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When chromatin structure meets *CD6* exon 5 Alternative Splicing: an encounter triggered by T cell activation

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"Há verdadeiramente duas coisas diferentes: saber e crer que se sabe. A ciência consiste em saber; em crer que se sabe reside a ignorância"

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Da Glória V.G., Martins de Araújo M., Leal R., de Almeida S.F., Carmo A.M. and Moreira A. (2014) T cell activation regulates CD6 alternative splicing by transcription dynamics and SRSF1. The Journal of Immunology. doi: 10.4049 (*in press*).

No cumprimento do Decreto-Lei supra mencionado, a autora desta dissertação declara que interveio na concepção e execução do trabalho experimental, na interpretação e discussão dos resultados e na sua redação. Todo o trabalho experimental foi realizado pela autora desta tese de doutoramento, Vânia Raquel Gomes Glória, com a excepção das experiências que envolveram a inibição das vias de sinalização de células T (capítulo II), experiências realizadas por Mafalda Martins de Araújo e as experiências de knockdown dos factores de splicing que foram realizadas por Rafaela Leal.

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ABSTRACT

In high eukaryotes alternative splicing is one of the most important steps of pre-mRNA processing as it allows the existence of a large and complex proteomic spectrum. It is also a valuable strategy for a rapid and efficient adaptation of the immune system to the environment stimuli. Several molecules of the immune system undergo alternative splicing of their pre-mRNA, giving rise to different protein isoforms. The T cell surface glycoprotein CD6 is a modulator of cellular responses and has been implicated in several autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and psoriasis. During antigen presentation, CD6 is targeted to the immunological synapse in a ligand binding-dependent manner, where CD6 domain 3 directly contacts CD166, expressed on the antigen-presenting cell. T cell activation results in the induction of CD6Δd3, an alternatively spliced isoform that lacks the ligand-binding domain and thus no longer localizes at the immunological synapse. In the present work the molecular mechanisms regulating the expression of *CD6Δd3* upon activation of human primary T cells were identified and characterized. By chromatin immunoprecipitation we observed an increase in RNA polymerase II occupancy along the *CD6* gene and augmented *CD6* transcription. We also showed that T cell activation results in transcription-related chromatin modifications, revealed by higher *CD6* acetylation levels. The usage of a histone deacetylase inhibitor to increase acetylation and modulate the chromatin structure caused an increase of exon 5 skipping. By directed mutagenesis we identified a splicing regulatory element in *CD6* intron 4 and we further showed that the splicing factors SRSF1, SRSF3 and hnRNP A1 bind to this regulatory element and modulate exon 5 inclusion. Concomitant with T cell activation-induced exon 5 skipping, we observed a down-regulation of SRSF1 expression and that in activated T cells the SRSF1 recruitment to *CD6* transcript is impaired. The results presented in this thesis show that that upon T cell activation there is an increase in *CD6* transcription and chromatin acetylation and SRSF1 becomes limiting. This causes insufficient SRSF1 recruitment to intron 4 resulting in an increase of *CD6* exon 5 skipping and *CD6Δd3* production. Regulation of *CD6* alternative splicing thus holds several players and represents a clear example of integration between transcription and pre-mRNA processing molecular mechanisms.

SUMÁRIO

Nos eucariotas superiores o splicing alternativo é um dos passos do processamento do pre-mRNA (pre-RNA mensageiro) que resulta na existência de um grande e complexo espectro proteómico. É também uma valiosa estratégia para o sistema imunitário permitindo uma rápida e eficiente adaptação aos estímulos do meio ambiente. O splicing alternativo ocorre em vários transcritos primários percursores de moléculas expressas em células do sistema imunitário, dando origem a diferentes isoformas proteicas. O CD6 é uma glicoproteína expressa na superfície de linfócitos T, modeladora de respostas celulares, implicada em várias doenças autoimunes tais como, esclerose múltipla, artrite reumatoide e psoríase. Durante a apresentação de um antigénio à célula T, o CD6 migra para a sinapse imunológica conectando através do seu terceiro domínio extracelular com o seu ligando CD166, expresso na célula apresentadora de antigénio. A activação da célula T induz a expressão de uma nova isoforma da proteína CD6, originada por splicing alternativo, que não possui o domínio de ligação ao CD166, comprometendo a exclusiva localização do CD6 na sinapse imunológica. No presente trabalho investigamos os mecanismos moleculares que regulam a expressão do *CD6Δd3* após a activação das células T. Em linfócitos T activados, observamos um aumento nos níveis de mRNA do *CD6* e também, por imunoprecipitação da cromatina um aumento da localização da RNA polymerase II no gene *CD6*. Mostramos também que a activação das células T leva a uma alteração da estrutura da cromatina devido a um aumento dos níveis acetilação do gene *CD6*. A modelação da estrutura da cromatina usando um inibidor de deacetilases de histonas causou um aumento da exclusão do exão 5. Por mutagenese direcionada identificamos uma sequência reguladora de splicing localizada numa região conservada no intrão 4 do *CD6*. Mostramos também que os factores de splicing SRSF1, SRSF3 e hnRNPA1 regulam o splicing alternativo do exão 5. Simultaneamente com a activação da célula T observamos a diminuição da expressão do SRSF1 e uma diminuição no recrutamento deste factor para a sequência reguladora existente no intrão 4. Este recrutamento mostrou-se igualmente diminuído após a indução de um aumento de acetilação da cromatina por tratamento com um inibidor de deacetilases. Os resultados presentes nesta tese mostram que após a activação das células T, o SRSF1 torna-se limitante, e a seu recrutamento torna-se insuficiente face ao aumento dos níveis de acetilação da cromatina e do consequente aumento do número de transcritos do gene *CD6*. A Regulação do splicing alternativo do *CD6* integra vários níveis de regulação,

representando assim um exemplo claro de integração entre os mecanismos moleculares de transcrição e processamento do pre-mRNA.

ABBREVIATIONS

Ag	Antigen
AGO1	Argonaute 1
ALCAM	Activated Leukocyte Cell Adhesion Molecule
APC	Antigen Presenting Cell
AS	Alternative Splicing
ATP	Adenosine Triphosphate
BBB	Blood Brain Barrier
BBP	Branchpoint Binding Protein
BP	Branch Point
Brm	Brahma
CD6	Cluster of Differentiation 6
cDNA	Complementary DNA
CHD1	Chromodomain Helicase DNA binding protein 1
ChIP	Chromatin Immunoprecipitation
CNS	Central Nervous System
CsA	Cyclosporine A
DNA	Deoxyribonucleic Acid
DP	Double Positive
Dscam	Down syndrome cell adhesion molecule
EDI	Extra Domain I
EGF	Epidermal Growth Factor
ERK	Extracellular signal-regulated kinase
ESE	Exonic Splicing Enhancer
ESS	Exonic Splicing Silencer
Ets	E26 transformation-specific
FACS	Fluorescence-Activated Cell Sorting
FASTK	Fas-Activated Serine/Threonine Kinase
FBS	Fetal Bovine Serum
FGFR2	Fibroblast Growth Factor Receptor 2
FTS	Farnesylthiosalisylicacid
GSK3	Glycogen Synthase Kinase 3
HDAC	Histone Deacetylase
hnRNP	Heterogeneous nuclear ribonucleoprotein

HP1γ	Heterochromatin Protein 1 *gamma*
ICAM	Intracellular Adhesion Molecule
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IgSF	Immunoglobulin superfamily
IL-2	Interleukin-2
IL-7Rα	Interleukin-7 receptor α-chain
IS	Immunological Synapse
ISE	Intronic Splicing Enhancer
ISS	Intronic Splicing Silencer
ITAM	Immunoreceptor Tyrosine-based Activation Motif
JNK	c-Jun N-terminal kinase
K	Lysine residue
LFA-1	Lymphocyte Function Associated-1
LPS	Lipopolysaccharides
LTA	Lipoteichoic Acid
MAPK	Mitogen-Activated Protein Kinase
MHC	Major Histocompatibility Complex
Mnase	Micrococcal nuclease
mRNA	messenger RNA
NCAM	Neuronal Cell Adhesion Molecule
NLS	Nuclear Localization Signal
NMD	Non-Mediated Decay
PBS	Phosphate Buffer Saline
PBMCs	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PHA	Phytohaemagglutinin
PKC	Protein Kinase C
PMA	Phorbol Myristate Acetate
Pol II	Polymerase II
PPT	Polypyrimidine Tract
PSF	PTB-associated Splicing Factor
qPCR	quantitative Polymerase Chain Reaction
RIP	RNA Immunoprecipitation
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute medium

RT	Reverse Transcription
RUNX	Runt related transcription factor
Sam 68	src-associated in mitosis 68 KDa
SFK	Src Family Kinase
SF1	Splicing Factor 1
siRNA	small interfering RNA
SLE	Systemic Lupus Erythematous
SMAC	Supramolecular Activation Clusters
SNP	Single-Nucleotide Polymorphism
snRNP	small nuclear Ribonucleoproteins
snRNA	small nuclear RNA
SP	Single Positive
SR	Serine/arginine-rich
SRCR	Scavenger receptor cysteine-rich
SRPK1	Serine/Arginine protein Kinase 1
SRSF1	Serine/arginine-rich Splicing Factor 1
SRSF3	Serine/arginine-rich Splicing Factor 3
SWI/SNF	switch/sucrose non fermentable
TCR	T Cell Receptor
TSA	Trichostatin A
U2AF	U2 Auxiliatory Factor
WMN	Wortmannin
WT	Wild Type
YAC	Yeast Artificial Chromosome
3’ ss	3’ splice site
5' ss	5’ splice site

Chapter I

Introduction

INTRODUCTION

1. The Immune System

1.1. Immune System and Immune Response

From a single cell organism to the more complex of the eukaryotes, all organisms have developed anti-pathogen strategies (1). In mammals the immune system is responsible for the ability of the host organism to fight foreign invaders. It includes an array of cells and molecules with specialized roles in defending the host against pathogenic microorganisms and cancer cells. The first lines of defense are the chemical and physical barriers of the organism. These are skin, mucociliary clearance mechanisms, low stomach pH, lysozyme in tears, saliva and other secretions (2). If the pathogen escapes this hostile environment it can be detected by the innate immune system, which is activated to eliminate the foreign body. This type of immunity is not specific to a particular pathogen and depends on germ line-encoded receptors that recognize highly conserved pathogen associated molecular patterns (PAMPs) that are characteristic of microbes such as bacteria, fungi and parasites. These receptors have therefore been termed pattern recognition receptors (PRRs) (3). PRRs can also recognize endogenous molecules released by damaged cells, called damage-associated molecular patterns (DAMPs). DAMPs can be a result of metabolic consequences of infection and inflammation (4).

If the innate immune response is not sufficient to eliminate the invader, the adaptive immune system is then activated. Adaptive immunity (also known as acquired immune response) is mediated by a specialized group of cells, the T and B lymphocytes, also known as T and B cells that respond to the challenge with a high degree of specificity to a diverse range of antigens (Ags). The adaptive immune system has the ability of generating an extremely large lymphocyte receptor repertoire capable of recognizing all potential invading pathogens (5). The T cell receptor (TCR) is generally composed by a heterodimer of α and β chains, however a small subset T cells can have TCR composed by γ and δ chains (6). The B cell receptor (BCR) is composed by two heavy (IgH) and two light (Igλ or Igκ) chains and recognizes soluble or membrane bound antigens (6). Basically, T cells can have cytolytic and helper function and B cells produce antibodies (7).

Finally, the immune system normally responds only to foreign antigens, indicating that it is capable of *self/nonself recognition.* The ability of the immune system to distinguish self from nonself and respond only to nonself molecules is essential, avoiding an inappropriate response to self-molecules – autoimmunity – that can be fatal (8).

1.2. T-cell Activation and Immunological Synapse

When the TCR encounters and interacts with MHC-Ag complex of mature APCs, there is an arrangement of membrane proteins in the area of contact between the two cells, which is a critical event, that leads to T-cell activation (9). TCR engagement initiates innumerous biochemical events such as kinase activation, protein and lipid phosphorylations, phospholipid turn-over, increases of intracellular calcium, among others, ultimately leading to the activation of transcription factors of specific genes within the nucleus (10). This results in a number of different cellular responses such as proliferation, differentiation and secretion of cytokines and growth factors.

The interface between T-cell and APC is called the Immunological Synapse (IS) due to its similarities to neuronal synapses (11).

During the formation of the IS, the T-cell suffers cytoskeleton rearrangements and polarization, and different cell surface receptors and signaling components migrate to the contact area (12). The mature synapse is an organized structure that is composed of two concentric regions having different macromolecular composition. They are called Supra-Molecular Activation Clusters (SMACs), the central area - cSMAC - which is enriched in TCRs, and the peripheral - pSMAC – which contains adhesion molecules, such as, lymphocyte function associated-1 (LFA-1) and cytoskeletal proteins like talin. APC surface components are also integral to these clusters, such that MHC-peptide complexes are found in the cSMAC, whereas ICAM-1, the LFA-1 ligand, is concentrated in the pSMAC (13, 14). Another more peripheral region named distal SMAC - dSMAC – is present, to where the large glycocalyx molecules such as CD43 and CD45 were shown to be localized upon TCR stimulation (14, 15).

1.3. T cell Surface Molecules

Currently there are approximately one hundred different T cell surface molecules, such as immunoreceptors, cytokine and chemokine receptors, as well as adhesion molecules (16). In figure I.1 are represented some of the protein molecules involved in T cell recognition.

T cells can be divided into two populations – Helper T cells, which express CD4, and cytotoxic T cells, which are CD8 positive. The major histocompatibility complex (MHC), a highly polymorphic membrane-bound protein complex that has the capacity to bind almost any peptide, presents the antigen to the T cell receptor. TCRs from helper T cells recognize antigenic peptides, of 12-20 amino acids, loaded on MHC class II molecules expressed at the surface of APCs, such as phagocytes, dendritic and B cells (17).

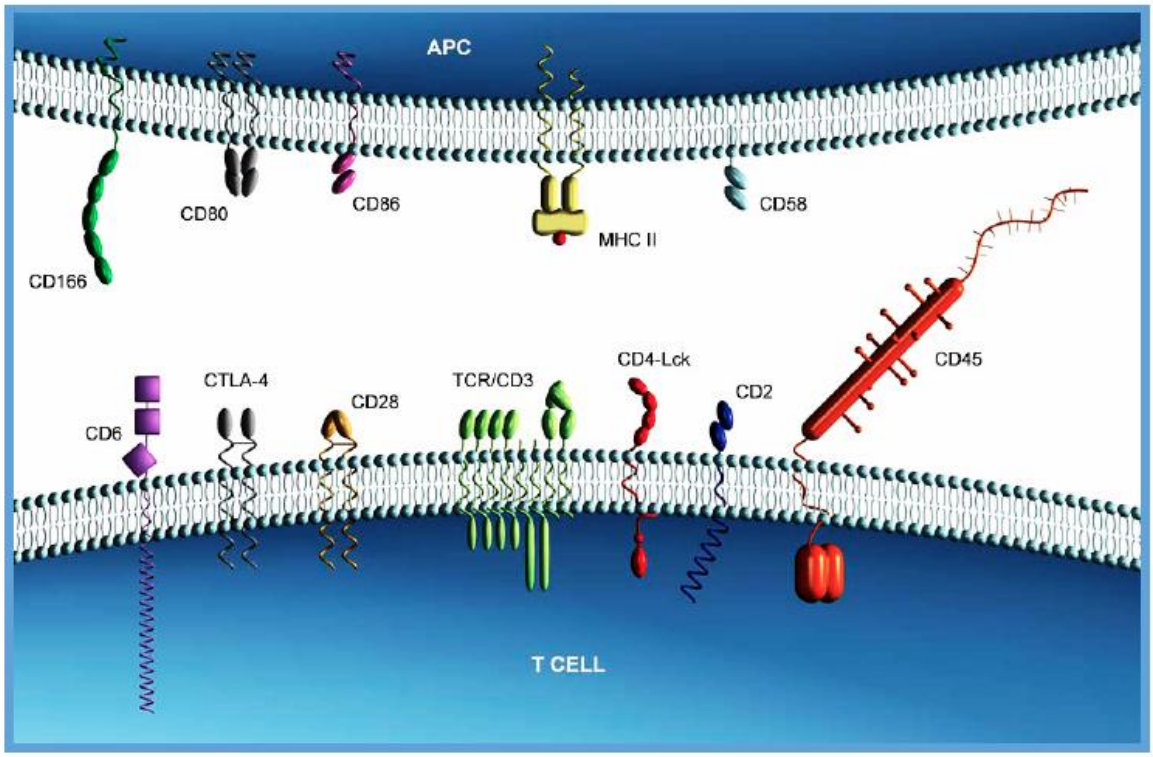

Figure I.1 – Immunological Synapse. Cell surface molecules involved in T cell antigen recognition. A schematic view of the architecture and dimensions of important cell surface proteins involved in T cell antigen recognition. Image adapted from Oliveira M, 2007 (18).

The CD4 positive lymphocyte population, upon antigen recognition, has the function of secreting cytokines and growth factors that lead to the proliferation and differentiation of other leukocytes in order to fight the invader (19). TCRs expressed in cytotoxic T cells recognize 8-9 amino acid-long antigenic peptides embedded in MHC class I molecules, present in the target cell (infected cell or invader cell) surface (17). This population kills the target cell through the production and secretion of granzyme and perforin (20).

It is important to note that T cell surface is not a restricted concept. T cells comprise diverse subsets that, at different activation stages, might display an altered profile due to lineage commitment or activation-induced expression of additional molecules or isoforms. More than a list of proteins that are expressed, it is critical to understand their complex interactions in the unique membrane environment; how they can be functionally regulated and how their interactions contribute to T cell Ag recognition.

1.4. T cell Signaling

The TCR engagement, during antigen recognition, leads firstly to the activation of the tyrosine kinase – Lck that will phosphorylate the ITAM motifs present in CD3ζ subunits. This promotes binding of another tyrosine kinase, ZAP-70 to the phosphorylated ITAMs (21). After ZAP-70 recruitment to the phosphorylated tyrosine residues of CD3ζ subunits, ZAP-70 phosphorylates LAT and SLP-76. The activation of LAT and SLP-76 leads to the phosphorylation of phospholipase Cγ1 (PLCγ1) and Vav1 (22, 23). PLCγ1 together with another intervenient – PI3 kinase (PI3K), catalyze the recruitment of other messengers such as inositol 1,4,5-triphosphate (IP3) and Diacylglycerol (DAG) that lead to cytosolic calcium flux, the initiation of MAPK signaling cascades and the activation of transcription factors that will determine the fate of T cells (24, 25).

1.5. CD6 – The Accessory Receptor

The human CD6 cell surface receptor is a type I glycoprotein of 105-130 kDa expressed on thymocytes, mature T and B1a cells (26), and in brain regions (27). CD6 belongs to the scavenger receptor cysteine-rich (SRCR) superfamily of protein receptors

based on the presence in its extracellular region of three cysteine-rich domains that are characteristic of that family (28). CD5 (29), SSC5D (30) and CD163 (31) are some examples of SRCR members. During thymocyte development, CD6-dependent signals contribute to thymocyte survival and positive selection (32). Nevertheless, the functional role of CD6 has not been definitively established. Whereas it is undeniable that increased expression of CD6 results in repression of T cell receptor (TCR)-mediated signaling (33, 34), direct binding of CD6 with specific antibodies or recombinant ligand may enhance cellular responses, presumably through the induced aggregation of protein kinases associated with the cytoplasmic tail of CD6 (33, 35, 36). Notwithstanding the uncertain nature of CD6, possibly even having a dual role, the fact is that CD6 impacts on cell growth and differentiation, and misregulation of the function of CD6 may result in physiological imbalances and autoimmunity. CD6 has been associated with several autoimmune diseases such as multiple sclerosis (37, 38), rheumatoid arthritis (39), psoriasis (40) and Sjögren's syndrome (41), and has been considered as a possible therapeutic target for some of these pathologies (42).

Additionally, it has been shown that CD6 may sense the presence of pathogen-associated molecular patterns, such as LPS and LTA, present on gram positive and gram-negative bacteria (43).

Human CD6 is encoded by 13 exons. The amino terminal sequence, extracellular region and transmembrane domains are encoded by the first seven exons (1-7), while the cytoplasmic domain is encoded by the remainder six exons (8-13). Each of the three extracellular SRCR domains is encoded by a separate exon. Fluorescence in situ hybridization studies and screening of chromosome-specific YAC (yeast artificial chromosome) library revealed that *CD6* gene is located on chromosome 11 at 11q13 in close proximity to the gene encoding the related molecule CD5 and within 600 Kb of CD20 (44).

CD6 closely resembles CD5, both in structure and expression pattern, and their genes are hypothesized to come from the duplication of a common ancestor. While some information is already available on the transcriptional regulation of CD5 in human (45) and mouse (46), very little is known about the *CD6* gene in any species. It has been described that *CD6* is transcriptionally regulated by RUNX and Ets transcription factors in T cells (47).

The known ligand for CD6 is the IgSF receptor CD166/ALCAM (Activated Leukocyte Cell Adhesion Molecule). CD166 is expressed on conventional antigen presenting cells (APC) and also in thymic epithelia, with the CD6-CD166 interaction being determinant on thymocyte selection (32). CD166 is also expressed in spleen, lymph nodes, tonsil,

digestive tract epithelia, breast, liver, pancreas, kidney, skin and brain (48-50). Interestingly, the interaction between CD6 and CD166 involves the binding of the membrane distal IgSF domain of CD166 to the membrane-proximal SRCR domain 3 of CD6 (49). This binding mode is unusual in that it occurs laterally, rather than in the “head to head” manner commonly seen in most cell surface contacts. Importantly, this interaction is fairly strong and can help strengthening and stabilizing T cell-APC contacts (51).

CD166 is additionally expressed in the epithelial layer of the blood-brain barrier and enables the transmigration of $CD4^+$ T cells into the brain (52). Besides CD166, other molecules from human epithelial cells, with 45 and 90 kDa, have been described to interact with CD6 through CD6-d1 or CD6-d2 (53-55).

On thymocytes and resting mature T cells, CD6 associates with CD5 and with TCR/CD3 complex at the central part of the mature IS (36, 56, 57) and are implicated in CD2 function (58-60) affecting the initial steps of T cell activation. CD6 can interfere in early T cell-APC contacts affecting the maturation of the IS and also T cell proliferative responses (57, 61).

The signaling pathway used by CD6 to influence T and B cell activation and maturation is mostly unknown. CD6 has a long cytoplasmatic region devoid of intrinsic enzymatic activity, but containing several consensus sequences related to signal transduction (62). The usage of CD6 mAb on both normal and leukemic human T cells leads to the activation of MAPK cascades (ERK1/2, p38 and JNK) (63). The cytoplasmic tail of CD6 has been found to directly interact with syntenin-1, an adaptor protein binding to cytoskeletal proteins and signal transduction effectors (64). Moreover, the costimulatory effect of CD6 on T cells upon ALCAM ligation is mediated through phosphorylation dependent binding of the CD6 cytoplasmatic tail to the adaptor protein SLP-76 (33).

The CD6 cell surface levels are tightly regulated during T and B cell development and activation. CD6 expression increases during the transition from immature to mature thymocytes. The level of CD6 expression in thymocytes has been related to the relative avidity of their respective TCR for MHC-antigen complexes and to higher resistance to apoptosis (32).

1.6. CD6Δd3 – A New CD6 Isoform Arised by Alternative Splicing

Castro and collaborators obtained from rat and human thymocytes and peripheral T cells, cDNAs of CD6 omitting exon 5, whose corresponding translated polypeptides, lack the SRCR domain 3 (65). This CD6 isoform, CD6Δd3, was present in all T lineage cells studied and is up-regulated upon T cell activation, paralleling a decline in the expression of full-length CD6. In this study it was shown that this CD6 isoform lacking the extracellular domain 3, could no longer bind its ligand CD166, because the missing domain 3 is exactly the domain involved in this ligation.

It was also observed that while CD6 full-length migrates to the contact area between the two cells during IS formation, CD6Δd3 does not specifically go to the IS but remains in all the cytoplasmatic membrane extension. The unaccomplished binding of CD6Δd3 to CD166 highlights the importance of the third domain in conduce CD6 to the IS, and the exclusion of domain 3 induced by T cell activation reveals a unusual way of positional control of cell surface receptors dependent on alternative pre-mRNA splicing. CD6Δd3 is expressed in 40 % of T lymphocytes, being the dominant isoform in a quarter of this sub-population in rat (65). However, upon CD3 + CD28 stimulation, nearly 90 % of T cells co-express CD6Δd3 together with full-length CD6, implying that a large proportion of CD6 molecules are not capable to remain positioned at the interface with the APC during physiological T cell activation. Double positive thymocytes show a decrease in the proportion of CD6Δd3, whereas in single positive CD4 or CD8 thymocytes CD6Δd3 is expressed in 50 % of the cells.

Analysis of CD6 isoforms at the protein level is made possible in human T cells due to the availability of mAbs against the different SRCR domains. Concordantly with the analysis in the rat system, activation of human T cells induces a large proportion of full-length CD6 protein to be replaced by the CD6Δd3 isoform. As the total amount of CD6 remains roughly constant, this suggests that a significant displacement of CD6 away from the immunological synapse occurs during the course of activation.

The down-modulation of domain 3 of CD6 thus reveals not only a very interesting mode of positional control of cell surface receptors in physiological processes such as cell activation and thymocyte selection, but can also have an impact on the development of pathology. A recent study showed that the multiple sclerosis associated SNP rs17824933^{GG} in intron 1 of *CD6* was associated with an increase of CD6Δd3 and decrease of full-length CD6 expression in T cells, which resulted in a diminished long-term proliferation of CD4+ T cells, suggesting the involvement of CD6Δd3 in the disease (66).

Other CD6 isoforms have also been reported that result from alternative splicing of exons coding for the cytoplasmatic domain (44, 62), but no specific physiological function has been attributed to any of these isoforms.

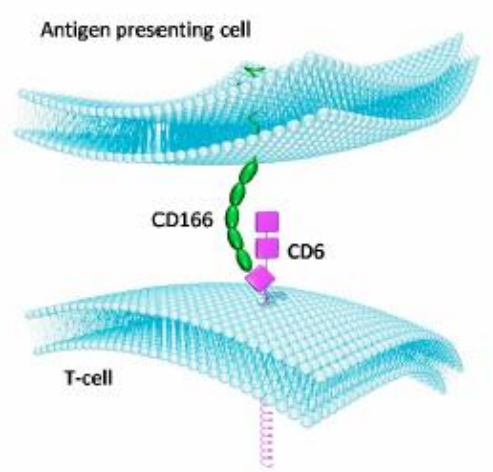

Figure I.2 – The interaction between CD6 and CD166. The 3rd extracellular SRCR domain (membrane proximal) of CD6 protein binds to the 1st IgG-like domain (membrane distal) of its ligand, CD166. Image taken from Oliveira M., 2007 (18).

2. Alternative Splicing

2.1. The Eukaryotic Constitutive Splicing

During mRNA maturation an important step of eukaryotic gene expression known as pre-mRNA splicing occurs. Pre-mRNA splicing consists in the removal of the non-coding regions, introns, from pre-mRNA sequence followed by the joining of the coding regions, exons. This process is carried out by the splicing machinery, the spliceosome – a macromolecular complex composed of five small nuclear ribonucleoprotein particles (snRNPs) assembled from proteins and uridine-rich snRNAs (U snRNAs: U1, U2, U4, U5 and U6), and many other proteins (67, 68). The assembly of the spliceosome on the nascent transcript requires recognition of highly degenerated short sequences present in the pre-mRNA, *cis*-acting elements located within the intron (Figure I.3): at the most 5'-

end is the 5' splice site (5'ss), which includes the conserved GU dinucleotide; and at the 3'-end, the 3' splice site (3'ss) containing the branch-point (BP) adenosine, followed by the polypyrimidine tract (PPT) and the conserved AG dinucleotide (69).

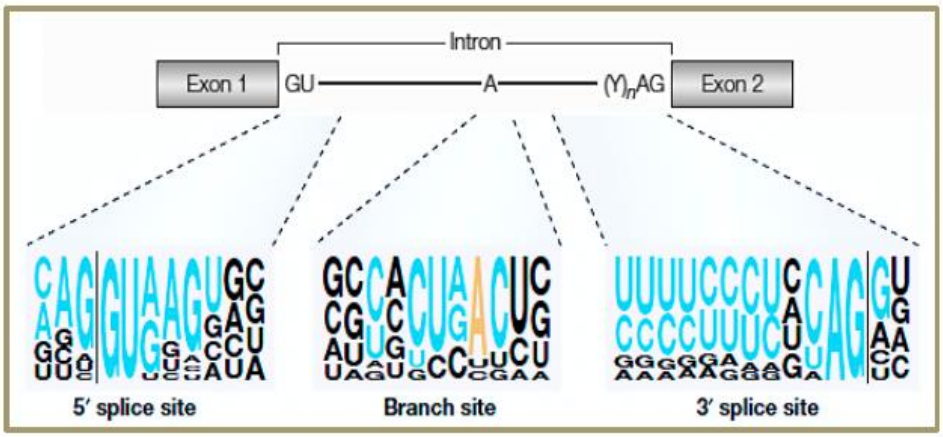

Figure I.3 – Consensus splicing signals. The nearly invariant GU and AG dinucleotides at the ends of the intron, the polypyrimidine tract (Y) before the 3' AG and the branchpoint adenosine residue (A) are represented. Below the represented two exon pre-mRNA are the sequence motifs that surround these conserved consensus nucleotides. For each consensus motif, the size of a nucleotide at a given position is proportional to the frequency of that nucleotide at that position in an alignment of conserved sequences from 1,683 human introns. Nucleotides that are part of the classic consensus motifs are shown in blue, except for the branch-point A, which is shown in yellow (Adapted from Cartegni *et al.,* 2002).

Within the assembled spliceosome, intron excision occurs in two chemical steps, that are two trans esterification reactions: (1) a nucleophilic attack by the 2'OH group of the BP adenosine on the phosphodiester bond at 5' splice site (donor site), releases the upstream exon and forms an intermediate lariat structure; and (2) the 3'OH group of the upstream exon attacks the phosphodiester bond at 3' splice site (acceptor site) leading to the ligation of the two exons and the excision of the intron lariat which is subsequently degraded (70, 71).

In vitro studies in mammalian and *S. cerevisiae* extracts revealed that the spliceosome components assemble on the pre-mRNA in a series of complexes (E, A, B and C) (72, 73). The spliceosome assembly begins with the ATP-independent formation of E (early) complex. During this step U1 snRNP recognizes and interacts by RNA base

pairing with the 5' splice site (74, 75) while the protein factors SF1/BBP and U2AF bind to the BP and polypyrimidine tract/3' splice site, respectively (76). U2AF (U2 Auxiliatory Factor) is a dimer composed by two subunits: one of 35 kDa, which specifically binds to the 3' splice site, and another with 65 kDa which binds to the polypyrimidine tract, promoting the stable association of U2 snRNP during A complex assembly. All subsequent steps are ATP-dependent. The pre-spliceosome, or A complex, forms upon stable ATP-dependent interaction of U2 snRNP with the pre-mRNA BP region that leads to BP adenosine protrusion, facilitating subsequent nucleophilic attack on the 5' splice site (77, 78). U2 snRNP-pre-mRNA association leads to the displacement of SF1/mBBP from the branch site (79). The $U2AF^{65}$ RS domain (RS - arginine and serine rich domain) promotes base pairing between U2 snRNA and the BP. The positively charged RS domain of $U2AF^{65}$ contacts with the BP sequence neutralizing the negatively charged phosphate sequence and stabilizing the base pairing between U2 snRNA and BP (80). Binding U4/U6.U5 tri-snRNP particle to the 5' splice site follows and produces the B complex. Although B complex contains all the snRNP components for splicing, it lacks an active site. Structural rearrangements are required to activate the spliceosome. The conformational changes destabilize U1 and U4 snRNP interactions to produce B* complex, which is poised to catalyze the first chemical step of splicing (81). In the B* complex U6 and U2 interact by base pairing and U6 also interacts with the 5' splice site, contributing to the fidelity of 5' splice site recognition and reinforcing the contact with the BP. The conformational rearrangements resulting from a reaction cascade involving the NTPase activity of three U5 snRNP components: Prp28, Brr2 and Snu114, are essential for the generation of activated B* complex and, finally, for the first chemical step of splicing, resulting in the release of a free 5' exon and a lariat-3' exon intermediates (82, 83).

A further set of rearrangements generates the C complex, in which the second step of splicing (cleavage at the 3' splice site and ligation of exons) occurs (84). The products of the first step of splicing must be realigned to displace the lariat and position the 5' exon for a nucleophilic attack on the phosphodiester bond at the 3' splice site (85). Within C complex, the 5' and 3' splice sites are held in close proximity by bridging interactions involving U2 and U6 snRNAs and also a stem loop of U5 snRNA, which interacts with both 3' and 5' exons (86). The cleavage occurs at the 3' splice junction and the 5' phosphate of the downstream exon is joined to the 3' OH of the upstream exon.

After the completion of the second step, additional structural reorganizations are required to release the spliced exons and disassemble the splicing machinery so that it

can engage the next substrate pre-mRNA. In figure I.4 is depicted the typical steps for introns removal.

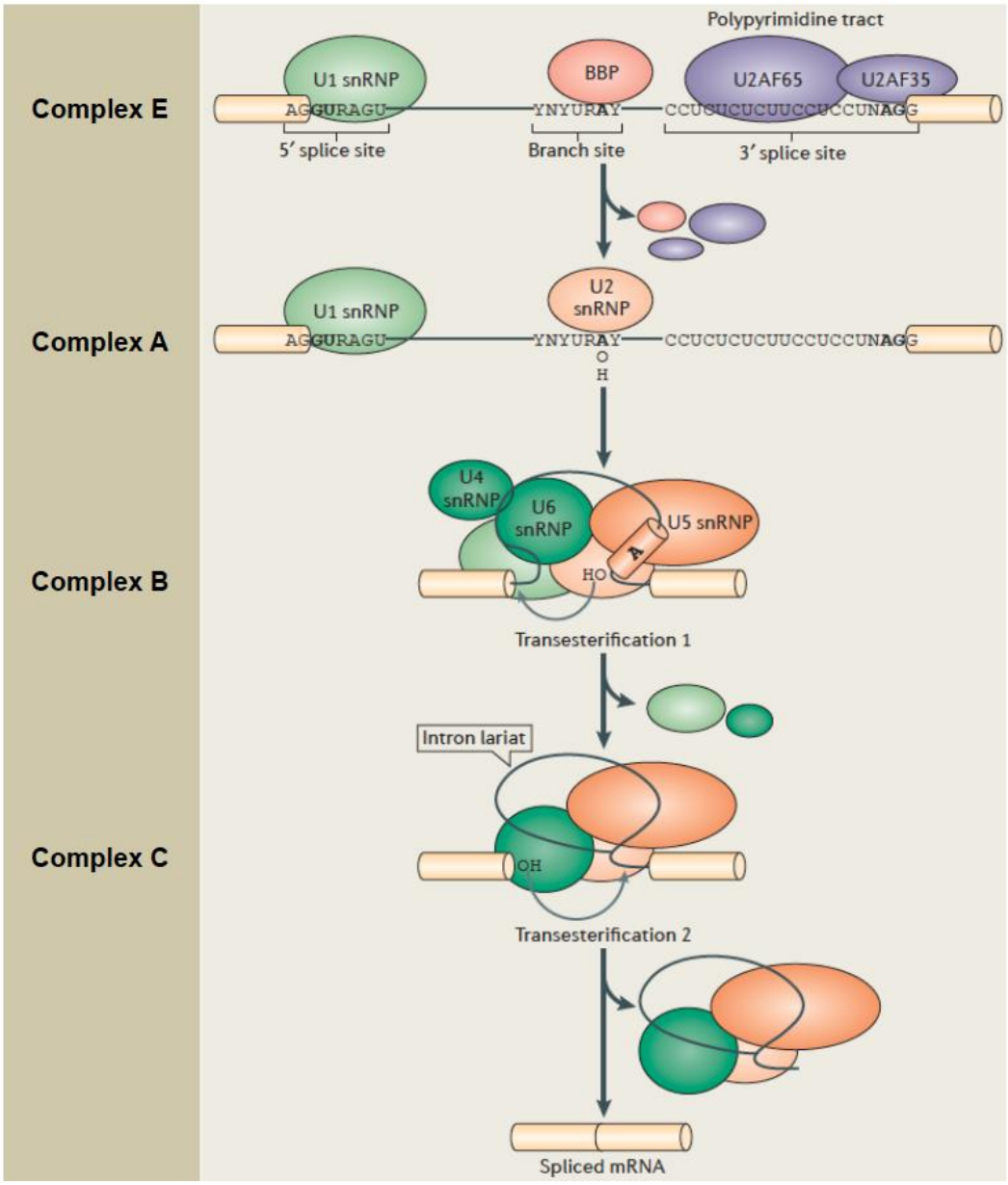

Figure I.4 – Spliceosome assembly and catalytic events during eukaryotic constitutive pre-mRNA splicing. The trans-esterification pathway of pre-mRNA splicing consists, in a first step, in the 5' splice site attack by the 2'-hydroxyl of the branch site adenosine, releasing the upstream exon and forming a branched intron "lariat" still attached to the downstream exon. In the second step, the 3' splice site is attacked by the 3'-hydroxyl of the free upstream exon. The final products are the spliced mRNA and the excised intron in a "lariat" form. Adapted from Kornblihtt *et al.,* 2013 (87).

2.2. Alternative Splicing – "One Gene, Many Proteins"

In contrast to the central dogma of molecular biology, which stated that "one gene gives rise to one protein", it was suggested in 1977 that variations in splicing, such as the joining of different 5' and 3' splice sites in a pre-mRNA sequence, leads to the production of different mRNAs (messenger RNAs) (88, 89). Thus, alternative splicing events allow an individual gene to express distinct protein isoforms with different and even antagonist functions, revealing what is likely to be the primary source of human proteomic diversity. Nearly 95% of human genes undergo alternative splicing (AS) (90, 91) but this process not only affects protein diversity but also controls gene expression by removing or inserting regulatory elements controlling translation, mRNA stability and degradation, or localization (92).

One of the best examples of the complexity of alternative splicing comes from the *Drosophila Down syndrome cell adhesion molecule* (Dscam) gene, which potentially generates 38,016 isoforms by the alternative splicing of 95 variable exons (93). Dscam is conserved in all insects and has important functions in both Nervous and Immune systems (94-96). Dscam is a cell adhesion molecule that belongs to the immunoglobulin (Ig) family of receptors presenting 18,000 alternative isoforms in the immune-competent cells of Drosophila. Watson and colleagues have detected secreted protein isoforms of Dscam in the hemolymph and that the efficiency of phagocytic uptake of bacteria was impaired by hemocyte-specific loss of Dscam, possibly due to reduced bacterial binding (96).

A large fraction of alternative splicing undergoes tissue-specific regulation in which splicing events are regulated according to cell type, developmental stage, gender, or in response to external stimuli (97-102).

2.3. Different Patterns of Alternative Splicing

The term *alternative splicing* is used to describe any situation in which a primary transcript can be spliced in more than one pattern to generate multiple, distinct mRNAs.

Most common AS events include exon inclusion/skipping (cassette exons), mutually exclusive exons, alternative selection of competing 5' or 3' splice sites and intron retention. However, there are also the alternative promoter/first exon and alternative

terminal exon due to multiple poly(A) sites (103). Figure I.5 depicts the different patterns of alternative splicing.

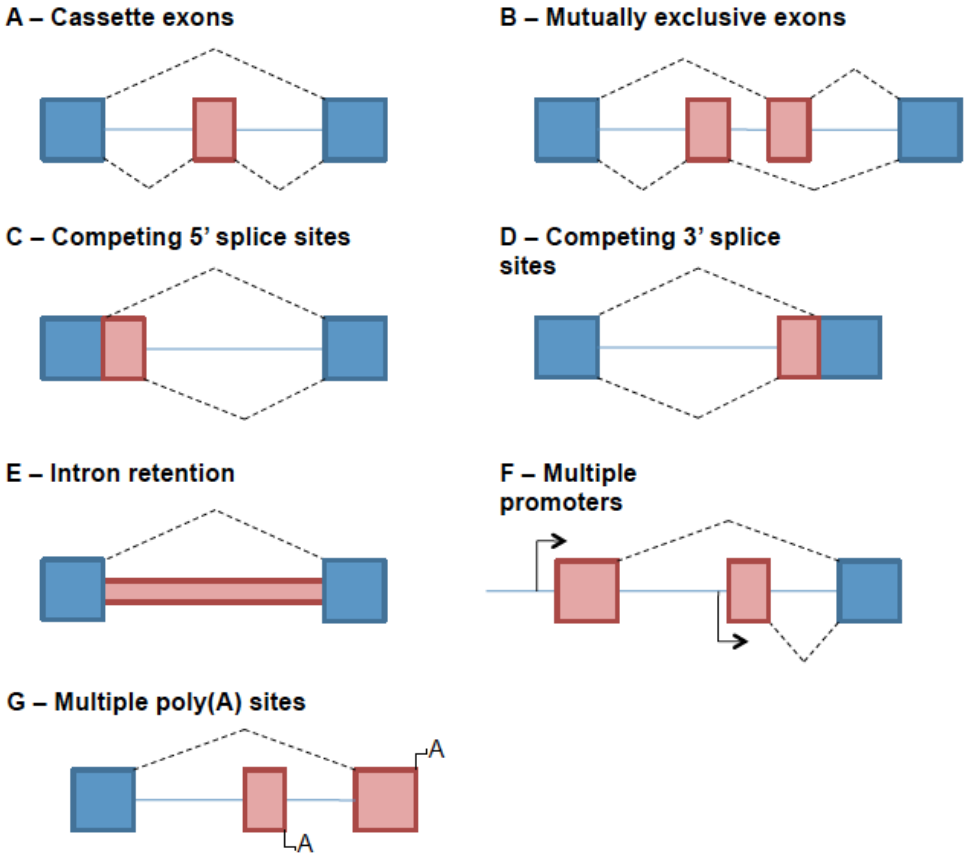

Figure I.5 – Patterns of Alternative splicing. Constitutive sequences are represented as blue boxes; alternative sequences are represented as pink boxes. A - cassette exons, B – mutually exclusive exons, C - competing 5' splice sites, D – competing 3' splice sites, E – intron retention, F – multiple promoters, G – multiple poly(A) sites.

3. Alternative Splicing Regulation

Both constitutive and alternative splicing requires the assembly of the basal splicing machinery on consensus regulatory sequences present at the boundaries between introns

and exons (the 5' and 3' splice sites). However, the efficiency with which the spliceosome acts on an exon is determined by a balance of several features, such as the strength of a splice site, exon size, the presence of auxiliary *cis* elements, the formation of RNA secondary structures that can expose or hide these elements, and the concentration or post-translation modifications of specific *trans*-acting factors (104, 105).

In humans, as in all vertebrates, a typical gene contains relatively short exons (50-250 base pairs in length) between much larger introns (typically, hundreds to thousands of base pairs) that on average account for > 90% of the primary transcript (106). This transcript geometry, is consistent with the idea that, in mammals, splice sites are predominantly recognized in pairs across the exon, involving interaction between splicing factors and the 5' splice site (5'ss) and the upstream 3' splice site (3'ss), in the so-called "exon definition model" (107, 108). In contrast, in lower eukaryotes a gene usually contains larger exons and much smaller introns and the interactions therefore occurs first across the intron between factors recognizing the 5'ss and the downstream 3'ss – in an intron definition strategy (109).

The sequences constituting the splice sites provide insufficient information to distinguish true splice sites from the greater number of cryptic splice sites existent within the pre-mRNA. There is additional information used for exon recognition in a large number of positively or negatively acting elements that lie both within the exons and in the adjacent introns, which have an important role regulating alternative splicing (110).

Barash and collaborators have thus proposed a "splicing code" that could usefully predict how a transcript can be spliced in different tissues. (91). The authors mapped genome-wide splicing factor binding, combined with genome-wide RNA expression. They used data profiling 3,665 cassette-type alternative exons in different tissues, such as central nervous system, muscle, digestive system and embryos. They also compiled approximately 1,000 of RNA features, including putative binding sites for splicing factors, validated positive and negative acting elements in both exons and introns, conserved short sequences (5-7 nucleotides) that are present in introns flanking alternative exons, exon and intron length, RNA secondary structures and also considered if the inclusion or exclusion of an exon could introduce premature stop codons, leading to nonsense-mediated mRNA decay (NMD). Nevertheless, this study has excluded other types of alternative splicing regulation such as epigenetic marks, chromatin structure and transcription dynamics. The real picture is more complex because as splicing is mostly co-transcriptional and pre-mRNA processing is coupled with transcription, factors that regulate RNA polymerase II transcription may also modulate splicing (111-113).

3.1. Auxiliary *Cis* Elements

Generally, the auxiliary *cis* elements responsible for the regulation of alternative splice sites choice by the spliceosome have in common typical features, such as: a short and variable sequence, individually weak and may exist in several copies. Although they are normally single stranded, secondary structure can occur and affect the function of the sequence elements (110, 114). These auxiliary regulatory sequences are frequently conserved across species, however occasionally they can be somewhat degenerated. They may be located in introns, laying upstream, downstream, or flanking the alternative exon or they can be located in the alternative exon (Figure I.6). Although intronic auxiliary *cis* elements are often near the alternative exon, they may also be located within 100 - 1000 nucleotides from the regulated exon. Moreover, auxiliary *cis* elements function by enhancing or inhibiting the recognition of the splice sites by the spliceosome. Thus, depending on their location and their effect on splice sites choice, they are identified as ESE (Exonic Splicing Enhancers), ESS (Exonic Splicing Silencers), ISE (Intronic Splicing Enhancers) or ISS (Intronic Splicing Silencers) (104). These additional regulatory elements function by recruiting *trans*-acting regulatory factors.

However, several lines of evidence suggest that the influence of *cis*-regulatory elements exerted on splice site choices is context-dependent, and consequently the term “ESE” (or any other) is correct only in so far as the context is considered. In fact, these sequences are present and repeated in all the genome, suggesting a high degree of redundancy and that only a few of them are in fact involved in alternative splicing regulation (87). Many of these sequences, enhancers and silencers, can act in both ways depending on the sequence and the position of the target site (115-117) for example, Ule and collaborators showed that the outcome of alternative splicing is determined by the position of Nova binding sites in pre-mRNA sequence. In this study, an RNA map was derived, combining bioinformatics, genetics and biochemistry approaches, showing that two main Nova splicing enhancers were intronic, located downstream of the alternative exon regulated by this splicing factor and the silencer sequences that are also binding sites for Nova were located immediately upstream of the alternative exon, or near the upstream constitutive exon (115).

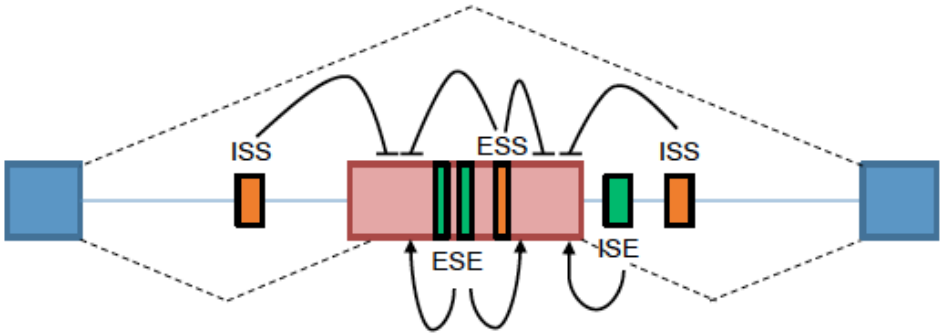

Figure I.6 – Alternative splicing regulatory *cis*-elements. Image adapted from Matlin AJ. *et al*., 2005 (118).

3.2. *Trans*-acting Splicing Factors

3.2.1 SR Proteins

Typically, the splicing enhancers (ESE and ISE) are bound by members of the SR proteins family (119). These proteins have an arginine-serine (RS)-rich domain that can interact with other proteins for example; they may recruit a number of different splicing factors to the pre-mRNA during spliceosome assembly (120), and other domain that interact with the RNA (121). RS domain is also found in many other proteins, which are collectively referred to as RS-related factors, for example, U2AF35 and U2AF65 (122). The great majority of SR proteins regulate splicing by binding ESE through their N-terminal RRM domains (RNA Recognition Motifs).

SR proteins can play an important role in splice site recognition for instance by recruiting U1 snRNP to the 5'splice site and U2AF complex and U2snRNP to the 3' splice site (Figure I.7a) or can antagonize the effect of an inhibitory protein bound to a silencer element, an RS-domain independent function (Figure I.7b) (69, 123-125).

In addition, several SR proteins can regulate mRNA nuclear export (126), nonsense-mediated mRNA decay (127) and translation (128) genome stability (129, 130) and cell-cycle progression (131).

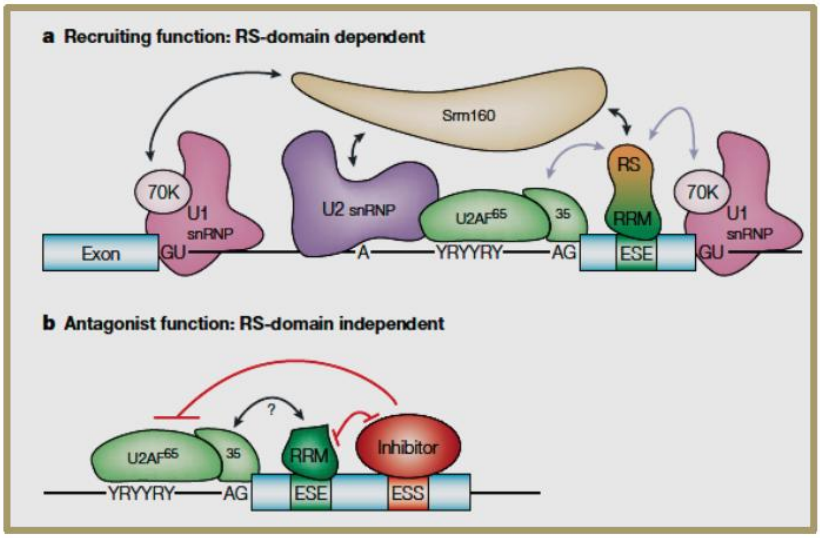

Figure I.7 – Models for SR proteins regulation of splice sites recognition. Image adapted from Cartegni *et al*., 2002**.**

SRSF1, also known as SF2/ASF, is a well-characterized member of the SR family of proteins. It contains an arginine-serine (RS)-rich sequence at the C-terminal domain, important for nuclear-cytoplasmic shuttling and two RRMs (RNA Recognition Motifs) at the N-terminal domain (132-134). SRSF1 is known to regulate constitutive and alternative splicing (135-139), however it can also regulate nuclear export, nonsense-mediated mRNA decay (NMD), translation, genome stability and miRNA processing (126-129, 140, 141).

CLIP-seq analysis revealed that SRSF1 binds preferentially to exons (142, 143) in consensus sequences rich in adenine and guanine, AG-rich elements (144, 145). In order to regulate splicing, SRSF1 binds ESE elements present on target pre-mRNAs and favors exon definition and the usage of proximal 5'ss or 3' ss (139, 142). Cho and collaborators showed that the RRMs of SRSF1 interacts with the U1-70K subunit to recruit U1 snRNP to the 5'ss, forming the early (E) spliceosomal complex. In this work it has also been shown that SRSF1 phosphorylation state affects its function. The RS domain of hypophosphorylated SRSF1 interacts with its own RRM1/2, conferring a close conformation to SRSF1. Upon hyperphosphorylation of SRSF1, the intramolecular connection is lost and the RRMs of SRSF1 become available to bind to the RRM of U1-70K, by RRM-RRM interaction (146).

Several studies have revealed an important function for SRSF1 in alternative splicing of physiological relevant pre-mRNAs. Spinal Muscular Atrophy (SMA) results from lack of expression of the *SMN1* gene and from the transition of a translationally silent C to T in exon 7 of the *SMN2* gene (147). Cartegni and collaborators have shown that this single nucleotide change occurs in within an ESE, located in exon 7, which in *SMN1* sequence is a putative binding site for SRSF1 (148). Additionally in this study, the authors generated a minigene with a compensatory mutation in exon 7, creating a SRSF1 binding site and restoring exon 7 inclusion (148). Martins de Araújo and colleagues showed that the C to T transition present in exon 7 of *SMN2,* drastically decreases U2AF in vitro binding to the 3' ss in intron 6. This suggests that this difference in U2AF recruitment impairs splice site choice and therefore may be responsible for the exon 7 skipping. In this study it was also shown that SRSF1 promotes U2AF recruitment (149).

Krainer and collaborators have shown that SRSF1 levels influences 5' ss selection: high concentrations of SRSF1 favors the usage of a proximal 5' ss in *in vitro* splicing assays with pre-mRNAs templates that contain multiple *cis*-competing 5' splice sites (137). It was also described that hnRNP A1 can competes with SRSF1 in a concentration-dependent manner and antagonizes exon inclusion (138, 150, 151). Caceres and collaborators have overexpressed SRSF1 and hnRNP A1 in HeLa cells, and showed that high levels of SRSF1 promote the usage of proximal 5' ss and inclusion of a neuron specific exon whereas high levels of hnRNP A1 promote the usage of distal 5' ss (150).

Pollard and collaborators have shown that the expression of hnRNP A1 and SRSF1 is spatially and temporally regulated in the human myometrium during fetal maturations (152). In this work it was reported that SRSF1 expression levels are increased in the lower uterine region, associated with a decreased expression of hnRNP A1 in the same uterine region. Contrarily, in the upper uterine region, it was observed the opposite pattern, hnRNP A1 was predominantly expressed in comparison to SRSF1. The authors have also shown that SRSF1 expression levels in lower uterine region are highest in pregnant women, and decrease during labor. In the case of hnRNP A1, the expression levels in the upper uterine region are increased in pregnant women and remain high during labor. In this work it was suggested that the variations of expression of these two splicing factors, could be regulating the expression of several myometrium specific protein isoforms, as for example G proteins isoforms, during gestation and parturition (152).

Eperon and collaborators have shown that SRSF1 and hnRNP A1 modulate *in vitro* the binding of U1 snRNP to the 5' ss. SRSF1 was shown to promote the binding of U1 snRNP to the 5' ss whereas hnRNP A1 has an opposite effect inhibits the recognition of 5'ss by the U1 snRNP (153).

The tyrosine kinase receptor for macrophage-stimulating protein – Ron, involved in cell dissociation, mobility and invasion of extracellular matrices also undergoes alternative splicing regulation (154). Cells expressing the alternative isoform ΔRon, lacking exon 11 present higher mobility (155). Ghigma and collaborators have identified two regulatory elements, an ESE and an ESS, in exon 12 of *Ron.* By UV-crosslinking and immunoprecipitation assays SRSF1 was identified binding to the ESE. Overexpression and knockdown of SRSF1 shown in this case that SRSF1 plays a different role in AS regulation, in contrast to the events mentioned above, silencing exon 11 and leading to the expression of ΔRon (156).

3.2.2. hnRNP Proteins

The splicing silencers (ESS and ISS) are frequently bound by splicing repressors of the hnRNP (heterogeneous nuclear ribonucleoprotein) family, a diverse group of RNA binding proteins containing one or more RNA recognition motifs (RRMs) that bind pre-mRNA sequence, and protein-protein interaction domains such as glycine-rich motifs, RGG (Arginine-Glycine-Glycine) (157, 158).

As SR proteins, hnRNPs bind preferentially to specific sequence motifs on the pre-mRNA, however they can also bind many other sequences with less complementarity (159).

The strategies used by hnRNP proteins to control splice site selection may be summarized as following (160):

a) The binding of hnRNP proteins close to splicing signals can occlude the binding of U1 snRNP or U2AF. Likewise, the binding of hnRNP proteins to exonic sequences can antagonize the interactions of SR proteins with ESEs (Figure I.8 A);

b) The propagation of hnRNP binding from a site of high-affinity located in an exon may occlude the binding of SR proteins. A similar situation has been proposed to occur when PTB (also known as hnRNP I) binds to an intron sequence (161) (Figure I.8 B);

c) Inhibition of exon definition when bound to an exon (Figure I.7 C, left) or inhibition of intron definition when bound to an intron (Figure I.8 C, right);

d) Interactions between bound hnRNP proteins that may loop out portions of a pre-mRNA can promote exon skipping (Figure I.8 D, left) or stimulate intron definition (Figure I.8 D, right);

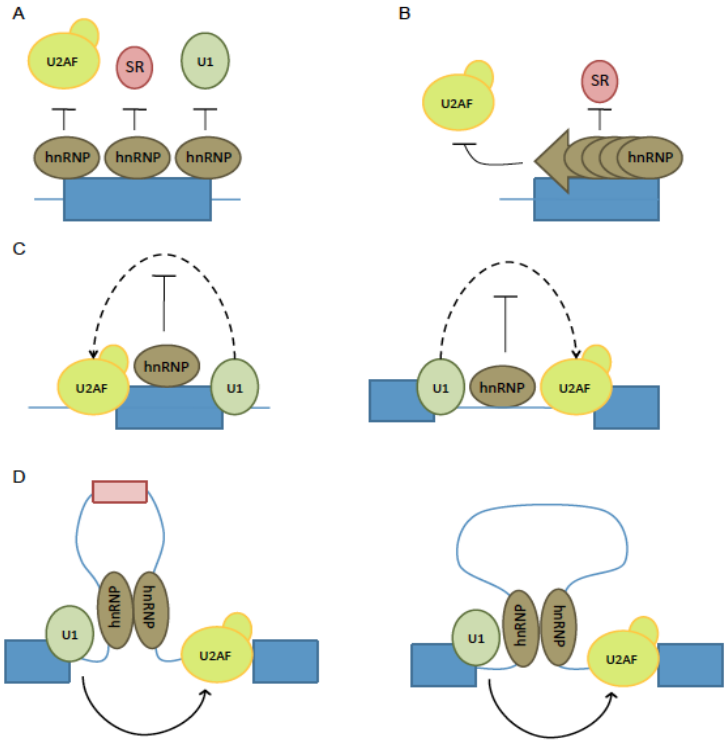

Figure I.8 – Strategies used by hnRNP proteins to control splice site selection. Adapted from Martinez-Contreras *et al.,* 2007.

A well-studied member of the hnRNP family is hnRNP I, commonly known as PTB (Polypyrimidine tract-binding protein) (162, 163). PTB is structurally composed by four RRMs (RNA recognition motifs) and a N-terminal NLS (Nuclear Localization Signal) necessary for nuclear-cytoplasmic shuttling (164). Functionally PTB was associated with CU-rich elements, repressing alternative spliced exons (165-167), and also modulating 3' end processing (168-170) mRNA stability, translation and localization (171-173). The repressive effect of PTB on alternative exons is achieved by multiple ways. It may antagonize exon definition, *via* direct competition with U2AF65 for the binding to the

polypyrimidine tract, it may bind and “coat” the exon, or it may loop out portions of RNA of containing the alternative exon by binding to elements flanking the exon (161, 167, 174, 175). Nevertheless, CLIP-seq analysis showed that PTB can also lead to exon inclusion in some alternative splicing events (176).

In non-neuronal cells, high levels of PTB expression leads to the exclusion of the *c-src* N1 exon, whereas in neurons PTB is expressed in lower levels and thus the N1 exon is included in the *c-src* mRNA (175, 177). The binding of PTB to the N1 exon has been characterized using extracts of neuronal and non-neuronal cells (177, 178) and revealed that PTB binding does not disturb the binding of U1 snRNP to the N1 5’ ss, but blocks the interaction between U1 snRNP and U2AF, preventing the binding of U2AF to the 3’ ss and the formation of the E complex (179). Recently, Sharma and collaborators have shown that repression of N1 exon by PTB was due to its binding to CU-rich elements flanking the N1 exon and also to the interaction between PTB and U1 snRNP stem-loop 4 (SL4), which prevents the interaction of U1 with the spliceosome complex at the downstream 3’ ss (180). Another event regulated by PTB is the *FAS* exon 6 alternative splicing (181).

Inclusion of *FAS* exon 6 is activated by TIA-1 RNA binding protein, by promoting the binding of U1 snRNP to the 5’ ss on intron 6 and helping in exon definition. This effect is antagonized by the binding of PTB to an ESS located in exon 6, which although does not inhibit the binding of U1 snRNP to the 5’ ss, blocks its interaction with the 3’ss complex leading to exon skipping (181).

The alternative splicing of *α-actinin* mutually exclusive exons SM (Smooth Muscle) and NM (Non Muscle) is also PTB-regulated (182). The SM exon is excluded in the majority of cells whereas NM exon is included (183); in smooth muscle cells the SM exon is included and NM exon is excluded. Southby and collaborators have shown that in *in vitro* splicing assays using HeLa nuclear extracts, SM exon is skipped in primary transcripts, unless the repressive elements present between the exon and its upstream distant branch point are removed or unless a depletion of PTB occurs (184). Matlin and collaborators have shown that PTB molecules spread throughout the α-actinin intron localized upstream of the SM exon, which represses its inclusion (182).

SM and NM alternative exons are also regulated by other proteins, in particular by CELF proteins (CUG-BP and ETR3-like factors). CUG-BP directly competes with PTB for the binding to the 3’ end of the PPT upstream of the of SM exon and favors the binding of U2AF65, leading to splicing of the intron and SM inclusion (185).

Although SR proteins mainly bind to enhancer sequences and hnRNP proteins to silencer sequences, many of them can act in both ways, depending on the sequence and position of the target site in the primary transcript (115).

Table 1 – Alternative splicing events regulated by SR and hnRNP splicing factors.

Splicing Factor	Domains	Target genes (regulated exon)	AS event	References
SRSF1	2 RRM 1 RS	*FN* (exon EDI)	inclusion	(148, 150, 156, 186-188)
		SMN2 (exon 7)	inclusion	
		Ron (exon 11)	skipping	
		c-src (exon N1)	inclusion	
		MCL1 (exon 2)	inclusion	
		CASP-9 (exon 4)	inclusion	
SRSF2 (SC35)	1 RRM 1 RS	*CD44* (exon v6)	Inclusion	(189-191)
		Tau (exon 10)		
		β-tropomyosin (exon 6B)		
SRSF3 (SRp20)	1 RRM 1 RS	*CD44* (exon v9)	inclusion	(192-195)
		SRSF3 (exon 4)	inclusion	
		CASP-2 (exon 9)	skipping	
		INSR (exon 11)	inclusion	
SRSF5 (SRp40)	2 RRM 1 RS	*GR* (exon 9α)	skipping	(196-198)
		CFTR (exon 9)	skipping	
		PKC (exon βII)	inclusion	
SRSF6 (SRp55)	2 RRM 1 RS	*CD45* (exon 4)	inclusion	(140, 199)
		FGFR1 (exon α)		
SRSF7 (9G8)	1 RRM 1 RS	*Tau* (exon 10)	skipping	(192, 200)
		CD44 (exon v9)	inclusion	

Splicing Factor	Domains	Target genes (regulated exon)	AS event	References
hnRNP A1	2 RRM 1 RGG	*c-src* (exon N1)	skipping	(150, 201-205)
		MAG (exon 12)		
		Rac1 (exon 3b)		
		INSR (exon 11)		
		SMN2 (exon 7)		
hnRNP I (PTB)	4 RRM	*c-src* (exon N1)	skipping	(177, 181, 182, 206, 207)
		FAS (exon 6)		
		α-actin (exon SM)		
		FGFR2 (exon IIIb)		
		α-tropomyosin (exon 3)		
hnRNP H	3 RRM	*CHRNA1* (exon P3A)	skipping	(208-211)
		FGFR2 (exon IIIc)	skipping	
		β-tropomyosin (exon 7)	skipping	
		c-src (exon N1)	inclusion	
hnRNP F	3 RRM	*INSR* (exon 11)	inclusion	(204, 211, 212)
		FGFR2 (exon IIIc)	skipping	
		c-src (exon N1)	inclusion	
hnRNP L	4 RRM	*CD45* (exon 5)	skipping	(213, 214)
		CD45 (exon 4)		

3.3. Transcription Machinery and RNA Polymerase II Elongation Rate

Subsequently to the observation of co-transcriptional splicing firstly described in 1988 by Beyer and Osheim, several studies have shown indeed a co-transcriptional recruitment of splicing factors to the primary transcript. However, in the majority of the cases the complete excision of the intron did not occur before transcript release (215-218).

The first evidence that alternative splicing was coupled to transcription was described in 1997 by Cramer and collaborators (219) and followed by Pagani and collaborators (220). In these studies it was shown that the outcome of alternative splicing was affected by the promoter structure used. Also transcription factors (221, 222), co-activators (223, 224), transcription enhancers (225) and chromatin remodelers (226) were also shown to affect the outcome of alternative splicing.

The finding that RNA Polymerase (Pol) II could recruit in its C-terminal domain (CTD) some RNA processing factors such as splicing factors (113, 227-229) and that other factors involved in transcription could affect splicing (226, 230-233) supported that not only splicing could occur co-transcriptionally but also that it could be coupled to transcription.

Co-transcriptional means that splicing occurs, or is committed to occur, before transcript release by RNA Pol II. Coupling implies that splicing and transcription machineries interact with each other.

3.3.1. RNA polymerase II: The Importance of CTD for the Recruitment of Splicing Factors

The eukaryotic RNA Pol II is the nuclear enzyme responsible for the transcription of mRNA, some small nuclear RNAs and microRNAs. It is composed by multisubunits and an extension that evolved from the largest subunit, Rpb1 that has been shown by X-ray crystallography to be the unique tail-like C-terminal repeat domain (CTD) (234). The CTD consists of highly conserved heptapeptide repeats (52 in mammals) of the consensus sequence YSPTSPS (Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7) (235).

During transcription CTD residues undergo differential and reversal post translation modifications, such as phosphorylation, glycosylation and proline isomerization (236-238). CDK7, the cyclin-dependent kinase subunit of TFIIH (transcription factor II human),

phosphorylates Ser5 and Ser7 at the promoter region of the gene, an important step for transcription initiation (239) and for the 5' end capping of the primary transcript (240). The entry of RNA Pol II into the elongation phase of transcription occurs with a change in the CTD phosphorylation pattern, characterized by an enrichment of Ser 2 and Thr4 phosphorylation, achieved mainly by CDK9, the cyclin-dependent kinase subunit of P-TEFb (positive transcription elongation factor b) (241, 242). The Ser 5 phosphorylation decreases while Ser2 increases towards the 3'end of the gene, favoring the recruitment of factors important for transcription elongation, such as Spt6, and also for pre-mRNA processing (243).

The simultaneous phosphorylation of Ser2/Ser5 leads to the recruitment of the methyltransferase Set2 that methylates lysine 36 of histone H3, thus increasing H3K36me3 levels at the body of the gene (244). As long as the polymerase approaches the poly(A) site, Tyr 1 phosphorylation levels decrease enabling the recruitment of important factors for pre-mRNA 3' end cleavage, polyadenylation and transcription termination, such as Rtt103 and Pcf11 (245-247).

The capacity of the residues of this heptapeptide to be modified post translationally during the different stages of the transcription process is an important feature of CTD, that enables its interaction with different other molecules, making the CTD an essential docking platform for a great diversity of factors necessary not only for transcription but also for mRNA maturation, such as 5' end capping, splicing and polyadenylation (237, 240, 248). In respect to splicing, David and collaborators have shown that the Ser2 and Ser5 double phosphorylation leads to the recruitment of the splicing factor U2AF65 that binds directly to the phosphorylated CTD (249). U1 snRNP and the SR proteins, SRSF1 and 9G8, are also co-transcriptionally recruited to the nascent pre-mRNA, in association with RNA Pol II (228).

Recently, Huang and collaborators have shown that the mediator subunit MED23, known to be responsible for the recruitment of the elongation factor P-TEFb through the binding of CDK9 (250), has also the capacity to interact, *in vivo* and *in vitro*, to RNA processing factors, such as hnRNP L, U1/U2 snRNPs and U2AF65 (230). Moreover, in this study it was shown, by minigene reporters and exon array analysis, that MED23 regulates a subset of alternative splicing, alternative cleavage and polyadenylation events (230).

These studies show that the recruitment of splicing factors by RNA Pol II CTD, to the local where the nascent pre-mRNA is being transcribed, is an important strategy to facilitate spliceosome assembly and increase splicing efficiency (228, 249).

3.3.2. RNA Polymerase II Elongation Rate

The extensive work of Kornblihtt's lab added a significant insight of how RNA Pol II elongation rate, and its kinetics could regulate alternative splicing in the fibronectin gene (221, 225, 251, 252). The authors showed that usage of SV40 large T antigen, a replication activator that induces DNA replication, led to the inclusion of EDI (Fibronectin Extra Domain I) alternative exon of fibronectin gene. On the other hand, the increase of RNA Pol II elongation rate, induced by the usage of the VP16, an activator of transcription initiation and elongation, had the opposite effect leading to the skipping of exon EDI (221, 225). In this case the exclusion of the alternative exon EDI from mRNA sequence occurs due to a suboptimal 3' ss of the upstream intron compared to the stronger 3' ss of the downstream intron.

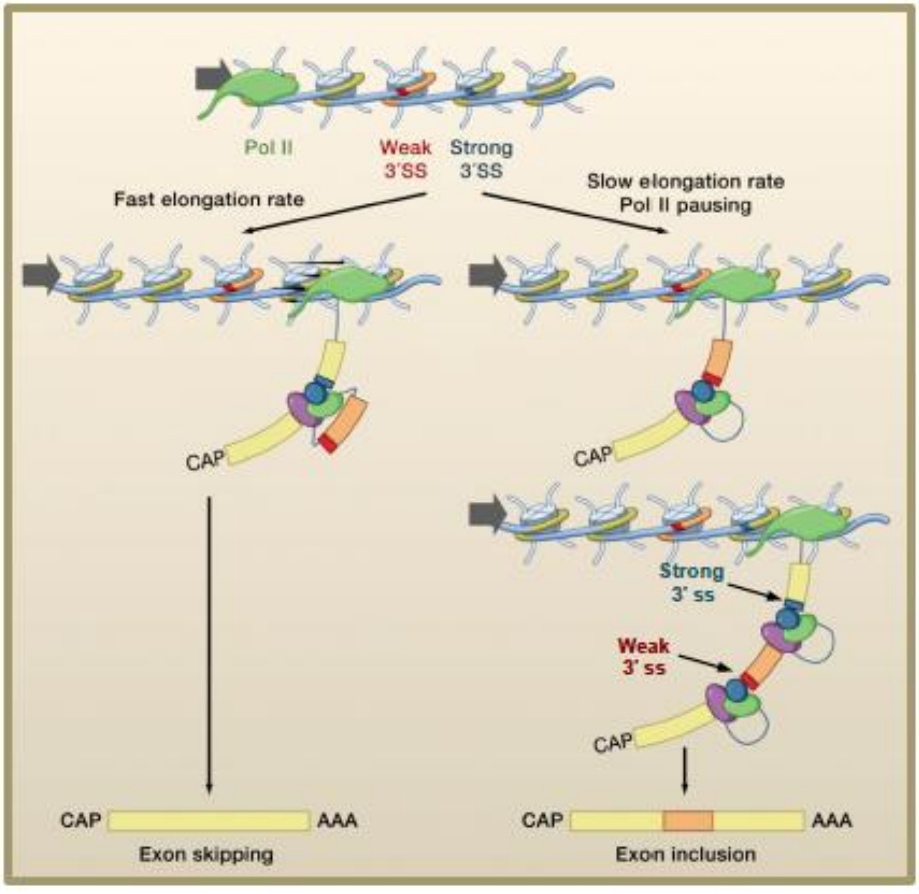

Figure I.9 – RNA polymerase II kinetic model for alternative splicing. Adapted from Luco et al., 2011.

If a decreased RNA Polymerase II elongation rate occurs in this region, the synthesis of the downstream intron will be delayed favoring the recognition of the upstream weak 3' ss and exon EDI inclusion. However, in an opposite scenario, when an increased RNA Pol II elongation rate is induced, only the strong splice site located downstream is recognized leading to exon skipping (252-254). The model proposed for the regulation of alternative splicing by RNA Pol II kinetics is depicted in figure I.9.

To study the role of RNA Pol II transcription in alternative splicing De la Mata and collaborators used a mutant RNA Pol II with a point mutation at the large subunit Rbp1 that confers low processivity to the polymerase, that they named "slow" RNA Pol II. Human hepatoma cells (Hep3B) were transfected with a plasmid coding for this mutant slow polymerase, which is resistant to α-amanitin, together with a wild type (WT) RNA Pol II also α-amanitin resistant, and a fibronectin minigene containing the alternative exon EDI. The RT-PCR analysis of the EDI splicing pattern showed that the transcripts that have been synthetized by the slow polymerase presented higher inclusion of the EDI exon in comparison to the ones that have been transcribed by the WT polymerase (251), confirming the previous observed effects of RNA polymerase elongation rate on EDI alternative splicing (221, 225).

Generally, when a slow elongation rate is induced the inclusion of an alternative exon is generally promoted (222, 252, 255). Slow RNA Pol II elongation rates can be induced by sequences that cause RNA Pol II pausing (256), or drugs that affect elongation (252, 255), like for example Camptothecin, that creates topoisomerase I-DNA adducts which physically interfere with RNA Pol II elongation (257), and D-ribofuranosylbenzimidazole (DRB) that inhibits kinases that phosphorylate CTD domain of RNA Pol II and thus impair RNA Pol II elongation (255). On the other hand, a rapid RNA Pol II elongation leads to more exon skipping. A fast RNA Pol II elongation rate can be induced by drugs that promote a more opened chromatin structure, such as the histone deacetylase (HDAC) inhibitor Trichostatin A (TSA) (258, 259) or the usage of transcriptional activators such as, Sp1, CTF/NF1, HIV-1 Tat, GAL4-VP16 and SV40 enhancer that favor elongation (222).

In another study, Munoz and collaborators have shown that CTD Ser5 and Ser2 hyperphosphorylation induced by UV irradiation, led to a decrease of RNA Pol II elongation rate and consequently to an increase of the inclusion of fibronectin alternative exon EDI, confirming a role for RNA Pol II elongation rate in alternative splicing, *via* CTD phosphorylation. In this study it was also shown that UV irradiation induced an increased expression of proapoptotic isoforms of the genes *Bcl-x* and *caspase 9* (231).

Montes and collaborators have also supported the model of alternative splicing control by RNA Pol II elongation rate, by showing that the elongation factor TCERG1 was

necessary to induce the expression of the pro-apoptotic *Bcl-x* alternative isoform. *Bcl-x* gene has competing 5' splice sites at the 3' end of exon 2. The usage of the proximal 5' ss lead to the expression of a shortest and pro-apoptotic isoform (Bcl-x_S) whereas the recognition of the distal 5'ss give rise to the longest and anti-apoptotic isoform (Bcl-x_L). The existence of a negative regulatory element – SB1- upstream of Bcl-x exon 2 proximal 5' ss, inhibits the recognition of this splice site and favors the usage of the distal 5' ss. Upon TCERG1 overexpression, the transcriptional elongation of the *Bcl-x* gene is stimulated, providing less time for the binding of a negative trans-acting factor to the SB1 element, thus favoring the usage of the proximal 5' ss, and producing the shortest and pro-apoptotic isoform (260).

The recently identified complex DBIRD binds directly to RNA Pol II and is enriched in genomic regions characterized by high frequency of adenines and thymines (A+T) that are typically difficult to transcribe (233). These (A+T)-rich regions were shown to be located upstream and downstream of the splice sites of included exons. Moreover, DBIRD-depleted HEK293 cells presented increased RNA Pol II occupancy levels at the genomic region of regulated exons of *RAD50* and *SLC36A4* genes. Montes and collaborators proposed that the DBIRD complex could act as an elongation factor that enhances transcription elongation in (A+T)-rich regions, affecting the inclusion of alternative exons present in these regions (233).

3.4. Chromatin Structure and Histone Modifications

Fox-Walsh & Hertel described in 2009 that transcripts that contain splicing *cis*-elements with errors in their sequence could often recruit with accuracy the splicing machinery to a given exon (261). Notwithstanding differences in RNA Pol II elongation rate have been shown to modulate alternative splicing events, how RNA Pol II processivity is modulated and how its elongation rate is controlled is still not completely understood. These two open questions suggested that other mechanisms are involved in AS regulation.

The answer was given during the last decade, when several researchers in the pre-mRNA splicing field showed that chromatin structure and epigenetic histone modifications played a role in those processes and could regulate AS.

The finding that fibronectin exon E33, inclusion could be modulated by TSA treatment and by a more compacted chromatin structure of a replicated reporter plasmid was the first evidence that chromatin structure was important for AS regulation (221, 222).

Interestingly, the average size of a mammalian exon coincides with the length of the DNA wrapped around the octamer of histone proteins that constitutes a nucleosome that is 147 bp, suggesting a role for nucleosome positioning in exon definition (262, 263).

Schwartz and collaborators used the micrococcal nuclease (MNase) digestion assay combined with computational prediction methods to map the nucleosome positioning in different species and shown that nucleosomes are particularly enriched at intron-exon junctions in comparison to intronic regions, thus “marking” the exons (262). These results indicate that the basis for this differential nucleosome occupancy along the genes is the DNA sequence itself and that splicing signals are also important at the DNA level, organizing the chromatin landscapes of exons and introns. It has been also shown that excluded alternative spliced exons are less enriched in nucleosomes than the included ones (262).

Tilgner and collaborators found that the nucleosome density is higher in exons defined by weak splice sites in comparison to exons defined by strong splice sites. Moreover in this study it has been shown that pseudoexons that despite being defined by strong splice sites are skipped from mRNAs, show nucleosome depletion (263). These evidences highlight the role of nucleosome positioning in exon definition and also in the regulation of splicing.

Zhou and collaborators have shown that the splicing regulators Hu proteins interact with RNA Pol II and histone deacetylases. Hu proteins were shown to inhibit HDAC2, leading to increased chromatin acetylation in the genomic region of *FAS* alternative exon 6 and *Nf1* alternative exon 23a. The change in chromatin structure created by the local hyperacetylation induced by HDAC2 inhibition, enhanced RNA Pol II elongation rate in ES-derived neurons and a consequently increased skipping of *Fas* and *Nf1* alternative exons (264).

Another study showed that the chromatin remodeler SWI/SNF binds to *CD44* gene and its Brm subunit associates with the splicing factor Sam68, that forms a roadblock to RNA Pol II, leading to increased inclusion of alternative exons (226).

3.4.1. Histone Modifications and Adaptor Proteins

In the cell nucleus, on average 147 bp of DNA is packed and wrapped around histones composing one nucleosome. This state of DNA compacted with proteins form the chromatin. Each nucleosome, the fundamental unit of chromatin, is composed by an octamer of four core histones (H2A, H2B, H3 and H4). Generally, the N-terminal ends of histones can be post-translationally modified on several sites. It has been identified several different types of histone modifications such as methylations, acetylations, phosphorylations, ubiquitylations and sumoylations among others, that can play a role in transcription, DNA repair, replication and chromatin condensation. There are 60 residues at histone tails that can be post-translation modified, but in great majority the ones that are known to have an impact on splicing regulation are the methylation and acetylation of lysine residues (K). Methylations at lysines can be complex because they may have increasing levels of modification forms: mono-, di- or trimethyl- if there are one, two or three methylated lysine residues respectively (265, 266).

Recently, histone post-translation modifications emerged as major regulators of alternative splicing (267-269). The human *fibroblast growth factor receptor 2* (*FGFR2*) gene is an example of how chromatin conformation and histone modifications can change the splicing outcome. *FGFR2* has two mutually exclusive alternative exons FGFR2 IIIb and IIIc, giving rise to two different and tissue-specific isoforms: in epithelial cells exon IIIb is predominantly included whereas in mesenchymal cells IIIb is repressed and IIIc is exclusively used (270). Luco and collaborators showed that in mesenchymal cells, the *FGFR2* region where these two alternative exons are located is enriched in H3K36me3 and H3K4me1 histone marks leading to exon IIIc inclusion, and in epithelial cells this region is enriched in H3K27me3 and H3K4me3 leading to exon IIIb inclusion. Moreover, the modulation of the expression levels of SETD2 and ASH2 histone methyltransferases, lead to the changes in the alternative splicing pattern of IIIb and IIIc exons (207). Schor and collaborators have shown that upon human neuronal cells depolarization, an increase of H3K9 acetylation and H3K36 methylation in the genomic region around the alternatively spliced exon 18 of *NCAM* gene was observed, leading to exon skipping. However, no changes in acetylation levels of *NCAM* promoter were detected (268).

Another study has shown that high levels of the epigenetic mark H3K9me3 were a characteristic of alternative exons of several genes including *CD44*. On *CD44* gene this enrichment of H3K9me3 levels at the variant exons region creates a binding site to HP1γ,

and the accumulation of HP1γ in this chromatin region facilitates the inclusion of CD44 alternative exons conceivably through a decreasing of RNA Pol II elongation rate (271).

The example described above, pointing to a modulation of chromatin structure by histone modifications, suggest that this effect on chromatin structure can regulate alternative splicing through a consequent modulation of the RNA Pol II elongation rate.

Another possible mechanism involving chromatin histone marks in alternative splicing regulation is through an adapter system that can recruit splicing factors during transcription. Such mechanisms are very well illustrated by the PTB-dependent alternative splicing events (207). It had already been described that the repression of exon IIIb of *FGFR2* in human mesenchymal cells was dependent on the polypyrimidine tract-binding protein (PTB) splicing factor, by binding to a pyrimidine-rich ISS present in the intron upstream of exon IIIb (272). Luco and collaborators showed that in these cells PTB is recruited to the primary transcript through the binding to an adaptor protein called MRG15. MRG15 is a component of the retinoblastoma binding protein 2 (RBP2) H3-K4 demethylase complex and binds directly to the histone mark H3K36me3 that is present in high levels at the *FGFR2* gene in human mesenchymal cells. Thus, by modulating the levels of H3K36me3 and MRG15 it was possible to modulate the binding of PTB to the nascent RNA and control alternative splicing of exon IIIb (207). Another study showed *in vivo* and *in vitro* that H3K4me3 is specifically recognized by the chromodomain of the chromatin adaptor protein 1 (CHD1) and that CHD1 can interact with components of the U2 snRNP complex (273). Loomis and collaborators performed a protein microsequencing analysis and showed that the chromatin binding protein HP1α/β bind to the methylated lysine 9 of histone H3 (H3K9me3) and that HP1 associates with SRSF1 in mitotic HeLa cells. Moreover SRSF1 knockdown led to retention of HP1 on mitotic chromatin (274).

3.5. Non-coding RNAs (ncRNAs)

Small interfering RNAs (siRNAs) can be used in order to change the splicing pattern of an alternative exon (269). Allo and collaborators have shown that it is possible to interfere with an AS event using a siRNA sequence targeting the intron downstream of the alternative exon. The binding of the siRNA to the intron involves the recruitment of the RNA-associated protein Argonaute 1 (AGO1) inducing high intragenic levels of silencing histone marks such as H3K9me2 and H3K27me3 that could afterwards lead to the binding

of heterochromatin binding protein 1 α (HP1α) to the condensed chromatin structure. This creates a roadblock to RNA Pol II, slowing down transcription elongation rate and increasing the inclusion of the given alternative exon (269).

MicroRNAs can regulate alternative splicing simply controlling the expression levels of important splicing factors as in the case of miR-124 that regulates the expression of PTB (275).

Another different way of regulation by ncRNAs is the case of the long non-coding RNA MALAT-1. Tripathi and collaborators have shown that MALAT-1 can bind to SR proteins, such as SRSF1, SRSF2, SRSF3 and SRSF5, sequestering these proteins at nuclear speckles. Downregulation of MALAT-1 expression in HeLa cells led to an increased availability of the SR proteins at the nucleoplasm and increased inclusion of alternative exons in different genes, such as *CDK7* (cyclin-dependent kinase 7), *SAT1* (spermidine/spermine N1-acetyltransferase 1), *MGEA6* (meningioma expressed antigen-6) (276).

An additional study has shown that smaller variants of a brain specific small nucleolar RNA (snoRNA) HB-52II bind to a silence element in exon Vb of the serotonin receptor 5-HT2C pre-mRNA. The binding of Hb-52II snoRNAs will presumably inhibit the binding of repressor splicing factors to the silencer element and lead to the inclusion of exon Vb (277, 278).

3.6. Signal Transduction and Alternative Splicing Regulation

Changes in the extracellular environment can lead to the alteration of splice site selection as an adaption of cells to specific stimuli such as growth factors, cytokines, hormones and depolarization of cell membrane potential. Consequently alternative splicing regulators and the kinases that regulate them can be affected by signaling molecules. Post-translation modifications such as phosphorylation and changes in subcellular localization of splicing factors can be a consequence of signal transduction triggered by an extracellular stimuli (279).

In humans, there are some examples of signal-induced alternative splicing events that occur for example upon neuronal cell depolarization, T cell activation and insulin signaling (268, 280-283).

The example of the T cell phosphatase CD45 alternative splicing, shows that in resting T cells the glycogen synthase kinase 3 (GSK3) directly phosphorylates the splicing factor PSF (PTB-associated splicing factor) promoting the interaction of PSF with TRAP 150 (an arginine/serine-rich subunit of the transcription regulatory complex TRAP/mediator) and preventing the binding of PSF to CD45 pre-mRNA. Upon T cell stimulation and activation of the RAS pathway, GSK3 activity is reduced, and consequently PSF phosphorylation levels decrease, leading to the release of PSF from TRAP150 allowing PSF to bind to CD45 pre-mRNA and repress CD45 exon 4 inclusion (284, 285).

The CD44 cell receptor, which is critical for organ development, neuronal axon guidance, immune functions, haematopoiesis and tumor development is another example of signal-induced AS (286). The Ras-Raf-MEK-ERK signaling cascade was described as being involved in the inclusion of variable exon 5 (v5) of CD44 upon T cell activation (287). The RNA binding protein Sam68 has been described being the final target of ERK. Phosphorylated Sam68 binds to a *cis*-acting element in CD44 pre-mRNA and leads to the inclusion of v5 exon (288).

In skeletal muscle cells, the activation of the insulin receptor by insulin recruits p85/p110 phosphatidylinositol 3-kinase (PI3K) and leads to the synthesis of phosphatidylinositol 3,4,5-triphosphate. This substance activates several serine-threonine kinases resulting in the phosphorylation of SRp40 leading to the inclusion of an alternative exon βII of protein kinase C (PKC) pre-mRNA (197).

The splicing factor SRSF1 may be modulated by the AKT signaling pathway promoting changes in alternative splicing in response to epidermal growth factor (EGF) signaling. AKT activation leads to auto-phosphorylation and activation of SR protein kinase 1 (SRPK1) which phosphorylates SRSF1 and other splicing factors members of the SR family (289).

Stress caused by pH changes, osmotic or temperature shock can affect AS regulation (279), as illustrated by muscle-specific alternative splicing of $F_1\gamma$ exon 9, which is altered by acidic treatment. Human fibrosarcoma HT1080 cells cultured in acidic DMEM exhibited the expression of the $F_1\gamma$ muscle specific isoform that excludes exon 9. This event possibly occurs due to the expression and binding of a negative regulatory splicing factor to a regulatory sequence present in $F_1\gamma$ pre-mRNA, in response to an environment with lower pH (290). Another study have shown that the exposure of NIH-3T3 cells to osmotic stress, induced by sorbitol, led to the activation of the MKK376-p38-Mnk1/2 signaling cascade and to hnRNPA1 accumulation in the cytoplasm affecting the splicing pattern of the E1A reporter minigene (291). Denegri and collaborators have shown that the heat shock of HeLa cells for 1h at 42^{o}C, lead to the formation of HAT (hnRNPA1 interacting

protein) bodies. HAT stressed-induced bodies also presented accumulation of different splicing factors such as SRSF1 and 9G8, thus altering the subnuclear distribution of these splicing factors and culminating in the perturbation the splicing pattern of the E1A reported gene (292).

Recently, it has been described that depolarization of neuroblastoma cell membrane potential causes an accumulation of several SR proteins, such as SRSF1, SRSF2 and SRSF3, in the nuclear speckles impairing alternative splicing (259).

The myriad of examples described above show that alternative splicing is not only a huge source of protein diversity, but its regulation at different levels contribute to a specific and fine-tuning of cellular responses.

4. Alternative Splicing and the Immune System

It is remarkable the plasticity that immune cells ought to have in order to respond to a changing environment that challenges the immune system with a giant number of different antigens and pathogens. As a result, immune cells are able to change their function very rapidly and precisely, in response to a given stimulus. The stimuli captured at the cell surface, through the T cell receptor (TCR) is transmitted by signaling cascades through the cytoplasm to the nucleus that will lead to cell proliferation, cytokines and cytotoxins secretion, cell migration and changes of morphology and a myriad of effector functions (10).

Alternative splicing (AS) is a very important process that produces a vast diversity of transcripts and protein isoforms from the code present in a single gene. Given the diverse number of responses and flexibility needed, it is expectable that cells of the immune system rely on alternative splicing in order to control their protein levels and function (293). Despite the importance of alternative splicing in the immune system, very little is still known about which signaling pathways, splicing factors, and sequences are involved in the regulation of this mRNA maturation process. However, a growing list of autoimmune diseases and other immune-related pathologies have been correlated with misregulated splicing events (294, 295).

4.1. Molecules of the Immune System Affected by Alternative Splicing: Physiological Consequences

Alternative splicing in cells of the immune system is physiologically relevant, as it allows a diversified number of cellular responses and results in a rapid adaptation to the challenging environment (293). However, the molecular mechanisms involved in some of these alternative splicing events, in particular those occurring during T cell activation, are mostly uncharacterized. One of the first described examples of AS in the immune system was the immunoglobulin heavy chain (IgH), which encodes both membrane-associated and secreted proteins through alternative RNA processing reactions (296, 297).

More recently, Lynch and collaborators have identified several AS splicing events in immune cells using a microarray and RNAseq profiling of naïve and PMA (phorbol myristate acetate) stimulated T cells (99, 298).

The AS of exon 8 CD3ζ has an impact on the expression of this protein receptor chain impairing T cell activation as CD3ζ couples Ag recognition with intracellular signaling pathways (299). Also the 3' UTR of CD3ζ has an intron that is preferentially removed in resting T cells and retained upon T cells activation (299). When this intron is removed by AS, two important AU-rich elements (ARE1 and ARE2) are lost at the 3'UTR, reducing the stability and translation of CD3ζ chain pre-mRNA (300). Skipping of this intron has been associated with the autoimmune disease systemic lupus erythematous (SLE). Patients with SLE express more CD3ζ isoform that lacks this 3'UTR intron and consequently have decreased expression of the CD3ζ chain, which compromises T cell signaling and the expression of proliferation cytokine interleukin-2 (IL-2) (301, 302). Nambiar and collaborators have cloned CD3ζ chain gene in a eukaryotic expressing vector and transfected this DNA plasmid into T cells isolated from patients with SLE. They have shown that restoring CD3ζ expression levels by overexpressing CD3ζ, rescue the IL-2 production and decreases the severity of SLE disease (303).

Another example is the already referred CD44 molecule, a cell surface glycoprotein important in cell-cell adhesion, T cell activation, cell migration and metastasis progression (304, 305). CD44 has several alternative isoforms depending on the AS of 10 different variable (v) exons (v1-v10) that code for the extracellular region (306, 307).

It has been shown that T cell stimulation with α-CD3 and α-CD28 antibodies lead to the increased expression of several CD44 alternative spliced isoforms and that the inclusion of the variable exon 5, is regulated by the Mek-Erk signaling pathway (287). Recently, the CD44 variant isoforms CD44v4 and CD44v6 were shown to be augmented

in $CD4^+$ and $CD8^+$ T cells from patients with SLE. Moreover, the expression of these isoforms was correlated with the extent of disease activity and the presence of nephritis (308).

The T cell transmembrane tyrosine phosphatase CD45 is also another well-characterized example of an immune molecule modulated by AS. Exons 4 and 6, that encode part of the extracellular region of CD45, are alternatively spliced in resting T cells: upon T cell activation the majority of the transcripts lack exons 4, 5 and 6, giving rise to the shortest isoform of CD45 (CD45R0) (284, 309). The CD45 extracellular region encoded by these alternative exons is highly glycosylated preventing CD45 homodimerization in a resting state. However, upon T cell activation, the skipping of these alternative exons results in the homodimerization of this phosphatase, which affects T cell signaling through the TCR (310, 311). Lynch and collaborators have identified an important regulatory element, an exonic splicing silencer (ESS) named ESS1, present in CD45 exon 4, which is responsible for exon 4 skipping (312). Interestingly, when a multiple sclerosis-associated polymorphism (C77G) is present in this element, it disrupts the ESS1 leading to a higher level of exon 4 inclusion (284, 312). hnRNP LL and PSF were shown to bind to this ESS1 in activated T cells, thus increasing exon 4 skipping (313-315).

The *IL-7 receptor α chain (IL-7Rα)* pre-mRNA also undergoes alternative splicing giving rise to different protein isoforms and has been implicated in a number of diseases. When a SNP (rs6897932; C/T, Thr244Ile), which is associated with multiple sclerosis, is present in exon 6, this exon is skipped and the receptor loses the transmembrane domain. This results in a decrease of the membrane bound IL-7Rα in PBMCs of multiple sclerosis patients, and consequently an increase in the soluble isoform (316). IL-7Rα is important for the survival of peripheral CD4 and CD8 positive T cells as well as in the generation of a memory phenotype (317, 318). The expression of IL-7Rα has also been related to human immunodeficiency virus (HIV), as CD4 and CD8 positive T cells of HIV-infected individuals expressed low levels of IL-7Rα and presented increased immune activity and cell apoptosis (319). Additionally, Crawley and collaborators have shown that HIV-1- positive individuals have higher levels of soluble IL-7Rα in their plasma correlating with increasing circulating IL-7 levels in this patients, indicating that the soluble receptor could bind IL-7, decreasing the levels of free IL-7 to bind to the transmembrane IL-7 receptor, thus affecting T cell surveillance (320). A higher expression of soluble isoforms of IL-7Rα has also been detected in patients with acute lymphoblastic leukemia (321).

The Fas receptor, also known as CD95, that mediates cell apoptosis when bound to Fas ligand (CD95L) and is expressed in T cells and macrophages (322, 323). *Fas*

receptor produce two different protein isoforms by alternative splicing: a receptor with a pro-apoptotic function is produced when exon 6, which encodes a transmembrane domain, is included. When exon 6 is skipped, a secreted receptor with an anti-apoptotic function is expressed (324, 325). Izquierdo and collaborators showed that exon 6 alternative splicing is regulated by the splicing factors TIA-1/TIAR (T cell intracellular antigen 1/TIA-1 related) and by PTB (Polypyrimidine Tract Binding protein) (181). After Fas receptor triggering, FAST K (Fas Activated Serine/Threonine Kinase) was shown to play an important role in exon 6 inclusion, through TIA-1/TIAR phosphorylation (326).

Upon T cell activation, CTL4 competes with CD28 and binds to CD80/CD83 presented at the APC surface, counteracting the costimulatory signals resulting from the binding of CD28 to CD80/CD83, and thus blocking a prolonged T cell activation (327). In resting T cells, *CTL4* exon 3, that codes for the transmembrane domain, is skipped by alternative splicing from the majority of the transcripts, resulting in the expression of a soluble protein isoform (328). However, upon activation of human blood mononuclear cells with PHA or α-CD3 α-CD28 antibodies, the splicing pattern of CTL4 pre-mRNA is changed, leading to the inclusion of the alternative exon and producing the transmembrane isoform of CTL4 (329). Importantly, expression of CTL4 transmembrane domain on activated T cells resulting from the inclusion of the alternative spliced exon 3, ensures that CTL4 is retained at the T cell membrane, being able to bind to its ligand CD80/CD83 and preventing a hyperstimulated T cell state (329). Accordingly, the soluble CTL4 isoform is expressed in high levels in patients with autoimmune thyroid disease (330) and also in patients with other autoimmune diseases such as acute infectious thyroiditis (AIT), celiac disease, Crohn's disease and biliary cirrhosis (331).

The complete knowledge of the splicing regulation processes in the immune system is therefore fundamental to understanding the normal immune function and also useful to determine the mechanisms behind several immune diseases.

Aims of the thesis

Given the importance of the differential expression of the 3rd extracellular domain of CD6 for the localization of this molecule at the immunological synapse and as CD6 is involved in several autoimmune diseases it became very important to study *CD6* exon 5 alternative splicing. Therefore a better understanding of the regulatory intervenients as well as the T cell signaling pathways involved in this process contribute to dissect the mechanisms that regulates this alternative splicing event. Therefore the main objective of this thesis is to extend the knowledge and understanding of the regulatory mechanisms that dictate the inclusion or exclusion of the alternative exon 5 upon T cell activation with a special focus on:

- The role of RNA *cis*-regulatory elements involved in *CD6* exon 5 AS.
- The role of chromatin state in the regulation of *CD6* exon 5 AS event.
- To identify the splicing factors necessary for *CD6* exon 5 AS.
- To unveil the T cell activation-triggered signaling pathway that regulates *CD6* exon 5 AS.

.

Chapter II

Research Work

T Cell Activation Regulates CD6 Alternative Splicing by Transcription Dynamics and SRSF1

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MATERIAL AND METHODS

Cell isolation and drugs treatment

Buffy coats from healthy donors were provided by Hospital São João, Serviço de Imunohemoterapia, Porto, and peripheral blood mononuclear cells (PBMC) were isolated by density gradient separation using Lympholyte-H (Cedarlane). T cells were isolated from PBMCs using Easysep Human T cell enrichment Kit (Stemcell). Cells were maintained in RPMI 1640 Glutamax with 10% FBS, 1% sodium pyruvate and 1% Penicillin-Streptomycin (Life Technologies). T cells were stimulated for an overnight period using, phytohaemagglutinin-P (PHA-P) at 10 µg/mL, or with a combination of α-CD3 (OK73) at 2 µg/mL and α-CD6 (MEM-98) at 10 µg/mL. In chromatin modulation experiments, 6×10^6 T cells were treated with 0.5 µM Trichostatin A (TSA) or with 10 µM Camptothecin for 1 h at 37 ºC.

Plasmids

To construct the CD6 minigene, the genomic DNA region from exon 4 to exon 6 was amplified by PCR and cloned into the pCMVdi vector (kindly given by Juan Valcarcel, Centre de Regulació Genòmica, Barcelona). Intron 4 mutants were made by PCR site-directed mutagenesis using Phusion DNA polymerase (Fynnzymes). T7 epitope tagged vectors pCGT7SF2, pCGT7SRp20 and pCGT7A1 used for overexpression of the splicing factors SRSF1, SRSF3 and hnRNPA1 respectively, were a kind gift from Javier Cáceres, MRC, Edinburgh.

Directed Mutagenesis

The PCR amplification of CD6 minigene was carried out in a 100 µl reaction, using Phusion DNA polymerase from Finnzymes and the respective primer for each mutant (Table 2). PCR conditions were: 1 min at 98^{o}C for initial denaturation, followed by 29 cycles: [10 sec at 98^{o}C; 30 sec at the respective annealing temperature; 3 min at 72^{o}C] and 10 min at 72^{o}C for final extension. In each PCR reaction, one of the primers was phosphorylated to create a phosphate group in the 5' end allowing the DNA fragment to ligate. After PCR amplification, 1 µl of *Dpn* I enzyme was added to the PCR reaction

products to digest the methylated plasmid template, incubating 1h at 37^{o}C. All the enzymes and buffers were removed by Phenol-Chloroform extraction and DNA was Ethanol precipitated. Pellet was resuspended in 8 µl distillated water. DNA ligation was performed adding 1 µl of T4 DNA ligase from Invitrogen to 4 µl of precipitated DNA in 10 µl reaction with 1x ligase buffer. The mixture was incubated overnight at 14^{o}C. DH5α competent Bacteria from Invitrogen were transformed with 2 µl of ligation product and then plated on LB rich medium. Plates were incubated overnight at 37^{o}C and clones were sent for sequencing.

Transfections

Cells were transfected by nucleofection using the Amaxa human T cell nucleofactor kit (Lonza). 2 µg of each CD6 minigene mutant or T7 tagged expression vectors were used for cell transfections (Figure II.14b). For the knocking-down experiments, PBMCs were transfected with siRNAs (Sigma) (Table 1) at a final concentration of 50 nM for SRSF1 and SRSF3, and 300 nM for hnRNPA1 (Figure II.15b and c). Total RNA and protein extracts were isolated after 48 h of incubation at 37 ºC, 5 % CO_2.

Cell fractionation and RNA extraction

Cells were washed twice with ice-cold PBS and centrifuged for 5 min at 290 × g at 4 ºC. The cell pellet was resuspended in 1 mL of RSB buffer (10 mM Tris-Cl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$) and incubated 3 min on ice. Cells were pellet at 1500 × g for 3 min at 4 ºC, the supernatant was discarded and the cells were lysed by gentle resuspension in 150 µL of RSBG40 buffer (10 mM Tris-Cl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 10 % glycerol, 0.5 % NP-40). Samples were centrifuged at 4500 × g for 3 min at 4 ºC. The supernatant (cytoplasmic fraction) was collected in a new eppendorf and 1 mL of Trizol was added to extract cytoplasmic RNA according to manufacturer's protocol. The nuclei pellet was also resuspended in 1 mL of Trizol to extract the nuclear RNA.

RT-PCR

Total RNA from human primary T cells was isolated using Trizol (Invitrogen) and 500 ng of RNA per each condition were treated with DNAse I (Roche). cDNA was synthetized using

Superscript III reverse transcriptase (Invitrogen) according to manufacturer's protocol. 25 % of the RT reaction volume was used to analyze endogenous *CD6* exon 5 alternative splicing pattern by PCR amplification with Go Taq DNA polymerase (Promega). In the case of CD6 minigene mutants, cDNA was synthesized with a plasmid -RT primer and radiolabeled primers were used in a low cycle (20 cycles) PCR reaction. qPCR reactions were performed with a 1:10 cDNA dilution using IQ SYBR Green Supermix (Biorad) and following the manufacturer's instructions. Primer sequences are in Table 2. Due to donor variability, the resting *CD6* exon 5 splicing pattern was confirmed prior to analysis.

Western Blotting

Whole-cell lysates were prepared, resolved and transferred with the iBlot gel transfer device (Life Technologies). Incubations with primary antibodies diluted in Tris-Buffered Saline, 0.1% Tween 20 (TBS-T) containing 3 % non-fat dried milk were followed by washes with TBS-T, incubation with the appropriate secondary antibodies in TBS-T/dried milk and by detection using enhanced luminescence, ECL Prime (Amersham/GE Healthcare). Antibodies used: anti-SRSF1 (AK96, a kind gift from Adrian Krainer, Cold Spring Harbor Laboratory), anti-hnRNP A1 (9H10, kind gift from Gideon Dreyfuss, Howard Hughes Medical Institute), and anti-SRp20 (7B4 – sc13510, Santa Cruz Biotechnology).

Chromatin Immunoprecipitation (ChIP) and RNA Immunoprecipitation (RIP)

Chromatin immunoprecipitation was performed using human T cells as previously described (332). The relative occupancy of the immunoprecipitated protein at each DNA site was estimated as follow: $2^{Ct\ (input)–Ct\ (IP)}$ where Ct (input) and Ct (IP) are mean threshold cycles of qRT-PCR done in duplicate on DNA samples from input and specific immunoprecipitations, respectively. Gene-specific and intergenic-region primer pairs are presented in Table 2, supplementary data. Antibodies used: rabbit polyclonal anti–Pol II (N20, Santa Cruz Biotechnology); anti–histone H3 (ab1791, Abcam); anti-H3K36me3 (ab9050, Abcam); anti-H3K9me3 (ab8898, Abcam), and anti-H3K9Ac (ab10812, Abcam).
RNA immunoprecipitation was performed using the EZ-Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Merck Millipore). A rabbit monoclonal antibody anti-SRSF1 (ab133689, Abcam) was used. The relative occupancy of the immunoprecipitated protein at each RNA site was estimated as described above for the ChIP assay.

Micrococcal Nuclease assays (MNase)

The assay was performed as described (333) using resting or PHA-stimulated primary human T cells. DNA was digested with 10 U of micrococcal nuclease (MNase, Fermentas) for 5, 10 and 15 min before the addition of a stop buffer. Mononucleosome-sized DNA was obtained after 15 min of digestion, by gel purification of the band with approximately 200 bp, and non-digested (t0) were used for RT-qPCR (32). The amount of MNase-resistant DNA at each gene region analyzed was estimated as follows: $2^{(Ct\ t0\ -\ Ct\ t20)}$, where Ct t0 and Ct t20 are mean threshold cycles of RT-qPCR done in duplicate on DNA samples from non-digested (t0) and 20 min MNase-digested (t20) samples, respectively. Results were further normalized to the amount of MNase-resistant DNA measured with primers for an intergenic region (estimated using the same $2^{(Ct\ t0\ -\ Ct\ t20)}$ formula). The sequences of gene-specific and intergenic primers are shown in Table 2.

UV-crosslinking Immunoprecipitation assays

5000 cpm of intron 4 pre-mRNA probes were incubated in 50 µl reactions mixtures containing 100 µg of Peripheral Blood Leucocytes nuclear extracts, 32 mM HEPES (pH 7.9), 1.56 mM $MgCl_2$, 0.5 mM ATP, 20 mM Creatine Phosphate and 2.6 % polyvinyl alcohol. Samples were incubated at 30^0 C for 20 min, UV-cross-linked in Hoefer UVC 500 UV Crosslinker (254 nm, for 9 min, 4 cm from light source) and treated with 5 µg RNase A (1 µg/µl) at 37^0 C for 30 min. A 10% aliquot of the cross-linked samples was loaded on a 12% SDS-PAGE. For immunoprecipitation, 100 µl of tissue culture supernatant of monoclonal α-SRSF1 antibody (AK96) and 35 µl Protein A sepharose beads 50% slurry (Sigma # CL-4B-200) was added to 80% cross-linked material, 0,1 M KCl Buffer D was added to a final volume of 200 µl. For hnRNP A1 and SRSF3 Immunoprecipitation it was used 10 µl of α-hnRNPA1 antibody (ab5832) and 25 µl of α-SRSF3 antibody (SC-13510). The mixture was incubated overnight at 4^0 C in a rotative wheel. Beads were washed two times with ice cold Binding Buffer I (20 mM HEPES pH 7.9, 150 mM NaCl, 0,05% Triton X-100) and two times with ice cold Binding Buffer_II (20 mM HEPES pH 7.9, 150 mM NaCl, 1% Triton X-100). After the last wash, the supernatant was completely removed and beads were resuspended in 35 µl 2x SDS-loading dye and boiled for 5 min at 95^oC. After centrifugation the supernatant was loaded on a 12% SDS-PAGE. Gels were fixed for 35 min at room temperature in a fixing solution before drying. Gels were exposed to a radiographic film [128].

Flow cytometry

10^6 per condition were washed with PBS and incubated for 20 min on ice and protected from light with FITC conjugated antibody α-CD69 at 1:50 dilution in FACS Buffer (PBS, 0,1% BSA and 0,1% Azide). Cells were washed twice with FACS buffer and resuspended in 500 µl of the same buffer for posterior flow cytometry analysis. Fluorescence for 10,000 live cells was collected on a FACSCanto (BD Bioscience California, USA) and the data were analyzed using FlowJo software (Treestar, Ashland, OR).

Immunofluorescence

Cell were washed with PBS and resuspended in RPMI 1640 without FBS, and 3 x 10^5 cells per condition were let to adhere to poly-lysin coated glass coverslips for 30 min at 37ºC, and then fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were washed 3 times with PBS and permeabilized with PBS containing 1% Triton-X100. After 30 min blocking at room temperature with PBS containing 10% FBS and 0,1% Triton-X100, cells were incubated for 1h with primary antibody, anti-SRSF1 (AK96) monoclonal antibody at 1:50 dilution. Cells were washed 3 times with PBS and incubated for 1h with secondary antibody anti-mouse, Alexa Flour 568 conjugate (Molecular Probles) at 1:500 dilution. Cells were mounted in Vectashield medium containing DAPI (Vector Laboratories). Stained preparations were observed with an AxioImager Z1 microscope (Carl Zeiss), and images acquired with Axiocam MR v.3.0 camera (Carl Zeiss). Images were processed with Image J program (334).

Table 1 – Sequences of the siRNAs oligonucleotides used in the knockdown experiments.

	siRNA (5'-3')	siRNA_as (5'-3')
SRSF1	ACGAUUGCCGCAUCUACGU (dT)	ACGUAGAUGCGGCAAUCGU (dT)
SRSF3_siRNA1	CGAAGUGUGUGGGUUGCUA (dT)	UAGCAACCCACACACUUCG (dT)
SRSF3_siRNA2	GAGAAGUGGUGUACAGGAA (dT)	UUCCUGUACACCACUUCUC (dT)
hnRNP A1_siRNA1	CCACUUAACUGUGAAAAAGAUAUUU (dT)	AAAUAUCUUUUUCACAGUUAAGUGG (dT)
hnRNP A1_siRNA2	CUUUGGUGGUGGUCGUGGA (dT)	UCCACGACCACCACCAAAG (dT)

Table 2 – Sequences of the primers used in mutagenesis, semi-quantitative and quantitative PCR.

	Forward Primer (5'-3')	Reverse Primer (5'-3')
ChIP CD6 Ex1	CAAGATGGTGCTTCCCACAG	CCAGTGATCCCGAAGAAGAG
ChIP CD6 Ex4	GGGCCGAAGCTTACCTGT	CTCTTTGTGGCCGCAGTAGT
ChIP CD6 Ex5	CTTGTCCGGCAGGATGTACT	TGCAGAGGTTGGAGTTGTTG
ChIP CD6 Ex6	TCCCTGCAAGTGTTCAGACA	AGCTTCTGAGAAACCGCGTA
ChIP CD6 Ex7	AAGAAGAAGGGTGAGTGCCC	GTCACAGAAGATTCTGGAAACAAGA
ChIP CD6 I4	GAGGAGGCTGTTGGTCTGTC	CCCCATCTCCTGCTTATCAA
Intergenic	GGCTAATCCTCTATGGGAGTCTGTC	CCAGGTGCTCAAGGTCAACATC
SRSF1	CATGCGTGAAGCAGGTGATG	TCCGTACAAACTCCACGACA
SRSF3	ACGGAATTGGAACGGGCTTT	CAAAGCCGGGTGGGTTTCTA
hnRNP A1	CAGGGGCTTTGGGTTTGTCA	CCTTGTGTTGGCCTTGCATTC
GAPDH	CAAATTCCATGGCACCGTCAA	ATGGTGGTGAAGACGCCAGTG
18S	GCAGAATCCACGCCAGTACAAGA	CCCTCTATGGGCCCGAATCTT
qPCR CD6 Full length	GTGTGCTCAGAGCACCAGTCC	CTCGGAAGTGTACCTCCACCTG
qPCR CD6Δd3	CGGTGTGCTACGCTTCCCG	CTATAGTGACTGTCTGAACACTTGCAGC
RIP_A	GCATTCAAGCAGCAAGGGG	CAAAGAGAGGCACTGGCTGGTC
RIP_B	GACCAGCCGGTGCCTCTCTTT	ACTCTGGAGCTCTGCCTCAG
RIP_C	CTGCGTTTCTAACAAGATCCCAG	TGTGATAAGTGTGGTCTGT
Endogenous CD6 PCR	ACTACTGCGGCCACAAAGAG	GGAGCATTAGCTCCCGAGATT
Minigene PCR	ACTACTGCGGCCACAAAGAG	GCTTATCGATACCGTCGACCTCG
i4D1	TGGCTGCATTGGTGCCTGC	(Phos)-CCTGACTGCCTTTCTTGGG
i4D6	TGGCTGCATTGGTGCCTGC	(Phos)-TGGCCAACTCCCACTCGGG
i4D6 A	GACCAGCCGGTGCCTCTCTTT	(Phos)-TGGCCAACTCCCACTCGGG
i4D6 B	CTGCGTTTCTAACAAGATCCCAG	(Phos)-CAGAACCTTCTGGCCCCACCTGG
i4D6 C	TGGCTGCATTGGTGCCTGC	ACTCTGGAGCTCTGCCTCAG
i4D6 AB	CTGCGTTTCTAACAAGATCCCAG	(Phos)-TGGCCAACTCCCACTCGGG
i4D6 BC	TGGCTGCATTGGTGCCTGC	(Phos)-CAGAACCTTCTGGCCCCACCTGG
i4D6 D	GCAGGGTTGGCCAGAGCAAATG	(Phos)-TCAGAATCTACATTTTAACACC

RESULTS

Modulation of chromatin structure affects CD6 exon 5 AS in human primary T lymphocytes

It has been shown that skipping of exon 5 of *CD6* is induced by T cell activation, resulting in an increase of the *CD6Δd3* mRNA isoform, which is translated into a CD6 polypeptide that lacks the domain of interaction with its ligand, CD166 (schematically represented in Figure II.1) (65).

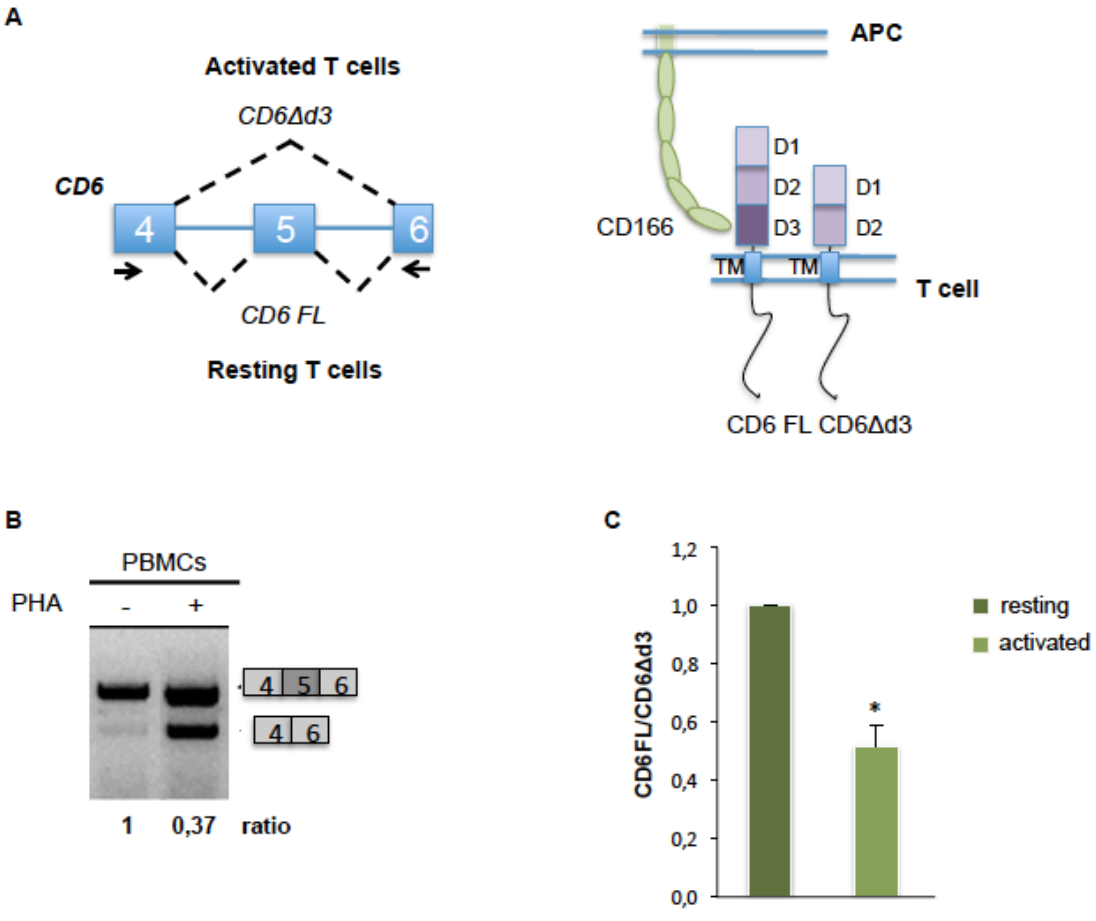

Figure II.1 – CD6Δd3 is upregulated upon T cell activation. (A) Schematic representation of *CD6* exon 5 alternative splicing pattern in resting and activated T cells (left panel). Representation of the proteins encoded by CD6 full-length (CD6 FL) and CD6Δd3 alternative splicing isoform, CD6Δd3, protein representation (right panel). (**B)** Semi-quantitative RT-PCR analysis of *CD6* exon 5 alternative splicing pattern in resting and PHA-stimulated PBMCs. The ratio between both mRNA isoforms (*CD6FL/CD6Δd3*) was calculated and then normalized to resting condition **(C)** Graphic representation

showing the ratio between exon 5 containing isoform (FL) and the isoform that lacks exon 5 (Δd3) in resting and activated state. Error bars represent s.e.m and asterisk is statistical significant (p<0,05 student's t-test), n=3.

To investigate the molecular mechanisms regulating T cell activation-dependent alternative splicing of *CD6*, we first confirmed the pattern of exon 5 skipping in human peripheral blood mononuclear cells (PBMCs) activated with the mitogenic lectin phytohemagglutinin-P (PHA-P) at 10 µg/ml. As expected, PHA induced a switch in the exon 5 alternative splicing pattern, resulting in a 2-fold decrease of the ratio between the CD6 full-length mRNA isoform (*CD6FL*) and the isoform omitting exon 5 (*CD6Δd3*) (Figure II.1B and C). This switch is also observed in $CD4^+/CD8^+$ T cells when stimulated with PHA or with α-CD3 and α-CD6 antibodies (Figure II.2A). Different concentrations of PHA (5,10,15 and 20 µg/ml) were tested and the expression of the activation marker CD69 was analyzed by flow cytometry (Figure II.2B). The shift observed in the expression of CD69 indicates that T cells are activated with all the PHA concentrations tested and 10 µg/mL were then used in all activation conditions.

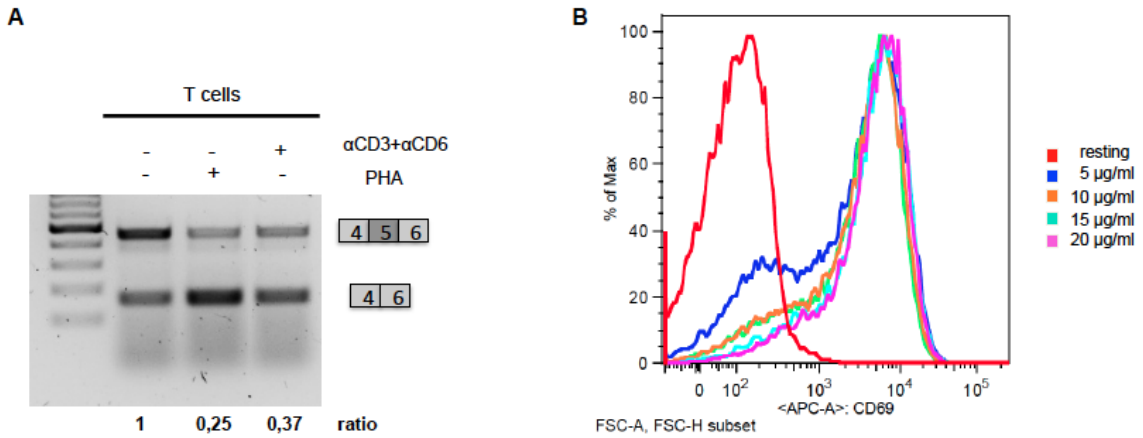

Figure II.2 - T cell activation and *CD6* exon 5 alternative splicing pattern. (A) Semi-quantitative RT-PCR analysis of *CD6* exon 5 alternative splicing pattern in resting, PHA stimulated and αCD3/αCD6 stimulated primary $CD4^+/CD8^+$ T cells. The ratio between both mRNA isoforms (*CD6FL/CD6Δd3*) was calculated and then normalized to resting condition **(B)** Histogram representing CD69 expression in resting and T cells activated with different concentrations of PHA.

It was also apparent that activation with PHA induced an increment in the levels of the full-length mRNA isoform, suggesting a general transcriptional induction (Figure II.1B). To understand how transcription of *CD6* was regulated upon T cell activation, we purified T lymphocytes from peripheral blood and analyzed changes in RNA Pol II occupancy in the *CD6* gene, using chromatin immunoprecipitation (ChIP), occurring in resting and activated T cells and we also analyzed *CD6* expression levels by RT-qPCR in the same conditions (Figure II.3).

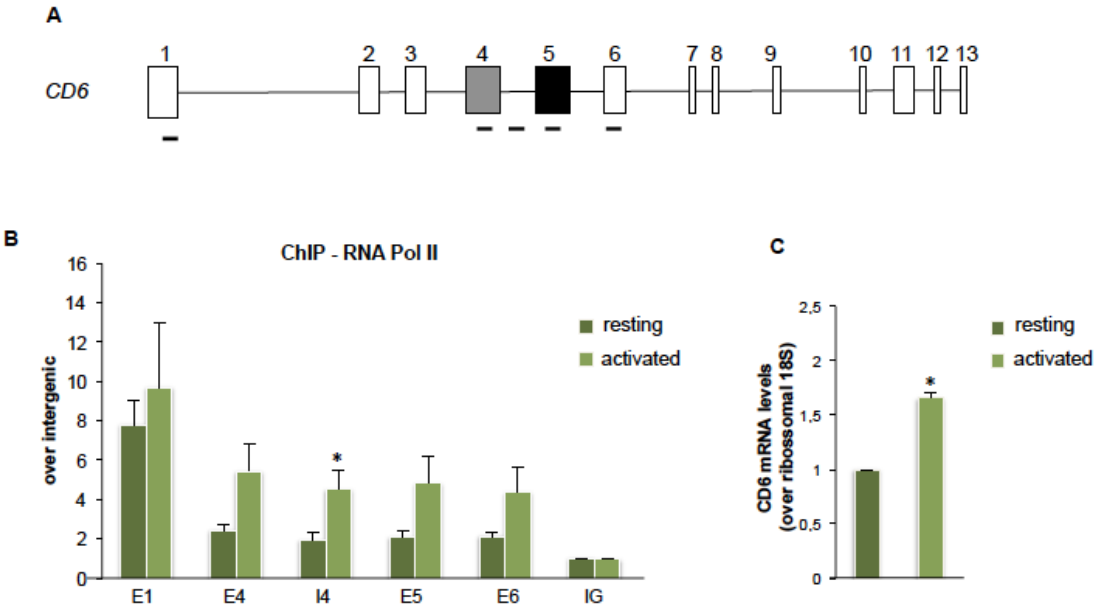

Figure II.3 – Characterization of RNA pol II occupancy in the *CD6* gene and its expression levels in resting and activated T cells. (A) Schematic representation of human *CD6* locus, exons are represented by boxes and introns are shown as a thin line. The region for specific qPCR primers pairs is underlined and primers sequences are in table 2 of the Materials and Methods section. **(B)** Graphic representation of RNA polymerase II occupancy levels in *CD6* gene in resting and activated T cells evaluated by Chromatin Immunoprecipitation assays showing an increase of RNA polymerase II occupancy upon T cell activation (n=5). IG-intergenic region **(C)** *CD6* expression levels in resting and activated PBMCs were analysed by qPCR revealing an increase in expression upon cell activation with PHA (n=3). Error bars represent s.e.m and asterisk is statistical significant ($p<0,05$, student's t-test).

Upon T cell activation an overall increase of RNA Pol II occupancy throughout the *CD6* gene was observed (Figure II.3B), concurring with an increase in CD6 expression (Figure II.3C). As exon-definition is in part determined by increased nucleosome

positioning in exons (262), we assessed nucleosome occupancy of the *CD6* gene using micrococcal nuclease assay (MNase) that consists in the enzymatic digestion of chromatin at the mononucleosome level. Using specific primers for the different exons we performed qPCR analysis (Figure II.4B). Although the overall nucleosome occupancy did not change between resting and activated T cells, it is clear that exon 5 possesses higher levels of nucleosomes than exon 4, 6 and 7 (Figure II.4B). This could suggest a poor definition of exon 5, which may be due to weak splice sites as described by Tilgner *et al.* (263), causing alternative splicing of this exon.

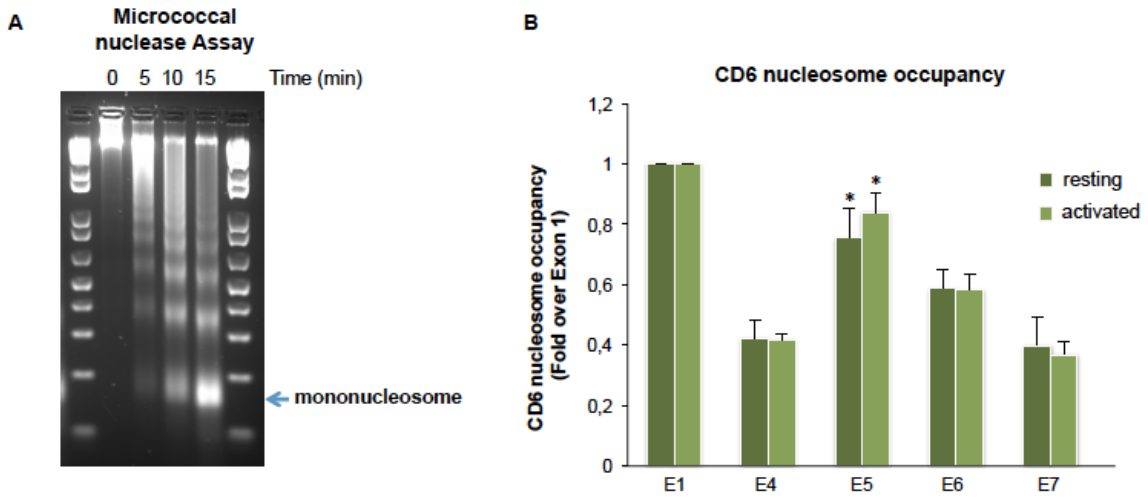

Figure II.4 – Characterization of *CD6* nucleosome occupancy in resting and activated T cells. (A) T cells chromatin digested in different time periods (5,10 and 15 min) was loaded and run in an agarose gel. It is visible that after 15 min of digestion the majority of the DNA fragments have 100-200 bp (mononucleosome) **(B)** qPCR analysis to determine the nucleosome occupancy levels on different exons of the *CD6* gene revealed by micrococcal nuclease (MNase) digestion assay**.** Primer sequences are in table 2 of the Material and Methods section. Error bars represent s.e.m and asterisk is statistical significant ($p<0,05$, Student's t-test).

Accumulating evidence indicates that alternative splicing is influenced by chromatin histone modifications (266), in particular H3K36me3 and H3K9me3 (207, 271). To investigate whether these regulatory mechanisms could also govern *CD6* exon 5 alternative splicing, we analyzed by ChIP the pattern of H3K36me3 and H3K9me3 in the *CD6* gene, in resting and activated T cells. H3K36me3 levels were increased in the body

of the gene comparing with the first exon and the intergenic region (Figure II.5A), as it occurs in actively transcribed genes (335) indicating that CD6 is being actively transcribed. The H3K9me3 levels that were described to be enriched in a subset of alternative exons (271) were decreased in the body of the *CD6* gene in comparison with the intergenic region (Figure II.5B). However, there were no differences in H3K36me3 and H3K9me3 levels either between resting and activated T cells, or comparing exon 5 with the neighbouring exons. This indicates that these epigenetic marks are not modulated in the *CD6* gene by T cell activation and they do not have a function in *CD6* exon 5 alternative splicing.

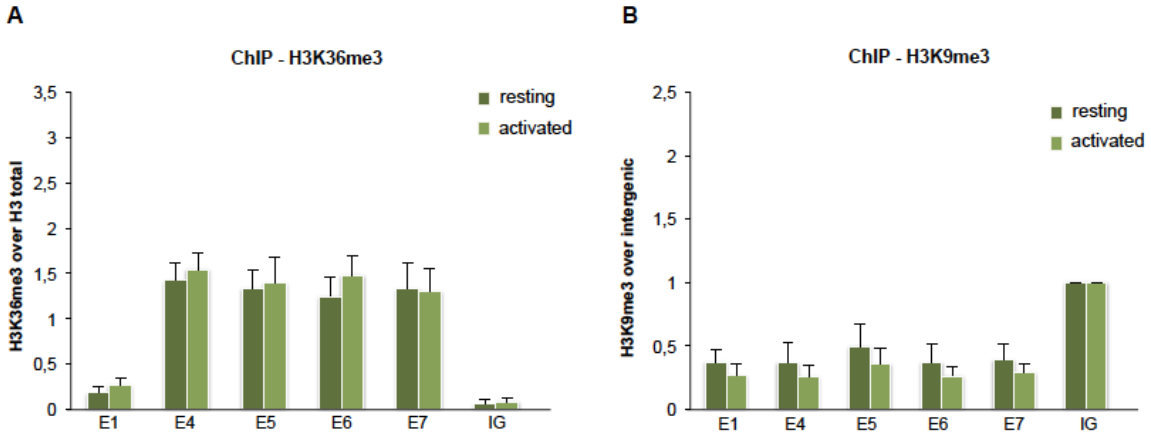

Figure II.5 – H3K36me3 and H3K9me3 marks in *CD6* gene. Chromatin Immunoprecipitation (ChIP) with H3K36me3 (A) and H3K9me3 (B) antibodies, using primer pairs for the *CD6* gene as in figure II.3A. IG-intergenic region.

Changes in chromatin acetylation levels between resting and activated T cells have been already shown (336). We thus characterized H3K9ac levels in *CD6* in resting and activated T cells (Figure II.6A). In contrast with the methylation marks analyzed in Figure II.5, we detected an overall increase in H3K9Ac in activated T cells. These differences are statistically significant at the 5' end of the gene and also in exon 5. Treatment of HeLa cells with inhibitors of histone deacetylases (HDAC) have been shown to cause alterations in the splicing pattern of alternatively spliced exons (337). We thus hypothesized that upon T cell activation the *CD6* gene could undergo alterations in the chromatin structure, in particular in H3K9ac, to facilitate the accessibility of the transcription machinery resulting in the increased *CD6* mRNA levels and exon 5 skipping observed. To investigate

the role of acetylation in this mechanism we treated T cells with an inhibitor of HDACs (Trichostatin A). An increase in the levels of transcripts lacking exon 5 was observed (Figure II.6B), suggesting that acetylation and an open chromatin state promotes skipping of exon 5. To further understand the role of the chromatin structure and Pol II elongation rate on *CD6* alternative splicing, we treated T cells with an inhibitor of topoisomerase I, Camptothecin, which hampers RNA Pol II elongation, the opposite effect in the alternative splicing pattern was observed: inclusion of exon 5 was promoted (Figure II.6B). We quantified both *CD6* mRNA isoforms and confirmed that TSA as well as Camptothecin have significant but opposite effects in the splicing pattern of exon 5 (Figure II.6C), suggesting that the RNA pol II transcription rate plays a determinant role in the skipping or inclusion of exon 5 in *CD6* pre-mRNA.

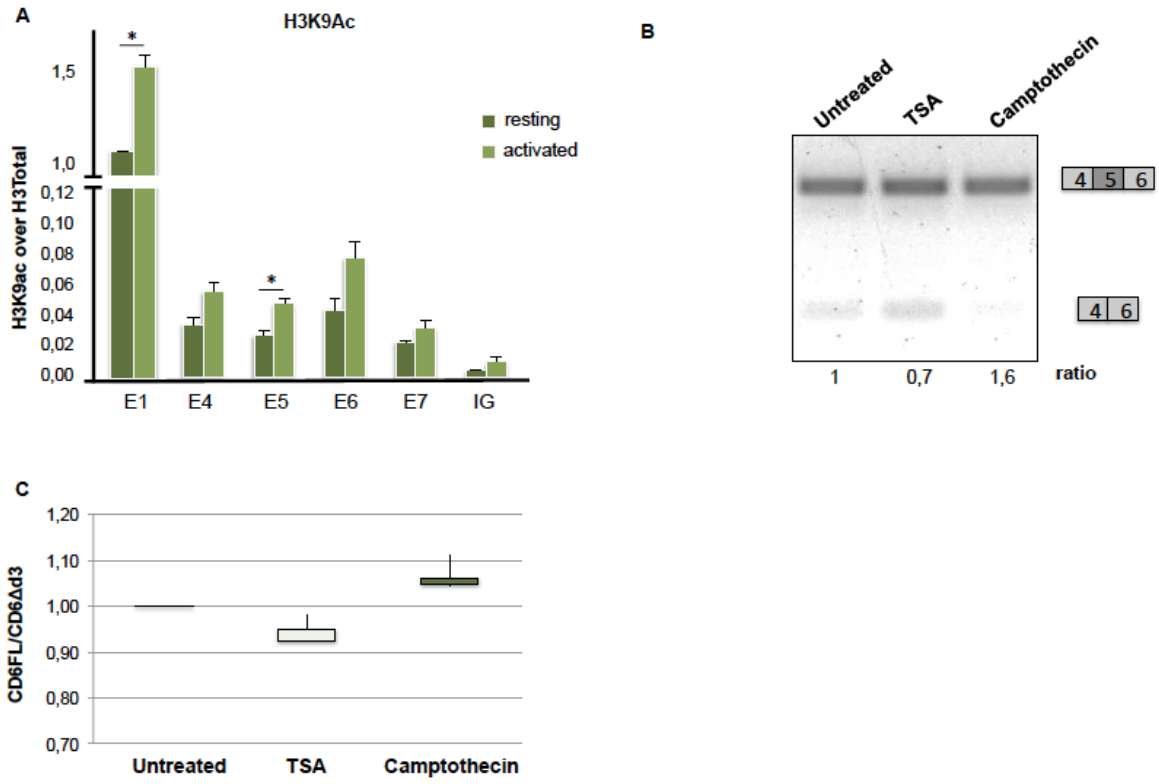

Figure II.6 – *CD6* H3K9ac levels in resting and activated T cells and *CD6* chromatin structure modulation. **(A)** Graphic representation of H3K9ac levels in *CD6* gene in resting and activated T cells evaluated by ChIP showing an increase of acetylation upon T cell activation. Primer pairs for the *CD6* gene are depicted in figure II.3A. IG-intergenic region **(B)** Semi-quantitative RT-PCR showing the Trichostatin A and Camptothecin effect in *CD6* exon 5 AS pattern. The ratio between both isoforms (*CD6FL/CD6Δd3*) was calculated and then normalized to untreated condition **(C)** *CD6* mRNA isoforms expression levels were analysed by qPCR. Primers were designed to span the 4-5 and 4-

6 exon junction regions. Error bars represent s.e.m and asterisk is statistical significant ($p<0,05$, student's t-test), n=3.

CD6 intron 4 contains a complex set of splicing regulatory elements

Using the UCSC Genome Browser we found several peaks of conservation in intron 4, distant from the splice sites. These are conserved in 46 species of vertebrates, suggesting the presence of important regulatory elements (Figure II.7).

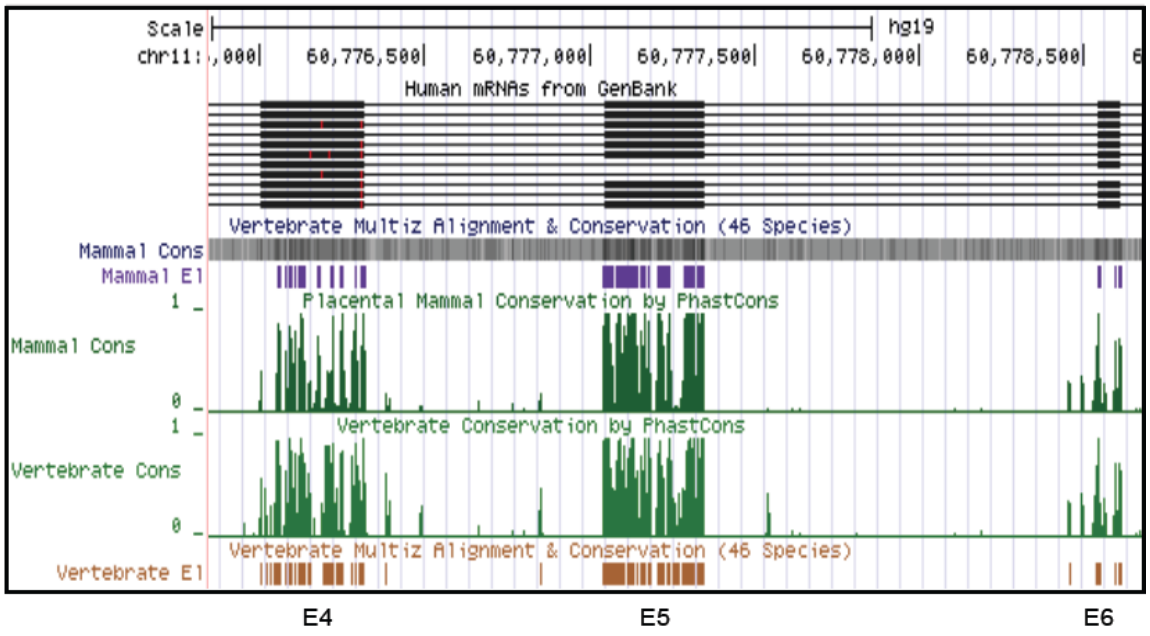

Figure II.7 – *In silico* analysis of *CD6* exon 5 genomic region conservation (a) Conservation analysis (comparative genomics tool, UCSC genome browser), of *CD6* exon 5 genomic region revealed several peaks of conservation in intron 4.

We therefore generated a functional minigene, containing the genomic fragment spanning exons 4 to 6, to characterize the *cis* elements involved in *CD6* alternative splicing (Figure II.8A). Importantly, when transfected into PBMCs, this minigene recapitulates the alternative splicing pattern of the endogenous *CD6* gene, with the majority of transcripts containing exon 5 (Figure II.8B, left panel). Moreover, upon PHA-induced T cell activation there is the same enrichment of *CD6Δd3* over *CD6FL* as

observed in endogenous *CD6* (Figure II.8B, right panel). This indicates that the minigene contains all the necessary sequences to promote *CD6* exon 5 alternative splicing and can be used to characterize the *cis* regulatory elements involved.

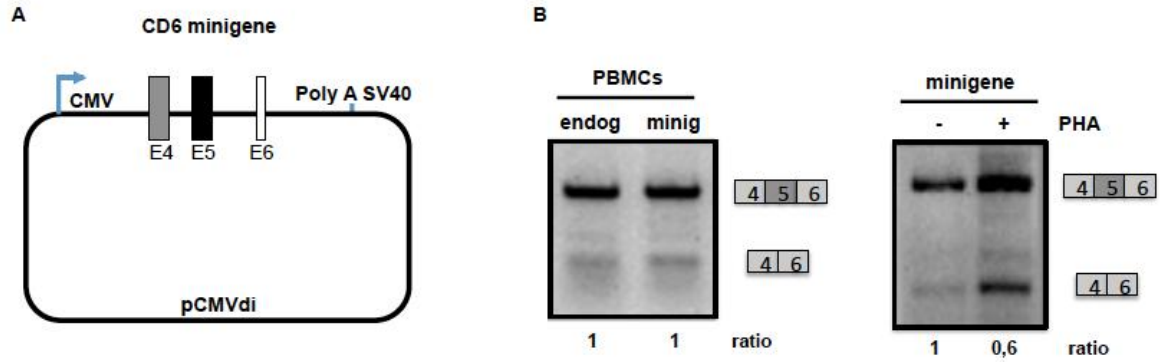

Figure II.8 – Analysis of *CD6* exon 5 alternative splicing using a minigene system **(A)** The genomic region from exon 4 to exon 6 was cloned into a mammalian expression vector carrying a CMV promoter and a SV40 polyadenylation signal. **(B)** Peripheral blood mononuclear cells of healthy donors were transiently transfected with the *CD6* minigene and the exon 5 alternative splicing pattern of minigene and endogenous transcripts was analysed by RT-PCR. PHA-stimulation of minigene transfected cells reproduced *CD6* exon 5 activation-induced alternative splicing. To distinguish the minigene transcripts from *CD6* endogenous transcripts a plasmid-specific primer was used in the RT reaction. The ratio between both mRNA isoforms (*CD6FL/CD6Δd3*) was calculated and then normalized to resting condition. Primers sequences are in table 2 of the Materials and Methods section.

To characterize the conserved region in intron 4, we engineered the minigene to introduce specific deletions originating the constructs depicted in figure II.9a. Minigene mutant i4Δ1, containing a 436 nt deletion in the central region of intron 4 (nt 193 to 629) (Figure II.9A), was transfected into resting T cells and the splicing pattern was analysed by RT-PCR. A complete switch in exon 5 alternative splicing was observed (Figure II.9B), with over a 3-fold reduction in the mRNA isoforms ratio (relative increase of *CD6Δd3* over *CD6FL*) mean of 3 independent experiments (Figure II.9D).

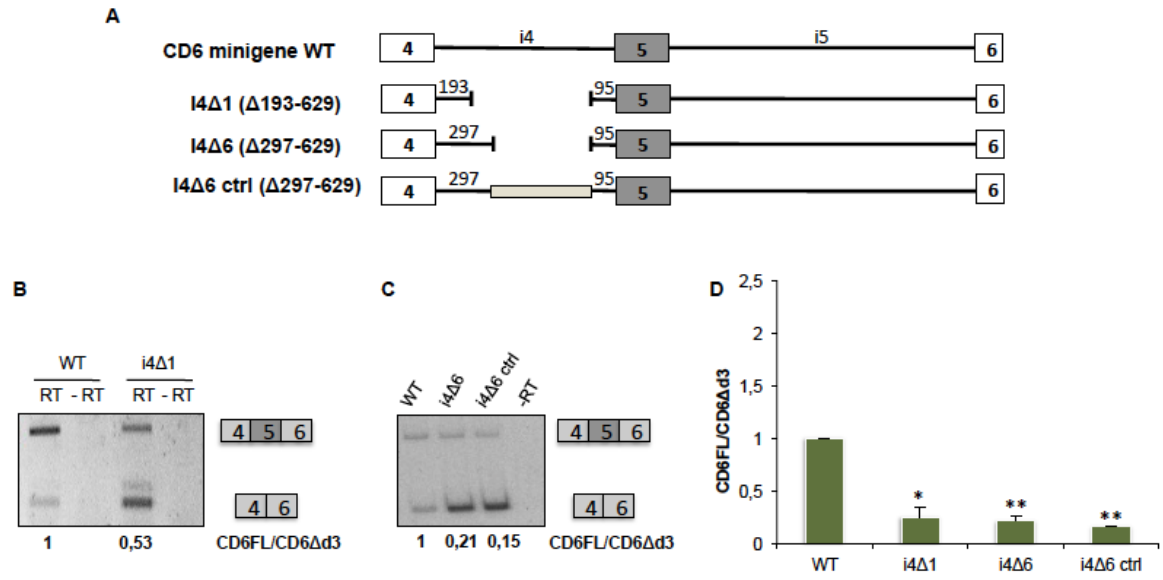

Figure II.9 – *CD6* intron 4 contains an intronic splicing enhancer (ISE). (A) Different mutants, each one having different deletions in intron 4 were created by PCR-directed mutagenesis and used for transient transfection in human T cells. **(B)** and **(C)** RT-PCR analysis of *CD6* exon 5 AS pattern**.** The ratio between both isoforms (*CD6FL/CD6Δd3*) was calculated and then normalized to *CD6 wild type (WT)* **(D)** Graphic representation of the *CD6FL/CD6Δd3* ratios in intron 4 mutants and *WT* minigene determined by semi-quantitative RT-PCR. Error bars represent s.e.m and asterisks are statistical significant (* p<0,05, ** p<0,01 student's t-test), n=3.

A mutant having a smaller deletion (i4Δ6) induced a statistically significant similar reduction, indicating that an intronic splicing enhancer (ISE) for exon 5 inclusion is contained within the region between nt 297 and 629 of intron 4 (Figure II.9C and D). This is a sequence-specific effect, as confirmed by replacement of the i4 297-629 sequence by an unrelated sequence, derived from part of the intron of RG6 minigene (338) (i4Δ6 crtl) (Figure II.9C and D).

To fine map the ISE identified, three mutants containing deletions of approximately 100 nt were generated, each one lacking one third of the i4Δ6 deleted region (A, Δ297-383; B, Δ384-481; C, Δ482-629), and transfected into T cells (Figure II.10A). Deletion of any of the three regions (A, B and C) induced only a partial 2-fold reduction in the *CD6FL*/*CD6Δd3* ratio when compared with wild-type intron 4 (Figure II.10B and C), which suggests that several regulatory elements may be present.

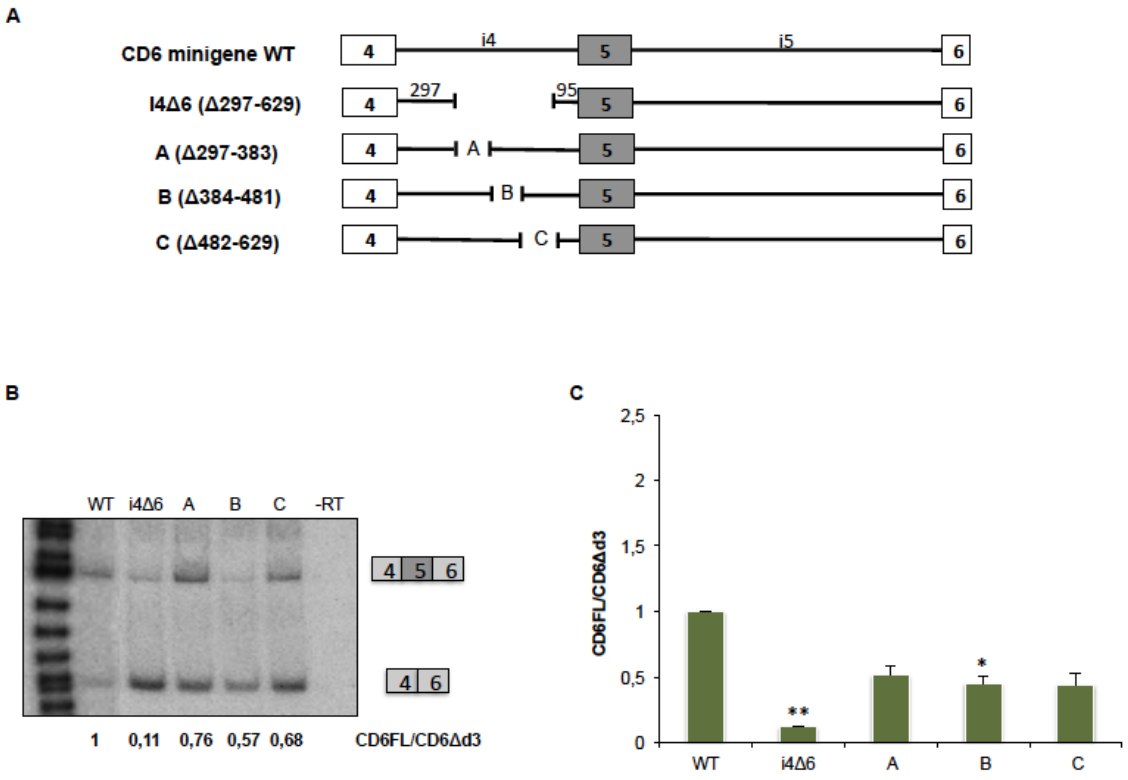

Figure II.10 – Characterization of the ISE in *CD6* intron 4. (A) Different mutants, each one having different deletions in intron 4 were created by PCR-directed mutagenesis and used for transient transfection in human T cells. **(B)** RT-PCR analysis of *CD6* exon 5 AS pattern**.** The ratio between both isoforms (*CD6FL/CD6⊿d3*) was calculated and then normalized to *WT* condition **(C)** Graphic representation of the *CD6FL/CD6Δd3* ratios in intron 4 mutants and *WT* minigene determined by semi-quantitative RT-PCR. Error bars represent s.e.m and asterisks are statistical significant (* p<0,05, ** p<0,01; student's t-test), n=3.

To further dissect this regulatory region, mutant minigenes having combined or overlapping deletions in the nt 297-629 region were generated (Figure II.11A), transfected in T cells and their splicing pattern analyzed (Figure II.11B and C). Deletion of nt 297 to 481 (AB) induced a reduction of nearly 3-fold in the *CD6FL*/*CD6Δd3* isoforms ratio, comparing with wild-type i4. In addition, deleting regions A and C together introduced a 2-fold reduction in the *CD6FL*/*CD6Δd3* ratio (Figure II.11C). By contrast, deletion of regions B and C together in mutant BC (Δ383-629) did not induce any significant differences in the ratio comparing to the WT (Figure II.11C) suggesting that both a silencer and an enhancer elements coexist in region BC as contrasting with the effect (increased exon 5 skipping) of deleting regions B or C independently (Figure II.10C). We therefore constructed an

additional mutant (D, Δ451-544) whose deleted sequences comprise part of region B and part of region C. Interestingly, this deletion causes a significant increase in exon 5 inclusion (2-fold increase in the *CD6FL*/*CD6Δd3* ratio), suggesting that it contained an inhibitory sequence for exon 5 inclusion (Figure II.11C).

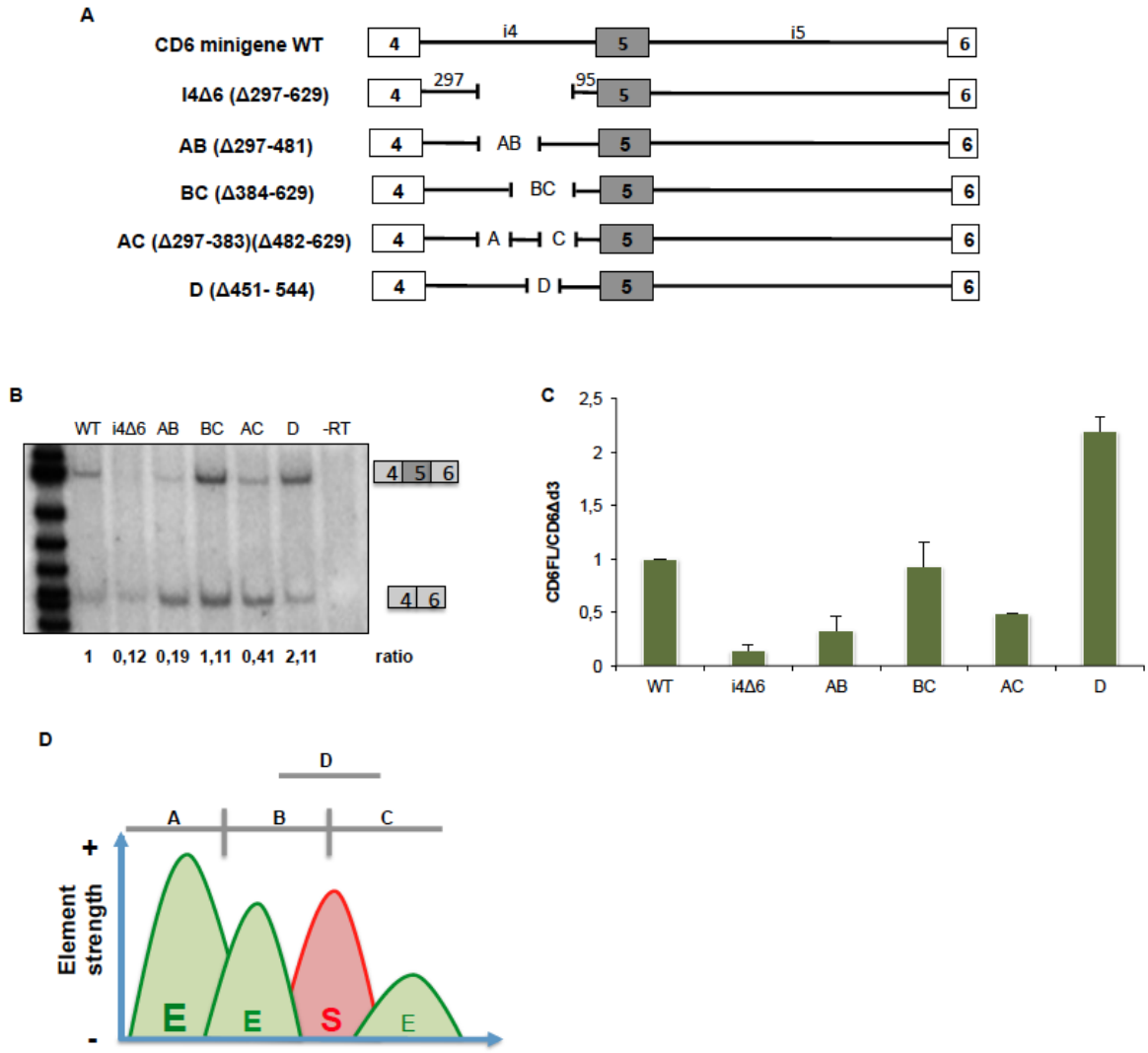

Figure II.11 – Mapping of regulatory regions in intron 4. (A) Different mutants, each one having different deletions in intron 4 were created by PCR-directed mutagenesis and used for transient transfection in human T cells. **(B)** RT-PCR analysis of *CD6* exon 5 AS pattern in *WT* and intron 4 mutants. The ratio between both mRNA isoforms (*CD6FL/CD6Δd3*) was calculated and then normalized to *WT* **(C)** Graphic representation of the *CD6FL/CD6Δd3* mRNA ratios in intron 4 mutants and *CD6 WT* minigene determined by semi-quantitative RT-PCR. Error bars represent s.e.m (n=2). **(D)** Schematic representation of intron 4 regulatory elements. E – enhancer; S- silencer.

CD6 exon 5 alternative splicing is regulated by SRSF1, SRSF3 and hnRNPA1

As the alternative splicing of *CD6* exon 5 is regulated by T cell activation, we investigated variations in the expression of relevant splicing factors in resting and activated T cells, by RT-qPCR and immunoblotting. In order to identify possible splicing factors that could bind to the ISE present in *CD6* intron 4 we performed and *in silico* analysis using SFmap tool (http://sfmap.technion.ac.il/index.html). We found that within the nt 297-629 regulatory sequence in intron 4 there are several putative binding sites for splicing factors such as SRSF1, SRSF2, SRSF3, SRSF5, SRSF6, hnRNPA1, hnRNPAB, hnRNPH/F and PTB as determined by bioinformatics analyses (Figure II.12). We focused on those presenting the highest SFmap algorithm scores, ie SRSF1 (average score: 0,8), SRSF3 (average score: 0,7) and hnRNPA1 (average score: 0,9) with a cutoff value of 0,6.

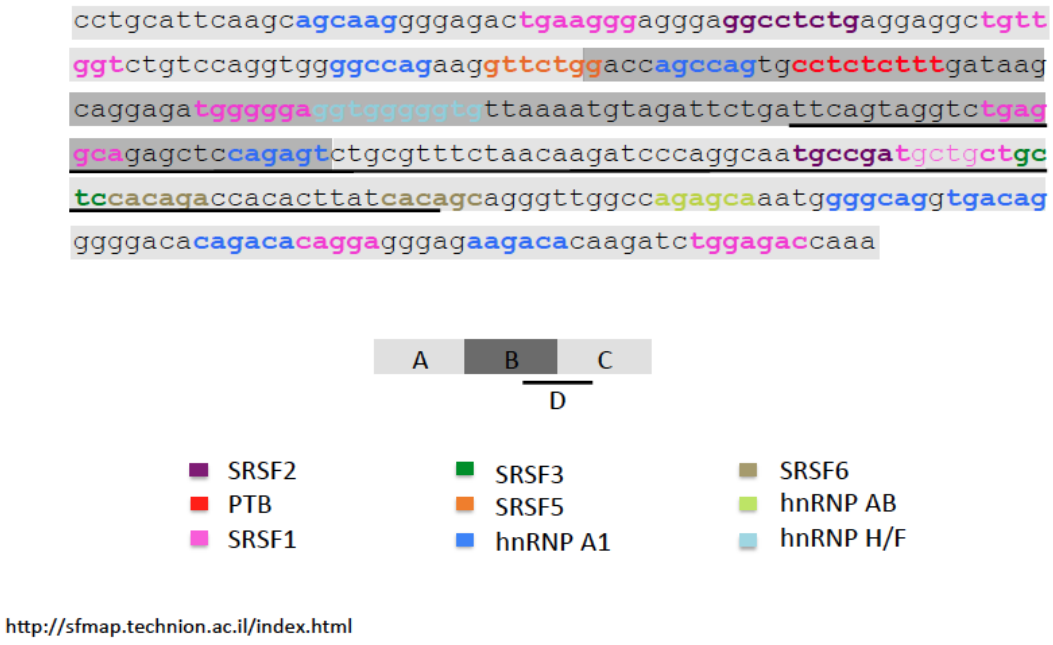

Figure II.12 – Putative binding sites for RNA binding proteins in the region deleted in i4Δ6. *In silico* analysis using the SFmap bioinformatics tool (http://sfmap.technion.ac.il/index.html) revealed putative binding sites for several splicing factors, represented in different colours.

Upon PHA-induced T cell activation, SRSF1 mRNA and protein levels were decreased by ~50 %, however there were no significant alterations in mRNA or protein expression levels of either hnRNP A1 or SRSF3 (Figure II.13A and B).

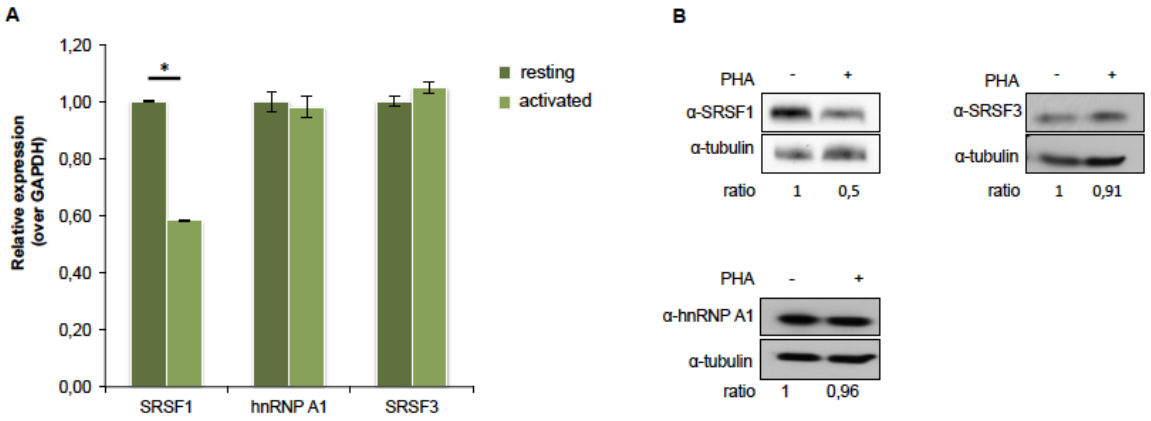

Figure II.13 – SRSF1 expression decreases upon T cell activation. (A) Quantitative PCR analysis of *SRSF1*, *hnRNP A1* and *SRSF3* expression levels in resting and activated T cells. **(B)** SRSF1, hnRNP A1 and SRSF3 protein levels in resting and activated T cells. Error bars represent s.e.m and asterisks are statistical significant (* p<0,05, student t-test), n=3.

To investigate the role of the differential SRSF1 levels observed upon T cell activation in *CD6* alternative splicing, we used overexpression and siRNA depletion assays in PBMCs (Figure II.14 and II.15). Remarkably, overexpression of SRSF1 resulted in 75% reduction in the expression of the *CD6Δd3* isoform (Figure II.14A). Overexpression of hnRNPA1 and SRSF3 also induced significant changes in *CD6Δd3* expression (1.5-fold increase and 2-fold decrease, respectively), suggesting that changes in their protein expression, when existent, may also affect *CD6* alternative splicing (Figure II.14A).

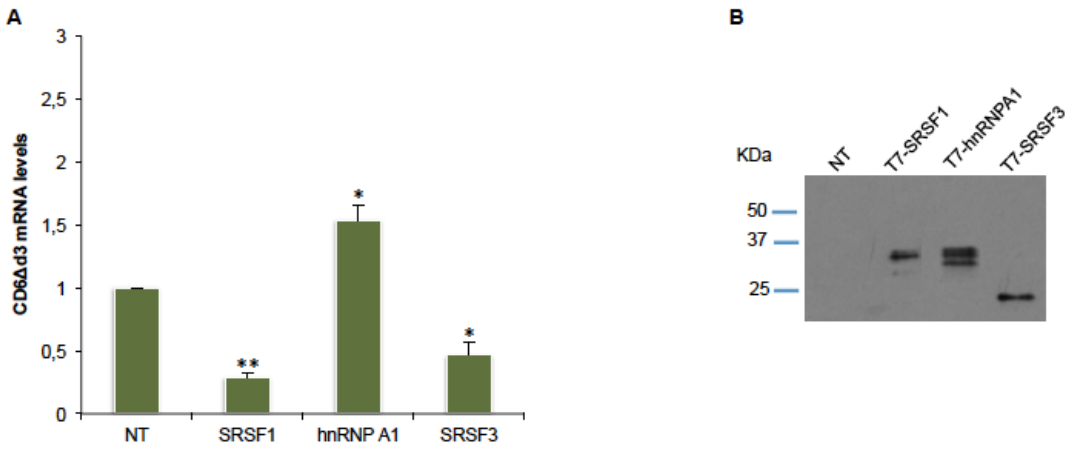

Figure II.14 – Effect of SRSF1, SRSF3 and hnRNP A1 overexpression on *CD6Δd3* mRNA levels. (A) Graphic representation of the quantification of *CD6Δd3* mRNA isoform

expression levels upon SRSF1, hnRNP A1 or SRSF3 overexpression in PBMCs. **(B)** 48 hours after transfection with T7-epitope-tagged plasmids containing the respective cDNA sequence to overexpress SRSF1 (pCGT7SF2,), hnRNP A1 (pCGT7A1) and SRSF3 (pCGT7SRp20), cell lysates were prepared for immunoblotting with an anti-T7 epitope antibody. Error bars represent s.e.m and asterisks are statistical significant (* p<0,05, ** p<0,01, student's t-test), n=3.

To confirm these results, the same splicing factors were siRNA knocked-down individually (Figure II.15A and B). To knockdown SRSF1 we used only one siRNA oligonucleotide sequence, the same that has been used by Das and collaborators (339), however to knockdown hnRNP A1 two different siRNA oligonucleotides were used as it has been described by Huelga and collaborators (340).

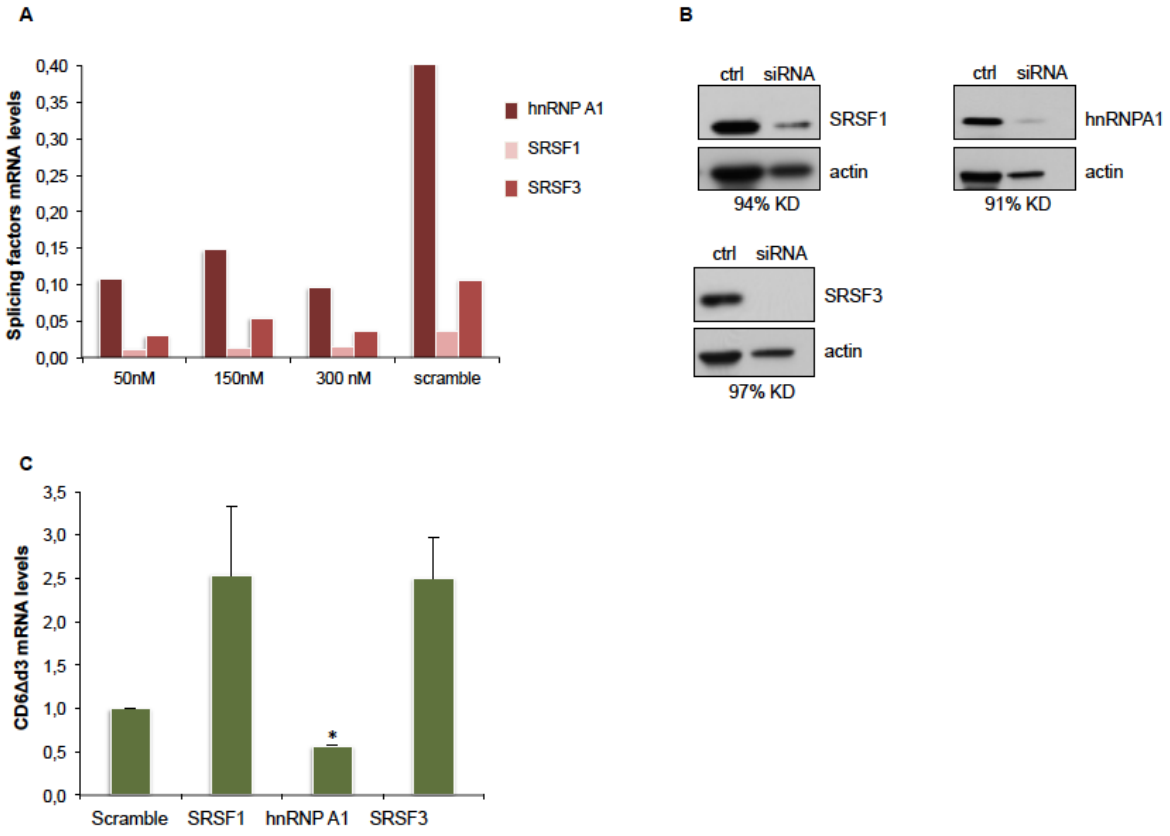

Figure II.15 – Effect of SRSF1, hnRNP A1 and SRSF3 knockdown on *CD6Δd3* mRNA levels. (A) *SRSF1*, *hnRNP A1* and *SRSF3* mRNA levels upon transfection of siRNAs at three different concentrations (50, 150 and 300 nM). **(B)** SRSF1, SRSF3 and hnRNP A1 protein levels upon siRNAs transfection and respective knockdown efficiency. Ctrl (control) – cells transfected with scramble siRNA **(C)** Graphic representation of the quantification of *CD6Δd3* mRNA isoform expression levels upon SRSF1, hnRNP A1 or

SRSF3 knockdown. Error bars represent s.e.m and asterisks are statistical significant (* p<0,05, student's t-test), n=3.

In order to decrease SRSF3 expression levels we also used two different siRNA oligonucleotides that have been designed by Sigma Aldrich. Cells were transfected with three different concentrations of siRNAs (50, 150 and 300 nM) and the mRNA expression levels of SRSF1, hnRNP A1 and SRSF3 were analyzed by qPCR (Figure II.15A).

The chosen conditions were 50 nM of siRNA to target SRSF1, 50 nM of the combination of both sequences that target SRSF3 and 300 nM of the combination of both sequences that target hnRNP A1 and then knockdown efficiencies were analyzed by western blot (Figure II.15B). In relation to the SRSF1, hnRNP A1 and SRSF3 knockdown results, we observed a conversed effect in *CD6⊿d3* isoform expression (Figure II.15C). Taken together these results indicate that these 3 splicing factors, SRSF1, hnRNP A1 and SRSF3 play an important role in the regulation of *CD6* exon alternative splicing more concretely SRSF1 and SRSF3 are important for exon 5 inclusion whereas hnRNP A1 is important for exon 5 skipping.

SRSF1 and hnRNP A1 bind to *CD6* intron 4 ISE

It was important to understand if the three splicing factors described above, having an effect in *CD6* exon 5 alternative splicing regulation, could bind to the intronic splicing enhancer (ISE) identified in *CD6* intron 4. Therefore, UV crosslinking and immunoprecipitation assays were performed using the sequence deleted from i4Δ6 as a pre-mRNA template, nuclear protein extracts prepared from PBMCs and antibodies against SRSF1, hnRNP A1 and SRSF3 (Figure II.16A). As it can be seen, the splicing factor SRSF1 binds to this element in intron 4 (Figure II.16B). We also detected the binding of hnRNP A1 to the intron 4 ISE however we could not detect the binding of SRSF3 to this region (Figure II.16B).

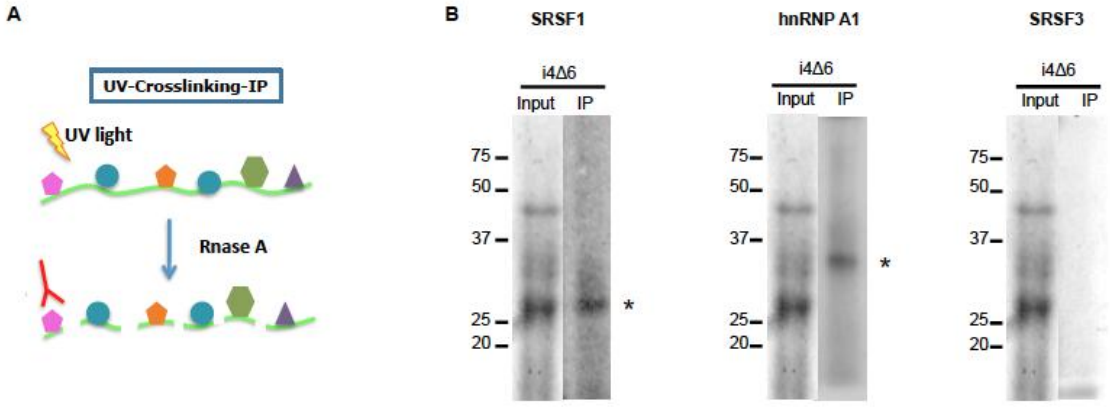

Figure II.16 – SRSF1 and hnRNP A1 bind to the ISE in intron 4. (A) Schematic representation of UV-crosslinking and immunoprecipitation assay. PBMCs nuclear extracts were incubated with ^{32}P-radiolabeled RNA template and cross-linked by UV light. RNase A digested all the RNA sequence that was not bound to protein. Using a specific antibody it was possible to immunoprecipitate the protein of interest. **(B)** UV-crosslinking and immunoprecipitation with specific antibodies showing SRSF1 and hnRNP A1 binding to the i4Δ6 sequence. Asterisks point out the band of the immunoprecipitated protein.

SRSF1 recruitment to *CD6* primary transcript is affected my chromatin hyperacetylation

Given the observed decrease in SRSF1 expression upon T cell activation and the effect of this factor on *CD6* exon 5 AS, we hypothesized that the recruitment of SRSF1 to the regulatory element of intron 4 was the limiting factor for exon 5 inclusion. To investigate this we performed RNA immunoprecipitation (RIP) with a specific antibody for SRSF1 and *CD6* primers targeting the ISE of intron 4 (Figure II.17A). Indeed, upon cell stimulation with PHA, a marked decrease of the recruitment of SRSF1 to the intron 4 was evident when compared with untreated cells (Figure II.17B), which could be due to the decreased expression of SRSF1 in activated T cells.

As it has been previously shown (Figure II.6A) T cell activation induces an increase of *CD6* acetylation levels and TSA treatment *per se* induces exon 5 skipping (Figure II.6B). Thus, we asked if chromatin acetylation could have an impact in SRSF1 recruitment. We therefore treated PBMCs with TSA and performed RIP as previously. Importantly, the recruitment of SRSF1 to *CD6* pre-mRNA was also prevented by TSA treatment (Figure II.17B).

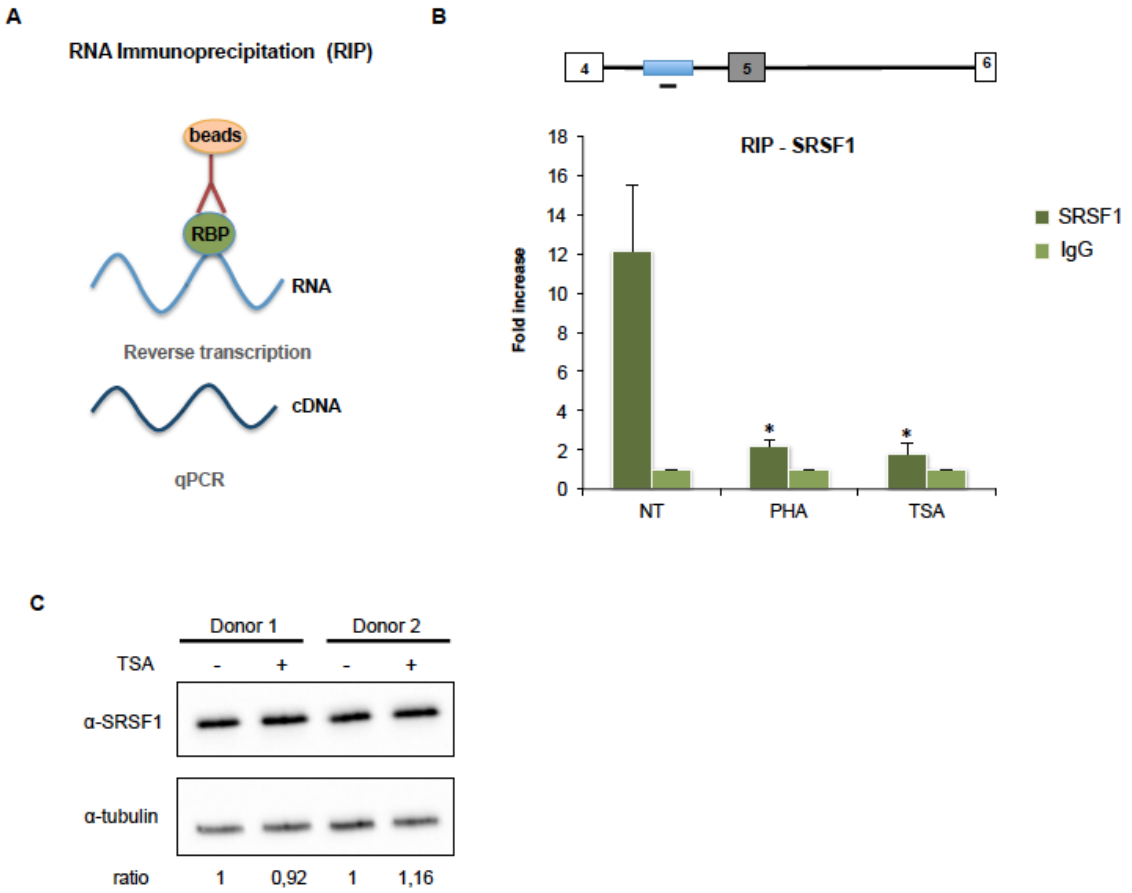

Figure II.17 – SRSF1 is less recruited to *CD6* pre-mRNA upon T cell activation and this effect is acetylation dependent. (A) Schematic representation of RNA Immunoprecipitation assay (RIP). A RNA binding protein (RBP) is immunoprecipitated together with it the target RNA. After RNA isolation and cDNA synthesis a qPCR analysis is done using specific primers **(B)** RIP showing a decrease in SRSF1 recruitment levels to the *CD6* primary transcript in PHA or TSA treated PBMCs (* $p<0,05$, student's t-test), n=3. **(C)** SRSF1 protein expression levels determined by western blot do not change in untreated (-) and TSA treated (+) T cells.

As TSA induces chromatin hyperacetylation this result indicates that an increased chromatin acetylation level also impairs SRSF1 recruitment to *CD6* pre-mRNA or that TSA could have a direct effect in SRSF1 expression leading to a decrease of SRSF1 levels in treated cells. To investigate this possibility we analyzed SRSF1 protein levels in untreated and TSA treated T cells isolated from healthy donors. We observed no significant changes in the expression of this splicing factor upon TSA treatment (Figure II.17C). This confirms that the diminished recruitment of SRSF1 to *CD6* pre-mRNA observed by RIP assays

(Figure II.17 B) is not due to alterations in SRSF1 protein levels upon T cell activation, but that the increased chromatin acetylation levels drastically decrease this recruitment.

Some splicing factors have been shown to lose their nucleoplasm localization and localize at nuclear speckles upon induction of chromatin acetylation (259), therefore we performed immunofluorescence microscopy to investigate if there was any differential nuclear localization of SRSF1 upon T cell activation. After T cells activation with PHA, it is visible that SRSF1 is more agglomerated in bright dots in the nucleus and less scattered at the nucleoplasm in comparison to resting T cells (Figure II.18).

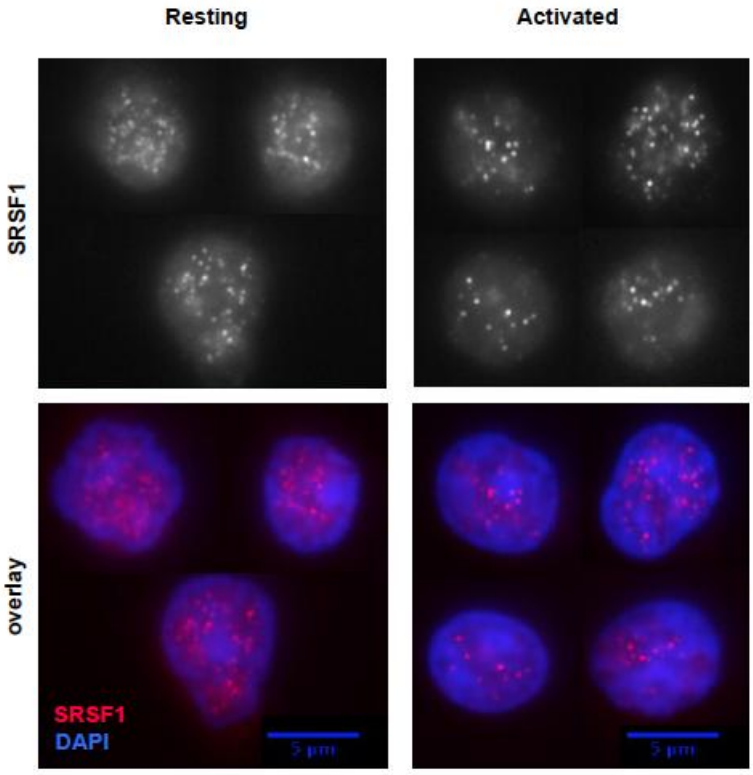

Figure II.18 – SRSF1 nucleoplasmic localization decreases upon T cell activation. Immunostaining for endogenous SRSF1 (in red) in human primary T cells. DNA was stained with DAPI (blue).

This effect on SRSF1 intranuclear localization is probably due to the increased chromatin acetylation induced by T cell activation and it is possible that this delocalization of SRSF1 modulates *CD6* exon 5 alternative splicing.

Overall, our results indicate that not only SRSF1 is less expressed upon T cell activation, but also that it is less recruited to an ISE present in intron 4 of the *CD6* primary transcript, by a mechanism that is dependent on chromatin acetylation levels, all resulting in a significant increase in exon 5 skipping.

The ERK pathway is involved in the regulation of *CD6* exon 5 alternative splicing

Alternative splicing is extensively regulated in the immune system, but the T cell signaling pathways directly involved are still poorly understood. To identify the signaling cascades that regulate splicing and culminate in *CD6* exon 5 skipping upon T cell activation, we stimulated T cells in the presence of inhibitors of key effectors of different signaling pathways (Figure II.19A), then isolated total RNA and analysed the splicing pattern of CD6 exon 5 in each condition. First, we used the Src family kinase (SFK) specific inhibitor PP2 (4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) (341). The conditions were optimized using three different concentrations (0,5; 2 and 20 μM) (Figure II.19B). As previously shown, activation with PHA induced a marked decrease in the *CD6FL*/*CD6Δd3* mRNA ratio that is visualized by the increment of the *CD6Δd3* mRNA isoform (Figure II.19B). However, using PP2 this effect was partially and completely cancelled at 2 and 20 μM PP2, respectively. This indicates that Lck or Fyn, or eventually both, are critical mediators of the signals that induce exon 5 skipping.

In order to deepen the identification of the signaling intervenients in the regulation of *CD6* exon 5 AS, we used other inhibitors: cyclosporin A (CsA) that inhibits calcineurin (342), U0126 that inhibits MEK1/2 (343), wortmannin (WMN) that inhibits PI3K (344) and farnesylthiosalicylic acid (FTS) that inhibits Ras signaling (345). Downstream and radiating from the SFK-mediated effects, it seems that no single pathway is fully accountable for the regulation of *CD6* alternative splicing, although it appears that calcineurin, PI3-kinase and Ras are not involved (Figure II.19C and D). Using a MEK1/2 inhibitor (U0126), exon 5 skipping was partially cancelled with a reduction in mRNA isoforms ratio of up to 2-fold (Figure II.19C and D). These results indicate that the ERK pathway is involved in the delivery of T cell activation signals to the splicing machinery involved in *CD6* exon 5 alternative splicing.

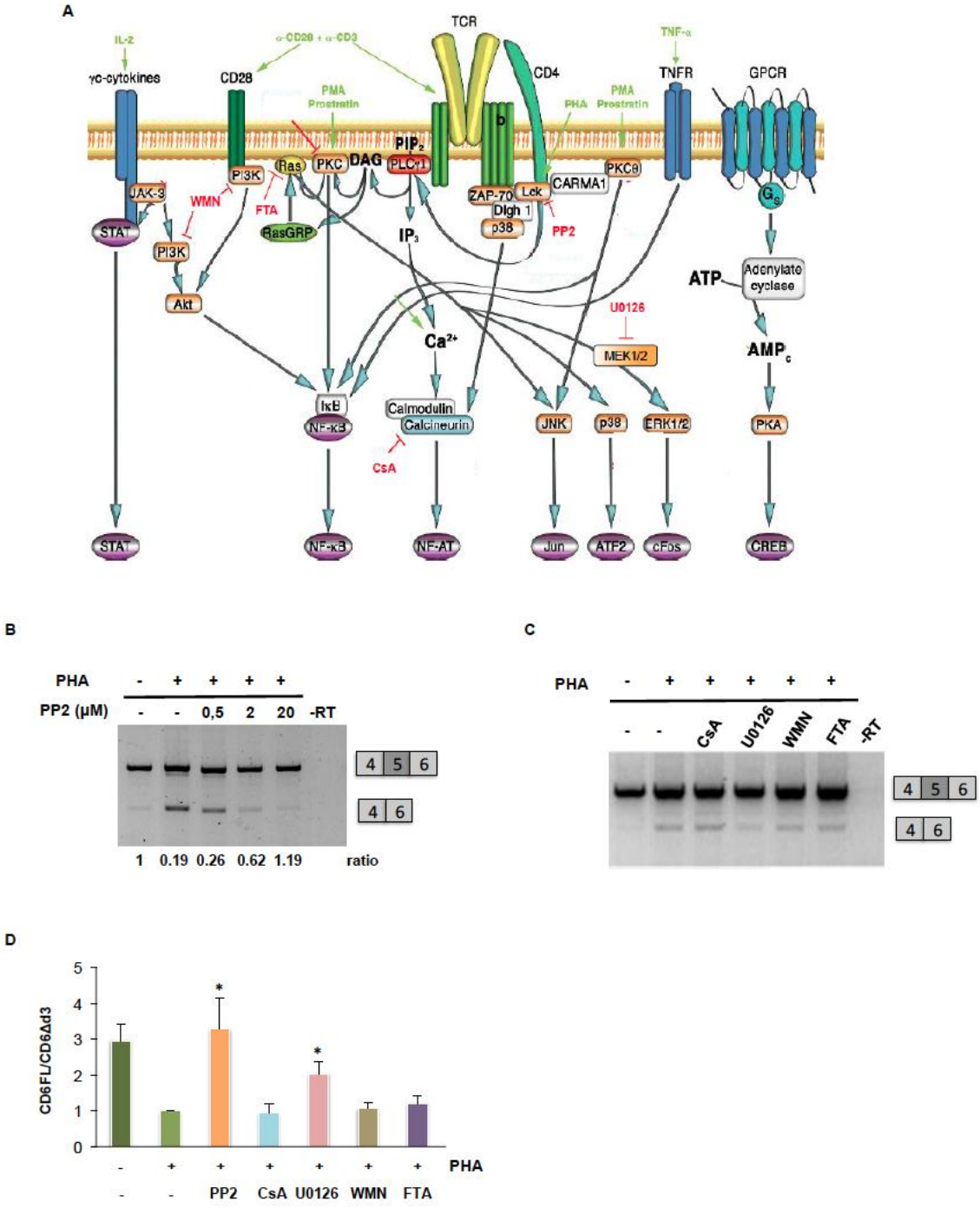

Figure II.19 - *CD6* exon 5 activation-induced alternative splicing is mediated by src-kinases and the ERK pathway. (A) T cell signaling pathways indicating the inhibited signal intervenients and respective inhibitors (image adapted from Bosque and Planelles 2009 (346) **(B) and (C)** Semi-quantitative RT-PCR analysis of *CD6* exon 5 AS pattern in resting, PHA activated and signaling inhibited PBMCs**.** The ratio between both mRNA isoforms (*CD6FL/CD6⊿d3*) was calculated and normalized to the resting cells condition. **(C)** Graphic representation of *CD6FL/CD6Δd3* ratio quantification in PBMCs treated with 2 μM PP2, 10 μg/ml cyclosporin A, 10 μM U0126, 50 nM wortmannin, 75 μM

farnesylthiosalicylic acid (FTA). Error bars represent s.e.m and asterisk is statistical significant (* p<0,05 student's t-test), n=3.

Chapter III

Discussion

DISCUSSION

Upon antigen recognition, the balance between activatory and inhibitory signals determines the activation state of a T cell. The first type of signals include the affinity of a given T cell receptor to a specific antigenic peptide presented in the context of MHC complexes displayed at the surface of antigen presenting cells, and also all the signaling machinery that relays the information received at the cell surface down to the nucleus. This consists on a well-structured pathway that sequentially activates effector enzymes and adapters, such as the Src-family kinases Lck and Fyn that phosphorylate the ITAMs of the CD3 complex, the kinase ZAP-70 that upon binding to phosphorylated ITAMs becomes activated and phosphorylates the membrane adapter LAT, which in turn serves as a docking platform for many downstream effectors such as PLC-γ, PI3-kinase, GADS, Grb2/Sos1 connecting to the Ras-MAPK pathway, Itk and SLP-76 among others (347).

On the other hand, there are a few receptors such as CD5 and CD6 that limit TCR-mediated responses at the onset of activation. In the case of CD5, this is achieved through the phosphorylation-dependent recruitment of signaling inhibitory enzymes such as the phosphatase SHP-1, the ubiquitin ligase Cbl, and the Ras GTPase-activating protein (348, 349). The kinases that promote these associations by phosphorylating tyrosine residues in the cytoplasmic tail of CD5 are the same Lck and Fyn that induce positive signaling *via* the TCR/CD3 complex (350, 351). Regarding CD6, it is still not clear how signaling attenuation is achieved, as none of the CD5-associated inhibitors have been reported to associate with CD6. On the contrary, upon activation and phosphorylation, CD6 becomes packed with effector enzymes and adapters such as Lck, Fyn, ZAP-70, Itk and SLP-76, all connoted with productive signaling and full T cell activation (33, 36).

One hypothesis to explain the inhibitory properties of CD6 is that the receptor may function as a decoy adapter, having high affinity for the signaling mediators and thus removing much of the activation potential away from the sites of TCR-mediated signaling initiation. On this line, it is attractive to ponder that upon T cell activation, to promote the skipping of the exon that encodes the ligand-binding domain is a very enticing way of removing this inhibitory receptor away from the immunological synapse and thus favor a stronger stimulus. The present thesis focused on the identification of *CD6* exon 5 alternative splicing regulatory elements and factors that are induced following T cell activation and have a striking impact on the subcellular localization of CD6, or more appropriately, on the alternative isoform CD6Δd3.

Although this strategy to remove inhibition from the sites of activation may appear complex and intricate, the molecular mechanisms utilized to induce exon 5 skipping revealed to be surprisingly and paradoxically simple.

The role of chromatin structure modulation upon T cell activation in *CD6* exon 5 alternative splicing

The importance of chromatin structure in alternative splicing regulation has been extensively documented, in particular the impact that these structural changes have in RNA polymerase II elongation rate. Compact chromatin structures can difficult RNA Pol II transcription whereas a relaxed chromatin structure is in principle more permissive to transcription, representing an open path for RNA Pol II along the gene.

In other to find out if *CD6* gene could undergo transcriptional changes we performed gene expression analysis that revealed an increase of *CD6* expression due to T cell activation. This result that was also corroborated by Chromatin Immunoprecipitation analysis that showed an augmented RNA Pol II occupancy upon T cell activation (Figure II.3).

This increased RNA Pol II occupancy on the *CD6* gene led us to hypothesize, that a T cell activation-dependent chromatin structural change could occur. Performing chromatin immunoprecipitation assay using an antibody against H3K9ac we observed a boost in histone acetylation levels on the *CD6* gene after T cell activation (Figure II.6A), which would lead to a more accessible chromatin state and thus facilitate *CD6* transcription and exon 5 skipping.

Chromatin structure and histone modifications can affect alternative splicing outcome and the opposite is also true. Indeed, De Almeida and colleagues showed that intron-containing genes have higher levels of H3K36me3 in comparison to intron less genes and that inhibition of splicing causes a decrease in the H3K36me3 levels together with a decrease in the HYPB/Setd2 methyltransferase recruitment (332). Despite the already described association of H3K36me3 and H3K9me3 with the regulation of alternative splicing events (207, 268, 271, 274), the results of chromatin immunoprecipitation experiments presented in this thesis do not show a correlation between CD6 exon 5 alternative splicing and those histone marks.

Tilgner and collaborators have shown in a high-throughput study that there is a relationship between nucleosome positioning and exon definition. They found higher

nucleosome occupancy in human and *Caenorhabditis elegans* exons with weak splice sites. Conversely, they have found that pseudoexons - intronic sequences that are not included in mRNAs but are flanked by strong splice sites show nucleosome depletion (263). The current view is therefore that nucleosomes act as an extra signal for spliceosome to recognize the exons that have weak splice sites and are thus more vulnerable to skipping (263, 266, 352, 353). In our studies, by micrococcal nuclease digestion, we observed an increase of nucleosome occupancy in *CD6* exon 5 region, confirming a possible poor definition of exon 5 that makes it more susceptible to be skipped (Figure II.4).

RNA Pol II transcription elongation rate has been demonstrated to affect alternative splicing (87) and an effect on alternative polyadenylation has been previously shown (354). According to the RNA Pol II kinetic coupling model that integrates alternative splicing with transcription kinetics, the RNA Pol II elongation rate affects alternative exon inclusion, depending on the strength of the splice sites. Considering the scenario of an intron with weak 3' splice site followed by a strong 3' splice site in the downstream intron, a high RNA Pol II elongation rate, allows the presentation of both splice sites to the spliceosome machinery at the same time, promoting the competition between them and resulting in the recognition of the strongest splice site by the spliceosome, leading to exon skipping. A lower RNA Pol II elongation rate allows more time for the spliceosome machinery to recognize the weak 3' splice site before transcribing the downstream 3' ss and thus leading to the inclusion of the first exon (252, 253, 355).

Kadener *et al.* showed that a compact chromatin structure of a replicated reporter plasmid acted as a barrier to RNA polymerase II elongation, leading to a higher exon inclusion. This effect was reverted by inhibition of histone deacetylases (HDAC), using Trichostatin A, which promotes histone acetylation and a subsequent chromatin "opening" (221, 222). Schor and collaborators characterized a change in chromatin structure upon neuronal cell depolarization that induces the skipping of the alternative exon 18 of *NCAM* mRNA (268). These results and in particular the relationship between an extracellular stimuli, in this case from another neuron, with the regulation of alternative splicing through the modulation of the chromatin structure led us to hypothesize that these mechanisms could also apply to exon 5 alternative splicing and prompted us to investigate the role of acetylation and chromatin structure changes in *CD6* gene. The results of the present work showed that T cell treatment with the HDAC inhibitor, TSA, results in increased skipping of exon 5, mimicking the effect of T cell activation on *CD6* alternative splicing (Figure II.6B and C). Conversely, the decreased in RNA Pol II elongation rate induced by Camptothecin treatment promotes the inclusion of exon 5 (Figure II.6B and C).

Taken together, our results indicate that alterations in the chromatin structure that occur upon T cell activation and an increase in the transcription rate of *CD6* may favor exon 5 skipping from primary transcripts. The fact that the most mature rat SP (Single Positive) thymocytes, which have higher *CD6* expression than DP (Double Positive) thymocytes, present increased levels of exon 5 skipping (32, 65) seems to corroborate our interpretation that the levels and rate of transcription of the *CD6* gene are co-responsible for the generation of an alternative transcript.

RNA *cis*-elements and *trans*-acting factors regulate *CD6* exon 5 splice site choice

The selection of 5' and 3' splice sites in pre-mRNA is governed in part by RNA sequences – the "splicing code" - and RNA binding proteins (106, 356, 357). SR and hnRNP proteins, through the binding to regulatory elements present in the primary transcript play an important role, regulating the recognition of the splice sites by the spliceosome machinery. To indentify the RNA motifs and the *trans*-acting factors in the regulation of *CD6* exon 5 alternative splicing, we used a minigene that mimics the endogenous *CD6* exon 5 splicing event (Figure II.8). We identified a highly conserved regulatory element localized in intron 4, containing a complex set of splicing enhancers and silencers that strongly regulates exon 5 inclusion (Figure II.9, II.10 and II.11). An *in silico* analysis revealed the presence of several putative binding sites for different RNA binding proteins from SR and hnRNP families (Figure II.12). This indication led us to quantify the expression of some of these splicing factors in resting and activated T cells (Figure II.13). We observed that the expression of SRSF1 was decreased upon T cell activation while that of hnRNP A1 and SRSF3 remained approximately constant (Figure II.13). It was important then to evaluate if changing the concentration levels of these splicing factors in T cells could affect the *CD6* exon 5 alternative splicing pattern. By overexpression (Figure II.14) and knockdown (Figure II.15) experiments, we showed that all three factors modulate *CD6* exon 5 alternative splicing (SRSF1 and SRSF3 promote exon 5 inclusion whereas hnRNP A1 increases exon 5 skipping), but only SRSF1 had its expression effectively decreased in activated T cells. Performing UV-crosslinking and immunoprecipitation assays we identified SRSF1 and hnRNPA1 binding to the ISE located in the *CD6* intron 4 pre-mRNA sequence. However we could not detect the

binding of SRSF3 to this element, probably because it is binding to another element (Figure II.16).

SRSF1 is a prototype member of the SR protein family of splicing factors with a role in alternative splicing (139, 358-360). SRSF1 has been extensively studied and several target transcripts have been identified (148-150, 156, 186-188).

It has been described that SRSF1 and hnRNP A1 can compete and regulate the binding of U1 snRNP to a 5' splice site, thus regulating the inclusion or skipping of an alternative exon, respectively (123, 153).

In a T cells resting state, with a basal transcription rate of *CD6* gene, the alternative isoform *CD6⊿d3* is also expressed, although in low levels. In this way, we could envision a scenario in which hnRNP A1 could competes with SRSF1 for the binding to the ISE regulating *CD6* exon 5 alternative splicing in resting T cells.

Our results indicate that, upon T cell activation, a deficit of SRSF1 dictate exon 5 exclusion. Taking into account the higher levels of *CD6* transcription in activated T cells SRSF1 is most likely the limiting factor, leading to an increase in *CD6Δd3* mRNA production.

T cell activation impairs SRSF1 recruitment to CD6 primary transcript

We detected a decrease in SRSF1 recruitment to the *CD6* primary transcript in activated T cells (Figure II.17B) that could be explained not only by a decrease in SRSF1 expression, but also by increased chromatin acetylation levels, as shown by others (259). Our ChIP experiments for H3K9ac revealed an increase of *CD6* acetylation level upon T cell activation. Accordingly, when we treated cells with an HDAC inhibitor (TSA), we observed less SRSF1 recruitment to *CD6* primary transcript (Figure II.17B). By inducing chromatin hyperacetylation we were recreating the effect that T cell activation has at the chromatin level, and thus we observed less SRSF1 recruitment to the CD6 primary transcript (Figure II.17B).

There was the possibility that TSA could have an effect on SRSF1 expression decreasing the levels of this splicing factor and thus leading to exon 5 skipping. However by western blotting analysis we also clarified that TSA treatments do not affect SRSF1 protein expression levels (Figure II.17C) supporting that chromatin acetylation decreases SRSF1 availability at the *CD6* primary transcript level. A similar effect was observed in neuroblastoma cells by Schor and collaborators, showing that upon cell membrane

potential depolarization, chromatin acetylation lead to an accumulation of SRSF1 in the nuclear speckles, being less recruited to the primary transcripts and compromising the splicing of nascent RNAs (259). To investigate if this phenomenon could also happen in T cells we performed immunofluorescence microscopy using resting and activated T cells. In resting T cells, although some aggregates of this protein can be seen, SRSF1 is scattered in the nucleus. In activated T cells it is visible that SRSF1 is less present at the nucleoplasm and the speckle dots are brighter and more pronounced (Figure II.18).

It has been shown that the long noncoding RNA (lncRNA) MALAT-1, one of the components of nuclear speckles, could bind to SRSF1 and other SR proteins and act as a "molecular sponge" regulating the levels of nucleoplasmic SRSF1 (276). However, Schor and collaborators shown that the total levels of MALAT-1 in HeLa cells remain similar after TSA treatment, suggesting that the accumulation of splicing factors in the nuclear speckles is not due to increased presence of MALAT-1 in the granular compartment rulling out the hypothesis that increased levels of chromatin acetylation could increase MALAT-1 expression to act as a "sponge" of free SRSF1 (259). It is possible that upon T cell activation and increased chromatin acetylation levels, MALAT-1 may sequester SRSF1 at the nuclear speckles controlling its nuclear distribution and thus affecting *CD6* exon 5 alternative splicing. However, this needs to be fully investigated, and in particular it is not known if the levels of MALAT-1 may vary upon activation of primary human T cells.

ERK pathway signals for *CD6* exon 5 alternative splicing

The weight of a particular signal transduction pathway in the regulation of alternative splicing is still poorly understood. Nevertheless, some concepts have begun to emerge on how extracellular stimuli can be communicated to specific RNA-binding proteins that control splice site selection by the spliceosome (283, 293). To identify the signaling pathways involved in *CD6* exon 5 alternative splicing upon T cell activation we made use of inhibitors for key signaling effectors of different signaling cascades. We found that the Src-family kinases Lck and/or Fyn are key transducers of the information leading to alternative *CD6* splicing. Further downstream, the MAPK-ERK pathway also seems to be utilized to induce alternative splicing of *CD6* (Figure II.19). Interestingly, we showed (Figure II.2) that stimulation of CD6 receptor conjugated with stimulation of CD3 receptor in primary T cells increases *CD6* exon 5 skipping as it happens when T cells are activated with PHA. It has been shown that CD6 stimulation (with the direct binding of specific

mAbs) induced Erk1/2 activation (63). In this way it is possible that CD6 signals the stimulus that will regulates the expression of its third extracellular domain through the activation of MAPK-ERK pathway.

Curiously, previous studies have shown that acetylation of histone H3 is regulated by the Erk pathway (361, 362) and we found an increase in H3 acetylation at the body of the *CD6* gene in activated T cells. We could hypothesize that Erk signaling promotes the increase in *CD6* acetylation levels upon T cell activation.

The working model we propose for the regulation of *CD6* exon 5 alternative splicing upon T cell activation is depicted in figure II.20.

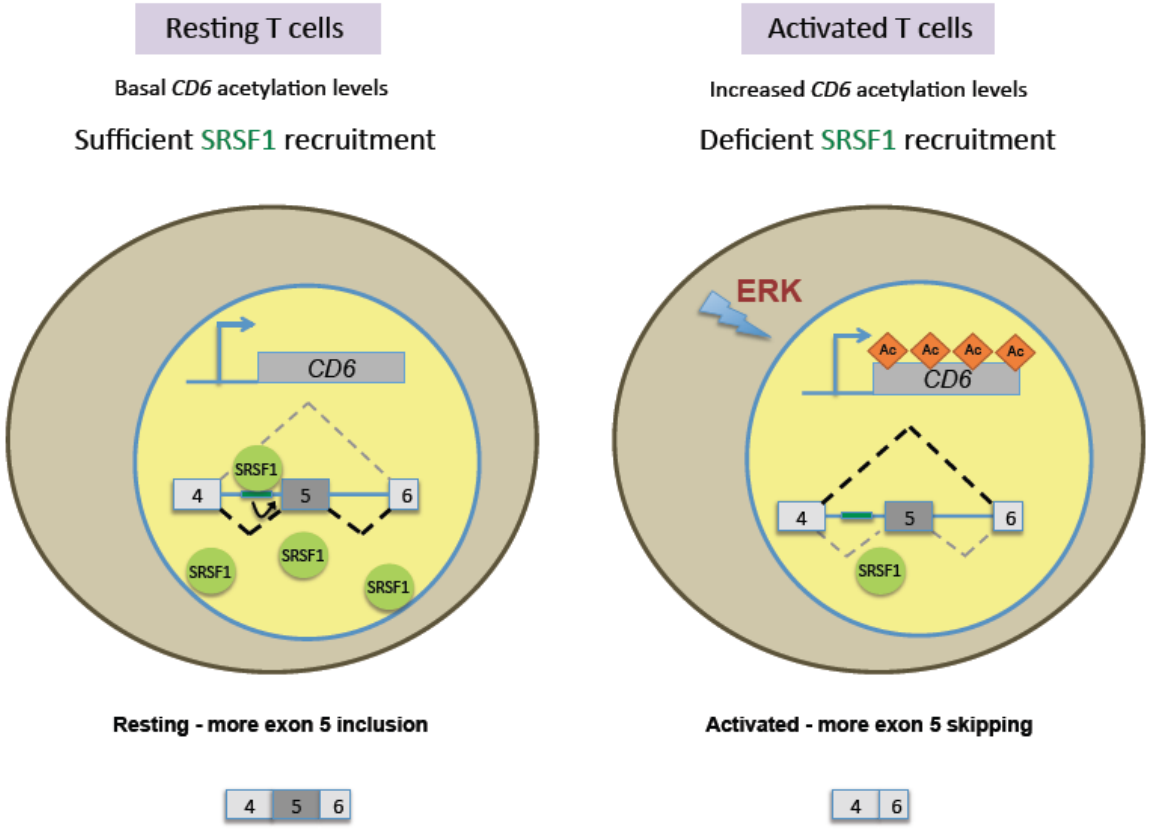

Figure II.20 - Proposed model of *CD6* exon 5 alternative splicing regulation upon T cell activation. Resting T cells present basal *CD6* H3K9 acetylation levels and SRSF1 is efficiently recruited to an intron 4 enhancer (green rectangle) leading to preferential inclusion of exon 5 in the *CD6* pre-mRNA. In activated T cells, there is an increase in *CD6* expression levels together with an increase of *CD6* H3K9 acetylation levels and a decrease in the recruitment of SRSF1 to the ISE causing an increase in exon 5 skipping and *CD6Δd3* mRNA production.

In resting conditions, T cells have a basal *CD6* transcription level, with sufficient SRSF1 levels to bind to the intron 4 regulatory element. In these conditions, SRSF1 promotes the inclusion of exon 5 in the majority of mature mRNAs. Upon T cell activation, the Erk signal transduction pathway is activated (363, 364) leading to an increase of *CD6* acetylation and consequently to a chromatin structure modification that facilitates *CD6* transcription. Under these activation conditions, there is a deficit of SRSF1 and a decrease in its recruitment to *CD6* pre-mRNA necessary for exon 5 inclusion, and this exon is thus skipped producing *CD6Δd3*. Taken together, we showed that the human T cell activation-induced alternative splicing of the *CD6* is regulated at multiple levels, with chromatin acetylation, transcription rate and SRSF1 having fundamental roles. As *CD6* has been associated with several autoimmune diseases, the molecular mechanisms regulating *CD6* alternative splicing upon activation of human T cells provide new insight in a physiologically relevant molecule.

Chapter IV

Concluding Remarks & Future Perspectives

CONCLUDING REMARKS & FUTURE PERSPECTIVES

Given the potential of alternative splicing to broadly shape cellular function the knowledge of the mechanisms that are involved in its regulation in the immune system is of extreme importance, and may help to elucidate some disease scenarios in the future.

Alternative splicing of exon 5 in the *CD6* transcript is physiologically and functionally relevant, as the differential expression of the membrane-proximal SRCR domain coded by that exon determines CD6 and CD166 localization or exclusion from the immunological synapse.

CD6 is an important molecule that modulates T cell activation. In one hand, CD6 works as an adhesion molecule, stabilizing the T cell-APC contact and can trigger co-stimulatory signals in association with signaling molecules such as Lck, Fyn, Itk and ZAP-70, important for T cell activation (34, 36). In the other hand, cells expressing CD6 have been shown to present reduced Ca^{2+} responses, less IL-2 production and less T cell proliferation (34). T cells that are incubated with cells expressing CD6 ligand, CD166, proliferate less than those incubated with cells lacking CD166, showing the importance of the CD6-CD166 interaction in the reduction of T cell proliferation (34). In this way it is plausible that the increased skipping of the exon that codes for the ligand-binding domain, that occurs after T cell activation may be a mode to escape this inhibitory stimuli and increase the potential of the T cell for proliferation. Taking this into consideration it would be interesting to assess if, when incubated with cells expressing CD166, T cells expressing only the CD6Δd3 isoform, that lacks the ligand-binding domain, proliferate more than cells expressing the CD6 full length isoform only.

Moreover, it would be very important to investigate at the protein level the effect on *CD6* exon 5 AS that we have shown in this work at the mRNA level, by simply modulating the expression levels of SRSF1, SRSF3 and hnRNP A1, in resting T cells and under basal *CD6* transcription levels. It would be expectable that resting T cells overexpressing SRSF1, for example, would produce higher levels of CD6 full length isoform and proliferate less, upon interaction with CD166, than T cells overexpressing with hnRNP A1 that would produce more CD6Δd3 isoform, and should present an increased proliferation.

Although increased chromatin acetylation induced by TSA treatments is not sufficient to increase MALAT-1 expression in HeLa cells (259), it is not known if MALAT-1 expression increases upon T cell activation, which should cause an increased SRSF1 localization at the speckles. If this were the case, then chromatin acetylation would regulate *CD6* exon 5 AS, by simply modulating chromatin structure and facilitating RNA Pol II transcription, increasing *CD6* transcripts levels, whereas MALAT-1 could be the

responsible for sequestering SRSF1. Therefore, it would be important to evaluate MALAT-1 expression and a possible interaction with SRSF1 in resting and activated T cells as well as expression changes of MALAT-1 in both conditions.

Concerning the differential expression of *CD6* during thymic maturation, an increased *CD6* expression together with an increase of exon 5 skipping in SP thymocytes in comparison to DP thymocytes that express lower levels of CD6, it is easily questionable if there is any difference in the *CD6* acetylation levels between these two thymocytes populations and also if there is any differential recruitment of SRSF1 to the *CD6* primary transcript or differential SRSF1 nuclear localization in SP and DP thymocytes. Chromatin and RNA immunoprecipitations as well as Immunofluorescence assays using SP and DP thymocytes could reveal new interesting results.

A previous study have shown that cross-linking of a CD6 mAb induced the activation of the Erk1/2, JNK and p38 kinases, showing the involvement of CD6 in MAPK signaling (63). In this thesis, the T cell stimulation with α-CD6 and α-CD3, led to an increased in the skipping of *CD6* exon 5 similarly to what happens upon T cell stimulation with PHA (Figure II.2). Considering the involvement of CD6 in the activation of Erk pathway (63) and that inhibition of this pathway can restore the resting pattern of *CD6* exon 5 alternative splicing, increasing exon 5 inclusion (Figure II.19) we may suggest that CD6 can exhibits a self-regulation, being responsible for signaling the stimuli that leads to the loss of its third extracellular domain. Having this in mind, it would be interesting to determine firstly if CD6 stimulation induces increased *CD6* acetylation and increased *CD6* expression levels and secondly if inhibition of Erk pathway with U0126 counteracts those effects.

The recent findings relating *CD6* exon 5 alternative splicing with multiple sclerosis has magnified the importance of the present work.

The multiple sclerosis patients homozygous for a risk allele characterized by a SNP rs7824933^{GG} in intron 1 of *CD6,* showed less expression of CD6 full length isoform and increased expression of the alternative isoform lacking exon 5, CD6Δd3. This group of patients with increased expression of CD6Δd3 also presented less proliferative $CD4^+$ T cells (66). Expression of CD166 has been shown to be upregulated in the endothelial cells in the blood-brain barrier (BBB) of patients with multiple sclerosis, who also presented increased leukocyte migration across the BBB into the central nervous system (CNS) (52). The trafficking into the CNS of a subset of $CD4^+$ T cells known to suppress immune response, Tregs, has been suggested to occur through the interaction of CD6 with CD166 (66). The increased expression of CD6Δd3 avoiding CD6-CD166 interaction due to the loss of the third extracellular domain, could prevent Tregs from entering the CNS. This

therefore explains how the SNP rs7824933^{GG} could contribute to the loss of suppressor immune function and promote brain inflammation, typical in multiple sclerosis (66). Considering the regulatory mechanisms involved in *CD6Δd3* expression unveiled in the present work, it would be very interesting to evaluate, in patients with multiple sclerosis the acetylation levels of *CD6* gene as well as the *CD6* mRNA levels, in comparison to healthy individuals. The expression levels and nuclear localization of the splicing factor SRSF1, that has been shown to be important for *CD6* exon 5 inclusion, should also be addressed. It would be also interesting to see if the inhibition of the Erk pathway in CD4^{+} T cells of multiple sclerosis patients, could diminish the expression of the CD6Δd3 isoform, resulting in a consequent increase in the expression of CD6FL.

The work in this thesis is an example of how an extracellular stimulus affects alternative splicing in the immune system, having a striking physiological consequence in CD6 membrane localization. Our results show that activation-induced *CD6* exon 5 skipping in T cells involves a deficit of the SRSF1 splicing factor on the primary transcript that together with changes in chromatin conformation and an increase in the RNA Pol II transcription rate of the *CD6* gene, cooperate to the production of a *CD6* mRNA isoform devoid of the ligand-binding domain being a contribution to the understanding of *CD6* expression regulation in health and disease.

Chapter V

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