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Regulation of alternative splicing and the case of *Bcl-x*

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Abstract: Almost every human pre-messenger RNA is alternatively spliced to produce mRNA variants that often encode proteins with distinct functions. How regulation of alternative splicing is achieved is still poorly understood. The goals of this article is to review our current knowledge of how regulatory factors orchestrate splice site selection, particularly in the context of cancer. As our own investigations into this process in the last 10 years have focused on the apoptotic gene *Bcl-x*, we will indicate how this experimental model system has helped us gain insight into the complexity of molecular strategies and the role of unexpected regulators in the control of splice site selection. Notably, our recent work has also revealed how the DNA damage pathway and upstream signalling routes are converging to regulate alternative splicing decisions.

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Alternative splicing as a generator of diversity

Almost every human pre-messenger RNA (pre-mRNA) is remodeled through alternative splicing (Figure 1) to produce an average of eight to ten splice mRNA variants that can encode proteins with distinct functions[1-5]. Alternative splicing can also impact mRNA stability by producing mRNAs that are more sensitive or more resistant to miRNAs or to nonsense-mediated RNA decay[6,7]. Recent genomic efforts have confirmed the remarkable functional diversity generated by alternative splicing[8]. Genes containing tandem repeats, a subset of them known as minisatellites or VNTRs (for Variable-Number of Tandem Repeats) are also alternatively spliced. VNTRs change remarkable fast in the human population, with point mutations observed at each generation[9]. We have shown that VNTRs can be part of transcribed genes, often located in coding sequences. The presence of splice sites in VNTRs leads to high rate of AS[10]. The instability of VNTRs may therefore create novel mRNA and protein variants with distinct functional determinants. The combination of intrinsically unstable elements in an unstable nuclear environment (as in cancer cells or under stress) may provide a unique mechanism to produce splice variants that help cells and organisms to adapt rapidly to challenging environments.

Alternative splicing and cancer

Perturbations in alternative splicing are associated with many human diseases, and particularly cancer[8,11]. All the processes that control cell proliferation, invasion, metabolism, angiogenesis, resistance to anticancer drugs and anti-apoptotic mechanisms are regulated by alternative splicing[12-14]. Overall, more than 15,000 tumor-associated splicing changes have been compiled[14-17]. We found that 10% (4 of 41 tested) alternative splicing events associated with breast and/or ovarian cancer contribute to cancer cell survival[14] indicating that splicing alterations can drive the cancer phenotype. Other telling examples of splicing alterations that contribute to tumorigenesis include the production of a variant of the tyrosine kinase RON that promotes cell motility and invasion[18] and of a variant of pyruvate kinase M that is critical for tumor metabolism[19].

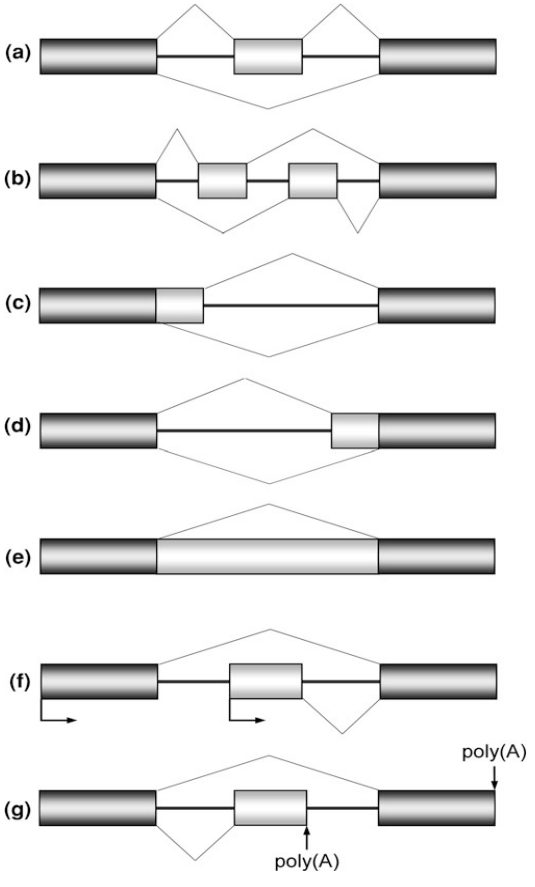

Figure 1: Patterns of alternative splicing. Different ways to splice a gene. Lighter gray boxes represent exons or exonic fragments that are alternatively spliced. **a** cassette exon, **b** mutually exclusive exons, **c** alternative 5' splice sites, **d** alternative 3' splice sites, **e** intron retention, **f** alternative promoters can affect the identity of the first exon, and **g** alternative polyadenylation sites can impact the structure of the terminal exon (modified from Shkreta et al, 2013). One gene can have multiple alternative splicing units.

Alternative splicing, apoptosis and the cell-cycle

Failure of cells to undergo apoptosis is one of the best studied hallmark of cancer, with many examples of variants with opposite functions such as the cell surface receptor Fas, and regulators such as the APAF-1, *Bcl-x* and caspases[20]. Although anticancer drugs often elicit apoptosis (and are often used for that purpose), their impact on the alternative splicing of apoptotic genes had never been investigated until we conducted a study with 20 drugs in several cancer cell lines[21]. While many drugs affected the splicing of apoptotic genes, the targets, amplitude and direction of the changes varied between compounds and cancer cell lines, underscoring the importance of an in-depth understanding of the molecular principles that coordinate splicing decisions.

Apoptosis and cell-cycle control are often linked, for example through the activity of transcription factors such as p53 and FOXO that control the expression of genes involved in both processes (Figure 2a)[22,23]. Coordination between apoptosis and cell-cycle progression also occurs through signal transduction. For example, the aurora kinase A controls mitosis and prevents the production of pro-apoptotic variants of *Bcl-x*, *Mcl1* and *caspase-9*[24]. Genes involved in cell-cycle control are alternatively spliced (including *p53*, *PTEN* and *CDKs*), but the functional impact of the splice variants have not been studied as much as apoptosis genes[25,26].

DNA damage elicits a cellular response that affects the cell-cycle because cell division needs to be stopped for repair. Apoptosis may then be triggered if DNA damage is too extensive. Given the links between cell-cycle and apoptosis (Figure 2a), and the fact that many apoptotic genes produce pro- and anti-apoptotic splice variants, it would not be unexpected to find that some aspects of the cellular response to DNA damage impact the alternative splicing of genes controlling both apoptosis and the cell-cycle. As preliminary data in support of this view, we identified alternative splicing events in cell-cycle genes that are altered by DNA damage and that are controlled by the same splicing factors that control the splicing of apoptotic genes[27].

A functional interface between RNA processing and genotoxic stress is also suggested by screens that uncovered RNA processing genes involved in the DNA damage response[28-31]. The only study that looked at the global impact of DNA damage (gamma rays) on alternative splicing identified many alterations in apoptotic and cell-cycle genes in human lymphoblastoid cell lines, although fewer than ten of these were validated[32]. While it is likely that most of these alterations are mediated by changes in the abundance or activity of splicing factors, little is known about upstream layers of regulation, such as signalling, that control splicing following DNA damage. UV light elicits the hyperphosphorylation of RNA polymerase II, changing its transcriptional elongation rate, a process that affects AS[33].

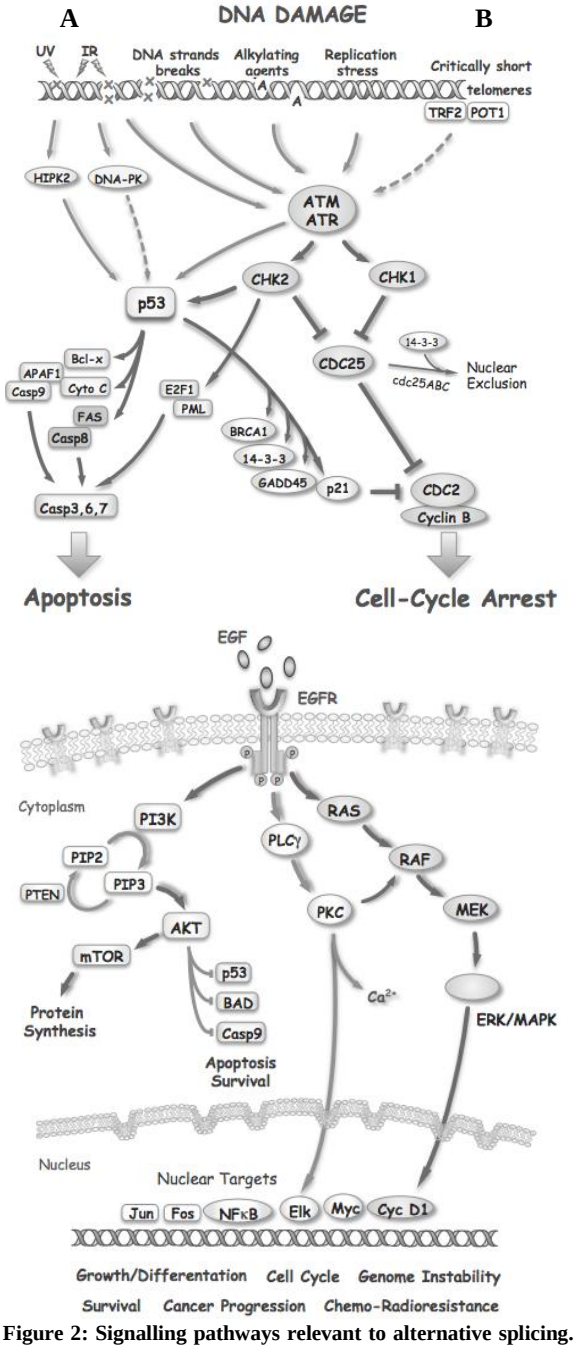

Figure 2: Signalling pathways relevant to alternative splicing. **A.** DNA damage pathways converging towards the control of apoptosis and cell-cycle progression. Shown are effects on proteins (expression or stability) but not on splicing. **B.** EGF signalling pathway; the PKC axis is relevant to *Bcl-x* splicing; the PI3K/AKT and the KRAS/BRAF axis may also affect *Bcl-x* splicing by regulating SRSF1 and Sam68, respectively.

As stated above, we began exploring the relationship between DNA damage and alternative splicing by focusing on DNA-damaging anticancer agents and found them to affect the splicing of apoptotic genes[21]. This initial study was followed by showing that oxaliplatin and cisplatin trigger an ATM/CHK2/p53-dependent splicing switch that neutralizes the homeostatic signalling and stimulates the production of pro-apoptotic *Bcl*-xS[34].

On the other hand, we know almost nothing about how DNA damage affects the activity of splicing regulators. A few studies have reported that DNA damage can alter the phosphorylation and deacetylation of SR proteins with an impact on RNA binding and a handful of alternative splicing events[35-41]. Upon DNA damage, hnRNP K is de-ubiquitinated and sumoylated, allowing it to cooperate with p53 to activate genes such as 14-3-3 and p21, ultimately causing cell-cycle arrest[42]. Other hnRNP proteins are sumoylated after DNA damage[43], but the impact of these modifications on alternative splicing has not been investigated.

Altered signalling pathways affect alternative splicing in cancer

Signalling pathways and splicing control are closely intertwined. One type of cancer where this intersection is becoming well documented is colorectal cancer (CRC). Alterations in the WNT, EGFR/RAS (Figure 2b) and/or TGF-□ signalling pathways frequently occur in CRC[44], and CRC-associated splicing alterations are linked to these signalling pathways. One example in the WNT/□-catenin pathway[45,46] is the transmembrane protein SLC39A14 which displays cancer-specificity and whose production is regulated by WNT through the activity of splicing factor SRSF1[47,48]. WNT signalling also causes elevated C-MYC expression, which is linked to the aberrant splicing of splicing factor FIR[49]. Importantly, the EGFR signalling pathway is misregulated in the majority of CRC[44]. Although disruption of the AKT-SRPK-SR protein axis has the potential to affect a plethora of splicing events, its disruption in CRC is largely undocumented, and most CRC-associated splicing alterations in the EGFR pathway are associated with the RAS/BRAF axis (which includes NRAS ad KRAS)[50]. For example, inclusion of exon v5 in the transmembrane adhesion glycoprotein *CD44* is upregulated by the RNA binding proteins SRm160 and Sam68, as part of the activated KRAS/BRAF/MEK/MAPK axis[51,52]. Exon v5 is an early and specific tumor marker, detectable in dysplastic polyps whose inclusion correlates with enhanced malignancy and invasiveness. CD44 exon v6 inclusion is also associated with tumor progression and reduced survival[53], and its inclusion requires SRSF2[54]. The exon v6-containing variant of CD44 can act as a co-receptor for the HGF/Met and VEGF/VEGFR complexes that activate KRAS[55,56]. KRAS signalling in turn promotes exon v6 inclusion through a positive feedback loop, providing a clear example of how signalling-dependent alternative splicing can stimulate mitogenic progression[51]. Also linked to KRAS signalling is FGFR2; overexpression of the FGFR2IIIc variant is observed in 27% of carcinomas and correlates with metastasis and poor prognosis. Downstream of KRAS is the BRAF kinase for which gain-of-function mutations occur in 50% of melanomas and 5-10% of colon carcinomas[44]. BRAF regulates MAPK signalling. Interestingly, the drug vemurafenib has remarkable clinical potency against melanomas by inhibiting mutant BRAF, but resistance frequently arises because of the production of a shorter, dimer-forming splice variant of BRAF. A functional role for alternative splicing in the epithelial to mesenchymal transition (EMT), a cellular process involved in cancer progression and metastasis, is underscored by the role of several splicing factors such as ESRPs and RBFOX2 in controlling the expression of key EMT regulators, and their aberrant expression in tumor tissues[11,17]. Thus and importantly, while signalling pathways can affect alternative splicing by modulating the activity of splicing regulators, alternative splicing can also regulate the activity of components of signalling pathways[14,57]. Despite these observations, the interconnections between alternative splicing and activated signalling pathways in cancer remain largely incomplete. For all cancers, a more comprehensive and systematic analysis of the function of the signalling pathways and their impact on tumor growth is needed to produce a useful model for tumor development and cancer progression.

Other pathways converge to regulate alternative splicing

In addition to signalling, other processes can affect splicing decisions. Promoter identity can elicit the recruitment of specific splicing regulatory proteins[58]. The speed of transcription can affect co-transcriptional splice site selection by dictating the time that a splicing regulatory complex has to assemble before another splice site is used as an alternate. Changes in the proportion of splice variants in cancer cells can also be caused by miRNA-induced degradation, especially since miRNA expression has been associated with different steps of the tumorigenic process[59]. Moreover, 3'UTRs, the region usually targeted by miRNAs, are extensively restructured by alternative splicing in cancer cells (Sandberg et al, 2008). While miRNAs have been linked to the expression of

splicing regulators (e.g. PTB) [60], the relevance of these observations to splicing regulation in cancer has not yet been investigated.

One of the major findings of recent genome analysis efforts is the discovery of long noncoding RNAs (lncRNAs); nearly 15 000 lncRNAs have been annotated so far[61]. One of the first to be discovered was MALAT1 (for metastasis-associated lung adenocarcinoma transcript 1) which regulates metastasis in lung cancer[62,63]. MALAT1 mutations occur in CRC mostly at the 3' end where splicing factors such as RNPS1 (an EJC auxiliary component) are binding[64]. This region regulates alternative splicing[65] and can stimulate the growth and migratory properties of CRC cell lines[66].

Altered splicing regulation in cancer by RNA binding proteins

Although mutations at splice sites and splicing regulatory elements of alternatively spliced genes have been linked to familial forms of cancer, it is unclear whether such mutations frequently contribute to sporadic cases of cancer[8]. Genes encoding components of the core splicing machinery are often mutated in myelodysplasic syndromes, which can evolve into acute myelogenous leukemia[67]. Whether similar genetic alterations occur in other forms of cancer has not yet been reported, although a recent study has found that mutations in the constitutive splicing factor U2AF65 occur in many cancers[68]. On the other hand, the expression and activity of RNA binding proteins (RBPs) that control alternative splicing are almost always misregulated in cancer[11,14,69,70,71]. For example, overexpression of SRSF1 in breast cancer favors the production of the RON kinase variant involved in cell motility and invasion[18]. The expression of hnRNP A1 and PTB proteins is also tightly linked to the production of a splice variant of pyruvate kinase M that promotes aerobic glycolysis in cancer cells[19,72]. We found that repressing RBFOX2 is essential to establish the epithelial splicing signature of more than 100 pre-mRNAs in breast and ovarian cancers[73,74]. RBFOX2, in association with hnRNP H and F proteins, also controls splicing of the mutually exclusive exons IIIb (epithelial) and IIIc (mesenchymal) in FGFR2, conferring distinct binding preferences to ligands[75]. hnRNP H is often overexpressed in tumors and also controls splicing of A-Raf[76]. We have recently found that RBFOX2 and QKI combinatorially control alternative splicing decisions in the microenvironment of ovarian tumors[77].

Molecular mechanisms of alternative splicing control

A process that produces proteins with distinct functions should be tightly controlled. Indeed, splicing regulation can occur at every step of spliceosome assembly, but most examples of regulation involve the early recruitment of splicing factors to splice sites[78]. Repression can occur through a variety of mechanisms including the blocking of splice site recognition or through the assembly of complexes that prevent normal spliceosome assembly[79,80]. SR proteins were initially categorized as stimulating factors (Figure 3a)[81], while hnRNP proteins were branded as negative regulators (Figure 3b)[82]. This proposed division of labor was short-lived since SR proteins were quickly documented to display repressor function[83] (Figure 3c). Likewise for hnRNP proteins, as they were shown to antagonize the repression imposed by some SR proteins[84] (Figure 3c).

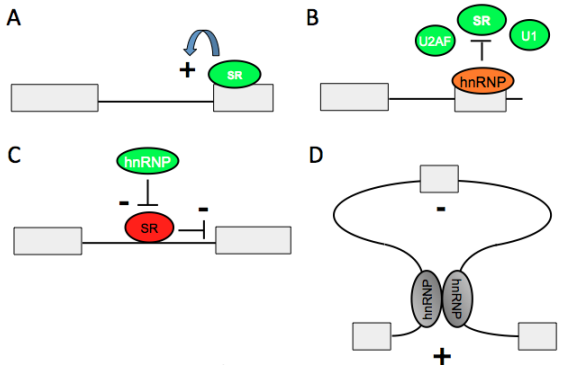

Figure 3: Examples of splicing regulation. A. An exonic splicing enhancer (ESE) bound by SR proteins stimulates U2 snRNP binding to the branchsite region (Lavigueur et al, 1993). The ESE is active when located less than 300 nt from the 3'ss. **B.** hnRNP proteins binding in an alternative exon or near splice sites can prevent the interaction of SR proteins or other factors involved in splice site recognition (e.g. U1 snRNP at the 5'ss and U2AF at the 3'ss) (Martinez-Contreras et al, 2007). **C.** SR protein binding in the intron can repress splicing (Simard & Chabot, 2002). This repression is abrogated by hnRNP I/PTB (Paradis et al, 2007). **D.** Looping occurs following the interaction between hnRNP proteins bound at distinct locations (Blanchette & Chabot, 1999; Chabot et al, 1997)). A splice site or an exon in the loop is repressed (Nasim et al, 2002), whereas splicing between the distal exons is stimulated because brought into closer proximity.

The distinction between activators and repressors becomes more ambiguous when we consider hnRNP proteins that bind on both sides of a splice site. In this case, their cross-interaction represses splicing, while the splicing between external exons is simultaneously stimulated because the loop brings them into closer proximity[82,85-87] (Figure 3d). Global studies performed on a handful of regulators suggest that the binding position of an RNA binding protein (RBP) determines its activity as an activator or a repressor, although how this affects splice site recognition remains unknown in most cases[88].

Our laboratory was one of the first two groups to document a physical connection between

transcription and splicing[89,90]. The role of this coupling in AS is now amply demonstrated (Kornblihtt et al, 2013); transcription complexes and modified chromatin components can recruit splicing factors or alter the elongation rate of RNA polymerase II, a process that in turn affects the selection of competing splice sites. Histone modifications can provide docking sites for interactions with splicing regulators[92,93]. Inhibitors of histone deacetylases affect splicing control and can display anti-cancer specificity[93]. Notably, siRNAs and AGO proteins, by altering histone marks on chromatin, will affect RNA polymerase elongation and alternative splicing of fibronectin and *CD44* model pre-mRNAs[94,95]. While miRNAs control the expression of some RBPs[60], long non-coding RNAs (lncRNAs) may modulate splicing by preventing the spreading of a histone mark that decreases the elongation speed of RNA polymerase II[96,97].

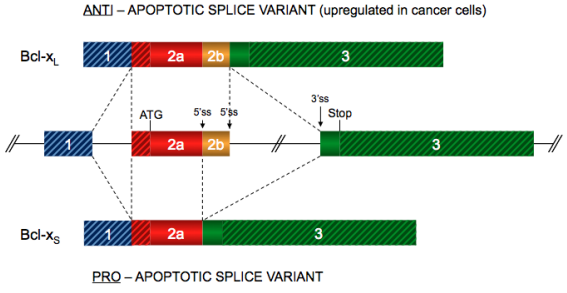

Figure 4: Bcl-x splicing. The long form (Bcl-xL; anti-apoptotic) and the short form (Bcl-xS; pro-apoptotic) are produced by the use of competing 5' splice sites separated by 189 nt.

SR and hnRNP proteins are equipped to be on the receiving end of signalling pathways because they can be phosphorylated, a modification that alters their activity and localization. For instance, phosphorylation of hnRNP A1 by the MNK kinases provokes its accumulation in the cytoplasm[99,100] whereas dephosphorylation of SRSF10 during mitosis or upon heat shock transforms it into an inhibitor of splicing[101]. Many kinases phosphorylate SR proteins, and a recent study showed that the AKT axis of the EGF signalling pathway (Figure 3a) controls a myriad of alternative splicing events, some of which relevant to cancer[50]. The RAS/MAPK axis (Figure 3a) is also known to control alternative splicing of *CD44* through phosphorylation of the splicing regulator Sam68[52]. In general however, little is known about how specific signals are transduced to regulate alternative splicing[101].

Bcl-x as a model gene to understand alternative splicing regulation

While studying individual splicing regulator proteins can yield valuable mechanistic insight, splicing decisions are often the result of a contribution involving multiple regulators. To better understand how combinatorial regulation is achieved, we focused on *Bcl-x*, a gene that produces two variants through alternative 5' splice site (5'ss) selection (Figure 4):

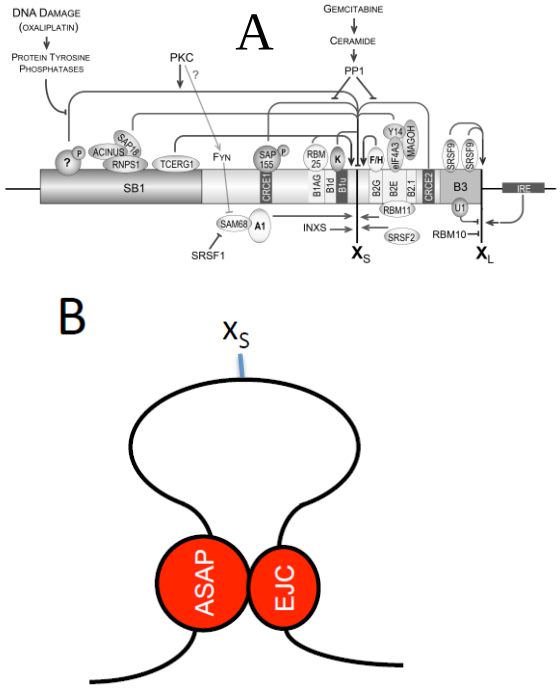

Figure 5: Controlling *Bcl-x* splicing. A. The IRE acts as an activator but the identity of splicing regulators mediating its activity is unknown. The B3 element is made up of binding sites for U1 snRNP that repress splicing. This repression is attenuated by the binding of SRSF9 (aka SRp30c) to stimulate U1 snRNP binding at the authentic 5'ss of Bcl-xL (Cloutier et al, 2008). RBM10 is proposed to bind to the Bcl-xL 5'ss to block its use (Inoue et al, 2014). eIF4A3, Y14 and Magoh make up the core exon-junction complex (EJC) that represses Bcl-xS splicing (Michelle et al, 2012). RNPS1, SAP18 and Acinus form the ASAP complex (Apoptosis and Splicing Associated Proteins) also repressing Bcl-xS but from the SB1 element (Michelle et al, 2012). hnRNP F/H stimulate the use of Bcl-xS (Garneau et al, 2005) while hnRNP K represses it (Revil et al, 2009). SAP155 binds to CRCE1 and is phosphorylated by protein phosphatase 1 (PP1) to repress the 5'ss of Bcl-xS (Massiello et al, 2006). CRCE2 negatively regulates Bcl-xS but the factor implicated is unknown (Chalfant et al, 2002). PP1 activity is controlled by ceramide whose production is induced by gemcitabine (Chalfant et al, 2002). RBM11 enhances the production of Bcl-xS by binding to sequences downstream of B2G element and antagonizing SRSF1 (Pedrotti et al, 2012). SRSF2 (aka SC35) has also been documented to stimulate the production of Bcl-xS (Merdzhanova et al, 2008). The long non-coding RNA INXS may stimulate use of the Bcl-xS 5′ss by recruitment of Sam68 (DeOcesano-Pereira et al, 2014). See text for more details. **B**. Alternative representation based on the postulated interaction of ASAP with EJC components. Looping the 5'ss of Bcl-xS would repress its use. **B**. Alternative representation based on the postulated interaction of ASAP with EJC components. Looping the 5'ss of Bcl-xS would repress its use.

Bcl-xL is anti-apoptotic[102], whereas the shorter *Bcl-xS* variant is pro-apoptotic[103-105]. Our work has provided a fundamental description of the complexity of splice site selection. In normally growing cells, the production of *Bcl-xS* is strongly repressed, but repression is lifted following DNA damage. Below we review our current knowledge of some of the sequences, regulatory factors and signalling routes that control *Bcl-x* splicing in normal growth conditions, and the changes that occur upon DNA damage.

a. Controlling 5´ss selection

A few elements and factors affect the 5´ss of Bcl-xL (sequence elements IRE and B3 in Figure 5a)[106]. However, most efforts have provided information on how the 5´ss of *Bcl-xS* is regulated. Our initial investigations identified hnRNP K as a repressor of *Bcl-xS* acting through the B1 element (Figure 5a)[107], and hnRNP F/H as activators of *Bcl-xS* recruited by the B2G element located downstream of the 5´ss (Figure 5a)[108]. The phosphatase 2A inhibitor protein SET interacts with hnRNP K to improve its binding and repress the production of *Bcl*-xS[110]. The mechanism by which hnRNP K itself represses splicing is for the moment unclear. As for hnRNP F/H, a collaborative study with the group of Frédéric Allain in Zurich suggests that hnRNP F/H may stimulate the 5'ss of Bcl-xS by preventing the formation of inhibitory G-quadruplexes in the G-rich environment of the *Bcl-xS* 5´ss[111].

A more surprising observation that we made was to identify eIF4A3 as a repressor of the 5´ss of *Bcl-xS*[27]. The surprise came from the fact that eIF4A3 was known as a component of the *core* exon-junction complex (EJC) deposited on mRNA concomitant with splicing to control mRNA stability, but not splicing[111]. Our study showed that eIF4A3 binds to the *Bcl-x pre-mRNA (*EJC in Figure 5a)[27]. Other EJC *core* components similarly repressed the production of Bcl-xS. In addition, the auxiliary EJC factors (RNPS1, Acinus and SAP18) which can form a complex called ASAP[113] also repressed the 5´ss of Bcl-xS. Intriguingly however, the core and auxiliary EJC factors bind to different sequences on the *Bcl-x* pre-mRNA. Indeed, we used a collection of mutants to link the activity of RNPS1 with the upstream SB1 element, while the activity of eIF4A3 was associated with an element downstream of the 5'ss of *Bcl-xS* (Figure 5a)[27]. Based on these observations, our working model is that an interaction between bound ASAP factors and bound core EJC components loops and represses the 5´ss of *Bcl-xS* (Figure 5b). This looping to repress a 5'ss is reminiscent of the way that we have proposed for hnRNP A1 to regulate 5'ss selection[87]. The role of EJC components regulating *Bcl-x* splicing is intriguing. What could be the advantage of using components of the EJC to control the alternative splicing of apoptotic regulators? Approximately 10% of the human transcriptome is regulated by NMD[113]. The NMD pathway eliminates mRNAs containing premature stop codons (PTCs) that arise through mutations or aberrant splicing[115]. NMD also eliminates unproductive splice variants that contain PTCs[115]. The production of such variants from genes encoding RNA binding proteins represents a strategy that controls the homeostatic levels of RNA binding proteins implicated in the processing of nearly all the mRNAs made by a cell[116]. Efficiently eliminating aberrant mRNAs and maintaining appropriate levels of RNA binding proteins therefore require that components of NMD machinery be produced in sufficient amounts. Indeed, NMD efficiency appears to be directly related to the concentration of RNPS1[117]. Producing appropriate levels of NMD components may be particularly critical for cancer cells because genomic instability most likely increases the level of toxic mRNA-encoded products. This may explain why the levels of Y14 and other EJC components are increased in high-grade serous ovarian cancers[118]. Thus, the regulation of alternative splicing by EJC components may function as a checkpoint to insure that NMD is fully operational; a decrease in the level of EJC components may alter the alternative splicing of key regulators to trigger apoptosis.

Another regulatory element named CRCE2 has been implicated in 5'ss regulation but its mechanism and the factor(s) that may associate with it are unknown[119]. Another set of recent findings concerns the implication of the RNA binding proteins Sam68 and hnRNP A1. Upregulation of Sam68 promotes *Bcl-xS* splicing. hnRNP A1 was found as a partner of Sam68, but the binding location of these proteins on the *Bcl-x* transcript was not examined[41]. More recently, the transcription factor FBI-1 was found to interact with Sam68 to reduce its binding to *Bcl-x* transcripts and the use of *Bcl-xS*[120]. Through a collaboration with the group of Carles Suñé in Granada (Spain), we also uncovered a link with transcription and showed that the upregulation of transcription elongation factor TCERG1 favor the production of the pro-apoptotic *Bcl-xS*[121]. If an overly processive RNA polymerase II is more mutagenic, as we can predict it could be if faulty nucleotides are incorporated, then *Bcl-x* splicing may be a sensor that monitors excessive TCERG1 activity.

Interestingly, a long non-coding RNA (lncRNA) of 1903 nt named INXS has been implicated in *Bcl-x* splicing regulation[122]. INXS is transcribed from the opposite genomic strand of *Bcl-x* and its expression increases the pro-apoptotic Bcl-xS splice variant.

Three apoptosis-inducing agents (UV-C light exposure, serum starvation and anti-cancer drug sulforaphane) increased INXS expression. It has been propsoed that INXS may help recruit Sam68 in the proximity of the *Bcl-xS 5′* ss[122].

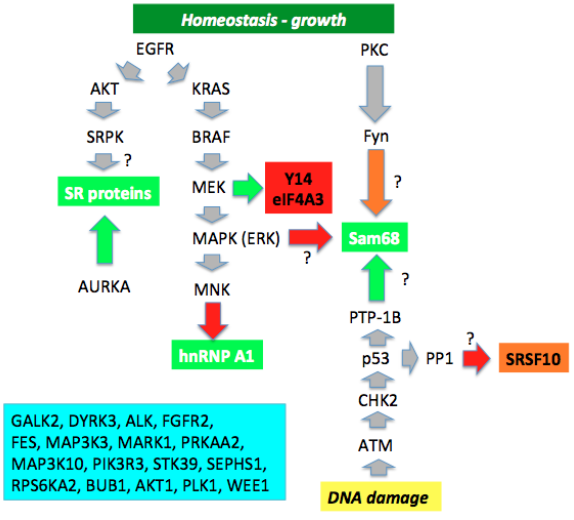

Figure 6: Signalling pathways and components relevant to Bcl-x splicing. The AKT axis as well as the cell-cycle kinase AURKA may maintain active SRSF1 whose depletion promotes Bcl-xS splicing (Hayes et al, 2007; Zhou et al, 2012). The KRAS axis can modulate Sam68 activity through ERK (Matter et al, 2002). The MNK kinase is also activated by the KRAS axis and phosphorylates hnRNP A1 which cannot migrate to the nucleus (Buxade et al, 2005; van der Houven van Oordt et al, 2000). hnRNP A1 collaborates with Sam68 to stimulate the production of Bcl-xS (Paronetto et al, 2007). PKC and DNA damage pathways are antagonistic (Revil et al, 2007; Shkreta et al, 2011) and may be converging on Sam68. Sam68 dephosphorylation is required for its activity as an enhancer of Bcl-xS (Paronetto et al, 2007). DNA damage activates the ATM/CHK2/p53 pathway that converges on the tyrosine phosphatase PTP-1B whose activation is required to lift the repression of Bcl-xS (Shkreta et al, 2011). A potential target for PTP-1B may be Sam68.

RBM25 and RBM10 have also been identified as modulators of *Bcl-x* splicing. The binding of RBM25 to a CGGGCA sequence in exon 2 helps the recruitment of U1 snRNP to the weak *Bcl-xS* 5' ss[50]. In contrast, RBM10 would bind to the 5'ss of Bcl-xL to block its use[123]. SRSF1 (aka ASF/SF2) and kinases that phosphorylate this SR protein also influence *Bcl-x* splicing by encouraging the production of *Bcl*-xL[24,41,106]. How SRSF1 achieves regulation of *Bcl-x* splicing is unknown. Finally, RBM11 enhances the production of Bcl-xS by binding to sequences in exon 2 (localized within the B2 element but downstream of B2G) and antagonizing SRSF1[124].

b. Signalling and DNA damage control Bcl-x splicing

Inhibiting protein kinase C (PKC) with staurosporine and other more specific inhibitors releases the repression of *Bcl-xS* in 293 cells[125]. The factor onto which PKC signalling converges is unknown but one possibility is Sam68 (Figure 6). PKC interacts with the tyrosine kinase Fyn[126], possibly preventing Fyn from phosphorylating Sam68, and hence repressing its binding to *Bcl-x* transcripts and the production of *Bcl-xS* (Figure 5a). Another potential target of PKC in 293 cells may be the EJC auxiliary factor RNPS1 since both RNPS1 and PKC act through the SB1 element[27,125]. It is intriguing to consider that this PKC-mediated repression observed in immortalized 293 cells has not been seen in any of the cancer cell lines that we have investigated[125]. In cancer cells, while the SB1 element is also important for repression, PKC inhibition did not affect *Bcl-x* splicing. This uncoupling of PKC signalling and *Bcl-x* splicing suggests the existence of other signalling routes that enforce the repression of Bcl-xS splicing in order to downregulate apoptosis in cancer cells[125]. It is therefore not totally surprising that a siRNA-based screen launched to identify genes controlling *Bcl-x* splicing reported more than 20 signalling components capable of affecting *Bcl-x* splicing in HeLa cells (Moore et al, 2010) (see Fig. 6). Moreover, the PP1 phosphatase was linked to *Bcl-x* splicing through SAP155, which interacts with the CRCE1 (Figure 5a) [119].

In all cancer cells that we have tested, the anti-cancer drugs oxaliplatin and cisplatin switch splicing in favor of the pro-apoptotic Bcl-xS splicing variant[21]. This splicing shift occurs through activation of the DNA damage-associated ATM/CHK2/p53 signalling axis. This axis converges on the activation of tyrosine phosphatases that regulate *Bcl-x* splicing through the SB1 element[34].

Interestingly, the PKC and the DNA damage response pathways are linked to the proteasome-mediated protein degradation pathway[34]. Because our results suggest that the repressor binding to SB1 may require to be phosphorylated and that phosphorylation has often been associated with protein stability, we monitored the impact of the proteasome inhibitors bortezomib and MG132 on *Bcl-x* splicing. We found that these compounds, while not affecting *Bcl-x* splicing in normal conditions, antagonize the impact of DNA damage and PKC inhibition[34]. If protein degradation regulates the level of the splicing regulator, we reasoned that inhibiting its production by blocking translation may produce a similar splicing shift. Another group had reported an effect of protein synthesis inhibitors (emetine and cycloheximide) on *Bcl-x* splicing but they attributed the shift as independent of protein synthesis[127]. When we blocked protein synthesis by incubating cells in a

medium lacking methionine, a strong shift in *Bcl-x* splicing was observed, and this shift was antagonized by bortezomide and MG132[34]. Thus, the SB1 repressor may rapidly become limiting when protein synthesis is inhibited allowing to couple global translation efficiency with the alternative splicing of *Bcl-x*. In conclusion, our results support a model in which a delicate balance between protein synthesis and degradation determines the appropriate level of a splicing repressor that controls a life-or-death splicing decision. The stability of this repressor would in turn be regulated positively through PKC signalling and negatively through dephosphorylation following DNA damage.

CONCLUSION

Our work has contributed to decipher the molecular mechanisms by which individual regulatory elements and RBPs enforce the alternative splicing of the apoptotic gene *Bcl-x*. A challenge that we are eager to tackle now is to determine how these individual elements collaborate, interact and possibly synergize to provide specific and dynamic regulation. A fully connected map describing how combinatorial sets of proteins converge to regulate *Bcl-x* splicing will also require that these processes be integrated with signalling pathways. We have initiated this work and identified some players but much remain to be done to produce a complete map of these converging interactions and how they can be reconfigured in different cellular states or during stress. As *Bcl-x* is one of many genes whose alternative splicing is of vital importance to cell survival and growth, it will be of paramount relevance to determine how the above regulatory circuits coordinately control the alternative splicing of other apoptosis and cell-cycle genes to produce a functional network. Our preliminary work in this direction is encouraging since we have identified other apoptotic genes whose splicing is regulated by EJC components including *Bim*, *Lig3*, *Capn3* and *Slit2*[51]. Likewise, we identified a few cell-cycle genes that shift the proportion of their splice variants when EJC components are depleted. Based on these data, we predict that alternative splicing control will turn out to be a functionally important cellular strategy used to coordinate apoptosis and cell-cycle progression. A better understanding of how alternative splicing is regulated in these gene categories in the normal and cancer states as well as following stresses like DNA damage should provide significant insight into molecular strategies for concerted regulation. This knowledge may ultimately provide new and unsuspected opportunities for therapeutic interventions pertinent to cancer.

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