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•REVIEW•

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Splicing dysregulation in cancer: from mechanistic understanding to a new class of therapeutic targets

Yongbo Wang[1*], Yufang Bao[1], Sirui Zhang[2] & Zefeng Wang[2*]

[1]*Department of Cellular and Genetic Medicine, School of Basic Medical Sciences, Fudan University, Shanghai 200032, China;*
[2]*CAS Key Laboratory of Computational Biology, CAS-MPG Partner Institute for Computational Biology, Shanghai Institute of Nutrition and Health, CAS Center for Excellence in Molecular Cell Science, University of Chinese Academy of Sciences, Chinese Academy of Sciences, Shanghai 200031, China*

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RNA splicing dysregulation is widespread in cancer. Accumulating evidence demonstrates that splicing defects resulting from splicing dysregulation play critical roles in cancer pathogenesis and can serve as new biomarkers and therapeutic targets for cancer intervention. These findings have greatly deepened the mechanistic understandings of the regulation of alternative splicing in cancer cells, leading to rapidly growing interests in targeting cancer-related splicing defects as new therapies. Here we summarize the current research progress on splicing dysregulation in cancer and highlight the strategies available or under development for targeting RNA splicing defects in cancer.

splicing, alternative splicing, cancer, RNA therapeutics

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Introduction

Splicing of RNA precursors (pre-RNA) is a key regulatory step of gene expression in eukaryotic cells. Through alternative splicing (AS), a process involving the selection and pairing of different splice sites, a single gene can generate multiple splice variants with distinct functions (Lee and Rio, 2015). The vast majority of human genes (>95%) undergo AS (Gonçalves et al., 2017; Koh et al., 2016; Wang and Burge, 2008), greatly expanding their functional diversity. Splicing regulation plays critical roles under physiological conditions, and its dysregulation is one of the major causes of various human diseases, including cancers and neurodegenerative diseases (Scotti and Swanson, 2016). Increasing evidence demonstrates that aberrant splicing is widespread in cancer and contributes to tumorigenesis by affecting cancer-associated genes (Dvinge et al., 2016; Song et al., 2018; Urbanski et al., 2018). In addition, the splicing defects resulting from splicing dysregulation can serve as new prognostic markers and therapeutic targets for cancer management (Agrawal et al., 2018; Lee and Abdel-Wahab, 2016). These findings provide profound mechanistic insights into splicing dysregulation in cancer and highlight the enormous potential of targeting cancer-related splicing defects. In this review, we aim to summarize the mechanistic understanding, and therapeutic targeting of splicing dysregulation in cancer. We first give a brief introduction of the general mechanisms of splicing and AS regulation, and then summarize the recent research progress in splicing dysregulation in cancer. We also discuss emerging connections between splicing and long non-coding RNAs as well as the roles of circular RNAs in cancer. Finally, we highlight the diverse strategies of targeting RNA splicing defects in can-

*Corresponding authors (Yongbo Wang, email: wangyongbo@fudan.edu.cn; Zefeng Wang, email: wangzefeng@picb.ac.cn)

cer, and discuss the possible roles of splicing dysregulation in cancer immunotherapy.

Catalysis of RNA splicing reactions

The splicing of precursor RNA involves multiple biochemical steps that lead to the removal of introns and ligation of exons. Since the discovery of RNA splicing in 1977 (Berget et al., 1977; Chow et al., 1977), extensive biochemical and structural studies have revealed that RNA splicing is a highly dynamic process catalyzed by the huge ribonucleoprotein complex called spliceosome (Shi, 2017; Wahl et al., 2009). The human major spliceosome contains five small nuclear RNAs (snRNAs): U1, U2, U4/U6, and U5 snRNAs, as well as more than 100 protein components (Wahl and Lührmann, 2015).

The detailed processes and molecular mechanisms involved in splicing have been thoroughly described in previous studies (Matera and Wang, 2014; Shi, 2017; Wahl et al., 2009). In brief, as shown in Figure 1A, the U1 small nuclear ribonucleoprotein (snRNP) recognizes the 5′ splice site (5′ss), and the U2AF2/U2AF1 heterodimer, and splicing factor 1 (SF1) recognize the 3′ splice site (3′ss), and branch point sequence (BPS) respectively, forming an early spliceosomal complex known as the E complex. The U2 snRNP subsequently displaces SF1 to form the pre-spliceosomal A complex. The U4/U6/U5 tri-snRNP then associates with the A complex to assemble into the pre-catalytic spliceosomal B complex, which is the first fully assembled spliceosome. Subsequently, the U1 and U4 snRNPs dissociate from the B complex to generate the activated B^{act} complex, which undergoes additional conformational rearrangements to become the catalytically activated spliceosomal B* complex that catalyzes the first transesterification reaction of splicing. Consecutively, the spliceosome is further rearranged through intricate changes of RNA-protein interactions, resulting in the spliceosomal C complex that sequentially catalyzes the step 2 splicing reaction. The post-splicing complex (P complex) and intron lariat spliceosome (ILS complex) are formed after the catalytic reactions. The snRNAs leaving the splicing process are recycled for new rounds of splicing reaction.

Alternative splicing regulation

AS occurs in almost all multi-exon human genes and is highly regulated (Lee and Rio, 2015). AS plays critical roles in various physiological contexts, such as cell proliferation, differentiation, and response to external stimuli (Baralle and Giudice, 2017). Generally speaking, as depicted in Figure 1B, AS outcomes are largely determined by interactions between regulatory *cis*-elements within pre-RNA and *trans*-acting splicing factors that either promote or repress the efficiency of basal splicing machinery (Lee and Rio, 2015; Matera and Wang, 2014). Based on distinct patterns of splice site selection, simple AS events can be categorized into cassette exon, alternative 5′ splice site, alternative 3′ splice site, mutually exclusive exons and retained intron (Figure 1C). There are also complicated AS events that involve combinations of these simple events.

In addition to the 5′ss, 3′ss, and BPS that are recognized by core components of the splicing machinery, numerous ancillary *cis*-elements in exons and introns are recognized by the regulatory splicing factors. Based on the locations and functions, these *cis*-elements are classified into exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs), and intronic splicing silencers (ISSs). Hundreds of splicing factors, mostly RNA binding proteins, participate in AS regulation of human genes. The well-characterized examples include serine/arginine-rich proteins (SR proteins) and heterogeneous nuclear ribonucleoproteins (hnRNPs) that typically promote and repress splicing, respectively (Han et al., 2010; Long and Caceres, 2009). More recent studies reveal that both SR and hnRNP proteins can either promote or repress splicing when binding to different positions in pre-mRNAs (Fu and Ares Jr, 2014; Geuens et al., 2016; Howard and Sanford, 2015), and such context-dependent activity in splicing regulation is also found in many other splicing factors (Gonçalves et al., 2017; Williamson et al., 2017; Zhou et al., 2012).

With the advent of powerful technologies to identify transcriptome-wide protein-RNA interactions and splicing alterations, the molecular mechanisms of a growing number of splicing factors have been elucidated (Lee and Ule, 2018; Lin and Miles, 2019; Stark et al., 2019). It is now clear that a single splicing factor often recognizes and regulates splicing of many pre-mRNA targets in a context-dependent fashion (Fu and Ares Jr, 2014), and that auto-regulation and cross-regulation frequently occur among splicing factors (Jangi and Sharp, 2014; Pervouchine et al., 2019; Sun et al., 2017). It should also be noted that RNA splicing is often coupled with and modulated by transcription since most splicing events happen co-transcriptionally (Herzel et al., 2017; Moore and Proudfoot, 2009). Other regulatory layers of gene expression, such as epigenetic modification and RNA modification, often play roles in splicing regulation (Braunschweig et al., 2013; Herzel et al., 2017; Martinez and Gilbert, 2018; Rahhal and Seto, 2019; Yang et al., 2015). Although significant advancement has been made in the mechanistic understanding of AS regulation, the intricate splicing regulatory networks are not well understood.

Splicing dysregulation in cancer

Splicing defects resulting from either mutations in splicing

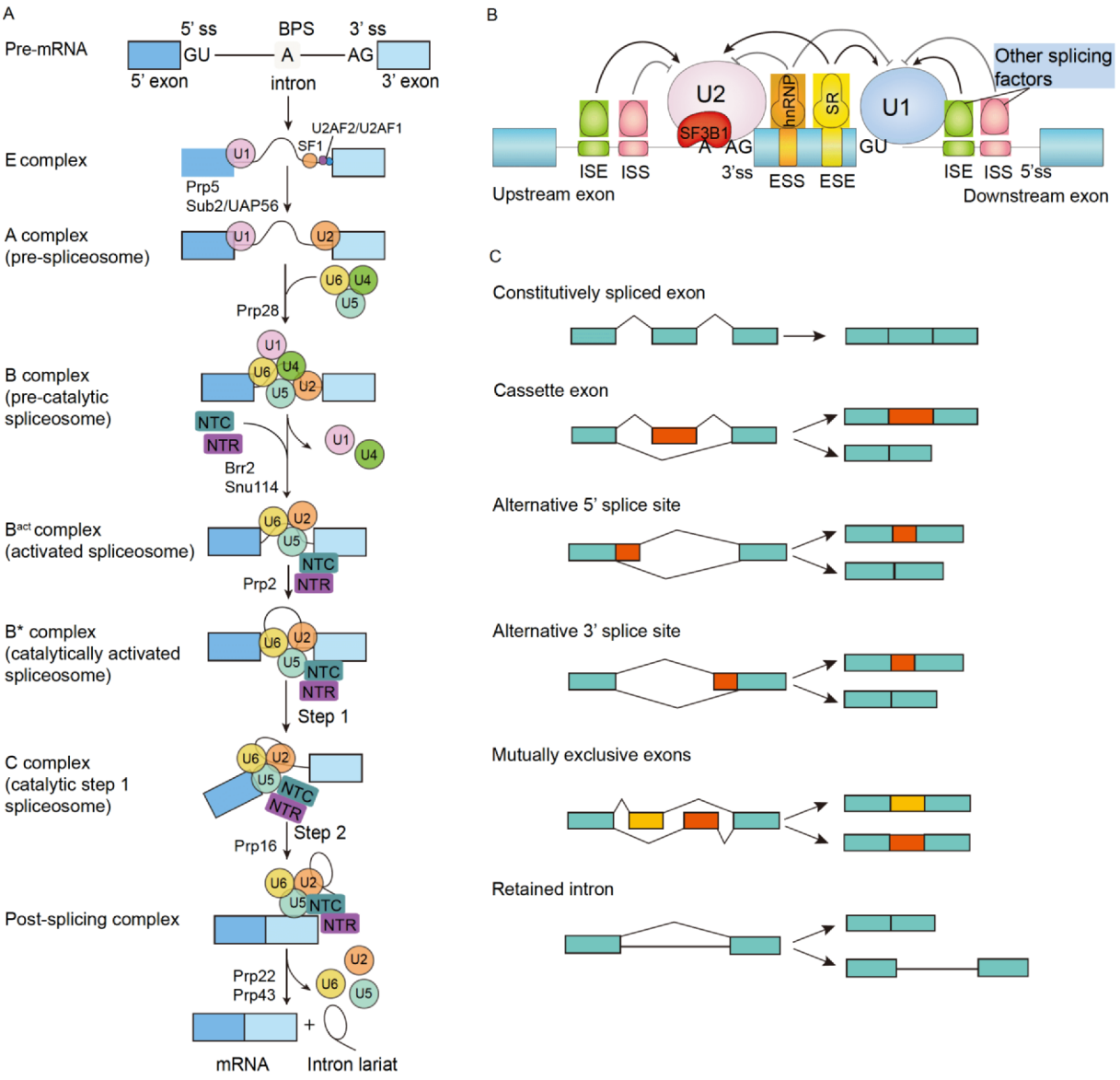

Figure 1 Mechanism of pre-mRNA splicing and alternative splicing regulation. A, Simplified schematic of the stepwise assembly of spliceosomal complexes on a pre-mRNA and the catalysis of splicing reaction. 5′ss: 5′ splice site; 3′ss: 3′ splice site; BPS: branch point sequence; NTC: the nineteen complex in yeast, also known as the PRP19–CDC5L complex in mammals; NTR: NTC-related complex. B, General molecular mechanisms of alternative splicing (AS) regulation. ESE: exonic splicing enhancer; ESS: exonic splicing silencer; ISE: intronic splicing enhancer; ISS: intronic splicing silencer; SR: serine/arginine-rich protein; hnRNP: heterogeneous nuclear ribonucleoprotein. C, Schematic of constitutive and distinct AS events.

cis-elements or mutation/dysregulation of splicing factors have been found to be widespread in cancer and significantly contribute to cancer development and progression (Figure 2A) (Anczuków and Krainer, 2016; Song et al., 2018). Increasing evidence demonstrates that cancer-associated splicing defects affect genes involved in almost every aspect of cancer biology, including cell proliferation, apoptosis, cell motility, epithelial-to-mesenchymal transition, angiogenesis, immune surveillance, and drug resistance (Siegfried and Karni, 2018; Sveen et al., 2016; Zhang and Manley, 2013). Cancer cells selectively express the potentially oncogenic splice variants of the affected genes to gain growth advantage and confer resistance to drug treatment (Figure 2B; see Table S1 in Supporting Information for a list of cancer-related splicing events). For these reasons, it is generally accepted that splicing dysregulation is a molecular hallmark of cancer and plays oncogenic roles in certain cancers (Dvinge et al., 2016; Oltean and Bates, 2014; Urbanski et al., 2018; Zhao et al., 2017). Despite intensive investigations, the functions and molecular mechanisms of the majority of AS events and splicing factors dysregulated in cancer remain largely unclear.

Mutation in splicing regulatory cis-elements

Mutations in splicing regulatory *cis*-elements of cancer-as-

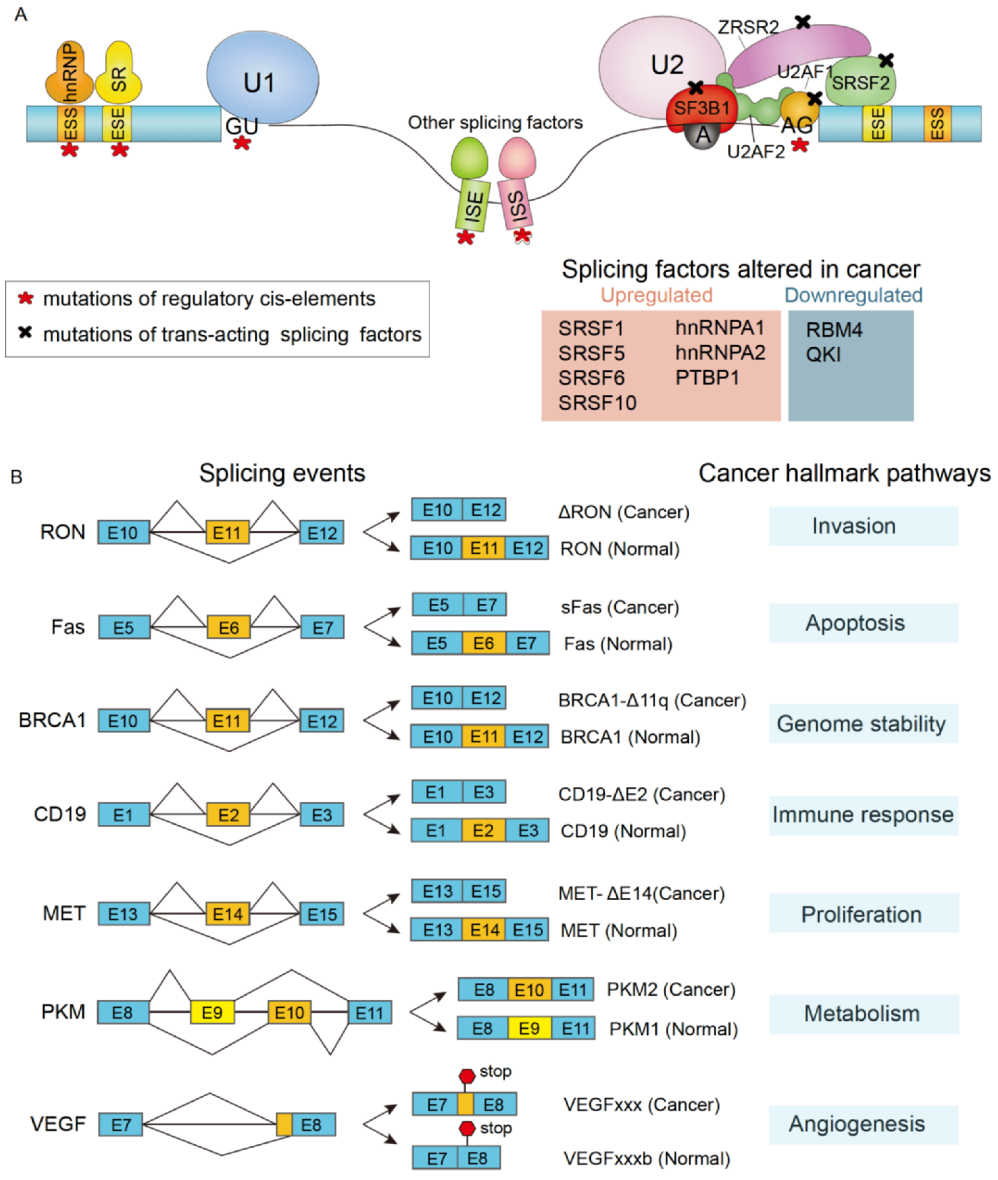

Figure 2 Splicing dysregulation in cancer. A, Mechanisms by which splicing is deregulated in cancer. Mutations in splicing regulatory *cis*-elements and *trans*-acting splicing factors are indicated by red asterisks and black crosses respectively in the diagram of a simplified splicing regulatory model. Representative examples of dysregulated splicing factors that have been shown with oncogenic or tumor suppressive functions are listed in the light red and light blue boxes respectively. B, Representative examples of splicing events altered in cancer and their affected cancer hallmark pathways. E: exon.

sociated genes can lead to splicing abnormalities that promote carcinogenesis. The well-characterized examples include splice site or intronic mutations that cause exon 14 skipping in proto-oncogene *MET*, a receptor tyrosine kinase involved in cell proliferation and migration (Pilotto et al., 2017). The exon 14 skipping in *MET* leads to an in-frame deletion in the juxtamembrane (JM) domain and produces an oncogenic splice variant with higher stability and prolonged signaling activation than the wild type *MET* (Pilotto et al., 2017). In addition, recurrent non-coding mutations in the 3′ untranslated region (3′ UTR) of *NOTCH1*, a transmembrane receptor that plays key roles in development, were reported to create new splice acceptor sites in the 3′ UTR and activate a cryptic splice donor site in the coding region of the last exon in chronic lymphocytic leukemia. These mutations produce an aberrantly active form of *NOTCH1* that lacks the PEST domain (Puente et al., 2015). Recent integrative analyses of genetic mutation and gene expression provide global insights into the impacts of genetic mutations on splicing (Climente-González et al., 2017; Jung et al., 2015; Kahles et al., 2018; Seiler et al., 2018a; Supek et al., 2014). Those studies showed that single nucleotide variations in splicing regulatory *cis*-elements lead to intron retentions that were enriched in tumor suppressor genes, such as *TP53*, *ARID1A*, and *PTEN* (Jung et al., 2015), and exon splicing alterations in proto-oncogenes, such as *PDGFRA* and *EGFR* (Supek et al., 2014).

Mutation in splicing factors or spliceosomal snRNA

Mutations in some spliceosomal genes are frequently identified in hematopoietic malignancies (Table S2 in Supporting Information), providing direct genetic evidence for the functional significance of splicing dysregulation in cancer (Agrawal et al., 2018; Dvinge et al., 2016). For example, *SF3B1*, *U2AF1*, *SRSF2*, and *ZRSR2* are the most commonly mutated spliceosomal genes in hematopoietic malignancies (Dvinge et al., 2016). Mutations in *SF3B1*, *U2AF1*, and *SRSF2* are almost always heterozygous missense mutations and tend to occur at specific locations (i.e., hotspot), indicating gain-of-function or change-of-function (Dvinge et al., 2016). Functional studies using cell lines, genetically engineered mouse models, and clinical samples revealed that those mutations exert cancer promoting functions by altering splicing (Dvinge et al., 2016). More specifically, *SF3B1* and *U2AF1* mutations respectively altered BPS and 3′ss recognition (Obeng et al., 2016; Shirai et al., 2015; Smith et al., 2019b; Wang et al., 2016; Yin et al., 2019; Zhang et al., 2019), and mutations in *SRSF2* changed the preference of the binding sequence in ESEs (Kim et al., 2015), leading to aberrant splicing of target genes. In addition, it has been shown that *SRSF2* mutation cooperates with mutation in *IDH2* (which encodes an important metabolic enzyme involved in epigenetic regulation) to drive leukaemogenesis, largely through synergistic effects on aberrant splicing of a member of the integrator complex *INTS3* (Yoshimi et al., 2019). In contrast to *SF3B1*, *U2AF2*, and *SRSF2*, mutations in *ZRSR2* spread across the entire gene and often lead to protein truncation, indicating a pathogenic pathway caused by loss-of-function (Madan et al., 2015). Interestingly, mutations in those cancer-associated spliceosomal genes occur in a mutually exclusive manner. A recent study showed that spliceosomal gene mutations are synthetically lethal and have convergent effects on common signaling pathways (Lee et al., 2018), explaining their mutual exclusivity in cancer.

Although spliceosomal mutations are less frequent in solid tumors, mutations in both spliceosomal genes and splicing regulatory factors have been observed in solid tumors (Table S2 in Supporting Information). For example, *SF3B1* mutations were found in uveal melanoma, bladder, pancreatic, breast, and lung cancers (Agrawal et al., 2018), and *U2AF1* and *RBM10* mutations were found in lung and pancreatic cancers (The Cancer Genone Atlas Research Network, 2014; Witkiewicz et al., 2015; Zhao et al., 2017). However, compared to hematopoietic malignancies, functional consequences and molecular mechanisms of splicing mutations in solid tumors remain largely uncharacterized. Interestingly, a recent study showed that various *SF3B1* mutations observed in different cancers consistently enhance a poison exon inclusion in *BRD9,* a component of a non-canonical BAF chromatin-remodeling complex, leading to its mRNA degradation and tumor progression (Inoue et al., 2019). This study suggests a common mechanism and potential therapeutic target for various *SF3B1*-mutated cancers (Inoue et al., 2019). Apart from affecting major functions in regulating splicing, mutations in splicing factors may also contribute to cancer progression by interfering with their non-canonical functions, such as the recently proposed *U2AF1*-mediated mRNA translation (Palangat et al., 2019).

In addition to mutations in splicing factors, two recent studies have found that hotspot mutations in U1 snRNA frequently occur in multiple cancers (Palangat et al., 2019) and are highly enriched in Sonic hedgehog (SHH) medulloblastomas (Suzuki et al., 2019). The hotspot U1 snRNA mutations were shown to alter the preference of U1 snRNA pairing with 5′ss, resulting in aberrant splicing in cancer genes (Palangat et al., 2019; Suzuki et al., 2019). Those findings provide new mechanisms of splicing dysregulation in cancer and highlight the importance of searching for non-coding driver mutations.

Dysregulation of splicing factors

Besides genetic mutations, dysregulation of splicing factors through expression and/or activity alteration has commonly been observed and significantly contributes to aberrant splicing in cancer. How splicing factors are dysregulated in cancers remains poorly understood. Oncogenic signaling pathways (e.g., EGF, PI3K-AKT, MAPK, Wnt and signals from tumor microenvironment) are recognized to play important roles in modulating splicing factors via diverse mechanisms, including transcriptional regulation, and/or post-translational modification (Figure 3; Gonçalves et al., 2017). For example, the proto-oncogene *c-MYC* is overexpressed via genetic amplification or activated by signaling cascades (e.g., RAS/RAF/MEK/ERK) in various cancers, which induces transcription of distinct splicing factors (e.g., SRSF1, hnRNPA1, hnRNPA2, PTBP1, and PRMT5) to promote the expression of potentially oncogenic splicing isoforms of cancer genes (e.g., BCL2L1, PKM1/2, RAC1, and DVL1) (Koh et al., 2016). The MEK/ERK signaling pathway was also reported to mediate DAZAP1 phosphorylation that is essential for its cytoplasm-to-nucleus translocation and splicing regulatory activity (Choudhury et al., 2014). In another instance, EGF signaling was reported to regulate splicing via AKT-SRPK1/2-SR protein phosphorylation (Zhou et al., 2012) and/or SPSB1-hnRNPA1 ubiquitination (Wang et al., 2017). It should be noted that aberrant splicing can affect key genes involved in oncogenic signaling pathways, such as *KRAS* (Tsai et al., 2015), *BRAF* (Poulikakos et al., 2011), and *TEAD4* (Qi et al., 2016), resulting in a feedback loop to drive oncogenesis. A lot more future efforts are required to understand the intricate interplay between oncogenic signaling and aberrant splicing in cancer.

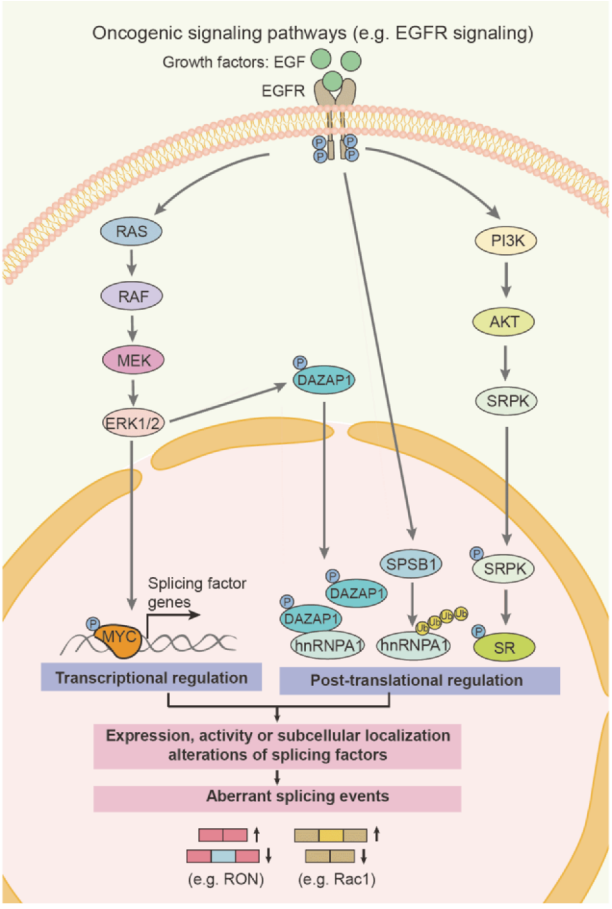

Figure 3 Oncogenic signaling pathways induce alterations of splicing factors and aberrant splicing events. Oncogenic signaling pathways (e.g., EGFR signaling) play important roles in dysregulation of splicing factors via diverse molecular mechanisms, including transcriptional regulation, and/or post-translational modification, which subsequently leads to aberrant splicing events that promote oncogenesis. P: phosphorylation; Ub: ubiquitination.

A variety of splicing factors deregulated in cancers have been shown to exhibit oncogenic or tumor suppressive functions (Anczuków and Krainer, 2016; Urbanski et al., 2018), which can be categorized into SR proteins (e.g., SRSF1, SRSF3, SRSF6, and SRSF10), hnRNP proteins (e.g., hnRNPA1, hnRNPA2/B1, hnRNPF, hnRNPH, PTBP1, and hnRNPK), and other splicing factors (e.g., RBM4, ESRP1, and QKI) (Table S3 in Supporting Information; Anczuków and Krainer, 2016; Dvinge et al., 2016; Urbanski et al., 2018). We discuss the roles of SRSF1, HNRNPK, and RBM4 as representative examples from each category in more detail below.

As the best-characterized oncogenic SR proteins, SRSF1 is overexpressed in a variety of types of cancer, including breast, lung, colon cancers, and glioblastoma, and correlates with adverse prognosis (Urbanski et al., 2018). Previous studies have demonstrated that moderate overexpression of SRSF1 leads to mammary epithelial cell transformation (Anczuków et al., 2012). Moreover, SRSF1 upregulation was shown to correlate with chemotherapy and radiotherapy resistance in lung cancer (Sheng et al., 2018). SRSF1 is positively regulated by MYC at the transcriptional level and acts cooperatively with MYC in breast and lung cancers (Das et al., 2012). In addition, SRSF1 can be phosphorylated at its RS domain by the SR protein kinase (SRPK) family members (SRPK1 and SRPK2) and the CDC2-like kinase family members (CLK1 to CLK4), and is hyper-activated in cancers (Gonçalves and Jordan, 2015). Overexpressed or hyper-activated SRSF1 exerts oncogenic functions by promoting oncogenic splice variants of target genes involved in diverse cellular pathways, including apoptosis (BCL2L1, BCL2L11, BIN1), cell proliferation and growth (MNK2, RPS6KB1, MYO1B), cell motility (RON), and DNA damage response (DBF4B) (Anczuków et al., 2012; Chen et al., 2017; Zhou et al., 2019). Notably, SRSF1 has pleiotropic molecular functions in addition to splicing, including regulating mRNA translation and stability (Das and Krainer, 2014). However, to what extent the non-splicing functions of SRSF1 contribute to its oncogenic functions remains poorly understood and warrant further investigation.

HNRNPK is a member of the hnRNP proteins that has regulatory functions in transcription, splicing, RNA stability, and translation (Gallardo et al., 2016). Previous studies have showed that hnRNPK can function as either a tumor suppressor or an oncoprotein in different cancers. Deletion of the 9q21.32 locus containing HNRNPK was found in acute myeloid leukemia (AML) patients, which is correlated with decreased HNRNPK expression (Gallardo et al., 2015). Consistently, heterozygous deletion of *Hnrnpk* in mice promoted hematologic and malignant phenotypes by directly inhibiting the C/EBPα p42 isoform and p21 expression (Gallardo et al., 2015), indicating that HNRNPK is a haploinsufficiency tumor suppressor for AML. Conversely, hnRNPK is reported to overexpress in breast, colorectal, and pancreatic cancers and possess potential oncogenic functions (Gallardo et al., 2016). Theoretically, the dichotomous roles of hnRNPK in tumorigenesis can be explained by different hnRNPK functions in different cellular contexts. However, the exact molecular mechanisms underlying hnRNPK functions in cancers are not clear, and require further investigation, particularly when considering its complex biological functions and regulation.

RBM4 has been proposed to function as a tumor suppressor in cancers by suppressing the anti-apoptotic splice variant of BCL-X and promoting the TEAD4 short isoform that inhibits the YAP activity and cell proliferation (Qi et al., 2016; Wang et al., 2014). In addition, RBM4 was shown to antagonize the oncogenic activity of SRSF1, thereby suppressing cancer progression (Wang et al., 2014). Interestingly, RBM4 was found to interact with translation regulator eIF4E2 under hypoxia, and thereby selectively promote translation of oncogenic proteins in cancer cells, including

EGFR, PDGFRA, and IGF1R (Uniacke et al., 2012). This observation indicates that RBM4 may have functions beyond splicing regulation to facilitate the adaption of cancer cells to stress.

Connections between splicing and non-coding RNAs in cancer

Tens of thousands of non-coding RNAs (ncRNAs), including long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs), have been identified, many of which have been shown to play important biological functions (Chen, 2016; Ransohoff et al., 2017). Dysregulation of lncRNAs or circRNAs contributes to cancer development and progression and can serve as potential biomarkers and therapeutic targets (Arun et al., 2018; Bach et al., 2019; Gutschner and Diederichs, 2012; Kristensen et al., 2017; Li et al., 2015a; Yan et al., 2015). It has been shown that the splicing of lncRNAs and circRNAs is often altered in cancer, which in turn affects the development and progression of cancer.

LncRNAs and splicing in cancer

LncRNAs can regulate splicing by diverse molecular mechanisms, such as interacting with or regulating splicing factors or base pairing with pre-RNAs (Romero-Barrios et al., 2018). For example, the metastasis associated lung adenocarcinoma transcript 1 (MALAT1) lncRNA co-localizes with SR splicing factors in the nuclear speckles (Tripathi et al., 2010), suggesting its functions in splicing regulation. MALAT1 has also been reported to act as an oncogenic transcript in multiple cancers by modulating splicing via phosphorylation and redistribution of SR proteins (Tripathi et al., 2010), transcriptional upregulation of the SR protein SRSF1 (Malakar et al., 2017), or sequestration of tumor suppressive function of the splicing factor SFPQ (Ji et al., 2014). In another example, the antisense transcript from intron 1 of the *Fas* locus, a lncRNA named as SAF, was reported to bind *Fas* pre-mRNA predominantly at exon 5–6 and exon 6–7 junctions, resulting in *Fas* exon 6 skipping (Villamizar et al., 2016). The Fas protein is an important death receptor on cell surface to induce apoptosis, and exclusion of exon 6 in *Fas* produces a soluble anti-apoptotic Fas isoform (sFas) that lacks the transmembrane domain (Inoue et al., 2019). As a result, SAF leads to the accumulation of production of sFas, which protects tumor cells against Fas ligand-induced apoptosis.

Similar to protein-coding genes, lncRNAs also undergo AS to generate different isoforms with distinct functions (Iyer et al., 2015; Jiang et al., 2019). In addition, AS of some protein-coding genes can generate lncRNA isoforms (Dhamija and Menon, 2018; Grelet et al., 2017; Williamson et al., 2017) that may have completely different functions or modulate their corresponding protein-coding isoforms. However, functions of most new splice variants of lncRNAs and lncRNAs produced by AS from canonical protein-coding genes are not well-characterized.

circRNAs in cancer

circRNAs are a large class of abundant and evolutionarily conserved non-coding RNAs primarily produced by back splicing, in which the downstream 5′ss joins with the upstream 3′ss to form a covalently-linked circle (Chen, 2016; Kristensen et al., 2019; Patop et al., 2019). It has been shown that a single gene locus can produce multiple circRNAs through alternative back splicing (Zhang et al., 2016), indicating the flexibility, and regulation of this process. circRNA has long been regarded as non-functional splicing byproducts since its discovery more than 20 years ago (Pasman et al., 1996). Although functions of most circRNAs are still enigmatic, emerging evidence demonstrates that circRNAs exert important biological and pathological functions (Kristensen et al., 2019; Patop et al., 2019).

circRNAs have been shown to be involved in various aspects of cancer biology via diverse mechanisms (Bach et al., 2019; Kristensen et al., 2017), including serving as microRNA sponges, RBP sponges, and scaffolds for protein complex assembly. For example, circFOXO3 was reported to bind both p53 and MDM2, and enhance sensitivity of breast cancer cells to cisplatin and doxorubicin (Du et al., 2016a). circFOXO3 was also found to bind cyclin-dependent kinase 2 (CDK2) and cyclin-dependent kinase inhibitor 1 (also known as p21) to form a ternary complex that suppresses cell cycle progression (Du et al., 2016b). As another example, a recent study showed that circTP63 is upregulated in lung squamous cell carcinoma and that elevated circTP63 promotes cell proliferation by competitively binding to miR-873-3p, thereby preventing miR-873-3p from decreasing the level of FOXM1 (Cheng et al., 2019).

Importantly, circRNAs have been considered as promising biomarkers for cancer diagnosis, prognosis, and treatment, mainly because they are abundant, and relatively stable compared to their linear mRNA counterparts. In addition, circRNAs are expressed in a specific manner with regard to both tissue and cell types (Salzman et al., 2012), also having differential expression patterns between cancer and adjacent non-cancerous tissues and among distinct cancer types (Vo et al., 2019). Moreover, circRNAs are observed in secreted extracellular vesicles and can be detected in body fluids and blood (Li et al., 2015b), further supporting the application of these RNAs as cancer biomarkers.

Besides functions as non-coding RNAs, several studies have shown that a subset of circRNAs can be translated in a 5′ cap independent manner (Legnini et al., 2017; Pamudurti

et al., 2017). The circRNA translation can be mediated by extensive modification of N6-methyladenosine (Yang et al., 2017), suggesting that translation of circRNAs is not a rare event. The molecular mechanisms underlying circular RNA translation and the functions of circRNA-translated proteins remain largely elusive. Given that many cancer-associated genes undergo 5′ cap independent translation in response to stress (Silvera et al., 2010), and that enhanced translation of circRNA has been observed under stress conditions (Yang et al., 2017), circRNA-encoded proteins may play important roles in cancer. Consistent with this notion, translation of circβ-catenin was recently reported to promote liver cancer cell growth by activating the Wnt pathway (Grelet et al., 2017).

Targeting splicing defects in cancer

Because splicing defects are widespread and functionally important in cancer, modulation of splicing as new types of cancer therapy has been intensively investigated. A diverse array of methods or drugs to target splicing defects in cancers have been developed or are under development, which can target the core components of the spliceosome, regulatory splicing factors, or specific aberrant splicing events (Figure 4; Table S4 in Supporting Information).

Targeting the core spliceosome

Multiple natural compounds derived from bacteria (e.g., pladienolides, herboxidienes, and spliceostatins) and their analogs have been shown to directly bind the SF3b complex of the U2 snRNP to interfere with early spliceosome assembly (Bates et al., 2017; Effenberger et al., 2017). Those compounds exhibit potent anticancer activities in preclinical studies but cannot be used therapeutically due to the lack of chemical stability (Bates et al., 2017; Effenberger et al., 2017). Several derivatives with improved stability were generated via further medicinal chemistry efforts, most notably E7107 (an analog of pladienolide B), spliceostatin A (SSA; from FR901464), and the Sudemycins (Figure 4; Table S4 in Supporting Information) (Bates et al., 2017; Effenberger et al., 2017). E7107 was tested in phase 1 of clinical trials for the treatment of solid tumors. Although this drug was generally well tolerated and caused splicing perturbation *in vivo*, the trial was suspended due to unexpected toxicity (Eskens et al., 2013). The molecular mechanisms responsible for the toxicity of E7107 are not well-understood, and further refinements to reduce toxicity and/or biomarker-guided patient stratification are required.

Cancers with certain molecular characteristics are more sensitive to spliceosome-targeting small molecules. A notable example is that MYC-driven cancers have been shown to be dependent on enhanced splicing activity and are preferentially vulnerable to splicing modulation (Hsu et al., 2015; Lee and Abdel-Wahab, 2016). Mechanistically, MYC directly promotes the expression core spliceosomal proteins (such as core snRNPs components (Koh et al., 2015)) or splicing regulators (such as SRSF1 (Das et al., 2012), PTBP1, hnRNPA1, and hnRNPA2 (David et al., 2009)) in distinct cancers, resulting in increased splicing activity that creates a therapeutic window for targeting the spliceosome.

Another exciting example is the recent findings that cancer cells bearing core spliceosomal mutations can be preferentially killed by spliceosome-targeting small molecules (Lee et al., 2016; Obeng et al., 2016; Shirai et al., 2017). Based on these findings, a phase 1 clinical trial is currently ongoing for an orally available compound modulating SF3b complex, H3B-8800, in advanced hematopoietic malignancies with spliceosomal gene mutations (Seiler et al., 2018b). Moreover, a recent study found that the long isoform of BCL-x (BCL-xL) confers resistance to spliceosome modulation via E7107 and that the combination of BCL-xL inhibitors and E7107 enhances cytotoxicity in cancer cells (Aird et al., 2019). Collectively, these results demonstrate the value of biomarkers in improving the effectiveness of spliceosome inhibition.

In addition to small molecules targeting the SF3b complex, various compounds targeting other components of core splicing machinery or different stages of spliceosome assembly have been identified, such as compounds targeting Brr2, an ATP-dependent RNA helicase in U5 snRNP (Figure 4; Table S4 in Supporting Information) (Iwatani-Yoshihara et al., 2017). With an expanding catalog of compounds and tailored patient groups, small molecules targeting the spliceosome are very likely to be successfully applied for cancer therapy in the near future.

Targeting regulatory splicing factors

The function of the core spliceosome is modulated by a plethora of regulatory splicing factors, of which dysregulation is commonly observed in cancer. Splicing factors have been found to promote oncogenesis via overexpression or increased activity, and thus may serve as potentially new targets of splicing modulation (Kole et al., 2012). One example is the inhibition of the phosphorylation of SR proteins, a family of splicing factors required for both constitutive splicing and AS (Figure 4; Table S4 in Supporting Information). The C terminus of SR proteins contain multiple consecutive RS-SR dipeptides and undergo extensive phosphorylation by multiple kinases, including the SRPK family members (SRPK1 and SRPK2) and the CDC2-like kinase family members (CLK1 to CLK4) (Giannakouros et al., 2011; Zhou and Fu, 2013). Phosphorylation of SR proteins plays critical roles in splicing regulation, and thus the SR

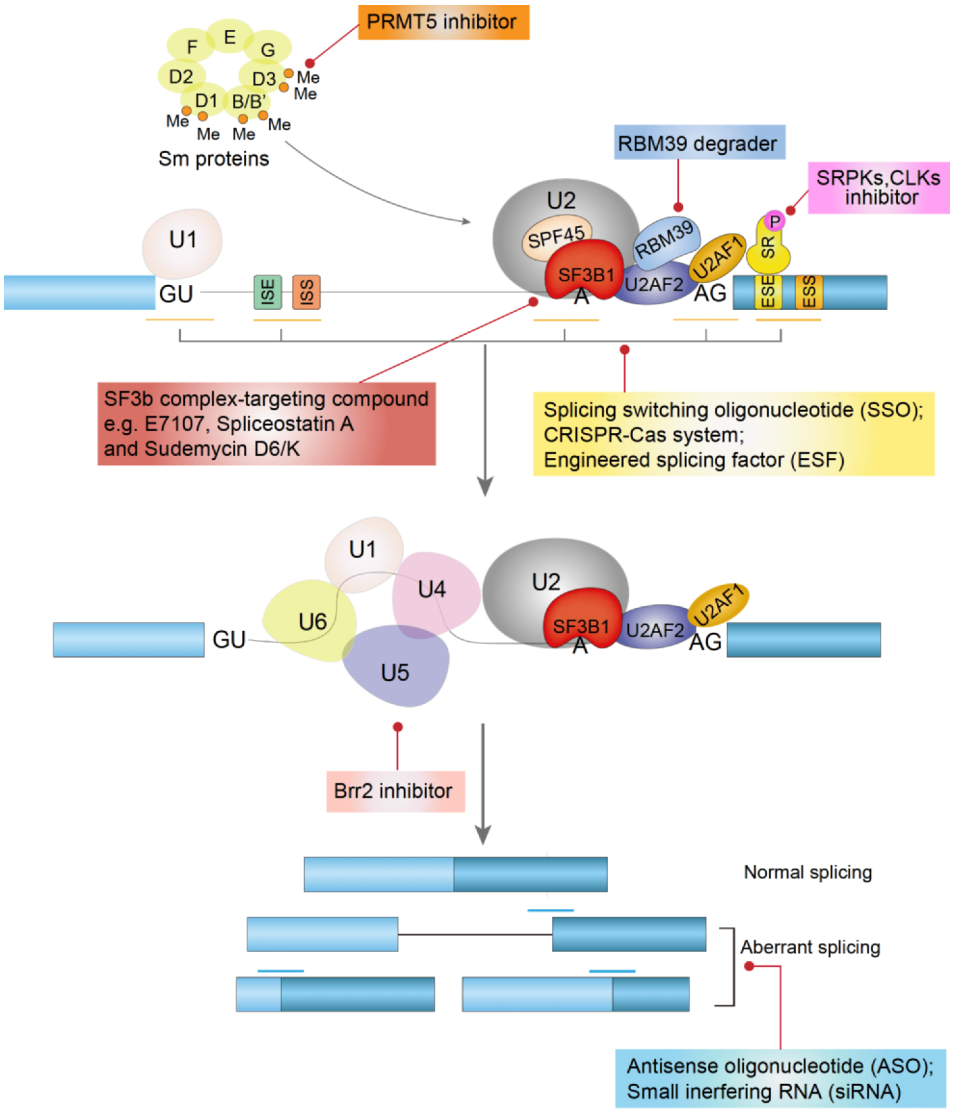

Figure 4 Strategies of splicing modulation for cancer therapy. Strategies based on targeting the core spliceosome (SF3b-complex targeting compound and Brr2 inhibitor), targeting splicing regulatory factors (PRMT5 inhibitor, RBM39 degrader and SRPKs/CLKs inhibitor) and modulating pathological splicing events (splicing switching oligonucleotide (SSO), clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) system (CRISPR-Cas), engineered splicing factor (ESF), antisense oligonucleotide (ASO) and small interfering RNA (siRNA)) are depicted in the diagram of simplified splicing regulatory model.

protein kinase inhibitors (such as CLK inhibitors TG003 and T-025; SRPK inhibitors SRPIN340; and CLK and SRPK inhibitors Cpd-1, Cpd-2, Cpd-3) can induce prominent splicing changes, resulting in the reductions of oncogenic splicing variants (such as S6K, FAS and VEGF) (Araki et al., 2015; Zhou and Fu, 2013). However, preclinical studies using these inhibitors in cancer cell lines showed limited anticancer activities. Compounds with higher potency and better selectivity as well as better predictors of treatment response are therefore needed to facilitate their successful application in cancer therapy. A recent study showed that an orally available and potent CLK inhibitor, T-025, induces skipped exons and suppresses tumor growth, and the sensitivity correlates with CLK2 expression or MYC amplification (Iwai et al., 2018). This study demonstrates the therapeutic value of this new CLK inhibitor, particularly in treating MYC-driven, or CLK2-overexpressed cancers.

Another interesting targetable splicing factor is RBM39, an RNA binding protein in the U2AF2 protein family (Figure 4; Table S4 in Supporting Information) (Kielkopf et al., 2004; Wu and Fu, 2015). Two independent studies found that anticancer sulfonamides modulate splicing by selectively promoting the recruitment of RBM39 to the CUL4-DCAF15 E3 ubiquitin ligase for degradation (Han et al., 2017; Uehara et al., 2017). A recent study reported that RBM39 is required for AML and that RBM39 degradation by an aryl sulfonamide, indisulam (also known as E7070), leads to broad anti-leukemic effects (Wang et al., 2019). Such anti-leukemic effects are more effective for AML bearing spliceosomal mutations (Wang et al., 2019). Indisulam has previously

shown very modest clinical responses in several phase I and phase II trials involving advanced-stage cancer patients (Haddad et al., 2004; Raymond et al., 2002). Based on the finding that the anticancer activity of sulfonamides depends, at least partially, on RBM39, DCAF15, and spliceosomal mutations, cancer patients with high expression of DCAF15 and/or bearing spliceosomal mutations could be selected in future clinical trials for these compounds.

Another promising splicing-related therapeutic target is the PRMT5, an arginine methyltransferase that methylates the Sm proteins of U2 snRNP (Figure 4; Table S4 in Supporting Information) (Blanc and Richard, 2017; Yang and Bedford, 2012). Pharmacologic inhibitors of PRMT5 have been developed (Chan-Penebre et al., 2015; Wang et al., 2018), and cancer cells are shown to be sensitive to PRMT5 inhibition, in part due to the general inhibition of splicing (Smith et al., 2018). Several molecular features have been identified to predict the sensitivity of PRMT5 inhibitors. For example, MYC directly upregulates PRMT5, and MYC-driven lymphoma in mice is dependent on PRMT5 expression (Koh et al., 2015), suggesting the potential application of PRMT5 inhibitors in MYC-driven lymphomas. In addition, a recent study reported that PRMT5 inhibition synergizes with PRMT1 inhibition or spliceosome inhibition and exhibits preferential activity in AMLs with spliceosomal mutations (Fong et al., 2019). It should be noted that PRMT5 has substrates other than Sm proteins and splicing factors (Blanc and Richard, 2017), which may also contribute to the effects of PRMT5 inhibition. Further investigations are needed to better understand the PRMT5 functions in various contexts and the molecular mechanisms underlying the anticancer effects via the pharmacologic inhibition of PRMT5, as well as to identify biomarkers that can predict treatment response in different cancers.

Modulation of aberrant splicing events in cancer

Given that aberrant splicing of cancer-related genes is common in various cancers and significantly contributes to tumorigenesis, modulating these pathological splicing events becomes an attractive strategy for cancer therapy. Compared to targeting the spliceosome or splicing factors that often results in broad splicing alterations, modulation of particular aberrant splicing events should achieve higher specificity.

The most commonly used approach to alter splicing is antisense oligonucleotide (ASO, also termed splicing switching oligonucleotide or SSO), which modulates splicing by pairing with splice sites or regulatory *cis*-elements in pre-mRNA to form steric hindrance (Figure 4) (Bennett, 2019; Shen and Corey, 2018). Alternatively, ASOs or small interfering RNAs (siRNAs) can also be used to block the translation or to degrade the oncogenic splicing variants (Figure 4) (Chakraborty et al., 2017; Shen and Corey, 2018). These oligonucleotide-based approaches, with the theoretical advantage of targeting any gene with high specificity, have been developed for decades. These efforts lead to the recent landmark approvals of ASOs and siRNAs by the US Food and Drug Administration for treating monogenic genetic diseases, including the ASO drug Eteplirsen1 to treat Duchenne muscular dystrophy and Nusinersen1 to treat spinal muscular atrophy, and an siRNA drug Patisiran to treat hereditary transthyretin-mediated amyloidosis (hATTR) (Bennett, 2019; Saw and Song, 2019). Promising preclinical results have also been achieved by targeting the oncogenic splicing variants or key oncogenic genes using ASOs. For instance, skipping of *MDM4* exon 6 by ASO was reported to reduce MDM4 expression, inhibit the growth of melanoma and diffuse large B cell lymphoma, and enhance sensitivity to MAPK-targeting therapeutics (Dewaele et al., 2016). This data demonstrated the enormous potential of targeting pathological splicing events or genes via oligonucleotide-based approaches in cancer; however, their clinical application in cancer treatment remains unclear. The major challenge is the delivery of the ASOs to cancer cells, particularly in the metastasis settings (Moreno and Pêgo, 2014).

In addition to oligonucleotides, clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) systems can be used to manipulate splicing (Figure 4) (Knott and Doudna, 2018; Pickar-Oliver and Gersbach, 2019). The CRISPR-Cas system can be designed to disrupt or edit a particular splice site using a single guide RNA (sgRNA), remove a particular exon or regulatory *cis*-element using a pair of sgRNAs, or correct splicing abnormality induced by genetic mutation using template-mediated homologous recombination (Anzalone et al., 2019; Gapinske et al., 2018; García-Tuñón et al., 2019; Ruan et al., 2017). Due to the advantages of versatile toolkits available and rapid advancements in various biological and disease treatment settings, CRISPR-Cas based splicing modulation holds great potential for cancer therapy. Similar to ASO, the effective delivery of CRISPR-Cas systems to cancer cells is a major obstacle to overcome before its successful application in cancer therapy (Fellmann et al., 2016; Lino et al., 2018).

Another approach to manipulate splicing is to use engineered splicing factors (ESFs) consisting of sequence-specific RNA binding modules (such as The PUF domain of human Pumilio1) and splicing effector domains (such as RS domains or Glycine-rich domains) (Figure 4) (Wang et al., 2009; Yoshimi et al., 2019). The RNA binding specificity of RNA binding module can be reprogrammed to recognize different RNA sequences (Pilotto et al., 2017; Qi et al., 2016; Zhao et al., 2018), enabling ESFs to modulate all types of AS events in different genes. For example, ESFs have been successfully used to shift the splicing of BCL-x from the anti-apoptotic long isoform (Bcl-xL) to the pro-apoptotic short isoform (Bcl-xS), leading to increased apoptosis and

the sensitization of multiple cancer cells to chemotherapies (Wang et al., 2009). This system was also shown to effectively modulate splicing in the central neural system of animal models (Dhamija and Menon, 2018). Compared to ASO or CRISPR-Cas, the ESFs recognize pre-mRNA directly without assembly with guide RNAs and thus can be delivered by using available gene therapy vectors. In addition, the engineered factors originate from human proteins and therefore should have less immunogenic effects compared to the CRISPR-Cas system.

Roles of splicing dysregulation in cancer immunotherapy

Immunotherapy is emerging as a revolutionary approach for cancer treatment (Tian et al., 2019; Zhao et al., 2020). Increasing evidence demonstrates close connections between splicing alternations in cancer and oncoimmunology (Frankiw et al., 2019). Studies have shown that splicing dysregulation directly affects genes with key roles in immune pathways, thereby compromising the effectiveness of cancer immunotherapy. For example, exon 2 skipping in *CD19* leads to a stable isoform that is not recognized by T cells expressing *CD19*-specific chimeric antigen receptors (CAR-T) and thus confers resistant to CD19 CAR-T treatment in B cell acute lymphoblastic leukemias (Sotillo et al., 2015). In another example, two secreted splicing variants of programmed death ligand 1 (PD-L1) were found to trigger resistance to a PD-L1 blockade in non-small cell lung cancer (Gong et al., 2019).

Widespread splicing alterations in cancer are predicted to produce cancer-specific protein isoforms that are probably a major source of cancer neoantigens. Tumor mutation burden has been found to positively correlate with the responses of immune checkpoint blockades (Chan et al., 2018; Samstein et al., 2019). This finding is of great clinical significance and is partially attributed to somatic mutation-derived cancer neoantigens that activate the adaptive immune response to kill cancer cells. Similar to somatic mutations, cancer-related splicing events with neoantigen-generating capacities have been proposed as predictors for the response of immunotherapy (Kahles et al., 2018; Smith et al., 2019a). In addition, the cancer neoantigens resulting from splicing alterations can serve as new targets of immunotherapy. For example, vaccines can be designed based on those neoantigens to trigger immune responses and kill cancer cells. Moreover, for cancer-specific splicing variants expressed in cell membranes, antibodies, or CAR-T against those splicing variants can be designed to specifically eliminate cancer cells. For these reasons, we believe that splicing dysregulation related to cancer immunology will attract more attention and warrant further investigations.

Conclusions and future perspectives

Splicing defects resulting from mutation in splicing regulatory elements or mutation/dysregulation of splicing factors are frequently observed in cancers and are thus considered to be a molecular hallmark of cancer. An increasing number of dysregulated splicing factors and splicing events in cancers have been studied in detail, providing critical insights into a mechanistic understanding of splicing dysregulation and its biological functions. Such advancements not only establish a solid basis for but also dramatically boost the interests of targeting oncogenic splicing defects. Various strategies have been developed to modulate cancer-associated splicing. With technological improvement in chemical modification/refinement and more effective drug delivery systems, as well as a biomarker-guided patient stratification, such strategies can be successfully applied in cancer therapy in the near future.

Despite exciting progress in this area, there are still a number of pressing challenges. First, functions of many splicing factors and splicing events deregulated in cancers are not characterized, calling for more efficient techniques for a systematic dissection of cancer-related splicing defects. Second, preclinical cancer models specifically designed for splicing defects are lacking, impeding an in-depth mechanistic study and the pace of drug development. Third, intricate interplay between oncogenic signaling pathways and splicing dysregulation are largely undetermined. Fourth, intercellular communications may play significant roles in splicing regulation (Georgilis et al., 2018; Pavlyukov et al., 2018) that are critical in cancer treatment, especially when considering the roles of the tumor microenvironment, but this topic remains nearly unexplored. Finally, the efficient delivery of splicing modulation drugs (e.g., ASO, CRISPR-Cas) remains to be achieved. Future efforts are clearly needed to address these challenges in order to ensure the successful application of targeting splicing for cancer therapy.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

Table S1 Cancer-related splicing events

Table S2 Splicing factors mutated in cancer

Table S3 Splicing factors dysregulated in cancer

Table S4 Inhibitors that modulate splicing

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