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Deregulation of splicing factors and breast cancer development

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ABSTRACT

It is well known that many genes implicated in the development and progression of breast cancer undergo aberrant alternative splicing events to produce proteins with pro-cancer properties. These changes in alternative splicing can arise from mutations or single nucleotide polymorphisms (SNPs) within the DNA sequences of cancer-related genes which can strongly affect the activity of splicing factors and influencing the splice site choice. However, it is important to note that absence of mutations is not sufficient to prevent misleading choices in splice site selection. There is now increasing evidence to demonstrate that the expression profile of ten splicing factors (including SRs and hnRNPs) and eight RNA-binding proteins changes in breast cancer cells compared to normal cells. These modifications strongly influence the alternative splicing pattern of many cancer-related genes despite the absence of any detrimental mutations within their DNA sequences. Thus, a comprehensive assessment of the splicing factors status in breast cancer is important to provide insights into the mechanisms that lead to breast cancer development and metastasis. Whilst most studies focus on mutations that affect alternative splicing in cancer-related genes, this review focuses on splicing factors and RNA-binding proteins that are themselves deregulated in breast cancer and implicated in cancer-related alternative splicing events.

INTRODUCTION

The majority of human protein-coding genes produce multiple mRNA transcripts by alternative splicing. This RNA processing event is mediated by a complex interplay of splicing factors (SFs) and defined RNA sequences (splice sites) within pre-mRNA transcripts. Splice sites are short conserved sequences located within the pre-mRNA that define the start/end of the exons and introns (Srebrow and Kornblihtt, 2006) (**Table1, Figure1**). Splice sites act as binding sites for the spliceosome - a multi component complex comprising of five small nuclear ribonucleoproteins (snRNPs) working with 100-200 different non-snRNPs (McManus and Graveley, 2011). Selection of different splice sites is under the control of the splicing factors to either promote or prevent the recruitment of the spliceosome to the splice sites (Cartegni et al., 2002) (**Figure 1**).

It is now established that aberrant alternative splicing events are associated with the onset and aggression of breast cancer (Dutertre et al., 2010). One such example is that of the *BRCA1* gene. *BRCA1* is a tumour suppressor and mutations within its genetic sequence are associated with breast cancer (Orban and Olah, 2003). Alternative splicing of *BRCA1* can generate variants which lack functional domains of the protein, thus compromising its tumour suppressor activity (Orban and Olah, 2003). The use of splicing assays reveals that mutations within specific sequences can affect the binding of splicing factors and this in turn strongly influences the splicing pattern of *BRCA1* (Raponi et al., 2014). It is not uncommon that point mutations that affect the binding of splicing factors to the pre-mRNA can lead to erroneous control over the spliceosome, resulting in inaccurate alternative splicing of cancer-related genes (Cartegni et al., 2002). As such, the production of proto-oncogene splice variants might be favoured over tumour suppressor splice variants. In addition to mutations at binding sites, changes in the nuclear concentration of splicing factors can also have repercussions on splice site selection.

Deregulation of splicing factors, and also other RNA-binding proteins, disrupts alternative splicing patterns of many genes associated with cancer (Grosso et al., 2008). This review focuses on splicing factors that have critical roles in regulating cancer-related genes associated with development and progression of breast cancer.

SPLICING FACTORS

Splicing factors are RNA-binding proteins that interact with specific RNA sequences, or motifs, known as exonic splicing enhancers or silencers (ESEs, ISEs) and intronic splicing enhancer and silencers (ISEs, ISSs) (**Figure 1**) (Cartegni et al., 2002). Once the splicing factors are bound to the pre-mRNA, they can either guide or block the interaction between spliceosome and pre-mRNA (McManus and Graveley, 2011). Some splicing factors are capable of playing a dual role either activating or inhibiting splicing depending on the sequence that they bind. To date over 30 splicing factors have been well described in human cells and tissues (Venables *et al.*, 2008; Twyffels *et al.*, 2011) and they include two families: serine-arginine proteins (SR) and heterogeneous ribonucleoproteins (hnRNP). SRs usually favour the definition of exons and introns by binding enhancer sequences (**Figure 1**) and recruiting the spliceosome to the pre-mRNA, thus promoting splicing (Cartegni et al., 2002; David and Manley, 2010). Conversely, hnRNPs usually inhibit the splicing process by binding to silencer sequences (David and Manley, 2010) and blocking the interaction between pre-mRNA and the spliceosome or antagonizing the effects of the SRs (Cartegni et al., 2002).

SR PROTEINS AND THEIR ROLE IN BREAST CANCER

The SR proteins are a family of RNA-binding proteins that contain a serine/arginine rich (RS) domain at the carboxy-terminal region. At the amino-terminal region they have one or more RNA-recognition motifs (RRMs), required for binding to RNA (Manley and Tacke, 1996; Twyffels et al., 2011). Many SR proteins are involved in other processes of gene regulation including RNA maturation, decay, transport and translation. SR proteins can be recruited onto the nascent transcript by RNA Polymerase II together with members of the spliceosome (Fu, 1995; Twyffels et al., 2011). To date, twelve SR proteins have been well described (Twyffels et al., 2011) and six of them (SRSF1,2,3,5,6 and 10) are overexpressed in breast cancer (Huang *et al.*, 2007; Dutertre *et al.*, 2010); these proteins regulate RNA processing events of numerous genes involved in cell cycle regulation, cell proliferation, apoptosis, epithelial-to-mesenchymal transition (EMT), angiogenesis and also drug resistance (He *et al.*, 2004; Ghigna *et al.*, 2005; Karni *et al.*, 2007; Amin *et al.*, 2011).

SRSF1 (SF2/ASF)

SRSF1, the most well characterised SR protein, is involved in splicing, nonsense mediated decay, RNA translation, nuclear export of the mature RNA and also genome stability (Zuo and Manley, 1993; Li and Manley, 2005). As a splicing regulator SRSF1 can interact with the polypyrimidine tract helping recruitment of snRNP U2 in proximity of the 3' splice site; SRSF1 also enables the selection of authentic 5' splice sites at the expense of cryptic splice sites (Krainer et al., 1990). SRSF1 has also been classified as proto-oncogene; it activates the Ras-Raf-MEK-ERK signalling pathway, commonly up-regulated in cancer, and its overexpression stimulates cell proliferation as well as inhibiting apoptosis (Karni *et al.*, 2007; Anczukow *et al.*, 2012; Das *et al.*, 2012; Shimoni-Sebag *et al.*, 2013). In breast cancer it is common to observe amplification of the *SRSF1* gene and increased levels of its protein providing further evidence to highlight its importance in cancer-associated alternative splicing events. High levels of SRSF1 strongly affect alternative splicing of many genes linked with cancer pathophysiology including the ribosomal-protein kinase *S6K1*, the tumour suppressor *BINI* and kinases *RON* and *MNK2* (Karni et al., 2007; Anczukow et al., 2012).

The *S6K1* gene (*RPS6KB1*) is frequently amplified in breast cancer resulting in a poor prognosis for the patient (Yamnik et al., 2009). S6K1 is a serine/threonine kinase and an important modulator of protein translation, cell growth and proliferation (Yamnik et al., 2009) (Ben-Hur et al., 2013). With relevance to breast cancer, S6K1 phosphorylates estrogen receptor α (ER α), thus regulating its transcriptional activity (Yamnik et al., 2009). Alternative splicing of *RPS6KB1* can produce a long splice variant (Iso-1) and a short splice variant (Iso-2). Iso-2, but not Iso-1, enhances cell motility and transformation (Ben-Hur *et al.*, 2013). SRSF1 strongly favours the production of Iso-2 by promoting inclusion of three alternative cassette exons of *RPS6KB1*: these are excluded in Iso-1 (Karni et al., 2007; Ben-Hur et al., 2013). As a result, generation of the Iso-2 variant, which contains an alternative C-terminal region and which has oncogenic activity, prevails over the tumour suppressor Iso-1.

The tumour suppressor *BINI* is another target of SRSF1. Overexpression of SRSF1 strongly promotes inclusion of exon 12A of *BINI* resulting in loss of its tumour suppressor activity (Karni *et al.*, 2007). The tumour suppressor role of Bin1 resides in its ability to inhibit the activity of the oncogene Myc which regulates cell cycle and proliferation. Interestingly, a lack of Bin1 has often been seen in malignant breast cancer where it leads to a hyper-activation of Myc and a poor prognosis of the disease (Ge et al., 1999; Ge et al., 2000).

SRSF1 also regulates alternative splicing of the proto-oncogene *RON* by promoting the production of a splice variant with even higher oncogenic properties compared to the wild type (Ghigna *et al.*, 2005). *RON* gene is commonly overexpressed in cancer, including breast, leading to cell growth and invasion (Cunha *et al.*, 2014). Wild type protein Ron is a tyrosine kinase receptor which plays a crucial role in epithelial to mesenchymal transition (EMT). During EMT, tumour cells acquire migratory and invasive properties which lead to a metastatic phase in different tumour tissues included breast (De Craene and Berx, 2013). Alternative splicing of the *RON* gene can generate a splice variant known as Δ Ron, which arises from exclusion of exon 11 (Ghigna *et al.*, 2005). Such an event can be triggered by SRSF1 via binding to an exonic splicing enhancer (ESE) located within exon 12 of *RON*, and enhanced skipping of exon 11. Compared to the wild type, the Δ Ron splice variant lacks a portion of the extracellular domain; this bestows an extension of its half life and, as such, increases its potential oncogenic activity (Ghigna *et al.*, 2005; Ghigna *et al.*, 2008; Cunha *et al.*, 2014). Moreover, Δ Ron is constitutively activated and can overcome proteolytic cleavage resulting in a more aggressive proto-oncogenic variant (Ghigna *et al.*, 2005).

Mitogen-activated protein (MAP) kinase-interacting kinases are a family of protein kinases which include splice variants Mnk2a and Mnk2b. Mnk2b is principally found in the nucleus and increases phosphorylation of the translational factor eIF4E (Scheper *et al.*, 2003). Increased levels of Mnk2b result in hyper-activation of eIF4E which, in turn, is positively related to cell growth and survival of breast cancer cells (Li *et al.*, 1997; Soni *et al.*, 2008). Overexpression of SRSF1 regulates the inclusion of an exclusive exon 13b of the *MKNK2* gene promoting production of the Mnk2b splice variant (Karni *et al.*, 2007).

The oncogenic transcription factor c-Myb is highly expressed in breast tumours classified as ER positive and is required for the proliferative, anti-apoptotic and invasive properties of tumour cells (Drabsch *et al.*, 2010; Thorner *et al.*, 2010; Quintana *et al.*, 2011; Knopfova *et al.*, 2012). In contrast, other studies claim that a deficiency of c-Myb increases tumorigenesis, suggesting that it may also have tumour suppressor activity (Thorner *et al.*, 2010). Two alternative splice variants of *c-Myb* have been described (Kumar *et al.*, 2003). The most common splice variant, with anti-oncogenic activity, is a 75kDa protein; the second 89kDa splice variant of *c-Myb*, with pro-oncogenic properties, results from inclusion of an alternative exon 9A (Kumar *et al.*, 2003). SRSF1 is able to regulate alternative splicing of c-

Myb, although the precise mechanism of action remains unclear (Karni *et al.*, 2007). Moreover, using qPCR it was revealed that several alternative splice variants of *c-Myb* mRNA exist from the inclusion of alternate exons: 8A, 9A, 9B, 10A, 13A, and 14A, although their precise roles, if any, have not as yet been described (O'Rourke and Ness, 2008). SRSF1 also regulates alternative splicing of apoptosis regulator Bcl-2 related genes. Bcl-2 family members include both pro-apoptotic and anti-apoptotic proteins. BIM and Mcl-1 are both two members of Bcl-2 family. In breast cancer cell lines, high expression of SRSF1 enhances inclusion of exon 2 of *Mcl-1*, promoting generation of anti-apoptotic variant of Mcl-1 (Gautrey and Tyson-Capper, 2012). SRSF1 are also promotes production of two new splice variants of *BIM* (*BIM* γ 1 and γ 2) which fail to block the activity of the anti-apoptotic Bcl-2 members (Anczukow *et al.*, 2012).

Vascular Endothelial Growth Factor (VEGF) is a protein that strongly promotes angiogenesis and its expression profile correlates with the estrogen receptor status of breast cancer cells (Adams *et al.*, 2000). The *VEGF* gene undergoes alternative splicing to produce two splice variant groups that are either angiogenic or anti-angiogenic; exon 8 of *VEGF* includes two 3' splice sites termed proximal 3' splice site and distal 3' splice site. From selection of the proximal 3' splice site, angiogenic splice variants, such as VEGF165, can be generated. Conversely, from the selection of the distal 3' splice site, anti-angiogenic splice variants, such as VEGF165b, are produced (Amin *et al.*, 2011). Interestingly, hyperphosphorylation of SRSF1 leads to an overproduction of the angiogenic variant VEGF165 by promoting selection of the proximal 3' splice site of the exon 8 (Amin *et al.*, 2011).

Another SRSF1 target is APC (Adenomatous polyposis coli); APC is a tumour suppressor that inhibits cell proliferation by blocking the transition to S phase of the cell cycle (Ho *et al.*, 1999). SRSF1 promotes inclusion of exon 14 of the *APC* gene resulting in a transcript with a premature stop codon; this leads to a deficiency of the mature tumour suppressor APC (Goncalves *et al.*, 2009).

The cyclin D1 gene (*CCND1*) is often overexpressed in different tumours included the breast (Roy and Thompson, 2006; Kim *et al.*, 2009). The resulting protein, cyclin D, is involved in cell cycle progression stimulating the transition from cell phase G to phase S by associating with cyclin-dependent kinase proteins and tumor suppressors (Roy and Thompson, 2006; David and Manley, 2010). The most common splice variant of *CCND1*, called cyclin D1a,

includes all 5 exons of the gene, whereas cyclin D1b arises from inclusion of intron 4 and skipping of exon 5 (Olshavsky et al., 2010) resulting in a protein lacking a nuclear export signal and retention of nuclear cyclin D1b (Kim et al., 2009; Paronetto et al., 2010). SRSF1 modulates formation of proto-oncogenic cyclin D1b (Olshavsky et al., 2010) and upregulation of cyclin D1b occurs in different cancers, included breast (Kim et al., 2009; David and Manley, 2010; Paronetto et al., 2010).

Rac1 is a GTPase that belongs to the Rho family and is mainly known for its role in cellular cytoskeleton growth and organization. Recent studies show that its activity also influences cell proliferation and its abundance is higher in breast tumour samples compared to normal tissue (Schnelzer *et al.*, 2000; David and Manley, 2010). During alternative splicing of the *Rac1* gene, SRSF1 promotes inclusion of exon 3b, resulting in a splice variant called Rac1b (David and Manley, 2010). Compared to Rac1, Rac1b is principally found in an activate state and it is positively associated to transcription of cyclin D and cell transformation (Zhou et al., 2013).

Whilst overexpression of SRSF1 is associated with breast cancer, its deficiency alters alternative splicing of the major hereditary breast cancer marker, *BRCA1*. To date many splice variants of *BRCA1* have been documented (Lixia et al., 2007; Tammaro et al., 2012); variants lacking either exon 9 or 10 are amongst the most commonly investigated in breast cancer (Orban and Olah, 2003) (Tammaro *et al.*, 2012). Knockdown of SRSF1 has shown to promote exclusion of both exons 9 and 10 of *BRCA1* (Karni et al., 2007) altering the function of this important tumour suppressor. Amongst its many roles, *BRCA1* is involved in DNA damage repair, chromatin remodelling and cell cycle control. It contains several domains for interacting with transcriptional factors, such as the tumour suppressor p53, and also with *BRCA1*-associated genome surveillance complex (BASC) which is also involved in DNA damage repair (Caestecker and Van de Walle, 2013).

SRSF2 (SC35)

SRSF2 is an SR protein with similar characteristics and properties to SRSF1 (Fu *et al.*, 1992). SRSF2 is involved in cell cycle progression and genomic stability maintenance (Xiao *et al.*, 2007). As a splicing factor, SRSF2, like SRSF1, is required for recruiting the spliceosome complex and initiating splicing (Fu *et al.*, 1992). Moreover, SRSF2 regulates alternative splicing of the tumour suppressor gene *KLF6*. *KLF6* is a member of the Kruppel-like

transcription factor family. Besides the full length KLF6 protein, alternative splicing of *KLF6* generates a splice variant, SpKLF6, which arises from inclusion of a cryptic exon located within intron 1; this is commonly excluded in KLF6. Although the function of SpKLF6 has yet to be defined, its transcript contains a premature stop codon which creates a protein lacking the DNA binding domain. SRSF2 promotes inclusion of the cryptic exon promoting formation SpKLF6 at the expense of the tumour suppressor KLF6 (Shi *et al.*, 2008).

SRSF3 (SRp20)

Another SR protein with multiple roles in transcription, alternative splicing, polyadenylation, nuclear transport and translation is SRSF3 (Jia *et al.*, 2010). One study evaluating SRSF3 expression in different stages of mammary tumorigenesis (normal, preneoplasia, neoplasia and tumorigenesis) showed a positive correlation between high levels of SRSF3 (both protein and mRNA) and severity of the disease (Stickeler *et al.*, 1999); these two studies collectively suggest that oncogenic SRSF3 plays a critical role in tumour initiation and progression of the disease. Moreover, SRSF3 overexpression can promote cell growth, transformation and increases production of FoxM1b-c, Cdc25B, and PLK1 which in turn accelerates the G2/M phase transition and cell proliferation (Jia *et al.*, 2010). Conversely, SRSF3 knockdown blocks cell cycle progression (Jia *et al.*, 2010). Different studies have focused on the involvement of SRSF3 in the regulation of *p53*, *MRP1*, and *FOXMI* which are strongly linked to cancer and also drug resistance.

P53 is a tumour suppressor implicated in cell cycle regulation and maintaining DNA stability to prevent mutation accumulation (Yang *et al.*, 2013). P53 activates cellular senescence and promotes apoptosis in cells subjected to mutations, thus, blocking any uncontrolled cell proliferation (Gasco *et al.*, 2002; Tang *et al.*, 2013). SRSF3 deficiency results in increased production of p53 β , a splice variant of p53 that is responsible for cellular senescence activity. p53 β arises from inclusion of exon i9 of the *p53* gene, an exon that is commonly skipped. SRSF3 inhibits the inclusion of exon i9 by binding specific motifs within the p53 pre- mRNA sequence (Tang *et al.*, 2013).

SRSF3 is also a splicing regulator of multidrug resistance-associated protein 1 (*MRP1*) (He *et al.*, 2004); expression of SRSF3 in ovarian cancer is associated with an increase in the number of *MRP1* splice variants which may be involved in resistance towards a commonly used chemotherapeutic drug, doxorubicin (He *et al.*, 2004). *MRP1* is frequently

overexpressed in breast carcinoma leading to poor survival and resistance towards many antineoplastic drugs (Nooter *et al.*, 1997; Filipits *et al.*, 1999; Kovalev *et al.*, 2013). Investigating the roles of SRSF3 in the regulation of *MRP1* would potentially help to further understand the mechanisms of drug resistance in breast cancer.

SRSF5 (SRp40)

Another SR overexpressed in breast tumours is SRSF5 (Huang *et al.*, 2007; Dutertre *et al.*, 2010); a strong association between SRSF5 expression and the production of oncogenic splice variants of *CD44* exists in some breast tumours (Huang *et al.*, 2007). *CD44* is a cell surface glycoprotein receptor that has many roles particularly in cell adhesion and migration. Furthermore, *CD44* is involved in cell growth, cell cycle regulation and cytoskeleton organization (Naor *et al.*, 1997; Cheng and Sharp, 2006; Olsson *et al.*, 2011). The *CD44* gene has 20 exons; amongst these, 10 are constitutive exons whilst the remaining are variable exons (“v” exons) and reside between the constitutive exons 5 and 6 (Cheng and Sharp, 2006). Variable exons are not included in the common *CD44* protein and their expression is mainly reported in different tumours, including breast. Moreover, their expression profile correlates with estrogen receptor status (Pind and Watson, 2003; Olsson *et al.*, 2011). Overexpression of SRSF5 has been linked to oncogenic variants of *CD44* that arise from the inclusion of the variable exons v2, v3, v5, and v6 (Huang *et al.*, 2007). V5 and v6 containing variants trigger the mechanisms that lead to metastasis and cell invasion (David and Manley, 2010). Similarly to SRSF1, SRSF5 can also promote formation of the anti-apoptotic Mcl-s splice variant through the regulation of the alternative splicing of the *Mcl-1* gene (Gautrey and Tyson-Capper, 2012).

SRSF6 (SRp55)

Both epidemiological and experimental evidence implicates a crucial role for estrogen and estrogen receptors ($ER\alpha$) in the progression of breast cancer; one SR protein deregulated by estrogen is SRSF6 (Lal *et al.*, 2013). Estrogen reduces the abundance of SRSF6 in MCF-7 cells resulting in exon skipping events within the corticotropin-releasing hormone receptor type 1 (*CRH-R1*) gene which, in turn, affect the receptor function and inhibit estrogen-induced cell proliferation (Lal *et al.*, 2013). SRSF6 also regulates alternative splicing events of *CD44* by promoting inclusion of the oncogenic variable exon v7 (Pind and Watson, 2003). However, SRSF6 can also act as a tumour suppressor by regulating the alternative splicing of

VEGFA and promoting the formation of the splice variant VEGF165b. VEGF165b is known to be less angiogenic compared to the common variant of VEGFA (David and Manley, 2010). Additionally, binding of SRSF6 onto pre-mRNA of *FGFR1* (fibroblast growth factor receptor) leads to inclusion of exon- α ; this splicing event promotes the formation of a splice variant of *FGFR1*, called FGFR1- α , whilst preventing the production of an oncogenic splice variant known FGFR1- β (Ghigna et al., 2008). The FGFR1- α variant contains three immunoglobulin-like domains on its extracellular domain, whilst FGFR1- β lacks one of them. As a result, FGFR1- β can bind the ligand FGF1 (fibroblast growth factor 1) with high affinity, thus stimulating cell growth and promoting acquisition of cancer properties (Groth and Lardelli, 2002). It is interesting to note that protein levels of FGFR1- β increase in breast cancer cells and the ratio FGFR1- α /FGFR1- β decreases comparing to normal cells (Luqmani et al., 1995).

SRSF10 (Tra2 β)

SRSF10 is more commonly referred to as Tra2 β . A paralog form of human *TRA2 β* gene exists and codes for another splicing factor known as Tra2 α (Best et al., 2014). High levels of one homologue of Tra2 β , called Tra2 β 1, were found in breast cancer, particularly at an invasive stage (Watermann et al., 2006; Dutertre et al., 2010). Tra2 β 1, together with YB-1 (an RNA-binding protein which also acts as splicing factor), influences alternative splicing events of *CD44* by promoting inclusion of both v4 and v5 exons and giving rise to oncogenic CD44 proteins (Watermann et al., 2006). Conversely, a study using the breast cancer cell line MDA-MB-231, reported that lack of both Tra2 α and Tra2 β misleads alternative splicing of the *CHEK1* gene through exclusion of its exon 3; this affects the normal translational process of CHEK1 resulting in loss of CHEK1-mediated DNA repair and cell cycle arrest in response of DNA damage (Best et al., 2014). Finally, using mini-gene splicing assays reveals that Tra2 β promotes inclusion of the 3.4 kb exon 11 of *BRCA1* gene which is needed for its full tumour suppressor activity (Raponi et al., 2014).

HnRNPs AND THEIR ROLE IN BREAST CANCER

HnRNPs are another well described family of splicing factors that are also involved in mRNA trafficking, stability and translation (He et al., 2005). HnRNPs present one, or more, RNA-binding domains and a domain for protein-protein interaction. HnRNPs, usually bind splicing silencer sequences (either ESSs or ISSs, **Figure 1**) preventing the splicing process

(Twyffels *et al.*, 2011). Different mechanisms of splicing inhibition by hnRNPs have been proposed: they can compete with the SR proteins for binding sites and block interactions of the spliceosome with pre-mRNA. Moreover, hnRNPs can interact with each other altering the structure of the pre-mRNA, and in doing so making some regions inaccessible to the spliceosome (Cartegni *et al.*, 2002). Numerous roles for hnRNPs in cancer development have been reported, including inhibition of apoptosis, promotion of EMT and angiogenesis; hnRNPs can control these important processes through regulation of alternative splicing highlighting their involvement with disease pathophysiology (Han *et al.*, 2013). The fact that hnRNPs, such as hnRNP A1, A2, I and K, are overexpressed in breast cancer highlights the importance of assessing splicing factors status for preventing the risk of cancer associated variants. The following section focuses on hnRNP A1, A2, I and K, particularly focusing on their roles in regulating alternative splicing of both oncogenes and tumour suppressors.

hnRNPA/B family

hnRNPA/B proteins are a subfamily of hnRNPs; their expression profile is related to different types of cancer included breast (Patry *et al.*, 2003; He *et al.*, 2005; Li *et al.*, 2009; David and Manley, 2010). hnRNPA/Bs include the homologous proteins hnRNPA1, A2, A3, A2/B1, B2, C1, C2 (Shi *et al.*, 2003; He *et al.*, 2005; Chaudhury *et al.*, 2010). Amongst these, hnRNPA1 is one of the most well studied splicing factors (Mayeda *et al.*, 1993; Pollard *et al.*, 2000; Patry *et al.*, 2003). HnRNPA1 is overexpressed in breast tumours together with other members of the hnRNP family (Patry *et al.*, 2003; He *et al.*, 2005; Li *et al.*, 2009). However, in contrast to SRSF1, hnRNPA1 can act as an onco-repressor by preventing exclusion of exon 11 of *RON*. This promotes formation of the splice variant Δ Ron and inhibition of epithelial-to-mesenchymal transition (EMT) (Bonomi *et al.*, 2013).

HnRNPA1 also regulates alternative splicing of *FAS* (*CD95*). The most common FAS protein is a trans-membrane receptor that stimulates apoptosis after binding its ligand, FasL. A splice variant of *FAS* lacks exon 6 (Δ E6 Fas); although Δ E6 Fas can still compete with the common FAS receptor for ligand binding activity, it lacks the trans-membrane domain resulting in a secreted protein which is unable to activate apoptosis (David and Manley, 2010; Oh *et al.*, 2013). HnRNPA1 binds a splicing silencer located within exon 5 of *FAS*; this promotes inclusion of exon 6 and, in turn, decreases levels of Δ E6 Fas (Oh *et al.*, 2013).

FGFR2 is a member of the fibroblast growth factor receptor (FGFR) family which is found amplified in breast cancer (Moffa and Ethier, 2007). Alternative splicing of *FGFR2* is also under the regulation of hnRNPA1. Immunoprecipitation and UV-cross-linking experiments reveal that hnRNPA1 can prevent production of a splice variant of *FGFR2*, known as FGFR2-IIIb, by binding an exonic splicing silencer located in proximity of exon IIIb (Baraniak et al., 2006). Additionally, specific single-nucleotide polymorphisms (SNPs), identified within intron 2 of the *FGFR2* gene, are closely linked to onset of disease (Hunter et al., 2007). From the alternative splicing of *FGFR2*, two splice variants arise from the inclusion of either exons IIIb or IIIc; whilst exon IIIb inclusion is exclusive to epithelial cells, exon IIIc characterises mesenchymal tissue (Baraniak et al., 2006). A change in the ratio of FGFR2-IIIb and FGFR2-IIIc occurs during the progression of prostate carcinomas and in metastatic breast carcinoma (Baraniak et al., 2006; Zhu et al., 2009), whilst another study observed that FGFR2-IIIb is overproduced in 5% of breast cancer cases (Moffa and Ethier, 2007).

It is noteworthy that the potential tumour suppressor activity of hnRNPA1 can prevent production of tumourigenic splice variants. However, hnRNPA1 can also act as oncoprotein by promoting inclusion of exon 9 of the tumour suppressor *Caspase-2* (*CASP2*), resulting in a premature stop codon. This splicing event leads to production of a splice variant known as Casp-2S (or Ich-1S) which displays anti-apoptotic activity at the expense of the pro-apoptotic variant of *CASP2*, Ich-1L (Jiang et al., 1998; David and Manley, 2010).

In addition, overexpression of hnRNPA1 can affect translation of different oncogenes; one study showed that hnRNPA1 promotes translation of cyclin D1 and c-Myc - both involved in cell proliferation - by regulating IRES (internal ribosomal entry site) activity (Jo et al., 2008). In turn, c-Myc stimulates transcription of hnRNPA1, resulting in a positive feedback loop between the two proteins (David and Manley, 2010). Interestingly, combinatory activities of hnRNPA1 and hnRNPA2 have been described; simultaneous overexpression of hnRNPA1 and hnRNPA2 reduces apoptosis by stabilizing telomeric DNA in breast cancer cells, whereas knockdown of both proteins induces cell death (Patry et al., 2003). As splicing factors, both hnRNPA1 and A2 regulate alternative splicing of *PKM* (pyruvate kinase enzyme). The *PKM* gene can generate two splice variants arising from inclusion of either exon 9 (PKM1 variant) or exon 10 (PMK2 variant). One of the splice variants PMK2 promotes aerobic glycolysis, typically exhibited by cancer cells, rather than oxidative

phosphorylation, which is most common in normal cells. Both hnRNPA1 and A2 can bind the exon 9 of *PKM*, repressing its inclusion, whilst promoting the production of the PMK2 splice variant (David et al., 2010).

Finally, another member of the hnRNPA/B family with relevant roles in cancer is hnRNPA2/B1 (Golan-Gerstl et al., 2011). Higher levels of hnRNPA2/B1 exist in breast tumours compared to normal breast tissue (Zhou et al., 2001). HnRNPA2/B1, similarly to SRSF1, regulates alternative splicing of both *RON* and *BINI* promoting production of oncogenic and anti-apoptotic splice variants (Golan-Gerstl et al., 2011; Bonomi et al., 2013).

PTB (hnRNP I)

PTB (polypyrimidine tract binding protein) is an hnRNP protein involved in numerous stages of RNA processing and translation (He et al., 2014). PTB is overexpressed in transformed human mammary epithelial cells (HMECs) as well as the following breast cancer cell lines MCF-7, T47D and MDA-MB231 (He et al., 2014). Moreover, a link between PTB expression and malignancy of breast cancer exists, and down-regulation of PTB reduces cell proliferation and invasion (He et al., 2014). During alternative splicing events, PTB commonly binds polypyrimidine-rich tracts blocking the interaction of U2AF with the pre-mRNA. Therefore, in most cases, PTB acts as a splicing inhibitor and promotes exon skipping (Brinkman, 2004; Chen and Manley, 2009; He et al., 2014). Similarly to hnRNPA1 and A2, PTB can repress the inclusion of exon 9 of *PMK*, promoting the production of the pro-cancer splice variant PMK2 (David et al., 2010; He et al., 2014).

PTB also favours the production of the oncogenic FGFR1 β variant at the expense of FGFR1 α (Ghigna et al., 2008). Furthermore, PTB can bind a consensus motif (uridine-rich) located within exon 6 of the *FAS* gene. As a result PTB hinders association between the spliceosome members U2AF and snRNP U2 and the 3' splice site upstream of the exon 6. This leads to inhibition of exon 6 inclusion and formation of the anti-apoptotic Δ E6 Fas (Izquierdo et al., 2005).

In addition, PTB presents anti-cancer properties by regulating alternative splicing of the tumour suppressor *Caspase-2*. PTB can repress inclusion of exon 9 of the pro-apoptotic *Caspase-2* whilst inhibiting production of the anti-apoptotic splice variant *Casp-2S* (Cote et

al., 2001). Moreover, together with hnRNPA1, PTB can repress the inclusion of exon IIIb of *FGFR2* by binding to intronic splicing silencer located either upstream or downstream the exon IIIb, thus preventing the formation of the splice variant FGFR2-IIIb (Baraniak et al., 2006). Finally, PTB can also promote translation of the oncogene c-Myc through the binding of c-Myc IRES (David and Manley, 2010).

HnRNP K

HnRNP K contains a conserved K Homology RNA-binding motif (KH domain) required for its DNA/RNA binding-protein activity and RGG boxes needed for RNA transcription, processing, shuttling and also translation (Mandal *et al.*, 2001; Tang *et al.*, 2014). Diverse studies have described the transforming potential of hnRNP K and its ability in stimulating cell cycle progression and tumour cell viability (Venables *et al.*, 2008; Zhou *et al.*, 2010; Tang *et al.*, 2014). Particularly interesting is the fact that expression of hnRNP K is under the regulation of the epidermal growth factor (EGF) (Mandal *et al.*, 2001; Zhou *et al.*, 2010). EGF activity stimulates cell proliferation by binding the epidermal growth factor receptor HER2 which is overexpressed in about 20-30% of breast cancer cases (Slamon *et al.*, 1987; Rubin and Yarden, 2001; Jackson *et al.*, 2013). As a splicing factor, hnRNP K regulates alternative splicing of the *Bcl-x* gene and, consequently, it promotes formation of the anti-apoptotic variant of Bcl-x (Bcl-xL) (Han et al., 2013). Bcl-x, is a member of the apoptosis regulator Bcl-2 family. Similarly to Mcl-1, Bcl-x undergoes alternative splicing which can generate two splice variants which have opposite effects on the signalling pathway: Bcl-x_s stimulates apoptosis whereas Bcl-xL inhibits that cellular process (Boise et al., 1993). Further genes involved in cell apoptosis, *APAF1* and *NALP1*, and genes associated to breast cancer aggressiveness, Osteopontin and Breast cancer-associated protein (*BCA3*), are also targets of hnRNP K (Venables *et al.*, 2008; Ortiz-Martinez *et al.*, 2014). Finally, also the ability of hnRNPK to bind the promoter of c-Myc and activate transcription of the proto-oncogene in breast cancer cells has been described (Mandal et al., 2001).

RNA-BINDING PROTEINS AND BREAST CANCER

Whilst the families of SRs and hnRNP proteins are the most well characterised splicing factors – other RNA-binding proteins, better known for their roles in RNA transcription, shuttling, stability and translation also have alternative splicing regulation activities.

Moreover, deregulation of their expression either directly or indirectly affect the alternative splicing of genes involved in breast cancer development. The following section will describe these RNA-binding proteins and their relevance to breast cancer.

Sam68 (KHDRBS1, Src-associated in mitosis)

Sam68, a member of Signal Transduction and Activation of RNA (STAR) family is activated by the proto-oncogene tyrosine-protein kinase (*c-Src*) (Fumagalli et al., 1994). Sam68 is overexpressed in breast cancer cell lines as well as tissues and can stimulate cell proliferation whilst its deficiency increases cell cycle inhibitors p27 and p21 (Song et al., 2010). Through the KH domain, Sam68 can bind its target RNAs. Amongst these targets, Sam68 regulates alternative splicing of *Cyclin D1*, *CD44* and *Bcl-X* (Song et al., 2010). Sam68 promotes production of the proto-oncogenic splice variant cyclin D1b, resulting in an increase in the cyclin D1b/cyclin D1 cellular ratio (Paronetto et al., 2010). Activation of Sam68 by GTPase RAS, leads to *CD44* exon v5 inclusion and formation of the cancer associated *CD44* splice variant (Cheng and Sharp, 2006).

Interestingly, Sam68 promotes expression of SRSF1 by binding to its 3'UTR (Valacca *et al.*, 2010); Sam68 also regulates alternative splicing of Bcl-x and promotes production of the pro-apoptotic splice variant Bcl-x(s) (Matter *et al.*, 2002; Paronetto *et al.*, 2007).

YB-1 (DNA-binding protein B1)

YB-1 is a DNA/RNA binding protein, member of the Y-box family. As a splicing factor, YB-1 is able to bind pre-mRNA of the *CD44* gene specifically on A/C-rich motifs. Once bound, YB-1 promotes the inclusion of the variable exon v4 of *CD44* (David and Manley, 2010). YB-1 plays also roles in both RNA transcriptional and translational processes (Swamynathan *et al.*, 2002) and is involved in cell cycle control (Swamynathan et al., 2002) and genomic instability in breast cancer (Davies *et al.*, 2011). YB-1 is overexpressed in breast cancer cells and in the surrounding tumour tissue (Janz et al., 2002; Rubinstein et al., 2002). Interestingly, YB-1 positively affects the expression of the breast cancer biomarkers HER2 and ER α (Fujii et al., 2008) and is associated with resistance towards breast cancer therapies, such as Lapatinib (Shibata et al., 2013); a link between YB-1 and the expression of intrinsic multidrug resistance gene 1 *MDR1*, is also documented (Mylona et al., 2014). YB-1 drives the expression of P-glycoprotein which blocks the permeability of drugs and toxic molecules into cells (Janz et al., 2002; Lin and Yamazaki, 2003). YB-1 is able to bind IRES sequence of

c-Myc promoting its translation (David and Manley, 2010). Overall, increased expression of YB-1 in human breast cancer is associated with severity of disease and poor clinical outcome (Bergmann et al., 2005; Evdokimova et al., 2009).

FOX2 (RBM9, Fxh)

Fox2 is another RNA-binding protein and regulator of tissue-specific splicing (Zhang et al., 2008). Fox2 interacts with ER α and acts as corepressor by blocking the tamoxifen-mediated transcriptional activity of ER α (Norris et al., 2002; Zhang et al., 2008). Expression of Fox2 is higher in both basal and claudin low breast cancer cells compared to luminal subtype cells (Lapuk et al., 2010). The same study has defined claudin-low tumors, generally as triple negative with low levels of adherens junction genes, such as E-cadherin, and high expression of EMT markers (Lapuk et al., 2010). Interestingly, Fox2 can influence 50% of splicing events occurring in breast cancer cells included genes involved in EMT (Venables et al., 2009; Venables et al., 2013). *FGFR2* is one of the target genes of Fox2; Fox2 promotes inclusion of exon IIIb of *FGFR2* resulting in the production of FGFR2-IIIb (Baraniak et al., 2006). Importantly, Fox2 can regulate alternative splicing of several factors including hnRNPs, SR proteins and itself (Yeo *et al.*, 2009). Additionally, Fox2 can also complex with hnRNP H and hnRNP F for inhibiting FGFR2-IIIc production (Mauger et al., 2008).

DAM1 ((DNA amplified in mammary carcinoma or BCAS2, SPF27)

Amplification of the *DAM1* gene has been described in MCF-7 and BT-20 breast cancer cell lines (Nagasaki et al., 1999; Maass et al., 2002). DAM1 protein binds both ER α , increasing ligand-mediated transcriptional activity of the receptor (Qi et al., 2005), and tumour suppressor p53, reducing its transcription activity (Kuo et al., 2009). Although *DAM1* has been classified as a proto-oncogene, some studies in mice show that DAM1 can exert tumour suppressor activity through activation of BLK, a strong pro-apoptotic Bcl-2 member (Lee et al., 2002). As a splicing regulator, DAM1 complexes with Prp19/Pso4 and directly interacts with the spliceosome, although its precise role has yet to be defined (Zhang et al., 2005). However, Prp19 can interact with U2AF65 and directly bind to the C-terminal domain of the RNA Polymerase II largest subunit (CTD); this interaction promotes CTD-dependent splicing activation (David et al., 2011).

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RBM5 (RNA-binding motif protein 5, LUCA15, H37)

Another RNA-binding protein classified as a tumour suppressor is RBM5. RBM5 promotes apoptosis and inhibits cell cycle (Rintala-Maki et al., 2007). The role of RBM5 as a splicing factor is well described; for example, RBM5 targets the pre-mRNA of the *Caspase-2* gene on U/C rich sequences promoting exclusion of exon 9 (Fushimi et al., 2008). RBM5, like PTB, promotes the formation of the anti-apoptotic splice variant of *FAS*, $\Delta E6$ Fas. Whilst PTB hinders interaction between U2AF and U2 – RBM5 interacts with snRNP U4/U5/U6 and blocks recruitment of the spliceosome complex on the pre-mRNA of *FAS* in proximity of the exon 6, promoting *FAS* exon 6 exclusion (Chen and Manley, 2009).

HuR (Human antigen R , ELAV-like protein 1)

HuR is a RNA-binding protein member of the ELAV like-proteins family, best known for their ability to stabilize mRNAs by binding AREs (AU-rich elements) (Denkert et al., 2004). Therefore, HuR has the capacity to regulate expression of many proto-oncogenes (Wurth, 2012). High levels of cytoplasmic HuR are found in breast carcinoma, where it stabilizes the RNA of Cyclooxygenase-2 - a prognostic factor of breast cancer (Denkert et al., 2004). A role for HuR in regulating alternative splicing has been recently described (David and Manley, 2010); HuR regulates *FAS* by binding a splicing silencer sequence located in exon 6. Thus, HuR blocks U2AF65 interaction with the 3' splice site of exon 6 and as a result, HuR also promotes production of anti-apoptotic splice variant of *FAS*, $\Delta E6$ Fas (Izquierdo, 2008).

PELP1 (Proline, glutamic acid, leucine-rich protein 1)

PELP1 is a proto-oncogene deregulated in breast cancer and involved in cell survival, metastasis and drug resistance (Mann et al., 2014). Additionally, PELP1 is a nuclear coactivator of ER α and enhances the transcriptional activity of the receptor in response to estrogen (Vadlamudi et al., 2001). Depletion of PELP1 has shown to affect the expression of several splicing factors, revealing that PELP1 has an indirect role in regulating splicing. Moreover, using immunofluorescence and immunoprecipitation shows co-localisation and interactions of PELP1 with SRSF2 (Mann et al., 2014); PELP1 can also directly promote *CD44* exon 4 and exon 5 exclusion. Finally, down-regulation of both PELP1 and RNA-binding protein PRMT6 directly affects the alternative splicing of *VEGF*, decreasing VEGF165 splice variant production whilst increasing the oncogenic variants VEGF121 and VEGF189 (Mann et al., 2014).

Protein arginine methyltransferase-6 (PRMT6, CARM1)

Like *PELP1*, *PRMT6* also acts as a proto-oncogene in breast cancer (Phalke et al., 2012); however, more recently a controversial study has revealed the tumour suppressor activities of *PRMT6* (Kim *et al.*, 2013). Whilst *PRMT6* is commonly known for its methyltransferase activity, knockdown of *PRMT6* effects transcription and alternative splicing events of hundreds of target genes (Dowhan *et al.*, 2012; Phalke *et al.*, 2012). Interestingly, *PRMT6* is also a co-activator of ER α (Dowhan et al., 2012). As a splicing factor, *PRMT6* directly promotes exclusion of exon v5 of *CD44*, thus, it negatively regulates the production of the *CD44* oncogenic splice variant. Moreover, *PRMT6* regulates methylation of several splicing factors, therefore influencing their activity (Cheng et al., 2007; Dowhan et al., 2012).

SPF45 (RBM17)

Increased levels of SPF45 were observed in breast cancer tissues (Sampath et al., 2003). Additionally, different studies correlate abundance of SPF45 with the severity of tumours and also drug resistance (Liu et al., 2013) (Sampath et al., 2003). SPF45 acts as splicing factor by complexing with spliceosome members SF1, U2AF65, and SF3b155. This complex is usually required for the selection of 3' splice sites and for the definition of exon 6 of *FAS*, which promotes its exclusion (Corsini et al., 2007).

Summary

This review has focused on SRs, hnRNPs and other RNA-binding proteins that are themselves deregulated in breast cancer, including genes classified as either proto-oncogenes (e.g. *SRSF1*, *SRFS3*, *DAM1*, *PELP1*) or tumour suppressors (e.g. *RBM*, *PRMT6*). Interestingly, some of these splicing factors can simultaneously regulate alternative splicing of common target genes. Both cooperation and competition of splicing factors can be observed; for instance, the anti-apoptotic splice variant of *FAS*, $\Delta E6$ Fas, is promoted by the putative tumour suppressor *RBM5* but negatively regulated by hnRNP A1.

The advent of high throughput systems such as LISA (layered and integrated system for splicing annotation) - a high-throughput reverse transcription-PCR-based platform – provide a means to reveal hundreds of cancer-associated alternative splicing events in different cancers including breast (Klinck *et al.*, 2008; Venables *et al.*, 2008a); this experimental approach can be also exploited by combining RNAi with LISA to work out how splicing

factors affect subsets of genes in different cellular contexts (Venables *et al.*, 2008). With relevance to this review Venables *et al.* compared a cohort of breast tumours differing in their estrogen receptor (ER) status and observed distinct changes in the alternative splicing events. ER α can alter alternative splicing of target genes by interacting and recruiting splicing machinery members, such as the splicing factor SF3a p120 (Auboeuf *et al.*, 2007) and by ER α transcriptional coregulators (Dowhan *et al.*, 2005). It is interesting to note that the structure of ER α homologous coregulators CAPER α and CAPER β is similar to that of the splicing factor subunit U2AF65 (Dowhan *et al.*, 2005). Through knockdown experiments, CAPER α can affect alternative splicing of the *VEGF* gene in a hormone-dependent way by changing the ratio VEDF-121/VEGF-189, the most predominant VEGF splice variants together with VEGF-165 (Catena *et al.*, 2007). Moreover, iCLIP combined with high-throughput sequencing is an emerging powerful tool to study splicing factor-RNA interactions in different cells and tissues (Konig *et al.*, 2011). The technique has been adopted to determine protein-RNA interactions *in vivo* on a global scale for splicing factors such as hnRNPC, SRSF3, SRSF4 and more recently Tra2 α /Tra2 β (Konig *et al.*, 2010; Anko *et al.*, 2012; Best *et al.*, 2014); the latter study also identified novel tra2-mediated splicing events that affect breast cancer cell viability (Best *et al.*, 2014).

In conclusion, further insights into the complexity and crosstalk within these splicing networks may help shed light on the mechanisms that lead to breast cancer development and metastasis.

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Table Legend

Table1. Splice sites and spliceosome members

Table shows consensus splice sites within pre-mRNA sequences, together with members of the spliceosome that specifically bind to the splices to trigger the splicing process. Nucleotide sequences and location of each splice site is also indicated.

Figure Legend

Figure 1. Regulation of splicing

Pre-mRNA contains four conserved sequences required for the splicing process; snRNP U1 binds the 5`ss therefore defining the end of the Exon 1; the small subunit of the dimeric protein U2AF (U2AF35) binds the 3`ss therefore defining the end of the intron and the beginning of Exon 2. Both U2AF35 and the larger subunit U2AF65, when bound to the polypyrimidine tract (defined by uridine repetition), supports the recruitment of snRNPU2 to the Branch Point (marked by an adenine). The figure highlights that the binding of U2AF to both the 3` splice site and the polypyrimidine tract is inhibited by a nearby Exonic Splicing Silencer (ESS) and an Intronic Splicing Silencer (ISS). This prevents the recruitment of snRNP U2 to the Branch point. Exon/intron splicing enhancers (ESEs, ISEs) and exon/intron splicing silencers (ESSs, ISSs) are defined recondition RNA for splicing factors which, in turn, either promote or prevent the splicing process. Splicing factors either guide or block the interactions between the spliceosome and splice sites. Different splicing factors can also compete with each other for binding the same motif, to influence the selection of the nearby splice sites.

Figure 2. Alternative splicing network of RNA-binding proteins and their target cancer-related genes

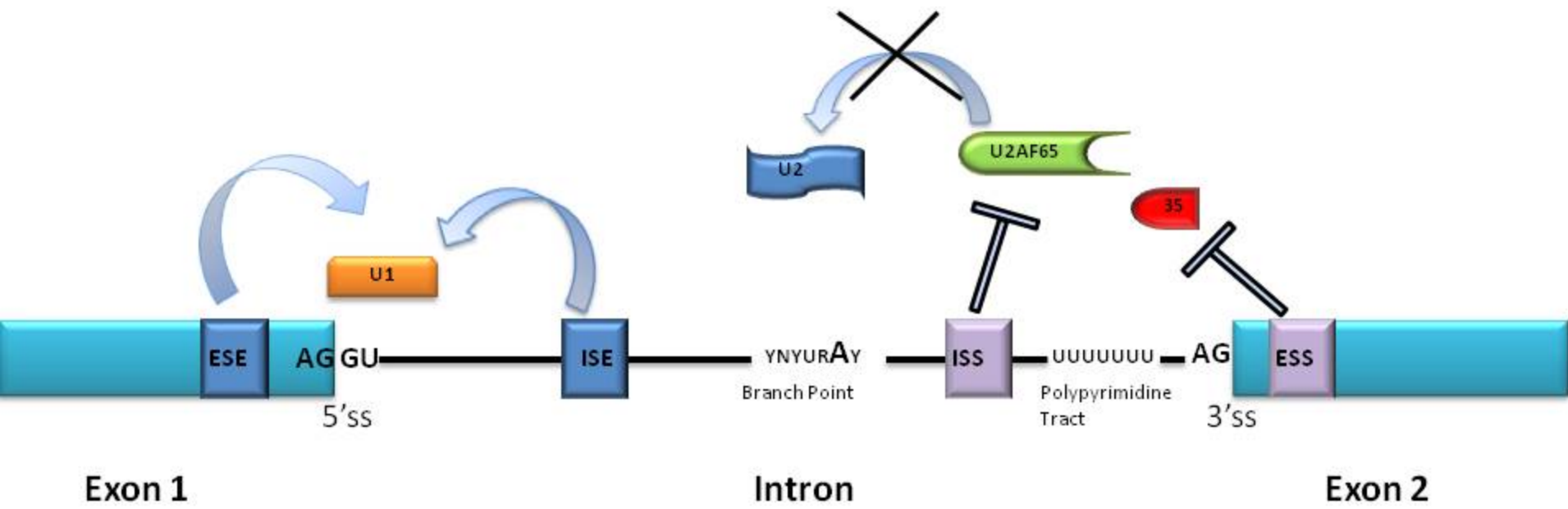
The network shows alternative splicing events that occur in a selection of cancer-related genes due to the activity of specific splicing factors: SRs (red circle), hnRNPs (green circle) and RNA-binding proteins (purple circle). Splice variants either produced (arrows) or prevented (bar) by SRs, hnRNPs and other RNA-binding proteins are indicated respectively.

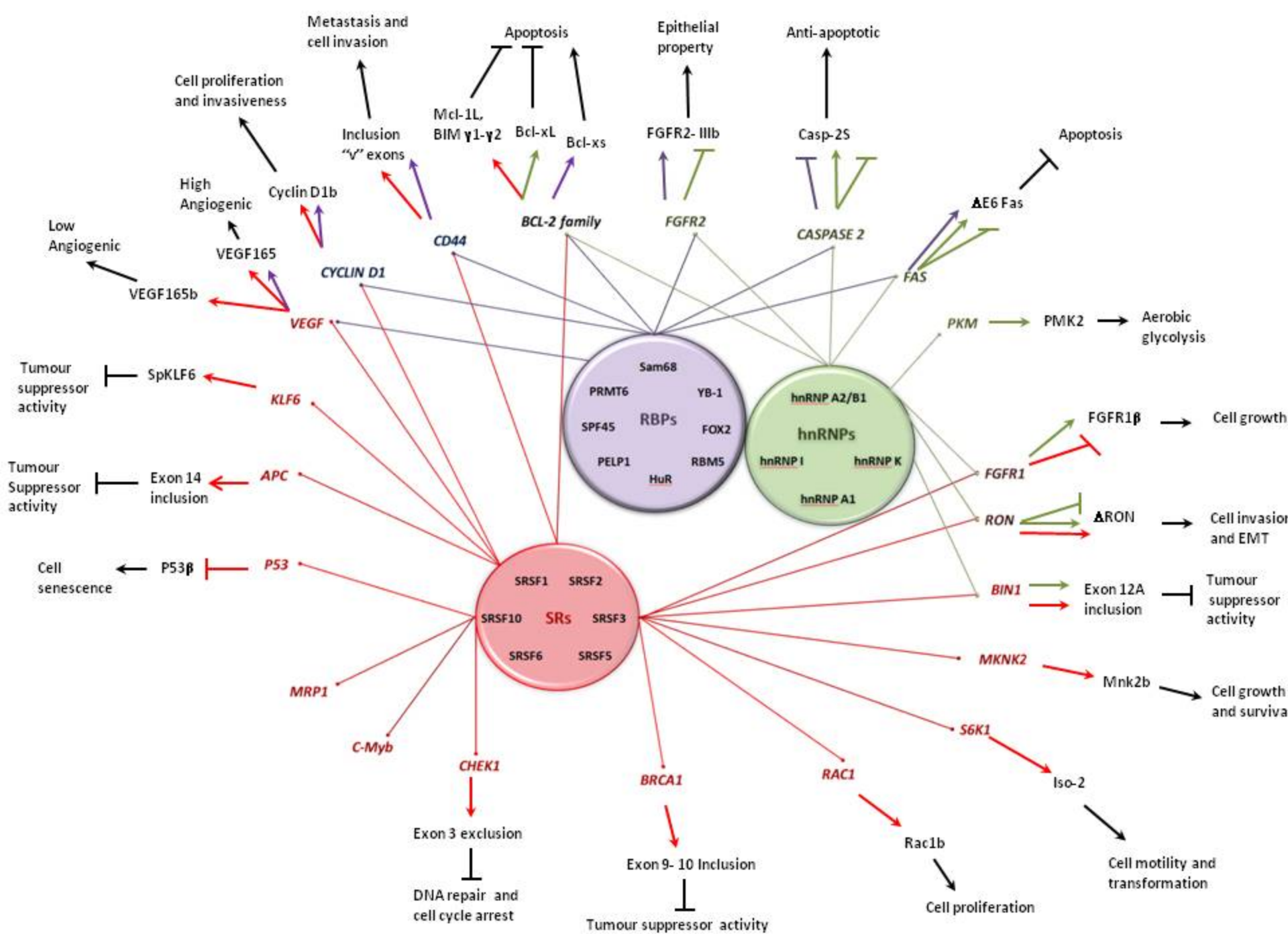
Figure 3. Splice variants and biological effects driven by overexpression/downregulation of SRSF1

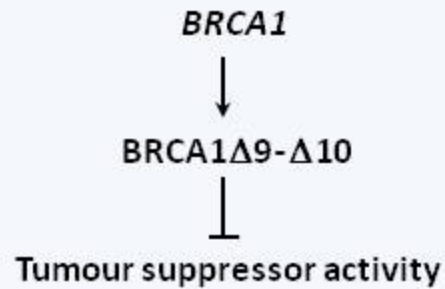
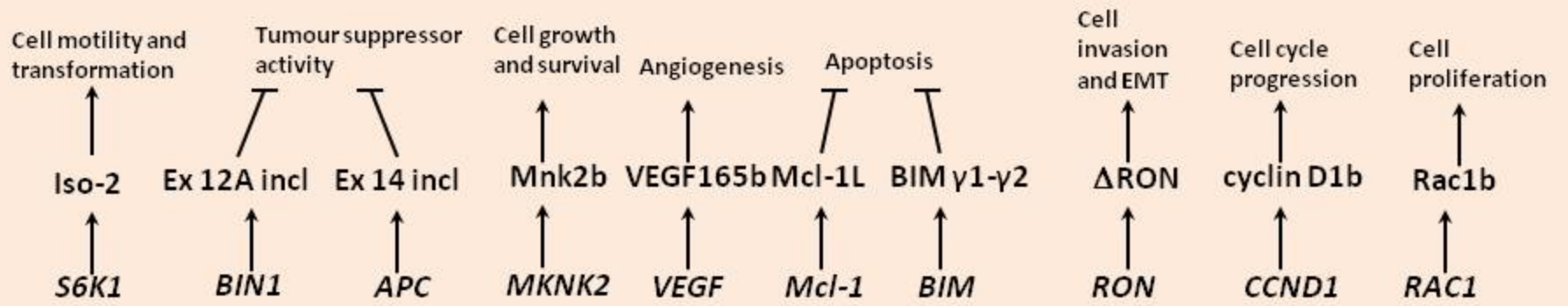
The figure shows specific splicing events and the resulting biological effects driven by deregulation of SRSF1. Splice variants and resulting biological effects either promoted (arrow) or prevented (bar) by overexpression of SRSF1 are reported (upper panel) whilst splice variants promoted by downregulation of SRSF1 are indicated underneath.

Figure 4. Splice variants and biological effects driven by overexpression of hnRNP A1

The figure shows specific splicing events and the resulting biological effects driven by overexpression of hnRNP A1. Splice variants and resulting biological effects either promoted (arrow) or prevented (bar) by overexpression of hnRNP A1 are reported (upper panel).

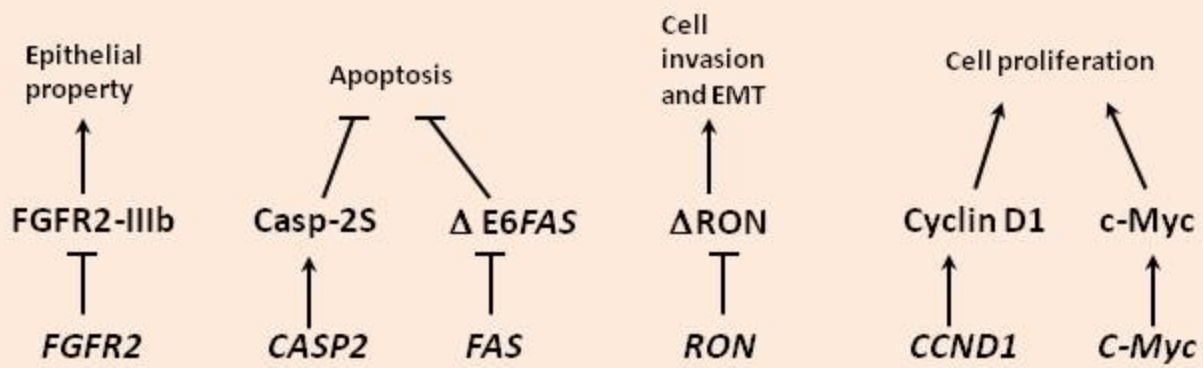






Overexpression

Downregulation



<i>SPLICE SITE</i>	<i>LOCATION</i>	<i>MOTIF</i>	<i>SPLICEOSOME MEMBER</i>
5' splice site (5'ss or donor splice site)	Exon/intron junction	AG/GURAGU /= Exon/intron boundary R= purine (either G or A)	Small nuclear ribonucleic particle (snRNP) U1
Branch point	Intron	YNYURAY N= any nucleotide Y= pyrimidine (either C or U) A= pivotal adenine for the splicing process (Srebrow, A. and Kornblihtt, A.R. 2006)	Small nuclear ribonucleic particle (snRNP) U2
Polypyrimidine Tract	Intron	15-20 uridine/cytosine repetition	U2AF large subunit (U2AF65)
3' splice site 3'ss or (acceptor splice site)	Intron/Exon junction	YAG/ Y= pyrimidine (either C or U) /= Intron/exon boundary (Collins and Guthrie, 2001)	U2AF small subunit (U2AF35)