in that it does not depend on BAX/BAK homodimerization for apoptotic activity. Interestingly, MCL1-ES neutralizes the effects of MCL1-L, and MCL-1ES apoptotic activation is enhanced by MCL1-L expression[275]. Conversely, MCL1-S apoptotic activity is inhibited by MCL1-L[266], suggesting that the different isoforms promote apoptosis via distinct mechanisms.

Isoforms rewiring cell metabolism

PKM—The two isoforms of pyruvate kinase, a key glycolytic enzyme, are formed by the splicing of one of two mutually exclusive exons that share 56 amino acids but differ at 22 residues[276]. Inclusion of *PKM* exon 9 produces the constitutively active PKM1, while inclusion of exon 10 encodes PKM2. Both PKM isoforms perform the same catalytic function, but PKM2 can switch between the active and inactive state[276]. PKM2 expression is

regulated either by repressing inclusion of exon 9 via binding of PTBP1, hnRNPA1, or hnRNPA2, or by promoting exon 10 inclusion via binding of SRSF3. Both splicing events increase expression of PKM2 relative to PKM1[73,148,150]. PKM2 is detected in most embryonic as well as proliferating adult tissues, with the exception of muscle, brain and bladder, which express only PKM1[276]. Increased PKM2 levels are reported in many human solid tumors and correlate with decreased patient survival, advanced stage and poor prognosis[285]. PKM2 knockdown inhibits tumor progression and metastasis *in vivo* and *in vitro* in ovarian, gastric, colon, liver, and esophageal cancer models[277,278,282,285]. Conversely, cancer cells engineered to express PKM1 in place of PKM2 convert from aerobic glycolysis to mitochondrial respiration and are unable to form tumors after xenotransplantation[286,287]. However, other studies suggest that PKM2 is not necessary for tumor growth in colon cancer cell lines or in a breast cancer mouse model[288–290]. PKM2 plays a role in cancer metabolism and activates the PI3K/Akt pathway[291]. The ability to inhibit PKM2 activity is important for cell proliferation *in vivo* and *in vitro*[292], and allows cells to respond to signaling and environmental cues[276].

Isoforms promoting angiogenesis

VEGFA—The growth factor VEGFA stimulates blood vessel formation by promoting proliferation and migration of endothelial cells. The *VEGFA* transcript undergoes alternative splicing in two distinct regions to produce protein isoforms of variable length. Inclusion of variable exons 6a, 6b, 7a, or 7b encodes the $VEGFA_{xxx}$ isoforms, where 'xxx' refers to the final number of amino acids[293]. In addition, inclusion of variable exon 8b, instead of exon 8a, at the 3′ end of the transcript produces the anti-angiogenic $VEGFA_{xxx}b$ isoforms[294]. $VEGFA_{xxx}b$ splicing is promoted by SRSF6 overexpression, whereas SRSF1 and SRSF5 overexpression promote $VEGFA_{xxx}$[295]. Adult human tissues express predominantly anti-angiogenic $VEGF_{xxx}b$ isoforms, such as the common $VEGF_{165}b$ isoform, but their expression often decreases as tumors progress[296]. Decreased expression of $VEGF_{165}b$ is found in human metastatic melanoma and prostate tumors[297,298]. A shift in expression from $VEGF_{165}b$ to $VEGF_{165}$ occurs in colon and squamous cell carcinoma tumors[80,299]. However neither the expression of $VEGFA_{xxx}$ nor of $VEGFA_{xxx}b$ correlates with patient survival in head and neck tumors[300]. Finally, overexpression of $VEGF_{165}b$ reduces tumor growth in mouse xenograft models of colon, renal, prostate, or soft tissue tumors[299,301,302]. *In vitro* $VEGFA_{165}b$ binds to the same receptor as do $VEGFA_{165}$ and with the same affinity; however, $VEGFA_{165}b$ is unable to stimulate the VEGF signaling pathway. Thus, anti-angiogenic $VEGFA_{xxx}b$ isoforms can inhibit $VEGFA_{xxx}$-mediated angiogenesis[298].

Isoforms enabling cell invasion and metastatic dissemination

CD44—CD44 is a transmembrane glycoprotein that binds hyaluronic acid and functions in cell division, survival, and adhesion. The *CD44s* isoform contains exons 1–5 and 16–20, whereas inclusion of any of the variable exons 6–10 generates one of the *CD44v* isoforms[303]. Regulators of *CD44* alternative splicing include ESRP1, hnRNPA1, and SRSF2, all of which promote *CD44v* splicing[126,304–306], whereas hnRNPL inhibits CD44v expression[307]. *CD44v* isoforms are expressed in both normal and tumor tissues, but their expression frequently increases in gastric, ovarian, bladder, colon, and prostate tumors[304,308–314]. *CD44v* expression is often associated with tumor progression, is

frequently found in recurrent tumors, and correlates with increased grading[312,313,315]. The role of *CD44v* isoforms in tumor progression remains a topic of discussion. Expression of exogenous *CD44v8-10* increases tumor initiation frequency in gastric cancer models[309], and, similarly, *CD44v9* facilitates invasion in prostate cancer[312,313]. However, some ovarian tumors express higher levels of *CD44s* than *CD44v,* and patients expressing *CD44v8-10* have longer survival rates[311,316]. Finally, EMT not only affects, but is also affected by *CD44* isoform expression. Epithelial cells express predominantly *CD44v*, but switch to *CD44s* after undergoing EMT in breast and ovarian tumor models[304,311]. *CD44s* is required for EMT in breast and ovarian cancer models, and its expression enhances migration[304,316]. *CD44s* expression also induces a mesenchymal phenotype, increases cell invasion, and results in poor differentiation and distant metastasis in gallbladder cancer models[317]. However, *CD44v* expressing gallbladder cancer cells are still highly tumorigenic even though exhibit decreased invasive potential[317].

ENAH—ENAH, also known as Mena, regulates actin nucleation and polymerization and modulates cell morphology and motility. Splicing of the *ENAH* transcript generates three main isoforms, which play different roles in tumor progression. Inclusion of exon INV produces *MENA-INV*, inclusion of exon 11a produces *MENA11a*, and skipping of exon 6 produces *MENAΔv6*[318]. Alternative splicing of *MENA* is regulated by ESPR1 and ESPR2[319]. The ratio of Mena isoforms varies between normal and tumor tissues. For example, breast tumors express high levels of Mena11a or MenaINV, while limited to no expression of these isoforms is detected in normal tissue. In addition, MenaINV expression increases with tumor grade, metastasis and tumor progression, and is accompanied by a decrease in Mena11a[320–322]. Expression of both pan-Mena and Mena11a increases in lung tumors compared to normal tissue; however, low Mena11a expression correlates with decreased survival rates in lung cancer patients, and patients expressing high levels of Mena11a do significantly better[323]. Expression of Mena11a also correlates with epithelial markers and decreased invasion, whereas MenaINV and MenaΔv6 expression correlate with mesenchymal markers, and increased invasion and metastasis[318,321,323–325]. Knockdown of Mena11a in breast cancer cell lines decreases cell migration, and ectopic Mena11a expression reduces lamellipodia protrusion[319].

MSTR1 (alias RON)—The receptor tyrosine kinase RON (*MSTR1*) is a member of the MET proto-oncogene family, which is implicated in tumor progression. Exons 5, 6, 11, and 19 undergo alternative splicing to produce four isoforms: *RONΔex11* (RONΔ165), *RONΔex5-6* (RONΔ160), *RONΔex5-6-11* (RONΔ155), and *RONΔex19* (RONΔ170)[326,327]. Splicing of RONΔ165 is regulated by SRSF1[59,328]. RONΔ165, RONΔ160 and RONΔ155 are constitutively active, whereas RONΔ170 is a kinase-defective isoform that inhibits tumorigenesis by other active RON isoforms[326,327,329]. RONΔ160 likely exerts its tumorigenic potential by increasing β-catenin expression[330]. RONΔ165, RONΔ160, or RONΔ155 are expressed in human primary colon, ovarian, breast, and brain tumors, as well as in gastric and lung cancer cell lines[59,328,329,331–333]. Ectopic expression of RONΔ160 or RONΔ155 promotes tumor formation and lung metastasis in NIH3T3 xenograft mouse models[331]. Additionally, expression of RONΔ160 or RONΔ155, but not RONΔ165, induces

anchorage-independent growth in colon cancer cells lines[330]. However, RONΔ165 expression increases motility and invasiveness in cancer cell lines[59,329].

RAC1—Rac1 is a member of the Rho GTPase family, which is involved in signaling for cell motility and proliferation. Inclusion of *RAC1* exon 3b produces the constitutively active RAC1b isoform, which contains 19 additional amino acids behind the switch II domain, a region important for Rac1 interaction with regulators and effectors[334,335]. SRSF1 is one of the regulators of *RAC1* alternative splicing[336]. Rac1b has accelerated guanosine diphosphate (GDP)/guanosine triphosphate (GTP) exchange and impaired GTP hydrolysis, thus leading to prolonged signaling activity[335,337,338]. Furthermore, RAC1b is unable to interact with RHO-GDI, to signal downstream PAK1 and JNK kinases, or to activate the RelB pathway[339,340], but can negatively regulate RAC1 activity[341]. *RAC1b* is expressed in breast, thyroid, colorectal, and lung tumors[336,342–344]. Increased expression of *RAC1b* in thyroid tumors correlates with metastasis and poor clinical outcome[343]. Rac1b expression in colon and thyroid cancer cell lines sustains cell survival by stimulating G1/S progression and protecting cells from apoptosis[345–347].

KLF6—KLF6 belongs to the Kruppel-like family of transcription factors which regulate cell proliferation, differentiation, and survival. *KLF6-SV1* uses an alternative 5′SS that causes a frame-shift and produces a protein isoform that contains 21 novel amino acids but lacks all three of the zinc finger domains[348]. Alternative splicing of *KLF6* is regulated by SRSF1, TGF-β1, and Ras signaling[349,350]. Increased KLF6-SV1 expression is observed in prostate, lung, ovarian, brain, breast, pancreatic, and liver tumors, and correlates with poor patient survival[349,351–356]. Full-length KLF6-FL can act as a tumor suppressor, whereas KLF6-SV1 is oncogenic. KLF6-SV1 knockdown in lung, ovarian, colon, and brain cancer cells increases apoptosis, whereas its overexpression promotes proliferation and survival[349,352,353]. Expression of KLF6-SV1 increases cell survival, migration and invasion in breast cancer cell lines, but has no effect on proliferation[354]. Ectopic KLF6-SV1 expression does not alter tumor size, but increases metastasis incidence, in mice xenograft experiments[354]. Furthermore, KLF6-SV1 knockdown prevents tumor formation, while knockdown of the full-length isoform increases tumor growth, in ovarian cancer xenograft models. Finally, expression of KLF6-FL is associated with epithelial markers, whereas KLF6-SV1 is associated with a mesenchymal phenotype[354].

Isoforms enabling drug resistance

BCL2L11 (alias BIM)—The BH3-only protein, BIM, is a pro-apoptotic protein encoded by *BCL2L11*. The three major isoforms, BIM-EL, BIM-L, and BIM-S, are pro-apoptotic but differ in their activity, BIM-S being most active[357]. In addition, two isoforms, BIMγ1 and BIMγ2, are generated by alternative splicing of exon 3, which contains a stop codon and results in a truncated protein that lacks the BH3 domain and thus lacks the pro-apoptotic activity[58]. SRSF1-overexpression induces alternative splicing of BIM to promote BIMγ1 and BIMγ2 splicing[58]. PTBP1 and hnRNPC promote exon 3 skipping and expression of the pro-apoptotic BIM isoforms[358]. The BIMγ isoforms are expressed in leukemia, lung, and breast cancer cells[58,359,360]. Ectopic expression of BIMγ1 reduces apoptosis levels in mammary epithelial cells[58]. Finally, expression of BIM isoforms has been linked to drug

response in tumors. High levels of BIM-EL correlate with a better induction of apoptosis in response to tyrosine kinase inhibitors in EGFR-mutant lung and HER2-amplified breast tumor models and predict responses in treatment-naïve patients[361]. In addition, expression of BIMγ isoforms in lung cancer patients with a BIM polymorphism increases resistance to tyrosine kinase inhibitors[360].

HER2 (alias ErbB2)—HER2 is a tyrosine kinase from the EGFR family, frequently amplified or overexpressed in breast tumors. Skipping of exon 20 encodes d16HER2, a constitutively active protein that lacks 16 amino acids in the extracellular domain, and is primarily detected in breast tumors[362–364]. Alternative splicing of *HER2* exon 20 is regulated by SRSF3 and hnRNPH1[365]. Expression of d16HER2 increases proliferation, induces EMT and invasion, and decreases sensitivity to the HER2-targeting antibody trastuzumab[363,366–370]. d16HER2 expression allows breast cancer cells to evade trastuzumab-induced apoptosis by upregulating Bcl-2 and activating SRC, a kinase involved in proliferation and migration[370,371].

SPLICING ALTERATIONS AND THE TUMOR MICROENVIRONMENT

Our current understanding of RNA splicing alterations relies on the expression of splicing isoforms and their regulators in tumor cells. However, solid tumors are composed of a mixture of cell types in addition to cancer cells, including fibroblasts, various immune cell types, and endothelial cells, all of which influence tumor progression and drug responses[372]. Although cell-type specific splicing has been described, we know very little about splicing alterations in these cell types in the tumor context.

Matrix stiffness and composition affects RNA splicing

The local microenvironment, or niche, plays important roles in cell fate, cancer onset, and malignant evolution[373]. A major component of the niche is the extracellular matrix (ECM), a complex network of macromolecules with distinctive physical, biochemical, and biomechanical properties that undergoes remodeling during metastasis. Yet, it remains unclear how the ECM composition impacts splicing isoforms and their regulators during tumor progression and metastasis. Interestingly, cells grown in 3D cultures on an ECM exhibit different splicing profiles compared to the same cells grown on plastic, suggesting that ECM stiffness and composition can influence splicing choices[64,374]. Matrix stiffness can alter splicing, for example, through differential phosphorylation and activation of splicing regulators from the SR protein family[375]. Additionally, signaling through ECM proteins and integrin engagement can impact tumor initiation and metastasis, and can also selectively alter splicing. For example, laminin 511 promotes self-renewal and tumor initiation by engaging the α6Bβ1 integrin splice variant[376]. The expression of the α6Bβ1 isoform is repressed by the splicing factor ESRP1 and depends on VEGF autocrine signalling[257]. Furthermore, ECM proteins are themselves regulated by splicing, and often undergo splicing switches during tumor progression. For example, the oncofetal ED-A and pro-angiogenic ED-B fibronectin isoforms differ in their integrin binding domain and show differential assembly into fibrils[377,378]. Malignant cells express high levels of ED-A fibronectin and its receptor, α5β1 integrin, both of which have been linked to radiation

resistance[379]. Interestingly, aberrant ECM can also alter fibronectin splicing in non-malignant cells[379]. Similarly, tenascin-c expresses unique alternative splice forms in breast tumors[380]. In both patient samples and cell culture models, these ECM splicing isoforms have been linked to invasiveness[379]. Osteopontin *SPP1* is another ECM protein that is overexpressed in various cancers and promotes oncogenic features. Splicing of *SPP1* generates multiple isoforms that play a role in cancer development and progression through their surface receptors CD44 and integrins[381]. Finally, metastatic lesions exhibit alterations in splicing isoforms that impact cell polarity, cell-cell interactions, and EMT. Examples of metastasis-specific splicing events include isoforms of fibronectin *FN1,* Tenascin C *TNC, CD44, ENAH,* and *RAC1*[321,382,383]. Alterations in upstream splicing regulators that control metastasis-associated splicing isoforms are found in human tumors[115,128].

Splicing isoforms and immune cell functions

Other key components of the tumor microenvironment are immune cells, which can either promote or inhibit tumor growth[384]. RNA splicing controls multiple regulatory steps in immune cell development and function[385]. Transcriptome-wide studies identified a repertoire of splicing isoforms expressed in specific immune-cell types, and linked many of these events with lineage differentiation[386]; however, it is not known how changes in the immune cell repertoire impact splicing patterns in human tumors.

Alternative splicing plays a role in the control of innate immunity. For example, SF3A1 regulates the splicing of genes involved in Toll-like receptor (TLR) signaling in macrophages, and controls the production of positive regulators of TLR signaling, IRAK1, CD14, and IKKβ, as well as negative regulators sTLR4 and Rab7b[387]. Another example is the splicing of the MyD88s isoform, which limits innate immune activation downstream of TLR signaling. MyD88s splicing is controlled by Eftdu2, SF3A1, and SF3B1[388,389]. Moreover, inclusion of an alternative *TLR4* exon generates a soluble isoform that inhibits TNF-α and NF-κB signaling in macrophages, thereby acting as a negative feedback mechanism. Similarly, soluble isoforms of membrane receptors, such as IL-4R, -5R, and -6R, are frequently generated by splicing in immune cells[385]. Furthermore, alternative splicing plays a role in class switch from IgM to IgD during B-cell differentiation and also impacts the generation of a secreted form of IgM[385]. In addition, splicing can increase the transcript diversity of IgE by generating isoforms that are either secreted or membrane-bound. Finally, loss of the RNA-binding protein HuR results in defective class-switching and leads to B-cell death[390,391].

The best-studied examples of functional splicing events in T-cell differentiation are the cell-surface glycoproteins *CD44* and *CD45 (PTPRC*). Splicing of *CD44*, which is regulated by Sam68, produces an alternative *CD44v* isoform that is involved in both lymphocyte activation and metastasis, as described above[385]. *CD45* isoforms, named RA/RB/RC/RO, are expressed in different patterns in functionally distinct T-cell populations, and *CD45* splicing serves as a feedback mechanism to maintain T-cell homeostasis[385]. Briefly, naïve T-cells express a *CD45* isoform that includes at least one of the variable exons 4, 5, and 6, each which encode an extracellular domain that is heavily glycosylated and thus prevents CD45 homodimerization[392]. Upon T-cell activation, skipping of *CD45* variable exons allows

homodimerization at the cell surface, which leads to an inactive form and decreased signaling through the T-cell receptor. HNRNPLL, HNRNPL, SRSF2, PTBP1, HNRNPE2, and HNRNPA1 have been all implicated in the regulation of *CD45* splicing[393–397]. Moreover, stimulation of the T-cell receptor induces splicing changes in immune-related targets including *CD45*, *Fyn*, *TRAF3*, *BRD8,* and *TRIM*[397–399].

SPLICING MODULATION AS CANCER THERAPEUTICS

Modulation of RNA splicing can provide novel therapeutic targets for oncology. Splicing modulation can be achieved either by fine-tuning the level or activity of splicing regulators, thus affecting the network of their downstream splicing targets, or by precisely targeting a single spliced isoform expressed in cancer cells (Figure 6).

Small molecules modulating the activity or levels of splicing regulators

Compounds that affect global splicing efficiency or SS selection have been identified over the years and their number is steadily increasing[400]. The molecular mechanisms of action of these agents are progressively being elucidated. The first group of compounds that impact splicing includes spliceostatins, sudemycins, and FD-895 and its parent pladienolides molecules or their derivatives (e.g. E7107 or FR901464), which all act directly on the core spliceosomal component SF3B1[401,402,403,404] (Figure 6A). Interestingly, only a fraction of the splicing events (~10%) are affected by SF3B1 inhibition, suggesting that some SS, likely weak SS, are more sensitive than others to spliceosomal inhibitors[17,29]. Isoginkgetin, another splicing inhibitor, acts by preventing recruitment of the U4/U5/U6 tri-snRNPs, which leads to accumulation of the spliceosomal complex A[405]. Additionally, several small molecules alter the activity of splicing-factors, for example by targeting their regulatory kinases. Molecules such as NB-506, SRPIN34, diospyrin D1, and TG003 reduce SR-protein phosphorylation through the inhibition of proteins from the SRPK, CLK, DYRK, or topoisomerase families and thus modulate splicing of SR-protein targets[406,407]. Finallysulfonamides, a class of cancer drugs that achieve efficacy in a subset of cancer patients, have been recently shown to act by reducing the expression of splicing factor RBM39 (alias CAPERα) through a novel mode of targeted proteasomal degradation[408,409]. Treatment of cancer cell lines with sulfonamides triggers the association of RBM39 with the CUL4- DCAF15 ubiquitin ligase, leading to RBM39 poly-ubiquitination and proteasomal degradation[408,409]. Degradation of RBM39 leas to aberrant pre-mRNA splicing in a set of target genes[408,409]. Interestingly, DCAF15 expression and copy number correlated with sulfonamides sensitivity, suggesting that regulators of splicing-factor degradation could constitute promising drug targets.

While effective, these small molecules often lack specificity, and their exact mechanisms of actions are not always well understood, which could potentially lead to off-target effects and limit their clinical application. Interestingly, *in vitro* and *in vivo* data suggest that cancer cells are more sensitive than normal cells to global splicing inhibition, thus providing a therapeutic window that could be exploited even when using broad-spectrum splicing inhibitors[151,152,410,411]. Even though initial trials of an SF3B1 inhibitor, E7107, in solid

tumors were suspended due to unexpected toxicity, newer inhibitors, such as H3B-8800, are currently being tested in phase I trials for hematological malignancies[412,413].

Splice-switching RNA-based therapeutics

RNA-based therapeutics offer the potential to target virtually any molecule, especially those lacking a catalytic activity that could be inhibited, or those not amenable to targeted antibody approaches[414]. FDA approval of Spinraza™, which is the first splicing-correcting therapy and uses antisense oligonucleotides (ASO) to treat spinal muscular atrophy (SMA), has opened the field for RNA-based approaches to target splicing defects[415]. Splice-switching ASOs are 15- to 30-mer long chemically modified RNA molecules that can redirect a specific splicing event in order to prevent the production of a truncated or mutated protein, or to generate a specific protein isoform. Their specificity comes from their complimentary binding to a unique sequence on the mRNA, thus affecting only the targeted spliced isoform. Splice-switching ASOs can be designed to specifically target (i) a 5′ or 3′SS, thus blocking its usage, (ii) a splicing enhancer sequence, thus preventing binding of a splicing activator and promoting exon skipping, or (iii) a splicing silencer sequence, thus preventing binding of a repressor and promoting exon inclusion[416] (Figure 6B). Another splice-switching strategy is the use of bifunctional oligonucleotides made of an antisense portion that determines target specificity, and a non-hybridizing tail that recruits proteins or RNA/protein complexes that modulate SS selection[417–419].

Natural unmodified DNA or RNA oligonucleotides are vulnerable to nuclease degradation and are unstable *in vivo*. Chemical modification of the phosphate backbone and/or the ribose ring can produce stable molecules with high substrate specificity, low toxicity, low immunogenicity, and that limit RNAse H degradation[420]. ASO designed to activate RNAse H cleavage will not be discussed here as they do not modulate alternative splicing but trigger degradation of their mRNA target. Several distinct ASO chemistries are currently used for splicing-modulation (Figure 6C). A common backbone modification uses phosphorothioates (PS) at the nucleotide link[420]. PS-ASOs are more hydrophobic, more nuclease resistant, and bind with higher affinity than ASOs with unmodified phosphodiester linkages[421]. PS-ASOs are often combined with ribose modifications such as 2′-O-(2-methoxyethyl) (2′-MOE) or 2′ O-methyl (2′-OMe)[420]. Uniformly modified 2′-MOE/PS ASOs are effective when administered in saline by nearly all routes of administration and their tissue half-lives ranges from 2 to 4 weeks, but can even achieve 6 months in the central nervous system[421]. Another type of modification uses locked nucleic acid (LNA), which increases binding affinity and reduces off-target effects by allowing the usage of shorter sequences that are less likely to partially hybridize to non-target sequences[416]. A distinct class of backbone chemistry uses phosphorodiamidate linkages in morpholino oligomers (PMO or morpholino)[416]. PMOs are neutrally charged and provide better specificity and display lower toxicity than PS-ASOs. However, PMOs often need to be conjugated to a delivery moiety for *in vivo* delivery. Finally, peptide nucleic acid (PNA) offer specificity similar to PMO, but their low water solubility limits their use[416].

However, efficient delivery to the target organ still remains one of the major challenges in the field of RNA-based therapeutics. The two challenging steps involve getting the ASO to

the tissue of therapeutic interest and then delivering it to the correct intracellular compartment[422]. In addition to naked formulations, ASO modifications, carriers and other approaches are currently being tested to increase splicing efficiency, lower the dosage, enable tissue-specific delivery, and limit toxicity and off-target effects[416]. *In vivo*, ASOs can be injected either systemically, or directly into the specific organ where the correction needs to be achieved[416]. For example, the FDA-approved 2′-MOE/PS ASO, Spinraza™, is delivered intrathecal in saline, and achieves a 4–6 months half-life in the cerebrospinal fluid after initial clearance[423]. Eteplirsen™, the first splice-switching PMO to received FDA-approval for Duchenne Muscular atrophy is delivered by intravenous infusion. Renal clearance plays a major role in ASOs pharmacokinetics and biodistribution[422]. PS-ASOs bind to plasma proteins and slow their renal clearance, thus allowing broader tissue distribution, whereas uncharged PMO are cleared much faster and accumulate at lower levels[422]. Finally, efforts to deliver ASOs to specific tissues are ongoing[422]. The most promising targeted approach utilizes ASOs conjugated with an N-acetylgalactosamine (GalNac) that allows effective uptake by hepatocytes via an asialoglycoprotein receptor dependent mechanism[422]. Novel ASO delivery strategies are rapidly emerging. Yet, ASOs delivery to tumors will certainly face similar challenges as the delivery of other cancer drugs and will require further optimization to efficiently delivery therapeutics to cancer patients.

ASO-mediated correction of cancer-associated splicing isoforms can be achieved *in vitro* in human cell lines and *in vivo* in xenograft tumor models (Table 1). For example, ASO targeting of a splicing enhancer that regulates inclusion of exon 23 of the transcription factor *STAT3* can shift expression from the *STAT3α* to the *STAT3β* isoform[424]. Induction of STAT3β, an isoform that lacks the C-terminal transactivation domain, leads to apoptosis and cell-cycle arrest in breast cancer cells, as well as to tumor regression in xenograft breast cancer models[424]. Another example is the ASO-mediated skipping of *MDM4* exon 6 to decreases MDM4 protein abundance, an oncoprotein that inhibits p53-mediated tumor suppression[425]. Tumors express high levels of MDM4 as a result of a splicing switch between the NMD-degraded *MDM4-S* isoform expressed in normal cells, and the full-length exon 6-containing *MDM4-L* isoform produced in cancer cells. Skipping of *MDM4* exon 6 decreases tumor growth in patient-derived xenograft models of melanoma and lymphoma[425]. RNA-based therapeutics are currently being tested in the clinic in lymphoma and lung cancer patients to downregulate *STAT3* expression[426].

Cancer drugs affecting RNA splicing

Alternative splicing is modulated by a variety of cellular responses, including body temperature changes, circadian rhythm, exposure to radiations, as well chemotherapies[427–430]. Transcriptome-wide studies identified a repertoire of splicing isoforms expressed after treatment with the cancer drugs camptothecin, doxorubicin, or cisplatin[82,431–433]. A large fraction of these transcripts function in pathways frequently disrupted in cancer, i.e., cell cycle, DNA repair, genetic instability, and replicative immortality[429]. Additionally, treatment with gemcitabine, a first line chemotherapy for pancreatic cancer, leads to drug-resistance and is associated with a splicing switch to the oncogenic isoforms *MKNK2-b* and *PKM2*, as well as with the upregulation of SRSF1 and PTBP1[434]. Finally, cancer drugs can be combined with splicing-modulating compounds; for

example amiloride potentiates the effect of imatinib in CML, and sudemycin enhances the effects of ibrutinib in CLL[435].

Interestingly, changes that affect SS selection can affect resistance to targeted cancer therapies. For example, treatment with vemurafenib, a $BRAF^{V600}$ inhibitor, selects resistant cells expressing an alternatively spliced *BRAF* isoform that lacks the RAS-binding domain that normally regulates BRAF dimerization and activation[436]. Similarly, the *BRCA1Δ11q* isoform, a variant lacking the majority of exon 11, promotes resistance to PARP inhibition and cisplatin[437]. Moreover, expression of the oncogenic *BARD1β* splicing isoform impairs homologous recombination and sensitize colon cancer cells to PARP inhibition even in *BRCA1* wild-type cells[438]. Finally, the selection for pre-existing alternatively spliced *CD19* isoforms bearing a compromised epitope explains resistance to CART-19 immunotherapy in B-ALL patients[439].

CONCLUSION

Since RNA splicing was discovered forty years ago, our understanding of its role in human diseases has been expanding, but many questions remain unanswered. The recent years have undoubtedly shown that alterations in RNA splicing are frequent in tumors and contribute to disease pathogenicity. Cancer screening panels currently include splicing-factor mutations among the mutated genes in hematological malignancies. Alterations in splicing-factor levels and dysregulation of downstream splicing targets are tumor characteristics shared by many cancers. Interestingly, these factors can act either as tumor suppressors or as oncogenes, depending on the tumor type, suggesting cell-type-specific functions and targets. Splicing alterations represent a novel and rich source of potential therapeutic targets, and several clinical trials are currently testing them in cancer patients, such as SF3B1 inhibitors in MDS patients as described above[412,413]. The advances in RNA-based therapeutics will likely accelerate the development of splicing-modulating compounds as cancer therapeutics. Additionally, advances in other fields may be applied to address current challenges in delivery and efficacy of RNA-based therapeutics. For example, specific delivery to leukocytes can been achieved by loading siRNA onto lipid-based nanoparticles coated with anti-CD38 monoclonal antibodies[440]. This approach was proven effective at inhibiting cyclin D1 *in vivo*, suppressing tumor growth and prolonging survival of mice xenografted with human lymphoma cells[440], thus opening a new avenue for the treatment of hematological malignancies. Similar targeted strategies could be utilized to deliver splice-switching ASOs to the cells of therapeutic interest and increase their efficacy in tumors.

Alterations in splicing-factor levels are often detected in human tumors, yet only a fraction of these tumors exhibit copy number changes. Thus understanding the transcriptional and post-transcriptional regulation of splicing factors is critically needed to open new direction for drug targets. The pathways that control splicing-factor homeostasis in relevant normal or tumor tissues are not well understood, and it remains unclear how they become dysregulated in tumors. Another underexplored area is how the coupling of alternative splicing with NMD impacts splicing-factor regulation in tumors. Given that cancer cells exhibit differences in the regulation of NMD, the link between these two regulatory pathways in tumorigenesis warrants further attention and may provide novel therapeutic opportunities[441,442]. In

addition, tumors often exhibit alterations in multiple splicing factors, and thus understanding the regulatory networks of RNA-binding proteins and their targets will be crucial for the development of effective splicing-factor inhibitors.

Importantly, all previous studies exploring the role of splicing in human cancer are based on bulk tumor material, which contains a majority of tumor cells together with other cell types that have been shown to impact tumor development and drug response. Yet, whether oncogenic splicing isoforms are present in each individual cell type remains unknown. Variations in splicing patterns have rarely been studied at the single-cell level, and these differences have the potential to contribute to the heterogeneity in drug response. Interestingly, a bimodal variation in splicing patterns was observed among single dendritic cells, suggesting that single cells can exhibit distinct splicing isoforms[443]. Dissecting splicing heterogeneity in tumors at the single-cell level will likely be required for to ensure the success of future splicing-modulating cancer therapies.

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ABBREVIATIONS

ASO	antisense oligonucleotides
BH	Bcl-2 homology domain
BPS	branch point site
CLL	chronic lymphocytic leukemia
CMML	chronic myelomonocytic leukemia
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
HEAT	Huntington, Elongation Factor 3, PR65/A, TOR domain
lncRNA	long non-coding RNA
MDS	myelodysplastic syndromes
miRNA	microRNA
NMD	nonsense-mediated decay
PMO	phosphorodiamidate morpholino oligomer
PTC	premature termination codon
RARS	refractory anemia with ringed sideroblasts

RCMD-RS	refractory cytopenia with multilineage dysplasia and ringed sideroblasts
RNA	ribonucleic acid
RRM	RNA-recognition Motif
SMA	spinal muscular atrophy
SR	serine/arginine-rich
sRNA	small nuclear RNA
snRNP	small nuclear ribonucleoproteins
SS	splice site
TLR	Toll-like receptor

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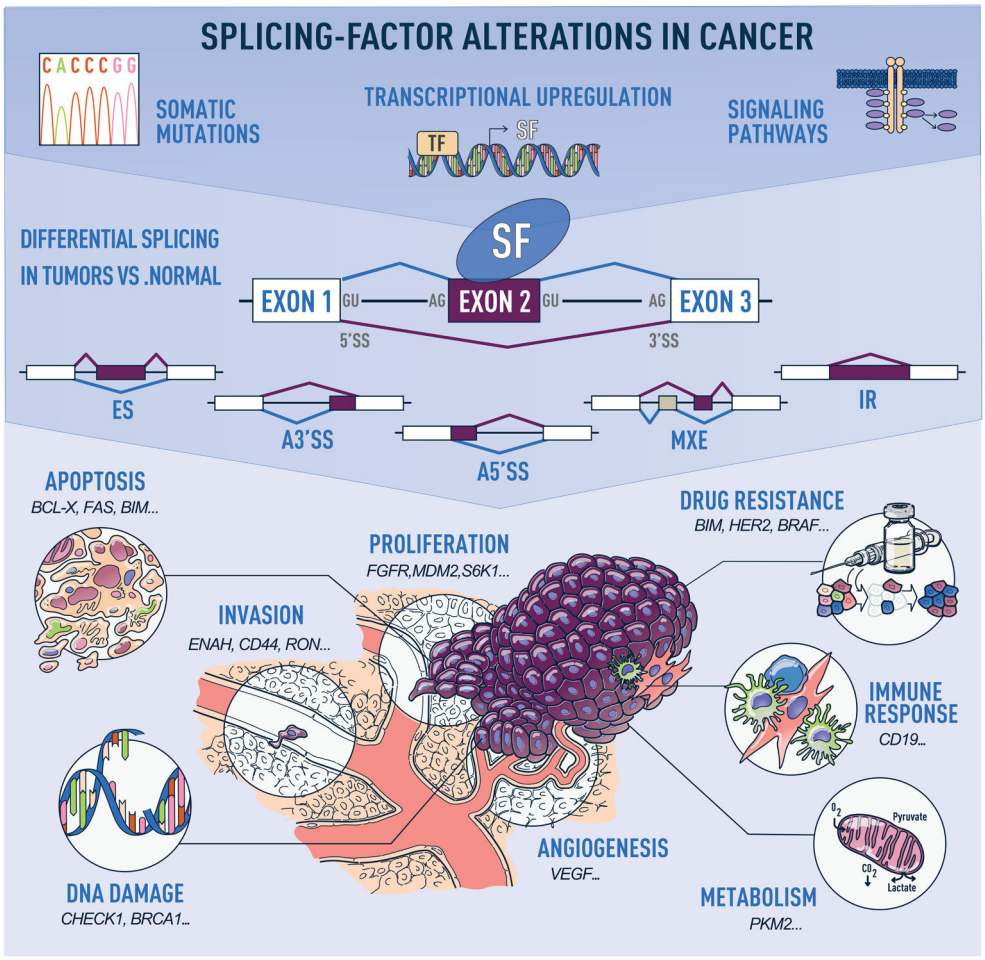


Figure 1. Alternative-splicing alterations in cancer

Human tumors exhibit recurrent mutations in, or changes in the levels of, splicing regulatory factors, the latter of which can occur due to copy number changes, or alterations in the transcriptional, post-transcriptional, or post-translational regulation of splicing factors in response to signaling changes (top panel). These changes in splicing-factor levels lead to alterations in the splicing of their downstream targets, promoting events that follow one of the following patterns: exon skipping (ES), alternative 5′ or 3′ splice site (SS) selection (A5′SS or A3′SS), inclusion of mutually exclusive exons (MXE), or intron retention (IR) (middle panel). Misregulated splicing of isoforms involved in key cellular pathways contributes to tumor initiation and progression. Examples of cancer hallmarks and associated tumor isoforms are indicated (bottom panel).

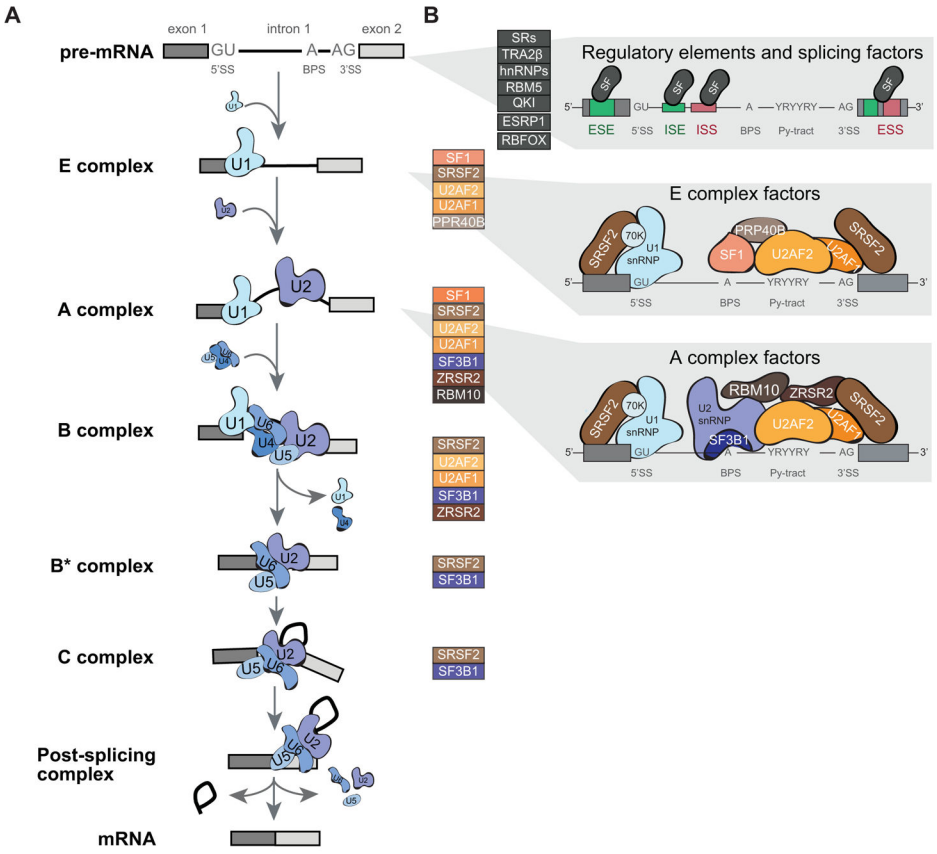


Figure 2. Components of the core and regulatory splicing machinery that exhibit alterations in human tumors

(A) Graphical representation of the stepwise assembly of spliceosomal complexes on a pre-mRNA molecule and catalysis of the splicing reaction to generate mature spliced mRNA. First, the ATP-independent binding of U1 snRNP to the 5′ splice site (5′SS) of the intron initiate the assembly of the "Early" or E complex on the pre-mRNA. In addition, SF1 and U2AF2 bind respectively to the branch point site (BPS) and the polypyrimidine tract (Py-tract). In the second step, the ATP-dependent interaction of U2 snRNP with the BPS leads to the formation of the A complex. This interaction is stabilized by the SF3a and SF3b protein complexes, as well as U2AF2 and U2AF1, and leads the displacement of SF1 from the BPS. Recruitment of the pre-assembled U4/U6/U5 tri-snRNP marks the formation of the catalytically inactive B complex. Major conformational changes, including release of U1 and U4, lead to spliceosome activation and formation of the B* complex. The first catalytic step of splicing, generates the C complex and results in the formation of the lariat. Complex C performs the second catalytic step of splicing, which results in the joining of the two exons. Post-splicing the spliceosome disassembles in an orderly manner, releasing the mRNA, as well as the lariat bound by U2/U5/U6. The snRNP are then further dissociated and recycled. **(B)** Spliceosomal core factors that exhibit recurrent somatic mutations in human tumors are listed next each complex (colored boxes) and are shown in more details for complexes E and A (right panels). In addition to core splicing factors, regulatory splicing factors (SF) that can bind to exonic or intronic splicing enhancer (ESE or ISE) or silencer (ESS or ISS) sequences to fine-tune splicing are also found altered in human tumors (grey boxes).

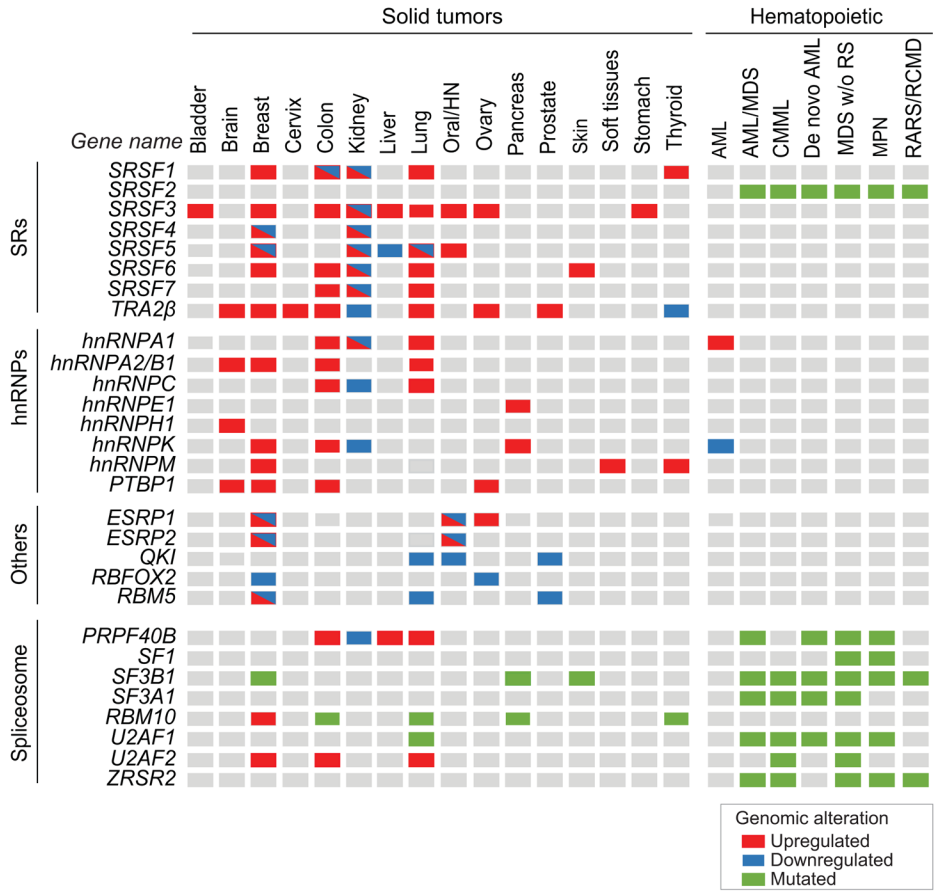


Figure 3. Recurrent splicing-factor alterations detected in human tumors

Genomic alterations including expression changes and recurrent somatic mutations in splicing factors detected in more than 2% of tumors in several cohorts of patients, including TCGA data, are indicated per tumor type. Splicing-factor upregulation are depicted in red, downregulation in blue, and somatic mutations in green (See legend for details). Several splicing factors can be found both upregulated and downregulated in tumors of the same tissue, suggesting that distinct splicing-factor genomic alterations are associated with distinct tumor subtypes within the same tissue. AML: acute myeloid leukemia; AML/MDS: acute myeloid leukemia myelodysplastic syndrome; CMML: chronic myelomonocytic leukemia; HN: head and neck; MDS w/o RS: myelodysplastic syndrome without ringed sideroblasts; RARS/RCMD: refractory anemia with ringed sideroblasts and refractory cytopenia with multilineage dysplasia and ringed sideroblasts; MPN: myeloproliferative neoplasm. See references in text.

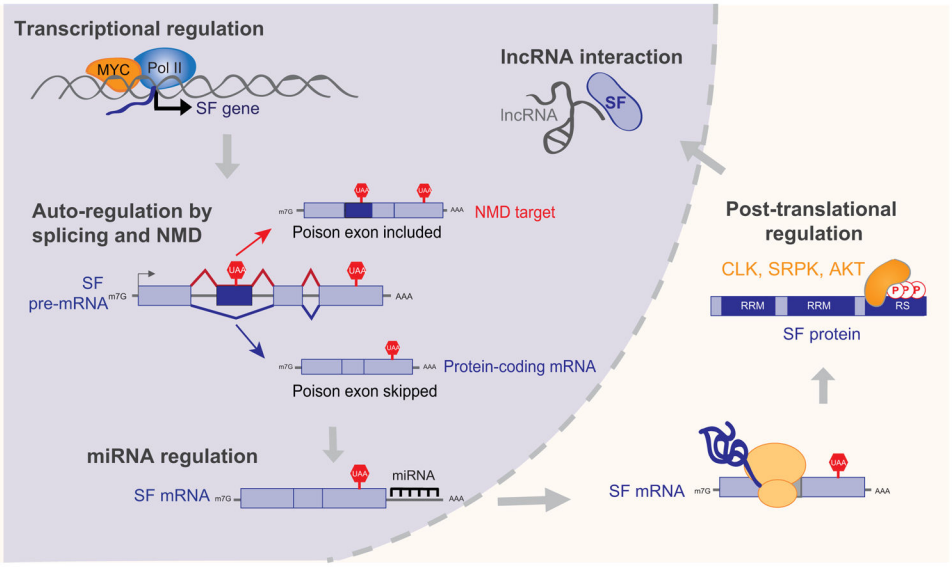
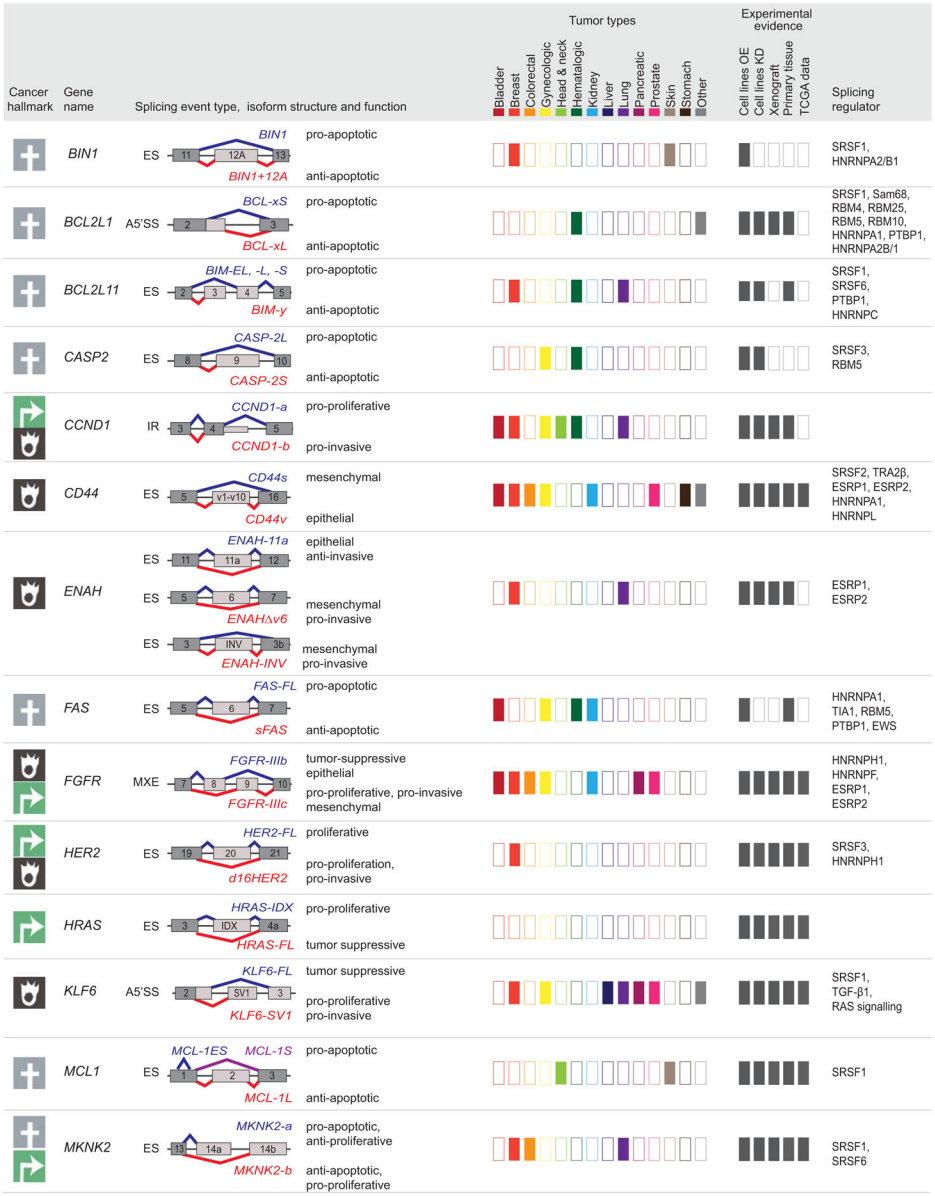


Figure 4. Defects in splicing-factor regulation lead to changes in splicing-factor levels, activity, and cellular localization

Schematic representation of the transcriptional, post-transcriptional, and post-translational steps that impact the expression of a splicing factor (SF). See text for specific examples and references.



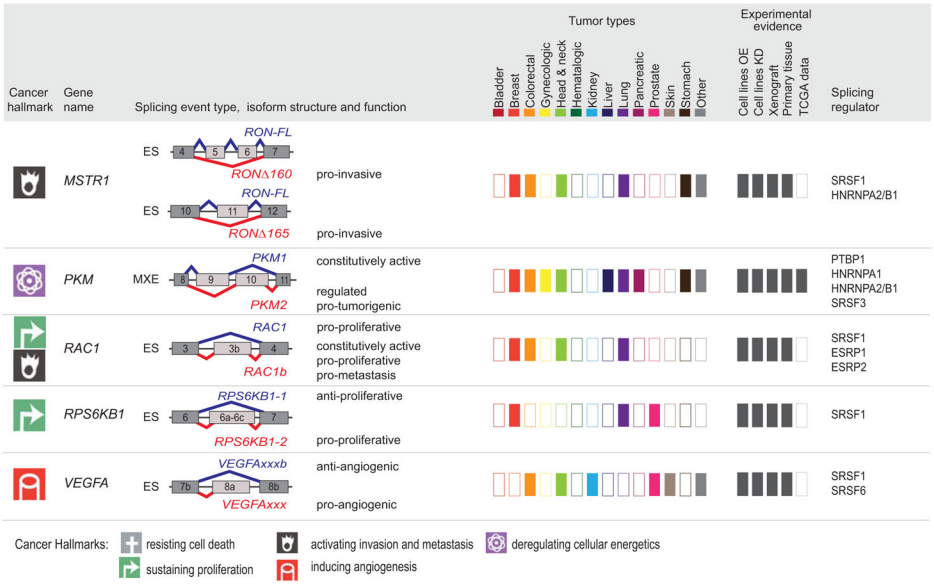


Figure 5. Tumor-associated isoforms representative of the cancer hallmarks

Splicing event type, isoform structure, tumor expression, and experimental evidence for selected alternative splicing isoforms detected in human tumors. For simplicity, only the alternatively spliced sequences and the flanking exons and are shown (not at scale). The type of splicing event is indicated: ES: exon skipping; MXE: mutually exclusive exons; 5′ASS: 5′ alternative splice site selection; IR: intron retention. The corresponding isoforms are shown in red or blue and their respective functions are indicated when known, to the right of the schematic figure of the isoform. The cancer hallmark associated with the red isoform is indicated in the left-hand column (See legend for details). ‘Tumor types,’ indicates, using dark-colored rectangles, the expression of tumor-associated isoforms in each of the indicated tumor types (‘other tumors’ include: adrenal, gallbladder, ampullary, bone, and brain; ‘gynecological tumors’ include: ovarian, cervical, and uterine; ‘head and neck’ tumors include: oral, head and neck, tongue, esophageal, and thyroid). ‘Experimental evidence’ indicates, using dark gray rectangles, the expression and functional evidence for each isoform based the following experiments: (i) overexpression (OE) or knockdown (KD) in cell lines, (ii) tumor xenografts, (iii) expression in primary tissue, or (iv) expression in TCGA RNA-sequencing data. Known splicing regulatory proteins are listed for each gene. See text for references.

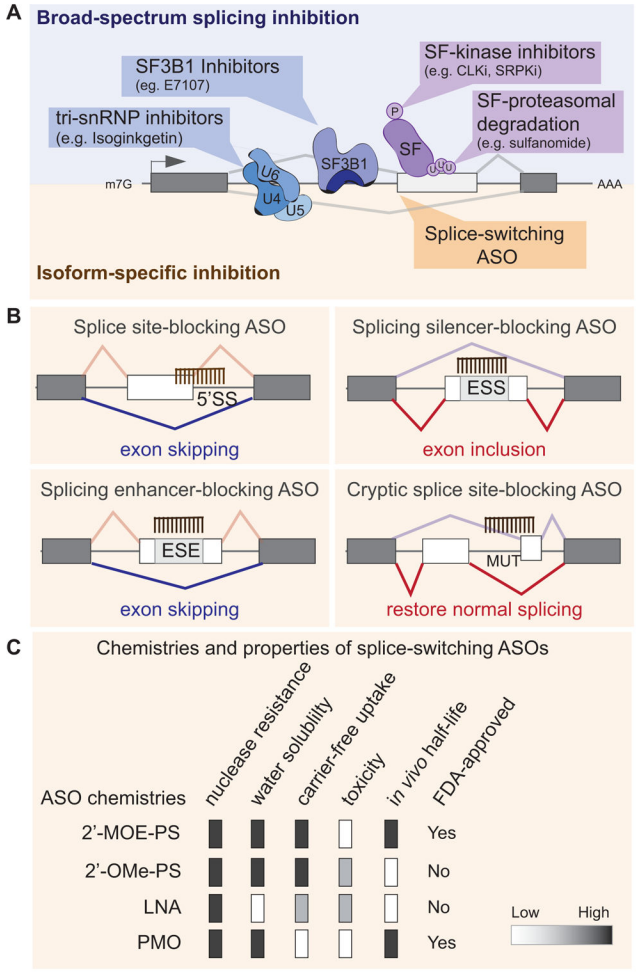


Figure 6. Therapeutic strategies to target splicing alterations in tumors rely either on broad-spectrum splicing inhibition or on isoform-specific modulation

(**A**) Small molecules targeting components of the spliceosome (e.g., SF3B1, or tri-sRNP) block their activity by preventing assembly of a functional spliceosome into the pre-mRNA, and thus globally inhibit splicing. Alternatively, broad splicing inhibition can be achieved by targeting the enzymes that modulating the activity of splicing regulatory factors (SF), using for example small molecules inhibitors of CLKs or SRPKs, two kinase families that regulate the phosphorylation and thus the activity of SR proteins. Compounds that affect splicing factor poly-ubiquitination and proteasomal degradation (e.g. sulfonamides) can also induce broad changes in splicing profiles. On the other hand, isoform specific inhibition can be achieved by using splice-switching antisense oligonucleotides (ASOs) that bind in a sequence-specific manner and modulate the outcome of a specific splicing isoform. **(B)** ASOs can promote exon skipping or inclusion by blocking the 5′SS, an exonic silencer (ESS), or enhancer element (ESE) or by preventing the usage of a mutant (MUT)/cryptic splice site. See text for details. (**C**) Properties of ASO chemistries are currently used for splicing-modulation. See text for details. 2′-MOE/PS: 2′-O-(2-methoxyethyl)/phosphorothioate; 2′-OMe/PS: 2′ O-methyl/phosphorothioate; PMO: phosphorodiamidate morpholino oligomer; LNA: locked nucleic acid.

Table 1

Cancer-associated human isoforms targeted by splice-switching ASO

Gene name	ASO chemistry	Type of splicing correction	Tumor type	Tested in cell lines	Tested *in vivo*	References
STAT3	PMO	exon 23 skipping	breast	√	√	424
MDM4	PMO	exon 6 skipping	skin, lymphoma	√	√	425
ERBB4	LNA	exon 26 skipping	breast	√	√	444
BCL2L1	2′-MOE/PS	exon 2 skipping	skin	√	√	239,240,445
GLDC	2′-MOE/PS	exon 7 skipping	lung	√	√	446
PKM2	2′-MOE/PS	exon 9 inclusion	brain	√	n.d.	447
MCL1	PMO	exon 2 skipping	skin	√	n.d.	271
MDM2	PNA	exon 4 skipping	uterine	√	n.d.	448
BRCA2	2′-OMe/PS	cryptic exon skipping	breast	√	n.d.	449
IL5R	2′-MOE/PS	exon 5 skipping	lymphoma	√	n.d.	450
FGFR1	PMO	exon α inclusion	brain	√	n.d.	451
MSTR1	PMO	exon 11 skipping	breast and stomach	√	n.d.	452
USP5	PMO	alternative 5′SS	brain	√	n.d.	453

PMO: phosphorodiamidate morpholino oligomer; LNA: locked nucleic acid; PNA: peptide nucleic acid; 2′-MOE/PS: 2′-O-(2-methoxyethyl)/phosphorothioate; 2′-OMe/PS: 2′ O-methyl/phosphorothioate; n.d.: not determined.