UNIVERSITY OF NOVA GORICA
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MODIFIED U1 RNAs AS SPLICING CORRECTORS IN HUMAN GENETIC DISORDERS

DISSERTATION

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ABSTRACT

The experimental work of this thesis was performed at the International Centre for Genetic Engineering and Biotechnology (ICGEB) in the Human Molecular Genetics Group, under the scientific direction of Prof. Franco Pagani. The project was developed during the academic years 2014-2017.

Modified U1 RNAs, also named Exon Specific U1s (ExSpeU1s) represent a novel class of small RNA-based molecules that correct exons splicing defects. To evaluate their therapeutic potential focused on Familial Dysautonomia (FD), a rare autosomal recessive disorder characterized by progressive degeneration of the sensory and autonomic nervous system. More than 99% of patients are homozygous for the T to C transition in position 6 of the *IKBKAP* intron 20 (c.2204+6T>C). This substitution modifies the exon 20 5' splice site (5'ss) inducing exon skipping in a tissue-specific manner and reducing the total amount of IKAP protein. The molecular mechanisms underlying the *IKBKAP* mis-splicing are not completely clear and there are no effective treatments.

In this thesis, I investigated the therapeutic potential of ExSpeU1s and the role of *cis*- and *trans*-acting factors that regulates *IKBKAP* splicing. Using a splicing functional assay, I identified ExSpeU1s that bind to intron 20 sequences and rescue the exon 20 skipping defect. Interestingly, their rescue activity was modulated by several splicing factors and requires a critical exonic splicing enhancer element. Transfection experiment showed the involvement of both enhancing (TIA1, PTBP1 and PTB4) and inhibitory (SRSF3, hnRNPA1, FOX and FUS) splicing factors in *IKBKAP* splicing. To better evaluate the ExSpeU1s therapeutic efficacy, I transduced FD patient's fibroblasts with a lentiviral vector expressing the most active ExSpeU1. This resulted in a complete rescue of the exon skipping defect and improvement in IKAP protein expression. Most importantly, intraperitoneal delivery of ExSpeU1s by AAV9 into a transgenic mouse model, that recapitulates the tissue-specific mis-splicing seen in FD patients, corrected the aberrant splicing patterns in several tissues increasing the amount of the corresponding IKAP protein.

All together, these results identify novel regulatory splicing factors involved in the *IKBKAP* exon 20 regulation and provide the proof of principle that ExSpeU1s delivered *in vivo* by AAV vectors represent a novel therapeutic strategy for FD.

KEYWORDS:
Familial Dysautonomia, *IKBKAP*, IKAP, splicing, splicing defects, ExSpeU1, U1 snRNA, mouse model, AAV

IZVLEČEK

Modificirane U1 RNA molekule za popravljanje spajanja eksonov pri humanih dednih obolenjih

Eskperimentalno delo je bilo opravljeno na Mednarodnem centru za genetski inženiring in biotehnologijo (International Centre fo Genetic Enginnerring and Biotechnology, ICGEB, Trst, Italija) v skupini za Humano molekularno genetiko, pod mentorstvom prof. Franco Pagani. Projekt je bil izveden v akademskem obdobju 2014-2017.

Družinska disavtonomija (and. Familial Dysautonomia, FD), oz. sindrom Riley-Day, je redko avtosomalno recesivno dedno obolenje, ki je karakterizirano s progresivnim odmiranjem senzoričnega in avtonomnega živčnega sistema. Večinoma je prisotno v populaciji Ashkenazi Judov. Več kot 99% bolnikov ima hogozigotno obliko tranzicije T v C na mestu 6 introna 20 v genu *IKBKAP* (c.2204+6T>C). Ta nukleotidna zamenjava spremeni 5' spojitveno mesto (5'ss) eksona 20, kar povzroči tkivno-specifično izključitev eksona v prepisu gena in posledično zmanjšano količino proteina IKAP. Molekularni mehanizmi, ki povzročajo spremembe v spajanju eksonov niso popolnoma poznani. Za bolezen ne obstajajo učinkovita zdravljenja.

V doktorskem delu sem raziskovala terapevtski potencial nove skupine malih RNA molekul, ki se imenujejo ekson-specifične U1 molekule (ExSpeU1) in vpliv *cis*- in *trans*-delujočih faktorjev, ki regulirajo *IKBKAP* spajanje eksonov. Z uporabo funkcionalne metode spajanja sem določila ExSpeU1 molekule, ki se vežejo na zaporedja introna 20 in tako popravijo izključitev eksona 20 iz prepisa gena. Ta aktivnost je regulirana z več faktorji spajanja ter zaporedjem elementa eksonskega ojačevalca spajanja. Transfekcija celičnih linij je pokazala, da so v proces spajanja *IKBKAP* vključeni tako ojačevalci spajanja (TIA1, PTBP1 in PTB4) ter prav tako inhibitorni faktorji (SRSF3, hnRNPA1, FOX in FUS). Z namenom boljše določitve terapevtskega potenciala ExSpeU1, sem transducirala fibroblaste, ki so bili pridobljeni iz bolnika z FD ter uporabo lentivirusnih vektorjev, ki so izražali najbolj učinkovito ExSpeU1. To je povzročilo popolno popravilo izključitve eksona

in tako izboljšanje izražanja proteina IKAP. Intraperitonalni prenos ExSpeU1 z AAV9 vektorjem v transgenem mišjem modelu, ki predstavlja tkivno-specifično okvaro spajanja podobno kot pri bolnikih FD, je popravilo vzorce okvarjenega spajanja v večih tkivih in tako povečalo izražanje pripadajočega IKAP proteina.
Rezultati so določili nove regulatorne faktorje spajanja, ki so udeleženi v regulaciji spajanja *IKBKAP* eksona 20 in tako predstavljajo potrditev načela domneve, da ExSpeU1 molekule prenesene *in vivo* z AAV vektorji predstavljajo ustrezno terapevstko strategijo za bolnike FD.

KLJUČNE BESEDE:
Družinska disavtonomija, *IKBKAP*, IKAP, spajanje eksonov, napake pri spajanju, ExSpeU1, U1 snRNA, model miške, AAV

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PUBLICATIONS

Publication arising from this thesis was submitted to Human Molecular Genetics Journal:

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Other previous publications not related to this thesis work:

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Jablonska, O., K. Kowalewska, A. Popławska, K. Rajkowska, A. Boroń, D. Juchno, A. Leska, A. Pecio, B. Cejko and R. Kowalski (2015). "Analysis of testicular germ cell apoptosis in Cobitis loaches (Cobitidae, Teleostei) from diploid-tetraploid populations". Front. Mar. Sci. Conference Abstract: XV European Congress of Ichthyology. doi: 10.3389/conf.FMARS.2015.03.00169

§ These Authors contributed equally to this work.

ABBREVIATIONS

The standard abbreviations used in this thesis follow IUPAC rules. The abbreviations are explained also in the text when they are used for the first time.

A	adenosine
AAV	adeno-associated virus
AAV1	adeno-associated virus serotype 1
AAV2	adeno-associated virus serotype 2
AAV3	adeno-associated virus serotype 3
AAV4	adeno-associated virus serotype 4
AAV5	adeno-associated virus serotype 5
AAV6	adeno-associated virus serotype 6
AAV7	adeno-associated virus serotype 7
AAV8	adeno-associated virus serotype 8
AAV9	adeno-associated virus serotype 9
AAV10	adeno-associated virus serotype 10
AAVS1	adeno- associated virus integration site 1
AAVS2	adeno- associated virus integration site 2
AAVS3	adeno- associated virus integration site 3
AdV	adenovirus
ApoB	apolipoprotein B
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
BBB	blood–brain barrier
BBP	branchpoint binding protein
bp	base pair
BP	branch point
BPS	branch point sequence
C	cysteine
CBC	Cap Binding Complex
CBP 20	Cap Binding Protein 20
CBP 80	Cap Binding Protein 80

CD4	cluster of differentiation 4
cDNA	complementary DNA
CERES	composite exonic regulatory elements of splicing
CFTR	cystic fibrosis transmembrane conductance regulator gene
CIP	alkaline phosphatase, calf intestinal
CLIP	*in vivo* cross-linking immunoprecipitation
CMV	cytomegalovirus
CPSF	cleavage and polyadenylation specificity factor
CTL	cytotoxic T-lymphocytes
CTD	C-terminal domain
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphate (adenine (A), guanine (G), cytosine (C) and thymine (T))
DRG	dorsal root ganglion
Dscam	Down Syndrome cell adhesion molecule
dsDNA	double-stranded DNA
DSE	distal sequence element
DTT	dithiothreitol
EDA	fibronectin extra domain A
EDTA	ethylenediaminetetraacetic acid
EGCG	(-)-epigallocatechin gallate
eIF4A	eukaryotic translation initiation factor
ESE	exonic splicing enhancer
ESS	exonic splicing silencer
ExSpeU1	exon-specific U1 snRNA
F7	coagulation factor VII gene
F8	coagulation factor VIII gene
FBS	fetal bovine serum
FD	Familial Dysautonomia
FDA	Food and Drug Administration
FL	full-lenght
FN	fibronectin
FOX1	hexaribonucleotide-binding protein 1

FOX2	hexaribonucleotide-binding protein 2
GABRG2	GABA A receptor, γ2
GFP	green fluorescent protein
GpppRNA	7-methylguanosine RNA cap
gRNA	guide RNA
hnRNP	heterogeneous ribonuclear protein
hnRNPA1	heterogeneous nuclear ribonucleoprotein A1
hnRNPA2	heterogeneous nuclear ribonucleoprotein A2
hnRNPK	heterogeneous nuclear ribonucleoprotein K
HRP	horseradish peroxidase
HSF	Human Splicing Finder
HSV	herpes simplex virus
I	inosine
ICGEB	International Centre for Genetic Engineering and Biotechnology
IG	infectious genomes
IKAP	IKK complex-associated protein
IKBKAP	inhibitor of kappa light polypeptide
IP	intraperitoneal injection
IRES	internal ribosome entry site
ISE	intronic splicing enhancer
ISS	intronic splicing silencer
ITR	inverted terminal repeat
kDa	kilodalton
LB	Luria-Bertani medium
LV	lentiviral vectors
M	adenine or cytosine
m^7G	7-methylguanosine
m^7GpppG	7-methylguanosine cap
m^7GMP	7-methylguanosine monophosphate
$m^7GpppRNA$	mature 7-methylguanosine RNA cap
MDa	Megadalton / million Dalton
miRNA	microRNA

mRNA	messenger RNA
MU	mutant
Nab	neutralizing antibody
ncRNA	non-coding RNA
NMD	nonsense-mediated decay
NOVA1	neuro-oncological ventral antigen 1
NOVA2	neuro-oncological ventral antigen 2
ORF	open reading frame
PABP-2	poly(A)-binding protein II
PAGE	polyacrylamide gel electrophoresis
PBP	phosphatidylethanolamine binding protein 1
PCR	polymerase chain reaction
PEG	poly(ethylene glycol)
piRNA	piwi-interacting RNA
PND	postnatal day
Pol I	RNA polymerase I
Pol II	RNA polymerase II
Pol III	RNA polymerase III
pppRNA	triphosphate-terminated mRNA
PPT	polypyrimidine tract
pre-mRNA	precursor messenger RNA
pre-miRNA-18a	microRNA 18a precursor
pre-snRNA	precursor small nuclear RNA
Prp8	pre-mRNA processing factor 8
Prp22	pre-mRNA processing factor 22
Prp31	pre-mRNA processing factor 31
PS	phosphatidylserine
PSE	proximal sequence element
PTB	polypyrimidine tract binding protein
PTC	premature translation-termination codons
PTF	proximal sequence element-binding transcription factor
qPCR	quantitative polymerase chain reaction / real-time PCR
R	adenine or guanine

rAAV	recombinant adeno-associated virus
RBD	RNA binding domain
RBMY	RNA binding motif, Y-linked
RE	response element
RNA	ribonucleic acid
RNP	ribonucleoprotein domain
RNU1	U1 snRNA gene
RRM	RNA recognition motif
rRNA	ribosomal RNA
RS	arginine/serine dipeptide-rich motif
RT-PCR	reverse transcription polymerase chain reaction
RV	retrovirus
SAM68	Src-associated in mitosis 68 KDa Protein
SD	standard deviation
SELEX	Systematic evolution of ligands by exponential enrichment
SF2/ASF	pre-mRNA-splicing factor SF2/alternative splicing factor 1
siRNA	small interfering RNA
Sm	Smith protein
SMA	spinal muscular atrophy
SMN	survival motor neuron
SNAPc	small nuclear RNA activating complex polypeptide 1
snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoprotein particles
snoRNA	small nucleolar RNA
SR	serine/arginine-rich protein
SRE	splicing regulatory element
SRp20	serine/arginine-rich protein of 20 kDa
SRp40	serine/arginine-rich protein of 40 kDa
SRp55	serine/arginine-rich protein of 55 kDa
SRp75	serine/arginine-rich protein of 75 kDa
SRp30c	serine/arginine-rich protein 30c
SRrp35	serine/arginine-rich repressor protein of 35 kDa
SRSF1	serine/arginine-rich splicing factor 1

SRSF2	serine/arginine-rich splicing factor 2
SRSF3	serine/arginine-rich splicing factor 3
SRSF4	serine/arginine-rich splicing factor 4
SRSF5	serine/arginine-rich splicing factor 5
SRSF6	serine/arginine-rich splicing factor 6
SRSF7	serine/arginine-rich splicing factor 7
SRSF9	serine/arginine-rich splicing factor 9
SRSF12	serine/arginine-rich splicing factor 12
ss	splice site
ssDNA	single-stranded DNA
TBE	tris-borate-EDTA
TBP	TATA-box binding protein
TER	transesterification reaction
TFIIA	transcription factor II A
TFIIB	transcription factor II B
TFIIC	transcription factor II C
TFIID	transcription factor II D
TIA1	cytotoxic granule-associated RNA binding protein
TOP	terminal oligopyrimidine tract
tra2-beta	transformer-2 protein homolog beta
tRNA	transfer RNA
U	unit
U	uracyl
U2AF	U2 snRNP auxiliary factor
UBF1	upstream binding factor
UCF	upstream control element
VP1	viral capsid protein 1
VP2	viral capsid protein 2
VP3	viral capsid protein 3
WT/wt	wild-type
Y	cytosine or thymine
(Y)n	polypyrimidine tract

1. INTRODUCTION

1.1. RNA processing and gene expression

The concept of gene expression refers to genes being activated and producing a product. The product could be an enzyme, a structural protein, or a control molecule. It is a very complex process leading to the formation of the mature, functional ribonucleic acid (RNA) molecules - protein-coding (messenger RNAs (mRNA), non-coding RNAs (ncRNA)), participating in transcription (e.g. 7SK RNAs), in RNA processing (small nuclear RNAs (snRNA) and small nucleolar RNAs (snoRNA)) in translation (ribosomal (rRNA) and transfer RNAs (tRNA)) or in gene regulation (microRNAs (miRNA), small interfering RNAs (siRNA), piwi-interacting RNAs (piRNA)) (Wen, Mohammed et al. 2014). Studies of gene expression usually measure the production of mRNAs. Most mechanisms that control gene expression do so by controlling transcription, the synthesis of mRNA. Precursor messenger RNAs (pre-mRNAs) are transcribed by the RNA polymerase II

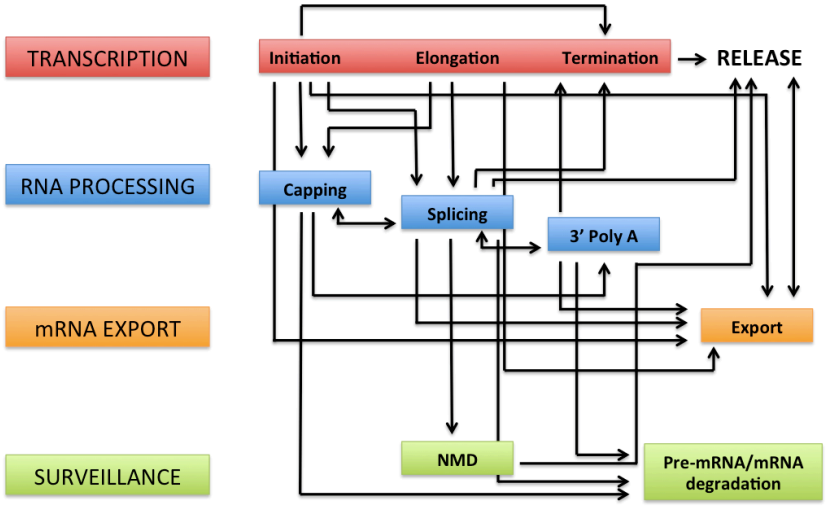

Figure 1.1. A complex network of coupled interactions in gene expression.

The main steps are indicated on the left and each stage in transcription is shown along the top in the red box. "Release" indicates release of the mature mRNA from the site of transcription. The steps of splicing are part of the RNA processing but they are also linked with other step, such as transcription, nonsense mediated decay (NMD), mRNA export and degradation. Black arrows indicate physical or functional interactions between components of gene expression machine. [Figure adapted from (Maniatis and Reed 2002)]

(Pol II) in the nucleus and subsequently are processed through several dynamic post-transcriptional events, like 5'-end capping, introns removal and exons splicing and the 3'-end processing and polyadenylation. All this in order to achieve different correctly processed and stable mature mRNAs (Figure 1.1). Complete mature mRNA is released from the nucleus to the cytoplasm, where it serves as a template for protein synthesis - translation (Maniatis and Reed 2002).

In eukaryotic organisms there are three types of DNA-dependent RNA polymerases. All of them are described as large protein complexes consisting from 8 to 12 subunits.

- **RNA polymerase I (Pol I)** - insensitive to α-amanitine (the most potent toxin present in fungi, binds strongly to RNA polymerase II, causing blocking elongation of RNA during synthesis), located in the nucleolus. Pol I leads transcription of all rRNA genes with the exception of 5S rRNA. Its activity requires the presence of Mg^{2+} ions and transcription factors like: upstream binding factor (UBF1) and upstream control element (UCF). Recognizes the promoters that are present upstream from the transcription start site (Moss, Stefanovsky et al. 2006);
- **RNA polymerase II (Pol II)** - very sensitive to α-amanitine. It is located in nucleoplasm and is responsible for the synthesis of messenger RNA and most of snRNA. It requires the presence of Mg^{2+} ions. The promoters of Pol II are composed of a core (TATA box - binding site for Transcription factor II D (TFIID)) and additional regulatory sequences, eg. CAAT sequence, enhancers and response elements (RE elements) (Woychik and Hampsey 2002);
- **RNA polymerase III (Pol III)** - sensitive to high concentrations of α-amanitine. Located in nucleoplasm, transcribes 5S rRNA genes, tRNA genes and snRNAs. It requires the presence of Mn^{2+} ions and general transcription factors like: transcription factor IIIA (TFIIIA), TFIIIB and TFIIIC forming pre-initiation complex. Genes transcribed by Pol III can be classified into three promoter types. First type requires transcription factors TFIIIA, TFIIIB and TFIIIC and promoter is located in 5S rRNA genes. Second type of promoters requires TFIIIB and TFIIIC, when the third one, requires snRNA activating complex polypeptide (SNAPc) and TFIIIB (Borchert, Lanier et al. 2006, Arimbasseri, Rijal et al. 2014).

All steps around the processes of the RNA maturation are well synchronized by the cellular machineries, and guarantee the right conversion of the information from the deoxyribonucleic acid (DNA) to the active RNA (Reed 2003, Kornblihtt, de la Mata et al. 2004, Bentley 2005, Schmid and Jensen 2008).

1.1.1. The 5'-end capping

All mRNAs have a cap-structure at their 5'-end added co-transcriptionally to the pre-mRNA, during the early stage of Pol II transcription (when the RNA polymerase has polymerized ~20-100 nucleotides). The cap-structure consists of a 7-methylguanosine (m^7G) linked to the first nucleotide through a 5'-5' triphosphate bridge and methyl groups (Figure 1.2). The cap is added by the enzyme guanylyl transferase, which may first interact with the RNA polymerase (Chiu, Ho et al. 2002).

The cap-structure interacts with two proteins in the nucleus - Cap Binding Protein 20 and 80 (CAP 20, CBP 80) (Izaurralde and Adam 1998, Kim, Yang et al. 2008). Together they are forming a Cap Binding Complex (CBC). It is known that CBP 20 binds to the cap-structure at the time of transcription (Lei, Krebber et al. 2001). The CBC influences splicing of the pre-mRNA and it may be concerned in transport into the cytoplasm of the pre-mRNA (Izaurralde and Adam 1998, Proudfoot, Furger et al. 2002). The CBC is, in addition, involved in the transport to the cytoplasm of various small nuclear ribonucleoparticles (snRNPs) (Sloan, Gleizes et al. 2016).

The capping process involves 3 steps: 5' triphosphate group of triphosphate-terminated mRNA (pppRNA), primary transcript, is converted to diphosphate-terminated mRNA (ppRNA) (Figure 1.2). The m^7G monophosphate (m^7GMP) is then linked to the RNA, through a 5'-5' binding, to make the capped RNA (GpppRNA). Finally, a methyltransferase methylates the 7 position of guanine to produce the mature cap ($m^7GpppRNA$) (Figure 1.2) (Chen, Walsh et al. 2005, Gu and Lima 2005, Lima 2014, Shuman 2015).

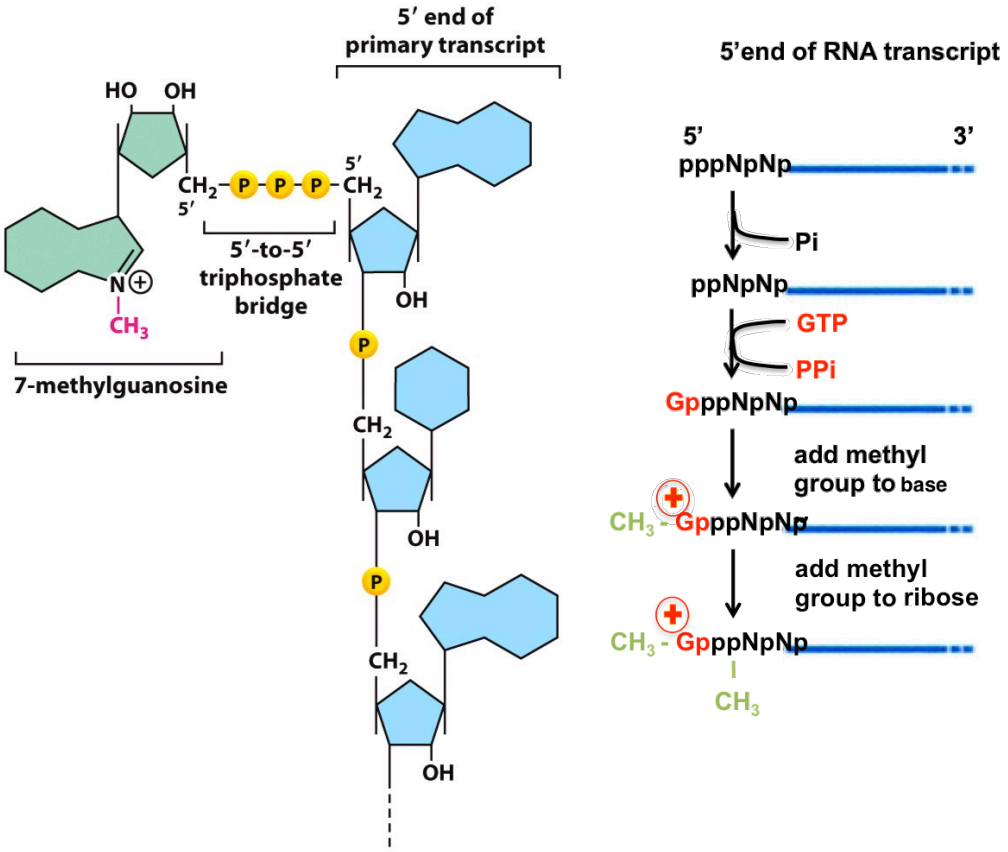

Figure 1.2. The 7-methyl guanine nucleotide forms the 5'mRNA cap.

Schematic representation of a bond between the 7-methylguanosine and 5'end of primary transcript. Schematic representation of the cap maturation steps. [Figure adapted from (Hayes 1964)]

It had been assumed that capping is a constitutive and efficient process for most mRNAs. However, existence of many factors that can stimulate capping of specific transcripts and the discovery of enzymes that are mediating the decay of partially processed RNA intermediates, suggest that capping is a dynamic and much more complex pathway (Chiu, Ho et al. 2002, Jiao, Xiang et al. 2010, Chang, Jiao et al. 2012, Jiao, Chang et al. 2013). The cap presence on the pre-mRNA is very important for the stability of this molecule, it prevents the degradation promoted by 5' - 3' exonucleases - protecting the mRNA (Tourrière, Chebli et al. 2002). The cap-structure is also involved in several aspects of pre-mRNA and mRNA metabolism, in most cases related to identifying the 5'-end of the RNA. In the cytoplasm, the cap-structure is important for the initiation and promoting of translation. The eukaryotic translation initiation factor (eIF4A) binds directly to the cap-structure (Lewis, Izaurralde et al. 1996, Gross, Moerke et al. 2003).

1.1.2. The 3'-end processing and polyadenylation

Mature eukaryotic mRNAs are generated in a series of processing steps from larger precursors - primary products of RNA Pol II in the cell nucleus. The 3'-end processing is one of those steps. All pre-mRNAs in eukaryotic organisms lose part of a sequence from their 3'-ends by an endonucleolytic cleavage. The next step is subsequent polyadenylation at the 3' end of the transcript (Figure 1.3).

The reaction of cleavage depends on sequences in the pre-mRNA - the hexanucleotide AAUAAA, located 10 - 30 nucleotides upstream of the cleavage site, stands out by its high degree of conservation. The cleavage and polyadenylation specificity factor (CPSF) binds specifically to the AAUAAA consensus sequence (Beaudoing, Freier et al. 2000), promoting cleavage at a 5'-CA-3' dinucleotide (Takagaki, Ryner et al. 1988, Lutz, Murthy et al. 1996). After the cleavage, polyadenylate polymerase adds a poly(A) tail composed of 200-250 adenine (A) residues. During this process there is intervention of poly(A)-binding protein II (PABP-2) that binds to the nascent poly(A) tail and plays a role during the elongation of poly(A) tails – this stabilizes and protects pre-mRNA from exonuclease digestion (Wahle 1991, Wahle, Lustig et al. 1993, Beaudoing, Freier et al. 2000, West, Gromak et al. 2004)

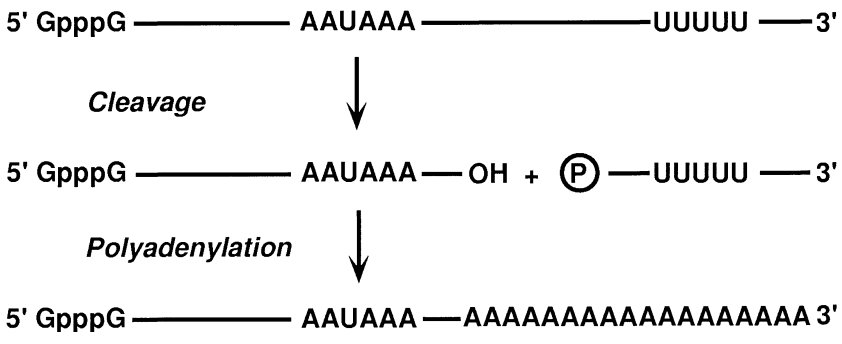

Figure 1.3. The two steps in 3P-processing.

Endonucleolytic cleavage between the AAUAAA sequence and the downstream element generates an upstream fragment terminating with 3P'OH and a downstream fragment starting with 5P' phosphate. The upstream fragment is polyadenylated, the downstream fragment is degraded. [Figure adapted from (Wahle and Keller 1996)]

The processing of pre-mRNA 3'-end has crucial functional importance in eukaryotes. Any disruption of this process has catastrophic effects on capacity of cell growth and viability (Zhao, Hyman et al. 1999). Firstly, 3'-end processing promotes the transport of mRNAs from the nucleus to the cytoplasm (Vinciguerra and Stutz 2004). Secondly, 3'-end processing promotes the stability of mRNAs – in the cytoplasm, mRNAs are degraded from the 3'-end first (Wickens, Anderson et al. 1997, Wilusz, Wormington et al. 2001, Wilusz and Wilusz 2004). The addition of the poly(A) tail and binding of PABP-2 prevents degradation in mammalian cells (Ford, Bagga et al. 1997). Thirdly, 3'-end processing intensifies the translation of mRNAs into proteins. The poly(A) tail and PABP-2 interact with the m^7GpppRNA at the 5'-end to promote translation (Sachs, Sarnow et al. 1997, Wickens, Anderson et al. 1997, Wilusz, Wormington et al. 2001, Chekanova and Belostotsky 2006).

Preiss and Hentze, showed that the presence of the poly(A) tail alone is sufficient to initiate efficient translation. However, the presence of both the poly(A) tail and the 5'-cap is the most favourable for translation (Preiss and Hentze 1998). Finally, 3'-end processing is largely coupled to the transcription and the splicing machineries (Hirose and Manley 2000, Maniatis and Reed 2002, Proudfoot, Furger et al. 2002, Zorio and Bentley 2004). Transcription factors and the C-terminal domain (CTD) of Pol II interact with 3′-end processing to help in the control of termination of the transcription. Essential for transcriptional termination is the proper poly(A) signal. Any alterations in these processes lead to improper polyadenylation and mRNA degradation (Proudfoot 2004).

1.1.3. Splicing

Splicing is an evolutionarily conserved mechanism, found in unicellular eukaryotes such as *S.cereviseae* as well as in complex metazoans (Ast 2004, Nilsen and Graveley 2010). The splicing reaction is an essential step occurring co-transcriptionally in the regulation of gene expression in eukaryotes. A typical

mammalian gene contains several relatively short coding sequences (exons) that are interrupted by multiple non-coding intervening sequences (introns) (Lander, Linton et al. 2001). The coding segments represent a minor portion of the genetic information, namely, no more than 1% of the entire genome. In order to generate mature transcripts introns must be precisely removed from the pre-mRNA and exons have to be joined together (Staley and Guthrie 1998). The translation machinery cannot discriminate introns from exons, which means that the introns have to be removed from the transcript before translation. This process is structurally and functionally associated with the nucleus and occurs through multiprotein complex named spliceosome (Maniatis and Reed 2002). Human genes typically have multiple introns and it has been estimated that at least 90% of them undergo alternative splicing. As a consequence of this event, a large number of mRNA transcripts can be generated from a single gene, increasing proteome diversity (Black 2003).

1.1.4. RNA editing

Some RNA molecules besides the aforementioned processing undergo less common process that involves changes in transcript. RNA editing is a particular process, involving chemical modifications, resulting with sequence of transcribed RNA not matching with DNA template (Benne, Van Den Burg et al. 1986). RNA editing is a modification at the RNA level without affecting the genomic information. There

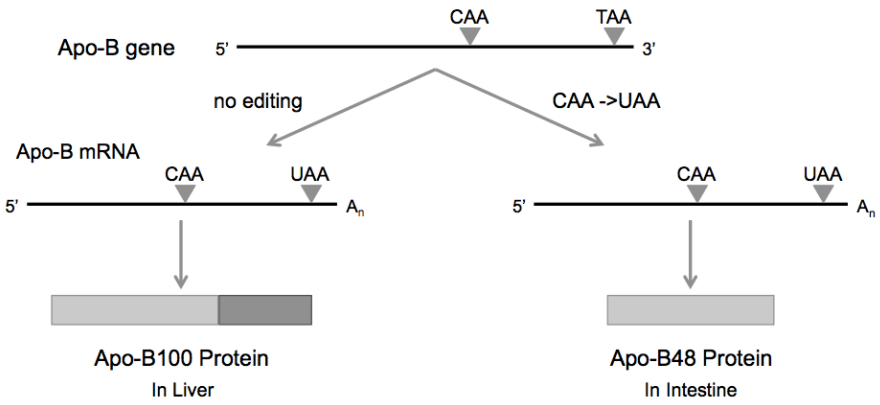

Figure 1.4. RNA editing of the Apo-B gene.

In mammals, the apo-B gene is expressed in hepatocytes and intestinal epithelial cells. In liver cells, its product is a 500 kD protein called Apo-B100 whereas in intestine cells its product is a smaller protein called Apo-B48. The Apo-B48 is synthesized from an mRNA whose sequence was edited by a specific enzyme. This enzyme changes a codon, CAA, in the middle of the original mRNA to the stop codon UAA, thereby causing early termination of the protein synthesis.

are two ways in which RNA can be edited: by substitution/conversion of base, or insertion/deletion. It occurs post-transcriptionally in different parts of the transcript, in the direction from the 3'-end to 5'-end. For example, one of the best known RNA editing process in mammalian cells, is deamination: conversion of the adenosine (A) into inosine (I) and the cytosine (C) into uracil (U) (Gott and Emeson 2000). Reaction is catalyzed by a family of enzymes called adenosine deaminases (Sommer, Köhler et al. 1991, Bass, Nishikura et al. 1997, Bass 2002). The importance of this process can be illustrated with the apolipoprotein B (Apo-B) case (Figure 1.4). In the intestine, mRNA undergoes, a tissue-specific C into U editing, which converts a CAA codon into a stop codon - UAA, leading to the formation of a shorter product - intestinal ApoB48 lipoproduct in comparison to ApoB100 protein, that is synthetized in the liver (Chen, Habib et al. 1987, Ashkenas 1997, Chan, Chang et al. 1997)

Insertion/deletion editing starts either post-transcriptionally or co-transcriptionally by creating a base-paired "anchor" duplex between pre-edited mRNA and guide RNA (gRNA) that directs the cleavage by editing endonuclease (Figure 1.5). In case

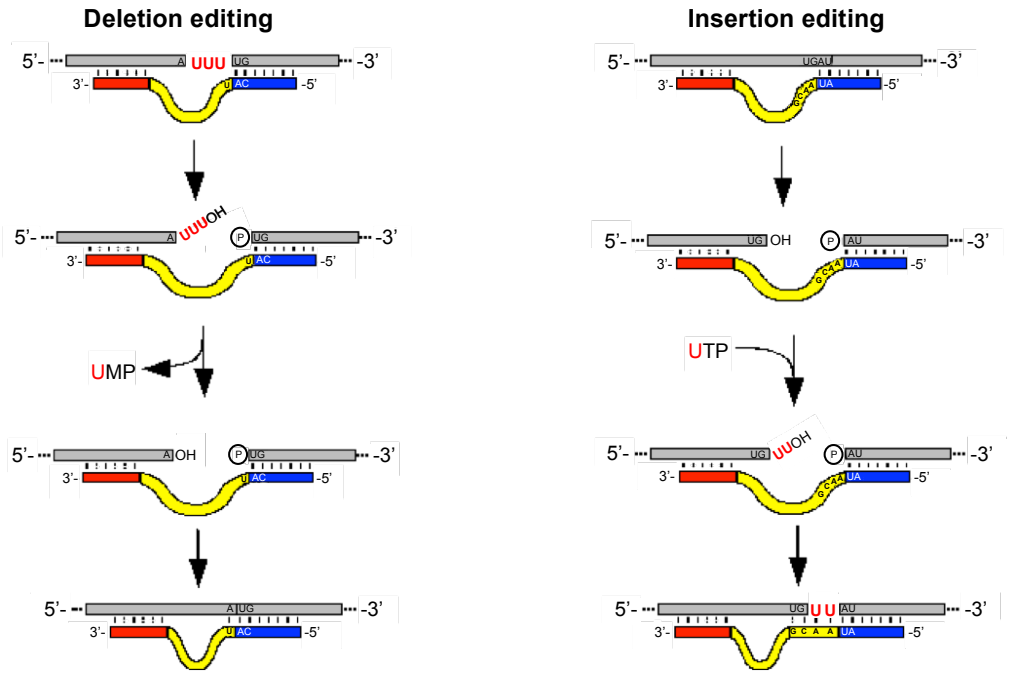

Figure 1.5. RNA editing by insertion/deletion mechanism.

Schematic representation of catalytic events in insertion and deletion editing. Pre-mRNAs (gray boxes) are edited 3' to 5' with each gRNA (red/yellow/blue boxes) specifying the editing of several sites. [Figure adapted from (Cruz-Reyes, Zhelonkina et al. 2001)]

of inserting the cleavage is acted upon terminal U-transferase (Frech and Simpson 1996), for deletion present is an U-specific exonuclease (Cruz-Reyes and Sollner-Webb 1996). Last step is ligation of the two mRNA fragments.

1.1.5. Degradation

The regulation of mRNA degradation and stability in the cytoplasm has been well characterized. This regulation is a critical step in changing the abundance of certain transcripts controlling various biological pathways (Raghavan and Bohjanen 2004, Keene 2010, Schoenberg and Maquat 2012).

Messenger RNA degradation is an important step in the regulation of gene expression: its rate varies more than 10-fold and can be modulated by endogenous or exogenous signals (Beelman and Parker 1995, van Hoof and Parker 1999, Tucker and Parker 2000, Mitchell and Tollervey 2001). In general, RNA is degraded at the end of its useful life, which is long for a ribosomal RNA but very short for excised introns or spacer fragments, and is closely regulated for most mRNA species. RNA molecules with defects in processing, folding, or assembly with proteins are identified and rapidly degraded by the surveillance machinery, like 5’ and 3’ exonucleases (Houseley and Tollervey 2009). The mRNA degradation occurs through distinct pathways, one primarily from the 5'-end of the mRNA and the second from the 3'-end. RNA exonucleases, which initiate degradation at the uncapped 5'-end of the nascent transcript, generated when the polyadenylation machinery cleaves the transcript, are involved in coupling polyadenylation and termination. A 3’ to 5’ exonuclease activity may quickly degrade the transcript back to the poly(A) signal (West, Gromak et al. 2004). During the course of mRNA production, specific RNA surveillance mechanisms operate to detect and eliminate defective pre-mRNA or mRNA (Maquat and Carmichael 2001). Frameshift or nonsense mutations, which result in the generation of premature translation-termination codons (PTCs) target the mRNA for efficient degradation by translation-coupled mechanism - a pathway known as a nonsense-mediated mRNA decay (NMD) in the cytoplasm (Hentze and Kulozik 1999, Brogna and Wen 2009).

1.2. Pre-mRNA processing

Eukaryotic pre-mRNAs are transcribed in the nucleus by RNA Pol II. The splicing reaction is a rapid and precise mechanism through which the introns are accurately removed in order to put the exons in the correct protein-reading frame (Roca, Krainer et al. 2013).

The first system used to study mRNA splicing and the structure of the pre-mRNA molecule was the late stage of adenovirus infection (Berget, Moore et al. 1977). The presence of introns was in fact discovered in the non-coding regions of the adenovirus (Berget, Moore et al. 1977, Chow, Gelinas et al. 1977). These studies have been soon after expanded to the entire eukaryotic world: Jeffreys and Flavell (Jeffreys and Flavell 1977) demonstrated a "large insert" in the coding sequence of the rabbit β-globin gene. Chambon and colleagues reported that the chicken ovalbumin gene contains seven coding sequences separated by six intervening sequences showing the exact positions where the coding sequences for ovalbumin mRNA are interrupted inside the genome (Breathnach, Benoist et al. 1978, Mandel, Breathnach et al. 1978). Moreover they found that the sequences at exon-intron boundaries carry common features, probably with the function of unique excision-ligation common points to all boundaries (Breathnach, Benoist et al. 1978). Interestingly, these consensus sequences were also conserved across vertebrates, plants and yeast suggesting that the splicing process is evolutionarily well conserved (Padgett, Grabowski et al. 1986, Nilsen and Graveley 2010).

This machinery has to work very accurately because even a single nucleotide change at exon joining will shift the reading frame, leading to abnormal proteins. To obtain this precision, the splicing machinery must efficiently recognize the intron-exon boundaries in the pre-mRNAs (Figure 1.6). The definition of the exons and the proper realization of the splicing reaction are mediated by a macromolecular machinery named spliceosome, which is composed of snRNAs and dozens of other pre-mRNA splicing factors and many associated proteins (Maniatis and Reed 2002, Rappsilber, Ryder et al. 2002, Wahl, Will et al. 2009).

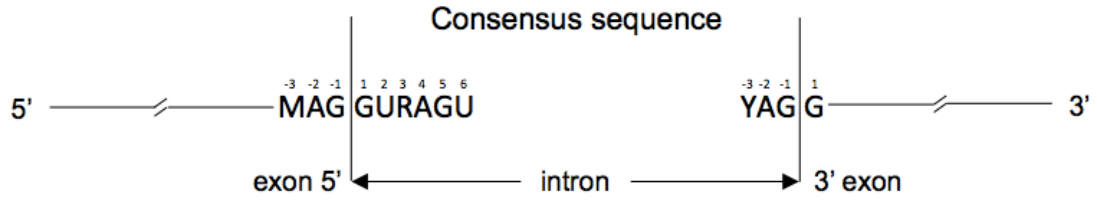

Figure 1.6. The consensus sequence for splicing.

Schematic representation of sequence at exon – intron borders. M – A or C; R – A or G; Y – C or T

In order to distinguish exons from introns, the spliceosomal machinery has to recognize several motifs that reside in proximity of the boundaries - sequences, which are known as 5' and 3' splice sites (ss). They exhibit a variable degree of conservation. Therefore the presence of auxiliary factors located on the sequence (*cis*-acting) and regulatory elements that recognize these sequences (*trans*-acting) are necessary for the proper recognition process (Cartegni, Chew et al. 2002).

The reaction occurs in two transesterification reactions (TER) (Figure 1.7) performed in the nucleus (Maniatis and Reed 2002). The components of the spliceosome complex have a dynamic and particular localization pattern, called the "speckled pattern". This pattern characterizes also the distribution of splicing factors around the cell (Spector, Fu et al. 1991, Neubauer, King et al. 1998). Splicing factors are cycling continuously between speckles and other nuclear localizations, such as transcription sites (Misteli, Caceres et al. 1997, Lamond and Spector 2003). Splicing factors are recruited from speckles to sites of transcription and when transcription is inhibited splicing factors accumulate in enlarged, rounded speckles. Nuclear speckles, can be called regulators of the pool of factors that are available to pre-mRNA splicing machinery (Misteli, Caceres et al. 1997).

In principle, the splicing process takes place in the nucleus however, some studies suggest that in particular cell types it can occur in other compartments (Meshorer and Misteli 2005), such as in rat neuronal dendrites (Glanzer, Miyashiro et al. 2005) or in human anucleate platelets (Denis, Tolley et al. 2005, Schwertz, Tolley et al. 2006).

1.2.1. The chemical reactions of splicing

Studying the chemistry of RNA splicing process, there are two noticeable consecutive S_N2-type transesterification steps, which are involving functional groups from three different regions in the pre-mRNA (Lamond 1993) (Figure 1.7). A bond is in effect transferred from one location to another. In order to initiate the splicing reaction conserved motifs are necessary: the GU dinucleotide at the 5'ss, the AG dinucleotide at the 3'ss (Figure 1.6), the A residue at the branch point (BP) and the polypyrimidine tract ((Y)n or PPT), both located upstream of the 3'ss (Moore and Sharp 1993).

The first step is a hydrophilic attack, the 2'-hydroxyl group of the A residue at BP attacks the phosphodiester bond at the 5'ss, leading to the cleavage of the 5'-exon from the intron and the concerted ligation of the intron 5'-end to the BP 2'-hydroxyl group in a lariat form: two reaction intermediates are then produced, a detached 5'-exon 1 and an intron-3' exon 2 fragment in a lariat configuration. A rearrangement of spliceosomal components must happen to allow the second transesterification reaction.

The second step occurs when the 3'-hydroxyl from the detached exon acts on the phosphate at 3'-end of the intron (Cech 1986, Moore and Sharp 1993). This step results in the ligation of two exons via a phosphodiester bond and the release of the intron in the form of a lariat. This lariat formation is debranched to give a linear intron. Afterwards, intron RNA is degraded in the nucleus, while snRNPs will be recycled (Konarska, Grabowski et al. 1985, Cech 1986, Chin and Pyle 1995).

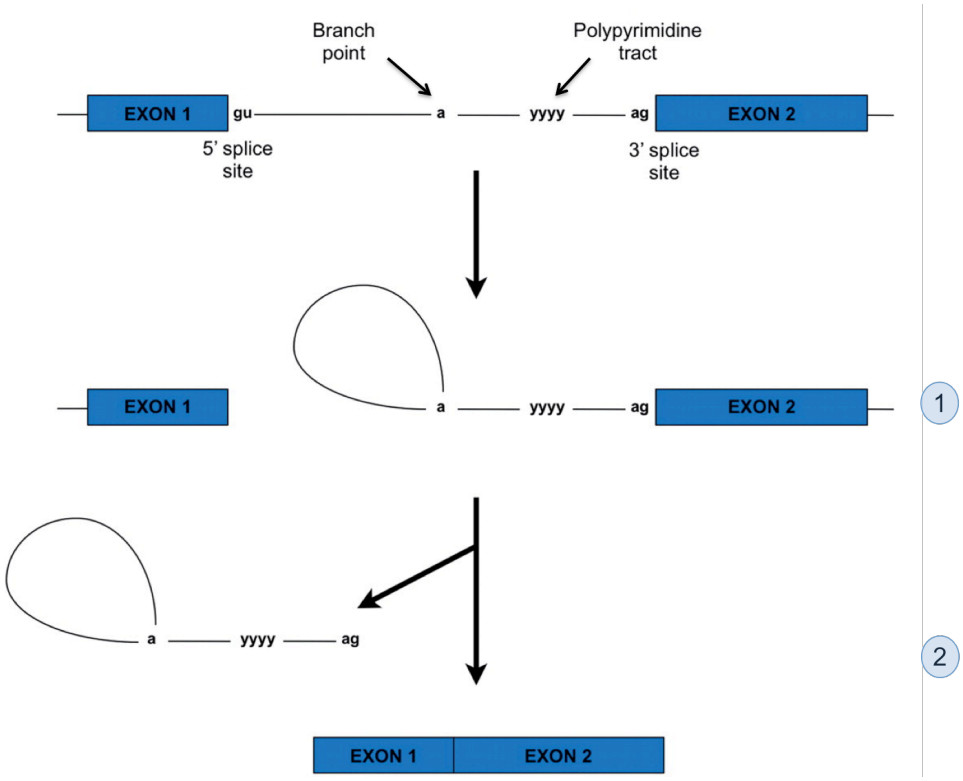

Figure 1.7. The basic splicing process.

The first transesterification reaction creates a lariat structure joined at the branch point. The second transesterification reaction releases the lariat intron and ligates the exons together. [Figure adapted from (Douglas and Wood 2011)]

1.2.2. The spliceosome

In the process of splicing involvs many proteins, forming a large conglomerate complex structure, called the spliceosome. It is estimated that the spliceosome, as a compact structure, contains more than 200 molecules of protein and five short (small) nuclear RNAs (Wu and Maniatis 1993, Schellenberg, Ritchie et al. 2008, Sperling, Azubel et al. 2008). The spliceosome is an organized and dynamic set of molecules in which there are protein-protein, protein-RNA and RNA-RNA interactions (Rappsilber, Ryder et al. 2002, Jurica and Moore 2003, Nicholls, Shields et al. 2004).

Among the components of the spliceosome, five uridine-rich (U) snRNPs – U1, U2, U4/U6 and U5, composed of snRNAs and proteins associated with them play a central role in the splicing of introns (Moore and Sharp 1993, Sperling, Azubel et al. 2008, Ritchie, Schellenberg et al. 2009). Uridine-rich snRNPs are quite abundant

in the nucleus, ranging from thirty thousand to one million copies per cell, depending on the snRNP (Baserga and Steitz 1993). Each U1, U2, U4/U6 and U5 snRNPs consist of one small stable snRNA (or two in case of U4/U6), a common set of seven Smith (Sm) proteins and a different number of complex-specific proteins. The number of these associated proteins varies greatly among the U snRNPs, from 3 only for U1 snRNP to more than 20 for U2 snRNP (Will and Luhrmann 2001, Fischer, Englbrecht et al. 2011). In addition, the human spliceosome interacts with numerous non-snRNPs proteins (from 150 to 300) (Lamm and Lamond 1993), such as heterogeneous nuclear RNPs (hnRNPs) and serine/arginine rich proteins (SR) (Guthrie and Patterson 1988, Steitz, Black et al. 1988, Bindereif and Green 1990, Lamond, Barabino et al. 1990).

The major spliceosome described above, containing the snRNAs U1, U2, U4, U5 and U6, is the dominant form in metazoans, plants, and fungi, and removes introns with GT-AG (as well as rarely AT-AC and GC-AG) boundaries. Another rare class of "non-canonical" introns with AT-AC (and rarely GT-AG (Sheth, Roca et al. 2006)) boundaries is excised by the minor spliceosome (Patel and Steitz 2003), which contains the snRNAs U11, U12, U4atac, U5, and U6atac (Patel and Bellini 2008). Just as in the major spliceosome, the minor spliceosome is present across most eukaryotic lineages and traces back to very early origins in the eukaryote evolution (Collins and Penny 2005, Lorković, Lehner et al. 2005, Russell, Charette et al. 2006). More recently it was found that the minor spliceosome can also act outside the nucleus and control cell proliferation (König, Matter et al. 2007).

The spliceosome assembly occurs in a stepwise manner, involving assembly/disassembly of different snRNPs and non-snRNP splicing factors on the pre-mRNA. In mammals, four distinct spliceosomal complexes, which form in the temporal order E, A, B and C, have been detected (Michaud and Reed 1991) (Figure 1.8). The E, A and B complexes contain unspliced RNAs, while the C complex comprise the catalytic product of the first transesterification reaction (exon 1 and lariat exon 2). The products of the second transesterification reaction are contained in two different complexes: the spliced exon is within complex D and the lariat intron in the I complex (Wahl, Will et al. 2009).

Complex E (Early)

The first step of spliceosomal assembly is the creation of complex E (early (E) complex or commitment complex). The 5' and 3'ss of an intron are recognized by the specific binding of the U1 snRNP and the protein U2 auxiliary factor (U2AF), respectively (Figure 1.8). The reaction can occur because of the base-pairing of the U1snRNA with the 5'ss in an adenosine triphosphate (ATP)-independent manner (Will, Rümpler et al. 1996). U1 snRNP associated proteins (U1-70k and U1-C) stabilize this transient interaction (Patel and Bellini 2008). The larger subunit U2AF 65 of the splicing factor U2AF recognizes the polypyrimidine tract at the 3'ss (Valcárcel, Gaur et al. 1996). The AG dinucleotide at the 3'ss interacts with the smaller U2AF35 subunit (Wu, Romfo et al. 1999). U2AF helps recruit U2 - auxiliary factor - to the branchpoint together with a branchpoint binding protein (Berglund, Abovich et al. 1998, Wu, Romfo et al. 1999, Schellenberg, Ritchie et al. 2008). Complex E contains the U2 snRNP as a component, which is essential for its formation (Yu, Shu et al. 1998, Das, Zhou et al. 2000, Dönmez, Hartmuth et al. 2004). Association of the U2 snRNP with the complex at this stage is weak; the underlying mechanism is not currently understood in detail. The pre-mRNA substrate is committed to the splicing pathway, that's why the splice sites are in close proximity (Michaud and Reed 1993, Kent and MacMillan 2002).

Complex A

The next to form is complex A (pre-spliceosome complex). In this complex, the U2 snRNP is bound stably by base-pairing to the branchpoint sequence, replacing the BBP factor and binds to BP in an ATP-dependent manner (Figure 1.8). The U2-associated proteins of the SF3A/B complexes are bound to the upstream site of the branchpoint and further stabilized the base-pairing interaction (Gozani, Feld et al. 1996). U2 snRNA base-pairs with the branchpoint sequences and, in so doing, bulges out the branchpoint adenosine, enhancing the first nucleophilic attack. This formation serves as a binding platform for the trimer of snRNPs U4/U6 and U5, which ends in the formation of complex B (assembled spliceosome complex). U4 does not directly interact with the pre-mRNA it plays an essential role in bringing U5 and U6 into the spliceosome (Will and Lührmann 2011).

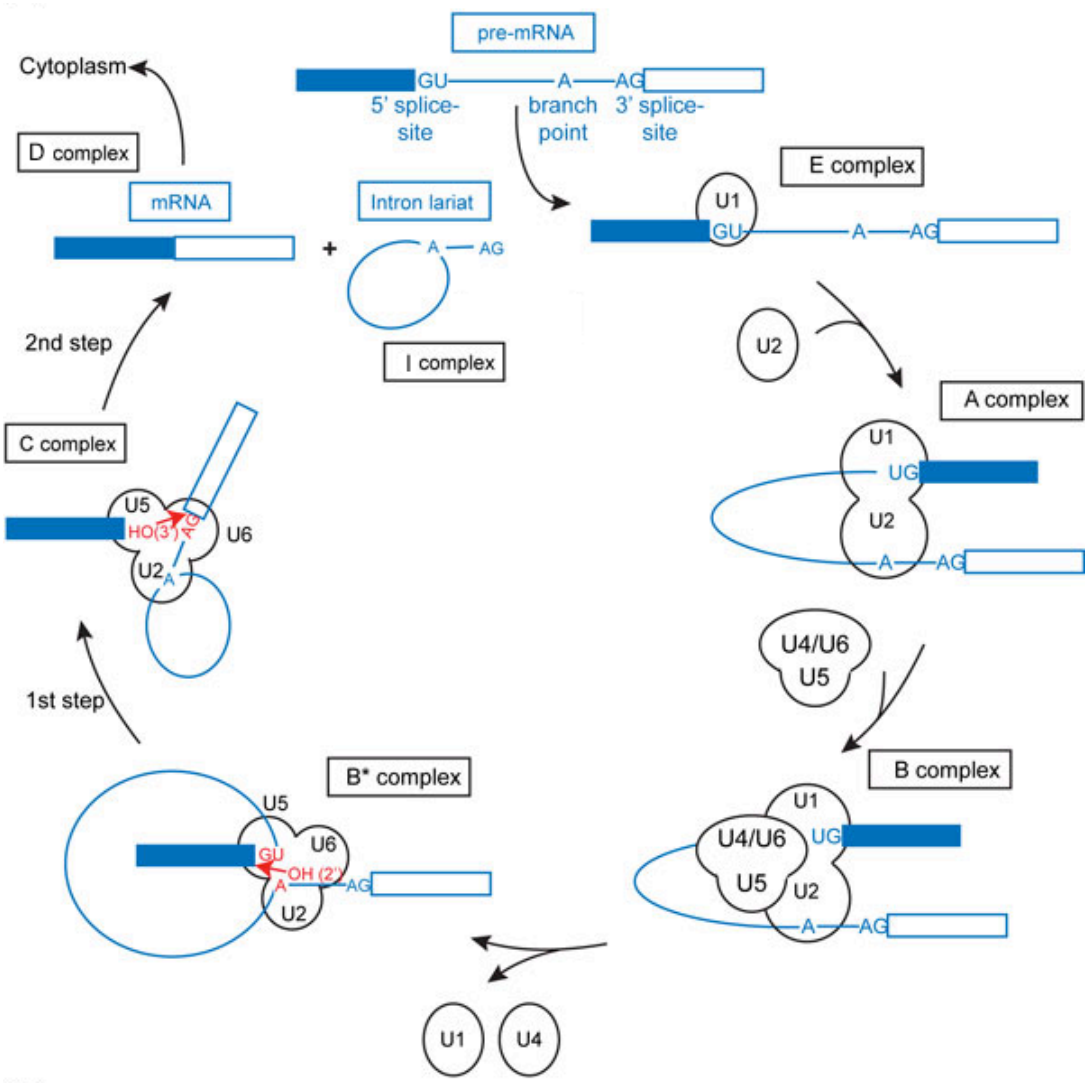

Figure 1.8. Spliceosomal assembly cycle and structures of spliceosomal complexes in the cycle.
U small nuclear ribonucleoproteins (snRNPs) assemble onto a precursor-mRNA (pre-mRNA) transcript and catalyze two transesterification reactions, which result in the splicing of two exons and the release of an intron in the form of a lariat-like structure. [Figure adapted from (Krummel, Nagai et al. 2010)]

Complex B

The fully formed spliceosome contains all five snRNPs and becomes the competent for splicing through a series of rearrangements. These rearrangements result in the dissociation of U1 (at the 5'ss by U6) and disruption of U4/U6 base-paring and the formation of the catalytic center for the first transesterification reaction – interactions between U2 and U6 snRNPs, in which the 5'ss of the exon is displaced and the lariat intron is formed. Both U2 and U6 not only interact with sites on the pre-mRNA, but importantly build three essential helices by intramolecular base-pairing with each other. (Madhani and Guthrie 1992, Turner, Norman et al. 2004). Furthermore,

U5 snRNA loop I base-pairs with exon 5'ss and later with exon sequence at 3'ss. The role of U5 is likely to be in positioning the exons for the second nucleophilic attack. The 5'and 3' splice site are also approached by the U5-220 KDa protein (Umen and Guthrie 1995), which has been reported to affect the tertiary interaction between the splice sites and U6 snRNA (Collins and Guthrie 1999). These conformational changes provide the structural basis to juxtapose the branch site and the donor splice site, promoting the formation of the activated B complex (B*) (Reed 2000, Boehringer, Makarov et al. 2004, Turner, Norman et al. 2004). The first step of the two splicing transesterification reactions occurs in the B* complex and generates two intermediates: the free 5' exon and the lariat-3'-exon (T O'Keefe, Norman et al. 1996). This event is followed by the formation of the C complex.

Complex C

The second transesterification reaction takes place in the presence of complex C (activated spliceosome complex). The U5, U2 and U6 snRNPs promote alignment of the exons for the second catalytic reaction (Figure 1.8). The U5-associated protein named pre-mRNA processing factor 8 (Prp8) is involved in the stabilization of these interactions (Umen and Guthrie 1995). The C complex contains the products of catalytic step I of the splicing reaction (exon 1 and lariat exon 2).

Complex I

The final product is a post-spliceosomal lariat-like structure between U6 and the branchpoint oligo – complex I (Figure 1.8) (T O'Keefe, Norman et al. 1996).

Complex D

Once the two exons are joined, the spliceosome dissociates to complex D (containing the spliced exons) (Figure 1.8). The release of the spliced product from the spliceosome is catalyzed by the DExD/H helicase Prp22 (Schwer and Gross 1998, Ilagan, Chalkley et al. 2013). Finally, the snRNPs are released and recycled for additional rounds of splicing.

The mRNA resulting from the splicing process leaves the nucleus and is directed to the cytoplasm, where it is subjected translation to the protein sequence (Will and Lührmann 2011). Which of the spliceosome elements finally catalyze the splicing reaction still remains unexplained, although the possibility that the mechanism is RNA-based is more likely. *In vitro* experiments demonstrate that U2 and U6 snRNA are able to base-pair even in absence of the associated proteins. In the presence of a branch point they are able to promote the nucleophilic attack of the adenosine at the branch point (Valadkhan and Manley 2001, Valadkhan, Mohammadi et al. 2009).

1.2.3. The canonical *cis*-acting elements for splice site recognition

The proper exon/intron splice site identification requires short consensus sequences located at the exon/intron boundaries on the pre-mRNA (Figure 1.9) (Shapiro and Senapathy 1987). These molecules are called *cis*-acting elements, they play a critical role in the exon-intron definition, promoting correct maturation of pre-mRNAs. Near the exon/intron boundaries, there are several canonical elements, which act as important signals for splicing reaction: the 5' splice site, the 3' splice site and the branch point site (Figure 1.6,Fifure 1.9). These elements are recognized by the basic components of the spliceosome machinery to promote the two transesterification reactions (Barash, Calarco et al. 2010).

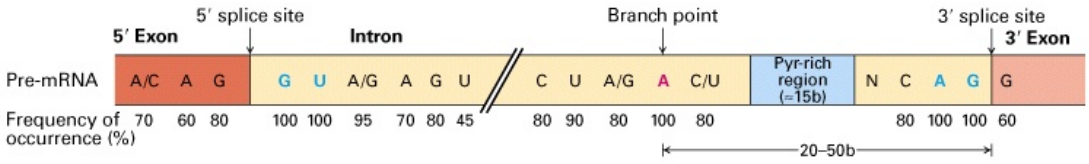

Figure 1.9. Schematic representation of exon-intron boundaries and consensus sequences for 5' and 3' splice sites and branch point.

The two exons and the intron are indicated. Pyr-rich region means region rich in pyrimidines. N means any base. The A in red is the branch point. The frequencies of each nucleotide in an alignment of conserved sequences from 1,683 human introns are indicated. To note that the universally conserved nucleotides are the dinucleotide cores of the 5' and 3' splice sites GU and AG respectively together with the branch point (A) (Zhang and Marr 1993). [Figure adapted from (Berk, Zipursky et al. 2000)]

1.2.3.1. The 5' splice site

The 5'ss defines the exon/intron junction at the 5'-end of the intron (Figure 1.9). In higher eukaryotes the motif surrounding the 5' splice site is composed of 9 nucleotides MAG/GURAGU (M indicates A or C, R indicates purins and the slash the exon/intron boundary), spanning from exonic position -3 to intronic position +6. The first two nucleotides of the intron in the position +1 and +2 (GU) are highly conserved as it was found in most of the human donor splice sites (98%). Mutations in this dinucleotide lead to splicing defects (Langford, Klinz et al. 1984). But even a correct GU are not sufficient for the recognition of the exon and require the presence of a partially degenerated sequence surrounding the donor splice site (Aebi, Hornig et al. 1987). The importance of the surrounding sequences is supported by the existence of a minority of 5'ss (<1%), that have a C to U substitution (GC dinucleotide) at the exon/intron boundary (Senapathy, Shapiro et al. 1990).

Recognition of the 5'ss is driven by a near perfect base-pairing between the sequence surrounding the donor site and the 5'-tail of the U1 snRNA (Figure 1.15). This is the first event that leads to the assembly of the spliceosomal machinery upon the intron (Horowitz and Krainer 1994). Crucial for the selection of the donor splice site is the RNA:RNA base-pairing of the 5'ss consensus sequence on the pre-mRNA with the snRNA U1 particle. Improvement in the recognition of weak splice sites by mutations have been described to promote constitutive inclusion when exons are spliced in the alternative manner (Muro, Iaconcig et al. 1998).

1.2.3.2. The 3' splice site

There are three main conserved intronic elements that take part in defining the 3' border of the intron (3'ss): the branch point, the polypyrimidine tract and the AG dinucleotide (or acceptor site) (Figure 1.9) (Gerke and Steitz 1986, Shapiro and Senapathy 1987, Reed 1989, Smith, Porro et al. 1989, Wu, Romfo et al. 1999).

The **branch point** is characterized by the presence of a conserved A, surrounded by a highly degenerated motif YNYURAC (Y indicates pyrimidines, R indicates purine and N any nucleotide) and is the site involved in the first transesterification reaction. It is commonly found 18-40 nucleotides upstream of the 3' acceptor site (Reed and Maniatis 1988, Smith, Porro et al. 1989, Wagner and Garcia-Blanco 2001). During the second step of the spliceosomal complex A formation, the U2 snRNA matches the branch point sequence (BPS) through an RNA:RNA interaction (Lamond, Barabino et al. 1990).

The **polypyrimidine tract** is composed of a stretch of pyrimidines, in particular uridines, and is located between the BPS and the terminal AG at the intron/exon junction (Castelo-Branco, Furger et al. 2004). This element of the 3'ss is recognized by the U2AF (through 65kDa subunit) during the creation of the complex E (Kielkopf, Lücke et al. 2004, Kent, Ritchie et al. 2005). Roscigno et al. showed that the length of the polypyrimidine tract is important and can influence the recognition of the 3' splice site. Progressive deletions of the PPT sequence significantly affect the recognition of the BPT, this can affect spliceosome process and formation of the lariat. In the case of elongation the polypirymide tract splicing efficiency can improve (Roscigno, Weiner et al. 1993).

The **AG dinucleotide** is located just downstream the PPT and defines the 3' border of the intron. This site is characterized by short YAG|G sequence (Y is a pyrimidine and | means the intron/exon boundary), where the AG is highly conserved and is fundamental for the second transesterification step of the splicing reaction (Reed 1989).

1.2.4. Additional *cis*-acting elements

The mammalian junctions that define an exon are weakly conserved and more degenerate with respect to yeast canonical *cis*-elements. These elements are necessary but are by no means sufficient to define exon/intron junctions. Early experiments in 1987 have shown that internal exonic sequences far from the 5' and 3'ss were essential for exon recognition (Mardon, Sebastio et al. 1987). These

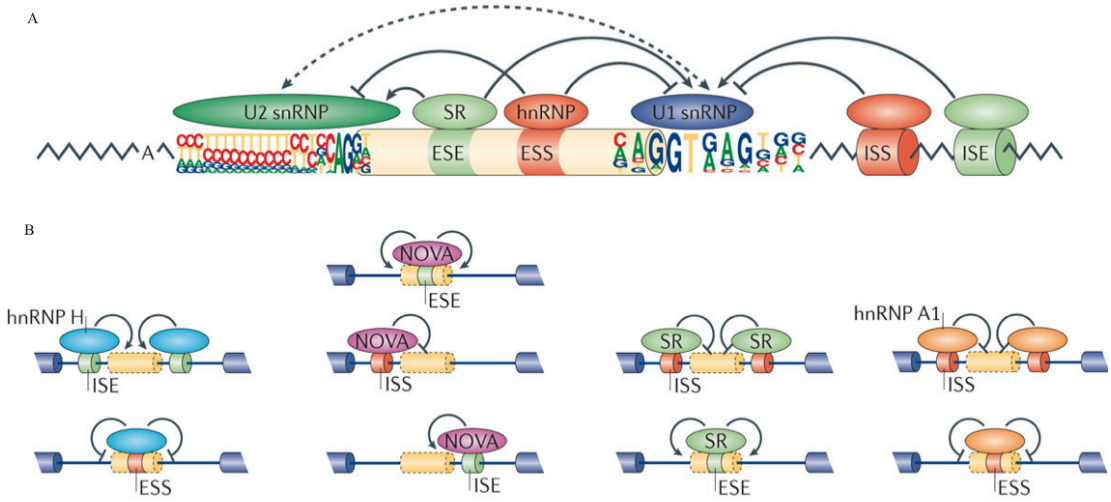

Figure 1.10. Auxiliary *cis*-acting elements in pre-mRNA splicing.

A) Splice site choice is regulated through *cis*-acting splicing regulatory elements (SREs) and *trans*-acting splicing factors. SREs are classified as exonic splicing enhancers (ESEs), intronic splicing enhancers (ISEs), exonic splicing silencers (ESSs) or intronic splicing silencers (ISSs). These SREs specifically recruit splicing factors to promote or inhibit recognition of nearby splice sites. Common splicing factors include SR proteins, which recognize ESEs to promote splicing, as well as various heterogeneous nuclear ribonucleoproteins (hnRNPs), which typically recognize ESSs to inhibit splicing. Both often affect the function of U2 and U1 small nuclear RNPs (snRNPs). The consensus motifs of splice sites are shown in the colored pictograph, with the height of each letter representing nucleotide frequency in each position. The dashed arrow represents the formation of the exon definition complex.

B) The activity of splicing factors and *cis*-acting SREs is context-dependent. Oligo-G tracts, recognized by hnRNPH, function as ISEs to promote splicing when they are located inside an intron (top), and as ESSs when located within exons (bottom). YCAY motifs, recognized by neuro-oncological ventral antigen (NOVA), act as ESEs when located inside an exon (top), as ISSs when located in the upstream intron of an alternative exon (middle) and as ISEs when located in the downstream intron (bottom). Binding sites for SR proteins and hnRNPA1 also have distinct activities when located at different regions on the pre-mRNA (Pagani and Baralle 2004). [Figure adapted from (Matera and Wang 2014)]

additional elements are located both in introns and in exons (Figure 1.10) and, depending on their position and function, are identified as exonic or intronic splicing enhancers (ESEs, ISEs) and silencers (ESSs, ISSs) (Cartegni, Chew et al. 2002, Fairbrother, Yeh et al. 2002). These elements are often conserved between different species, but show highly degenerate sequence motifs, making their identification difficult. It is noted that a *cis*-acting element can have overlapping functions as was shown for the cystic fibrosis transmembrane conductance regulator gene (CFTR) exons 9 and 12 (Pagani, Buratti et al. 2003). These have been called, composite exonic regulatory elements of splicing (CERES) (Pagani, Stuani et al. 2003).

1.2.4.1. Splicing enhancers

Splicing enhancers are regulatory elements that reside in both exonic and intronic regions and stimulate exon inclusion. **Exonic Splicing Enhancers** are thought to serve as binding sites for specific SR proteins (Blencowe 2000). SR proteins that are bound to ESEs can promote exon definition by directly recruiting the splicing machinery through their RS domain and/or by antagonizing the action of nearby silencer elements (Kan and Green 1999).

There are two models of splicing enhancement mechanisms: RS-domain-dependent and RS-domain-independent (Figure 1.11). In the first one an SR protein binds to an ESE through its RNA-recognition motifs (RRM) and contacts the splicing factor U2AF 35. The recruitment of the splicing factor U2AF, either directly through RS-RS domain interaction or indirectly through splicing co-activators (the protein that mediates splicing enhancement without binding directly to the pre-mRNA) seems to be important, especially in those cases in which recognition of a weak pyrimidine tract is a rate-limiting step in the splicing reaction (Cartegni, Chew et al. 2002). In the RS- domain-independent mechanism the main function of the SR protein that is bound to an ESE is to antagonize the negative effect on splicing of an inhibitory protein that is bound to a juxtaposed exonic splicing silencer (Cartegni, Chew et al. 2002). These models are not mutually exclusive, and the splicing of some introns might involve a combination of these mechanisms.

Enhancers localized in introns are also described (**Intronic Splicing Enhancers)** (Venables 2007), but a lot remain to be discovered because fewer large-scale studies have been conducted, compared with ESEs. ISEs have been identified mainly through studies on point mutations that cause a disease. Several of these mutations occur 20 - 40 bp downstream of the5' splice site and cause exon skipping (McCarthy and Phillips 1998, Lew, Fei et al. 2004). The subsequences can be fundamental for constitutive exon recognition or can be regulated in a tissue or developmental specific manner (Venables 2007). Well studied ISEs are the G run motifs, which enhance adjacent 5' or 3' splice site (McCullough and Berget 1997) or the intronic CA repeats that promote inclusion of upstream exons (Hui, Hung et al. 2005) and

the UGCAUG sequence present in downstream regions of neuron-specific exons that act as enhancer binding to the brain specific factor Fox-1 (Nakahata and Kawamoto 2005).

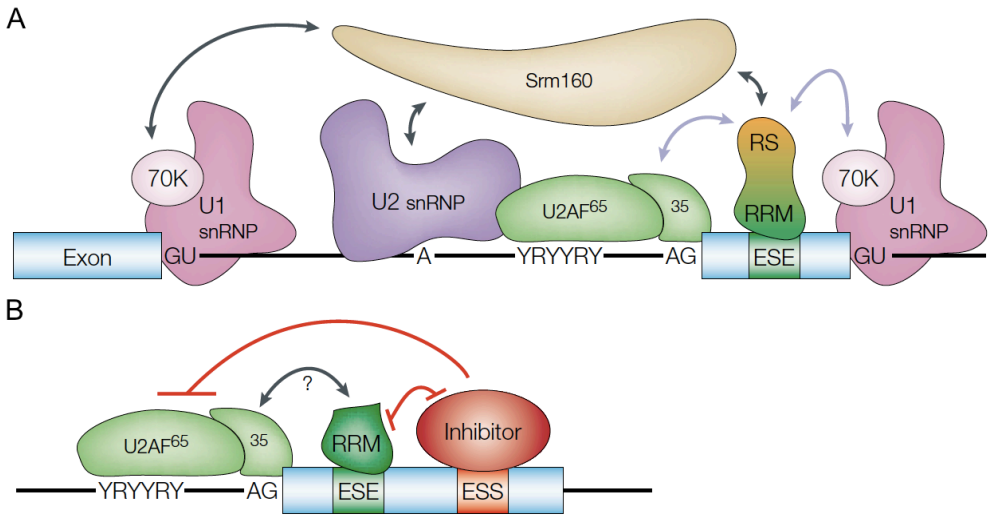

Figure 1.11. Models of SR protein action in exonic-splicing-enhancer-dependent splicing.

A) Recruiting function: RS-domain-dependent mechanism. An SR protein binds to an exonic splicing enhancer (ESE) through its RNA-recognition motifs and contacts the splicing factor U2AF35 (U2 auxiliary factor) and/or U1-70k at the adjacent splice sites through its RS domain. The U2AF splicing factor consists of two subunits (U2AF65 and U2AF35), and the large subunit binds to the polypyrimidine (Y) tract, which here is interrupted by purines (R) and is therefore part of a weak 3' splice site. U2AF65 also promotes binding of U2 snRNP to the branch site. U2AF35 recognizes the 3' splice-site AG dinucleotide. The U1 snRNP particle binds to the upstream and downstream 5' splice sites through base-paring of the U1 snRNA; the 70k polypeptide of each U1 snRNP particle is shown. For some ESE-dependent pre-mRNAs, indirect interactions (black arrows) are bridged by the splicing co-activator Srm160, which stimulates splicing of some ESE dependent pre-mRNAs and also interacts with the U2 small nuclear ribonucleoprotein (snRNP).

B) Antagonist function: RS-domain-independent mechanism. Here the main function of the SR protein that is bound to an ESE is to antagonize the negative effect on splicing of an inhibitory protein that is bound to exonic splicing silencer (ESS). [Figure adapted from (Cartegni, Chew et al. 2002)]

1.2.4.2. Splicing silencers

Splicing silencers are regulatory elements located in introns - **Intronic Splicing Silencers** or in exons - **Exonic Splicing Silencers** that are responsible for splicing inhibition. About one-third of randomly selected short, human DNA fragments showed splicing inhibitory activity *in vivo* when inserted into the middle exon of the three-exon minigene (Fairbrother and Chasin 2000).

ESSs seem to interact with negative regulators that often belong to the hnRNPs (Wagner and Garcia-Blanco 2001, Pagani and Baralle 2004). Alternatively, binding of the polypyrimidine tract binding protein (PTB) or hnRNPA1 to several sites around or within a silenced exon, followed by dimerization of the bound PTB proteins, might cause relevant portions of the pre-mRNA to loop out, which makes them unavailable for splicing (Cartegni, Chew et al. 2002). The decision of whether to include an exon reflects the intrinsic strength of the flanking splice sites and the combinatorial effects of positive and negative elements (Smith and Valcárcel 2000).

ISS are less systematically studied elements that are usually identified through the study of individual alternatively spliced genes (Zheng 2004). PTB normally recognizes pyrimidine-rich elements and functions either by antagonizing the action of U2AF65 or by creating a region of silencing across the down-regulated exon. Other factors can bind to ISS, as demonstrated by the alternative splicing of CFTR exon 9 that is inhibited when a SR protein is recruited to a silencing element present in intron 9 (Pagani, Buratti et al. 2000).

1.2.4.3. RNA secondary structure

The interaction between *cis*- and *trans*- acting elements is considered a key aspect in the constitutive and alternative splicing regulation. An additional factor suggested to modulate this interaction and influence splicing selection is represented by the RNA secondary structure. RNA molecules have a natural tendency to form highly stable secondary structures: these structures might act as *cis*-acting elements, influencing the splicing process (Brion and Westhof 1997, Conn and Draper 1998, Buratti and Baralle 2004). RNA secondary structure may hinder the accessibility of splicing factors to functional sequences, either splice sites or other regulatory sequences, present in the pre-mRNA, by sequestering them in stems or looping them out (Hiller, Zhang et al. 2007).

The secondary structure plays an important role in determining alternative splicing efficiency in the fibronectin (FN) extra domain A (EDA) (Buratti, Muro et al. 2004).

The peculiar secondary structure of the EDA exon localizes the ESE element binding alternative splicing factor 1/pre-mRNA-splicing factor SF2 (SF2/ASF) (now serine/arginine-rich splicing factor 1 (SRSF1)) in an exposed position as part of a loop region. The nearby ESS seems to be determinant for the RNA conformation, as it lies in a stem structure. Furthermore, the efficient function of the ESE element depends on the maintenance of the single-stranded configuration (Muro, Caputi et al. 1999). RNA secondary structure can also affect the relative distance between splicing elements determining a considerable variation in splice site recognition (Buratti and Baralle 2004) or, for example, the splicing process can be stimulated when silencer elements are trapped within a RNA structure.

1.2.5. *Trans*-acting factors

The proteins involved in the splicing process are two major groups: the snRNPs and the non-snRNPs. These splicing factors, independent of their functional characteristics, share some similar structural features such as the RNA-recognition motifs and/or protein binding domains and usually target short sequence elements adjacent to site of regulation.

The U1, U2, U4/U6 and U5 snRNPs are the main components of the spliceosome and catalyze the splicing reaction (Patel and Steitz 2003). Each snRNP is composed of a U-rich snRNA molecule, seven Sm or Sm-like proteins and several splicing factors (Will and Luhrmann 2001). The snRNAs are characterized by their small size, stability and show high level of sequence conservation (Kambach, Walket et al. 1999). RNA Pol II transcribes all U snRNAs with the exception of U6 snRNA that is transcribed by RNA Pol III (Will and Luhrmann 2001). Upon transcription, the assembly of U6 snRNP probably takes place in the nucleus, whereas the other snRNAs are transported to the cytoplasm where snRNP assembly initiates (Fischer, Sumpter et al. 1993). Following their export to the cytoplasm, the snRNA precursors bind seven Sm proteins in order to form the structural core of snRNPs. A properly assembled Sm core, together with cap hypermethylation and 3'-end processing of the snRNA, are required for nuclear import (Fischer, Sumpter et al. 1993). Apart

from the Sm and Sm-like proteins, that are required for U6 snRNP assembly, snRNPs also contain a certain number of other particle-specific proteins.

The non-snRNPs proteins are involved in the regulation of both general and tissue-specific splicing events. In particular, SR proteins and hnRNPs (Figure 1.10) have been found as components of distinct regulatory complexes with functional specificity in splicing (David and Manley 2008)

1.2.5.1. Heterogeneous ribonuclear proteins (hnRNPs)

The hnRNP protein family is a class of several RNA-binding proteins that associate with nascent pre-mRNAs allowing their maturation, export of mRNA from the nucleus to the cytoplasm and translation. The hnRNPs form a complex consists of 20 hnRNP nuclear proteins (Han, Tang et al. 2010); one of them is protein K (hnRNPK). Together with similar in function SAM68 (68 kDa-associated Src substrate during mitosis) (Taylor and Shalloway 1994) protein K represents a separate class of nucleic acid-binding factors that regulate gene expression and signal transduction involved in cell cycle (Bomsztyk, Denisenko et al. 2004).

Although these factors are predominantly localized in the nucleus, a subset of these proteins shuttle continuously between nucleus and cytoplasm: this indicates a role in mRNA export from nucleus to cytoplasm and in other cytoplasmic processes (Piñol-Roma and Dreyfuss 1992). These proteins are expressed in all tissues but the relative amount of different hnRNPs vary among cell types and show stage-specific expression patterns: some hnRNPs are extremely abundant (~100 million copies per nucleus), while others are present in a lower amount (Kamma, Portman et al. 1995)

The structure of hnRNPs contain one or more RRMs associated with auxiliary domains (Figure 1.12) that have been shown to mediate protein-protein interactions (Dreyfuss, Matunis et al. 1993). The hnRNP proteins usually mediate splicing inhibition, particularly through the interaction with ESS elements or by steric interference with other splicing factors (Cartegni, Chew et al. 2002), antagonizing

directly or indirectly SR proteins. Nevertheless, depending on the position of the splicing regulatory elements, hnRNPs can also associate with enhancer elements to help exon inclusion (Caputi and Zahler 2002), indicating that hnRNPs can have various roles in pre-mRNA splicing. However, assemblies of tissue-specific factors and proteins of the SR and hnRNP families can have positive or negative roles depending on their precise location, composition and state of modification of their components (Smith and Valcárcel 2000)

Protein hnRNPA1 is a nucleo-cytoplasmic shuttling protein (Piñol-Roma and Dreyfuss 1992). It is involved in many aspects of mRNA metabolism. It has been shown that hnRNPA1 together with other members of the hnRNP group function in alternative splicing regulation as an antagonist of the SR proteins, both *in vitro* and *in vivo* (Mayeda and Krainer 1992, Cáceres, Stamm et al. 1994, Mayeda, Munroe et al. 1994, Yang, Bani et al. 1994). This protein functions in various post-splicing activities like mRNA export (Izaurralde, Jarmolowski et al. 1997), internal ribosome entry site (IRES)-mediated translation (Bonnal, Pileur et al. 2005) and mRNA stability (Hamilton, Burns et al. 1997). Recent studies propose a model where hnRNPA/B and hnRNPF/H proteins may function in generic splicing by modulating the conformation of mammalian pre-mRNAs (Martinez-Contreras, Fisette et al. 2006).

Although hnRNPA1 is extensivly characterized, only a relatively small number of cellular and viral genes regulated by hnRNPA1 have been identified (Caputi, Mayeda et al. 1999, Matter, Marx et al. 2000, Hou, Lersch et al. 2002, Pollard, Krainer et al. 2002, Guil, Gattoni et al. 2003, Rooke, Markovtsov et al. 2003, Zhao, Rush et al. 2004, Han, Yeo et al. 2005), and only a small number of high-affinity binding sites have been obtained by SELEX (Systematic evolution of ligands by exponential enrichment) (Burd and Dreyfuss 1994). Guil and Cáceres in their study used an *in vivo* cross-linking and immunoprecipitation protocol (CLIP), they were looking for endogenous RNA targets of hnRNPA1. They reported, that this splicing factor binds specifically to human pri-miR-18a (the stem-loop precursor of miR-18a in the context of the primary RNA) and facilitates its Drosha-mediated processing. Thus, hnRNPA1 acts as an auxiliary factor for the processing of a specific miRNA substrate (Guil and Cáceres 2007).

The hnRNPA1 binds to exonic splicing silencer or intronic splicing silencer elements and thus repress the splicing (Figure 1.10B) of alternatively spliced exons (Smith and Valcárcel 2000, Cartegni, Chew et al. 2002). The mechanisms that hnRNPA1 use to mediate splicing repression are not fully understood.

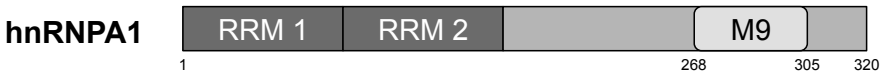

Figure 1.12. Structure of hnRNPA1 protein.

Human hnRNPA1 consists a single polypeptide chain of 320 amino acids. An N-terminal proteolytic fragment spanning the first 196 amino acids, known as unwinding protein 1 (UP1), contains two RNA-recognition motifs (RRM, also known as RNA-binding domain, RBD; and ribonucleoprotein consensus sequence, RNP-CS) separated by a short linker. The C-terminal region of hnRNPA1 is particularly rich in glycine residues and includes several Arg–Gly–Gly (RGG) tri-peptide repeats that also constitute an RNA-binding motif (Xu, Jokhan et al. 1997). Near the COOH terminus of the protein, between amino acids 268 and 305, the splicing factor contains a 38-amino acid sequence, termed M9, responsible for its nuclear import (Izaurralde, Jarmolowski et al. 1997). [Figure adapted from (Allemand, Guil et al. 2005)]

1.2.5.2. Serine-arginine rich proteins (SRs)

RNA binding proteins, called SR proteins (serine-arginine-rich proteins) are involved in forming the spliceosome. Recent studies have shown that SR proteins are also involved in other processes of RNA metabolism, such as transport of the transcript into the cytoplasm and translation. The proteins of this group are present in all tested tissue of animals and plants (Krainer, Conway et al. 1990, Ge, Zuo et al. 1991, Zahler, Lane et al. 1992, Lazar, Schaal et al. 1995, Lopato, Mayeda et al. 1996, Golovkin and Reddy 1999). In humans 10 - 12 SR proteins have been identified (Figure 1.13). SR proteins are characterized by the presence of one or two RRMs, RBD - RNA binding domain or RNP - ribonucleoprotein domain, located in the N-terminal and C-terminal RS-rich domains (Birney, Kumar et al. 1993). In SRSF1 protein RS residues constitute 76% of the total residues of the domain (Bourgeois, Lejeune et al. 2004).

It is believed that the main role of the RS domain is to mediate interactions with other proteins which also have RS domains (Wu and Maniatis 1993). Serine residues

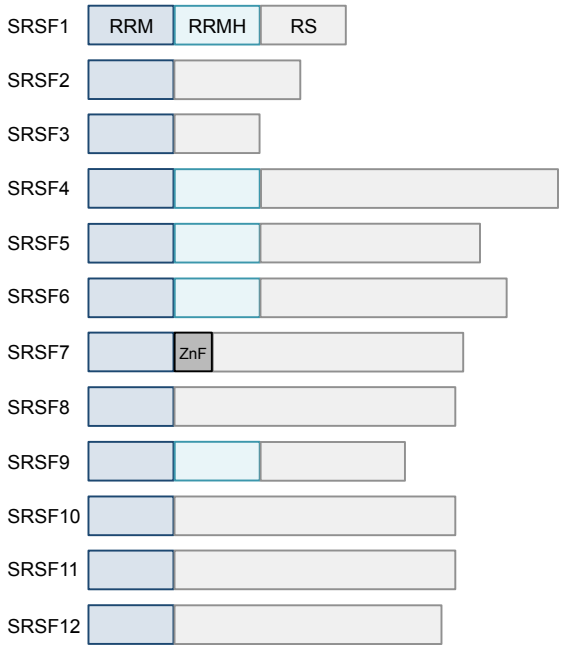

Figure 1.13. Human SR proteins.

RRM – RNA binding domain; RRMH - pseudo-RRM; RS – serine/argine rich domain; ZnF – zinc-finger motif. [Figure adapted from (Nayler, Stamm et al. 1997)]

in the RS domain are subject to intensive phosphorylation by several proteins Phosphorylation is a common post-translational modification of many proteins, but in the case of SR proteins it is of particular importance. This reaction is catalyzed by protein kinase and occurs in a relatively narrow region of serine and arginine rich domain. The accumulation of phosphate residues in the area of only several amino acid residues have a decisive influence on cellular localization of those proteins and the selection of processes in which they are going to be involved. This modification is essential for the functioning of the protein.

The physiological relevance of SR proteins was confirmed by many *in vivo* experiments. It has been shown that the protein SRSF1 is essential for the survival of chicken DT40 cell line (Wang, Takagaki et al. 1996), and its absence results in the loss of genomic stability and DNA fragmentation (Li and Manley 2005).

SR proteins are important in the process of pre-mRNA splicing due to their function as factors involved in the regulation of this process and participation in the formation of the spliceosome (Jurica and Moore 2003, Sanford, Ellis et al. 2005). They are involved in constitutive and alternative splicing process. In the first former SR proteins mediate interactions of the spliceosome components connected to 3'ss

and 5'ss. In alternative splicing they may regulate selection of 3'ss and 5'ss. By binding to specific ESE sequences they can stimulate splicing of the neighboring introns (Lam and Hertel 2002). Most likely this is achieved by RS domain of SR proteins that recruits other components of the spliceosome to weak splice sites (Wu and Maniatis 1993). This binding recruits and stabilizes the U1 snRNP and the U2AF binding to 5'ss and 3'ss respectively (Sanford, Ellis et al. 2005). Another idea proposes that SR proteins, by interacting with ESE, can antagonize the effect of silencer elements that are localized nearby (Kan and Green 1999).

1.3. U1 as small nuclear RNA (U1 snRNA) and small nuclear ribonucleoparticle (U1 snRNP)

The U1 spliceosomal RNA is a small nuclear RNA component of U1 snRNP, a ribonucleoprotein that cooperates with other components to assemble the spliceosome. Hodnett and Busch discovered the U1 snRNA in 1968. They isolated the uridylic acid-rich RNA from rat liver cells and used polyacrylamide gel electrophoresis (PAGE) method for characterization (Hodnett and Busch 1968). U1 is present in a variety of organisms including Animalia, Fungi, Protista and Plantae kingdoms (Hudson, Stark et al. 2015). In the early 1980's, U1 snRNA was discovered in *S. cerevisiae* (Lossky, Anderson et al. 1987, Siliciano, Jones et al. 1987). In 1983 there were reports about molecular weights and presence of 5' caps determined for U snRNAs in yeast (Wise, Tollervey et al. 1983).

Four stem-loop regions (I, II, III, IV) and two single-strand regions characterize the secondary structure of U1 snRNA (Figure 1.14). One of those single-strand regions (bases from 3 to 10) is a conserved sequence and play a big roles in the base-pairing and interaction with the 5' splice site of introns during pre-mRNA splicing. The other single-strand is also a conserved sequence for the Sm-binding sites (AAUUUGUGG). This sequence is recognized in the cytoplasm by a heteroheptameric ring of Sm proteins (Raker, Hartmuth et al. 1999, Paushkin, Gubitz et al. 2002). The stem-loops are recognized by the three U1 specific proteins: U1-70k that binds stem-loop I, U1-A that binds stem- loop II, and the U1-C protein that are probably connected by protein - protein interaction.

The U1 snRNP is the first snRNP that binds the splicing complex forming the commitment complex. At the next point the branch-points bridging protein binds to form the complex A (Seraphin and Rosbash 1989). For the whole spliceosome assembly the most crucial step is the commitment complex formation, the initiation step.

The main role of the U1 snRNP is identification of the donor splice site (Mount, Pettersson et al. 1983, Rinke, Appel et al. 1984). However in some cases, the recognition of the 5'ss can occur without the presence of the U1 particle, but in those cases splicing is less efficient (Du and Rosbash 2002). The U1-C protein

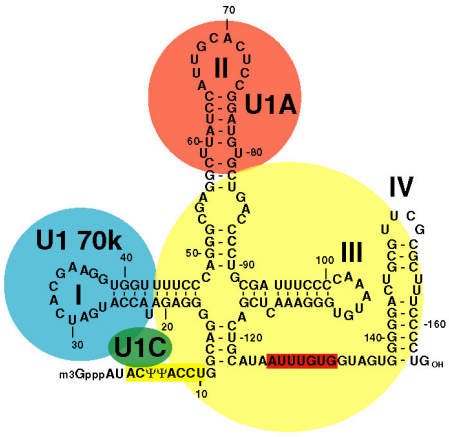

Figure 1.14. Structure of human U1 small nuclear ribonucleoprotein (snRNP).

Secondary structure of U1 snRNA (black) and general location of the seven Sm proteins (yellow), U1-70k (blue), U1-A (red), and U1-C (green). A consensus 5′ splice-site is base-pairing to the single-stranded 5′ end of U1 snRNA. (Rossi, Forné et al. 1996, Stark, Dube et al. 2001, Du and Rosbash 2002). [Figure adapted from (Krummel, Nagai et al. 2010)]

contributes to the 5'ss recognition by stabilizing base-pairing between the 5' tail of the U1 with the donor site of the pre-mRNA (Du and Rosbash 2002). All this promotes creation of complex E (Will, Rümpler et al. 1996). The U1-A protein is not required for splicing, *in vitro* experiments showed that deletion of the stem-loop II or depletion of the protein from the extract does not have influence on U1 snRNP splicing efficiency (Heinrichs, Bach et al. 1990, Will, Rümpler et al. 1996). Berg and colleagues identified a new role for U1 snRNPs. They showed that these particles play an important role in protecting pre-mRNA from polyadenylation at the cryptic polyadenylation signals (Berg, Singh et al. 2012).

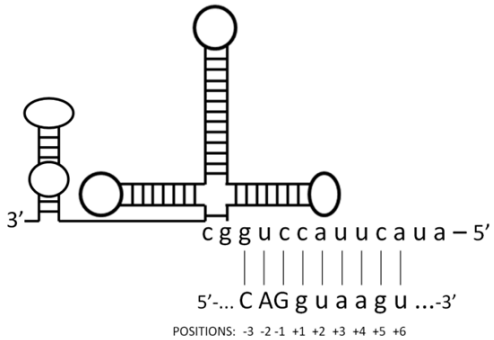

Figure 1.15. The mechanism of U1 snRNA binding to donor splice site.

Schematic representation of U1 snRNP base-pairing with RNA sequence. [Figure adapted from (Buratti and Baralle 2004)]

1.3.1. U1 gene

Splicing is based on the correct recognition of *cis*-acting factors by *trans*-acting factors, in particular U snRNAs. Among all of them, U1 is the first one to work. Human U1 snRNA gene (RNU1) is located on the short arm of chromosome 1 at position p36 (Naylor, Zabel et al. 1984). There are many genes with a high level of sequence similarity to this snRNA, mostly pseudogenes that are dispersed in the genome. However, they could produce functional variants of U1s (Lund and Dahlberg 1984, Bernstein, Manser et al. 1985). The canonical U1 snRNA gene is characterized by the presence of two essential promoter elements (Figure 1.16), the distal sequence element (DSE) and the proximal sequence element (PSE). The proximal promoter is recognized by the snRNA gene-specific factors: PSE-binding transcription factor (PTF) and SNAPc or phosphatidylethanolamine binding protein (PBP) (Hernandez 2001).

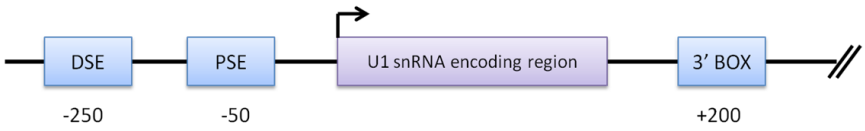

Figure 1.16. The structure of human U1 snRNA gene transcribed by Pol II.

The diagram shows the DSE and PSE *cis*-acting promoter elements and the 3' box, with their position relative to the transcription start site. The start site of transcription is marked with an arrow above the line. [Figure adapted from (Egloff, O'Reilly et al. 2008)]

The TATA-box binding protein (TBP) and several transcription factors like TFIIA, TFIIB, TFIIE and TFIIF, stimulate the transcription by RNA Pol II to yield short non-polyadenylated 3'-elongated pre-snRNAs, which are exported and further

processed in the cytoplasm (Huang, Jacobson et al. 1997). The proper formation of the 3'end is important for the stability of U1 snRNA, also for the transport trough the nuclear membrane (Terns, Dahlberg et al. 1993). The 3' end formation requires the presence of a 3'-box (conserved *cis*-acting element located 9-19 bases downstream of the 3' end of the RNA-encoding region), and phosphorylation of the

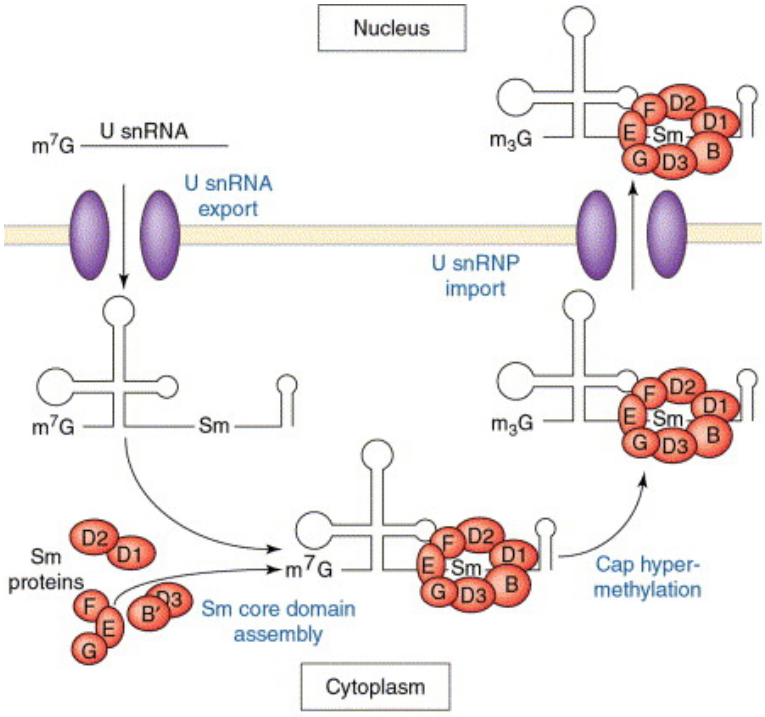

Figure 1.17. Biogenesis of spliceosomal U1 snRNPs.

The nuclear encoded and m^7G-capped U snRNA is transiently exported to the cytoplasm. The Sm proteins are stored in this compartment and assemble onto the exported U1 snRNA to form the Sm core domain. The m^7G cap of the U snRNA is subsequently hypermethylated to the m^3G cap. The assembled and m^3G-capped particle is then actively imported into the nucleus – the site of its function in splicing. The stage at which the U snRNP-specific proteins join the particle is not known in many cases. [Figure adapted from (Meister, Eggert et al. 2002)]

C-terminal domain of Pol II (Hernandez 1985, Hernandez and Weiner 1986, Uguen and Murphy 2003). The presence of a monomethyl guanosine cap structure (m^7GpppG) is also required for correct assembly into the large export complex (Figure 1.17). This cap structure is recognized by the cap-binding complex (CBC) (Ohno, Segref et al. 2000).

1.4. Alternative Splicing

Mechanisms that increase protein diversity include the use of multiple transcription start sites, alternative pre-mRNA splicing, polyadenylation, pre-mRNA editing, co-translational and post-translational protein modifications. Among these mechanisms, alternative pre-mRNA splicing is considered to be the most important source of protein diversity (Maniatis and Tasic 2002). In fact, as described above, mammalian genes are transcribed as precursor-mRNAs, which need to be maturated to produce mRNAs. The regulatory elements located within pre-mRNA sequences, indeed, follow this process to permit correct identification of canonical splice sites. However, this does not mean that one pre-mRNA encodes for only one mRNA, instead differential usage of splice sites due to alternative regulation of *cis*- and *trans*-acting elements could generate many different mature mRNAs from the same gene. Alternative splicing can generate multiple transcripts encoding proteins with subtle or opposing functional differences that can have profound biological consequences. For example *D. melanogaster* gene Down syndrome cell adhesion molecule (Dscam), can potentially synthesize 38,016 different mRNA isoforms (Schmucker, Clemens et al. 2000) produced by a single gene containing 95 cassette exons.

Recent studies indicate that 94% of human genes are alternatively spliced, demonstrating that RNA molecules are not simply passive intermediates in gene expression, increasing the complexity of the already complex regulation (Ward and Cooper 2010). Single cassette exons can reside between two constitutive exons such that the alternative exons are either included or skipped (Figure 1.18). Alternatively, multiple cassette exons can reside between two constitutive exons such that the splicing machinery must choose between them (Figure 1.18). Introns can be retained in the mRNA and become translated (Figure 1.18). Finally, they can have alternative promoters or alternative 5'ss (Figure 1.18) and alternative polyA site/alternative 3' splice site (Figure 1.18) (Black 2003). It is also known that most genes are alternatively spliced both, spatially and temporally. This results in the expression of different splice variants in different tissues, in different cells

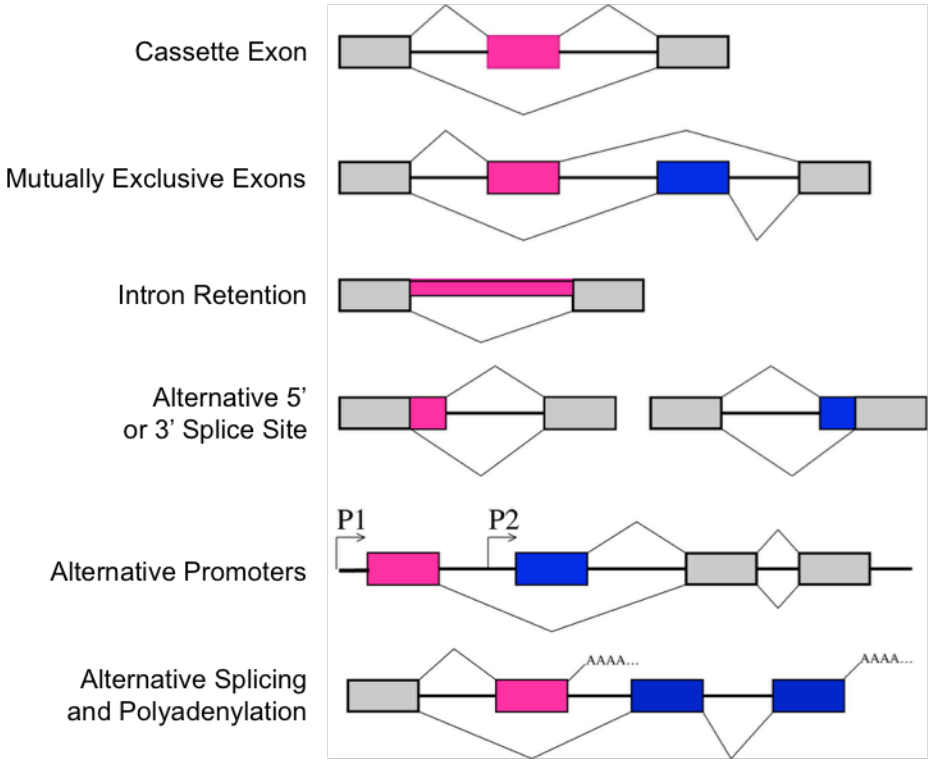

Figure 1.18. Alternative splicing patterns.

In each case, alternative splicing path is indicated in black line above. In complex pre-mRNAs, more than one of these modes of alternative splicing can apply to different regions of the transcript, and extra mRNA isoforms can be generated through the use of alternative promoters or polyadenylation sites. [Figure adapted from (Cartegni, Chew et al. 2002)]

of the same tissue, in the same tissue at different stage of development, or in response to external stimuli. The regulation of alternative splicing is very complex to generalize, it is a mechanism that requires an accurate coordination of both *cis*- and *trans*-acting factors, which can change on the basis of the gene considered. In human cells, in fact, there are several *trans*-acting elements, such as neuro-oncological ventral antigen 1 or 2 (NOVA) and hexaribonucleotide-binding protein 1 or 2 (FOX), which have been shown to act as either repressors or activators depending on the location of their binding site (Dredge, Stefani et al. 2005, Ule, Stefani et al. 2006, Mauger, Lin et al. 2008, Yeo, Coufal et al. 2009). For example, NOVA1 binds to an ISE in GABRG2 (GABA A receptor, γ2) pre-mRNA and promotes inclusion of exon 9 (Dredge and Darnell 2003), but it binds to the ESS in the alternative exon 4 of its own pre-mRNA and prevents exon 4 from being included (Dredge, Stefani et al. 2005). The final decision about how splicing will occur is determined by the concentration, localization and activity of each type of regulator.

1.4.1. Splicing mutations and disease

Aberrant splicing may result from mutations that affect *cis*-acting elements and *trans*-acting factors required for splicing regulation. These mutations can be the direct reason for a disease or can contribute to the susceptibility or severity of diseases (Cooper, Wan et al. 2009). The frequency of splicing mutations is significantly different between individual genes. It is considered that approximately 15% of pathogenic mutations cause disease through the defect they introduce in the splicing process (Krawczak, Reiss et al. 1992). These mutations can affect splicing in different ways:

1) mutations that affect the conserved canonical splice sites or polypyrimidine tract, directly interfering with the binding of U snRNPs;
2) mutations that affect intronic or exonic regulatory elements (ESE, ESS ISE, ISS), changing the bindings of positive and negative regulators;
3) mis-regulations or mutations that affect regulatory *trans*-acting factors.

In the spliceosomal formation, the first step of pre-mRNA splicing is the recognition of donor site (5'ss) mediated by U1 snRNP. As mentioned above, this particle is able to bind the consensus sequence through a complementary base-pairing interaction between the pre-mRNA and the 5' tail of U1. If this ability is lost because of the mutations that affect the 5'ss, it will cause a splicing defect. This is observed for example when the splice site mutation affects the invariant nucleotides GU of the donor site and AG at the acceptor site (Krawczak, Reiss et al. 1992, Krawczak, Thomas et al. 2007). Mutations occurring at other positions of the 5' and 3' splice sites can also lead to aberrant splicing, inducing exon skipping, activation of cryptic splice site or intron retention (Figure 1.18).

Other mutations that cause aberrant splicing can affect exonic or intronic enhancers and silencers sequences. One of the most studied diseases caused by an exonic mutation is the spinal muscular atrophy (SMA). Survival of motor neuron (SMN) is a ubiquitously expressed protein that plays a critical role in snRNP biogenesis and is essential for viability of all cells in different eukaryotes. In humans, there are two SMN genes, SMN1 and SMN2, both of which encode the same open reading frame. The majority of SMA patients have deletions of the SMN1

gene but retain SMN2, which has a single synonymous substitution in position +6 of exon 7 (c.840C>T) that induces the skipping of exon 7 and produces an inactive truncated protein (Lorson, Hahnen et al. 1999, Monani, Lorson et al. 1999). Thus, SMN2 is much less effective than SMN1 in producing SMN protein. The biochemical mechanism, by which the splicing is altered, provides two non-exclusive models, either through gain of an ESS or loss of an ESE. In the ESS-gain model, the mutation creates an ESS binding site for hnRNPA1 which functions as a splicing repressor (Kashima and Manley 2003), whereas in the ESE-loss model, the mutation disrupts an ESE that is bound by the SR protein SRSF1 (Cartegni, Hastings et al. 2006).

Mutations can also affect *trans*-acting factors. In fact, while disease-causing mutations that affect *cis*-acting sequences affect splicing of a single gene, mutations that affect components of the splicing machinery create the potential for multiple genes to be mis-spliced. The relative scarcity of examples for severe loss-of-function mutations in *trans*-acting factors may be an indication that mutations with widespread consequences are lethal during embryonic development or in individual cells. Mutations in splicing involving the protein Prp31 have been observed in retinitis pigmentosa, a progressive loss of photoreceptor cells during childhood (Vithana, Abu-Safieh et al. 2001). A deletion of RNA binding motif, Y-linked (RBMY) factor is a cause of azospermia (Venables, Elliott et al. 2000). Additionally, changes in the concentration or localization of splicing regulators are seen in tumorigenesis, in the case of ovarian cancer the levels of SRSF1, SRSF2 and transformer-2 protein homolog beta (tra2-beta) are altered (Fischer, Noack et al. 2004). Many general splicing factors and neuron-specific splicing factors, like NOVA-1 and NOVA-2, have changed levels of expression in Hodgkin's lymphoma (Relógio, Ben-Dov et al. 2005). Many other splicing events were altered, but connections with *trans*-acting factors are difficult to explain (Jeanteur 2006). I report these examples because it has became clear that different types of exonic or intronic variants can affect splicing and accordingly should be considered as a potential disease causing mutations (Baralle, Lucassen et al. 2009). Therefore, it is of crucial importance to study the effect of each mutation, to understand their effect on splicing processing (Cooper, Wan et al. 2009).

1.5. ExSpeU1s as novel therapeutic strategy to rescue exon skipping defects

As mention above, a significant number of mutations affect pre-mRNA splicing, inducing skipping of exons and leading to pathological phenotypes. Aberrant exon skipping is a common defect in the splicing process, caused by mutations that disrupt the exon definition. Mutations at the 5'ss reduce the complementarity with the U1 snRNA, while mutations at the polypyrimidine tract and within exons affect both *cis*- and *trans*-acting elements. The U1 snRNP is a critical player in recognition of the exon definition and the donor site. This activity is promoted by Watson-Crick interactions between donor site and the 5'-tail of U1. ~40, 22 and 5% of normal 5'ss contains, respectively, two, three or four mismatches toward the U1 snRNA (Carmel, Tal et al. 2004).

In the last few years, studies heve concentrated on modifying U1 snRNAs particls in order to create splicing correction approaches. There are published data showing modification of the binding site of U1 particle at suboptimal 5'ss (Sánchez-Alcudia, Pérez et al. 2011, Schmid, Glaus et al. 2011). Increasing the complementarity of the U1 snRNA tail according to the mutated 5'ss of a specific exon, the aberrant splicing of coagulation factor VII (*F7*) gene exon 8 was corrected (Pinotti, Rizzotto et al. 2008, Sánchez-Alcudia, Pérez et al. 2011, Schmid, Glaus et al. 2011, Schmid, Hiller et al. 2012). Nevertheless, this strategy appears to be limited to single mutations and also it can interfere with processing of other pre-mRNAs. Another novel therapeutic approach of correcting splicing defects was proposed by Fernandez et al., that is to engineer the 5'tail and direct loading of U1 onto non-conserved intronic regions downstream of the donor site of a specific exon (ExSpeU1), reducing undesired off-target events (Alanis, Pinotti et al. 2012). ExSpeU1s are able to correct exon skipping variants in Spinal Muscular Atrophy (Dal Mas, Rogalska et al. 2015), Netherton Syndrome (Figure 1.19) (Dal Mas, Fortugno et al. 2015), Cystic fibrosis (Alanis, Pinotti et al. 2012) and Hemophilia B (Alanis, Pinotti et al. 2012, Tajnik, Rogalska et al. 2016). It was possible to develop a large panel of ExSpeU1s that bind the non-conserved intronic sequences of each gene considered. The results, an effective rescue of aberrant splicing caused by mutations was observed,

promoting the inclusion of exon through the activity of ExSpeU1s in the four models. The strategy adopted by Fernandez et al. has two important advantages: first, the ExSpeU1s do not interact directly with normal donor sites; second, they can correct different types of splicing defects associated to exon skipping. The binding of the ExSpeU1s to intronic sequences will significantly reduce the possibility of off-targets events.

Furthermore, the therapeutic activity of ExSpeU1 was tested *in vivo* on the mouse model of SMA (Rogalska, Tajnik et al. 2016). It was shown that specific modification of a U1 core spliceosomal RNA particle is safe and active in an animal model. ExSpeU1 has no apparent side effects, it is non toxic *in vivo* and has high exon rescue efficiency.

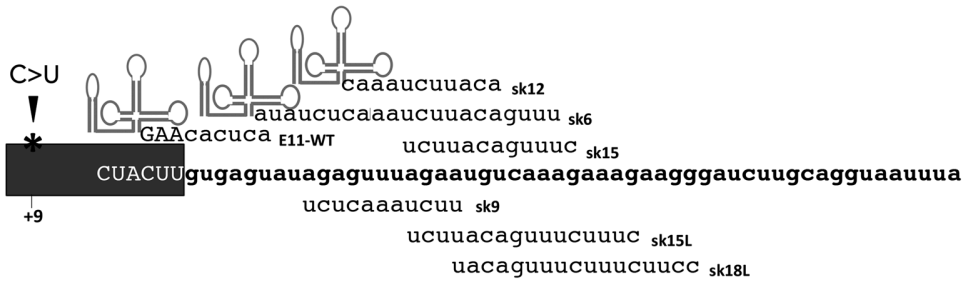

Figure 1.19. Schematic representation of possibilities for ExSpe U1s to bind in the SPINK5 sequence (Netherton syndrome).

[Figure adapted from (Dal Mas, Fortugno et al. 2015)]

In comparison to classical gene therapies, the ExSpeU1 strategy presents additional advantages. This approach could be helpful in mutations that affect several proteins that require expression at a specific differentiation stag. Moreover, the short length of the ExSpeU1s cassette (~500 bp) can also be useful in gene therapy of splicing mutations of large genes, such as CFTR and coagulation factor VIII (*F8*), whose full-length transcript can represent a limiting step for their insertion into viral vectors such as AAV. Another advantage regards dominant-negative mutations, where replacement therapy is not possible. Indeed, ExSpeU1s can correct the dominant-negative effect, correcting splicing defects and reduce the amount of the mutated toxic protein.

1.6. Familial dysautonomia

Familial dysautonomia (FD), also called hereditary sensory and autonomic neuropathy, type III or Riley-Day Syndrome (Riley, Day et al. 1949) is a rare autosomal recessive genetic disorder (Brunt and McKusick 1970) that occurs primarily in people of Ashkenazi Jewish descent (central or eastern European) (Slaugenhaupt, Blumenfeld et al. 2001). Estimated carrier frequency is about 1 in 32 to 1 in 18 individuals in this population. Live birth incidence is around 1 in 3600 in American and Israeli Jews (Maayan, Kaplan et al. 1987). The FD genetic screening programs and the recommendations of the American College of Obstetricians and Gynecologists have resulted in a decrease in the number of children born with FD (Lerner 2009). Familial dysautonomia is extremely rare in the general population (Blumenfeld, Slaugenhaupt et al. 1999, Dong, Edelmann et al. 2002, Lehavi, Aizenstein et al. 2003). The disorder is inevitably fatal, with only 50% of patients reaching the age of 30 years (Slaugenhaupt, Blumenfeld et al. 2001). FD affects the development and survival of primary afferent sensory neurons, sympathetic and some parasympathetic neurons (Norcliffe-Kaufmann, Axelrod et al. 2010, Macefield, Norcliffe-Kaufmann et al. 2011, Norcliffe-Kaufmann, Slaugenhaupt et al. 2017, Rubin and Anderson 2017). There are reported FD patients with neuronal deficits in the central nervous system (Mendoza-Santiesteban, Hedges III et al. 2012, Gutiérrez, Norcliffe-Kaufmann et al. 2015). Summarizing, FD individuals have diminished number of myelinated and non-myelinated axons in the sural nerve, reduced presence of nerves in dorsal root ganglia, depletion of axons in the spinal cord, diminished number of superior cervical ganglia and reduced presence of nerve fibers in the layers of epidermis, decreased cardiac and peripherial blood vessels sympathetic innervation, delayed brain stem reflexes and abnormal vestibular reflex responses, as well as retinal degeneration resulting in progressive blindness (Hilz, Axelrod et al. 2004, Goldstein, Eldadah et al. 2008, Mendoza-Santiesteban, Palma et al. 2017, Norcliffe-Kaufmann, Slaugenhaupt et al. 2017, Rubin and Anderson 2017, Gutiérrez, Kaufmann et al. 2018). The disorder mainly affects cells in the autonomic nervous system, which controls involuntary actions like breathing, digestion, tear production, and the regulation of body temperature or blood pressure. The sensory nervous system is also affected

so patients have abnormalities in sense of taste and the perception of pain, heat, and cold (Axelrod 2004).

Early signs and symptoms that appear during infancy include: poor growth, feeding difficulties, poor muscle tone (hypotonia), frequent lung infections, lack of tears, and difficulty in maintaining of the body temperature. Older babies and young children with FD may hold their breath for prolonged periods of time, which may cause a bluish appearance of the skin or lips (cyanosis) or fainting. Walking, speech and other developmental abilities are usually delayed, although some affected individuals show no signs of developmental delay (Brunt and McKusick 1970, Axelrod 2004).

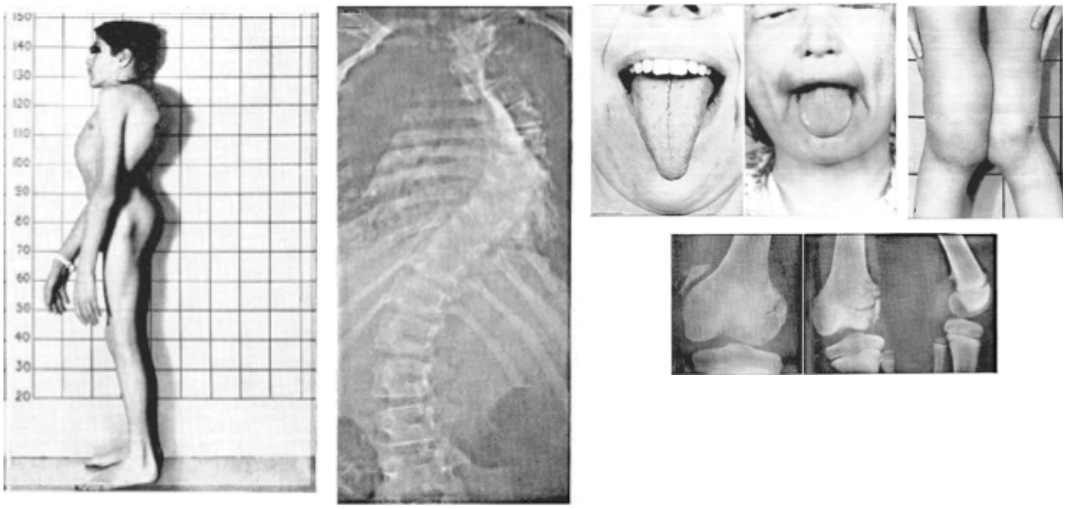

Figure 1.20. Symptoms of FD

Scoliosis in 16-year-old boy; absence of fungiform papillae on a child tongue and a control one; neuropathic knee joints in 11-year-old girl. [Figure adapted from (Brunt and McKusick 1970)]

School-age children can show additional symptoms like bed wetting, episodes of vomiting, reduced sensitivity to temperature changes and pain, poor bone quality and increased risk of bone fractures, poor balance, abnormal curvature of the spine (scoliosis) (Figure 1.20), and also kidney and heart problems. Affected individuals also have poor regulation of blood pressure. They may experience a sharp drop in blood pressure upon standing (orthostatic hypotension), which can cause dizziness, blurred vision, or fainting. They can also have episodes of high blood pressure when nervous or excited, or during vomiting incidents. About one third of children with this disorder have learning difficulties, such as a short attention span

(Brunt and McKusick 1970, Elkayam, Matalon et al. 2006, Gold-von Simson and Axelrod 2006, Norcliffe-Kaufmann, Axelrod et al. 2013).

Adults, which are affected, often have increasing difficulties with balance and walking unaided. Other problems that may appear include lung damage due to repeated infections, impaired kidney function, and worsening vision due to the shrinking size (atrophy) of optic nerves, which carry information from the eyes to the brain (Brunt and McKusick 1970, Gold-von Simson and Axelrod 2006, Norcliffe-Kaufmann, Axelrod et al. 2013). No effective treatment for FD is currently is available.

1.6.1. Mutation of the *IKBKAP* gene in Familial Dysautonomia

Familial Dysautonomia is caused by mutation of the inhibitor of kappa light polypeptide (*IKBKAP*) gene on chromosome 9q31 (Anderson, Coli et al. 2001, Slaugenhaupt, Blumenfeld et al. 2001). The gene codes for the IKK complex-associated protein (IKAP or inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein) (Slaugenhaupt, Blumenfeld et al. 2001). This protein is found in a variety of cells throughout the body, including brain cells, and generally in all eukaryotic organisms (Hawkes, Otero et al. 2002). Although the exact function of the IKAP protein is not clearly understood (Svejstrup 2007), it probably plays a role in transcription and elongation as well as in neuronal development (Close, Hawkes et al. 2006) and myelination during embryogenesis (Cheishvili, Maayan et al. 2007). Researchers have identified the IKAP protein as part of a six-protein complex (called the holo-elongator complex) (Hawkes, Otero et al. 2002) that interacts with enzymes necessary for transcription (Krogan and Greenblatt 2001, Dalwadi and Yip 2018). The Elongator complex is detected in both the nucleus and the cytoplasm. The IKAP protein probably performs other functions in the cell as well, such as responding to stress (Holmberg, Katz et al. 2002), cell migration and adhesion (Johansen, Naumanen et al. 2008, Creppe, Malinouskaya et al. 2009), intracellular trafficking (Rahl, Chen et al. 2005), tRNA modification (Lin, Shen et al. 2013, Karlsborn, Tükenmez et al. 2014), cytoplasmic kinase signalling (Holmberg, Katz et al. 2002) and p53 activation (Cornez, Creppe et al. 2008).

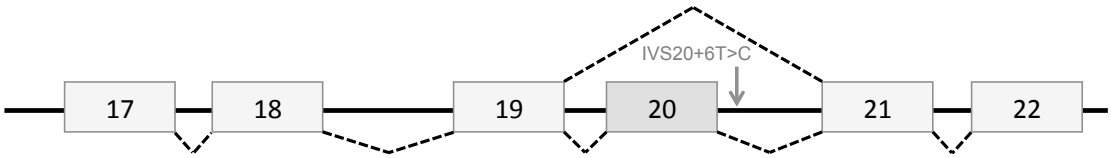

Figure 1.21. Normal and abnormal *IKBKAP* mRNA splicing.

Alternative splicing path is indicated by the upper dashed line. Star represents place of mutation in intron 20 (+6 T to C).

The most common FD-causing mutation occurs in intron 20 (Figure 1.21) of the donor splice site in the IKAP gene (present in 99.5% of patients). The T→C transition (c.2204+6T>C) at position 6 of the intron (single noncoding mutation) affects the donor splice site causing exon 20 skipping (Slaugenhaupt, Blumenfeld et al. 2001). Another less common mutation is a G→C transversion resulting in one amino acid substitution, where proline replaces the wild-type arginine. Only 4 patients have been reported with this change. The result of this mutation is disruption of a phosphorylation of I-kB-associated protein (Slaugenhaupt, Blumenfeld et al. 2001). A missense mutation - change from proline to leucine in exon 26 has also been reported in one non-Jewish patient (Cuajungco, Leyne et al. 2003, Leyne, Mull et al. 2003).

Despite the fact that FD is a recessive disease, homozygous mutant cells express both wild-type (WT) and mutant (MU) *IKBKAP* isoforms, and are therefore, capable of producing full-length functional IKAP protein. Thus, the mutation weakens but does not completely inactivate the 5'ss of exon 20. This suggests that a treatment based on modification of splicing efficiency will have the potential to benefit the vast majority of FD patients.

The not-conserved C nucleotide (c.2204+6T>C) at position +6 in the 5'ss is present in many introns that are spliced normally. The underlying basis causing the splicing defect in FD cases are not clear (Okubo, Horinishi et al. 2002, Yabuta, Shinmura et al. 2002). It could be possible, that this mutation destroyed or modified splicing regulatory elements, leading to mis-recognition of an exon by spliceosome machinery (Pagani, Stuani et al. 2003, Pagani and Baralle 2004). However, in this case exon 20 definition and 5'ss strength are impaired, which results in reduced efficiency of U1 snRNP binding (Carmel, Tal et al. 2004). A report from

2007, revealed that T to C change in intron 20 of the *IKBKAP* gene, decreases the potential number of base-pairs between the donor splice site and U1 snRNA from consensus nine MAGGTRAGT to non-consensus seven CTTGTAAGC and results in the production of an IKAP transcript lacking exon 20 (Ibrahim, Hims et al. 2007). There papers show that the sequences in and around exon 20 contain inherently weak ESS elements (Anderson, Qiu et al. 2003, Ibrahim, Hims et al. 2007). ESSs are insufficiently strong to affect wild-type, when the normal 5'ss is present, but in a single nucleotide change causing the absence of U1 binding they become more prominent and strongly reduce splicing in mutant (Anderson, Qiu et al. 2003). This suggests, that aberrant *IKBKAP* splicing can be corrected by improving base-pairing of the exon 20 5'ss to U1 snRNA (Ibrahim, Hims et al. 2007).

1.6.1.1. Tissue-specific defect

The IKAP skipping mutation is tissue-specific, which means it affects some tissue types more than others, for example brain tissue cells are affected more than white cells in the blood (Figure 1.22). This might explain why a defect in a gene with a ubiquitous expression mainly affects the nervous system (Slaugenhaupt, Blumenfeld et al. 2001, Cuajungco, Leyne et al. 2003). This suggests involvement of tissue-specific splicing factors that regulate splicing of *IKBKAP*. As discussed, regulation of alternative splicing relies on combinatorial interactions between multiple proteins, and that tissue-specific splicing decisions most likely result from differences in the concentration and/or activity of these proteins. There are very few known regulatory proteins that selectively control the splicing of specific genes. The brain-specific NOVA1 protein in mammalians is ubiquitously expressed and modulates splicing of several genes in distinct cell types in the majority of cases (Jensen, Dredge et al. 2000). A recent paper referring to FD, showed a correlation between high levels of NOVA 1 factor and low levels of IKAP protein (Hervé 2016). Splicing factors studies show changes in splicing in FD are tissue-specific (Slaugenhaupt, Blumenfeld et al. 2001).

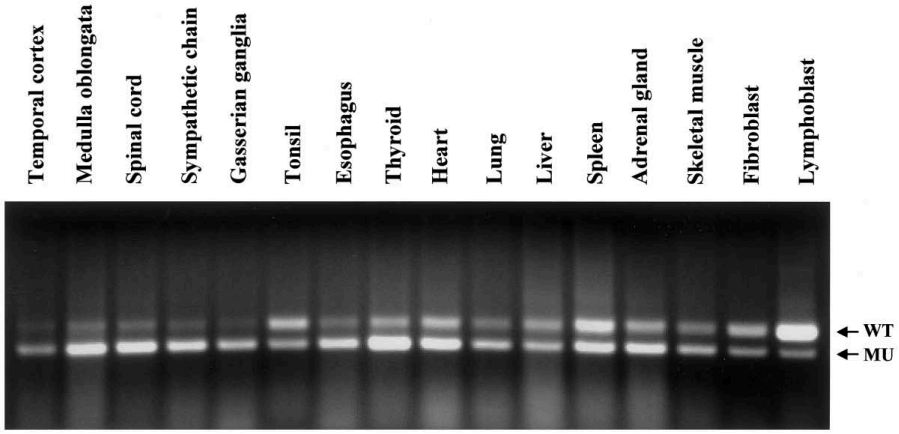

Figure 1.22. Expression of *IKBKAP* mRNA in postmortem FD tissue samples and cell lines.
The highest level of WT transcript in FD lymphoblast lines, lowest in FD nervous system tissues, and intermediate in other tissues. The distinctive reduction of relative WT message in most of the CNS and PNS tissues corresponds to the tissue-specific pathology observed among patients with FD. [Figure adapted from (Brunt and McKusick 1970)]

1.6.2. FD treatments in development

Over the past decade, several compounds have been investigated and documented to improve *IKBKAP* splicing (Slaugenhaupt, Mull et al. 2004, Axelrod, Liebes et al. 2011, Liu, Anderson et al. 2013, Yoshida, Kataoka et al. 2015).
The plant cytokinin kinetin (6-furfurylaminopurine), a dietary supplement, was identified as a potent splicing enhancer for *IKBKAP* in both FD lymphoblast and fibroblast cell lines with the ability to increase the level of wild-type *IKBKAP* transcripts, and IKAP protein to normal levels (Slaugenhaupt, Mull et al. 2004, Hims, Leyne et al. 2007). Kinetin was also tested on a transgenic mouse model expressing human *IKBKAP* gene, showing that mis-splicing can be corrected (Hims, Shetty et al. 2007). Kinetin improves also *IKBKAP* splicing in mouse N2A cells transiently expressing *IKBKAP* from the FD bacterial artificial chromosome (BAC) (Hims, Shetty et al. 2007). One month oral kinetin treatment was tested on FD patients and data demonstrates that also in this case kinetin can positively modify splicing (Axelrod, Liebes et al. 2011).

A reported plant-derived compound was picked by re-purposing pharmaceuticals that have already been approved by the US Food and Drug Administration (FDA) - cardiac glycoside digoxin. Digoxin-mediated inclusion of exon 20 in the *IKBKAP*

transcript is related to suppression of the levels of the splicing factor SRSF3 (Liu, Anderson et al. 2013). It was demonstrated that depletion of SRSF3 in the Hek293-FD minigene-bearing cell line caused a corresponding increase in the production of the exon-20-containing *IKBKAP* transcript (Liu, Anderson et al. 2013).

Phosphatidylserine (PS) is a FDA-approved food supplement able to elevate the amount of wild-type *IKBKAP* mRNA in FD cell lines (Keren, Donyo et al. 2010). Long-term treatment with PS on FD cells led to a significant increase in the amount of IKAP protein. Additionally, oral treatment of knock-in mice with the PS also increased levels of *IKBKAP* mRNA and IKAP protein (Bochner, Ziv et al. 2013). It was demonstrated that PS works though activation of the MAPK/ERK signaling pathway. Despite this, the full mechanism of action is still unknown (Donyo, Hollander et al. 2016).

Screening of chemical libraries identified a small molecule, named RECTAS (rectifier of aberrant splicing) that corrects the abnormal splicing of the *IKBKAP* gene. This molecule was able to increase IKAP protein expression in FD fibroblasts, and to recover the level of 5-carbamoylmethyluridine (ncm^5U), 5-methylcarbonylmethyl-2-thiouridine (mcm^5s^2U), and 5-methoxycarbonyl-hydroxymethyluridine ($mchm^5U$) in wobble positions of tRNAs that are typically reduced in FD patients, implying that the reduced expression of IKAP in patients who have FD results in the down regulation of tRNA modification (Yoshida, Kataoka et al. 2015).

In 2003 δ-tocotrienol was reported to increase transcription of *IKBKAP* mRNA in FD-derived cells. Corresponding increases in the transcripts with end without exon 20 and in levels of functional protein (Anderson, Qiu et al. 2003) was observed. δ-tocotrienol is a member of a group of eight molecules that make up the vitamin E family. It was tested on patients showing its effect on general transcription level (Cheishvili, Maayan et al. 2016). Responses to the treatment differed between the patients.

Down-regulation of the expression of hnRNPA2/B1 (Cáceres, Stamm et al. 1994, Yang, Bani et al. 1994, Fujimoto, Sueoka et al. 2002) and ability of the hnRNPs

to promote selection of intron-distal 5' splice sites (Mayeda and Krainer 1992), is a mechanism of action used by (-)-epigallocatechin gallate (EGCG), a polyphenol, that promotes an increase in the amount of wild-type *IKBKAP* transcript and functional protein in FD-derived cells (Anderson, Qiu et al. 2003). EGCG was tested in combination with δ-tocotrienol, resulting in synergistic production of the complete gene product and functional protein in FD-derived cell lines (Anderson, Qiu et al. 2003).

In 2017, a report evaluated different types of first and second generation proteasome inhibitors: bortezomib and carfilzomib, both FDA-approved drugs used in clinics for cancer treatment, as well as epoxomicin, that is used for *in vitro* proteasome inhibition (Hervé 2017). Cells treatment with these drugs favourably modified the *IKBKAP* transcript profile and induced a significant increase in the IKAP protein levels. The mechanism behind this observation is unclear, but can be related with interconnection between 26S proteasome and the spliceosome machinery (Bellare, Small et al. 2008, Bieler, Hammer et al. 2012) including SR proteins and hnRNPs as splicing factors (Moulton, Gillooly et al. 2014, Koumbadinga, Mahmood et al. 2015).

Despite the apparent effectiveness, none of the described compounds is selective for *IKBKAP* gene. They were not designed to target solely the region of exon 20, and this can mean high risks of off-targets effects.

1.7. Characteristic and therapeutic usage of adeno-associated virus vectors in gene therapy

Gene therapy applies viral and nonviral delivery systems. The first one includes vectors developed from retrovirus (RV), lentivirus (LV), herpes simplex virus (HSV), adenovirus (AdV) and adeno-associated virus (AAV) (Gould and Favorov 2003). The second system consists of naked DNA delivery and lipid or poly(ethylene glycol) (PEG)-assisted systems (Gould and Favorov 2003, Minato, Iwanaga et al. 2003, Omori, Maruyama et al. 2003, Lu 2004).

All the mentioned current methods of gene delivery have some limitations. The type of used vector is dictated by the need. For only short time expression of the gene (like in case of toxic gene products in cancer therapies) adenoviral vectors are ideal. However, for most genetic diseases, sustained expression is needed. In this case vector able to integrate, but not causing immunological problems would be suitable. Combination of synthetic delivery systems with properties of viral vectors would be desire. Such vector should have an easy and reproducible production system, high concentration during production (>10^8 viral particles / ml), ability of integration into a specific location in the host chromosome or to be stable episome, a transcriptional unit responding to manipulations, ability to target the expected cells, no effect on immune system. Although currently there is no this good vector, the vector based on recombinant AAV (rAAV) is one of those that are closest to the ideal one (Lu 2004).

1.7.1. The structure of AAV and life cycle

Adeno-associated virus is a small (25-nm), nonenveloped virus that packages a linear single-stranded DNA genome (4680 bp) with molecular weight of 5.5–6.2 million Daltons (MDa) (Muzyczka and Berns 2001). It belongs to the *Parvoviridae* family and is placed in the genus *Dependoparvovirus* what means that for the infection by AAV occurs only in the presence of helper virus, adenovirus or herpesvirus (Daya and Berns 2008). AAV genome consists of two open reading frames, Rep and Cap. They are flanked by two 145 bp inverted terminal repeats (ITRs), which are GC rich and capable of forming a palindromic structure, located at both the 5' and 3'-ends (Muzyczka and Berns 2001). ITR contains important *cis*-active sequences for the biology of AAV. The main role of ITRs concerns about the AAV DNA replication, they allow for synthesis of the complementary DNA strand. The left ORF – Rep gene – produces four proteins: Rep78, Rep68, Rep52, Rep40 - required for the AAV life cycle (Flotte 2004). All Rep proteins possess helicase and ATPase activity. The right ORF – Cap gene – produces three viral capsid proteins: VP1, VP2 and VP3, that are present in 1:1:10 ratio, respectively (Grieger, Johnson et al. 2007).

The infectious pathway of AAV vectors involves several steps. First, the viral particle attaches to the cell surface through binding to the receptor and co-receptors. At the next step, the viral particles penetrate the nuclear membrane and virus can replicate in the nuclear plasma (lytic pathway). For this necessary is helper virus (adenovirus (Myers, Laughlin et al. 1980) or herpesvirus (Handa and Carter 1979)) or genotoxic agents (UV irradiation or hydroxyurea) (Yalkinoglu, Heilbronn et al. 1988, Yakobson, Hrynko et al. 1989, Matsushita, Elliger et al. 1998). The lytic phase is initiated when cell containing AAV is infected also with a helper virus. AAV undergoes productive infection characterized by genome replication, viral gene expression, and virion production. When the DNA of AAV is relased into the nucleus, its single strand is converted into double strand. This initiates replication, described as unidirectional strand displacement mechanism (Cotmore and Tattersall 2005). Self complementary ITR anneals together and creates secondary harpin structure with free 3' hydroxyl group. Formed structure works as replication primer for unidirectional synthesis of secondary strand with the host cellular replication machinery (Li, Samulski et al. 1997). The ITR replication is complited by Rep78 and Rep68 that bind to the RBS leading to regeneration of 3' hydroxyl group (Brister and Muzyczka 2000). Co-infection with helper virus is necessary for viral promoter activation and transcription initiation of Rep and Cap genes (Lackner and Muzyczka 2002). Next, helper virus induces cell lysis, and the newly assembled AAV is released (Balakrishnan and R Jayandharan 2014).

In the absence of helper viruses, the replication of AAV is limited, viral gene expression is repressed, and the AAV genome can establish latency by integrating into a 4-kb region on chromosome 19 (q13.4) (lysogenic pathway), region named adeno-associated virus integration site 1 (AAVS1). Tissue culture experiments suggest that this is a safe integration site, and the frequency of integration is estimated to be 0.5% (McCarty, Young Jr et al. 2004, Mehrle, Rohde et al. 2004). The earliest evidence for the integration was shown in human bone marrow derived fibroblast-like Detroit 6 cells. Infection with AAV2 demonstrated the persistence of the viral DNA for ~47 passages, with a genome copy number between 3-5 per diploid cellular genome (Berns, Pinkerton et al. 1975). Study of integration sites for wild type AAV2 in human genome has revealed other integration positions – in chromosome 5 at position p13.3 (AAVS2) and in chromosome 3 at position

p24.3 (AAVS3) (Hüser, Gogol-Döring et al. 2010). Sequence around AAVS1 contains CpG sites (cytosine nucleotide followed by a guanine nucleotide) and tandem GCTC repeat elements, which serve as binding sites for two Rep proteins (Rep78 and Rep68). Additionally, AAVS1 contains a Rep specific nicking site called *trs* (Weitzman, Kyöstiö et al. 1994). Rep proteins stabilize binding to GCTC site and mediate formation of the complex between AAVS1 site and the ITR. Random integration of AAV DNA into the host genome is detectable but occurs at very low frequency (Daya and Berns 2008). Both lytic and lysogenic pathways are distinct and interchangeable (Lu 2004).

In contrast to the wild-type AAV, in the absence of helper virus, rAAV vector genomes do not integrate into chromosome 19 in human cells *in vitro* and persist in episomal form in animal models *in vivo* (Penaud-Budloo, Le Guiner et al. 2008). In order to produce recombinant AAV for gene therapy, the Rep and Cap genes have to be replaced with a transgene. This replacement deprives the virus of the ability to integrate (Verma and Somia 1997). In mice, the AAV genome has been observed persisting for long periods of time in quiescent tissues, such as skeletal muscles, in episomal form (Duan, Sharma et al. 1998). Functional rAAV genomes were present in nonhuman primates several years after one single intramuscular injection. Especially in non-dividing cells like skeletal muscles. rAAV episomals were remarkable stable and persist as supercoiled monomeric and concatemeric circles, in a chromatin-like structure (Rivera, Gao et al. 2005, Penaud-Budloo, Le Guiner et al. 2008). However, the precise mechanism of how AAV vector episomes persist and maintain long-term expression of a therapeutic transgene in tissues is still unclear.

1.7.2. AAV serotypes and tropism

AAV serotypes were isolated as contaminants of adenovirus preparations from primates and other species (e.g., avian and bovine). Currently, there are 12 serotypes and 108 new isolates (serovars) of AAV reported and recognized by the International Committee for Taxonomy of viruses (Gao, Alvira et al. 2002, Gao, Vandenberghe et al. 2004). They include: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7,

AAV8, AAV9, AAV10, AAV11, avian AAV, bovine AAV, canine AAV, equine AAV, ovine AAV and others (Flotte 2004, Balakrishnan and R Jayandharan 2014). AAV serotypes 2, 5, 6 and 9 were originally isolated from human clinical specimens. AAV6 is believed to form from the recombination of AAV2 and AAV1. AAV serotypes 1, 3, 4, and 7, 8, 10, 11 have nonhuman primate origins. AAV1 and 4 were originally found from simian sources, and AAV7 and 8 were isolated from rhesus monkey (Lu 2004), AAV9 was isolated from human tissues (Gao, Vandenberghe et al. 2004). The general organization of the parvovirus genome is conserved across the different serotypes, with a similar configuration of replication and structural genes, although there are some differences in transcription profiles (Weitzman and Linden 2012).

All AAV serotypes differ in their tropism or types of cells they infect. This is caused by presence of different cellular receptors and co-receptors. This makes AAV a very useful system, used for various diseases, able to preferentially transduce specific cell types. The chart below gives a summary of the tropism of some AAV serotypes.

AAV1	muscle, heart, ocular, CNS
AAV2	CNS, kidney, muscle, testes, liver, ocular
AAV4	lung, CNS
AAV5	lung, CNS, ocular, pancreas
AAV6	lung, heart, muscle
AAV7	muscle, liver
AAV8	liver, muscle, ocular, CNS, heart, pancreas
AAV9	lung, liver, muscle, heart, CNS, kidney, testes

1.7.3. Therapeutic usage of AAV vectors

First attempts to apply AAV as a delivery vector (AAV2) to transduce different mammalian cell cultures started in the mid-1980s. However, it turned out quickly that AAVs are more efficient *in vivo* than *in vitro*, especially when applied on terminally differentiated cells or non-dividing cells, such as muscle fibers, neuronal cells, retinal cells, airway epithelial cells, and hepatocytes (Lu 2004).

To date, AAV vectors have been used in over 117 clinical trials worldwide. Most of the trials are Phase I (study for the safety). One of the first among those was Cystic fibrosis where AAV was delivered into the lungs by bronchoscope or aerosol (Flotte, Carter et al. 1996, Moss, Rodman et al. 2004). Early trials showed lack of measurable toxicity and a very modest immune response, connected with increase in the levels of interleukin-10 and decrease in levels of interleukin-8. Promising results have been obtained from Phase 1 and Phase 2 trials for Hemophilia B (Nathwani, Tuddenham et al. 2011). Initial studies were performed by the intramuscular injection of an AAV2, and with AAV8 in next phases. AAV vectors were also used in Leber's congenital amaurosis (Bainbridge, Smith et al. 2008, Hauswirth, Aleman et al. 2008, Maguire, Simonelli et al. 2008), congestive heart failure (Jessup, Greenberg et al. 2011), spinal muscular atrophy (AveXis. Data from ongoing study of AVXS-101 in spinal muscular atrophy type 1 presented at World Muscle Congress), lipoprotein lipase deficiency (Libby and Wang 2014), X-linked adrenoleukodystrophy (Aubourg, Hacein-Bey-Abina et al. 2011), β-thalassaemia (Cavazzana-Calvo, Payen et al. 2010) and Parkinson's disease (LeWitt, Rezai et al. 2011).

1.7.4. Immune response to AAV vectors

For the successful gene therapy there are at least three steps that must be taken into the consideration. The first one is gene that has to be transferred, second – the target tissue, and the third one – vector. The active agent of the therapy is the gene, but it is the vector that in most cases is critical determinant of therapeutic success. The biggest challenge facing gene delivery with AAV is host immune response (Daya and Berns 2008). *In vivo* administration of a viral vector it risks causing immune response to foreign antigens (Mingozzi and High 2013). This response can be directed against the vector components or transgene, or both. Host immunity functions as a cell-mediated response and humoral immunity. First one involves cytotoxic T cells that are able to eliminate the transduced cells. The other immunity mechanism bases on production of neutralizing antibodies (Nab), that prevent readministration of vector (Daya and Berns 2008).

The main response to AAV follows a humoral pathway (Xiao, Li et al. 1996). Big problem can be preexisting Nab, from prior infection. In AAV trials for Cystic fibrosis 96% of patients and healthy subjects had antibodies, and 32% showed neutralizing ability in an *in vitro* experiments (Chirmule, Propert et al. 1999). This limits AAV transduction abilities in liver (Murphy, Li et al. 2008) and lung (Halbert, Standaert et al. 1998). However, studies show, that humoral response can be T-cell dependent. Anti-CD4 antibodies inhibit T-cells, and this results in limited formation of Nab, allowing vector readministration (Halbert, Standaert et al. 1998, Manning, Zhou et al. 1998, Chirmule, Xiao et al. 2000).
Cell-mediated response to AAV is mainly dependent from the AAV serotype (Wang, Figueredo et al. 2007) and administration protocol (Brockstedt, Podsakoff et al. 1999). Strong immune response was seen against AAV-ovalbumin after intraperitoneal, intravenous and subcutaneous injection, but not after intramuscular. Other then, AAV2 vectors that induce a weak cell-mediated immune response (Zhang, Cortez et al. 2007).

Experiments with animal models were very successful in prediction immunological response to the transgene product, but were insufficient to predict response against viral vectors (Manno, Pierce et al. 2006, Mingozzi, Maus et al. 2007). AAV treatments on Hemophilia B patients reviled connection between an immune response against AAV-transduced hepatocytes and vector doses (George, Sullivan et al. 2017). In this study, AAV vector expressing a high-specific-activity factor IX variant was administered at low doses to drive therapeutic factor IX expression and minimize the risk of an AAV capsid immune response. Any low incidence of capsid-directed immune response was controllable with oral glucocorticoids. Interestingly, two patients who showed signs of immune response also had the most rapid initial rise in factor IX coagulation activity (George, Sullivan et al. 2017).
This study brings a question, whether the immune response is able to facilitate vector expression and modulate therapeutic outcome. These early results require confirmation and further studies to better understand human immune response to AAV vectors.

The AAV treatments can result also in toxic effects. Liver toxicity was observed in clinical trials for Hemophilia B and was connected with cytotoxic T-lymphocytes (CTL) against AAV2 transduced hepatocytes (Manno, Pierce et al. 2006). Further studies revealed that T-cells activation was caused by the AAV2 capsid heparin binding motif (Vandenberghe, Wang et al. 2006).

Additionally, toxicity was very recently reported on the example of nonhuman primates and piglets administrated with AAV vectors expressing human SMN. In this trial it was observed acute systemic inflammation, coagulation defects and hepatic toxicity with damage of the hepatocytes at high dosage of AAV9 vectors (Hinderer, Katz et al. 2018).

Ongoing trial of high dose systemic AAV administration for SMA patients, describes clinically asymptomatic transaminase elevations (Mendell, Al-Zaidy et al. 2017). This can be early sign of systemic inflammation, caused by AAV toxicity (Hinderer, Katz et al. 2018).

2. AIM OF THE THESIS

Processing of the pre-mRNA is a complex pathway with many levels of regulation, so it should not be surprising that mutations affecting splicing signals, can cause aberrant splicing and finally trigger genetic diseases. Alternative splicing is a key regulator in gene expression. It generates numerous transcripts based on a single protein-coding gene and largely increases the use of genetic information. More than a quarter of alternative exons introduce premature stop codons in their mRNAs. This can result either in the formation of truncated proteins or in the degradation of the mRNA in nonsense-mediated decay.

Familial Dysautonomia is a syndrome caused by mutations in a 5' splice site that affects pre-mRNA processing of *IKBKAP* gene and provokes aberrant splicing. Till now, the available treatments for FD are only supportive and based on the attenuation of the symptomatology. In the last few years, several compounds were tested in order to correct *IKBKAP* splicing however, none of them is specifically designed to target this gene, which can result in many off-target effects.

A novel therapeutic approach based on modification of U1 snRNA - ExSpeU1s was developed in our laboratory. Those molecules bind downstream 5' splice site and recovers exon skipping, caused by different types of mutations. By targeting specific intronic sequence molecule becomes exon-specific and decrease possible off-targets. Taking into account, that *IKBKAP* splicing is altered due to a weak definition of exon 20 as well as the presence of regulatory elements in the sequence, the usage of modified U1 binding in non-conserved position could be a very valuable tool in FD therapy development.

In this thesis, I considered the c.2204+6T>C change in *IKBKAP* gene causing FD and evaluated the ExSpeU1 strategy to correct splice site mutation associated with FD.

Aims of the thesis:

- developing ExSpeU1s to rescue aberrant skipping of *IKBKAP* exon 20
- investigating the role of *cis*- and *trans*-acting factors involved in *IKBKAP* splicing and their relation to mechanism of ExSpeU1 action
- estimation of the potential therapeutic effect of ExSpeU1s in *ex vivo* and *in vivo* FD models

3. MATERIALS AND METHODS

3.1. Chemical reagents

General chemicals and kits were purchased from Sigma-Aldrich (St. Louis, MO, USA), Merck KGaA (Darmstadt, Germany), Thermo Fisher Scientific (Waltham, MA, USA), Roche (Basel, Switzerland), Qiagen (Hilden, Germany), Agilent Technologies (Santa Clara, CA, USA), and Promega (Madison, WI, USA).

3.2. Standard solutions

All solutions are identified in the text except for the following:

- 5x TBE: 53 g Tris-HCl, 27.5 g Boric acid, 20 ml 0.5 M EDTA, pH 8.0 in 1 l
- 6x DNA sample buffer: 0.25% w/v Bromophenol blue, 0.25% w/v Xylene Cyanol FF, 30% v/v glycerol in H_2O
- 5x Running buffer: 30 g Tris-HCl, 144 g glycine, 5 g SDS in 1 l
- 10x Blotting buffer: 30 g Tris-HCl, 144 g glycine, 20% methanol in 1 l
- 10x Protein sample buffer: 20% w/v SDS, 1 M DTT, 0.63 M Tris-HCl (pH 7.0), 0.2% w/v bromophenol blue, 20% v/v glycerol, 10 mM EDTA (pH 7.0)
- 1x PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4

3.3. Bacterial culture

The *Escherichia coli* K12 strain DH5α was transformed with the plasmids described in this study and used for their amplification. Plasmids were maintained as single colonies on agar plates at 4°C. Bacteria were amplified by an overnight incubation in Luria-Bertani medium (LB medium: 10 g BD Bacto™ Tryptone (Difco Laboratories, Becton Dickinson & Co, Franklin Lakes, NJ, USA), 5 g Oxoid™ Yeast Extract Powder (Thermo Fisher Scientific), 10 g NaCl, pH 7.5 in 1 l). Bacterial growth media were sterilized before use by autoclaving. Then ampicillin (Sigma-Aldrich) was added to the media at a final concentration of 100 µg/ml.

3.4. Preparation of bacterial competent cells

Bacterial competent cells were prepared following the method described by Chung et al. (Chung, Niemela et al. 1989). *Escherichia coli* strains were grown overnight in 10 ml of LB at 37°C. The following day, 150 ml of fresh LB were added and the cells were grown in the shaker at room temperature until the OD600 was 0.3-0.4. Then the cells were put on ice to stop the growth and centrifuged at 4°C, 1000 g for 1 hour. The pellet was resuspended in 1/10 volume of cold 1x TSS solution (10% w/v PEG 4000, 5% v/v DMSO, 35 mM $MgCl_2$, pH 6.5 in LB medium). The cells were aliquoted, rapidly frozen in liquid nitrogen and stored at -80°C. Competence was determined by transformation with 0.1 ng of pUC19 control DNA plasmid and was deemed satisfactory if this procedure resulted in more than 100 colonies.

3.5. Transformation of bacteria

Transformation of ligation reaction was performed using half of the initial reaction volume (10 µl). Transformation of positive clones was carried out using 1 ng of the DNA plasmid. The DNA was incubated with 65 µl of competent cells for 30 minutes on ice, followed by a heat shock at 42°C for 30 seconds and finally the cells were placed again on ice for 1 minute and then spread onto agarose plates containing the appropriate antibiotic concentration (100 µg/ml of ampicillin).
The plates were then incubated for 12-15 hours at 37°C. When DNA inserts were cloned into pUC19, 30 µl of IPTG 100 mM and 30 µl of X-Gal (4% v/v in dimethylformamide) were spread onto the surface of the agarose before plating to facilitate screening of positive clones (white colonies) through identification of β-galactosidase activity (blue colonies).

3.6. DNA preparation

3.6.1. Small scale preparation of plasmid DNA from bacterial cultures

Single bacterial colony was picked and transferred into 10 ml of LB medium containing 100 µg/ml of ampicillin. The culture was incubated overnight at 37°C in a shaking incubator. The Plasmid DNA purification kit (Macherey-Nagel, Düren, Germany) was used according to the manufacturer's instructions in order to obtain small amounts of pure plasmid DNA. The final pellet was resuspended in 50 µl of Nuclease-Free water. The quality of extracted plasmid DNA was verified on 0.8% agarose gels. The DNA was stored at -20°C. Routinely 3 µl of such DNA preparation were taken for the restriction enzyme digestion and 20 µl were taken for sequence analysis (GATC Biotech, Konstanz, Germany).

3.6.2. Medium scale preparation of plasmid DNA from bacterial cultures

For medium-scale preparations (Midiprep) of plasmid DNA that was necessary for the transfection experiments, NucleoBond Xtra Plasmid DNA Purification Kit (Macherey-Nagel) was used according to the manufacturer's instructions. In order to get a good amount of plasmid DNA, an overnight bacterial culture of 100 ml of LB medium was used.

3.7. Enzymatic modification of DNA

3.7.1. Restriction enzymes

Restriction enzymes were purchased from New England BioLabs Inc. (NEB, Ipswich, MA, USA). All buffers were also supplied by the same company and were used according to the manufacturer's instructions. 0.5x, 1x or 2x concentration of 10x OPA buffer (100 mM Tris-acetate, pH 7.5, 100 mM magnesium acetate,

500 mM potassium acetate) were used. For analytical digests 300 ng of DNA were digested in a volume of 20 µl containing the appropriate units (U) of the restriction enzyme per µg of DNA. The digestion was incubated 2 hours at the optimal temperature required by the enzyme used. Preparative digestions of vectors and inserts were made of 3 µg DNA using the appropriate condition needed by the restriction enzyme in 50 µl reaction volume. Enzymatic activity was then removed by heat inactivation.

3.7.2. Restriction enzymes DNA Polymerase I, Large (Klenow) Fragment T4 Polynucleotide Kinase

These enzymes, provided by NEB, were used to treat PCR products for blunt-end ligation during construction of recombinant plasmids.

The Large Fragment of DNA Polymerase I (Klenow) is a proteolytic product of *E. coli* DNA Polymerase I, which retains polymerization and 3' → 5' exonuclease activity, but has lost 5' → 3' exonuclease activity. This was useful for digesting specific residues added by Taq DNA Polymerase at the 3' terminus to create a compatible end for ligation. Briefly the DNA was dissolved in 1x NEBuffer 2.1 and supplemented with 25 µM dNTPs. 1 U of Klenow per µg DNA was added and the mixture (final volume 20 µl) was incubated 10 minutes at room temperature. The reaction was inactivated by heating at 70°C for 20 minutes.

T4 Polynucleotide Kinase catalyses the transfer and exchange of phosphate from ATP to the 5'-hydroxyl terminus of ds/ssDNA and RNA. It was useful for the addition of 5'-phosphate to PCR products to allow subsequent ligation. The proper Us of Kinase, its reaction buffer (70 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM Dithiothreitol (DTT), pH 7.6) and ATP 10 mM were added to the DNA and incubated at 37°C for 30 minutes. The enzyme was inactivated by incubation at 65°C for 20 minutes.

3.7.3. T4 DNA Ligase

T4 DNA Ligase from Promega, catalyses the joining of two strands of DNA between the 5'-phosphate and the 3'-hydroxyl groups of adjacent nucleotides in either a cohesive-ended or blunt-ended configuration. 20-40 ng of linearized vector were ligated with a 5-10 fold molar excess of insert in a total volume of 20 µl containing 1x ligase buffer and 1 U of enzyme. Reaction was carried out at room temperature for 3 hours.

3.7.4. Alkaline Phosphatase, Calf Intestinal (CIP)

Calf Intestinal Phosphatase (CIP), obtained from NEB, catalyses the removal of 5'-phosphate groups from DNA and RNA. Since CIP-treated fragments lack the 5'-phosphoryl termini required by ligases, they cannot self-ligate. This property can be used to decrease the vector background in cloning strategies. The standard reaction was carried out in a final volume of 50 µl using 0.5 U of enzyme per 1 µg DNA at 37°C for 30 minutes. The enzyme was inactivated by incubation at 85°C for 15 minutes.

3.8. Agarose gel electrophoresis of nucleic acids

DNA sample were size fractionated by electrophoresis in agarose gels ranging in concentration from 0.8% w/v (large fragments) to 3% w/v (small fragments). The gels contained ethidium bromide (0.5 µg/ml) in 1x TBE solution. Samples containing 1x DNA Loading Buffer (0.4% bromophenol blue, 60% glycerol, water) were loaded into submerged wells. Horizontal gels were used for fast analysis of DNA restriction enzyme digestions, estimation of DNA concentration, or DNA fragment separation prior to elution from the gel. A fast analysis of RNA samples was obtained by running samples on 0.8% agarose gels. The gels were electrophoresed at 50-90 mA in 1x TBE running buffer for a time depending on the expected fragment length and gel concentration. Sample was visualized by UV transillumination and the result recorded by digital photography.

3.9. Elution and purification of DNA fragments from agarose gels

The following protocol was applied for purification of both vectors and inserts of DNA for cloning strategies. The DNA samples were electrophoresed onto an agarose gel as previously described. The DNA was visualized with UV light and the DNA fragment of interest was excised from the gel and the QIAquick Gel Extraction Kit (Qiagen), was used for extraction according to the manufacturer's instruction. 30-50 µl of DNA was obtained: the amount of recovered DNA was estimated by UV fluorescence of intercalated ethidium bromide in agarose gel electrophoresis.

3.10. Amplification of selected DNA fragments

The polymerase chain reaction (PCR) was performed using both genomic and plasmid DNA as templates and following the basic protocol of Roche or NEB Taq DNA polymerase. The volume of the reaction was 25 µl or 50 µl and comprised: 1x Taq buffer, dNTPs mix (100 µM each), oligonucleotide primers (100 nM each), Taq DNA polymerase (2.5 U) and 100 ng of genomic DNA or 0.1 ng of plasmid DNA. The synthetic DNA oligonucleotides used for PCR amplification were purchased from Sigma-Aldrich and from Integrated DNA Technologies (Coralville, IA, USA). The standard amplification conditions were the following: 94°C for 5 minutes (initial denaturation), 94°C for 45 seconds (denaturation), 56°C for 45 seconds (annealing), 72°C for 45 seconds (extension) for 28-35 cycles and 72°C for 10 minutes (final extension). PCRs were optimized to be in the exponential phase of amplification and products were fractionated on agarose gel. The PCR reactions were performed on a Gene Amp PCR System (Applied Biosystem, Foster City, CA, USA).

3.11. Sequence analysis for cloning purpose

Sequence analyses of plasmid DNA were performed in GATC Biotech. 5 µl of DNA samples (100 ng/µl) in 1.5 ml tubes were shipped to company. The primers used for sequencing are either primers specific for plasmid vectors (universal primers) or primers binding to the cloned sequence that were added to DNA samples (5 µl of primer at concentration of 5 pmol/µl for each sample). The results of sequencing were analysed using the SnapGene software (GSL Biotech LLC, Chicago, IL, USA).

3.12. Construction of the minigene systems for splicing studies

Minigene reporters are widely used and well characterized systems for studying alternative splicing and *cis*- and *trans*-acting elements that influence the splicing process, to understand the cell-specific splicing pattern, to identify exonic and intronic elements that enhance or silence splicing and to determine whether a specific mutation can compromise the splicing and to establish the role of the splice sites in the exon recognition. They usually contain few exons and introns of the gene of interest and after transient transfection and RNA analysis, allowed us to study the splicing outcome, which can be tracked by semiquantitative (reverse transcription polymerase chain reaction (RT-PCR)) or quantitative (real-time polymerase chain reaction (qPCR)) methods.

3.12.1. pTB-*IKBKAP* hybrid minigene

To prepare the *IKBKAP* WT minigene, a genomic region spanning from intron 18 to intron 21 was used as a template for cloning. Human DNA was amplified with the IKAP-int18-NdeI_dir and IKAP-int21-NdeI_rev primers. The amplified fragment of 2157 bp includes the last 363 bp of intron 18, full-length of sequence from exon 19 till end of intron 21. The PCR product was digested with NdeI and cloned in the NdeI site into previously described pTB-NdeI minigene (Pagani, Buratti et al. 2003) under the SV40 promoter (Figure 3.1). *IKBKAP* IVS20+6T>C minigene was

created by site-directed mutagenesis of *IKBKAP* WT minigene using the QuikChange Site-directed Mutagenesis Kit II (Agilent Technologies) according to the manufacture's instructions. All minigene constructs were verified by DNA sequencing.

IKAP-int18-NdeI_dir	5'-GCGCATATGGCCAACTTATTTTCT-3'
IKAP-int21-NdeI_rev	5'-TGTCCTTTTTTTGGTAGCATATGGTCG-3'

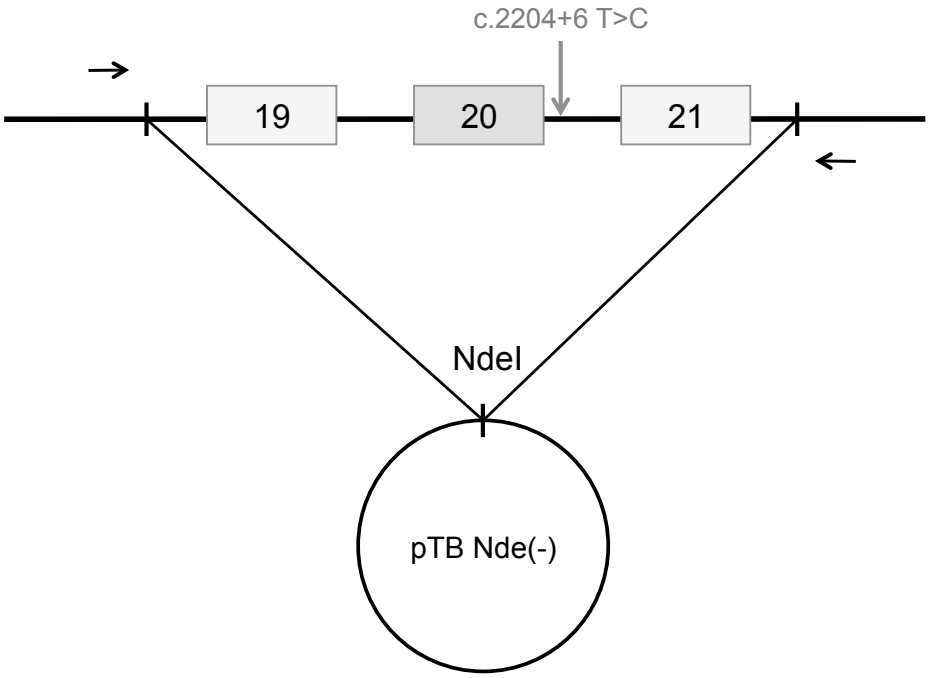

Figure 3.1. Schematic representation of pTB-*IKBKAP* minigene.

A DNA fragment of 2157 bp containing part of the human *IKBKAP* gene spanning from exon 19 till exon 21 (grey boxes), with intronic flanking regions (black line) was cloned in the unique NdeI restriction site of the pTB-Nde(-) minigene. Primers used for the amplifications (black arrows), the cloning restriction sites and the position of the mutation are indicated.

3.12.2. pcDNA3.1-TOPO-*IKBKAP* minigene deletion constructs

The WT-MG, FD-MG and all deletions mutants were created at Center for Human Genetic Research, Massachusetts General Hospital in Boston, MA, USA in the laboratory of S. A. Slaugenhaupt (Slaugenhaupt, Mull et al. 2004, Hims, Leyne et al. 2007). *IKBKAP* genomic DNA was amplified from an unaffected and an FD individual using primers in exon 19 and 21. The amplified products were cloned into pcDNA3.1/V5-His TOPO (Thermo Fisher Scientific) (Figure 3.2) and sequenced for verification.

EX19F	5'- CATTACAGGCCGGCCTGAG -3'
EX21R	5'- CAGCTTA GAAAGTTACCTTAG -3'

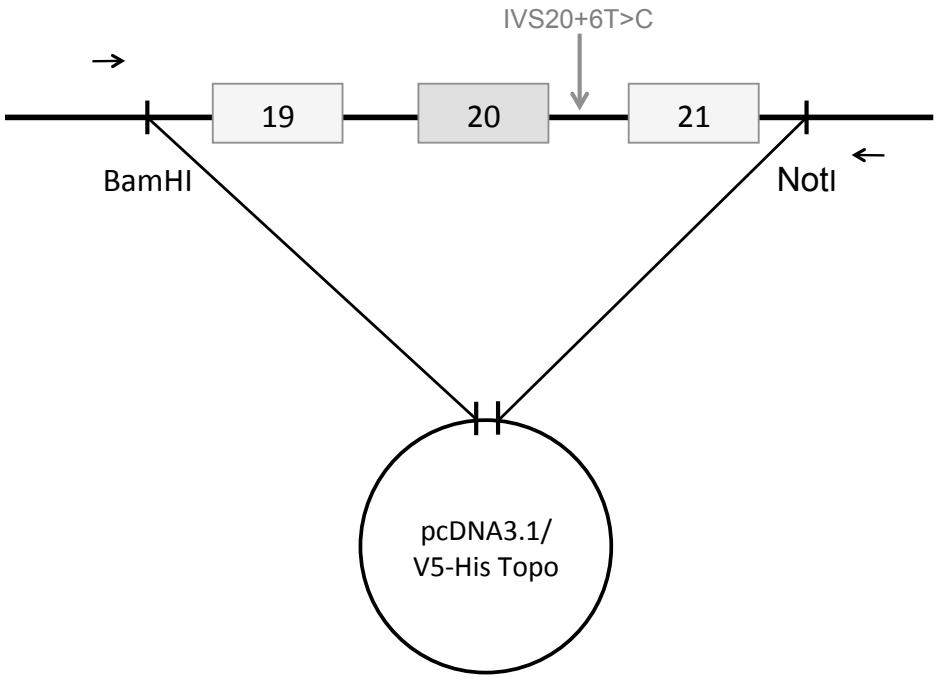

Figure 3.2. Schematic representation of pcDNA3.1-TOPO-*IKBKAP* minigene.
A DNA fragment of 1721 bp containing part of the human *IKBKAP* gene spanning from exon 19 till exon 21 (grey boxes), with intronic flanking regions (black line) was cloned in the pcDNA3.1/V5-His TOPO minigene using BamHI and NotI unique sites. Primers used for the amplifications (black arrows), the cloning restriction sites and the position of the mutation are indicated.

3.12.3. Expression vector for generation different ExSpeU1s

The vector used for the production of exon-specific U1s (ExSpeU1s) snRNAs is pSP64, which is a standard cloning vector. The wild-type U1 snRNA gene was cloned with BamHI restriction site into the vector (Figure 3.3). ExSpeU1s were created by replacing the sequence between the BclI and BglII sites with the oligonucleotides, as previously reported (Pagani, Buratti et al. 2002, Alanis, Pinotti et al. 2012). The sequences of PCR primers are the following:

U1IK$_{4}$_dir	5'-GATCTCATAAATGGCGCTGCAGGGGAGATACCAT-3'
U1IK$_{4}$_rev	5'-GATCATGGTATCTCCCCTGCAGCGCCATTTATGA-3'
U1IK$_{10}$_dir	5'-GATCTCATAGCAAACAGTACAATGCAGGGGAGATACCAT-3'
U1IK$_{10}$_rev	5'-GATCATGGTATCTCCCCTGCATTGTACTGTTTGCTATGA-3'
U1IK$_{15}$_dir	5'-GATCTCATAGCAAACAGTGCAGGGGAGATACCAT-3'
U1IK$_{15}$_rev	5'-GATCATGGTATCTCCCCTGCACTGTTTGCTATGA-3'
U1IK$_{34}$_dir	5'-GATCTCATACTTGTGATTTATGCAGGGGAGATACCAT-3'
U1IK$_{34}$_rev	5'-GATCATGGTATCTCCCCTGCATAAATCACAAGTATGA-3'
U1IK$_{46}$_dir	5'-GATCTCATAGTGTGAAGACAATGCAGGGGAGATACCAT-3'
U1IK$_{46}$_rev	5'-GATCATGGTATCTCCCCTGCATTGTCTTCACACTATGA-3'
U1IK$_{57}$_dir	5'-GATCTCATAATAAGTATTTTATGCAGGGGAGATACCAT-3'
U1IK$_{57}$_rev	5'-GATCATGGTATCTCCCCTGCATAAAATACTTATTATGA-3'
U1IK$_{72}$_dir	5'-GATCTCATACAATTTCGAGGCAGGGGAGATACCAT-3'
U1IK$_{72}$_rev	5'-GATCATGGTATCTCCCCTGCCTCGAAATTGTATGA-3'
U1IK$_{77}$_dir	5'-GATCTCATATCGAGAACTTATGCAGGGGAGATACCAT-3'
U1IK$_{77}$_rev	5'-GATCATGGTATCTCCCCTGCATAAGTTCTCGATATGA-3'
U1IK$_{99}$_dir	5'-GATCTCATAAGCCCTCATTACGCAGGGGAGATACCAT-3'
U1IK$_{99}$_rev	5'-GATCATGGTATCTCCCCTGCGTAATGAGGGCTTATGA-3'
U1IK$_{123}$_dir	5'-GATCTCATATCAGATTCTTAGGCAGGGGAGATACCAT-3'
U1IK$_{123}$_rev	5'-GATCATGGTATCTCCCCTGCCTAAGAATCTGATATGA-3'

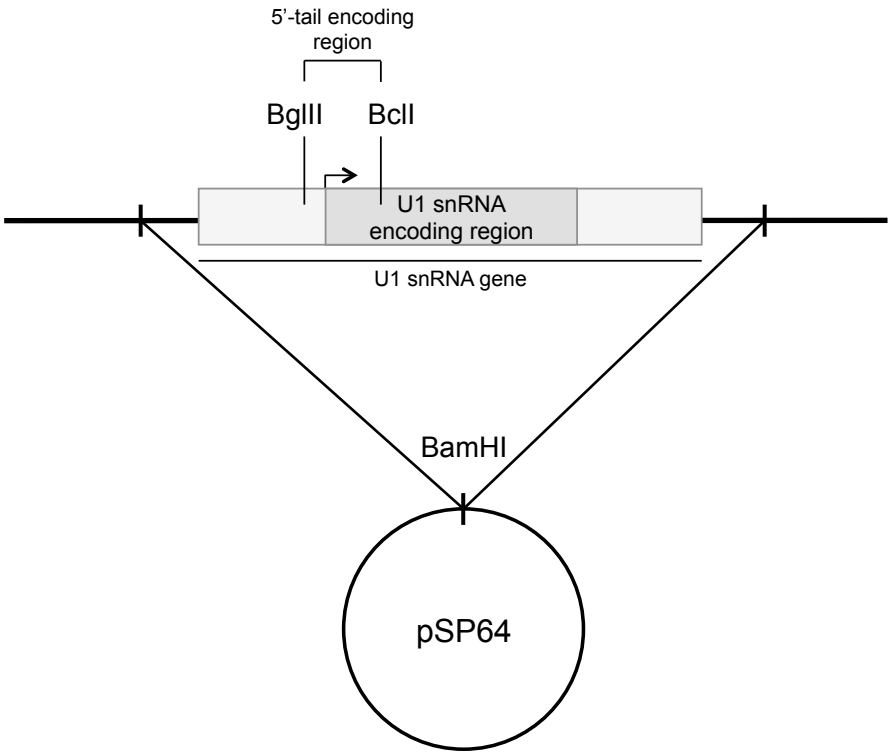

Figure 3.3. Schematic representation of cloning strategy for creation of modified U1 snRNA.
U1 snRNA wild-type gene sequence was inserted into BamHI site of pSP64 vector. For exon-specific U1s - BglII and BclI fragment was replaced with annealed DNA oligonucleotides.

The wild-type U1 snRNA gene was cloned in BamHI restriction site located in the multiple cloning site of pSP64 vector. The region between BglII and BclI sites encoding for the 5'-tail of the U1 was replaced by specific annealed DNA oligonucleotides to create engineered U1 snRNAs able to recognize specific target sequences with their modified 5'-tails.

3.12.4. Expression vector for generation of modified U7smOPT

The 570 bp HaeIII fragment of U7 gene was cloned with SmaI restriction site into the pSP64 vector (Figure 3.4). The original U7smOPT vector contains the murine U7 gene in which the wild-type U7-specific Sm binding site (AAUUUGUCUAG) was replaced with the consensus Sm OPT sequence (AAUUUUUGGAG) and a single StuI and HpaI sites have been inserted upstream and downstream of the U7 sequence. Modified U7 snRNAs were created by replacing the sequence of interest through PCR amplification of the U7SmOPT vector using specific primers U7 IK10F and SP6R.

U7 IK10F	5'-ACAGAGGCCTTTCCGCATCGCAAACAGTACAATAATTTTTGGAG-3'
SP6R	5'-ATTTAGGTGACACTATAGAA-3'

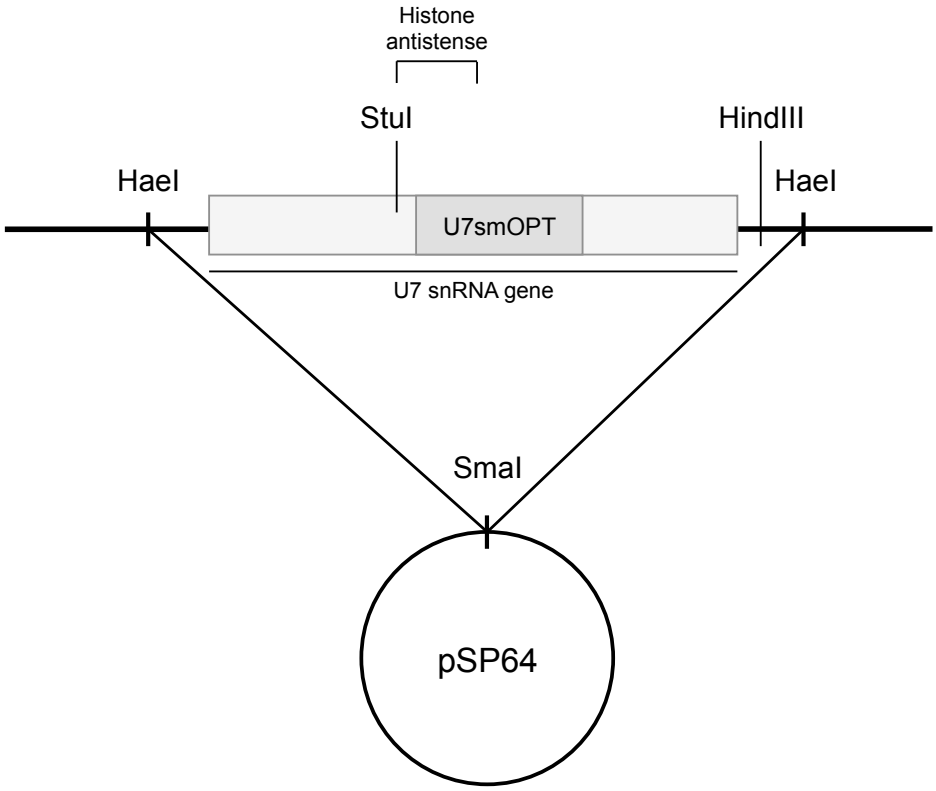

Figure 3.4. Schematic representation of cloning strategy for creation of modified U7 snRNA.
U7 snRNA gene sequence was inserted into SmaI site of pSP64 vector. For modified U7 - StuI and HindIII fragment was replaced with PCR amplification product.

The murine U7 snRNA gene was cloned in SmaI restriction site of the pSP64 polylinker. The modified U7 snRNA were generated by PCR amplification using mutagenic primers. The PCR products were digested and then cloned into the HindIII/StuI opened pSP64-U7smOPT vector.

3.13. Eukaryotic cell lines

The eukaryotic cell lines used for the experiments are: HeLa (human cervical carcinoma), Hek293T (adrenal embryonic kidney) and SHSY5Y (neuroblastoma) cells. Additionally, human FD fibroblasts were purchased from Coriell Institute (GM04959, GM04899).

3.13.1. Maintenance and analysis of cells in culture

HeLa, Hek293T, and SHSY5Y cells were grown in Dulbecco's Modified Eagle Medium (DMEM with glutamine, sodium pyruvate, pyridoxine and 4.5 g/l glucose) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (EuroClone, Pero, Italy) and antibiotic/antimycotic (Sigma-Aldrich) according to the manufacturer's instruction. FD primary fibroblast cells were maintained as suggested by supplier. Briefly, cells were grown in Dulbecco's Modiefied Eagle Medium, as described above with 10% FBS (EuroClone) and antibiotic/antimycotic (Sigma-Aldrich) according to the manufacturer's instruction. A standard 100 mm dish containing a monolayer of cells was washed twice with 1x PBS solution, in order to remove all the medium residues, as well as the dead cells, treated with 1 ml Trypsin (PBS containing 0.045 mM EDTA and 0.1% trypsin) and incubated at 37°C for 2 minutes or until cells were completely detached. After adding 5 ml of medium, blocking trypsin, cells were precipitated by centrifugation (1000 rpm for 5 minutes) and resuspended in pre-warmed medium. A sub-cultivation ratio of 1:6 to 1:8 was used. 1 ml of cell dilution was added fresh medium and plated in a new 100 mm dish.

3.14. Transfection of recombinant DNA

$3x10^5$ HeLa cells were plated as described above into 6-well cell culture dishes in order to reach a final confluence of 40-70%. The plasmid DNA used for transfection was prepared with Plasmid DNA purification kit (Macherey-Nagel) as previously described. Transfection of HeLa cells was performed by applying Effectene Transfection Reagent (Qiagen) according to manufacturer's instructions. For 6-well cell culture plates, 250 ng of *IKBKAP* IVS20+6T>C plasmid DNA were first mixed with 150 µl of EC buffer and 4 µl of Enhancer and the mixture was incubated at room temperature for 5 minutes to allow the condensation of DNA. 5 µl of Effectene Reagents were then added to the mixture and incubated for 10 minutes to allow Effectene-DNA complexes to form. After the addition of 500 µl of complete growth medium, the mixture was added to the cells in 1.5 ml of the same medium and incubated at 37°C. After 24 hours, cells were harvested and subjected to further analyses. Each transfection experiment was repeated at least three times in duplicate.

3.15. Co-transfection of ExSpeU1s and antisense U7 snRNAs

For the ExSpeU1 screening co-transfection experiment 250 ng of *IKBKAP* IVS20+6T>C minigene has been transfected with 500 ng of one from the following ExSpeU1s: U1 IK_4, U1 IK_{10}, U1 IK_{15}, U1 IK_{34}, U1 IK_{46}, U1 IK_{57}, U1 IK_{72}, U1 IK_{77}, U1 IK_{99}, U1 IK_{123}. Additionally, 500 ng of U1 wt minigene was used with 250 ng of *IKBKAP* IVS20+6T>C minigene as a control. The experiments were performed in SHSY5Y cell line.

For the inhibitory effect of antisense U7 co-transfection experiment 250 ng of *IKBKAP* IVS20+6T>C minigene has been transfected with 500 ng of U7 IK_{10}. The experiments were performed in Hek293T cell line.

For the ExSpeU1 efficiency co-transfection experiment 250 ng of *IKBKAP* IVS20+6T>C minigene has been transfected with 500 ng of antisense U7 IK_{10} and 500 ng of one from the mention above ExSpeU1s (U1 $IK_{4\text{-}123}$). The experiments were performed in Hek293T cell line.

3.16. Co-transfection of splicing factors

For the different splicing factors co-transfection experiments, 250 ng of the minigenes were transfected with 500 ng of the expressing plasmid. The splicing factors used for the co-transfections are SRSF1 (SF2/ASF), SRSF3 (SRp20), SRSF4 (SRp75), SRSF5 (SRp40), SRSF6 (SRp55), SRSF7 (9G8), SRSF9 (SRp30c), SRSF12 (SRrp35), TIA1, PTBP1, PTB4, U2AF1, U2AF2, hnRNPA1, hnRNPA2, and hnRNPH1, FOX Muscle, FOX Brain, NOVA C2, NOVA N2, DAZAP1, FUS coming from pCG expressing plasmid. The experiments were performed in three cell lines (HeLa, Hek293T and SHSY5Y).

For the ExSpeU1 efficiency co-transfection experiment 250 ng of *IKBKAP* IVS20+6T>C minigene was transfected with 300 ng of one from the above mentioned splicing factors and 300 ng of one from the ExSpeU1s (U1 $IK_{4\text{-}72}$). The experiment was performed in Hek293T cell line.

3.17. Lentiviral particle production and titration

The VSV-G pseudotyped lentiviral vectors (LV) were used in order to generate viral particles encoding snRNA U1 wt or ExSpeU1 IK_{10} (U1 IKAP), as previously described (Charrier, Ferrand et al. 2011). Briefly, LV expressing the U1 wt and U1 IKAP were prepared by cloning of the expression cassettes into the pRRL HIV-derived backbone through the BamHI and PstI restriction sites. At the next step the transient co-transfection was into the Hek293T cells using following plasmids: psPAX vector encoding for gag-pol, tet and rev proteins, pVSV-G encoding packaging proteins and modified pLVTHM plasmid encoding the green fluorescent protein (GFP) under the cytomegalovirus (CMV) promoter and U1snRNA wild-type or ExSpeU1 IK_{10} gene under their own promoter. The viral supernatant was collected from transfected cells after 48 hours and concentrated by ultracentrifugation at 25000 rpm for 90 minutes. The viral pellet was resuspended in 1x PBS, flash frozen in liquid nitrogen and aliquots were stored at -80°C until use. The titer of the virus was 7.8E8 infectious genomes (IG)/ml for U1-IKAP and 1.1E9 IG/ml for U1 wild-type.

3.18. Lentiviral transduction

Human FD fibroblasts were grown as described above. At day 0, cells were seeded at a density of $6x10^4$ cells/well. At day 1, cells were transduced with lentiviral ExSpeU1 IK_{10} and lentiviral U1 wt with increasing multiplicity of infection (MOI 0-20). In order to reduce the electronegativity between cell and virus surface polybrene (Sigma-Aldrich) was used at a concentration of 10 µg/ml. Cells were harvested 72 hours post-transduction for RNA and protein analysis.

3.19. AAV9 production and titration

Adeno-associated virus serotype 9 (AAV9). AAV vectors were produced by our collaborator Prof. F. Mingozzi from Genethon company. Briefly, vectors were produced by transient transfection of adherent human embryonic kidney (Hek293) cells grown in roller bottles with the three plasmids containing: the adenovirus helper proteins (pFdelta6), plasmid with the AAV Rep and Cap genes and the AAV plasmid containing the recombinant expression cassette flanked by packaging signals (inverted terminal repeats, ITRs). Seventy-two hours post-transfection cells were harvested, lyzed and treated with benzonase (Merck Millipore, Billerica, MA, USA). In order to purify AAV, centrifugation in cesium chloride density gradient was applied. Full capsids were collected in sterile phosphate buffered saline containing 0.001% of pluronic (Sigma Aldrich) in order to formulate the final product, that was stored at -80°C. AAV9 were titer matched to 7.10E12 VG/ml for the AAV9-ExSpeU1 IK_{10} and 5.30E12 VG/ml for the AAV9-ExSpeU1 IK_{15}. Titers were determined by quantitative qPCR with specific-primers.

3.20. Animal model

Familial Dysautonomia mouse model was previously described by Hims et al. (Hims, Shetty et al. 2007). This model carries the complete human *IKBKAP* locus with the FD IVS20+6T>C splice mutation from a human BAC. Presence of the FD mutation in the BAC causes mis-splicing of human *IKBKAP* in mice; further exon inclusion is seen in a tissue-specific manner that closely represents situation seen in FD patients. Mice do not show any pathological phenotype. For our purposes animals were kept in a controlled environment at 25°C with a 12 hours light/dark photoperiod. Animals were housed and handled according to institutional guidelines approved by International Centre for Genetic Engineering and Biotechnology (ICGEB) board. The experimental procedures were carried out with full respect to the European Union Directive 2010/63/EU for animal experimentation, and were approved by the Italian competent authorities and Ethical Committees.

3.21. Administration of AAV9-ExSpeU1s to FD mice

Newborn mice were intraperitoneally (IP) injected with 25 µl of the specific solution at two different time points: postnatal day (PND) 0 and 2. Four FD animals were treated with AAV9-ExSpeU1 IK_{10}, four FD mice with AAV9-ExSpeU1 IK_{15} and four were treated with saline. Injections were preformed with a 25 µl micro-syringe (Hamilton, Reno, NV, USA) with a 33-gauge removable needle. Mice were sacrificed at PND7 in order to collect tissues and organs (brain, heart, liver, kidney, spinal cord and muscle). RNA and protein extraction was preformed as described below.

3.22. RNA preparation from cultured cells and mice organs

Cultured cells were washed twice with 1x PBS and then 800 µl of RNA TRI Reagent (Thermo Fisher Scientific) was added. Cells in trizol were collected into 1.5 ml tubes. Mice tissues were transferred into FastPrep® Lysis Beads & Matrix Tubes for Sample Disruption (MP Biomedicals, Santa Ana, CA, USA). Each impact-resistant tube contains 1.4 mm ceramic spheres. Lysing Matrix D is used primarily for lysis of softer tissues. For tissue homogenization 1 ml of RNA TRI Reagent was added and MagNA Lyser Instrument (Roche) was used for automatic disruption. The instrument facilitates the production of a supernatant containing nucleic acids suitable for further extraction. For both, cells and homogenized tissues, 0.2 mL of chloroform per mL of trizol was added. Samples were vortexed for 15 seconds, and left for 15 minutes at room temperature. Chloroform ensures phase separation of the two liquids, because chloroform is miscible with phenol and has a higher density than phenol; it forces a sharper separation of the organic and aqueous phases thereby assisting in the removal of the aqueous phase.

After chloroform extraction, the RNA-containing supernatant (aqueous phases) was precipitated by addition of 1 volume of isopropanol and collected by centrifugation at maximum speed in a microcentrifuge at 10.000 g for 30 minuts. The pellet was rinsed in 75% ethanol. The final pellet was resuspended in H_2O and stored at -80°C. The RNA quality was checked by electrophoresis on a 0.8% agarose gel. In the case

of DNA contamination, the extracted RNA was treated with RNase-free DNase (Promega) in 1x DNase Buffer, according to manufacturer's instructions. The DNA digestion was performed at 37°C for 30 minutes. The reaction was stopped by adding DNase Stop Solution and incubating at 65°C for 10 minutes. At the next step the concentration of nucleic acids was measured with NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, LLC, Wilmington, DE, USA).

3.23. Genotyping

From organs harvested at PND7 RNA was extracted. Using RT-PCR with verified human-specific primers (Hs-spEx19F and Hs-sp-Ex22R), expression of *IKBKAP* mutant variant was confirmed. The thermal cycling protocol was:
94°C for 5 minutes, 30 cycles at 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds and 72°C for 10 minutes.

Hs-spEx19F	5'- AGCAGCAATCATGTGTCCCA-3'
Hs-sp-Ex22R	5'- GTGACATCTTCTTCTTTCAA-3'

3.24. The mRNA functional splicing analysis

3.24.1. cDNA synthesis

The first-strand cDNA synthesis was performed with the M-MLV Reverse Transcriptase Kit (Thermo Fisher Scientific) following manufacturer's instructions. 1μg of total RNA extracted from cells were mixed with 2 μl of random primers (100 ng/μl, Thermo Fisher Scientific) and diluted in water to the final volume of 12 μl. To denature the RNA, the mixture was put at 94°C for 2 minutes and quick chilled on ice. After denaturation 6 μl 5x First-Strand Buffer (250 mM Tris-HCl

(pH 8.3 at room temperature), 375 mM KCl, 15 mM $MgCl_2$), 3 µl 0.1 M DTT, 3 µl dNTPs, 0.5 µl M-MLV RT were added to the reaction. The final mixture was then incubated at 37°C for 90 minutes. Three µl of the reaction were used as a template for the PCR analysis.

3.24.2. PCR analysis

PCR reactions were performed using previously described protocol in a final volume of 25 µl. Oligonucleotides used for PCR analysis, specific for the minigene system used or for the endogenous genes of interest, are listed below. The results of all the transfections are the representative of at least three independent experiments. PCR products were resolved by agarose gel electrophoresis. Quantification of exon inclusion, exon skipping and intron retention was performed using ImageJ Software (NIH, Bethesda, MD, USA; http://imagej.nih.gov/ij).

IKBKAP minigene splicing was analysed by semiquantitative PCR using specific primers α2,3_dir and IKAP-ex21_rev. PCR conditions were: 94°C for 5 minutes, 40 cycles at 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 40 seconds and 72°C for 10 minutes. PCR products were analysed by electrophoresis on a 1.5% or 2% agarose gel. Semiquantitative analysis of ExSpeU1 IK_{10} expression was performed with U1-IK10_dir and U1-IK10_rev primers at the following conditions: 94°C for 5 minutes, 30 cycles at 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 15 seconds and 72°C for 10 minutes.

For the splicing pattern in FD fibroblast and mouse tissues, human-specific hIKAP-ex19_dir and IKAP-ex21_rev primers were used at the following conditions: 95°C for 2 minutes, 40 cycles at 95°C for 30 seconds, 69°C for 30 seconds, 72°C for 30 seconds and 72°C for 5 minutes.

α2,3 _dir	5'-CAACTTCAAGCTCCTAAGCCACTGC-3'
IKAP-ex21_rev	5'-GATTCTCAGCTTTCTCATGCATTC-3'
U1-IK10 _dir	5'-ATAGCAAACAGTACAATGC-3'
U1-IK10_rev	5'-CACTACCACAAATTATGCA-3'
hIKAP-ex19 _dir	5'-GGCCGGCCTGAGCAGCAATCATGTGTCC-3'

3.25. Real Time Quantitative PCR analysis - SYBR Green

SYBR Green-based quantitative PCR (qPCR) was performed using the iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in a CFX96 Real-Time PCR system (Bio-Rad Laboratories, Inc), following manufacturer's instructions. Briefly, 100 ng of cDNA were mixed with the master mix and the forward and reverse primers and filled with water to a final volume of 15µl. Primers were designed to target an amplicon size of 100-200 bp.

For qPCR, human-specific primers were used: IKBKAP-FL_dir and IKBKAP-FL_rev for full-length transcripts and IKBKAP-Δ20_dir and IKBKAP-Δ20_rev primers for transcripts lacking the exon 20. Total *IKBKAP* mRNA was quantified with human-specific primers Tot-IKBKAP_dir and Tot-IKBKAP_rev. GAPDH was used as housekeeping for normalization with human-specific primers: hGAPDH_dir and hGAPDH_rev in fibroblasts samples and mouse-specific primers: mGAPDH_dir and mGAPDH_rev were used in animal samples. Three replicates per RT reaction were performed. The thermal cycling protocol was: 94°C for 3 minutes, 40 cycles at 95°C for 10 seconds and 40 cycles at 59°C for 45 seconds.

IKBKAP-FL_dir	5'-GCAGCAATCATGTGTCCCA-3'
IKBKAP-FL_rev	5'-ACCAGGGCTCGATGATGAA-3'
IKBKAP-Δ20_dir	5'-CACAAAGCTTGTATTACAGACT-3'
IKBKAP-Δ20_rev	5'-GAAGGTTTCCACATTTCCAAG-3'
Tot-IKBKAP_dir	5'-GCTGTTCCCACACCCTGT-3'
Tot-IKBKAP_rev	5'-AGGGTCAGCACTTGGACAA-3'
hGAPDH_dir	5'-GACAGTCAGCCGCATCTTCT-3'
hGAPDH_rev	5'-TTAAAAGCAGCCCTGGTGAC-3'
mGAPDH_dir	5'-ATGGTGAAGGTCGGTGTGAA-3'
mGAPDH_rev	5'-GTTGATGGCAACAATCTCCA-3'

3.26. Small interfering (siRNA) transfection

The sense strand of RNAi oligos (Dharmacon, Lafayette, CO, USA), which were used for silencing the expression of different target genes, were the following:

SRSF3	5'-GAGUGGAACUGUCGAAUGG-3'
hnRNPA1	5'-CAGCUGAGGAAGCUCUUCA-3'
hnRNPA2	5'-GGAACAGUUCCGUAAGCUC-3'
FUS	5'-GAUCAAUCCUCCAUGAGUA-3'
	5'-GGACAGCAGAGUUACAGUG-3'
	5'-GGACAGCAGCAAAGCUAUA-3'
	5'-GAGCAGCUAUUCUUCUUAU-3'
Non-Targeting siRNA #2	5'-UAAGGCUAUGAAGAGAUAC-3'

$2.5x10^5$ cells were plated in 60 mm plates to achieve 40-50% confluence the following day when siRNA transfection was carried out preparing two reaction mixtures. The first comprised 10 µl of Oligofectamine Transfection Reagent (Thermo Fisher Scientific) combined with 20 µl of Opti-MEM medium (Thermo Fisher Scientific), whereas the second one consisted of 10 µl of 40 µM siRNA duplex oligos diluted in a final volume of 370 µl of Opti-MEM. The two mixtures were combined, left at room temperature for 20 minutes and finally added to the cells, which were maintained in 1.6 ml of Opti-MEM. After 24 hours the second treatment with siRNA was performed as described above. 6-8 hours later Opti-MEM was exchanged with DMEM and the cells were transfected with the minigene of interest using Qiagen Effectene Transfection Reagent. 1µg plasmid DNA was mixed with 8 µl of Enhancer and 10 µl of Effectene Reagent using previously described protocol. After addition of 500 µl of complete DMEM medium, the mixture was added to the cells in 4 µl of the same medium and incubated at 37◦C. After 24-72 hours cells were collected and divided in two fractions for protein and RNA extractions. RT-PCR from total RNA was performed as described above. The whole protein extracts were obtained by cell resuspension in 1x lysis buffer (100 mM KCl, 200 µM EDTA pH 8.0, 20 mM HEPES pH 7.5, 0.5 mM DTT, 1% NP-40, 6% glycerol, 1x proteinase inhibitor). Each siRNA experiment was repeated at least three times.

3.27. Protein extraction

Cells were silenced and transfected as described above. Cultured cells were washed with 1x PBS and collected in 1 ml of PBS in tubes. Then, after centrifuging, samples from cells, FD fibroblasts and mice were lysed in 75 μL of RIPA buffer (Sigma-Aldrich) and protease inhibitor cocktail (SIGMAFAST™ Protease Inhibitor Tablets, Sigma-Aldrich), in order to inhibit enzymes from degrading proteins. Then, samples were sonicated twice for 5 minutes at high impulse.

In order to measure the concentration of total proteins, Bradford assay was performed using Bradford Protein Assay kit (Bio-Rad Laboratories, Inc). Standard curve was made using bovine serum albumin (BSA) and spectrophotometer analysis was carried out at 595 nm wavelengths.

3.28. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

30 μg of cells protein and 50 μg of FD fibroblasts and mice tissues proteins were mixed with the appropriate volume of 2x Protein loading buffer (the max volume that can be loaded is 55 μl) and the mixture was incubated at 95◦C for 4 minutes before loading on 10% SDS polyacrylamide gel (SDS-PAGE). The SDS-PAGE gel was composed of 5% stacking gel (pH 6.8) and 10% running gel (pH 8.8). The gels were run at 35 mA in 2x Running buffer, until the desired bands are well separated. After running and separating on the gels, proteins were transferred onto a 0.45 μm polyvinylidene difluoride blotting membrane (Amersham™ Hybond™, GE Healthcare Life Sciences, Marlborough, MA, USA). Sandwich was prepared with 3 pieces of paper, the gel, the membrane (previously activated with 30 minutes in methanol, two times for 1 minute in H_2O, 5 minutes in 1x Blotting buffer) and other 3 pieces of paper and everything was wet with buffer. After elimination of air bubbles the sandwich was closed and put in the blotting support filled with 1x Blotting buffer. The sandwich was blotted at 200-250 mA for 75-150 minutes depending on the proteins that had to be transferred.

3.29. Western blotting

The membrane was blocked overnight with PBS buffer supplemented with 0.1% Tween-20 (PBS-T) and 5% low fat dry milk (Bio-Rad Laboratories, Inc.). Membranes were probed with different primary antibodies for 3 hours at room temperature, and then incubated with secondary antibodies conjugated with horseradish peroxidase (HRP) enzyme and the resulting immunoreactive bands were detected with the Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific). Membrane was re-probed with antibody against tubulin as a protein loading control. The primary antibodies that were applied in Western blot analysis are: for SRSF3 mouse monoclonal anti-SRp20 antibody (Thermo Fisher Scientific; 1:1000), rabbit polyclonal anti-TLS/FUS antibody (Abcam, Cambridge, England; 1:1000), rabbit polyclonal anti-hnRNPA1 antibody (Abcam; 1:000), rabbit polyclonal anti-hnRNPA2 antibody (Abcam; 1:1000), rabbit polyclonal anti-IKAP antibody (AnaSpec, Fremont, CA, USA; 1:500), mouse monoclonal anti-Tubulin antibody (Santa Cruz Biotechnology, Dallas, TX, USA; 1:5000) and mouse monoclonal anti-GAPDH antibody (Abcam; 1:1000). The secondary antibodies are respectively: polyclonal goat anti-rabbit immunoglobulins/HRP (Dako, Agilent Technologies; 1:2000), polyclonal goat anti-mouse immunoglobulins/ HRP (Dako, Agilent Technologies; 1:2000). Protein bands were detected as described above.

3.30. Bioinformatic analyses

All data are presented as mean ± standard deviation (SD). Statistical analysis was carried out using Prism Software (GraphPad Software, La Jolla, CA, USA). Analysis of splice site strength was performed using the Neural Network program (threshold 0.01) (http://fruitfly. org/seq tools/splice.html). The *in silico* splicing prediction of protein binding sites were performed using Human Splicing Finder (HSF) (Desmet, Hamroun et al. 2009) web-based program (www.umd.be/HSF3/HSF.shtml). Those data were combined with outputs of the ESS Hexamers (Wang, Rolish et al. 2004) web server (http://genes.mit.edu/fas-ess) that was used to predict and analyse ESSs present in the sequence, and with output of ESEfinder (Cartegni, Wang et al. 2003) web-based resource (http://krainer01.cshl.edu), which facilitates rapid identification of putative ESEs.

4. RESULTS

4.1. *In vitro* effect of ExSpeU1s on the aberrant splicing of the FD founder mutation c.2204+6T>C

To identify ExSpeU1s that rescue the exon 20 splicing defect associated to FD, I took advantage of a splicing assay based on hybrid minigenes. I created two minigenes: one containing the wild-type genomic sequence of the *IKBKAP* gene spanning from the intron 18 to intron 21 and the second carrying the intronic IVS20+6T>C substitution (Figure 4.1A). In order to test the effect on pre-mRNA processing, I transfected neuronal SHSY5Y cells with wild-type and mutant constructs. Total RNA was extracted 24 hours post-transfection and analysed by reverse transcriptase (RT-PCR) using the specific primers α2,3_dir and IKAP-ex21_rev. The resulting amplified products were separated by electrophoresis on a 1.5% agarose gel. In the wild-type minigene, the analysis of the PCR products showed the presence of only one band of 349 bp, corresponding to the transcript retaining exon 20 (Figure 4.1B, lane 1). On the other hand, analysis of mutant minigene showed the presence of an additional lower band of 275 bp, corresponding to transcripts lacking the exon 20 (Figure 4.1B, lane 2). The percentage of exon 20 inclusion in the mutant context was ~30%, confirming the effect of the mutation on the pathological FD mis-splicing.

To study the activity of ExSpeU1s in correction of the splicing defect caused by the c.2204+6T>C substitution, I prepared twelve bioengineered U1 snRNAs with their 5'-tail complementary to the intronic region downstream the *IKBKAP* exon 20 donor splice site. The ExSpeU1s were designed to cover the intronic sequence from position +4 to +219 (Figure 4.2A). To test the efficacy of these modified U1 snRNAs in rescuing FD aberrant splicing, I co-transfected SHSY5Y cells with both wild-type and mutant *IKBKAP* minigenes along with the different ExSpeU1s. After 24 hours, total RNA was extracted, retro-transcribed and amplified by PCR using

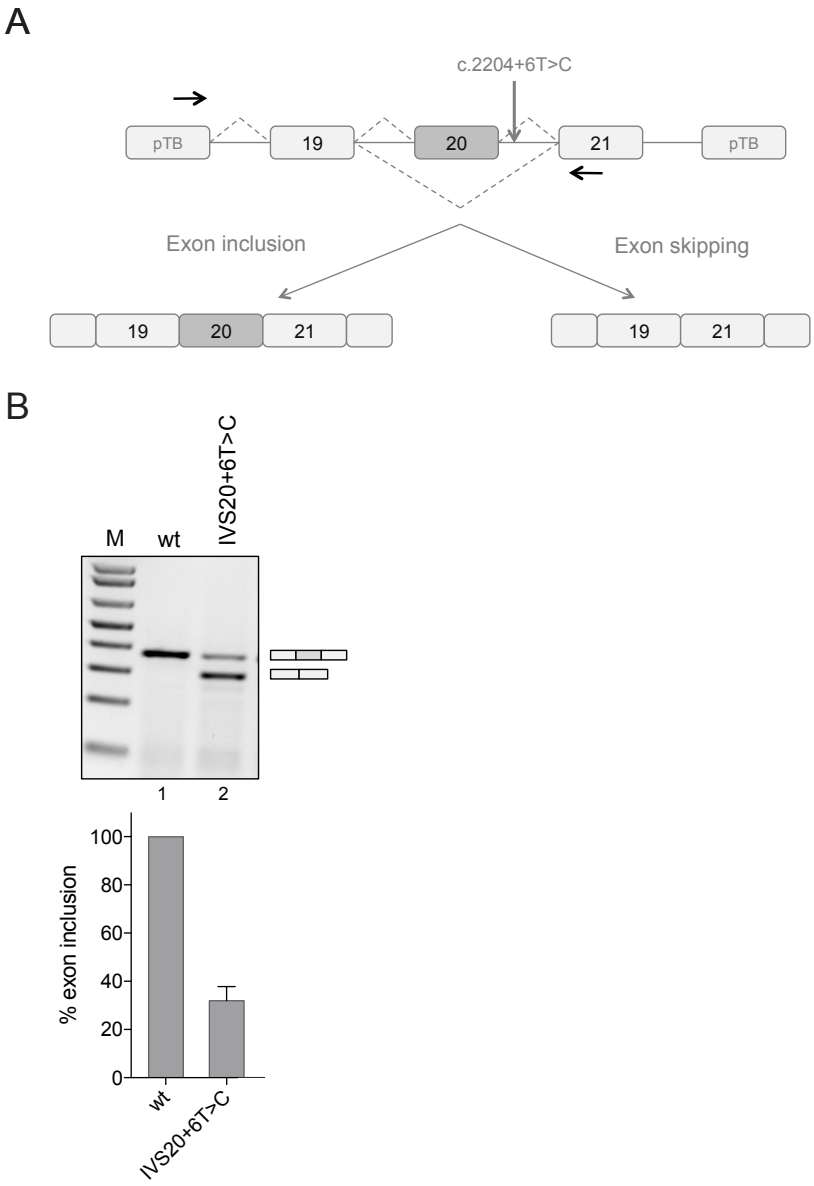

Figure 4.1. Splicing pattern of *IKBKAP* wt and IVS20+6T>C minigenes.

A) Schematic diagram of the pTB-*IKBKAP* minigenes used for functional splicing assays. Boxes represent exons and lines introns of *IKBKAP* gene. The intronic c.2204+6T>C mutation is indicated. Arrows upon the exons indicate the α2,3_dir and IKAP-ex21_rev primers location used for reverse transcriptase PCR (RT-PCR) and analysis of splicing products. Dashed lines indicate the possible patterns of splicing observed in *IKBKAP* minigenes. The two alternative transcripts are indicated.

B) SHSY5Y cells were transfected with 250 ng of indicated minigenes. After 24 hours, total RNA was extracted, mRNAs produced were analysed through reverse transcriptase PCR using α2,3_dir and IKAP-ex21_rev oligonucleotides. Amplified products were run on 1.5% agarose gel. Schematic representation of the splicing pattern is shown on the right of the RT-PCR gel analysis. The upper band corresponds to the transcripts that include *IKBKAP* exon 20 (349 bp), whereas the lower represent the exclusion of *IKBKAP* exon 20 (275 bp). The experiment was performed in triplicate and the identity of the bands was verified by direct sequencing. The graph represents the quantification analysis obtained with ImageJ software expressed as means ± SD.

α2,3_dir and IKAP-ex21_rev oligonucleotides (Figure 4.1A). The resulting amplified products were separated by electrophoresis on 1.5% agarose gel. The analysis of splicing patterns showed that ExSpeU1s are able to efficiently counteract the negative effect of the intronic mutation (Figure 4.2B). In particular, ExSpeU1s covering the intronic region from +4 to +81 are very efficient in rescuing abnormal processing, inducing complete inclusion of exon 20 (Figure 4.2B, lanes 4-10). ExSpeU1s that bind to deeper intronic positions, as well as the U1 wt, did not show any splicing improvement. (Figure 4.2B, lanes 11-15 and lane 3, respectively).

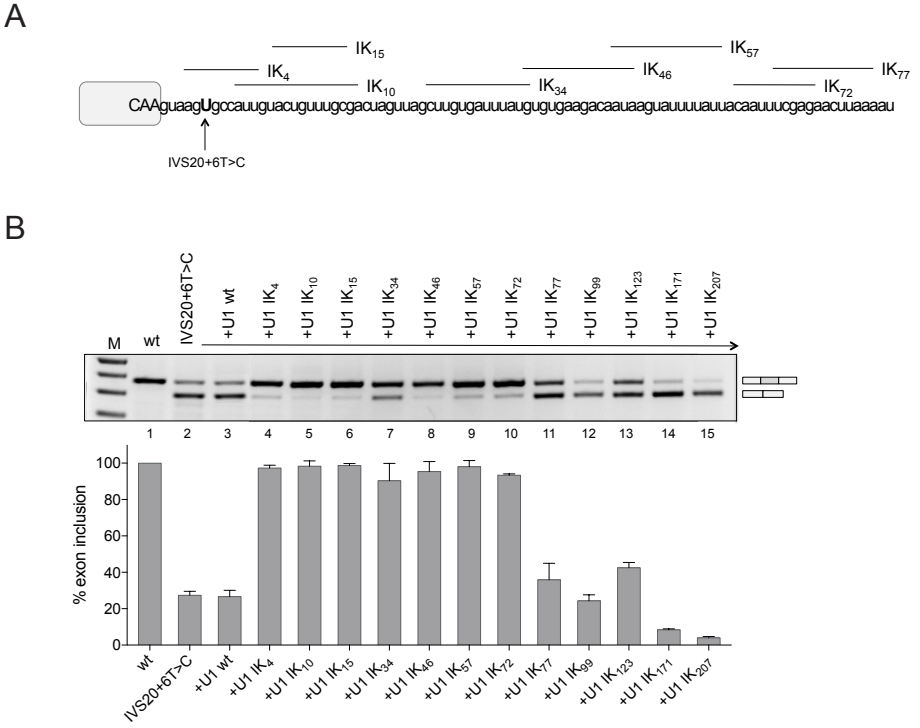

Figure 4.2. ExSpeU1s correct the FD splicing in *in vitro* splicing assay.

A) Schematic representation of the *IKBKAP*-ExSpeU1s binding regions in the intronic sequence downstream the *IKBKAP* exon 20 donor splice site. Exonic and intronic sequences are in upper and lower cases, respectively. Black lines up on the sequence represents the 5'-complementaty tail of the ExSpeU1s.

B) Co-transfection of *IKBKAP* minigenes along with *IKBKAP*-ExSpeU1s in SHSY5Y cells. Lines 1-2, cells transfected with 250 ng of *IKBKAP* wild-type (wt) and mutant (IVS20+6T>C) minigenes, respectively. Line 3, cells transfected with 250 ng of mutant (IVS20+6T>C) minigene and 0.5 µg of plasmid encoding for endogenous U1 snRNA, used as a control. Lines 4-15, cells transfected with 250 ng of mutant (IVS20+6T>C) minigene and 0.5 µg of plasmids encoding for modified U1 snRNAs. After 24 hours, total RNA was extracted, mRNAs produced were analysed through reverse transcriptase PCR (RT-PCR) using α2,3_dir and IKAP-ex21_rev oligonucleotides. The upper band of 349 bp corresponds to transcripts including the exon 20; the lower band of 275 bp to exon 20 skipping. Wild-type, mutant and ExSpeU1s (U1 IK4-207) are indicated. Schematic representation of the splicing pattern is shown on the right of the RT-PCR gel analysis. PCR products were resolved on 1.5% agarose gel and the bands intensity was quantified with ImageJ software. The graph represents the percentage of exon 20 inclusion and data are expressed as means ± SD of three independent experiments.

4.2. Identification of splicing factors that influence the exon 20 splicing

Previous studies showed that the change T>C at position +6 of the *IKBKAP* intron 20 causes skipping of exon 20 in a tissue-dependent manner, in particular in both the central nervous system (CNS) and peripheral nervous system (PNS) (Slaugenhaupt, Blumenfeld et al. 2001, Cuajungco, Leyne et al. 2003). In order to study possible regulatory mechanisms, I transfected wild-type and mutant *IKBKAP* minigenes into three cell lines of different origin: HeLa, SHSY5Y and Hek293T. After 24 hours, total RNA was extracted, retro-transcribed and amplified by PCR using α2,3_dir and IKAP-ex21_rev oligonucleotides (Figure 4.1A). Transfection of IVS20+6T>C minigene into different cell lines showed, as expected, different levels of exon inclusion for IVS20+6T>C minigene (Figure 4.3). The highest percentage of correct splicing is observed in HeLa epithelial cancer cells (~75%). In the Hek293T adrenal embryonic kidney cells inclusion of exon 20 is at the level of ~40%. The lowest level of exon inclusion (~30 %) is seen in the SHSY5Y neuronal cells. This is in agreement with the reported higher levels of exon skipping in the nervous system (Cuajungco, Leyne et al. 2003). Wild-type minigene samples showed a full inclusion of exon 20 in all cell lines (Figure 4.3).

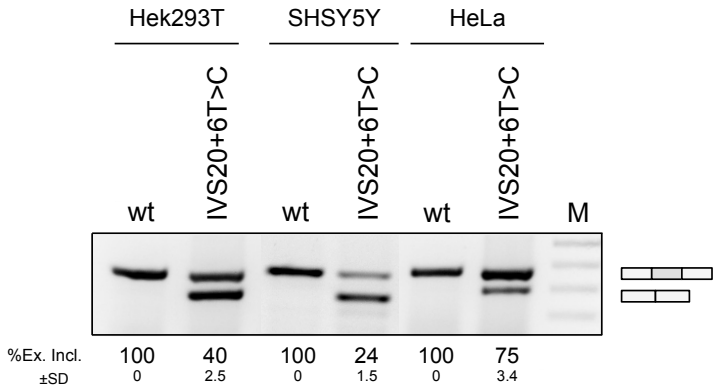

Figure 4.3. Cell type-dependent splicing pattern of IVS20+6T>C minigene.

Cells were transfected with 250 ng of indicated *IKBKAP* minigenes. Used cell lines are indicated on the figure. After 24 hours, total RNA was extracted, mRNAs produced were analysed through reverse transcriptase PCR (RT-PCR) using α2,3_dir and IKAP-ex21_rev primers. The upper band of 349 bp corresponds to transcripts including the exon 20; the lower band of 275 bp to exon 20 skipping. Schematic representation of the splicing pattern is shown on the right of the RT-PCR gel analysis. PCR products were resolved on 1.5% agarose gel and the bands intensity was quantified with ImageJ software. The graph represents the percentage of exon 20 inclusion and data are expressed as means ± SD of three independent experiments.

To identify splicing factors potentially involved in modulating the definition of mutant *IKBKAP* exon 20, I performed co-transfection experiments in three cell lines, mentioned above. Wild-type (Figure 4.4) and mutant (Figure 4.5) *IKBKAP* minigenes were co-transfected with a panel of splicing factors that are known to be involved in splicing regulation: serine/arginine-rich proteins (SR), heterogeneous ribonuclear proteins (hnRNPs) and factors acting in tissue-specific manner (Figure 4.4, Figure 4.5). In the wild-type minigene (Figure 4.5), co-transfection with all the splicing regulators, except for one, did not show changes in the splicing pattern and exon 20 remained completely included in the final transcript. Only in Hek293T cell line the splicing factor hnRNPA1 had a small effect, inducing exon skipping (~8%) (Figure 4.4).

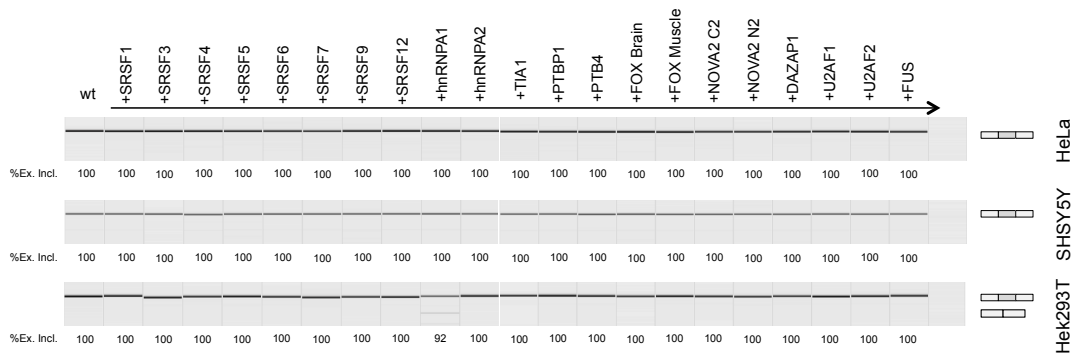

Figure 4.4. Effect of splicing factors overexpression in the wt *IKBKAP* minigene.

Co-transfection experiment of 250 ng of wild-type *IKBKAP* (wt) minigene with 0.5 µg of plasmids overexpressing a panel of splicing factors (indicated at the top) in HeLa, SHSY5Y and Hek293T cells. After 24 hours, total RNA was extracted, and mRNAs analysed through reverse transcriptase PCR (RT-PCR) using α2,3_dir and IKAP-ex21_rev primers. The upper band of 349 bp corresponds to transcripts including the exon 20; the lower band of 275 bp to exon 20 skipping. Schematic representation of the splicing pattern is shown on the right of the RT-PCR gel analysis. PCR products were analyzed with QIAxcel automated DNA electrophoresis. The percentage of exon 20 inclusion was calculated by the QIAxcel software and is indicated below.

In the mutant minigene, co-transfection of the different splicing factors induced significant changes in the pattern of splicing (Figure 4.5A). Analysis of the percentage of exon inclusion showed three factors with enhancing activity: PTBP1, PTB4 and TIA1 that are inducing exon inclusion (Figure 4.5B) whereas nine induce significant exon skipping. (Figure 4.5C). The inhibitory factors belong to the SR proteins (SRSF3, SRSF7, SRSF12), to the hnRNPs (hnRNPA1), to proteins involved in U2 recognition (U2AF1) or to the more specialized group of factors (FOX Muscle and Brain, NOVA2 C2 and FUS) (Figure 4.5C). Interestingly, hnRNPA1, a well characterized inhibitory splicing factor, showed the most pronounced inhibitory effect in one cell line Hek293T, whereas SRSF3 and FUS induced exon 20 skipping in all three cell lines tested (Figure 4.5C). The other nine factors tested did not show any significant influence on *IKBKAP* splicing (Figure 4.5D). The presence of a larger panel of splicing factors with inhibitory activity acting only in the mutated *IKBKAP* context suggest that they could be involved in modulating the severity of the exon skipping defect.

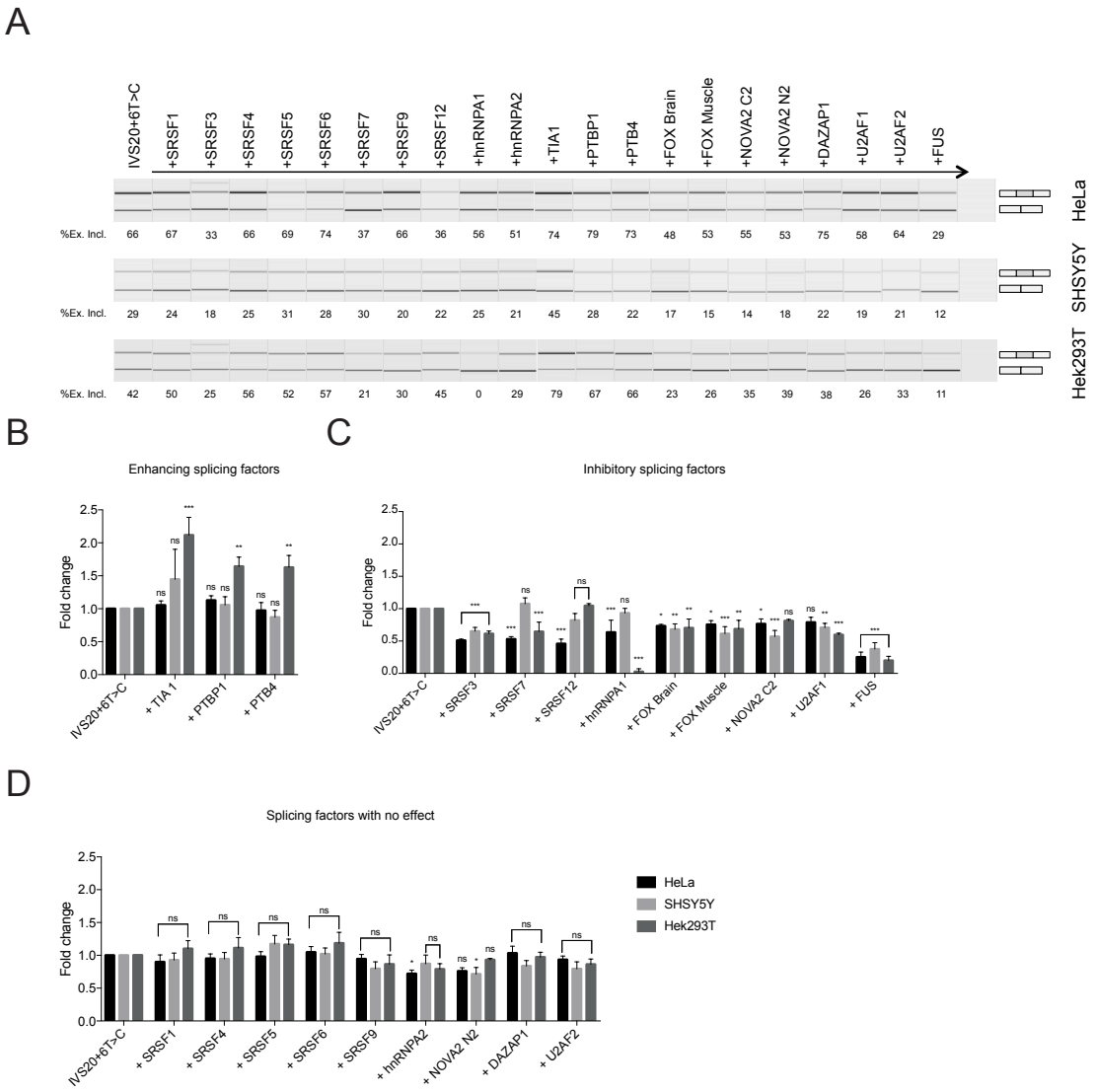

Figure 4.5. Effect of splicing factors overexpression in the *IKBKAP* IVS20+6T>C minigene.

A) Co-transfection experiment of 250 ng of mutant *IKBKAP* (IVS20+6T>C) minigene with 0.5 µg of plasmids overexpressing a panel of splicing factors (indicated at the top) in HeLa, SHSY5Y and Hek293T cells. After 24 hours, total RNA was extracted, mRNAs produced were analysed through reverse transcriptase PCR (RT-PCR) using α2,3_dir and IKAP-ex21_rev primers. The upper band of 349 bp corresponds to transcripts including the exon 20; the lower band of 275 bp to exon 20 skipping. Schematic representation of the splicing pattern is shown on the right of the RT-PCR gel analysis. PCR products were analyzed with QIAxcel automated DNA electrophoresis. The calculated percentage of exon 20 inclusion is indicated below.

B) Analysis of the fold splicing changes induced by splicing factors on the IVS20+6T>C minigene with enhancing activity. Transfection of the mutant *IKBKAP* minigene alone in all tested cells is set to one. Data are expressed as mean ± SD of three independent experiments. Statistical analysis was performed using a two-ways ANOVA (***p<0.0001, **p<0.001, *p<0.01, ns – not significant).

C) Analysis of the fold splicing changes induced by splicing factors on the IVS20+6T>C minigene with inhibitory activity. Transfection of the mutant *IKBKAP* minigene alone in all tested cells is set to one. Data are expressed as mean ± SD of three independent experiments. Statistical analysis was performed using a two-ways ANOVA (***p<0.0001, **p<0.001, *p<0.01, ns – not significant).
D) Analysis of the fold splicing changes induced by splicing factors with no significant effect on the IVS20+6T>C minigene. Transfection of the mutant *IKBKAP* minigene alone in all tested cells is set to one. Data are expressed as mean ± SD of three independent experiments. Statistical analysis was performed using a two-ways ANOVA (***p<0.0001, **p<0.001, *p<0.01, ns – not significant).

4.2.1. Effect of siRNA against hnRNPA1/A2, SRSF3 and FUS on *IKBKAP* exon 20 splicing pattern

To validate the regulatory role of some splicing factors on the defective exon 20, I focused on those that when overexpressed induce exon skipping. In order to confirm the inhibitory activity of the most significant negative SFs (hnRNPA1, SRSF3 and FUS) on *IKBKAP* exon 20 pattern of splicing, I decided to deplete these proteins from cells using specific siRNAs. The *in vivo* depletion of the candidate proteins was achieved through two rounds of transient transfection of siRNA oligonucleotides directed against the corresponding proteins. Cells treated with the siRNAs were transfected with IVS20+6T>C minigene, total RNA was extracted, retro-transcribed, amplified by PCR using α2,3_dir and IKAP-ex21_rev oligonucleotides (Figure 4.1A) and the pattern of splicing evaluated. Depletion of SRSF3 has a moderate effect on exon 20, leading to an increase of exon inclusion from ~50% to ~70% (Figure 4.6A, upper panel). Silencing of hnRNPA1/A2 increased exon 20 inclusion in the mutant construct from ~50% to ~70% (Figure 4.6B, upper panel). A slight increase in the percentage of exon inclusion, shift from ~20% to ~30% (Figure 4.6C, upper panel), was also observed after FUS depletion. Treatment of cells with Non-Targeting (NT) siRNA did not affect the pattern of splicing in all the contexts.

To confirm the siRNA efficacy, I performed immunoblot to check the reduction in protein expression level upon siRNA treatment. All the siRNA treatment significantly reduced the corresponding protein levels, ~70% for SRSF3 (Figure 4.6A, lower panel), ~60% for hnRNPA1 (Figure 4.6B, lower panel) and ~90% for FUS (Figure 4.6C, lower panel), whereas the cells transfected with NT siRNA did not affect the amount of the corresponding proteins.

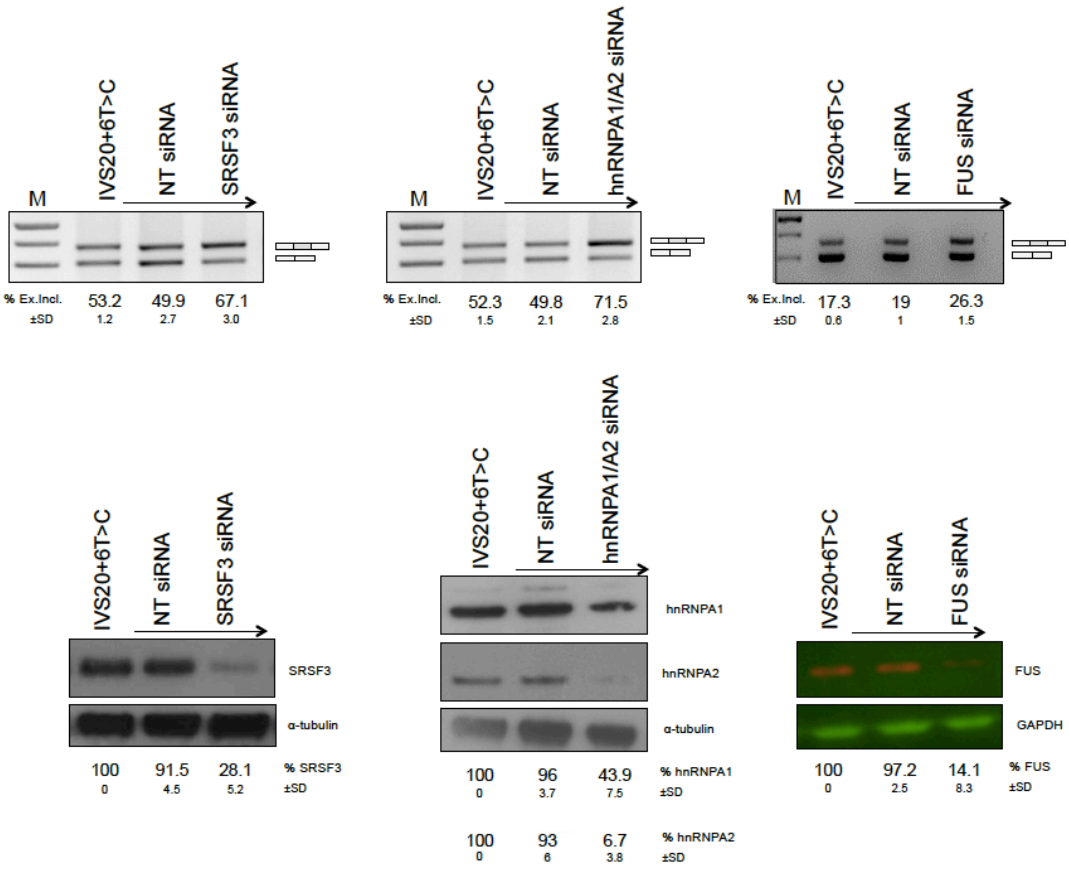

Figure 4.6. Silencing of inhibitory splicing factors improve *IKBKAP* exon 20 splicing.

Silencing of SRSF3, hnRNPA1/A2 and FUS in Hek293T cells transfected with IVS20+6T>C *IKBKAP* minigene. NT was a scramble siRNA used as a negative control.

A) Semiquantitative PCR of the *IKBKAP* splicing pattern using α2,3_dir and IKAP-ex21_rev primers. The upper band of 349 bp corresponds to transcripts including the exon 20; the lower band of 275 bp to exon 20 skipping. Schematic representation of the splicing pattern is shown on the right of the RT-PCR gel analysis. PCR products were resolved on 1.5% agarose gel and the bands intensity was quantified with ImageJ software. The graph represents the percentage of exon 20 inclusion and data are expressed as means ± SD of three independent experiments.

B) Western blot analysis of the silenced proteins, which are indicated in the upper part. A mouse monoclonal antibody for SRSF3 was used, for hnRNPA1/A2 rabbit polyclonal antibody, and for FUS rabbit polyclonal antibody. α-tubulin and GAPDH were used as reference for internal normalization.

4.2.2. Evaluation of effect of the negative splicing factors on the ExSpeU1-mediated splicing rescue activity

The most potent negative splicing factors as assessed previously in co-transfection experiments were used to test the ExSpeU1 rescue efficacy. I reasoned that the most potent ExSpeU1 should act not only in basal conditions but also in the presence of negative factors that reduce the exon definition. Therefore, Hek293T cells were transfected with IVS20+6T>C minigene and co-transfected with hnRNPA1, SRSF3 or FUS expressing vectors along with ExSpeU1s. I tested six ExSpeU1s (U1 IK_4, U1 IK_{10}, U1 IK_{15}, U1 IK_{46}, U1 IK_{57} or U1 IK_{72}), those that in previous transfection experiments in SHSY5Y cells promote complete exon inclusion (~100%) (Figure 4.2). After 24 hours, total RNA was extracted, retro-transcribed and amplified by PCR using α2,3_dir and IKAP-ex21_rev oligonucleotides (Figure 4.1A), then pattern of splicing was evaluated. Treatment with ExSpeU1s showed that all of them induce exon inclusion, but with different efficacy.

In the case of hnRNPA1, the most active was ExSpeU1 IK_{10}. The overexpression of hnRNPA1 resulted in ~10% of exon inclusion and ExSpeU1 IK_{10} rescued splicing to 100%. ExSpeU1 IK_{15}, the second most active U1 increases the percentage of exon inclusion to 88% whereas, the other ExSpeU1s reached at best a percentage of 80% (Figure 4.7). In the case of FUS, I did not observe significant differences among them as all ExSpeU1s completely restore exon 20 inclusion 100% (Figure 4.7). In the case of SRSF3 splicing factor, U1 IK_{10} and U1 IK_{15} induced 100% and 96% of exon inclusion, respectively, and ExSpeU1 IK_4 90%. The ExSpeU1 IK_{57} and U1 IK_{72} were less active raising exon inclusion to 42% and 33%, respectively (Figure 4.7). The result indicates, that IK_{10} and U1 IK_{15} showed the best rescue efficacy. This is particularly evident in the case of SRSF3 and hnRNPA1, where U1 IK_{10} and U1 IK_{15}, but not the other ExSpeU1s completely recovered exon 20 inclusion (Figure 4.7).

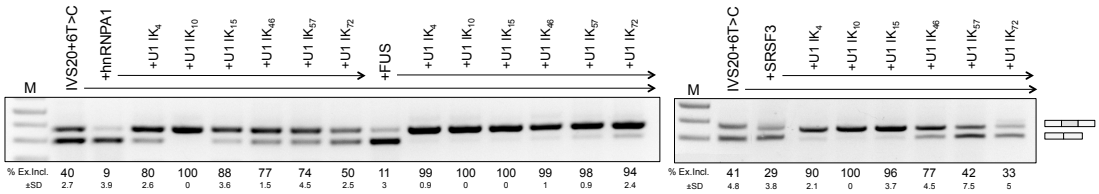

Figure 4.7. Identification of the most active ExSpeU1.

IVS20+6T>C minigene was co-transfected with different ExSpeU1s ($IK_{4-72)}$ along with plasmids coding for the indicated inhibitory splicing factors in Hek293T cells: hnRNPA1, FUS and SRSF3. After 24 hours, total RNA was extracted, mRNAs produced were analysed through reverse transcriptase PCR (RT-PCR) using α2,3_dir and IKAP-ex21_rev primers. The upper band of 349 bp corresponds to transcripts including the exon 20; the lower band of 275 bp to exon 20 skipping. Schematic representation of the splicing pattern is shown on the right of the RT-PCR gel analysis. PCR products were resolved on 1.5% agarose gel and the bands intensity was quantified with ImageJ software. The calculated percentage of exon 20 inclusion is indicated below the gel and is expressed as means ± SD of three independent experiments.

Since ExSpeU1 IK_{10} showed the strongest efficiency, I tested its activity by co-transfecting it with all the previously identified inhibitory splicing factors (Figure 4.5C). Hek293T cells were co-transfected with IVS20+6T>C minigene, ExSpeU1 IK_{10} and one of the inhibitory factors. Total RNA was extracted after 24hours, retro-transcribed and the pattern of splicing evaluated by RT-PCR with α2,3_dir and IKAP-ex21_rev oligonucleotides (Figure 4.1A). As shown in figure 4.8, ExSpeU1 IK_{10} was able to restore the aberrant splicing, inducing complete exon 20 inclusion.

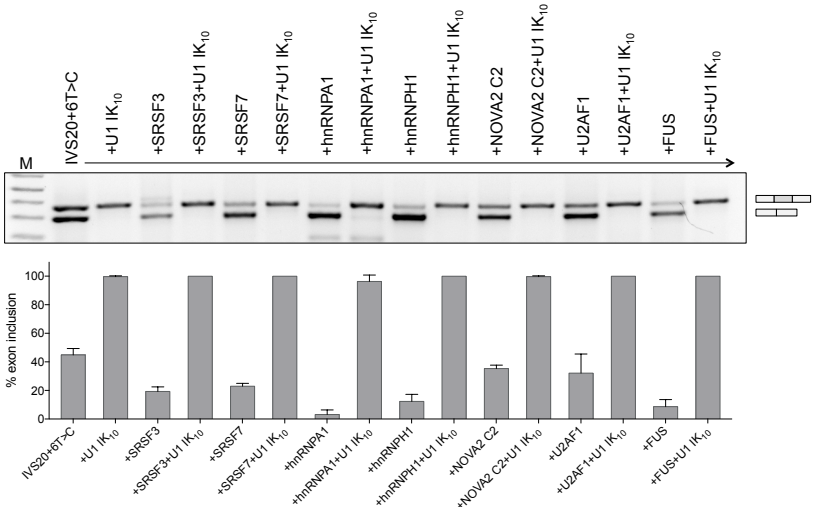

Figure 4.8. ExSpeU1 IK_{10} counteracts the inhibitory effect of splicing factors.

IVS20+6T>C minigene was co-transfected with ExSpeU1 IK_{10} along with splicing factors identified to have inhibitory effect (indicated at the top) on *IKBKAP* exon 20 splicing in Hek293T cell line. After 24 hours, total RNA was extracted, mRNAs produced were analysed through reverse transcriptase PCR (RT-PCR) using α2,3_dir and IKAP-ex21_rev primers. The upper band of 349 bp corresponds to transcripts including the exon 20; the lower band of 275 bp to exon 20 skipping. Schematic representation of the splicing pattern is shown on the right of the RT-PCR gel analysis. PCR products were resolved on 1.5% agarose gel and the bands intensity was quantified with ImageJ software. The graph represents the percentage of exon 20 inclusion and data are expressed as means ± SD of three independent experiments.

4.2.3. Effect of exonic elements on the ExSpeU1-mediated splicing rescue of *IKBKAP* exon 20

To identify exonic regulatory elements potentially involved in the ExSpeU1 rescue I evaluated *IKBKAP* exon 20 constructs that contain exonic deletions. These minigenes were previously described (Hims, Leyne et al. 2007) and contain 14-22 bp long consecutive deletions distributed throughout the entire exon (from ΔEX20-A to ΔEX20-D) (Figure 4.9A). In this minigene system, (obtained from Prof. Slaugenhaupt), the exon 20 is completely included in wild-type (WT-MG) and completely skipped in the mutant (FD-MG) (Figure 4.9B, lanes 1 and 2). As previously reported, the exonic deletions affected the splicing pattern. Based on the splicing changes, ΔEX20-B, ΔEX20-D1 and ΔEX20-D3 caused total exon 20 skipping (Figure 4.9B, lane 6, 12 and 16). Another four constructs (ΔEX20-A, ΔEX20-C, ΔEX20-D and ΔEX20-D2) disrupted splicing significantly, causing exon skipping with different efficiency (Figure 4.9B, lanes 4, 8, 10 and 14).

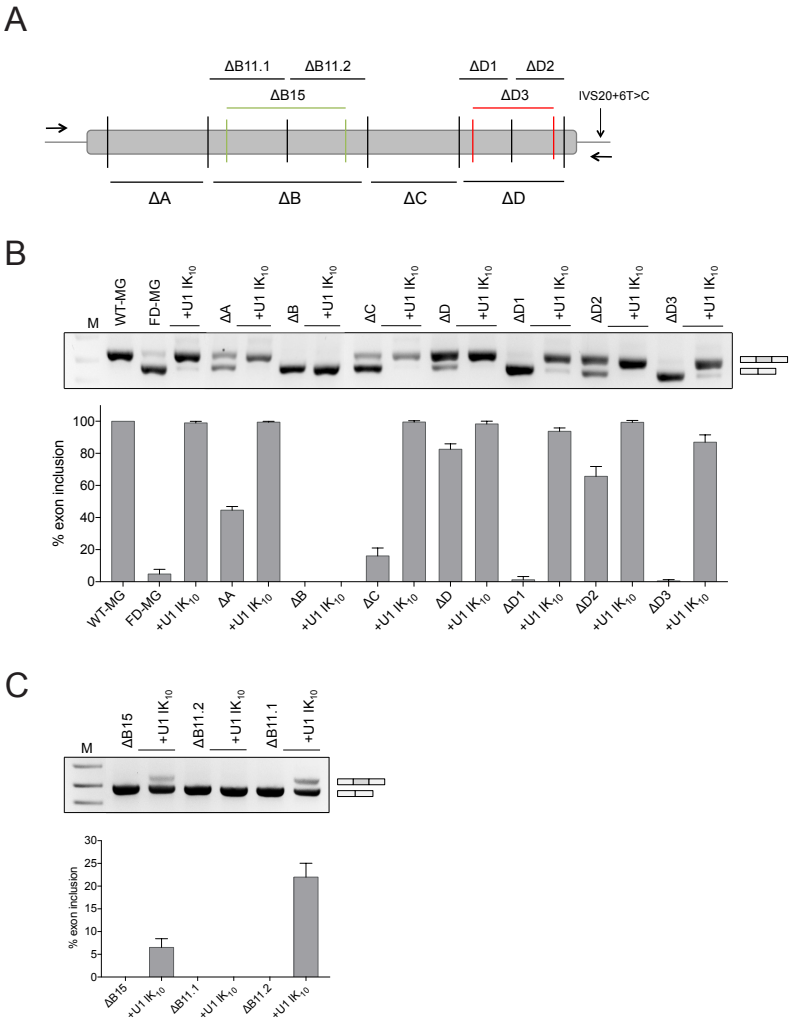

Figure 4.9. *IKBKAP cis*-acting elements and ExSpeU1 IK_{10} activity.

A) Schematic diagram of the pcDNA3.1-FD-MG minigene used for functional splicing assays, showing the location of the deletions (ΔA- ΔD3). Grey box represents exon 20 and lines flanking introns of *IKBKAP* gene. Arrows upon the exons indicate the T7_dir and BGH_rev vector specific primers location used for reverse transcriptase PCR (RT-PCR) and analysis of splicing products.

B) Hek293T cells were co-transfected with 250 ng of indicated minigenes along with ExSpeU1 IK_{10}. After 24 hours, total RNA was extracted, mRNAs produced were analysed through reverse transcriptase PCR (RT-PCR) using T7_dir and BGH_rev primers. Wild-type *IKBKAP* (WT-MG) and mutant *IKBKAP* (FD-MG) are indicated. Amplified products were run on 1.5% agarose gel. Schematic representation of the splicing pattern is shown on the right of the RT-PCR gel analysis. The upper band corresponds to the transcripts that include *IKBKAP* exon 20 (300 bp), whereas the lower represent the exclusion of *IKBKAP* exon 20 (226 bp). The experiment was performed in triplicate and the identity of the bands was verified by direct sequencing. The graphic represents the quantification analysis obtained with ImageJ software expressed as means ± SD.

C) Hek293T cells were co-transfected with 250 ng of indicated minigenes along with ExSpeU1 IK_{10}. After 24 hours, total RNA was extracted, mRNAs produced were analysed through reverse transcriptase PCR (RT-PCR) using T7_dir and BGH_rev primers. Deletions in ΔB region are indicated Amplified products were run on 1.5% agarose gel. Schematic representation of the splicing pattern is shown on the right of the RT-PCR gel analysis. The upper band corresponds to the transcripts that include *IKBKAP* exon 20 (300 bp), whereas the lower represent the exclusion of *IKBKAP* exon 20 (226 bp). The experiment was performed in triplicate and the identity of the bands was verified by direct sequencing. The graph represents the quantification analysis obtained with ImageJ software expressed as means ± SD.

I tested the ability of ExSpeU1 IK_{10} in rescuing exon 20 inclusion in those constructs. Co-transfection experiments showed that the ExSpeU1 IK_{10} was able to recover exon inclusion to the level of wild-type in all minigenes, except FD ΔEX20-B, where U1 IK_{10} did not show any rescue activity (Figure 4.9B, lane 7). This result suggests the presence of a regulatory *cis*-acting element in this region that is important for the ExSpeU1s activity. To investigate in more detail this sequence, I created additional deletion constructs that encompass the ΔB region (FD ΔEX20- B11.1, FD ΔEX20-B11.2, FD ΔEX20-B15) (Figure 4.9A). The two FD ΔEX20-B11.1 and FD ΔEX20-B11.2 deletions divide ΔB region in the half whereas FD ΔEX20-B15 deletes the middle 15 nucleotides. All these constructs caused complete exon 20 skipping (Figure 4.9C, lanes 1, 3 and 5) like the ΔEX20-B larger deletion. Co-transfection experiment using ExSpeU1 IK_{10} showed a slight rescue activity in FD ΔEX20-B15 and FD ΔEX20-B11.1 constructs. In fact, modified U1 IK_{10} promoted an increase in exon inclusion from 0% to ~8% and 25% for FD ΔEX20-B15 and FD ΔEX20-B11.1, respectively (Figure 4.9C, lanes 2 and 6). In contrast, the ΔEX20-B11.2 did not respond to the ExSpeU1 rescue (Figure 4.9C, lane 4). These results indicate the presence of a regulatory element in *IKBKAP* exon 20 affecting the ΔEX20-B11.2 sequence, critical for ExSpeU1 rescue activity.

4.2.4. Effect of U7 snRNAs targeting the regions bound by the ExSpeU1s

To better understand the mechanism by which ExSpeU1s improve exon 20 splicing, I took advantage of the U7smOpt molecule, an optimized RNA that has been broadly used as antisense carrier for masking pre-mRNA regions. To perform this analysis, I replaced the RNA binding sequence of the U7smOPT and I created an U7 antisense RNA (U7 IK_{10}) that covers the same binding region of the most active ExSpeU1 IK_{10} (Figure 4.10A). Hek293T cells were co-transfected with wild-type and mutant minigenes, along with U7 IK_{10} and ExSpeU1s. After 24 hours, total RNA was extracted, retro-transcribed and amplified by PCR using α2,3_dir and IKAP-ex21_rev oligonucleotides (Figure 4.1A). The resulting amplified products were separated by electrophoresis on 1.5% agarose gel. U7 IK_{10} induced complete

exon skipping in the context of the mutant minigene (Figure 4.10B, line 5). On the other hand, U7 IK_{10} did not affect the pre-mRNA processing of the wild-type minigene. In order to evaluate that the U7 IK_{10} is produced and targets the intronic region, I co-transfected the U7 IK_{10} molecule with different modified U1s. Co-transfection experiments showed competition between the U7 molecule and engineered U1s for splicing. In fact, the ExSpeU1 IK_{10} and U1 IK_{15} efficiently counteracted the U7 IK_{10} splicing inhibition increasing the percentage of exon inclusion from 0% up to ~100% and ~90%, respectively (Figure 4.10B, lanes 6 and 7); U1 IK_{46} has a reduced effect bringing exon inclusion up to ~62% (Figure 4.10B, lane 8), whereas U1 IK $_{57}$ and U1 IK_{72} were inactive (Figure 4.10B, lanes 9 and 10). Thus, masking of the same target intronic sequences using an U7 antisense induces exon skipping and the antisense effect activity, is efficiently counteracted by the ExSpeU1s.

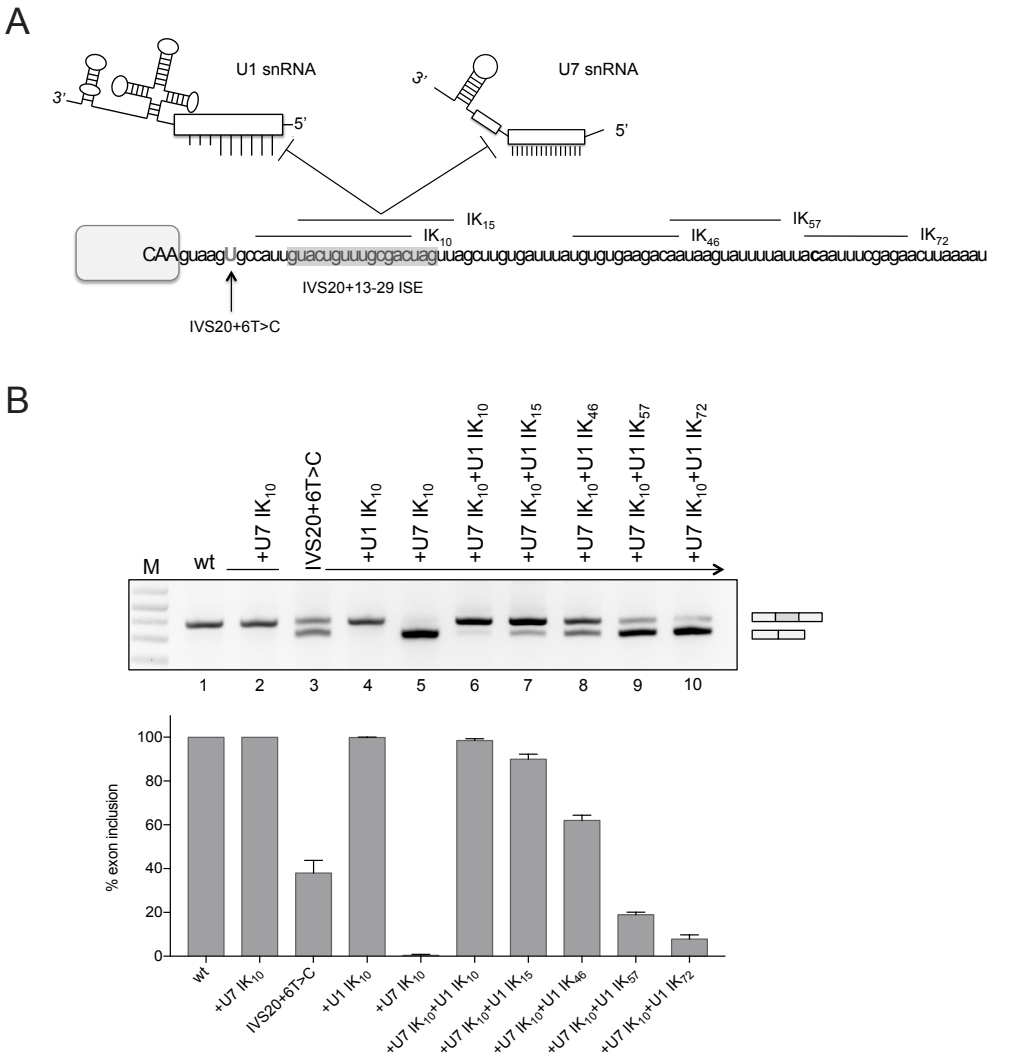

Figure 4.10. ExSpeU1s do not act as antisense molecules.

A) Schematic representation of the *IKBKAP*-ExSpeU1s and modified U7 snRNA (U7 IK_{10}) along with their binding regions in the intronic sequence downstream the *IKBKAP* exon 20 donor splice site. Exonic and intronic sequences are in upper and lower cases, respectively. Black lines above the sequence represent the 5'-complementaty tail of the ExSpeU1s. The recently described cryptic intronic splicing enhancer (ISE) is indicated.

B) Co-transfection of *IKBKAP* minigenes along with *IKBKAP*-ExSpeU1s (IK_{10-72}) in Hek293T cells. Lines 1 and 3, cells transfected with 250 ng of *IKBKAP* wild-type (wt) and mutant (IVS20+6T>C) minigenes, respectively. Line 2, cells transfected with 250 ng of wt minigene and 0.5 µg of plasmid encoding for U7 IK_{10}. Line 4 cells transfected with 250 ng of mutant (IVS20+6T>C) minigene and 0.5 µg of plasmids encoding or U1 IK_{10}, line 5 – cells transfected with 250 ng of mutant (IVS20+6T>C) minigene and 0.5 µg of plasmids encoding for U7 IK_{10}. Lines 6-10, cells transfected with 250 ng of mutant (IVS20+6T>C) minigene and 0.5 µg of plasmids encoding for U7 IK_{10} and different ExSpeU1s. After 24 hours, total RNA was extracted, mRNAs produced were analysed through reverse transcriptase PCR (RT-PCR) using α2,3_dir and IKAP-ex21_rev oligonucleotides. The upper band of 349 bp corresponds to transcripts including the exon 20; the lower band of 275 bp to exon 20 skipping. Schematic representation of the splicing pattern is shown on the right of the RT-PCR gel analysis. PCR products were resolved on 1.5% agarose gel and the bands intensity was quantified with ImageJ software. The graph represents the percentage of exon 20 inclusion and data are expressed as means ± SD of three independent experiments.

4.3. Role of endogenous U1 snRNP in *IKBKAP* exon 20 splicing rescue induced by ExSpeU1s

To further investigate how ExSpeU1 IK rescues exon skipping, I considered the role of endogenous U1 snRNP. In order to do this, I used a decoy system, which has been previously used to obtain a functional suppression of U1 snRNP in the context of SMN2 exon 7 (Hua, Vickers et al. 2008, Roca and Krainer 2009). To determine whether the ExSpeU1 molecules require the presence of the endogenous U1 at the canonical 5' splice site, I decided to check efficacy of the ExSpeU1 IK_{10} on the IVS20+6T>C minigene when the endogenous U1 particle is functionally inactivated in the cells. HeLa cells were transfected with wild-type and mutant *IKBKAP* minigenes and co-transfected with U1 snRNA specific decoy (D1), a plasmid encoding an RNA decoy, previously described to interact by complementarity with the endogenous U1 RNA (Figure 4.11A). The resulting functional inactivation of U1 snRNP (Figure 4.11B) caused exon skipping in the case of SMN gene (Roca and Krainer 2009, Dal Mas, Rogalska et al. 2015). As a control, I used a U1 decoy that contains a mis-match in the base-pairing with the U1 (D3) (Figure 4.11A), which has no effect on SMN splicing pattern (Roca and Krainer 2009, Dal Mas, Rogalska et al. 2015). After 24 hours, total RNA was extracted, retro-transcribed and amplified by PCR using α2,3_dir and IKAP-ex21_rev oligonucleotides (Figure 4.1A). The resulting amplified products were separated by electrophoresis on 1.5% agarose gel. Co-transfection experiments showed that the U1 snRNA decoy (D1) affects *IKBKAP* splicing, decreasing the percentage of exon 20 inclusion from 83% to 64% (Figure 4.11C). Control U1 decoy (D3) did not change percentage of exon 20 inclusion (Figure 4.11C). Interestingly, ExSpeU1 IK_{10} was able to counteract the negative effect of D1, resulting in a complete exon 20 inclusion.

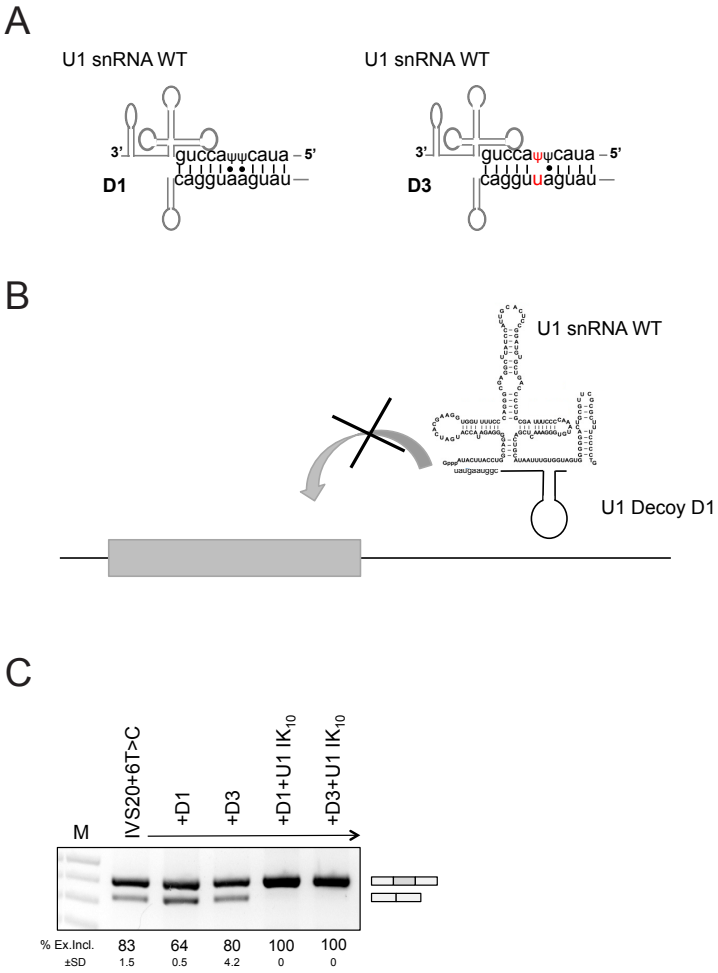

Figure 4.11. Role of the endogenous U1 snRNP on the ExSpeU1-mediated splicing rescue of FD exon 20.

A) Schematic representation of the base-paring between the 5'tail of the U1 snRNA wt and the tail of the RNA U1 decoy (D1). U1 snRNA decoy with a mismatch in position +3 (D3).

B) Schematic representation of the inhibition of the U1 snRNA through the U1 decoy.

C) HeLa cells were co-transfected with IVS20+6T>C minigene alone or in combination with U1 snRNA D1, D3 or ExSpeU1 IK_{10}. After 24 hours, total RNA was extracted, mRNAs produced were analysed through reverse transcriptase PCR (RT-PCR) using α2,3_dir and IKAP-ex21_rev primers. The upper band of 349 bp corresponds to transcripts including the exon 20; the lower band of 275 bp to exon 20 skipping. Schematic representation of the splicing pattern is shown on the right of the RT-PCR gel analysis. PCR products were resolved on 1.5% agarose gel and the bands intensity was quantified with ImageJ software. The calculated percentage of exon 20 inclusion is indicated bellow the gel and is expressed as means ± SD of three independent experiments.

4.4. ExSpeU1-mediated correction of *IKBKAP* exon 20 skipping in *ex vivo* cellular model

As shown above, several ExSpeU1s efficiently promote *IKBKAP* exon 20 inclusion in minigene splicing assays. To evaluate their therapeutic potential in genomic context, I selected the most effective ExSpeU1 IK_{10} (in *in vitro* experiments) and tested it on FD patient's fibroblasts. Primary cells have low transfection efficiency with conventional methods and for this reason we expressed the ExSpeU1 by lentiviral (LV) transduction. We designed LV to express the non-coding U1 IK_{10} and control U1 snRNA wt. We transduced primary FD fibroblasts with increasing concentrations (multiplicity of infection - MOI) of LV particles (MOI 0-20). After 72 hours from infection cells were harvested and rescue efficacy was determined at the RNA level through the analysis of the *IKBKAP* exon 20 splicing pattern using semiquantitative RT-PCR assay with human-specific primers hIKAP-ex19_dir and IKAP-ex21_rev (Figure 4.12A). Transduction of FD fibroblasts with U1 IK_{10} MOI 1, MOI 10 and MOI 20 induced a significant rescue of splicing and increased exon 20 inclusion percentage from ~26% to 54%, 95% and 99%, respectively (Figure 4.12A). Lentiviral particles expressing U1 wt did not affect splicing (Figure 4.12A). The expression of ExSpeU1 IK_{10} in transduced cells was checked through a RT-PCR with a specific set of oligonucleotides (U1-IK10_dir and U1-IK10_rev). Analysis in lentiviral infected fibroblasts showed progressive expression of U1 IK_{10}, corresponding with increasing concentrations of LV IK_{10} (Figure 4.12B). Improvement of the *IKBKAP* exon 20 inclusion was evaluated by Real-Time PCR. Quantitative analysis confirmed the ExSpeU1 IK_{10} efficacy, showing a significant ~3, ~16 and ~16 fold increase in the amount of full-length (FL) *IKBKAP* mRNAs in cells treated with MOI 1, MOI 10 and MOI 20 of LV IK_{10}, respectively (Figure 4.12C), in comparison to untreated cells. In parallel with the increase in the FL, the amount of transcript lacking exon 20 (D20) was gradually reduced in relation to the increase of MOI (Figure 4.12C). Overexpression of control U1 wt did not influence the level of *IKBKAP* transcripts (Figure 4.12C). In order to evaluate if the significant increase of full-length transcripts was due to the splicing correction, I analysed the total amount of *IKBKAP* mRNAs by quantitative Real-Time PCR of a constitutive-not affected region using

specific primers (Figure 4.12A,C). Analysis revealed a ~3 times fold increase of total *IKBKAP* mRNA in cells treated with MOI 10 and MOI 20 of ExSpeU1 IK_{10} (Figure 4.12C), whereas I did not sae any significant increase with MOI 1 or with LV expressing U1 wt (Figure 4.12C). To investigate, if the correction at the RNA level results in a corresponding increase in the protein level, I evaluated the IKAP protein levels in FD patient's fibroblasts after ExSpeU1 lentiviral mediated transduction, as well as the level of the protein present in untreated fibroblasts. The IKAP protein levels were evaluated through western blot using a rabbit polyclonal anti-IKAP antibody. I was able to detect IKAP protein only in the cells treated with MOI 10 and MOI 20 of the ExSpeU1 IK_{10} (Figure 4.12D). Quantification of IKAP bands intensity showed that ExSpeU1 IK_{10} restored biosynthesis of IKAP protein in those cells where expression of ExSpeU1 was high (MOI 10 and MOI 20). The protein was undetectable in untreated, treated with MOI 1 of LV IK_{10} and treated with LV U1 wt cells (Figure 4.12D). α-tubulin was used as a housekeeping loading control.

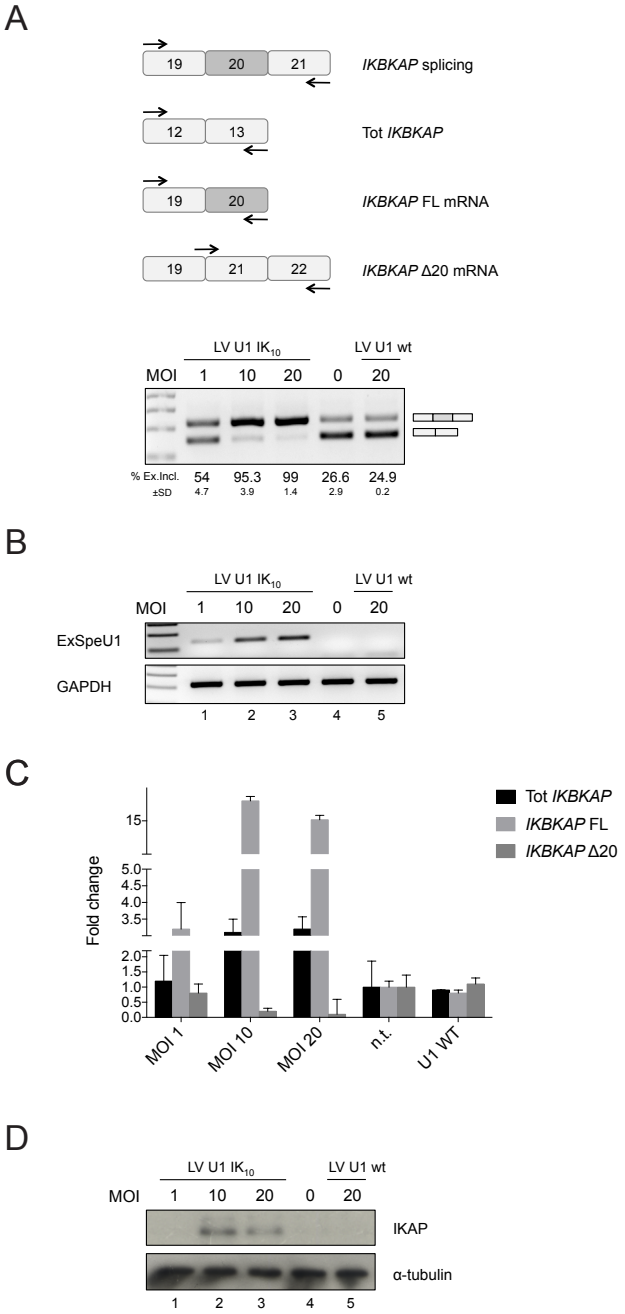

Figure 4.12. Lentiviral delivery of ExSpeU1 rescues *IKBKAP* splicing and IKAP protein in FD patients fibroblasts.

A) Upper panel represents schematic representation of *IKBKAP* transcripts along with PCR primers used for semiquantitative and quantitative analysis of *IKBKAP* mRNA isoforms. Lover panel represents semiquantitative analysis of the *IKBKAP* splicing pattern in FD patient's fibroblasts after lentiviral transduction. FD fibroblasts were treated with lentiviral particles expressing the ExSpeU1 IK_{10} (LV U1 IK_{10}) and the wild-type U1 snRNA (LV U1 wt) at different Multiplicity of Infection (MOI). After 24 hours, total RNA was extracted, mRNAs produced were analysed through reverse transcriptase PCR (RT-PCR) using ELP1-Ex19_dir and ELP1-Ex21_rev primers. The upper band of 202 bp corresponds to transcripts including the exon 20; the lower band of 128 bp to exon 20 skipping. Schematic representation of the splicing pattern is shown on the right of the RT-PCR gel analysis. PCR products were resolved on 2% agarose gel and the bands intensity was quantified with ImageJ software. The calculated percentage of exon 20 inclusion is indicated bellow the gel and is expressed as means ± SD of three independent experiments.

B) In the upper panel semiquantitative analysis of the ExSpeU1 IK_{10} in FD fibroblasts untreated and transduced with increasing concentrations (MOI indicated on the figure) of lentiviral particles expressing the ExSpeU1 IK_{10} (LV U1 IK_{10}) and lentiviral particles expressing the wild-type U1 snRNA (LV U1 wt). In the lower panel semiquantitative analysis of GAPGH used as reference for internal normalization.
C) Quantitative analysis of *IKBKAP* mRNAs isoforms by real-time PCR (qPCR). The histograms represent the fold change of total (Tot *IKBKAP* FL), full-length (*IKBKAP* FL) and exon 20 skipping (*IKBKAP* Δ20) *IKBKAP* mRNAs compared to the untreated cells (n.t.). Statistical analysis was performed using a two-ways ANOVA (***p<0.0001, **p<0.001, *p<0.01; ns, not significant).
D) IKAP protein detection by Western blot with human-specific primary antibody. α-tubulin was used as reference for internal normalization.

4.5. *In vivo* activity of ExSpeU1 in FD mouse model

In order to evaluate the therapeutic potential of the ExSpeU1s *in vivo*, I used the adeno-associated virus serotype 9 (AAV9) carrying U1 IK_{10} and U1 $IK_{15,}$ the two most active molecules. The AAV-ExSpeU1s were tested in the *TgFD9* transgenic mouse, an available FD model carrying the entire human *IKBKAP* gene with the major splice mutation c.2204+6T>C. This model show a tissue-specific *IKBKAP* splicing pattern similar to that reported in FD patients. The AAV-ExSpeU1s were produced by our collaborators from Prof. F. Mingozzi group in Genethon company. Newborn animals (N = 6) were injected into the peritoneum (IP) at two time points, postnatal day 0 and 2 (PND0 and PND2). Control treatment was done with saline solution. Mice were sacrificed at PND7 and organs collected for splicing analysis: brain, liver, heart, kidney, muscle and spinal cord. To analyse the *IKBKAP* exon 20 splicing RNA was extracted from the tissues, and the pattern of splicing analysed using semiquantitative RT-PCR assay with specific primers (hIKAP-ex19_dir and IKAP-ex21_rev). Treatment with AAV-ExSpeU1s induced a significant rescue of splicing increasing the exon 20 inclusion percentage in several tissues: in liver from ~60% to ~97%; in heart from ~65% to ~90% and in muscle from ~52% to ~75% (Figure 4.13A-C). In the spinal cord I observed small, statistically not significant correction in the percentage of exon 20 inclusion from ~24% to ~32% (Figure 4.13F). In brain and kidney, I did not observe any significant improvement (Figure 4.13D,E). The overall splicing correction mediated by both ExSpeU1s was similar in both tissues (Figure 4.13). In order to confirm that increase

in the full-length transcripts seen in splicing PCR was due to the splicing correction, I analysed tissues samples by quantitative Real-Time PCR with human specific oligonucleotides. In liver, I observed a 6- and 4-fold increase in the amount of wild-type *IKBKAP* transcript, for U1 IK_{10} and U1 IK_{15}, respectively. At the same time, I observed significant decrease in the amount of mutated *IKBKAP* transcript for both ExSpeU1s (Figure 4.13A). In heart, I found ~3-fold increase in the amount of the correct transcript (Figure 4.13B), whereas for muscle ~1.5-fold increase (Figure 4.13C), for both U1s. Also in these tissues, I was able to see a decrease in the amount of mutated transcript (Figure 4.13B,C). In brain, kidney and spinal cord no significant improvement was observed in splicing (Figure 4.13D-F). As done for the *ex vivo* experiment, I also checked the amount of the total mRNAs using human specific primers (hIKAP-ex19_dir and IKAP-ex21_rev). Interestingly, AAV-ExSpeU1s treatment increased also in this case, the total amount of *IKBKAP* transcripts in liver (~3-fold) and in heart tissue (~2-fold) (Figure 4.13A-F).

In order to investigate, if the correction at the RNA level results in an increase of the IKAP protein, I evaluated the protein levels in mice tissues after AAV injections, and saline control treatment. The IKAP protein levels were evaluated through Western blot using a rabbit polyclonal anti-IKAP antibody. Quantification of IKAP bands intensity from the Figure 4.14 showed that ExSpeU1 IK_{10} increased ~3.5 times the amount of IKAP protein in liver (Figure 4.14A,F) and ~3 times in heart (Figure 4.14B,F). In those two tissues ExSpeU1 IK_{15} nearly doubled the amount of the protein (Figure 4.14A,B,F). In all the other tissues: brain, muscle and kidney I did not see an increase in the amount of IKAP protein (Figure 4.14C-F). α-tubulin was used as a housekeeping loading control. All together these results demonstrate that ExSpeU1s represent a valid strategy for the treatment of the splicing defect present in FD.

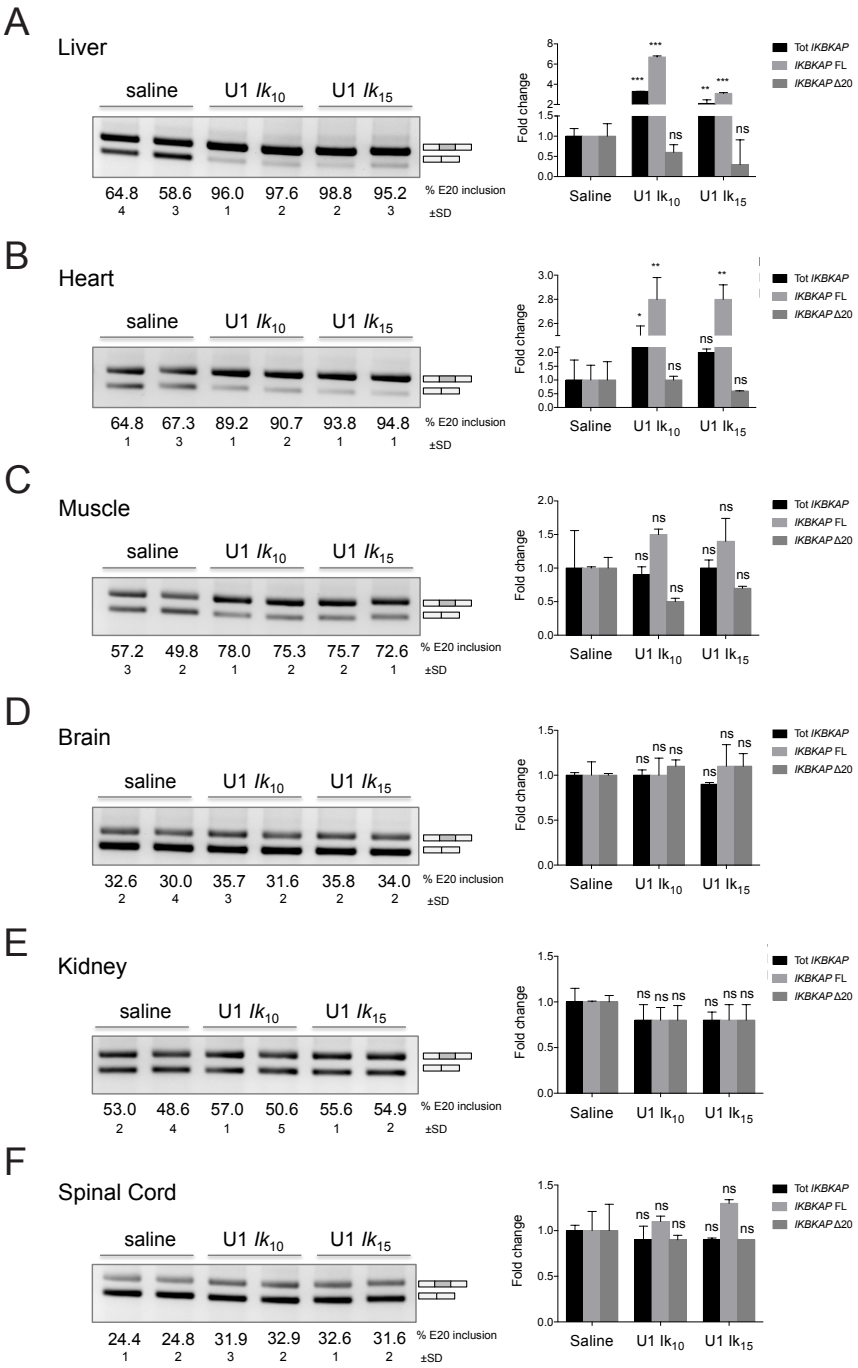

Figure 4.13. AAV9-mediated delivery of ExSpeU1s IK_{10} and IK_{15} rescues *IKBKAP* splicing defect in FD mouse model.

A-F) Semiquantitative (left) and quantitative (right) analysis of *hIKBKAP* mRNAs' isoforms in tissues of transgenic FD mice treated with saline, 3.55E11 VG/mouse of AAV9-ExSpeU1 IK_{10} (U1 IK_{10}) and 2.65E11 VG/mouse of AAV9-ExSpeU1 IK_{15} (U1 IK_{15}). A representative semiquantitative PCR of two samples per condition resolved on a 2% agarose gel is shown and the intensity of the bands was quantified with ImageJ software. The upper band of 202 bp corresponds to exon 20 inclusion and the lower band of 128 bp corresponds to exon 20 skipping. The percentage of exon inclusion is expressed as mean ± SD of groups of four animals per condition. The histograms on the right represent the quantitative fold change of *IKBKAP* mRNAs' isoforms (total *IKBKAP*, *IKBKAP* full-length and *IKBKAP* Δ20) compared to saline-treated of four animals per condition. Statistical analysis was 20 performed using a two-ways ANOVA (***p<0.0001, **p<0.001, *p<0.01; ns – not significant).

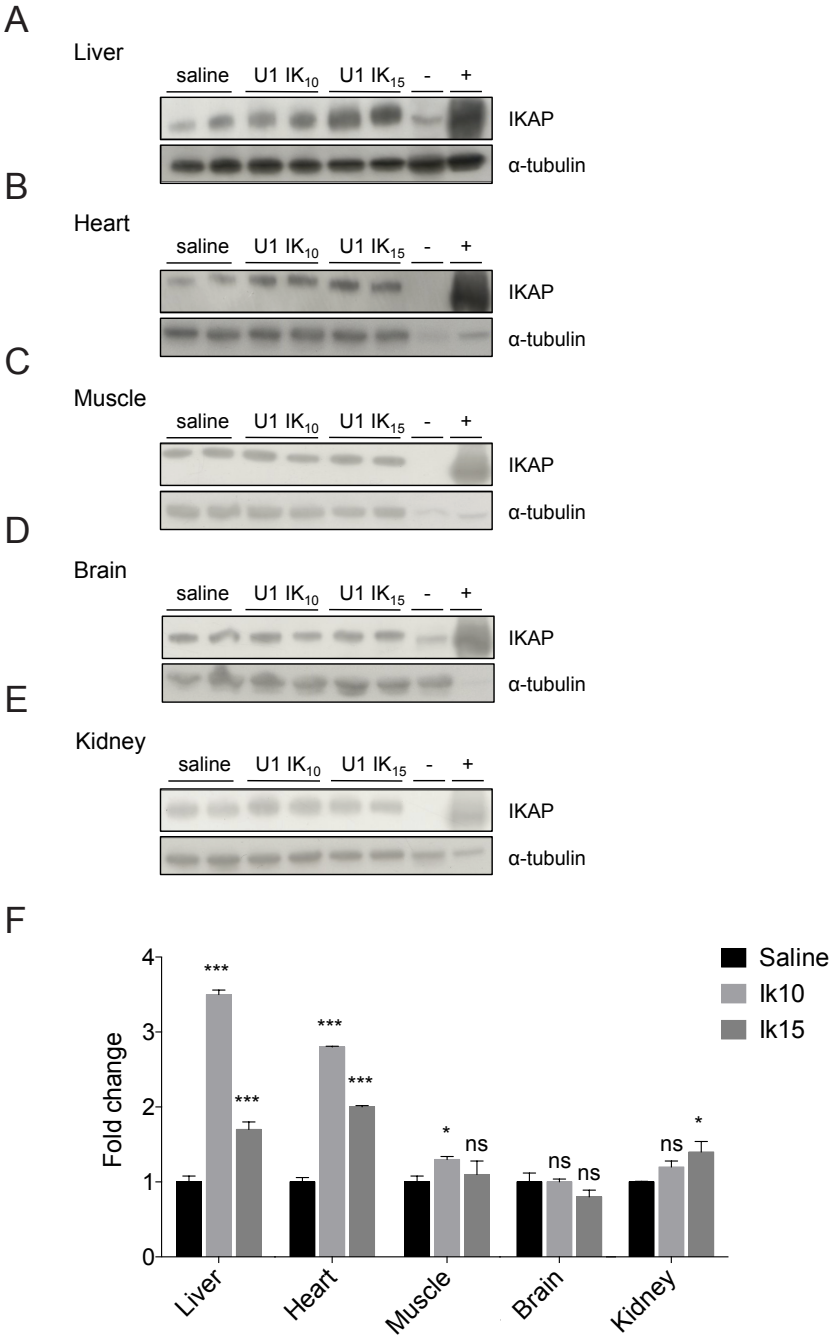

Figure 4.14. ExSpeU1 IK_{10} and IK_{15} increase IKAP biosynthesis in FD mouse tissues.

A-E) IKAP Western blots of mouse tissues samples (indicated on the left) of FD mice treated with saline, 3.55E11 VG/mouse of AAV9-ExSpeU1 IK_{10} (U1 IK_{10}) and 2.65E11 VG/mouse of AA9-ExSpeU1 IK_{15} (U1 IK_{15}). α-tubulin was used as reference for internal normalization.

F) The histogram represents the fold change of IKAP protein amount obtained from densitometric analysis compared to tissues treated with saline. Each group includes two animals and data are expressed as mean ± SD. Statistical analysis was performed using a two-ways ANOVA (***p<0.0001, **p<0.001, *p<0.01; ns - not significant).

5. DISCUSSION

Exon-specific U1 snRNAs (ExSpeU1s) represent a novel RNA-based strategy that act on pre-mRNA promoting definition of defective exons. To evaluate their splicing rescue activity I focused on Familiar Dysautonomia. FD is a rare, severe and lethal autosomal recessive disease affecting autonomic and sensory nervous system. Available treatments are only supportive and based on the attenuation of the symptomatology (Dietrich and Dragatsis 2016, Norcliffe-Kaufmann, Slaugenhaupt et al. 2017). In this dissertation, I provide evidence that ExSpeU1-based approach can be a valid therapeutic treatment for FD patients. *In vitro* system screening based on minigenes, *ex vivo* analysis in primary affected cells transduced with lentiviral vectors and *in vivo* analysis with AAV9-ExSpeU1s in the *TgFd9* mouse model show the efficacy of the approach. This is mainly demonstrated by the ExSpeU1-mediated rescue of the *IKBKAP* exon 20 aberrant skipping and recover of the IKAP protein in the primary cells and in the animal model. In addition, I also provide interesting observations regarding the ExSpeU1-mediated rescue mechanism and the involvement of regulatory splicing factors. As ExSpeU1-rescue activity entirely depends on an exonic regulatory element, does not require endogenous U1 snRNA and is not working as an antisense mechanism, my results strongly suggest that these molecules specifically improve the *IKBKAP* exon 20 definition. I also identified novel splicing factors that regulate the *IKBKAP* exon 20 splicing and might be involved in the tissue-specific expression of the defect.

5.1. ExSpeU1 represents a novel therapeutic strategy for FD

ExSpeU1s are U1 snRNAs molecules with engineered 5'-tail that specifically target the intronic region downstream of the 5' splice site of defective exons. These molecules have been reported to correct several exon skipping events (Alanis, Pinotti et al. 2012, Dal Mas, Fortugno et al. 2015, Dal Mas, Rogalska et al. 2015, Tajnik, Rogalska et al. 2016). To identify the active splicing-switching ExSpeU1s

in the FD context, I used a minigene system that, recapitulating the pathological FD mis-splicing, allowed me to screen a large number of ExSpeU1s. Indeed, through co-transfection experiments, I have identified a panel of active ExSpeU1s that induce a significant splicing correction in basal conditions (Figure 4.2). I reasoned that the most potent ExSpeU1 should act not only in basal conditions, but also in the presence of negative factors that reduce the exon definition. For this reason, and to evaluate the most interesting molecules in terms of splicing rescue efficiency, I performed co-transfection experiments with negative splicing factors (Figure 4.4, Figure 4.5). The overexpression of the negative splicing factors (hnRNPA1, SFSF3 and FUS) resulted in the selection of two ExSpeU1s, IK_{10} and IK_{15}. These two molecules showed the strongest ability to overcome the inhibitory effect of the splicing factors, promoting the complete inclusion of exon 20 in the final transcript (Figure 4.7). Interestingly, they also fully recovered the exon skipping induced by an antisense particle that binds to the same intronic region (Figure 4.10) further indicating their higher rescue activity.

To provide evidence that ExSpeU1s have a positive effect on the defective *IKBKAP* transcript and IKAP protein abundance, I tested their effect in available FD primary cells (i.e. fibroblasts) (Figure 4.12). Previous studies in SMA fibroblasts and SPINK5 keratynocytes showed the positive effect of ExSpeU1s in patients' derived cells (Dal Mas, Fortugno et al. 2015, Dal Mas, Rogalska et al. 2015). With lentiviral transduction I demonstrate that the ExSpeU1s efficiently correct the *IKBKAP* exon 20 skipping defect and increase the amount of the corresponding IKAP protein (Figure 4.12A,D). Based on these positive results, I then evaluated the ExSpeU1s in the FD mouse model (Figure 4.13, Figure 4.14). To evaluate the therapeutic potential of the *IKBKAP*-ExSpeU1s *in vivo*, I chose the previously described *TgFD9* transgenic mouse carrying the entire human *IKBKAP* gene with the major FD splicing mutation (Hims, Shetty et al. 2007). This FD mouse model does not have any phenotypic abnormality but displays the same tissue-specificity of exon skipping observed in FD patients. AAV ExSpeU1 treatment corrects the *IKBKAP* exon 20 skipping and increases the IKAP protein in several mouse tissues (Figure 4.13, Figure 4.14). As FD onset is from birth (Haynes 1965), I decided to perform intraperitoneal administration at PND0 and PND2. At PND7

treated animals were sacrificed and several organs were collected for analysis. This gave the opportunity to analyse ExSpeU1s effect at early stages of disease. Results of the experiment demonstrate that both modified U1s are able to correct the *IKBKAP* exon 20 skipping in several mouse tissues *in vivo* and that the overall effect obtained with the two ExSpeU1s used is comparable indicating that this RNA based approach might be an effective therapeutic option for FD.

To evaluate effect of AAV9 expressing ExSpeU1s on splicing of *IKBKAP* in the mouse model we performed the semiquantitative assay with RT-PCR and more quantitative analysis with qPCR. Even if those two methods cannot be directly compared, I observed a good correlation between the semiquantitative assay and the quantitative assay (Figure 4.13). Interestingly, in both FD fibroblasts and in mouse tissues (Figure 4.12, Figure 4.13) ExSpeU1s treatment not only improves FD splicing pattern but also increases the total amount of *IKBKAP* mRNA transcript. The increase in the total amount of *IKBKAP* transcript could be due to two not-mutually exclusive mechanisms.

The first is related to nonsense-mediated mRNA decay (NMD). NMD is a mechanism responsible for quality-control, able to degrade mRNA harbouring a premature termination codon in order to prevent the synthesis of truncated proteins (Maquat 2004, Conti and Izaurralde 2005). In the case of *IKBKAP* exon 20 skipping, the c.2204+6T>C mutation, introduces a premature termination codon (Yoshida, Kataoka et al. 2015) and the resulting transcripts are degraded by NMD (Mendell and Dietz 2001). Treatment with ExSpeU1s results in correction of aberrant splicing with the resulting transcript not subjected to NMD. However, it is also possible that the increase in total amount of mRNA could be due to a direct effect of the ExSpeU1 particle on *IKBKAP* pre-mRNA stability, as previously reported for the SMN2 gene (Dal Mas, Rogalska et al. 2015). Indeed, the ExSpeU1-mediated correction of SMN2 exon 7 skipping resulted in increased stability of the pre-mRNA intermediate, suggesting a more direct effect of snRNP on pre mRNA processing. U1 snRNA is involved in many cellular processes, for example transcripts protection from premature polyadenylation signals (Kaida, Berg et al. 2010) or promotion of transcription when bound to the first exon (Damgaard, Kahns et al. 2008) Thus, it could be possible that ExSpeU1s like endogenous U1 particles,

have a positive effect not only on splicing, but more in general on mRNA processing by promoting transcription, affecting cryptic polyadenylation sites and finally stabilizing the mRNAs. This effect can be particularly seen on the figure 4.13, where for liver and heart amount of total transcript significantly increases. However, detailed studies are required to understand in detail how ExSpeU1s affect processing of pre-mRNA transcripts.

Splicing correction therapies present interesting advantages over classic substitutive gene therapies. First, the splicing correction maintains the regulation of the gene expression in the correct cell-specific chromosomal context under control of endogenous transcription and pre-mRNA processing regulatory elements. In addition, the short length of the ExSpeU1s cassette (650 bp) can be useful in gene therapy of splicing mutations in large genes such as FD whose full-length transcript represents a limiting step for insertion in viral vectors such as AAV. On the other hand, to achieve optimal therapeutic rescue, the expression level could be modulated *in vivo* by varying the number of ExSpeU1 cassettes in the viral vector. Moreover, binding of the ExSpeU1s to intronic sequences, which are not conserved, will significantly reduce the possibility of off-target events. In the last few years, several compounds have been reported to correct *IKBKAP* splicing, but none of them work specifically for the exon 20 mutation. The plant cytokinin kinetin has been shown to rescue *IKBKAP* splicing in FD cell lines (Cuajungco, Leyne et al. 2003, Slaugenhaupt, Mull et al. 2004, Lee, Papapetrou et al. 2009) and *in vivo* in both a transgenic mouse model (Hims, Shetty et al. 2007) and in FD patients (Axelrod, Liebes et al. 2011). The glycoside digoxin, possibly through the inhibition of SRSF3 expression, also corrects *IKBKAP* splicing in FD cells (Liu, Anderson et al. 2013). Phosphatidylserine (PS), a FDA-approved food supplement, is also active in FD cellular and mouse models where it increases *IKBKAP* transcription (Keren, Donyo et al. 2010, Bochner, Ziv et al. 2013, Naftelberg, Abramovitch et al. 2016). RECTAS (rectifier of aberrant splicing), a small molecule with a chemical structure similar to kinetin, has also been shown to improve *IKBKAP* splicing in FD cells (Yoshida, Kataoka et al. 2015). Despite their activities, none of these compounds is designed to specifically target *IKBKAP* exon 20, and thus they have high risks of off-target effects. In addition, the initial clinical study performed with kinetin showed that high

doses are required to improve splicing and that the compound is not well tolerated by the patients (Axelrod, Liebes et al. 2011). However, the recruitment to the II Phase of the clinical trials is currently in progress (NCT02274051), and aim to determine the safety and tolerability of kinetin in FD patients. It is important to note that also phosphatidylserine is now in the process of recruitment for the clinical trial Phase II (NCT02276716).

5.2. Therapeutic usage of AAV vectors

AAV vectors represent a safe and effective delivery system for classical substitutive gene replacement therapies and for this reason they are actively explored in several diseases and clinical trials (Mingozzi and High 2011, Nathwani, Reiss et al. 2014, Bennett, Wellman et al. 2016, Scoto, Finkel et al. 2017).The AAV strategy has not been previously considered in FD as the AAV particles cannot easily accommodate the large size of the *IKBKAP* transcript (5.9-kb cDNA; GenBank, gene accession number AF153419), and associated regulatory elements (Daya and Berns 2008, Mingozzi and High 2011, Nathwani, Reiss et al. 2014, Bennett, Wellman et al. 2016, Scoto, Finkel et al. 2017). In contrast, our ExSpeU1 approach that is based on splicing correction uses a very short cassette of ~650 bp that contains the entire regulatory elements for expression and can be easily accommodated in the AAV backbone. In addition, AAV vectors appear to be one of the most promising agents in gene therapies of nervous system associated diseases (Weinberg, Samulski et al. 2013). AAV vectors have already been used in several Phase I and Phase II clinical trials (Grieger and Samulski 2005, Mingozzi and High 2011, Nathwani, Reiss et al. 2014, Bennett, Wellman et al. 2016, Scoto, Finkel et al. 2017) and represent a safe and effective delivery system, when used with low doses (George, Sullivan et al. 2017). AAV9 serotype has been shown to achieve widespread gene transfer to the CNS after systemic delivery (Barkats 2008), cross the blood-brain barrier (BBB) and infect cells of the CNS, including primary neurons (Zhang, Yang et al. 2011, Merkel, Andrews et al. 2017). Additionally, AAV9 was shown to be very efficient in delivering genes to skeletal and cardiac muscle in various animal models (Su, Yeghiazarians et al. 2008, Zincarelli, Soltys et al. 2008, Kotchey, Adachi et al.

2011, Hadri, Kratlian et al. 2013, Greenberg, Yaroshinsky et al. 2014, Wang, Zhong et al. 2014). This is very important taking in consideration that FD affects the development and survival of sensory, sympathetic, and parasympathetic neurons and accordingly brain, muscles and spinal cord should be the optimal target therapeutic tissues. In fact FD is a neurodevelopmental and neurodegenerative disorder, whose main therapeutic targets are represented by the afferent neurons in dorsal root ganglia (DRGs) and cranial nerve ganglia (Dietrich and Dragatsis 2016, Norcliffe-Kaufmann, Slaugenhaupt et al. 2017). In addition, retina should also be considered. Progressive blindness is a major debilitation that affects the quality of patients' life and recent studies highlighted the importance of the retinal dysfunction in the onset of blindness in FD (Mendoza-Santiesteban, Hedges III et al. 2012, Mendoza-Santiesteban, Hedges III et al. 2014). Reduction in retinal nerve fiber thickness, which is due to loss of retinal ganglion cells, was observed in retinas of FD patients (Mendoza-Santiesteban, Hedges III et al. 2012, Mendoza-Santiesteban, Hedges III et al. 2014). As AAV represents a suitable and efficient system for gene delivery in the eye, it could be considered also for the ExSpeU1s. In my experiments performed with IP injections, I observed only modest rescue of the splicing pattern in brain and spinal cord. I observed an increase in the percentage of exon 20 inclusion (and in corresponding IKAP protein) mainly in liver (from ~60% up to ~97%), heart (from ~65% up to ~90%) and muscle (from ~52% up to 75%) and no significant effect in neuronal tissues (Figure 4.13 and Figure 4.14). Similar results were also evident in previous experiments with AAV9 performed in the SMA mice (Dal Mas, Rogalska et al. 2015) suggesting that the AAV serotype and/or the delivery route might be involved. Further experiments are required to evaluate the AAV biodistribution and also to test different delivery systems (intracerebroventricular, in the eye or systemic) or eventually different AAV serotypes (Tanguy, Biferi et al. 2015). For example, AAV serotype rh10 serotype has been shown to transduce with high efficiency several tissues including the CNS (Hu, Busuttil et al. 2010, Zhang, Yang et al. 2011) and with a higher transduction efficiency in the brain (Zhang, Yang et al. 2011).

5.3. Identification of novel splicing regulatory factors in FD

To identify splicing factors potentially involved in regulating FD aberrant splicing, I performed overexpression experiments (Figure 4.4, Figure 4.5). This analysis identified several splicing factors with both negative and positive effect on FD exon 20. I focused my attention on negative factors and in particular on hnRNPA1, SRSF3 and FUS (Figure 4.5C) where silencing experiments confirmed their regulatory role (Figure 4.6). SRSF3 has been previously implicated in exon 20 regulation as its digoxin-mediated reduction was shown to induce exon 20 inclusion (Liu, Anderson et al. 2013) whereas hnRNPA1/A2 is a well-established negative splicing factor that regulates other exon skipping events (Kashima and Manley 2003, Hua, Vickers et al. 2008) . FUS is a splicing factor that binds along the whole length of the nascent RNAs, with preference for single stranded RNA just upstream of structured GC-rich regions and with increased binding in introns around the repressed exons (Rogelj, Easton et al. 2012). Hundreds of alternative splicing events have been shown to be regulated by FUS in neuronal cells (Masuda, Takeda et al. 2016), in mouse ES cell-derived neurons (Nakaya, Alexiou et al. 2013), in primary cortical neurons (Ishigaki, Masuda et al. 2012) and in mice (Rogelj, Easton et al. 2012). FUS can directly interact with spliceosome components, such as U1 snRNP (Yamazaki, Chen et al. 2012) or SR proteins (Yu and Reed 2015) but the exact mechanism of splicing regulation is obscure. In addition this protein has an important role in the pathogenesis of neurodegenerative diseases. Cytoplasmic accumulation and aggregation along with nuclear clearance have been associated with FUS-specific subset of amyotrophic lateral sclerosis and frontotemporal dementia (Rogelj, Easton et al. 2012) (Vance, Rogelj et al. 2009). In ALS patients, mutations in FUS enhance interaction with SMN protein that results in a reduced association with U1 snRNP (Sun, Ling et al. 2015). This information in combination with knowledge that FD alternative splicing is increased in neuronal cells makes FUS an interesting candidate for the *IKBKAP* splicing regulation.

In co-transfection experiments I also identify splicing factors that promoted exon 20 inclusion. This information is interesting, as previous studies showed that SMN2 mRNA processing can be modified *in vitro* and *in vivo* by drug-mediated increase of splicing factors levels, offering a potential for the treatment of SMA in humans

(Hofmann, Lorson et al. 2000) (Andreassi, Jarecki et al. 2001) (Chang, Hsieh-Li et al. 2001). Although the exon-skipping mechanism in SMA is different from that in FD, these observations clearly demonstrate that certain *trans*-acting factors have the potential to prevent skipping of *IKBKAP* exon 20. This gives a basis for the identification of cellular splicing modulators, such as drugs and/or splicing factors that enhance the efficiency of inclusion of exon 20 in *IKBKAP* transcripts. Between those very efficient enhancers I have identified TIA1 (Figure 4.5B), which was also reported to act as alternative splicing regulator in several systems (David and Manley 2008) (Del Gatto-Konczak, Bourgeois et al. 2000). Future studies should be done to evaluate the splicing factors involved in more detail and the regulatory mechanism to establish their contribution in tissue-specific regulation of the FD splicing defect.

5.4. ExSpeU1 promotes exon definition

Previous studies in other gene systems suggested a model in which ExSpeU1 rescues exon skipping, promoting exon definition (Rogalska, Tajnik et al. 2016). According to this model, ExSpeU1 binding at the intronic sequences downstream the donor site promote exon definition through the formation of the correct network of interactions between the splice sites. In this work on FD exon 20, several observations are in line with this mechanism. In particular, ExSpeU1s need to be in proximity of the defective exon and necessitate the presence of a critical ESE.

Considering the proximity of the ExSpeU1s to the defective exon, ExSpeU1s that bind ~80 bp downstream of the donor site lose their splicing rescue activity (Figure 4.2). It is possible that the distance between the U1 snRNP and the U2- associated factors need to be in a fixed range and when ExSpeU1 is too far away these connections are lost. In addition, as the ExSpeU1-rescue activity is strictly dependent on the presence of an ESE (Figure 4.9) potential splicing factors that interact with this ESE might be involved in promoting the network of interactions on the exon. In addition, my results suggest that ExSpeU1s are not antisense molecules (Figure 4.10) and do not require the endogenous U1 snRNPs (Figure 4.11). In order to understand whether ExSpeU1s could potentially mask an intronic splicing silencer I used modified U7 snRNA. These molecules have been

used as antisense carriers to mask regulatory sequences (Brun, Suter et al. 2003) (Madocsai, Lim et al. 2005) (Marquis, Meyer et al. 2007) (Goyenvalle, Wright et al. 2012). I addressed the ExSpeU1-mediated mechanism by placing the target sequence of the active ExSpeU1 IK_{10} in the U7 snRNA. This molecule, in contrast to the ExSpeU1 that targets the same intronic sequences, induced complete exon 20 skipping (Figure 4.10). This result indicates, as also shown in other gene systems (Dal Mas, Rogalska et al. 2015), that ExSpeU1 does not act as antisense. In addition, the negative effect of the U7 suggests the presence of an intronic splicing enhancer located in position IVS20+13-29 ISE. Indeed, in this region an ISE was recently reported (Ohe, Yoshida et al. 2017). This ISE was shown to interact with RBM24 and promote recognition of the defective 5'ss (Ohe, Yoshida et al. 2017). Interestingly, a competition experiment showed that ExSpeU1s binding at the same intronic element completely rescue the U7 negative effect (Figure 4.10) further indicating that the ExSpeU1s does not act as antisense molecule and promotes exon definition.

6. CONCLUSIONS

In this thesis, I identified for the first time a novel splice-switching strategy based on Exon Specific U1, a small RNA molecule that, by targeting by complementary intronic sequences downstream *IKBKAP* exon 20, specifically corrects the underlying splicing defect. In FD cellular and mouse models, the ExSpeU1-mediated splicing correction results in significant rescue of IKAP protein levels. These results, coupled with the fact that AAV vectors can be used for efficient *in vivo* delivery, suggest that the *IKBKAP*-ExSpeU1 strategy has strong therapeutic potential in FD. Although FD patients show early signs of the disease already at birth, there is continued neurodegeneration throughout life (Dietrich, Alli et al. 2012). Therefore, potential treatments should start as soon as possible and a small improvement in the amount of *IKBKAP* might be sufficient to reduce the degenerative processes (Dietrich and Dragatsis 2016). AAV vectors are currently used to target different tissues and evidence from animal models and clinical trials indicate that they can potentially reach the most important target tissues affected in FD, including DRG, brain and retina (Foust, Poirier et al. 2008, Machida, Kuwahara et al. 2013, Bennett, Wellman et al. 2016). Further experiments are required to evaluate whether the small improvements that we observed in neuronal tissues are sufficient to improve the pathological phenotype and/or to evaluate a more specific delivery system (intracerebral or systemic) or different AAV serotypes (Tanguy, Biferi et al. 2015). The development of a mouse model that recapitulates the FD splicing defect and the typical FD phenotype has proved difficult in the past due to embryonic lethality of the endogenous *IKBKAP* knockout (Chen and Manley 2009). Very recently, a humanized mouse that presents both the FD phenotype and the mis-splicing has been developed (Morini, Dietrich et al. 2016). This mouse model would represent the ideal system to test the efficacy and safety of the ExSpeU1 strategy, providing the necessary preclinical evidence for therapeutic development.

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