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Steering research on mRNA splicing in cancer towards clinical translation

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Abstract

Splicing factors are affected by recurrent somatic mutations and copy number variations in several types of haematologic and solid malignancies, which is often seen as prima facie evidence that splicing aberrations can drive cancer initiation and progression. However, numerous spliceosome components also 'moonlight' in DNA repair and other cellular processes, making their precise role in cancer difficult to pinpoint. Still, few would deny that dysregulated mRNA splicing is a pervasive feature of most cancers. Correctly interpreting these molecular fingerprints can reveal novel tumour vulnerabilities and untapped therapeutic opportunities. Yet multiple technological challenges, lingering misconceptions, and outstanding questions hinder clinical translation. To start with, the general landscape of splicing aberrations in cancer is not well defined, due to limitations of short-read RNA sequencing not adept at resolving complete mRNA isoforms, as well as the shallow read depth inherent in long-read RNA-sequencing, especially at single-cell level. Although individual cancer-associated isoforms are known to contribute to cancer progression, widespread splicing alterations could be an equally important and, perhaps, more readily actionable feature of human cancers. This is to say that in addition to 'repairing' mis-spliced transcripts, possible therapeutic avenues include exacerbating splicing aberration with small-molecule spliceosome inhibitors, targeting recurrent splicing aberrations with synthetic lethal approaches, and training the immune system to recognize splicing-derived neoantigens..

Introduction

In 1977, two groups led by Richard Roberts and Phillip Sharp simultaneously observed the presence of "amazing [loop-like] sequence arrangements" in adenoviral DNA–RNA heteroduplexes, suggesting that the mature messenger RNA (mRNA) molecule is produced by intramolecular joining of short segments of "split genes"[1,2]. In doing so, they discovered mRNA splicing, a key step in transcript processing, wherein non-contiguous exons are transcribed to form a long pre-mRNA precursor followed by the removal of intervening intronic sequences. Shortly thereafter, introns were identified in many eukaryotic organisms, with the number of spliced genes increasing on average with organismal complexity.

Of note, exons are not always spliced together precisely or sequentially: common alterations include alternative 5' and 3' exon boundaries, exon skipping, and intron retention. In humans, it is estimated that over 95% of multi-exon genes undergo alternative splicing[3,4], a subset of which can encode proteins with distinct, and sometimes even opposing functions[5]. In fact, alternative splicing plays a critical role in many cellular processes. They include cell differentiation, development, and proliferation, intracellular signaling and metabolism, and intrinsic and extrinsic apoptosis[6–11]. It is therefore not surprising that alterations in RNA splicing have been linked to human diseases, including cancer.

Over the past twenty years, the discovery of highly recurrent mutations in genes encoding splicing factors across a variety of tumor types has provided compelling genetic evidence for a direct causal relationship between splicing dysfunction and cancer[12]. Even in tumors without splicing factor mutations, mRNA splicing patterns are patently aberrant, resulting in the profound re-shaping of cancer transcriptomes and proteomes[13,14]. This allows aberrant mRNA splicing to enable virtually all recognized 'Hallmarks of Cancer'[15], impacting not only tumor initiation, but also progression, metastatic spread, drug responses, etc[12]. These far-reaching effects informed the development of first-in-class approaches to target cancer-promoting spliced isoforms or regulators thereof, raising early hopes of translating mRNA splicing research to the clinic[12].

10+ years later, our still incomplete understanding of how dysregulated splicing contributes to cancer pathogenesis remains the chief obstacle to achieving this goal. At first glance, the havoc that mutant splicing factors wreak upon pre-cancerous cells appears indiscriminate, with thousands of exons per tumor assembled out of order and thousands of introns retained in otherwise mature transcripts. There might be a method to this madness, *i.e.*, a small number of aberrantly spliced mRNAs that are key to neoplastic transformation. However, currently there is no clear consensus on how these driver events might be identified and whether they could and should be reversed, either genetically or pharmacologically. Building such a consensus would require insights from experts in computational biology, RNA biology, structural/molecular biology, medicinal chemistry, immunology, and hematology/oncology.

Most recent effort in that direction was expended at the Forbeck Forum on Therapeutic Targeting of mRNA Splicing in Cancer held September 21–24, 2023, at the Asilomar Conference Grounds, the site of the 1975 scientific summit that had yielded the influential "Summary Statement of the Asilomar Conference on Recombinant DNA Molecules"[16]. Not having to worry any longer about hefty recombinant *DNA* biosafety issues allowed the 2023 Forum participants to focus on "recombinant" (*i.e.*, mis-spliced) *RNA* molecules and their role in cancer. The resultant Roadmap article is not meant to serve as a comprehensive catalogue of all recent developments in this dynamic field, which have been summarized previously[12]. Rather, our goal was to highlight basic science advancements, ranging from long-read RNA-sequencing to single-cell proteomics, that could facilitate clinical use of compounds targeting - or taking advantage of - aberrant splicing patterns in cancer.

Dysfunction of splicing regulators in cancer

RNA splicing is a highly regulated process performed by the spliceosome - a very large complex consisting of both small nuclear RNA (snRNA) **[G]** and protein components - along with additional regulatory splicing factor proteins that fine-tune its activity. The spliceosome recognizes core regulatory sequences in the pre-mRNA, which include the 5' and 3' splice sites (5' and 3' SS) that mark intron-exon boundaries and flank the branch point site **[G]** (BPS) and the polypyrimidine tract **[G]** (Py-tract) (Figure 1a). Splicing reactions are carried out by either of the two spliceosomal complexes, major U2-type and minor U12-type, which differ in a subset of snRNA components and in the splice site sequences they recognize. The major U2-type spliceosome preferentially recognizes

GT-AG splice sites and is responsible for the removal of ~99% of introns. It contains over 300 components – including snRNAs that interact with Sm proteins **[G]** and additional proteins to form small nuclear ribonucleoprotein **[G]** (snRNP) particles. The minor U12-type spliceosome recognizes both GT-AG and AT-AC sites and is involved in the removal of less than 1% of introns. It utilizes distinct spliceosomal snRNA and protein components, including ZRSR2. Remarkably, as detailed below, both RNA and core protein components of the spliceosome are altered in human tumors (Figure 1b).

Pleiotropic mutations in core spliceosomal proteins

Landmark studies performed concurrently and published almost simultaneously in 2011 described recurrent driver mutations in human genes encoding core components of the spliceosome **[G]**, namely splicing factor 3b subunit 1 (SF3B1), serine and arginine-rich splicing factor 2 (SRSF2, previously known as SC35), zinc finger CCCH-type RNA binding motif and serine/arginine rich 2 (ZRSR2), and U2 small nuclear RNA auxiliary factor 1 (U2AF1)[17–20]. These mutations, found in both hematologic malignancies and solid tumors, for the first time implicated splicing factors as *bona fide* cancer drivers[21] (Figure 1a,b). All four proteins play key roles during the splicing reaction and are critical for 3'SS recognition: SF3B1 is a component of the U2 snRNP that is involved in BPS recognition and assembly of the pre-spliceosomal complex A, SRSF2 interacts with U1 and U2 snRNPs and mediates recognition of the 5' and 3'SS, U2AF1 binds to the AG dinucleotide at the 3'SS and is critical for U2 snRNP binding, and ZRSR2 has been implicated in 3'SS recognition for both U2-and U12-type introns. Follow-up molecular studies have revealed that hotspot mutations at specific amino acid residues in *SF3B1*, *SRSF2*, and *U2AF1* either lead to a gain of function or alter the spliceosome specificity, resulting most frequently in the selection of alternative 3' splice sites[22–26]. In contrast, mutations in *ZRSR2*, which are distributed across the whole gene, lead to a loss of function and improper retention of U12-type introns[27,28].

Up until recently, it was assumed that mutations in these genes impact tumor formation and progression mostly by affecting RNA splicing. However, it is now well-understood that mutations in spliceosome components can have additional tumorigenic properties, since their targets are involved in numerous cellular processes beyond splicing. Since splicing occurs on nascent pre-mRNA molecules, it is intimately linked to transcript elongation, with both processes affecting each other[29]. One important aspect of this crosstalk is that splicing factors U2AF1 and SRSF2 regulate both transcription elongation and nucleocytoplasmic export[30–32]. SRSF2 additionally has been implicated as a reader of the 5-methylcytosine RNA modification, which in turn affects RNA stability and nuclear export[33]. Of note, imbalanced levels of nuclear mRNAs (spliced or un-spliced) result in the formation of R-loops **[G]**, triple-stranded structures consisting of a DNA:RNA hybrid and a displaced strand of DNA[34]. These structures need to be resolved as they are causally connected to double-stranded DNA damage, chromosomal instability, and aneuploidy[35]. Independently, SRSF2 is involved in the formation of the exon junction complex (EJC)[36] **[G]**, a key step required for nonsense-mediated mRNA decay (NMD) **[G]**, a co-translational process that eliminates transcripts containing premature stop codons[37]. Therefore, cells with mutations in core splicing factors might be expected to have multiple potentially tumorigenic features,

namely defects in transcription elongation, R-loop resolution, DNA damage repair, NMD, and translational regulation.

This indeed turned out to be the case. For example, a recent study found that the $SF3B1^{K700E}$ mutation reduces the elongation rate of RNA polymerase II along gene bodies and its density at promoters in leukemia cell models[38]. This elongation defect results from disrupted pre-spliceosome assembly due to impaired protein-protein interactions of mutant SF3B1. This $SF3B1^{K700E}$-mediated transcriptional elongation defects also reduces chromatin accessibility and H3K4me3 marks at promoters[38], suggesting that modulation of histone tail modifications could counteract the effects of mutant $SF3B1^{K700E}$. Furthermore, mutations in splicing factors *SF3B1*, *SRSF2* and *U2AF1* were all found to augment the formation of R-loops, most likely due to prolonged transcriptional pausing and the availability of free RNA ends generated during splicing-related RNA cleavage events[39–43]. Importantly, suppression of R-loops has been shown to partially correct the proliferation defect of hematopoietic progenitor cells with mutations in splicing factors, suggesting that these mutations, at least in myelodysplastic neoplasms, lead to chronic insult to the genome and ensuing cell cycle arrest. In turn, there is evidence that DNA damage-induced splicing aberrations preferentially affect hundreds of genes involved in DNA repair, cell cycle progression, and programmed cell death[44].

Cancer cells with mutations in key splicing factors also exhibit significant perturbations in NMD. *SRSF2*-mutants cancer cell lines were found to exhibit enhanced mRNA decay[45]. Mechanistically, binding of mutant SRSF2 to RNA facilitates the deposition of the EJC downstream from premature stop codons to a greater extent than that of wild-type SRSF2. This in turn boosts the association of key NMD factors with the transcript and augments mRNA decay[45]. Similarly, cells with mutations in *SF3B1* or *U2AF1* not only exhibited elevated DNA replication stalling, DNA damage, and chromosomal instability but were more sensitive to NMD inhibition compared to their wild-type counterparts[46]. Finally, U2AF1 bearing the S34F mutation was shown to directly affect translation of hundreds of transcripts via direct mRNA binding in the cytoplasm[47].

Collectively, these findings highlight the complex functions of splicing regulators. Especially when mutated, they can have pleotropic effects, both indirectly (via mRNA splicing and altered proteomes) and directly, by "moonlighting" in a variety of cellular processes. This routine repurposing of RNA-binding proteins[48] make seemingly distinct steps in gene expression, from transcriptional regulation to post-translational control, functionally interconnected[49,50].

Wide-reaching mutations in snRNAs

Beyond its protein constituents, critical components of the spliceosome are the snRNAs, whose deregulation can profoundly affect splicing patterns. U1 and U2 snRNAs are primarily responsible for the recognition of 5′ splice sites and branch points, respectively, which are critical steps for defining the beginning and the end of introns that will be excised by the major spliceosome (Figure 1a). As snRNAs are short in length and lack poly(A) tails **[G]**, they are not detected by many routinely used RNA-sequencing protocols. However, more targeted approaches designed to capture short noncoding RNAs have revealed that

snRNA genes exhibit variable expression levels in human cell lines and adult and fetal tissues[51]. This suggested that by impacting the efficiency of snRNPs assembly, they play important roles in controlling tissue- and cell-type–specific splicing patterns. Comparable variations in snRNA levels have been detected also across tumors. For example, U6atac snRNA, which regulates the activity of the minor U12 spliceosome, is elevated in primary and metastatic prostate cancer samples, where its levels correlate with metastatic spread[52]. Mechanistically, U6atac has been shown to control a cluster of genes involved in cell proliferation[53], potentially explaining its association with disease progression. And just like core splicing factors, snRNAs influence many steps of RNA processing, not just splicing. For example, U1 precludes selection of proximal polyadenylation signals in introns and last exons (an activity dubbed telescripting[54,55]), and in doing so, promotes cancer cell migration and invasion *in vitro*[56].

Thus, it comes as no surprise that recurrent mutations in U1 and U2 genes have been detected across several liquid and solid tumor types, namely in blood, liver, prostate, and pancreatic cancers, as well as in pediatric brain tumors[57–59]. While the impact of common U2 mutations (e.g., U2 c.28) hasn't been extensively studied, U1 mutations typically change the preferred A-U base-pairing between this snRNA and the 5'SS to the C-G base-pairing, redirecting splicing towards cryptic 5'SS events. In addition, mutations in U11 snRNA, which is responsible for 5′SS recognition of minor introns, have been reported in the Sonic Hedgehog subtype of medulloblastomas[57]. Collectively, tumors with U1 or U11 snRNA mutations exhibit widespread splicing alterations, with a bias towards cryptic 5'SS, including those mapping to known oncogenes and tumor suppressors[57,58].

Aberrant expression of regulatory splicing factors

While recurrent mutations in core spliceosome components of the splicing machinery are common in hematological malignancies (30–80% of patients), these are much less common in solid tumors (1–4% of patients), with the exception of *SF3B1* mutations in uveal melanoma[12]. Solid tumors, on the other hand, exhibit frequent changes in expression levels in regulatory splicing factors that fine-tune the activity of the spliceosome (Figure 1b), most notably RNA-binding proteins of serine/arginine-rich (SR) **[G]** and heterogeneous nuclear ribonucleoprotein (HNRNP) **[G]** families. These proteins participate in the splicing of canonical exons, but are particularly adept at regulating inclusion or skipping of non-constitutive "cassette" exons[60]. To perform these functions, they recognize and bind *cis*-acting exonic and/or intronic sequences and strengthen or weaken the spliceosome's recognition of the corresponding splice sites.

In tumors, changes in levels of regulatory splicing factors are sometimes due to focal gene amplifications or "deep" bi-allelic deletions[61–65] (which could credential them as cancer drivers), but more commonly due to more elusive "soft-wired" gene expression changes at the transcriptional, post-transcriptional, or post-translational levels[63,66–70] (Figure 1b). For example, in B- and T-cell acute lymphoblastic leukemias (B-/T-ALL), where mutations in the core spliceosome components are not observed, many members of the SR protein family are dysregulated at the post-transcriptional level whereby the inclusion of a stop codon-containing poison exon **[G]** regulates their protein levels[71,72]. Furthermore, in T-ALL

SRSF6 protein levels can be sustained via the activity of ubiquitin-specific peptidase 7 (USP7)[72]. Similarly, the levels of multiple splicing factors, including SR proteins, are upregulated in breast, lung, kidney, melanoma and others tumors, as a result of increased poison exon skipping in their respective transcripts[73,74] (Figure 1b).

Many of these splicing factor up- and down-modulation events could be traced back to *bona fide* cancer genes dysregulated in the majority of all cancers, such as MYC[75] and TP53[76]. Indeed, evidence shows that tumors with alterations in *MYC* and *TP53* have widespread splicing aberrations[63,66–68,77]. They can occur by a diverse range of direct and indirect mechanisms (Figure 1a). For example, the MYC oncoprotein is known to bind directly to the promoter regions and regulate the expression of assorted splicing factors, including members of the SR and HNRNP protein families[63,68,78–80]. Most notably, it transcriptionally upregulates both snRNP assembly genes and their upstream regulators[67] such as PRMT5, an arginine methyltransferase that methylates Sm and other RNA binding proteins. These coordinated regulatory effects are key for the biogenesis of snRNPs, effective pre-mRNA splicing, cell survival and proliferation[67]. Consequently, the MYC–PRMT5 signaling axis is critical to splicing fidelity of exons with weak 5'SS. Therefore, it is a targetable vulnerability of SF3B1 and SRSF2-mutant cancers[81]. Moreover, as MYC selectively amplifies pre-mRNA synthesis[82,83], it increases the pressure on the spliceosome to process more intron-containing transcripts[66,67]. Thus, MYC-dependent tumors are particularly sensitive to inhibition of the splicing machinery, providing opportunities for synthetic lethal approaches for these aggressive cancers. Although MYC is notable for its ability to profoundly dysregulate tumor cell transcriptomes[84], many other cancer drivers have outsized impacts on mRNA splicing. For example, the PI3K–AKT pathway, which is frequently hyperactivated in multiple cancers, regulates multiple SR proteins, both directly[85] and indirectly, through the SR protein kinases SRPKs[86]. As a result, PI3K hotspot mutations drive not only widespread transcriptomic changes but also aberrant splicing that has been shown to support tumor growth in breast cancer models[87].

More tissue-restricted oncogenes are also known to influence the landscape of their respective cancers. For instance, in pancreatic cancer, *TP53* hotspot missense mutations giving rise to the oncogenic form of p53 are associated with aberrant splicing, when compared to truncating, tumor-suppressive *TP53* mutations[77]. Specifically, *TP53* mutant cells upregulate the expression of splicing factor HNRNPK, which preferentially binds to C-rich exonic sequences. As a result, cytosine-rich exons corresponding to KRAS-suppressing GTPase-activating proteins are more frequently included in mutant tumors, leading to heightened KRAS activity and more robust tumor growth[77]. Similarly, adult gliomas driven by mutant isocitrate dehydrogenase (IDH) dysregulate splicing factors PTBP1, SRSF3, and RBFOX1 compared to their IDH wild-type counterparts, with ensuing changes to splicing patterns[88,89].

The effects of splicing factor up- and down-modulation events on tumor phenotypes vary significantly, depending on histology and unique properties of individual family members. In breast tumors, upregulation of specific SR protein family members promotes tumor formation, while others impact cell migration and metastasis[63,79]. Very recently, deep deletions of SRSF3 and HNRNPU were observed in B-ALL with acquired resistance to

chemotherapy, but not in paired diagnostic samples[61]. There are many other examples of dysregulated splicing factors promoting distinct tumor phenotypes[90–93]. While such observations could be instrumental in elucidating novel oncogenic and tumor suppressive pathways, the task of identifying key affected transcripts remains challenging, due primarily to the multitude of downstream splicing events.

Mapping splicing alterations in tumors

Unlike mutations in many classical cancer genes (*e.g.*, Ras family members or the PI3 kinases), mutations affecting the spliceosome function have unusually broad, pleiotropic effects on cancer transcriptomes. This puts an emphasis on the precise resolution of RNA isoforms, which is a much more challenging task than merely analyzing difference in gene expression at the level of whole transcripts.

Isoform detection with short-read RNA-sequencing

Over the past twenty years, high-throughput approaches to profile RNA isoforms, from exon-level microarrays to deep short-read RNA-sequencing, have enabled researchers to catalog differences in alternative splicing patterns in cancer cell lines and patients samples with some degree of confidence[12]. Most cancer studies to date, particularly those based on large-cohorts like The Cancer Genome Atlas (TCGA) and Therapeutically Applicable Research to Generate Effective Treatments (TARGET), have used short-read RNA-sequencing to map and measure splicing in tumor samples[14,94]. These approaches rely on mapping millions of short reads onto reference or *de novo*-reconstructed transcriptomes, identifying sequences that are differentially included or skipped, and then quantifying splicing differences between cancer cohorts (Figure 2). Yet, accurate detection and quantification of splicing remains challenging, especially in clinical samples. While most researchers acknowledge that shallow RNA-sequencing (<30 million reads per sample) is not sufficient for accurate splicing detection and isoform mapping, there is growing evidence that even very deep short-read RNA-sequencing (>100 million reads) is not sufficient to adequately define the splicing landscape in certain tissues[95].

On the biological side, the problem is that the highly dynamic nature of RNA processing often results in the abundance of rare splicing events, which are increasingly being detected and reported as more samples are sequenced[96,97]. On the technical side, the heavy reliance on complementary DNA (cDNA) sequencing opens the door for reverse transcription artifacts. For example, the so-called 'falsitrons' **[G]** are artifactual alternative splicing events characterized by missing exonic fragments, which are only detected by reverse transcription-based protocols and, unlike true 'exitrons'[98,99] **[G]**, cannot be experimentally validated using direct long-read RNA-sequencing of native RNA molecules[100] (see the next section). Finally, on the computational side, there are almost as many methods to identify spliced isoforms as there are laboratories studying splicing. The most commonly used ones are replicate multivariate analysis of transcript splicing (rMATS), mixture-of-isoforms (MISO), LeafCutter, Modeling Alternative Junction Inclusion Quantification (MAJIQ), SUPPA, and Vertebrate Alternative Splicing and Transcription Tools (VAST-TOOLS)[101–108]. They rely on either 'event-centric' approaches, which focus on identifying and characterizing

individual alternative splicing events within genes, or more challenging 'transcript-centric' calculations, which consider entire transcript isoforms resulting from alternative splicing as units of analysis. However, even the event-centric approaches often disagree with one another, which is not to say that some are always right or always wrong[109]. Comparative analyses have shown that each method has its strengths and weaknesses with regard to specific splicing events, and those called by one method but missed by another can be validated using orthogonal approaches[104].

Nonetheless, short-read RNA-sequencing approaches are clearly sub-optimal for the reconstruction of full-length isoforms and are even less adept at profiling intron retention[109,110]. This is because accurate quantification of intron retention events would require reads that span the full length of introns. Such coverage is usually not feasible, given that the mean length of human introns is >3kb[111]; and reads that partially overlap with introns may not provide sufficient information to accurately estimate the abundance of retained introns. It is also challenging to determine whether any given set of reads maps to translatable protein-coding mRNAs, to long non-coding RNAs, or in fact to NMD substrates. Finally, most computational tools for RNA splicing analysis lack batch correction methods, thus limiting our ability to compare data across studies and models. To the best of our knowledge, the recently developed MOCCASIN is the only tool that addresses this challenge by accounting for both known and unknown confounders in mRNA splicing analysis[112].

Isoform detection with long-read RNA-sequencing

Advances in long-read approaches have enabled the detection of end-to-end, full-length RNA molecules using the Pacific Biosciences (PacBio) or Oxford Nanopore Technologies (ONT) RNA-sequencing platforms. These approaches have two important advantages: they do not rely on transcriptome reconstruction required for short-read data and they facilitate the discovery of novel isoforms that are not present in reference transcriptomes (Figure 2). Additionally, by enabling direct long-read RNA-sequencing, ONT can detect chemical (epitranscriptomic) modifications in the RNA (described further below). Unsurprisingly, recent long-read profiling experiments have revealed the increased complexity of the splicing repertoire in tumor samples compared to normal tissues and uncovered novel tumor-specific isoforms missed by current short-read sequencing[113–116].

While long-read RNA-sequencing approaches hold the promise of a harmonized view of splicing aberrations in cancer, there is no single universally appropriate method for isoform calling. Detection of *de novo* events, absent from reference transcriptomes, remains particularly challenging[96]. This is partly due to technical issues, such as persistent errors in base calling encountered with ONT sequencing. Consequently, accurate mapping and alignment of reads in the context of tumors, which often exhibit novel isoforms absent in reference transcriptomes and tumor-specific gene fusions and rearrangements, can be problematic. To some extent, this challenge can be addressed computationally with algorithms like Full-Length Alternative Isoform analysis of RNA (FLAIR) and Error Statistics PRomoted Evaluator of Splice Site Options (ESPRESSO), which account for splice site motifs and correct identified base calling errors accordingly[117,118]. An

additional limitation of long-read RNA-sequencing is the bias against very long transcripts (those greater than 6 kilobases), therefore making it more challenging to capture retained introns[96,119]. On the opposite side of the length spectrum a commonly encountered problem is the mapping of microexons **[G]**, a class of exons shorter than 30 nucleotides in size that play key roles in brain development and neuropathologies[120,121]. Recent studies suggest that microexons are also expressed in non-neuronal tissues and tumors, can encode short peptides and putative neoantigens, and are associated with survival in patients with cancer[122–126]. It is anticipated that dedicated local alignment methods can improve the mapping of these frequently misaligned exons[127,128].

However, the most basic limitation of current long-read approaches is that, until recently, they yielded far fewer reads per sample, thus limiting their utility for isoform quantification across transcriptionally diverse cancer tissues[96]. To address this limitation, studies have combined long-read RNA-sequencing for isoform identification with short-read RNA-sequencing for isoform quantification, an effective but complex and expensive approach for characterizing the splicing repertoire in tumors[113–115,119]. Additionally, targeted long-read RNA-sequencing, which uses probe captures to enrich for isoforms of interest, can help to increase read coverage for the targets of interest and enable isoform quantification using only long-read data. A recent study focusing on 468 clinically actionable cancer genes (including frequently mutated genes as well as approved and pre-clinically druggable targets) discovered novel isoforms of tumor suppressor genes (*e.g.*, *TP53*) that are targeted by NMD in breast cancer cell lines, revealing a common RNA-associated mechanism for tumor suppressor gene inactivation[129]. Finally, recent advances in ONT technologies as well as concatenation methods for PacBio workflows are increasing the throughput and one day might reach more than 100 million reads per sample, replacing short-read technologies.

To sum up, for this relatively new field of long-read RNA-sequencing there are not only two distinct sequencing platforms *(i.e.*, PacBio vs. ONT) but also a variety of library generation protocols with their inherent limitations and biases (*e.g.*, direct RNA *vs.* cDNA sequencing) and a rapidly growing number of computational tools. Recently, the Long-read RNA-Seq Genome Annotation Assessment Project (LRGASP) consortium evaluated the use of these technologies and computational approaches for isoform analysis[96]. In brief, the LRGASP study revealed that libraries with longer, more accurate reads produce better annotated catalogues than those with increased read depth, whereas greater read depth improved transcript quantification. Moreover, when aiming to detect rare and novel transcripts or when using reference-free approaches, incorporating additional orthogonal data and replicate samples is advisable. The key message is that no one method is a clear winner, and the choice of experimental and computational approaches should be driven by the project's aims[96]. The LRGASP consortium also experimentally validated many novel transcripts detected by long- and short-read RNA-sequencing methods in individual samples, confirming the existence of an important pool of rare RNA isoforms[96]. This effort provides a benchmark for current practices, opens the door for improved and more standardized pipelines, and suggests future directions for method development[96].

Profiling RNA modifications and dynamics

Our understanding of the role of RNA processing in cancer relies on our ability to define mRNA repertoires in tumors, profiling their overall structure, stability, dynamics, and last but not least, post-transcriptional modifications (Figure 2). The latter is now known to modulate the expression of oncogenes and tumor suppressor genes by affecting nuclear export, stability, and translation[130]. For example, high levels of the m6A RNA modification destabilize mRNAs encoding metabolic enzymes in the citric acid (TCA) cycle, setting up a decrease in oxidative-phosphorylation metabolism in hematopoietic stem and progenitor cells and favoring the development of leukemia [131]. Perhaps not surprisingly, deposition of the m6A mark onto mRNA (usually by METTL3–METTL14 methyltransferases) also affects splicing patterns. One known mechanism is based on the m6A reader YTHDC1 promoting exon inclusion via recruitment of the pre-mRNA splicing factor SRSF3[132]. In addition to these 'hyperlocal' *cis* effects, m6A can affect splicing transcriptome-wide by virtue of being deposited onto the U6 snRNA by METTL16[133]. Of note, METTL3, 14 and 16 are all essential genes in human acute myeloid leukemia cell lines[134], suggesting that these enzymes could be potential therapeutic targets in AML and leading to the clinical development of STM2457, a highly selective first-in-class inhibitor of METTL3[135].

Additional RNA modifications are likely to play their own roles in splicing of cancer-related genes, pseudouridylation of alternatively spliced regions being one recent example[136]. The growing spectrum of such modifications can be captured by ONT-based platforms, which enable direct sequencing of the native RNA molecules, rather than the resulting cDNA[137]. While very promising, these technologies still require large amounts of RNA, and may not be generally applicable to clinical samples for which RNA material may be limited. Therefore, high-throughput profiling of RNA modifications and associated splicing events in larger cohorts of cancer patients remains a largely unmet need, especially with respect to disease-specific signatures and tumor evolution.

Capturing splicing heterogeneity at the single cell level

Most splicing studies published to date have relied on bulk RNA-sequencing, where a small (usually frozen) piece of the tumor is ground in a homogenizer, and pools of mRNAs from different compartments are extracted and sequenced. However, bulk approaches are unable to resolve which cells within a given tissue express the detected isoforms. Thus, we have a very limited understanding of isoform heterogeneity and clonality within the neoplastic compartment or within the other cell types present in the tumor microenvironment. Also largely unknown is how intratumoral heterogeneity of alternative splicing impacts disease progression and drug responses. For this, a transition to single-cell and spatial approaches is urgently needed, since only these approaches can fully unravel the role of RNA splicing in tumor evolution and therapeutic resistance (Figure 2). One frequently encountered problem is that the commonly used single-cell RNA-sequencing or spatial transcriptomics 10x Genomics platforms, at least as standardly implemented, only yield sequences less than 100 nucleotides from either 5' or more commonly 3' ends of mRNA. Therefore, they don't capture information about full transcripts and their internal exons. Another key problem is obtaining sufficient read coverage for the quantification of isoforms in the limited material obtained from a single cell or a spatial spot, often requiring amplification steps

that can introduce major biases[138–141]. Finally, some of the current spatial transcriptomic approaches rely on pre-designed transcript probes that are unable to distinguish between spliced isoforms.

While original dedicated single-cell approaches, such as Smart-seq and Smart-seq2, were able to generate read coverage along the whole transcript, their throughput was limited to only several hundred cells[142,143]. This lower cell number (as compared to the 10X Genomics platform, which interrogates 5,000–10,000 cells per run) sharply reduced the number of cell types and states that can be captured in one experiment. Several newer approaches are being developed to map spliced isoforms in single cells at scale[144–148], but these have not yet been extensively applied to tumor tissues. For example, Smart-seq3 combines full-length transcriptome coverage with a 5′ unique molecular identifier for duplication correction, and has greatly increased sensitivity compared to Smart-seq2. It typically allows to detect and reconstruct in silico thousands more transcripts per cell[144]. Other workflows implement two-step approaches. Examples include single-cell isoform RNA-Seq (ScISOr-Seq) which combines short-read 3′ sequencing with long-read RNA-sequencing (PacBio or ONT)[145]; single-cell COrrected Long-Read sequencing (scCOLOR-seq), which is a single-cell corrected long-read RNA-sequencing approach which overcomes the high error rate inherent in ONT reads[146]; and single cell Nanopore sequencing analysis of Genotypes and Phenotypes Simultaneously (scNanoGPS), which performs independent deconvolution of error-prone long-reads into single-cells and single-molecules and calculates both genotypes and phenotypes in individual cells[147]. Finally, Genotyping of Transcriptomes (GoT)-Splice enables genotyping of transcriptomes with long-read single-cell RNA-sequencing and proteogenomics for single-cell profiling of transcriptomes, surface proteins, somatic mutations, and RNA splicing[148].

These single-cell and long-read approaches are being increasingly used to characterize the splicing landscape of human tumors across cell types. Recent studies revealed cell type-specific isoforms and alternative polyadenylation site usages in tumor, immune and/or stromal cells in dissociated single cells from ovarian and kidney tumor samples[147,149]. In addition, at the tissue level, a handful of studies have combined spatial transcriptomics with long-read RNA-sequencing to understand how splicing contributes to tissue development and disease *in situ*; yet these have mostly been used in the context of brain development and neurodegeneration[150,151], and are yet to be applied to tumors.

Both single-cell and spatial transcriptomic approaches suffer from gene drop-out even at the whole transcript level, which is likely exacerbated at the isoform level. Future technological advances that decrease cost, increase read depth, and improve accuracy and throughput would aid our ability to map splicing in tumors. For example, Multiplexed Arrays Sequencing (MAS-seq) library preparation concatenates transcripts to optimally utilize PacBio long-read RNA-sequencing and increase transcript yield by 5–10-fold[152]. Thus, more tumor studies benefiting from these advantages are expected in the near future, assisting with the development of splicing-based prognostic and predictive biomarkers.

Functional relevance of splicing aberrations

The rapid advances in sequencing technologies will continue to improve our ability to profile splicing aberrations with great resolution and accuracy. However, as the catalog of recurrent splicing events grows, so will the list of questions about any functional roles that these isoforms might (or might not) play in cancer. There are excellent examples of alternatively spliced mRNA isoforms that have been functionally validated[153,154], but the extent to which splicing-derived proteoforms **[G]** are actually expressed and display distinct functions has been the subject of debate[155–157]. Therefore, considerable efforts have been expanded to develop tools to assess the functional impact of cancer-associated splicing events, including their utility as prognostic or predictive biomarkers.

Annotation of splicing-derived proteoforms

While splicing events have been suggested to play functional roles in tumor progression[67,77,80,158–164], it remains challenging to determine which isoforms are clinically relevant. Improvements are needed for both computational predictions and high-throughput functional testing in pre-clinical model systems. On the computational side, novel methods for prioritizing and assessing the functional relevance of recurrent spliced isoforms found in large-scale transcriptomic datasets are rapidly emerging. These include methods for functional annotation and profiling at the isoform level[165], for inferring interaction networks that connect alternative splicing events to functional annotations and to co-regulated genes or pathways[166], for predicting the functional impact of the altered splicing patterns[13,14], and for integrating with large-scale proteomics datasets[167,168]. Still needed are databases that integrate splicing signatures from large sets of independent studies. A first example of such a database is MAJIQlopedia, a MAJIQ-based encyclopedia of splicing variations that encompasses 86 human tissues and 41 cancer datasets. It serves as a unique resource for RNA researchers seeking to understand what splicing variations in their transcripts of interest exist across tissues or cancers, and for translational scientists aiming to catalog mRNA isoform usage in tissues/cancers under investigation, all through a user-friendly web-based interface[169].

On the experimental side, there is a pressing need to assess which alternatively spliced mRNA isoforms are actually being translated into proteins. Part of the difficulty in addressing this problem lies in the limitations of the methods used to identify proteins or translated mRNAs, particularly for molecules with low expression levels. Theoretically, integrating RNA-sequencing results with large-scale proteomics datasets would be a great way to address this question[167,168]. However, measurements of protein diversity by mass spectrometry[156] are affected by the biases of the digestion enzymes used in the process, which can limit variant detection[170]. Nonetheless, aberrant peptides produced by cancer-specific splicing alterations can be validated using proteomics[14]. More recent studies reflecting efforts to increase the diversity of proteases and depth of coverage reveal that 64% of the frame-preserving splicing events of relatively highly expressed genes detected by transcriptomics are translated and present at the protein level[157]. Additionally, top-down proteomics methods can help identify intact protein isoforms from the same gene[171]. However, their sensitivity needs to be improved for detecting the full complexity of the

proteome, especially in tissues with increased complexities of mRNA isoforms, greater sample-to-sample variability, and limited material availability, such as cancers.

A useful surrogate measurement of translation efficiency is ribosome or polysome profiling. These transcriptome-wide sequencing methods detect RNAs actively engaged by ribosomes and therefore likely to be translated into proteins. However, given that these approaches are typically based on short reads, their outputs often suffer from low coverage and insufficient depth, with only ribosome-protected RNA positions being captured. Thus, profiling translational dynamics at the transcript isoform level is currently challenging. Nevertheless, there is evidence supporting ribosome activity on regions that are unique to spliced isoforms for >50% of the measured events, even after accounting for variation across conditions, such as cell cycle progression, diversity of tissue types, and 'tumor *vs.* normal' comparisons[172–174].

While these studies generally support a direct impact of alternative splicing on protein production, additional strategies to address technological challenges are being developed. Their salient features are improve sequencing depths, more precise biochemical selection of ribosome-protected fragments, leveraging multiple datasets to increase consistency across replicates and conditions, combining RNA-seq and Ribo-seq datasets, and generating both comprehensive and cell type-specific transcriptomes[172,174]. Yet, ribosome or polysome profiling are not routinely used in cancer research, mainly because they are costly and time-consuming, require specialized equipment, and cannot easily be scaled to hundreds of samples.

Functional interrogation of spliced isoforms

Assuming that the majority of polyadenylated mRNA isoforms without retained introns or poison exons are translated into proteins, the next challenge is to assign them functional significance in the context of neoplastic transformation. This task can be approached either at the level of individual transcripts or at scale. One incredibly powerful tool to interrogate the function of specific cancer-associated isoform switches is the use of splice-switching antisense oligonucleotides **[G]** (ASOs). These short, chemically modified RNA oligonucleotides are complementary to the target sequence in a pre-mRNA, thereby preventing its interaction with the core spliceosome as well as positive or negative regulators of splicing (*e.g.*, SR and HNRNP proteins). Therefore, they can be used to up- or down-regulate any splicing module, including cassette exons, alternative splice sites, mutually exclusive exons, or retained introns. Rational design has made it possible to identify relatively easily ASOs that efficiently modulate splicing at the endogenous level, and demonstrate that a specific splicing event is required for cancer cell growth and/or therapeutic resistance both *in vitro* and *in vivo*[67,73,163] (Figure 3 and Table 1). For example, in B-ALL cell culture models, it has been demonstrated that ASOs forcing the skipping of the first AUG-containing exon 2 of the *CD22* gene makes leukemic cells resistant to the CD22-directed antibody-drug conjugate inotuzumab ozogamycin[175]. Similarly, the use of ASOs to force inclusion of the poison exon in the *FPGS* gene renders them resistant to methotrexate[61].

Several studies also have demonstrated that splice-switching ASOs can delay tumor formation or metastasis in pre-clinical models[12] (Table 1). For example, *SF3B1*-mutant tumors include a poison exon in the bromodomain containing 9 (*BRD9*) gene leading to *BRD9* transcript degradation. An ASO that forces exon skipping increase BRD9 protein levels and decreased tumor volume in uveal melanoma mouse models[176]. The main benefit of such splice-switching ASOs is that, at least theoretically, they can be used not only for pre-clinical testing in model organisms, but also as viable therapeutic modalities in humans. In fact, several ASOs have been approved by the US Food and Drug Administration (FDA) for non-cancerous genetic diseases[177,178] and could one day reach the oncology space. The main drawback of using splice-switching ASOs for functional interrogation is that this approach is not scalable, and candidate ASOs need to be tested individually. Screening hundreds or thousands of them one by one to prioritize candidate splicing events would be time-consuming and expensive, to the point of not being feasible in an academic setting. The same can be said about overexpression or knockdown approaches.

Fortunately, approaches to interrogate splicing isoforms at scale do exist. The impact of hundreds of spliced isoforms can be simultaneously tested in model systems using paired guide RNAs for alternative exon removal[74] or single-base editors to program exon skipping in cultured cells or xenograft models of cancer[179,180] (Figure 3). However, these approaches target DNA sequences and therefore have the potential to impact both genomic and epigenomic regulatory elements or alter gene transcription. RNA-targeting CRISPR approaches are an attractive alternative strategy that can be used either to sterically block a splice site and prevent its usage, or to guide the recruitment of artificial CRISPR-fused splicing factors to modulate either exon inclusion or skipping. In fact, these approaches have been used successfully for combinatorial targeting of multiple exons[73,181–183] (Figure 3).

The main limitations of genome- and transcriptome-editing approaches are three-fold. One is the need for efficient delivery of both the Cas proteins and the guide RNAs. The second is the relatively low efficiency of the current editing approaches. The third and perhaps the most problematic one is the fact that the majority of studies focus on exon-skipping events, which represent less than half of the splicing alterations detected in tumors. Cassette exons studies are also biased in favor of NMD-inducing events, which are easier to model, and given their putative loss-of-function consequences, easier to interpret functionally. Future improvements will be needed to model the impact of other splicing alterations, including intron retention, alternative 5' or 3' splice site choices, mutually exclusive exons, as well as combinations of multiple splicing events in the same transcript.

Interpreting splicing signatures

Independently of our ability to pinpoint alternative splicing events of singular significance, there is growing appreciation of the prognostic utility of complex splicing signatures. Several pan-cancer studies demonstrated that alternative splicing-based survival predictors outperform gene expression-based ones. Specifically, splicing signatures can accurately predict survival in breast[184], gastric[185], pancreatic[186], lung[187], and ovarian[188] cancer patients as well as in adults with high-grade gliomas[88]. These discoveries underscore the need to integrate clinical and gene expression data with alternative splicing profiles for the

most accurate risk stratification and survival prediction in cancer patients[189,190]. It could also inform treatment decisions, leading to improved patient outcomes.

This being said, the use of splicing signatures in cancer patients has several caveats. One such caveat is the growing evidence suggests that splicing changes can occur in pre-neoplastic tissues. The case in point is clonal hematopoiesis, a common preleukemic condition. Recent data suggest that mutations in splicing factors occur very early during disease initiation and can act as founder mutations[27,191,192]. Similarly, studies of bladder cancer evolution have reported that mutations in splicing factor RNA binding protein 10 (RBM10) are frequent in most tumor clones, suggesting that splicing alterations could occur early during solid tumor formation and clonal expansion[193]. It remains unknown whether these events predict progression to frank malignancies more accurately than genomic or epigenetic alterations.

With regard to risk factors, one of the main ones is age, at least in adult cancers. Yet its impact on splicing remains understudied. Interestingly, both spliced isoforms and splicing regulators have been reported to change with age in human and mouse tissues as well as in the context of age-related neurological disorders[194], emphasizing splicing dysregulation as a hallmark of aging[195,196]. While many of the splicing changes seem to be largely tissue specific, a number of splicing patterns, including aberrant intron retention, have been found across aged tissues in multiple studies[197–200]. Similarly, a loss of splicing fidelity at specific intronic sequences has been seen in age-related neurodegenerative diseases[198] and in cancers[201]. In addition, mutant splicing factors have been causally linked with longevity in model systems[199,202–205]. Despite the links between aging, cancer and splicing, most studies ignore the aging dimension when modeling and measuring the impact of splicing in cancer. This gap in knowledge has profound implications for our understanding of the mechanistic origins of splicing aberrations in solid tumors and whether they play a role in clonal cell expansion in pre-neoplastic tissues.

Therapeutic targeting of mRNA splicing

Following the identification and functional validation of key splicing aberrations, the development of therapeutic approaches could be approached from two broad angles: mis-splicing normalization and mis-splicing exploitation. The first approach entails identifying individual stand-out "driver" splicing events and reversing them *in vivo* with ASOs, CRISPR-mediated DNA or RNA editing; or else via modulation of the splicing machinery, as part of systemic splicing normalization (Figure 3). While splicing normalization might sound like an attractive scenario conceptually, many practical hurdles would complicate the development of such therapies, including but not limited to i) the pan-essential function of several components of the spliceosome[206]; ii) the difficulties in targeting RNA binding proteins, which lack traditional druggable pockets and often contain low-complexity unstructured domains; iii) the loss-of-function nature of many splicing factor mutations (*e.g.*, deep deletions); and iv) the lack of drugs selective for the mutant splicing factor variant in the cases of neomorphic mutations. A radically different second approach is to consider aberrant splicing as a desirable therapeutic vulnerability and to take advantage of it with dedicated targeted or immuno-therapies (Figure 3).

Correcting individual mis-spliced isoforms

The success of this approach is predicated on the assumption that while individual tumors might have thousands of splicing alterations, the vast majority of them are "passenger" events, with only one or two "driver" events being germane to cancerous growth. Such events would likely affect genes already classified as cancer drivers based on accumulation of acquired mutations or copy number variations in tumors of the same origin. Examples of such events may include splicing variants of mutant *BRAF* with the hotspot V600E amino acid substitution. These splice variants, lacking exons encoding for the Ras binding domain, are often detected in melanoma patients with acquired resistance to B-Raf inhibitor vemurafenib[207]. Similarly, it has been proposed that the exon-16-skipping *HER2* variant has oncogenic activity in breast carcinomas, where *HER2* is a known driver[208]. In these scenarios, one might envision employing therapeutic strategies to target individual spliced isoforms. Such strategies might utilize RNA-based drugs, such as ASOs, to reprogram specific splicing switches and/or to target tumor-associated spliced isoforms that are otherwise undruggable with classical small-molecule compounds[209]. Because of their potential to restore normal splicing patterns or to selectively target cancer-specific splicing events, these therapies, hold promise for precision oncology.

In preclinical settings, there have been some success stories (Table 1). Splice-switching ASOs have been used to target splicing of the *MDM4* transcript. Normally, its translation product suppresses p53 only in highly proliferating cells such as embryonic stem cells, but it is often re-expressed in cancers. ASOs promoting *MDM4* exon 6 skipping led to MDM4 protein downregulation, p53 reactivation, and decreased tumor growth *in vivo* in a *TP53* wild-type melanoma PDX model [163]. Similarly, splice-switching ASOs can rewire cancer cell metabolism by targeting the M2 pyruvate kinase (PKM2) isoform, which is upregulated in most cancers. In contrast, PKM1 is expressed in terminally differentiated, non-proliferating cells. This is because PKM2, but not PKM1, promotes the Warburg effect, which favors aerobic glycolysis over oxidative phosphorylation for energy metabolism[153]. Both *PKM1* and *PKM2* transcripts are derived from the *PKM* gene through alternative splicing of two mutually exclusive exons: *PKM1*-specific exon 9 and *PKM2*-specific exon 10. Splice-switching ASOs promoting a switch from exon 10 to exon 9 usage led to a protein switch from PKM2 to PKM1 and delayed liver cancer cell growth *in vitro* and *in vivo* in xenograft models[164]. Lastly, ASOs modulating myosin phosphatase RHO-interacting protein, a splicing target of the tumor suppressive RNA binding protein fox-1 homolog 2 (RBFOX2), limited pancreatic metastases by altering cytoskeletal organization and focal adhesion[162].

The RBFOX2 study also introduced the broader concept of a metastatic signature of alternative splicing, based, at least in part, on the epithelial-mesenchymal transition (EMT)[210]. Indeed, EMT, broadly recognized as one of the hallmarks of cancer[15], is a program activated during cancer cell dissemination to distant organs. Importantly, a combinatorially regulated EMT-associated RNA splicing signature faithfully predicts breast cancer patient survival[184]. This is because EMT involves multiple transcripts undergoing isoform switching: *CD44*, *FGFR2*, *Rac1*, and *ENAH*, among others[158,211–213]. In the case of *CD44*, the epithelial CD44v isoform sustains Ras-MAPK signaling and in

doing so promotes cell proliferation[214]. Conversely, the alternatively spliced mesenchymal CD44s isoform activates PI3K signaling, leading to enhanced survival, invasion, cancer stem cell properties, and ultimately, cancer metastasis[158,160,215,216]. Besides RBFOX2[217], several regulatory splicing factors have been reported to contribute to EMT, either as activators (hnRNPM and QKI[218,219]) or as repressors (ESRP1, AKAP8, hnRNPF, and RBM47[70,159,161,220,221]). ASOs could be very instrumental in determining which of their targets (beyond the usual suspects) might contribute to EMT and metastatic spread.

Beyond ASOs, desirable mRNA-induced changes in splicing might be achieved by targeting distinctive features of RNA sequences at RNA-protein interfaces[222,223]. This is the mechanism by which Risdiplam, an FDA-approved drug that targets *SMN2* splicing to restore the production of viable SMN protein (and the first oral medication approved for the treatment of spinal muscular atrophy), appears to function[224]. While some small molecules targeting RNA structures have advanced to the clinic[225], none of them was designed to modulate splicing for therapeutic use in the oncology space.

In summary, few success stories notwithstanding, both the selection of a single target that would strongly impact the tumor phenotype and the intra-tumoral delivery of splice-switching drugs remains challenging. While splice-switching ASOs have shown promise in pre-clinical models, not a single one has led to complete tumor regression as a monotherapy. It could well be that the underlying issues are merely technical in nature, and that better chemistry and better delivery tools will yield promising results. However, a more sober view is that one target might never be enough, as aberrant splicing contributes to oncogenesis by dysregulating multiple pathways and gene networks, causing the failure of various checkpoint mechanisms. In that case, it might make more sense to consider splicing aberrations a therapeutic vulnerability, where the attainable goal would be not to "fix" but rather to exploit them.

Exploiting splicing-related vulnerabilities

Targeting the splicing machinery—Current strategies to target the core spliceosome in tumors where some of its components are already mutated are based on the idea that there are limits to how much splicing dysregulation a tumor cell can tolerate[12]. Indeed, there is ample preclinical evidence that cancers with splicing factor mutations and imprecisely functioning splicing machineries can be targeted by direct spliceosome inhibitors and/or inhibition of enzymes that regulate splicing factors via post-translational modifications (Table 1 and references therein). This, in turn, affects both disease pathogenesis and therapeutic responses. By systemically inhibiting the already dysfunctional cancer cell spliceosomes (manifested by alternative 3'SSs, widespread intron retention, etc.), we can find a therapeutic window within which normal tissues with unperturbed splicing will be largely spared.

Clinical-grade small molecules that modulate or inhibit aberrant RNA splicing have been developed and are currently being tested to treat oncological patients in pre-clinical studies and clinical trials, both for blood cancers and solid tumors[12] (Table 1). These include molecules that disrupt the splicing reactions, such as inhibitors of the core spliceosome component SF3B1[226–228], as well as compounds that prevent the recruitment of the

U4/U5/U6 tri-snRNP[229–231]. H3B-8800 and E-7107 are first-in-class splicing modulators targeting SF3B1, which showed promise in preclinical studies and a phase 1/2 clinical trial, where treatment with H3B-8800 resulted in transfusion independence in a subset of patients with myelodysplastic neoplasms bearing splicing factor mutations[228]. Other approaches to directly target splicing factors include degraders of the splicing factor RBM39[232], as well as ASOs that either act as decoys to sequester splicing factors and attenuate their activity[233] or block their production at the level of transcription[73] (Table 1).

Moreover, the biogenesis and activity of many splicing factors are regulated by post-translational modifications. Consequently, the activity of select splicing factors could be down-modulated by inhibitors of specific protein kinases (*e.g.*, CLKs, SPRKs, dual specificity tyrosine-phosphorylation-regulated kinases DYRKs)[234–236], or arginine methyltransferases PRMTs[81,237,238]. Ongoing Phase 1 clinical trials are exploring PRMT5 inhibitors and an RBM39 degrader in patients with splicing factor-mutant myelodysplastic neoplasms [see US National Library of Medicine's https://clinicaltrials.gov/study/NCT03573310 (2018), https://clinicaltrials.gov/study/NCT03886831 (2019), and https://clinicaltrials.gov/study/NCT05024994 (2021)].

Targeting the splicing machinery, although elegant in principle, is not without its challenges. Early clinical trials of SF3B1 inhibitors were terminated due to on-target, off-tumor toxicity in the retina[227], and the more recent SF3B1 inhibitor H3B-8800 induced cardiac toxicities[228]. Follow-up studies of SF3B1 inhibitors in low-risk splicing factor-mutant leukemias have recently been closed after disappointing phase I/II trial results [see US National Library of Medicine's https://clinicaltrials.gov/study/NCT02841540 (2016)]. While recently developed inhibitors might be more promising, it is still unclear how cytotoxicity can be managed. Moreover, as stem cells share many alternative splicing similarities with cancer cells[65], the therapeutic window separating cancer cells from stem cells and other healthy cells remains elusive. Lastly, it remains to be determined whether targeting the regulatory splicing machinery rather than the core spliceosome, would elicit less toxicity.

Discovering splicing-based synthetic lethalities—Given that so far spliceosome inhibitors have not transformed the therapeutic landscape of hematological malignancies, one can ask whether we should turn our attention to discovering unique vulnerabilities arising from splicing, but not directly involving splicing regulators. Indeed, there is emerging evidence that splicing alterations might drive resistance to conventional chemotherapy while creating new vulnerabilities. In B-ALL, inclusion of a microexon into the 5'-nucleotidase, cytosolic II (*NT5C2*) mRNA was recently shown to confer resistance of leukemic cells to thiopurine while simultaneously rendering them sensitive to the immunosuppressive drug mizoribine (and quite possibly the more widely used mycophenolate mofetil)[61,71]. Similarly, in refractory AML with *U2AF1* mutations there was increased inclusion of a poison exon in transcripts encoding the eukaryotic translation initiation factor 4A2 (EIF4A2). This elicited the integrated stress response (ISR) and rendered AML cells sensitive to the ISR inhibitor ISRIB combined with chemotherapy[239]. More broadly speaking, there is emerging evidence that splicing factor mutations confer sensitivity to drugs targeting not only the core spliceosome[240], but also the RNA binding motif protein 39 (RBM39)[241], the Sin3–histone deacetylase (HDAC) pathway[38], and protein

arginine methyltransferases[81]. In murine models of AML, mutations in *SF3B1* and *SRSF2* independently converged on nuclear factor κB (NFκB) signaling[242], making this pathway a potential dependency. Conversely, DNA damage repair-deficient cancer cells (*e.g.*, those bearing cohesin mutations) were reported to be exquisitely sensitive to SF3B1 inhibitors, and this treatment can sensitize them to subsequent treatment with poly ADP ribose polymerase (PARP) inhibitors or chemotherapy[243].

Other vulnerabilities stemming from aberrant splicing are likely to be discovered through the use of carefully designed CRISPR screens. Alternatively, such vulnerabilities can be artificially created. An elegant approach illustrating this concept was recently developed based on the use of cell-death inducing synthetic introns, which are only processed in cells bearing neomorphic mutations in splicing factors. Inserting these cassettes in the herpes simplex virus-thymidine kinase (HSV-TK) gene allowed TK expression only in *SF3B1*-mutant cancers, and subsequent treatment with gancyclovir suppressed growth lethal xenografts and improved mouse host survival[244]. How close such gene therapy-based approaches are to entering clinical trials only the future will show.

Weaponizing splicing-derived neoepitopes—There is considerable evidence that at least in hematological malignancies alternative splicing can contribute to epitope loss and cause immunotherapy failures. Examples include loss of CD19[245–248], CD22[175], CD20[249], and CD33[250]. One the other hand, alternative splicing has the well-recognized, but still largely untapped propensity to create new epitopes. The approaches closest to clinical translation entail either designing immunotherapies targeting abnormally expressed proteoforms or improving responses to immune checkpoint blockade by increasing the production of splicing-derived neoantigens. Such shared splicing-derived neoantigens have been identified in melanoma[251,252], breast and ovarian[253] and lung cancers[254]. However, in most cases identifying shared major histocompatibility complex (MHC)-presented neoantigens for T cell receptor (TCR)-based therapies remains challenging and laborious, in part because human leukocyte antigens (HLAs) are highly polymorphic in the human population and restrict the repertoires of peptides presented to T cells. It is estimated that an average tumor harbors very few, if any, abnormal splicing-derived peptide[253]. It may therefore be more feasible to devise therapies targeting surface proteins with chimeric antigen receptor (CAR) T cells or antibody drug conjugates, which do not rely on MHC and are HLA-agnostic. A number of computational tools exist to predict splicing-derived neo-epitopes, such as Isoform peptides from RNA splicing for Immunotherapy target Screening (IRIS) and ISOform-guided prediction of epiTOPEs in cancer (ISOTOPE)[123,255]. There are also examples in the literature of antibodies raised against splice isoforms of surface proteins that have the potential to become good therapeutic targets, such as CD22 in B-cell in human B-ALL[175] and collagen 11A in patient-derived xenograft models of pediatric osteosarcoma, Ewing's sarcoma, and rhabdomyosarcoma[256]. However, single-cell data is needed to assess intratumoral heterogeneity and to identify neo-epitopes generated by tumor cells themselves and not by other cell types within the tumor microenvironment.

In addition, splicing-based therapies could be aimed at reprogramming the immune system. This approach takes advantage of the fact that alternative splicing plays a crucial role in determining the functional properties of immune cell receptors, cytokines, and signaling

molecules[257–263]. By selectively promoting the generation of splicing isoforms associated with enhanced cytotoxicity, cytokine secretion, and memory formation, therapies altering splicing can augment the ability of immune cells to recognize and eliminate cancer cells. Furthermore, modulating splicing could regulate immune checkpoint pathways to improve immune responses. For example, by targeting alternative splicing events that control the expression of checkpoint receptors (*e.g.*, PD-1, CTLA-4) and their ligands, it might be possible to fine-tune immune responses and to prevent checkpoint blockade[264–267]. For example, creating decoy PD-1 through forced skipping of exons encoding the transmembrane domain could prevent its cell surface expression[264,268]. Alternatively, splice-switching ASOs could target key regulatory networks to relieve immunosuppression in cancer. The prototype preclinical study utilized ASOs that mediate knockdown of FOXP3, achieving reduction of suppressive functions of Tregs in mouse models of lymphoma[269]. A comprehensive understanding of the splicing regulatory landscape in immune cells and its dysregulation in cancer is therefore crucial for rational design and optimization of splicing-based immunotherapies.

Conclusions

The past 15 years witnessed the wide-spread adoption of next-generation sequencing technologies, including short-read RNA-sequencing[270]. The availability of large transcriptomics datasets along with companion bioinformatics tools led to the cataloging of thousands of non-canonical mRNA isoforms, many of which corresponded to known cancer drivers. This in turn raised hopes that, unlike "hard-wired" somatic mutations and copy number alterations, the "soft-wired" splicing aberration could be reversed with ASOs and other RNA-targeting therapeutics. In parallel, the discovery of splicing factor mutations in human cancers and the concurrent development of SF3B1 inhibitors (E-7107 and its successors) generated a surge in optimism over the clinical use of spliceosome inhibitors, at least against cancers with splicing factor mutations.

So far, both attempts at clinical translation have achieved rather modest successes. Splice-switching ASO drugs proved to be very useful tools for preclinical studies, with greater specificity and less off-target toxicity than traditional small-molecule inhibitors. Moreover, there are documented examples of localized delivery of ASOs to brain, skeletal muscle, and especially liver[178]. However, whether these nucleic acid-based drugs could bypass the hepatic reticuloendothelial system following intravenous administration or be delivered to disseminated tumors remains to be determined. Small molecule SF3B1 inhibitors have not become FDA-approved cancer medicines, due to their limited efficacy and both on- and off-target toxicities, especially in the retina and the heart.

Despite these setbacks, we are far from being pessimistic and do believe that the time for clinical translation of mRNA splicing research will come. However, before this can happen, both technological and conceptual innovations would need to be implemented, and the two will likely arrive hand in hand. Improvements in long-read single-cell and spatial RNA-sequencing will enrich our understanding of tumor heterogeneity and various neoplastic cell states. Advances in CRISPR-based DNA engineering and especially RNA editing will allow us to interpret with greater confidence the functional significance of individual splicing

variations and broad splicing signatures. Creating better *in vivo* delivery vehicles for ASOs and related compounds will facilitate the credentialing and prioritization of therapeutic targets. It will also enable longitudinal studies of mRNA splicing and the mapping of evolutionary trajectories of clones with splicing aberrations. Large-scale CRISPR/Cas9 and chemical library screens will uncover new vulnerabilities of cancer cells with dysregulated splicing. Finally, new approaches to antibody discovery and protein engineering will allow us to generate and test at scale novel immunotherapeutics directed against splicing-derived proteoforms. On the diagnostic side, better mRNA profiling panels will allow us to identify novel prognostic and predictive biomarkers, beyond somatic mutations in *SF3B1* and other core spliceosome components. All this will undoubtedly take time, as the much-needed technologies won't mature overnight. Nevertheless, we believe that the progress towards clinical translation is best achieved not through experimental and therapeutic shortcuts, but rather through steady investments in cross-pollinating branches of biomedical research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

GLOSSARY

Antisense oligonucleotide (ASO)

short synthetic chemically modified single-stranded RNA molecules that can bind to specific RNA sequence and alter their function

Branch point site (BPS)

a nucleotide that performs a nucleophilic attack on the 5' splice site in the first step of splicing

Exitrons

non-constitutive introns located within annotated protein-coding exons

Exon junction complex (EJC

protein complex assembled on the spliced mRNA at the junction of two exons

Falsitrons

artifactual alternative splicing events characterized by missing exonic fragments, which are only detected by reverse transcription-based protocols

Heterogeneous nuclear ribonucleoproteins (hnRNPs)

a large family of RNA-binding proteins that regulate multiple RNA processing steps including alternative splicing

Microexons

a class of exons shorter than 30 nucleotides

Nonsense-mediated mRNA decay (NMD)

a translation-coupled quality control mechanism that removes mRNAs with premature termination codons

Polypyrimidine tract

a pyrimidine (C or T)-rich motif upstream of many 3' splice sites that is bound by U2AF2 to facilitate 3' splice site recognition

Poly(A) tail

long chains of adenine nucleotides added to the 3' of mRNA molecules to increase stability, export and contribute to their translation

Poison exon

an exon that introduces a premature termination codon when included in the spliced mRNA

Proteoform

a variant of a protein, including variation due to alternative splicing

R-loop

a three-stranded nucleic acid structure consisting of an RNA molecule that has invaded duplex DNA

Serine/arginine-rich (SR) proteins

a large family of RNA-binding proteins that contain one or two serine/arginine-rich domains and regulate multiple RNA processing steps including alternative splicing

Sm proteins

family of small proteins that bind to RNA and are part of the spliceosome

Small nuclear ribonucleoprotein (snRNP)

RNA-protein complex that are part of the spliceosome

Small nuclear RNA (snRNA)

RNA component of the spliceosome

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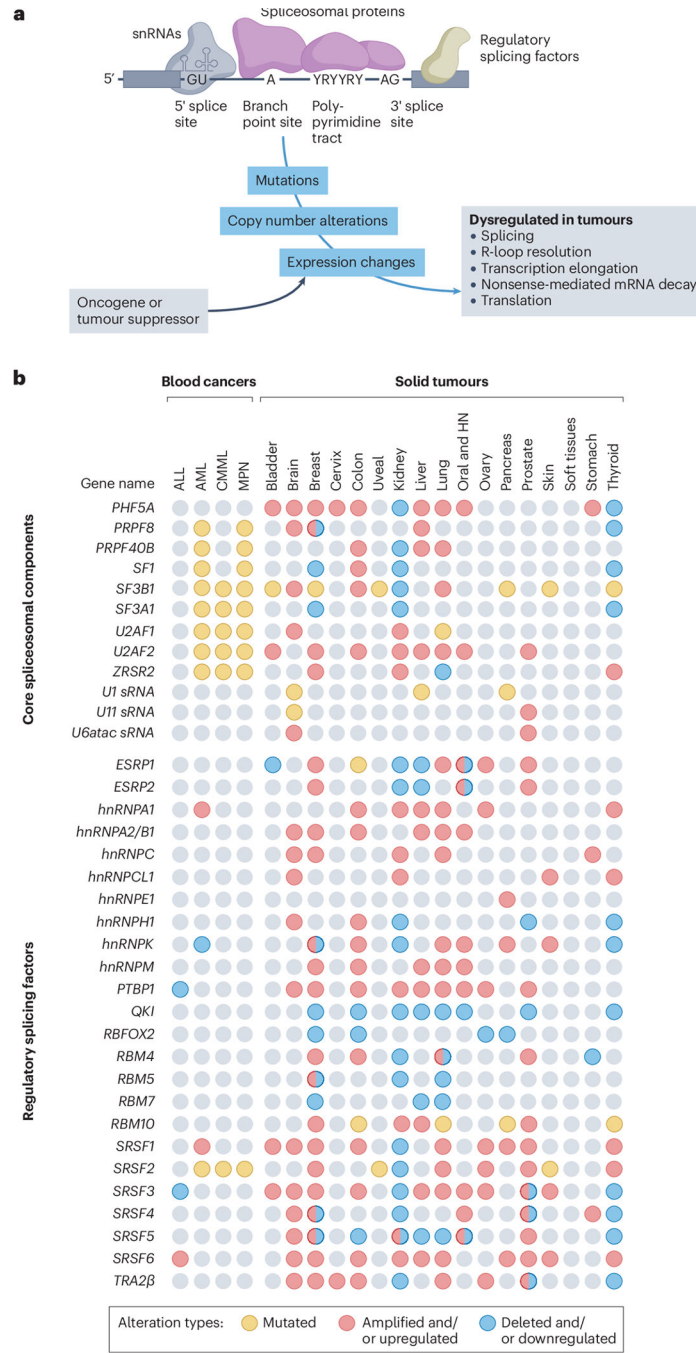
Figure 1. Splicing factor alterations in human tumors.
a, Core spliceosomal proteins (purple) and small nuclear RNAs (snRNAs) (grey) are critical components of constitutive RNA splicing, whereas regulatory splicing factors (tan) have a role in alternative splicing. These are depicted assembled on a pre-mRNA molecule composed of exons (grey rectangles) and introns (black lines). The 5′ and 3′ splice sites are depicted along with highly conserved dinucleotides GU and AG, which define intron boundaries, the adenosine residue serving as the branch point site, and the polypyrimidine tract. Tumours exhibit splicing factor mutations, copy number alterations, or expression

changes. In some tumours, these splicing factor expression changes are induced directly or indirectly by oncogenes or tumour suppressors. Splicing factor alterations lead to dysregulation of alternative splicing but also of other processes listed on the right.

b, Genomic events that affect expression and function of selected splicing factors in blood (left) and solid (right) cancers. They affect both select core (top) and regulatory (bottom) spliceosome components. The data presented have been curated from the literature and represent key splicing factors that are frequently altered in primary or metastatic human tumours compared with normal tissues (see references in Supplementary Table 1). Splicing factors can be mutated (single nucleotide changes), upregulated with or without gene amplification, or downregulated with or without gene deletion. A limitation of these data is that given their non-focal nature, amplification and shallow deletions are more difficult to functionally interpret than deep deletions. Note that for some splicing factors, individual studies have reported them to be either upregulated or downregulated in the same tumour type. These discrepancies can often be explained by differences in tumour subtypes, tumour stages, and even spatial localization within the tumour tissues (for example, carcinoma in situ versus the invasive tumour front). ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; CMML, chronic myelomonocytic leukaemia; HN, head and neck cancers; MPN, myeloproliferative neoplasms.
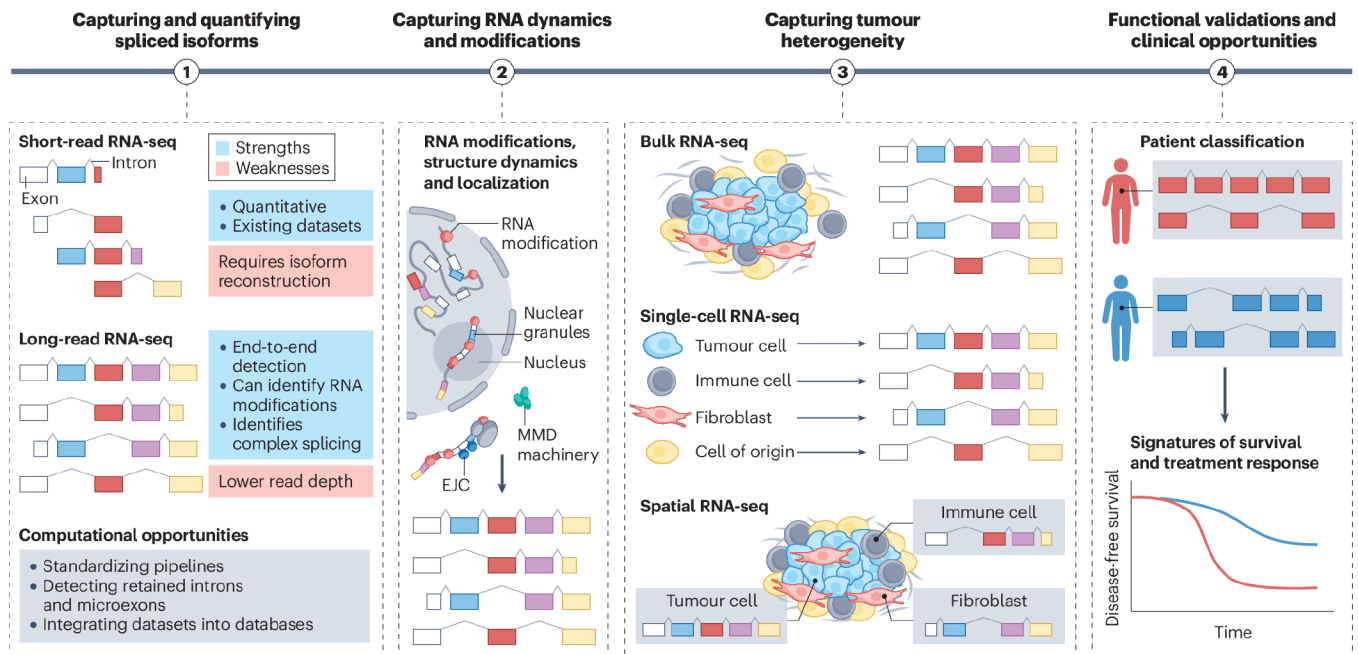
Figure 2. Four key steps towards clinical translation of mRNA splicing research.
The first step towards translating mRNA splicing research is to capture and quantify
isoforms. The strengths and weaknesses are listed for current RNA-sequencing (RNA-
seq) approaches, along with computational challenges that remain be addressed. Second,
understanding the role of RNA processing in cancer requires profiling of complex
repertoires of RNA isoforms, their structure and folding, any chemical modifications
(including m^{7}G, N^6-methyladenosine (m^{6}A) and m^{5}C ψ), A-I editing (represented by red
bubbles), the cellular localization (to specific nuclear comportments or phase-separated
granules), the stability and turnover (including cytoplasmic degradation by the nonsense
mediated mRNA decay (NMD) pathway of transcripts containing an exon junction complex
(EJC) downstream of their stop codon), and the RNA dynamics. All of these factors can
impact splicing, stability, nuclear export and translation. Third, capturing spliced isoform
heterogeneity and their spatial localization will enable isoforms expressed in tumour cells to
be distinguished from those originating in the tumour microenvironment. This is critical for
splicing-directed therapies that are currently being developed, often with the assumption that
all cells within the tumour express the same isoform. Lastly, identifying spliced isoforms
in tumours offers multiple clinical opportunities. For example, classifying patients based on
their isoform profile might reveal novel tumour subtypes, some of which might be associated
with distinct survival patterns, prognosis, and drug responses.

Correcting individual mis-spliced isoforms

- Splice-switching ASOs
- RNA-targeting CRISPR
- Engineered snRNAs
- CRISPR–Cas9 editing
- CRISPR–Cas9 deletion
- RNA-targeting small molecules

Exploiting splicing-related vulnerabilities

Targeting the splicing machinery
- SF3B1 inhibitors
- Tri-snRNP inhibitors
- PRMT inhibitors
- Kinase inhibitors
- Splicing factor degraders
- Splicing factor targeting ASOs
- Decoy oligos for splicing factors

Discovering synthetic lethalities
- Collateral sensitivities to anti-cancer drugs
- Cell death-inducing synthetic introns

Weaponizing splicing-derived neoepitopes
- Targeting splicing-derived neoantigens
- Immune reprogramming
- Re-evaluating immune checkpoint inhibitors

Patient selection
- Measuring splicing factor status
  - Mutations
  - Copy number alterations
  - Expression
- Measuring splicing signatures
  - RNA-seq
  - Targeted PCR

Figure 3. Strategies that target mRNA splicing in cancer.

Transcript-centric therapeutic strategies (blue box) target individual mis-spliced mRNAs that are expressed in tumour cells, primarily to alter their splicing patterns. These strategies yield distinct protein isoforms, which either gain tumour-suppressive properties or lose oncogenic ones. Alternatively, they yield non-productive transcripts that are degraded by nonsense-mediated mRNA decay (NMD). Splice-switching can be achieved, for example, by using antisense oligonucleotides (ASOs) that block splicing regulatory elements. Similarly, exogenous small nuclear RNAs (snRNAs) can be engineered to match specific splice sites and in doing so increase inclusion 'weak' exons. For genome editing, Cas9 proteins are often paired with single guide RNAs (sgRNAs) to delete a specific exon in a given transcript or to base-edit a specific splice site to restore or to compromise its utilization, leading to exon inclusion or skipping, respectively. Finally, recent studies reported the discovery of small molecules that can recognize a specific RNA sequence or structure and modulate splicing of a specific exon. Spliceosome-centric strategies (light purple box), focus on proteins that controls splicing of multiple downstream transcripts, instead of targeting specific RNA isoforms. These approaches include small-molecule inhibitors that block the activity of the core spliceosomal protein splicing factor 3b subunit 1 (SF3B1), prevent the recruitment of the U4/U5/U6 tri-snRNP, or block the activity of protein kinases or protein arginine methyltransferases (PRMTs) which regulate the activity of splicing factors at the post-translational level. Alternatively, the protein levels of a specific splicing factor can be directly targeted using dedicated protein degraders or splice-switching ASOs that,

by modulating poison exon skipping or inclusion, can increase or decrease splicing factor protein levels. Finally, an ASO with sequence complementarity to a splicing factor binding motif can be used as decoy to sequester the splicing factor away from its cognate mRNA targets. In parallel, numerous approaches can exploit synthetic lethalities (pink box), as specific splicing alterations often create new vulnerabilities, including sensitizing cancer cells to conventional anti-cancer drugs. Alternatively, a set of specific introns were found to be spliced exclusively in cancer cells with *SF3B1* mutations, opening the door to the use of synthetic intron sequences to enable *SF3B1* mutation-dependent expression of exogenous cell death-inducing genes. The last class of splicing-based therapies leverages splicing-derived neo-epitopes produced by cancer cells or even creates new ones (dark purple box). These strategies often involve chimeric antigen receptor (CAR) T cells that target abnormally expressed surface protein isoforms or induce immune reprogramming by selectively promoting spliced isoforms associated with enhanced cytotoxicity, cytokine secretion and the generation of memory T cells. The success of these strategies in the clinic will require appropriate patient selection, which should include more systematic molecular measurements of both the splicing factor status and RNA isoform signatures (grey box). This will help to identify patients who would benefit from therapies targeting specific mRNA isoforms versus those that would respond to more global approaches exploiting splicing-related vulnerabilities. RNA-seq, RNA sequencing; snRNP, small nuclear ribonucleoprotein.

Table 1.

Therapies targeting splicing in preclinical studies and clinical trials

Target	Approach	Clinical Trial	Tumor Type	Ref.
SF3B1	Small molecule inhibitor (H3B-8800 and E-7107)	Yes	MDS	228,271
PRMT5	Small molecule inhibitor (EPZ015666, GSK3326595, JNJ-64619178, PRT543, PRT811)	Yes	MDS, blood cancer, solid tumors	81,272
Pan type-I PRMTs	Small molecule inhibitor (MS023)	No	Blood	81
RBM39	Degrader (E7820)	Yes	MDS	241
U4/U5/U6 tri-snRNP	Small molecule inhibitor (Isoginkgetin)	No	Skin	229–231
SRPK1	Small molecule inhibitor (SRPKIN-1, SRPIN340, MVRL09, and SHPINX)	No	Solid tumors	235
CLK1	Small molecule inhibitor (TG003)	No	Colon	234–236
DYRKs & pan-CLK	Small molecule inhibitor (SM08502)	Yes	Solid tumors	236
U2AF complex	Small molecule stabilizer (NSC-194308)	No	Blood	273
BRD9	Splice-switching ASO	No	Skin	176
ERG	Splice-switching ASO	No	Prostate	274
FGFR1	Splice-switching ASO	No	Brain	275
MCL1	Splice-switching ASO	No	Skin	276
MDM4	Splice-switching ASO	No	Skin	163
MST1R (RON)	Splice-switching ASO	No	Breast, Stomach	277
STAT3	Splice-switching ASO	No	Breast	278
USP5	Splice-switching ASO	No	Brain	279
ERBB4	Splice-switching ASO	No	Breast	280
MDM2	Splice-switching ASO	No	Uterine	281
ATM	Splice-switching ASO	No	Blood	282
BRCA2	Splice-switching ASO	No	Breast	283
EZH2	Splice-switching ASO	No	Blood	81
MKNK2	Splice-switching ASO	No	Brain	284
BCL-X	Splice-switching ASO	No	Solid tumors	285
BIM	Splice-switching ASO	No	Blood	286
GLDC	Splice-switching ASO	No	Lung	287
IL5R	Splice-switching ASO	No	Blood	288
PKM2	Splice-switching ASO	No	Brain	289
SRSF3	Splice-switching and splicing factor inhibition ASO	No	Breast	290
TRA2β	Splice-switching and splicing factor inhibition ASO	No	Breast	73
RBFOX	Decoy ASO and splicing factor inhibition	No	Others	233
SRSF1	Decoy ASO and splicing factor inhibition	No	Others	233
PTBP1	Decoy ASO and splicing factor inhibition	No	Others	233

Abbreviations: ASO, antisense oligonucleotide, ATM, ataxia-telangiectasia mutated; BCL-X, Bcl-2-like protein 1; BIM, Bcl-2-like protein 11; BRCA2, breast cancer type 2 susceptibility protein; BRD9, bromodomain-containing protein 9; CLK1, dual specificity protein kinase CLK1; DYRK, dual-specificity tyrosine phosphorylation-regulated kinase; EZH2, histone-lysine N-methyltransferase; FGFR1, fibroblast growth factor receptor 1; GLDC, Glycine dehydrogenase; IL5R, interleukin 5 receptor; MKNK2, MAP kinase-interacting serine/threonine-protein kinase 2; MDS, myelodysplastic syndrome; MST1R (aka RON), Macrophage Stimulating 1 Receptor; PKM2, Pyruvate kinase PKM; PRMT5, Protein arginine N-methyltransferase 5; PTBP1, polypyrimidine tract-binding protein 1; RBFOX, RNA-binding protein fox-1 homolog; RBM39, RNA-binding motif protein 39; SF3B1, splicing factor 3B subunit 1; SRSF1, serine/arginine-rich splicing factor 1; SRPK1, SRSF protein kinase 1; STAT3, signal transducer and activator of transcription 3; TRA2β, transformer-2 protein homolog beta; USP5, Ubiquitin carboxyl-terminal hydrolase 5.