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OPINION

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Fusion transcripts: Unexploited vulnerabilities in cancer?

Carla Neckles | **Soumya Sundara Rajan** | **Natasha J. Caplen**

Functional Genetics Section, Genetics Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, DHHS, Bethesda, Maryland

Correspondence
Natasha J. Caplen, Functional Genetics Section, Genetics Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, DHHS, Bethesda, MD.
Email: ncaplen@mail.nih.gov

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Abstract

Gene fusions are an important class of mutations in several cancer types and include genomic rearrangements that fuse regulatory or coding elements from two different genes. Analysis of the genetics of cancers harboring fusion oncogenes and the proteins they encode have enhanced cancer diagnosis and in some cases patient treatment. However, the effect of the complex structure of fusion genes on the biogenesis of the resulting chimeric transcripts they express is not well studied. There are two potential RNA-related vulnerabilities inherent to fusion-driven cancers: (a) the processing of the fusion precursor messenger RNA (pre-mRNA) to the mature mRNA and (b) the mature mRNA. In this study, we discuss the effects that the genetic organization of fusion oncogenes has on the generation of translatable mature RNAs and the diversity of fusion transcripts expressed in different cancer subtypes, which can fundamentally influence both tumorigenesis and treatment. We also discuss functional genomic approaches that can be utilized to identify proteins that mediate the processing of fusion pre-mRNAs. Furthermore, we assert that an enhanced understanding of fusion transcript biogenesis and the diversity of the chimeric RNAs present in fusion-driven cancers will increase the likelihood of successful application of RNA-based therapies in this class of tumors.

This article is categorized under:
RNA Processing > RNA Editing and Modification
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alternative splicing, fusion-driven cancers, oncogenic fusion transcripts, RNA-based therapeutics

1 | INTRODUCTION

Gene fusion events that deregulate protein expression or generate a chimeric protein are associated with the pathology of several cancer types, accounting for approximately 20% of tumors overall (Mitelman, Johansson, & Mertens, 2007). Most tumorigenic gene fusions comprise of regulatory and protein-encoding sequences from two different genes and, depending on the fusion gene, one or both the partner genes can contribute to oncogenesis (Mertens, Johansson, Fioretos, & Mitelman, 2015). Examples of pathologically relevant gene fusion events include the juxtaposition of promoter and enhancer sequences close to a proto-oncogene, the disruption of a tumor suppressor gene, or the creation of a fusion protein with aberrant functionality

(Mertens et al., 2015). In the latter case, gene fusions can act as the primary driver mutation in several subtypes of hematological (Gianfelici, Lahortiga, & Cools, 2012; Gough, Slape, & Aplan, 2011; Roberts & Mullighan, 2015; Wright & Vaughan, 2014) and soft tissue tumors (Mertens, Antonescu, & Mitelman, 2016). Next-generation sequencing efforts have also shown the importance of gene fusions in the development of some epithelial cancer types (Kumar-Sinha, Kalyana-Sundaram, & Chinnaiyan, 2015; Matissek et al., 2018). Significantly, tumor-specific gene fusion studies have advanced our understanding of the mechanistic basis of neoplasia and, in the case of fusion kinases, have impacted the treatment of many cancer types (Cuellar, Vozniak, Rhodes, Forcello, & Olszta, 2018; Druker et al., 2001; Holla et al., 2017; Thomas & Heiblig, 2016).

Structural genomic rearrangements (e.g., inter- and intra-chromosomal translocations, insertions, inversions, deletions) are the most common events that generate gene fusions (Mertens et al., 2015). There are rare reports of fusion transcripts generated by non-structural genomic rearrangement mechanisms (e.g., transcription read-through), but the functions of these molecules are poorly defined (Jividen & Li, 2014; H. Li, Wang, Ma, & Sklar, 2009; Varley et al., 2014; Zhang et al., 2012). The best-characterized fusion oncogenes occur as a result of chromosomal rearrangements with over 500 genes that recurrently fuse to one or more gene partners in defined tumor types (Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer, 2019). Transcriptomic profiling using deep-sequencing technologies and new computational tools has resulted in an enormous increase in the reports of gene fusion events in individual tumors (Kumar, Razzaq, Vo, Gautam, & Li, 2016). In most cases, the fusion events identified by sequencing are non-recurrent and are thus less likely to contribute to tumorigenesis (Johansson et al., 2019). However, the accurate detection of many recurrent, clinically relevant fusion transcripts is a critical tool needed for the diagnosis and treatment of a subset of cancers (Kumar et al., 2016). For example, a recent RNA sequencing analysis of over 9,500 tumor samples from 33 cancer types detected the presence of *TMPRSS2-ERG* fusions in 38.2% of prostate adenocarcinoma; *FGFR2-BICC1* fusions in 5.6% of cholangiocarcinoma; *CCDC6-RET* fusion in 4.2% of thyroid carcinomas; *FGFR3-TACC3* fusions in 1–2% of bladder, cervical, and lung cancers; and *EML4-ALK* fusions in ~1% of lung adenocarcinoma (Gao et al., 2018). Another recent study by Heyer et al. (2019) showed that targeted RNA sequencing of fusion transcripts improves the diagnostic rate from 63 to 76% compared to conventional approaches in a cohort of 72 clinical patient samples. This clinical cohort represented a wide range of cancer samples, comprising of 40 solid tumors and 32 hematological malignancies.

Advanced detection methods of fusion transcripts can provide quantitative expression data for multiple fusion genes in a single clinical sample, which can aid in both fusion oncogene diagnosis and classification of cancer subtypes. However, the use of this information for the development of therapeutic strategies has proven challenging to date, even though only tumor cells express the fusion oncogene products—RNA and protein—and thus, in many cases represent ideal molecular targets. Although the targeting of fusion kinase proteins has proven clinically beneficial, the occurrence of drug resistance has complicated the success of even this approach (Rossari, Minutolo, & Orciuolo, 2018). Some of the first studies aiming to use RNA-based technologies using antisense oligonucleotides (ASOs) or mediators of RNAi targeted fusion transcripts (e.g., *BCR-ABL* and *EWS-FLI1*; Dohjima, Lee, Li, Ohno, & Rossi, 2003; Skorski et al., 1994). In fact, the success of early basic and pre-clinical studies targeting the *BCR-ABL* fusion transcript stimulated some of the first clinical trials of these approaches (de Fabritiis et al., 1998; Koldehoff, Steckel, Beelen, & Elmaagacli, 2007), but unfortunately, no long-term strategies emerged for targeting these RNA-related vulnerabilities found in fusion-dependent cancers. Nevertheless, there has been significant achievements within the past few years applying RNA-focused strategies to treat genetic diseases (Crooke, Witztum, Bennett, & Baker, 2018; Setten, Rossi, & Han, 2019). This recent success leads us to ask the question—*Is this the time to reassess the feasibility of similar therapeutic strategies to directly target fusion transcripts in cancers that depend on their translated products*?

Here, using case studies, we highlight how the genetic organization of fusion oncogenes impacts the generation of translatable mature mRNAs. We discuss how the complexity of fusion transcripts expressed in specified tumor types may influence disease pathology and treatment, and how this may complicate the application of RNA-based strategies to target fusion-driven cancers. We also emphasize how little we know about the proteins required for processing transcripts that contain regulatory sequences derived from two different genes. Furthermore, using our recent studies of the *EWS-FLI1* fusion oncogene, we demonstrate how functional genetic approaches could be used to address this lack of knowledge. Finally, we aim to show that with careful assessment of RNA-related vulnerabilities inherent to fusion-driven cancers, it should be possible to develop RNA-based therapies against fusion transcripts for some tumors that are dependent on the activity of their translated proteins.

2 | ONCOGENIC FUSION TRANSCRIPTS—THE JOINING OF PARTS

Despite a significant increase in the ability to characterize the genomic structure of fusion genes and detect the transcripts they express, the complex molecular processes required for the generation of a translatable product from these atypical genes remains relatively unexplored. Most factors that modulate splicing of the wild-type counterpart transcripts will affect the

processing of the corresponding fusion transcripts. However, it is currently ambiguous how exon/intron definition around fusion transcript junctions influence the RNA splicing outcomes required for the expression of an in-frame fusion product. Furthermore, many fusion genes express transcript variants that potentially impact disease pathology or the response to treatment. But, the proteins responsible for regulating the alternative splicing events that give rise to these transcript variants remain poorly characterized. Bridging this gap could have profound implications, as it would allow for the development of new therapies that block the biogenesis of the fusion transcript and deplete expression of the oncoprotein it encodes. Before discussing the layer of complexity observed when a cancer cell expresses a fusion precursor messenger RNA (pre-mRNA), we will briefly describe the processes utilized to generate the diversity of mRNAs expressed in all human cells.

Precursor messenger RNA splicing is a vital process that intricately regulates gene expression and the protein diversity observed in complex organisms (Braunschweig, Gueroussov, Plocik, Graveley, & Blencowe, 2013; Irimia & Blencowe, 2012; Nilsen & Graveley, 2010). Splicing of pre-mRNAs involves the recognition and removal of noncoding regions (intron excision) and joining of coding regions (exon ligation) to generate a mature mRNA. The spliceosome, a dynamic ribonucleoprotein machine that incorporates the stepwise assembly and disassembly of several hundred proteins and five small nuclear RNAs, catalyzes this process. For further details, please see the following reviews: Jurica and Moore (2003), Shi (2017), and Sperling (2017). The architecture of pre-mRNAs determines whether the spliceosome components recognize splice sites across introns or exons and modifies spliceosome assembly (Berget, 1995; Zhu et al., 2009). If exons are long and separated by short introns (<250 bp), then the splicing machinery is more likely to form across introns—the intron definition model. In contrast, if exons are short and separated by long introns (>250 bp), then the splicing machinery can form across exons—the exon definition model. The efficiency of juxtaposing smaller units of either exons or introns compared to larger units is considered a critical determinant of these splice-site pairing mechanisms. In addition to the importance of splice-site recognition and pre-mRNA architecture in spliceosome assembly, short degenerate sequences found within introns and exons can recruit trans-splicing auxiliary proteins, such as members of the serine/arginine-rich (SR) or heterogeneous nuclear ribonucleoproteins (hnRNPs) protein families to regulate alternative splicing. These sites are exonic/intronic splicing enhancers and exonic/intronic splicing silencers, which can synergistically or antagonistically change the efficiency of spliceosome formation. For more information on elements that influence spliceosome assembly and pre-mRNA splicing, we refer the reader to the following advanced reviews by De Conti, Baralle, & Buratti, 2013 and Keren, Lev-Maor, & Ast, 2010.

RNA processing of transcripts that arise from gene fusions adds another layer of complexity to the splicing process. The intronic and exonic regulatory sequences derived from two different genes need to be compatible with the efficient formation of the spliceosome to initiate canonical and alternative splicing. This compatibility is essential at regulatory sites nearby and surrounding the fusion junction sites. Figure 1 depicts plausible splicing events that may lead to the generation of the predominant and/or variant fusion transcripts.

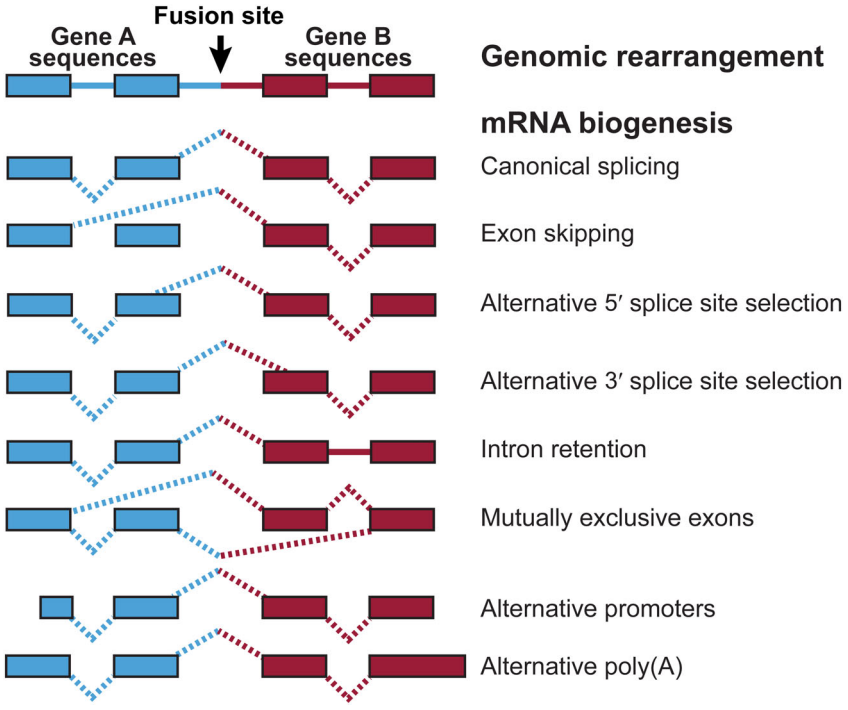

FIGURE 1 Canonical and alternative splicing events for a representative fusion transcript. Boxes and solid lines indicate exons and introns, respectively. The dashed lines indicate possible splicing events in the context of exons adjacent to a fusion breakpoint

Analysis of the mRNAs expressed from many fusion oncogenes has shown the expression of variant transcripts resulting from alternative splicing, including those expressed by the *BCR-ABL1*, *RUNX1-RUNX1T1*, and *TMPRSS2-ERG* fusion genes. These findings are not surprising since ~95% of multiexon genes are alternatively spliced (Pan, Shai, Lee, Frey, & Blencowe, 2008). In fact, to date, there are over 11,000 chimeric transcripts associated with cancer in the ChiTaRS database (Gorohovski et al., 2017). While many represent minor transcript species, in some cases, the dominant oncogenic fusion transcript that encodes the protein required for the initiation and maintenance of tumorigenesis depends on a specific RNA processing event (e.g., exclusion of one or more exons). Thus, dissecting the RNA and protein factors that antagonistically and synergistically decide the fate of exon/introns in these fusion transcripts could lead to potential targetable vulnerabilities to treat various cancer types. Overall, these factors are potentially driven by (a) distinctive RNA sequence and/or structural features within fusion transcripts and (b) protein factors that participate in a discrete splice-site pairing when processing the fusion transcript. The following sections discuss some of these features in the context of representative fusion genes associated with different cancers and their potential relevance to disease development and current or future treatment.

3 | ONCOGENIC FUSION TRANSCRIPTS—PARTNERS IN CANCER

3.1 | *BCR-ABL1* in leukemia

The discovery of fusion oncogenes began with the study of hematological malignancies that harbor recurrent gross chromosomal rearrangements (Rowley, 1984), the first being *BCR-ABL1* (Ben-Neriah, Daley, Mes-Masson, Witte, & Baltimore, 1986). *BCR-ABL1* is generated from a reciprocal translocation, t(9;22)(q34;q11), and is a hallmark of chronic myeloid leukemia (CML) (Figure 2a). Soon after the identification of the *BCR-ABL1* fusion oncogene, reports of variant transcripts derived from the use of alternative *ABL* 5′ exons emerged (Shtivelman, Lifshitz, Gale, Roe, & Canaani, 1986). However, further studies demonstrated that while translocation of the 5′ end of the *ABL* gene can result in the inclusion of different first exons at a genomic level, upon RNA processing, the majority of transcripts include one 5′ *ABL1* exon, which is typically referred to as *ABL1* exon a2 (reviewed in Barnes & Melo, 2002; Kurzrock, Gutterman, & Talpaz, 1988; Melo, 1996). In contrast, the three regions in the *BCR* locus that are the target of rearrangements can lead to the expression of different transcripts encoding the principal BCR-ABL1 isoforms defined by their approximate molecular weights, p210, p190, and p230. Each BCR-ABL1 isoform persistently enhances tyrosine kinase activity resulting in altered downstream signaling pathways and transformation (Branford, Rudzki, & Hughes, 2000; Chan et al., 1987; Demehri et al., 2005; Hochhaus et al., 1996; LaFiura et al., 2008; S. Li, Ilaria, Million, Daley, & Van Etten, 1999; Saglio et al., 1996).

The variant *BCR-ABL1* transcripts predominantly observed in CML involve exons 13 or 14 of *BCR* (*13a2* or *14a2*) that encode the p210 isoforms. Some tumors can express both the *13a2* and *14a2* transcripts as a result of alternative splicing. The *e1a2 BCR-ABL1* fusion is observed in CML but is found more frequently in acute lymphoblastic leukemia. The p230-coding transcripts that result from breakpoints in intron 19 of *BCR* are relatively rare, as are other transcripts that account for less than 2% of *BCR-ABL1* positive tumors (Weerkamp et al., 2009). A recent retrospective analysis of the *BCR-ABL1* fusion transcripts expressed in over 45,000 newly diagnosed CML cases showed that 37.9% of tumors express *e13a2-BCR-ABL1* transcripts and 62.1% of tumors express *e14a2-BCR-ABL1* (Baccarani et al., 2019). Depending on the cohort, between ~2 and 19% of tumors co-express the *e13a2* and *e14a2-BCR-ABL1* transcripts. Curiously, though many studies have defined the complex

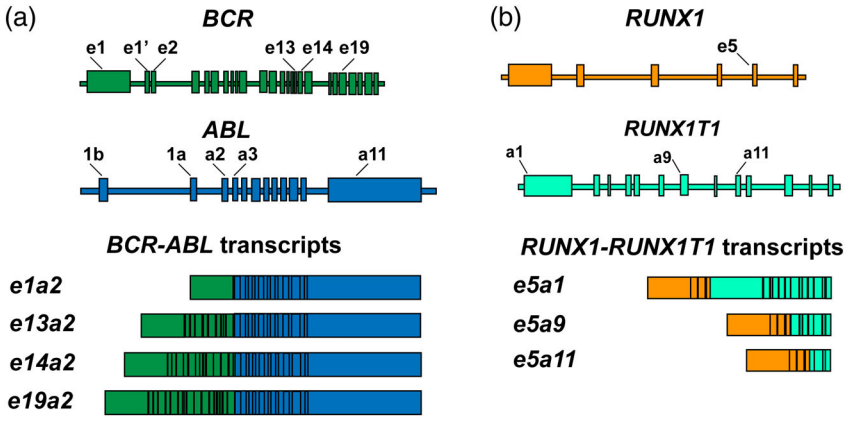

FIGURE 2 Schematic depictions of representative transcripts expressed from fusion genes generated by chromosomal rearrangements that disrupt (a) the *BCR* and *ABL* genes (t(9;22)(q34;q11)). (Reprinted with permission from Deininger, Goldman, and Melo (2000). Copyright 2000 American Society of Hematology). (b) The *RUNX1* and *RUNX1T1* genes (ins(21;20)(q22;q11q11)). Genes and transcripts (not to scale) are shown 5 to 3′. (Adapted with permission from Lam and Zhang (2012))

structure of the in-frame transcripts that express the different versions of the BCR-ABL1 oncoprotein, to date, the proteins that contribute to the biogenesis of these oncogenic transcripts are unknown.

Although the use of tyrosine kinase inhibitors (TKIs), such as imatinib, nilotinib, and dasatinib that target BCR-ABL1 have proven enormously successful, drug resistance occurs in 10–20% of patients with CML. In most cases, point mutations are responsible for TKI resistance, but the detection of new or altered levels of variant *BCR-ABL1* transcripts in addition to the wild-type *BCR-ABL1* in drug-treated CML samples has led to consideration of alternative splicing as another possible mechanism of resistance (Laudadio, Deininger, Mauro, Druker, & Press, 2008; Lee et al., 2008; Ma et al., 2009; Marin et al., 2008; Talpaz et al., 2006; White et al., 2006, 2010). Mutations in *BCR-ABL1* that alter the processing of the transcript include a 35-nucleotide insertion of sequences between *ABL1* exons 8 and 9, first identified in a patient with imatinib resistance (Ma et al., 2009) that results in the expression of a truncated protein. One follow-up study detected *BCR-ABL1*^{ins35bp} transcripts in only four of 20 patients, and the presence of this mutation did not track with treatment response (O'Hare et al., 2011). Furthermore, heterologous expression of BCR-ABL1^{ins35bp} mutant protein failed to induce resistance to imatinib (O'Hare et al., 2011). However, a second retrospective review of over 250 cases of CML that analyzed the presence of kinase mutations detected *BCR-ABL1*^{ins35bp} in 23% of samples, all of which came from patients that exhibited disease progression or treatment resistance (Berman et al., 2016). Using long-range sequencing of the *BCR-ABL1* transcripts present at different phases of disease, the *BCR-ABL1*^{ins35bp} variant was also detected in one patient that represented 12% of total *BCR-ABL1* mRNA 7 months after diagnosis and 38% after 13 months. Imatinib failed to mediate a clinical response in this patient, but nilotinib therapy proved efficacious. Other *BCR-ABL1* isoforms detected by long-range sequencing of samples from this patient included isoforms that retained additional *BCR*-derived sequences or deletion of part of *ABL1* exon 7. A previous study also observed alternative splicing events that remove *ABL1-exon 7* sequences, as well as the presence of rare *BCR-ABL1-e6a2* and *e19a2* transcripts (Gruber et al., 2012). While the biological significance of these *BCR-ABL1* variant molecules is unknown, the recurrent nature of some of these variant mRNAs suggests that changes in the processing of the *BCR-ABL1* pre-mRNA during disease progression and treatment warrants further study.

3.2 | *RUNX1-RUNX1T1* in acute myeloid leukemia

One of the earliest discovered chromosomal aberrations after *BCR-ABL1* was a consistent nonhomologous balanced translocation between chromosomes 8 and 21 in leukemic patients. The t(8;21) translocation is one of the most common genetic defects in acute myeloid leukemia, and this chromosome abnormality results in the *RUNX1-RUNX1T1* fusion gene (formerly called *AML1-ETO*) (Muller, Duque, Shizuru, & Lubbert, 2008; Figure 2b). The RUNX1-RUNX1T1 fusion protein (also known as RUNX1-ETO or RUNX1-CBFA2T1) is thought to interact with other proteins to repress transcription and cooperates with additional secondary events, including mutations in c-*KIT*, *FLT3*, or *PDGFRα/β* to trigger leukemogenesis in myeloid progenitor cells (Beghini et al., 2000; Hatlen, Wang, & Nimer, 2012; Higuchi et al., 2002; Yuan et al., 2001).

Interestingly, several studies have demonstrated a wide range of variant protein-coding and noncoding transcripts generated from the *RUNX1-RUNX1T1* gene related to the utilization of different *RUNX1* promoters and alternative *RUNX1T1* exons (Era et al., 1995; Erickson et al., 1992; Kozu, Fukuyama, Yamami, Akagi, & Kaneko, 2005; Kozu et al., 1993; LaFiura et al., 2008; Mannari, Gascoyne, Dunne, Chaplin, & Young, 2010; M. Yan et al., 2006). The breakpoints in the *RUNX1* gene cluster in intron 5 and the breakpoints in *RUNX1T1* cluster in intron 1. One *RUNX1-RUNX1T1* transcript variant uses an alternative *RUNX1T1* exon 9 (9a) and expresses a protein lacking the C-terminal domain that inhibits the transcriptional activation of RUNX1 (M. Yan et al., 2006). Expression of this variant fusion protein in a mouse model enhanced leukemogenesis (M. Yan et al., 2006). Another variant that occurs as a consequence of the alternative use *RUNX1T1* exon 11 (11a) also results in the expression of a fusion protein lacking a C-terminal domain (Kozu et al., 2005). However, the impact of either variant on disease progression is unclear. Other rare variants detected in acute myeloid leukemia (AML) samples include transcripts containing a partial deletion of *RUNX1T1*-exon 3 with or without other changes (LaFiura et al., 2008; Mannari et al., 2010).

Similar to *BCR-ABL*, we know little about the proteins that facilitate the extensive alternative splicing of the *RUNX1-RUNX1T1*. One study attempted to begin to address this issue by examining the diversity of fusion transcripts present in AML cell lines and tumor samples and linking this to the recognition sequence motifs of different RNA processing proteins (Grinev et al., 2015). An analysis of over 100 possible *RUNX1-RUNX1T1* variant transcripts revealed a diverse pool of full-length and truncated mRNA products formed from over 150 splicing events that map to 23 reference exons. Next, to identify *cis*-regulatory elements that could mediate the alternative splicing of *RUNX1-RUNX1T1*, Grinev and coworkers determined the enrichment for sequence motifs within each exon and flanking intron sequences. Their results highlighted the presence of RBFOX3 binding sites in the flanking regions of introns of some *RUNX1-RUNX1T1* exons, and the study also showed AML

cell lines and tumor samples express *RBFOX3* mRNA, whereas control samples do not. However, this analysis did not link any RNA processing factor to specific *RUNX1-RUNX1T1* transcripts such as the 9a variant.

3.3 | *EML4-ALK* in non-small cell lung cancer

An alternative approach to the discovery of fusion oncogenes involves the use of cDNA expression libraries generated using RNA harvested from tumor samples. In 2007, this strategy identified *EML4-ALK* as a novel fusion oncogenic driver of a subset of non-small cell lung carcinoma (NSCLC) (Soda et al., 2007). Subsequent studies showed a chromosomal inversion on chromosome 2 (inv2) (p21:p23) fuses parts of the *EML4* and *ALK* genes (Figure 3a). This inversion places the kinase domain of the receptor tyrosine kinase ALK under control of the constitutive promoter of *EML4*, enabling activated ALK signaling through well-established downstream pathways and malignant transformation (Rikova et al., 2007). The initial description of *EML4-ALK* fusion transcripts identified fusion of exon 13 or exon 20 of *EML4* to exon 20 of *ALK*. The study of further NSCLC samples showed that the breakpoints in the *ALK* gene predominantly map to intron 19, but the breakpoints in *EML4* can occur in several introns, including 6 (variant 3), 13 (variant 1), and 20 (variant 2) (Choi et al., 2008; Soda et al., 2007). Alternative splicing can generate two forms of variant 3; variant 3a contains *EML4* exon 6a and variant 3b contains *EML4* exons 6a and 6b. There are also reports of two variants (5a and b) involving breakpoints in *EML4* intron 2. The variant *EML4-ALK* 5 fusions involve the same gene rearrangement in *EML4* intron 2, but the processing of the variant 5b transcript includes an alternative splicing event that incorporates 117 nucleotides from the intron 2 into the fusion mRNA (Lin et al., 2018; Rikova et al., 2007; Soda et al., 2007; Yoshida et al., 2016).

Overall, 4–5% of NSCLC tumors contain ALK-rearrangements (Boland et al., 2009; Shaw et al., 2009; Soda et al., 2007; Takeuchi et al., 2008), which has enabled the clinical use of ALK TKI, such as crizotinib or alectinib, to treat this sub-group of patients. However, the clinical response to ALK TKI is variable, leading to speculation that ALK isoforms may exhibit different sensitivities to drug inhibition (Ali et al., 2016; Heuckmann et al., 2012; Woo et al., 2017; Yoshida et al., 2016). More recently though, an interim analysis of a Phase III clinical trial in ALK-positive NSCLC indicated no association between the clinical efficacy of alectinib or crizotinib and the EML4-ALK fusion type, although in some cases the sample numbers remain low and further analysis is ongoing to further investigate this question (Camidge et al., 2019). Also, while the availability of ALK inhibitors has proven a significant step forward for the treatment of at least a proportion of lung cancer patients (Kwak et al., 2010 and reviewed in Sasaki, Rodig, Chirieac, & Janne, 2010), resistance mechanisms are emerging. In the long-term, an improved understanding of how *EML4-ALK* fusion pre-mRNAs are processed could assist with the development of new treatment strategies for primary and refractory disease.

3.4 | *NUT*-fusions in NUT-midline carcinoma

Nuclear protein in testis (NUT)-midline carcinoma is a rare and highly aggressive cancer typically caused by the translocation t(15;19). In most cases, the testis-specific nuclear gene *NUTM1* is fused to the bromodomain-containing protein 4 (BRD4)

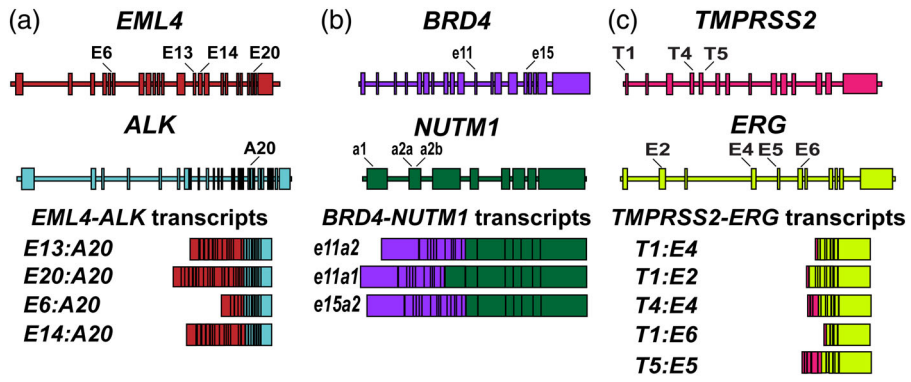

FIGURE 3 Schematic depictions of representative transcripts expressed from fusion genes generated by chromosomal rearrangements that disrupt (a) the *EML4* and *ALK* genes (inv(2)(p21p23)). (Adapted from Sabir, Yeoh, Jackson, and Bayliss (2017)), (b) the BRD4 and NUTM1 genes (t(15;19)(q14;p13)) (Adapted with permission from Thompson-Wicking et al. (2013). Copyright 2013 Springer Nature), and (c) the *TMPRSS2* and *ERG* genes (del(21)(q22q22)). Genes and transcripts (not to scale) are shown 5 to 3′. (Adapted with permission from Clark et al. (2007). Copyright 2007 Springer Nature)

gene (French et al., 2003; Figure 3b). There are also rarer rearrangements involving *BRD3*, *NSD3*, ZNF532, or unidentified 5′ partner genes (French, 2018). Unfortunately, patients with t(15;19)-positive carcinomas respond poorly to standard chemotherapeutic treatment and this cancer subtype rapidly metastasizes (French, 2012, 2018). The fusion protein BRD4-NUT drives this disease by disrupting cell differentiation and promoting the growth of carcinoma cells through several molecular mechanisms (French et al., 2008; R. Wang & You, 2015; J. Yan, Diaz, Jiao, Wang, & You, 2011). For instance, the fusion partner BRD4 can tether NUT to acetylated chromatin via the bromodomain and then the putative transcriptional domain in NUT can subsequently recruit p300 and activate its histone acetyltransferase activity (Alekseyenko et al., 2015; Reynoird et al., 2010).

Most *BRD4-NUT* fusion mRNAs involve alignment of exons 1–11 of *BRD4* to *NUTM1* exons 2–7, but recent studies have detected variants that include a dependency on particular splicing events to generate in-frame transcripts. For example, nested polymerase chain reaction (PCR)-based studies by Thompson-Wicking et al. (2013), identified fusion genomic breakpoints upstream of the first exon of *NUT*. Critically, this exon must be removed to generate the in-frame protein-coding transcripts. In another case, a rearrangement that fuses *BRD4* exon 15 to the last part of *NUTM1* exon 2 results in the use of a cryptic splice site that serves to maintain an open reading frame. To date, it is unknown which splicing factors contribute to the recognition or resolution of these splicing events.

3.5 | *TMPRSS2-ERG* in prostate cancer

The fusion gene *TMPRSS2-ERG* is prevalent in prostate cancer and places the expression of the transcription factor ERG under the control of the androgen-regulated *TMPRSS2* promoter (Figure 3c; Tomlins et al., 2005). Fusion-driven events result in increased aberrant expression of ERG that regulates diverse cellular process associated with cancer metastasis (for reviews, please see Adamo & Ladomery, 2016; Kumar-Sinha, Tomlins, & Chinnaiyan, 2008; Shah & Chinnaiyan, 2009). Other *TMPRSS2* fusions include alignment of *TMPRSS2* sequences with either *ETV1* or *ETV4* (Perner et al., 2006; Tomlins et al., 2005). *TMPRSS2-ERG* fusions can arise as a consequence of either an intrachromosomal translocation or deletion as the *TMPRSS2* and *ERG* genes map to the same arm of chromosome 21. The position of the breakpoints in each fusion partner and alternative splicing of *ERG* exons both contribute to the diversity of *TMPRSS2-ERG* fusion transcripts observed in fusion gene positive prostate cancer. Distinct fusion transcripts include exons 1, 2, and 3 of *TMPRSS2* spliced to *ERG* exons 2, 3, 4, or 6 that can generate either full-length ERG protein, N-terminal truncated ERG proteins or a TMPRSS2-ERG fusion protein (Clark et al., 2007; Hagen et al., 2014; Hu et al., 2008; Perner et al., 2006; Tomlins et al., 2005). A study of localized and invasive prostate cancers also showed an increase in the exclusion of two cassette exons (72- and 81-bp exon) within *ERG* (Hagen et al., 2014). The retention of the 72-bp exon in *ERG* leads to increased cell proliferation and invasion (J. Wang et al., 2008). Moreover, a recent study utilized splice-switching oligonucleotides to target the 3′ splice site of the 72-bp exon in *ERG* and induce exon skipping in VCaP prostate cancer cells (Jumbe et al., 2019). Induction of exon skipping reduced both cell invasion and proliferation and stimulated apoptosis. Unfortunately, as with other fusion transcripts, we still know little about the proteins and other recognition sequences that regulate the processing of *TMPRSS2-ERG* pre-mRNAs.

3.6 | *EWS-FLI1* and *EWS-ERG* in Ewing sarcoma

The primary oncogenic event in most Ewing sarcoma (EWS) tumors involves either a t(11;22) or t(21;22) translocation that fuses the 5′ end of the *EWSR1* gene to the 3′ end of either the *FLI1* (~85% of cases) or *ERG* (~10% of cases) genes, generating the fusion genes *EWS-FLI1* or *EWS-ERG*. (Delattre et al., 1992; May et al., 1993; Sorensen et al., 1994). The most common *EWS-FLI1* fusion transcripts involve alignment of the first seven exons of *EWSR1* spliced to either exons 5–9 (type 2 fusion subtype) or 6–9 (type 1 fusion subtype) of *FLI1*, while the most *EWS-ERG* fusion transcripts align *EWSR1* exons 1–7 to exons 8 or 11 of *ERG* (COSMIC, 2019 database; Figure 4a). The transcripts expressed by these fusion genes encode different variants of the chimeric transcription factor EWS-FLI1 or EWS-ERG. These transcription factors upregulate genes in cell cycle, invasion and proliferation pathways (Bailly et al., 1994; Braun, Frieden, Lessnick, May, & Denny, 1995; Dauphinot et al., 2001; Nagano et al., 2010), and repress the expression of tumor suppressor genes (Hahm et al., 1999). In some cases, expression of an in-frame *EWS-FLI1* fusion transcript requires exon exclusion. For example, retention of *EWSR1* exon 8 at the genomic level necessitates the removal of this exon during pre-mRNA processing to generate an in-frame fusion transcripts (Berger et al., 2013; Crompton et al., 2014; Patocs et al., 2013; Zoubek et al., 1994; Zucman et al., 1993). One study showed 15 of 42 EWS tumors and four cell lines harbor translocations in which the *EWSR1* exon 8 is spliced out at a posttranscriptional level (Berger et al., 2013), consistent with an overall frequency of approximately one-third of EWS-FLI1 positive

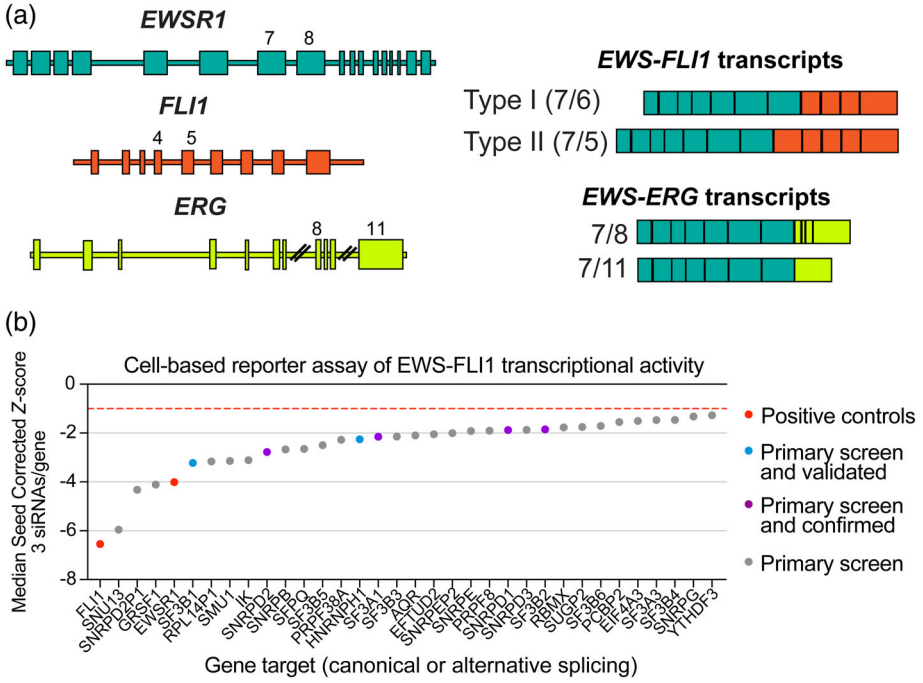

FIGURE 4 (a) Schematic depictions of representative transcripts expressed from fusion genes generated by chromosomal rearrangements (t11;22)(q24;q12) and t(21;22)(q21;q12) that disrupt the *EWSR1* and *FLI1* or *ERG* genes. (Adapted with permission from Sankar and Lessnick (2011). Copyright 2011 Elsevier) and COSMIC resources (COSMIC). (b) Candidate genes associated with canonical or alternative splicing identified by a RNAi screen of EWS-FLI1 activity. The median seed corrected *Z*-score obtained by the difference between Z_{NROB1} and Z_{CMV} (three siRNAs per gene) for 35 genes that exhibited a selective decrease (*Z*-score < -1) in the TC32-NR0B1-luc reporter when silenced. (See Pubchem BioAssay AID: 1159506 and Grohar et al., (2016))

tumors (Hawkins et al., 2011). Critically, study of this particular variant has offered one of the first opportunities to identify a protein required for the biogenesis of an oncogenic fusion transcript.

4 | THE MAKING OF A FUSION TRANSCRIPT: INSIGHTS FROM A FUNCTIONAL GENETIC SCREEN

Recently, our group performed a genome-wide RNAi screen in the TC32 EWS cell line modified to report a readout of EWS-FLI1 activity. The screen identified over 30 candidate genes that selectively decreased EWS-FLI1 activity and are associated with canonical or alternative splicing (Figure 4b; Grohar et al., 2016). One of the lead candidate genes identified by this screen was HNRNPH1, a member of the hnRNP family of RNA binding proteins that include H2 and F. The HNRNPH/F proteins are involved in pre-mRNA processing, particularly alternative splicing (Geuens, Bouhy, & Timmerman, 2016). The TC32 cell line used for the RNAi screen harbors a translocation in which the *EWSR1* exon 8 is spliced out at a posttranscriptional level (Berger et al., 2013). We thus hypothesized that the exclusion of *EWSR1* exon 8 in TC32 cells requires HNRNPH1. Analysis of *HNRNPH1*-silenced TC32 cells revealed that exclusion of *EWSR1* exon 8 requires HNRNPH1 and loss of HNRNPH1 results in the retention of *EWSR1* exon 8. These results were confirmed in a second EWS cell line, SKNMC that retains *EWSR1* exon 8 at the genomic level. The failure to splice out *EWSR1* exon 8 in these cell lines results in reduced expression of *EWS-FLI1* mRNA and protein, and reversal of EWS-FLI1 driven expression. EWS cell lines harboring Chr. 22 breakpoints in *EWSR1* intron 7 (TC71 and RD-ES) exhibited no dependence on the expression of HNRNPH1. Furthermore, immunoprecipitation of HNRNPH1-bound RNA indicated direct binding of HNRNPH1 to *EWSR1* exon 8, and an in vitro binding assay showed HNRNPH1 binds G-rich RNA sequences at the 3′ end of *EWSR1* exon 8 (Grohar et al., 2016). Together, our results suggest that the inhibition of the *EWSR1* exon exclusion event regulated by HNRNPH1 could block the expression of the EWS-FLI1 fusion oncoprotein expressed in about a quarter to a third of cases of EWS.

Interestingly, the RNAi screen also identified many components of the spliceosome as selectively required for EWS-FLI1 activity including, *SF3A1*, *SF3B1*, *SF3B2*, *SNRPD1*, and *SNRPD2* (Figure 4b). To understand the mechanistic basis for this finding, we focused our first study on the gene encoding the catalytic component of the spliceosome, SF3B1. We observed a decrease in *EWS-FLI1* mRNA levels in *SF3B1*-silenced EWS cell lines representing different breakpoints and fusion types; however, we also detected at least one EWS-FLI1 protein variant in SF3B1-depleted ES cells. The detection of EWS-FLI1 protein variants in SF3B1-depleted ES cells led us to hypothesize that altered spliceosome activity results in reduced expression of full-length *EWS-FLI1* and the expression of mis-spliced in-frame *EWS-FLI1* variant transcripts. Using PCR-based analysis, we established that SF3B1-depleted EWS cells mis-splice *EWS-FLI1* and that some of the mis-spliced products are in-frame, explaining the presence of at least one EWS-FLI1 protein variant. To further understand the consequences of

inhibiting spliceosome function on the splicing of *EWS-FLI1*, we next employed a pharmacological inhibitor of the SF3b spliceosome subunit, Pladienolide B (PlaB). In EWS cells, treatment with PlaB phenocopied the effect of silencing *SF3B1*, including changes in the splicing of the *EWS-FLI1* pre-mRNA, and altered expression of the EWS-FLI1 protein.

One reason for the observed sensitivity to the inhibition of canonical splicing may relate to differences in the exon–intron architecture of the fusion pre-mRNA versus the wild-type counterpart genes. Most of the breakpoints in *EWSR1* occurring in either intron 7 or 8, which are 1,471 and 2,775 nucleotides in length, respectively. The breakpoints in the *FLI1* locus most commonly occur in introns 4 and 5, which are 8,972 and 23,342 nucleotides, respectively. The fusion intron that combines sequence elements from both *EWSR1* and *FLI1* genes are thus typically over 10,000 nts and includes splice site and branch site sequence derived from two genes (Figure 5a). There is also a complexity of overlapping putative splicing factor motifs identified within the exons surrounding the fusion junction that will affect splicing outcomes (Figure 5b; Desmet et al., 2009). Given the importance of the relative size of exons and introns as a determinant of splicing, the study of how this relationship is disrupted in many fusion pre-mRNAs may yield interesting information about the splicing process and, with the development of splicing inhibitors with in vivo efficacy, a potential therapeutic approach in some fusion-driven tumor types.

5 | SPLICING MODULATION AS A RISING THERAPEUTIC STRATEGY AGAINST FUSION-DRIVEN CANCERS?

There is a range of investigational or approved therapeutic modalities that could be employed to target fusion transcripts. Using the *EWS-FLI1* transcript as an exemplar fusion transcript, we have depicted some of these potential therapeutic approaches in Figure 6. Most of these approaches involve the application of nucleic acid analogues such as ASOs (Figure 6a),

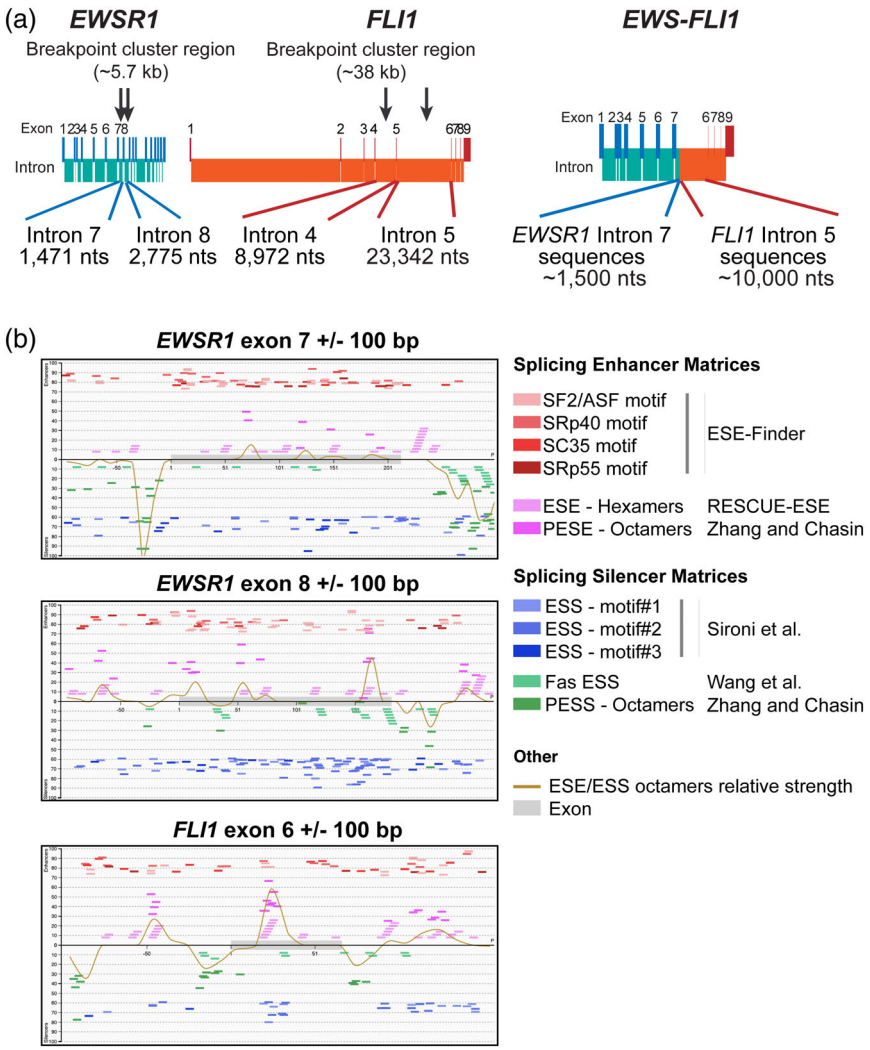

FIGURE 5 (a) Schematic depictions of the *EWSR1*, *FLI1*, and *EWS-FLI1* genes. Indicated are the relative size of each exon (upper) and each intron (lower part of each panel). Genes and transcripts (not to scale) are shown 5 to 3′. (b) Schematic depiction of the putative splicing motifs in regions including and adjacent to *EWSR1* exons 7 and 8 and *FLI1* exon 6 that are involved in *EWS-FLI1* type 1 fusions. Representations are provided by Human Splicing Finder 3.1 (Desmet et al., 2009) for indicated regions within ENST00000406548 (*EWSR1*) or ENST00000527786 (*FLI1*)

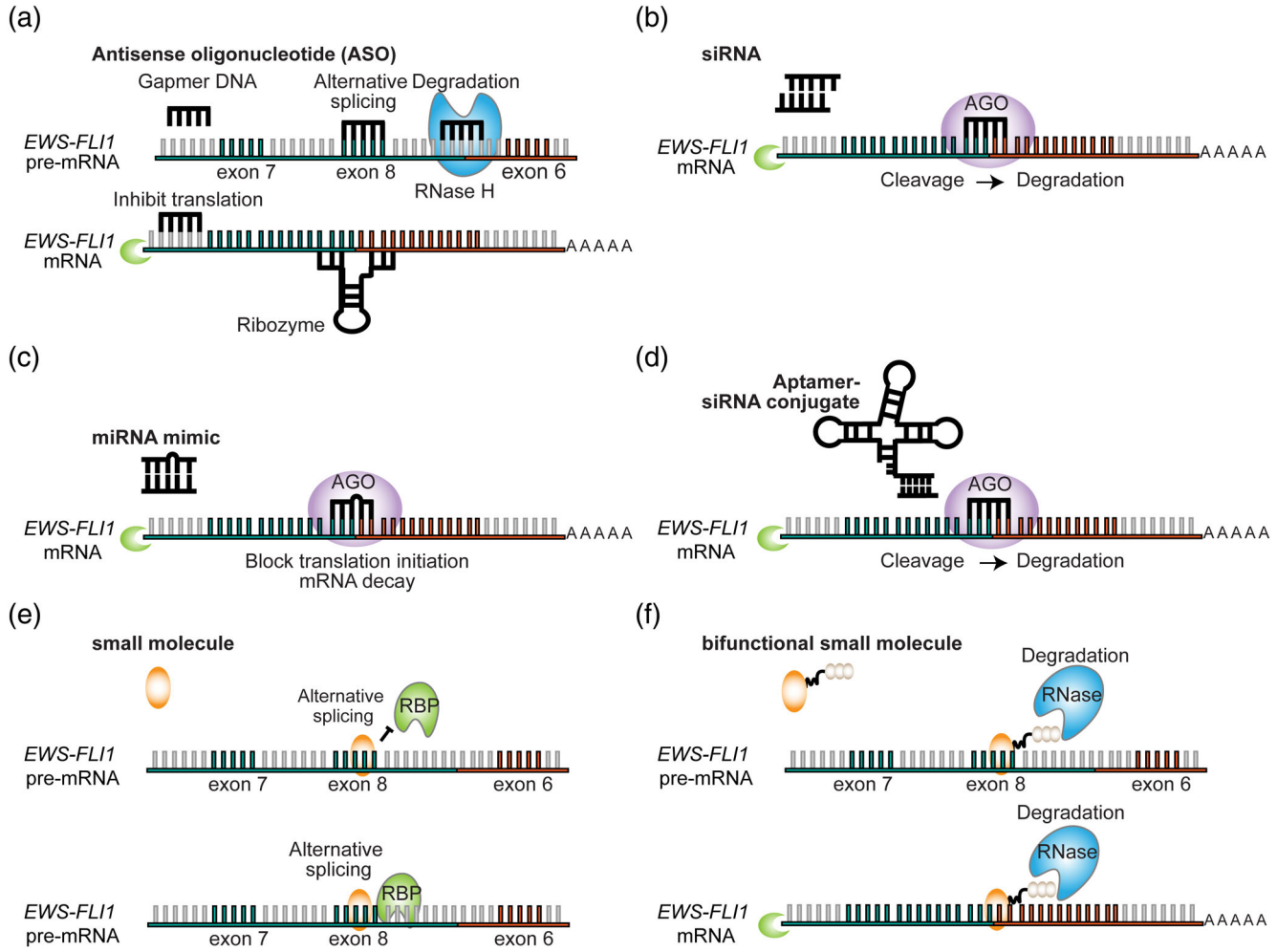

FIGURE 6 Potential therapeutic approaches to target *EWS-FLI1* transcripts using nucleic acid analogues or small molecules. The representations of the mechanism of action of nucleic acid analogues are adapted with permission from Lieberman (2018). Copyright 2018 Springer Nature and encompass some of the major classes of RNA-based drugs. (a) Antisense oligonucleotides that are typically single-stranded with a central DNA gapmer region; (b) double-stranded siRNAs; (c) miRNA mimics; and (d) aptamer-based analogues such as aptamer-siRNA conjugates. Representations of the mechanism of actions for (e) RNA-binding small molecules that modulate the binding of an RNA binding protein (RBP) and (f) bifunctional small molecules that recruit a ribonuclease (RNase)

small interfering RNAs (siRNAs) (Figure 6b), or microRNA (miRNA) mimics (Figure 6c). These nucleic acid analogues undergo complementary base-pairing with its target and modulate endogenous RNA processes. For example, splice-switching ASOs have been shown to base-pair with the pre-mRNA and alter splicing by blocking RNA:RNA base-pairing or protein–RNA interactions (Bauman, Jearawiriyapaisarn, & Kole, 2009; Havens & Hastings, 2016). Critical to the success of small RNA-based therapeutic approaches in recent years were the many breakthroughs in the chemical composition of the phosphate backbone and sugar components of oligonucleotides. Such modifications are needed to enhance the binding affinity, increase in vivo stability, and improve cellular uptake and release of ASOs, siRNAs, and miRNAs (reviewed in Evers, Toonen, & van Roon-Mom, 2015; Saleh, Arzumanov, & Gait, 2012; Sharma & Watts, 2015). Another nucleic acid-based therapeutic strategy involves the use of aptamers. Nucleic-based aptamers fold into distinct tertiary structures and bind to specific molecular targets with high affinity. One application of aptamer-based approaches for the targeting of a fusion transcript could involve the use of an aptamer to enhance the delivery and molecular recognition of an interfering RNA-based drug (Soldevilla, Meraviglia-Crivelli de Caso, Menon, & Pastor, 2018; Figure 6d). Alternatively, an aptamer-based strategy has the potential to act as an agonist or antagonist of an endogenous RNA process by targeting an RNA-binding protein. For more information about RNA-based drug design, we refer the reader to the reviews by Burnett and Rossi (2012) and Lieberman (2018).

Other promising approaches for targeting specific transcripts involves the application of small molecules that selectively interact with RNA (Figure 6e,f). As discussed above, some splicing factors interact with a pre-mRNA to antagonistically or

synergistically decide the fate of particular exon or introns. Studies have shown that the in vitro binding specificities of RNA-binding proteins can differ based on RNA sequence, structure, and contextual features (Dominguez et al., 2018). Thus, the identification of small molecules that bind specific RNA elements within a fusion pre-mRNA could displace the interaction of an RNA-binding protein required for the generation of the mature fusion transcript (Figure 6e, top). Although RNA structures are flexible and dynamic, one can identify RNA-binding small molecules through diverse structurally guided experimental approaches that trap thermodynamically favorable RNA conformations, blocking the recruitment of an RNA-binding protein at a specific sequence (reviewed by Connelly, Moon, & Schneekloth, 2016). Small molecules could also form ternary complexes with fusion pre-mRNAs and RNA-binding proteins, which may activate or inhibit alternative splicing (Figure 6e, bottom). For example, RG-7916 is currently in Phase II clinical trials for various types of spinal muscular atrophy and it functions by modulating pre-mRNA splicing of the survival motor neuron-2 (*SMN2*) gene (Poirier et al., 2018; Ratni et al., 2018; Sturm et al., 2019; and clinicaltrials.gov; NCT03779334). It was shown recently that binding of RG-7916 analogues to *SMN2* pre-mRNA enhances the recruitment of two splicing activators (FUBP1 and KHSRP) and the formation of these ternary complexes contribute to its mechanism of action to enhance SMN2 splicing (J. Wang, Schultz, & Johnson, 2018). Another small molecule-based approach relevant to the targeting of fusion transcripts could involve the use of a bifunctional molecule that both selectively interacts with a transcript and facilitates the localized recruitment of an RNase, triggering the degradation of the targeted RNA (Figure 6f). This approach can be advantageous because linking a ribonuclease recruitment module to an RNA-binding small molecule may enhance selectivity and potency. Recent studies by Costales, Suresh, Vishnu, and Disney (2019) demonstrated the use of a bifunctional molecule as a chimeric recruitment strategy, in which their compound TGP-210-RL interacted with hypoxia-associated noncoding RNA and induced RNA degradation via the recruitment of RNase L. Overall, we anticipate a rise of RNA splicing modulators, both nucleic acid analogues and small molecules, that can selectively redirect alternative splicing as potential fusion-driven anti-cancer therapeutics.

6 | CONCLUSION

Some of the first studies that assessed the feasibility of RNA-based therapeutic strategies targeted the mRNAs expressed by fusion oncogenes. Unfortunately, no active drug development programs emerged from these early studies. The recent approval of the first RNA-based molecules that target transcripts for degradation suggests that this may be the time to relook at oncogenic fusion transcripts as viable candidate targets. However, before embarking on such efforts, we need to more fully appreciate the inter- and intra-tumoral diversity of oncogenic fusion transcripts. In this study, we highlighted the complexity of the fusion transcripts expressed in six cancer subtypes, showing how variations in the position of chromosomal breakpoints and alternative splicing can contribute to the generation of a more heterogeneous population of fusion transcripts than many diagnostic-based assays will detect. We also highlighted our limited knowledge of the proteins that regulate the expression of fusion transcripts and how we overcame this in one case by using a functional genetic approach. We hope our work will stimulate the employment of similar strategies to probe the biogenesis of other fusion mRNAs. With a clearer appreciation of the unusual RNA-related vulnerabilities present in fusion-driven cancers, we consider that this class of tumors will once again be a focus of efforts that will target the lethal transcripts they express.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

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