INTRODUCTION

RNA splicing is the ingenious process that produces mature mRNA transcripts by removing introns from pre-mRNA. A growing body of evidence has underlined the involvement of recurrent point mutations in the major RNA splicing factors SF3B1, SRSF2, and U2AF1 in the pathogenesis of cancers. These mutations in genes encoding SF mutations are frequently identified in a variety of hematopoietic malignancies1-3 as well as in solid tumors such as breast cancers, lung cancers, and pancreatic cancers,4-8 strongly suggesting that aberrant RNA splicing is a new class of hallmark that contributes to the initiation and/or maintenance of cancers. In parallel, some studies have demonstrated that cancer cells with global splicing alterations are dependent on the transcriptional products derived from wild-type spliceosome for their survival, which potentially creates a therapeutic vulnerability in cancers with a mutant spliceosome. It has been c. 10 y since the frequent mutations affecting splicing factors were reported in cancers. Based on these surprising findings, there has been a growing interest in targeting altered splicing in the treatment of cancers, which has promoted a wide variety of investigations including genetic, molecular and biological studies addressing how altered splicing promotes oncogenesis and how cancers bearing alterations in splicing can be targeted therapeutically. In this mini-review we present a concise trajectory of what has been elucidated regarding the pathogenesis of cancers with aberrant splicing, as well as the development of therapeutic strategies to target global splicing alterations in cancers.

KEYWORDS
antisense oligonucleotide, cancer, RNA binding protein, SF3b, splicing factor

1 INTRODUCTION

SF mutations are frequently identified in a variety of hematopoietic malignancies1-3 as well as in solid tumors such as breast cancers, lung cancers, and pancreatic cancers,4-8 strongly suggesting that SF mutations plays an essential role in cancer pathogenesis (Figure 1A-C). In this mini-review we will summarize how SF mutations cause global alterations in splicing, how they contribute to cancer pathogenesis, and whether they can be therapeutically targeted.

Abbreviations: ASO, antisense oligonucleotide; BPS, branchpoint sequence; CLL, chronic lymphocytic leukemia; CMML, chronic myelomonocytic leukemia; ESE, exonic splicing enhancer; ESS, exonic splicing silencer; hnRNP, heterogeneous nuclear ribonucleoprotein; ISE, intronic splicing enhancer; ISS, intronic splicing silencer; MDS, myelodysplastic syndrome; MDS-RS, MDS with ring sideroblasts; PDX, patient-derived xenograft; RBP, RNA binding protein; SF, splicing factor; snRNP, small nuclear ribonucleoprotein particle; SR, serine and arginine; ss, splice site; SSO, splicing switching oligonucleotide; TCGA, The Cancer Genome Atlas; UVM, uveal melanoma.

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RNA splicing is essential for processing pre-mRNA in >90% of human protein-coding genes that contain more than 1 exon. The primary function of splicing is to remove non-coding introns and essential splicing sequences include the 5′ss, the BPS and a 3′ss (Figure 2A). Upon recognition of these sequences, the spliceosome catalyzes the splicing reaction. The core enzymatic machinery of splicing is accomplished by the major spliceosome that consists of 5 snRNP particles, U1, U2, U4, U5, and U6. The excision of >99% of human introns is carried out by this core enzymatic machinery.
Each constituent snRNP contains a Sm or Sm-like protein complex that is essential for the formation of the mature snRNP complex and proteins specific to each snRNP.\textsuperscript{1,11,12}

In addition to core splicing sequences recognized by the spliceosome, both cis- and trans-acting factors regulate the splicing pathways. The cis-acting factors are splicing regulatory elements including the ESEs and ESS, and the ISEs and ISS (Figure 2A).\textsuperscript{9} Trans-acting RBPs recognize these sequences and recruit or repress the core splicing enzymatic machinery to regulate splicing. RBPs include SR-rich proteins that generally bind to ESEs to promote the splicing, and the hnRNP family that recognize ESS and ISS and inhibit splicing.\textsuperscript{9} Transcripts from almost all human multi-exon genes are alternatively spliced, where a particular 5′ss can be joined to different 3′ss (or vice versa), frequently in a regulated fashion.\textsuperscript{13} Alternative splicing events are further classified based on how they affect the exonic structure of the mature mRNA (Figure 2B).

3 | MUTATIONS AFFECTING RNA SPLICING FACTORS IN CANCERS

3.1 | Mutations in SF3B1

SF3B1 is the most commonly mutated splicing factor across cancers. Particularly, mutations in SF3B1 have an enrichment in several cancer types including MDS, CLL and UVM. Interestingly, SF3B1 mutations have specific values as biomarkers in certain types of cancer. For example, SF3B1 mutations are identified in >90% of patients with MDS-RS. This form of MDS is characterized by anemia, iron-laden mitochondria surrounding the nuclei of erythroid precursors and a favorable prognosis.\textsuperscript{2,3,14} Because SF3B1 mutations have >97% positive predictive value for patients suspected to have MDS-RS,\textsuperscript{15} mutations in SF3B1 are currently part of the diagnostic criteria for MDS-RS. Mutations in SF3B1 are generally early events in the disease progression of myeloid malignancies, whereas SF3B1 mutations in CLL are most commonly subclonal and enriched in more advanced and aggressive disease.\textsuperscript{16,17}

SF3B1 is a member of the U2 snRNP complex that physically associates with PHF5A, SF3B3 and the U2 snRNA (Figure 2A). The U2 snRNP complex is important in recognizing the BPS within the intron. In harmony with this, splicing analysis based on RNA sequence data from cancer cells and mouse models with mutations in SF3B1 revealed that SF3B1 mutant cells exhibit usage of an aberrant intron-proximal 3′ss compared with canonical splicing (Figure 3A,B).\textsuperscript{18-21} Mutations in SF3B1 are enriched in the 4th to 7th HEAT repeat domains, suggesting that mutant SF3B1 has altered conformation of the HEAT repeat domains. In fact, recent crystal structure analysis of the mutant SF3B1 protein clarified that SF3B1 mutations alter the conformation of the HEAT repeat domains that affects the interactions with U2AF2 or the SF3b complex.\textsuperscript{22}

SF3B1 is affected by some distinct hotspot mutations, and our recent pan-cancer splicing analysis unveiled differential splicing events based on lineage as well as SF3B1 mutant allele.\textsuperscript{20} The most common mutation in SF3B1 is the SF3B1\textsuperscript{K700E} mutation, which is found in myeloid malignancies, CLL and many solid tumors including breast cancers, pancreatic ductal adenocarcinomas, and others. In addition, there are several hotspot mutations in SF3B1 including SF3B1\textsuperscript{R625K} mutations (enriched in melanomas) and SF3B1\textsuperscript{E902K} mutations (enriched in bladder carcinomas). The biological and molecular bases for the lineage specificity of these hotspots still remain to be clarified. A recent paper has revealed that SF3B1\textsuperscript{K700E} loses physical interaction with a poorly studied splicing protein, SUGP1, during BPS recognition. Loss of the interaction leads to aberrant usage of upstream BPSs that was partly rescued upon SUGP1 restoration.\textsuperscript{23} This report also showed that the loss of interaction between mutant SF3B1 and SUGP1 is commonly observed among disease-associated mutant SF3B1 proteins other than K700E, suggesting that the loss of interaction is one of the key mechanisms that promote aberrant usage of 3′ss by mutant SF3B1.

3.2 | Mutations in SRSF2 and U2AF1

In addition to mutations in SF3B1, extensive studies have revealed more about how hotspot mutations in SRSF2 and U2AF1 impact splicing and disease development.

SRSF2 is an auxiliary splicing factor that binds ESEs to recruit the core spliceosome to promote splicing. Whereas wild-type SRSF2 physically binds CCNG and GGNG sequences equally,\textsuperscript{24} hotspot mutations in SRSF2 affecting proline 95 alter this preference.\textsuperscript{18,25-27} As a result, mutant SRSF2 promotes splicing of exons with C-rich sequences over G-rich sequences (Figure 3C). Although the frequency of mutations in SRSF2 was originally estimated as low (<2%) in AML,\textsuperscript{28} our group reanalyzed TCGA dataset and identified that 95% (18/19) of patients with SRSF2 mutations were missed in the previous TCGA publication,\textsuperscript{26} which makes SRSF2 one of the most frequently mutated genes in AML. This was probably due to the markedly GC-rich sequence around the SRSF2 mutational hotspot, leading to low coverage around this region in the next-generation sequencing result. Interestingly, genomic analysis of the TCGA AML cohort using refined SRSF2 genotyping revealed the frequent and significant co-occurrence in mutations affecting SRSF2 and IDH2 in AML. Further functional and biological studies clarified that aberrant RNA splicing and mutant IDH2-mediated DNA hypermethylation closely cooperate with each other to drive leukemogenesis. One of the most robust splicing changes in IDH2/SRSF2 double-mutant AML is the combined intron retention and exon skipping events in Integrator 3 (INTS3), whose loss results in dysregulated gene expression programs associated with hematopoietic cell differentiation and multiple signaling pathways, and blockade of myeloid differentiation leading to the development of myelodysplastic/myeloproliferative neoplasms in vivo.\textsuperscript{26} Mutant SRSF2 also generates an EZH2 isofrom that undergoes nonsense-mediated decay, leading to loss of a key hematopoietic regulator.\textsuperscript{25}

U2AF1 is part of a heterodimeric U2AF complex with its partner, U2AF2, that recruits the U2 snRNP to the BPS (Figure 2A). U2AF1
associates with the AG dinucleotide at the 3′ss, whereas U2AF2 binds the polypyrimidine tract. Hotspot mutations in U2AF1 mainly affect the S34 or Q157 residue, each of which is located in 1 of its 2 zinc fingers. Similar to the hotspot mutations in SF3B1, U2AF1 mutations are linked to specific lineages of cancer. One of the examples is lung adenocarcinoma, in which U2AF1S34F/Y mutations are recurrent whereas U2AF1Q157P/R mutations favor inclusion of cassette exons with a G-nucleotide at the “+1” position.

3.3 | Mutations in other splicing factors

Mutations in ZRSR2 are identified in c. 10% of MDS patients. These mutations are located sporadically across the coding regions, suggesting that these mutations are loss-of-function mutations distinct from the change-of-function mutations in SF3B1, SRSF2, and U2AF1. In addition, a recent study investigating mutations in 404 SFs from 33 cancer types in TCGA identified potential driver mutations in 119 SFs. Functional roles of these mutations in cancers remain to be addressed.

4 | STRATEGIES FOR TARGETING SPLICING ALTERATIONS IN CANCERS

4.1 | Inhibition of SF3b binding

Several natural products from bacterial species and their analogs that bind the SF3b complex have been discovered. These compounds, including E7107 (an analog of pladienolide B), spliceostatin A, and the sudemycins inhibit the binding of the branchpoint binding region of U2 snRNP to the BPS and block the essential conformational change in U2 snRNP (Figure 4A). H3 Biomedicine Inc and our group developed an orally bioavailable, clinical-grade analog of E7107, H3B-8800, and demonstrated that H3B-8800 modulates splicing of wild-type and mutant spliceosomes in vitro and preferentially kills spliceosome-mutant cells. Sequentially, we added studies for the evaluation of spliceosome inhibitor in isogeneic murine MDS/AML models and, more importantly, in preclinical settings using PDX models with mutant SF3B1 expression. In mice engrafted with SF3B1K700E PDX, 10 d of H3B-8800 treatment significantly reduced leukemic burden relative to that in vehicle-treated mice. Given that mutations in SRSF2 are prevalent (46.9%) in CMML, we have also tackled the generation of the first CMML PDX models.
that are robust enough to perform preclinical tests. Combined use of CD34-enriched leukemia cells, intrafemoral injection, and NSG mice as recipients enabled us to generate robust CMML PDX models with or without mutations in \textit{SRSF2}.\footnote{41} Notably, the results that AML cells with mutations in spliceosome are more sensitive to H3B-8800 were recapitulated in the CMML PDX models with or without \textit{SRSF2} mutations.\footnote{39}

These studies suggested that survival of spliceosome-mutant cells may depend on the activity of the residual wild-type allele and that cells heterozygous for spliceosomal mutations may therefore have increased sensitivity to spliceosomal inhibition.\footnote{39} Following this preclinical study, clinical trials to evaluate the therapeutic potential of SF3b inhibitors have been initiated. Very recently, the results from the Phase I First-in-Human dose escalation study of H3B-8800 was reported (Table 1).\footnote{42} According to this study, H3B-8800 treatment was associated with mostly low-grade treatment-related adverse events and induced red blood cell transfusion independence in some patients, especially in MDS patients with SF3B1 mutations and patients with high pre-treatment splicing alterations in TMEM14C (a target of SF3B1 splicing encoding a mitochondrial porphyrin transporter). Future efforts, including the exploration of other dosing schedules will be needed to understand the safety and potential therapeutic efficacy of H3B-8800 and other SF3b inhibitors.

\section*{4.2 \textbf{Sulfonamides and RBM39}}

Clinical trials of anticancer agents, sulfonamides, have shown only limited efficacy in cancer patients.\footnote{43,44} However, these trials were performed before the discovery of frequent SF mutations in cancers. Two studies have identified that RBM39, an RBP that plays a key role in regulating splicing, is the molecular target of sulfonamides including E7820, indisulam, and chloroquinoxaline.\footnote{45,46} Consonant with this, depletion of RBM39 resulted in global splicing changes such as increased exon skipping and intron retention. Sulfonamides function as molecular glue that connects RBM39 to the CUL4-DCAF15 ubiquitin complex and induces ubiquitination-mediated proteasomal degradation of RBM39 protein (Figure 4B). Following these observations, our group confirmed that leukemia cells with SF mutations are preferentially sensitive to pharmacological inhibition of RBM39.\footnote{47} These results may motivate further clinical trials to address whether anticancer sulfonamides preferentially kill cancer cells with SF mutations.

\section*{4.3 \textbf{Spliceosome inhibition in MYC oncogene-dependent cancers}}

Some reports have demonstrated that cancer cells with activated MYC are also preferentially sensitive to pharmacological spliceosome inhibition. For instance, multiple splicing regulatory proteins, such as hnRNP A1 and hnRNP A2, are transcriptionally regulated by MYC and promote tumorigenesis.\footnote{48} In addition, an analysis of the MYC transcriptional targets in lymphomas revealed that MYC directly upregulates some snRNP genes and snRNP assembly genes including PRMT5 (an arginine methyltransferase that methylates the Sm proteins of U2 snRNP).\footnote{49} Depletion of Prmt5 in MYC-driven lymphoma cells led to global splicing changes and abrogated lymphomagenesis. Given the recent development of pharmacological inhibitors of PRMT5,\footnote{50} these data seem encouraging because activated MYC may create a therapeutic vulnerability to pharmacological inhibition of spliceosome and PRMT5 inhibitors through its effects on global splicing and the dependency on PRMT5.
Related to the above, our comprehensive splicing and transcriptomic analysis across cancers identified that SF3B1<sup>K700E</sup> mutations induce a robust splicing change in PPP2R5A in CLL, which encodes one of the B-subunits of the PP2A serine/threonine phosphatase complex. SF3B1 mutations promote decay of the PPP2R5A transcripts and increase MYC phosphorylation at serine 62, which in turn enhances MYC protein stability. Considering that activated MYC may sensitize cells to pharmacological inhibition of spliceosome, combined treatment with a spliceosome inhibitor with a PP2A activating agent would be a promising therapeutic option for CLL patients bearing SF3B1<sup>K700E</sup> mutations.

### 4.4 | Modulating splicing regulatory proteins

SR-rich proteins belong to a family of RBPs that regulate splicing by recognizing splicing enhancer motifs. Post-translational modifications of SR proteins and other SFs regulate the formation of spliceosome and splicing catalysis (Figure 4C). For example, phosphorylation of SR proteins controls the nuclear shuttling of SR proteins. Based on these observations, various kinase inhibitors and methyltransferase inhibitors that block CDC-like kinases (CLKs), SR protein kinases (SRPKs), and PRMTs have been evaluated as splicing modulators. Among such newly developed agents is an orally available CLK inhibitor called CTX-712, which inhibits phosphorylation of multiple SR proteins including SRSF3, SRSF4, SRRSF5, and SRSF6. In a preclinical study using 5 AML PDX models, leukemia cells were completely abrogated in 2 cases, including 1 SRSF2 mutant model. Another class of compound to modulate splicing is the PRMT inhibitors. As mentioned above, genetic ablation or chemical inhibition of PRMT5 (which catalyzes symmetric arginine demethylation) leads to splicing inhibition and anticancer effects across cancer types. Considering these observations, it would be important to determine whether inhibition of type I PRMT enzymes (which catalyze asymmetric arginine dimethylation) causes splicing alterations similar to PRMT5 inhibition. Of note, some of the PRMT5 inhibitors (such as GSK3326595, PF06939999 and JNJ-64619178) and at least 1 type I PRMT inhibitor (GSK3368715) are in clinical trials for patients with relapsed/refractory malignancies (Table 1). Interestingly, a recent study has shown that the antitumor effects of the type I inhibitor synergizes with PRMT5 inhibition, which is consistent with the notion that each has distinct substrates within the spliceosome. These studies will provide an opportunity to assess the safety and efficacy of PRMT inhibition in vivo.

#### 4.5 | Oligonucleotide-based approach

Another approach to therapeutically target aberrant splicing in cancers is to design and use specific ASOs (or antisense SSOs) that bind complementarily to RNA through base pairing (Figure 4D). This class of therapies aims to target the RNA for degradation or to be used to affect splicing via hybridization to RNA, and has been recently approved in the United States for the treatment of neurodegenerative diseases such as spinal muscular atrophy and Duchenne muscular dystrophy. Although these approaches have not yet been applied successfully to cancers, there have been some promising experimental results for modulating STAT3 or Bcl-x splicing in which switching in the usage of a specific exon by ASO treatment resulted in different or opposite functions from the original transcripts. For example, an ASO targeting the splicing enhancer of STAT3 successfully modified alternative splicing of STAT3 to enforce exon 23 skipping, resulting in the expression of a proapoptotic isoform Bcl-<sup>x</sup>S that consequently increasing cell death and tumor regression. Similarly, an ASO targeting the alternative splice site of Bcl-<sup>x</sup> caused exon 6 skipping, resulting in the expression of proapoptotic isoform Bcl-<sup>x</sup>S that suppressed tumor load in preclinical models in vivo. Furthermore, it has been revealed that diverse SF3B1 mutations converged on reexpression of BRD9, which is a core component of the non-canonical BAF chromatin-remodeling complex, and that treatment with ASO targeting cryptic exon inclusion of BRD9 increased the level of BRD9 protein and suppressed tumor growth.

Technologically, synthetic oligonucleotides composed of subunits with a morpholine ring (termed morpholino) were developed to improve the stability of ASOs. The morpholino lacks the negatively

| Target | Molecular target | Agent | Clinical trial |
|--------|-----------------|-------|----------------|
| SF3B1 inhibitor | SF3b | H3B-8800 | NCT02841540 |
| Sulfonamide-related agents | RBM39 | E7820, Indisulam/E7070, chloroquinolaxine | |
| PRMT inhibitor | PRMT5/MEP50 | GSK3326596 | NCT03614728, NCT02783300 |
| PRMT5 | PF06939999, JNJ-64619178 | NCT03573310 |
| Type I PRMT | GSK3368715 | NCT03666988 |
| Kinase inhibitor | CLK | CTX-712 | JapicCTI-184188, NCT03355066 |
| SRPK | SRPIN340 | | |
charged backbone of traditional ASOs that may nonspecifically bind to other components of the cell and therefore may reduce the toxicity of ASOs. This technology is also suitable for targeting splicing because it is not recognized by RNase H and, therefore, does not cause direct degradation of the targeted pre-mRNA.

Despite these technological advances, the utility of ASOs in the treatment of cancers remains challenging due to the lack of sufficient understanding of which mis-splicing events are key to initiating and/or maintaining specific types of cancer, and to the lack of technological progress to efficiently deliver ASOs to the cancer cells in vivo.

5 | CONCLUSION

Although our understanding of the genomics, molecular biology, and therapeutic implications of altered RNA splicing in cancer has been greatly improved since the frequent SF mutations in cancers were identified in 2011, standard treatment strategies targeting cancers bearing splicing alterations have not yet been established. In addition, the full contribution of aberrant RNA splicing to cancer pathogenesis has not been elucidated. The major direction of the field so far is the use of splicing modulators aimed at synthetic lethality in cancers with SF mutations. The tactics to inhibit regulatory proteins, such as CLKs, SRPKs and PRMTs, and RBPs including RBM39, are also being explored as therapeutic avenues. Although emerging technologies and the rapid development of treatment strategies using ASOs still have many challenges to be resolved before clinical use, these therapeutic strategies will expand the treatment options for cancers with aberrant splicing. Interestingly, the recent pan-cancer TCGA splicing analysis suggested that cancer-specific changes to RNA splicing may be an additional source of neoepitopes. In fact, an elegant study has shown that the pharmacologic perturbation of splicing suppresses tumor growth in vivo, depending on host T cells and tumoral MHC I-presented peptides. The authors provided evidence that pharmacological modulation of global splicing enhances the effects of immune checkpoint blockade, providing a way to improve response to checkpoint blockade. We are just beginning to understand how neoepitopes generated by dysregulated alternative splicing through SF mutations and pharmacological perturbation overcomes immune tolerance to elicit an anticancer response. In summary, there still remains many unsolved problems in terms of the pathogenesis of spliceosome-mutant cancers and the development of therapeutic avenues for cancers with aberrant splicing. In parallel, the ongoing efforts to modulate splicing will hopefully address the molecular and clinical questions of whether pharmacological intervention in global splicing is efficacious and safe in patients with cancers.

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DISCLOSURE

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