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Review

Alternative splicing and cell survival: from tissue homeostasis to disease

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Most human genes encode multiple mRNA variants and protein products through alternative splicing of exons and introns during pre-mRNA processing. In this way, alternative splicing amplifies enormously the coding potential of the human genome and represents a powerful evolutionary resource. Nonetheless, the plasticity of its regulation is prone to errors and defective splicing underlies a large number of inherited and sporadic diseases, including cancer. One key cellular process affected by alternative splicing is the programmed cell death or apoptosis. Many apoptotic genes encode for splice variants having opposite roles in cell survival. This regulation modulates cell and tissue homeostasis and is implicated in both developmental and pathological processes. Furthermore, recent evidence has also unveiled splicing-mediated regulation of genes involved in autophagy, another essential process for tissue homeostasis. In this review, we highlight some of the best-known examples of alternative splicing events involved in cell survival. Emphasis is given to the role of this regulation in human cancer and in the response to chemotherapy, providing examples of how alternative splicing of apoptotic genes can be exploited therapeutically.
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Facts

- The complexity of the apoptotic program is achieved through a broad spectrum of proteins acting at transcriptional, post-transcriptional and post-translational levels.
- Through alternative processing of their precursor mRNAs (pre-mRNAs), many apoptotic genes encode for multiple protein variants displaying opposite functions.
- Dysregulation of apoptotic splice variants contributes to the pathogenesis of human diseases.
- Modulation of splicing by small molecules is emerging as a valuable therapeutic approach to human diseases.

Open Questions

- What are the specific splicing factors that contribute to key alternative splicing events in apoptotic genes of relevance for normal development or human diseases?
- Is alternative splicing of apoptotic genes targetable for therapeutics purpose?
- What is the contribution of alternative splicing to the regulation of autophagy?

The balance between cell proliferation and death insures dynamic tissue remodeling during development and maintains tissue homeostasis in adult organisms. When necessary, cells must respond to negative environmental cues and/or endogenous signals by activating a 'cell death' program named apoptosis. If these rules normally in act in tissues are ignored, pathological conditions can arise.[1–3]

The ability to surgically remove erroneous and potentially deleterious cells is an indispensable requirement for the physiology of tissues.[1] Through apoptosis, damaged cells trigger their own destruction with little effect on the surrounding tissue.[1–4] Apoptosis occurs via two interlinked pathways: the extrinsic pathway, typically activated by pro-apoptotic receptors at the cell surface; the intrinsic pathway, triggered by mitochondrial signals within the cell (Figure 1). These two pathways converge at the level of common effectors, which orchestrate and execute the apoptotic program.[4]

An example of how apoptosis participates in development and tissue homeostasis is provided by the nervous system. Extensive elimination of neurons through apoptosis is an essential step for the functional and structural maturation of the brain.[5] Viability of neurons during embryogenesis depends on trophic factors, such as nerve growth factor,[6] secreted by neighboring cells and competition for these cues results in survival of some neurons and death of others.[7] Another example of developmentally programmed cell death program is follicular atresia in ovary. Factors secreted by granulosa cells, such as estradiol and insulin-like growth factor, are essential for

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Abbreviations: AS, alternative splicing; snRNP, small nuclear ribonucleoprotein; hnRNP, heterogeneous nuclear ribonucleoprotein; SRSF, serine arginine-rich splicing factor; Pre-mRNA, precursor messenger RNA; PTC, premature termination codon; NMD, nonsense-mediated decay; LncRNA, long non-coding RNA; UTR, untranslated regions; BH, BCL-2 family-homology; ASO, antisense oligonucleotide; PCa, prostate cancer; PDAC, pancreatic ductal adenocarcinoma; AML, acute myeloid leukemia; SMA, spinal muscular atrophy
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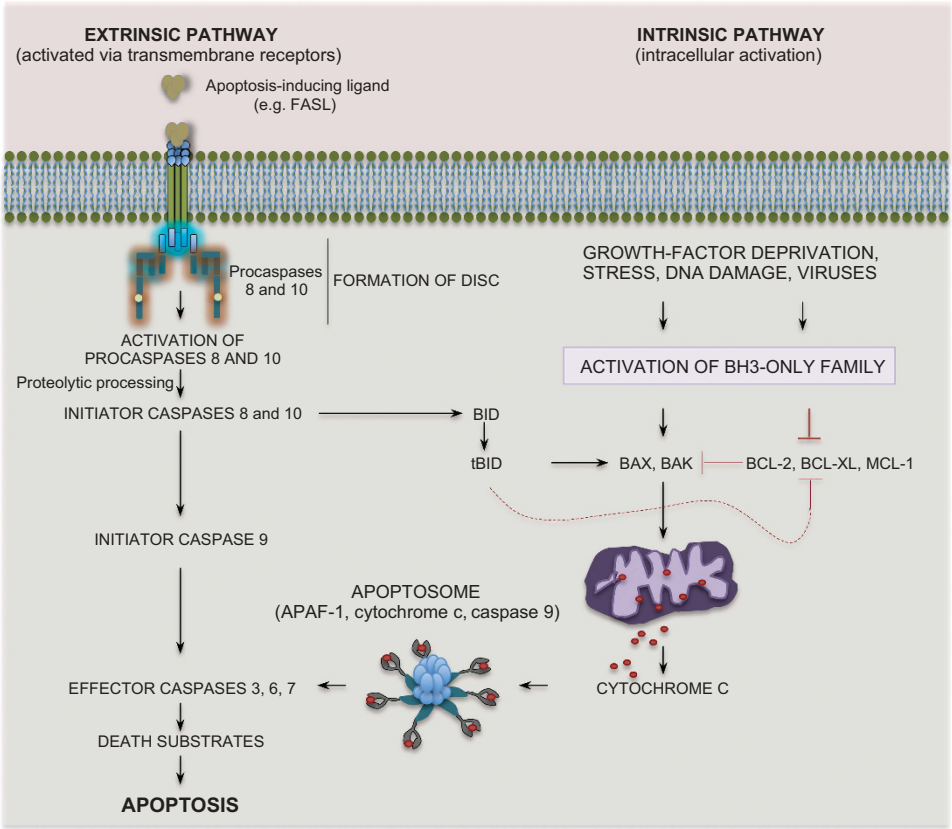


Figure 1 The apoptotic pathways. Schematic representation of the the extrinsic (death receptor) pathway (left) and of the intrinsic (mitochondrial) pathway of apoptosis (right). Binding of ligands of the TNF family leads to oligomerization of death receptors and subsequent recruitment and activation of initiator caspases (caspase-8 and -10) via adaptor proteins. The initiator caspases target effector caspases for proteolytic cleavage and activation. In response to an apoptotic stimulus pro-apoptotic BCL-2 proteins lead to permeabilization of the outer mitochondrial membrane and release of cytochrome c, which then binds APAF-1. Next, a conformational change leads to recruitment of an initiator caspase (caspase-9) and formation of the apoptosome. Caspase-9 in turn activates effector caspases (for example, caspase-3). In both pathways the activated caspases cleave selected nuclear and cytoplasmic target proteins for the accomplishment of the apoptotic program

follicle growth and deprivation of them or stimulation of death receptors, is the main cause of follicle regression before birth.[8]

To avoid adverse effects, apoptotic pathways are precisely regulated at multiple levels and interconnected with checkpoints monitoring the crucial events in the cell life. Regulation is achieved through a broad spectrum of proteins that act at multiple layers.[1–4] Notably, many apoptotic genes encode for protein variants with opposite functions through alternative splicing (AS) of the precursor mRNA (pre-mRNA),[9] suggesting that this step in the regulation of gene expression is particularly suited for the fine-tuned control required to properly execute apoptosis.

Alternative Splicing and Apoptosis

Eukaryotic genes are characterized by short exons interspersed between long non-coding introns. Splicing of introns is catalyzed by the spliceosome, a macromolecular machinery composed of five small nuclear ribonucleoprotein particles (U1, U2, U4, U5 and U6 snRNP) and many auxiliary proteins.[10] When the splice sites display high levels of sequence conservation, exons are almost always included in the mRNA (constitutive exons). Nevertheless, many exons lack strong exon–intron consensus sequences and are subjected to regulation (alternative exons). In this case, their recognition is tuned by splicing factors that recognize enhancer and silencer sequences in the pre-mRNA.[11] It is the interplay between antagonistic splicing factors to determine whether a target exon is included or skipped from the mature mRNA. This flexibility in splicing regulation allows alternative assortment of exons in the transcripts encoded by virtually every multi-exon gene, thus greatly expanding the coding potential and plasticity of genomes.[12]

Regulation of apoptosis is a typical example of the impact of AS in the modulation of biological processes. Several apoptotic genes encode splice variants having opposite roles.[9] For example, the long isoforms of BCL-X (BCL-X_L) and APAF1 (APAF1$_L$) protect cells against apoptosis, whereas their short isoforms (BCL-X_S and APAF1$_S$) promote it.[13,14] Conversely, the long isoform of CASP2 (ICH-1L) induces apoptosis, whereas the short isoform (ICH-1S) inhibits it.[15]

Dysregulation of apoptotic splice variants expression contributes to human diseases, as cancer and neurodegenerative disorders.[1–3] For instance, use of alternative promoters and AS generates a complex pattern of pro- and anti-apoptotic isoforms of the tumor-suppressor *TP73* gene. Transcription

from the P1 promoter yields the full-length protein with pro-apoptotic activity. However, usage of the P2 promoter in intron 3 and/or AS of exons 2 and 3 produce variants that lack the N-terminal trans-activation domain and counteract the tumor-suppressor activity of p53 and p73.[16] Likewise, differential expression of *TP53* splice variants can switch astrocyte function from neuroprotective (Δ133p53) to neurodegerative (p53*β*). An altered ratio of these variants in favor of the neurotoxic p53*β* isoform was detected in brain of patients affected by Alzheimer's disease and amyotrophic lateral sclerosis,[17] thus linking *TP53* AS to common forms of neurodegenerative diseases.

Herein, we will illustrate molecular mechanisms involved in AS regulation of three well-studied apoptotic genes (*FAS*, *CASP9* and *BCL2L1*) encoding antagonistic variants. Moreover, we will describe the impact of apoptotic genes splicing in cancer progression and chemoresistance, providing examples of how AS modulation can be envisioned as therapeutic tool for human cancer.

Alternative Splicing Regulation: Lessons from Apoptotic Genes

Although most genes undergo AS and many biological processes are likely affected by it,[12] in few cases the specific impact of AS on gene functions is well illustrated as for apoptosis. For this reason, genes like those encoding the membrane-bound death receptor FAS, the apoptotic modulator BCL-X and the protease caspase 9 have been employed as model systems to investigate the basic rules of AS regulation (Figure 2).

FAS. FAS (CD95/APO-1/TNFRSF6) belongs to the tumor necrosis factor receptor family.[18] Binding of FAS to its ligand FASL (CD95L/CD178/TNFSF6) triggers apoptosis.[18] FAS and FASL are involved in T-cell-mediated cytotoxicity and in feedback mechanisms dampening immune reactions. FAS-mediated apoptosis is implicated in the maintenance of immune cell homeostasis and in autoimmunity,[18,19] indicating that a tight balance between FAS and FASL insures a proper immune response.

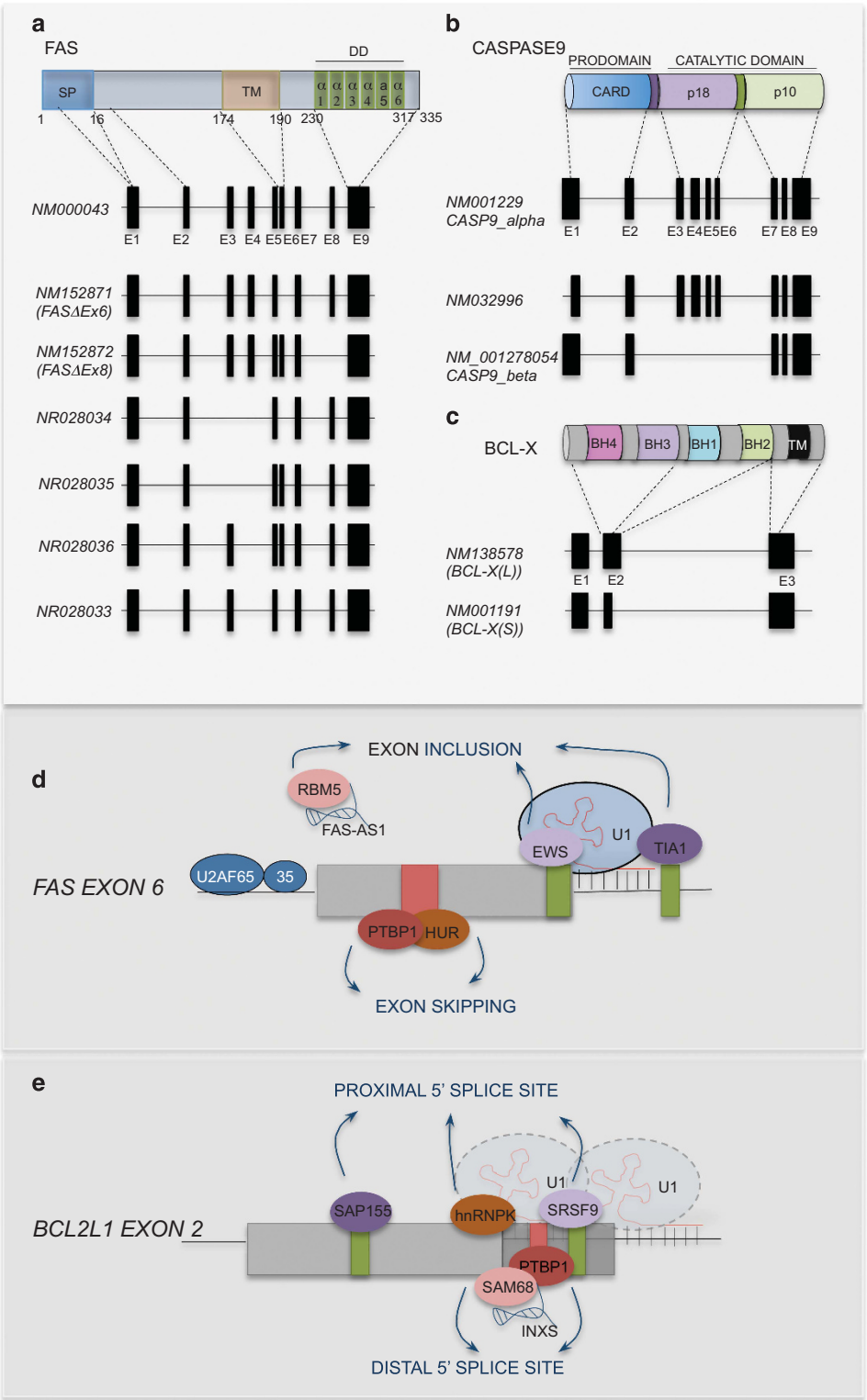
In addition to FAS full-length mRNA, shorter transcripts exist (Figure 2a). In most variants, AS alters the open reading frame and introduces premature termination codons (PTCs), resulting in transcripts that are candidates for nonsense-mediated decay (NMD) and likely not translated into proteins. However, in-frame skipping of exon 6 yields a soluble protein that lacks the transmembrane domain and inhibits FASL-mediated apoptosis[20] (Figure 2a). Because of its functional relevance, this event has been intensively studied. Binding of the T-cell intracellular antigen (TIA)-1 to intron 6,[21] or of the Ewing Sarcoma protein (EWS) within the exon,[22] favors splicing of the full-length variant. Both splicing factors interact with U1C and promote U1 snRNP assembly,[21–25] possibly facilitating recognition of the 5'-splice site by the spliceosome (Figure 2d). This regulation is modulated by the FAS-activated serine/threonine kinase (FAST K), which phosphorylates TIA-1 and enhances U1 snRNP recruitment.[26] Thus, FAS signaling can modulate splicing of its own transcript, suggesting that this mechanism forms an autoregulatory loop amplifying FAS response at occurrence, as during sustained T-cell activation. Conversely, the polypyrimidine tract-binding protein 1 (PTBP1)[25] and the Hu antigen R protein (HuR)[27] bind to an exonic splicing silencer and inhibit association of the U2 snRNP auxiliary factor (U2AF) and U2 snRNP with the 3' splice site, thus impairing exon 6 inclusion (Figure 2d). A different mechanism was described for the tumor-suppressor RBM5/Luca-15/H37. RBM5 directly contacts components of the U4/5/6 tri-snRNP and inhibits catalytic activation of the spliceosome after exon 6 definition by the U1 and U2 snRNPs.[28] The long non-coding RNA (lncRNA) FAS-AS1, corresponding to an antisense transcript of *FAS*, sequesters RBM5 and interferes with skipping.[29] It is likely that competition between all these splicing regulators determines the outcome of *FAS* splicing in any given cell, predisposing it to survive or die to different cues. In this regard, genome-wide screening for regulators of exon 6 splicing identified several cellular pathways involved in its modulation,[30] highlighting its multifactorial nature in human cells.

Splicing factors recruitment to *FAS* pre-mRNA could offer therapeutic perspectives in diseases associated with FAS function, like cancers displaying poor clinical outcomes (that is, Hodgkin's lymphomas, autoimmune lymphoproliferative syndromes). For instance, forced expression of EWS increased FAS and enhanced FAS-induced death in Ewing Sarcoma cells.[22] Thus, development of small molecules mimicking EWS function may result useful to directly impair cancer cell survival.

Caspase 9. Apoptosis culminates with the activation of caspases (Figure 1), a family of proteases that cleave substrates at the carboxyl side of specific aspartate residues.[31] Caspases share similarities in sequence, structure, and substrate specificity. They are synthesized as single-chain procaspases (30–50 kDa), comprising an NH2-terminal domain, a large (18–20 kDa) and a small subunit (~10 kDa)[31,32] (Figure 2b). Activation involves proteolytic processing between domains and binding to cofactors. Procaspase-9 activation is crucial for the accomplishment of the classic apoptotic program, and requires formation of a complex with APAF-1 and cytochrome c.[31-33] Knockout of the caspase 9 gene (*Casp9*) in mice caused perinatal death, due to excess cell survival causing malformations during brain development.[34] Moreover, *Casp-9* knockout thymocytes, ES cells, and fibroblasts are resistant to apoptosis,[34] confirming its key role in the execution of apoptosis.

The human *CASP9* gene encodes at least three protein-coding splice variants generated by either inclusion/exclusion of the exon 3,4,5,6 cassette or by different assortment of first exons (Figure 2b). In the latter case, the variants contain different 5'-untranslated regions (UTR) and lack the canonical start codon, with translation initiating at downstream codons and yielding shorter N-termini. On the other hand, skipping of exons 3,4,5,6 yields caspase-9 short (caspase-9S), which behaves as dominant-negative by counteracting death induced by the main isoform.[35] Caspase-9S lacks the catalytic domain but retains the caspase APAF-1-association domain and the small subunit (Figure 2b). Hence, it competes with caspase-9 for binding to APAF-1 and contributes to resistance to apoptosis.[35]

Binding of the splicing factor SRSF1 to a purine-rich cis-element in intron 6, 24 bp downstream of the splice site, promotes inclusion of the exon 3,4,5,6 cassette.[36] Instead, heterogeneous nuclear ribonucleoprotein L (hnRNP L) recognizes a purine-rich exonic splicing silencer in exon 3.[37] Phosphorylation of hnRNP L modulates *CASP9* AS. When hnRNP L is phosphorylated on Serine 52, such as in non-small cell lung cancers (NSCLC), its splicing activity is induced, leading to skipping of the cassette.[37] Candidate survival/oncogenic kinases (i.e., GSK3β, AKT, and casein kinase II) are



predicted to phosphorylate hnRNP L, suggesting that this mechanism is part of their oncogenic program. HnRNP U competes with hnRNP L for the same sequence, but produces opposite effects on the splicing outcome.[38] Since AKT-dependent phosphorylation of hnRNP L alters this balance and promotes splicing of caspase 9S,[37,38] targeting it by specific kinase inhibitors might represent an attractive approach for treatment of NSCLC and other cancers.[36–38]

BCL-X. The B-cell lymphoma-2 (BCL-2) protein family contains pro- and anti-apoptotic members. Pro-apoptotic proteins, such as BAX, cause formation of pores in the mitochondrial outer membrane eliciting its permeabilization.[1–4] Anti-apoptotic BCL-2 proteins protect cells by interacting with and preventing the activities of pro-apoptotic family members.[1–4]

BCL-X (*BCL2L1* gene) is an essential member of the BCL-2 family. *Bcl2l1*-deficient mice die around embryonic day 13, show death of hematopoietic cells in the liver, shortened lifespan of immature lymphocytes and extensive apoptosis of neurons in brain.[39] *BCL2L1* encodes two main splice variants:[13] the anti-apoptotic BCL-X_L (233 amino acids) and the pro-apoptotic BCL-X_S, which lacks 63 internal amino acids comprising two BCL-2 family-homology regions (BH1 and BH2) (Figure 2c). Accordingly, mutations in the BH1 or BH2 regions of BCL-2 and BCL-X_L disrupt their ability to heterodimerize with BAX and to inhibit apoptosis.[41] Other *BC2L1* splice variants with pro- (BCL-Xβ) and anti-apoptotic (BCL-$X_{\Delta TM}$, BCL-Xγ) functions have been described in mouse and human,[42–44] however the mechanisms underlying their AS regulation are still unknown.

BCL- X_L and X_S originate from two alternative 5'-splice sites in exon 2. Selection of the proximal site at the end of the exon yields BCL-X_L, whereas BCL-X_S originates from usage of a distal cryptic site ~200 bp upstream.[13] HnRNP F, H,[45] and I (PTBP1),[40] SAM68,[46] RBM11[47] and RBM25[48] promote the pro-apoptotic BCL-X_S variant, whereas SAP155,[49] SRSF9,[50] hnRNP K[51] and SRSF1[46,52] enhance splicing of BCL-X_L (Figure 2e). Site-specific and deletion mutagenesis identified two regions that are important for BCL-X splice site selection. One upstream of the proximal 5'-splice site enforces BCL-X_L splicing, while the other region, located immediately downstream of the distal 5'-splice site, favors BCL-X_S production.[45] HnRNP F/H proteins bind guanosine stretches in this second element and stimulate splicing of BCL-X_S.[45,53] Moreover, PTBP1 binds to a polypyrimidine tract between the two alternative 5' splice sites, in proximity of the hnRNP F/H binding site, displacing SRSF1 from the BCL-X_L 5' splice site and repressing its selection.[40]

Activation of signaling pathways modulate binding of splicing factors to *cis*-regulatory elements in the BCL-X pre-mRNA. Ceramide, a regulator of stress and proliferation, affects BCL-X_L 5' splice site selection by recruiting SAP155 to a purine-rich *cis*-element in exon 2.[49] Ceramide also induced extensive dephosphorylation of SR proteins,[54] thus influencing AS of *BCL2L1* and *CASP9*.[55] These findings are in line with the observation that ceramide induces cell cycle arrest, differentiation and apoptosis,[56] and suggest that AS modulation by signaling pathways could be exploited for therapeutic purpose. Another splicing factor regulated by post-translational modifications is SAM68.[57] When it is upregulated, SAM68 favors splicing of BCL-X_S and triggers cell death. Tyrosine phosphorylation by FYN disrupted SAM68-hnRNP A1 interaction and binding to RNA, reverting BCL-X splicing.[46] Interaction with the transcription factor FBI-1 also decreased SAM68 recruitment to the pre-mRNA and restored splicing of BCL-X_L.[58] Thus, modulation of SAM68 phosphorylation and/or protein–protein interactions can finely tune cell survival through AS of antagonistic BCL-X variants.

A genome-wide screening identified the mitotic aurora kinase A (AURKA) as regulator of *BCL2L1* splicing.[52] Mitotic arrest and/or AURKA inhibitors promoted splicing of BCL-X_S by increasing the turnover of SRSF1, which normally enhances BCL-X_L splicing.[52] These observations revealed a novel connection between AS regulation and apoptosis in response to activation of a cell cycle checkpoint.

Recruitment of splicing factors to the BCL-X pre-mRNA can be also regulated by lncRNAs. INXS is transcribed from the *BCL2L1* locus in antisense direction and its expression is lower in cancer cells.[59] Apoptosis-inducing agents upregulate INXS and favor splicing of BCL-X_S. INXS interacts directly with SAM68, suggesting that it may function by recruiting it to the BCL-X pre-mRNA. When INXS is absent or expressed at low levels the predominant output of splicing is BCL-X_L. Intratumor injections of a plasmid encoding this lncRNA, however, increased expression of BCL-X_S and reduced tumor burden in a mouse xenograft model.[60] Hence, downregulation of INXS in cancer might contribute to resistance to apoptosis, and its

Figure 2 Regulation of alternative splicing in apoptotic genes. (**a**) FAS protein scheme showing the three main domains (upper): signal peptide (SP), transmembrane domain (TM) and death domain (DD). DD is a protein module composed of a bundle of six α-helices. The lower panel shows the annotated FAS transcripts. Exons encoding each domain are indicated. NM000043 is the longest mRNA variant. The NM152871 variant 2 (FASΔEx6) lacks an in-frame exon encoding the transmembrane region. The NM152872 variant 3 (FASΔEx8) lacks a coding segment, which leads to a translation frameshift and a distinct, shorter C-terminus compared with isoform 1. The NR028034 (5), NR028035 (6), NR028033 (4) and NR028036 (7) variants lack, respectively, three (5), two (6 and 4) or one (7) alternative coding exons compared with variant 1 and are candidates for NMD. (**b**) In the upper panel, the three main domains of the CASP9 protein are depicted: CARD domain and the two catalytic domains (p18 and p10). The lower panel shows the annotated CASP9 transcripts. Exons encoding each protein domain are indicated. The NM001229 variant (α) encodes the longest isoform. The NM032996 variant differs in the 5'-UTR, lacks a portion of the 5'-coding region and yields a shorter N-terminus. The NM001278054 variant (β) lacks four alternative in-frame exons in the coding region and encodes the caspase-9 S isoform. (**c**) BCL-X comprises four BCL-2 homology (BH) domains. The anti-apoptotic BCL-X_L isoform (NM138578 variant) contains all BH domains and a transmembrane (TM) domain that anchors the protein to cellular membranes, including the mitochondrial outer membrane. The pro-apoptotic BCL-X_S isoform (NM001191 variant) uses an alternate in-frame 5'-splice site in exon 2, yielding a smaller protein that lacks BH2. (**d**) Alternative splicing of FAS is controlled by TIA-1, EWS, PTB and HUR, which directly bind the pre-mRNA and either promote or inhibit exon 6 inclusion. The lncRNA FAS-AS1 affects FAS alternative splicing by sequestering RBM5 and preventing its inhibitory effect of FAS exon 6 inclusion. (**e**) Regulation of *BCL2L1* alternative splicing. Enhancer and silencer *cis*-elements are indicated in either in green or in red. Representative splicing factors (SAP155, hnRNP K, hnRNP F/H, PTBP1 and SRSF9) and lncRNAs (INXS) affecting the recognition of the proximal (upper factors) or distal (lower factors) 5' splice site are depicted

targeted induction could be exploited for the development of a RNA-based therapy.

Alternative Splicing and Cancer Cell Survival

AS of genes involved in cell survival is often altered in cancer and contributes to the ability of neoplastic cells to withstand hostile environments.[9,61–64] In most cases, transformed cells opt for mechanisms that promote splice variants with pro-survival activity. This paradigm is exemplified by the regulation of the *TP53* gene.[65] *TP53* encodes for a transcription factor (p53) whose upregulation exerts tumor-suppressor function by promoting apoptosis and senescence in response to cellular stresses.[65] However, *TP53* is often mutated in cancer cells, thus relieving this negative feedback and providing them with an opportunity to by-pass checkpoints activated by cellular or genetic alterations. Notably, in the remaining cancer cells where the *TP53* gene is still functional, its aberrant splicing often yields loss of function or antagonistic variants that repress this apoptotic response.[62,65] Another way to keep p53 expression low is through its degradation. The MDM2 ubiquitin ligase that promotes p53 degradation is often upregulated in cancer cells.[66] More than 70 MDM2 splice variants have been described in cancer cells.[66] These variants potentially encode proteins with different functions, further expanding the splicing control of *TP53* in cancer. Moreover, the homologous MDM4 protein levels are normally kept low by exon 6 skipping and degradation of the transcript by NMD.[59] However, SRSF3-mediated inclusion of this exon in cancer cells yields full-length MDM4 expression and consequent suppression of p53.[59] Since antisense oligonucleotide (ASO)-mediated skipping of exon 6 reduced tumor burden in mouse models of melanoma and lymphoma,[59] it is conceivable that regulation of this AS event might represent a therapeutic target of clinical relevance (Figure 3a).

BCL2L1 splicing also has a role in human cancer. Expression of BCL-X_L correlates with increased chemoresistance of cancer cells[67] and upregulation of SRSF1 might underlie its preferential splicing.[52] BCL-X_L splicing is enhanced when SRSF1 is phosphorylated by NEK2,[68] a mitotic kinase frequently overexpressed in cancer.[68,69] Conversely, SRSF1 activity is counteracted by RBM4, which promotes BCL-X_S splicing and apoptosis. RBM4 is downregulated in cancer patients and its expression correlates with better prognosis.[70] Thus, upregulation of the NEK2-SRSF1 axis and/or RBM4 downregulation might lead to high expression of BCL-X_L in cancer. This feature can be exploited therapeutically by switching splicing toward the pro-apoptotic BCL-X_S through splice site-specific ASOs[67,71] (Figure 3a). Delivery of such ASOs by lipid nanoparticles was efficacious to reduce tumor size and metastasis in a mouse model of melanoma,[72] indicating the potential of this approach.

Alternative Splicing in Chemoresistance

Loss of checkpoints, dysregulation of cell cycle progression and/or enhanced response to growth factors underlie the unrestrained proliferation of cancer cells. Although chemotherapy represents an effective approach to counteract these features, cancer cells often develop resistance. Thus, elucidation of the molecular mechanisms involved in the acquisition of resistance is strategic to develop more efficacious therapies for human cancer. AS contributes to chemoresistance by enhancing plasticity of gene expression during the adaptive response to stress.[64] In this section, we report some splicing events and factors that are involved in such response. Other examples have been recently illustrated in extensive reviews on this topic.[73,74]

Genotoxic stress imposed by chemotherapy or irradiation induces changes in AS that affect cell survival.[75–78] This scenario suggests that cancer cells may develop resistance by modulating the expression or activity of splicing factors required for cell survival and/or by selection of cells that express the repertoire of splicing factors conferring pro-survival features. For instance, although most drugs increased splicing of BCL-X_S in non-neoplastic cells, only a subset of cancer cells showed the same regulation.[75] Thus, identification of splicing factors involved in splicing of oncogenic variants may pave the path to new therapeutic approaches.

SRSF1 upregulation causes neoplastic transformation, at least in part, through splicing of pro-oncogenic variants involved in proliferation and survival.[72] SRSF1 was upregulated upon short-term treatment of pancreatic adenocarcinoma (PDAC) cells with gemcitabine. SRSF1 promoted splicing of MNK2b, which in turn caused constitutive activation of the MAPK pathway and phosphorylation of the translation initiation factor eIF4E, thus enhancing cell survival.[79] These observations linked SRSF1 expression to the adaptive response to genotoxic stress (Figure 4).

AS contributes also to selection of PDAC cells that are stably resistant to drugs. Cells isolated after chronic exposure to gemcitabine displayed an altered splicing pattern of the pyruvate kinase (*PKM)* gene.[80] *PKM* encodes for two splice variants generated by usage of mutually exclusive exons 9 (PKM1) or exon 10 (PKM2). PKM2 is expressed during embryogenesis and promotes glycolytic metabolism. However, its expression is restored in cancer cells.[81] Gemcitabine-resistant clones showed higher PKM2/PKM1 ratio with respect to parental cell lines, and reversion of *PKM* splicing by an ASO-mediated approach rescued drug sensitivity.[80] Among the factors involved in the regulation of *PKM* splicing,[82] PTBP1 was upregulated in drug-resistant clones and favored PKM2 splicing by binding intron 8 and repressing exon 9 inclusion. Knockdown of PTBP1 expression reverted the PKM2/PKM1 ratio and rescued sensitivity to gemcitabine,[80] suggesting that targeting the PTBP1/PKM axis may counteract acquisition of chemoresistance in PDAC cells (Figure 4).

HnRNP H contributes to chemoresistance by altering splicing of the Thymidine phosphorylase (*TP*) gene. TP is an enzyme required for the conversion of capecitibine to 5-fluorouracil. HnRNP H binds intron 1 and exon 6 sequences in TP pre-mRNA, causing intron retention and degradation by NMD.[83] Accordingly, leukemia cells expressing high levels of hnRNP H showed lower expression of TP protein and increased chemoresistance with respect to parental cells.[83] Hence, modulation of the expression of a splicing factor during chemotherapy may favor survival by altering the metabolic route required for the effect of the drug.

SAM68 is upregulated in prostate cancer (PCa)[84] and other human cancers,[57,85] where it contributes to cell proliferation

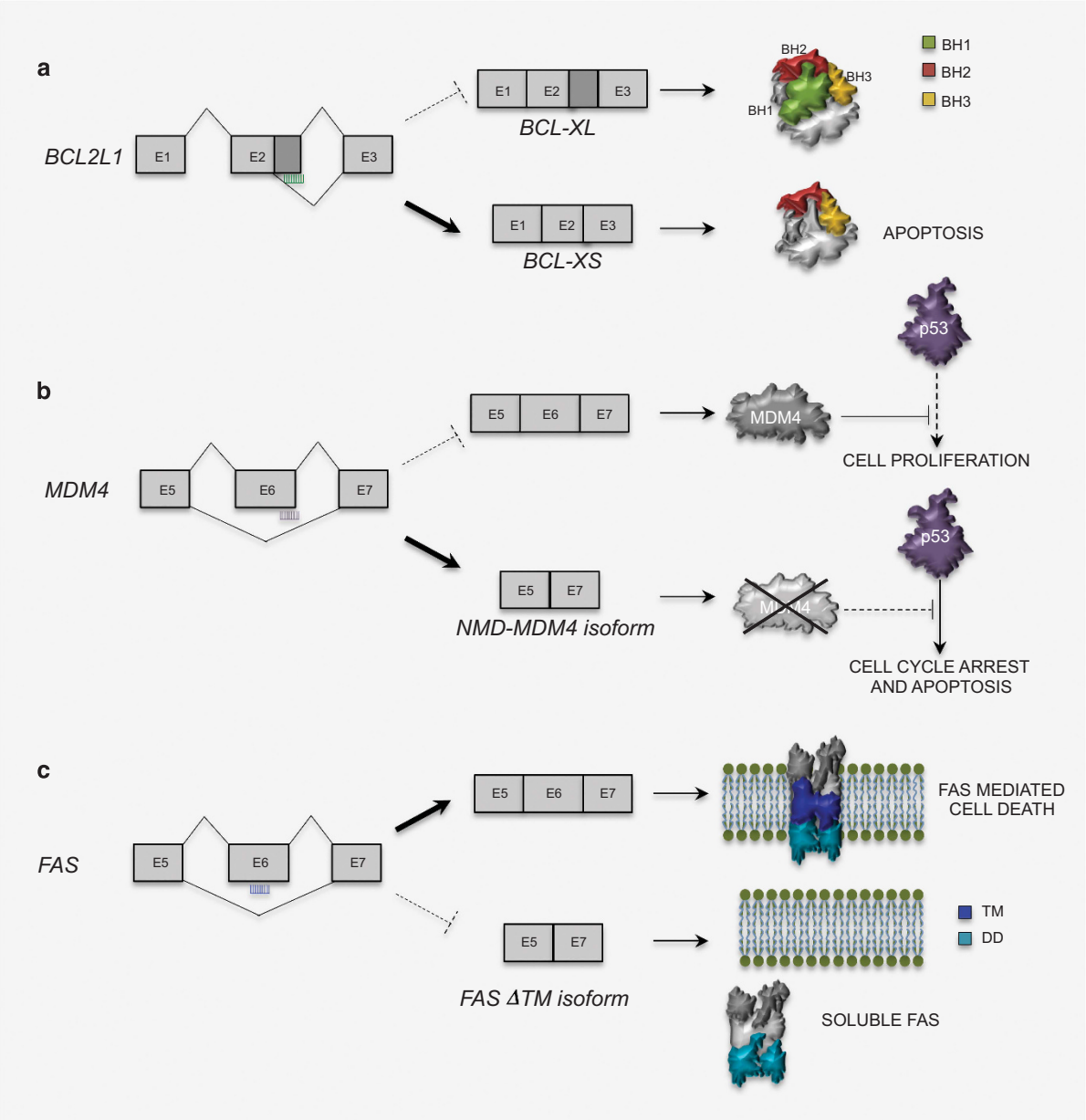


Figure 3 Cancer therapies based on antisense oligonucleotide-mediated splicing modulation. (**a**) Treatment of cancer cells with an ASO masking the proximal 5' splice site effectively switches BCL-X splicing and induces apoptosis. (**b**) MDM4 protein is highly expressed in embryonic tissues and in cancers as a result of enhanced exon 6 inclusion, driven mainly by SRSF3. ASO-promoting skipping of exon 6 is a suitable approach to inhibit p53 degradation by MDM4 in cancer cells. (**c**) Example of the hypothetical application of ASO therapy to FAS. An ASO masking the PTBP1-binding site in FAS exon 6 could be rescue FAS exon 6 inclusion, thus enhancing FAS-mediated cell death of cancer cells

and survival. SAM68 modulates AS of cancer-relevant genes, as *CD44,*[86] *BCL2L1,*[46] *CCND1*[87] and *AR,*[88] generally favouring oncogenic splice variants. Thus, its upregulation might represent a general feature of transformed cells, which confers an advantage by setting in motion an oncogenic splicing program. For instance, upon genotoxic stress, SAM68 clustered to foci of active transcription and promoted oncogenic CD44 variants.[89]

In some cases, the splicing factors responsible for AS events involved in chemoresistance are still unknown. Mutation of BAX in cancers characterized by microsatellites instability generally confers resistance to cell death.[90] However, a unique combination of microsatellite deletion in exon 3 with exon 2 skipping generates the BAXΔ2 isoform in some microsatellite unstable tumors, which is a potent death-inducer that sensitizes cells to chemotherapy.[91,92] Furthermore, genotoxic stress induces inclusion of the alternative exon 6A of the DHX9 helicase in Ewing Sarcoma cells. DHX9 interacts with the oncogenic EWS-FLI1 transcription factor and enhances its recruitment on target gene promoters.[78] Exon 6A contains a PTC and leads to NMD, thus reducing DHX9 protein expression and sensitizing cells to apoptosis.[78] These findings indicate that an NMD-linked splicing event

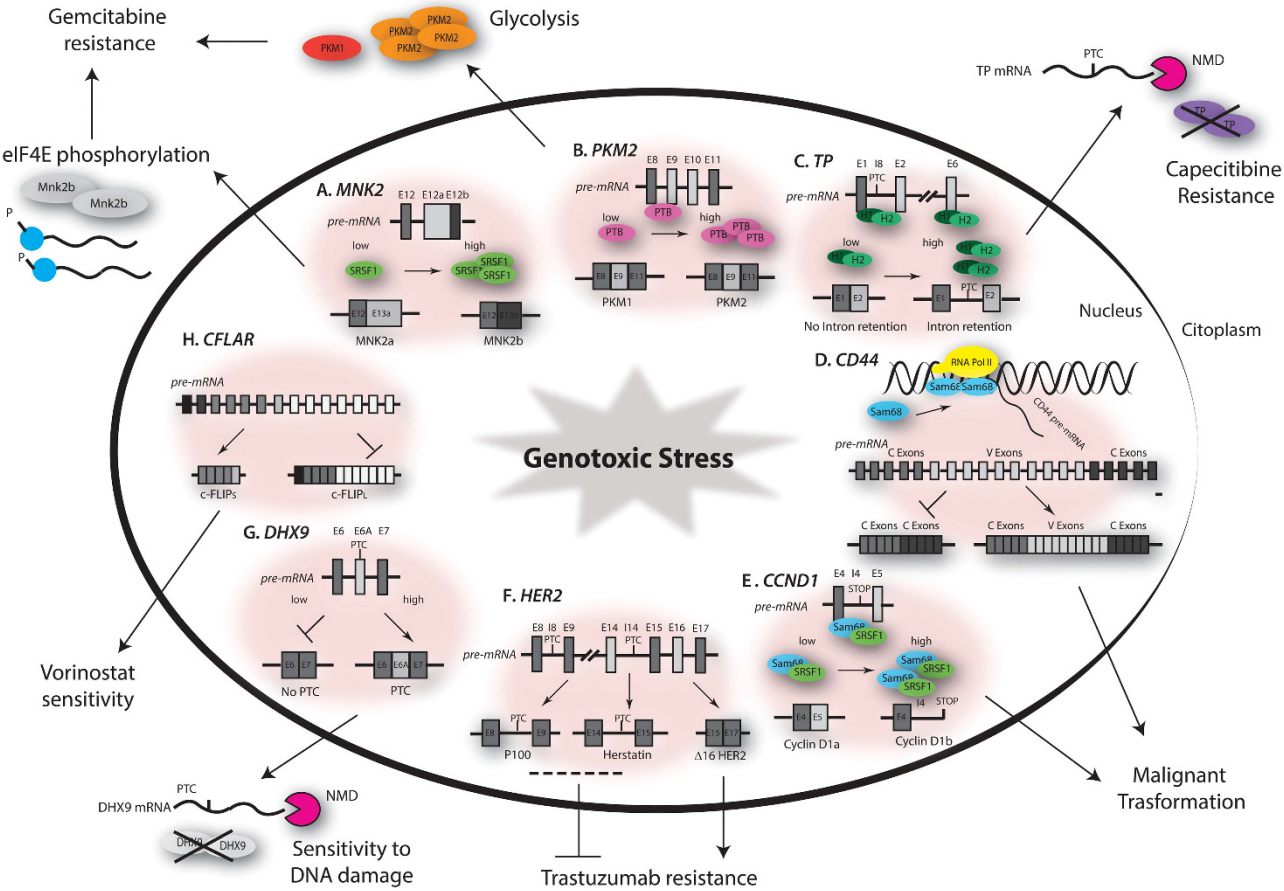


Figure 4 Alternative splicing and chemoresistance. Schematic representation of representative splicing events involved in genotoxic stress response. Gemcitabine resistance in pancreatic cancer is favored by short-term upregulation of SRSF1, MNK2b splicing (A) and eIF4E phosphorylation, or by long-term upregulation of PTBP1 and splicing of the pro-oncogenic PKM2 isoform (B). In AML, hnRNPs H1/H2 cause NMD-linked splicing of TP (C), leading to resistance to capecitibine. In prostate cancer, mitoxantrone causes a relocalization of SAM68 in active transcription sites (D), and leads to the inclusions of CD44 variable exons which confers pro-oncogenic features. In prostate cancer, malignant transformation correlates also with the upregulation of SAM68 and SRSF1, which promote the pro-oncogenic Cyclin D1b variant (E). In breast cancer, alternative splicing of HER2 confers different sensitivity to trastuzumab (F). Genotoxic stress causes the inclusion of exon 6 in DHX9 transcript in Ewing sarcoma cells (G), leading to NMD of DHX9 mRNA and sensitizing cells to apoptosis. In AML, expression of the shorter isoform of *CFLAR* gene, c-FLIPs, correlates with sensitivity to Vorinostat (H)

exerts a direct impact on EWS-FLI1 oncogenic activity in sarcomas (Figure 4).

The splicing signature of cancer cells can be used to predict response to chemotherapy. In Acute Myeloid Leukemia (AML), patients with high expression of the long splice variant of c-FLIP (c-FLIP$_L$) displayed lower overall survival.[93] C-FLIP$_L$ inhibits cleavage of caspase 8 and negatively regulates apoptosis. Treatment with the drug Vorinostat downregulated c-FLIP$_L$ and enhanced cell death induced by combined treatment with TNF-related apoptosis-inducing ligand (TRAIL).[93] These findings suggest that modulation of a single splicing event could represent a therapeutic approach for AML.

Another example of splice variant correlating with poor prognosis in patients is cyclin D1b. It originates from *CCND1* intron 4 retention and transcription termination upstream of exon 5.[94] Cyclin D1b is retained in the nucleus and more stable than the canonical variant.[94] It was associated with tumor progression and poor outcome in PCa,[95] while cyclin D1b expression in mice induced cellular transformation and tumor growth, confirming its oncogenic potential.[96] Since SAM68[87] and SRSF1[97] promote this splice variant in PCa cells and are upregulated in patients,[84,97] it is likely that dysregulation of *CCND1* AS is part of their oncogenic program in the prostate (Figure 4).

HER-2, a member of the epidermal growth factor receptor (EGFR) family, is frequently upregulated in breast cancers. The monoclonal anti-HER-2 antibody (trastuzumab) represents the elective therapy for these patients.[98] Three HER-2 splice variants differently influence response to chemotherapy and cell survival in breast cancer. Δ16HER2 originates from exon 16 skipping and is associated with malignant transformation and trastuzumab resistance.[99] The other splice variants, P100 and Herstatin, derive from retention of intron 14 and intron 8, respectively, and formation of a PTC. Both encode truncated proteins that interact with HER2 and impair its activity,[100,101] conferring a disadvantage for cancer progression (Figure 4). Thus, characterization of HER-2 splice variants in breast cancer patients could contribute to prognosis and be predictive of the efficacy of chemotherapy.

Emerging Implication of Alternative Splicing in the Regulation of Autophagy

Autophagy is an evolutionarily conserved mechanism employed by cells to adapt to hostile conditions.[102] Autophagy maintains cellular homeostasis in the absence of essential nutrients and its regulation is sensitive to changes in cell metabolism.[103] Under starvation or other stressful conditions, specific signal transduction pathways trigger the formation of membrane vesicles (autophagosomes) that encapsulate molecules, macromolecules or organelles. Autophagosomes fuse with lysosomes and the entrapped cellular components are degraded to fuel ATP synthesis and support metabolic routes under adverse conditions.

Regulation of autophagy involves many proteins that act in coordinated fashion in response to stimuli. It mainly relies on rapid post-translational mechanisms that directly influence the activity, stability or localization of autophagic proteins.[102,103] However, modulation of gene expression also contributes to fine-tune this process, as starvation activates a transcriptional program that coordinates the autophagic response with lysosomal biogenesis.[104]

Changes in transcription are often linked to changes in AS[76] and recent studies have unveiled the contribution of AS to autophagy regulation. BECN1 is a key component of the multiprotein complex that promotes nucleation of autophagosome precursors.[102,103] AS converts BECN1 from an inducer of nonselective macroautophagy to a regulator of mitochondria-selective autophagy.[105] BECN1s, a shorter splice variant originating from skipping of exons 10 and 11, selectively localizes in the outer membrane of mitochondria and contributes to mitophagy in response to starvation or mitochondrial membrane depolarization.[105] New splice variants were also identified in members of the autophagy-related gene 8 (ATG8) family (LC3B, GABARAP and GABARAPL1).[106] They encode proteins that are not integrated in the autophagosomes, suggesting that they could limit autophagy. In the case of LC3B, the LC3B-a variant originates from AS of intron 3, leading to a single amino acid deletion (Arg68).[106] This study highlights the power of AS to generate variants differing for single amino acids but displaying opposite activities.

AS can influence the autophagic response of cancer cells. DU145 PCa cells lack the canonical ATG5,[107] an essential protein for autophagy,[102] but express two alternative mRNAs that lack either exon 6 or exon 3 and 6, leading to premature termination of translation.[108] As a consequence, autophagy was not induced in DU145 cells by treatment with a chemotherapeutic agent that normally induces autophagy. Likewise, oncogenic U2AF35 mutants were shown to promote transformation by aberrant processing of ATG7 pre-mRNA, leading to an autophagy defect.[108]

Although very preliminary, these observations suggest that autophagy, like apoptosis, is likely modulated by AS through production of variants displaying different activities. Given the emerging role of autophagy in human diseases,[102,103] a better understanding of the impact of AS on the autophagic response will highlight new paths for the development of tools that modulate this process.

Conclusions and Perspectives

The complexity of the human genome is exemplified by the flexibility with which each gene is regulated through AS.[12] Nevertheless, this flexibility is also a risk factor, as many human diseases are linked to defective splicing. Thus, comprehension of the molecular basis of its regulation is believed to offer therapeutic perspective. Some approaches in this sense are already being developed. In Spinal Muscular Atrophy (SMA), rescue of *SMN2* exon 7 splicing represents a valuable therapy. An ASO interfering with binding of splicing repressors corrected *SMN2* splicing and rescued viability in mouse models.[109] This ASO is in clinical trial for SMA (ISIS 396443; www.clinicaltrials.gov), a disease for which no cure is available,[110] indicating that aberrant splicing is a 'druggable' defect. In principle, redirection of splicing outcome by ASOs could be applied to other genes implicated in disease (Figure 3). Genes involved in cell survival represent good candidates for human cancers. In some cases, such as for the 'unhealthy' splice variants of *BCL2L1,*[71] *PKM*[80] and *MDM4,*[59] preclinical evidence of ASOs efficacy has been provided and this paves the ground for clinical oriented studies. Improvement in ASO chemistry and administration protocols has recently ameliorated their efficacy in patients. An ASO directed against SMAD7 elicited positive clinical responses, and in some cases disease remission, in patients affected by Crohn's disease.[111] Elucidation of the AS events associated with cell survival and autophagy in human diseases will offer new therapeutic targets, in addition to representing diagnostic and prognostic tools. With these premises, development of splicing-directed therapies have the potential to become standard approaches to counteract cancer development and/or the adaptive response of cancer cells to chemotherapy.

Conflict of Interest

The authors declare no conflict of interest.

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