REGULATION OF ALTERNATIVE SPLICE SITE SELECTION

Inaugural-Dissertation
zur Erlangung des Doktorgrades der
Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf

vorgelegt von
KUSUM KUMARI SINGH
aus
Samastipur, Indien
Düsseldorf, 2009

Aus dem Institut für Molekulare Medizin
der Heinrich-Heine Universität Düsseldorf

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf

Referent: Prof. Dr. Klaus Schulze-Osthoff
Koreferent: Prof. Dr. Heinz Mehlhorn

Tag der mündlichen Prüfung: 20 Jan 2009

TO MY PARENTS

ACKNOWLEDGEMENTS

Many people have contributed to this dissertation in innumerable ways, and I am grateful to all of them. Firstly, I would like to extend my sincere gratitude to **Prof. Klaus Schulze-Osthoff** for his generosity with his time, encouragement and advice concerning the project. Without his support, this project would not have been possible.

I thank **Prof. Heinz Mehlhorn** for his helpful advices, supervision and for making it possible for me to present this thesis.

My special thanks to my supervisor **Dr. Christian Schwerk**, the main driving force behind the projects. I deeply appreciate for his invaluable discussions, advices, and encouragements throughout this work.

I thank our collaborators, **Prof. Heiner Schaal** and **Steffen Erkelenz**, University of Duesseldorf for kindly providing me an HIV-minigene construct. They have been instrumental in adding a novel role of SAP18 in splicing process. I would also like to extend my gratitude to **Dr. Ingo Schmitz** and **Nana Ueffing** for working together on the FLIP project and I am grateful to all the collaborators who were generous in providing us with Follicular Lymphoma patient samples.

Mrs. **Andrea Mayer** offered excellent technical support and I am grateful to her for all her help.

This work was supported by the **DFG** (Deutsche Forschungsgemeinschaft; GRK1089, SFB725) and I am deeply grateful to them for the same.

I sincerely thank all my colleagues at the Institute of Molecular Medicine, for offering very conducive environment in the lab. I thank them for their advice, comments and time. I would like to specially thank **Dr. Ute Fischer** for offering help in correcting the thesis. I would also like to thank **Dr. Frank Essmann** and **Dr. Dennis Sohn** for their support and assistance.

I would like to extend my deepest gratitude to my family members, especially my parents. They have always provided unwavering love and encouragement and believed in me.

Figure Index

Table Index

Abbreviations

%	percentage
°C	grad celsius
A	adenine
aa	amino acid
Acinus	Apoptotic chromatin condensation inducer in the nucleus
Amp	ampicillin
Apaf-1	apoptotic protease activating factor-1
APS	ammonium persulphate
ASAP	apoptosis and splicing associated protein
ASF/SF2	alternative splicing factor/splicing factor 2
ATP	adenosine triphosphate
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X-protein
Bcl-2	B cell leukemia-2
bp	base pair
BPS	branch point sequence
BSA	bovine serum albumin
BSE	bidirectional splicing enhancer
C	cytosine
c-FLIP	cellular-FLICE-like inhibitory protein
CHX	cycloheximide
C-terminal	carboxy terminal
DAPI	4',6'-diamidino-2-phenylindole
dATP	deoxy adenosine triphosphate
DD	death domain
DED	death effector domain
dH_2O	deionized water
DISC	death inducing signaling complex
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DR	death receptor
ds	double spliced RNA

dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
DTT	dithiothreitol
E.coli	Escherichia coli
ECL	enhanced chemo luminescence
EDTA	ethylenediamine tetraacetic acid
EJC	exon junction complex
env	gene coding for the envelope protein of HIV-1
ER	endoplasmic reticulum
ESE	exonic splicing enhancer
ESS	exonic splicing silencer
EtBr	ethidium bromide
EtOH	ethanol
FADD	Fas associated protein with death domain
FasL	Fas ligand
FCS	fetal calf serum
Fig.	figure
G	guanine
gag	gene coding for structure protein of HIV-1
GAR	Guanine-Adenine-rich
h	hour
HDAC	histone deacetylases
hGH	human growth hormone
HIV-1	human immunodeficiency virus type-1
hnRNP	heterogenous nuclear ribonucleoprotein
IAP	inhibitor of apoptosis protein
IB	immunoblot
IF	immunofluorescence
IgV	immunoglobulin variable region
ISE	intronic splicing enhancer
ISS	intronic splicing silencer
kb	kilo base
kDa	kilo Dalton
l	liter
LB	Luria broth
LTR	long terminal repeat

M	molar
mg	milligram
min	minutes
mRNA	messenger RNA
nef	gene for negative factor
NGFR	nerve growth factor receptor
NLS	nuclear localization signal
NMD	nonsense-mediated mRNA decay
NP-40	nonidet P-40
N-terminal	amino terminal
OD	optical density
ORF	open reading frame
ori	origin of replication
pA	polyadenylation signal
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pmol	picomole
PMSF	phenylmethylsulfonylfluoride
pol	gene for polymerase enzyme of HIV-1
PPT	Polypyrimidine-tract
PTC	premature termination codon
rev	gene for the regulator of viral protein expression of HIV-1
RNA	ribonucleic acid
RNAPII	RNA-polymerase II
RNAse	ribonuclease
RNPS1	RNA-binding protein with serine-rich domain
rpm	rounds per minute
RRE	Rev responsive element
RRM	RNA recognition motif
RS domain	arginine/serine-rich domain
RT	room temperature
RT-PCR	reverse transcriptase-polymerase chain reaction
SA	splice acceptor
SAP18	Sin3-associated polypeptide of 18 kDa

SC35	spliceosome component of 35 kDa
SD	splice donor
SDS	sodium dodecyl sulfate
sec	seconds
sk	splice product with exon-skipping
snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoprotein particle
SR	serine/arginine
SR-rp	serine/arginine-related protein
SRp40	SR-protein (spliceosome component of 40 kDa)
SRp54	SR-protein (spliceosome component of 54 kDa)
ss	single spliced RNA
STS	staurosporine
SV40	Simian Virus 40
T	thymine
Taq	Thermus aquaticus
TAR	trans-activation responsive sequence
Tat	Trans-activator of transcription
TBE	Tris / boric acid / EDTA
TE	Tris / EDTA
TNF	Tumor necrosis factor
TNFR	TNF receptor
TRAF	TNF-receptor associated factor
TRAIL	TNF-related apoptosis inducing ligand
U	unit
U2AF	U2 snRNP auxiliary factor
us	unspliced
UV	ultraviolet
vif	gene for viron infectivity factor of HIV-1
vpr/*vpu*	gene for the association of HIV-1 Protein
wt	wild type
µg	microgram
µl	microlitre

1. INTRODUCTION

Advances in animal genome sequencing have led to many questions concerning the very nature and number of genes and how they help to promote the diversity of cellular and organismal functions [214]. One surprising observation resulting from the analysis of the human genome sequence is that we may possess less than 25,000 genes [192], which are only about two times as many as the developmentally much simpler fruit fly, *Drosophila melanogaster* [1]. How can the much greater complexity of humans be encoded in only twice the number of genes required by a fly? The solution to this paradox is not completely understood, but it appears that humans and other mammals may be more adept than other organisms at encoding many different proteins from each gene. One way to accomplish the expansion of the proteome is through "alternative splicing", a special form of precursor mRNA (pre-mRNA) processing to generate more than one type of protein from the common pre-mRNA.

1.1. Pre-mRNA Splicing

A typical feature of eukaryotic cells is that the primary transcription products of their structural genes, the so-called pre-mRNA, experience a variety of post-transcriptional modifications to produce the functional mature RNA (mRNA) [75, 214].

One of these modifications is the process of splicing (Fig. 1). The genes of most eukaryotes contain long stretches of sequences that have no protein-coding information. These sequences are termed introns, which interrupt the protein-coding sequences, termed exons [204]. After a gene has been copied, or transcribed, into pre-mRNA, the introns are removed from its pre-mRNA, and the exons are linked together to form an mRNA, consisting of one contiguous protein-coding sequence. Splicing is usually constitutive, which means that all exons are joined together in the order in which they occur in the pre-mRNA. Mature mRNAs contain additional untranslated regions (UTRs) upstream and downstream of the part encoding for the amino acid sequence. Although these

sequences do not encode protein, they help to regulate the translation of the mRNA into protein.

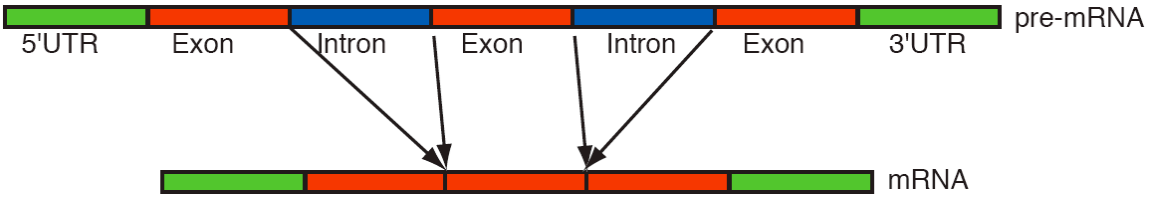

Figure 1. Pre-mRNA splicing. A process during which introns (in blue) are removed from the precursor mRNA (pre-mRNA) and exons (in red) are retained in mature RNA (mRNA). Green boxes indicate the untranslated regions (UTRs) that are non-coding parts of exons present at both the ends of mRNA.

1.1.1. Biochemistry of splicing

Pre-mRNA splicing is a two-step process (Fig. 2) that takes place inside the nucleus [101, 70, 144]. Only a few short nucleotide sequences present within the primary transcript provide the cell with clues of which parts are to remove before the RNA transcript leaves the nucleus.

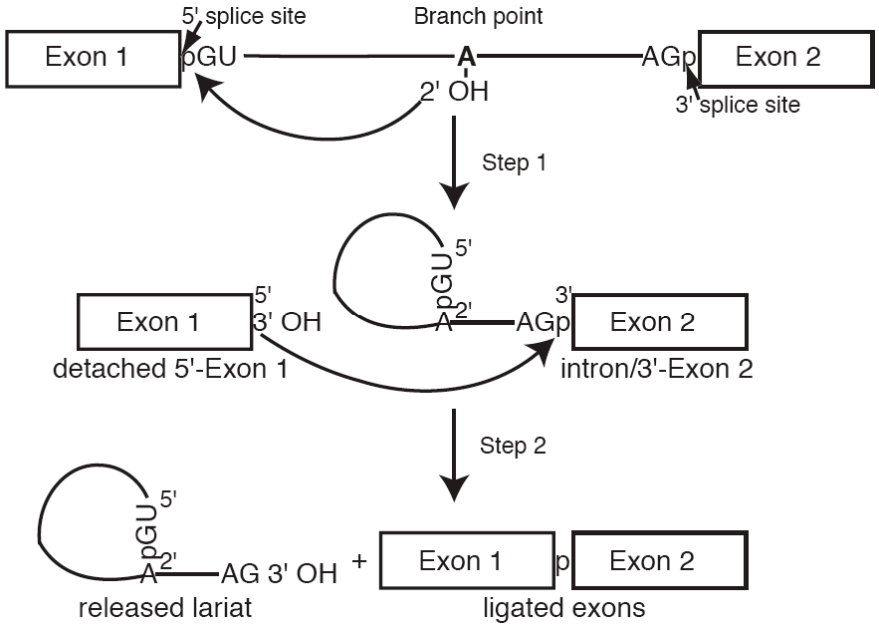

Figure 2. The catalytic steps of pre-mRNA splicing. Splicing takes place in two transesterification steps. The first step results in two reaction intermediates: the detached 5' exon-1 and the intron/3'-exon-2 fragment in a lariat structure. The second step ligates the two exons and releases the intron lariat.

In the first step, the 2'-hydroxyl group of the adenosine at the branch site attacks the phosphate at the 5' splice site. This leads to cleavage of the 5' exon from the intron and the concerted ligation of the guanosine at the introns 5' terminal to the branch site adenosine in a 2',5'-phosphodiester bond. This step generates two splicing intermediates: a detached 5' exon and an intron/3'-exon intermediate in a branched circular, or lariat, configuration [95]. The second transesterification step proceeds by attack of the 3' hydroxyl group of the 5' exon on the phosphate bond at the 3' splice site, resulting in the displacement of the lariat intron and the ligation of the exons [11].

1.1.2. Splice sites

Splicing is a process that demands remarkable accuracy because a mistake of just one nucleotide can lead to an mRNA that encodes a non-functional protein by changing the reading frame. Therefore, to ensure that the coding information is not lost or altered and that the exons are correctly spliced from within tens of thousands of intronic nucleotides, each splicing reaction takes place with a high degree of fidelity.

This accuracy is ensured by short nucleotide sequences, called splice sites that are necessary for intron removal and are restricted to the exon/intron borders [183]. The consensus sequence at the 5' splice site in higher eukaryotes is AG|GURAGU (the splice site is denoted by a vertical bar and invariant nucleotides are underlined; R=purine, Y=pyrimidine, N=any nucleotide), whereas the 3' splice site is characterized by the sequence YAG|. Another sequence element, the branch site, is usually located at a distance of 18 to 40 nucleotides upstream of the 3' splice site and displays the degenerate sequence YNCUR**A**Y (the site of branch formation is shown in bold). Within a pre-mRNA a number of sequences termed pseudo splice sites exist, which closely resemble classical splice sites, but do not function as those [117, 48]. The bona fide splice sites are distinguished from these pseudo splice site sequences by auxiliary cis-elements, known as exonic and intronic splicing enhancer (ESE and ISE) and exonic and

intronic splicing silencer (ESS and ISS) sequences, which aid in the recognition of exons [150].

1.1.3. Spliceosome assembly

Pre-mRNA splicing is a biochemical reaction that takes place in a macromolecular machinery, called the spliceosome (Fig. 3). The components of the spliceosome include five uridine-rich small nuclear ribonucleoprotein particles (U snRNPs), namely, U1, U2, U4, U5 and U6 and a large number of non-snRNP proteins. Each U snRNP consists of a short RNA stably bound by several proteins. In addition, other splicing factors are transiently less stably associated with the RNA-protein complex. Spliceosome assembly is a highly dynamic process, with multiple RNA-RNA, RNA-protein, and protein-protein interactions. It occurs in a stepwise manner involving more than 200 proteins, which are not necessarily associated with U snRNPs [69].

The spliceosome assembly begins with the ATP-independent formation of the E (early) complex, during which U1 snRNP binds to the 5' splice site via efficient base pairing between the 5' splice site and U1 snRNA [150]. The 3' splice site elements are bound by a specific set of proteins. SF1/mBBP, a branch-point binding protein, binds to the branch point sequence and the 65 kDa subunit of the dimeric U2 auxiliary factor (U2AF) binds to the polypyrimidine (Py)n tract. The 35 kDa subunit of U2AF binds to the 3' splice site at the intron/exon junction. Mutual stabilization of contacts with the U2AF-3' splice site and the downstream U1-5' splice site can be mediated by members of the serine/arginine (SR) protein family that bind to ESEs [36, 190]. This stabilization involves protein-protein interactions mediated by the (arginine/serine) RS-rich domains of SR proteins [219, 94]. For example the RS domain of U2AF interacts with the RS domain of SR proteins to enhance the recognition of weak splice sites. The RS domains of SR proteins also contact the pre-mRNA during spliceosome assembly [206, 174]. Thus SR proteins play important roles in the formation of the E complex, which is

considered to be a commitment step that directs the nascent pre-mRNA transcript to the splicing pathway.

Subsequent to E complex formation, the A (prespliceosome) complex is built. The A complex is formed upon stable, ATP-dependent interaction of U2 snRNP with the pre-mRNA branchpoint [31, 160]. The tight association of U2 snRNP with the pre-mRNA coincides with the displacement of SF1/mBBP from the branch site [119, 160]. Base pairing of U2 snRNA to the branch point is facilitated by the RS domain of U2AF65 kDa bound to the polypyrimidine tract [199]. Subsequently, the RS domain of an ESE-bound SR protein apparently stabilizes the prespliceosome [173, 174].

In the next step the U4/U6•U5 tri-snRNP associates with the A complex to form the B complex [157]. This progression from A to B complex is also an ATP-dependent process, where a role of SR proteins in escorting the tri-snRNP to the assembling machinery has been suggested [157].

The B complex undergoes a complicated rearrangement to form the C (Catalysis) complex, in which the U1 snRNP interaction at the 5' splice site is replaced with the U6 snRNP and the U1 and U4 snRNPs are lost from the complex [167, 166]. It is the C complex that catalyzes the two chemical steps of splicing (Fig. 2), [30, 152, 198]. After the catalysis, splicing products, ligated exons and the lariat intron are released from the spliceosome. Whereas exons are exported to the cytoplasm, several enzymes degrade lariat introns and many spliceosomal components are recycled to form new spliceosomes [127, 29].

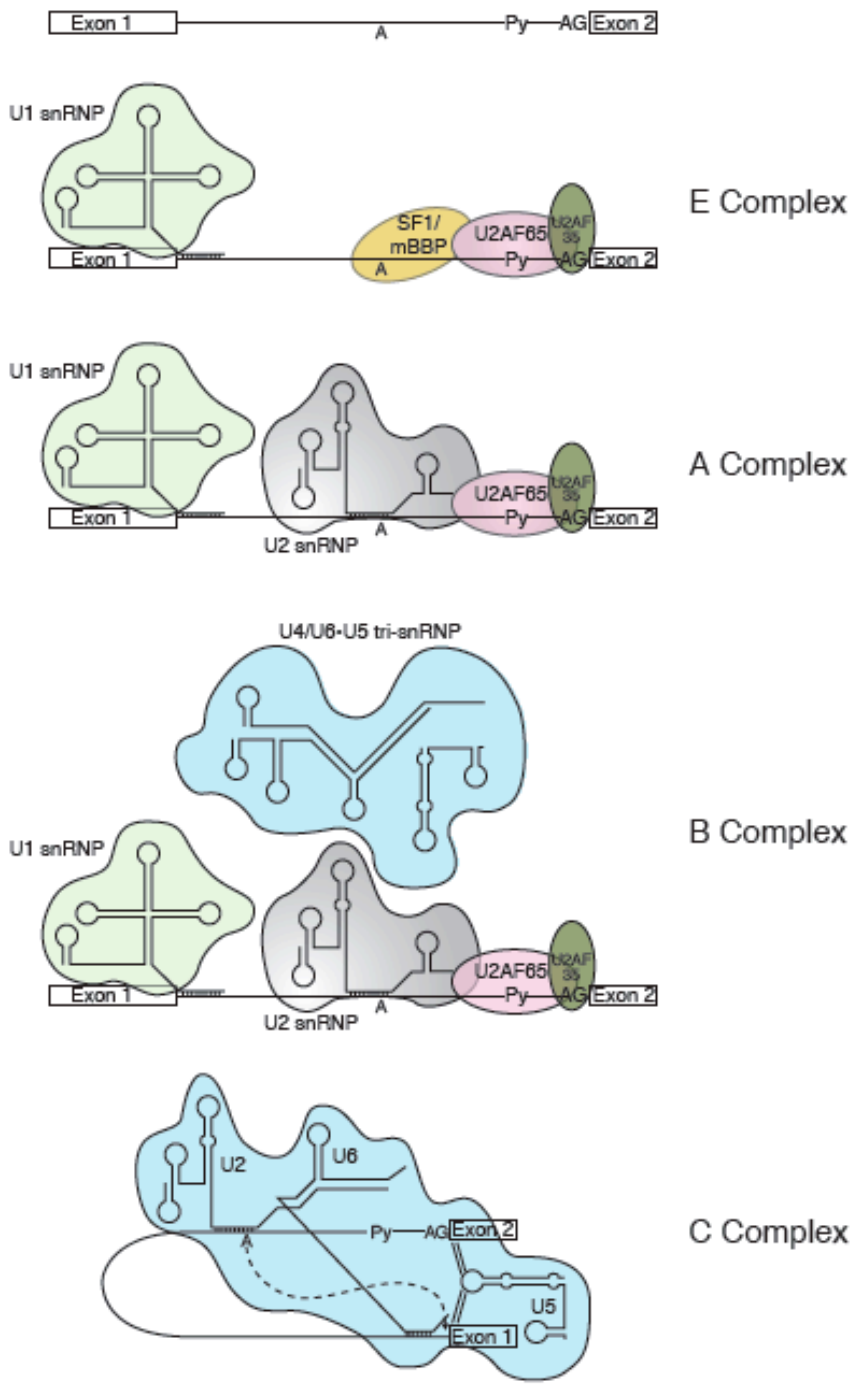

Figure 3. Spliceosome assembly pathway. In the first step of spliceosome assembly, U1 snRNP binds to the 5' splice site, SF1/mBBP binds to the branch point, and U2 auxiliary factor (U2AF) binds to the pyrimidine tract and 3' YAG. This complex commits the pre-mRNA to the splicing pathway and is called the E, or early, complex. Next, the E complex is converted to A complex when the U2 snRNP binds to the branchpoint. Subsequently, B complex is formed when the U4, U5, and U6 snRNPs enter the spliceosome as a tri-snRNP particle. Finally, a massive rearrangement occurs in which U6 replaces U1 at the 5' splice site, U6 and U2 interact, U5 bridges the splice sites, and U1 and U4 become destabilized. This rearranged spliceosome is called the C complex and is catalytically active. Figure modified from Graveley, 2000, [69].

1.2. Alternative splicing

The central dogma of molecular biology states that one gene produces one protein, which carries out a specific function. In 1978, Walter Gilbert hypothesized that variations in splicing, i.e. alternative splicing, can generate functionally distinct protein products from a single gene by joining of different 5' and 3' splice sites (Fig. 4) [62].

Alternative splicing is a ubiquitous regulatory mechanism of protein function [10]. Up to 74% of human genes generate multiple mRNAs by alternative splicing [84, 109] and ~80% of alternative splicing results in changes in the encoded protein [138], revealing what is likely to be the primary source of human proteomic diversity. Alternative splicing generates variable mRNA segments that can insert or remove amino acids from the protein product. The process of alternative splicing can shift the reading frame of the protein and it can also introduce a termination codon into the protein (Fig.4) [151, 179, 185]. A large fraction of alternative splicing events undergoes cell specific regulation, in which splicing pathways are modulated according to cell type, developmental stage, gender, or in response to external stimuli [48, 209].

Alternative splicing is regulated by the binding of trans-acting protein factors like SR protein family members to cis-acting sequences present within the pre-mRNA leading to a differential use of splice sites. Many such sequences have been identified and are grouped as either enhancer (ESEs/ISEs) or suppressor (ESSs/ISSs) elements [107] depending on their function. These elements are generally short (8-10 nucleotides long) and are less conserved than those present at exon-intron junctions.

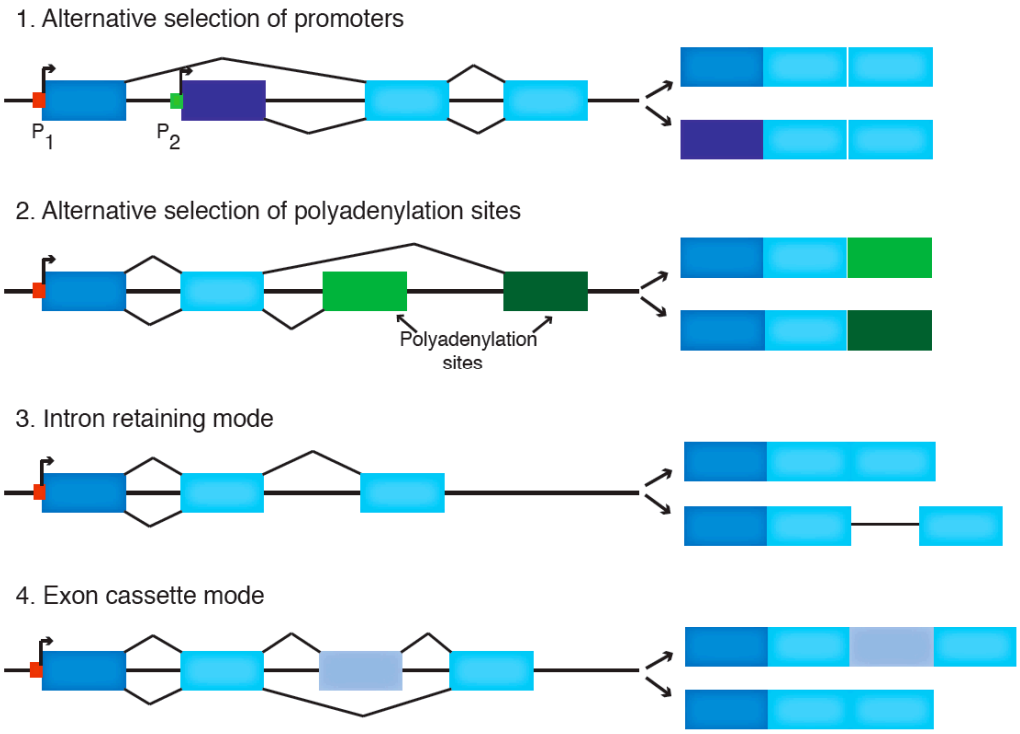

Figure 4. Various modes of alternative splicing. 1. Alternative selection of promoters: This is a step of splicing, which can produce an alternative amino-terminal domain in proteins. In this case, different sets of promoters can be spliced with certain sets of exons. An example for this mode of splicing is the myosin primary transcript. 2. Alternative selection of cleavage/polyadenylation sites: In the presented model, this step of splicing produces an alternative carboxyl-terminus in proteins. In this case, different sets of polyadenylation sites can be spliced with the other exons. An example of alternative poly-A site selection is the tropomyosin transcript. 3. Intron retaining mode: In this mode, instead of being spliced out, the intron is retained in the mRNA transcript. However, the intron's code must be properly expressible, otherwise a stop codon or a shift in the reading frame will cause the protein to be non-functional. This kind of mode has been documented for the transposase primary transcript. 4. Exon cassette mode: In this case, certain exons are spliced out to alter the sequence of amino acids in the expressed protein. The troponin primary transcript is an example for exon cassette mode. Exons are indicated in different shades of blue boxes, introns as lines. Promoters are indicated as P_1 and P_2 and polyadenylation sites are shown in shades of green boxes.

1.3. The Serine/Arginine-rich protein family (SR proteins)

The SR proteins represent a family of splicing factors that are remarkably conserved in vertebrates and invertebrates and have also been found in plants [9, 110, 120]. Members of the SR family in mammalian cells were discovered in the early 1990s by the identification of factors associated with purified spliceosomes [53, 54]. Later, SR proteins were independently discovered by a number of research groups taking very different approaches. For instance, SF2/ASF (Splicing Factor 2/Alternative Splicing Factor) [59, 100] was purified

from HeLa cell nuclear extracts as a factor required to reconstitute splicing in cytoplasmic extracts (S100). Cytoplasmic extracts lack components that are vital for the splicing process. It was demonstrated that addition of SF2/ASF into the S100 extract activated constitutive splicing [99] as well as influenced the 5' splice sites within the pre-mRNA [98, 58]. In another approach, monoclonal antibodies directed against purified spliceosomes were used to identify SR proteins. In one case, this led to the identification of the 35 kDa (SC35) and 9G8 components of the spliceosome [53, 54]. The structural domain of 9G8 contains a zinc knuckle domain that plays a role in the determination of RNA-binding specificity [28]. Another group used a monoclonal antibody (mAb104) that binds active sites of RNA polymerase II transcription [159, 211] to identify an entire family of related proteins (SRp20, -30, -40, -55 and -75 kDa), which they termed SR proteins because they contained a domain rich in arginine and serine residues [158, 211]. Subsequently, additional members of the SR protein family were identified by several different research groups [5, 41, 212]. Some of the important human SR protein family members are shown in Fig. 5 [69]. The SR proteins are vital for pre-mRNA splicing and have dual roles in constitutive and alternative splicing, hence they also influence the regulation of alternative splicing [56, 69, 19, 19].

1.3.1. Structural organization of SR proteins

Members of the SR protein family have a characteristic structural organization. They have a modular structure consisting of one or two copies of an RNA recognition motif (RRM) at the amino terminal followed by a carboxy-terminal domain rich in alternating serine and arginine residues, known as the RS domain. RRMs ascertain RNA-binding specificity, whereas the RS domain functions as a protein-protein interaction module by recruiting components of the core splicing apparatus to promote splice site pairing [206]. It has also been demonstrated that the RS domain can directly contact interaction partners at both the pre-mRNA branch point and the 5' splice site [174]. SR proteins can interact with both exonic and intronic regulatory elements of the pre-mRNA. Based on the

interaction of SR proteins with regulatory sequences like ESEs or ESSs, they can either enhance or suppress splicing. In addition, when artificially tethered to the pre-mRNA, the RS domains of several human SR proteins are sufficient to activate enhancer-dependent splicing [68], an activity that presumably requires protein interactions.

Within the cell, the RS domain of SR proteins acts as a nuclear localization signal mediating the interaction with the SR protein nuclear import receptor, known as transportin-SR, and determines nucleocytoplasmic shuttling of individual SR proteins [22, 23]. The extensive serine phosphorylation of the RS domain plays an important role in regulating the activities and localization of SR proteins [164].

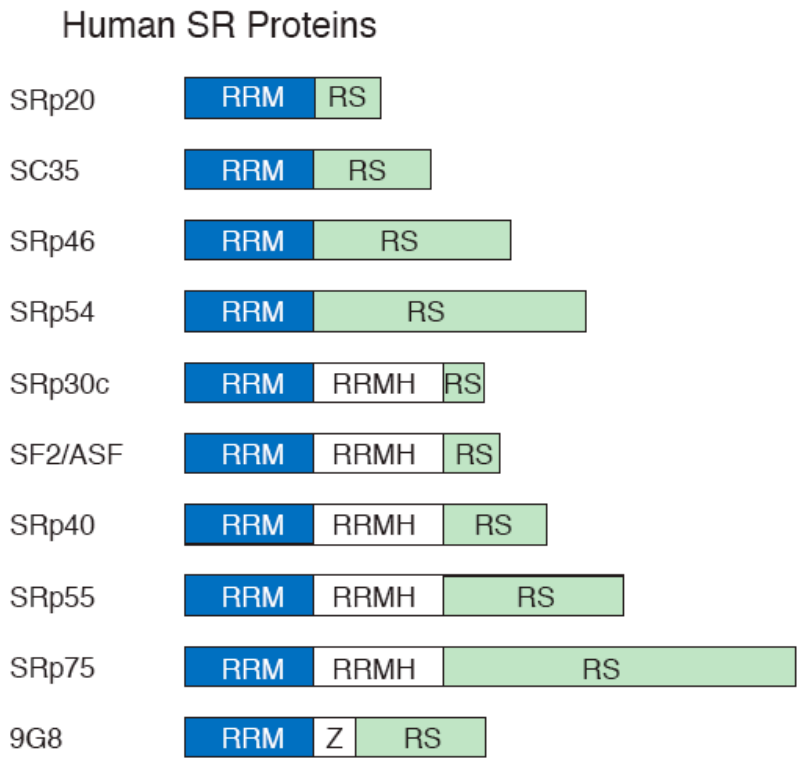

Figure 5. Schematic diagram of human SR proteins. The domain structures of the known members of the human SR protein family are depicted. RRM: RNA recognition motif; RRMH: RRM homology; Z: zinc knuckle, RS: arginine/serine-rich domain. Figure modified from Graveley, 2000, [69].

1.3.2. SR-related proteins (SR-rps)

There are a number of additional RS-domain containing proteins, distinct from the classical SR proteins, which are required for pre-mRNA splicing (Fig. 6). These are collectively referred as serine/arginine-related proteins (SR-rps) [13]. Some examples of SR-rps include U2AF35 kDa, U2AF65 kDa, U1-70K, Sip1, U5-27K and U5-100K [69]. Most of the SR-rps do not contain an RNA-binding domain and even though many of the SR-rps are essential splicing factors, they cannot complement S100 extracts.

One of the best-characterized SR-rp is U2AF65 kDa. It contains three RNA-binding domains responsible for binding to the p(Y)tract and an amino-terminal RS domain, which is believed to facilitate binding of U2 snRNP to the branch point [215, 199]. The RS domain of U2AF35 kDa stabilizes U2AF65 kDa binding to the p(Y)tract and is proposed to mediate interactions with SR proteins at the 3´ splice site [206, 219]. Although U2AF35 kDa does not contain a canonical RNA-binding domain, it contacts the 3´splice site AG [69].

The U1-70K protein has an RNA-binding domain, which is required to tether the RNA (U1 snRNA) of the U1 snRNP protein as well as an RS domain, which is needed for the interaction with SR proteins [24, 94, 140]. Another SR-rp is the splicing co-activator SRm160/300, which contains one RS domain [12, 15]. SRm160/300 has been shown to bind an ESE to promote splicing of a pre-mRNA by interacting with the U2 snRNP and SR proteins [14]. Other examples of SR-rps include splicing regulators, like isoforms of hTra2 (hTra2α, hTra2β), a protein that was originally shown to modulate splicing of the Drosophila sex determination gene doublesex (*dsx*) [37]. Later it was demonstrated to activate pre-mRNA splicing in a sequence specific manner and to interact with RNPS1 (RNA-binding protein with serine-rich domain) and other splicing factors [187, 163, 194]. SR-rps also include RNA helicases, e.g. hPrp16 [69]. A protein motif termed DEXD/H box in RNA helicases is suggested to mediate ATP-dependent proofreading and RNA conformational rearrangement between step 1 and 2 of splicing. The SR-rp Clk/Sty is an example for a protein kinase, which catalyses

phosphorylation of SR proteins at multiple sites in the RS domain, but with different substrate specificity [201, 34, 74].

Figure 6. Schematic representation of human SR-related proteins. The domain structure of important SR-rps that participate in pre-mRNA splicing are depicted; RRM: RNA recognition motif; RS: arginine/serine-rich domain; RS/P: arginine/serine/proline-rich domain; Zn: zinc finger; DEXD/H Box: motif characteristic for RNA helicases.

1.3.3. Role of SR proteins in constitutive and alternative splicing

The SR proteins participate at several steps in the assembly of the spliceosome. They are acknowledged to play a critical role in both constitutive (Fig. 7A-C) and alternative splicing (Fig. 7D). They are among the first components that interact with the pre-mRNA and possess the ability to commit the pre-mRNA to the splicing pathway [55]. SR proteins are incorporated into the spliceosome at the time of E complex assembly and stimulate the formation of this complex when added to a nuclear extract [180]. SR proteins enhance the binding of U1 snRNP to 5' splice sites [46, 94, 213] and U2AF at the 3' splice site [135]. This step is necessary and the process is known as exon definition (Fig. 7A) [153, 18]. When present at high concentrations, SR proteins can abrogate the need for U1 snRNP in splicing, suggesting an early and essential role in spliceosome assembly and splice site selection [36, 190, 191]. Selection of splice sites depends on the interaction of SR proteins (as well as numerous other RNA-binding proteins) with cis-acting elements within exonic or intronic sequences. Interestingly, the effects of cis-acting elements are position-dependent. Often SR-protein-binding sites within exons (ESE) exert a positive effect on splice site selection, whereas intronic binding sites for SR proteins (ISS) repress splice site selection [168, 87].

SR proteins have also been suggested to form a network of protein-protein interactions across introns to juxtapose the 5' and 3' splice site early in spliceosome assembly (Fig. 7B). This intron bridging is mediated by simultaneous interactions of SR proteins with the U1-70K at the 5' splice site and with the U2AF35 at the 3' splice site, via their RS domains. Additionally, subsequent to the formation of the E complex, SR proteins can recruit U2 snRNP to the branch site of a RNA substrate and they are also required for the transition of complex A into complex B, a step that involves the incorporation of U4/U6•U5 snRNP into the spliceosome. SR proteins support this step by facilitating the recruitment of the U4/U6•U5 tri-snRNP (Fig. 7C) [157]. Several research groups have utilized MS2 tethering system to describe the functions of SR proteins and

their RS domains, in the splicing machinery. For example, in a study by Furuyama and Bruzik, the 9G8-specific ESE region was replaced by a specific RNA-binding site for the bacteriophage MS2 protein to analyze fusion proteins. They found out that addition of the MS2-RS proteins increased the formation of spliceosomal complexes and helped in the recruitment of U4/U6•U5 tri-complex into the pre-mRNA [19].

Owing to their prominent role in splice site selection, SR proteins are key players in the control of alternative splicing. Often alternative exons are designated by weak splicing signals; for instance, many alternative 3' splice sites contain poor polypyrimidine tracts, which are recognized inefficiently by U2AF, resulting in exon skipping. However, SR proteins bound to ESEs can compensate for a weak polypyrimidine tract by recruiting U2AF (Fig. 7D, upper panel). The control of alternative splicing also entails the specific repression of splice sites. This involves silencer elements that are typically bound by hnRNPs (heterogeneous nuclear ribonucleoproteins). The hnRNPs are RNA-binding proteins and they form complexes with hnRNA (heterogeneous nuclear RNA/pre-mRNA) [11, 44]. It has been demonstrated that hnRNPs could antagonize the activity of SR family proteins in alternative splicing regulation in a concentration dependent manner [129, 21]. Alternatively, ESE-associated SR proteins may promote alternative exon inclusion by antagonizing the negative activity of hnRNPs bound to ESS elements (Fig. 7D, lower panel) [14, 75, 165]. Thus the relative level and activity of members of the SR and hnRNP family of proteins may represent an important determinant of alternative splicing regulation [154].

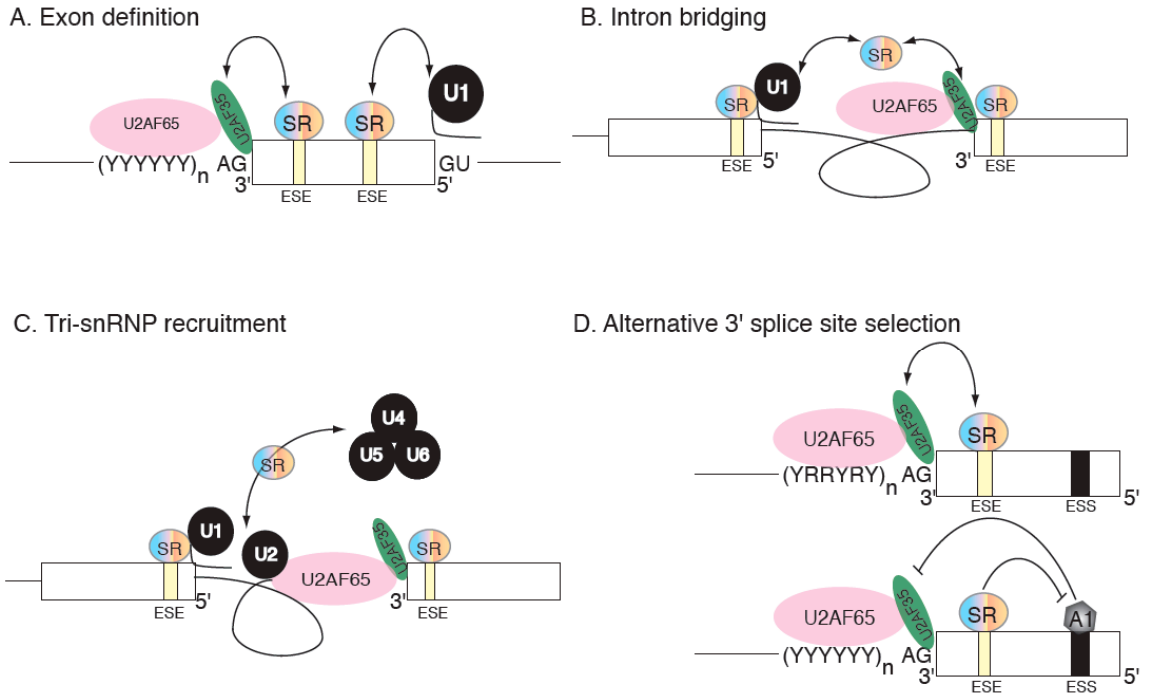

Figure 7. Roles of SR proteins in constitutive and alternative splicing. A) Exon definition by U2AF65 (pink oval) and U2AF35 (green oval) at an upstream 3' splice site and U1 snRNP (black circle) at a downstream 5' splice site is facilitated by SR proteins bound to an exonic splicing enhancer (ESE, light yellow boxes). The strong polypyrimidine tract $(YYYYYY)_n$ adjacent to the 3' splice site is depicted. B) 5' splice site and 3' splice site can be juxtaposed early in the splicing reaction by intron bridging interactions between SR proteins and the RS domain containing subunits of U1 snRNP and U2AF. C) SR proteins can recruit the U4/U6•U5 tri-snRNP to the pre-spliceosome. D) SR proteins bound to ESEs can promote alternative 3' splice site selection by recruiting U2AF to a suboptimal 3' splice site. The weak polypyrimidine $(YRRYRY)_n$ tract is shown. Alternatively, exonic splicing silencers (ESS, black boxes) can recruit splicing repressor proteins such as hnRNPA1 and block 3' splice site selection by U2AF. SR proteins can antagonize the effects of splicing repressors, thus promoting splice site selection. Figure modified from Sanford et al., 2005, [165].

1.4. Apoptosis and splicing associated protein (ASAP) complex

Additional regulatory proteins and protein complexes, which can be associated with the functional spliceosome, are possibly involved in RNA processing. An example is the ASAP complex, which includes the proteins RNPS1, SAP18 and distinct isoforms of Acinus [170].

Acinus was observed to play a critical role in apoptotic chromatin condensation and DNA fragmentation and was therefore named "Apoptotic chromatin condensation inducer in the nucleus" [161, 80, 85]. Acinus has also been mentioned to fulfill important functions in RNA processing [170], cell cycle

control and transcriptional regulation [85, 83, 202]. Alternative splicing generates three different isoforms of Acinus: Acinus-L, Acinus-S, and Acinus-S' (Fig. 8), with approximate molecular masses of 220, 98, and 94 kDa, respectively [170]. Acinus-L consists of 1341 amino acids, whereas Acinus-S has a short sequence at the amino terminus, which is followed by residues 767-1341 of Acinus-L. Acinus-S' corresponds to residues 767-1341 of Acinus-L. All three isoforms of Acinus contain a region, which shows considerable similarity to the *Drosophila* splicing regulator sex-lethal (*Sxl*) and suggests a possible role of Acinus in RNA metabolism [161]. Acinus has also been identified as a component of functional spliceosomes, which facilitates intron removal during mRNA processing [148, 218]. Furthermore, analysis of the purified exon junction complex (EJC) by mass spectroscopy and co-precipitation experiments has revealed that Acinus, RNPS1 and SAP18 stably associate with each other and co-purify with other EJC components. The components of the mammalian EJC include numerous splicing coactivators/alternative splicing factors, mRNA export factors and nonsense-mediated mRNA decay (NMD) factors [189]. The multiprotein EJC is deposited on mRNAs upstream of exon-exon junctions as a result of pre-mRNA splicing. According to the accepted model, EJC core components activate eukaryotic NMD, causing specific degradation of aberrant mRNAs with truncated open reading frame [109] due to the presence of an in-frame premature termination codon (PTC) >25-30nt upstream of an EJC deposition site [82, 113, 123].

All isoforms of Acinus contain two RS-like domains in their carboxy terminal part (Fig. 8). The presence of RS-rich domains and a region similar to an RNA-binding domain in the protein sequence of Acinus indicates its prospective potential to act as a splicing regulatory protein [19, 203, 125]. Recently, the potential to self-associate and to form aggregates has been attributed to RS domain containing proteins, including Acinus. The RS domains of Acinus have also been demonstrated to associate mainly with RNA [142]. Acinus localizes in nuclear speckles/interchromatin granule clusters, which act as storage compartments for splicing factors. Acinus has been shown to colocalize together

with other splicing factors like SC35 and ASF/SF2 [83].

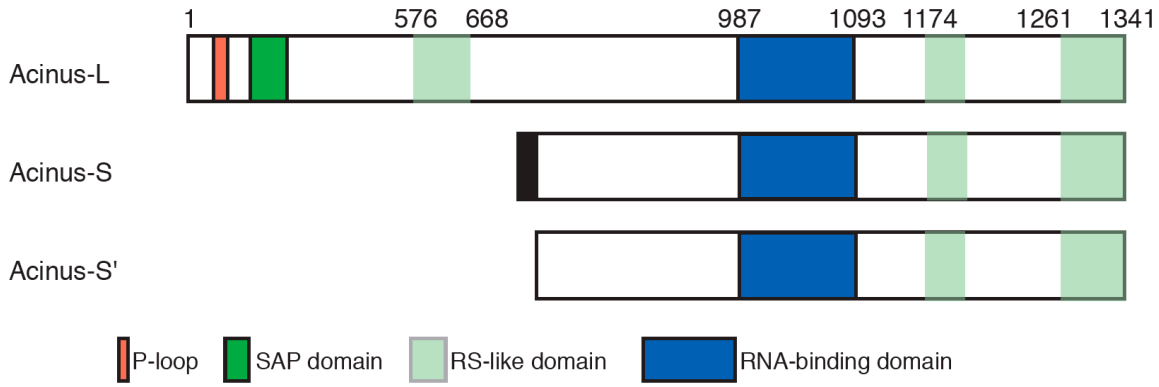

Figure 8. Schematic representation of the domain structure of Acinus. The three isoforms of Acinus (L, S and S') are depicted. Arginine/serine (RS)-rich domains are colored in light green, RNA-binding domains in dark blue. A sequence similar to the P-loop, a nucleotide-binding domain, is colored in red and the SAP domain is presented as a dark green box. The short N-terminus of Acinus-S is shown in black box.

The RNPS1 (RNA-binding protein with serine-rich domain) protein, another subunit of the ASAP complex, is a well-known general activator of pre-mRNA splicing [130] and recently it has been added into the list of SR proteins [16]. A possible role of RNPS1 in mRNA export and NMD has also been mentioned [112, 111, 122, 123]. As its name suggests, RNPS1 contains a serine-rich domain (S-rich domain) spanning from amino acid 69-121 and an RRM domain, which lies between amino acid 163-243. At the carboxy terminus it possesses a domain rich in RS/P residues (Fig. 9).

It has been demonstrated that RNPS1 interacts with factors that are directly or indirectly involved in pre-mRNA splicing and different domains of RNPS1 are required for these interactions. For example, the S-rich domain interacts with p54, a member of the SR protein family. In contrast, the RS/P domain interacts with hTra2β, a purine-rich ESE binding factor and the RRM domain binds to pinin, a protein which localizes predominantly in nuclear speckles and has been suggested to associate with U2 snRNP proteins [163]. RNPS1 acts as a versatile factor that regulates alternative splicing of a variety of pre-mRNAs. For instance when RNPS1 was overexpressed in HeLa cells, it induced exon skipping in a

model β-globin pre-mRNA and hTra2β pre-mRNA, whereas coexpression of RNPS1 with p54 stimulated exon inclusion in an ATP synthase γ-subunit pre-mRNA [163, 216]. Furthermore, RNPS1 colocalizes with SR proteins to the nucleus in an intense speckled pattern, which is characteristic of many splicing factors [122, 130]. Taken together, these data strongly support a role of RNPS1 in alternative splicing.

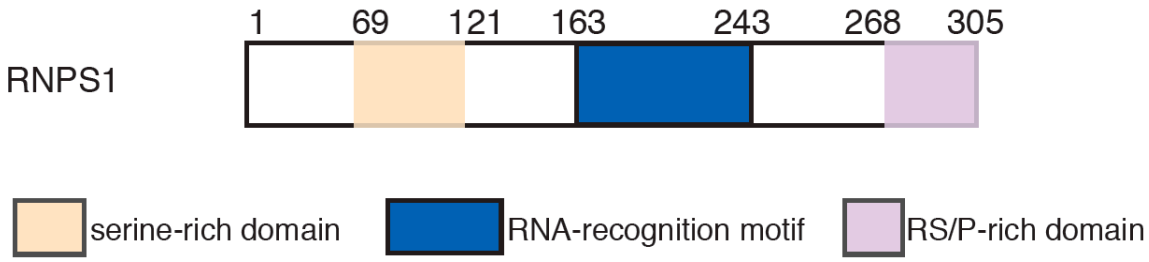

Figure 9. Schematic representation of the domain architecture of RNPS1. The serine-rich domain (residues 69-121) and the RNA recognition motif (RRM, residues 163-243) are shown in light yellow and dark blue, respectively. The carboxy terminal arginine-serine/proline (RS/P)-rich domain is represented in light violet.

The functional role of the third ASAP complex protein, SAP18, has not yet been defined clearly. SAP18 was originally identified within a complex together with the mammalian transcriptional repressor Sin3 and histone-deacetylases HDAC1 and 2 [217]. The Sin3-HDAC complex has been reported to cause transcriptional repression by deacetylating histones at the promoter regions [155, 186]. A number of other distinct proteins, including Gli, Su(fu) and Bicoid [143, 217], have been described to bind SAP18, however the functional consequences are not yet known.

Human SAP18 is 153 amino acids in length and it is highly conserved among higher eukaryotes with an identity of approximately 98, 60, and 54% in sequence alignments between the human polypeptide and homologues from mouse, *D. melanogaster*, and *Caenorhabditis elegans*, respectively [131]. Unlike Acinus and RNPS1, SAP18, which lacks both an RS and RRM domain, does not display typical structural properties of splicing factors. The structure of SAP18 contains an ubiquitin-like fold and it resembles ubiquitin and Elongin like proteins [131].

The amino acids aspartate at position 118 and threonine at position 121 of SAP18 are conserved in the proteins SAP18, ubiquitin, SUMO and Elongin B and are located in the centre of the ubiquitin-like fold structure. A β-grasp fold contained in ubiquitin and several other proteins is thought to be responsible for protein-protein interactions and might function in various cellular processes. Often ubiquitin-like fold containing proteins serve as cofactors in the recognition of interaction partners or in the assembly of multiprotein complexes [90]. The ubiquitin-like fold in SAP18 suggests its functional role as a protein-protein adapter module.

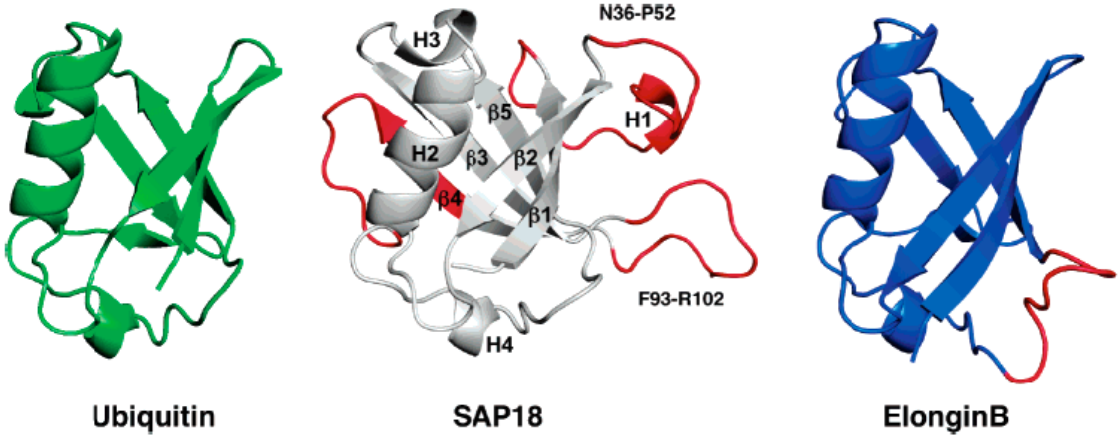

Figure 10. Structural domains of SAP18. A ribbon representation of residues 25-139 of SAP18 is shown together with the structurally related proteins ubiquitin and Elongin B. Inserts within the ubiquitin-fold architecture are shown in red for SAP18 and Elongin B. Figure is taken from McCallum et al., 2006, [131].

1.5. Alternative splicing and Apoptosis

The process of alternative splicing can serve to fine tune important and complex cellular events. There is increasing evidence that this mode of regulation of gene expression also plays a major role in the control of programmed cell death or apoptosis [207].

1.5.1. Apoptosis

Apoptosis is a morphologically distinct, genetically controlled form of cell death by which unnecessary or abnormal cells are eliminated in multicellular

organisms during embryonic development, immune system and during the maintenance of tissue homeostasis [89, 208]. Apoptosis is a programmed way of cell death during which the apoptotic cell undergoes several changes. These changes are a consequence of controlled and coordinated molecular and biochemical events that result in cell shrinkage, cleavage of the cellular deoxyribonucleic acid (DNA) into oligonucleosomal fragments, condensation of the nuclear content, membrane blebbing and the eventual packing of the cellular content and the compacted chromatin into numerous membrane-enclosed structures called 'apoptotic bodies' that are finally disposed by macrophages without inducing an inflammatory response [89]. Apoptosis is an active form of cell death and requires energy [208]. Dr. Stanley Korsmeyer had shown that the apoptosis program is suppressed in Bcl-2 overexpressing B cell lymphoma and that its suppression enhances the development of B cell lymphoma [97]. Since then innumerous studies have accumulated to support the idea that the acquired resistance to apoptosis is a hallmark of most or perhaps all types of cancer [73].

The defining molecular feature of apoptosis is the activation of a family of cysteinyl aspartate-specific proteases, called caspases. Caspases are initially synthesized as inactive zymogens, called procaspases, which upon induction of apoptosis are cleaved to form the active enzyme [33]. The active form of caspases is a heterotetramer containing two heterodimers and two active sites. Based on their order of activation, caspases are classified into two families: initiator caspases (also known as apical caspases; caspase-8, -9, -2 and -10) and effector caspases (also known as executioner caspases; caspase-3, -6 and -7) [49, 57]. Initiator caspases are activated by dimerization and autoprocessing of the procaspases inside a macromolecular structure, such as the death inducing signaling complex (DISC) or the apoptosome [141, 17]. Regarding caspase-9, the proteolytic activation is accomplished in the apoptosome complex composed of Apaf-1 (apoptotic peptidase activating factor-1) and cytochrome c (Fig. 11). Effector caspases are proteolytically activated by initiator caspases. Once activated, effector caspases degrade more than 280 cellular proteins and thereby

execute the cell death process [49, 141]. Several members of the caspase family are alternatively spliced and generate isoforms that display antagonistic functions during cell death. For example, a long isoform of caspase-2 can cause apoptosis, whereas the short variant inhibits cell death [171].

Apoptosis can be triggered in a cell by various signals, which could be either extracellular or intracellular in origin. Two distinct pathways based on the origin of the death stimulus are the extrinsic and the intrinsic apoptotic pathway [169]. Triggers that are extracellular in origin are mediated by activation of the so called 'death receptors', which are cell surface receptors that transmit apoptotic signals after ligation with the corresponding ligand. Death receptors form a subgroup of the tumor necrosis factor (TNF) receptor superfamily that includes Fas (also known as Apo-1/CD95), TNF-R1 and receptors binding to the TNF-related apoptosis-inducing ligand (TRAIL) [52, 205, 124]. All death receptors are characterized by an intracellular motif called the death domain (DD). Upon ligand binding, the death receptors interact via their DD with the DD of adapter proteins such as FADD (Fas-associating protein with death domain) [4]. These adapter proteins also contain a second protein interaction motif, the death effector domain (DED), that facilitates their binding to the initiator caspase-8 (or its relative caspase-10) to form the death inducing signaling complex (DISC) (Fig. 11) [210]. DISC formation activates caspase-8 through a proximity-induced dimerization mechanism [50]. Subsequently, caspase-8 cleaves and activates caspase-3, resulting in further cleavage of cellular targets, which ultimately perform the process of cell death.

In addition to extrinsic apoptotic pathways, death signals originating from cellular stress, including radiation and chemotherapeutic drugs, activate an intrinsic apoptotic program that is mediated largely by the mitochondria [50]. Mitochondrial release of cytochrome c into the cytoplasm induces the formation of an oligomeric complex containing cytochrome c and Apaf-1 (Fig. 11). This complex, called the apoptosome, supports the catalytic activation of caspase-9, which further cleaves and activates the effector caspase-3 resulting in the

subsequent degradation of cellular death substrates.

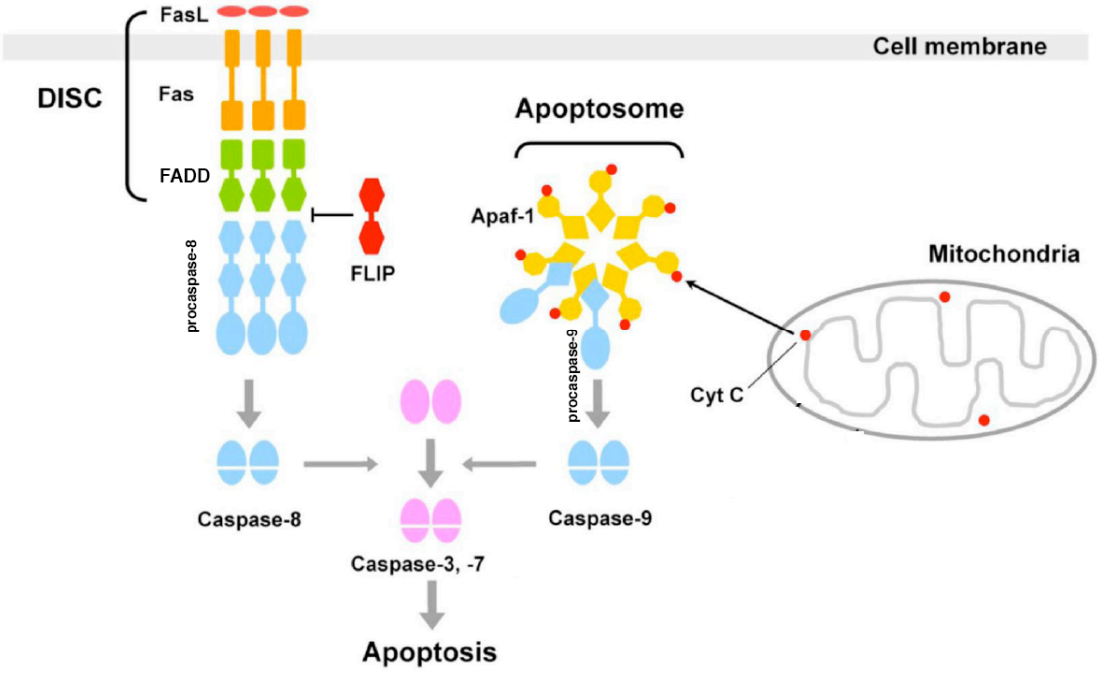

Figure 11. Two major routes to apoptosis exist in mammalian cells. In the extrinsic pathway of apoptosis binding of ligands of the tumor necrosis factor (TNF) family leads to oligomerization of death receptors and subsequent recruitment and activation of initiator caspase (caspase-8) via adaptor proteins (FADD). The activated initiator caspases target effector caspases like caspase-3, -7, -6 for proteolysis and activation. In the intrinsic (mitochondrial) pathway an apoptotic stimulus like DNA damage leads to permeabilization of the outer mitochondrial membrane and the release of cytochrome c. Subsequently, the cytochrome c binds to Apaf-1 and induces a conformational change that is responsible for recruitment of an initiator caspase (caspase-9) and formation of the caspase-activating apoptosome complex. Active caspase-9 in turn activates effector caspases. In both pathways the activated caspases cleave selected nuclear and cytoplasmic target proteins for execution of apoptosis. Figure modified from Yang, 2008, [210].

Proteins of the Bcl-2-family are crucial checkpoints of the intrinsic mitochondrial death pathway [35]. Members of this family can either induce or inhibit the release of mitochondrial apoptotic factors and are therefore classified as either pro- or anti-apoptotic proteins. It has been mentioned that the mitochondrial apoptotic pathway is inhibited by high concentrations of anti-apoptotic Bcl-2 family proteins like Bcl-2 itself or Bcl-x_L. Overexpression of those proteins is frequently observed in tumors of diverse origin, for example inhibition of apoptosis by overexpressed Bcl-2 proteins has been identified in B cell lymphoma. The *bcl-2* gene isolated from B cell lymphoma often shows a

t(14;18)(q32;q21) translocation, which brings the *bcl-2* gene under transcriptional control of the highly active immunoglobulin heavy chain promoter. Similarly, transgenic mice overexpressing Bcl-2 develop polyclonal follicular hyperplasia of long-lived B cells [132, 133].

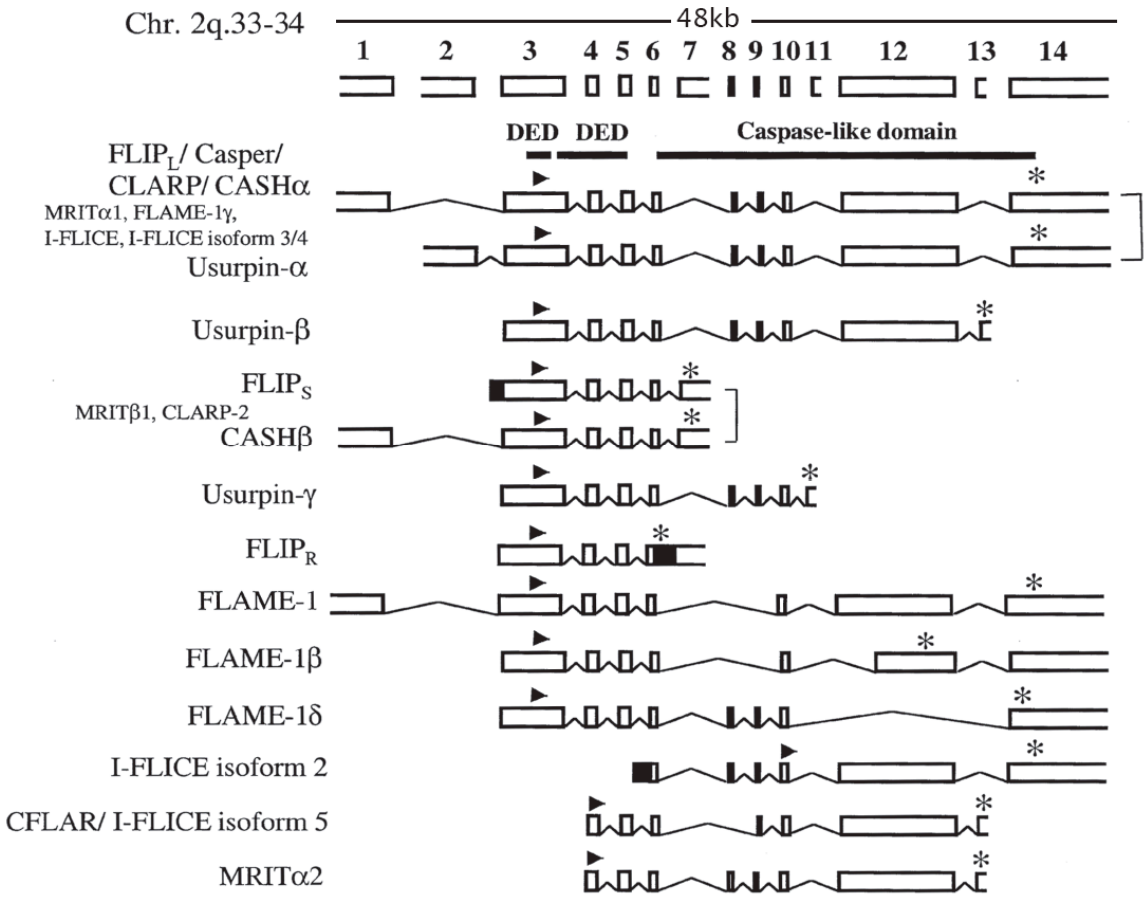

Figure 12. Different isoforms of c-FLIP at mRNA level. Schematic representation of the structure of the FLIP gene of 48 kb comprising a minimum of 14 exons. The generation of the 11 isoforms by alternative splicing is shown. The start and stop sites for the translation of the various splice forms are indicated as arrowheads and asterisks, respectively. Transcripts belonging to the same isoform are grouped by a square bracket and black boxes (present in $FLIP_S$, $FLIP_R$ and I-FLICE isoform 2) represent intron sequences. Figure is taken from Djerbi et al., 2001, [42].

A protein with significant homology to caspase-8, also termed FLICE (FADD-like interleukin-1β-converting enzyme), was identified in human cells and was named c-FLIP (cellular FLICE-inhibitory protein). Like caspase-8, c-FLIP possesses two DEDs but lacks a functional caspase domain due to a mutation of a critical cysteine to tyrosine in the enzymatic domain. c-FLIP acts as a

competitive inhibitor for the recruitment of caspase-8, or caspase-10 to the DED of FADD following Fas ligation and prevents downstream activation of apoptosis.

Although 11 distinct splice variants of c-FLIP have been described at the mRNA level (Fig. 12), only three isoforms have been detected at the protein level: c-$FLIP_{Long}$ (c-$FLIP_L$; 55 kDa), c-$FLIP_{Short}$ (c-$FLIP_S$; 26 kDa) and c-$FLIP_{Raji}$ (c-$FLIP_R$; 25 kDa) [42, 64]. The production of c-$FLIP_L$ and c-$FLIP_S$ is regulated by an alternative splicing event leading to either inclusion or skipping of exon 7. Inclusion of exon 7 leads to the production of c-$FLIP_S$, which is truncated at the C-terminus and lacks the inactive caspase-like domain, due to the introduction of a stop codon into the c-FLIP open reading frame. In contrast, skipping of exon 7 allows the synthesis of c-$FLIP_L$. The third isoform of c-FLIP was originally identified in Raji cells, and is therefore named c-$FLIP_R$. The expression of c-$FLIP_R$ requires inclusion of intron 6 into the mRNA of c-FLIP. Since read through into intron 6 leads to a rapid encounter of a stop codon, a second short form of c-FLIP is produced, which is very similar in size to c-$FLIP_S$ and differs only at the final C-terminal amino acids (Fig. 13) [42]. All three isoforms of c-FLIP are recruited to the DISC through their tandem DEDs interacting with the DED of FADD and thereby excluding procaspase-8 from the DISC. This may be the common mechanism of various c-FLIPs to inhibit the activation of caspase-8.

Although c-$FLIP_L$ has been described as an inhibitor of caspase-8 activation in the DISC, other studies have shown that c-$FLIP_L$ can activate caspase-8 [210, 104]. To initiate activation of caspases, c-$FLIP_L$ can form dimers with procaspase-8 that are proteolytically active and thus can also act as a pro-apoptotic protein. However, all short variants of c-FLIP have been reported to be solely anti-apoptotic, because they lack the caspase-like domain that is only present in the long form of c-FLIP [43].

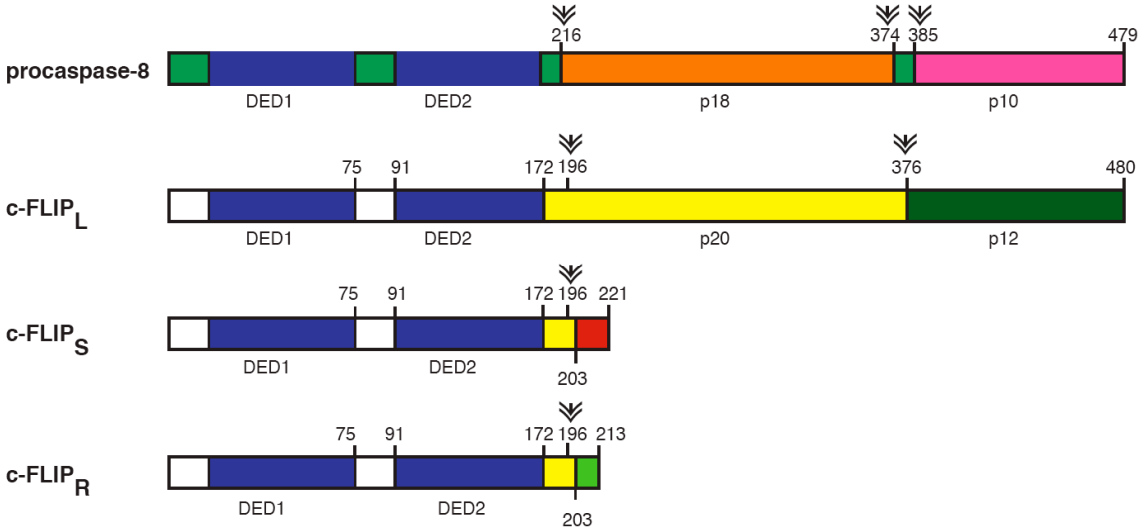

Figure 13. Different protein isoforms of c-FLIP. Structural similarities between procaspase-8 and different isoforms of c-FLIP. The c-FLIP isoforms differs in their carboxy-terminal that are differently colored: c-FLIP$_L$ in dark green, c-FLIP$_S$ in red and c-FLIP$_R$ in green. Arrows denote the sites cleaved by caspase-8.

1.5.2. Function of c-FLIP in the B cell immune response

The immune system is a society of interacting cells consisting of T lymphocytes and B lymphocytes, natural killer (NK) cells, macrophages and their various subclasses. Cellular components of the immune system are derived from precursors in the bone marrow. B lymphocytes, NK cells and macrophages mature in the bone marrow and in the fetal liver. T lymphocytes mature in the bone marrow and in the thymus [102].

Each B cell expresses cell membrane receptors, immunoglobulins (Igs /antibodies) with single antigen specificity. Millions of different B cells produce antibodies and can potentially capture millions of antigens. The sum of all antibody specificities is called ‘the antibody repertoire’. B cells are selected in the bone marrow on the basis of the affinity of their antibodies: cells with high affinity for proteins derived from ‘self’ tissues are eliminated via negative selection. Mature B lymphocytes leave the bone marrow and populate the secondary lymphoid organs, like spleen and lymph nodes [102]. Once a B cell encounters its cognate antigen, it is ingested and digested by the B cell to peptides and nestled inside a class II MHC (major histocompatibility) protein. Subsequently,

this complex is moved to the outside of the B cell membrane. The macrophages are activated to deliver multiple signals to a specific T cell (helper T cells) that recognizes the peptide-MHC II complex presented on the surface of the B cell. The T cell is then stimulated to produce cytokines that activate the B cells. The interaction of CD40 (present on the B cell membrane) with CD154 (present on activated T cells) is one of the main co-stimulators required for the activation of B cells. The activated B cells then can adopt one of the two fates: 1) movement into extra follicular areas followed by proliferation and terminal differentiation into short-lived plasma cells that secrete antibodies or 2) movement into B cell follicles of secondary lymphoid organs followed by proliferation and the establishment of germinal centres (GCs) [2].

The B cells germinal centre is formed within a few days after antigen recognition and reaches a maximum size within two weeks. At the peak of B cell development, the GC contains two compartments: a dark zone consisting almost exclusively of densely packed proliferating B cells known as centroblasts, and a light zone comprised of smaller, non-dividing centrocytes placed within a mesh of follicular dendritic cells (FDCs), T cells and macrophages [93].

Meanwhile, activated follicular B cells in the centroblasts proliferate and undergo a process of affinity maturation through the introduction of somatic mutation (somatic hypermutation, SHM) in the variable antigen-binding region of the BCRs, so that the resulting B cells have increased diversity of BCR affinities. These high-affinity B cells then migrate to light zone/centrocytes of the GC, which contains the FDC network. Here, high-affinity centrocytes are positively selected if they bind specifically to antigen trapped on the surface of FDCs. The positively selected centrocytes are then differentiated into long-lived plasma cells and memory B cells. The plasma cells and memory B cells can be supplemented by somatic recombinations (or class switch recombination, CSR); an isotype switching from IgM and IgD to other isotypes like IgG, IgA or IgE [200, 93], which alters the effector functions of an antibody that can be specifically required to clear a particular antigen [200]. Many of the long-lived plasma cells migrate into

the bone marrow, where they secrete antibody for several weeks or longer [2].

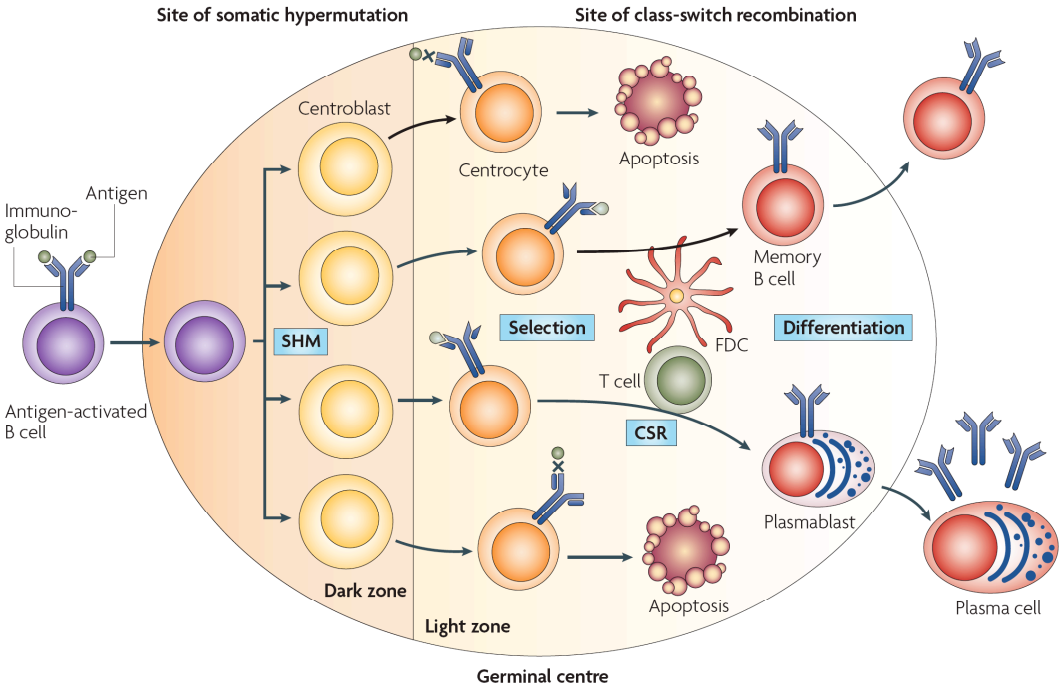

Figure 14. The germinal centre microenvironment. Antigen-activated B cells differentiate into centroblasts that undergo clonal expansion in the dark zone of the germinal centre. During proliferation, the process of somatic hypermutation (SHM) introduces base-pair changes into the V(D)J region of the rearranged genes encoding the immunoglobulin variable region (IgV) of the heavy chain and light chain; some of these base-pair mutations lead to a change in the amino-acid sequence. Centroblasts then differentiate into centrocytes and move to the light zone, where the modified antigen receptor, with help from immune helper cells including T cells and follicular dendritic cells (FDCs), is selected for improved binding to the immunizing antigen. Newly generated centrocytes that produce an unfavorable antibody undergo apoptosis and are removed. A subset of centrocytes undergoes immunoglobulin class-switch recombination (CSR). Cycling of centroblasts and centrocytes between dark and light zones seems to be mediated by a chemokine gradient. Antigen-selected centrocytes eventually differentiate into memory B cells or plasma cells. Figure is taken from Klein and Dalla-Favera, 2008, [93].

Human GC B cells show a typical apoptosis-sensitive phenotype; they express low levels of the anti-apoptotic protein Bcl-2 and high levels of the pro-apoptotic proteins Fas, Bax and c-Myc. Human GC B cell apoptosis relies on the rapid activation of caspase-8 at the level of the Fas DISC. c-FLIP$_L$ is one of the most important regulators of human GC B cell apoptosis because it controls the activity of caspase-8. It is subject to developmental regulation in the mature B cell compartment and it is expressed at low levels in naive and memory B cells, but at

high levels in GC B cells. Immunoprecipitation of Fas or FADD performed on freshly isolated human GC B cells has revealed that these cells contain a preformed Fas DISC, consisting of FADD, procaspase-8 and c-FLIP$_L$, resulting in a nonfunctional Fas DISC (Fig. 15a). *In vitro* experiments conducted on isolated human GC B cells have shown that c-FLIP$_L$ disappears rapidly from GC B cells when they are removed from their microenvironment, resulting in a functional Fas DISC that activates caspase-8 (Fig. 15b) [200]. Three types of stimuli known to prevent apoptosis in GC B cells also promote the sustained expression of c-FLIP$_L$ in these cells: ligation of the BCR, ligation of CD40 and signals delivered by FDCs. Thus, the ability to maintain the survival of GC B cells seems to be well correlated with the potential to prevent the disappearance of c-FLIP$_L$. The decay of c-FLIP$_L$ in unstimulated GC B cells is fast (completed within 30 minutes of culture), whereas approximately two hours of CD40L stimulation are required to detect reassociation of c-FLIP$_L$ with the Fas DISC (Fig. 15c) [78]. The fact that re-expression of c-FLIP$_L$ precedes the restoration of the integrity of the plasma membrane in CD40L-stimulated GC B cells supports the notion that c-FLIP$_L$ determines the fate of these cells.

Lack of apoptosis in the immune system or its deregulation can lead to the emergence of serious immune diseases such as formation of malignant lymphomas. Hodgkin's disease and non-Hodgkin's lymphomas are traditionally classified among malignant lymphoma. The vast majority of non-Hodgkin's lymphomas (NHL) show somatically mutated variable-region genes, indicating its origin from the germinal centre B cells [126].

Follicular lymphoma (FL) is one of the most common forms of B-NHL. The disease usually follows an indolent course. The initial symptoms of follicular lymphoma include painless swelling in one or more parts of the body's lymph nodes, particularly in the neck, armpit, or groin area; called adenopathy. In later stages the gastrointestinal tract, the spleen and the bone marrow are also affected [67].

In the majority of cases (85-90%) FL cells overexpress the Bcl-2 protein, an

anti-apoptotic factor involved in the mitochondrial pathway of apoptosis. The overexpression occurs as a consequence of a translocation t(14;18)(q32;q21) of *bcl-2* into the immunoglobulin heavy-chain locus, which indicates its origin from the GC during SHM [45]. Overexpression of Bcl-2 suppresses apoptosis, this is supported by the increased tumour incidence in *bcl-2* transgenic mice that develop polyclonal follicular hyperplasia of long-lived B cells [132].

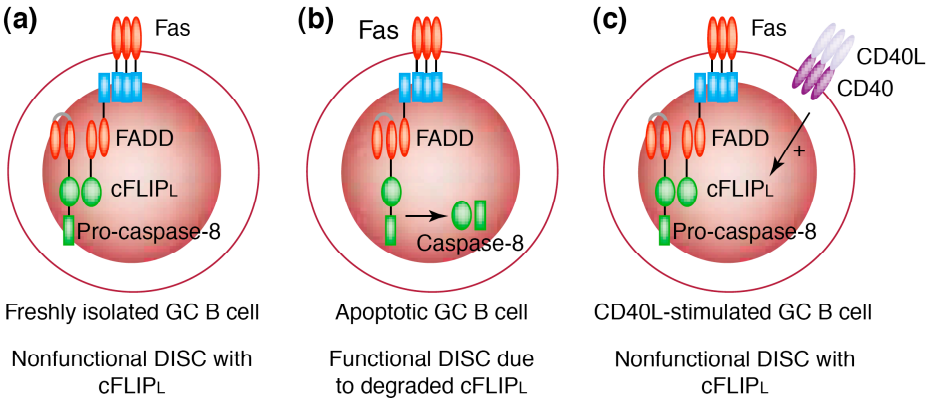

Figure 15. Regulation of apoptosis in GC B cells by c-FLIP$_L$. A) Freshly isolated GC B cells are not apoptotic because they contain a nonfunctional DISC composed of Fas, FADD and c-FLIP$_L$. The presence of c-FLIP$_L$ precludes activation of caspase-8. B) The rapid loss of c-FLIP$_L$ from the Fas DISC in unstimulated GC B cells renders the DISC functional, resulting in the activation of caspase-8. C) A nonfunctional DISC is generated as a result of ligation of CD40 on GC B cells. CD40 signals induce expression of c-FLIP$_L$ and thereby prevent activation of caspase-8. Figure is taken from Eijk, et al., 2001, [200].

1.6. Single Nucleotide Polymorphism (SNP)

A single nucleotide polymorphism (SNP) is a natural DNA sequence variation occurring when a single nucleotide - A, T, C, or G - in the genome differs between members of a species or between paired chromosomes in an individual. For example, a sequenced DNA fragment from two different individuals contains a difference in a single nucleotide (e.g.: AAGC**C**TA to AAGC**T**TA). Each of the two possibilities is called an allele. In this particular case the two alleles are C and T.

SNPs may fall within coding sequences of genes, non-coding regions of genes, or in the intergenic regions between genes. SNPs within a coding

sequence will not necessarily change the amino acid sequence of the protein that is produced, due to degeneracy of the genetic code. A SNP in which both forms lead to the same polypeptide sequence is termed synonymous (or a silent mutation), if a different polypeptide sequence is produced they are nonsynonymous. A nonsynonymous change may either be missense or "nonsense", where a missense change results in a different amino acid, while a nonsense change results in a premature stop codon. SNPs that are not located in protein-coding regions may still have consequences for gene splicing, transcription factor binding, or the sequence of non-coding RNA.

SNPs in the DNA sequences of humans can lead to the development of human diseases and can also predispose humans towards different responses to pathogens, chemicals, drugs, vaccines, and other agents. The international Haplotype Map project, in which research groups from Canada, China, Japan, Nigeria, the United Kingdom, and the United States collaborate together to develop a haplotype map (HapMap) of the human genome, describes the common pattern of human SNPs. So far over 3.1 million SNPs have been identified in humans [51]. This project provides a framework to study the connection between DNA sequence variations and human disease.

2. AIM OF THE STUDY

Alternative splicing plays a major role in the generation of protein diversity of higher organisms. The key for an accurate accomplishment of alternative splicing events lies in the correct selection of splice sites, a process involving interplay of a vast amount of proteins. The aim of this study was to elucidate the mechanisms by which splicing factors mediate splice site selection and to shed light on the role played by the consensus sequences involved, the splice sites.

In the first part, components of the apoptosis and splicing associated protein (ASAP) complex were examined for their impact on splice site selection. Whereas a role of the ASAP subunit RNPS1 has been well defined in both constitutive and in alternative splicing [163, 116, 195], the other subunits of the ASAP complex, Acinus and SAP18, have not been studied in detail for their function during splice site selection.

The specific objectives of this study were:

- To determine the potential of full-length Acinus, RNPS1 and SAP18 proteins to act as splice regulatory factors.
- To analyze the significance of RS domains existing in Acinus and RNPS1 for the selection of splice site.

In the second part, the role of splice site during the generation of different c-FLIP isoforms was studied. Like many other apoptotic factors, c-FLIP is also subjected to alternative splicing [64]. Although a great deal of knowledge has been gained regarding the function of different isoforms of apoptotic proteins, only limited information is available concerning the molecular mechanisms underlying the regulation of splice site choice. Potentially, splice site selection can be determined by the presence of natural genetic variations like single nucleotide polymorphisms (SNPs) [139, 121]. Importantly, the existence of a SNP can have a great impact on the isoform generation and in disease development [38].

The aims of this study were:

• To investigate the production of short isoforms (c-FLIP$_S$ and c-FLIP$_R$) of c-FLIP.

• To examine differences in c-FLIP$_S$ and c-FLIP$_R$ expression with correlation to function.

• To analyze whether variations in c-FLIP short isoform productions are associated with human diseases like follicular lymphoma.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals and reagents

All standard chemicals, which are not mentioned in the list, were purchased from Carl Roth (Karlsruhe, Germany).

Substance	Supplier
β-glycerophosphate	Sigma (Munich, Germany)
ATP	Roche (Mannheim, Germany)
Boric acid	Merck (Darmstadt, Germany)
Bovine serum albumin (BSA)	Sigma
Crystal violet	Merck
Digitonin	Sigma
Dimethylsulfoxide (DMSO)	Sigma
Dithiothreitol (DTT)	Sigma
FLAG M2-agarose	Sigma
Glucose	Sigma
Glutaraldehyde	Sigma
Isopropanol	Merck
Non-fat dry milk	Applichem (Darmstadt, Germany)
Nonidet P-40 (NP-40)	Calbiochem (La Jolla, USA)
Paraformaldehyde	Merck
Phenol:Chloroform:Iso-amyl alcohol	Sigma
Phosphocreatine	Sigma
Potassium chloride (KCl)	Merck
Potassium ferricyanide [$FeK_3(CN)_6$]	Sigma
Potassium ferrocyanide	Fluka Analytical, Sigma-Aldrich Chemie
[$K_2Fe(CN)_6.3H_2O$]	GmbH, (Taufkirchen, Germany)

Protease inhibitor cocktail tablets	Roche
Sodium dodecyl sulphate (SDS)	Merck
Sodium phosphate	Merck

3.1.2. Enzymes and proteins

Product	Manufacturer
Proteinase K	Sigma
Restriction enzymes	New England Biolabs GmbH (Frankfurt, Germany)
RNase A	Sigma
FLAG-peptide	Sigma
Creatine kinase	Sigma
DNAse I	Roche
T-4 DNA ligase	New England Biolabs
Taq polymerase	Applied Biosystems (Foster City, CA, USA)
Pfx Taq polymerase	Invitrogen
SuperScript III RNAse H reverse transcriptase	Invitrogen

3.1.3. Antibodies

Antibody	Origin	Provided by	Working dilution
Anti-Actin	Mouse (monoclonal)	Sigma	1:5000
Anti-His.H8	Mouse (monoclonal)	Santa Cruz Biotechnology	1:1000
Anti-Flag	Rabbit (polyclonal)	Sigma-Aldrich	1:1000
Anti-Flag	Mouse (polyclonal)	Stratagene	1:1000
Anti-RNPS1	Rabbit (polyclonal)	Krainer laboratory (Cold Spring Harbor)	1:500

Anti-SAP18	Goat (polyclonal)	Santa Cruz Biotechnology	1:1000
Anti-Acinus	Rabbit (polyclonal)	Upstate Biotechnology	1:1000
Anti-NF6/ (c-FLIP)	Rabbit (polyclonal)	Alexis Biochemicals	1:1000

Secondary antibodies, anti-mouse and anti-rabbit coupled to horseradish peroxidase, were purchased from Promega, Mannheim, Germany. An anti-goat antibody coupled to horseradish peroxidase was purchased from Molecular Probes, Leiden, The Netherlands.

3.1.4. Kits

Product	Manufacturer
QIAgen HiSpeed plasmid maxi kit	Qiagen (Hilden, Germany)
QIAquick gel extraction kit	Qiagen
QIAquick PCR purification kit	Qiagen
RNeasy kit	Qiagen
RT-PCR kit	Invitrogen (Karlsruhe, Germany)
innuPREP DNA mini kit	Analytik jena AG (Jena, Germany)
DNeasy® Blood and Tissue kit	Qiagen
ECL Western blotting detection reagent	Amersham (Braunschweig, Germany)

3.1.5. Consumables

Product	Manufacturer
Bacterial culture tubes	Greiner Bio-One GmbH (Essen, Germany)
Hyperfilm ™ MP	GE Healthcare UK Ltd (Buckinghamshire, UK)
Cryotubes	Nalgene (Rochester, NY, USA)
Disposable plastic cuvettes	Sarstedt (Nuembrecht, Germany)
Eppendorf tubes (1,5 and 2 ml)	Eppendorf (Hamburg, Germany)

Sterile tubes (15ml and 50 ml)	Greiner Bio-One GmbH (Essen, Germany)
PCR tubes	Eppendorf
Polyvinylidene difluoride (PVDF) membranes	Amersham Pharmacia (Braunschweig, Germany)
Quartz cuvette	PerkinElmer LAS GmbH (Juegesheim, Germany)
Tissue culture dishes	Greiner Bio-One GmbH

3.1.6. Equipments

Product	Manufacturer
Autoclave-Varioklav	Thermo Electron corporation (Waltham, MA, USA)
Axiovert135 microscope	Zeiss (Goettingen, Germany)
Certomat® BS-1 (Bacterial shaker)	Sartorious AG (Goettingen, Germany)
Lambda Fluoro 320 Plus plate reader	MWG Biotech (Eberberg, Germany)
Cell culture hood	Heraeus Holding GmbH (Hanau, Germany)
CO_2 incubator	Heraeus Holding GmbH
Deep freezer (-80°C)	Heraeus Holding GmbH
Hypercassette™ (Developing cassettes)	GE Healthcare UK Ltd
Curix 60 (Developing machine)	Agfa (Cologne, Germany)
Horizon 58-Horizontal gel electrophoresis tank	Life technologies (Gaithersburg, MD, USA)
FACSCalibur	Becton Dickinson (Heidelberg, Germany)
LAS-3000 (Gel documentation system)	Fuji Photo Film Europe GmbH (Duesseldorf, Germany)

Millipore H_2O-production unit	Millipore GmbH (Schwalbach, Germany)
pH meter-Thermo Orion 55A	Thermo Electron corporation (Waltham, MA, USA)
Pipettes (p-10, p-20, p-200, p-1000)	Eppendorf
Power packs-EPS 1000	GE Healthcare UK Ltd
Sorvall RC5B Plus centrifuge	Thermo Electron corporation (Waltham, MA, USA)
Lambda 20, UV/VIS Spectrometer	PerkinElmer LAS GmbH
Eppendorf Concentrator 5301	Eppendorf
GeneAmp PCR System 9700	Applied Biosystems
Ultracentrifuge-Optima LE-80K	Beckman Coulter GmbH (Krefeld, Germany)
UV illuminator	Carl Roth GmbH (Karlsruhe, Germany)
Wallac Victor 1420-Multilabel counter	PerkinElmer LAS GmbH

3.1.7. Cell line

Cell line	Description
HeLa Tet-Off	HeLa cells stably expressing the tetracycline-controlled transactivator (tTA) protein

3.1.8. Cell culture reagents

Product	Supplier
Blasticidin	Invitrogen (Karlsruhe, Germany)
Dulbecco's modified Eagle's medium with high glucose (DMEM)	PAA Laboratories (Coelbe, Germany)
Fetal calf serum	PAA Laboratories
Penicillin/Streptomycin	PAA Laboratories
Sterile PBS	PAA Laboratories

Tetracycline	Sigma
Tetracycline-reduced fetal bovine serum	PAA Laboratories
Trypsin-EDTA	PAA Laboratories

3.1.9. Other reagents

Products	Supplier
DEVD-AMC (N-acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin)	Bachem (Heidelberg, Germany)
1kb DNA ladder	Bio Rad Laboratories (Munich, Germany)
6x DNA loading dye	Fermentas GmbH (St. Leon-Rot, Germany)
Fugene 6 reagent	Roche Diagnostics

3.1.10. MS2 tethering system

To analyze potential splice regulatory activities of the individual ASAP subunits, a MS2 tethering system was employed. This tethered system consists of: **a)** An HIV-1 based splice reporter construct "2xMS2" and **b)** Fusion proteins containing individual subunits of ASAP complex.

a) The splice reporter (2xMS2) construct is an exon-inclusion/skipping model. It contains 3 exons and 2 introns, and is a derivative of the HIV-1 (SV-1-env) plasmid (GenBank Accession No. M19921). The coding sequences for structural proteins and enzymes were deleted from the HIV-1 genome to generate the source plasmid (SV-1-env). Remaining 5' and 3' regions were joined together via a nucleotide linker and inserted into an eukaryotic expression vector (Fig. 16). SV-1-env contains a cis-acting domain, which is rich in purine residues. This region is an exonic splicing enhancer (ESE)/Guanine-Adenine-rich (GAR) sequence and is located in the 5' part of the HIV-1 exon-5 [86]. The HIV derived sub-genomic reporter 2xMS2 was constructed by substituting the GAR-ESE containing *EcoR*I-*Sac*I fragment with two RNA stem loops recognized specifically

by the RNA-binding domain of the MS2 bacteriophage coat protein [25]. The generated reporter construct “2xMS2” is depicted in Fig. 16B. Reporter construct contains the alternative internal exons-4c, -4a, -4b and exon-5 in between two introns.

b) The experimental system further uses fusion proteins (Fig. 17A) containing a nuclear localization signal (NLS) and the protein sequence of interest fused to the RNA-binding domain of the MS2 bacteriophage coat protein [25]. This RNA-binding domain can specifically bind to the RNA stem loops introduced into the reporter construct. Therefore, the tethering system can be used for targeting specific SR proteins or their RS domains to the MS2 stem loop structures via an MS2 RNA-binding domain. Upon specific binding of the MS2-fusion proteins to the transcribed MS2 loops, they potentially recruit splicing factors and the splicing machinery to both 5’ and 3’ splice sites of the middle exon of the 2xMS2 pre-mRNA. After the recruitment of the splicing machinery, the process of splicing follows during which several spliced transcripts can be generated (depicted in Fig. 17B). The sizes of the spliced transcripts are dependent on the selected splice acceptor sites and the potential splice site usage on the other hand is dependent on the MS2-fusion proteins. The generated spliced transcripts can be detected with the help of reverse transcriptase polymerase chain reaction (RT-PCR) using appropriate primers. Thus, the tethered system allows the analysis of the splicing regulatory potential of the selected proteins or protein domains.

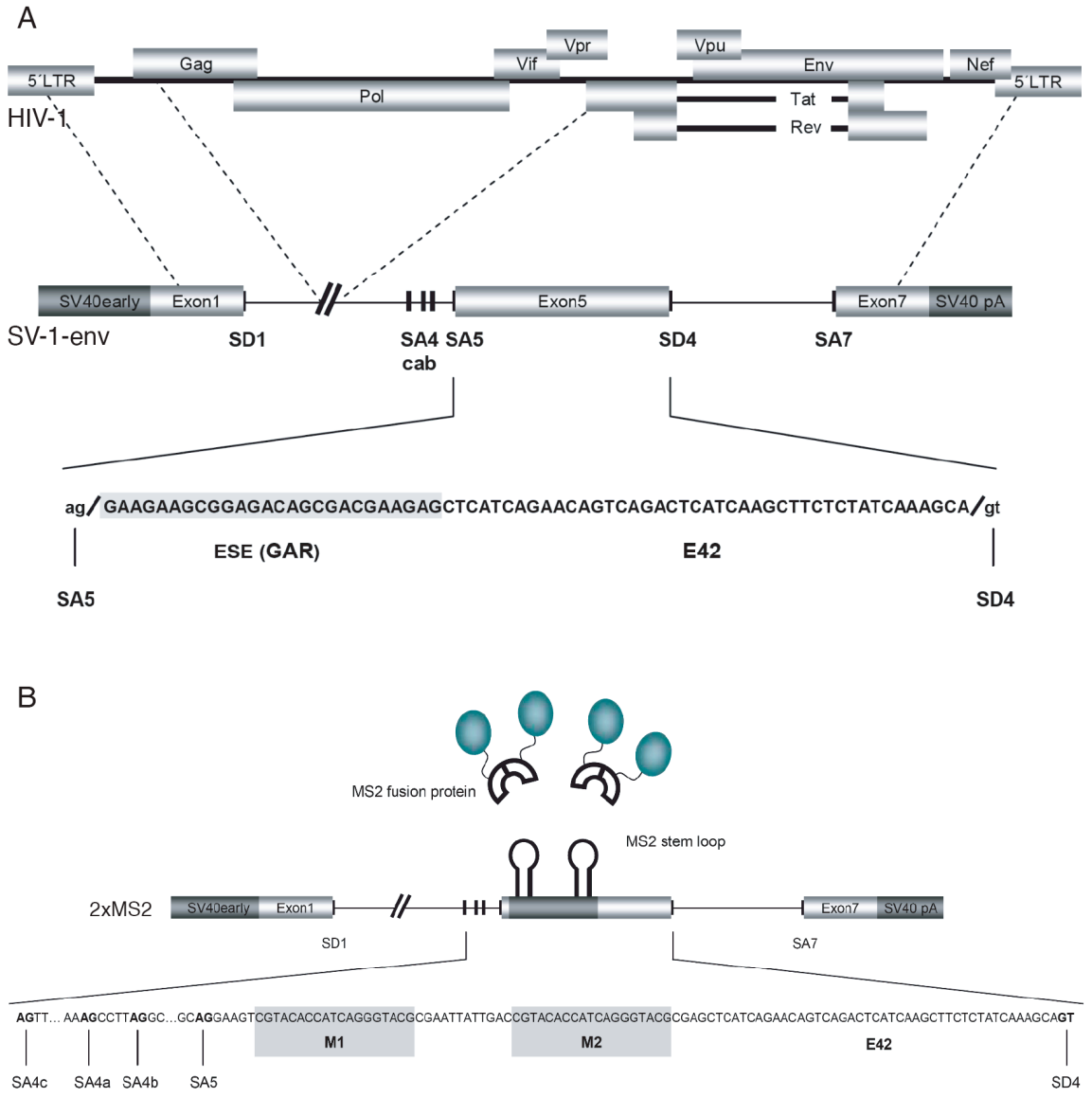

Figure 16. Schematic representation of the HIV-1 derived minigene SV-1-env/2xMS2 and MS2-fusion proteins A) Top: A schematic depiction of the HIV-1 genome with open reading frames (ORFs) and long terminal repeats (LTRs) shown as grey boxes. Centre: The structure of the SV-1-env minigene. Exons are indicated as grey boxes, introns as lines. The SV40 promoter (SV40) and polyadenylation site (pA) are depicted as dark grey boxes. The position of splice acceptor (SA) and splice donor (SD) sites is indicated. Bottom: The nucleotide sequences of the GAR splicing enhancer sites. B) A schematic representation of the 2xMS2 reporter construct, where the ESE region is replaced by MS2 stem loops. MS2-fusion proteins specifically bind to the stem loops. The nucleotide sequences of the MS2 loops are shown in grey boxes. The position of SA4c, SA4a, SA4b, SA5 and SD4 are depicted.

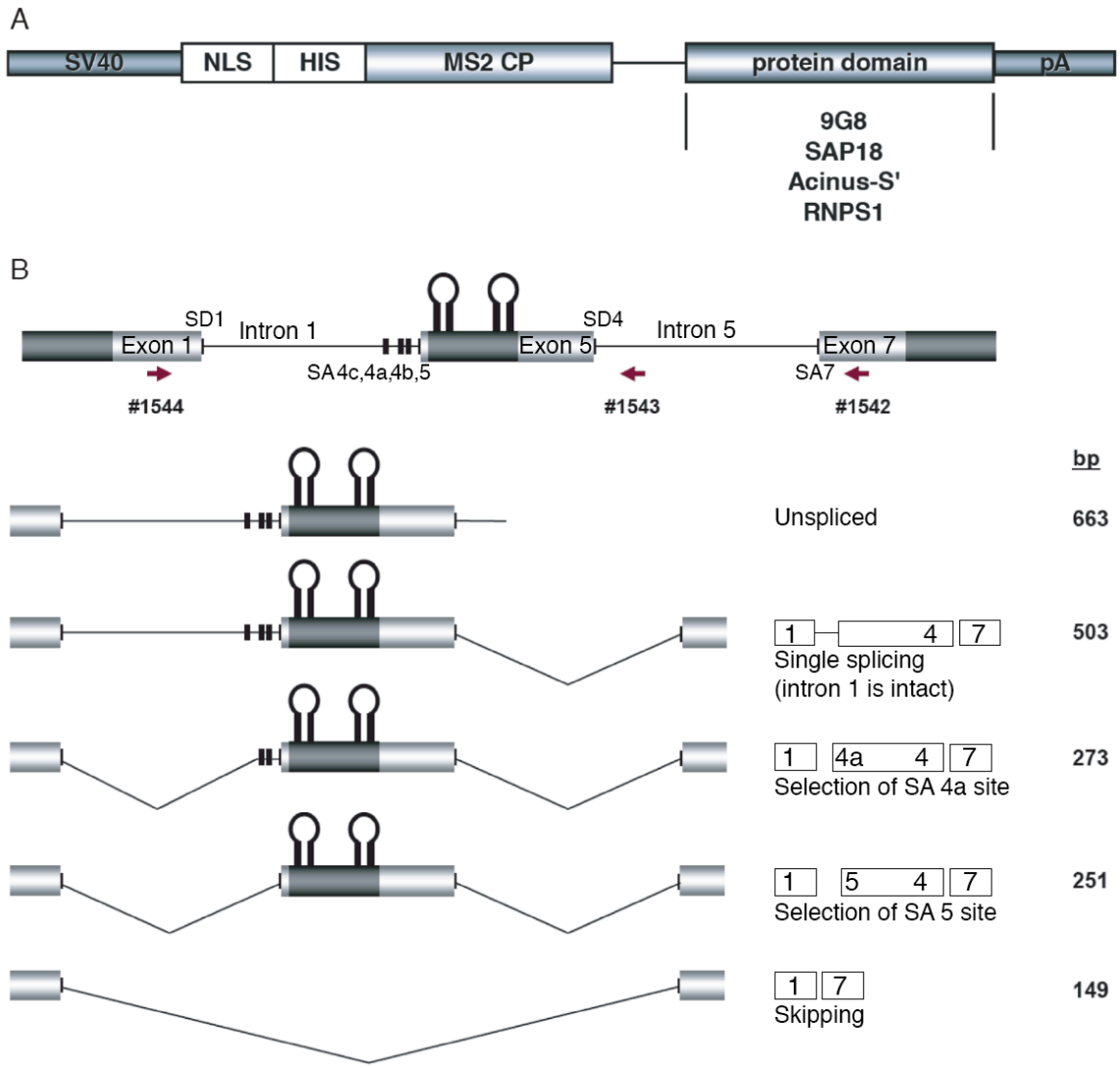

Figure 17. Representation of the MS2-fusion proteins and splicing events. A) Schematic representation of the structure of the MS2-fusion proteins expressed in the splicing assays. The fusion proteins comprise of a nuclear localization signal (NLS), a histidine tag (HIS) and the RNA-binding domain of the MS2 bacteriophage coat protein (MS2 CP) fused to protein domain of choice. The SV40 promoter (SV40) and polyadenylation site (pA) are indicated. B) The 2xMS2 reporter construct and the resulting transcripts from the alternative splicing process are displayed. Transcript sizes are indicated at the right; primers employed for RT-PCR analyses are shown as red arrowheads.

3.1.11. Expression vectors

pCI-neo, pcDNA3.1, and SV-NLS-MS2 9G8 [25] are the basic plasmids used to clone all the constructs used in the present study. Each construct was generated according to the table below. The constructs were validated via sequencing (Qiagen Genomic Services, sequencing services, Hilden).

Plasmids	Features
SV-NLS-MS2-9G8	A 4.9 kb human 9G8 gene with a linked SV40 enhancer and T7 promoter [25]. Kind gift from Prof. Dr. Schaal, University of Duesseldorf, Germany.
SV-NLS-MS2-ΔRS	A 4.6 kb plasmid in which all RS domains of the human 9G8 gene were deleted from the sequence [25]. Kind gift from Prof. Dr. Schaal.
SV-NLS-MS2-Acinus (576-668), SV-NLS-MS2-Acinus (1174-1341), SV-NLS-MS2-Acinus (1261-1341)	The *Bam*HI-*Xho*I fragment of SV-NLS-MS2-9G8 was substituted with PCR-amplified Acinus amino acid residues spanning 576-668, 1174-1341, and 1261-1341 using forward and reverse primers containing *Bam*HI and *Xho*I restriction site, respectively.
SV-NLS-MS2-RNPS1 (69-121), SV-NLS-MS2-RNPS1 (268-305)	The *Bam*HI-*Xho*I fragment of SV-NLS-MS2-9G8 was substituted with PCR-amplified RNPS1 fragments spanning 69-121 and 268-305 amino acids using forward primer and reverse primers

	having *Bam*HI and *Xho*I restriction site, respectively.
pcDNA3.1zeo(-)Flag-MS2-Acinus-S', pcDNA3.1zeo(-)Flag-MS2-SAP18, pcDNA3.1zeo(-)Flag-MS2-RNPS1, pcDNA3.1zeo(-)Flag-MS2-hnRNPA1, pcDNA3.1zeo(-)Flag-MS2-MLN51	The amplified sequences were cloned into the *Not*I-*BamH*I sites of pcDNA3.1zeo(-) vector [189]. Kind gifts from Prof. Dr. Moore. University of Massachusetts Medical School, USA.
pCIneo-FlagRNPS1, pCIneo-FlagRNPS1 Δ1-68, pCIneo-FlagRNPS1 Δ69-121, pCIneo-FlagRNPS1 Δ163-243	RNPS1 sequence was amplified from HeLa cDNA and cloned into the *Nhe*I-*Xho*I sites of pCI-neo vector [61]. Plasmids were kind gifts from Dr. Gehring, University of Heidelberg, Germany.

3.1.12. Oligonucleotides

DNA oligonucleotides were ordered from Invitrogen. PCR primers were obtained in desalted, lyophilized form and were dissolved in dH_2O.

3.1.12.1. PCR primer list

Name	Sequence (5' → 3')
Acinus (576-668) fwd	GGT GGA TCC TCA TCC TCT AGC CGG TCC
Acinus (576-668) rev	AGA CTC GAG TTA GCT GGT GCT GCT GTC
Acinus (1174-1341) fwd	GGT GGA TCC GAG CGG GAG AAG GAA GC
Acinus (1174-1341) rev	AGA CTC GAG CTA GCG GCG CCC ACC C
Acinus (1261-1341) fwd	GGT GGA TCC CGT TCC CGA TCA AGG TCC
Acinus (1261-1341) rev	AGA CTC GAG CTA GCG GCG CCC ACC C

RNPS1 (69-121) fwd	GGT GGA TCC AAT AAA AAG TCC AGC ACT AGG
RNPS1 (69-121) rev	AGA CTC GAG TTA TCT GCG CCG AGA AGG
RNPS1 (268-305) fwd	GGT GGA TCC CCC TGG CCT AGG CCA C
RNPS1 (268-305) rev	AGA CTC GAG TTA TCG GGA GTT GGA G
SAP18 fwd	GGT GGA TCC ATG GCG GTG GAG TCG CGC GTT ACC
SAP18-126 rev	AGA CTC GAG TTA CTT CTG CGA CTG CAG GG
SAP18-121 rev	AGA CTC GAG TTA GGT CAT GGA ATC ATC AGT CCC
SAP18-103 rev	AGA CTC GAG TTA AAC TCG ATA GCC AGG TCT TTT AAC ATC

3.1.12.2. Primers used for site-directed mutagenesis

The following primers were used to generate double mutant SAP18(Asp118→Ala, Thr121→Ala) expression plasmid.

Name	Sequence (5' → 3')
Double mutant SAP18 fwd	GGC AGA AAG GGG ACT GAT GCT TCC ATG GCGCTG CAG TCG CAG AAG
Double mutant SAP18 rev	CTT CTG CGA CTG CAG CGC CAT GGA AGC ATCAGT CCC CTT TCT GCC

3.1.12.3. Primers used for sequencing

Name	Sequence (5' → 3')
1816	ACC TTA GAG CTT TAA ATC TCT
T7	TAA TAC GAC TCA CTA TAG GG
T3	CAA TTA ACC CTC ACT AAA G
SP6	ATT TAG GTG ACA CTA TAG AA

3.1.12.4. Primers used for the reverse transcriptase-PCR

Name	Sequence (5' → 3')
SA7 1542	CAC CTT CTT CTT CTA TTC CTT
SD4 1543	CCC CCA TCT CCA CAA
SD1 1544	CTT GAA AGC GAA AGT AAA GC

3.2. Methods

3.2.1. Preparation of competent *E.coli* for transformation

For the preparation of competent *E.coli* DH5α, bacteria from frozen stock were streaked onto LB agar (10 g tryptone, 5 g yeast extract, 10 g NaCl, 8 g agar) and incubated at 37°C overnight. A single colony was inoculated into 5 ml LB medium and allowed to proliferate overnight at 37°C. 1 ml of the bacterial suspension was transferred to 100 ml LB medium (see 3.2.3) and incubated at 37°C until the suspension reached an OD_{600} of 0.6. The culture was cooled on ice, and the bacterial cells were pelleted by centrifugation at 1200x g for 5 min at 4°C. The bacterial pellet was resuspended in 10 ml of ice-cold TSS (85% LB medium, 10% PEG, 5% DMSO, 50 mM $MgCl_2$ pH 6.5) aliquoted in pre-cooled reaction tubes and stored at -80°C. Aliquots were tested by transformation with serial dilutions of plasmid DNA. Transformation efficiency of at least 10^6 colonies per μg of plasmid DNA was obtained.

3.2.2. Transformation of competent bacteria

50 ng of plasmid DNA were mixed with 100 μl of competent E.*coli* DH5α, incubated on ice for 30 min and heat-shocked for 90 s at 42°C. The suspension was chilled on ice, 500 μl of LB medium were added after 3 minutes and the suspension was incubated for another 30 minutes at 37°C with rotation. The transformed bacteria were then streaked on LB agar plates containing 100 μg/ml ampicillin and incubated at 37°C overnight.

3.2.3. Culturing bacteria

Luria Broth (per liter)

10 g	Tryptone
5 g	Yeast extract
10 g	NaCl

To maintain bacterial stocks of plasmids and propagating plasmid constructs, competent *E.coli* DH5α strains were transformed with the plasmids and cultured overnight in LB medium, containing the appropriate concentrations (Amp. 100 μg/ml or Kan. 50 μg/ml) of the respective antibiotic, at 37°C with shaking at 200 rpm.

3.2.4. Isolation of plasmid DNA from bacteria

Small-scale plasmid isolation

Solution I		Solution II	
50 mM	Glucose	0.2 N	NaOH
25 mM	Tris Cl, pH 8.0	1% (w/v)	SDS
10 mM	EDTA, pH 8.0		
0.5 mg/ml	RNase A		

Solution III

3 M	potassium acetate
11.5% (v/v)	glacial acetic acid

Plasmids were propogated in *E.coli* DH5α. Single colonies were picked from an agar plate and incubated in 3 ml LB medium, containing the respective antibiotic and incubated overnight at 37°C with constant shaking at 220 rpm. The bacterial cells were pelleted by centrifuging at 500x g for 5 min at room temperature in a tabletop centrifuge (Biofuge fresco, Heraeus). Pellets were resuspended in 200 μl of solution I and lysed with 100 μl of solution II. Addition of 150 μl of solution III precipitated proteins leaving the plasmid DNA in solution. The proteins were separated from the DNA by phenol chloroform extraction. The

precipitate was pelleted by centrifugation at 18000x g for 15 min at 4°C and the supernatant was collected. 600 µl of isopropanol was added and the samples chilled at -80°C for 30 minutes to precipitate the DNA. Precipitated DNA was pelleted by centrifugation at 18000x g for 15 min at 4°C and was washed in 500 µl of ice cold 70% ethanol. The precipitated DNA was pelleted and dried in a speed vac. Finally, the dried pellet was resuspended in 40 µl of dH_2O and the concentration of DNA was spectrophotometrically measured at 260 nm (A_{260}) in dH_2O (pH 8.0). Measurements were performed in quartz cuvettes. The yield was estimated using the standard conversion that 50 µg/ml DNA corresponds to an OD of 1.0 at 260 nm. The ratio of OD_{260}/OD_{280} was determined to assess the purity of the sample. Visualizing on the ethidium bromide stained agarose gel controlled the plasmid concentration.

Large-scale plasmid isolation (Maxi-prep)

A pre-culture was prepared by inoculating a single colony from an agar plate in 2 ml of LB medium, containing the respective antibiotic, at 37°C for 8 hours. This was used to subsequently inoculate 250 ml of LB broth containing the respective antibiotic. After overnight incubation in a bacterial shaker at 37°C, the cells were pelleted at 1700x g for 10 min at 4°C. Plasmid purification was subsequently carried out using the Hispeed plasmid purification kit (Qiagen) according to the manufacturer's instructions. DNA was eluted in 1 ml of dH_2O and its concentration was quantitated spectrophotometrically.

3.2.5. Restriction enzyme digests

Plasmids and DNA fragments generated by PCR were digested with appropriate enzymes (supplied by NEB Biolabs). Optimal reaction buffers, as recommended by the manufacturer were used. Reactions were typically carried out in a total volume of 50 µl, using 1 U of each enzyme per µg DNA. BSA was added where required. The reaction was carried out for 3 to 5 hours in an

incubator at 37°C. Restriction fragments were visualized by agarose gel electrophoresis (see 3.2.7) and purified using the QIAGEN Gel Extraction kit according to the manufacturer's direction.

3.2.6. Ligations

A linearized vector and a gene fragment with compatible ends were ligated using T4-DNA ligase (NEB Biolabs). Generally, 50 ng of linearized plasmid were ligated with (1:3) molar ratios of insert. Ligations were performed in a 20 µl reaction containing 1 µl T4 DNA ligase (400 U/ml) and 2 µl 10x T4 DNA ligase buffer (100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP, 300 mM Tris-HCl, pH 7.8). Ligation reactions were performed for 2 hours at room temperature. 10 µl of the ligation reaction mixture was subsequently transformed into competent bacterial cells.

3.2.7. Agarose gel electrophoresis

0.5x TBE buffer (per liter)	
5.4 g	Tris base
2.75 g	Boric acid
2 ml	0.5 M EDTA, pH 8.0

Electrophoresis was performed in 0.8 to 2% (w/v) agarose gels in 0.5x TBE buffer depending on the expected fragment sizes. Agarose gels were electrophoresed in a horizontal electrophoresis tank submerged in 0.5x TBE buffer at 1-5 V/cm. Ethidium bromide stained gels were visualized and photographed.

3.2.8. Purification of DNA fragments from agarose gels

DNA fragments generated by PCR or by restriction digestion were purified depending on their sizes on 0.8% to 1.5% agarose gels. Subsequently, excised DNA fragments were extracted using the QIAquick Gel Extraction Kit (Qiagen)

according to the manufacturer's instructions. DNA was eluted from the silica-based purification column with 30 μl dH_2O and stored at -20°C.

3.2.9. Standard Polymerase Chain Reaction (PCR)

DNA fragments were amplified from plasmid DNA constructs or genomic DNA to obtain selective amplification of the desired fragments. For each PCR reaction, a total volume of 50 μl was prepared as follows:

5 μl 10 x PCR-buffer (eppendorf)

2 μl Fwd-primer (10 pmol/μl)

2 μl Rev-primer (10 pmol/μl)

1 μl 10 mM dNTPs

1 μl plasmid DNA (10 ng/ μl) or PCR product (10 ng/μl) or bacterial clones (tooth picks), or 1 to 5 μl of genomic DNA solution

0.5 μl Taq-polymerase (eppendorf)

d H_2O till 50 μl

PCR reactions were performed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The optimal number of PCR cycles and the annealing temperature were determined empirically for each PCR reaction. A standard PCR programme was as follows:

94 °C	3 min of initial denaturing
94 °C	30 sec (25 times) of denaturing
TA	30 sec (25 times) of primer annealing
72 °C	30 sec (25 times) of DNA synthesis
72 °C	10 min of final extention
4 °C	storage temperature

3.2.10. Site directed mutagenesis

To mutate aspartate 118 and threonine 121 amino acids of SAP18 to alanines, site directed mutagenesis was used (Quick change site directed

mutagenesis kit, Stratagene). Using the following procedure, base pairs were successfully changed. The reaction was prepared as follows:

5 μl of 10x reaction buffer

2 μl (10 ng) of pFlag-MS2-SAP18 control plasmid (5 ng/μl)

1.25 μl (125 ng) of double mutant SAP18 fwd primer

1.25 μl (125 ng) of double mutant SAP18 rev primer

1 μl of dNTP mix

1 μl of *PfuTurbo* DNA polymerase (2.5 U/μl)

38.5 μl of distilled water (dH_2O) to a final volume of 50 μl

Programme

Segment	Cycles	Temperature	Time
1	1	95°C	30 seconds
2	18	95°C	30 seconds
		55°C	60 seconds
		68°C	3 minutes

After the PCR cycle, the reaction mixture was subjected to *Dpn* I digestion. 1 μl of *Dpn* I restriction enzyme was added to the reaction mixture and incubated at 37 °C for 1 h. 2 μl of the reaction mixture were transformed into Top10 competent cells.

3.2.11. DNA extraction from paraffin embedded tissue samples

Genomic DNA was extracted from paraffin embedded tissues according to the manufacturer's protocol (innuPREP DNA mini Kit, analytik jena bio solutions). Paraffin samples were first de-paraffinized in xylene. After a brief centrifugation for 5 min at room temperature supernatants were discarded. These samples were then washed with ethanol and subsequently incubated at 37°C for 15 minutes to evaporate the residual ethanol. Incubating the samples at 50°C with lysis solution (400 μl) and proteinase K (25 μl) for 5-10 hours using a pulsed vortexer (Genie-2, Scientific Industries) lysed the tissue. Lysed samples were

mixed with 400 µl of binding solution. These samples were applied to a spin filter located in a 2.0 receiver tube and centrifuged at 10000x g for 2 minutes. Spin filters were washed twice and finally DNA was eluted in 50 µl of elution buffer.

3.2.12. TaqMan® single nucleotide polymorphism (SNP) Genotyping Assay

The SNP genotyoing was performed on the c-FLIP gene of the control and patient samples. Genomic DNA from healthy persons, urothelial carcinoma patients and from follicular lymphoma patients was used for this assay. Genotyping of rs10190751 was performed using custom TaqMan®SNP Genotyping Assays (Assay ID: C_30472738), which consist of a sequence specific forward and reverse primer to amplify the polymorphic sequence of the c-FLIP gene. It also contains two TaqMan® probes: one probe labeled with VIC® dye detects the Allele 1 sequence and the other probe labeled with FAM™ dye detects the Allele 2 sequence.

PCR was performed using 12.5 µl of TaqMan® Universal Master Mix, 10 ng (2 µl) of DNA, 1.25 µl of custom TaqMan® SNP Genotyping Assay (20-fold mix) and 9.25 µl of DNase-free water to bring the final reaction volume to 25 µl. A control sample without added DNA and DNA controls with known genotypes were used as negative and positive controls, respectively. PCR plates were read on a 7900HT Fast Real-Time PCR system (Applied Biosystems).

3.2.13. Growth and maintenance of mammalian cell lines

RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin was used to maintain HeLa tet-off cells in culture. Every 3-4 days, cells were sub-cultured by dilution of the cells to a concentration of $1x10^5$ cells/ml.

HEK293T human embryonic kidney cells were cultured in Dulbecco's modified Eagle's medium with high glucose supplemented with 10% fetal calf serum and 50 µg/ml of each penicillin and streptomycin.

Chimpanzee (LTR2008, EB176) and Bonobo cell lines were cultured in Iscove's modified DMEM (IMDM, PAA laboratories) supplemented with 15% fetal calf serum (BioWest, Frickenhausen, Germany), 2 mM glutamine, 0.1 mM MEM non-essential amino acid solution (Invitrogen), and 50 μg/ml of each penicillin and streptomycin (Invitrogen). All cell lines were incubated at 37°C in a humidified incubator with 5% CO_2.

3.2.14. Preparation of liquid nitrogen stocks

Cells cultured to approximately 70% confluency were harvested by trypsinization and resuspended in 4 ml growth medium. Cells were pelleted at 1200x g for 2 min at 4°C. The cell pellet resuspended in 2 ml freezing medium (growth medium containing 20% fetal bovine serum and 10% DMSO) was aliquoted into cryotubes and then frozen at -80°C overnight. On the following day the tubes were transferred to a liquid nitrogen tank.

3.2.15. Transfection of mammalian cell lines

Mammalian cells were transfected for transient expression using FuGENE 6 reagent according to the manufacturer's instructions. For transient transfection, efficiency of transfection was monitored by control transfection using a GFP-SAP18 expressing construct, which was transfected in a separate well. Total cell extracts were collected after 24 hours of transfection.

3.2.16. Purification of total cellular RNA

Total cellular RNA was extracted from 6 x 10^5 cells seeded in 6 cm plates. Cells were harvested by scraping and washed once in cold PBS. Subsequently, RNA extraction was performed using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The amount of total RNA in the final eluate was determined spectrophotometrically.

3.2.17. Reverse transcriptase PCR (RT-PCR)

Prior to reverse transcription, RNA was subjected to DNA digestion. 1 μl of DNAse I (RNase-free 10 U/μl, Roche Molecular Biochemicals) was incubated with 2 μg of RNA together with dH_2O in a total volume of 10 μl at 37°C for 1 hour. After DNAse I inactivation at 95°C for 5 min, 4.5 μl of the DNAse digested RNA samples were used for the reverse transcription. 1 μl of oligo-$(dT)_{12-18}$ (7.5 μM, Invitrogen), 4 μl of deoxyribonucleoside triphospahtes (25 mM/dNTPs, Applied Biosystems) and 3.5 μl of dH_2O were added to the DNAse treated RNA and was incubated at 65°C for 5 min. After incubation, 4 μl 5xfirst strand buffer (Invitrogen), 1 μl DTT (0.1 M, Invitrogen), 1 μl ribonuclease inhibitor (20-40 U/μl, Promega) and 1 μl SuperScript III RNAse H-reverse transcriptase (200 U/μl, Invitrogen) were added to the samples. The reverse transcription was carried out at 50°C for 60 min followed by 70°C for 15 min. The prepared cDNA were used for the PCR.

PCR was carried out in a total volume of 50 μl. In addition to 3 μl of cDNA, 5 μl 10xPCR buffer (Applied Biosystems), 4 μl dNTP (2.5 mM/dNTP, Applied Biosystems), 10 μM forward (1544) and reverse (1543, 1542) primers and 0.25 μl AmpliTaq (5 U/μl, Applied Biosystems) were added and PCR reactions were performed at the following conditions:

94°C	3 min		
	94°C	30 sec	27 cycles
	52°C	1 min	
	72°C	1 min	
72°C	10 min		

The spliced products; double-spliced, partially spliced and skipped RNAs were detected with primer pairs employing primers 1544, 1543 and 1542 (see Fig. 17). The hGH mRNA was amplified with the primer pair 1225 and 1224. PCR products were separated on a 6% non-denaturating polyacrylamide gel (29 ml H_2O, 10 ml 40% PAA, 10 ml 5xTBE, 370 μl APS and 20 μl TEMED), stained with

ethidium bromide (10 min) and visualized with a Lumi-Imager (LAS3000; Fujifilm).

3.2.18. PROTEIN STUDIES

3.2.18.1. Preparation of cell extracts

High salt lysis buffer

350 mM NaCl

20 mM HEPES pH 7.9

1.0 mM $MgCl_2$

0.5 mM EDTA

0.1 mM EGTA

0.5 mM DTT

1.0% NP-40

20% glycerol

About 5 $x10^5$ cells were collected from the cell culture dish by scraping and pelleted down at 500x g at 4°C for 5 min. Cells were washed once in PBS and then lysed in a high-salt buffer containing Complete protease inhibitor cocktail and centrifuged at 18000x g for 15 min at 4°C to pellet the cell debris. The supernatant was collected and the protein concentration quantitated using Bio Rad Protein assay according to the manufacturer's instructions.

3.2.18.2. Protein gel electrophoresis (SDS-PAGE)

Since the range of apparent molecular weights of proteins to be analyzed were between 18 and 220 kDa, 4-15% gradient SDS-PAGE gels were used to separate proteins. The composition of 4 and 15% gradient gels and the stacking gel is given in the following table.

Composition of polyacrylamide gels

Stacking	Acrylamide (40%) (ml)	0.5 M Tris/HCl pH 6.8 (ml)	H_2O (ml)	10% SDS (μl)	10% APS (μl)	TEMED (μl)
4%	1.35	1.25	7.15	100	100	10

Gradient	Acrylamide (40%) (ml)	0.5 M Tris/HCl pH 8.8 (ml)	50% glycerol (ml)	H_2O (ml)	10% SDS (μl)	10% APS (μl)	TEMED (μl)
4%	0.666	1.25	-	3	50	33.3	3.3
15%	2.5	1.25	1	0.166	50	33.3	3.3

These gradient gels were loaded with 30 μg of protein samples. Gels were run at 200 V and the separated proteins were subsequently transferred to the polyvinylidene difluoride membrane (PVDF, Amersham Pharmacia) via Western blotting.

3.2.18.3. Western blotting

Laemmli's running buffer

0.25 M Tris

1.92 M glycine and 1% SDS

5x SDS sample buffer

60 mM Tris-Cl (pH 6.8)

24% glycerol

2% SDS

14.4 mM β-mercaptoethanol

1% bromophenol blue

Transfer buffer

39 mM glycine

48 mM Tris base

0.037% SDS

20% methanol

Protein samples for SDS-PAGE were prepared by mixing 30 μg of cell extract with 5x SDS sample buffer. Samples were heated to 95°C for 5 min and cooled immediately on ice before loading. Equal amounts of proteins were separated under reducing conditions by SDS-PAGE on 4-15% gradient gels. Gels were run at 200V. The separated proteins were subsequently blotted onto a PVDF membrane (Amersham Pharmacia) using a wet blot apparatus (Bio Rad) at 1 mA per cm^2 of the membrane. Membranes were blocked with blocking buffer (5% non-fat dry milk in PBST buffer; 10 mM phosphate, pH 7.4, 2.7 mM KCl, 137 mM NaCl and 0.05% Tween-20) for 1 hour and then incubated overnight at 4°C with the primary antibody against the protein of interest. Blots probed with primary antibody were washed in PBST three times at 5 min interval. Then the membranes were incubated with their respective HRP conjugated secondary antibodies for 2 h at room temperature. Blots were extensively washed with PBST buffer to remove unbound antibodies. ECL solution (Amersham Biosciences) was used to activate the conjugates for chemiluminescence. The blots were immediately exposed to photosensitive films (Amersham Bioscience) and developed.

3.2.18.4. Immunoprecipitations

Buffer A

10 mM HEPES pH 7.9

5 mM $MgCl_2$

0.25 M sucrose

10 mM β-mercaptoethanol

Buffer B

10 mM HEPES pH 7.9

25% (v/v) glycerol

1.5 mM $MgCl_2$

0.1 mM EDTA

10 mM β-mercaptoethanol

Buffer C

20 mM Tris-HCl pH 7.9

0.25 mM EDTA

10% (v/v) glycerol

0.5 mM DTT

0.2 mM phenylmethylsulfonyl fluoride

Extracts for immunoprecipitation were prepared as follows: 1 x 10^7 cells were collected and the cell pellets were resuspended in buffer A containing Complete protease inhibitor cocktail (Roche Diagnostics). NP-40 was added to a final concentration of 0.1%, and the cells were lysed by freezing and thawing followed by incubation on ice for 10 min. The supernatant or cytosolic fraction was collected after centrifugation in a microcentrifuge at 20,000x g for 5 min at 4°C. The pellet was dissolved in buffer B containing Complete protease inhibitor cocktail. NaCl was added to a final concentration of 400 mM and the sample was incubated on ice for 30 min. The sample was freeze-thawed, centrifuged at 20,000 rpm for 15 min at 4°C and the supernatants used as input material for immunoprecipitations. FLAG M2-agarose beads (Sigma) were equilibrated in buffer C containing 100 mM KCl. The beads were incubated for 12 hrs with 100 μl (300 μg) of the input material at 4°C. Following four washes with buffer C containing 500 mM KCl and 0.05% NP-40, protein complexes were eluted from the beads with 2x SDS-sample buffer and analyzed by SDS-PAGE and Western blotting.

3.2.18.5. Immunofluorescence microscopy

HeLa cells were grown on 16 mm coverslips in a 12-well dish. Cells were transiently transfected with 1 μg of the respective plasmid, using FuGENE6 reagent as described in the manufacturer's protocol. 24h post-transfection, cells were rinsed with phosphate buffer saline (PBS). Cells were fixed and permeabilized with 100% methanol for 5 min at -20ºC. After permeabilization, cells were blocked for 1h with block buffer (4% BSA in PBS containing 0.05% saponin). Cells were then incubated overnight at 4°C with the primary antibody (anti-FLAG; anti-His; anti Acinus; each 1:500) dilution in blocking buffer. After rinsing twice with PBS, cells were incubated at room temperature for 2h with the appropriate secondary antibodies coupled to Alexa Flour 488 or Alexa Flour 594

(1:1000). For nuclear staining, cells were stained with 4´,6´-diamidino-2-phenylindole dihydrochloride (DAPI, 10 ng/ml). Cells were then mounted in mounting medium (DakoCytomation, Hamburg, Germany) and analyzed under a confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) at a magnification of 630x.

4. RESULTS

4.1. Regulation of exon inclusion by ASAP complex components.

Acinus and RNPS1 are components of the ASAP complex that contain RS and RRM domains. The presence of RS and RRM domains lead to the assumption that Acinus and RNPS1 might interact with RNA or other proteins and influence RNA splicing. However, SAP18 contains an ubiquitin-like fold in its structure and no RS or RRM domain. Nevertheless, all subunits of the ASAP complex have been shown to be associated with the functional spliceosomes [148, 218]. Therefore, to characterize the role of ASAP complex proteins in pre-mRNA splicing more closely, the MS2-tethering system was utilized (Fig. 16, 17) and the potential of these proteins to act as splicing regulators was examined on the HIV-1-based substrate.

4.1.1. RNPS1 and SAP18 mediate exon inclusion in an HIV-based *in vivo* splicing system.

At first full-length MS2-fusion constructs of Acinus-S', RNPS1 and SAP18 were analyzed for their splicing activity. For this purpose HeLa cells were transiently cotransfected with the reporter "2xMS2" construct together with the respective MS2-fusion proteins. As revealed by RT-PCR analysis of the transiently transfected cells (Fig. 18B), either transfection of the splicing reporter construct alone (lane 3) or cotransfection of the splicing reporter and a construct expressing the MS2-fusion protein lacking any RS domain (lane 4) did not lead to detectable levels of exon inclusion. Only processing of transcripts lacking an internal exon could be observed. In contrast, cotransfection of a control MS2-fusion protein containing the RS domain of the splicing factor 9G8 that was used as a positive control led, as expected, to internal exon inclusion (lane 5). The analysis of ASAP subunits expressed as MS2-fusion proteins revealed that both full-length RNPS1 (lane 6) as well as, surprisingly, SAP18 (lane 8) mediated efficient internal exon inclusion. In contrast, a fusion protein consisting of the

MS2 domain and full-length Acinus-S' displayed only background splicing regulatory activity comparable to the background activity of the splicing silencer hnRNPA1 (cf. lanes 7 and 9). To normalize for any differences in the transfection efficiency or RNA recovery between samples a plasmid expressing hGH was cotransfected.

To rule out that lack of a detectable splicing regulatory activity was due to lack of proper protein expression, Western blot analysis was performed, which revealed that all ASAP subunits were efficiently expressed as MS2-fusion proteins in HeLa cells (Fig. 18C).

To demonstrate that not all nuclear proteins mediate exon inclusion of an HIV-based reporter construct, the human protein MLN51, which is an RNA-binding protein present in the ribonucleoprotein complexes, was examined for its splice regulatory activity [60, 40]. As can be seen in Fig. 19, cotransfection of the splicing reporter gene with a construct expressing the fusion protein MS2-MLN51 does not lead to the inclusion of middle exon (lane 5), but rather leads to the exon skipping. Again, full-length RNPS1 (lane 3) and SAP18 (lane 4), but not Acinus-S' (lane 7) lead to middle exon inclusion.

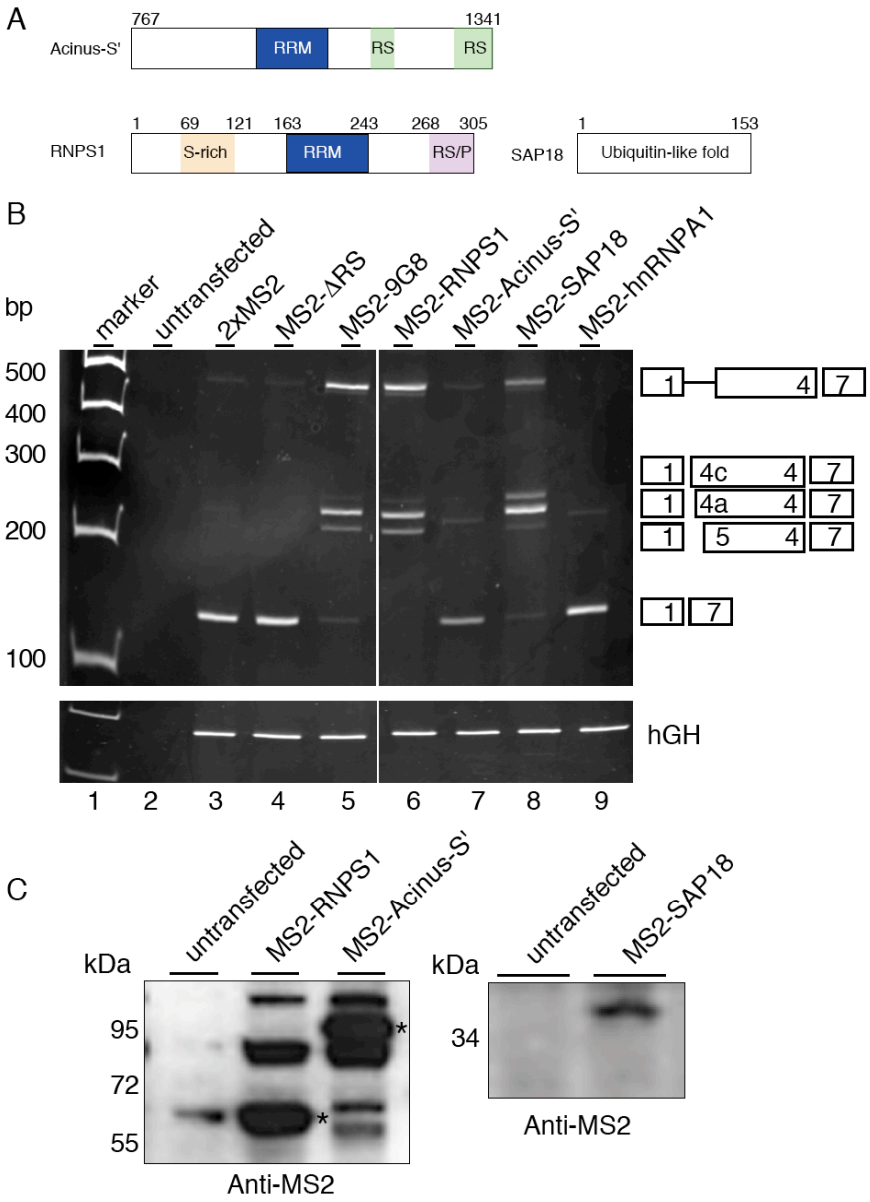

Figure 18. Exon inclusion activity of ASAP subunits in an HIV-1-based reporter system. A) Schematic representation of the subunits Acinus-S', RNPS1 and SAP18 present in the ASAP complex. B) RT-PCR assay of HeLa cells transfected with the splicing reporter 2xMS2 and the MS2-fusion proteins are indicated at the top (lanes 3 to 9). Untransfected control cells are shown in lane 2, marker bands are loaded in lane 1. Spliced products (indicated schematically at the right) were analyzed with specific primers mentioned in Fig. 17B. An internal control, pXGH5 plasmid expressing human growth hormone (hGH) served as control for transfection efficiency. PCR products were separated on a nondenaturing 6% polyacrylamide gel and stained with ethidium bromide. C) Western blot analysis of MS2-fusion proteins. Nuclear extracts of HeLa cells transfected with the MS2-fusion proteins indicated at the top were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). MS2-SAP18 (left panel) as well as MS2-RNPS1 and MS2-Acinus-S' (right panel) were expressed as detected by Western blotting with an antibody against the MS2-domain. Lysates from untransfected cells served as controls.

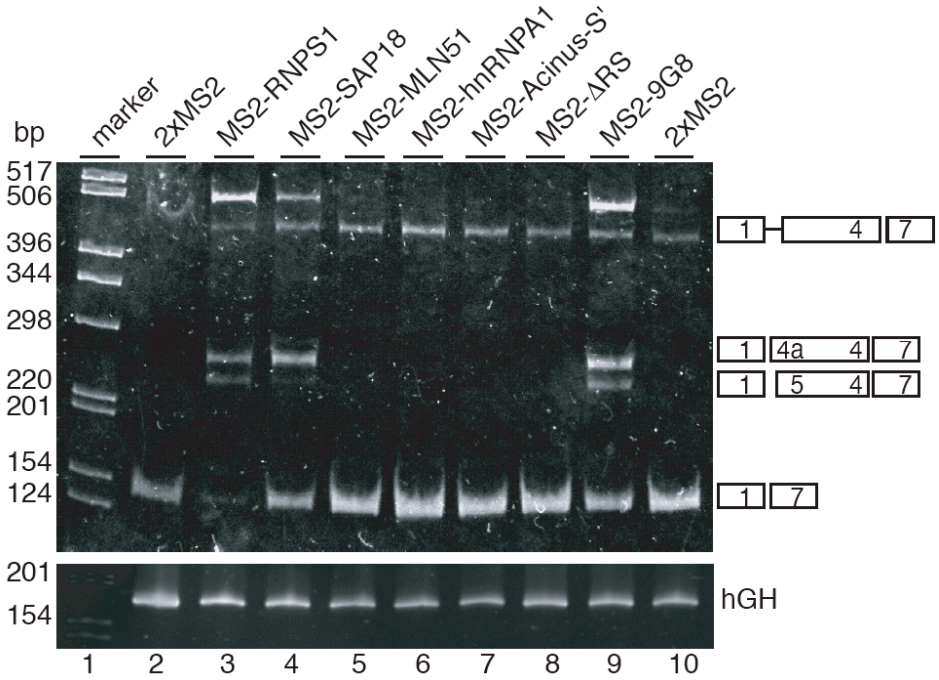

Figure 19. MLN51 shows no exon inclusion activity in an HIV-1-based reporter system. RT-PCR assay of HeLa cells transfected with the splicing reporter 2xMS2 and the MS2-fusion proteins indicated at the top (lanes 2 to 10). Molecular weight marker is loaded in lane 1. Spliced products are indicated schematically at the right. The lower panel shows hGH mRNA level, which served as an internal control.

4.1.2. Individual RS domains of the ASAP complex proteins have the potential to activate splicing of the HIV-minigene.

It has been demonstrated that the RS domains of several human proteins lead to the activation of the pre-mRNA splicing when used in the tethering assay [22, 79]. To determine if the individual RS domains present in Acinus and RNPS1 are sufficient to regulate alternative splicing of the HIV-based substrate, the RS-rich domains of Acinus and RNPS1, were cloned into the MS2-fusion protein expression vector. These domains contained the amino acid residues spanning 576 to 668, 1174 to 1341 and 1261 to 1341, respectively, of Acinus-L. Similarly, the serine-rich domain (amino acid residues spanning 69 to 121) and the RS/P domain (amino acid residues spanning 268 to 305) of RNPS1 were cloned into the MS2-fusion protein expression vector. The influence of co-expression of these MS2-fusion proteins containing the individual RS domains was examined employing RT-PCR.

Interestingly, Acinus (576-668), Acinus (1174-1341) and Acinus (1261-1341) domains significantly augmented selection of an alternative 3' splice acceptor site to mediate inclusion of the middle exon (Fig. 20, lane 5, 6 and 7). Strikingly, Acinus (576-668) preferred splice acceptor 5, whereas MS2-9G8 preferentially supported the splice site 4a (cf. lane 5 and 10). Co-expression of Acinus (1174-1341) caused middle exon inclusion via selection of the 4c acceptor site (lane 6), in addition to splice acceptor 5 and 4a sites. The presented results show that the RS domains of Acinus can regulate the selection of splice sites, suggesting a role of these RS domains in alternative splicing.

To determine the influence of the serine-rich (S-rich) region and the arginine-serine/proline (RS/P) domain of RNPS1, experiments were performed in a similar fashion. Co-expression of the MS2-RNPS1 (69-121) fusion protein together with the HIV minigene stimulated strong inclusion of the internal exon inclusion by selecting splice acceptor 4a and 5 (lane 8). In contrast, co-expression of the MS2-RNPS1 (268-305) fusion protein induced only little exon inclusion (lane 9). To show that the effects of exon inclusion were specific to the respective cotransfections, appropriate controls were performed. Untransfected HeLa cells generated no signals (lane 2), the reporter construct (2xMS2) mediated no exon inclusion (lane 3) and the negative control (MS2-ΔRS), as expected, induced no middle exon inclusion (lane 4). Finally, the positive control MS2-9G8 stimulated exon inclusion (lane 10), as expected.

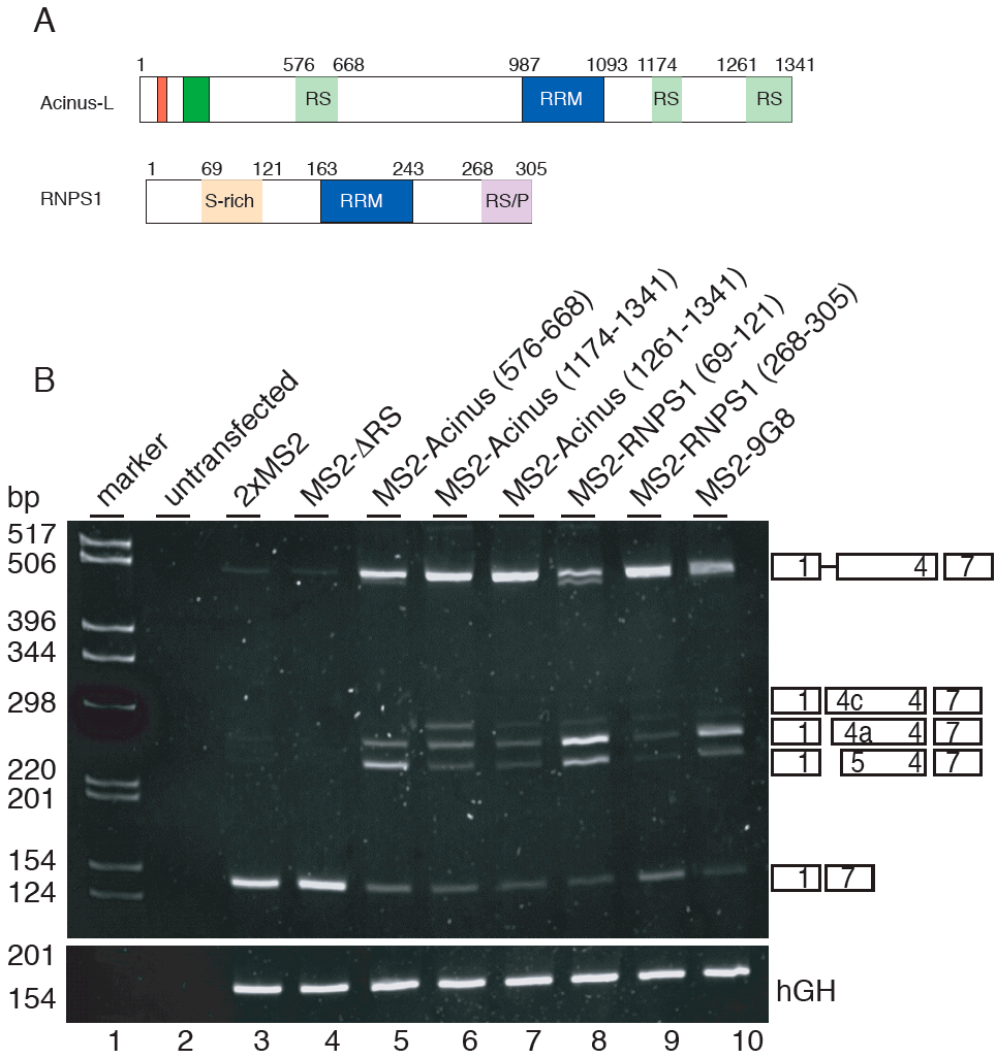

Figure 20. Individual RS domains of Acinus and RNPS1 modulate alternative splicing of the HIV-based pre-mRNA. A) Schematic representation of Acinus-L and RNPS1 protein. B) RT-PCR assays were carried out to monitor the amplified spliced mRNAs. HeLa cells transfected with individual RS domains are indicated on top of lanes 5-10. The RS domains of Acinus (lane 5, 6 and 7) and RNPS1 (lane 8 and 9) mediate splicing via selection of splice acceptor sites indicated schematically at the right. Molecular weight marker was loaded in lane 1.

4.1.3. The S-rich domain of RNPS1 is required for HIV-1 internal exon inclusion.

As mentioned previously, the distinct functional domains of RNPS1 are involved in multiple functions during splicing regulation. Interestingly, the S-rich domain of RNPS1, which interacts with SRp54, stimulated exon inclusion of an ATP synthase γ-subunit pre-mRNA [163]. To analyze whether the S-rich domain is also critical for the internal exon inclusion of the HIV-1 minigene, the 2xMS2

(minigene) together with RNPS1-deletion constructs expressed as MS2-fusion proteins were cotransfected into HeLa cells and the splicing products were analyzed by RT-PCR (Fig. 21B). As expected, the positive control MS2-9G8 (lane 5) as well as full-length RNPS1 mediated strong internal exon inclusion (lane 6). Of note, whereas MS2-9G8 preferentially supported exon 4a inclusion, MS2-RNPS1 shifted the splicing pattern towards enhanced exon A5 inclusion (cf. lanes 5 and 6). This finding is similar to SF2/ASF and SRp40, which are the splicing factors regulating internal exon inclusion by binding to the authentic GAR ESE [25]. The splicing patterns of deletion mutants of RNPS1 that lacked either the first 68 amino acids (Δ1-68) (lane 7) or the region containing the RNA recognition motif (Δ163-243) (lane 9) resembled that of full-length RNPS1. In contrast, cotransfection of a construct expressing an MS2-fusion protein of RNPS1 with the S-rich domain deleted (Δ69-121) displayed a nearly complete loss of internal exon inclusion (lane 8). The reporter alone (2xMS2) or a fusion protein lacking an RS domain (MS2-ΔRS) served as negative control and did not cause internal exon inclusion (lanes 3, 4).

To determine that all MS2-RNPS1 constructs were expressed in HeLa cells (Fig. 21C), Western blot analysis was performed. Although the (Δ69-121) and (Δ163-243) deletion mutants were expressed at a lower level compared to the (Δ1-68) mutant and full-length RNPS1, (Δ163-243) was, in contrast to (Δ69-121), fully capable of mediating internal exon inclusion in the HIV-1 reporter construct, showing that the lower expression level was not the cause of exon skipping.

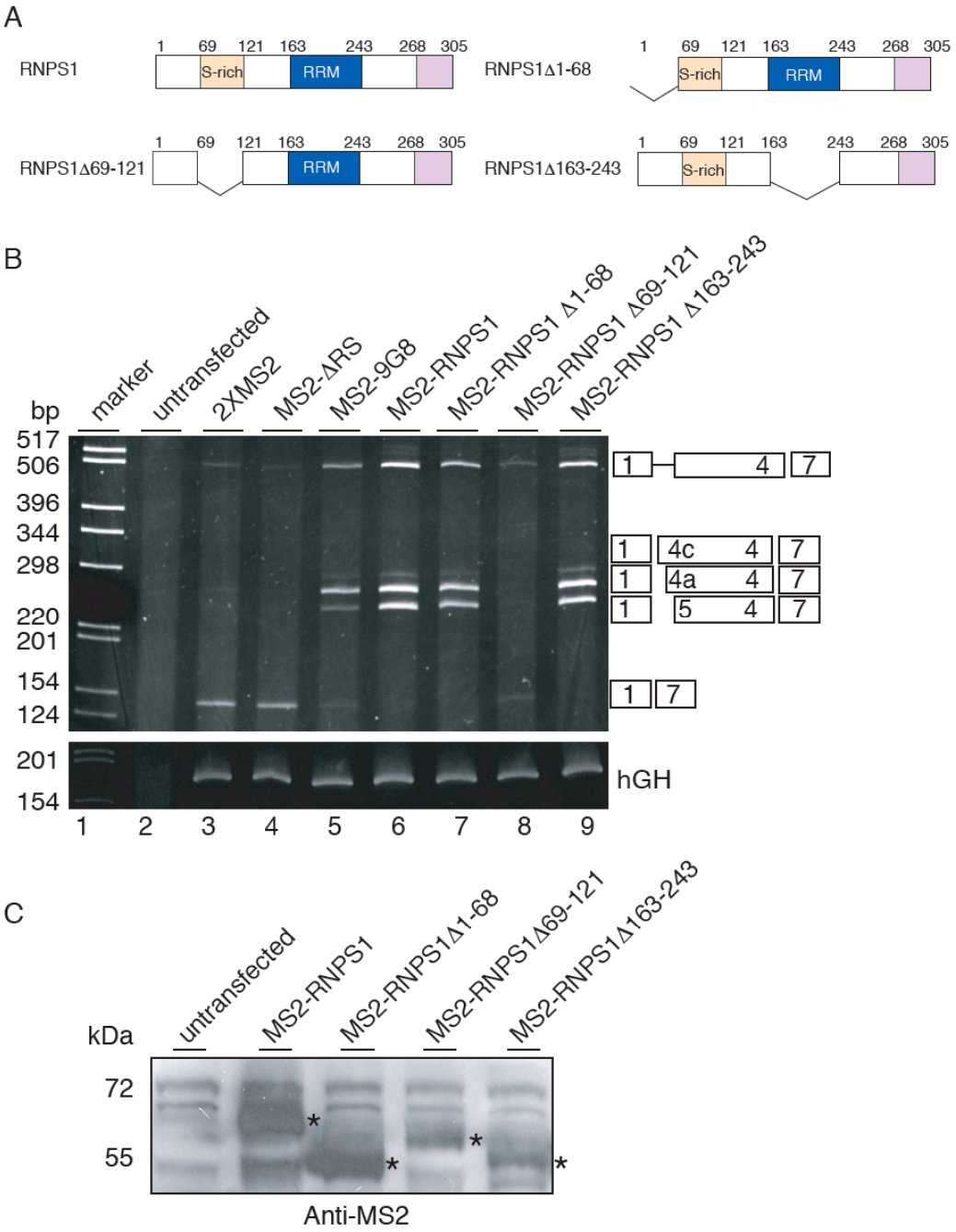

Figure 21. The S-rich domain mediates exon inclusion of the HIV-1 substrate by RNPS1. A) Schematic depiction of RNPS1 and its deletion mutants. B) RT-PCR assay of HeLa cells transfected with the splicing reporter 2xMS2 and the MS2-fusion proteins indicated at the top. MS2-RNPS1 (Δ1-68) (lane 7), MS2-RNPS1(Δ69-121) (lane 8) and MS2-RNPS1(Δ163-243) (lane 9) lack the indicated amino acids. Untransfected control cells are shown in lane 2, marker bands are loaded in lane 1. Spliced products are indicated schematically at the right. C) Nuclear extracts of MS2-RNPS1 fusion proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and protein expression was detected with an antibody against the MS2-domain.

4.1.4. Individual RS domains of Acinus and RNPS1 are localized in the nucleus.

It is known that SR proteins such as ASF/SF2, SC35 and 9G8 are localized in nuclear speckles/splicing speckles, which are thought to be sites of storage of mRNA splicing factors [108, 162]. For participation in the splicing reaction the SR proteins are recruited to sites of mRNA production [137]. Although it has been demonstrated that the RS domains present in these SR proteins can serve as nuclear localization signals [114, 76] experiments involving ASF/SF2 indicated that these RS domains are neither necessary nor sufficient to target SR proteins to nuclear speckles [22].

Since the individual RS domains of the ASAP complex proteins generated differential splice products in the model system, it was interesting to examine if these domains (RS domains of Acinus and RNPS1) are also localized in nuclear speckles. For this purpose HeLa cells were transiently transfected with the respective plasmids. The cellular localization of the expressed proteins was analyzed with an antibody against the fused His-tag and an antibody specific for SC35. Immunofluorescence analyses showed that the RS domains of Acinus (1174-1341) and RNPS1 (268-305) were present in nuclear speckles and colocalized with SC35 (Fig. 22, second and fifth panel). Interestingly, Acinus (1261-1341) was mainly localized in the nucleolus (Fig. 22, third panel). Acinus (576-668) and RNPS1 (69-121) were strongly expressed and distributed throughout the nucleus (except nucleoli) and also colocalized with SC35 (Fig. 22 first and fourth panel). All hybrid RS domains of Acinus and RNPS1 were able to mediate splicing, irrespective of their cellular localization.

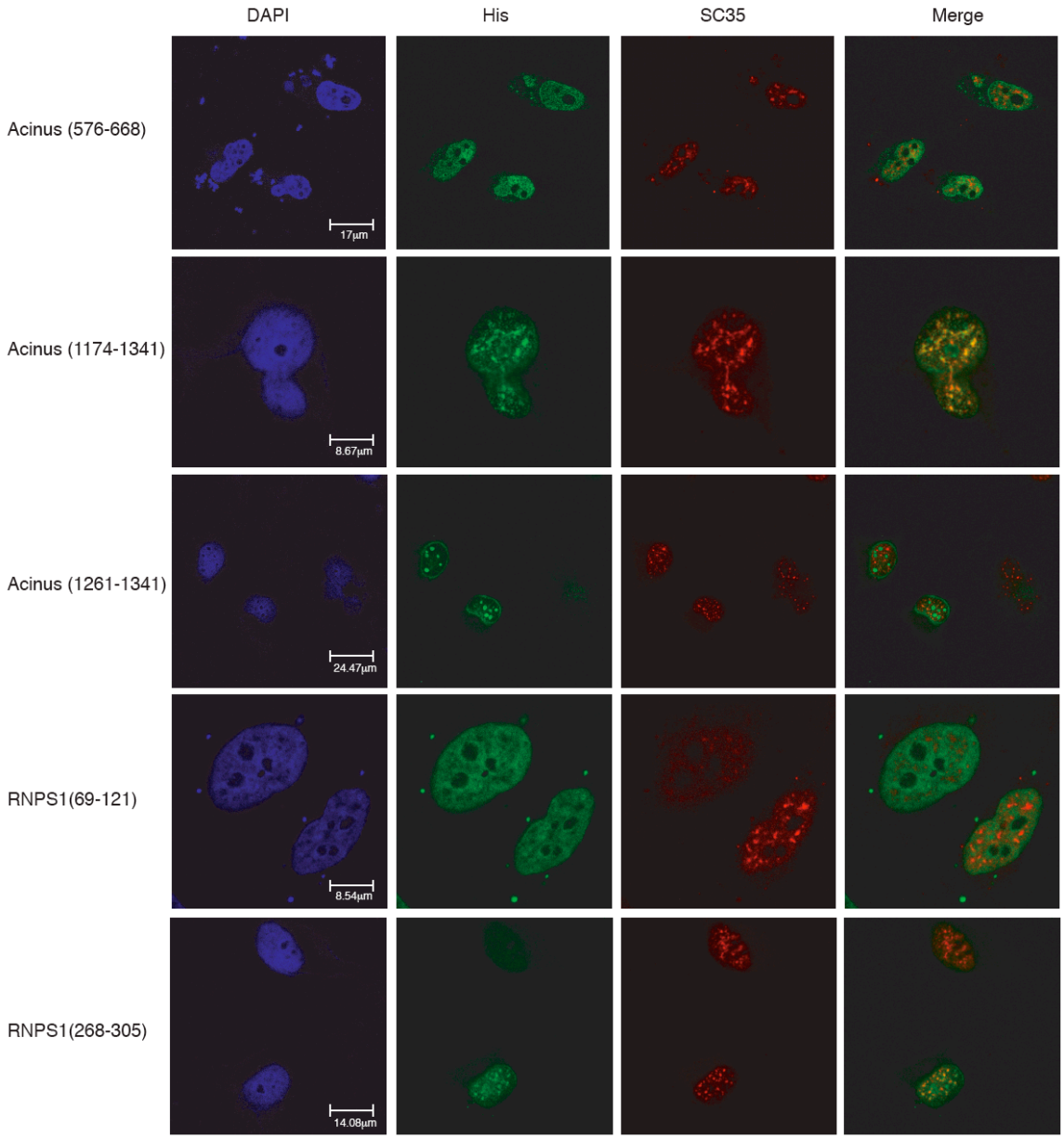

Figure 22. Cellular localization of the individual RS domains of Acinus and RNPS1. HeLa cells were transiently transfected with a Acinus(576-668), Acinus(1174-1341), Acinus(1261-1341), RNPS1(69-121) or a RNPS1(268-305) construct, respectively. Localization of fusion proteins and endogenous SC35 was determined with antibodies against the His-tag and against the SR protein SC35. Whereas Acinus(1174-1341) and RNPS1(268-305) colocalize with SC35 in nuclear speckles, Acinus(576-668) and RNPS1(69-121) are distributed evenly in the nucleus. The Acinus(1261-1341) domain is distributed in the nucleoli and does not colocalize with SC35. Nuclei were stained with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI). Magnifications are indicated by scale bars.

4.1.5. SAP18 mediates exon inclusion of the reporter construct through its ubiquitin-like fold.

The solution structure determination of SAP18 had disclosed that SAP18 forms an ubiquitin-like ß-grasp fold related to the structure of proteins like ubiquitin, sumo or Elongin B [131]. Since ubiquitin-like fold-containing proteins often fulfill functions as adapter proteins or during the assembly of multiprotein complexes [90], the requirement of the ß-grasp fold structure of SAP18 for splicing regulation was investigated. Analysis of MS2-SAP18 fusion proteins with gradual deletions of amino acids from the C-terminus of SAP18 into the ubiquitin-like fold shows that only full-length SAP18 can mediate internal exon inclusion (Fig. 23B, lane 6). The amino acids aspartate at position 118 (Asp118) and threonine at position 121 (Thr121) of SAP18 are conserved in the proteins SAP18, ubiquitin, sumo and Elongin B and are located in the centre of the ubiquitin-like fold structure. These two amino acids were point-mutated into alanines and a double mutant MS2-SAP18(dm) construct was created. Most interestingly, mutation of Asp118 and Thr121 completely abolishes exon inclusion by MS2-SAP18 (cf. lane 6 and 10).

The lack of exon inclusion was not caused by low expression of the mutated construct since wild type MS2-SAP18 [MS2-SAP18(wt)] and the mutants of SAP18 were expressed at a similar level, as determined by Western blotting (Fig. 23C) with an antibody against the MS2-domain.

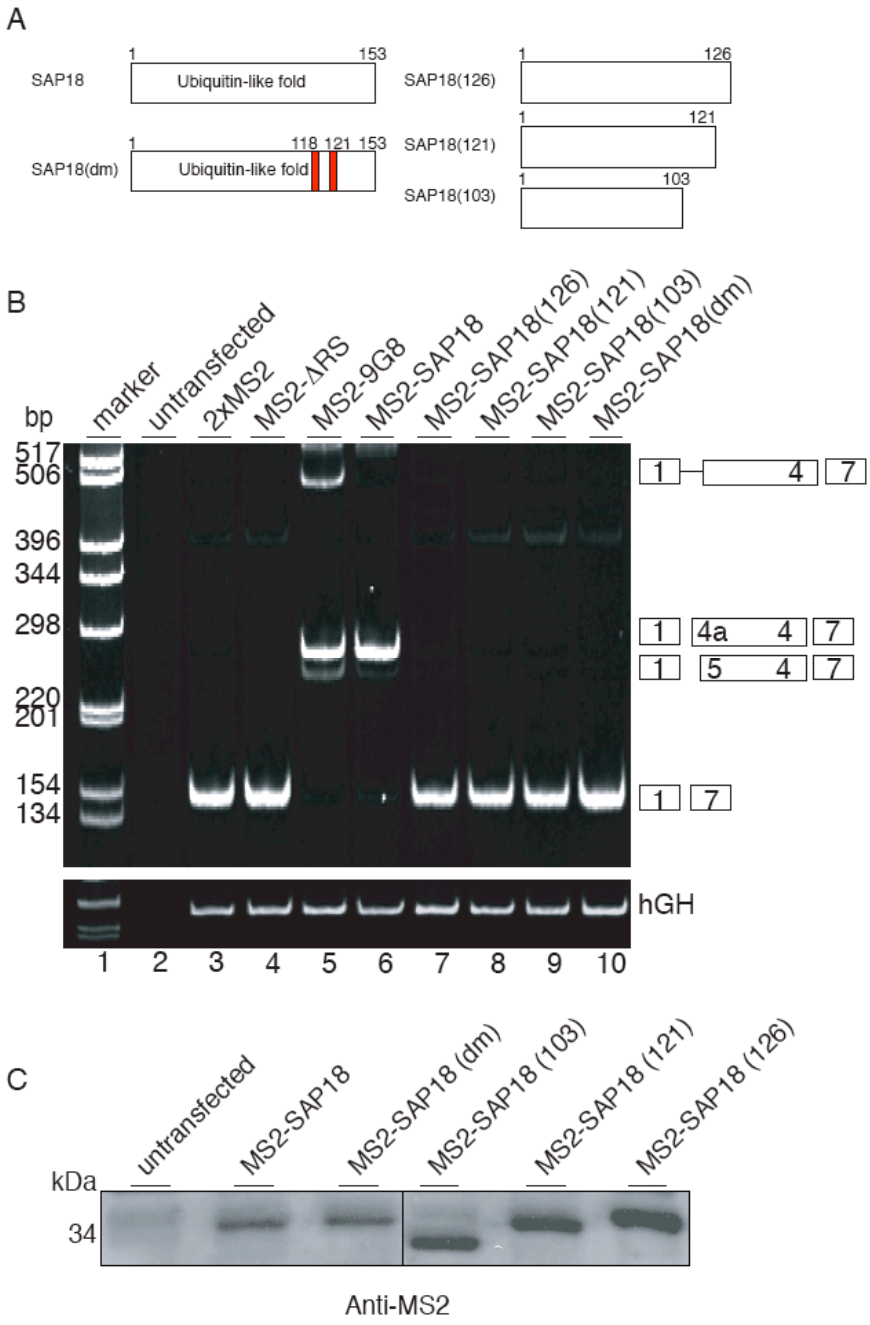

Figure 23. The ubiquitin-like fold structure of SAP18 is required for exon inclusion. A) Schematic depiction of SAP18 and its deletion mutants. B) RT-PCR assay of HeLa cells transfected with the splicing reporter 2xMS2 and the MS2-fusion proteins indicated at the top. MS2-SAP18(126) (lane 7), MS2-SAP18(121) (lane 8) and MS2-SAP18(103) (lane 9) contain the MS2-RNA-binding domain fused to the first 126, 121 or 103 amino acids of SAP18, respectively. Asp118 and Thr121 (indicated in red box; Fig. 23A) were mutated to alanine in MS2-SAP18(dm) (lane 10). Untransfected control cells are shown in lane 2, marker bands are loaded in lane 1. Spliced products are indicated schematically at the right. C) Western blot analysis of MS2-SAP18 fusion protein expression. Nuclear extracts of HeLa cells transiently transfected with the MS2-fusion constructs indicated at the top were separated by SDS-PAGE and protein expression was detected with an antibody against the MS2-domain.

4.1.6. Mutation of the ubiquitin-like fold prevents nuclear speckle localization of SAP18.

It is well known that SR proteins and other splice regulatory factors localize to nuclear compartments termed nuclear speckles or interchromatin granule clusters [108]. Nuclear speckle localization of SAP18 together with the ASAP subunit Acinus and the SR protein SC35 has been shown before [170]. Since the mutation of Asp118 and Thr121 of SAP18 to alanine abolished splicing regulation by SAP18, studies were conducted to determine if a Flag-tagged MS2-fusion protein of SAP18 carrying the two mutations [FlagMS2-SAP18(dm)] can still be found in nuclear speckles. For this purpose HeLa cells were transiently transfected with either wild-type (wt) or double mutated (dm) FlagMS2-SAP18 constructs. The cellular localization of fusion proteins was analyzed with an antibody against the Flag-tag and colocalization studies were carried out using antibodies specific for Acinus and SC35. Immunofluorescence analyses confirmed that wild type SAP18 is present in nuclear speckles and colocalizes with Acinus (Fig. 24A, upper panels) and SC35 (Fig. 24B, upper panels). In contrast, and in agreement with the results obtained in the splicing assay, FlagMS2-SAP18(dm) colocalized neither with Acinus (Fig. 24A, lower panels) nor with SC35 (Fig. 24B, lower panels), suggesting that no interaction takes place between the splicing machinery (splicing factors/ASAP complex) and the delocalized SAP18(dm). The results also indicate that the ubiquitin-like domain of SAP18 is required for the speckle localization. Acinus and SC35 were detected in nuclear speckles regardless whether wild type or mutant SAP18 was transfected.

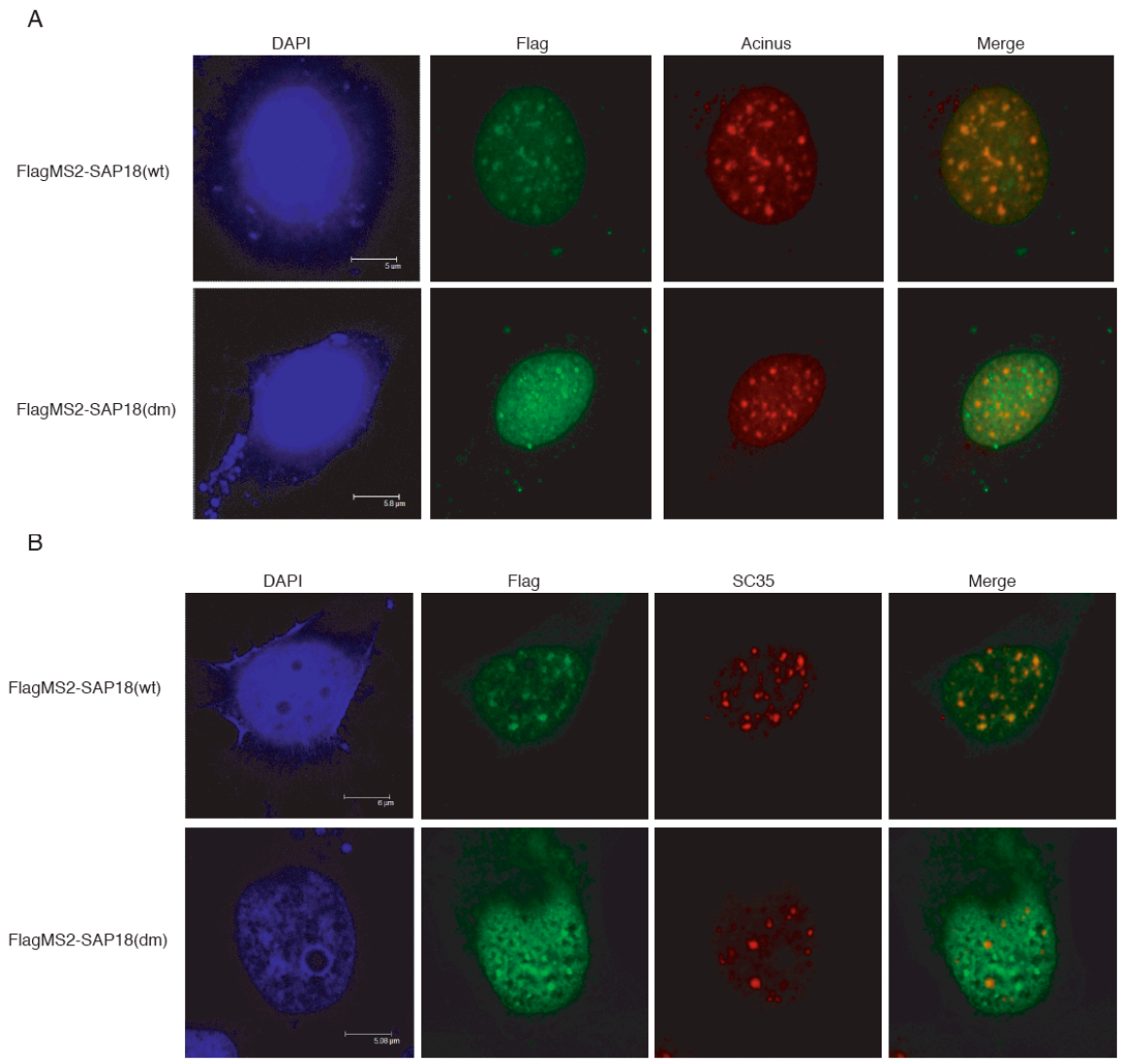

Figure 24. Nuclear speckle localization of SAP18 requires an intact ubiquitin-like fold structure. A) HeLa cells were transiently transfected with a FlagMS2-SAP18(wt) or a FlagMS2-SAP18(dm) construct, respectively. Localization of FlagMS2-SAP18 proteins and endogenous Acinus was determined with antibodies against the Flag-tag and Acinus. Whereas FlagMS2-SAP18(wt) colocalizes with Acinus in nuclear speckles, FlagMS2- SAP18(dm) is distributed evenly in the nucleus and does not colocalize with Acinus. B) HeLa cells transfected as in A) were analyzed with antibodies against the Flag-tag and against the SR protein SC35. Only FlagMS2-SAP18(wt) colocalizes with SC35 in nuclear speckles. Nuclei in A) and B) were stained 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI). Magnifications are indicated by scale bars.

4.1.7. SAP18 assembles the ASAP complex via the ubiquitin-like fold.

SAP18 has been shown before to interact with Acinus and RNPS1 to form the ASAP complex, which is also found as substructural component of the exon-junction-complex [189]. Since the point-mutated SAP18(dm) does not colocalize with Acinus in HeLa cells (Fig. 24), the potential of SAP18(dm) to interact with Acinus and RNPS1 was investigated. For this purpose HeLa cells were transiently transfected with FlagMS2-SAP18(wt) and FlagMS2-SAP18(dm) constructs. After 24 h the nuclear extracts from the transfected cells were prepared and used for the immunoprecipitation. The Flag-fusion proteins were immunoprecipitated with a resin directed against the Flag-tag and the coprecipitating proteins were analyzed with the help of Western blotting. As can be seen in Fig. 25, whereas FlagMS2-SAP18(wt) coprecipitated RNPS1 and Acinus, FlagMS2-SAP18(dm) did not bind to either Acinus or RNPS1. The result that FlagMS2-SAP18(dm) did not interact with the other ASAP subunits, i.e. Acinus and RNPS1, is in agreement with the observation that FlagMS2-SAP18(dm) did not colocalize with Acinus in nuclear speckles (Fig. 24). Control immunoprecipitations performed with nuclear extracts from HeLa cells transiently transfected with FlagMS2-RNPS1 and FlagMS2-Acinus-S' showed that both proteins bound to their respective partners in the ASAP complex (Fig. 25B). It is noteworthy that FlagMS2-Acinus-S' precipitated significantly lower amounts of ASAP subunits than FlagMS2-RNPS1, providing an explanation for the lack of internal exon inclusion activity for MS2-Acinus-S' observed in Fig. 18B, lane 7.

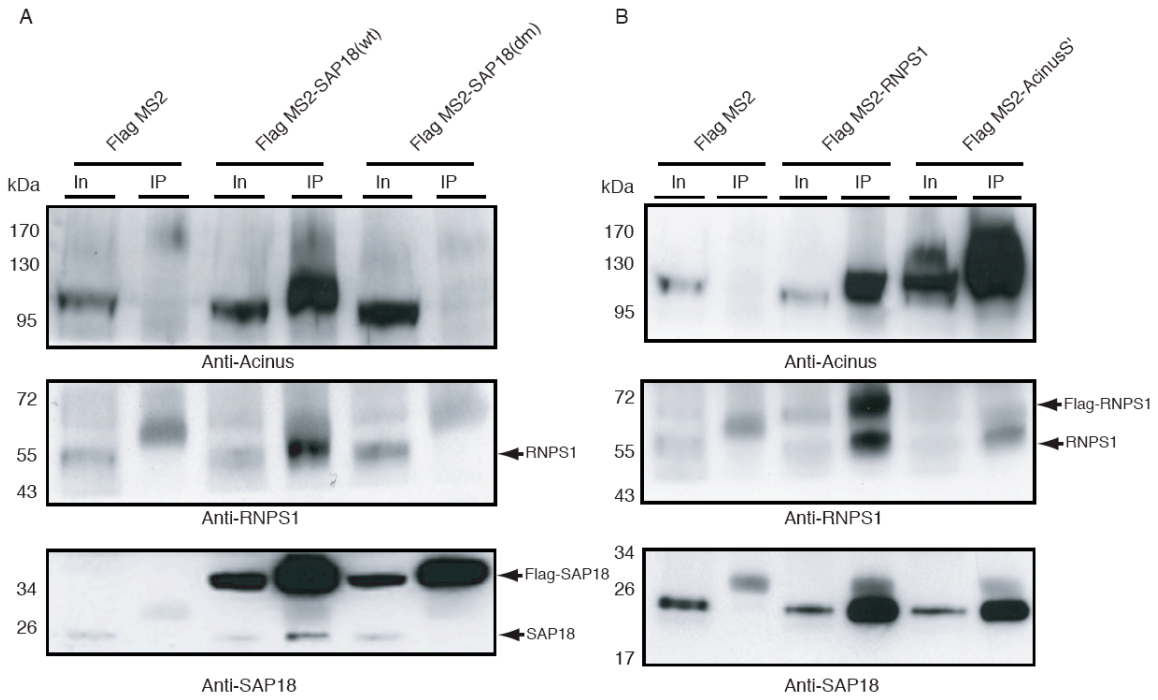

Figure 25. The ubiquitin-like fold structure of SAP18 is required for ASAP assembly. A) HeLa cells were transiently transfected with constructs expressing FlagMS2, FlagMS2-SAP18(wt) or FlagMS2-SAP18(dm), respectively. After preparation of nuclear extracts the Flag-tagged SAP18 proteins were precipitated with a resin directed against the Flag tag. Precipitated proteins (IP) (lanes 2, 4 and 6) were separated by SDS-PAGE and subsequently detected by Western blotting with antibodies against Acinus, RNPS1 and SAP18, respectively. 10% of the respective input (In) is loaded in lanes 1, 3 and 5. Only FlagMS2-SAP18(wt) containing an intact ubiquitin-like fold structure coprecipitates Acinus and RNPS1. B) HeLa cells were transiently transfected with constructs expressing FlagMS2, FlagMS2-RNPS1 and FlagMS2-Acinus-S', and analyzed as described in A). Although all ASAP subunits are precipitated with both Flag-tagged fusion proteins, only a fraction of FlagMS2-Acinus-S' is incorporated into ASAP.

4.1.8. SAP18 interacts with the RNA-binding domain of RNPS1.

RNPS1 contains several functional domains including a serine-rich domain (S-rich domain), an RNA recognition motif (RRM) and an arginine-serine/proline (RS/P)-rich domain [6, 130, 163]. In addition to Acinus and SAP18, RNPS1 interacts with several other splicing-associated proteins. Different domains of RNPS1 are responsible for these interactions, for example, whereas the S-rich domain binds the SR protein SRp54, the region containing the RRM associates with the nuclear speckle protein pinin [163].

To identify the domains of RNPS1, which mediate the interaction with Acinus and SAP18, HeLa cells were transfected with constructs expressing full-length

RNPS1 as well as different deletion constructs as Flag-tagged fusion proteins and analyzed protein-protein interactions by immunoprecipitation studies. As demonstrated in Fig. 26, full-length RNPS1 and its mutants lacking the first 68 amino acids (Δ1-68) or the S-rich domain (Δ69-121) coimmunoprecipitated both Acinus and SAP18. In contrast, only an RNPS1 deletion mutant lacking the region containing the RRM (Δ163-243) did not precipitate SAP18 or Acinus.

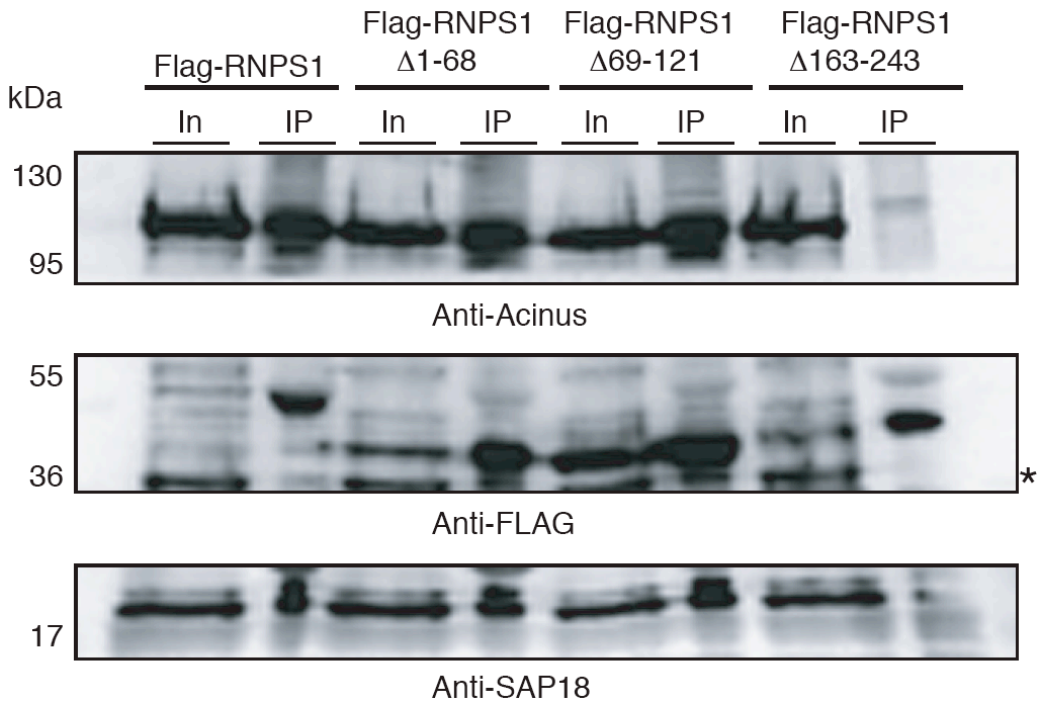

Figure 26. The region containing the RRM is required for ASAP assembly by RNPS1. HeLa cells were transiently transfected with constructs expressing full length Flag-RNPS1, Flag-RNPS1(Δ1-68), Flag-RNPS1(Δ69-121) or Flag-RNPS1(Δ163-243), respectively. After preparation of nuclear extracts the Flag-tagged RNPS1 proteins were precipitated with a resin directed against the Flag-tag. Precipitated proteins (IP) (lanes 2, 4, 6 and 8) were separated by SDS-PAGE and subsequently detected by Western blotting with antibodies against Acinus, Flag and SAP18, respectively. 10% of the appropriate input (In) is loaded in lanes 1, 3, 5 and 7. High-molecular-mass weight standards are indicated at the left. An unspecific band recognized by the FLAG antibody is indicated by an asterisk.

4.2. Investigation of c-FLIP isoform production.

The genomic structure of the human c-FLIP protein is significantly different from mouse (*Mus musculus*) c-FLIP [197]. The murine c-FLIP gene encodes two isoforms of c-FLIP: the long isoform c-FLIP$_L$ and a truncated protein c-FLIP$_R$. Similarly, the c-FLIP gene of humans produces the homologous isoforms c-FLIP$_L$ and c-FLIP$_R$. However, the human c-FLIP gene contains an additional exon that can be alternatively spliced to generate another truncated FLIP protein termed c-FLIP$_S$ (Fig. 27). The mouse c-FLIP gene lacks a homologous exon 7 and therefore does not produce a c-FLIP$_S$ isoform. For this reason humans express two truncated FLIP isoforms (c-FLIP$_R$ and c-FLIP$_S$), whereas mice produce only c-FLIP$_R$ (Fig. 27). The differences in the genomic structure of the c-FLIP gene between mice and man raises questions regarding control of c-FLIP isoform production and the role of splice sites in mammals.

4.2.1. Analysis of truncated c-FLIP isoforms in mammals.

To examine the occurrence of short isoforms of c-FLIP in different organisms, the isoform production of c-FLIP proteins in diverse mammals was investigated. For this purpose the c-FLIP sequences from several selected species were compared (Fig. 28). Similar to the described murine isoforms [197], the rat (*Rattus norvegicus*) expresses c-FLIP$_R$ as the only short isoform. This might not be surprising, since the genomic sequence of the rat displays an intron-exon structure related to that of the mouse. An isoform homologous to c-FLIP$_R$ has also been described for wild boar (*Sus scrofa*) and dog (*Canis lupus familaris*) [66]. Furthermore, to analyze the occurrence of c-FLIP short isoforms during the course of evolution, the intron-exon structure of the c-FLIP gene in the common chimpanzee (*Pan troglodytes*) was examined. Interestingly, although the c-FLIP genomic sequence of *P. troglodytes* deposited in the database is highly similar in sequence and retains the human intron-exon structure, production of c-FLIP$_S$ is not possible due to a nucleotide exchange (G→A) at position 2 in the 3' splice site of intron 6, changing the dinucleotide consensus sequence from AG to AA.

Thus, according to the database, c-FLIP$_R$ would be the only short isoform expressed in *P. troglodytes*. The putative amino acid sequence of the c-FLIP$_R$ isoform expressed by *P. troglodytes* is identical to human c-FLIP$_R$.

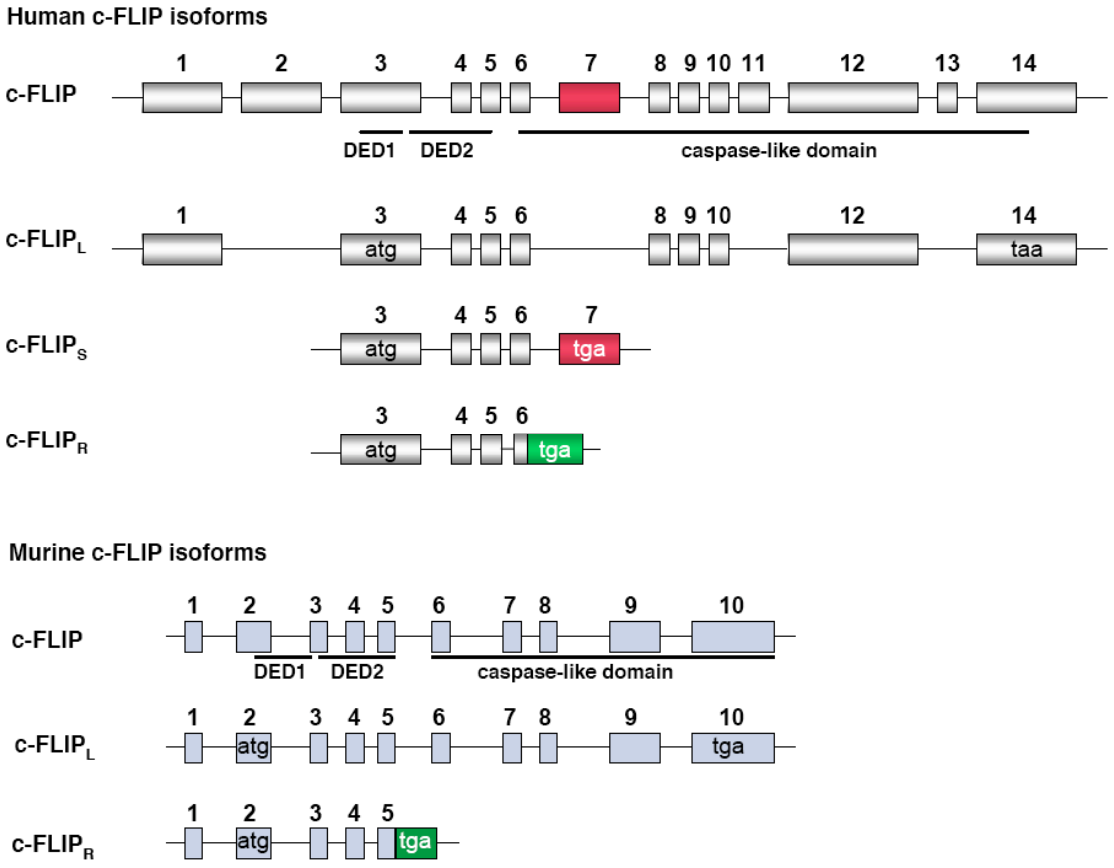

Figure 27. Intron-exon structure of the human and mouse c-FLIP gene locus. Exons containing the translation start and stop codons are labeled with 'atg', 'taa' and 'tga', respectively. Regions encoding for the first DED, the second DED and the caspase-like domains are indicated. The human-specific exon 7, which is contained in the c-FLIP$_S$ mRNA, is marked in red, and the intronic sequences are marked in green.

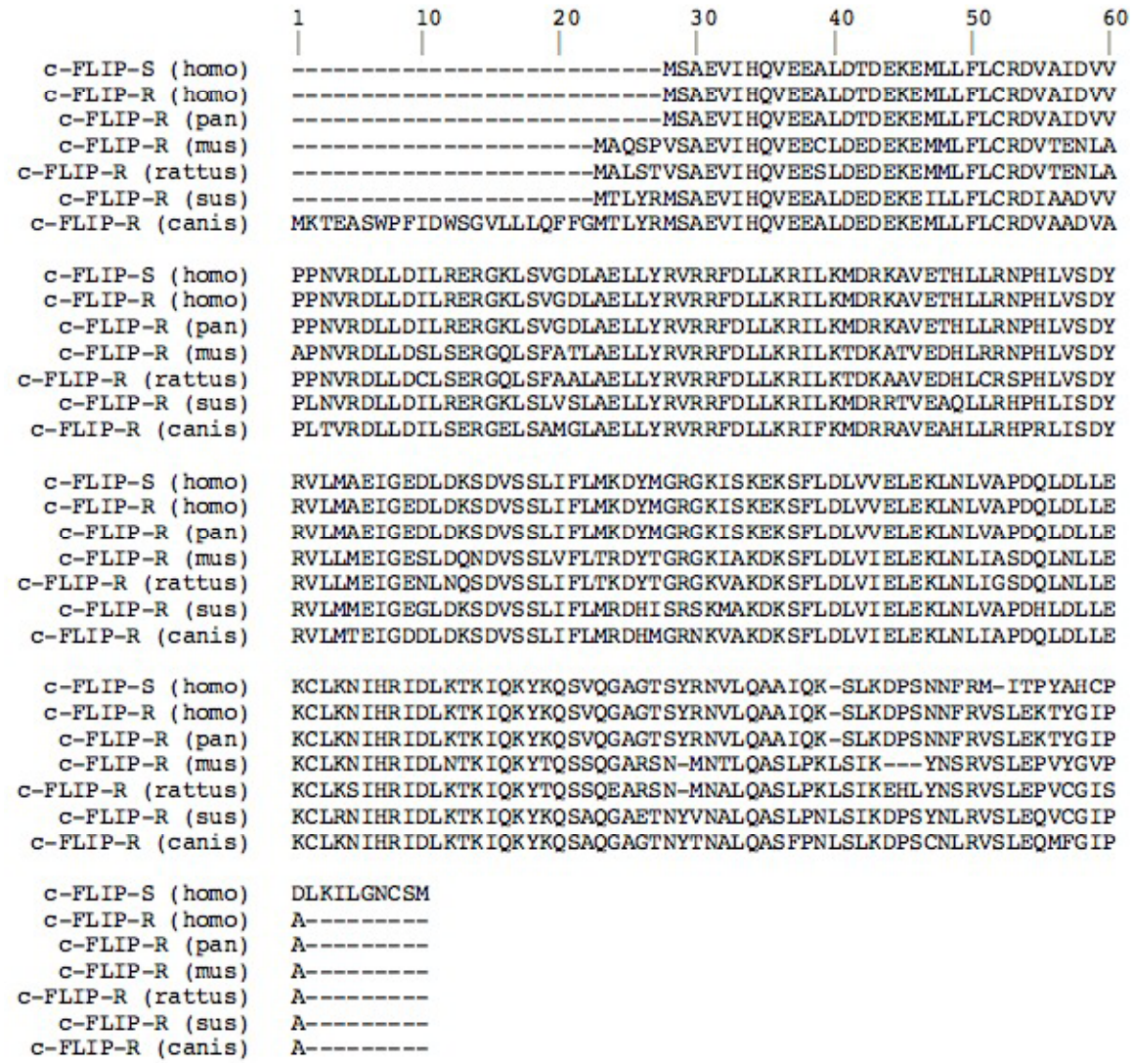

Figure 28. Sequence alignment of mammalian c-FLIP$_S$ and c-FLIP$_R$ proteins. Alignments were performed using the Geneious software (http://www.geneious.com). Sequences are shown in single-letter amino acid code.

4.2.2. Presence of a functional SNP in the 3' splice site of intron 6 of the human c-FLIP gene.

While studying the c-FLIP genomic sequences of *P. troglodytes* deposited in the database, the presence of nucleotide exchange at the 3' splice site of intron 6 was observed. This observation led to the question whether the existence of a similar nucleotide exchange could be involved in the definition of c-FLIP isoform production in humans. To evaluate this hypothesis genomic DNA of selected human cell lines, which express either c-$FLIP_S$ (HeLa, HuT78, J16, BJAB) or c-$FLIP_R$ (Raji, SKW6.4, BL60, NALM6) [42, 64] (Fig. 29A) was carefully analyzed. Additionally, genomic DNA of two *P. troglodytes* cell lines and one cell line obtained from its close relative, the Bonobo (*Pan paniscus*) was also analyzed. The analysis revealed that all three-chimpanzee cell lines carried the nucleotide variation from G→A at the 3' splice site of intron 6 (Fig. 29A), as expected following the study of genomic sequences. Most interestingly, all human cell lines described to produce c-$FLIP_R$ displayed a nucleotide exchange in the 3' splice site of intron 6 identical to that of the chimpanzee species. In contrast cells expressing c-$FLIP_S$ had an intact splice acceptor (AG) site.

The detection of two different nucleotides in the splice acceptor site of intron 6 in different human cell lines strongly pointed to the possibility of a single nucleotide polymorphism (SNP). SNPs are frequently occurring genetic variations defined by the mutation of a single nucleotide that can be identified in more than 1% of the population [91]. Indeed, as expected, screening of the NCBI (National Centre for Biotechnology Information) SNP database disclosed the presence of a SNP (rs10190751 A/G) at this position. Notably, the data of the International Haplotype Map (HapMap) project (2003) showed that rs10190751 A/G is variably distributed in the different populations analyzed (Fig. 29B). For example, a high prevalence of c-$FLIP_R$ isoform can be observed especially in Yoruba people of Ibadan, Nigeria. In Asian populations (Han Chinese; HCB and Japanese; JPT), a far higher incidence of the c-$FLIP_S$ isoform but only a very small proportion of c-$FLIP_R$ can be found. A similar distribution is seen in the American residents with

northern and western European ancestry (CEU), who display a low incidence of the c-FLIP$_R$ isoform and increased prevalence of c-FLIP$_S$. A distribution tendency similar to that observed for the CEU population was obtained following a SNP analysis of the genomic DNA of about 200 healthy-donors from Europe, confirming the level of prevalence for c-FLIP$_S$ in European donors (Table 2).

A

Human:	HeLa	TAC **AG** ATG
T cells	HuT78	TAC **AG** ATG
	J16	TAC **AG** ATG
B cells	BJAB	TAC **AG** ATG
	Raji	TAC **AA** ATG
	SKW6.4	TAC **AA** ATG
	BL60	TAC **AA** ATG
	NALM6	TAC **AA** ATG
Chimp.:	Pan.t (EB176)	TAC **AA** ATG
	Pan.t (LTR2008)	TAC **AA** ATG
	Pan.p (Bonobo)	TAC **AA** ATG

Splice site

B

	A/A	A/G	G/G	A	G
CEU	0.067	0.300	0.633	0.217	0.783
HCB		0.089	0.911	0.044	0.956
JPT		0.045	0.955	0.023	0.977
YRI	0.183	0.583	0.233	0.475	0.525

Figure 29. Expression of different c-FLIP short isoforms in mammals. A) Analysis of c-FLIP genomic sequences of selected cell lines by sequencing of genomic DNA. Shown is the region covering the 3' splice site of intron 6. The AG dinucleotide consensus (bold letters) and the position of rs10190751 A/G (arrow) are indicated B) Distribution of the variants of rs10190751 A/G in the different populations analyzed in the HapMap project (CEU, Utah residents with ancestry from northern and western Europe; HCB, Han Chinese in Beijing, China; JPT, Japanese in Tokyo, Japan; YRI, Yoruba in Ibadan, Nigeria).

Table 1. List of cell lines and their respective data obtained from genomic sequencing, RT-PCR and Western blot analysis.

Cell line	Cell type	State	Genomic DNA	RNA	Protein
Bonobo	chimpanzee relative species, B cells	-	AA at splice site	c-FLIP$_L$ and c-FLIP$_R$	c-FLIP$_L$ and c-FLIP$_R$
EB 176	chimpanzee, B cells	-	AA at splice site	c-FLIP$_L$ and c-FLIP$_R$	c-FLIP$_L$ and c-FLIP$_R$
LTR 2008	chimpanzee, B cells	-	AA at splice site	c-FLIP$_L$ and c-FLIP$_R$	c-FLIP$_L$ and c-FLIP$_R$
B cells	human, primary, isolated from peripheral blood	mature B cells	AG and/or AA at splice site	c-FLIP$_L$, c-FLIP$_S$ and/or c-FLIP$_R$	c-FLIP$_L$, c-FLIP$_S$ and/or c-FLIP$_R$
BL60	human B lymphocyte	Burkitt lymphoma	AA at splice site	c-FLIP$_L$ and c-FLIP$_R$	c-FLIP$_L$ and c-FLIP$_R$
SKW6.4	human B lymphocyte	sIgM+	AA at splice site	c-FLIP$_L$ and c-FLIP$_R$	c-FLIP$_L$ and c-FLIP$_R$
Raji	human B lymphocyte	Burkitt lymphoma	AA at splice site	c-FLIP$_L$ and c-FLIP$_R$	c-FLIP$_L$ and c-FLIP$_R$
Nalm6	human B lymphocyte	B cell precursor leukemia, acute lymphoblastic leukemia (ALL)	AA at splice site	c-FLIP$_L$ and c-FLIP$_R$	c-FLIP$_L$ and c-FLIP$_R$
Med B1		medastinal	AG at splice site	c-FLIP$_L$ and c-FLIP$_S$	c-FLIP$_L$ and c-FLIP$_S$

		lymphoma			
Ramos	human B lymphocyte	sIgM +, Burkitt lymphoma	AG at splice site	c-FLIP$_L$ and c-FLIP$_S$	c-FLIP$_L$ and c-FLIP$_S$
Daudi	human B lymphocyte	Burkitt lymphoma	AG at splice site	c-FLIP$_L$ and c-FLIP$_S$	c-FLIP$_L$ and c-FLIP$_S$
Bjab	human B lymphocyte		AG at splice site	c-FLIP$_L$ and c-FLIP$_S$	c-FLIP$_L$ and c-FLIP$_S$
L-1236	human B lymphocyte	Hodgkin lymphoma	AG and AA at splice site	c-FLIP$_L$, c-FLIP$_S$ and/or c-FLIP$_R$	c-FLIP$_L$, c-FLIP$_S$ and/or c-FLIP$_R$
L-428	human B lymphocyte	Hodgkin lymphoma	AG and AA at splice site	c-FLIP$_L$, c-FLIP$_S$ and c-FLIP$_R$	c-FLIP$_L$, c-FLIP$_S$ and c-FLIP$_R$
T cells	human, primary, isolated from peripheral blood	mature T cells	AG and/or AA at splice site	c-FLIP$_L$, c-FLIP$_S$ and/or c-FLIP$_R$	c-FLIP$_L$, c-FLIP$_S$ and/or c-FLIP$_R$
J16	human T lymphocytes	acute lymphoblastic leukemia (ALL)	AG at splice site	c-FLIP$_L$ and c-FLIP$_S$	c-FLIP$_L$ and c-FLIP$_S$
HuT 78	human T lymphocytes	lymphoma, cutaneous T cell, mature T cell	AG at splice site	c-FLIP$_L$ and c-FLIP$_S$	c-FLIP$_L$ and c-FLIP$_S$
CEM	human T lymphocytes	acute lymphoblastic leukemia (ALL)	AG at splice site	c-FLIP$_L$ and c-FLIP$_S$	c-FLIP$_L$ and c-FLIP$_S$

4.2.3. rs10190751 A/G variation determines c-FLIP short isoform production in humans.

After genomic sequence analysis, a correlation between the presence of a SNP and the production of specific c-FLIP short isoforms was observed. Therefore, to analyze the impact of this SNP on RNA and protein production, studies were performed to determine the c-FLIP isoform production on both protein and RNA level. A selected set of cell lines was analyzed by reverse transcriptase PCR (RT-PCR) and Western blotting. Among these were cell lines, which, according to genomic sequencing and SNP assay analysis, were homozygous for an intact splice acceptor site (rs10190751 G; J16, Hut78, CEM, MED-B1, Ramos, Daudi, Bjab), homozygous for the splice defect allele (rs10190751 A; BL60, SKW6.4, Raji, Nalm6) or contained both alleles (L428, L1236). Cells containing copies of the splice intact allele should have the potential to express both c-FLIP$_S$ and c-FLIP$_R$. Strikingly, all cell lines homozygous for an intact splice acceptor produced only c-FLIP$_S$ on RNA and protein level. However, on RNA (Fig. 30A) as well as on protein level (Fig. 30B) expression of c-FLIP$_R$ was detected only in those cases, where cells were either heterozygous or homozygous for the splice dead phenotype. The long isoform c-FLIP$_L$ was, as expected, expressed in all cell lines analyzed.

Due to the fact that c-FLIP proteins are mainly expressed in the immune system [149], further emphasis was placed on lymphocytes (B and T cells). To obtain an overview of the rs10190751 A/G variation in these cell types, human B and T cell lines were analyzed by genomic sequencing and SNP assay (Table 1). Additionally, primary B cells heterologous for rs10190751 A/G as determined by genomic sequencing (Table 1) displayed synthesis of mRNAs for both short c-FLIP isoforms (Fig. 31A, left panel). Also, the expression of both c-FLIP isoforms was detected in primary T cells after stimulation with phytohaemagglutinin-L (PHA-L), which is used to activate T cells (Fig. 31A, right panel). For analysis on protein level, primary cells expressing both short c-FLIP isoforms and cells, which were homozygous for either c-FLIP$_S$ or c-FLIP$_R$ expression, were selected.

To maximize c-FLIP production, primary B and control cells were stimulated with lipopolysaccharide (LPS) or phorobol12-myristate13-acetate/ionomycin (PMA/I), and primary T cells were treated with PHA, respectively (Fig. 31B). In all primary B and T cells that were analyzed for their rs10190751 A/G status, only the c-FLIP short protein isoforms corresponded to the respective SNP variants. The *P. troglodytes* cell lines EB176 and LTR2008 displayed expression of the c-FLIP$_R$ isoform at the RNA and protein level, as expected (Fig. 31A and B).

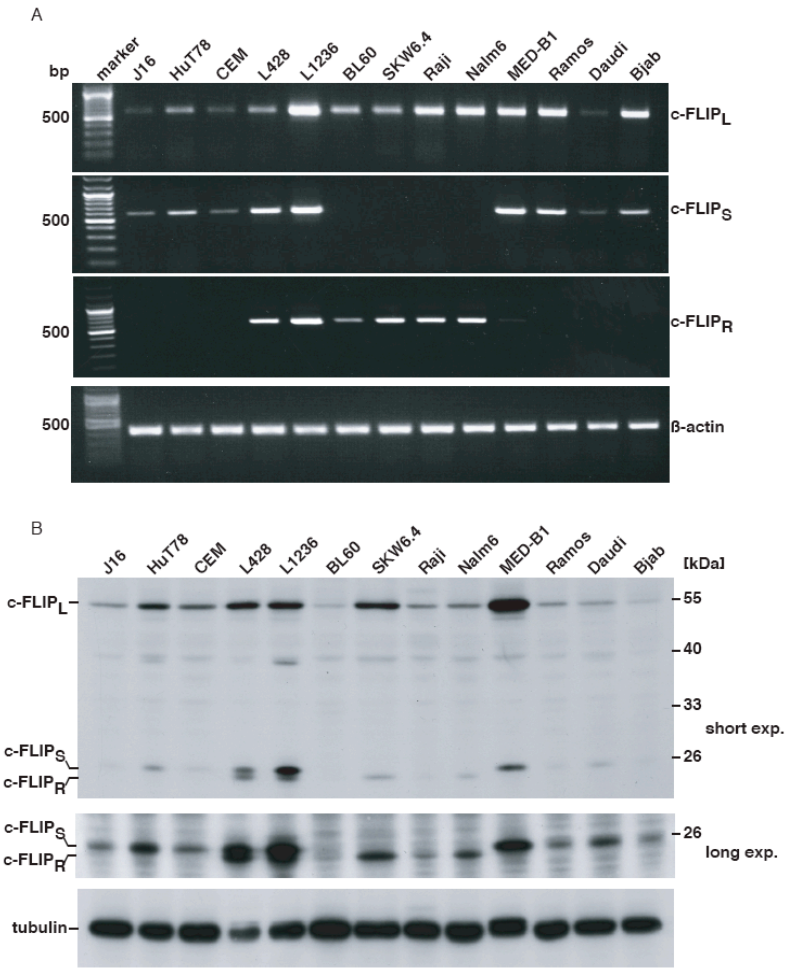

Figure 30. Differential expression of the c-FLIP isoforms in selected cell lines. A) Reverse transcriptase polymerase chain reaction (RT-PCR) was used to determine the abundance of specific different c-FLIP RNA isoforms expressed in the different human cell lines tested. β-actin served as an internal control. B) Analysis of c-FLIP protein expression of the selected cell lines by Western blotting. The human T cell lines J16, HuT78, and CEM were stimulated for 16 h with 20 ng/ml PMA and 1 µM ionomycin. Daudi cells were kept in co-culture with L cells stably expressing the CD40L for 72 h. For a better visualization of short c-FLIP isoform levels a long exposure (10 min) is shown additionally. As loading control tubulin expression levels are presented.

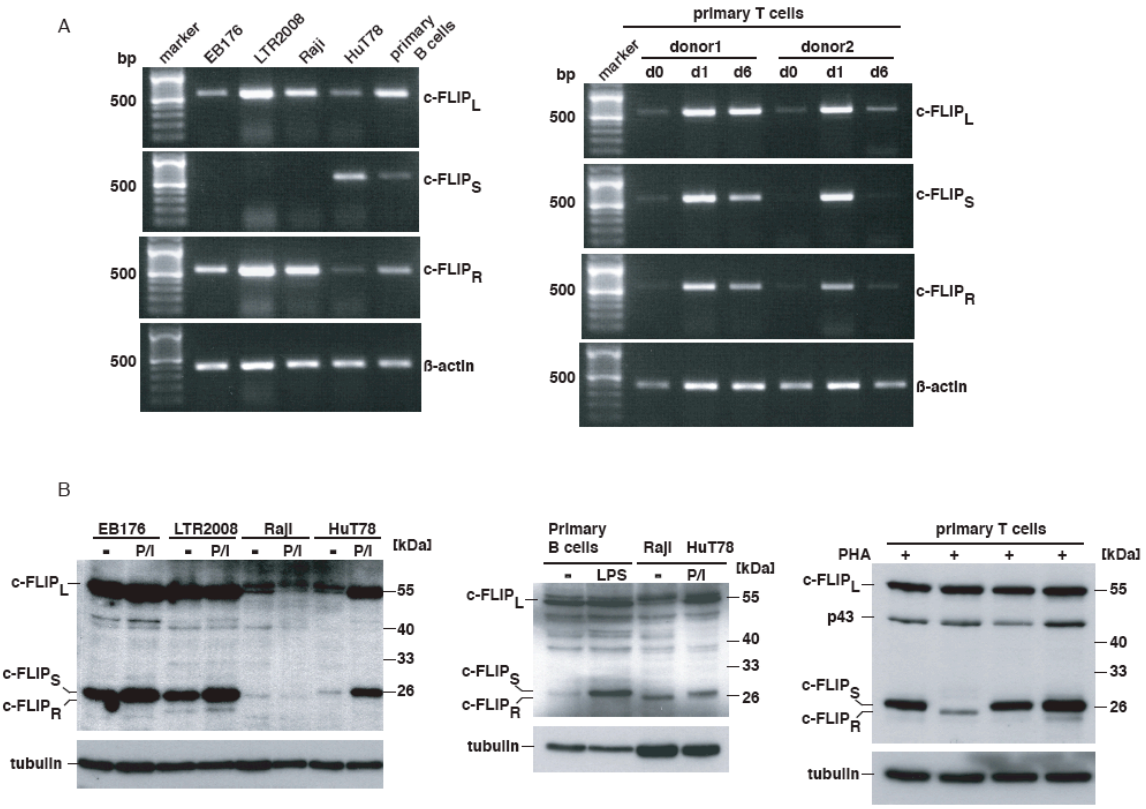

Figure 31. Variation of the expression of short c-FLIP isoform expression in chimpanzee cells as well as primary B and T cells. A) Expression of c-FLIP isoforms in EB176, LTR2008, primary B cells (left panel) and primary T cells (right panel) was analyzed by reverse transcriptase PCR. Human peripheral T cells (donor 1 and donor 2) were either left untreated or stimulated with PHA-L (5 µg/ml) for 16 hours. Activated T cells (d1) were washed with PBS and stimulated with IL-2 (25 U/ml) for another 5 days (d6). The indicated cell lines expressing c-FLIP$_L$ and c-FLIP$_R$ were used as control. B) Freshly prepared human peripheral B cells (middle panel) or T cells of four different blood donors (right panel) were either left untreated or stimulated with LPS (10 µg/ml) or PHA-L (5 µg/ml) for 16h. EB176 and LTR2008 cells (left panel) were left untreated or stimulated for 16 h with 20 ng/ml PMA and 1 µM ionomycin. c-FLIP expression was determined by Western blot analysis. Tubulin served as a loading control.

4.2.4. c-FLIP$_R$ is lower expressed than c-FLIP$_S$.

In Western blot analyses the protein levels of c-FLIP$_S$ seemed to be higher than those of c-FLIP$_R$, which was especially evident when both isoforms were expressed in the same cell (Fig. 30B, L428 and L1236). Increased protein expression of c-FLIP$_S$ compared to c-FLIP$_R$ had also been noted before in primary human T cells [64]. A similar turn-over rate of c-FLIP$_S$ and c-FLIP$_R$ proteins after treatment with a protein translation inhibitor, cycloheximide (CHX), had been described before in Boe cells [64] and Fig. 32A, confirmed this

observation in L428 cells. The L428 cells express c-FLIP$_R$ to a significant extent, although still on lower levels than c-FLIP$_S$. Additionally, similar stabilities of c-FLIP$_S$ and c-FLIP$_R$ mRNAs were observed in L428 cells after treatment with the transcription inhibitor actinomycin D (Fig. 32B).

To analyze the expression levels and turnover rates of c-FLIP short forms expressed exogenously from cDNA templates 293T cells were transiently transfected with expression plasmids encoding c-FLIP$_S$ or c-FLIP$_R$ followed by treatment with CHX. Like the endogenous short c-FLIP isoforms, exogenously expressed c-FLIP$_S$ and c-FLIP$_R$ displayed similar half lifes (Fig. 32C). The protein levels achieved by c-FLIP$_R$ were not as high as those achieved by c-FLIP$_S$, even after transfecting the ten-fold amount of expression plasmid. Furthermore, 293T cells were treated with actinomycin D and cotransfected with equal amounts of c-FLIP$_S$ and c-FLIP$_R$ constructs (Fig. 32D). The c-FLIP$_S$ and c-FLIP$_R$ mRNAs derived from cDNA expression plasmids were equally expressed and demonstrated comparable stabilities. Treatment of L428 cells or transfected 293T cells with the transcription inhibitor Actinomycin D showed that the increased protein expression of c-FLIP$_S$ is not due to an increased mRNA stability of c-FLIP$_S$ mRNA compared to c-FLIP$_R$.

To investigate the protein expression levels of c-FLIP$_S$ and c-FLIP$_R$ more quantitatively, 293T cells were transiently transfected with green fluorescent protein (GFP) fusion constructs encoding either GFP-c-FLIP$_S$ or GFP-c-FLIP$_R$. As can be seen in Fig. 33A, transfection with identical amounts of the plasmids (0.5 μg) led to a stronger production of c-FLIP$_S$ compared to c-FLIP$_R$. Immunofluorescence analysis revealed that both c-FLIP$_S$ and c-FLIP$_R$ localize to the cytoplasm (Fig. 33B). So called “death effector filaments”, which can be caused by overexpression of c-FLIP proteins [177, 193], could only be observed in GFP-c-FLIP$_S$ transfected cells (Fig. 33B, middle panel), again underlining the higher expression levels of this c-FLIP short isoform.

Although no differences in transcription of c-FLIP short isoforms could be detected, higher protein expression levels of c-FLIP$_S$ compared to c-FLIP$_R$ were

found, leading to the presumption that the mRNA of the c-FLIP$_S$ isoform may be translated more efficiently.

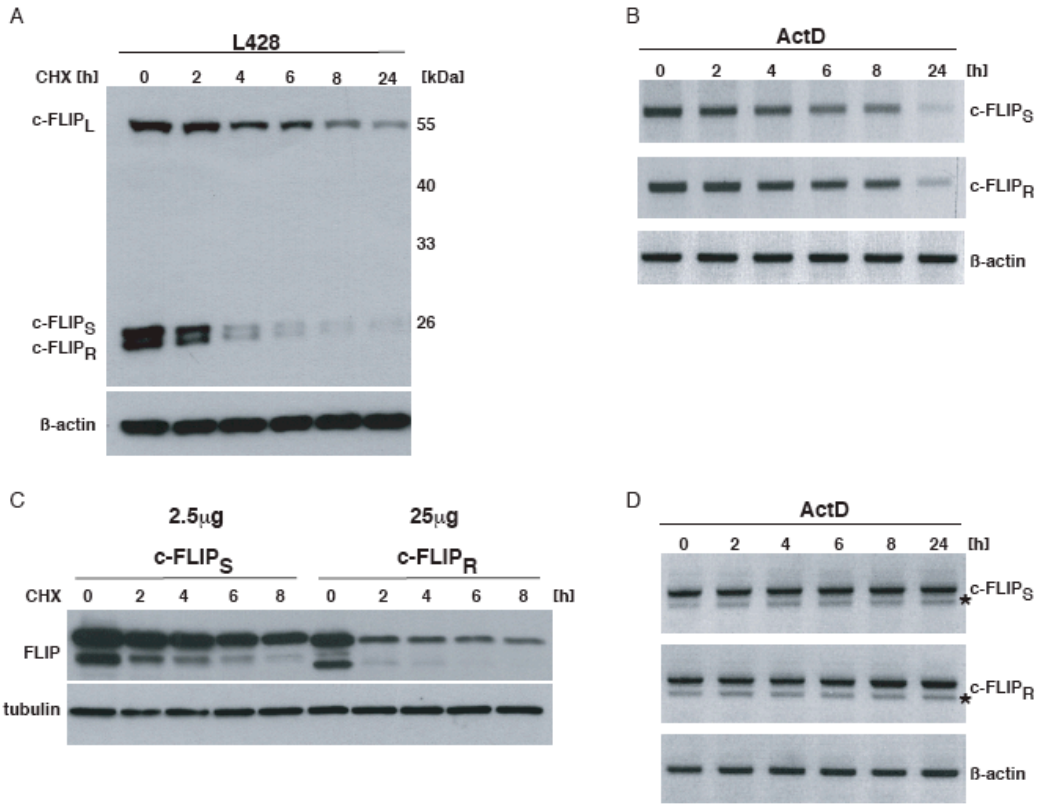

Figure 32. Increased protein expression of c-FLIP$_S$. A) L428 cells were treated for the indicated times with 10 µg/ml cycloheximide. c-FLIP expression was analyzed by Western blot. β-actin served as a loading control. B) L428 cells were treated for the indicated times with actinomycin D (ActD, 1 µg/ml). Endogenous c-FLIP isoform mRNA stability was detected by RT-PCR. C) 293T cells were transiently transfected with the indicated amounts of c-FLIP$_S$ or c-FLIP$_R$. 24 h post transfection cells were treated for up to 8h with 10 µg/ml cycloheximide. Protein stability of the two c-FLIP isoforms was monitored by Western blot analysis. Tubulin was analyzed to control equal protein loading. D) 293T cells were cotransfected with equal amounts of c-FLIP$_S$ and c-FLIP$_R$. 24 h later, cells were stimulated for the indicated times with actinomycin D (1 µg/ml). mRNA stability was assessed by RT-PCR. The asterisk marks a band of unclear identity, which appears specifically in the transfected cells.

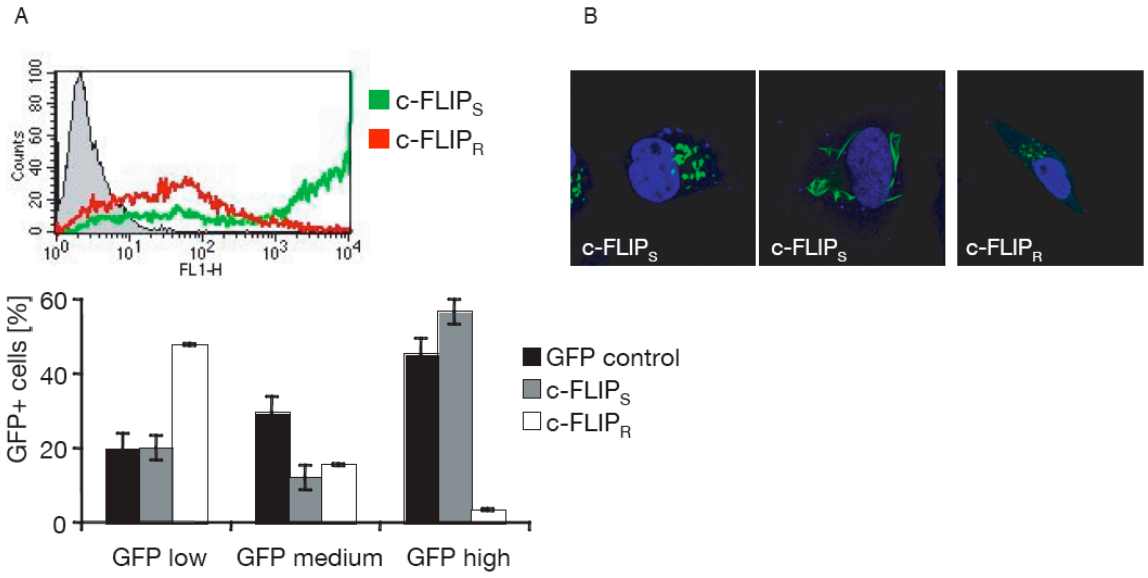

Figure 33. Analysis of GFP-c-FLIP short isoforms. A) GFP-tagged c-FLIP$_S$ or c-FLIP$_R$ was transiently overexpressed in 293T cells. Differences in protein expression were determined by evaluating the GFP fluorescence intensity via FACS analysis. B) HeLa cells were transiently transfected with either GFP-c-FLIP$_S$ or GFP-c-FLIP$_R$. 24 h post transfection the cells were fixed and stained with DAPI (blue). Subsequently, samples were analyzed by confocal laser scanning microscopy.

4.2.5. Enhanced expression levels of c-FLIP$_S$ is achieved on the level of protein translation.

The expression of c-FLIP$_S$ and c-FLIP$_R$ at the transcription level was similar. Therefore, to verify if the observed higher expression of c-FLIP$_S$ is indeed achieved during protein translation, coupled *in vitro* transcription/translation assays were performed. Interestingly, about five times higher expression level of c-FLIP$_S$ compared to c-FLIP$_R$ were obtained when equal amounts of expression plasmid were added into the coupled transcription/translation system (Fig. 34, left panel). Importantly, a similar effect was observed when c-FLIP short isoform production was initiated by the addition of *in vitro* transcribed RNA encoding either c-FLIP$_S$ or c-FLIP$_R$ to translation competent extracts excluding differences in transcription efficiency of the c-FLIP short isoform RNAs (Fig. 34, right panel). When translation was performed with c-FLIP RNAs preheated at 65°C an identical result was obtained, indicating that differences in protein translation are not caused by secondary RNA structures.

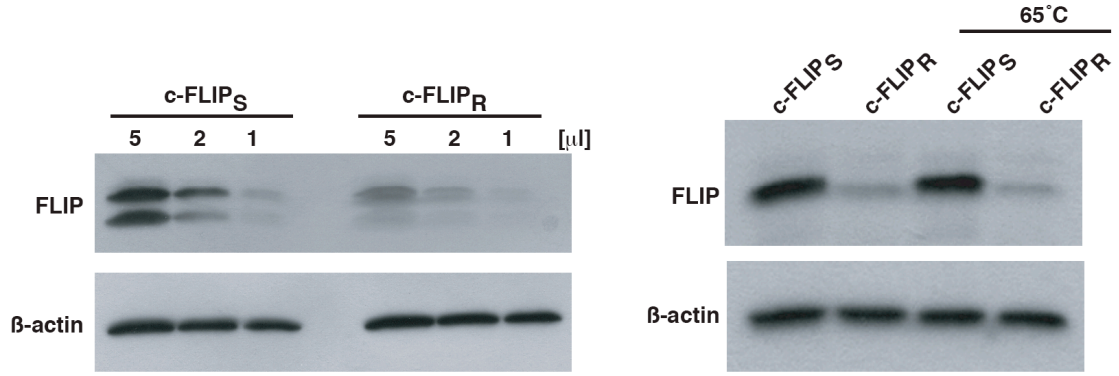

Figure 34. Coupled *in vitro* transcription/translation of c-FLIP$_S$ and c-FLIP$_R$. Left panel: 5, 2, or 1 µl of translated proteins were separated via SDS-PAGE and analyzed by Western blot. Right panel: c-FLIP$_S$ or c-FLIP$_R$ were *in vitro* transcribed and then equal RNA amounts were applied for an *in vitro* translation assay. Western blot determined protein levels of the two c-FLIP isoforms.

4.2.6. rs10190751 A and c-FLIP$_R$ expression is associated with follicular lymphoma.

SNPs are the most common DNA variation in the human genome [91] and can be found in both coding as well as non-coding areas of the genome. SNPs can have a significant impact on the expression or function of the encoded protein, e.g. a SNP can change the efficiency of transcription or translation in a positive or negative manner. It is therefore assumed that SNPs play a crucial influence on the predisposition to a variety of diseases. In addition, SNPs allow the identification of disease-associated genes using them as molecular markers [172]. Therefore, it is of importance to analyze whether the rs10190751 A/G variation in the human c-FLIP gene and the corresponding differential expression of its short isoforms correlates with the development of specific cancers [149].

The frequent appearance of rs10190751 A and concomitant expression of c-FLIP$_R$ in B cell lymphoma cell lines led on to investigate the frequency of rs10190751 A in lymphoma patients (n=183) compared to healthy subjects (n=233). Additionally, a set (n=130) of previously described urothelial carcinomas samples was analyzed [182], since a role of c-FLIP in this type of cancer has been proposed [96, 181]. As mentioned above (Fig. 30B), variants of rs10190751 A/G are unevenly distributed in different populations. Therefore, control and

carcinoma samples were carefully controlled regarding ethnical background. Samples were obtained from 233 healthy control individuals, 183 follicular lymphoma patients and 130 urothelial carcinoma patients. The control samples were 33.9% male and 66.1% female and of an average age of 42 ± 17 years. The follicular lymphoma samples were 44.3% male and 55.7% female and of an average age of 61 ± 13 years. All the subjects were of Caucasian origin.

Statistical analysis was conducted by using the SAS software (version 9.1). Genotype distribution, association with rs10190751 A and the allelic frequency have been addressed using the standard χ^2 test, for analysis of genotype distribution additionally the Conchran-Armitage was applied. Furthermore, odds ratios including 95% confidence intervals have been calculated. A p-value of < 0.05 was considered significant.

As can be seen in Table 2, a significant difference is detected between control samples and follicular lymphoma in genotype distribution (χ^2 test: p=0.0431; Cochran-Armitage Trend test: p=0.0131). A significant association of rs10190751 A with follicular lymphoma was also detected (p=0.0137; odds ratio=1.6358; 95% confidence interval, 1.1051-2.4213). Furthermore, a significant difference in allelic distribution between follicular lymphoma and the control samples was found (p=0.0185; odds ratio=1.4689; 95% confidence interval, 1.06658-2.0246). In contrast, no significant differences were observed comparing the urothelial carcinoma and control samples (Table 2).

***Note:** The study for the analysis of c-FLIP isoforms, were done together with Dr. Ingo Schmitz and Nana Ueffing.
Figure. 30B, 31B, 32A, C, D, 33 and 34 were performed by Nana Ueffing.

Table 2. Genotype distribution, association with rs10190751 A and allelic frequency in urothelial carcinoma (UC) samples, follicular lymphoma (FL) samples and controls.

UC = urothelial carcinoma; FL = follicular lymphoma; OR = odds ratio; CI, 95% = confidence interval.

	Controls	UC	FL
Genotype distribution			
GG	144 (62%)	86 (66%)	91 (49.7%)
GA	82 (35%)	36 (28%)	83 (45.4%)
AA	7 (3%)	8 (6%)	9 (4.9%)
n	233	130	183
χ^2		p=0.1575	p=0.0431
Cochran-Armitage Trend		0.8471	0.0131
Association with A			
GG	144 (62%)	86 (66%)	91 (49.7%)
GA+AA	89 (38%)	44 (34%)	92 (50.3%)
n	233	130	183
χ^2		p=0.409	p=0.0137
OR (CI)		0.8278 (0.5282-1.2973)	1.6358 (1.1.051-2.4213)
Allelic frequency			
G	370 (97.5%)	208 (80%)	265 (72.4%)
A	96 (20.5%)	52 (20%)	101 (27.6%)
n	466	260	366
χ^2		p=0.8472	p=0.0185
OR (CI)		0.9635 (0.6604-1.4059)	1.4689 (1.0658-2.0246)

5. DISCUSSION

5.1. RS and non-RS domain containing subunits of ASAP mediate exon inclusion.

Concomitant with the evolution of higher eukaryotes, cells were required to raise their protein diversity in order to cope with the increasingly broad spectrum of functional and behavioral complexity. One of the major approaches to achieve this task is the generation of multiple transcript species from a common mRNA precursor. This phenomenon is termed alternative splicing and adds considerable complexity to the gene expression process by generating discrete protein isoforms [171, 11].

Alternative splicing takes place inside the nucleus of the cells and involves a macromolecular machinery called spliceosome, which constitutes the basal splicing machinery [11]. For an error free splicing, it is important that 5' and 3' splice sites are accurately recognized. To fulfill this task, the basic splicing machinery is assisted by a large number of protein components, most notably members of the hnRNP and SR proteins [125, 69]. These factors, together with various other RNA-binding proteins, are also substantial in regulating alternative splicing by modulating spliceosome assembly and splice site choice.

The apoptosis and splicing associated protein (ASAP) complex, which consists of Acinus, RNPS1 and SAP18, was identified as a component of functional spliceosomes [148, 218], suggesting its possible role in RNA metabolism. Whereas the function of RNPS1 is well characterized as a general activator of splicing, the role of Acinus and SAP18 during pre-mRNA splicing process remains unclear. Functional investigation of RS domain containing (Acinus and RNPS1) and non-RS domain containing (SAP18) proteins in pre-mRNA splicing by utilizing HIV-based substrate demonstrated that both types of subunits were able to mediate exon inclusion (Fig. 18B). During the exon inclusion process several SR and non-SR proteins are potentially involved, which may play a role during the final transcript(s) production. Collectively, the data

suggested that the ASAP complex is involved in the regulation of the splice site selection process.

5.1.1. Subunits of the ASAP complex and their individual RS domains act as splicing regulatory factors.

The splicing regulatory activities of the subunits of the ASAP complex have been analyzed using an HIV-1-based splicing reporter measuring internal exon inclusion (Fig. 18B). The obtained findings have provided evidence that the RS domains of Acinus and RNPS1 modulate splice site selection. The present study demonstrates for the first time that the RS domains of Acinus regulate the modulation of alternative splice acceptor sites of the HIV-based pre-mRNA supporting its splice regulatory functions in mRNA processing.

The individual RS domains of Acinus showed internal exon inclusion of the reporter substrate. Exon inclusion varied in magnitude when different RS domains were used demonstrating differential intrinsic stimulatory potential inherent in the individual domains. Also, different splice acceptor sites were preferred by the individual RS domains, e.g. the amino acids 1174-1341 of Acinus specifically stimulated internal exon inclusion using the distal splice acceptor 4c site in addition to SA 5 and SA 4a sites (Fig. 20B). Importantly, there are different isoforms of Acinus (Fig. 8), containing various sets of RS domains, pointing to distinct splice regulatory potential of individual Acinus proteins. Since proteins and RS domains involved in the splicing regulation are generally localized in the nuclear speckles [108, 142], it was interesting to investigate the cellular localization of the RS domains of Acinus. All RS domains of Acinus showed nuclear localization (Fig. 22), which further supports their potential role in nuclear reactions, like pre-mRNA splicing or protein-RNA interaction [146, 156].

It was surprising to find that despite having several RS domains, MS2-Acinus-S' mediated no exon inclusion (Fig. 18B, lane7). One possible reason could be that the RS domains present in the MS2-Acinus-S' are masked due to its conformation and cannot be exposed efficiently to mediate protein-protein

interactions with other splicing factors available inside the nucleus of HeLa cells, or it might be interacting with repressor proteins, which do not activate splicing. From the immunoprecipitation studies (Fig. 25) it can be noted that FlagMS2-Acinus-S' precipitated significantly lower amounts of ASAP subunits, including RNPS1, than FlagMS2-RNPS1. This might be another explanation for the lack of internal exon inclusion caused by MS2-Acinus-S' (Fig. 18B).

RNPS1 had previously been reported to be a general activator of splicing [130]. Later, various domains of RNPS1 were analyzed on a β-globin minigene construct using a cotransfection system. Whereas it was found that the S-rich domain stimulates exon skipping, the RS/P domain was determined to be necessary for splicing repression on a β-globin substrate [163]. However, in the presented experiment (Fig. 21B) splicing of the HIV-based pre-mRNA *in vivo* indicated that the S-rich domain is necessary for exon inclusion. Deletion of this domain completely abolishes exon inclusion (Fig. 21B, lane 8), suggesting that the S-rich domain by itself, at least on the HIV minigene, does play a significant role in modulating splice site selection.

5.1.2. SAP18 mediates assembly of a splicing regulatory multiprotein complex via its ubiquitin-like fold.

Efficient exon inclusion activity was detected for SAP18 (Fig. 18B, lane 8), which was surprising, since SAP18 does not contain protein motifs like RS or RS-like domains, which would account for a function during regulation of mRNA processing. In contrast, the solution structure of SAP18 reveals an ubiquitin-like ß-grasp fold, a protein motif involved in protein-protein interactions and the assembly of multiprotein complexes [90, 131]. The presence of an ubiquitin-like fold agrees with the identification of SAP18 as a subunit associated with diverse multiprotein complexes [170, 189, 217]. Indeed, it is demonstrated here that the integrity of the structure of SAP18 is required for formation of the ASAP complex (Fig. 23B, lane 10).

The solution structure of the SAP18 ubiquitin-like fold highly resembles that of

ubiquitin, SUMO and Elongin B. As suggested by Fairbrother and coworkers [131], the structural relationship of SAP18 to ubiquitin and SUMO raises the intriguing question of whether SAP18 could serve as an adapter molecule to recruit a similar molecular machinery to target proteins. In this regard a novel ubiquitin-like domain (termed DWNN domain) has been identified in the splicing-associated RBBP6 protein, further implicating an involvement of ubiquitin-like pathways during RNA metabolism [147]. In addition, several members of the splicing regulatory proteins (hnRNPs) are modified by SUMOylation, which results in decreased affinity for mRNA [115].

To prove that the integrity of the ubiquitin-like fold is required for the splicing regulatory activity of SAP18 two amino acids (Asp118 and Thr121), which are conserved in the ß-grasp folds of SAP18, ubiquitin, SUMO and Elongin B [131], were mutated. Interestingly, mutation of the ubiquitin-like fold completely abrogated exon inclusion mediated by SAP18 (Fig. 23B, lane 10). It is conceivable that the structure of SAP18 is a prerequisite for interaction with further components of the splicing machinery that support the regulation of mRNA processing by SAP18. The other ASAP subunits, most notably RNPS1, might play a major role in this context. Exon inclusion activity has been demonstrated for RNPS1 in cooperation with the SR protein SRp54 [163]. SRp54 interacts with the 65 kDa subunit of the splicing factor U2AF and promotes the use of the most distal splice site on the E1A pre-mRNA [216]. In the presented study, compared to the splicing factor 9G8, RNPS1 tended to enhance use of the proximal (A5) splice site (Fig. 21B), hinting at the possibility that additional splicing regulatory proteins might be involved. It has been suggested that the interaction with SRp54 is required for proper nuclear speckle localization of RNPS1. In agreement, nuclear speckle localization of SAP18 was abolished by mutation of the ubiquitin-like fold, too (Fig. 24A and 24B, second row), implying that correct nuclear targeting of SAP18 is mediated by interaction with RNPS1 and other splicing factors, presumably involving SRp54. Direct or indirect associations with RNA-binding proteins must be involved to target SAP18 to sites

of RNA processing, since SAP18 itself does not contain an obvious RNA recognition motif.

Putative models of activation of internal exon inclusion by SAP18, which are not mutually exclusive, are presented in Fig. 35. It is possible that SAP18 functions mainly as a scaffold protein and is responsible for stabilizing multiprotein complexes by protein-protein interactions via its ubiquitin-like fold (Fig. 35A). These interactions involve RNPS1 and Acinus for ASAP formation, but possibly additional spliceosomal components. On the other hand, SAP18 could also play a rather active part during splicing regulation functioning as an adapter molecule. In this model SAP18 is recruited to the site of RNA processing by interaction with a hypothesized RNA-binding protein X (Fig. 35B). The model further proposes that SAP18 subsequently mediates assembly of ASAP via its ubiquitin-like fold.

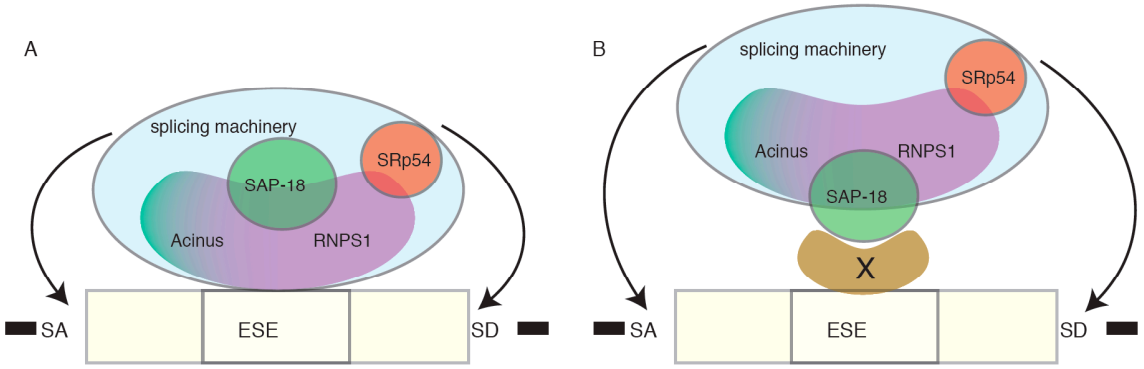

Figure 35. Putative models of internal exon inclusion mediated by SAP18. A) SAP18 functions as a scaffold protein by stabilizing multiprotein complexes, including ASAP, via protein-protein interactions mediated by the ubiquitin-like fold. B) In its function as adapter molecule SAP18 is targeted to splice sites by interaction with a hypothetical RNA-binding protein X. Subsequently, SAP18 recruits Acinus and RNPS1 for ASAP formation via the ubiquitin-like fold. RS and RS-like domains of Acinus and RNPS1 mediate assembly of a functional splicing regulatory complex, presumably containing SRp54. The spliceosomal complexes are targeted to the splice sites via exonic splicing enhancers (ESEs). Interactions of the splicing machinery with splice acceptor (SA) and splice donor (SD) sites lead to activation of exon inclusion.

Integration of RNPS1 into the ASAP complex requires a region of RNPS1 comprising amino acids 163 to 243 or the RRM domain (Fig. 26). Since SAP18 does not contain domains with potential in splicing regulation presumably the RS

and RS-like domains of RNPS1 and potentially Acinus function during splice site selection. It is likely that the S-rich domain of RNPS1, which is responsible for the recruitment of SRp54 and required for internal exon inclusion in the HIV-1 splicing reporter construct (Fig. 21B, lane 8), is also involved in splicing regulation by SAP18. Via their RS and RS-like domains RNPS1 and Acinus could mediate formation of an extended splicing regulatory competent multiprotein complex probably involving SRp54. It has been proposed that RS domains interact with other RS domain-containing splicing factors in a phosphorylation-dependent manner [69, 134]. Additionally, sequence-unspecific interactions of RS domain proteins with pre-mRNAs might play a role in spliceosome assembly [79, 175]. Noteworthy, a tendency of RS domain proteins including Acinus for self-aggregation has been proposed [142]. The extended complex is supposed to assist the basic splicing machinery in choosing appropriate splice sites for exon inclusion and this process would involve interactions with selected downstream splice donor (SD) and upstream splice acceptor (SA) sites [3].

Within the splicing reporter 2xMS2, the HIV-1 exonic splicing enhancer (GAR ESE) sequence had been substituted by two binding sites for the MS2 RNA-binding domain. Thus, the data demonstrate that a protein devoid of RS or RS-like domains can also mediate internal exon inclusion at least as efficiently as the well-characterized splicing factor 9G8, when targeted to the appropriate site on the mRNA precursor molecule. Besides an RNA-binding domain this process requires a domain functional in recruiting splicing regulatory proteins. Noteworthy, these observations implicate that spliceosome-associated factors like SAP18 can be manipulated to influence splice site choice of target genes.

5.2. A SNP in the c-FLIP gene determines protein isoform production and is associated with risk of follicular lymphoma.

About 90% of the human genetic variation is ascribed to single nucleotide polymorphisms (SNPs), which occur at frequencies of more than 1%. This characteristic makes SNPs the most common natural genetic variation occurring

in the human genome. SNPs can have a significant impact on the expression or even in the function of the encoded protein. Fairbrother and coworkers have mentioned that SNP density decreases significantly near splice sites, where the ESE sequences tend to cluster, especially within the region 10-20 nt from the splice site [47]. In contrast, analyzing the human c-FLIP genome revealed a SNP present at the 3' splice site of intron 6, which leads to the generation of the c-$FLIP_R$ isoform. It has been shown that in addition to the generation of different isoforms, SNPs can lead into the development of diseases [38]. Employing a SNP genotyping assay a higher existence of the c-$FLIP_R$ isoform in follicular lymphoma patients was observed, suggesting a role of the c-$FLIP_R$ isoform in the development of follicular lymphoma.

5.2.1. A SNP decides the production of c-$FLIP_R$ isoform.

Selected mammals were investigated in detail for their described isoforms of the c-FLIP protein. Of all species analyzed only humans were able to express c-$FLIP_S$ as a short isoform (Fig. 28). In several species the missing c-$FLIP_S$ expression is due to the lack of an equivalent of exon 7, e.g. in mouse and rat [197]. The appearance of exon 7 or an equivalent in higher mammals (humans, chimpanzee species) suggests that c-$FLIP_R$ is the original ancestral short isoform of c-FLIP and that expression of c-$FLIP_S$ arose later in evolution.

Although the common chimpanzee *Pan troglodytes* contains an exon 7 equivalent, at least the sequence provided in the database as well as the *P. troglodytes* cell lines analyzed (EB176, LTR2008) lack the potential to express c-$FLIP_S$ due to a nucleotide exchange in the second position of the AG dinucleotide of the splice acceptor consensus sequence. Interestingly, an identical exchange constitutes a SNP (rs10190751 A/G) in humans (Fig. 29A and B). An intact splice acceptor would theoretically allow production of both short isoforms, but the c-$FLIP_R$ isoform was detected only in cells containing at least one splice dead allele (rs10190751 A), strongly indicating that the rs10190751 A/G variant status is the critical factor determining the short c-FLIP isoform(s) expressed.

Here a potential model for the short c-FLIP isoforms (c-FLIP$_S$ and c-FLIP$_R$) production is presented (Fig. 36). The c-FLIP$_S$ isoform is generated when the 3' splice acceptor site (AG) of the intron 6 is intact (Fig. 36A), which leads to the recognition and inclusion of adjacent exon 7 into the final transcript of c-FLIP$_S$. On the other hand, the presence of a nucleotide exchange from G→A at the splice site destroys the 3' splice acceptor consensus sequence (AG→AA) and renders the splice site inactive, leading to inhibition of exon 7 recognition (Fig. 36B). In this case the splicing machinery reads through intron 6, including a part of this intron in the coding sequence, which is translated to generate c-FLIP$_R$ protein.

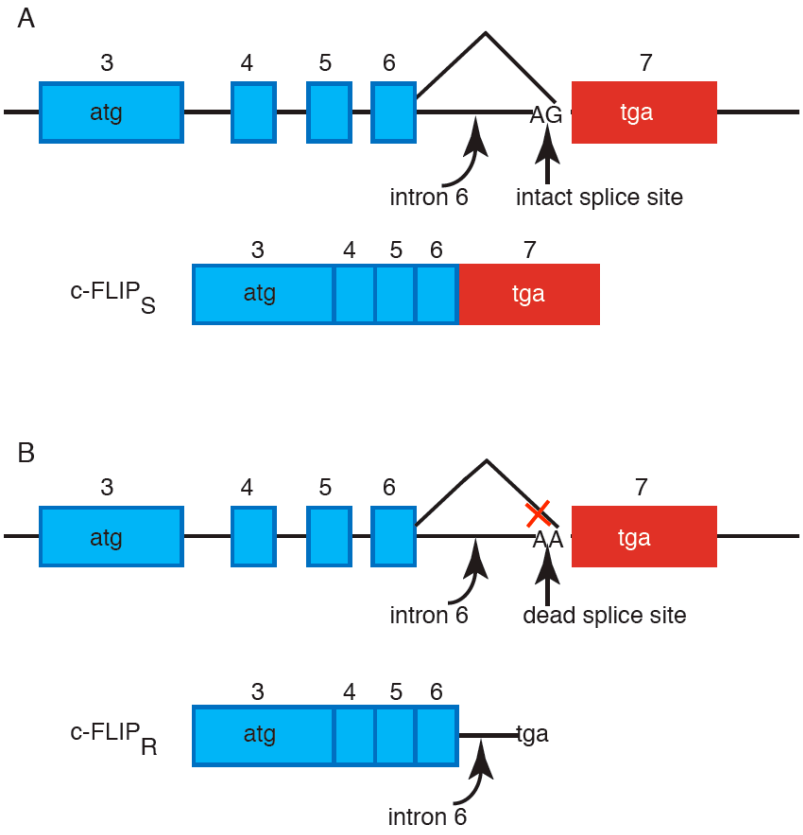

Figure 36. An alternative splicing process leads to the production of the distinct short isoforms of c-FLIP. A) Intron 6 is efficiently spliced out due to the presence of an intact (AG) splice acceptor site, which allows inclusion of exon 7 (in red) and hence production of c-FLIP$_S$. B) When the splice site is not intact, due to the presence of a nucleotide variation from AG→AA at the splice site, exon 7 could not be included. In this case splicing machinery read through the intron 6 including a part of intron 6 in the coding sequence, which is translated to generate c-FLIP$_R$ protein.

Independently of the SNP variant(s) present, transcription of the c-FLIP gene must have the potential to proceed through the whole locus, since the expression of c-$FLIP_L$ was present in all cell lines analyzed. Although only the splice dead variant was detected in *P. troglodytes* and *P. paniscus* cell lines, the existence of a SNP identical to rs10190751 A/G cannot be excluded. Therefore, chimpanzee species might have the potential to express c-$FLIP_R$ as well as c-$FLIP_S$ isoforms.

5.2.2. Lower expression of c-$FLIP_R$ is regulated on the level of protein translation.

Apoptotic signaling pathways are regulated through a variety of pro- and anti-apoptotic mediators and many of these apoptotic regulators are alternatively spliced [171]. The different spliced isoforms not only differ in their mRNA sequence but can also have modified functions in the regulation of apoptotic signaling processes. An example is the pre-mRNA of the Fas death receptor, where an alternatively spliced variant contains a rendered transmembrane domain, which can not be anchored on the cell [27]. Different c-FLIP proteins are also generated via alternative splicing; of the 11 identified FLIP-mRNA isoform, only three isoforms could be demonstrated at the protein level: the long version, c-$FLIP_L$ and two short versions, c-$FLIP_S$ and c-$FLIP_R$ [64, 176, 81]. All expressed isoforms of the c-FLIP protein are effective inhibitors of the death receptor-mediated apoptosis [88]. In contrast, c-$FLIP_L$ can also activate the autocatalytic processing of procaspase-8 in the DISC [104], by generating a heterodimeric complex with the caspase-like domain of procaspase-8 [72, 104, 136, 43]. This observation points out that the alternatively spliced isoforms of c-FLIP are functionally different.

A reduced translation efficiency of the c-$FLIP_R$ isoform was observed in the coupled *in vitro* transcriptional/translational analysis, but it is unclear why the c-$FLIP_R$ isoform is translated less efficiently than c-$FLIP_S$. One possibility might be the presence of rare codons and a lower incidence of their respective tRNAs, thereby leading to a delay in translation of the c-$FLIP_R$ [92], but an accumulation

of rare codons was not observed in the c-FLIP$_R$ transcript. Since the two short isoforms of c-FLIP only differ at their C-terminal, it is quite possible that the C-terminus of c-FLIP$_R$ binds more efficiently with the protein mediators, like P bodies. P bodies are aggregates of transcriptionally repressed mRNPs associated to certain translation repression and mRNA decay machinery [145]. A putative association of c-FLIP$_R$ with P bodies might prevent FLIP$_R$-mRNA translation or even lead to the degradation of its mRNA. Further investigations are needed to understand the clear mechanisms that play a role in lower protein expression of c-FLIP$_R$.

5.2.3. Increased c-FLIP$_R$ is associated with the increased risk of follicular lymphoma.

An accumulation of the c-FLIP$_R$ directing SNP variant rs10190751 A in tumorigenic B cell lines (Table 1) was observed. In agreement, increased appearance of c-FLIP$_R$ in B and also T cells has been noted before [64]. Analysis of the distribution of rs10190751 A/G variant in B cell lymphoma patients revealed an association of rs10190751 A with an increased risk for follicular lymphoma (Table 2). In contrast, investigation of a set of urothelial carcinoma samples did not reveal an association, pointing out that the association with rs10190751 A is specific for follicular lymphoma and not a general attribute of cancer cells.

The initiating genetic event of follicular lymphoma is the t(14:18) translocation, which occurs in ~90% of follicular lymphoma and causes constitutive expression of the antiapoptotic protein Bcl-2 [7, 32, 196]. Although expression of Bcl-2 is thought to be an essential factor during the development of follicular lymphoma, overexpression of Bcl-2 alone is not sufficient to cause follicular lymphoma in mice [132, 133] and the presence of sporadic t(14:18) bearing B cells has been reported in a substantial proportion of healthy individuals [118]. Therefore, secondary events, including the accumulation of genetic alterations and clonal selection, must contribute to the development of follicular lymphoma [8, 39].

Ongoing somatic mutation and cytological features, including a follicular growth pattern, identify follicular lymphoma as a germinal centre B cell tumor [106, 105]. In agreement with the important role of FLIP during the germinal centre reaction [78, 93], perturbance of the c-FLIP isoform composition could contribute to the development of follicular lymophoma. Furthermore, changes in c-FLIP protein levels have been described in several distinct B cell malignancies [20, 188]. Importantly, a higher protein expression of c-FLIP$_S$ compared to c-FLIP$_R$ (Fig. 31, 32 and 33) was detected, an observation also noted in human T cells [64]. Differential levels in c-FLIP short isoform expression would also lead to a shift in the proportion of long and short c-FLIP molecules, which in turn would effectively influence the amounts of the different isoforms in the DISC. Noteworthy, varying roles in the DISC have been described for c-FLIP$_L$ and the short c-FLIP isoforms [104].

Recent findings have pointed to an important role of the immunological microenvironment for the development of follicular lymphoma, including functions of T cells and FDCs [8, 39]. Studies from different groups have pointed to a growth supportive role of CD4-positve T helper cells in at least a subset of follicular lymphoma [45, 63, 128]. Most interestingly, transgenic mice expressing Bcl-2 in multiple hematopoietic lineages developed lymphomas reminiscent of follicular lymphoma. These mice displayed a significantly enlarged T cell compartment, consistent with a high Bcl-2 expression in the T cells [45]. A functional modulation of CD4-positive T cells was also suggested in rapidly transforming follicular lymphoma, which presented differences in the spatial distribution and activation of this T cell subset. These results are consistent with the fact, that c-FLIP proteins play a major role during T cell survival [103]. The expression profile of c-FLIP isoforms might influence the risk for follicular lymphoma not only by affecting B cell homeostasis, but also rather by modulating survival of T cells within the micro-environment of follicular lymphoma.

The data presented show that a SNP in the human c-FLIP gene leads to the production of c-FLIP$_R$ isoform, which is associated with a risk for follicular

lymphoma. A recent study, which demonstrates an increased risk of follicular lymphoma associated with a SNP in the human CD40 gene, supports the presented work [178]. Stimulation of B cell-expressed CD40 by T cell-expressed CD154 is highly important for the delivery of B cell survival signals, including upregulation of c-FLIP [71, 77, 78, 93, 200]. These observations underline the potential impact of SNPs in genes involved in lymphocyte regulation for the development of follicular lymphoma.

5.3. Distinct regulation of splice site choice determines protein isoform production.

The recognition of splice site consensus sequences and their usage for the removal of introns during RNA processing is a determining factor for protein isoform production The splice site consensus sequences are helped by additional sequence elements, like ESEs, ESSs, ISEs and ISSs, that are required for the regulation of splice site selection [184]. These elements are bound by splice regulatory and SR proteins [36, 125], putatively including components of the ASAP complex, which seem to promote exon inclusion and splice sites selection. Interestingly, as described in this thesis, proteins with or without RS domains can be capable of choosing splice sites and mediating exon inclusion (Fig. 18B).

Depending on the selected splice sites, distinctive transcripts of various sizes will be produced (Fig. 18B). Different SR proteins may influence splice site selection in both positive and negative fashion [87] and some SR proteins are more productive in promoting splice site selection than others [65].

Importantly, approximately 50% of disease-causing point mutations affect pre-mRNA splicing, pointing out that integrity of the splice site consensus sequences (GU; 5' splice site and AG; 3' splice site) is vital [26]. Mutations at these sites can significantly contribute to human genetic diseases including cancer. In the presented study a single nucleotide polymorphism located in a 3' splice site consensus is responsible for the generation of the c-FLIP$_R$ isoform of c-FLIP. Most interestingly, an association of the splice site variation and

concomitant c-FLIP$_R$ production with an increased risk for a specific type of cancer, follicular lymphoma, was found.

Collectively, the presented data show that distinct “modes” of protein isoform production involving splice site recognition exist. These include selection of splice sites via the function splice regulatory proteins, but also genetic variations of splice sites can determine the production of distinctive protein isoforms.

6. SUMMARY

During alternative splicing introns are spliced out in various ways from the precursor RNA, resulting in one gene producing several different mRNAs and protein products. The process of alternative splicing requires accurate selection of splice sites, which play a key factor in the generation of different transcripts. Several regulatory protein complexes take active part to aid in the selection of splice sites, like small nuclear ribonucleoprotein complexes containing U1, U2, U4/U6 and U5 subunits. The serine/arginine-rich protein family members (SR proteins) and SR-related proteins (SR-rps) also have the capability to assist in the splice site selection process. In addition, various other regulatory protein complexes might be involved in the alternative splicing process. In this regard, the role of the subunits existing in the apoptosis and splicing associated protein (ASAP) complex during splice site selection was analyzed.

An HIV-based reporter construct was utilized to understand the effects of this trimeric complex on the splice site selection. The presented data show that the full-length Acinus-S' isoform displayed only limited splicing regulatory activity, whereas both RNPS1 and surprisingly, SAP18 mediated strong exon inclusion. Mutational analysis revealed that the ubiquitin-like fold of SAP18 was required for efficient exon inclusion and for the localization of SAP18 in the nuclear speckles. The deletion mutants of RNPS1 demonstrated that the serine-rich domain is mainly responsible for the exon inclusion. The individual RS and RS-like domains of Acinus and RNPS1 also showed the ability to modulate the selection of alternative splice acceptor sites. Taken together, the data demonstrate that the proteins of the ASAP complex are able to mediate internal exon inclusion of the HIV-based construct. However, further investigations are necessary to understand the exact molecular mechanisms involved in the specific selection of splice sites by these fusion proteins.

In the second section, the isoforms of the cellular-FLICE inhibitory protein (c-FLIP) were investigated. c-FLIP is a modulator of death receptor mediated apoptosis and plays a major role in T and B cell homeostasis. The c-FLIP gene

transcript is subject to alternative splicing and so far three different c-FLIP isoforms (c-FLIP$_L$, c-FLIP$_S$ and c-FLIP$_R$) have been demonstrated on the protein level. Investigation of the c-FLIP gene revealed the presence of a single nucleotide polymorphism (SNP) at the 3' splice site of the intron 6. The SNP leads to either an intact (AG) splice site or a dead (AA) splice consensus. Whereas an intact splice site directs exclusively production of c-FLIP$_S$ via inclusion of exon 7, the splice dead variant causes production of c-FLIP$_R$ due to read through into intron 6. Comparison of c-FLIP short isoform protein sequences in several mammals identifies c-FLIP$_R$ as the ancestral short version of c-FLIP. Employing *in vivo* and *in vitro* expression studies, it was observed that the c-FLIP$_S$ isoform was expressed strongly compared to c-FLIP$_R$. With the help of RT-PCR studies and coupled *in vitro* transcription/translation assays, it was found that the higher expression of c-FLIP$_S$ is achieved on the protein level. Analysis of diverse human cell lines points to an increased frequency of c-FLIP$_R$ in cancerogenous B cell lines. Importantly, analysis of follicular lymphoma patients reveals an increased risk associated with the rs10190751 A genotype causing disruption of the splice consensus and expression of c-FLIP$_R$. Taken together, the described data show that c-FLIP$_R$ is generated due to the presence of a SNP at the 3' splice site and that c-FLIP$_R$ production is associated with an increased risk of follicular lymphoma.

Zusammenfassung

Beim alternativen Spleißen werden die Introns auf verschiedene Art und Weise aus der Vorläufer-RNS herausgespleißt, wodurch aus einem Gen zahlreiche unterschiedliche Boten-RNS-Moleküle und Proteine produziert werden können. Der Vorgang des alternativen Spleißens erfordert eine akkurate Auswahl von Spleißstellen, welche eine Schlüsselrolle bei der Generierung der verschiedenen Transkripte spielen. Verschiedene regulatorische Proteinkomplexe sind aktiv an der Auswahl der Spleißstellen beteiligt, unter Anderem “kleine nukleäre” Ribonukleoproteinkomplexe mit U1, U2, U4/U6 and U5 Untereinheiten. Mitglieder der Serin/Arginin-reichen Proteine (SR-Proteine) sowie SR-verwandte Proteine (SR-rps) können ebenfalls in den Spleißstellen-Selektionsprozess eingreifen. Zusätzlich können weitere regulatorisch tätige Proteine in den Prozess des alternativen Spleißens involviert sein. In diesem Zusammenhang wurde nun die Rolle der Untereinheiten des Apoptose- und spleißassoziierten Protein (ASAP) Komplexes während der Auswahl von Spleißstellen untersucht.

Um den Einfluss dieses trimeren Komplexes auf die Spleißstellen-Selektion zu verstehen, wurde ein Reporterkonstrukt, welches auf dem HI-Virus basiert, eingesetzt. Die vorgestellten Daten zeigen, dass RNPS1 und, überraschenderweise, SAP18 starken Exon-Einschluss vermitteln können, während ein Volllänge-Acinus-S’-Konstrukt nur eine sehr begrenzte spleißregulatorische Aktivität aufweist. Mutationsanalysen zeigten auf, dass die Ubiquitin-ähnliche Struktur von SAP18 für effizienten Exon-Einschluss sowie die Lokalisierung von SAP18 in Kern-„speckles“ benötigt wird. Mit Hilfe von RNPS1-Deletionsmutanten wurde nachgewiesen, dass die Serin-reiche Domäne von RNPS1 hauptsächlich für den RNPS1-vermittelten Exon-Einschluss verantwortlich ist. Ebenfalls wiesen individuelle RS- und RS-ähnliche Domänen von Acinus und RNPS1 die Fähigkeit auf, die Auswahl alternativer Spleißstellen zu beeinflussen. Zusammengefasst zeigen diese Daten, dass die Proteinuntereinheiten des ASAP Komplexes in der Lage sind internen Exon-

Einschluss auf dem HIV-basierenden Reporterkonstrukt zu vermitteln. Dennoch sind weitere Untersuchungen notwendig, um die exakten molekularen Mechanismen zu verstehen, welche in die Spleißstellen-Selektion durch diese Proteine involviert sind.

In einem zweiten Abschnitt wurden die Isoformen des zellulären FLICE-inhibitorischen Proteins (c-FLIP) untersucht. c-FLIP kann die Todesrezeptor-vermittelte Apoptose modifizieren und spielt eine wichtige Rolle während der T- und B-Zellhomöostase. Das c-FLIP-Gen wird dem Prozess des alternativen Spleißens unterworfen und drei verschiedene c-FLIP-Isoformen (c-$FLIP_L$, c-$FLIP_S$ und c-$FLIP_R$) konnten bis jetzt auf Proteinebene demonstriert werden. Eine analyse des c-FLIP-Gens offenbarte einen „single nucleotide“ Polymorphismus (SNP) an der 3’-Spleißstelle des Introns 6. Dieser SNP verursacht entweder eine intakte (AG) oder defekte (AA) Spleißkonsensussequenz. Während eine intakte Spleißstelle über den Einschluss Exon 7 ausschließlich Expression von c-$FLIP_S$ bewirkt, verursacht die defekte Variante die Produktion von c-$FLIP_R$, da bei der Abschrift der RNS in das Intron 6 hineingelesen wird. Ein Vergleich der Proteinsequenzen der kleinen c-FLIP-Isoformen in verschiedenen Säugern identifierte c-$FLIP_R$ als die ursprüngliche kleine Version von c-FLIP. Mit Hilfe von *in vivo* und *in vitro* Expressionsstudien konnte beobachtet werden, dass c-$FLIP_S$ im Vergleich zu c-$FLIP_R$ erheblich stärker exprimiert wird. Weiterhin wurde mit Hilfe von RT-PCR Studien und gekoppelten *in vitro* Transkriptions-/Translationsexperimenten herausgefunden, das die höhere Expression von c-$FLIP_S$ auf der Ebene der Proteintranslation erreicht wird. Eine Analyse diverser humaner Zelllinien deutete auf eine gesteigerte Häufigkeit der Expression von c-$FLIP_R$ in kanzerogenen B-Zelllinien hin. Interessanterweise offenbarte die Untersuchung von follikulären Lymphompatienten eine Assoziation der rs10190751 A Variante des SNP, welche eine Zerstörung des Spleißstellenkonsensus und die Expression von c-$FLIP_R$ bewirkt, mit einem erhöhten Risiko für follikulare Lymphome. Zusammengefasst zeigen die beschriebenen Daten, dass die Expression von c-$FLIP_R$ durch einen SNP am 3’-

Spleißkonsensus des Intron 6 bewirkt wird, und dass die c-FLIP$_R$-Produktion mit einem Risiko für follikuläre Lymphome assoziiert ist.

7. BIBLIOGRAPHY

1. **Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G., Scherer, S.E., Li, P.W., Hoskins, R.A., Galle, R.F. *et al*.** The genome sequence of Drosophila melanogaster. Science **287**:2185-2195. 2000.

2. **Allen, C.D., Okada, T., Tang, H.L., Cyster, J.G.** Imaging of germinal center selection events during affinity maturation. Science **315**:528-531. 2007.

3. **Asang, C., Hauber, I., Schaal, H.** Insights into the selective activation of alternatively used splice acceptors by the human immunodeficiency virus type-1 bidirectional splicing enhancer. Nucleic Acids Res **36**:1450-1463. 2008.

4. **Ashkenazi, A., Dixit, V.M.** Apoptosis control by death and decoy receptors. Curr Opin Cell Biol **11**:255-260. 1999.

5. **Ayane, M., Preuss, U., Kohler, G., Nielsen, P.J.** A differentially expressed murine RNA encoding a protein with similarities to two types of nucleic acid binding motifs. Nucleic Acids Res **19**:1273-1278. 1991.

6. **Badolato, J., Gardiner, E., Morrison, N., Eisman, J.** Identification and characterisation of a novel human RNA-binding protein. Gene **166**:323-327. 1995.

7. **Bakhshi, A., Jensen, J.P., Goldman, P., Wright, J.J., McBride, O.W., Epstein, A.L., Korsmeyer, S.J.** Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. Cell **41**:899-906. 1985.

8. **Bende, R.J., Smit, L.A., van Noesel, C.J.** Molecular pathways in follicular lymphoma. Leukemia **21**:18-29. 2007.

9. **Birney, E., Kumar, S., Krainer, A.R.** Analysis of the RNA-recognition motif and RS and RGG domains: conservation in metazoan pre-mRNA splicing factors. Nucleic Acids Res **21**:5803-5816. 1993.

10. **Black, D.L.** Protein diversity from alternative splicing: a challenge for bioinformatics and post-genome biology. Cell **103**:367-370. 2000.

11. **Black, D.L.** Mechanisms of alternative pre-messenger RNA splicing. Annu Rev Biochem **72**:291-336. 2003.

12. **Blencowe, B.J., Issner, R., Nickerson, J.A., Sharp, P.A.** A coactivator of pre-mRNA splicing. Genes Dev **12**:996-1009. 1998.

13. **Blencowe, B.J., Bowman, J.A., McCracken, S., Rosonina, E.** SR-related proteins and the processing of messenger RNA precursors. Biochem Cell Biol **77**:277-291. 1999.

14. **Blencowe, B.J.** Exonic splicing enhancers: mechanism of action, diversity and role in human genetic diseases. Trends Biochem Sci **25**:106-110. 2000.

15. **Blencowe, B.J., Bauren, G., Eldridge, A.G., Issner, R., Nickerson, J.A., Rosonina, E., Sharp, P.A.** The SRm160/300 splicing coactivator subunits. RNA **6**:111-120. 2000.

16. **Blencowe, B.J., Graveley, B.R.** Alternative splicing in the postgenomic era. Book **623**:2007.

17. **Boatright, K.M., Salvesen, G.S.** Mechanisms of caspase activation. Curr Opin Cell Biol **15**:725-731. 2003.

18. **Boukis, L.A., Liu, N., Furuyama, S., Bruzik, J.P.** Ser/Arg-rich protein-mediated communication between U1 and U2 small nuclear ribonucleoprotein particles. J Biol Chem **279**:29647-29653. 2004.

19. **Bourgeois, C.F., Lejeune, F., Stevenin, J.** Broad specificity of SR (serine/arginine) proteins in the regulation of alternative splicing of pre-messenger RNA. Prog Nucleic Acid Res Mol Biol **78**:37-88. 2004.

20. **Budd, R.C., Yeh, W.C., Tschopp, J.** cFLIP regulation of lymphocyte activation and development. Nat Rev Immunol **6**:196-204. 2006.

21. **Caceres, J.F., Stamm, S., Helfman, D.M., Krainer, A.R.** Regulation of alternative splicing in vivo by overexpression of antagonistic splicing factors. Science **265**:1706-1709. 1994.

22. **Caceres, J.F., Misteli, T., Screaton, G.R., Spector, D.L., Krainer, A.R.** Role of the modular domains of SR proteins in subnuclear localization and alternative splicing specificity. J Cell Biol **138**:225-238. 1997.

23. **Caceres, J.F., Screaton, G.R., Krainer, A.R.** A specific subset of SR proteins shuttles continuously between the nucleus and the cytoplasm. Genes Dev **12**:55-66. 1998.

24. **Cao, W., Garcia-Blanco, M.A.** A serine/arginine-rich domain in the human U1 70k protein is necessary and sufficient for ASF/SF2 binding. J Biol Chem **273**:20629-20635. 1998.

25. **Caputi, M., Freund, M., Kammler, S., Asang, C., Schaal, H.** A bidirectional SF2/ASF- and SRp40-dependent splicing enhancer regulates human immunodeficiency virus type 1 rev, env, vpu, and nef gene expression. J Virol **78**:6517-6526. 2004.

26. **Cartegni, L., Chew, S.L., Krainer, A.R.** Listening to silence and understanding nonsense: exonic mutations that affect splicing. Nat Rev Genet **3**:285-298. 2002.

27. **Cascino, I., Fiucci, G., Papoff, G., Ruberti, G.** Three functional soluble forms of the human apoptosis-inducing Fas molecule are produced by alternative splicing. J Immunol **154**:2706-2713. 1995.

28. **Cavaloc, Y., Popielarz, M., Fuchs, J.P., Gattoni, R., Stevenin, J.** Characterization and cloning of the human splicing factor 9G8: a novel 35 kDa factor of the serine/arginine protein family. Embo J **13**:2639-2649. 1994.

29. **Cheng, H., Dufu, K., Lee, C.S., Hsu, J.L., Dias, A., Reed, R.** Human mRNA export machinery recruited to the 5' end of mRNA. Cell **127**:1389-1400. 2006.

30. **Chew, S.L., Liu, H.X., Mayeda, A., Krainer, A.R.** Evidence for the function of an exonic splicing enhancer after the first catalytic step of pre-mRNA splicing. Proc Natl Acad Sci U S A **96**:10655-10660. 1999.

31. **Chiara, M.D., Gozani, O., Bennett, M., Champion-Arnaud, P., Palandjian, L., Reed, R.** Identification of proteins that interact with exon sequences, splice sites, and the branchpoint sequence during each stage of spliceosome assembly. Mol Cell Biol **16**:3317-3326. 1996.

32. **Cleary, M.L., Sklar, J.** Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. Proc Natl Acad Sci U S A **82**:7439-7443. 1985.

33. **Cohen, G.M.** Caspases: the executioners of apoptosis. Biochem J **326 (Pt 1)**:1-16. 1997.

34. **Colwill, K., Pawson, T., Andrews, B., Prasad, J., Manley, J.L., Bell, J.C., Duncan, P.I.** The Clk/Sty protein kinase phosphorylates SR splicing factors and regulates their intranuclear distribution. Embo J **15**:265-275. 1996.

35. **Cory, S., Huang, D.C., Adams, J.M.** The Bcl-2 family: roles in cell survival and oncogenesis. Oncogene **22**:8590-8607. 2003.

36. **Crispino, J.D., Blencowe, B.J., Sharp, P.A.** Complementation by SR proteins of pre-mRNA splicing reactions depleted of U1 snRNP. Science **265**:1866-1869. 1994.

37. **Dauwalder, B., Amaya-Manzanares, F., Mattox, W.** A human homologue of the Drosophila sex determination factor transformer-2 has conserved splicing regulatory functions. Proc Natl Acad Sci U S A **93**:9004-9009. 1996.

38. **De Gobbi, M., Viprakasit, V., Hughes, J.R., Fisher, C., Buckle, V.J., Ayyub, H., Gibbons, R.J., Vernimmen, D., Yoshinaga, Y., de Jong, P. *et al***. A regulatory SNP causes a human genetic disease by creating a new transcriptional promoter. Science **312**:1215-1217. 2006.

39. **de Jong, D.** Molecular pathogenesis of follicular lymphoma: a cross talk of genetic and immunologic factors. J Clin Oncol **23**:6358-6363. 2005.

40. **Degot, S., Le Hir, H., Alpy, F., Kedinger, V., Stoll, I., Wendling, C., Seraphin, B., Rio, M.C., Tomasetto, C.** Association of the breast cancer protein MLN51 with the exon junction complex via its speckle localizer and RNA binding module. J Biol Chem **279**:33702-33715. 2004.

41. **Diamond, R.H., Du, K., Lee, V.M., Mohn, K.L., Haber, B.A., Tewari, D.S., Taub, R.** Novel delayed-early and highly insulin-induced growth response genes. Identification of HRS, a potential regulator of alternative pre-mRNA splicing. J Biol Chem **268**:15185-15192. 1993.

42. **Djerbi, M., Darreh-Shori, T., Zhivotovsky, B., Grandien, A.** Characterization of the human FLICE-inhibitory protein locus and comparison of the anti-apoptotic activity of four different flip isoforms. Scand J Immunol **54**:180-189. 2001.

43. **Dohrman, A., Russell, J.Q., Cuenin, S., Fortner, K., Tschopp, J., Budd, R.C.** Cellular FLIP long form augments caspase activity and death of T cells through heterodimerization with and activation of caspase-8. J Immunol **175**:311-318. 2005.

44. **Dreyfuss, G., Matunis, M.J., Pinol-Roma, S., Burd, C.G.** hnRNP proteins and the biogenesis of mRNA. Annu Rev Biochem **62**:289-321. 1993.

45. **Egle, A., Harris, A.W., Bath, M.L., O'Reilly, L., Cory, S.** VavP-Bcl2 transgenic mice develop follicular lymphoma preceded by germinal center hyperplasia. Blood **103**:2276-2283. 2004.

46. **Eperon, I.C., Ireland, D.C., Smith, R.A., Mayeda, A., Krainer, A.R.** Pathways for selection of 5' splice sites by U1 snRNPs and SF2/ASF. Embo J **12**:3607-3617. 1993.

47. **Fairbrother, W.G., Holste, D., Burge, C.B., Sharp, P.A.** Single nucleotide polymorphism-based validation of exonic splicing enhancers. PLoS Biol **2**:E268. 2004.

48. **Faustino, N.A., Cooper, T.A.** Pre-mRNA splicing and human disease. Genes Dev **17**:419-437. 2003.

49. **Fischer, B., Coelho, D., Dufour, P., Bergerat, J.P., Denis, J.M., Gueulette, J., Bischoff, P.** Caspase 8-mediated cleavage of the pro-apoptotic BCL-2 family member BID in p53-dependent apoptosis. Biochem Biophys Res Commun **306**:516-522. 2003.

50. **Fischer, U., Schulze-Osthoff, K.** New approaches and therapeutics targeting apoptosis in disease. Pharmacol Rev **57**:187-215. 2005.

51. **Frazer, K.A., Ballinger, D.G., Cox, D.R., Hinds, D.A., Stuve, L.L., Gibbs, R.A., Belmont, J.W., Boudreau, A., Hardenbol, P., Leal, S.M.** ***et al***. A second generation human haplotype map of over 3.1 million SNPs. Nature **449**:851-861. 2007.

52. **French, L.E., Tschopp, J.** Protein-based therapeutic approaches targeting death receptors. Cell Death Differ **10**:117-123. 2003.

53. **Fu, X.D., Maniatis, T.** Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. Nature **343**:437-441. 1990.

54. **Fu, X.D., Maniatis, T.** Isolation of a complementary DNA that encodes the mammalian splicing factor SC35. Science **256**:535-538. 1992.

55. **Fu, X.D.** Specific commitment of different pre-mRNAs to splicing by single SR proteins. Nature **365**:82-85. 1993.

56. **Fu, X.D.** The superfamily of arginine/serine-rich splicing factors. RNA **1**:663-680. 1995.

57. **Fuentes-Prior, P., Salvesen, G.S.** The protein structures that shape caspase activity, specificity, activation and inhibition. Biochem J **384**:201-232. 2004.

58. **Ge, H., Manley, J.L.** A protein factor, ASF, controls cell-specific alternative splicing of SV40 early pre-mRNA in vitro. Cell **62**:25-34. 1990.

59. **Ge, H., Zuo, P., Manley, J.L.** Primary structure of the human splicing factor ASF reveals similarities with Drosophila regulators. Cell **66**:373-382. 1991.

60. **Gehring, N.H., Neu-Yilik, G., Schell, T., Hentze, M.W., Kulozik, A.E.** Y14 and hUpf3b form an NMD-activating complex. Mol Cell **11**:939-949. 2003.

61. **Gehring, N.H., Kunz, J.B., Neu-Yilik, G., Breit, S., Viegas, M.H., Hentze, M.W., Kulozik, A.E.** Exon-junction complex components specify distinct routes of nonsense-mediated mRNA decay with differential cofactor requirements. Mol Cell **20**:65-75. 2005.

62. **Gilbert, W.** Why genes in pieces? Nature **271**:501. 1978.

63. **Glas, A.M., Knoops, L., Delahaye, L., Kersten, M.J., Kibbelaar, R.E., Wessels, L.A., van Laar, R., van Krieken, J.H., Baars, J.W., Raemaekers, J.** ***et al***. Gene-expression and immunohistochemical study of specific T-cell subsets and accessory cell types in the transformation and prognosis of follicular lymphoma. J Clin Oncol **25**:390-398. 2007.

64. **Golks, A., Brenner, D., Fritsch, C., Krammer, P.H., Lavrik, I.N.** c-FLIPR, a new regulator of death receptor-induced apoptosis. J Biol Chem **280**:14507-14513. 2005.

65. **Goren, A., Ram, O., Amit, M., Keren, H., Lev-Maor, G., Vig, I., Pupko, T., Ast, G.** Comparative analysis identifies exonic splicing regulatory sequences--The complex definition of enhancers and silencers. Mol Cell **22**:769-781. 2006.

66. **Goto, Y., Matsuda-Minehata, F., Inoue, N., Matsui, T., Maeda, A., Manabe, N.** Porcine (Sus scrofa) cellular FLICE-like inhibitory protein (cFLIP): molecular cloning and comparison with the human and murine cFLIP. J Reprod Dev **50**:549-555. 2004.

67. **Goval, J.J., Thielen, C., Bourguignon, C., Greimers, R., Dejardin, E., Choi, Y.S., Boniver, J., de Leval, L.** The prevention of spontaneous apoptosis of follicular lymphoma B cells by a follicular dendritic cell line: involvement of caspase-3, caspase-8 and c-FLIP. Haematologica **93**:1169-1177. 2008.

68. **Graveley, B.R., Maniatis, T.** Arginine/serine-rich domains of SR proteins can function as activators of pre-mRNA splicing. Mol Cell **1**:765-771. 1998.

69. **Graveley, B.R.** Sorting out the complexity of SR protein functions. RNA **6**:1197-1211. 2000.

70. **Green, M.R.** Pre-mRNA splicing. Annu Rev Genet **20**:671-708. 1986.

71. **Guzman-Rojas, L., Sims-Mourtada, J.C., Rangel, R., Martinez-Valdez, H.** Life and death within germinal centres: a double-edged sword. Immunology **107**:167-175. 2002.

72. **Han, D.K., Chaudhary, P.M., Wright, M.E., Friedman, C., Trask, B.J., Riedel, R.T., Baskin, D.G., Schwartz, S.M., Hood, L.** MRIT, a novel death-effector domain-containing protein, interacts with caspases and BclXL and initiates cell death. Proc Natl Acad Sci U S A **94**:11333-11338. 1997.

73. **Hanahan, D., Weinberg, R.A.** The hallmarks of cancer. Cell **100**:57-70. 2000.

74. **Hanes, J., von der Kammer, H., Klaudiny, J., Scheit, K.H.** Characterization by cDNA cloning of two new human protein kinases. Evidence by sequence comparison of a new family of mammalian protein kinases. J Mol Biol **244**:665-672. 1994.

75. **Hastings, M.L., Krainer, A.R.** Pre-mRNA splicing in the new millennium. Curr Opin Cell Biol **13**:302-309. 2001.

76. **Hedley, M.L., Amrein, H., Maniatis, T.** An amino acid sequence motif sufficient for subnuclear localization of an arginine/serine-rich splicing factor. Proc Natl Acad Sci U S A **92**:11524-11528. 1995.

77. **Hennino, A., Berard, M., Casamayor-Palleja, M., Krammer, P.H., Defrance, T.** Regulation of the Fas death pathway by FLICE-inhibitory protein in primary human B cells. J Immunol **165**:3023-3030. 2000.

78. **Hennino, A., Berard, M., Krammer, P.H., Defrance, T.** FLICE-inhibitory protein is a key regulator of germinal center B cell apoptosis. J Exp Med **193**:447-458. 2001.

79. **Hertel, K.J., Graveley, B.R.** RS domains contact the pre-mRNA throughout spliceosome assembly. Trends Biochem Sci **30**:115-118. 2005.

80. **Hu, Y., Yao, J., Liu, Z., Liu, X., Fu, H., Ye, K.** Akt phosphorylates acinus and inhibits its proteolytic cleavage, preventing chromatin condensation. Embo J **24**:3543-3554. 2005.

81. **Irmler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J.L., Schroter, M., Burns, K., Mattmann, C. *et al***. Inhibition of death receptor signals by cellular FLIP. Nature **388**:190-195. 1997.

82. **Ishigaki, Y., Li, X., Serin, G., Maquat, L.E.** Evidence for a pioneer round of mRNA translation: mRNAs subject to nonsense-mediated decay in mammalian cells are bound by CBP80 and CBP20. Cell **106**:607-617. 2001.

83. **Jang, S.W., Yang, S.J., Ehlen, A., Dong, S., Khoury, H., Chen, J., Persson, J.L., Ye, K.** Serine/arginine protein-specific kinase 2 promotes leukemia cell proliferation by phosphorylating acinus and regulating cyclin A1. Cancer Res **68**:4559-4570. 2008.

84. **Johnson, J.M., Castle, J., Garrett-Engele, P., Kan, Z., Loerch, P.M., Armour, C.D., Santos, R., Schadt, E.E., Stoughton, R., Shoemaker, D.D.** Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays. Science **302**:2141-2144. 2003.

85. **Joselin, A.P., Schulze-Osthoff, K., Schwerk, C.** Loss of Acinus inhibits oligonucleosomal DNA fragmentation but not chromatin condensation during apoptosis. J Biol Chem **281**:12475-12484. 2006.

86. **Kammler, S., Leurs, C., Freund, M., Krummheuer, J., Seidel, K., Tange, T.O., Lund, M.K., Kjems, J., Scheid, A., Schaal, H.** The sequence complementarity between HIV-1

5' splice site SD4 and U1 snRNA determines the steady-state level of an unstable env pre-mRNA. RNA **7**:421-434. 2001.

87. **Kanopka, A., Muhlemann, O., Akusjarvi, G.** Inhibition by SR proteins of splicing of a regulated adenovirus pre-mRNA. Nature **381**:535-538. 1996.

88. **Kataoka, T.** The caspase-8 modulator c-FLIP. Crit Rev Immunol **25**:31-58. 2005.

89. **Kerr, J.F., Wyllie, A.H., Currie, A.R.** Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer **26**:239-257. 1972.

90. **Kiel, C., Serrano, L.** The ubiquitin domain superfold: structure-based sequence alignments and characterization of binding epitopes. J Mol Biol **355**:821-844. 2006.

91. **Kim, K.S., Kim, G.S., Hwang, J.Y., Lee, H.J., Park, M.H., Kim, K.J., Jung, J., Cha, H.S., Shin, H.D., Kang, J.H. *et al***. Single nucleotide polymorphisms in bone turnover-related genes in Koreans: ethnic differences in linkage disequilibrium and haplotype. BMC Med Genet **8**:70. 2007.

92. **Kimchi-Sarfaty, C., Oh, J.M., Kim, I.W., Sauna, Z.E., Calcagno, A.M., Ambudkar, S.V., Gottesman, M.M.** A "silent" polymorphism in the MDR1 gene changes substrate specificity. Science **315**:525-528. 2007.

93. **Klein, U., Dalla-Favera, R.** Germinal centres: role in B-cell physiology and malignancy. Nat Rev Immunol **8**:22-33. 2008.

94. **Kohtz, J.D., Jamison, S.F., Will, C.L., Zuo, P., Luhrmann, R., Garcia-Blanco, M.A., Manley, J.L.** Protein-protein interactions and 5'-splice-site recognition in mammalian mRNA precursors. Nature **368**:119-124. 1994.

95. **Konarska, M.M., Query, C.C.** Insights into the mechanisms of splicing: more lessons from the ribosome. Genes Dev **19**:2255-2260. 2005.

96. **Korkolopoulou, P., Goudopoulou, A., Voutsinas, G., Thomas-Tsagli, E., Kapralos, P., Patsouris, E., Saetta, A.A.** c-FLIP expression in bladder urothelial carcinomas: its role in resistance to Fas-mediated apoptosis and clinicopathologic correlations. Urology **63**:1198-1204. 2004.

97. **Korsmeyer, S.J.** Chromosomal translocations in lymphoid malignancies reveal novel proto-oncogenes. Annu Rev Immunol **10**:785-807. 1992.

98. **Krainer, A.R., Conway, G.C., Kozak, D.** The essential pre-mRNA splicing factor SF2 influences 5' splice site selection by activating proximal sites. Cell **62**:35-42. 1990.

99. **Krainer, A.R., Conway, G.C., Kozak, D.** Purification and characterization of pre-mRNA splicing factor SF2 from HeLa cells. Genes Dev **4**:1158-1171. 1990.

100. **Krainer, A.R., Mayeda, A., Kozak, D., Binns, G.** Functional expression of cloned human splicing factor SF2: homology to RNA-binding proteins, U1 70K, and Drosophila splicing regulators. Cell **66**:383-394. 1991.

101. **Kramer, A.** The structure and function of proteins involved in mammalian pre-mRNA splicing. Annu Rev Biochem **65**:367-409. 1996.

102. **Krammer, P.H.** CD95's deadly mission in the immune system. Nature **407**:789-795. 2000.

103. **Krammer, P.H., Arnold, R., Lavrik, I.N.** Life and death in peripheral T cells. Nat Rev Immunol **7**:532-542. 2007.

104. **Krueger, A., Schmitz, I., Baumann, S., Krammer, P.H., Kirchhoff, S.** Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. J Biol Chem **276**:20633-20640. 2001.

105. **Kuppers, R., Goossens, T., Klein, U.** The role of somatic hypermutation in the generation of deletions and duplications in human Ig V region genes and chromosomal translocations. Curr Top Microbiol Immunol **246**:193-198. 1999.

106. **Kuppers, R., Klein, U., Hansmann, M.L., Rajewsky, K.** Cellular origin of human B-cell lymphomas. N Engl J Med **341**:1520-1529. 1999.

107. **Ladd, A.N., Cooper, T.A.** Finding signals that regulate alternative splicing in the post-genomic era. Genome Biol **3**:reviews0008. 2002.

108. **Lamond, A.I., Spector, D.L.** Nuclear speckles: a model for nuclear organelles. Nat Rev Mol Cell Biol **4**:605-612. 2003.

109. **Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W. *et al***. Initial sequencing and analysis of the human genome. Nature **409**:860-921. 2001.

110. **Lazar, G., Schaal, T., Maniatis, T., Goodman, H.M.** Identification of a plant serine-arginine-rich protein similar to the mammalian splicing factor SF2/ASF. Proc Natl Acad Sci U S A **92**:7672-7676. 1995.

111. **Le Hir, H., Izaurralde, E., Maquat, L.E., Moore, M.J.** The spliceosome deposits multiple proteins 20-24 nucleotides upstream of mRNA exon-exon junctions. Embo J **19**:6860-6869. 2000.

112. **Le Hir, H., Gatfield, D., Izaurralde, E., Moore, M.J.** The exon-exon junction complex provides a binding platform for factors involved in mRNA export and nonsense-mediated mRNA decay. Embo J **20**:4987-4997. 2001.

113. **Lejeune, F., Maquat, L.E.** Mechanistic links between nonsense-mediated mRNA decay and pre-mRNA splicing in mammalian cells. Curr Opin Cell Biol **17**:309-315. 2005.

114. **Li, H., Bingham, P.M.** Arginine/serine-rich domains of the su(wa) and tra RNA processing regulators target proteins to a subnuclear compartment implicated in splicing. Cell **67**:335-342. 1991.

115. **Li, T., Evdokimov, E., Shen, R.F., Chao, C.C., Tekle, E., Wang, T., Stadtman, E.R., Yang, D.C., Chock, P.B.** Sumoylation of heterogeneous nuclear ribonucleoproteins, zinc finger proteins, and nuclear pore complex proteins: a proteomic analysis. Proc Natl Acad Sci U S A **101**:8551-8556. 2004.

116. **Li, X., Niu, T., Manley, J.L.** The RNA binding protein RNPS1 alleviates ASF/SF2 depletion-induced genomic instability. RNA **13**:2108-2115. 2007.

117. **Lim, L.P., Burge, C.B.** A computational analysis of sequence features involved in recognition of short introns. Proc Natl Acad Sci U S A **98**:11193-11198. 2001.

118. **Limpens, J., Stad, R., Vos, C., de Vlaam, C., de Jong, D., van Ommen, G.J., Schuuring, E., Kluin, P.M.** Lymphoma-associated translocation t(14;18) in blood B cells of normal individuals. Blood **85**:2528-2536. 1995.

119. **Liu, Z., Luyten, I., Bottomley, M.J., Messias, A.C., Houngninou-Molango, S., Sprangers, R., Zanier, K., Kramer, A., Sattler, M.** Structural basis for recognition of the intron branch site RNA by splicing factor 1. Science **294**:1098-1102. 2001.

120. **Lopato, S., Waigmann, E., Barta, A.** Characterization of a novel arginine/serine-rich splicing factor in Arabidopsis. Plant Cell **8**:2255-2264. 1996.

121. **Lorson, C.L., Hahnen, E., Androphy, E.J., Wirth, B.** A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. Proc Natl Acad Sci U S A **96**:6307-6311. 1999.

122. **Loyer, P., Trembley, J.H., Lahti, J.M., Kidd, V.J.** The RNP protein, RNPS1, associates with specific isoforms of the p34cdc2-related PITSLRE protein kinase in vivo. J Cell Sci **111 (Pt 11)**:1495-1506. 1998.

123. **Lykke-Andersen, J., Shu, M.D., Steitz, J.A.** Human Upf proteins target an mRNA for nonsense-mediated decay when bound downstream of a termination codon. Cell **103**:1121-1131. 2000.

124. **MacFarlane, M.** TRAIL-induced signalling and apoptosis. Toxicol Lett **139**:89-97. 2003.

125. **Manley, J.L., Tacke, R.** SR proteins and splicing control. Genes Dev **10**:1569-1579. 1996.

126. **Marafioti, T., Hummel, M., Anagnostopoulos, I., Foss, H.D., Huhn, D., Stein, H.** Classical Hodgkin's disease and follicular lymphoma originating from the same germinal center B cell. J Clin Oncol **17**:3804-3809. 1999.

127. **Martin, A., Schneider, S., Schwer, B.** Prp43 is an essential RNA-dependent ATPase required for release of lariat-intron from the spliceosome. J Biol Chem **277**:17743-17750. 2002.

128. **Martin, I., Bonnefoix, T., Roucard, C., Perron, P., Lajmanovich, A., Moine, A., Leroux, D., Sotto, J.J., Garban, F.** Role of autologous CD4+ T cell clones in human B non-Hodgkin's lymphoma: aborted activation and G1 blockade induced by cell-cell contact. Eur J Immunol **29**:3188-3195. 1999.

129. **Mayeda, A., Krainer, A.R.** Regulation of alternative pre-mRNA splicing by hnRNP A1 and splicing factor SF2. Cell **68**:365-375. 1992.

130. **Mayeda, A., Badolato, J., Kobayashi, R., Zhang, M.Q., Gardiner, E.M., Krainer, A.R.** Purification and characterization of human RNPS1: a general activator of pre-mRNA splicing. Embo J **18**:4560-4570. 1999.

131. **McCallum, S.A., Bazan, J.F., Merchant, M., Yin, J., Pan, B., de Sauvage, F.J., Fairbrother, W.J.** Structure of SAP18: a ubiquitin fold in histone deacetylase complex assembly. Biochemistry **45**:11974-11982. 2006.

132. **McDonnell, T.J., Deane, N., Platt, F.M., Nunez, G., Jaeger, U., McKearn, J.P., Korsmeyer, S.J.** bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. Cell **57**:79-88. 1989.

133. **McDonnell, T.J., Korsmeyer, S.J.** Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14; 18). Nature **349**:254-256. 1991.

134. **Mermoud, J.E., Cohen, P.T., Lamond, A.I.** Regulation of mammalian spliceosome assembly by a protein phosphorylation mechanism. Embo J **13**:5679-5688. 1994.

135. **Michaud, S., Reed, R.** A functional association between the 5' and 3' splice site is established in the earliest prespliceosome complex (E) in mammals. Genes Dev **7**:1008-1020. 1993.

136. **Micheau, O., Thome, M., Schneider, P., Holler, N., Tschopp, J., Nicholson, D.W., Briand, C., Grutter, M.G.** The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. J Biol Chem **277**:45162-45171. 2002.

137. **Misteli, T., Spector, D.L.** Protein phosphorylation and the nuclear organization of pre-mRNA splicing. Trends Cell Biol **7**:135-138. 1997.

138. **Modrek, B., Lee, C.** A genomic view of alternative splicing. Nat Genet **30**:13-19. 2002.

139. **Monani, U.R., Lorson, C.L., Parsons, D.W., Prior, T.W., Androphy, E.J., Burghes, A.H., McPherson, J.D.** A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2. Hum Mol Genet **8**:1177-1183. 1999.

140. **Nelissen, R.L., Will, C.L., van Venrooij, W.J., Luhrmann, R.** The association of the U1-specific 70K and C proteins with U1 snRNPs is mediated in part by common U snRNP proteins. Embo J **13**:4113-4125. 1994.

141. **Nicholson, D.W., Thornberry, N.A.** Caspases: killer proteases. Trends Biochem Sci **22**:299-306. 1997.

142. **Nikolakaki, E., Drosou, V., Sanidas, I., Peidis, P., Papamarcaki, T., Iakoucheva, L.M., Giannakouros, T.** RNA association or phosphorylation of the RS domain prevents aggregation of RS domain-containing proteins. Biochim Biophys Acta **1780**:214-225. 2008.

143. **Paces-Fessy, M., Boucher, D., Petit, E., Paute-Briand, S., Blanchet-Tournier, M.F.** The negative regulator of Gli, Suppressor of fused (Sufu), interacts with SAP18, Galectin3 and other nuclear proteins. Biochem J **378**:353-362. 2004.

144. **Padgett, R.A., Grabowski, P.J., Konarska, M.M., Seiler, S., Sharp, P.A.** Splicing of messenger RNA precursors. Annu Rev Biochem **55**:1119-1150. 1986.

145. **Parker, R., Sheth, U.** P bodies and the control of mRNA translation and degradation. Mol Cell **25**:635-646. 2007.

146. **Philipps, D., Celotto, A.M., Wang, Q.Q., Tarng, R.S., Graveley, B.R.** Arginine/serine repeats are sufficient to constitute a splicing activation domain. Nucleic Acids Res **31**:6502-6508. 2003.

147. **Pugh, D.J., Ab, E., Faro, A., Lutya, P.T., Hoffmann, E., Rees, D.J.** DWNN, a novel ubiquitin-like domain, implicates RBBP6 in mRNA processing and ubiquitin-like pathways. BMC Struct Biol **6**:1. 2006.

148. **Rappsilber, J., Ryder, U., Lamond, A.I., Mann, M.** Large-scale proteomic analysis of the human spliceosome. Genome Res **12**:1231-1245. 2002.

149. **Rasper, D.M., Vaillancourt, J.P., Hadano, S., Houtzager, V.M., Seiden, I., Keen, S.L., Tawa, P., Xanthoudakis, S., Nasir, J., Martindale, D. *et al***. Cell death attenuation by 'Usurpin', a mammalian DED-caspase homologue that precludes caspase-8 recruitment and activation by the CD-95 (Fas, APO-1) receptor complex. Cell Death Differ **5**:271-288. 1998.

150. **Reed, R.** Initial splice-site recognition and pairing during pre-mRNA splicing. Curr Opin Genet Dev **6**:215-220. 1996.

151. **Resch, A., Xing, Y., Modrek, B., Gorlick, M., Riley, R., Lee, C.** Assessing the impact of alternative splicing on domain interactions in the human proteome. J Proteome Res **3**:76-83. 2004.

152. **Rhode, B.M., Hartmuth, K., Westhof, E., Luhrmann, R.** Proximity of conserved U6 and U2 snRNA elements to the 5' splice site region in activated spliceosomes. Embo J **25**:2475-2486. 2006.

153. **Robberson, B.L., Cote, G.J., Berget, S.M.** Exon definition may facilitate splice site selection in RNAs with multiple exons. Mol Cell Biol **10**:84-94. 1990.

154. **Rooke, N., Markovtsov, V., Cagavi, E., Black, D.L.** Roles for SR proteins and hnRNP A1 in the regulation of c-src exon N1. Mol Cell Biol **23**:1874-1884. 2003.

155. **Roopra, A., Sharling, L., Wood, I.C., Briggs, T., Bachfischer, U., Paquette, A.J., Buckley, N.J.** Transcriptional repression by neuron-restrictive silencer factor is mediated via the Sin3-histone deacetylase complex. Mol Cell Biol **20**:2147-2157. 2000.

156. **Ropers, D., Ayadi, L., Gattoni, R., Jacquenet, S., Damier, L., Branlant, C., Stevenin, J.** Differential effects of the SR proteins 9G8, SC35, ASF/SF2, and SRp40 on the utilization of the A1 to A5 splicing sites of HIV-1 RNA. J Biol Chem **279**:29963-29973. 2004.

157. **Roscigno, R.F., Garcia-Blanco, M.A.** SR proteins escort the U4/U6.U5 tri-snRNP to the spliceosome. RNA **1**:692-706. 1995.

158. **Roth, M.B., Zahler, A.M., Stolk, J.A.** A conserved family of nuclear phosphoproteins localized to sites of polymerase II transcription. J Cell Biol **115**:587-596. 1991.

159. **Roth, M.J., Schwartzberg, P., Tanese, N., Goff, S.P.** Analysis of mutations in the integration function of Moloney murine leukemia virus: effects on DNA binding and cutting. J Virol **64**:4709-4717. 1990.

160. **Rutz, B., Seraphin, B.** Transient interaction of BBP/ScSF1 and Mud2 with the splicing machinery affects the kinetics of spliceosome assembly. RNA **5**:819-831. 1999.

161. **Sahara, S., Aoto, M., Eguchi, Y., Imamoto, N., Yoneda, Y., Tsujimoto, Y.** Acinus is a caspase-3-activated protein required for apoptotic chromatin condensation. Nature **401**:168-173. 1999.

162. **Saitoh, N., Spahr, C.S., Patterson, S.D., Bubulya, P., Neuwald, A.F., Spector, D.L.** Proteomic analysis of interchromatin granule clusters. Mol Biol Cell **15**:3876-3890. 2004.

163. **Sakashita, E., Tatsumi, S., Werner, D., Endo, H., Mayeda, A.** Human RNPS1 and its associated factors: a versatile alternative pre-mRNA splicing regulator in vivo. Mol Cell Biol **24**:1174-1187. 2004.

164. **Sanford, J.R., Longman, D., Caceres, J.F.** Multiple roles of the SR protein family in splicing regulation. Prog Mol Subcell Biol **31**:33-58. 2003.

165. **Sanford, J.R., Ellis, J., Caceres, J.F.** Multiple roles of arginine/serine-rich splicing factors in RNA processing. Biochem Soc Trans **33**:443-446. 2005.

166. **Sawa, H., Abelson, J.** Evidence for a base-pairing interaction between U6 small nuclear RNA and 5' splice site during the splicing reaction in yeast. Proc Natl Acad Sci U S A **89**:11269-11273. 1992.

167. **Sawa, H., Shimura, Y.** Association of U6 snRNA with the 5'-splice site region of pre-mRNA in the spliceosome. Genes Dev **6**:244-254. 1992.

168. **Schaal, T.D., Maniatis, T.** Selection and characterization of pre-mRNA splicing enhancers: identification of novel SR protein-specific enhancer sequences. Mol Cell Biol **19**:1705-1719. 1999.

169. **Schulze-Osthoff, K., Ferrari, D., Los, M., Wesselborg, S., Peter, M.E.** Apoptosis signaling by death receptors. Eur J Biochem **254**:439-459. 1998.

170. **Schwerk, C., Prasad, J., Degenhardt, K., Erdjument-Bromage, H., White, E., Tempst, P., Kidd, V.J., Manley, J.L., Lahti, J.M., Reinberg, D.** ASAP, a novel protein complex involved in RNA processing and apoptosis. Mol Cell Biol **23**:2981-2990. 2003.

171. **Schwerk, C., Schulze-Osthoff, K.** Regulation of apoptosis by alternative pre-mRNA splicing. Mol Cell **19**:1-13. 2005.

172. **Shastry, B.S.** SNPs in disease gene mapping, medicinal drug development and evolution. J Hum Genet **52**:871-880. 2007.

173. **Shen, H., Kan, J.L., Ghigna, C., Biamonti, G., Green, M.R.** A single polypyrimidine tract binding protein (PTB) binding site mediates splicing inhibition at mouse IgM exons M1 and M2. RNA **10**:787-794. 2004.

174. **Shen, H., Kan, J.L., Green, M.R.** Arginine-serine-rich domains bound at splicing enhancers contact the branchpoint to promote prespliceosome assembly. Mol Cell **13**:367-376. 2004.

175. **Shen, H., Green, M.R.** RS domains contact splicing signals and promote splicing by a common mechanism in yeast through humans. Genes Dev **20**:1755-1765. 2006.

176. **Shu, H.B., Halpin, D.R., Goeddel, D.V.** Casper is a FADD- and caspase-related inducer of apoptosis. Immunity **6**:751-763. 1997.

177. **Siegel, R.M., Martin, D.A., Zheng, L., Ng, S.Y., Bertin, J., Cohen, J., Lenardo, M.J.** Death-effector filaments: novel cytoplasmic structures that recruit caspases and trigger apoptosis. J Cell Biol **141**:1243-1253. 1998.

178. **Skibola, C.F., Nieters, A., Bracci, P.M., Curry, J.D., Agana, L., Skibola, D.R., Hubbard, A., Becker, N., Smith, M.T., Holly, E.A.** A functional TNFRSF5 gene variant is associated with risk of lymphoma. Blood **111**:4348-4354. 2008.

179. **Sorek, R., Shamir, R., Ast, G.** How prevalent is functional alternative splicing in the human genome? Trends Genet **20**:68-71. 2004.

180. **Staknis, D., Reed, R.** Direct interactions between pre-mRNA and six U2 small nuclear ribonucleoproteins during spliceosome assembly. Mol Cell Biol **14**:2994-3005. 1994.

181. **Steele, L.P., Georgopoulos, N.T., Southgate, J., Selby, P.J., Trejdosiewicz, L.K.** Differential susceptibility to TRAIL of normal versus malignant human urothelial cells. Cell Death Differ **13**:1564-1576. 2006.

182. **Steinhoff, C., Franke, K.H., Golka, K., Thier, R., Romer, H.C., Rotzel, C., Ackermann, R., Schulz, W.A.** Glutathione transferase isozyme genotypes in patients with prostate and bladder carcinoma. Arch Toxicol **74**:521-526. 2000.

183. **Stephens, R.M., Schneider, T.D.** Features of spliceosome evolution and function inferred from an analysis of the information at human splice sites. J Mol Biol **228**:1124-1136. 1992.

184. **Sterner, D.A., Berget, S.M.** In vivo recognition of a vertebrate mini-exon as an exon-intron-exon unit. Mol Cell Biol **13**:2677-2687. 1993.

185. **Sugnet, C.W., Kent, W.J., Ares, M., Jr., Haussler, D.** Transcriptome and genome conservation of alternative splicing events in humans and mice. Pac Symp Biocomput 66-77. 2004.

186. **Sun, Z.W., Hampsey, M.** A general requirement for the Sin3-Rpd3 histone deacetylase complex in regulating silencing in Saccharomyces cerevisiae. Genetics **152**:921-932. 1999.

187. **Tacke, R., Tohyama, M., Ogawa, S., Manley, J.L.** Human Tra2 proteins are sequence-specific activators of pre-mRNA splicing. Cell **93**:139-148. 1998.

188. **Takahashi, H., Feuerhake, F., Kutok, J.L., Monti, S., Dal Cin, P., Neuberg, D., Aster, J.C., Shipp, M.A.** FAS death domain deletions and cellular FADD-like interleukin 1beta converting enzyme inhibitory protein (long) overexpression: alternative mechanisms for

deregulating the extrinsic apoptotic pathway in diffuse large B-cell lymphoma subtypes. Clin Cancer Res **12**:3265-3271. 2006.

189. **Tange, T.O., Shibuya, T., Jurica, M.S., Moore, M.J.** Biochemical analysis of the EJC reveals two new factors and a stable tetrameric protein core. RNA **11**:1869-1883. 2005.

190. **Tarn, W.Y., Steitz, J.A.** SR proteins can compensate for the loss of U1 snRNP functions in vitro. Genes Dev **8**:2704-2717. 1994.

191. **Tarn, W.Y., Steitz, J.A.** Modulation of 5' splice site choice in pre-messenger RNA by two distinct steps. Proc Natl Acad Sci U S A **92**:2504-2508. 1995.

192. **Thigpen, K.G.** International sequencing consortium. Environ Health Perspect **112**:A406. 2004.

193. **Tibbetts, M.D., Zheng, L., Lenardo, M.J.** The death effector domain protein family: regulators of cellular homeostasis. Nat Immunol **4**:404-409. 2003.

194. **Tran, Q., Roesser, J.R.** SRp55 is a regulator of calcitonin/CGRP alternative RNA splicing. Biochemistry **42**:951-957. 2003.

195. **Trembley, J.H., Tatsumi, S., Sakashita, E., Loyer, P., Slaughter, C.A., Suzuki, H., Endo, H., Kidd, V.J., Mayeda, A.** Activation of pre-mRNA splicing by human RNPS1 is regulated by CK2 phosphorylation. Mol Cell Biol **25**:1446-1457. 2005.

196. **Tsujimoto, Y., Finger, L.R., Yunis, J., Nowell, P.C., Croce, C.M.** Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. Science **226**:1097-1099. 1984.

197. **Ueffing, N., Keil, E., Freund, C., Kuhne, R., Schulze-Osthoff, K., Schmitz, I.** Mutational analyses of c-FLIPR, the only murine short FLIP isoform, reveal requirements for DISC recruitment. Cell Death Differ **15**:773-782. 2008.

198. **Umen, J.G., Guthrie, C.** The second catalytic step of pre-mRNA splicing. RNA **1**:869-885. 1995.

199. **Valcarcel, J., Gaur, R.K., Singh, R., Green, M.R.** Interaction of U2AF65 RS region with pre-mRNA branch point and promotion of base pairing with U2 snRNA. Science **273**:1706-1709. 1996.

200. **van Eijk, M., Defrance, T., Hennino, A., de Groot, C.** Death-receptor contribution to the germinal-center reaction. Trends Immunol **22**:677-682. 2001.

201. **Velazquez-Dones, A., Hagopian, J.C., Ma, C.T., Zhong, X.Y., Zhou, H., Ghosh, G., Fu, X.D., Adams, J.A.** Mass spectrometric and kinetic analysis of ASF/SF2 phosphorylation by SRPK1 and Clk/Sty. J Biol Chem **280**:41761-41768. 2005.

202. **Vucetic, Z., Zhang, Z., Zhao, J., Wang, F., Soprano, K.J., Soprano, D.R.** Acinus-S' represses retinoic acid receptor (RAR)-regulated gene expression through interaction with the B domains of RARs. Mol Cell Biol **28**:2549-2558. 2008.

203. **Wang, Z., Burge, C.B.** Splicing regulation: from a parts list of regulatory elements to an integrated splicing code. RNA **14**:802-813. 2008.

204. **Weiner, A.M.** mRNA splicing and autocatalytic introns: distant cousins or the products of chemical determinism? Cell **72**:161-164. 1993.

205. **Wiley, S.R., Schooley, K., Smolak, P.J., Din, W.S., Huang, C.P., Nicholl, J.K., Sutherland, G.R., Smith, T.D., Rauch, C., Smith, C.A. *et al*.** Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity **3**:673-682. 1995.

206. **Wu, J.Y., Maniatis, T.** Specific interactions between proteins implicated in splice site selection and regulated alternative splicing. Cell **75**:1061-1070. 1993.

207. **Wu, J.Y., Tang, H., Havlioglu, N.** Alternative pre-mRNA splicing and regulation of programmed cell death. Prog Mol Subcell Biol **31**:153-185. 2003.

208. **Wyllie, A.H., Kerr, J.F., Currie, A.R.** Cell death: the significance of apoptosis. Int Rev Cytol **68**:251-306. 1980.

209. **Xing, Y., Lee, C.** Evidence of functional selection pressure for alternative splicing events that accelerate evolution of protein subsequences. Proc Natl Acad Sci U S A **102**:13526-13531. 2005.

210. **Yang, J.K.** FLIP as an anti-cancer therapeutic target. Yonsei Med J **49**:19-27. 2008.

211. **Zahler, A.M., Lane, W.S., Stolk, J.A., Roth, M.B.** SR proteins: a conserved family of pre-mRNA splicing factors. Genes Dev **6**:837-847. 1992.

212. **Zahler, A.M., Neugebauer, K.M., Lane, W.S., Roth, M.B.** Distinct functions of SR proteins in alternative pre-mRNA splicing. Science **260**:219-222. 1993.

213. **Zahler, A.M., Roth, M.B.** Distinct functions of SR proteins in recruitment of U1 small nuclear ribonucleoprotein to alternative 5' splice sites. Proc Natl Acad Sci U S A **92**:2642-2646. 1995.

214. **Zahler, A.M.** Alternative splicing in C. elegans. WormBook 1-13. 2005.

215. **Zamore, P.D., Patton, J.G., Green, M.R.** Cloning and domain structure of the mammalian splicing factor U2AF. Nature **355**:609-614. 1992.

216. **Zhang, W.J., Wu, J.Y.** Functional properties of p54, a novel SR protein active in constitutive and alternative splicing. Mol Cell Biol **16**:5400-5408. 1996.

217. **Zhang, Y., Iratni, R., Erdjument-Bromage, H., Tempst, P., Reinberg, D.** Histone deacetylases and SAP18, a novel polypeptide, are components of a human Sin3 complex. Cell **89**:357-364. 1997.

218. **Zhou, Z., Licklider, L.J., Gygi, S.P., Reed, R.** Comprehensive proteomic analysis of the human spliceosome. Nature **419**:182-185. 2002.

219. **Zuo, P., Maniatis, T.** The splicing factor U2AF35 mediates critical protein-protein interactions in constitutive and enhancer-dependent splicing. Genes Dev **10**:1356-1368. 1996.

8. CURRICULUM VITAE

KUSUM KUMARI SINGH
Institute of Molecular Medicine, Duesseldorf, Germany
Phone: +49-17663131181; Email: kusum.singh@uni-duesseldorf.de

PERSONAL INFORMATION

Date of Birth	:	06.10.1979
Place of Birth	:	Samastipur (India)
Gender	:	Female
Marital Status	:	Single
Nationality	:	Indian
Personal email:		kusi79in@gmail.com

EDUCATIONAL QUALIFICATION

- **Ph.D student**, under the supervision of Prof. Klaus Schulze-Osthoff, Institute of Molecular Medicine, University of Duesseldorf, GERMANY. April 2005 till date.

- **Diploma** in Microbiology, **DSER** (Diplome superieur d'etudes et de Recherches), from Institute Jules Guyot, University of Bourgogne, Dijon, FRANCE. 2002-2003; with Grade A (tres honorable).

- **Masters** in Biotechnology, from Deen Dayal Upadhyay Gorakhpur University, Uttar Pradesh, INDIA. 2000-2002; with first class.

- **Bachelors** in Biotechnology, from St. Xavier's College Ahmedabad, University of Gujarat, INDIA. 1997-2000; with first class.

- All Inida Senior School Certificate Examination (**12th**), from Central Board of Secondary Education. 1997; with first class.

- All Inida Seconday School Examination (**10th**), from Central Board of Secondary Education. 1995; with first class.

PROJECT PROFILES

- **Post-transcriptional and post-translational modifications in apoptosis.**
  Institute of Molecular Medicine, Heinrich-Heine University of Duesseldorf, Duesseldorf, GERMANY, April 2005 - Present
  Guide: Prof. Dr. Klaus Schulze-Osthoff

- **Functional characterization of aldehyde dehydrogenase genes from *Arabidopsis thaliana* and identification of their substrates.**
  Institute of Molecular Physiology and Biotechnology of Plants, University of Bonn, Bonn, GERMANY, July 2003 - March 2005
  Guide: Prof. D. Bartels.

- **Contribution of different species of *Pythium* as biocontrol agent against *Botyris cinerea* pathogen of the Vineyards.**
  Institute Jules Guyot, University of Bourgogne, Dijon, FRANCE, November 2002 - December 2003
  Guide: Prof. Bernard Paul

LIST OF PUBLICATIONS

- Singh, K. K., Erkelenz, S., Schaal, H., Schulze-Osthoff, K., Schwerk, C. Human SAP18 mediates assembly of a splicing-regulatory multiprotein complex via its ubiquitin-like fold. Under preparation (2008).
- Ueffing, N.*, Singh, K. K.*, Christians, A., Feller, A.C., Fend, F., Heikaus, S., Marx, A., Zotz, R.B., Schulz, W.A., Schulze-Osthoff, K., Schmitz, I., Schwerk, C. A SNP in the cellular FLICE-inhibitory protein (c-FLIP) gene determines protein isoform production and is associated with risk of follicular lymphoma in humans. submitted (2008). * equal first authorship.
- Singh, K. K., Mathew, R., Emmanuel, M.I., Bernard, P. ITS region of the rDNA of *Pythium rhizosacchurum* sp Nov., isolated from sugarcane roots: taxonomy and comparison with related species. FEMS Microbiology Letters. 2003; (221), 233-236.
- Mathew, R., Singh, K. K., Bernard, P. *Pythium campanulatum* sp. Nov., isolated from rizosphere of maize, its taxonomy, ITS region of rDNA, and comparison with related species. FEMS Microbiology Letters. 2003; (226), 9-14.

ABSTRACT/MEETING/POSTERS PRESENTED

- November 2005: Autumn session of the SFB-612 and GRK-1089 (04.-05.11.2005). Wermelskirchen, Germany.
  Poster presentation: “ASAP complexes are involved in RNA processing and Apoptosis”. Singh, K. K., Schwerk, C., Schulze-Osthoff, K.
- June 2006: Spring Workshop of the GRK 1089 (09.-10.06.2006), Bensberg, Germany.
  Talk and Poster presentation: "Alternative splicing and cardiovascular diseases". K. K. Singh
- December 2006: Autumn session of the SFB-612 and GRK-1089 (11.-12.12.2006), Wermelskirchen, Germany.
  Poster presentation: "Characterization of ASAP complexes in RNA processing and Apoptosis”. Singh, K. K., Schwerk, C., Schulze-Osthoff, K.
- June 2007: Spring workshop of the GRK 1089 (29.-30.06.2007). Muenster, Germany.
  Talk: "Splicing and Cardiovascular Diseases". K. K. Singh.
- October 2007: Autumn session of the SFB-612, GRK-1089 and SFB-688 (30.09.-2.10.2007). Kaiserswerth, Duesseldorf.

Poster presentation: “Serine/arginine-rich domains of the apoptosis and splicing-associated protein complex: Functional analysis of splice site selection”. K.K. Singh, C. Schwerk and K. Schulze-Osthoff

- June 2007: Symposium on “Day of scientific talent” at the Heinrich-Heine-University of Duesseldorf (22.07.2007).
  Poster presentation: “Role of serine/arginine domains of the ASAP complex in regulation of the HIV-1 alternative RNA splicing”. Singh, K. K., Erkelenz, S., Schaal, H., Schulze-Osthoff, K., Schwerk, C

- September 2007: Cell Death Meeting at Cold Spring Harbor Laboratory, NY, USA (26.-30.09.2007).
  Poster presentation: “Role of Serine/Arginine domains of the apoptosis and splicing-Associated Protein (ASAP) complex in regulation of HIV-1 alternative RNA splicing”. Singh, K. K., Erkelenz, S., Schaal, H., Schulze-Osthoff, K., Schwerk, C. Abstract book page 71.

Date and Place **Signature**

Erklärung

Die hier vorgelegte Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den

Kusum Kumari Singh