Chapter 13
The Potential of Targeting Splicing for Cancer Therapy

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Abstract Many molecules currently used to treat cancer patients target proteins encoded by transcripts that are alternatively spliced. As a consequence, the treatment may simultaneously block isoforms with different and sometimes opposing biological activity, thus reducing its efficacy. Recent studies highlight the role of splicing regulation in cancer progression and the importance of the splicing machinery as a therapeutic target. In reviewing this emerging field of cancer biology, we describe very exciting novel findings that illustrate the range of scenarios in which alternative splicing can contribute to all cancer hallmarks, from avoidance of apoptosis to angiogenesis, invasion and acquired resistance to drug therapy. Finally, we address cancer-selective approaches that are being developed to interfere with the splicing machinery and modulate splicing decisions.

Keywords Alternative splicing • Splicing factors • Cancer biology • Cancer therapy • Drug targets

13.1 Introduction

Although cancer is a genetic disease, no single gene defect causes a tumor. Rather, it is only when several genes are altered that cancer arises. Moreover, cancer evolves through successive genetic changes that become advantageous to a cell. In essence, defective genes responsible for tumorigenesis belong to three groups: oncogenes, tumor-suppressor genes and genome stability genes [1]. Defects in oncogenes

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render the gene constitutively active or active under conditions in which the normal gene is not, whereas tumor-suppressor and stability genes become inactivated. These defects in gene function can result from chromosomal translocations, deletions or insertions, amplifications or intragenic mutations. An additional, recently recognized mechanism of oncogene activation or tumor-suppressor and genome stability gene inactivation is alternative splicing. Most human genes produce multiple mRNA isoforms through alternative splicing, and altered splicing is a major contributor to cancer progression. This chapter focuses on the role of alternative splicing in cancer and highlights the therapeutic potential of targeting and modulating cancer-specific splicing isoforms.

13.2 Pre-mRNA Splicing and Its Regulation

RNA splicing was discovered in 1977 as a new mechanism for the biosynthesis of adenovirus mRNA in mammalian cells [2, 3]. Shortly after, cellular genes were also shown to be split into exons and introns. The first examples included the globin [4, 5], the ovalbumin [6] and the immunoglobulin [7] genes. Next, it was recognized that at each intron boundary there are consensus sequences common to vertebrate, plant and yeast cells, suggesting the splicing process was evolutionary conserved [8, 9].

Today we know that the vast majority of human protein-coding genes contain up to 90 % of non-coding sequence in the form of introns that must be spliced from the primary transcripts synthesized by RNA polymerase II (pre-mRNAs). There are over 200,000 different introns in the human genome, ranging in size from <100 to >700,000 nucleotides (nts), with median intron and exon lengths of 1,800 and 123 nts, respectively.

Excision of introns with single nucleotide precision relies on the spliceosome, one of the largest and most elaborate macromolecular machines in the cell [10]. The building blocks of the spliceosome are uridine-rich small nuclear RNAs (UsnRNAs) packaged as ribonucleoprotein particles (snRNPs) that function in conjunction with over 100 distinct non-snRNP auxiliary proteins [11]. The major spliceosomal small nuclear ribonucleoprotein particles comprise the U1, U2, U4, U5 and U6 snRNAs. In addition, human cells have a minor variant form of the spliceosome responsible for excision of about 800 so-called U12-dependent introns that are characterized by a distinct set of splice-site sequences [12–14]. Much of our current understanding of the role of snRNPs in splicing was triggered by studies using human autoantibodies from patients with systemic lupus erythematosus that selectively react with the spliceosomal RNA-protein complexes [15].

The spliceosomal snRNAs recognize, through base pairing, four short consensus sequences termed the exon-intron junctions (5′ splice site and 3′ splice site), the branch point sequence, and the polypyrimidine tract (Fig. 13.1a). Spliceosomes build anew on every intron that is synthesized and then disassemble for the next round of splicing (Fig. 13.1b–e). Assembly of the spliceosome starts with the ATP-independent binding of the U1 snRNP through base-pairing interactions of the 5′ end of the U1 snRNA to the 5′ splice site of the intron. This is followed by the binding of the SF1 protein and the heterodimeric U2 auxiliary factor (U2AF) to the

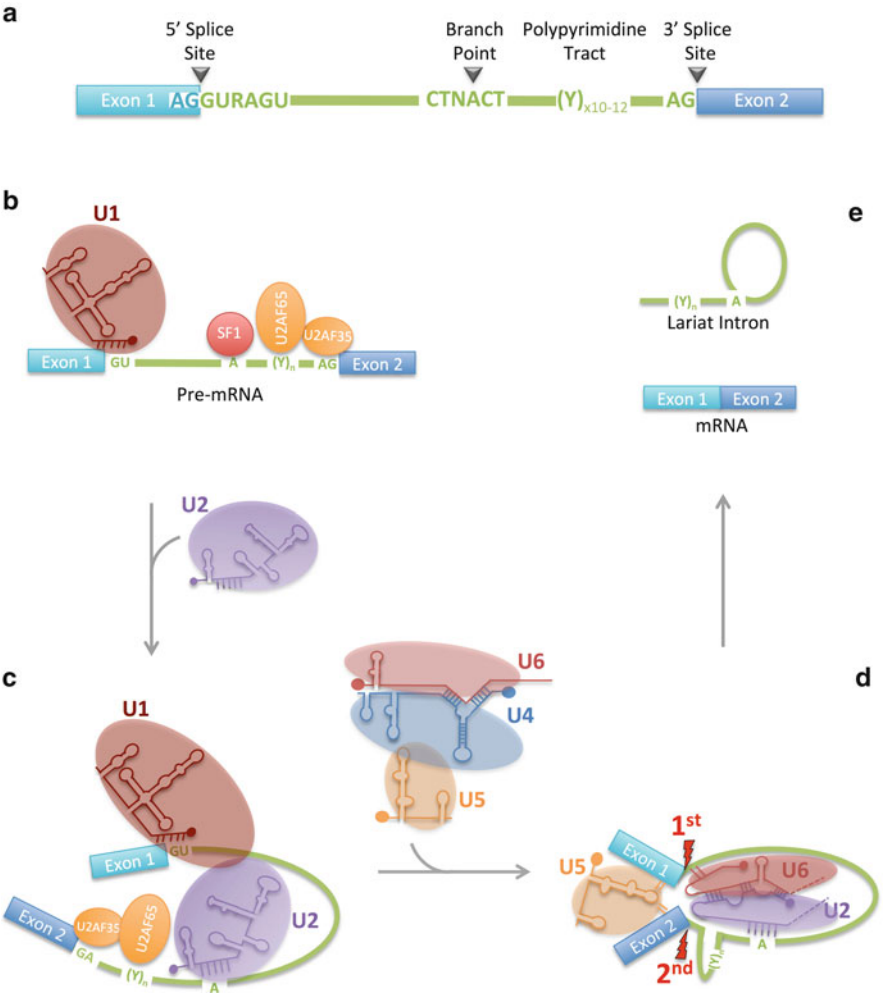

Fig. 13.1 Intron removal by splicing. (**a**) In humans, most introns are removed by the major spliceosome that recognizes conserved sequence elements located at the 5′ splice site, branch point (BP) and 3′ splice site. The polypyrimidine tract is a pyrimidine-rich stretch located between the BP and the 3′ splice site. The panel depicts two exons (*blue*) separated by an intron (*green*). N represents any nucleotide, R a purine, and Y a pyrimidine. (**b**) Spliceosome assembly initiates by binding of the U1 snRNP to the 5′ splice site and recruitment of the U2AF65/U2AF35 heterodimer to the 3′ splice site. The U2AF65 subunit binds to the polypyrimidine tract, the U2AF35 subunit to the AG dinucleotide at the 3′ splice site, and the SF1 protein binds to the branch point sequence. (**c**) In an ATP-dependent reaction, the U2 snRNP displaces SF1 and binds to the branch point. At this stage, the 5′ splice site, branch point sequence, and 3′ splice site, are in close spatial proximity. Bending of the polypyrimidine tract induced by interaction with U2AF brings the 3′ splice site into juxtaposition with the branch point sequence. Both the 5′ and 3′ splice sites are close to the 5′-end of the U2 snRNA, which later assembles with U6 snRNA forming the catalytic center of the spliceosome. (**d**) Catalytic activation occurs subsequent to addition of the U4/U6.U5 tri-snRNP and requires several rearrangements, including departure of the U1 and U4 snRNPs. The splicing reaction consists of two consecutive transesterification (replacement of one phosphodiester linkage for another) events. (**e**) After the two chemical steps of splicing are complete, the spliced exons are released, the spliceosome disassembles and the excised intron is degraded

branch point sequence and the downstream polypyrimidine tract, respectively. These proteins bind cooperatively, with SF1 interacting with the large subunit of U2AF (U2AF65), whereas the small subunit (U2AF35) binds the AG dinucleotide of the 3′ splice site (Fig. 13.1b). Next, the U2 snRNA engages in an ATP-dependent base-pairing interaction with the branch point sequence, displacing SF1 (Fig. 13.1c). Subsequently, the U4, U5 and U6snRNPs are recruited as a preassembled U4/U6.U5 tri-snRNP. With all snRNPs present, the spliceosome undergoes major conformational rearrangements that lead to release of U1 and U4. The spliceosome is now competent to catalyze the first transesterification step of splicing (Fig. 13.1d): the phosphodiester bond at the 5′ splice site is attacked by the 2′-hydroxyl of the adenosine at the branch point sequence, generating a free 5′ exon and an intron lariat-3′ exon intermediate. After additional rearrangements, the spliceosome catalyzes the second transesterification reaction: the 3′-hydroxyl of the 5′ exon attacks the phosphodiester bond at the 3′ splice site, leading to exon ligation and excision of the lariat intron. Then the spliceosome dissociates, releasing the mRNA (Fig. 13.1e).

Most of the functionally important RNA-RNA interactions formed within the spliceosome are weak and require the assistance of auxiliary proteins that bind weakly to specific sequences in exons and introns. This combination of multiple weak interactions is crucial for the flexibility of the spliceosome, in particular during regulated splicing decisions. Recently, fluorescence microscopy has been used to follow assembly of individual yeast spliceosomes in real time. The results indicate that spliceosomal components associate with pre-mRNA sequentially, but each step in the assembly pathway is reversible [16]. This implies that potentially any step during spliceosome formation might be subject to regulation. Spliceosome assembly is indeed highly regulated: depending on the combinatorial effect of proteins that either promote or repress the recognition of the core splicing sequences, splice sites in pre-mRNA can be differentially selected to produce multiple mRNA isoforms (Fig. 13.2). This process is called alternative splicing.

13.3 The Importance of Alternative Splicing

Diverse forms of mRNA are created by the differential use of splice sites (reviewed in [17]). Exons that are always included in the mRNA are called constitutive exons, and exons that are sometimes included and sometimes excluded from the mRNA are called cassette exons (Fig. 13.3). Some pre-mRNAs contain multiple cassette exons that are mutually exclusive, producing mRNAs that always include one of several possible exon choices. Exons can also be lengthened or shortened by altering the position of one of their splice sites alternative 5′ and alternative 3′ splice site selection; (Fig. 13.3). The 5′ and 3′-terminal exons can further be switched by combining alternative splicing with the use of alternative promoters or alternative polyadenylation sites, respectively (Fig. 13.3). Finally, certain intronic sequences may persist in the final mRNA, a splicing pattern called intron retention (Fig. 13.3).

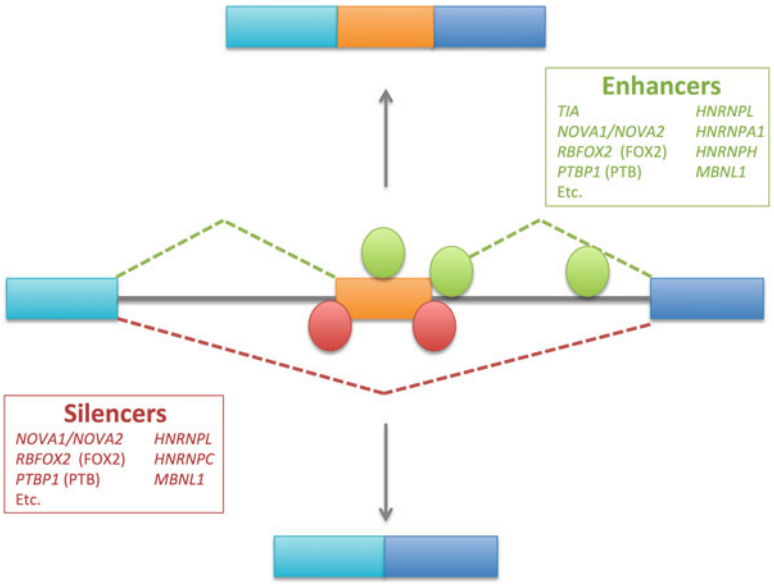

Fig. 13.2 Splicing is regulated by positional binding of RNA-binding proteins. The diagram depicts a model for mechanism of splicing activation or repression by RNA-binding proteins (RBPs). Two constitutively spliced exons (*blue*) are separated by an alternative or cassette exon (*orange*). Pending on the positions at which RBPs bind to the pre-mRNA, the alternative exon is either included (*top*) or excluded (*bottom*). Certain RBPs (depicted *red*) bind at intronic positions close to the 3′ and 5′ splice sites of the alternative exon to silence its inclusion. In contrast, binding of enhancer RBPs (*depicted green*) within the exon or in the downstream intron promotes inclusion of the alternative exon

Alternative splicing was first reported in 1980, when it was discovered that membrane-bound and secreted antibodies are encoded by the same gene [18, 19]. During the 1980s and 1990s many biologically important alternative splicing events were identified and characterized. Yet, the prevalence and general importance of this process was far from clear. More recently, the application of genome-wide profiling technologies coupled with bioinformatic approaches resulted in major advances in our understanding of alternative splicing. In particular, high-throughput massively parallel short-read sequencing provided for the first time unambiguous and unbiased detection of expressed RNA sequences. Compared to microarray-based systems for profiling alternative splicing, short-read sequencing offers a more accurate method for quantifying relative levels of different transcripts. Analysis of human tissue RNA sequencing (RNA-Seq) data revealed that approximately 95 % of human pre-mRNAs that contain more than one exon are spliced to yield multiple mRNAS, and that most isoforms display variable expression across tissues [20, 21]. Genes with few exons typically encode a small number of mRNA isoforms, while tremendously diverse mRNA repertoires can be produced from genes containing numerous exons. For example, the human gene *UTY* has 61 exons and can generate 129 mRNA isoforms (according to UCSC Known Gene annotations [22]).

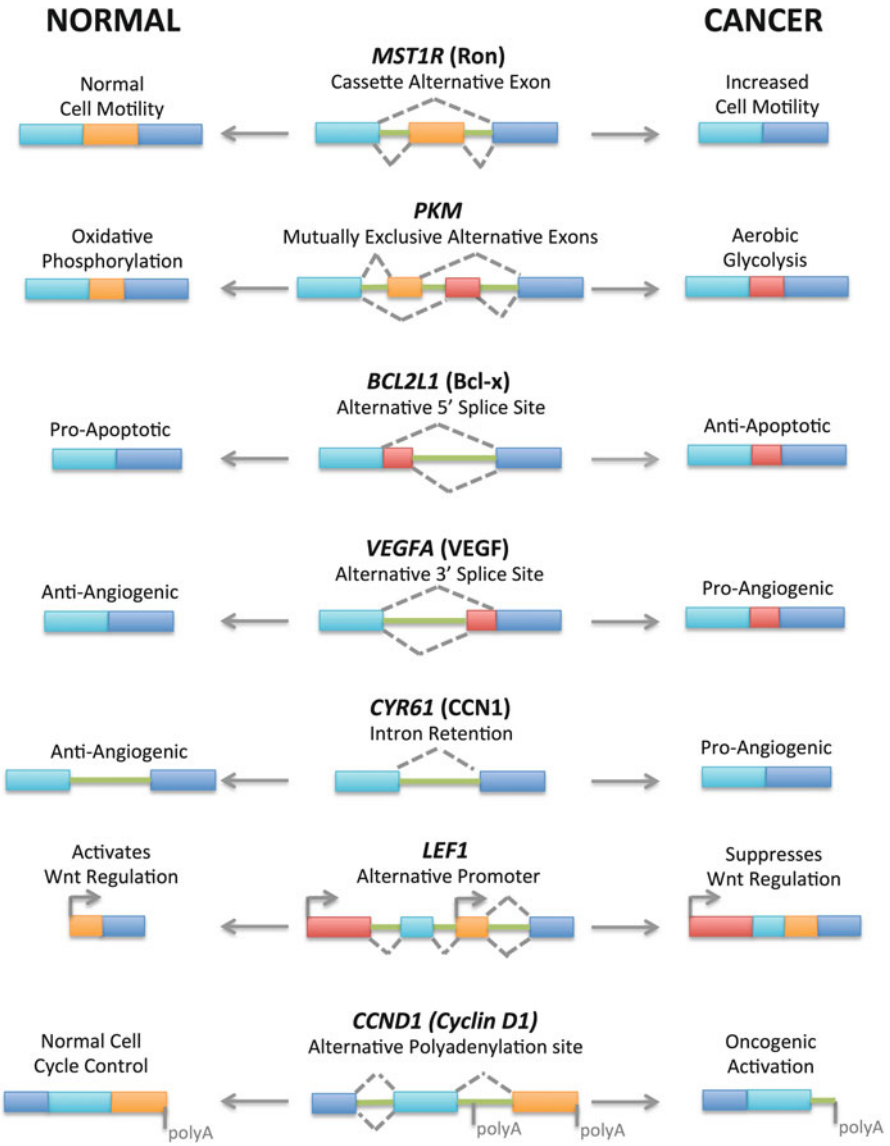

Fig. 13.3 Alternative splicing events in cancer. The basic types of alternative splicing include cassette-exon inclusion or exclusion, alternative 5′ or 3′ splice site selection, intron retention, alternative selection of transcription initiation (alternative promoter) and alternative selection of 3′ end processing sites (alternative polyadenylation). Alternative splicing events that have positive effects on cancer progression are shown. Constitutively spliced exons are depicted in *blue*

Overall, the high prevalence of alternative splicing combined with the finding that many mRNA isoforms represent low-abundant, non-conserved transcripts argue that they may be devoid of functional impact. Yet, recent studies are revealing a rapidly growing number of physiologically important splicing events.

Distinct splicing patterns of a given pre-mRNA can be observed pending on the cellular environment. For example, some mRNA isoforms are specifically expressed in certain tissues or developmental stages and other are triggered in response to external stimuli such as signaling pathways (reviewed in [23, 24]) or depolarization of neurons [25]. The diversity of mRNA isoforms appears to be higher in embryonic stem cells compared to differentiated cells [26] and some isoforms specifically detected in embryonic stem cells have recently been shown to play a key role in pluripotency [27–30]. Remarkably, a single splicing event can function to control an entire transcriptional network. This is well illustrated by alternative splicing of the transcription factor *FOXP1*. *FOXP1* mRNAs transcribed in embryonic stem cells contain a specific exon that is skipped in differentiated cells [30]. Inclusion of this exon determines the DNA binding properties of the encoded protein and is required for stimulating the expression of several pluripotency transcription factor genes [30].

Presumably, the intron-exon structure of genes played an important role in the generation of new genes during evolution. Moreover, alternative splicing seems to be rapidly evolving, particularly among physiologically equivalent organs from vertebrate species [31]. A remarkable example of how species-specific alternative splicing evolved was found in bats [32]. In order to detect warm-blooded prey, vampire bats express a splice isoform of the transient receptor potential cation channel V1 (*TRPV1*) gene. This isoform produces a channel with a truncated carboxy-terminal cytoplasmic domain capable of detecting infrared radiation. In contrast, the protein isoform expressed in fruit-feeding bats has a much higher thermal activation threshold [32].

Around 10–30 % of all alternatively spliced exons have inclusion levels that differ across tissues and are therefore called tissue-specific exons. Many of these exons play an important role in cell differentiation, for example during brain and heart development [33]. Recent large-scale computational analysis revealed that genes with tissue-specific exons tend to have more interaction partners compared to the other genes [34, 35]. Tissue-specific exons tend to encode flexible protein segments without a well-defined three-dimensional structure that likely form conserved interaction surfaces [34, 35]. Using a high-through put coimmunoprecipitation assay, inclusion of tissue-specific exons was shown to both promote and disrupt partner interactions [35]. Thus, tissue-specific splicing can potentially mediate new molecular interactions in a cell type-specific manner.

How human cells control more than 100,000 alternative splicing decisions remains incompletely understood. Clearly, there are multiple mechanisms involved, including RNA-binding proteins that interact with pre-mRNAs and modulate the efficiency of splice-site recognition by the spliceosome, formation of secondary structures in the RNA, the transcription rate and epigenetic modification of the template chromatin [36, 37]. A relatively small number of splicing regulators has been identified, and most are ubiquitously expressed although their relative abundances can fluctuate in different tissues [38]. A few, however, are tissue-specific

RNA-binding proteins, such as *NOVA1* and *NOVA2* [39], *PTBP2* (nPTB) [40, 41], *RBFOX1* (FOX-1) and *RBFOX2* (FOX-2) [42, 43], *ESRP1* and *ESRP2* [44] and *SRRM4* (nSR100) [45]. Currently, large-scale quantification of alternative splicing has been combined with genome-wide identification of in vivo binding sites of splicing regulators (Fig. 13.2) to create maps identifying all pre-mRNAs regulated by a single RNA-binding protein. RNA splicing maps are providing an unprecedented view of the global principles guiding splicing regulation [46].

13.4 Numerous Alterations in Splicing Occur in Cancer Cells

Recent high-throughput transcriptome sequencing studies revealed that different splicing variants are commonly found in cancer tissue compared to the normal surrounding tissue. This type of information has already proven useful in the classification of ovarian and breast cancer [47, 48]. In the case of prostate cancer, alternative splicing signatures are more reliable for diagnostic purposes than are gene expression signatures [49], and in osteosarcoma, changes in relative expression of splicing isoforms of the *TP53* (p53) inhibitor *MDM2* (HDMX) is a more effective prognostic biomarker than *TP53* mutation [50].

Direct causes of splicing alterations in cancer can be grouped into two main categories: *cis*-acting mutations in the pre-mRNA sequence and *trans*-acting changes in expression or activity of regulatory splicing factors. The first category encompasses mutations or polymorphisms in splice sites or regulatory sequence motifs. For example, in breast and ovarian cancer, mutations in the tumor suppressor breast cancer 1, early onset (*BRCA1*) often disrupt constitutive splice sites, leading to the production of inactive protein isoforms [51]. Splice site mutations in the *TP53* gene have also been described in various cancers [52]. In infant B-precursor leukemia, intronic mutations were found in the *CD22* gene that affect target motifs for splicing factors *HNRNPL* (hnRNP-L), *PTBP1* (PTB) and *PCBP1* leading to deletion of exon 12 and expression of a truncated and functionally defective *CD22* coreceptor protein unable to transmit apoptotic signals [53]. Recent systematic surveys identified 106 acquired somatic splice site mutations associated with aberrant splicing in lung cancer [54] and 158 essential splice site mutations in breast cancer [55].

In addition to inherited and acquired mutations, the human genome contains approximately 1,200 single nucleotide polymorphisms (SNPs) expected to modify splicing decisions [56]. A subset of these splicing-related SNPs may be functionally relevant in the context of cancer susceptibility and cancer progression, as shown by a recent study that identified a splicing polymorphism in the germline as predictor of response to targeted therapies [57]. A common intronic deletion polymorphism in the *BCL2L11* (BCL2-like 11, BIM) gene switches splicing from exon 4 to exon 3, leading to expression of *BCL2L11* isoforms that lack the pro-apoptotic BCL2-homology domain 3. The presence of this polymorphism explains why some individuals with chronic myeloid leukemia and epidermal growth factor receptor-mutated non-small-cell lung cancer have inferior responses to tyrosine kinase inhibitors [57].

Changes in expression or activity of *trans*-acting protein factors are caused by defects in components of the spliceosome or splicing regulatory factors. These can be induced by either genetic mutations and amplifications, or transcriptional and post-transcriptional mis-regulation. For example, *SRSF1* (SF2/ASF), a member of the SR protein family of splicing regulators, is frequently upregulated in many cancers. In some tumors, the gene locus is amplified accounting for the elevated levels of the protein [58]. Altered transcriptional regulation by *MYC* (c-Myc), which binds directly to E-boxes in the *SRSF1* gene promoter, is an alternative cause for *SRSF1* protein over expression in cancer [59]. *MYC* can also control expression of additional splicing regulators, namely hnRNP proteins [60]. In addition to *MYC*, other transcription factors control the expression of splicing proteins. Namely, mutations in the Wilms's tumor suppressor gene, *WT1*, abrogates binding of the *WT1* protein to the *SRPK1* promoter, causing over-expression of this SR-protein kinase and hyperphosphorylation of splicing regulator *SRSF1*; this in turn resulted in altered splicing of *VEGFA*, stimulating angiogenesis [61]. Direct binding of the transcription repressor Snail to the *ESRP1* promoter was also shown to cause reduced expression of this epithelial-specific splicing factor, thus promoting isoform switching of several genes involved in epithelial-to-mesenchymal transition [62]. Recurrent mutations in genes encoding essential components of the splicing machinery such as *U2AF1*, *ZRSR2*, *SRSF2*, *SF3A1* and *SF3B1* were recently found in patients with myelodysplastic syndromes [63–66]. More recently, mutations in *U2AF1*, *U2AF2*, and *SF3B1* genes were also detected in lung cancer patients [67]. Remarkably, most of these mutations affect proteins involved in 3′-splice site recognition during the early stages of spliceosome assembly (Fig. 13.1b, c). This, together with the finding that mutations were detected in a mutually exclusive manner, strongly suggests that the compromised function of early spliceosome complexes is a hallmark of cancer.

In general, factors involved in splicing regulation are RNA binding proteins that interact with particular sequence motifs, albeit with relatively low specificity. Therefore, most alternative splicing decisions are controlled by the cooperative binding of several protein factors to short redundant RNA motifs. As a consequence, each alternative splicing event is frequently regulated by multiple factors, and each factor may control several splicing events [46]. A striking example was recently reported for the tissue-specific splicing factors *RBFOX1* (FOX-1) and *RBFOX2* (FOX-2). Unlike most other known splicing regulators, the FOX proteins bind exclusively two defined sequence motifs: UGCAUG and AGCAUG [68]. The expression of *RBFOX2* was found downregulated in ovarian cancer and *RBFOX2* binding sites were detected downstream of one-third of the exons alternatively spliced in this type of cancer [69]. Importantly, reducing the expression of *RBFOX2* in cell lines recapitulated the cancer-associated splicing signature, suggesting that the reduced level of *RBFOX2* causes the changes in splicing [69]. This study further showed that ovarian and breast cancers share a common splicing signature. Although *RBFOX2* transcripts were not downregulated in breast cancer, they were alternatively spliced producing an inactive form of the protein [69]. This illustrates how changes in splicing of a splicing factor can change its regulatory activity, leading to further changes in splicing of its target pre-mRNAs.

Although splicing is primarily controlled by sequence elements in the pre-mRNA that recruit trans-acting splicing factors, recent work make it clear that alternative splicing is also sensitive to transcriptional rate and chromatin conformation (reviewed in [70]). Since many proteins that control DNA and histone modification show aberrant expression or altered activity in tumors [71], it is likely that epigenetics represents a third cause of splicing abnormalities in cancer. Of note in this regard, variations in the methylated status of the *MST1R* (RON) promoter correlate with transcription of a short isoform of the enzyme that is constitutively active and drives cell proliferation [72].

While the majority of cancer-specific alternative splicing events may have just coincidently occurred during tumor development, a few bestow a growth advantage on the tumor. To date, several splicing isoforms that are specifically expressed in cancer have been demonstrated to contribute to cellular malignant phenotypes such as avoidance of apoptosis, angiogenesis, limitless replication potential, and invasion [73], as detailed below (see also Table 13.1 and Fig. 13.3).

13.4.1 Apoptosis

Transcripts from numerous genes involved in apoptosis are alternatively spliced, often resulting in isoforms with opposing roles in promoting or preventing cell death. Well-characterized examples include the *BCL2L1* (Bcl-x), *CASP2* (Caspase-2), *CASP9* (Caspase-9), and *FAS* (Fas) genes. In general, isoforms that enhance survival tend to be up-regulated in cancer and correlated with clinical staging (reviewed in [73]). Another protein that promotes apoptosis upon DNA damage is *AIMP2* (Aminoacyl-tRNA synthetase-interacting multifunctional protein 2). A splicing variant of *AIMP2* was found highly expressed in lung cancer, leading to increased resistance to cell death, and the relative expression of this isoform correlated with cancer stage and survival of patients [75].

13.4.2 Angiogenesis

Primary transcripts encoding Vascular Endothelial Growth Factor (*VEGFA*), which plays a key role in promoting the formation of new blood vessels, undergo extensive alternative splicing. As a result, two families of splicing isoforms are produced with either pro-angiogenic or anti-angiogenic functions. Anti-angiogenic isoforms are expressed in normal tissues and are downregulated in many cancers (reviewed in [124, 125]). Another protein involved in angiogenesis that is regulated by alternative splicing is *CYR61* (cysteine rich 61, CCN1). While normal cells express a variant with retention of an intron that most likely targets the transcripts for degradation, in cancer cells the intron is spliced leading to an accumulation of active protein [85].

Table 13.1 Alternative splicing (AS) events that favor cancer progression

	Gene	AS event type	Altered regulatory proteins	Functional implications	References
DNA damage	*ABL1*	Cassette exon	*EWSR1* (EWS)	Response to DNA damage	[74]
	CHEK2	Cassette exon	*EWSR1* (EWS)	Response to DNA damage	[74]
	MAP4K2	Cassette exon	*EWSR1* (EWS)	Response to DNA damage	[74]
Apoptosis	*AIMP2*	Cassette exon	–	Inhibition of apoptosis	[75]
	BCL2L1 (Bcl-x)	Alternative 5′ splice site	–	Inhibition of apoptosis	[76]
	BCL2L11 (BIM)	Cassette exon	*SRSF1* (ASF/SF2)	Inhibition of apoptosis	[77]
	CASP2 (Caspase-2)	Cassette exon	–	Inhibition of apoptosis	[78]
	CASP9 (Caspase-9)	Cassette exon	*HNRNPL* (hnRNP L)	Inhibition of apoptosis	[79]
	CD22	Cassette exon	*HNRNPL*, *PTBP1*(*PTB*), *PCBP1* (PCB)	Inhibition of apoptosis	[53]
	CFLAR (FLIP)	Cassette exon	–	Inhibition of apoptosis	[80]
	FAS	Cassette exon	*TIA1*, *PTBP1*	Inhibition of apoptosis	[81]
	STAT3	Alternative 3′ splice site	–	Inhibition of apoptosis	[82]
	TP53	Alternative promoter	–	Inhibition of apoptosis	[83]
	TP73	Alternative promoter	–	Inhibition of apoptosis	[84]
Angiogenesis	*CYR61*	Intron retention	–	Inhibition of angiogenesis	[85]
	FLT1 (VEGFR1)	Intron retention	–	Inhibition of angiogenesis	[86]
	KDR (VEGFR2)	Alternative polyadenylation	–	Inhibition of angiogenesis	[87]
	VEGFA	Alternative 3′ splice site	*SRSF1*	Activation of angiogenesis	[88]
Proliferative potential	*CCND1* (Cyclin D1)	Alternative polyadenylation	*EWSR1*	Oncogenic activation	[89]
	CCND1 (Cyclin D1)	Alternative polyadenylation	*SRSF1*	Oncogenic activation	[90]
	CD44	Cassette exon	*ESRP1*	Evasion from oxidative stress	[91]
	CYP19A1	Alternative promoter	–	Activation of tumor growth	[92]
	ERBB2 (HER2)	Cassette exon	–	Activation of tumor growth	[93]
	ERBB4	Cassette exon	–	Activation of tumor growth	[94]
	FYN	Mutually exclusive exons	–	Activation of tumor growth	[95]
	H2AFY (macroH2A.1)	Mutually exclusive exons	*QKI*	Activation of tumor growth	[96]

(continued)

Table 13.1 (continued)

	Gene	AS event type	Altered regulatory proteins	Functional implications	References
	KLF6	Cassette exon	*SRSF1*, *SRSF3*	Activation of tumor growth	[97]
	MDM2	Cassette exon	*EWSR1*	Genotoxic stress response	[98]
	MET	Cassette exon	–	Activation of tumor growth	[99]
	MTOR	Cassette exon	–	Activation of tumor growth	[100]
	LEF1	Alternative promoter	–	Activation of tumor growth	[101]
	PKM	Mutually exclusive exons	*PTBP1*, *HNRNPA1*, *HNRNPA2B1*	Aerobic glycolysis	[60]
	RASSF1	Alternative promoter	–	Activation of tumor growth	[102]
	RPS6KB1 (S6K1)	Cassette exon	*SRSF1*	Oncogenic activation	[58]
	TP63	Alternative promoter	–	Activation of tumor growth	[103]
	TPM1 (tropomyosin)	Intron retention	*DEK*	Activation of tumor growth	[104]
Invasion and metastasis	*ADAM8*	Cassette exon	–	Invasive cell migration	[105]
	CD44	Cassette exon	*SRRM1* (SRm160)	Invasive cell migration	[106]
	CD44	Cassette exon	*ESRP1*	Induction of EMT	[107]
	CPE	Alternative promoter	–	Invasive cell migration	[108]
	CTNND1 (p120)	Alternative promoter	–	Invasive cell migration	[109]
	ENAH (Mena)	Cassette exon	–	Invasive cell migration	[110]
	FGFR2	Mutually exclusive exons	*ESRP1*, *ESRP2*	Induction of EMT	[44]
	GLI1	Cassette exon	–	Invasive cell migration	[111]
	MST1R (Ron)	Cassette exon	*HNRNPA2B1* (hnRNPA2/B1)	Oncogenic activation	[112]
	MST1R (Ron)	Cassette exon	*SRSF1*	Oncogenic activation	[113]
	MST1R (Ron) and *MADD* (IG20)	Cassette exon	*HNRNPH1* (hnRNPH)	Oncogenic activation	[114]
	NCOA3 (AIB1)	Cassette exon	–	Invasive cell migration	[115]
	RAC1	Cassette exon	*SRSF1*, *SRSF3*	Oncogenic activation	[116]
	SENP7	Cassette exon	–	Induction of EMT	[117]
	TP53INP2	Cassette exon	*HNRNPA2B1*	Invasive cell migration	[118]
Cancer therapy	*AR*	Cassette exon	–	Resistance to hormonal therapy	[119]
	BCR	Cassette exon	–	Resistance to drug therapy	[120]
	BRAF	Cassette exon	–	Resistance to drug therapy	[121]
	MKNK2 (MNK2)	Mutually exclusive exons	*SRSF1*	Resistance to drug therapy	[122]
	MS4A1 (CD20)	Cassette exon	–	Resistance to drug therapy	[123]
	TP53 (p53)	Cassette exon	–	Resistance to chemotherapy	[52]

13.4.3 Proliferative Potential

Proliferating cells reprogram their metabolism to engage in aerobic glycolysis (the Warburg effect), in part through alternative splicing of the pre-mRNA that encodes the enzyme pyruvate kinase M, *PKM*. Normal cells express the splicing isoform PKM1, whereas all tumors express PKM2. Importantly, replacing PKM2 with PKM1 in cancer cells reduced tumor growth (reviewed in [126]). Cancer-associated changes in alternative splicing can also result in activation of proto-oncogenes such as *CCND1* (Cyclin D1) [reviewed in 127].

13.4.4 Invasion and Metastasis

A significant reprogramming of alternative splicing occurs during the epithelial-to-mesenchymal transition (EMT), a process by which cancer cells acquire invasive capabilities and become metastatic. EMT-associated changes in splicing affect genes such as *MST1R* (Ron), *RAC1*, *CD44*, *FGFR2*, *CTNND1* (p120-catenin), and *ENAH* (Mena) (reviewed in [128, 129]). Among these, the *CD44* transmembrane protein was one of the first genes for which splicing variants were found associated with metastasis. The expression of specific *CD44* splicing variants correlates with aggressive behavior in several cancer cell types [130–132], and one particular isoform (CD44v8-10) potentiates the ability of cancer cells to defend themselves against reactive oxygen species [91, 133]. Alternative splicing can further contribute to regulate the onset of EMT in cancer cells. Indeed, it was recently found that normal breast epithelia express two splice variants of sentrin/small ubiquitin-like modifier (SUMO)-specific protease 7 (*SENP7*), and breast cancer cells express predominantly the isoform that promotes EMT initiation [117]. Additional examples of alternatively spliced isoforms that promote cancer cell migration and invasion via a gain-of-function mechanism include the truncated glioma-associated oncogene homolog 1, *GLI1* [111, 134], the steroid receptor coactivator 3 (*NCOA3*, AIB1) with a deletion of exon 4, SRC-3Δ4 [115, 135], truncated forms of *ADAM8* (a disintegrin and metalloprotease) [105] and *CPE* (carboxypeptidase E) [108], and alternative inclusion of an exon in the five untranslated region of tumor protein p53 inducible nuclear protein 2 *TP53INP2* [118].

In addition to generating protein isoforms with different biological activities, alternative splicing can also regulate gene expression level through inclusion of premature translation termination codons that target the mRNA for degradation by nonsense-mediated decay [136]. This mechanism causes downregulation of proteins involved in tumor development such as *NFAT5* transcription factor [137] and *CDH1* (E-cadherin) in chronic lymphocytic leukemia [138].

Finally, several lines of recent evidence reveal that splicing contributes for acquired resistance to chemotherapeutic drugs. For example, patients with metastatic melanoma are currently treated with vemurafenib, a newly approved drug that

selectively binds monomers of the most prevalent oncogenic mutation of *BRAF* (B-RAF, V600E), inhibiting its kinase activity. However, most patients acquire resistance within a year of treatment. Different mechanisms have been identified that counteract vemurafenib effectiveness, and one of them consists in expression of truncated forms of the *BRAF* (V600E) protein generated by abnormal pre-mRNA splicing. These splicing isoforms lack the RAS-binding domain and dimerize in a RAS-independent manner therefore rendering the enzyme insensitive to RAF inhibitors [121]. Another example is gemcitabine, the drug used for pancreatic ductal adenocarcinoma. Gemcitabine induces overexpression of splicing factor *SRSF1*, leading to formation of a mitogen activated protein kinase interacting kinase *MKNK2* (MNK2) splicing variant that overrides upstream regulatory pathways and confers resistance to the drug [122]. In chronic myeloid leukemia, one of the mechanisms responsible for resistance to tyrosine kinase inhibitors is the expression of an alternatively spliced *BCR* (BCR-ABL) pre-mRNA that lacks the drug-targeted kinase domain [120, 139] and in B cell malignancies, a splicing isoform of *MS4A1* (CD20) produces a *truncated* protein that loses membrane anchorage and causes resistance to rituximab [123]. Splicing variants of the androgen receptor *AR* may also contribute to the development of castration-resistant prostate cancers [119], and in ovarian cancer expression of a particular *TP53* (p53) splicing isoform correlates with impaired response to primary platinum-based chemotherapy [52].

13.5 Targeting Splicing for Cancer Therapy

In cancer research, much effort is focused on the identification of molecular pathways that are specific to tumor cells and essential for their survival. Cancer-specific splice variants are therefore emerging as highly attractive therapeutic targets, since only cancer cells will be targeted with minimum toxicity towards normal cells. However, in contrast with diagnostic and prognostic purposes for which any discriminating isoform can be a valuable biomarker, the selection of splicing isoforms as drug targets requires detailed functional studies to evaluate their potential in ablating cancer cells. RNA interference (RNAi) screens specifically targeted to silence tumor-associated splicing variants currently represent a valuable tool for identification of isoforms essential for cancer cell survival. Recently, a systematic isoform-specific functional screen of 41 alternatively spliced variants associated with breast and ovarian cancer revealed that targeting the spleen tyrosine kinase *SYK* isoform induced apoptosis, whereas global knockdown of the same gene had no effect [140]. Clearly, the functional contribution of splicing isoforms to tumor behavior should be considered when designing anticancer strategies. This is well illustrated by the limited efficacy of currently used molecules like Bevacizumab that target angiogenesis but do not distinguish between the pro- and the anti-angiogenic splicing isoforms of *VEGFA* [125]. It remains to be studied whether targeting specifically the pro-angiogenic *VEGFA* isoform or treating patients with Bevacizumab only in cases where the anti-angiogenic isoforms are absent will be more beneficial [125].

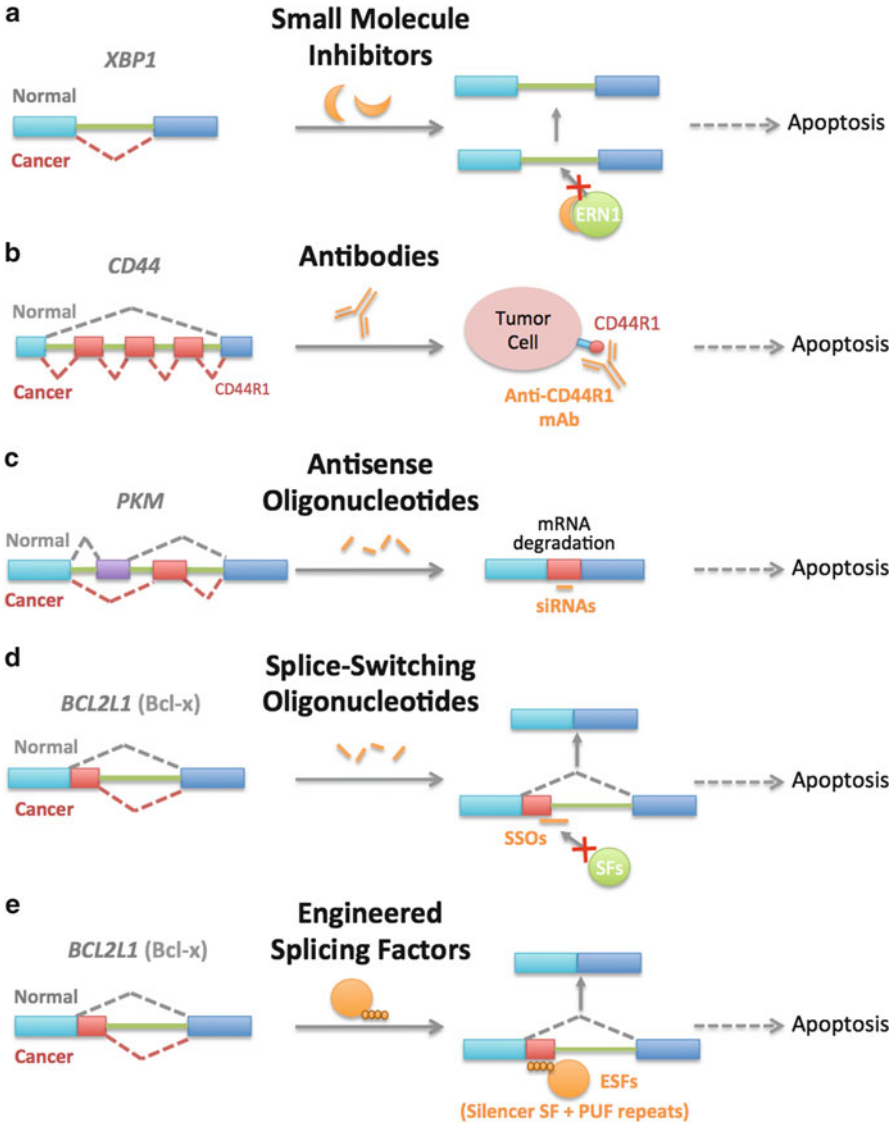

Fig. 13.4 Strategies for splicing-targeted cancer therapies. (**a**) Small molecule inhibitors capable of altering cancer-associated splicing. (**b**) Monoclonal antibodies that recognize unique protein epitopes encoded by cancer-associated mRNA isoforms. (**c**) Antisense oligonucleotides that induce RNAi-mediated knockdown of oncogenic mRNA isoforms. (**d**) Splice-switching oligonucleotides that redirect splicing decisions, thereby reducing oncogenic mRNA isoforms. (**e**) Instead of using oligonucleotides, splicing reprogramming can be achieved by engineered proteins that combine sequence-specific RNA-binding domains with functional domains that regulate splicing

Multiple strategies have been envisioned to therapeutically target cancer-associated splicing. These include small molecule inhibitors, antibodies, and antisense oligonucleotides, as described below in more detail (see Fig. 13.4).

13.5.1 Small Molecule Inhibitors

Different types of molecules capable of altering alternative splicing have emerged from several chemical screens. Many of these substances act by either blocking histone deacetylases or by inhibiting the kinases that phosphorylate SR splicing factors (reviewed in [141]). For example, amiloride can revert cancer-specific splicing events and this effect is likely mediated by changes in amount and phosphorylation status of SR proteins [142, 143]. Similarly, a small-molecule inhibitor of *XBP1* splicing may be a promising therapeutic option in multiple myeloma [144].

Using a different approach, natural products derived from distantly related bacteria were found to target a core component of the spliceosome, the SF3B1 protein, suggesting that interfering with splicing may be a mechanism by which bacteria compete with eukaryotes. Remarkably, mutations in the *SF3B1* gene were found in some cancers [66] and anti-SF3B1 compounds demonstrated dramatic, selective antitumor activity in human tumor xenograft models (reviewed in [145]). The mechanism responsible for such selective antitumor activity is unknown, but one intriguing possibility is that proliferating cancer cells are more vulnerable than normal cells to splicing inhibitors. Consistent with this view, several lines of evidence suggest that RNA splicing is functionally coupled to cell-cycle progression (see [146] and references therein). Moreover, interfering with the splicing machinery leads to activation of *TP53* [147] and induces an alternatively spliced isoform of *TP53* that promotes cellular senescence [148]. Altogether these observations suggest that activation of p53 may contribute to the observed selective anti-tumor activity. Thus, targeting the spliceosome might be a viable approach for development of novel anticancer drugs [145].

13.5.2 Antibodies

An alternative strategy that is being explored consists of raising antibodies against epitopes that are uniquely present in the cancer-associated protein isoforms. A recent example is a fully human monoclonal antibody that recognizes the extracellular domain of a *CD44* isoform expressed on the surface of various epithelial cancers [149].

13.5.3 Antisense Oligonucleotides

Oligonucleotides designed to bind defined sequence elements in the pre-mRNA can induce either an RNAi-mediated specific knockdown of a particular splicing isoform, or redirect splicing decisions. For example, alterations in glucose metabolism mediated by pyruvate kinase (*PKM*) activity are likely to confer a selective advantage for tumor cells to grow in hypoxic environments. Because PK activity is

modulated by alternative splicing, inhibition of the PKM2 isoform that is commonly expressed at high levels in tumor cells appears as a promising target of broad therapeutic applicability. By screening a tiling siRNA library, Goldberg and Sharp recently identified sequences that discriminate between the M1 and M2 splicing isoforms of pyruvate kinase and produce a potent and specific knockdown of the M2 isoform. This resulted in decreased viability and increased apoptosis in multiple cancer cell lines but less so in normal fibroblasts or endothelial cells. Moreover, when the selected siRNAs were delivered as nanoparticles to established xenografts, a substantial reduction of tumor volume was observed [150]. Oligonucleotides can also be designed to redirect splicing decisions through blocking access to the transcript by the spliceosome. Splice-switching oligonucleotides (SSOs) are chemically modified to ensure stability and increase their binding affinity for the target sequence. SSOs have been applied to restore correct splicing of an aberrantly spliced transcript, induce expression of a novel splice variant with therapeutic value, or manipulate alternative splicing from one splice variant to another (reviewed in [151]). The latter mechanism can induce downregulation of a deleterious transcript while simultaneously upregulating expression of a preferred isoform, making it an attractive anti-cancer molecular therapy. Although the application of SSOs is still hindered by poor in vivo delivery to tumor cells, promising results were reported for antisense compounds directed at either inducing the pro-apoptotic splicing variant Bcl-x(S) at the expense of the more abundant survival Bcl-x(L) isoform of *BCL2L1* gene [152], or redirecting splicing of the signal transducer and activator of transcription 3 (*STAT3*) transcripts to produce a truncated isoform lacking the transactivation domain [82]. Enhanced delivery of SSOs to the cell nucleus can be achieved through aptamers that bind nucleolin, a protein that is found on the surface of rapidly proliferating tumor cells and traffics from the cell surface to the nucleus [153].

In principle, splicing can also be modulated using engineered proteins instead of antisense oligonucleotides. A recently proposed strategy relies on direct recognition of the pre-mRNA targets through protein-RNA interaction. Splicing reprogramming is achieved through engineering artificial splicing factors that combine sequence-specific RNA-binding domains with functional domains that regulate splicing [154]. Artificial factors targeted to the human endogenous gene *BCL2L1* (Bcl-X) increased the amount of the pro-apoptotic splicing isoform and promoted apoptosis of cancer cells [154].

13.6 Concluding Remarks

Molecular studies are increasingly used to guide therapeutic decisions for cancer patients, as clinical trials demonstrate superior efficacy of targeted treatments compared to “classical” chemotherapy. Recent advances in high-throughput DNA and RNA sequencing will ultimately lead to a comprehensive characterization of the genome and transcriptome of most cancers. New activating mutations and translocations in oncogenes will be discovered and pursued as drug targets. Additionally, a

more insightful perspective on the contribution of post-transcriptional regulation of gene expression in cancer complexity and diversity will be gained. The complete landscape of splicing alterations will be described for each cancer type and their functional impact on cell growth, metabolism, viability, apoptosis, invasiveness, angiogenesis and drug resistance will be established with the help of RNAi screens. The molecular mechanisms responsible for the functionally relevant cancer-associated splicing events will also be identified. Doubtless, these studies will significantly increase the list of potential cancer therapeutic targets in the near future. The challenge ahead will be to further develop innovative approaches to selectively and efficiently interfere with the splicing machinery and modulate splicing decisions. There is growing optimism that this research area may enable new opportunities for cancer patients.

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