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Molecular Pathways: Understanding and Targeting Mutant Spliceosomal Proteins

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Abstract

Splicing of precursor messenger RNA is a critical step in regulating gene expression and major advances are being made in understanding the composition and structure of the enzymatic complex which performs splicing, termed the spliceosome. In parallel, there has been increased appreciation for diverse mechanisms by which alterations in splicing contribute to cancer pathogenesis. Key among these includes change-of-function mutations in genes encoding spliceosomal proteins. Such mutations are amongst the most common genetic alterations in myeloid and lymphoid leukemias, making efforts to therapeutically target cells bearing these mutations critical. To this end, recent studies have clarified that pharmacologic modulation of splicing may be preferentially lethal for cells bearing spliceosomal mutations and also may have role in the therapy of MYC-driven cancers. This has culminated in the initiation of a clinical trial of a novel oral spliceosome modulatory compound targeting the SF3B complex and several novel alternative approaches to target splicing are in development as reviewed here. There is therefore now a great need to understand the mechanistic basis of altered spliceosomal function in cancers and to study the effects of spliceosomal modulatory compounds in pre-clinical settings and in well-designed clinical trials.

Background

Basic Mechanisms of pre-mRNA splicing

Aberrant regulation of gene expression is a well-known hallmark of cancer cells. As such, mRNA splicing, the process of removing introns from precursor messenger RNA (pre-mRNA) represents a critical step in the post-transcriptional regulation of gene expression. More than 95% of human genes are capable of generating multiple RNA species through

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Potential Conflicts of Interest

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alternative splicing and this process enables cells to generate a diversity of functionally distinct proteins from a single gene. mRNA splicing is carried out inside the nucleus by an enzymatic complex known as the spliceosome. The spliceosome is a metalloribozyme that consists of 5 small nuclear ribonucleoproteins (snRNPs; U1, U2, U4, U5, and U6 snRNPs), each of which contains its own small nuclear RNA (snRNA) complexed to a group of proteins, and more than 200 related proteins. Recent utilization of cryo-electron microscopy has enabled an unprecedented high-resolution view of each step in splicing (1–5). Although splicing is a complex multistep process (reviewed in refs (6–10) in detail), the crux of splicing catalysis consists of 2 sequential transesterification reactions (Figure 1A). Base-pairing of snRNAs to conserved sequences on pre-mRNA as well as interactions of numerous splicing accessory proteins and RNA-protein interactions are essential in guiding the massive spliceosomal complex to regions of pre-mRNA for splicing of the correct segments of RNA (Figures 1B-D). Here we present a simplified summary of the spliceosome assembly pathway and the factors required for exon definition (Figure 1B).

An intron is defined by four consensus elements: (i) the 5’ splice site (5' SS; located at the 5’ end of the intron), (ii) the 3’ SS (located at the 3’ end of the intron), (iii) the branch point sequence (BPS) (located upstream of the 3’ SS), (iv) the polypyrimidine tract (located between the BPS and the 3’ SS) (Figure 1C). These sequences are critical in allowing the spliceosome to recognize nucleotide sequences as introns and to distinguish introns from exonic sequence. For the majority of introns, the 5' SS is characterized by a GU dinucleotide while the 3' SS contains an AG dinucleotide. These 2 sequences are not sufficient by themselves to define an intron in most cases and a variable stretch of pyrimidine nucleotides, called the polypyrimidine tract, further helps define the 3' SS. The polypyrimidine tract is situated between the 3' SS and the BPS, and also serves to recruit splicing factors to the 3' SS and BPS. The BPS, so-called as it consists of a nucleotide which initiates a nucleophilic attack on the 5′ SS to create a "branch" like structure, contains a conserved Adenosine nucleotide required for the first step of splicing (Figure 1A).

The early steps of spliceosome assembly are then achieved by binding of the 5’ SS and BPS by U1 and U2 snRNPs, respectively, through base-pairing interactions. U2 snRNP consists of SF3A, SF3B, and a 12S RNA subunit in which SF3B1 is involved in the binding to the BPS. The likelihood of splicing at a particular site is influenced by additional proteins outside of the core spliceosome. For example, members of the serine/arginine (SR) family proteins generally promote splicing by recognizing specific sequences in pre-mRNA named exonic and intronic splicing enhancers (ESE and ISE) (Figure 1D). SR proteins generally act as enhancers of splicing from nearby splice sites by interacting with these sequences and recruiting the U1 snRNP and U2AF to 5’ and 3’ SS, respectively. In contrast, heterozygous nuclear ribonucleoprotein particle proteins (hnRNPs) generally suppress splicing by interacting with exonic and intronic splicing silencers (ESS and ISS).

Altered mRNA splicing in cancer

Growing evidence has revealed that mis-splicing of pre-mRNA can promote cancer initiation, maintenance, and/or progression. Genetic alterations in cancer that contribute to mis-splicing fall into 2 categories: (i) mutations falling within the mRNA sequence that is

being spliced and thereby influencing splicing (so-called "*cis*-acting" mutations) and (ii) alterations in the level of expression or mutations in splicing factors which promote splicing of pre-mRNA (so-called "trans-acting" splicing factors).

Cis-acting mutations include those affecting the 5' SS, 3' SS, BPS, or splicing enhancer or silencer elements. Mutations with pathologic effects on splicing may therefore occur within introns or exons and include synonymous as well as non-synonymous mutations. Such mutations represent common mechanisms of inactivation of tumor suppressor genes (11). For example, recurrent synonymous mutations within *TP53* occur adjacent to splice sites resulting in intron retention or activation of a cryptic splice site to produce a frameshifted mRNA subjected to nonsense mediated decay (12). Similarly, recurrent somatic mutations in *APC* resulting in exon skipping (13) or creation of a new splice site (14) in colon and lung cancer, respectively.

In 2011, recurrent somatic mutations affecting *trans*-acting spliceosome components were reported in hematopoietic malignancies (15, 16) and are currently among the most common class of mutations in patients with myelodysplastic syndromes (MDS) (15) and chronic lymphocytic leukemia (CLL) (17). These mutations occur predominantly in *SF3B1* and *U2AF1* (core spliceosomal components important in 3' SS recognition), *SRSF2* (an SR protein), and *ZRSR2* (which serves a function in the minor (U12-dependent) spliceosome in a role analogous to U2AF1) (recently reviewed (18, 19)). Mutations in these splicing factors have also been identified in solid tumors and include *SF3B1* mutations in uveal melanoma (15–19%) (20–22), pancreatic ductal adenocarcinoma (4%) (23), and breast cancer (2–4%) (24, 25), as well as *U2AF1* mutations in lung adenocarcinoma (3%) (26).

Mutations in *SF3B1, U2AF1*, or *SRSF2* alter mRNA splicing preferences in a manner distinct from loss-of-function (27–31). Consistent with this change-of-function effect, mutations in *SF3B1, U2AF1*, and *SRSF2* are invariably found as heterozygous point mutation at restricted amino acids and occur in a mutually exclusive manner with one another. We and others recently identified that cells bearing spliceosomal mutations depend on wildtype splicing function for survival (32–34), which appears to create a therapeutic window between spliceosomal-mutant cancer cells and normal cells for pharmacologic modulation of splicing.

In addition to mutations in splicing factors, mis-regulated expression of regulatory factors in the splicing machinery can also impact alternative splicing and promote cancer development. For example, SRSF1 is known to be upregulated in multiple cancers and transform cells by modulating alternative splicing of target genes, such as *Ron* (35) and *S6K1* (36). Genetic alteration and/or mis-regulated expression of *RBM5, RBM6,* and *RBM10* are also frequently observed and involved in the pathogenesis of cancers of the lung and other tissues (26, 37–39). These observations connect mis-regulation of RNA splicing to cancer pathogenesis.

Clinical-Translational Advances

As mentioned above, recent studies have suggested that spliceosomal-mutant malignancies are preferentially sensitive to pharmacologic or genetic modulation of splicing compared to spliceosomal-wildtype cancers or normal cells. To this end, natural products from several bacteria species and their analogs have been discovered that bind SF3B1 (and possibly other components of U2 snRNP) and block early spliceosome assembly. These compounds, which include E7107 (an analog of pladienolide B) (40), spliceostatin A (41), and the sudemycins (42), are thought to inhibit the exposure and binding of the branch point binding region of U2 snRNP to the BPS, thereby blocking the essential conformational change in U2 snRNP required for the transition from complex A to complex E (34, 43–45) (the activities and properties of these compounds have been reviewed recently in detail (46)). Although the downstream changes in the transcriptome and protein expression caused by these drugs are still largely unknown, results from preclinical evaluation of these compounds in genetic subsets of cancer are promising.

We recently demonstrated that *in vivo* treatment with E7107 increases retention of both constitutive and alternative introns as well as cassette exon skipping, consistent with E7107 inhibiting splicing catalysis. However, the magnitude of splicing inhibition following E7107 treatment was more severe in myeloid leukemias with *Srsf2*-mutant versus wildtype leukemias, resulting in decreased disease burden in both isogenic murine leukemia models and AML patient-derived xenograft (PDX) models with or without *SRSF2* mutations (34). Similar preferential sensitivity was seen in *Sf3b1*K700E mutant hematopoietic cells after *in vivo* treatment with E7107 (47). An orally bioavailable analog of E7107, H3B-8800, has shown promising preclinical results in isogenic *SRSF2* and *SF3B1*-mutant leukemias (48). These data have resulted in initiation of a phase I dose-escalation study of H3B-8800 for patients with spliceosomal-mutant MDS, AML, and CMML (clinicaltrials.gov identifier NCT02841540).

Given the frequency and adverse prognosis of *SF3B1* mutations in CLL (49), several studies have examined the zpotential efficacy of spliceosome inhibition in CLL. *In vitro* exposure of primary CLL cells to FD-895 (50), pladienolide B (50), or spliceostatin A (51) results in increased apoptosis of CLL cells compared with normal B-cells, regardless of *SF3B1* mutational status. However attempts to study the efficacy of these compounds *in vivo* in the context of CLL have largely been limited by the lack of stable and robust PDX models of CLL (52) as well as genetically engineered CLL models with *Sf3b1* mutations. One issue to consider in therapeutic targeting of spliceosomal-mutant CLL is that, distinct from myeloid malignancies where *SF3B1* mutations are usually in the predominant clone, *SF3B1* mutations in CLL are frequently subclonal (49, 53). Therefore, estimation of *SF3B1* mutant allele frequencies may be needed to assess the impact of targeting the spliceosome in CLL.

To date there have been no studies testing the efficacy of SF3B1 binding agents based on the presence of spliceosomal gene mutations in epithelial cancers. However, several studies using unbiased approaches have revealed that a wide-range of MYC-dependent cancers are preferentially vulnerable to spliceosomal modulation. A genome-wide MYC-synthetic lethal screen in mammary epithelial cells identified several components of the spliceosome as

preferentially required in cells with *MYC* overexpression (54). This observation motivated the authors to hypothesize that oncogenic MYC depends on normal spliceosomal functions for cell survival. Similarly, a genome-wide siRNA screen in patient-derived glioblastoma multiforme stem cells (GSCs) (55) identified *PHF5A* as differentially required for survival of GSCs over normal neuronal stem cells. PHF5A is known to form a bridge between the U2 snRNP and ATP-dependent RNA helicases and be involved in RNA splicing. In fact, knockdown of PHF5A resulted in GSC-specific intron retention and exon skipping events in hundreds of genes as well as preferential cell cycle arrest and loss of viability in GSCs, but not in untransformed neural stem cells. Intriguingly, these observations in GSCs were phenocopied by overexpression of *MYC* in untransformed neural stem cells. Taken together, therapeutic intervention with spliceosomal inhibition in MYC-driven cancers appears to be a promising approach to target a wide variety of solid and liquid tumors.

In addition to the use of spliceosome modulators, several recent clinical trials have highlighted potential therapeutic approaches for spliceosomal-mutant cancers by targeting biological processes not directly linked to splicing. For example, a pilot study of the telomerase inhibitor imetelstat for patients with the myeloproliferative neoplasm myelofibrosis demonstrated preferential effects of imetelstat in patients with *SF3B1* or *U2AF1* mutations versus patients without these mutations (complete response rate, 38% vs 4%, p=0.04) (56). However, testing of imetelstat in forms of MDS where >80% of patients harbor *SF3B1* mutations, termed refractory anemia with ring sideroblasts (RARS) and RARS-t (a variant of RARS with thrombocytosis), revealed only modest effects in these patients. For these reasons and the need to define its therapeutic efficacy further, results from larger clinical trials of imetelstat in myeloid malignancy patients are clearly needed.

While spliceosomal gene mutations are a recently discovered feature of MDS, one of the oldest hallmarks of MDS is the presence of ineffective erythropoiesis associated with erythroid hyperplasia and apoptosis of red blood cell (RBC) precursors in the bone marrow. Recent data has identified excessive SMAD2/3 signaling as casually linked to pathologic erythropoiesis in MDS patients (57, 58). Consistent with this, lower-risk MDS patients treated with the SMAD2/3 inhibitor, luspatercept (ACE-536), achieved hematologic improvement and reduced RBC transfusion independence in a phase II, multicenter, open-label study (59, 60). Higher response rates were observed in patients with RARS MDS and *SF3B1* mutations in this study. Luspatercept is a fusion protein containing a modified extracellular domain of the human activin receptor type IIB linked to a human IgG1 Fc domain, which sequesters TGF-β superfamily ligands to suppress SMAD2/3 activation (a so-called "ligand trap") (58). It is currently unclear if the improvements in anemia in low-risk MDS patients are due to an unrecognized link between *SF3B1* mutations and TGF-β signaling or if the effects of luspatercept on erythropoiesis are unrelated to *SF3B1* mutations. Several clinical trials of luspatercept are currently ongoing for MDS patients now (clinicaltrial.gov identifiers NCT02631070, NCT02268383, NCT01749514) and may clarify this association.

Open questions

Systematic analyses of mutations in cancer have shown that >50% of human tumors possess one or more mutational hotspots (61). These data underscore the importance of developing therapeutic strategies to target cancer cells bearing such gain-of-function mutations. Of these hotspots, 81% arise in two or more tumor types, suggesting that many hotspot mutations confer a selective advantage across diverse lineages. *SF3B1* and *U2AF1* mutations are included among such newly defined hotspots and further efforts to define the functionally relevant downstream mis-spliced events present in spliceosomal-mutant cancers will be essential in furthering our understanding of these mutations and developing therapies targeting cells bearing these mutations. Although much has been learned about how mutations in *SRSF2* and *U2AF1* alter RNA recognition and splicing, more effort to define the allele-specific effects of different *SF3B1* mutational hotspots on splicing and gene expression will be critical. Moreover, understanding the effects of spliceosomal gene mutations in the context of mutations commonly co-occurring with them, such as commonly co-existing mutations in *SRSF2*/*IDH2* and *U2AF1*/*ASXL1* as well as enrichment of *SF3B1* mutations in patients with inv(3) MDS/AML and del(13q) CLL may reveal novel contributions of splicing mutations to cancer (62–64).

Given the preferential sensitivity of spliceosomal-mutant cells to SF3B1 binding agents, further effort to decipher the mechanistic effects of these compounds on gene expression and splicing are now needed. In addition, ongoing efforts may soon determine the potential efficacy of candidate compounds with effects on splicing beyond SF3B1 binding agents. Increasing evidence supports a role for protein arginine methyltansferase (PRMT) family proteins as splicing regulators. PRMT5 has been shown to play an essential role in regulating splicing (65) as deletion of *Prmt5* in several cell types results in reduced methylation of Sm proteins, suboptimal maturation of snRNP complexes, as well as aberrant constitutive and alternative splicing of mRNAs (66). Importantly, PRMT5 suppression in MYC-driven lymphomas results in exon skipping and intron retention coincident with loss of tumor maintenance (67). These findings strongly suggest that targeting PRMTs may have importance for spliceosomal-mutant malignancies as well as MYC-driven tumors. In addition, inhibition of phosphorylation of SR proteins may represent another method to perturb splicing pharmacologically. SR proteins have conserved arginine- and serine-rich domains, which are subject to phosphorylation by multiple kinases, including the SR protein kinases and the CDC2-like kinases. Although the role of phosphorylation of these domains remains to be clarified, modulation of SR protein phosphorylation clearly impacts splicing (68, 69). Characterization of the effects of these new classes of compounds on splicing and potential effects on spliceosomal-mutant malignancies may represent novel therapeutic approaches for conquering malignancies with aberrant spliceosomal catalysis.

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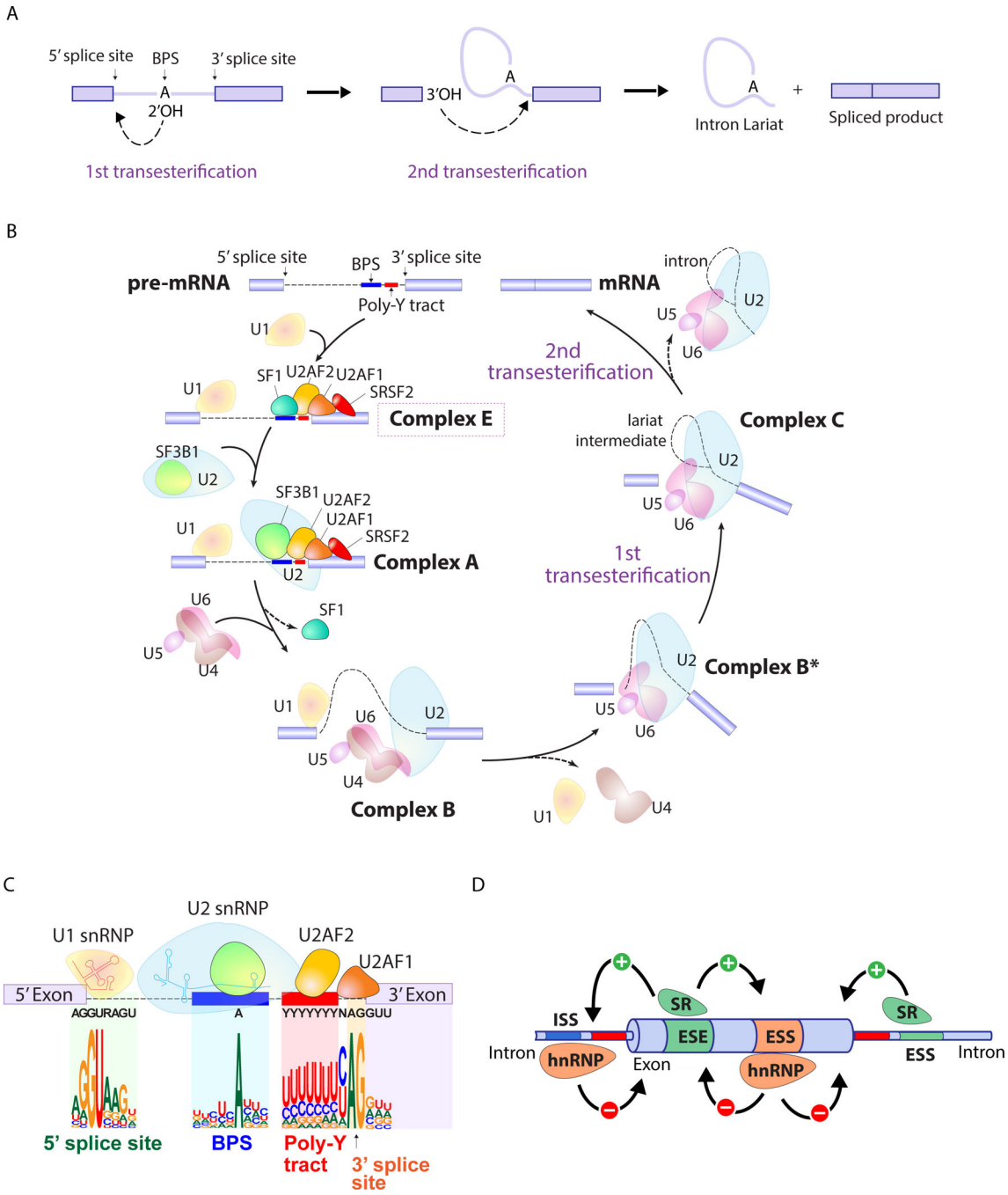

Figure 1. Splicing catalysis, the spliceosome assembly pathway, and mechanisms of splice site selection

(A) Diagram of the 2 sequential transesterification reactions that represent the crucial catalytic steps in intron removal during splicing. An adenine nucleotide (termed the "invariant adenine") of the branch point sequence (BPS) initiates the first transesterification and generates a free 5' exon and an intron-3' exon lariat. The 3' end hydroxyl of the free 5' exon then attacks the intron-3' exon junction, completing the splice and releasing a lariat RNA intron. **(B)** Pre-mRNA splicing is a dynamic process that involves several distinct

spliceosomal complexes. The earliest complex (complex E) is established by binding of (i) U1 snRNP to the the 5' splice site (SS), (ii) splicing factor 1 (SF1) to the BPS, (iii) U2AF2 (also known as U2AF65) to the polypyrimidine tract, (iv) U2AF1 (also known as U2AF35) to the 3' SS. Formation of complex E in turn enhances the recruitment of U2 snRNP to the BPS and leads to the formation of complex A. SF3B1, a component of U2 snRNP, is involved in the binding to the BPS. The pre-assembled U4/U6.U5 tri-snRNP complex joins and the U1/U4 snRNPs are released to form the catalytically active complex B (complex B*), followed by the further conformational rearrangements that results in the formation of complex C. Complexes B and C catalyze the first and second esterification reactions, respectively, and mediate excision of the intron and ligation of the proximal and distal exon to synthesize mature mRNA. **(C)** A focus on complex E highlights consensus sequence elements recognized by U1 and U2 snRNPs as well as the U2AF complex. An intron is defined via (i) the 5' SS, (ii) the 3' SS, (iii) the branch point sequence (BPS), and (iv) the polypyrimidine (Poly-Y) tract. The definition of an intron depends on recognition of the 5' SS and BPS by U1 and U2 snRNPs, respectively. The consensus sequences shown are those recognized by the major (U2-dependent spliceosome) which processes >95% of introns (as opposed to the minor U12-dependent spliceosome which recognizes different consensus sequences than those shown here). **(D)** In addition to sequences in mRNA recognized by the core spliceosome and the U2AF complex, accessory splicing regulatory proteins are essential in promoting or repressing splice site usage. Members of the serine/arginine (SR) family proteins control the pattern of alternative splicing by recognizing specific sequences in pre-mRNA named exonic and intronic splicing enhancers (ESE and ISE). SR proteins generally act as enhancers of splicing from nearby splice sites by interacting ESE and ISE, while heterozygous nuclear ribonucleoprotein particle (hnRNP) suppresses splicing by interacting with exonic and intronic splicing silencers (ESS and ISS).