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The splicing program of SRSF1 transcripts is finely tuned by cell metabolism

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Abstract

One of the best-studied splicing regulator is SRSF1 (formerly known as SF2/ASF, Manley *et al.*, 2010), a prototypical SR protein involved in both constitutive and alternative splicing. SRSF1 promotes exon definition and the use of proximal alternative 5' splice sites or 3' splice sites in a concentration-dependent manner, in part through recognition of ESE sequence elements in the pre-mRNA targets. SRSF1 regulates other aspects of RNA metabolism, including mRNA stability (Lemaire *et al.*, 2002), nuclear export (Huang *et al.*, 2003), nonsense-mediated mRNA decay (Zhang *et al.*, 2004), translation, and miRNA processing (Wu *et al.*, 2010). It has been shown that the *SRSF1* gene, located on Chromosome 17q23, is amplified in some tumors (Sinclair *et al.*, 2003) and that increased *SRSF1* expression is associated with the redistribution of β -catenin, reorganization of actin cytoskeleton, and downregulation of the epithelial marker E-cadherin, a tumor and invasion suppressor in human carcinomas. These morphological and molecular changes represent the hallmarks of the epithelial to mesenchymal transition (EMT), a process occurring during embryonic development but also required for cell invasiveness and metastatic properties of carcinomas in vivo (Thiery J.P. 2002). To avoid these deleterious effects on cells and organisms, the *SRSF1* gene expression is tightly regulated through mechanisms operating at different levels, from transcription to translation.

SRSF1 is able to recognize splicing regulatory sequence elements on its own transcripts, leading to alternative splicing. Alternative splicing plays a role in this regulation leading to the production of six different transcripts. Isoform I, encodes the full-length protein, and is characterized by a long 3'UTR (Ge H *et al.*, 1991, Krainer *et al.*, 1991). Isoforms II and III retain the third intron

(the last in the coding part of the gene). Moreover, Isoforms III and IV undergo splicing of intron 5 in the 3'-UTR. Isoform II, III, and IV are retained in the nucleus. Hence, these molecules can not be translated (Sun *et al.*, 2010) and can be considered as long non-coding RNAs. Isoform IV can undergo further splicing of intron 4 in the 3'-UTR leading to the production of Isoform V, which is exported in the cytoplasm and degraded by the NMD pathway. Similarly, through skipping of exon 4, Isoform III can be matured into Isoform VI, which is exported in the cytoplasm and subjected to NMD (Sun *et al.*, 2010). During the three years of my Ph.D. program, my research activity

concerned on the analysis of signals and factors that control the complex SRSF1 splicing program and the function of lncRNAs products, the signals and the mechanisms underlying the choice between the production of the protein coding Isoform I and the expression of the lncRNAs the mechanism and signal controlling alternative splicing of SRSF1 transcripts.

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I. Review of the literature

1.1 Splicing and regulation gene expression

Eukaryotic gene expression is a complex process that involves several biochemical reactions from transcription of the pre-mRNA in the nucleus to protein translation in the cytoplasm. The pre-mRNA molecule must undergo a number of processing events (5'-end capping, splicing and 3'-end cleavage/polyadenylation) before being exported to the cytoplasm as a mature mRNA for translation. Moreover, the mRNA is subject to quality controls, which affect its stability and translability. All of these events are tightly controlled and coordinated in a tissue-specific and temporal manner, in order to determine the proper protein profile of the cell (Das & Krainer., 2014). Most genes in higher eukaryotes are composed of exons (coding sequences) separated by introns (non-coding sequences). Both exonic and intronic sequences are transcribed into a pre-mRNA molecules. The removal of introns and the precise joining of exons is called splicing and gives rise to a mRNA molecule consisting of the protein-coding open reading frame and the 5' and 3' untranslated regions (UTRs), (Wahl, M.C. *et al.* 2009) (Figure.1).

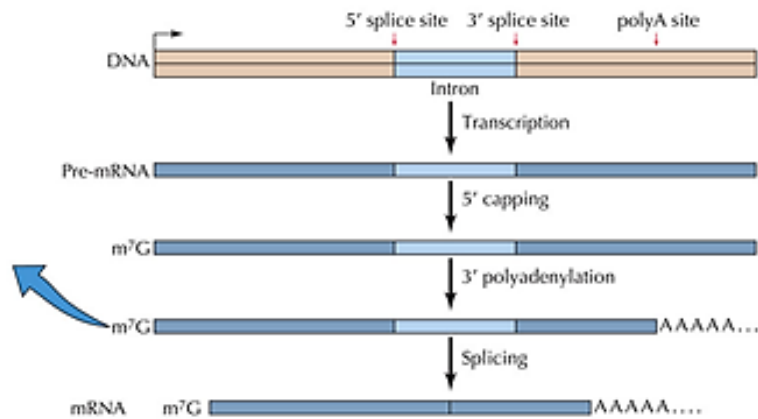


Figure.1 Phases of maturing a messenger RNA. (THE CELL, 4^o Edition).

The removal of intronic sequences occurs with the accuracy and *precision* of the single *nucleotide* through two successive transesterification reactions, each involving a nucleophilic attack on terminal phosphodiester bonds of the intron (Black DL., 2003). These reactions are directed by particular sequence elements: the 5' and 3' splice sites, also called donor splice site and acceptor splice sites at the intron/exon junctions and the branch point sequence (BPS), which is typically located within 30–50 nucleotides upstream of the 3' splice site. All these elements conform to consensus sequences. The majority of splice sites include invariant dinucleotides at each end of the intron: GU at the 5' end, and AG at the 3' end. At the 3' end of the intron there are two additional conserved sequence elements. One is the polypyrimidine tract, a 15-20 nucleotide region, rich of pyrimidines immediately upstream of the terminal AG (Berglund JA *et al.*, 1997). The second one is the branch point (consensus sequence YNCURAY), where Y represents any pyrimidine, R any purine and N any nucleotide. These sequences contain the information

required for precise exon joining (Lim & Burge., 2001) and are recognized through RNA-RNA and RNA-protein interactions with components of the spliceosome (Figure.2).

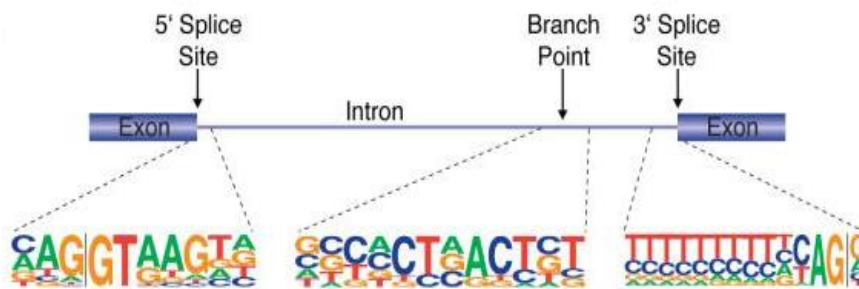


Figure.2 In the picture are shown consensus sequences for the splice site at 5', branch point and splice site at 3'(McManus & Graveley, 2011).

During splicing, the 2'OH of the Adenosine at the branch point attacks the 5' phosphate of the first nucleotide of the intron. The 3'OH end of the upstream intron then initiates a second nucleophilic attack on the 3' acceptor splice site, releasing the intron in the form of a lariat structure and covalently combining the two exons.

1.2 The spliceosome

The splicing reaction is carried out by a complex molecular machinery, the spliceosome, which is composed of 5 small nuclear RNAs (snRNAs) (U1, U2, U4/U6, and U5) assembled in small nuclear ribonucleoprotein complexes (snRNPs) and numerous (more than 100) protein factors (Brody & Abelson 1985; Butcher & Brow 2005). These protein factors include RNA-binding proteins (RBPs) and enzymes (helicases/RNPsases, kinases and phosphatases, etc.). These modulate the structure and the orderly stepwise assembly of the spliceosome, facilitate the splicing reaction, proofreading, and substrate release (Nilsen., 2003; Staley & Guthrie., 1998; Will & Luhrmann., 2001; Hoskins AA & Moore MJ., 2012).

An exhestensive biochemical characterization allowed the identification of different steps in the splicing reactuaion. The first step is the ATP-independent assembly of the E complex, which involves the recognition of the 5' splice site by snRNP U1 and the binding of splicing factor 1 (SF1) to the branch point. This is followed by the recruitment of the U2 auxiliary factor (U2AF) to the polypyrimidine tract and 3' terminal AG (Kent *et al.*,2005). The E complex is then switched to the pre-spliceosomal A complex in an ATP-dependent step that involves the replacement of SF1 with U2 snRNP at the branch point (Zamore PD & Green MR., 1989). Thereafter the U4/U5/U6 tri-snRNP joins the A complex to form the B complex. Rearrangement of this structure with the displacement of U4 and U1 snRNPs produces the C complex: the catalitically active spliceosome (Figure.3).

Metazoan genes also contain a minor class of introns with non-canonical consensus sequences that accont for less than 0,5% of all introns and are excised by a distinct splicing machinery (Jackson, I. J 1991; Hall & Padgett.,

1994; Turunen *et al.*, 2013). Frequently, these introns initiate with AT and end with AC, from which the name of ATAC introns . Splicing of minor group of introns is determined by longer and more tightly constrained consensus sequences at the 5' splice site and branch site, as well as by the lack of a polypyrimidine tract upstream of the 3' splice site (Burge *et al.*, 1998; Dietrich *et al.*, 1997). They are processed by a specific U12-dependent spliceosome, which is similar to, but distinct from, the major spliceosome. U12-type introns are spliced somewhat less efficiently than the major introns, and it is believed that this limits the expression of the genes containing such introns (Dietrich *et al.*, 1997). The low-abundance spliceosome that is responsible for the excision of these introns includes four snRNPs (U11, U12, U4atac and U6atac) that are different from, but functionally analogous to, the well-characterized U1, U2, U4 and U6 snRNPs, respectively (Tarn & Steitz 1996). The U5 snRNP is shared by both spliceosomes. Because the excision of these minor-class introns is dependent on the U12 snRNP, they are referred to as U12-type introns, whereas the canonical, major-class introns that require the analogous U2 snRNP are referred to as U2-type introns (Tarn & Steitz 1996). Although U12-type are less abundant than U2 introns, their persistence throughout virtually all of metazoan species indicates that they have an ancient origin and an important cellular function (Patel AA & Steitz JA., 2003).

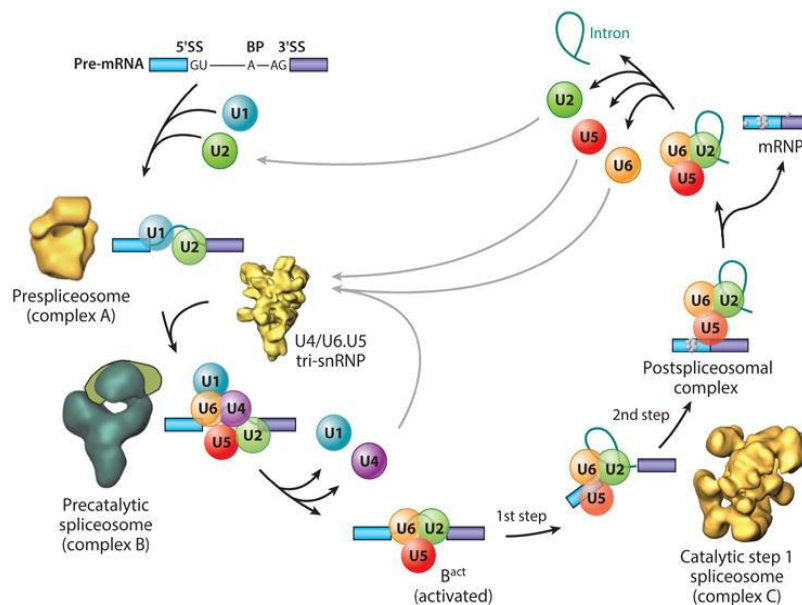


Figure.3 (A) Principal steps of reactions catalyzed by the U2-type spliceosome. Exons are represented as rectangles and introns as lines. The conserved sequences that enable recognition of the RNA by the spliceosome are: the 5' splice site (GU), the 3' splice site (AG), the Adenosine (A) of the branch point (BP) and the polypyrimidine tract B) During the first step of spliceosome assembly (E complex), U1 base-pairs with the 5'-splice site, whereas U2 base-pairs with the branch-point. Then, the tri-snRNP complex U4, U5 and U6 associates with the forming spliceosome (now called B complex). However, this multi-megadalton complex still has no catalytically active site. The subsequent catalytic activation of the spliceosome involves dramatic structural rearrangements that lead to changes in the conformations and biochemical composition. During this process, a complex network of RNA–RNA interactions is formed between the pre mRNA and the snRNAs U2, U5 and U6. U4 is ejected and this allows U6 to replace U1 at the 5' splice site (C complex) and leads to a U6–U2 interaction that gets close together to the 5'-splice site and the branch point, allowing for a transesterification step. At the end, U5 brings near the two exons and allows for the second step of splicing, joining the two exons (Lee & Rio, 2015).

1.3 Alternative Splicing

Before to the completion of the human genome project, the human genome was thought to have a greater number of genes as it seemed structurally and functionally more complex than other simpler organisms. Indeed, it was estimated that humans express more than 90,000 different types of proteins, hence it was assumed that a corresponding number of genes would be present in the human genome (Valdivia HH., 2007). It was also reasoned that since the human genome is structurally and functionally more complex than that of simpler organisms, it would contain a higher number of genes. However, it came as a surprise to discover that humans had fewer than 25,000 genes while a much simpler organism such as corn (*Zea mays*) contains approximately 40,000 genes (Valdivia HH., 2007). This mismatch in the ratio of gene to protein formation is explained through the mechanisms of alternative splicing (AS) which challenges the ‘one gene - one protein’ paradigm.

In 1977, Phillip Sharp and Richard Roberts almost simultaneously discovered the concept of “split genes” in the Adenovirus (Berget SM., 1995; Berget *et al.*, 1977). After the discovery of exons and introns, Walter Gilbert proposed that different combinations of exons could be spliced together (‘alternative splicing’) to produce different mRNA Isoforms (Gilbert W., 1978; Modrek B & Lee C.,2002).

Alternative splicing is a ubiquitous regulatory mechanism of gene expression that allows generation of more than one mRNA species from a single gene. Initially it was postulated that AS occurred only in about 5% of all genes (Sharp PA., 1994). Thanks to high-throughput sequencing studies it is now clear that at least 90% of human genes are transcribed in pre-mRNA undergoing alternative splicing (Merkin *et al.* 2012, Barbosa-Morais *et al.*, 2017).

One function of alternative splicing is to expand the human proteome (Nilsen & Graveley 2010). Alternative splicing can impact several biological processes, from sex determination and diversity of neuronal wiring in the fruit fly to determination of the physiological function of membrane-bound receptors in the mammalian nervous system (Kelemen *et al.*, 2013). Alternative splicing influences a number of protein features such as subcellular distribution, protein interactions or catalytic activity (Stamm *et al.*, 2005). It can also impact mRNA stability. This happens when a premature stop codon is introduced because of an alternative splicing event and the mRNA is degraded by the non-sense mediated RNA decay (NMD) pathway. AS has been found to play important roles in human diseases such as Mediterranean anemia (Radmilovic *et al.*, 2010), type II diabetes (Wen *et al.*, 2010), Alzheimer's disease (Donev *et al.*, 2007), Duchenne–Aran disease (Malcovati *et al.*, 2015), retinitis pigmentosa (Singh RK & Cooper TA. 2012), and cancer (Lokody I., 2014; Tazo *et al.*, 2014;). Many studies have associated AS with the occurrence, development, and metastasis of multiple cancer types. This suggests that AS could be a useful target for cancer diagnosis, treatment, and prognosis prediction (Zhao *et al.*, 2015).

Five major alternative splicing events are distinguished (Figure.4):

- Exon skipping (cassette exon): an exon that can be included or excluded from the mature mRNA. This is the most frequent alternative splicing event in mammalian cells (Sharp PA 2005; Gupta *et al.*, 2004; Kan *et al.*, 2002).
- Intron retention: the intron is retained in mature mRNA and it can encode amino acids in frame with the neighboring exons, or it can generate stop codon or a shift in the reading frame causing the protein to be non-functional. This is the less frequent type of AS event in mammals.

- Mutually exclusive: two or more adjacent cassette exons are spliced such that only one exon in the group is included at a time.
- Alternative acceptor and/or donor sites: this will result in a slightly modified mRNA sequence (Sharp, 2005; Gupta *et al.*, 2004a; Kan *et al.*, 2002).
- Alternative splicing can also be associated with alternative polyadenylation or alternative promoter, thanks to alternative splice sites, in upstream alternative 3' terminal exons. These events can modify the sequence of the 3' UTR and bring differential regulation by mRNA stability or translation factors (Warzecha CC& Carstens RP., 2012).

In some cases, as in the neurexin and CD44 loci, a gene portion consists of a number of exons that can undergo complex AS programs to produce several proteins encoded by a single locus (Missler M & Südhof T.,1998). Another striking example, the *Drosophila* gene encoding the Dscam axon guidance receptor can potentially generate 38,016 different protein Isoforms. The Dscam gene has 24 exons, with 12 alternative versions of exon 4, 48 versions of exon 6, 33 versions of exon 9, and two versions of exon 17. Only one version of a particular exon is chosen, with the exclusion of all the others, so that by the combinatorial use of alternative exons (Schmucker *et al.*, 2000). Often several splicing patterns are combined in processing a single pre-mRNA and occur in the same cell; the ratio between the different splice forms is usually characteristic for a certain cell type or tissue.

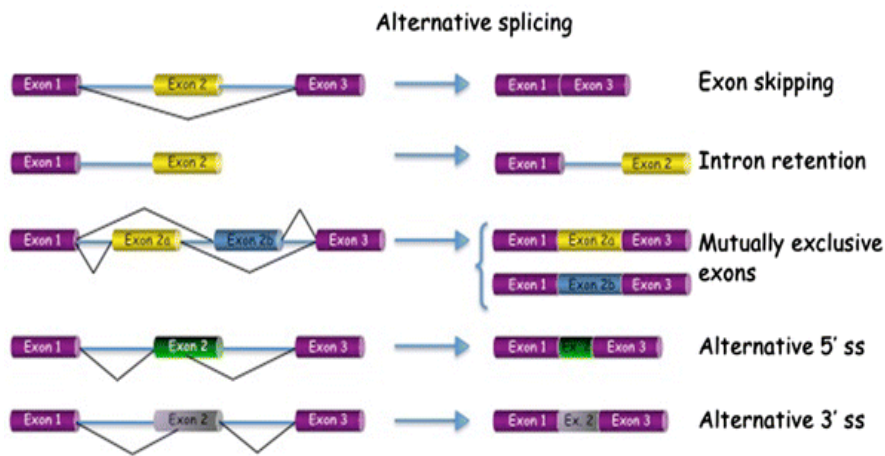


Figure.4 The five types of alternative splicing: exon skipping (or cassette exon), intron retention, mutually exclusive exons, alternative 5' splice sites and alternative 3' splice sites (Biamonti *et al.*, 2012).

1.3.1 Cis-acting elements

Despite the large potential for errors, the splicing process appears to occur with very high fidelity, implying the widespread involvement of splice signals in splice site selection. It is now clear that the presence of well-defined cis elements, namely the 5' and 3' splice sites, the branch point and the poly-pyrimidine tract, are necessary but not sufficient to define intron-exon boundaries (Lim, L.P. & Burge, C.B., 2001). In vertebrates exons are usually short. They may be flanked either by short or long introns that can be even many kilobases in length. It has been proposed that two different splicing strategies operate in the case of short or long introns. Short introns are the rule in lower eukaryotes and their splicing involves an intron recognition

mechanism. On the contrary, in the case long introns, which are more frequent in mammals, splicing involves an exon definition model. The concerted recognition of multiple weak elements located within exons as well as in intronic regions is nowadays thought to provide the necessary splicing information (*cis*-acting elements). Sequences having a role in splicing regulation are generally referred to as either enhancers or silencers, depending on the effect they exert. *Cis*-acting elements include enhancers and silencers of splicing, which can be classified in four categories: exonic splicing enhancers (ESEs) and intronic splicing enhancers (ISE), exonic splicing silencers (ESSs) and intronic splicing silencers (ISSs). Enhancers act by recruiting trans-acting factors, such as SR proteins (serine/arginine-rich family of nuclear phosphoproteins), that promote the recognition of a splice site by the spliceosome, whereas silencers are bound by negative acting factors, such as heterogeneous nuclear ribonucleoproteins (hnRNPs) (Black DL., 2003; Castle JC, *et al.*, 2008) (Figure.5). Splicing is usually controlled by combinatorial or competitive effects of both activators and inhibitors. The decision whether an exon is included is determined by the concentration of the regulator or by its activity which is modulated by a number of post-translational modifications.

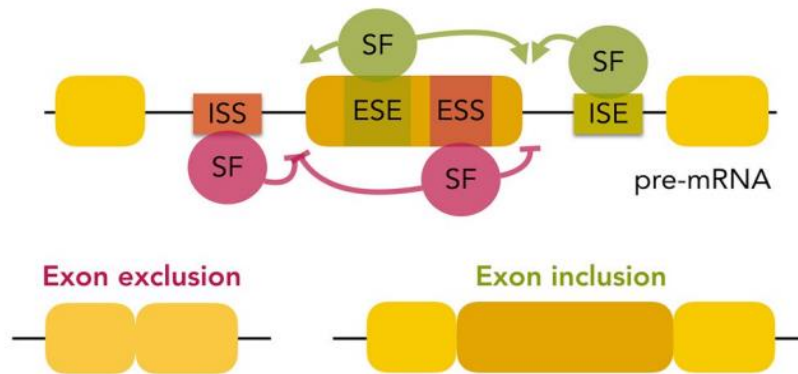


Figure 5. Alternative splicing is regulated by *Cis* and *Trans* factors. Motif sequences ESE (Exonic splicing enhancer) and ISE (intronic splicing enhancer) are bound by specific RNA binding proteins (RBPs) that promotes the inclusion of the alternative exon, whereas binding of given factor to motif sequences in ISS (intronic splicing silencer) or ESS (exonic splicing silencer) inhibits the splicing of alternative exon. (Gallego-Paez *et al.*, 2017)

1.3.2 Trans-acting splicing factors

Trans-acting splicing factors, which bind enhancer and silencer sequences elements, function in a combined manner to create the distribution of alternatively spliced products in a given cell type. As each cell type expresses a distinct array of splicing regulators, the interpretation of regulatory information on a given RNA target is exceedingly dependent on the cell type (Busch & Hertel, 2012).

The best characterized families of splicing regulators, such as SR factors and heterogeneous nuclear ribonucleoproteins (hnRNPs), are ubiquitously expressed, although their relative abundances can change in different tissues.

Other factors such as NOVA, nPTB (also known as PTBP2) (Polydorides *et al.*, 2000; Markovtsov *et al.*, 2000), FOX1 and FOX2 (also known as A2BP1 and A2BP2) (Underwood *et al.*, 2005) ESRP1 and ESRP2 (Warzecha *et al.*, 2009), and nSR100 (also known as SRRM4), all expressed in a tissue-specific manner (tab 1).

Class	Function	Examples*
SR and SR-related proteins	Typically activate splicing, by recruiting components of the splicing machinery	nSR100 (SRRM4), SC35, SF2 (ASF), SRM160 (SRRM1), SRp30c, SRp38, SRp40, SRp55, SRp75, TRA2 α , TRA2 β
hnRNPs	Typically repress splicing, by a variety of poorly understood mechanisms	hnRNP A1, hnRNP A2/B1, hnRNP C, hnRNP F, hnRNP G (RBMX), hnRNP H, hnRNP L, nPTB (PTBP2), PTB (PTB1)
Other RNA-binding proteins	Activate or repress splicing U1 snRNP is essential for constitutive splicing but can also repress splicing	CELF4 (BRUNOL4), CUGBP, ESRP1, ESRP2, FOX1 (A2BP1), FOX2 (A2BP2), HuD, MBNL1, NOVA1, NOVA2, PSF (SPFQ), quaking, SAM68 (KHDRBS1), SLM2 (KHDRBS3), SPF45 (RBM17), TIA1, TIAR (TIAL1), U1 snRNP

*Synonyms are listed in parentheses.

Tab.1 Type and examples of splicing regulatory protein (Nilsen & Graveley., 2010)

SR factors exert a positive affect on splicing through protein interactions involving the C-terminal domain rich in arginine and serine (RS) dipeptides, from which they get their name. Indeed, the SR domain establishes protein-protein interactions with other proteins containing an SR domain including components of the spliceosome (Fredericks *et al.*, 2015 Chenc & Manley 2009). By contrast, hnRNPs, which are also RNA-binding proteins, are generally thought to repress splicing by interfering with the ability of the core splicing machinery to engage splice sites (Martinez-Contreras *et al.*, 2007). The existence of 'antagonistic' classes of splicing regulatory factors, and the

evidence that these factors are involved in a large number of splicing decisions, has led to the elaboration of a model of alternative splicing (Fu XD., 2004). In this model, splice-site usage is determined by the number of positively acting sites (splicing enhancers that are bound by SR proteins) and negatively acting sites (splicing silencers bound by hnRNPs). When positive sites outnumber negative sites, splicing occurs; when negative sites predominate, splicing is inhibited (Zhang *et al.*, 2009; Blanchette *et al.*, 2009).

Inhibition of splice site recognition can be achieved in many ways. When splicing silencers are located close to splice sites or to splicing enhancers, inhibition can occur by sterically blocking the access of snRNPs or of positive regulatory factors. An example is the modulation of alternative splicing of gene transcripts by the polypyrimidine tract binding protein (PTB). PTB, indeed, promotes exon skipping by binding to an exonic splicing silencer and inhibiting the association of U2AF and U2 snRNP with the upstream 3' splice site (Izquierdo *et al.*, 2005).

In addition, tissue-specific splicing factors FOX1 and FOX2 inhibit the formation of the E complex by binding to an intronic sequence and preventing SF1 from binding to the branch site of CAICA (calcitonin-related polypeptide- α) pre-mRNA (Zhou HL & Lou H., 2008). Some silencers can be over 100–200 bp away from enhancers, and a simple 'bind and block' model thus cannot explain their inhibitory effect. One explanation for the activity of such splicing inhibitors is that they function by masking splice site recognition through multimerization along the RNA (Spellman R, & Smith CW., 2006).

Another model proposes that the alternative exon might be 'looped out' in a process involving protein–protein interactions between RNA-binding proteins bound at sites spanning the alternative exon (Spellman R & Smith

CW., 2006; Sharma *et al.*, 2006) and that this loop formation may sterically interfere with further spliceosome assembly, even though splice site recognition may not be inhibited (Nasim *et al.*, 2002). As mentioned above, splicing is usually controlled by combinatorial or competitive effects of both activators and inhibitors. In particular it was shown that the ratio between SRSF1 and hnRNP A1 regulates splicing outcomes. A model that explains these observations is that under limiting concentrations of SRSF1, U1 snRNP binds only to functionally stronger splice sites. Thus, a weak 5' splice site might not be selected, despite its proximity to the 3' splice site. Higher levels of SRSF1 promote full occupancy of all 5' splice sites by U1 snRNP, and under these conditions the 5' splice site closest to the 3' splice site is selected (Chabot, B. 1996; Cáceres & Krainer., 1997). By contrast, hnRNP A1 antagonizes this activity of SR proteins, causing a shift toward distal 5' splice sites. The molecular mechanisms underlying the effects of hnRNP A1 are less well understood. Access to the proximal site by SRSF1 could be blocked by hnRNP A1. Indeed, binding of hnRNP A1 to certain exon splicing silencers (ESS) inhibits the use of adjacent 3' splice sites (Del Gatto-Konczak *et al.*, 1999; Caputi *et al.*, 1999). In conclusion, the ratio of SRSF1 to hnRNP A1 regulates alternative splicing through the antagonistic effects of these proteins on splice site selection (Mayeda & Krainer 1993).

Sometimes, the position of the cis-active elements, relatively to regulated exons affects the splicing outcome. Location-dependent activity reflects the flexibility of splicing regulatory factors in their interactions with core splicing machinery. Many proteins can act as either repressors or activators depending on the location of their binding site. A possible explanation of this phenomenon is that these proteins promote exon inclusion when, upon binding to the regulatory sequence they favour the recognition of the splice sites of the alternative exon by changing the local RNA structure. On the other

hand, when bound to silencers they may compete with components of the splicing machinery or may change the structure of the RNA to prevent splice site recognition (Chen M & Manley JL.,2009).

The protein NOVA1 has been shown to act as either repressor or activator depending on the location of its binding site. Thus, it can regulate alternative splicing of the GABA A receptor $\gamma 2$ (GABRG2) by binding to an ISE in GABRG2 pre-mRNA and promoting inclusion of exon 9, but it is also able to regulate alternative splicing of its own transcript by binding to the ESS in the alternative exon 4 of its pre-mRNA and preventing exon 4 from being included. As shown in Figure 6, NOVA1 can bind to a high-affinity site within an alternative exon, thus repressing exon inclusion. In contrast NOVA 1 can also bind within the intron downstream of an alternative exon, thus enhancing exon inclusion (Dredge *et al.*, 2005).

Similarly to NOVA1, hnRNP I can activate or repress upstream alternative exons, and this probably depends on the location of its binding site relative to the regulated 5' splice site (Hui *et al.*, 2005). hnRNP H promotes the formation of ATP-dependent spliceosomal complexes when it binds to G-rich sequences (G runs) downstream of the 5' splice site (Schaub *et al.*,2007), but it inhibits splicing when the G-rich sequences are located in exons (Caputi M & Zahler AM 2001). There are usually patterns and rules that summarize this location-dependent activity.



Figure. 6 Position dependent action of NOVA 1 on alternative splicing (Dredge BK *et al.*, 2005).

1.4 SR Proteins

The SR protein family comprises a number of phylogenetically conserved and structurally related proteins. SR proteins exist in all metazoan species (Zahler *et al.*, 1992) as well as in some lower eukaryotes.

The SR proteins were first discovered as splicing factors in the early 90s (Zahler *et al.*, 1992). A protein domain rich in arginine and serine dipeptides, termed the RS domain, was originally observed in three *Drosophila* splicing regulators, SWAP (suppressor-of-white-apricot) (Chou *et al.*, 1987), Tra (transformer) (Boggs *et al.*, 1987) and Tra-2 (transformer-2) (Amrein *et al.*, 1988). Subsequent identification of SRSF1 (splicing factor 2/alternative splicing factor) (Ge H *et al.*, 1991) and SC35 (spliceosomal component 35) (Fu & Maniatis., 1990) revealed that these proteins contained an RS domain, which is also present in the U1 snRNP-associated protein, U1-70K (Spritz *et al.*, 1987; Theissen *et al.*, 1986).

In humans, 12 SR proteins, called Serine / Arginine-rich Splicing Factors (SRSF 1 to 12) have been identified (Busch & Hertel, 2012) (Figure.7).

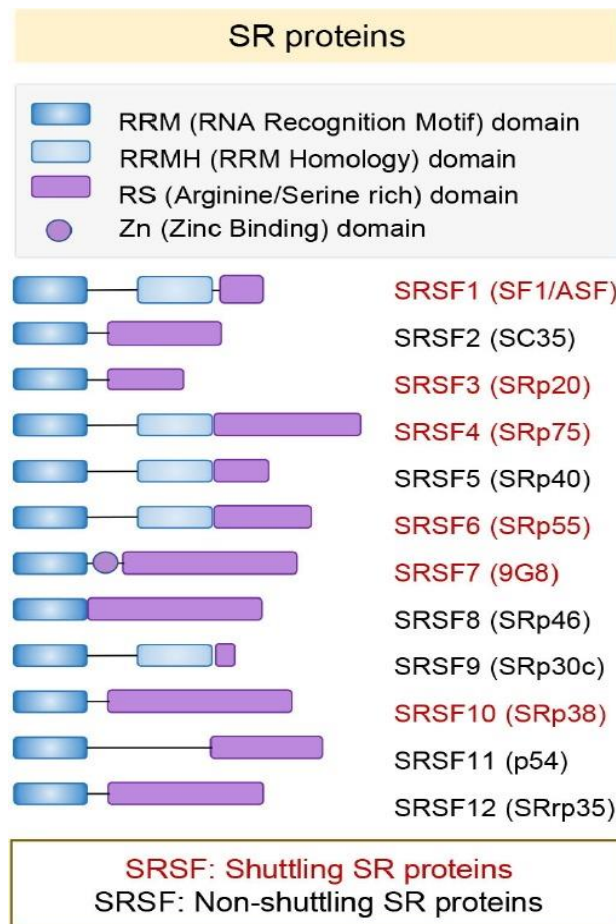


Figure.7 The human SR protein family. The structural organization of the 12 human SR proteins is shown. Alternative names are in parentheses. RRM: RNA recognition motif; RRMH: RRM homology; Zn: zinc-knuckle; RS: arginine/serine-rich domain. 6 SR proteins (red letters) are reported to shuttle between nucleus and cytoplasm, whereas the others (black letters) are exclusively nuclear (Sunjoo Jeong., 2017)

All SR proteins share two main structural features: the RS domain at the C-terminal that acts to promote protein-protein interactions to facilitate recruitment of the spliceosome and at least one RRM (RNA recognition motif) at the N-terminus that provides RNA-binding specificity. For the majority of SR proteins with two RNA-binding domains, the second is a poor match to the RRM consensus and is referred to as an RRM homolog (RRMH). The RS domains of SR proteins participate in protein interactions with a number of other RS-domain-containing splicing factors (Wu JY, & Maniatis T 1993; Kohtz *et al.*, 1994). These include other SR proteins, SR-related proteins (Blencowe *et al.*, 1999), and components of the general splicing machinery (Teigelkamp *et al* 1997; Cáceres *et al.*, 1997). The RS domain can also contact the pre-mRNA directly via the BP and the 5' ss, suggesting an alternative way to promote spliceosome assembly (Shen H *et al.*, 2008; Shen H & Green M. R., 2004). Furthermore, the RS domain acts as an NLS (nuclear localization signal), affecting the subcellular localization of SR proteins by mediating the interaction with the SR protein nuclear import receptor, transportin-SR (Caceres *et al.*, 1997; Lai *et al.*, 2000).

SR proteins exhibit dual functionality in constitutive and alternative splicing. One function of SR proteins is to promote the binding of U1 snRNP to 5' splice sites (Eperon *et al.* 1993; Kohtz *et al.* 1994); this appears to require protein-protein interactions between SR proteins bound at or near a 5' splice site, and U1-70K, a protein of snRNP U1. It has also been reported that SR proteins play a role in the association of the U4/U6·U5 tri-snRNP with the pre-spliceosome (Rosigno & Garcia-Blanco.,1995). Finally, SR proteins may also aid in the correct pairing of 5' and 3' splice sites by simultaneously interacting with U1-70K and U2AF35 (Wu & Maniatis 1993), which are bound near the 5' and 3' splice sites, respectively.

SR proteins also play important roles in establishing exon-intron boundaries in large metazoan genes. The process of “exon definition” is hypothesized to solve a significant problem related to finding relatively short exons within the context of long intronic sequences (Berget SM., 1995). Two main models have been proposed to explain the mechanism by which SR proteins regulate exon inclusion. The ‘recruitment model’ focuses on the ability of ESE-bound SR proteins to recruit and stabilize interactions between the U1 snRNP at the 5' ss and U2AF65 at the 3' ss (Graveley *et al.*, 2001; Robberson *et al.*, 1990). In the ‘inhibitor model’, ESE-bound SR proteins may act by antagonizing the negative activity of hnRNP proteins recognizing ESSs (Zhu *et al.*, 2001). SR proteins also contribute to intron-definition through a series of protein-protein interactions, mediated by the RS domain linking U1 snRNP at the 5'ss to U2AF35 at the 3'ss (Wu JY & Maniatis., 1993).

In contrast to the robust splicing enhancing effect in constitutive splicing, the role of SR factors in alternative splicing regulation is more complex and subtle. Alternative exons generally hold shorter length and weaker 5' spliced sites, so SR proteins induce their inclusion by increasing the recognition of weak splice sites by splicing machinery. Since the regulatory roles of RBPs in alternative splicing are position- and context-dependent, locations of SR-RNA interaction influence splicing outcome. For example, exon-bound SR proteins act as enhancers, but intron-bound SR proteins may function as suppressors (Shen & Mattox, 2012). So the location of SR-RNA interactions affect spliceosome assembly and splice site selection (Erkelenz *et al.*, 2013). In addition, SR proteins can act as activators or repressors in a context-dependent manner with other RBPs (Fu & Ares, 2014; Han *et al.*, 2011).

In addition, SR proteins can have long-range effects on regulation of alternative exons. Indeed, they can associate with constitutive exons adjacent to alternative exons (Howard JM & Sanford JR., 2015). In this way they can

modulate the strength between 3'splice site in adjacent exons that compete for a common upstream 5'splice site.

1.5 SRSF1

Serine/Arginine Splicing Factor 1 (SRSF1) is the founding member of the SR protein family. It has been originally isolated thanks to two of its activities: it promotes spliceosome assembly and constitutive pre-mRNA splicing in a S100 HeLa cell extract (SF2: splicing factor 2) (Krainer AR *et al.*, 1990) and regulates alternative splicing of the SV40 early pre-mRNA in vitro (ASF: Alternative Splicing Factor) (Ge H& Manley JL., 1991; Wang *et al.*, 2005). Later, SRSF1 has been found to regulate different other aspects of RNA metabolism in addition to splicing: mRNA export to the cytoplasm, translation, mRNA stability, nonsense-mediated decay (NMD). Moreover, SRSF1 is involved in mRNA-independent processes, such as protein sumoylation, miRNA processing, and the nucleolar stress response. Not surprisingly, SRSF1 can act as an oncoprotein, emphasising the importance of alternative splicing in tumorigenesis (Karni *et al.*, 2007; Anczuków *et al.*, 2007; Das *et al.*, 2014) (Figure.8).

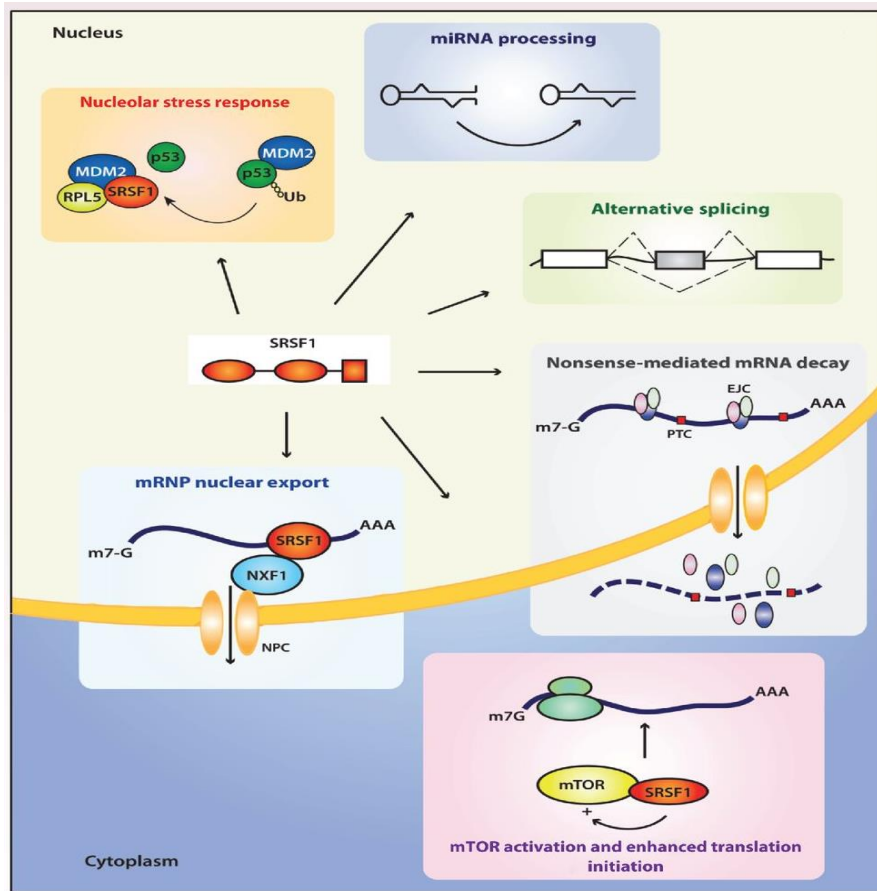


Figure.8 A schematic representation of the different SRSF1's functions. (Das & Krainer 2014).

1.5.1 SRSF1 Structure

SRSF1 is a protein with an apparent molecular mass on SDS-PAGE of approximately 33 kDA. The human *SFRS1* gene spans 6424 bases on the minus strand of the long arm of chromosome 17 (q 22) and encodes for 248 aminoacids.

Similarly to other SR proteins, SRSF1 contains two functional modules (Figure. 9):

- an arginine-serine rich region (RS domain) at C-terminus that it is shorter than in most of SR factors and where the bulk of SRSF1 adjustment takes place; in addition the RS domain is also required for nuclear-cytoplasmic shuttling of SRSF1 and regulates its sub-nuclear localization and its interaction with other splicing factors (Mayeda *et al.*, 1999);
- two RNA recognition motifs (RRMs), through which SRSF1 interacts with RNA and other splicing factors. The first RRM is located at N-terminus and is followed by a pseudo-RRM, which has a dominant role in dictating substrate specificity in vivo (Caceres *et al.*, 1997, Mayeda *et al.*, 1999). The consensus sequence 5'-ACGCGCA-3' was determined by SELEX in the presence of the N-terminal canonical RRM alone (RRM1), whereas more recent NMR structural analysis showed that RRM2 specifically binds a GGA motif. The RRM2 domain either activates or inhibits splicing by competing with binding of the splicing repressor hnRNP A1 (Mayeda A & Krainer AR., 1993; Eperon *et al.*, 2000) to a 5'-AGGA-3' motif, which contains overlapping binding sites for both proteins (5'-AGG-3' and 5'-GGA-3' for hnRNP A1 and SRSF1 pseudo-RRM, respectively).

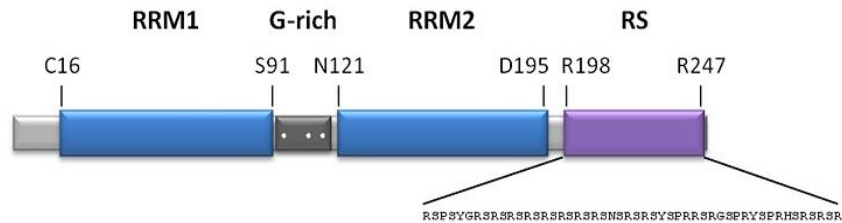


Figure.9 Scheme of the SRSF1 structure. The two RRM domains (blue) are separated by a glycine-rich hinge (dark grey), where three arginine residues (white dots) can be dimethylated. The sequence of the RS domain (purple) is also indicated. (Blanco & Bernabéu.,2012)

1.5.2 Posttranslational modifications of the SRSF1 protein

RS domain of SRSF1 is heavily phosphorylated and its phosphorylation status influences the interaction with other proteins, the subcellular distribution and the RNA binding properties of SRSF1 (Misteli *et al.*,1998; Xiang *et al.*, 2013). The SR domain is target of many kinases, including members of the Clk/Sty family, in the nucleus, and SRPKs, in the cytoplasm (Colwill *et al.*, 1996; Nayler *et al.*, 1997; Gui *et al.*,1994;). SRPK1 was shown to phosphorylate approximately 12 Ser residues in the N-terminal portion of the RS domain (Colwill *et al.*, 1996). This promotes nuclear import of SRSF1 through the association with transportin-SR2. The imported protein localizes into nuclear splicing speckles, namely the highly dynamic nuclear subcompartments that act as depots for a number of splicing factors (Ngo J. C *et al.*, 2005).

Once in the nuclear speckles SRSF1 is further phosphorylated on the C-terminal portion of the RS domain by Clk/Sty to generate a hyperphosphorylated form of the protein. Hyperphosphorylated SRSF1 leaves the nuclear speckles and is recruited to active sites of transcription, where it promotes the splicing reaction. During splicing, SRSF1 becomes dephosphorylated and this is the prerequisite for its export to the cytoplasm bound to spliced mRNA. Other protein kinases have been reported to phosphorylate SRSF1 (Figure. 10).

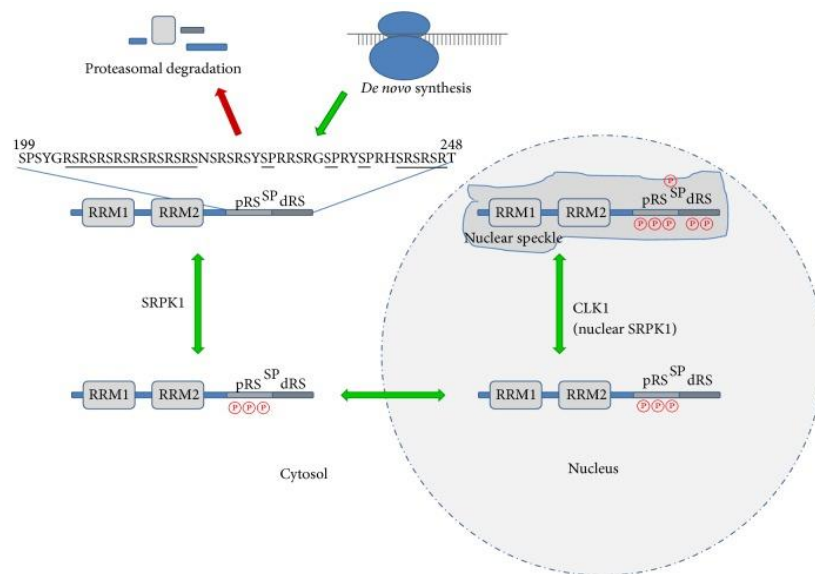


Figure. 10 SRSF1 protein is phosphorylated by the cytosolic protein kinase SRPK. This phosphorylation step is required for nuclear import of SRSF1 and accumulation in nuclear speckles. Further phosphorylation by Clk/Sty is necessary for the recruitment to the splicing complex. SRSF1 dephosphorylation induces its nuclear to cytoplasmic translocation (Gonçalves & Jordan., 2015).

Another SRSF1 kinase is Topoisomerase I (TopoI). Human DNA topoisomerase I plays a dual role in transcription, by controlling DNA supercoiling and by acting as a specific kinase for the SR-protein family of splicing factors. Human DNA topoisomerase I has also been described to phosphorylate SRSF1 (Rossi *et al.*, 1996; Labourier *et al.*, 1998). The formation of R-loops is promoted by an excessive negative supercoiling generated behind the transcription complex during elongation which results in partial strand separation and in the annealing of the nascent RNA molecule to the template (Aguilera A & García-Muse T.,2012). This torsional stress is relieved by Topo I in collaboration with SRSF1 (Tuduri *et al.*, 2009) and the activity of the PAR-Polymerase (Rossi *et al.*, 1996). The formation of a complex between PAR and SRSF1 promotes Topo I to switch from its protein kinase to DNA relaxation activity (Malanga *et al.*, 2008).

Protein kinase A (PKA) can phosphorylate SRSF1 on serine 119 in vitro and modulate its activity as a splicing factor (Shi *et al.*, 2011; Aksaas *et al.*, 2011). This phosphorylation occurs in the so-called pseudo-RNA recognition motif (RRM) and was described to change the RNA-binding properties of SRSF1 and reduce its capacity to activate splicing.

In addition, SRSF1 is also methylated at three Arg residues in the inter-RRM linker region (Sinha *et al.*, 2010). It has been shown that differential methylation modulates SRSF1 nuclear-cytoplasmic shuttling and protein functions.

1.5.3 SRSF1 Regulation

SRSF1 levels vary widely among cell types (Blencowe BJ., 2000) and tight control of its expression is important for normal cell and organismal physiology. The SRSF1 gene is essential, and protein depletion, by gene knock-out triggers genomic instability, cell-cycle arrest, and apoptosis (Xu *et al.*, 2005; Li X & Manley JL., 2005), while moderate (2-3 fold) SRSF1 overexpression is sufficient to transform immortal rodent fibroblasts, which acquire the ability to form sarcomas in nude mice. Therefore, in order to prevent destructive consequences caused by its misregulation, the level of SRSF1 is tightly controlled by mechanisms that operate at transcriptional, post-transcriptional and translational levels.

Interestingly, SRSF1 can negatively autoregulates its own expression through various post-transcriptional and translational mechanisms (Sun *et al.*, 2010; Wu *et al.*, 2010) in order, to maintain its homeostatic levels. Through alternative splicing SRSF1 pre-mRNA can generate 6 different molecules only one of which is protein coding (Figure.11). The remaining splicing products are either nuclear or are subject to NMD in the cytoplasm. This regulation involves 2 introns in the 3'-UTR where also 2 ultra-conserved elements are located. The molecular mechanisms underlying this regulation are still poorly defined.

The major Isoform, I, encodes the full-length protein, and has a long 3'UTR (Krainer *et al.*, 1991). Isoforms II and III retain the third intron (the last in the coding part of the gene). Moreover, Isoforms III and IV undergo splicing of intron 5 in the 3'-UTR.

Isoform II, III, and IV are retained in the nucleus and hence are not translated (Sun *et al.*, 2010). Isoform IV can undergo further splicing of intron 4 in the

3'-UTR leading to the production of Isoform V, which is exported in the cytoplasm and degraded by the NMD pathway. Similarly, through skipping of exon 4 Isoform III can be matured into Isoform VI, which is exported in the cytoplasm and subjected to NMD (Sun *et al.*, 2010).

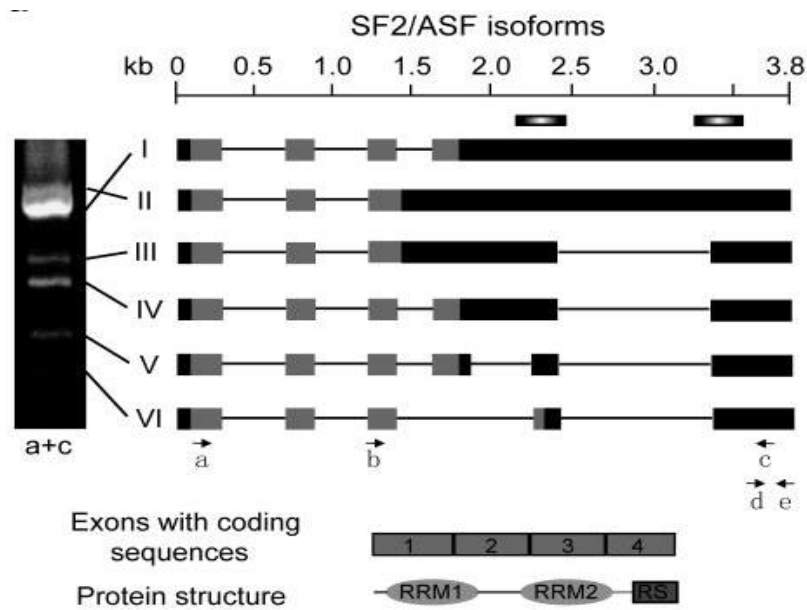


Figure. 11. Alternative splicing Isoforms of SRSF1 transcripts. Protein-coding regions are represented in gray, while UTRs are in black. The two short bars below the genomic scale denote the position of ultra-conserved elements (Sun *et al.*, 2010).

AS can affect the RNA stability by inclusion of premature translation termination codons (PTCs) that activate the nonsense-mediated mRNA decay (NMD) pathway (Isken & Maquat., 2008). In mammals, a termination codon is recognized as premature when located >50–55 nt upstream of the exon–exon junction marked by the exon junction complex (EJC). EJCs downstream of PTCs recruit essential NMD factors, including Upf1/Rent1, that in turn

promote mRNA degradation (Isken & Maquat, 2008). Initially, NMD was considered a mechanism for disposing of aberrant mRNAs arising from nonsense codon-containing alleles. It is now evident that NMD is involved in quantitative posttranscriptional regulation of gene expression through specific AS events in a processed calle Alternative splicing coupled to NMD or AS-NMD (Hillman *et al.*, 2004).

Recently, it has been found that AS-NMD regulates the expression of all SR splicing factors and several other RNA-binding proteins (Lareau *et al.*, 2007; Ni *et al.*, 2007; Saltzman *et al.*, 2008). In particular, this mecanism operates on splicing products V and VI of the SRSF1 gene (Lareau *et al.*, 2007). This mechanism serves both as a negative feedback loop, in which increased SR protein levels promote an increase in unproductive splice variants, and as a target for regulation.

A mechanism that can contribute to SRSF1 regulation involves miRNAs, which regulate gene expression by controlling the translation or stability of target mRNAs.

Micro-RNAs (miRNAs) are processed from longer precursors called pri-miRNAs, transcribed by RNA Pol II (He L & Hannon., 2004). With few exceptions (Vasudevan *et al.*, 2007), miRNAs typically bind to the 3' untranslated regions (UTRs) of mRNAs producing translational repression and/or target mRNA degradation. Alterations in miRNA expression often lead to severe pathological consequences and are frequently observed in human diseases (Esquela-Kerscher & Slack, 2006; Lee & Dutta, 2009; He *et al.*, 2007). miRNAs regulator of SRSF1 translation have been identified, including miR-28, miR-505, miR-10a, and miR-10b (Meseguere *et al.*,2010). A recent publication described the increased complexity of *SFRS1* gene expression regulation by unveiling a negative feedback loop in which SRSF1 promotes miR-7 processing and mature miR-7 could bind to the 3'-UTR of

SRSF1 to repress its translation (Wu *et al.*, 2010). Other miRNAs, including miR-221 and miR-222, may also be regulated by *SRSF1* expression through a similar mechanism. These results underscore a function of *SRSF1* in pri-miRNA processing and highlight the potential coordination between splicing control and miRNA-mediated gene repression in gene regulatory networks.

1.5.4 *SRSF1* and Cancer

SRSF1 is a potent prooncogene (Karni *et al.*, 2007; Anczuków *et al.*, 2012). Despite the above-mentioned regulatory mechanisms designed to maintain constant *SRSF1* levels, the protein is overexpressed in many different cancer types, such as colon, thyroid, small intestine, kidney, lung, liver, pancreas, and breast (Xu *et al.*, 2005). The *SRSF1* dysregulation can be due to several alternative mechanisms.

First (Karni *et al.*, 2007), *SRSF1* is overexpressed in some breast cancers due to amplification of the *SRSF1* gene at Chr. 17q23, a chromosomal rearrangement associated with breast malignancies with poor prognosis (Sinclair *et al.*, 2003).

Second, the *SRSF1* gene is a target of MYC (c-Myc), an important oncoprotein and transcription factor overexpressed in many different tumor types (Tansey W. P. 2014). MYC binds directly to the *SRSF1* gene promoter and activates transcription (Mao *et al.*, 2003). The resulting increase in *SRSF1* protein is sufficient to modulate alternative splicing of a subset of transcripts, such as the signaling kinase MKNK2 and the transcription factor TEAD1.

In addition, it has been showed that SRSF1 promotes phosphorylation of eIF4E, and this factor is known to enhance MYC's translation (Lin *et al.*, 2008). MYC and eIF4E cooperate to induce tumorigenesis: eIF4E antagonizes MYC-induced apoptosis, whereas MYC overrides eIF4E-induced senescence (Ruggero *et al.*, 2004). In addition, SRSF1 regulates AS of BIN1 (Karni *et al.*, 2007), a tumor suppressor that interacts with MYC and inhibits its ability to induce proliferation (Elliott *et al.*, 1999). Thus, SRSF1, being frequently overexpressed in breast tumors, may play an important role in regulating BIN1 and promoting the expression of Isoforms that fail to interact with MYC (Ge *et al.*, 1999; Karni *et al.*, 2007). Therefore SRSF1 can be considered a MYC target that contributes to its oncogenic capability by enabling MYC to regulate the expression of specific protein Isoforms through alternative splicing (Das *et al.*, 2014).

Third, *SRSF1* expression is also regulated at the level of splicing, by splicing factor Sam68 (Valacca C. *et al.*, 2005). Phosphorylated Sam68 inhibits the production of the *NMD* sensitive RNA Isoforms and promotes the formation of protein coding transcripts, thus leading to increased *SRSF1* protein levels (Valacca *et al.*, 2010). Sam68 phosphorylation depends on ERK/MAP kinase activity, which is frequently augmented in human tumors. Thus, Sam68 appears to link SRSF1 gene expression to proliferation signals. Both MYC- and SAM68- mediated increased expression of SRSF1 is associated with the acquisition of oncogenic features, such as a higher rate of cell proliferation, cell motility and invasion, and epithelial to mesenchymal transition (Das *et al.*, 2012; Valacca *et al.*, 2010). In addition, SRSF1 enhances translation of β -catenin through mTOR activation, leading to activation of the Wnt signaling pathway (Fu Y *et al.*, 2013) (Figure 12).

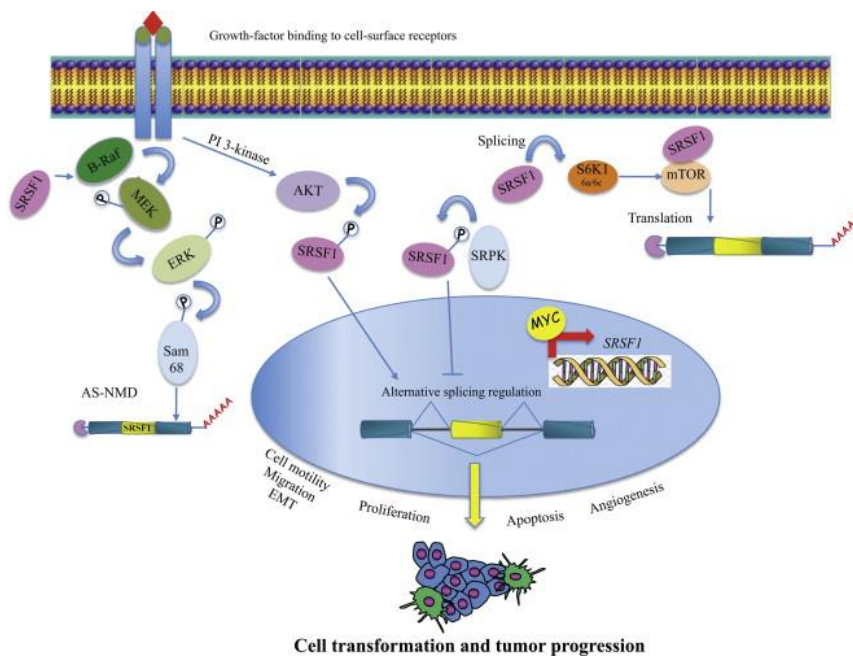


Figure.12. SRSF1 and oncogenic signaling pathways. On the left: SRSF1 activates the MEK-ERK kinases. Phosphorylation of Sam68 by ERK controls the expression levels of SRSF1. Center: SRSF1 phosphorylation by Akt and SRSPK kinases has opposite effects in the regulation of AS events relevant to cancer progression. On the right. The SRSF1 gene is a transcriptional target of the c-myc. The expression level of SRSF1 modulates the activity of mTOR kinase (Biamonti *et al.*, 2014)

1.6 EMT

The epithelial-to-mesenchymal transition is a biological process in which epithelial cells undergo a profound reorganization that converts them into

mesenchymal cells, with enhanced migratory capacities, invasiveness, and resistance to apoptosis (Kalluri R & Neilson EG 2003).

Epithelial and mesenchymal cells represent two main cell types in mammals.

Epithelial cells are characterized by:

- cohesive interactions among cells;
- three membrane domains: apical, lateral and basal;
- presence of tight junctions between lateral domains;
- apicobasal polarized distribution of the various organelles and cytoskeleton components;
- lack of mobility of individual epithelial cells with respect to their local environment.

Instead, mesenchymal cells have:

- loose or no interactions among cells, so that no continuous cell layer is formed;
- no apico-basal polarized distribution of organelles and cytoskeleton components;
- motile and invasive properties.

Moreover epithelial cells express high level of E-cadherin that sequester β -catenin in the cytoplasm, while mesenchymal cells express N-cadherin, fibronectin and vimentin and β -catenin is nuclear (Nieto MA 2013).

Under certain conditions epithelial and mesenchymal cell phenotypes are reversible and epithelial cells can switch to a mesenchymal status by means of a tightly regulated process defined as the EMT (epithelial to mesenchymal transition), which is associated with a number of cellular and molecular events. In some cases, EMT is reversible and cells undergo the reciprocal mesenchymal to epithelial transition (MET) (Larue & Bellacosa 2005).

There are different types of Epithelial-Mesenchymal Transition classified on the basis of the biological context where they occur (figure.13): Type 1 EMTs occurs during embryo implantation, embryo formation, and organ development to generate cells with common mesenchymal phenotypes. For example, during gastrulation, cells of the invaginating epithelium undergo EMT and migrate inward to form the primary mesenchyme. Mesenchymal cells can subsequently undergo the reverse process - MET - to form epithelial structures, a process that has been well documented in formation of the renal tubular epithelium during renal organogenesis (Dressler GR., 2006). Type 2 EMTs occur during wound healing, tissue regeneration, and organ fibrosis. In particular, the process ends up with the generation of fibroblasts and other related cells in order to reconstruct damaged tissues (Arnoux *et al.*, 2008). Finally, cancer cells undergo a type of EMT (type 3) after an initial clonal expansion of the primary tumor (Thiery JP, 2008). Through EMT cancer cells may acquire invasive features that are necessary to produce metastases.

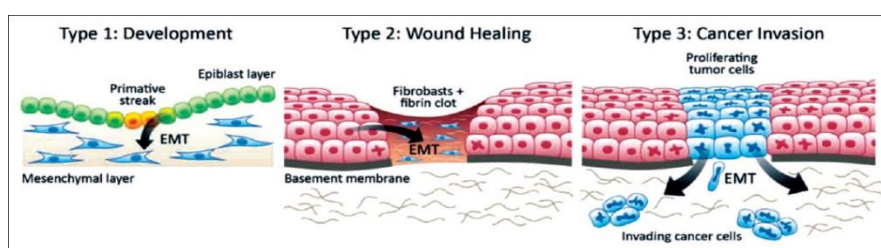


Figure.13 Three types of EMT are recognized. Type 1 EMT occurs early during embryonic development. Type 2 EMT occurs during persistent injury. Type 3 EMT is part of the metastatic process, whereby epithelial tumor cells leave a primary tumor nodule, migrate to a new tissue site, and reform as a secondary tumor nodule (Punnya V Angadi & Alka D Kale., 2015)

An important aspect of cancer cell biology is cell communication with the surrounding stroma.

The vast majority of the signaling pathways known to trigger EMT converge on specific aspects (Voulgari A & Pintzas A., 2009; Zavadil & Bottinger EP., 2005). First, cell-cell adhesion disintegrates with the loss of epithelial markers such as E-cadherin and the gain of mesenchymal markers such as vimentin. Next, there is a loss of baso-apical polarization and the acquisition of front-rear polarization. Finally, cell-matrix adhesion is altered, with activation of proteolytic enzymes such as matrix metalloproteases. Note that the process of metastasis formation also consists of multiple steps (Chambers *et al.*, 2002; Woodhouse *et al.*, 1997). Initially, cells detach from the primary tumor and invade the surrounding tumor stroma. They subsequently enter into the circulation and reach new metastatic sites. Finally they have to revert to an epithelial phenotype to give rise to the metastasis.

At the cellular level, pathological EMTs are very similar to physiological EMTs in that they are governed by similar signaling pathways, regulators, and effector molecules.

EMT is associated with the enhanced expression and redistribution of β -catenin due to mTOR activation. This in turn leads to activation of the Wnt signaling pathway, an important contributor to oncogenesis, and down-regulation of the epithelial marker E-cadherin, a component of adherens junctions, which inhibits invasion and the formation of metastasis. Down-regulation of E-cadherin is accompanied by increased expression of N-cadherin and vimentin and by the nuclear accumulation of β -catenin. In the nucleus β -catenin acts as a transcription factor with members of the lymphoid

enhancer factor (Lef)/T-cell factor family and promotes the expression of transcription factors, such as Snail and Slug, that inhibit E-cadherin production (Polyak & Weinberg, 2009).

EMT is also frequently marked by increased expression and/or activity of SRSF1, which regulates splicing of transcripts encoding the tyrosine kinase receptor Ron. High SRSF1 levels inhibit inclusion of exon 11, thereby promoting expression of the constitutively active Δ Ron Isoform that induces EMT (Ghigna *et al.*, 2005; Dow *et al* 2007) (Figure.14).

A very important aspect for the growth of tumors is the ability of the cells to gain access to nutrient supply via blood vessels. Thus, cancer cells release angiogenic signals, as for instance the vascular endothelial growth factor VEGF, that activate endothelial cells. SRSF1 is involved in promoting proximal splice site selection in C-terminal exon 8 of VEGF transcripts, resulting in the generation of pro-angiogenic Isoforms (Kalsotra & Cooper., 2011).

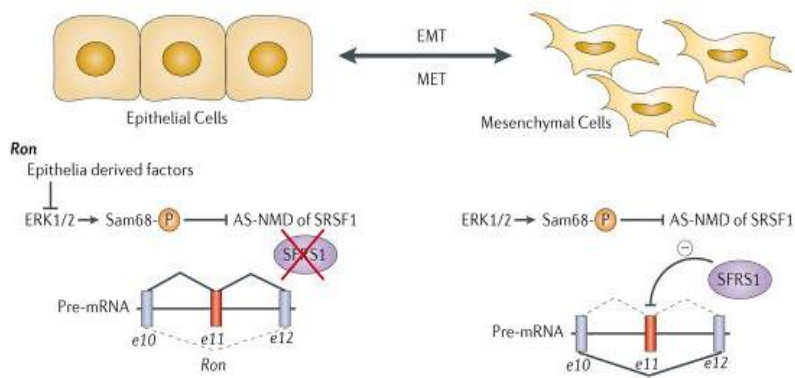


Figure.14 SRSF1 controls alternative splicing of *Ron* proto-oncogene by repressing exon 11 inclusion. This results in the production of the Delta-RON, a constitutively

active Isoform. This splicing event ultimately influences EMT by promoting cell motility and invasion, typical features of mesenchymal cells (Kalsotra & Cooper., 2011).

1.7 Metabolism and Cancer

To support the high rate of cell growth and proliferation cancer cells require precursors for the biosynthesis of macromolecules that normal cells. This is obtained by reprogramming glucose metabolism, a process first recognized by Otto Warburg nearly a century ago and now recognized as a hallmark of cancer cells. Warburg observed that cancer tissues have high rates of glycolysis even in the presence of oxygen and “aerobic glycolysis”, is often referred to as the Warburg Effect (Warburg Otto., 1956., Frezza & Gottlieb., 2009) (Figure 15).

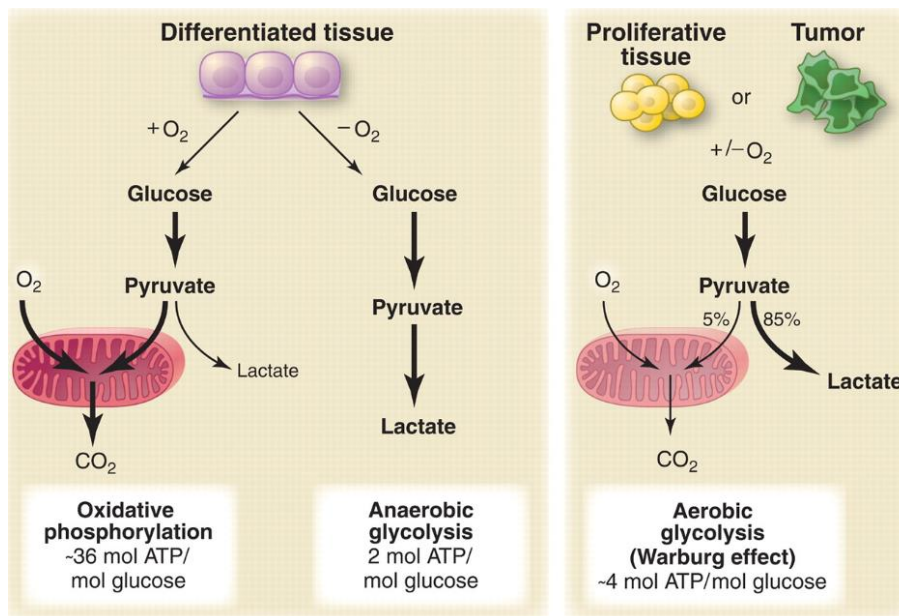


Figure.15 Differences between oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis (Warburg effect). In the presence of oxygen, differentiated tissues metabolize glucose into pyruvate through glycolysis and then completely oxidize most of that pyruvate in the mitochondria to CO₂ during oxidative phosphorylation. Oxygen is essential to completely oxidize glucose because it is the final electron acceptor. Cells grown under low oxygen tension or hypoxia stop using pyruvate in oxidative phosphorylation and generate lactate (anaerobic glycolysis). This allows glycolysis to continue (by cycling NADH back to NAD⁺), but results in minimal ATP production when compared with oxidative phosphorylation. Warburg observed that cancer cells convert most glucose to lactate regardless of whether oxygen is present (aerobic glycolysis) (Matthew *et al.*, 2009).

The oxidation of glucose in the glycolytic pathway generates pyruvate, which is preferentially transformed to lactate by lactate dehydrogenase (LDH) in the cytosol than oxidized through the TCA cycle (Vander Heiden *et al.*, 2009; Warburg Otto.,1956;).

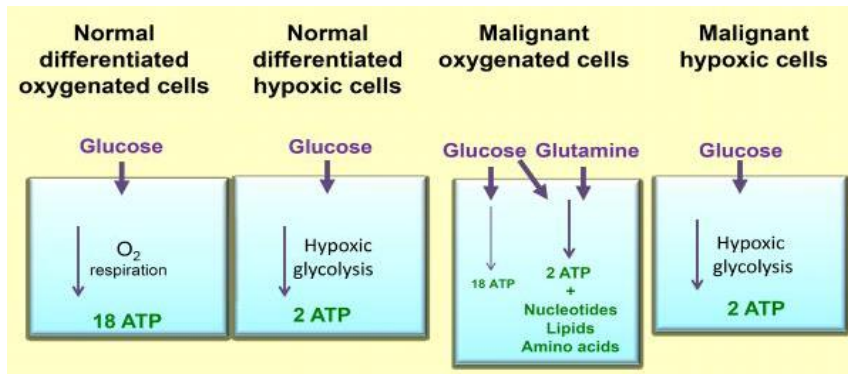


Figure. 16 Scheme illustrating the production of ATP by normal and malignant cells under different conditions of oxygenation (Teicher *et al.*, 2012).

In addition, glycolysis, as opposed to complete metabolism of glucose to water and CO₂ during the TCA cycle, provides carbon sources to other key metabolic pathways required for nucleotide, lipid, and amino acid synthesis, building blocks that are essential for the anabolic pathway in highly proliferative cells (Figure. 17).

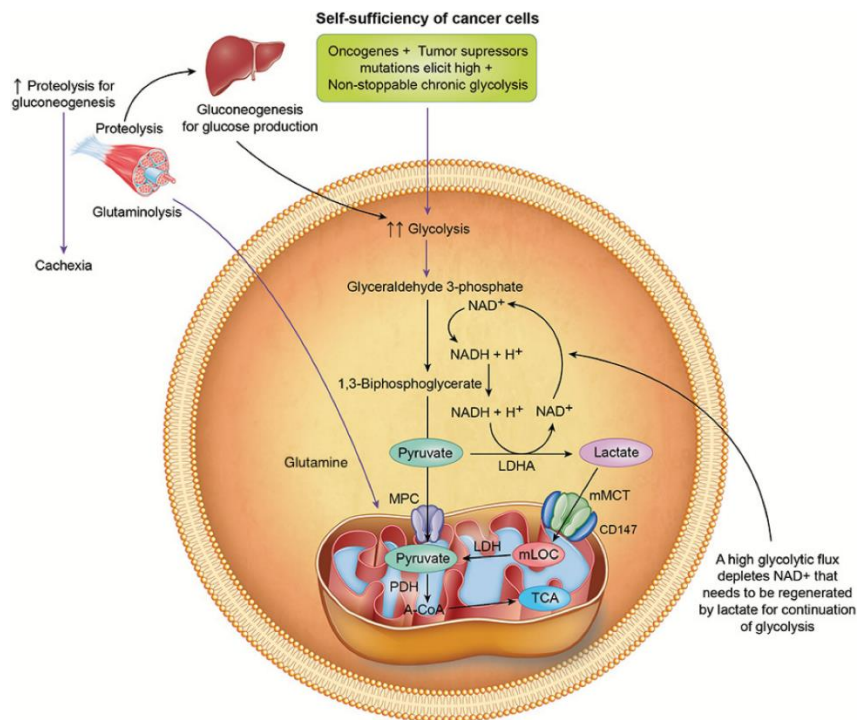


Figure 17. Representation of self-sufficiency of cancer cells. Accelerated glycolysis in the cancer cells depletes NAD⁺. The reduction of pyruvate to lactate replenishes cytosolic levels of NAD⁺ and regulates the NADH/NAD⁺ balance that is necessary to sustain glycolysis. Lactate can enter mitochondria to be oxidized to pyruvate and then acetyl CoA (A-CoA) (San-Millán I & Brooks GA., 2017).

1.7.1 Role of glutamine

The seemingly wasteful metabolism of glucose in cancer cells is mirrored by a similarly inefficient metabolism of glutamine (DeBerardinis & Cheng., 2009). Cancer cells do not survive in the absence of exogenous glutamine, and exhibit what is known as “glutamine addiction” (Eagle H., 1955). The

switch of glutamine from a nonessential to an essential amino acid (EAA) as an artifact of in vitro culture. Glutamine serves as a source of carbon to replenish the tricarboxylic acid (TCA) cycle. Glutamine is first converted to glutamate by glutaminase and then deaminated to produce α -ketoglutarate (α -KG) to enter the TCA, to generate ATP through production of NADH and FADH₂ (Figure 18).

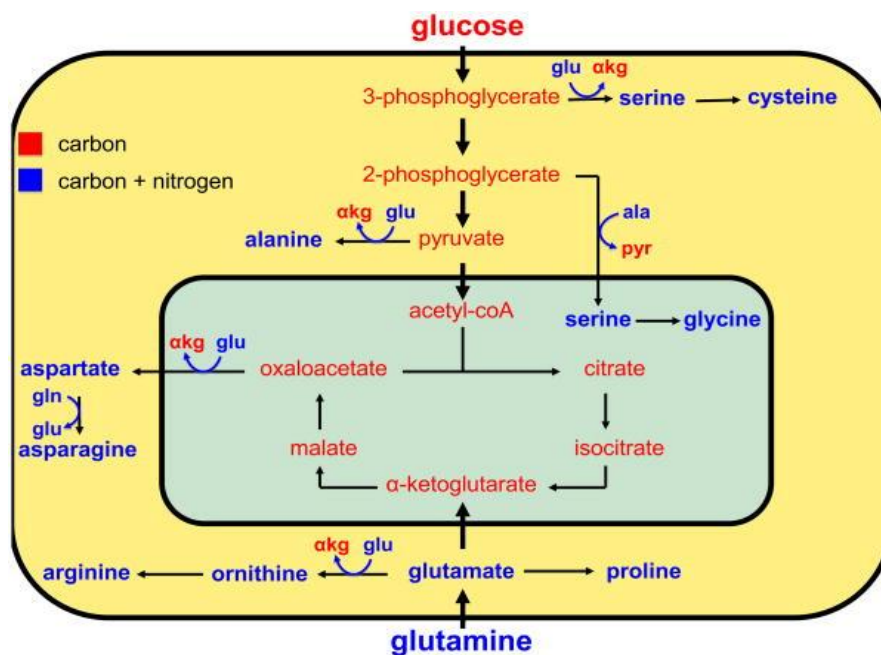


Figure 18. Glucose and glutamine (gln) provide the growing cell with a large pool of carbon and nitrogen for the biosynthesis of the nonessential amino acids. Carbon precursors derived from glycolysis (3-phosphoglycerate, 2-phosphoglycerate, pyruvate) and glutaminolysis (oxaloacetate, glutamic acid γ -semialdehyde) serve as the carbon substrate for amino acid biosynthesis (Wise & Thompon., 2010)

Glutamic acid is the primary nitrogen donor for the synthesis of non-essential amino acid - NEAAs (Young & Ajami., 2001). Transaminases transfer the amine group from glutamic acid to α -ketoacids. The α -ketoacids used to generate NEAAs are the carbon catabolites of glucose or glutamine: pyruvate, 3-phosphoglycerate, oxaloacetate, and glutamic acid gamma-semialdehyde. In addition, glutamine serves as an activator of mTOR1, which is a central cell growth controller (Crespo JL, *et al.*, 2002). As such, mTOR activity must be tightly controlled to prevent inappropriate cell growth, and glutamine and amino acid availability have a direct role in this regulation. Arginine and leucine are two amino acids that can together almost fully stimulate mTOR complex 1 (mTORC1) (Sancak Y *et al.* , 2008; Kim *et al.*, 2013). Glutamine can contribute to mTORC1 activation by being exchanged for essential amino acids, including leucine, through the large neutral amino acid transporter 1 (LAT1; a heterodimer of SLC7A5 and SLC3A2) antiporter (Nicklin *et al.*, 2009). Glutamine promotes mTOR localization to the lysosome and thus activity through the RAS family member ADP ribosylation factor 1 (ARF1) in a poorly understood mechanism (Jewell *et al.* 2015; Kim, S. G. *et al.* 2013). In addition, glutamine contributes directly to the availability of cysteine and glycine for production of glutathione, an endogenous antioxidant, to maintain the cellular redox homeostasis.

The MYC oncogene, the third most commonly amplified gene in human cancer, has been associated with upregulated glutamine metabolism (Zack, T. I. *et al.* 2013). The discovery that Myc-induced metabolic reprogramming triggers cellular dependency on exogenous glutamine as a source of carbon and macromolecular synthesis increased the interest in glutamine metabolism (Yuneva *et al.*, 2007; DeBerardinis *et al.*, 2007). Glutamine depletion of Myc-transformed cells leads to a profound reduction in the levels of TCA cycle

metabolites despite abundant extracellular availability of glucose, supporting the importance of glutamine in the maintenance of mitochondrial anaplerosis (Yuneva M, *et al.* 2007). These findings suggest Myc transformation might also suppress the ability of tumor cells to use glucose as an anapleurotic substrate perhaps through upregulation of LDH-A.

1.7.2 HIF

One key mediator of the Warburg effect is transcription factor Hypoxia-inducible factor-1 (HIF1). HIF1 is a heterodimeric transcription factor with HIF1 α or HIF2 α (also known as ePAS1) as the O₂ responsive subunit, and HIF1 β (also known as ARnT) as the constitutively expressed subunit (Jiang *et al.*, 1996) (Figure 19). The HIF1 α subunit was first identified as a protein that respond to a decreases oxygen availability or hypoxia. HIF1 α is not only controlled by low O₂ tension but also by other factors such as oncogene activation or loss of tumour suppressors, and pseudo-hypoxia (Bardos JI. & Ashcroft M., 2004; Costa *et al.*, 2014).

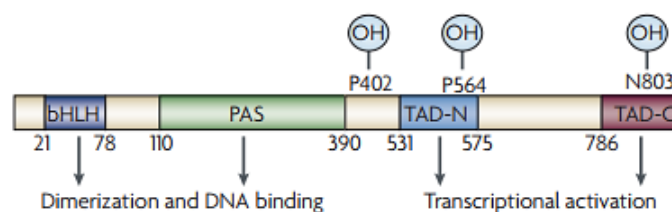


Figure 19 HIF1 α domain structure. Sites of proline hydroxylation are indicated in the O₂. (Denko NC., 2008)

Under normal oxygen tension, HIF1 α protein is degraded by the proteasome due to ubiquitination by a pathway involving von Hippel–Lindau protein (pVHL), a tumor suppressor protein and one of the recognized components of an E3 ubiquitin protein ligase (Stickle *et al.*, 2004). Two proline residues (P402/ P564) of HIF1 α are substrates of prolyl-4-hydroxylases (PHDs) (Bruick *et al.*, 2001; Jaakkola *et al.*, 2001). These are 2-oxoglutarate (2-OG)-dependent dioxygenases that require oxygen for their hydroxylation action, in addition to other co-factors such as iron and ascorbate. PHDs catalyse the conversion of a prolyl residue, molecular oxygen and α -ketoglutarate to hydroxyprolyl, carbon dioxide and succinate in a reaction dependent on ferrous iron and ascorbate as cofactors. Hence, hydroxylation of proline residues by PHDs occurs only in the presence of sufficient oxygen (Maxwell *et al.*, 1999). Moreover, another residue of HIF-1 α , lysine K532, is acetylated by an enzyme called arrest-defective-1 (ARD-1), an acetyl transferase enzyme. Modified HIF-1 α with hydroxylated and acetylated moieties is preferably recognized by pVHL and is tagged for proteasomal degradation. Since both the hydroxylation action from PHDs and ARD-1 expression require the presence of oxygen, under hypoxic condition, neither hydroxylation nor acetylation of HIF-1 α occurs, resulting in HIF-1 α stabilization (Jeong JW *et al.*, 2002).

Pseudohypoxic stabilization of HIF α refers to conditions in which PHD is inhibited not by a lack of O₂, but rather by a low level of 2-oxoglutarate, by the chelation of iron or by high levels of succinate. Low levels of exogenous ROS can also inhibit PHD during normoxia, presumably by oxidizing ferrous iron to the ferric state (Guzy *et al.*, 2005). An excess of succinate, a TCA cycle intermediate with structural similarities to 2-oxoglutarate and a product of the reaction catalyzed by PHDs, can also inhibit PHD (Selak *et al.*, 2005). All of these mechanisms determine an accumulation of the α subunit and

promote the formation of the HIF1 heterodimer. The active heterodimer translocates to the nucleus and binds, with co-activators p300 and CBP, to hypoxia-responsive elements (HREs) located in promoters hypoxia-inducible genes resulting in their transcriptional activation. The long list of genes controlled by HIF1 include genes involved in glucose metabolism and angiogenesis which are required for survival to hypoxia in normal and malignant tissues (Bertout *et al.*, 2008) (Figure 20).

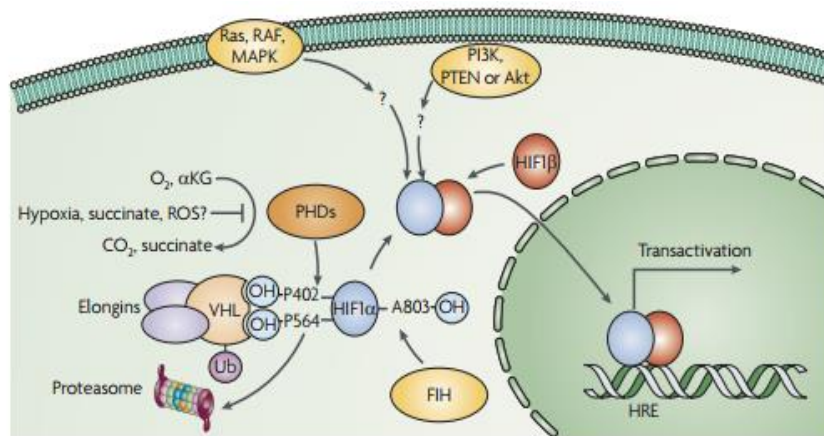


Figure 20. The classical O₂ sensing pathway is through O₂ dependent enzymatic hydroxylation at Pro 402 and/or Pro 564 on HIF-1 α . This modification mediates recognition of the HIF1 α by vHL and hence HIF1 α targeting for proteasomal degradation. Oncogenic activation, associated with activation of the RAS–RAF–MAPK, phosphoinositide 3-kinase, PTEN or Akt pathways, can also cause HIF1 α accumulation through unknown mediators. Tricarboxylic acid cycle intermediates such as succinate and fumarate, or perhaps mitochondrial reactive oxygen species (ROS), can inhibit the activity of PHDs, also stabilizing HIF1 α . Stabilized HIF1 α associates with HIF1 β , and the HIF1 heterodimer binds to cognate hypoxia-responsive elements (HREs) in target genes (Denko., 2008).

1.7.3 Succinate

The phenomenon of enhanced glycolysis in tumours has been acknowledged for decades, but biochemical evidence to explain it is just beginning to emerge. In the last decade, the discovery that inherited and acquired alterations in some enzymes of tricarboxylic acid (TCA) cycle have a causal role in carcinogenesis indicate that the altered metabolism as the underlying hallmark of neoplastic transformation (Oermann *et al.*, 2012). Recently, mutations in the gene encoding succinate dehydrogenase (SDH) (also known as complex II) have been implicated in several cancers, and SDH is now regarded as a tumor suppressor (Wu, W. & Zhao, S., 2013). The SDH complex resides in the inner mitochondrial membrane where it acts as a core component of both the Krebs cycle and of the electron transport chain that couples the conversion of succinate to fumarate with the reduction of ubiquinone. SDH is composed of four core subunits, SDHA, SDHB, SDHC, and SDHD and two assembly factors, SDHAF1 and SDHAF2, all of which are encoded by the nuclear genome. Mutations in SDHA, SDHB, SDHC, as well as the assembly factor SDHAF2 can also drive different types of tumors by destabilizing the complex and causing loss of enzymatic activity (Korpershoek *et al.*, 2011) (Figure 21).

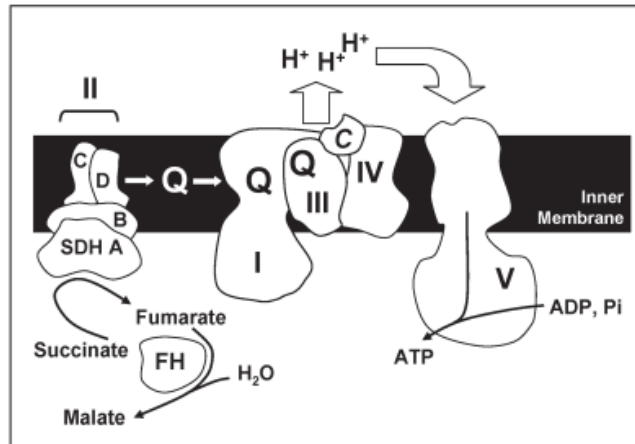


Figure 21. Schematic view of the respiratory chain (Brière *et al.*, 2005)

In particular, dominant mutations in the SDH subunits SDHB, SDHC and SDHD predispose carriers to carotid body paragangliomas and adrenal gland pheochromocytomas (Baysal BE, *et al.* 2000; Baysal *et al.* 2002). Dominant defects in SDH5 were also found to predispose individuals to paragangliomas (Hao *et al.*, 2009). A germline SDHA mutation has also been found in a patient presenting with paraganglioma (Burnichon *et al.*, 2010). Finally, mutations in SDHB are associated with renal cell carcinoma and T-cell acute leukemia (Vanharanta *et al.*, 2004; Baysal BE., 2007), and mutations in SDHB, SDHC and SDHD are associated with gastrointestinal stromal tumors (Stratakis CA & Carney JA., 2009).

The observation that many tumors arising from mutations in SDH genes show pseudo-hypoxia has suggested that the activation of the HIF-1 α could play a supportive role in the tumorigenic processes induced by TCA cycle dysfunctions. The link between the loss of mitochondria tumour suppressors

and pseudo-hypoxia is indicated by several studies. Initial studies by Gimenez-Roqueplo *et al.* (2003) proved that in some tumours with SDH mutations, the HIF pathway and consequently the angiogenic response are activated. More recently, an extensive gene expression analysis of 76 pheochromocytomas with different genetic lesions revealed a 'HIF signature' pattern of gene expression in a cluster of pheochromocytomas with SDHB, SDHD or VHL mutations (Dahia *et al.*, 2005). The causal link between TCA cycle dysfunction and HIF-1 α activation was initially suggested by Selak and coworkers demonstrating that the loss of SDH activity leads to accumulation of its substrate, succinate, which causes the inhibition of PHDs.

Succinate accumulated in mitochondria of SDH-deficient or SDH-inactive cells leaks out in the cytosol. As mentioned above, prolyl hydroxylation of HIF-1 α by PHD is the first step in tagging HIF-1 α for degradation, enabling its interaction with pVHL. PHDs are α -ketoglutarate dependent dioxygenases which couple the hydroxylation of the substrates with the oxidation of α -KG to succinate in reactions that are dependent on O₂ and Fe²⁺ (Ozer A& Bruick RK., 2007). For that reason, increase levels of succinate in SDH deficient cells impairs PHDs activity and leads to HIF-1 α stabilization under normoxic conditions (Kim, W. Y., & W. G. Kaelin., 2004). Thus, succinate is not only a substrate for SDH in the mitochondria, but also a product of PHDs in the cytosol. The accumulation of succinate in the cancer tissues as well as the PHD inhibition and pseudohypoxia was confirmed both in HLRCC leiomyomas and PGL tissues by Pollard et colleagues (Pollard *et al.*, 2005). Accumulation of α -KG, on the other hand, may have an opposite effect leading to the constitutive destabilization of HIF-1 α . Accordingly, Gottlieb and his group have shown that the increase in α -KG alone is sufficient to oppose succinate and hypoxia-mediated activation of HIF1 α . This resulted in

the reversal of enhanced glycolysis and cell death (Frezza C.& Gottlieb E., 2009). Hence, the levels of α -KG, even in hypoxia, may be sufficient to foster PHD activity and prevent the hypoxic response. Recently, biochemical studies showed that PHD activity is competitively inhibited by succinate and, therefore, the ratio between α -ketoglutarate and succinate rather than the absolute concentrations of these metabolites dictates PHD activity (MacKenzie *et al.*, 2007).

2 Aims of the research

Previous studies in our laboratory have established that the splicing profile of SRSF1 transcripts is modulated by cell density. When cells are grown at low density a protein coding transcript (Isoform I) is generated with modest levels of other RNAs. At high cell density, however, several alternative splicing events occur leading to the preferential expression of five long non-coding RNAs (lncRNAs) called Isoform II to VI. The production of these molecules involves splicing of two introns in the 3'-UTR portion of the gene (introns 4 and 5), retention of intron 3 (the last one in the protein coding portion of the gene) and skipping of exon 4 (containing the natural stop codon). Isoform II, III and IV are exclusively nuclear while Isoforms V and VI are exported to the cytoplasm and degraded by the non-sense mediated RNA decay (NMD) pathway. Retention of intron 3 distinguishes the proteincoding Isoform I from Isoform II. The latter is the precursor of nuclear Isoform III (generated through splicing of intron 5) that is the main Isoform detectable in high-density cells. Isoform III is then processed to Isoform IV via a splicing event between the donor splice site of intron 3 and the acceptor site in intron 4, which results in skipping of exon 4. My thesis focuses on the analysis of signals and factors that control this complex splicing program prompts questions about the function of these lncRNAs, the signals and the mechanisms underlying the choice between the production of the protein coding Isoform I and the expression of the lncRNAs the mechanism and signal controlling alternative splicing of SRSF1 transcripts, which is the main subject of my thesis.

3 Materials and methods

3.1 Cell culture

Cells are grown under sterile conditions in a humidified 37°C incubator with 5% CO₂

Medium is changed three times a week. Cell line used are:

- SW480 (ATCC® CCL228™) cell line;
- Clone 2: a clone of SW480 cells, in which the 3' splice site of intron 3 of the SRSF1 gene (**Q07955**) has been deleted by Vezzoni's group by means of the CRISPR-Cas9 technology.

Medium: RPMI (RPMI Medium, Sigma-Aldrich)

10% FBS (Fetal Bovine Serum, Euroclone)

2mM L-Glutamine (Euroclone)

25mM Hepes

Gentamycin 1X

Our experimental model consists of two different growth conditions: Low and High density. To obtain Low density (LD) cells were seeded at 500 cells/mm² while for High density (HD) 8000 cells/mm².

3.2 RNA purification using the RNeasy Mini Kit (Qiagen)

Cells grown in Petri dish are washed with PBS and treated for one minute with 1X Trypsin. After that cells are collected in complete medium 10% FBS to inhibit Trypsin activity and transferred to an Eppendorf tube, centrifuged for 10 minutes at 1400 rpm at 4°C washed in PBS (Phosphate Buffered Saline) and centrifuged for 10 minutes at 1400 rpm at 4°C.

The cellular pellet is now processed with the RNeasy Mini Kit (Qiagen) for RNA extraction.

- Resuspend each pellet in the proper volume of RTL buffer by vortexing.
- Add 1 volume of 70% ethanol and, after mixing gently, transfer the content of the Eppendorf to an RNeasy spin column, inside a 2ml collection tube. Samples are centrifuged at 13000 rpm for 15 seconds at 4°C and discard the flow through.
- Add 700 µl of RW1 buffer to the RNeasy spin column and centrifuge for 15 seconds at 13000 rpm at 4°C.
- Add 500 µl of RPE buffer to the RNeasy spin column and centrifuge at 13000 rpm for 15 seconds. Add another 500 µl aliquot of RPE and centrifuge at 13000 rpm for 15 seconds. After discarding the flow through, the Eppendorf tube is centrifuged at 13000 rpm for 2 minutes, in order to clean the filter inside the RNeasy spin column.
- Transfer the column to a new 1.5ml collection tube and add 30-40 µl of free-RNase water directly at the centre of the membrane.
- After waiting for 1 minute at room temperature, the column is centrifuged at 13000 rpm for 1 minute and the RNA is now eluted.

- RNA is then treated with DNase, using TURBO DNA-free kit (Life Technologies) to remove traces of DNA.

3.3 DNase treatment

DNase treatment is performed using the TURBO DNA-free kit (Life Technologies).

- Add 0.1 volume of DNase Reaction Buffer 10X to the RNA preparation, together with 1 μ l of DNase.
- Samples are incubated at 37°C for 20 minutes.
- Stop the reaction by adding 0.1 Volume of Inactivation Reagent and incubate for further at 5 minutes at room temperature.
- Samples are then centrifuged for 2 minutes at 13000 rpm to pellet the Inactivation Reagent. The supernatant containing the RNA is then removed and transferred to a new Eppendorf tube.

3.4 RNA reverse transcription

The concentration of the purified RNA is determined by means of a NanoPhotometer (Implen). Then, the RNA is used as template in a retrotranscription reaction with a Superscript IV 1st strand kit (Life Technologies).

1st reaction: Annealing

	stock solution conc.	
H2O RNase free		Up to 13 μ l
Oligo dT	50 μ M	1 μ l
dNTPs	10 mM	1 μ l
RNA		1-2 μ g

5'65°C

2nd reaction: cDNA synthesis

	stock solution conc.	
RT Buffer	5x	4 μ l
DTT	0.1 M	1 μ l
RNase OUT	40u/ μ l	1 μ l
RT	200u/ μ l	1 μ l

10' at 50°C, then 10' at 80°C. Add 1 μ l RNase H (2u/ μ l) and incubate 20' at 37°C

3.5 PCR

In order to perform the Polymerase Chain Reaction (PCR), the GoTaq G2 Flexi DNA Kit (Promega) is used together with specific primers.

Reagent	Final concentration
GoTaq Flexi Buffer	1X
MgCl ₂	2.5 mM
PCR Nucleotide Mix	0.2 mM
Primer Forward	0.2 μM
Primer Reverse	0.2 μM
cDNA	1 μl
GoTaq G2 Flexi DNA	1.5 u
H ₂ O	to 25 μl

The reaction is carried out in a thermal cycler using specific programs with different temperatures and cycles according to the primers set sequence.

Step	Time	Temperature	Cycle
Denaturation	2'	95°C	1
Denaturation	30''-1.5'	95°C	} n cycles
Annealing	30''-1.5'	Specific A.T.	
Extension	30''-1.5'	72°C	
Final extension	5'	72°C	1

For SRSF1 we used an annealing temperature (AT) of 60.5°C and 25 cycles, while for GAPDH the annealing temperature is 60°C and cycles are 16.

3.5.1 Primers used for PCR:

-SRSF1

Fw: CACTGGTGTCGTGGAGTTTGTACGG

Rw: GGGCAGGAATCCACTCCTATG

-GAPDH

Fw: ACCACAGTCCATGCCATCAC

Rw: TCCACCACCCTGTTGCTGTA

-SRSF1 (intron 2)

Fw: ATAAACGAGGATTGCTGC

Rw: CACGTTAAGCTGGTAAGG

3.6 Electrophoresis

PCR amplified products are analyzed by agarose gel. Agarose concentration depends on the size of the band to be detected. Agarose (Sigma-Aldrich) is melted in TBE 1X, which is also the running buffer in the electrophoretic chamber.

TBE 10X (pH 8.0)

Tris base 0.89 M

Boric acid 0.89 M

EDTA 0.02 M

DNA bands are visualized by staining with to Ethidium Bromide at the final concentration of 0.5 µg/ml.

Samples are made 1X Green Go Taq Flaxi Buffer (Promega) to the PCR products in order to increase the density of the samples. The dimension of the amplified PCR products is determined by comparison with the Ready load 1kb plus DNA ladder (Invitrogen), used as size marker. At the end of the electrophoresis bands are visualized with Gel Doc EZ Imager (Biorad), which visualizes PCR products under UV light. The image is then analyzed by the software Image Lab TM (Bio-Rad).

3.7 Quantitative PCR

We determined the relative expression of a gene of interest in different samples, by means of the QuantiTect SYBR Green PCR kit (Qiagen) using the Light Cycler 480 (Roche) apparatus. The reaction mix contains:

QuantiTect SYBR Green Master Mix 2X	10 µl
Primer Forward (100 µM)	0.1 µl
Primer Reverse (100 µM)	0.1 µl
cDNA	1 µl
H ₂ O	to 20 µl

PCR conditions:

Step	Time	Temperature	
Denaturation	15'	90°C	
Denaturation	15''	90°C	} n cycles
Annealing	20''	Specific A.T	
Extension	30''	72°C	
Melting curve		40°C	
		90°C	
		40°C	

Annealing temperature used to analyze *SRSF1*, *GINS2*, *VEGFA*, and *E-cadherin* gene transcripts is 60°C with 50 cycles.

Primers sets:

-SRSF1

Fw:

TTGGTGTACATGTGGGTACAGGGT

Rw:

ACGCTCTATCAGTCACACAGAGGA

-GINS2 (DNA replication
protein)

Fw:

TAACCCTGGTTTACCCGTGGAAGT

Rw:

TTCCATGTAGTAAGGGCTGGGCAT

-VEGFA (Vascular
endothelial growth factor
A)

Fw:

TCGGGCCTCCGAAACCATGAACTTT

Rw:

TTCGTGATGATTCTGCCCTCCTCCT

-E-cadherin

Fw:

CTGGGACTCCACCTACAGAAAGTT

Rw: GAGGAGTTGGGAAATGTGAGCA

3.8 Protein extraction

Lysis buffer for protein extraction is composed of 1X sample buffer, 1X phosphatase inhibitors “PhosSTOP” (Roche), and 1X protease inhibitors cocktail “Complete-EDTA free” (Roche).

Sample buffer 4x composition	final concentration
-glycerol	40%
-SDS	8%
-Tris HCl pH 6.8	240 mM
-Bromophenol Blue	0.04%
-β-MercaptoEthanol	5%

Proteins samples are kept on ice during the entire extraction procedure. Protein extraction is carried out by resuspending the cell pellet in 4-5 volumes of 1X lysis buffer and pipetting till the suspension becomes fluid. Then the sample is sonicated twice (Bioruptor plus, Diagenode) at 20-60 kHz. Each round of sonication is set on “low intensity” and involves 3 cycles each of 45’’ pulses separated by a 45’’ pause. If still viscous, the solution can be passed through 21Gx1/2’’gauge needle.

3.9 Western blot (WB)

Cell extracts are heated at 100°C for 10’, spinned and then analyzed through SDS-PAGE. We routinely use either 4-20% or 4-15% acrylamide pre-cast gels depending on the molecular weight of the protein we want to analyze (Biorad Mini-protean TGX - stain free). The acrylamide gel is put in the electrophoretic chamber, which is appropriately filled with a Running solution (25mM Tris, 190 mM glycine, 0.1% SDS). Precision Plus dual color marker from Biorad is used for molecular size evaluation. A current of 15 mA and 40 V for 10’ is applied allowing the samples to migrate through the stacking gel, afterwards the potential is raised to 100 mV and run is continued for 1.5 h. At the end of electrophoretic separation, proteins are transferred from the gel onto a nitrocellulose membrane by means of a Mini Trans-Blot® Cell (Bio-Rad) by applying a constant current of 1.3A up to 25 V for 10 min. In order to verify protein transfer and to compare the amount of proteins in the different lanes the membrane is stained with a Ponceau solution (0.1% Ponceau S w/v, 5% acetic acid v/v). The filter is then washed in TBST buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20) to remove excess

of dye. The membrane is incubated for 1 hr at room temperature in a blocking solution composed of Milk powder (SigmaAldrich) dissolved in TBST buffer at a final concentration of 5%. This step is designed to prevent aspecific binding of the antibody. Therafter, the membrane is hybridized to a primary antibody directed against the protein of interest and used at the proper dilution in TBST made 5% skimmed milk.

We used the following primary antibodies:

- Anti-SRSF1 (Sigma-Aldrich) (diluted 1:2000, incubated for 16-18h at 4°C),
- Anti- β -actin (Sigma-Aldrich) (diluted 1:2000, incubated for 1h at RT)
- Anti-GAPDH (Thermo scientific) (diluted 1:10000, incubated for 1h at RT)
- Anti-Jmjd6 (Abcam) (diluted 1:1000, incubated for 1 h at RT)
- Anti α -tubulin (Sigma-Aldrich) (diluted 1:5000, incubated for 1h at RT)
- Anti HIF1 α (Thermo Scientific) (diluted 1:500, incubated fot 16-18h at 4°C)

The nitrocellulose filter is then washed 3 times for 6-7 minutes each with TBST buffer and incubated with the secondary antibody; secondary antibodies are conjugated to with HRP (horseradish peroxidase) and are directed against the primary antibody. The following secondary antibodies were used: Anti-mouse,1:30.000 (SantaCruz Biotechnology) used to detect antibodies against GAPDH, β -actin, α -tubulin and SRSF1. Anti-rabbit,1:10.000 (Cell Signaling Technology) used for Jmjd6 primary antibody.

The membrane is incubated with the appropriate secondary antibody diluted in 5% skimmed milk TBST buffer for 1 hr at room temperature

The membrane is then washed 3 times for 6-7 minutes each in TBST buffer.

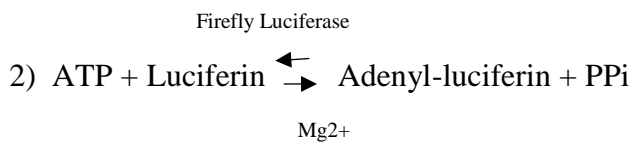
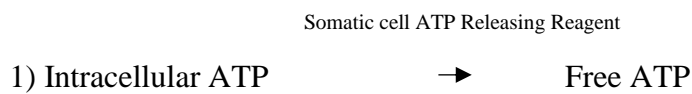
The filter is then incubated with a chemiluminescent substrate. To visualize SRSF1 and HIF1 α proteins the filter was incubated with Ultra solution (Cyanagen), while for β -actin, GAPDH and α -tubulin Pico solution (Thermo-Scientific) was used. The membrane is then placed in a cassette in contact with an X-Ray film which is then developed to get an image of the intensity of band which is a measure of the protein content.

3.10 Subcellular fractionation

Cells are pelleted by centrifugation for 5 min at 1200 rpm at 4°C. The cell pellet is then resuspended in Lysis buffer (10 mM HEPES pH 7.4, 3 mM MgCl₂, 0.5% NP-40, 10mM NaCl, 0.4 U/ml RNase OUT). After 5 min on ice, nuclei are observed under a phase contrast microscope to verify the release of intact nuclei free of cytoplasm. Then, samples are vortexed for 15'', passed through a 21Gx1/2'' gauge needle and spinned for 10 min at 5000 rpm at 4°C. The supernatant (cytosol) is transferred to a new tube. The nuclear pellet containing nuclei is washed in lysis buffer, and incubated 5 min on ice. Afterwards the samples are spinned at 13000 rpm 5' 4°C and nuclear pellet is used for RNAs and proteins extraction.

3.11 ATP-CONCENTRATION ASSAY

To determine levels of ATP released from a suspension of viable somatic cells, the Adenosine 5'triphosphate (ATP) bioluminescent somatic cell assay kit (Sigma Aldrich) was used. The method evaluates the average ATP concentration per cell. Viable somatic cell ATP is determined as follows:



When ATP is the limiting reagent, the light emitted is proportional to the ATP present, which is in turn proportional to the number of somatic cells in the sample.

The assay uses several solutions.

- ATP Assay Mix Dilution Buffer is made dissolving the content of the ATP Assay Mix Dilution Buffer already present in the kit, in 50ml of ultrapure water.
- ATP Assay Mix Stock Solution is prepared dissolving the contents of the ATP Assay Mix vial provided with the kit, in 5ml of ultrapure

water to generate a stock solution with pH 7.8. This solution must be kept on ice for at least 1 hour before use.

- ATP Assay Mix Working Solution is a 25-fold dilution of ATP Assay Mix Stock solution in 10 ml of ultrapure water.
- ATP Standard Stock Solution is prepared by dissolving the ATP Standard vial in 10ml of ultrapure water.
- ATP Standard Working Solutions is obtained using serial dilutions of the ATP Standard Stock Solution with ultrapure water.
- Somatic Cell ATP Releasing Reagent comes from a 10-fold dilution in ultrapure water of the Somatic Cell ATP Releasing Reagent present in the kit.

Cell samples (2×10^5) are taken for assay directly from a cell culture. To dissolve the solid sample 0.2 ml filtered ultrapure water is used.

It is important to keep pH at 7.8. 0.1 ml of ATP Assay Mix Working Solution is added to a reaction vial and is then vortexed and kept at room temperature for 3 minutes. Here, the endogenous ATP will be hydrolyzed, decreasing the background. To a separate vial containing 0.1 ml of Somatic Cell ATP Releasing Reagent, 0.05 ml of ultrapure water are added. Then 0.05 ml of the cell sample to be assayed are added and, after a quick swirl, 0.1 ml are transferred to the reaction vial. The amount of light emitted [L(SAM)] is immediately measured with a luminometer. The amount of ATP released is determined by running an internal standard. 0.1 ml of ATP Assay Mix Working Solution is added to a reaction vial and allowed to stand at room temperature for 3 minutes. To a separate vial containing 0.1 ml of Somatic Cell ATP Releasing Reagent, 0.05 ml of an appropriate ATP Standard is added together with 0.05 ml of cell sample. After a quick swirl, 0.1 ml are transferred to the reaction vial and the amount of light emitted [L(SAM+IS)]

is measured with a luminometer. The amount of ATP in the cell sample is calculated by the following equation:

$$\text{ATP (SAM)} = (\text{ATP(IS)} * \text{L(SAM)}) / (\text{L(SAM+IS)} - \text{L(SAM)})$$

Where ATP(SAM) are moles of ATP in the cell sample, ATP(IS) are moles ATP in the added internal standard, L(SAM) is the light emitted by the cell sample and L(SAM+IS) is the light emitted by the cell sample plus the internal standard.

3.12 RNA interference

Knockdown of JMJD6 was performed using the following protocol:

Cells were seeded in 10 cm dishes in Opti-MEM (Life Technologies) reduced serum medium (10 % FBS) without antibiotic. 24 hours later, when cells are 60 - 80 % confluent, the cells are washed with 5- ml 1x PBS (phosphate buffer solution, Lonza) and after 10 ml of medium with distinctive composition was added to the cells. In the first sample (control with no siRNA) was added the initial medium (Opti-MEM - 10 % FBS). For each sample targeted for transfection either with scrambled or specific siRNAs, the following steps were applied.

50 μ l of the lipofectamine RNAi MAX (Life Technologies) was dissolved in 1.5 ml of Opti-MEM medium. In a separate tube siRNA (500 pmol) was added to 1.5 ml of Optimem medium, and after 5 minute added to the tube containing lipofectamine to a final volume of 3 ml. After 5' the solution is

added drop by drop to cells. After 24h from the first transfection, a second round was performed.

- After 5h, the cells were seeded at different cell densities and 18 h later cells were harvested for RNA and protein extraction.

The efficiency of the silencing procedure is assessed by quantitative real time PCR (qPCR) and Western blotting.

siRNAs used in my thesis (commercial):

Target sequence for Jmjd6 (Gene ID: 23210) supplied by Dharmacon:

J-010363-10: 5'-GGAGAGCACUCGAGAUGAU-3'

J-010363-11: 5'-GGACCCGGCACAACUACUA-3'

J-010363-12: 5'-GGUAUAGGAUUUUGAAGCA-3'

J-010363-13: 5'-GGAUAACGAUGGCUACUCA-3'

Non-targeting control siRNA (Dharmacon):

5'-UGGUUUACAUGUCGACUAA-3'

5'-UGGUUUACAUGUUGUGUGA-3'

5'-UGGUUUACAUGUUUCUGA-3'

5'-UGGUUUACAUGUUUCCUA-3'

3.13 Conditional medium with glucose or glutamine

Low density SW480 cells (2×10^4 cells/cm²) were seeded in 10 cm dishes in pre-conditioned medium (CM) from High Density cells. When required CM was supplemented with glucose (2g/l), glutamine (2mM) or both. CM derives

from high density SW480 cultures in RPMI and 10% FBS . Medium was taken after 24 h and centrifuged to discard cell debris.

3.14 Dimethyl succinate treatment

SW480 cells ($2,5 \cdot 10^6$) were seeded in 10 cm dishes.

After 24h cell culture was made 60mM Dimethyl succinate (Sigma-Aldrick). 24h later cells were harvested for RNA and protein extraction.

3.15 α -ketoglutarate treatment

Cells ($7,5 \cdot 10^6$) were seeded in 3 cm dishes in medium made 0,6mM α -ketoglutarate (Cayman Chemical). After 18h cells were harvested for RNA and protein extraction.

4 Results

4.1 A complex splicing profile of SRSF1 transcripts.

SRSF1 is a highly conserved RNA-binding protein with an oncogenic potential and its expression needs to be tightly controlled for normal cell physiology. Post-transcriptional regulation of splicing factors occurs at multiple levels, one of these being alternative splicing. Krainer's group identified six alternatively spliced SRSF1 transcript Isoforms in HeLa cells, using primers positioned at the end of the first and last exon (Figure 22A, ref Sun *et al.*, 2010). The canonical Isoform, Isoform I, being by far the most abundant, encodes the full-length protein, and has a long 3'UTR (Ge *et al.*, 1991; Krainer *et al.*, 1991). Isoform II retains the third intron (Krainer *et al.*, 1991). A third Isoform differs from Isoform II for a splicing event that removes a large intron (intron 5) in the 5'UTR. Isoforms IV and VI are not listed in the UCSC or ENSEMBL browsers. Isoform IV, is identical to Isoform I except for the splicing of intron 5. Further processing of Isoform IV with splicing of intron 4 in the 3'-UTR, results in the production of Isoform V. Isoform III can mature into Isoform VI through skipping of exon 4 (Figure. 22B, ref Sun *et al.*, 2010).

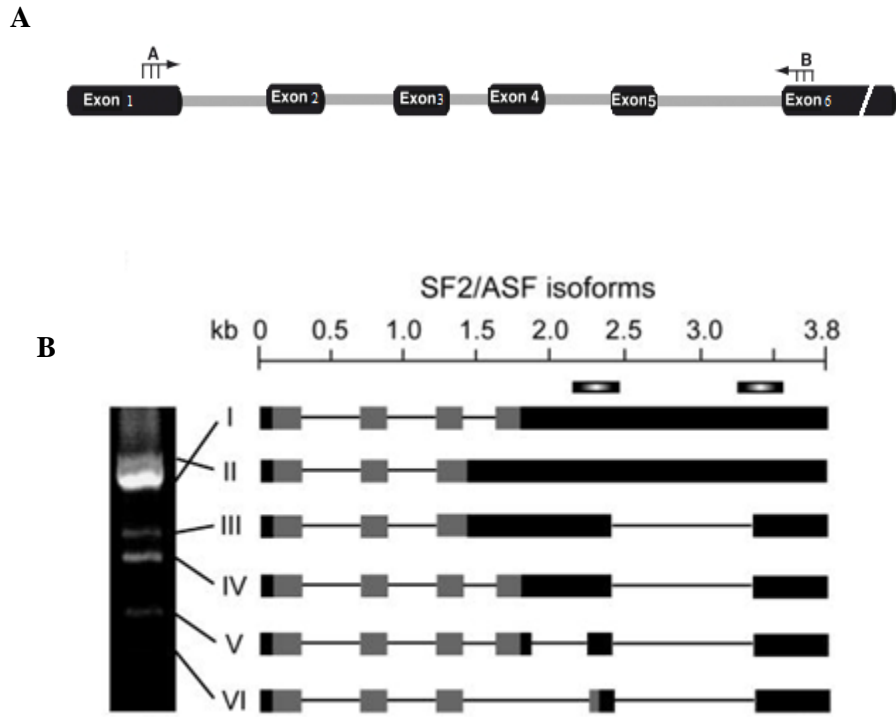


Figure 22 **A)** Scheme of the SRSF1 (SF2/ASF) gene structure. Primers used in RT-PCR analysis are indicated. **B)** Six alternative splicing Isoforms were identified by RT-PCR with primers A and B. Their structure, as determined by sequencing, is shown on the right. Spliced introns are indicated by a line. Black rectangles indicated non coding regions while grey rectangles indicate protein coding sequences.

We have previously shown that in cultured adenocarcinoma SW480 cells undergo morphological and gene expression changes typical of EMT in response to cell density (Valacca *et al.*, 2010). Indeed, low density (LD; 2×10^4 cells/cm²) cells display a morphology and express markers typical of mesenchymal cells thus resembling the invasive front of the tumor. On the

contrary high density cells (HD; 5×10^5 cells/cm²) exhibit an epithelial-like phenotype and some features similar to cells in the central part of the tumor (Valacca *et al.*, 2010; Brabletz *et al.*, 2001). Under these conditions, the level of SRSF1 appears to be controlled through alternative splicing events in the 3'-UTR of *SRSF1* transcripts that promote the production of a mRNA molecule target of the NMD pathway (Valacca *et al.*, 2010).

Alternative splicing in the 3'UTR region has been previously visualized in our laboratory by RT-PCR by means of a primer set flanking intron 5 (Figure 23A and ref Valacca). By this approach it was possible to show that LD cells express mainly the Isoforms in which intron 5 is retained, whereas splicing of this intron, resulting in the production of NMD+ sensitive transcripts, is predominant in HD cells.

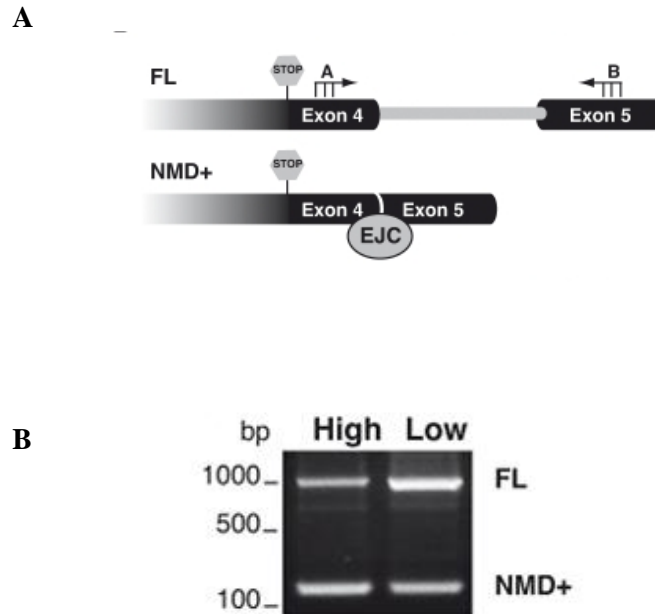


Figure 23 **A)** Schematic representation of AS in the 3' UTR of *SRSF1* transcripts. The relative position of RT-PCR primers is indicated. **B)** Splicing profile of *SRSF1* transcripts in HD and LD cells with primers A and B flanking the intron 5.

4.2 Splicing of *SRSF1* transcripts is modulated by cell density

As previously mentioned, Sun and colleagues described the existence of six alternative *SRSF1* transcripts (Sun *et al.*, 2010) (Figure 22). We observed that this program can be modulated by cell density. As shown in Figure 23 A) LD SW480 cells preferentially express the protein-coding Isoform I, while the remaining splicing products are barely detectable. When seeded at high

density, a drastic reduction of Isoform I is detectable accompanied by increased expression of the remaining splicing products, with a prevalence of Isoform III. Although slight variations among experiments can occur concerning the relative abundance of the 6 bands, this trend is usually observed. Interestingly, this drastic reprogramming of splicing is not accompanied by a comparable change in SRSF1 protein level indicating that additional control mechanisms operate to maintain the homeostatic level of the protein. At the same time this discrepancy raises a question about the meaning of this complex splicing program and about the function of Isoform III.

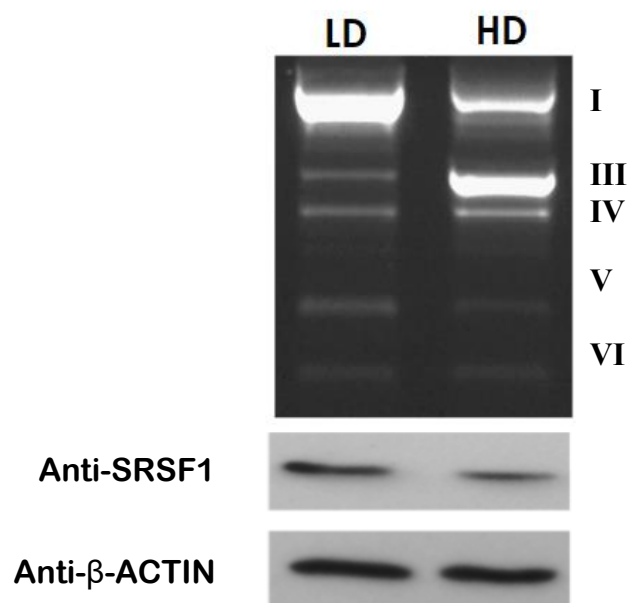


Figure 24 RT-PCR analysis of the splicing profile of *SRSF1* transcripts in SW480 cells. Sw480 cells grown at Low density (LD) mainly express Isoform I, while alternative splicing Isoforms, particularly Isoform III, become more abundant at high density (HD). Western Blot analysis on SW480 cells grown at low and high density.

Low density cells display higher SRSF1 (33 kDa) protein levels. β -actin (40 kDa) was used as a loading control.

In agreement with Sun *et al.*, cell fractionation experiments indicate that Isoform III is exclusively nuclear while splicing products IV, V and VI, although mainly nuclear, are detectable also in the cytoplasm. Isoforms V and VI (Figure 25) (Sun *et al.*, 2010). Krainer and colleagues previously demonstrated that NMD pathway is involved in regulation of SRSF1. Indeed, when they inhibited NMD with the inhibitor of protein synthesis cycloheximide (CHX), the level of Isoforms V and VI increased substantially (Sun *et al.*, 2010). This data is in agreement with data obtained by Biamonti and colleagues, that showed that CHX increases the level of NMD+ transcripts in SW480 cells in LD and HD (Valacca *et al.*, 2010).

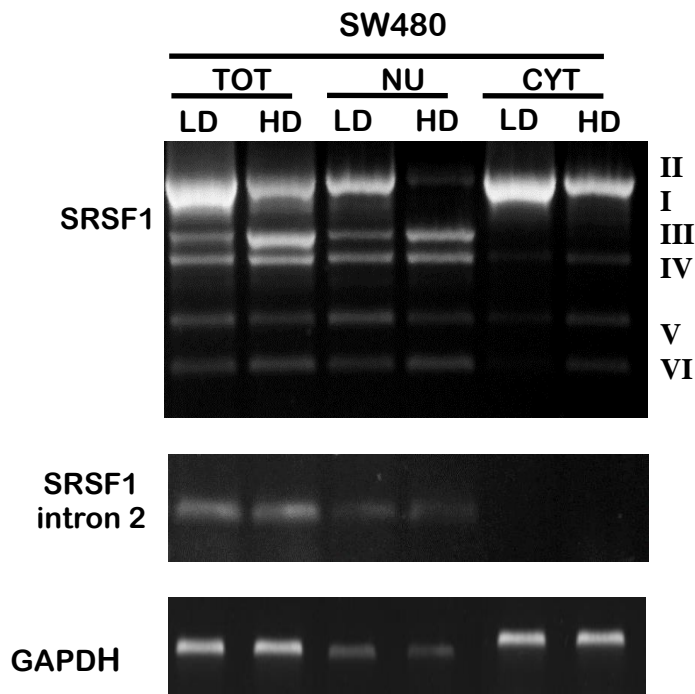


Figure 25. Cell fractionation was performed to separate nucleus (Nu) and cytoplasm (Cyt). RT-PCR was performed on total RNAs and on RNA purified from nuclear and cytoplasmic fractions. RT-PCR for SRSF1 intron 2 and GAPDH was used as control for the nucleus/cytoplasmic fractionation.

4.3 Regulation of alternative splicing of SRSF1 transcripts is part of a conserved response to cell density

In order to understand whether this modulation of the splicing profile of *SRSF1* transcripts was specific of SW480 cells or could be part of a general response to cell density, we verified its occurrence in different cell types: 1) colon adenocarcinoma SW620 cells deriving from a metastatic lymph node

in the same patient from whom primary adenocarcinoma SW480 cells were obtained 2) epithelial colon adenocarcinoma HT-29 cells, 3) HEK-293 cells, 4) normal human diploid WI-38 fibroblasts and 5) mouse NIH-3T3 cells. As shown in Figure 26, in all these lines, cell density induces a drop in the level of Isoform I accompanied by the accumulation of transcripts III to VI. These results point to the existence of a similar regulation of the splicing profile of SRSF1 transcripts in response to cell density in different cells type.

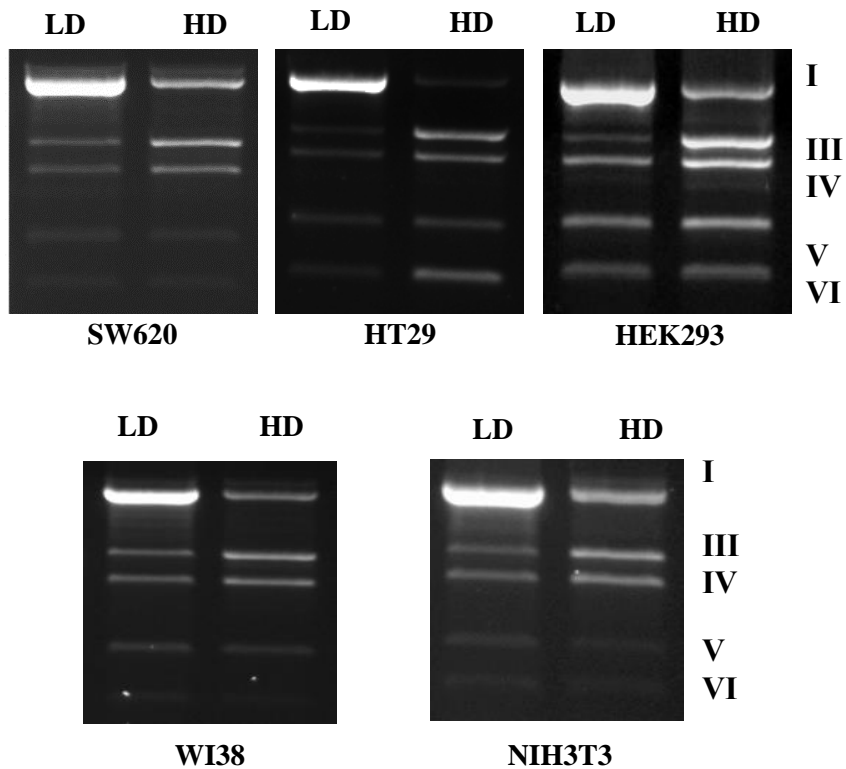


Figure 26. Splicing profile of *SRSF1* transcripts in different cell lines.

4.4 Reversible splicing profile of *SRSF1* transcripts in response to cell density

So far we have compared two extreme conditions, LD vs HD cells. We decided to investigate whether the splicing profile of *SRSF1* transcripts could be modulated even in response to small differences of cell density and we seeded the cells at progressively higher concentrations.

As shown in Figure 27 A, as cell density grows from 0,2 to 5×10^6 cells /cm² a progressive reduction in the abundance of the protein-coding Isoform I takes place which is accompanied by an increase of the other splicing products, with Isoforms III and IV being approximately equimolar. At higher cell densities Isoform III becomes prevalent in parallel with the appearance of Isoform II.

Our data indicate that splicing of intron 5 in the 3'UTR is already activated at 1.5×10^5 cells /cm² while retention of intron 3, which generates Isoforms II and III, becomes more important when density exceeds 2×10^6 cells /cm². Activation of the 3'-ss of intron 4, to generate Isoforms V (splicing with the 5'-ss of intron 4) and VI (splicing with the 5'-ss of intron 3 and skipping of exon 4), represents the last obligate step to produce transcripts exported to the cytoplasm and eventually degraded by the NMD pathway. Notably, it occurs only after splicing of intron 5. Thus, generation of Isoforms V and VI involves initial production of splicing products (Isoforms II, III and IV) that accumulate in the cell nucleus and characterized by detained introns.

To verify if the effect of high density on the splicing profile of *SRSF1* transcripts is reversible SW480 cells were grown at high density for 18 hours, and then diluted at low density and harvested at different time points (1, 6, 12 and 24 hours) for RNA analysis. RT-PCR analysis reveals a very rapid change

of the splicing profile already one hour after dilution. In particular it is detectable an immediate drop in the intensity of band III with a concomitant appearance of band VI indicating the removal of the detained intron, suggesting that activation of the 3'ss of intron 4 could be one of the first events occurring upon dilution (figure 27 B). At 12 hours the splicing profile typical of low density cells is re-established.

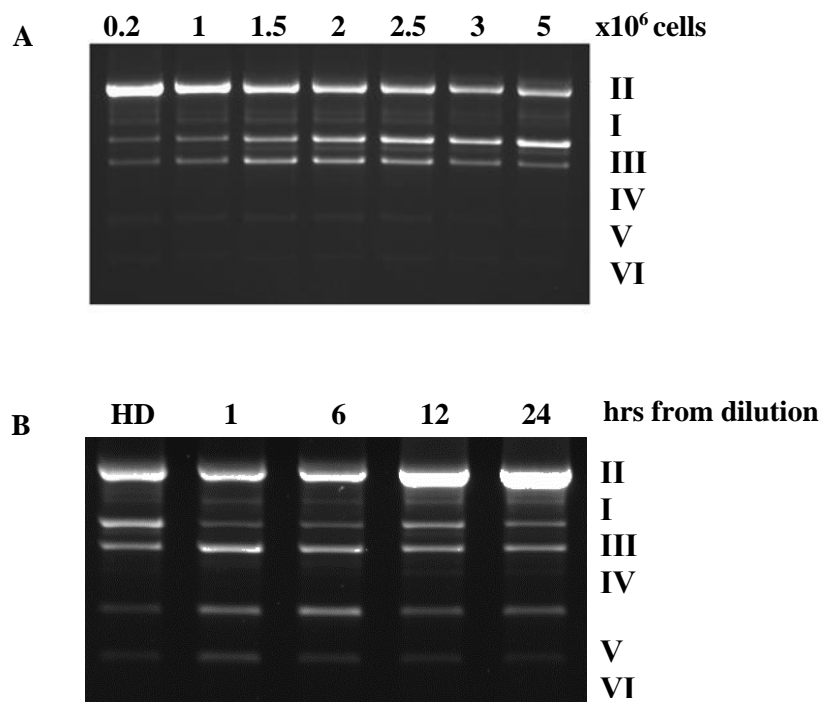


Figure 27 Effect of cell density on alternative splicing of *SRSF1* transcripts. **A)** RT-PCR analysis shows a progressive change of the splicing profile as cell density increases from 0,2 to 5 x 10⁶ cells /cm². **B)** RT-PCR analysis shows the time course modulation in the splicing profile of *SRSF1* transcripts after diluting HD cells to low density.

Since Isoforms II, III and IV are exclusively nuclear one can propose a that the production of the six Isoforms derives from two independent splicing programs. One program produces Isoforms I, IV and V while the other gives rise to the remaining Isoforms. The discrimination between the two pathways is the initial retention of intron 3.

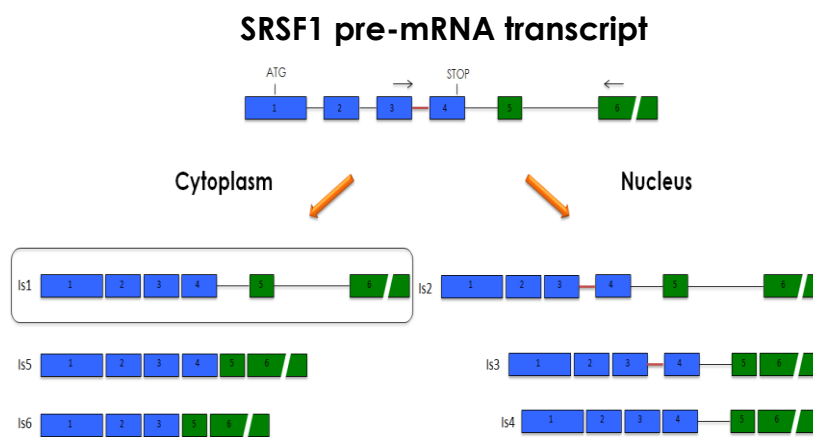


Figure 28 The scheme shows the six Isoforms produced through alternative splicing of the *SRSF1* pre-mRNA

Interestingly, the change in the splicing program of *SRSF1* transcripts in response to cell density is accompanied by a progressive reduction in the proliferation rate of SW480 cells. The histogram in Figure 29 indicates that the fraction of replicating cells, identified by BrdU incorporation, drops from more than 70% at $0,2 \times 10^5$ cells to about 20% at 2×10^5 cells/cm². No further reduction, nor induction of apoptosis, is observed at higher concentrations.

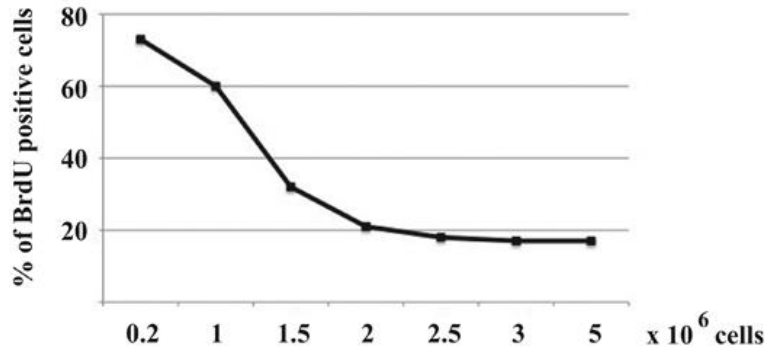


Figure 29. Histogram BrdU incorporation.

4.5 Specific gene expression pathways are affected by cell density

In order to gain further insight into this cell system, Biamonti and colleagues performed a gene expression array with total RNAs from LD and HD cells. A total of 1279 and 1432 genes were respectively DOWN- and UP-regulated ($|\text{LogFC}| > +1$) in HD cells.

Bioinformatics analysis performed with the Ingenuity program, of gene differentially expressed in LD and HD cells suggested that biosynthetic pathways are inhibited in HD cells. In agreement with the reduction in the fraction of replicating cells (figure 29), genes for proteins involved in DNA synthesis, as for instance GINS2, and cell cycle progression are down-regulated in HD cells, while negative regulators, such as cyclin-G, are up-regulated.

Concerning genes for factors involved in pre-mRNA processing, 42 genes (35 DOWN and 7 UP) out of 50 considered by Ingenuity, are affected by cell

density. In particular, the expression of the hnRNP A1 gene, which antagonizes the activity of SRSF1 in controlling the splicing profile of the *Ron* proto-oncogene transcripts, increases (LogFC = 1.5) while the level of *SRSF1* mRNA is reduced in HD cells (LogFC = -1.35). Bioinformatics analysis of among genes differentially expressed in LD and HD cells identified by the array analysis, there is the whole set of genes regulated by transcription factor HIF1 (hypoxia inducible factor) that includes genes for proteins involved in the uptake of carbon sources (glucose and glutamine) and extrusion of catabolites such as lactic acid. Up regulation of these genes in response to high density suggests that HD cells experience an energy stress. Altogether changes in gene expression profiles are compatible with the idea that HD cells, similarly to cells in the central mass of the tumor, are exposed to a stressing inhibitory environment that affects their proliferation and anabolic pathways. This is suggested also by the up-regulation of genes required for the cell adaptation to hypoxia whose activation depends on the hypoxia inducible factor 1 (HIF1) (**Table 1**). HIF1 is a heterodimeric transcription factor with HIF1 α as the O₂ responsive subunit, and HIF1 β (also known as ARnT) as the constitutively expressed subunit. HIF1 recognizes similar hypoxia-responsive elements (HRes) in the promoters of target genes. Among genes regulated by HIF, there is the gene encoding the vascular endothelial growth factor A (*VEGF-A*), which stimulates the formation of blood vessels and whose expression increases of about 10 fold in HD cells.

Response to hypoxia	Gene Name	Gene ID	logFC	adj.P.Val
	peroxisome proliferator-activated receptor alpha	5465	1.87	6.18E-08
ATP1B1	ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide	481	1.89	2.65E-08
CAV1	caveolin 1, caveolae protein, 22kDa	857	1.45	1.89E-05
CLDN3	claudin 3	1365	1.26	2.02E-06
PDGFA	platelet-derived growth factor alpha polypeptide	5154	4.18	6.08E-07
PDK1	pyruvate dehydrogenase kinase, isozyme 1	5163	2.58	1.79E-06
SLC2A1	solute carrier family 2 (facilitated glucose transporter), member 1	6513	1.49	2.10E-02
PDIA2	protein disulfide isomerase family A, member 2	64714	1.19	1.56E-05
ALDOC	aldolase C, fructose-bisphosphate	230	3.64	2.33E-08
STAT5B	signal transducer and activator of transcription 5B	6777	1.27	1.81E-03
EGLN3	egl nine homolog 3 (C. elegans)	112399	2.16	1.85E-05
PML	promyelocytic leukemia; similar to promyelocytic leukemia protein isoform 1	5371	1.08	2.35E-06
JAG2	jagged 2	3714	1.61	4.49E-07
EGLN1	egl nine homolog 1 (C. elegans)	54583	2.07	1.03E-06
CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	10370	1.45	2.58E-04
TGFB2	transforming growth factor, beta 2	7042	1.03	8.18E-04
HMOX2	heme oxygenase (decycling) 2	3163	-1.22	1.68E-05
PLOD1	procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1	5351	1.11	4.01E-04
CXCR4	chemokine (C-X-C motif) receptor 4	7852	1.21	1.11E-06
ANG	angiogenin, ribonuclease, RNase A family, 5	283	3.25	4.99E-09
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	5352	2.36	2.89E-08
HMOX1	heme oxygenase (decycling) 1	3162	1.02	5.51E-05
CAMK2D	calcium/calmodulin-dependent protein kinase II delta	817	1.55	1.69E-06
NOS2	nitric oxide synthase 2, inducible	4843	1.01	2.46E-06
MT3	metallothionein 3	4504	1.18	5.96E-05
ANGPTL4	angiotensin-like 4	51129	3.65	2.52E-06
SMAD9	SMAD family member 9	4093	1.55	5.46E-06
APOLD1	apolipoprotein L domain containing 1	81575	1.34	2.47E-06
MMP14	matrix metalloproteinase 14 (membrane-inserted)	4323	1.01	1.19E-05
DDIT4	DNA-damage-inducible transcript 4	54541	4.29	3.84E-06
TFRC	transferrin receptor (p90, CD71)	7037	-1.77	4.81E-07
ADM	adrenomedullin	133	4.87	6.87E-08
VEGFA	vascular endothelial growth factor A	7422	3.59	4.05E-06
PDE5A	phosphodiesterase 5A, cGMP-specific	8654	1.53	1.02E-03
NPPC	natriuretic peptide precursor C	4880	2.31	1.96E-08
ALDH2	aldehyde dehydrogenase 2 family (mitochondrial)	217	1.18	8.93E-06
CYB5R4	cytochrome b5 reductase 4	51167	-0.89	1.02E-04
PGF	placental growth factor	5228	0.87	1.22E-05
BCL2L1	BCL2-like 1	598	-0.78	3.21E-05
ASL	argininosuccinate lyase	435	-0.96	2.35E-05
ADA	adenosine deaminase	100	0.85	2.49E-04
ARNT	aryl hydrocarbon receptor nuclear translocator	405	0.69	8.17E-03
CD24	CD24 molecule	100133941	0.78	8.41E-03
CABC1	chaperone, ABC1 activity of bc1 complex homolog (S. pombe)	56997	0.9	5.76E-05
NOL3	nucleolar protein 3 (apoptosis repressor with CARD domain)	8996	0.75	8.31E-04
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	1543	0.69	1.09E-04
NF1	neurofibromin 1	4763	0.91	1.99E-04
BIRC2	baculoviral IAP repeat-containing 2	329	0.88	1.36E-04
UBE2B	ubiquitin-conjugating enzyme E2B (RAD6 homolog)	7320	-0.77	1.17E-03
SOD3	superoxide dismutase 3, extracellular	6649	0.79	4.25E-05
ITPR1	inositol 1,4,5-triphosphate receptor, type 1	3708	-0.64	1.55E-05
HSP90B1	heat shock protein 90kDa beta (Grp94), member 1	7184	0.73	1.34E-05
HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	3091	-0.62	4.26E-02
CA9	carbonic anhydrase IX	768	0.75	2.16E-03
ADAM17	ADAM metalloproteinase domain 17	6868	0.76	1.63E-04
ABAT	4-aminobutyrate aminotransferase	18	0.74	1.19E-02
PLAU	plasminogen activator, urokinase	5328	0.9	1.54E-04
VLDLR	very low density lipoprotein receptor	7436	0.98	9.77E-06

Table 1. Hypoxia is one of the most altered biological process in response to cell density.

In order to understand if the activation of the HIF1 pathway in HD cells can be relevant for the switch in the splicing profile of *SRSF1* transcripts, I down-regulated the expression of the ARNT gene, which encodes the constitutive β -subunit of the HIF1 complex (Figure 30A). As expected, ARNT down-regulation reduces the activation of the VEGF-A. Remarkably, it also affects the splicing profile of *SRSF1* transcripts in HD cells by reducing the relative abundance of Isoforms III and IV with the contemporary increase in Isoforms I and II. Altogether these experiments indicate that HD cells do suffer a sort of hypoxia even though they are grown in normal oxygen tension. At the same time they suggest a link between the activation of the HIF1 and the splicing program of *SRSF1* transcript. In particular, the result in Figure 30 suggests the involvement of the HIF1 pathway in triggering efficient splicing of intron 5 in HD cells. Indeed, the Isoform III and IV that are produced by splicing of intron 5 are reduced after ARNT down-regulation, while the abundance of Isoform I and II, that retain the intron, increases.

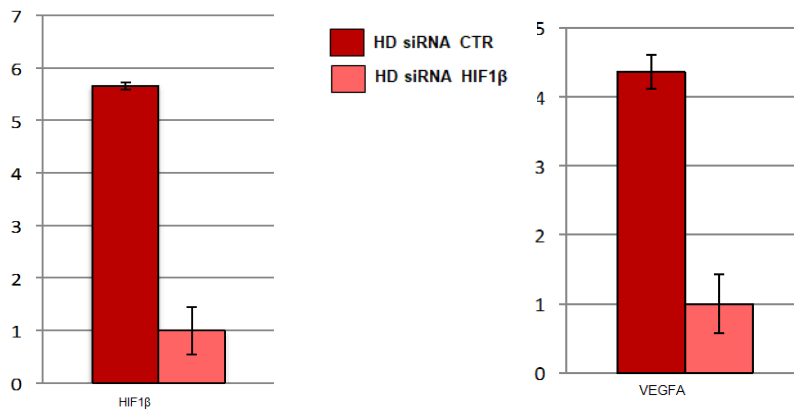
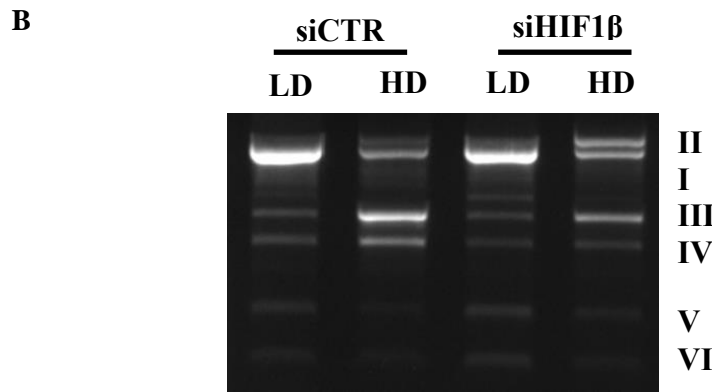
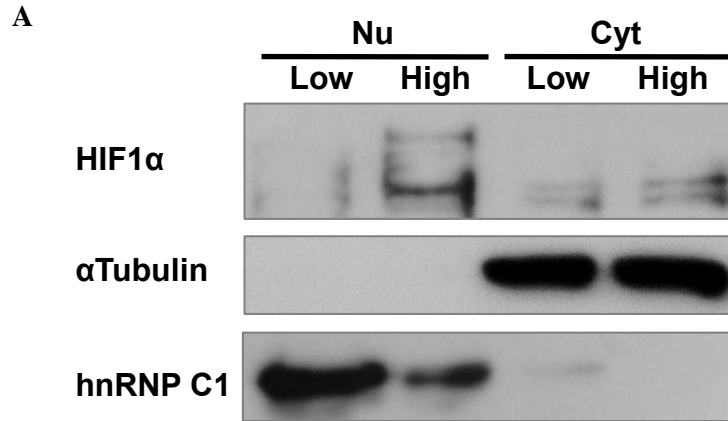


Figure 30. A) Nuclear and cytoplasmic fractions from Low and High-density SW480 cells were analyzed in Western blotting with antibodies against HIF1 α and

α -tubulin and hnRNP C1 (as cells fractionation control). **B)** RT-PCR and qPCR analysis of total RNAs extracted from cells transfected with siRNAs against HIF1 β (siHIF1 β) or with a control oligo (siCTR). HIF1 β downregulation inhibits splicing of intron 5 in the 3'UTR of *SRSF1* transcripts and prevents VEGFA induction driven by cell density.

4.6 The splicing program of SRSF1 transcripts is modulated by cell metabolism

Both tumor hypoxia and dysregulated metabolism are classical features of cancer. Recent analyses have revealed interconnections between oncogenic activation, hypoxia signaling systems and metabolic pathways that are dysregulated in cancer. These studies have demonstrated that metabolic and hypoxia signaling pathways are also directly connected to oncogenic signaling mechanisms at many points. The major regulator of the hypoxia response is the transcription factor hypoxia inducible factor (HIF) (Selak *et al.*, 2005). Overall these studies have defined multiple means by which HIF is up-regulated in cancer and multiple effects of HIF activation on metabolism in cancer (Masson & Ratcliffe., 2014). Furthermore, HIF acts (directly or indirectly) on genes encoding a wide range of enzymes that contribute to metabolic dysregulation in cancer. The relationships between hypoxia, the TCA cycle and tumor formation are complex.

Bioinformatics analysis of genes differentially expressed in LD and HD cells, has identify that a large set of genes that encode for proteins involved in the uptake of carbon sources (glucose and glutamine) and extrusion of catabolites such as lactic acid are up-regulated in HD cell.

Indeed, it is plausible that the higher expression of genes involved in the uptake of nutrients and extrusion of toxic catabolites reflects the strategy used by the cells to cope with the decreased content of glucose and glutamine in the medium. In order to prove that this is indeed the case I determined levels of glucose and glutamine and lactic acid in the medium of LD and HD cells after an overnight incubation (Figure 31 A, B).

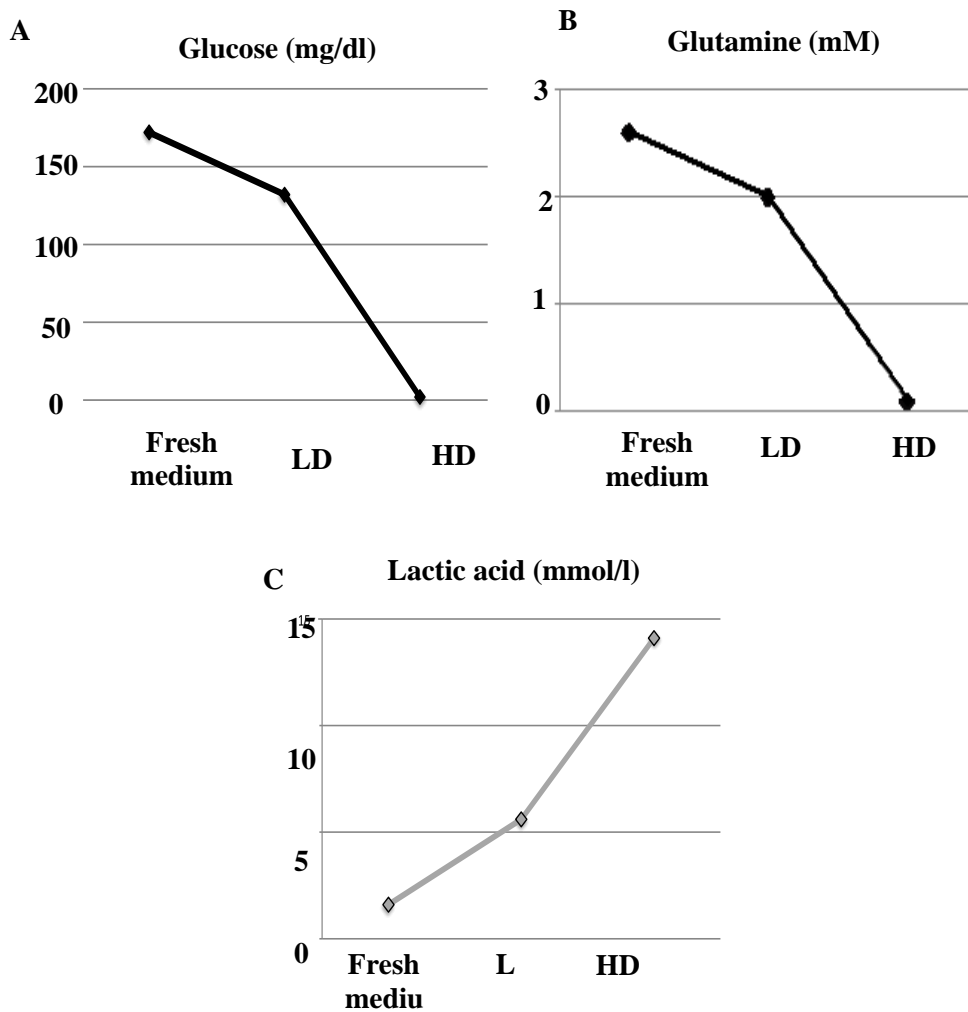


Figure 31. Concentrations of **A)** glucose, **B)** glutamine and **C)** lactic acid in fresh medium and after 18h of cell growth at Low (LD) or high (HD) density.

Otto Warburg was the first to observe, more than 80 years ago, that tumor cells consume glucose at a surprisingly high rate compared with normal cells, and accumulate a significant amount of lactate rather than oxidizing glucose (Warburg Otto., 1956., Frezza & Gottlieb., 2009). Indeed, cancer cells decrease dependence on aerobic respiration and cell metabolism is shifted toward glycolysis even in the presence of oxygen. Glycolysis, as opposed to complete metabolism of glucose during the TCA cycle, provides carbon sources to other key metabolic pathways required for nucleotide, lipid, and amino acid synthesis, building blocks that are essential for the anabolic pathway in highly proliferative cells. This metabolic difference is a distinctive marker of tumor cells that has been exploited in tumor diagnosis. In addition to glucose, increased glutamine uptake is another tumor-specific metabolic alteration (Vander Heiden *et al.*, 2009; Wise & Thompson., 2010). Indeed, it was reported that glutamine could fully sustain the oxidative TCA cycle for energy production, even in the absence of glucose (Le *et al.*, 2012). The high demand of proliferating tumor cells for glutamine was first described by the American physiologist Harry Eagle in 1950s, who demonstrated that the optimal growth of cultured HeLa cells requires a 10- to a 100-fold molar excess of glutamine in culture medium relative to other amino acids (Eagle, 1955). Glutamine is first converted to glutamate by glutaminase and then deaminated to produce α -KG to enter the TCA. Glutamine also serves as an activator of mTOR1, which is a central cell growth controller, to promote cell growth. A consequence of switched between aerobic respiration to glycolysis is the drop in the ATP level. Aerobic respiration produces far more ATP molecules, 32 per molecule of glucose, than anaerobic respiration, which produces a mere two. To verify the hypothesis that HD cells prefer glycolysis to aerobic respiration we assessed the ATP concentration in HD and LD cells by means of the bioluminescent ATP-concentration assay. In agreement with

our prediction, a 4.4 fold drop in the ATP level was detectable in HD ($1.6 \cdot 10^{-15}$ mol/cell) vs LD ($7 \cdot 10^{-15}$ mol/cell).

To sum up, HD cells suffer a deprivation of carbon sources that produces a drop in the level of cellular ATP. I wondered whether these aspects could be related to the switch in the splicing profile of *SRSF1* transcripts. To explore this possibility I took advantage of a previous observation made in our laboratory concerning the effect of pre-conditioned medium (CM) from HD cells (Valacca). Indeed when LD cells are grown for 18 h in the presence of CM the splicing profile of *SRSF1* transcripts becomes similar to that observed at medium density (Figure 32) with a drop in the level of Isoform I and an increase in other splicing products but without a prevalence of Isoform III. In other word CM activates splicing of intron 5 but fails to induce retention of intron 3, which is the hallmark of HD cells. We have previously suggested that growth factors in the CM can direct the partial reprogramming in the splicing profile of *SRSF1* transcripts. It is plausible that LD cells fail to acquire a complete HD profile simply because they have no enough carbon sources (and hence ATP). To explore this possibility, I analyzed RNAs from LD cells grown in CM, supplemented with either glucose or glutamine. Surprisingly we noticed a complete different effect of glucose and glutamine. As shown in Figure 32, glucose partially reverts the effect of CM and promotes the production of Isoform I. On the contrary glutamine induces the massive production of Isoform III. This result points to critical role of glutamine metabolism in this splicing switch.

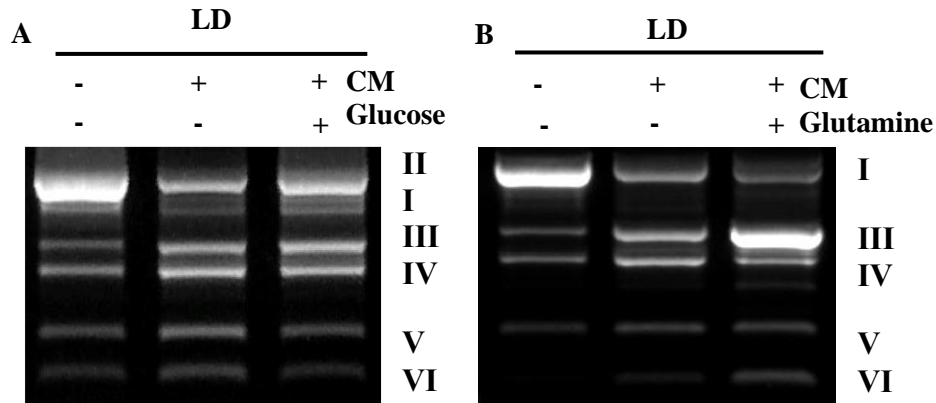


Figure 32. RT-PCR analysis of total RNAs extracted from LD SW480 cells untreated or grown in pre-conditioned medium (CM) from High-density SW480 cells, supplemented with glucose 2g/L **A**) or glutamine 2mM **B**).

4.7 Role of the TCA cycle intermediary metabolite in the hypoxia pathway

The essential role of glutamine for the splicing profile switch from LD to HD cells, prompted us to investigate the connection between glutamine, the tricarboxylic acid (TCA) cycle and HIF1 activation. Indeed, through anaplerotic reactions glutamine is converted first to glutamate by glutaminase and then deaminated to produce α -ketoglutarate (α -KG, also called oxoglutarate), an intermediate of the TCA cycle (Figure 33).

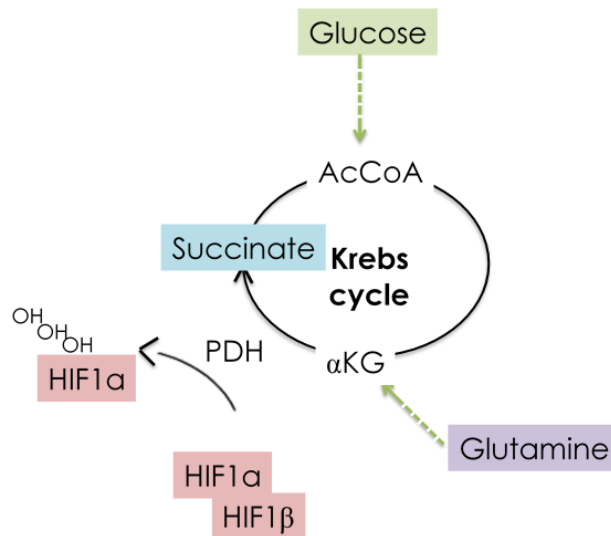


Figure 33 Role of the TCA cycle intermediary metabolite α -ketoglutarate and succinate in the hypoxia inducible pathway. Glutamine is converted into α -ketoglutarate. Succinate inhibits the activity of HIF-prolyl hydroxylase enzyme (PHD), which tags HIF-1 α for degradation by VHL and the ubiquitin pathway. Accumulated HIF-1 α dimerizes with HIF-1 β and translocates to the nucleus to upregulate hypoxia-responsive genes.

This may induce Hif1-alpha stabilization through a process known as pseudo-hypoxia. The level of HIF-1 α is controlled at the post-translational level via hydroxylation and successive ubiquitination that direct protein degradation by the proteasome pathway. Crucial for this process is a group of enzymes called prolyl-4-hydroxylases (PHDs) or HIF-1 prolyl hydroxylases (HPH), which belong to a large family of 2-oxoglutarate-dependent dioxygenases (2-OGDDs). These enzymes share the same basic reaction mechanism, in which the substrate is hydroxylated by molecular oxygen in the presence of a

divalent metal cofactor (most commonly Fe^{2+}). In this reaction the α -KG co-substrate is decarboxylated to succinate and CO_2 (Chowdhury *et al.* 2011; Xu *et al.* 2011). Succinate has been shown to inhibit several 2-OGDDs competitively with respect to α -KG. Thus, a higher succinate/ α -KG ratio stabilizes the HIF α -subunit leading to pseudo-hypoxia. (O’Flaherty *et al.*, 2010; Selak *et al.*, 2005). While these observations clearly place the TCA cycle upstream of the hypoxia response, it is worth underlying that one of the consequences of the hypoxia/pseudo-hypoxia response is a decreased supply of pyruvate flow to the TCA cycle and an increase in glutamine catabolism that feeds the α -ketoglutarate pool (Vander Heiden *et al.*, 2009). The TCA cycle and the hypoxia response therefore form a complex regulatory network in which each of the pathways reciprocally affects the other.

To understand whether the splicing profile of *SRSF1* transcripts could be modulated through a similar mechanism, we treated HD cells with the α -KG for 18 hr. As shown in the Figure 34, α KG promotes a splicing profile comparable to that observed in LD cells. In agreement with the fact that α -KG activates OGDD enzymes, including PHD, we observed a functional inhibition of HIF1 activity measured as VEGF-A down-regulation, a HIF1 target whose expression increases in response to cell density. Interestingly, α -KG treatment also inhibits E-cadherin expression, which is normally up-regulated in HD cells. Although the mechanism through which the expression of the E-cadherin gene is controlled by cell density is still poorly understood, collectively these results indicate that in the presence of α -KG HD cells become more similar to cells grown at low density

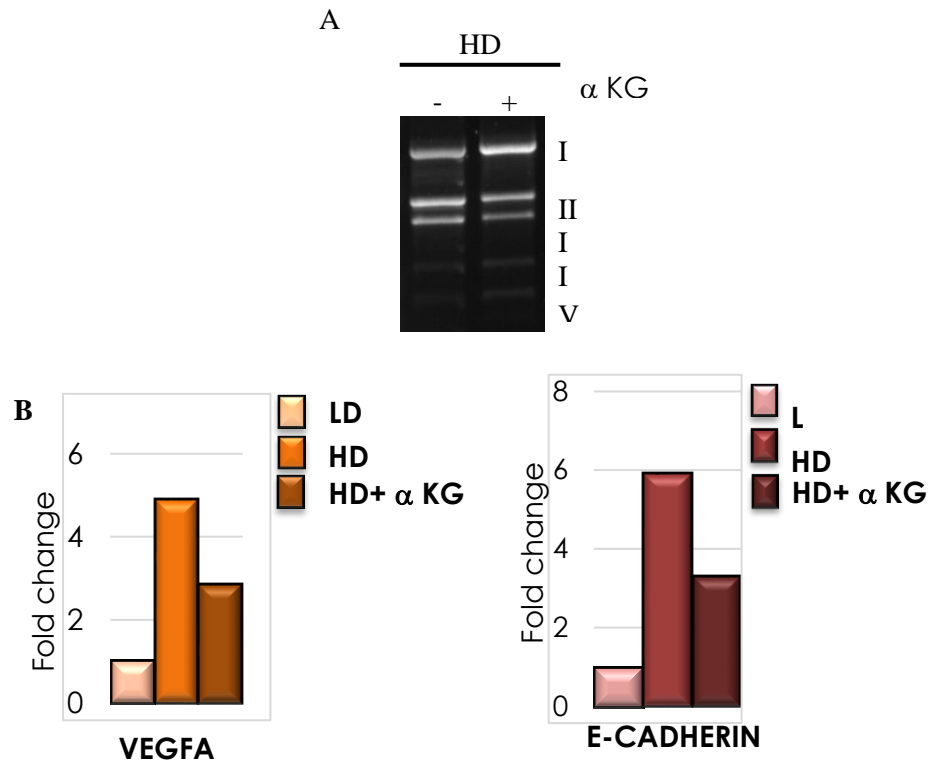


Figure 34. To understand whether α KG is involved in modulating the splicing profile of *SRSF1* transcripts, HD cells were treated with 0,6 mM α KG. **A)** RT-PCR and **B)** qPCR analysis of total RNAs indicate that α KG treatment promotes a switch in the splicing profile of *SRSF1* transcripts, decreases VEGFA and E-cadherin expression to level similar to those observed in LD cells.

To verify whether the α -KG effect is mediated by OGDD activities we performed the reciprocal experiment in which LD cells were grown in the presence of succinate, which is both a substrate for SDH in the mitochondria and a product of PHDs in the cytosol. High levels of succinate antagonize α -

KG, inhibit OGDDs including PHD and lead to HIF α stabilization and activation of the HIF complex. We use a dimethyl-succinate that thanks its methyl-groups is cell membrane permeable and is then converted to succinate within the cell through a still poorly defined mechanism.

As a preliminary experiment, we treated cells with increasing concentrations of succinate. 24 hours after seeding the cells at low density, succinate was added to the medium and cells were collected 18 hours later. We investigated by RT-PCR the effect of this treatment on the splicing profile of *SRSF1* transcripts (Figure 35).

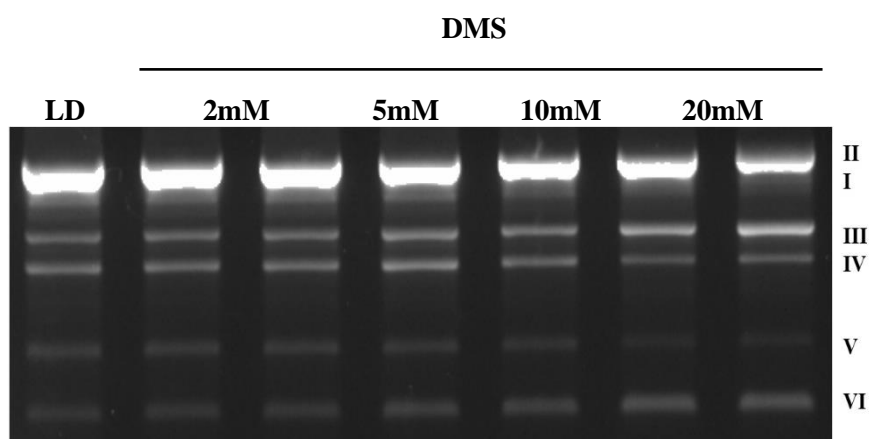


Figure 35 *SRSF1* splicing profile in LD SW480 cells treated with increasing concentrations of dimethyl succinate.

As shown in Figure 35, the splicing profile of treated cells progressively changed toward a pattern similar to that observed in HD cells in which the level of Isoform I declines while with abundance of Isoforms III and VI increases. This observation indicates the ability of succinate to drive a switch typical of high density cells.

Moreover, DMS up-regulates VEGF-A and E-cadherin genes expression in LD cells (Figure 36). Thus, DMS and α -KG have opposite effects on cell behavior. While treatment of HD cells with α -KG induces a behavior similar to that observed in LD cells, DMS promotes the acquisition of HD features by LD cells.

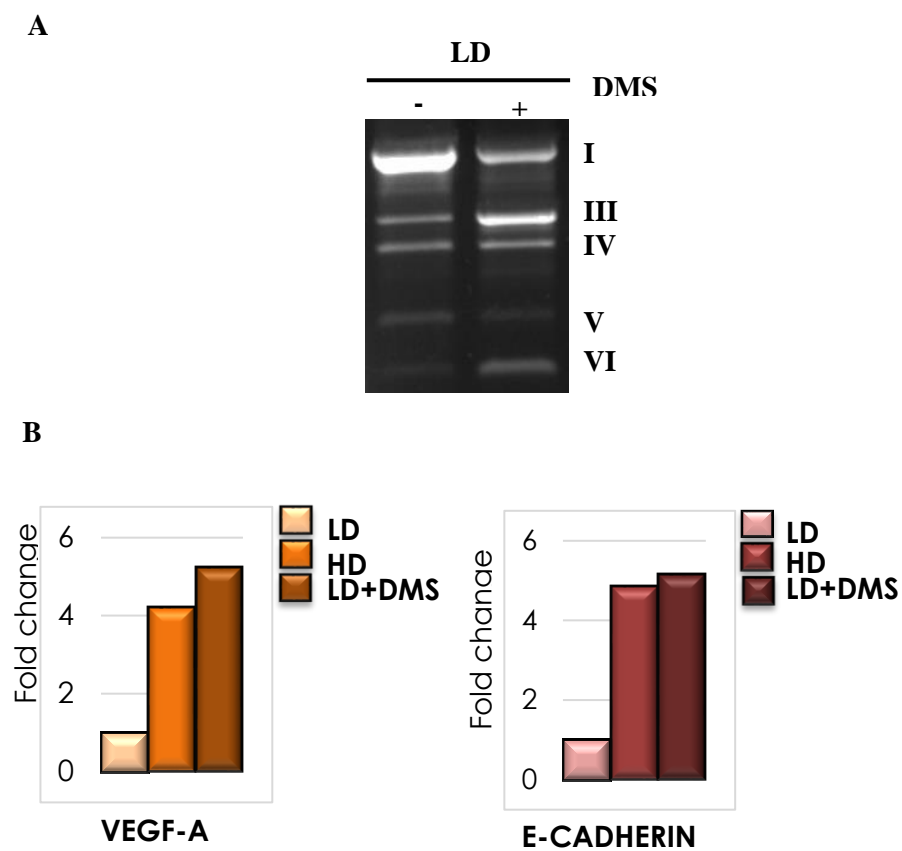


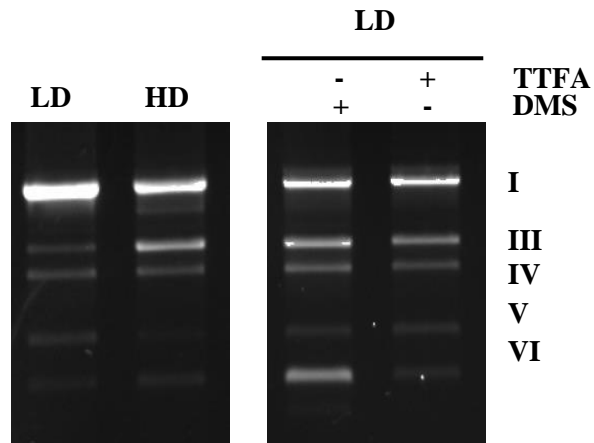
Figure 36. Succinate is one intermediate of the Krebs cycle that promotes stabilization of HIF-1 α . To understand whether this compound modulates the splicing profile of *SRSF1* transcripts LD were treated or not with 60 mM dimethyl succinate (DMS) for 18h). **A)** RT-PCR analysis of total RNAs indicate that DMS treatment promotes a splicing profile similar to that detected in HD cells, **B)** q-PCR

analysis indicates that DMS treatment increases VEGF-A and E-cadherin expression to a level comparable to that observed in HD cells.

4.8 Complex II inhibition

In the hypothesis that Succinate can modulate the splicing profile of *SRSF1* transcripts one should expect to observe the same effect upon inhibition of the succinate dehydrogenase (Sdh) complex or respiratory Complex II that catalyzes the oxidation of succinate to fumarate with the reduction of ubiquinone to ubiquinol. Indeed, Complex II dysfunction leading to accumulation of succinate has been proved to cause HIF-1 α stabilization (Majmundar *et al.*, 2010). Decreased Sdh activity should cause an accumulation of succinate that can transit to the cytosol through the dicarboxylate carrier. In the cytoplasm, succinate potentially can inhibit PHD and cause HIF- α stabilization in a process known as a pseudo-hypoxia (Pollard *et al.*, 2005). To verify this prediction I treated the cells with the Complex II inhibitor Thenoyltrifluoroacetone (TTFA). As shown in the Figure 37, upon treatment with TTFA the splicing profile of *SRSF1* transcripts in LD cells becomes similar to that of LD cells incubated in the presence of DMS.

A



B

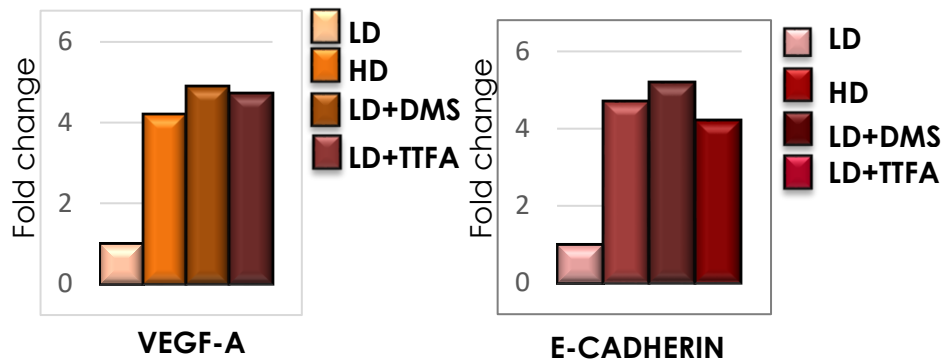


Figure 37. A) RT-PCR analysis of total RNAs indicate that TTFa treatment promotes a switch in the splicing profile similar to cell density B) q-PCR analysis indicates an increase in VEGF-A and E-cadherin expression similar to that produced by cell density or by DMS.

4.9 JMJD6: a new regulator for srsf1 splicing?

Our data indicate that succinate promotes the production of Isoform III. At the same time succinate it is known to stabilize HIF1 α via pseudo-hypoxia. On the other hand, HIF-1 β down-regulation impacts splicing of intron 5 without affecting the production of Isoform III, i.e. retention of intron 3. Thus, we wondered whether the effect of high density and succinate on the splicing profile of *SRSF1* transcripts could be mediated by some other factor. The family of OGDDs controlled by α -KG and succinate includes Jmjd6, an enzyme recently shown to control RNA metabolism, RNA processing and RNA splicing. The expression profile of JMJD6 has been investigated by several groups in different types of cancers (Poulard *et al.*, 2016). Using tissue microarrays, Wang and colleagues found that JMJD6 is upregulated in all types of carcinoma including breast, liver, lung, renal, pancreatic, colon, esophageal, rectal and gastric cancers (Wang *et al.*, 2014). However, the highest expression was recorded in colon adenocarcinoma, and a negative correlation exists between the level of JMJD6 expression and survival time (Wang *et al.*, 2014).

Jmjd6, a jumonji C (JmjC) domain-containing protein demethylase and hydroxylase, can catalyze protein demethylation through an oxidative mechanism requiring Fe (II) and 2-oxoglutarate (2OG) as cofactors. A bioinformatics analysis of jmjd6 protein in *Hydra vulgaris* led to the identification of three nuclear localization signals (NLS), a JmjC domain and a DNA binding domain (AT-hook domain) (Figure 38A). In silico analysis also uncovered a high level of homology with FIH-1 protein (factor inhibiting HIF), which possesses a 2OG-and Fe (II)-dependent oxygenase activity (Cikala *et al.*, 2004), suggesting that jmjd6 could display a similar oxidative

enzymatic activity (Figure 38B) and a function in the cellular response to hypoxia.

In addition, it has been shown that *jmjd6* interacts with and hydroxylates multiple serine/arginine-rich (SR) proteins and SR related proteins, all of which are known to function in splicing regulation. Different groups then reported the role of *jmjd6* in the regulation of RNA splicing (Boeckel *et al.*, 2011, Heim *et al.*, 2014, Palmer *et al.*, 2016). *Jmjd6* protein has been recently shown to interact with splicing factor U2AF65; however, very few splicing events in a limited number of genes were attributable to JMJD6 expression (Webby *et al.*, 2009). In endothelial cells, alternative splicing of VEGF receptor (Flt1) by U2AF65 promoted endothelial cell migration, and siRNA-mediated knockdown of JMJD6 in endothelial cells led to decreased migration (Boeckel *et al.*, 2011). Based on X-ray crystallographic data, it was predicted and shown that apart from its enzymatic activity, *jmjd6* protein can bind single-stranded RNA (Hong *et al.*, 2010). These diverse findings predict a range of versatile functions for *jmjd6*, at the transcriptional, splicing, post-transcriptional, and biochemical levels, impacting embryonic development (Bose *et al.*, 2004; Kunisaki *et al.*, 2004, Lee *et al.*, 2012), cell cycle progression (Wang *et al.*, 2014), cellular proliferation and motility (Lee *et al.*, 2012; Chen *et al.*, 2014) and development of various types of cancers. However, very little is known about the role of JMJD6 in cancer and the molecular pathways that may impinge on disease initiation and prognosis.

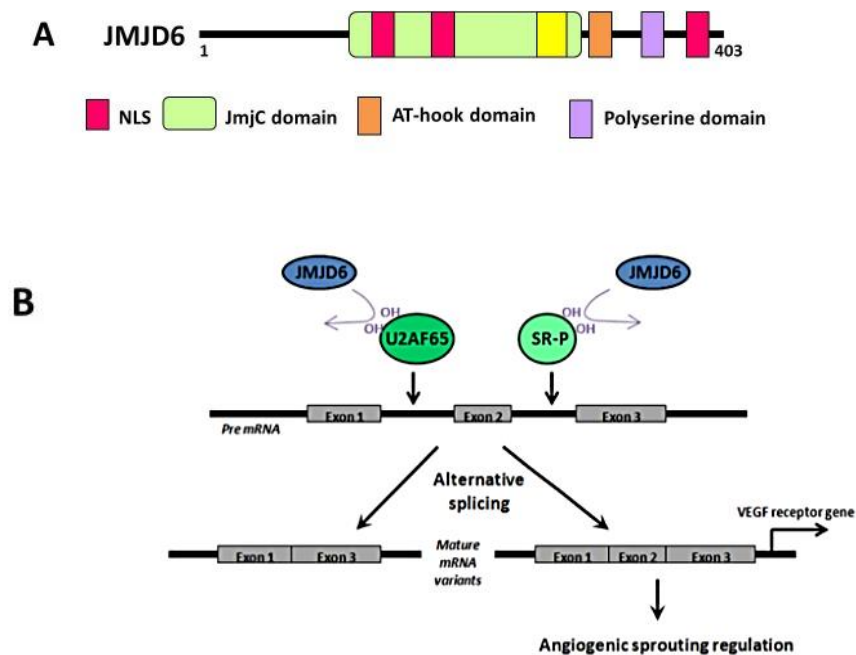


Figure 38. A) Schematic representation of *jmjd6* structure highlighting the different functional domains of the protein. NLS: Nuclear localization signal, AT-hook domain: DNA binding domain. B) Pre-mRNA splicing regulation. Lysine hydroxylation of U2AF65 (K15, K276) and SR-like proteins (SR-P) by *jmjd6* regulates alternative splicing. For example, VEGFR receptor gene splicing is regulated by U2AF65 hydroxylation (Poulard *et al.*, 2016).

We decided to test whether *jmjd6* could have a role in controlling the splicing profile of *SRSF1* transcripts. To this, we knocked down JMJD6 by small interference RNA (siRNA). JMJD6 down-regulation drastically impacts the splicing profile of *SRSF1* transcripts leading to a reduction in the level of the protein-coding Isoform I with the concomitant an increase in Isoform VI produced through skipping of the last coding exon (exon 4). Unexpectedly, silencing of JMJD6 also leads to the appearance of an extra band (Figure

39A). Sequence analysis proved that this band corresponds to a transcript that retains intron 5 and lacks the fourth exon. As shown in the scheme this molecule could be a precursor of Isoform VI, thus accounting for the higher level of this Isoform upon JMJD6 down-regulation (figure 39B). This result clearly indicates an involvement of *jmjd6* in regulating the splicing profile of SRSF1 transcripts, specifically by preventing skipping of exon 4 and inhibiting splicing of intron 4.

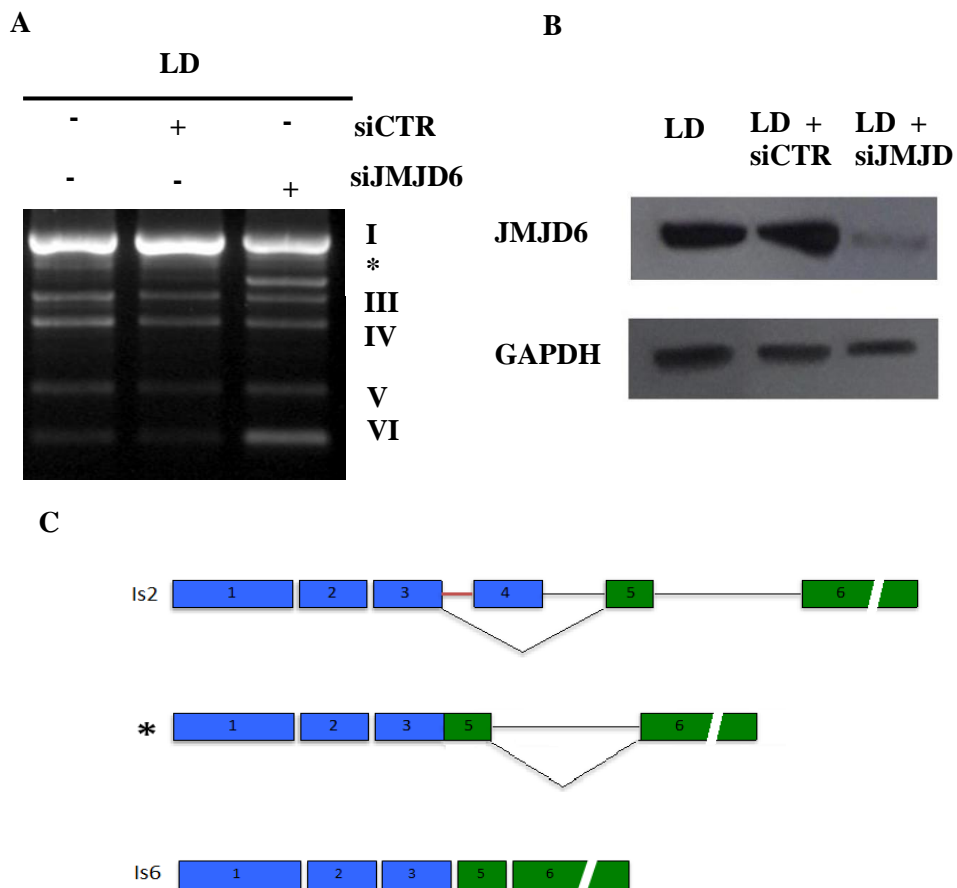


Figure 39. A) Silencing JMJD6 and the effect on the splicing profile SRSF1 transcripts. RT-PCR analysis of SRSF1 transcripts. B) Western blotting analysis of *jmjd6* protein in LD cells, in cells transfected with a control siRNA or with siRNA

specific for JMJD6. GAPDH was used as a loading control C) The star Isoform (*) corresponds to a molecule obtained through skipping of exon 4 but still containing intron 5. Through splicing of intron 5 Isoform star generates Isoform VI.

In order to characterize more in detail the property of Isoform III and understand if it may contribute to cell physiology we decided to force its production. In collaboration with Vezzoni's group, we applied the CRISPR-Cas9 approach to edit the splicing acceptor site of intron 3 of the *SRSF1* gene. Vezzoni's group targeted a genomic sequence within intron 3 of the *SRSF1* locus with the following target sequence: 5'GAAAGTTCTGTCTATCCAATAGG-3' where the underlined AG is the 3'-ss of the intron. They selected a clone, called Clone2, which harbors a 32 bp deletion at the 3' end of intron 3, which also removes the 3' splice site of this intron. Notably, Clone 2 cells have 3 copies of the *SRSF1* gene, only one of which mutated. None out of 20 analyzed clones had more than one mutated copy of the gene, as if this condition were not compatible with cell proliferation. On the other hand, because of this arrangement (two gene copies wild type and 1 mutated) we expected to observe only a partial modification of the *SRSF1* splicing profile. Indeed, the overall picture appears similar to that of wild type cells and Clone 2 cells conserve the splicing switch driven by cell density (Figure 40), with Isoform I being the main products in LD cells and Isoform III prevalent in HD cells. However, a new Isoform (Isoform *) is detectable in Clone 2. The relative abundance of Isoforms I and * in LD cells is likely to reflect the fact that only one gene out of three is mutated. Sequence analysis showed that Isoform * in Clone 2 is identical to Isoform* detectable after JMJD6 down-regulation, i.e. a molecule produced through skipping of exon 4 and maintaining intron 5 (see Figure 40). Thus,

mutation of the 3' splice site of intron 3 and down-regulation of JMJD6 have the same effect on splicing of SRSF1 transcripts. This result is compatible with a model whereby *jmjd6* may have a major role in controlling the usage of the 3' splice site of the intron 3.

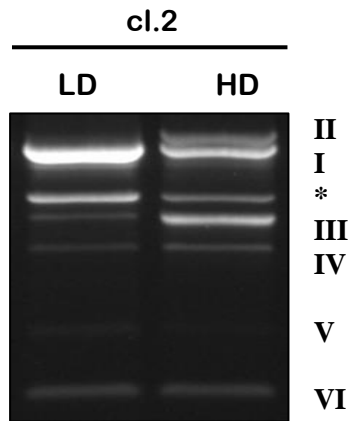


Figure 40. RT-PCR analysis of the splicing profile of *SRSF1* transcripts in Clone 2 cells at low and high density

5 Discussion

The early steps in carcinoma metastases involves a highly conserved developmental program called epithelial-to-mesenchymal transition (EMT). Crucial to the metastatic process is the reversibility of EMT, namely the mesenchymal-to-epithelial transition (MET) that occurs at the final metastatic sites (Thiery JP 2002). Tumor microenvironment appears to have a crucial role in altering tumor cell behavior by affecting gene expression programs. In particular, a role for post-transcriptional regulation of EMT is increasingly evident (Brown *et al.*, 2011; Ghigna *et al.*, 2008).

We have used cell density as a model to mimic the EMT process. Colon adenocarcinoma SW480 cells are seeded at Low density (LD) to mimic the mesenchymal cells at the metastasizing front of the carcinoma, with protruding lamellipodia and low level of E-cadherin accompanied by nuclear accumulation of β -catenin, and at High density (HD) to simulate the epithelial cells at the center of the tumoral mass, a condition associated with upregulation and a membranous localization of E-cadherin.

The general notion is that cells at the core of solid tumors experience hypoxia and nutrient end energetic stress (Majmundar *et al.*, 2010)

Crucial for the ability of cancer cells to mount an adaptive response to hypoxic stress is transcription factor HIF1. HIF1 is a heterodimeric transcription factor with HIF1 α as the O₂ responsive subunit, and HIF1 β (also known as ARnT) being constitutively expressed. Previous bioinformatics analysis performed in our laboratory revealed that the whole set of genes regulated by HIF1 is activated in response to high density, indicating that HD cells actually suffer a sort of hypoxia although growing under normal oxygen tension.

Bioinformatics analysis also suggests that biosynthetic pathways are inhibited by high cell density. This is consistent, for instance, with the observation that the fraction of replicating cells drops in parallel with cell density, even though replicating SW480 cells can be detected even at the highest tested density. Collectively, changes in gene expression profiles are compatible with the idea that HD cells, similarly to cells in the central mass of the tumor, are exposed to a stressing inhibitory environment that affects their proliferation and anabolic pathways. High glucose uptake benefits the cancer cells both in bioenergetics and biosynthesis. In addition to glucose, increased glutamine uptake is another tumor-specific metabolic feature. A consequence of the switch from aerobic respiration to glycolysis is the drop in the ATP level. This is in agreement with the drop (4,4 fold) in the ATP level in HD cells vs LD cells.

Interestingly, metabolic stress produced by cell density is accompanied by a change in the splicing program of *SRSF1* transcripts (Figure 23B). Whereas the protein-coding Isoform I prevails in highly proliferating cells, the abundance of the remaining non-coding and NMD sensitive isoforms progressively increases with cell density and nuclear Isoform III becomes the most abundant *SRSF1* transcript in HD cells.

Three major questions stem from this splicing reprogramming.

The first one concerns the function of this alternative splicing program by which a protein-coding gene produces long non-coding RNAs (lncRNAs).

The SRSF1 protein is encoded by Isoform I that is mainly cytoplasmic and contains the entire 3'UTR. Two splicing events in the 3'UTR generate Isoform IV and V: Isoform IV is produced through splicing of intron 5, the last of the gene, while splicing of introns 4 and 5 is required to produce Isoform V. Both molecules, particularly Isoform IV, are mainly nuclear.

Since the 5' ss of intron 4 is immediately upstream of the natural stop codon, Isoform V can potentially encode a protein that differs from SRSF1 for the last amino acids. However, this RNA molecule is a target of the NMD pathway due to the fact that its STOP codon falls in the penultimate exon. The remaining three Isoforms (II, III and VI) are generated through alternative splicing events that involve the last intron in the protein-coding part of the gene, i.e. intron 3. Isoforms II and III are exclusively nuclear and differ from Isoforms I and IV for retention of intron 3. Isoform VI is produced through skipping of exon 4, is exported to the cytoplasm and, similarly to isoform V, is degraded by the NMD pathway. This isoform can potentially encode a protein lacking the entire SR domain, coded by exon 4, which is involved in protein-protein interaction. However, there are no evidences so far about the expression of this form of SRSF1 protein deleted of the SR domain. Based on a number of considerations we propose that the production of the six molecules involves two independent splicing programs, as schematically depicted in Figure 28. The first one determines the production of Isoforms I, IV and V while the other generates Isoforms II, III and VI. The discrimination between the two programs is splicing of intron 3. The observation that this complex program is conserved in evolution from mouse (NIH-3T3 cells) to human seems to suggest a function for all these non-coding RNAs. However, only hypothesis can be presently raised. For instance, one can speculate about a role of nuclear *SRSF1* lncRNAs. They may sequester RNA binding proteins in this way modulating alternative splicing of other genes. Alternatively, retention of intron 4 and 3 may be required to establish a pool of nuclear molecules matured to Isoform V and VI on demand. However, the last two molecules appear to be degraded by the NMD pathway and their coding capacity is questionable. Whatever their

function, these splicing products are detectable in all human tissues, although their relative abundance shows tissue specific differences.

The second main question concerns the reason why this splicing program is modulated. This may be linked to the possible function of the five non-coding molecules that, as stated above is still hypothetical. Another possibility is that this complex splicing program is only a mechanism to control the level of protein-coding Isoform I. As a matter of fact, SRSF1 protein has been suggested to modulate splicing of its own gene transcripts by producing a sort of feedback control mechanism in which high levels of SRSF1 protein induce the expression of *SRSF1* lncRNAs (Gonzalez *et al.*, 2015). Moreover, phosphorylation of Sam68 by ERK appear to control splicing of intron 5 and hence the level of Isoforms III to VI (Valacca *et al.*, 2010). However, the observation that the reduction of isoform I with the concomitant production of alternative splicing products only modestly influences SRSF1 protein level (Valacca *et al.*, 2010) seems to argue against this possibility.

Interestingly, we have observed that the splicing profile of SRSF1 transcripts is modulated in response to cell density. Protein-coding Isoform I prevails in sparse low density cells with a mesenchymal morphology and gene expression profile, while nuclear Isoform III is the most abundant species in epithelial-like high density cells. Whether or not this nuclear transcript has a function of its own or simply represents a reservoir for the ensuing production of Isoform VI is still matter of investigation. According to a recent work by Phil Sharp (Boutz PL *et al.*, 2015), detained intron, as for instance intron 3, would identify stable nuclear RNAs, as Isoform III, that are processed on demand to meet the cell needs. Interestingly enough, when HD cells are

diluted back to LD the level of Isoform III very rapidly drops with the appearance of isoform VI.

The third question concerns the mechanisms and signals controlling alternative splicing of SRSF1 transcripts, which is the main subject of my thesis. As stated above the splicing profile of *SRSF1* transcripts is modulated in response to cell density. Interestingly HD cells suffer metabolic and energetic stress due to exhaustion of carbon sources (glucose and glutamine) and activate the hypoxic response as indicated by the stabilization of HIF1 α and the up-regulated expression of the whole set of genes controlled by HIF1 (Table 1). Numerous data in my thesis indicate that this stressing condition directly impacts the *SRSF1* splicing program at several levels. First of all, I have found that down-regulation of HIF1 β is accompanied by a reduction in the relative abundance of isoforms III and IV with the contemporary increase in isoforms I and II (Figure 30). Although the molecular details are still to be clarified, the experiment in Figure 30 suggests that HIF1 activity in HD cells is required to promote splicing of intron 5.

The most relevant part of my work concerns the effect of glutamine metabolism and pseudo-hypoxia on splicing of intron 3 and thus in the production of isoforms III and VI.

When LD cells are grown in pre-conditioned medium (CM) a partial reprogramming of the splicing profile is detectable. However, when CM is supplemented with glutamine, but not with glucose, as carbon source a complete switch occurs with the massive production of Isoform III

Glutamine appears to be a crucial player in our experimental system. In addition to affect the splicing profile of *SRSF1* transcripts it is also connected to the tricarboxylic acid cycle and to HIF1 activation.

Indeed, through anaplerotic reactions glutamine is converted in α - ketoglutarate (α -KG, also called oxo-glutarate), an intermediate of the TCA cycle. This may induce HIF1- α stabilization through a process known as pseudo-hypoxia. HIF-1 α protein is controlled at the post-translational level via hydroxylation and successive ubiquitination that direct its degradation by the proteasome pathway. Crucial for this process is a group of enzymes called prolyl-4-hydroxylases (PHDs) or HIF-1 prolyl hydroxylases (HPH), which belong to a large family of 2-oxoglutarate-dependent dioxygenases (2-OGDDs). These enzymes share the same basic reaction mechanism, in which the substrate is hydroxylated by molecular oxygen in the presence of a divalent metal cofactor (most commonly Fe^{++}). In this reaction the α -KG co-substrate is decarboxylated to succinate and CO_2 (Chowdhury *et al.* 2011; Xu *et al.* 2011). The product of this reaction, namely the succinate molecule, has been shown to inhibit several 2-OGDDs competitively with respect to α -KG. Thus, a high succinate/ α -KG ratio stabilizes the HIF α -subunit leading to pseudo-hypoxia. (O'Flaherty *et al.*, 2010; Selak *et al.*, 2005).

The family of OGDDs controlled by α -KG and succinate includes JMJD6, an enzyme recently shown to control RNA metabolism, RNA processing and RNA splicing.

My hypothesis that ODGGs play a role in controlling splicing of intron 3 is supported by several experimental data:

- 1) treatment of HD cells with α -KG, by promoting the activity of OGDDs, induces a splicing profile more similar to that observed in LD cells. Moreover this treatment reduces the expression of VEGF-A, a HIF1 target whose activity increases in response to cell density. Interestingly, α -KG treatment also inhibits E- cadherin expression, which is normally up-regulated in HD cells (Figure 34).

2) treatment of LD cells with succinate inhibits PHD leading to HIF α stabilization and VEGF-A expression. Moreover, it produces a splicing profile of *SRSF1* transcripts similar to that observed in HD cells characterized by a drop in the level of Isoform I and by an increase of Isoforms III and VI. This observation indicates the ability of succinate to drive a splicing program typical of high density cells. Finally, succinate induces the up-regulation of the E-cadherin gene (Figure 36).

3) Inhibition of mitochondrial SDH activity by the complex II inhibitor Thenoyltrifluoroacetone (TTFA) in LD cells promotes a splicing profile of *SRSF1* transcripts similar to that observed in HD cells. Inhibition of SDH activity is known to result in succinate accumulation that can transit from mitochondria to the cytosol through the dicarboxylate carrier SLC25A10. Treatment of LD cells with TTFA makes the splicing profile of *SRSF1* transcripts similar to that of LD cells incubated in the presence of succinate and produces the increased expression of VEGF-A and E-cadherin genes (Figure 37).

The activation of the hypoxia response is a common feature of tumors. OGDD activities appear to have a pivotal role in this phenomenon. The ODGG family includes several members that share the same basic reaction mechanism, in which the substrate is hydroxylated by molecular oxygen in the presence of a divalent metal cofactor (most commonly Fe⁺) and the 2-oxoglutarate (2-OG) co-substrate is decarboxylated to succinate and CO₂. These enzymes control gene expression by modulating CpG and histone demethylation. Interestingly, one member of this family is JMJD6 protein Lysil- Arginine demethylase and Lysyl hydroxylase. JMJD6 is homologous to the hypoxia-inducible factor (HIF) asparaginyl-hydroxylase, suggesting a function in cellular response to hypoxia. In addition, it has been shown that JMJD6 interacts with and hydroxylates multiple serine/arginine-rich (SR)

proteins and SR related proteins, including U2AF65, all of which are known to function in alternative splicing regulation. However, whether JMJD6 is widely involved in alternative splicing and the molecular mechanism underlying JMJD6-regulated alternative splicing events are still incompletely understood. (Boeckel *et al.*, 2011, Heim *et al.*, 2014, Palmer *et al.*, 2016).

I found that *JMJD6* down-regulation deeply affects splicing of *SRSF1* transcripts in LD cells by promoting skipping of exon 4 and the appearance of a new splicing product (Isoform *) that lacks exon 4 and retains intron 5. Although this result clearly implicates, JMJD6 in the regulation of intron 3 splicing in sparse low-density cells, the splicing profile after *JMJD6* down-regulation is different from that observed when JMJD6 activity in LD cells is inhibited by the cell membrane permeable Dimethyl-succinate (DMS). This difference can have several explanations. DMS is known to affect the whole family of 2-OGDD activities, and other members may contribute to splicing regulation of *SRSF1* transcripts. Another possibility is that JMJD6 is part of a multi protein complex that controls splicing of intron 3. In this scenario, when JMJD6 is active (low succinate/ α -KG ratio) the complex efficiently recognizes the 3'ss of intron 3 and promotes inclusion of exon 4. Notably, the list of JMJD6 targets includes a number of RNA binding proteins involved in recognition of 3' splice sites. When JMJD6 is depleted by siRNAs the complex does not form and the 3'ss is no longer recognized. The outcome is skipping of exon 4. On the contrary, when cells are treated with DMS (high succinate/ α -KG ratio), JMJD6 is inhibited. Under this condition, an inactive complex is formed that stalls the splicing reaction. According to the model in Figure 28, the 3'ss of intron 3 would have a major role in controlling the choice between inclusion vs skipping of intron 3 and thus in the production of Isoforms II, III and VI. Interestingly, bioinformatic analysis of the *SRSF1* gene sequence with the Max Entropy Scan (MES) program indicates that this

splice site is extremely weak, a condition frequently associated with regulated splice sites. Moreover, deletion of this splice site by the CRISPR-Cas9 approach (performed in collaboration with Vezzoni's group) results in the production of the same Isoform* detectable upon silencing of *JMJD6*. Interestingly, Isoform * is a possible precursor of Isoform VI, whose level actually increases both after *JMJD6* down-regulation and after mutation of the 3'splice site. We are presently testing by RNA-IP experiment whether *JMJD6* does interact with *SRSF1* transcripts.

Altogether my analysis has unveiled an unexpected impact of intermediates of the Krebs cycle on the splicing profile of *SRSF1* transcripts, opening a new perspective to analysis of splicing programs.

Understanding the effects played by metabolites such as succinate or α -KG on gene expression regulatory networks, including splicing, could provide exciting perspectives for future therapeutic intervention.

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