Università degli Studi di Ferrara

DOCTORAL COURSE IN

"Biomedical sciences and biotechnology"

CYCLE XXXIII

DIRECTOR Prof. Paolo Pinton

U1snRNA-mediated rescue of splicing defects: exon definition determinants and application in Cystic Fibrosis

Scientific/Disciplinary Sector (SDS) BIO / 11

Candidate

Dott. Stefano Donegà

Supervisor

Prof. Franco Pagani

Academic Year 2017, 2021

Università degli Studi di Ferrara

DOCTORAL COURSE IN

"Biomedical sciences and biotechnology"

CYCLE XXXIII

DIRECTOR Prof. Paolo Pinton

U1snRNA-mediated rescue of splicing defects: exon definition determinants and application in Cystic Fibrosis

Scientific/Disciplinary Sector (SDS) BIO / 11

Candidate

Dott. Stefano Donegà

(signature)

Supervisor

Prof. Franco Pagani

(signature)

Academic Year 2017, 2021

Abstract

Splicing defects can be the direct cause or contribute to the severity of several diseases and exon skipping represents the most frequent pathological event. Splicing mutations that induce exon skipping can affect different *cis*-acting elements including the splice site consensus sequences (3'ss and 5'ss), the polypyrimidine tract or exonic/ intronic regulatory elements.

Modified U1snRNA represents an interesting and promising tool to rescue exon skipping mutations for therapeutic purposes. Modified U1s, named Exon Specific U1s (ExSpeU1s), are based on the genetic engineering of their 5'tail that bind by complementarity to intronic sequences downstream the donor site. This loading on defective exons has been shown to promote their definition but the determinants that regulate this rescue are not known. In this study, using *Factor IX* and *FANCA* as models, I explored the elements present in defective exons that negatively affect their response to ExSpeU1s. In addition, I evaluated in detail the therapeutic potential of ExSpeU1s in Cystic Fibrosis (CF) studying a representative panel of splicing mutations.

To study the determinants involved in ExSpeU1-mediated rescue of defective exons I focussed on *Factor IX* (FIX) exon 5 and *FANCA* (FAN) exon 8 that differ in their ExSpeU1 rescue efficiency. Through the analysis of a series of chimeric *FIX-FANCA* minigenes and site-directed mutagenesis experiments I have identified a critical *cis*-acting exonic elements, named ExSpeU1 Inhibitory Element (ExSpeU1-IE) present in the FANCA context that inhibits the ExSpeU1 response. In addition, co-transfection experiments with splicing factors suggested that this element has a context-dependent splicing regulatory mechanism: depending on the exon it might either counteract the function of enhancing splicing factors or promote the activity of silencing factors.

To provide a useful rescuing approach in a specific disease, I focussed on Cystic Fibrosis (CF). In the last few years, CF research has been very successful in developing personalised therapies based on the correction of the underlying basic

mutations at the protein level. This led to the discovery of drugs that act on specific *CFTR* protein mutants either as potentiators or correctors. However, CF individuals with mutations that disrupt *CFTR* RNA processing cannot benefit of these personalized therapies.

To prove the potential therapeutic activity of ExSpeU1 on *CFTR* and to establish a useful platform that can be applied to this disease, I focussed on ten relatively frequent splicing mutations that cause skipping of corresponding exons 5, 10, 13, 16 and 18. Splicing mutations 711+3A>C/G and 711+5G>A are located in the 5'ss consensus of exon 5; 1863C>T (p.Y577Y) and 1898+3A>G in an exonic regulatory element and in the 5'ss consensus of exon 13, respectively; 2789+5G>A and 3120G>A are located at the 5'ss consensus of exon 16 and 18 respectively, whereas TG13T3, TG13T5, TG12T5 are variants at the polypyrimidine tract of exon 10. Using minigene splicing assay, I show that ExSpeU1s can efficiently correct the splicing defects in all these mutations, restoring the CFTR protein levels.

This result represents the first step towards the development of a personalized approach based on the ExSpeU1 strategy for rescuing *CFTR* splicing mutations. With ten mutations and five exons analysed, it is the first time that an exon rescue strategy is shown to work efficiently in a single gene on several exon skipping mutations and different exons.

Acknowledgements

I want to express my gratitude to Prof. Franco Pagani for giving me the opportunity to work in his Lab, a multi-cultural environment where I had the possibility to grow as a researcher and as human being.

As well, I want to thank all my lab members: Erica Bussani for all the technical suggestions and the supportive moments, Giulia Romano and Irving Donadon for the important advices, and all former members I had the opportunity to know as Ivana Petrovic, Katarzyna Rajkowska, Giulia Pianigiani, Chiara Mattioli and Malgorzata Rogalska. This PhD experience would have been different without all lab students thus I thank the colleagues Federico Riccardi, Antonio Vicidomini, Laura Peretto and Cecilia Alessandri. All staff in ICGEB was really kind, thus I want to express my gratitude to all the people working in this Research Centre.

For supporting me in all good and less god moments, I want to thank Lucia Gennaro, as well as my family, my parents Gianni and Antonella, my sister Valentina and her husband Matteo together with my nephews Alice and Francesco.

Index

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.

3' UTR	Untranslated Region At 3'end
aa	Amino Acid
ALS	Amyotrophic Lateral Sclerosis
AS	Alternative Splicing
ASL	Airway Surface Layer
ASOs	Antisense Oligonucleotides
AZLI	Aztreonam For Inhalation Solution
bp	Base Pair
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CERES	Composite Exonic Regulatory Regulatory Elements
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CTD	Carboxyl-Terminal Tail Domain
DMD	Duchenne Muscular Dystrophy
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleoside Triphosphate (A, G, C And T)
DSE	Downstream Element
dsRBP	Double-Stranded RNA-Binding Protein
dsRNA	Double-Stranded RNA
DTT	Dithiothreitol
EDTA	Ethylenediamine Tetra-Acetic Acid
ENaCs	Epithelial Sodium Channels
ESE	Exonic Splicing Enhancer
ESS	Exonic Splicing Silencer

ExSpeU1	Exon Specific U1
FIX	Factor IX
hnRNP	Heterogeneous Ribonuclear Protein
HTS	High Throughput Screening
ISE	Intronic Splicing Enhancer
ISS	Intronic Splicing Silencer
Kb	Kilobase
kDa	Kilodalton
m7G	7-Methylguanylate Cap
m7GpppG	Monomethyl Guanosine Cap Structure
mRNA	Messenger RNA
MSD	Membrane Spanning Domains
N	Nucleotide
NBD	Nucleotide Binding Domains
NE	Nuclear Extract
NMD	Nonsense-Mediated Decay
NS	Netherton Syndrome
nt	Nucleotides
PBP	Phosphatidylethanolamine Binding Protein
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PI	Pancreatic Insufficiency
pi RNA	Piwi-Interacting RNA
PPT	Polypyrimidine Tract
pre mRNA	Precursor Messenger RNA
PTF	PSE-Binding Transcription Factor
R	Purine (G Or A)
rhDNase	Recombinant Human Dnase
RNA	Ribonucleic Acid
RNA PolII	RNA Polymerase II

RNA PolIII	RNA Polymerase III
RPGR	Retinitis Pigmentosa Gtpase Regulator
RRM	RNA Recognition Motif
rRNAs	Ribosomal RNA
RS	Arginine-Serine Rich Motif
RT	Room Temperature
SDS	N-Lauroylsarcosine Sodium Salt
si RNA	Small Interfering RNA
SMA	Spinal Muscolar Atrophy
SMN	Survival Motor Neuron
SNAPc	Snrna Activating Complex Polypeptide
snRNA	Small Nuclear Ribonucleic Acid
snRNP	Small Nuclear Ribonucleoprotein Particles
SR	Arginine-Serine Rich Protein
Ss	Splice Site
TBE	Tris-Borate-EDTA Buffer
TCIRG1	T Cell Immune Regulator 1
TF	Transcription Factor
TIP	Tobramycin Inhalation Solution
tRNA	Transfer RNA
U2AF	U2 Snrnp Auxiliary Factor
USE	Upstream Element
UTR	Untranslated Region
Wt	Wild Type
Y	Pyrimidine (T Or C)

List of figures

1. Introduction

1.1. **RNA processing pathway**

Cellular integrity is maintained in eukaryotic cells by a fine-tuned organized pathway of RNA synthesis, processing and surveillance.

This complex series of steps is an essential requirement to get protein coding RNAs (messenger RNAs or mRNA) or non-coding RNAs. In fact, RNA molecules such as microRNA, small interfering RNA and piwi-interacting RNA (miRNAs, siRNAs, piRNAs) are necessary for gene regulation. As well, small nuclear RNAs (snRNAs) and other molecules are indispensable for RNA processing and transcription. Translation is guaranteed by ribosomal and transfer RNAs (rRNAs and tRNAs). This regulated series of molecular steps begins in the nucleus. Here, DNA sequences are transcribed in precursor mRNAs (pre mRNA) by three DNA-dependent RNA polymerases (RNAP): I, II and III. Polymerases have specific targets: RNAPI transcribes rRNA, RNAPII mainly protein-coding genes, whereas RNAPIII refers to short structural tRNAs with the exception of 5S-rRNA (Grummt, 2003; Schramm & Hernandez, 2002; Woychik & Hampsey, 2002). After transcription, several processing steps are required to obtain a final functional mRNA: 5'end capping, 3′ cleavage with polyadenylation and splicing (Fig. 1). As well, in rare cases, minor changes as substitution/conversion or insertion/deletion can be generated in newly generated RNA transcript, by a process named RNA editing (Benne et al., 1986). From the nuclear transcription site, mRNA is then exported to the cytoplasm, where it is checked by an RNA surveillance system to eliminate wrongly spliced, mutant and aberrantly processed pre mRNA (Fig. 1). It also operates to remove noncoding RNAs produced by RNAPII (Schmid & Jensen, 2008).

The vast network of cellular machineries guarantees the right conversion of the information carried by the DNA to the active RNA (Maniatis & Reed, 2002).

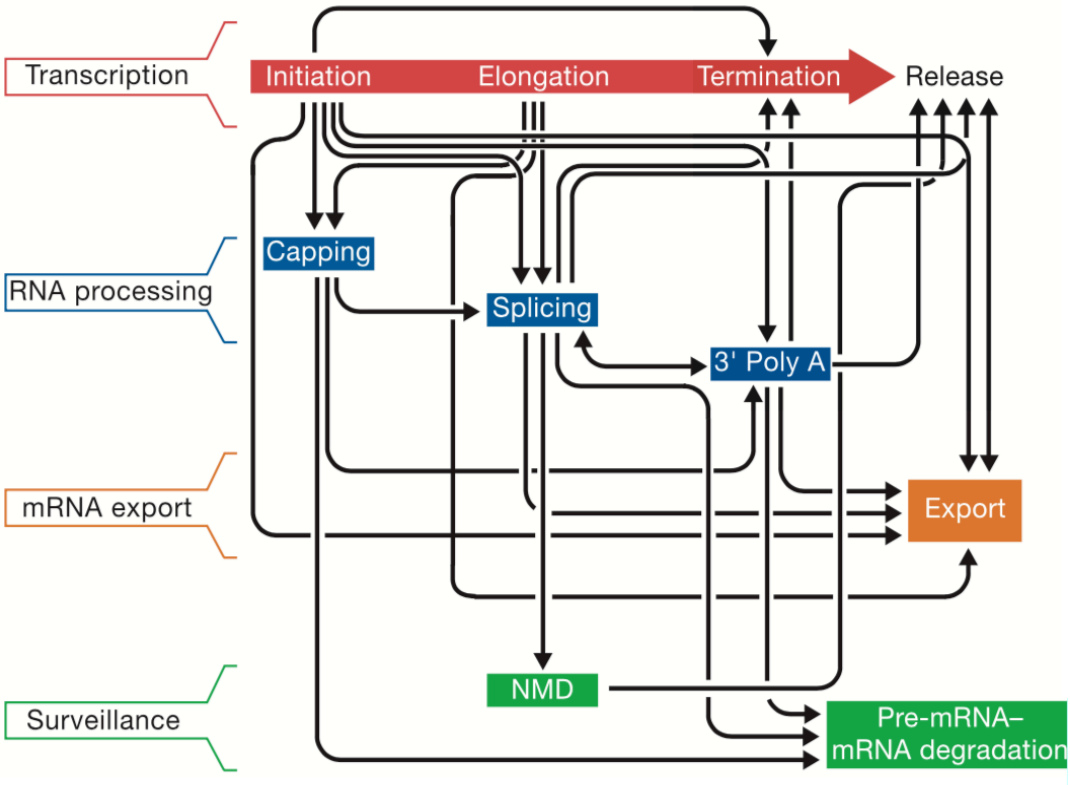

Figure 1: Complex regulatory network of coupled interactions in gene expression. On the left the main gene expression steps are indicated. Physical and/or functional coupling between two steps in gene expression are represented by black arrows. From (Maniatis & Reed, 2002).

1.1.1. **5'end capping**

The main goal of capping reaction is preventing the nascent transcript from cellular degradation. The other functions are: I) 5′ proximal intron excision (Konarska et al., 1984); II) translation promotion (Banerjee, 1980); III) exonuclease degradation prevention (Burkard & Butler, 2000); IV) nuclear export regulation (Lewis & Izaurralde, 1997).

Capping reaction is triggered after the beginning of transcription, about when the primary transcript reaches length of ca 30 nucleotides (Fig. 2a). During this initial step, pre mRNA 5' triphosphate group of triphosphate-terminated mRNA (pppRNA) is converted to diphosphate-terminated mRNA (ppRNA, Fig. 2b). Through a 5'-5' binding, m^7G monophosphate (m^7GMP) is linked to the RNA to create the capped RNA (GpppRNA). Lastly, a methyltransferase methylates the 7 position of guanine to produce the mature cap ($m^7GpppRNA$). This is consequently methylated by a methyltransferase (Marcotrigiano et al., 1997), referring thus to the a 7-methylguanylate cap, abbreviated m7G (Fig. 2). The enzyme is linked with the carboxyl-terminal tail domain (CTD) of RNA polymerase II. Additionally, one or two methyl groups are present in this binding at specific positions.

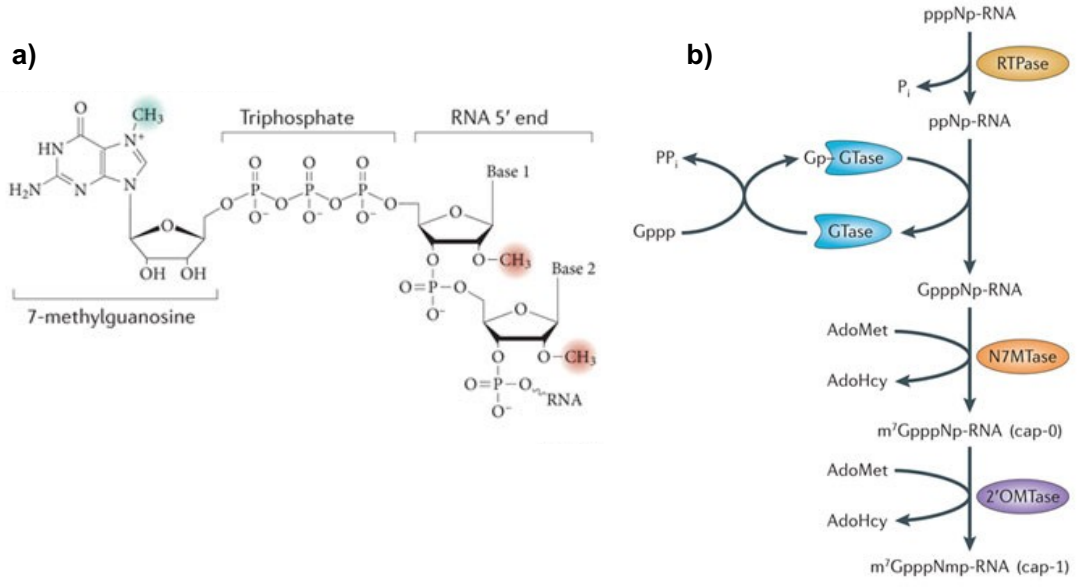

Figure 2: 5'end capping a) Chemical structure of RNA cap. b) RNA cap reaction pathway. Modified and edited from (Decroly et al., 2012)

1.1.2. **3'end polyadenylation and cleavage**

Polyadenylation occurs in almost the entire known eukaryotic mRNA sequences. It is an indispensable stage of RNA processing throughout the formation of mature mRNA. This process involves two stages: cleavage of the primary transcript and polymerization of an adenosine tail at the downstream of the cleaved mRNA in the case of protein-coding transcripts (Fig. 3).

Two elements orchestrate the cleavage of nascent transcripts: an upstream element (USE) and one downstream (DSE) from the cleavage site (Elkon et al., 2013). USE is a 10-30 nucleotides sequence preceding the site of endonucleolytic cleavage and has a conserved hexanucleotide sequence, called polyadenylation signal (PAS). A large proportion of genes contains more than one polyadenylation site: the canonical is AAUAAA, which is present in ~60% polyadenylated mRNAs while AUUAAA is the most important variant which appears in ~15% of poly(A) sites (Wahle & Keller, 1992). DSE sequence contains a consensus like YGUGUUYY, where Y stands for pyrimidine – GU-rich element required for 3'end cleavage (Scorilas, 2002). To promote export from the nucleus and degradation prevention, by acting as a binding site for polyA binding protein,a poly-adenylic acid tail (polyA) is finally added by PolyA polymerase (Coller et al., 1998).

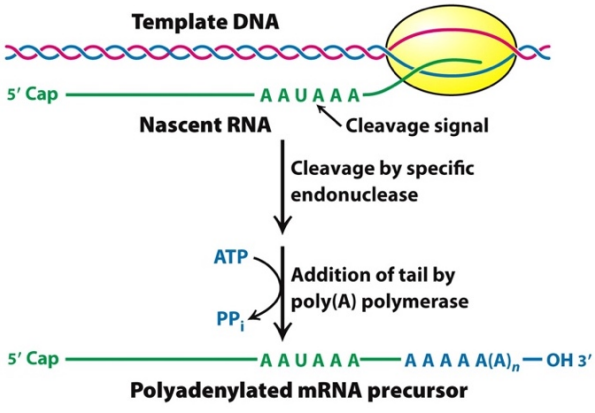

Figure 3: RNA cleavage and polyadenylation. From (J. Berg, 2012)

1.2. **Pre-mRNA Splicing**

1.2.1. **Spliceosome formation**

The main process leading to the maturation of messenger RNA is splicing, during which introns are removed and exons are joined together. The whole splicing reaction is catalyzed by the spliceosome, a multi-megadalton ribonucleoprotein (RNP) complex composed by five snRNPs (namely U1, U2, U4, U5 and U6 snRNPs) and a set of corresponding proteins. All snRNP are constituted by an snRNA (or two in the case of U4/U6) and a common pool of seven Sm proteins (namely B/B′, D3, D2, D1, E, F, and G) with a variable number of particle-specific proteins (Will & Lührmann, 2011) .

Splicing reaction acts in the sheltered environment of spliceosome active core, forming a continuous surface deep within the large spliceosomal cavity. U snRNPs combine with other proteins to form RNA-protein complexes (mainly SM proteins, a family of nuclear proteins), only minor conformation changes are needed to accommodate additional spliceosomal components. Firstly, the sequence at the 5'ss must be recognized by U1snRNP through complementary binding to the last three bases of the exon and to the first six bases of the intron (Fig. 4b). Only in rare contexts, less efficient splicing was reported in literature when the recognition of the 5'ss can occur without presence of the U1 particle (H. Du & Rosbash, 2002). Consequently, Splicing Factor 1 (SF1) binds to the branch point sequence (BPS) in the intron. Meanwhile, two U2 auxiliary proteins (U2AF1 and U2AF2) bind to 3'ss and to the polypyrimidine tract, respectively. U2snRNP forms the pre-spliceosome by substituting the SF1 at the BPS. Then, U4snRNP and U5snRNP link together, binding to U6 snRNP to form the fully assembled but catalytically inactive complex B. Major structural and compositional changes then produce the catalytically active complex B* to trigger the first trans-esterification reaction. Changes include dissociation of U1 snRNP from the 5′ splice site, unwinding of the extensively base-paired U4/U6 snRNA duplex (BΔU1). This process leads to loss of U4 snRNA together with its

associated proteins, recruitment of large protein complexes and formation of a new base-pairing interaction between U2 and U6. The spliceosome then undertakes additional modifications for the second trans-esterification reaction (complex C). At the end of this reaction, the residual intron lariat spliceosome (ILS) is disassembled after the spliced mRNA product is released. Finally, snRNPs are recycled for new splicing events and the excised intron lariat is degraded.

An exception to the described U2-type major spliceosome splicing, is the U12-type minor spliceosome, comprising ~0.9% of human splice sites (Parada et al., 2014; Sheth et al., 2006). It is composed by four main snRNP proteins, U11, U12, U4atac, and U6atac, together with U5 and other snRNPs still not fully investigated.

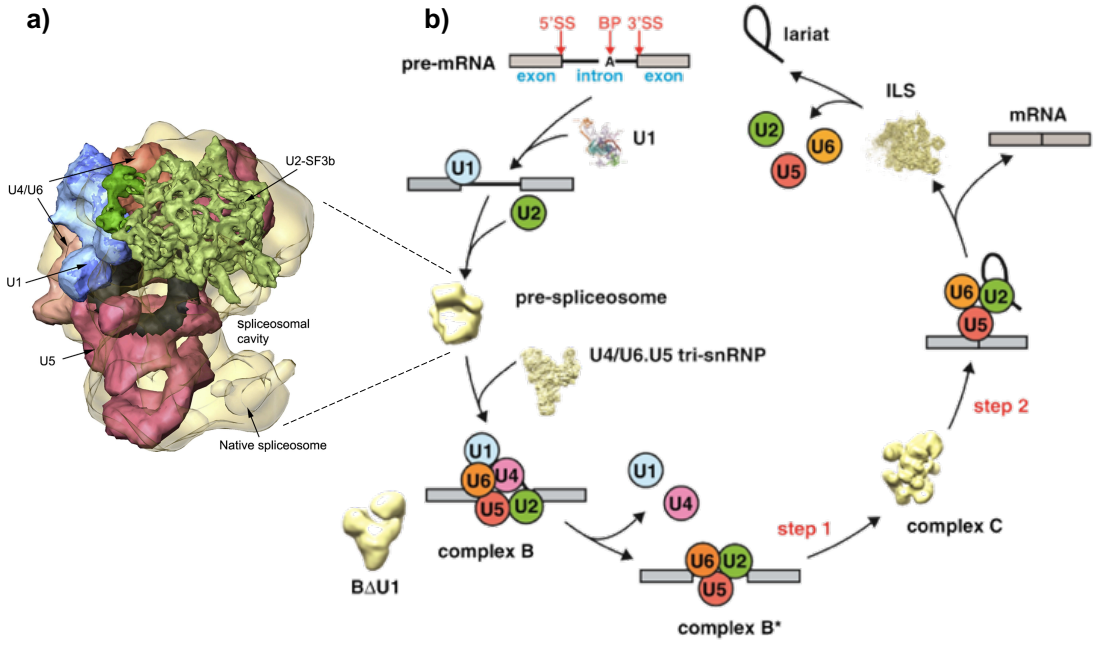

Figure 4: Spliceosomal dynamics during pre-mRNA splicing. a) Representative image of the spliceosome assembly at sites of transcription. Adapted from (Frankenstein et al., 2012). b) Spliceosome steps during splicing. Modified From (Nguyen et al., 2016)

1.2.2. **Splicing reaction**

From a biochemical perspective, splicing reaction consists of two phosphoryl-transfer steps (Fig. 5a). In the first step, the 5′ intron phosphate (the 5′ splice site) is attacked by a 2′ hydroxyl specified within the intron (the branch point A). In the second step, the 3′ intron phosphate (the 3′ splice site) is attacked by the 3′ hydroxyl of the cleaved 5′ exon. The final products of the splicing reaction are ligated exons, and the excised intron in a branched, 'lariat' form. U1, U2, U4, U5, U6 snRNPs assemble in the intron substrate in a stepwise fashion, directed by interactions with intron consensus sequences (Fig. 5b). According to the previously described spliceosome dynamics, during catalytic activation U1 and U4 snRNPs become destabilized, while U2, U5, and U6 snRNPs constitute the active spliceosome necessary to trigger the catalytic reactions.

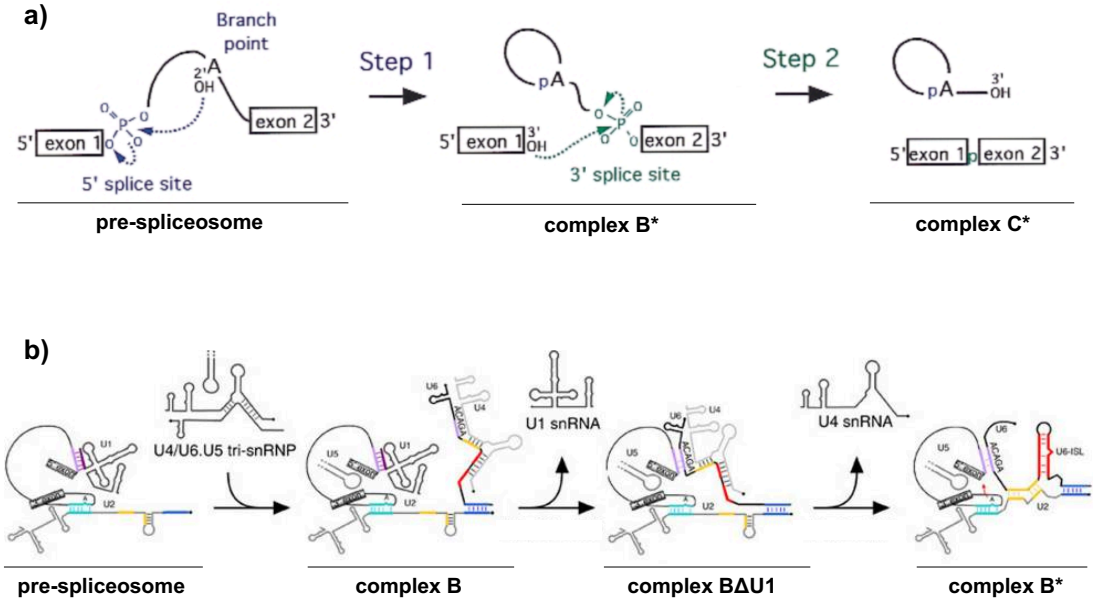

Figure 5: Splicing reaction mechanisms. a) Biochemical perspective of the reaction. Modified from (Collins & Guthrie, 2000). b) U snRNPs bindings during splicing reaction. Modified and edited from (Chandler, 2011)

1.2.3. **Alternative splicing**

Pre-mRNA is transcribed from one single gene leading to a single mature mRNA molecule. However, one gene can lead to several and relatively different protein isoforms, structurally and functionally different, increasing its coding capacity and functionality in a biochemical process named Alternative Splicing (AS).

It is nowadays clear the central role of AS in gene expression in almost every aspect of protein function, since it was evolutionary built in order to increase the quantity and the biodiversity of proteins produced by the same single gene (Black, 2003). In fact, large intron numbers and 'weak' 5′ss were found in our eukaryotic ancestors, which is associated with frequent alternative splicing in modern organisms (Irimia et al., 2007).

During development, AS strategy was found to be essential in tissue-specific gene expression and splicing factors regulation (Kalsotra et al., 2008; Nilsen & Graveley, 2010; Rossbach et al., 2014). Focusing on brain development, by the use of genome-wide transcriptome analysis, it was recently discovered the importance of AS in neuronal differentiation and morphogenesis, including the establishment of delicate synapses (Norris & Calarco, 2012; Su et al., 2018; Zheng & Black, 2013).

The main occurrence in alternative splicing is exon skipping, in which an exon is entirely skipped producing a final product lacking an entire exon (Fig. 6). In presence of two skippable exons, it is possible to display two variants in which only one is included as in the case of "mutually exclusive exons".

In relation to the strength of the sequence at the 5'ss and 3'ss, alternative donor site and an alternative acceptor site may be detected thus increasing or shortening the length of the processed mRNA by adding an intronic contribution or removing an exonic component, correspondingly.

Another form of AS is Intron retention, in which the total intronic sequence is translated, causing in most of the cases the protein to be non-functional. Comparable to AS, another mechanism still partially unknown is alternative poly(A), which permits from a single gene to encode multiple mRNA transcripts.

If the 3'UTR length is altered, this could potentially influence the destiny of mRNAs in several ways, altering the availability of RNA binding sites for protein and microRNA (Y. Wang et al., 2015).

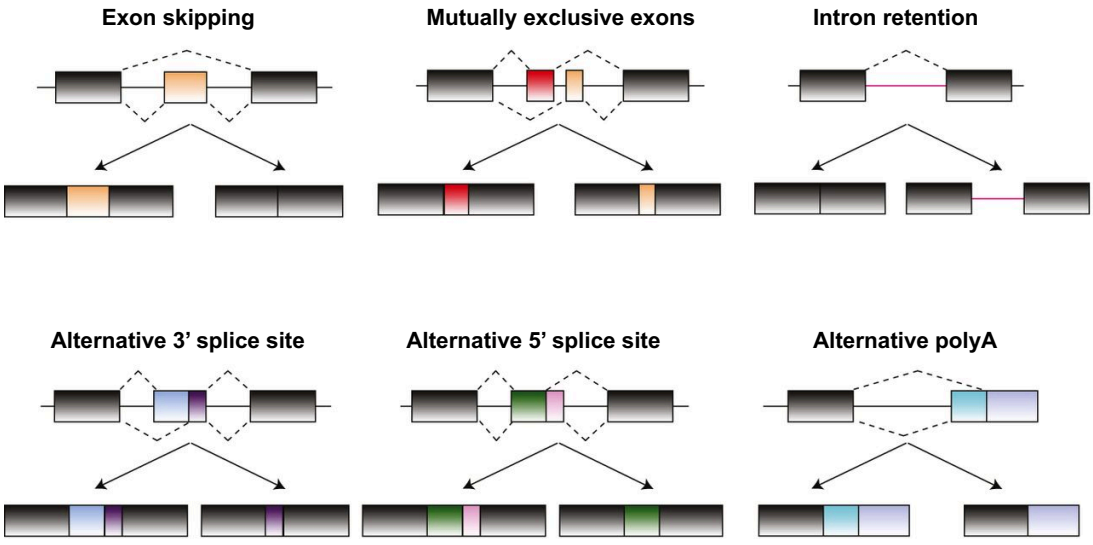

Figure 6: Alternative splicing. Modified and edited from (Blue et al., 2018)

1.3. **Splicing regulatory elements**

The identification of exons on nascent pre-mRNA requires core splicing regulatory elements, mainly categorized as trans-acting factors binding to cis-acting sequences. Regulatory components may be located within exons or along intronic sequences, interacting with specific molecules. The interplay among them may result in promoting or inhibiting splicing activity from nearby splice sites (enhancer and silencers, respectively).

1.3.1. **Cis-acting sequences**

Canonical cis-acting elements are:

I) the donor site - 5'splice site (ss);

II) acceptor site - 3'ss;

III) poly-pyrimidine tract near the BPS (Fig.7).

The donor site is located in the 5'end of the intron, characterized by a GT invariant sequence, whereas the acceptor site is characterized by the AG sequence at the 3'end of the intron. The polypyrimidine sequence is located upstream the 3'ss and is an enriched sequence in C and T/U, near the branch site, involved in the lariat formation through a specific adenine nucleotide.Several non-canonical splicing regulatory elements are important for correct exon recognition. Since they may enhance or suppress the splicing reaction (enhancer and silencer motifs, respectively), categorization was done according to their function. Exonic/Intronic Spicing Enhancers (ESE, ISE), and Exonic/Intronic Splicing Silencers (ESS, ISS) are considered binding site for different trans-acting factors thank to their relatively short sequence (4-7 bases). They are considered relatively separated entities, however in different contexts they overlap with both enhancer and silencer functions such as in Composite Exonic Regulatory Elements (CERES, Baeza-Centurion et al., 2019; Julien et al., 2016; Pagani, Stuani, et al., 2003).

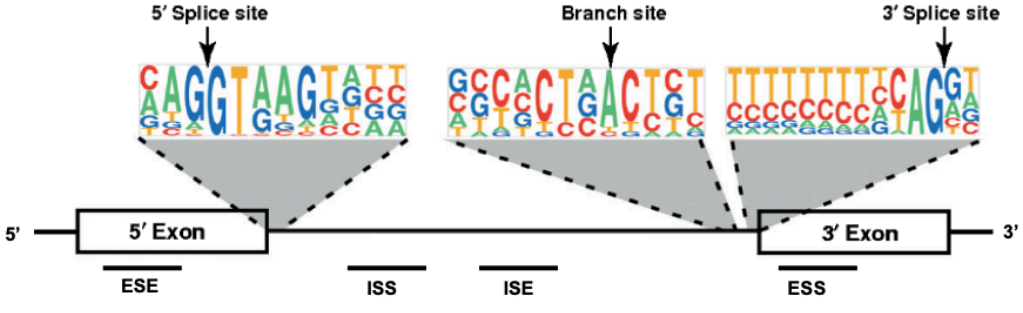

Figure 7: Cis-acting elements regulating splicing. Modified from (Padgett, 2012)

1.3.2. **Trans-acting factors**

The final splicing decision is made based on the complex and combinatorial interactions that occur between the positive and negative regulatory elements present on the pre mRNA with the corresponding trans-acting factors (Valerio, 2014).
Trans-acting factors are divided in three subgroups:
I) Serine Arginine rich proteins (SR proteins);
II) heterogeneous nuclear RiboNucleo-protein Particles (hnRNPs);
III) snRNPs components of the spliceosome (previously described in chapter 1.2.1).
Splicing factors are activated either by chemical modification (phosphorylation) or by ligand binding. They are ubiquitous or tissue-specific allowing a differential control of alternative splicing (Grosso et al., 2008). As well, they are characterized by temporal modulation, essential in development (Kanadia et al., 2008). Such regulatory elements present at C-terminus a domain of variable length rich in serine-arginine dipeptides (RS domain) and at N-terminus a modular structure with one or two RNA recognition motifs (RRM) interacting with the pre-mRNA (Fig. 8, Pradella et al., 2017). SR proteins, are important regulators for several activities other than splicing, such as mRNA nonsense-mediated mRNA decay and mRNA nuclear export (Long & Caceres, 2009). HnRNP proteins have a main repressor and silencer activity role in splicing. These factors are among the most abundant nuclear proteins and they are involved in mRNA export, localization, translation, and stability (Chaudhury et al., 2010). HnRNPs expression level is altered in many types of cancer, suggesting their role in tumorigenesis, as well as in neurodegenerative diseases, such as spinal muscular atrophy (SMA), amyotrophic lateral sclerosis (ALS), Alzheimer's disease and fronto-temporal lobe dementia (Geuens et al., 2016).
Thus, not only the nature of the sequence itself but also the complex combinatorial interplay between regulatory elements and their cognate factors determines exon definition and regulation (Pradella et al., 2017).

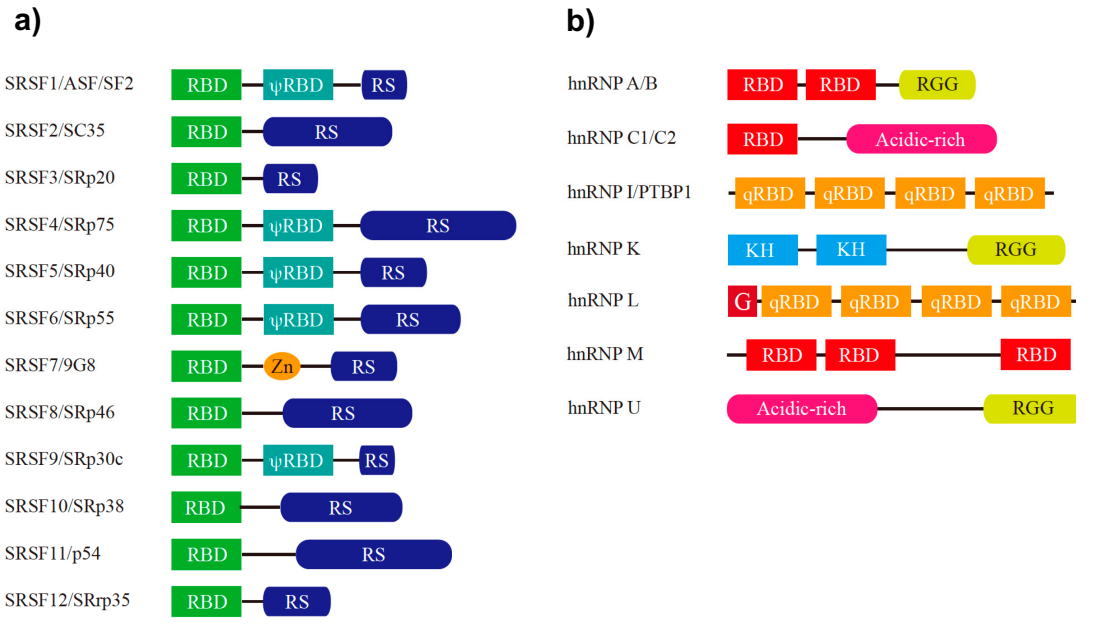

Figure. 8: Major splicing factors representation. a) Structure of human SR proteins. Current serine-arginine-rich splicing factor (SRSF) names are shown on the left together with the protein aliases. RBD: RNA-binding domain (also known as RNA recognition motif (RRM)), RS: arginine-serine dipeptide repeat-rich region, Zn: Zn-binding domain, ψRBD: RBD homology domain. b) Structure of human hnRNPs. RBD: RNA-binding domain, RGG: arginine-glycine-glycine repeat-rich region, Acidic-rich: acidic amino acid residue-rich region, qRBD: non-canonical RBD, G: Glycine rich region, KH: K homology domain. From (Nakayama & Kataoka, 2019)

1.4. **Exon skipping in disease-causing mutations**

Splicing regulation is a precisely structured series of interactions between proteins and the mRNA transcript in particular developmental stages and tissues. Dissecting the complexity in human tissues using mRNA-Seq data, a study reported that mostly 95% of human genes are differently spliced (Pan et al., 2008). In several diseases, a single nucleotide substitution can affect splicing, which can be the direct cause of the disease

or may contribute to its severity, as in the case of Cystic Fibrosis, Fanconi Anemia, Hemophilia B (Factor IX gene).

Mutants may affect different cis-acting elements including the canonical splice site consensus sequences, the polypyrimidine elements or exonic/ intronic regulatory elements. If mutations are located near the 5'ss or the 3'ss, the outcome may not only lead to exon skipping, but also result in cryptic splice site activation leading to intron retention. Examples of different splicing mutations and relative diseases have been discussed in literature (Abramowicz & Gos, 2018; Wimmer et al., 2007).

Leading to alternative splicing in a similar way, mutations affecting exonic or intronic regulatory elements (ESE, ESS ISE, ISS), will change the bindings of positive and negative regulators (Fig. 9). This could potentially develop in destructive conditions, as shown in patients affected by Spinal Muscular Atrophy with C/T transition altering ESE and ESS functions (Kashima & Manley, 2003).

Mis-regulations or mutations that affect regulatory trans-acting factors will also be a triggering cause for a disease, for example in retinitis pigmentosa, a progressive loss of photoreceptor cells due to mutation in *PRPF31* gene (Vithana et al., 2001).

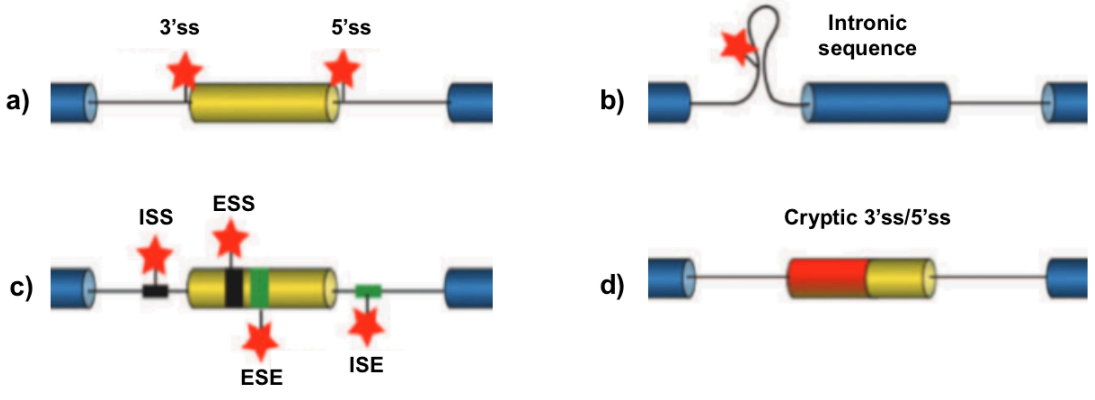

Figure 9: Disease-causing mutations affecting splicing. Modified (Pistoni et al., 2010)

1.5. **Engineered U1snRNPs as RNA-based gene therapy**

1.5.1. **U1snRNA gene and RNA secondary structure**

Human U1 snRNA gene (RNU1, MIM #180680) is located in the short arm of chromosome 1p36, present in all living animal kingdoms (Naylor et al., 1984).
The elements that characterize the canonical U1 snRNA gene are three: firstly two essential promoters, namely distal sequence element (DSE) and proximal sequence element (PSE), then the U1 coding sequence (164 bp) and lastly the 3'box (Fig. 10a).
U1 snRNP has a well-defined secondary structure characterized by four stem-loops and two single stranded regions and interact at least with 10 proteins. These are 7 Sm proteins together with U1-70K, U1-A and U1-C protein - binding respectively to U1snRNA stem loops region I, II III (Fig. 10b).
Fundamental for biogenesis and stability of the snRNA, Sm proteins compose the so-called Sm core ribonucleoproteins (RNPs) and might be involved in the stability or maturation of RNA polymerase III transcripts (Achsel et al., 2001; Salgado-Garrido et al., 1999). U1-70K is encoded by *SNR70* gene (Spritz et al., 1990) and was shown to interact with several families of trans-acting factors such as SF2, SRPK1 and ZRANB2 (H. Y. Wang et al., 1998; Xiao & Manley, 1998). U1-C protein is encoded by the *SNRPC* gene (H. Du & Rosbash, 2002) and contributes to the 5'ss recognition by stabilizing the base-pairing between the 5' tail of the U1 with the donor site of the pre-mRNA, as well as involvement in the formation of complex E in Spliceosome (Sillekens et al., 1988). U1-A protein is encoded by *SNRPA* gene and deletion of the protein from the extract does not have influence on U1 snRNP splicing efficiency from in-vitro experiments (Heinrichs et al., 2006).
In the cytoplasm, the U1 stability necessary for the transport between the nuclear membrane due to the proper formation of 3'end is guaranteed by the 3' Box, a conserved acting element located 9-19 bases downstream of the 3' end of the RNA-encoding region (Terns et al., 1993).

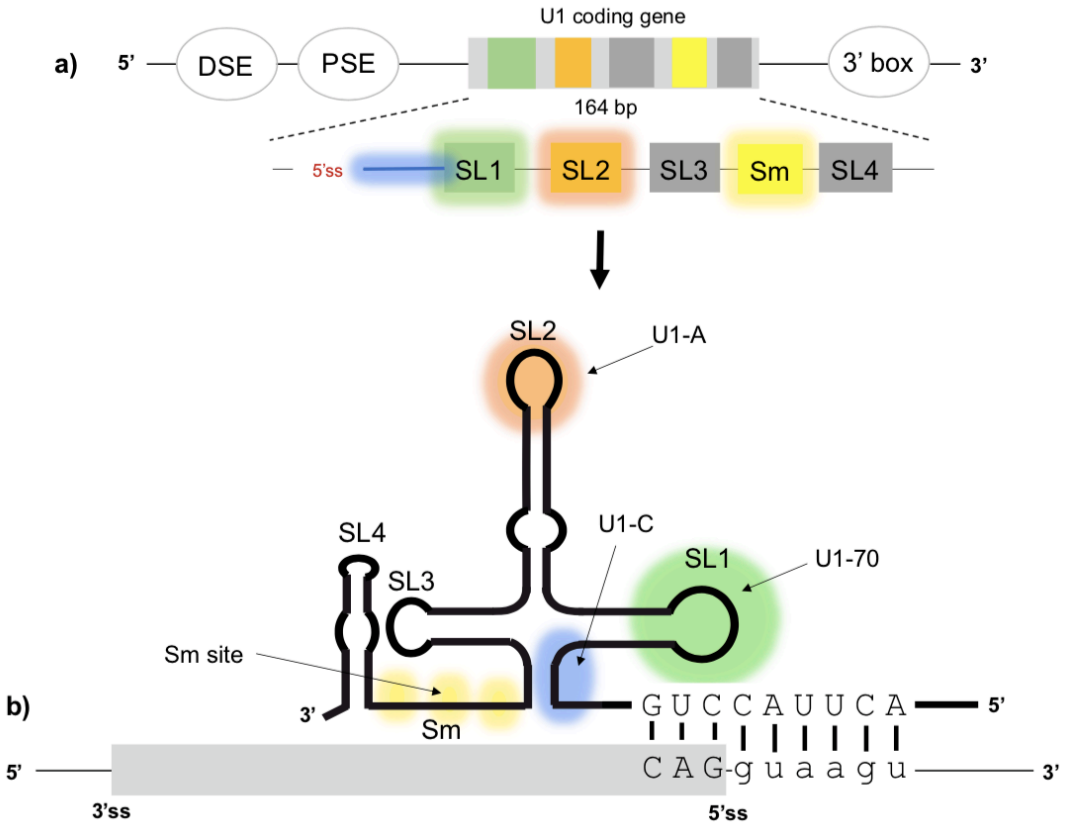

Figure 10: U1snRNA gene and corresponding RNA secondary structure. a) U1 gene representation. B) U1 snRNP secondary structure. The line 5'-3' represent intronic sequence, with exon in gray box. Colours represents the ring of Sm proteins (yellow), U1-70K (green), U1-A (orange) and U1-C (blue)

1.5.2. **Biogenesis of U1 snRNPs**

U1, U2, U4, U5 and U6 snRNPs are RNA protein complexes located within the splicing speckles of Cajal bodies in the eukaryotic cell nucleus. Their biogenesis has been accurately studied.

Molecule export is ensured by a monomethyl guanosine cap structure (m^7GpppG), recognized by the cap-binding complex (CBC, Ohno, Segref, Bachi, Wilm, & Mattaj, 2000). m^7GpppG-UsnRNP complexes are transitorily exported to the cytoplasm where the Sm proteins are accumulated (B/B′, D1, D2, D3, E, F, G). Then, Sm proteins bind to a conserved sequence found in the U snRNAs called the Sm site creating a 'ring'-shaped Sm core domain usual to all U snRNPs, providing a binding site for an RNA methyltransferase. The enzyme catalyses the formation of the trimethylguanosine (m3G) cap of the U snRNA (Branlant et al., 1982). Finally, the newly assembled particle is transported actively to the nucleus.

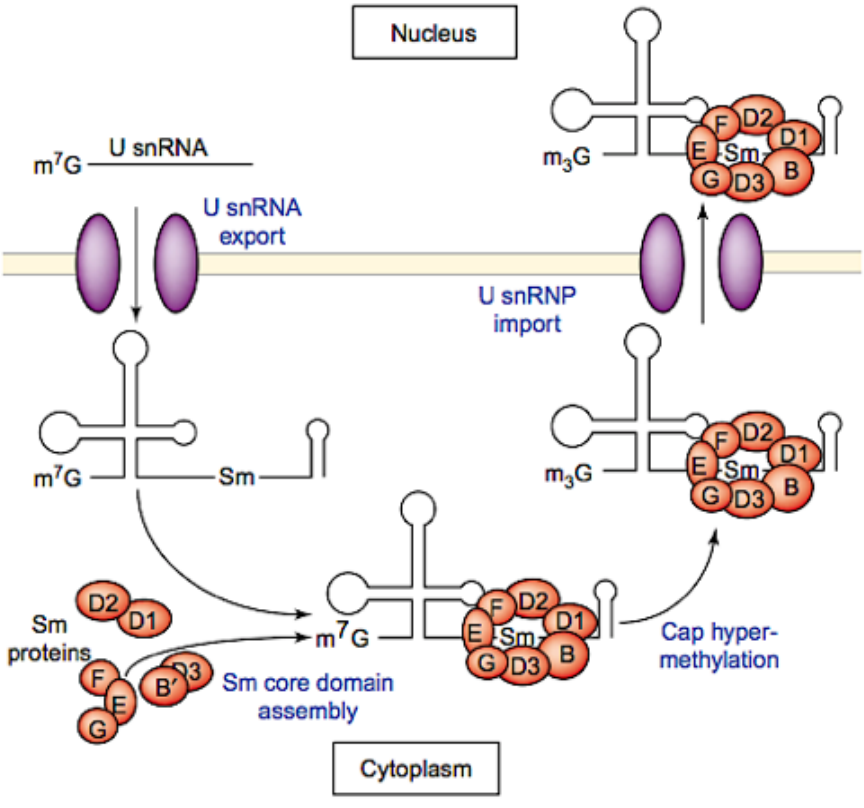

Figure 11: Biogenesis pathway of U1 snRNPs. From (Meister et al., 2002)

1.5.3. **Exon Specific U1s**

The recognition of donor site (5'ss) mediated by U1 snRNP through a complementary interaction between the pre-mRNA and the 5' tail of U1 is the first step of pre-mRNA splicing. Thus, if the binding is disrupted, the correct consensus recognition will be defective, (Fig.12). In case of a mutation affecting the GU of the donor site or the AG of the acceptor site, this will cause splicing defect, mostly resulting in exon skipping. Nearly two decades ago, U1snRNA have been tested to manipulate pre-mRNA processing for therapeutic purposes to promote exon inclusion in the presence of exon-skipping mutations.

The first generation of engineered U1snRNA had a modified 5′ tail with increased complementarity to defective 5′ss. U1 5' tail was modified to pair the splice site sequence in order to make it match perfectly with the altered donor site to efficiently correct the mutation (Baralle, 2003). This strategy was adopted more and more efficiently in different contexts: coagulation factor VII, mild Bardet-Biedl syndrome, retinitis pigmentosa GTPase regulator gene (RPGR), and TCIRG1-dependent recessive osteopetrosis (Pinotti et al., 2008, 2009; Susani et al., 2004; Tanner et al., 2009).

Based on U1snRNP binding in a non-conserved intronic regions downstream of the donor site of a specific exon, another strategy was proposed to reduce undesirable off-target effects. Essentially, if binding between engineered U1s and mRNA involves intronic sequences, it was thought possible to specifically rescue diverse kinds of splicing deficiencies associated to exon skipping, because the canonical donor site is not involved. Using this approach, specific developmental-dependent expression proteins could be ideally rescued by acting at the splicing correction level. Thus, researchers designed specific engineered U1snRNAs, namely Exon Specific U1 (ExSpeU1) to bind the non-conserved intronic sequences of each gene considered. Several cell models for exon-skipping diseases were rescued, as Haemophilia B

Coagulation Factor IX (FIX), Cystic Fibrosis (CF) and Spinal Muscular Atrophy (SMA, Alanis et al., 2012; Donadon et al., 2019; Rogalska et al., 2016).

To comprehend the mechanism by which ExSpeU1 stimulates the definition of defective exons, composition of the resulting ExSpeU1 particles was investigated (Rogalska et al., 2016). ExSpeU1 snRNPs form particles that resemble U1 snRNP containing the U1-specific proteins U1A, 70K and U1C. Particularly, RNA mutant experiments showed that most of splicing correction acts through the 70K and stem loop IV elements whereas U1A protein is unessential.

ExSpeU1 approach in SMA mouse models showed high efficiency without toxic concerns since no apparent side effect were detected (Rogalska et al., 2016). ExSpeU1 treatment was found long lasting in several post-mitotic tissues by rescuing a severe SMA phenotype (Donadon et al., 2019). In the ExSpeU1 treated SMA mouse model, significant improvement in splicing and in protein rescue was detected in liver, heart and muscle, with minor effects on the central nervous system.

In a familial dysautonomia (FD) mouse model, to correct ELP1 exon 20 definition, proof of principle of ExSpeU1s-adeno-associated virus particles was described (Donadon et al., 2018). ExSpeU1's treatment in FD fibroblasts recovered 80% of the protein level. In the same study, by using modified U7snRNAs with the same binding site for ExSpeU1, the authors suggested ExSpeU1s do not have antisense activity.

Taken these results together, exon Specific U1s look to represent an innovative strategy to correct different types of splicing mutations associated with malfunctioning splicing pattern, in numerous human diseases.

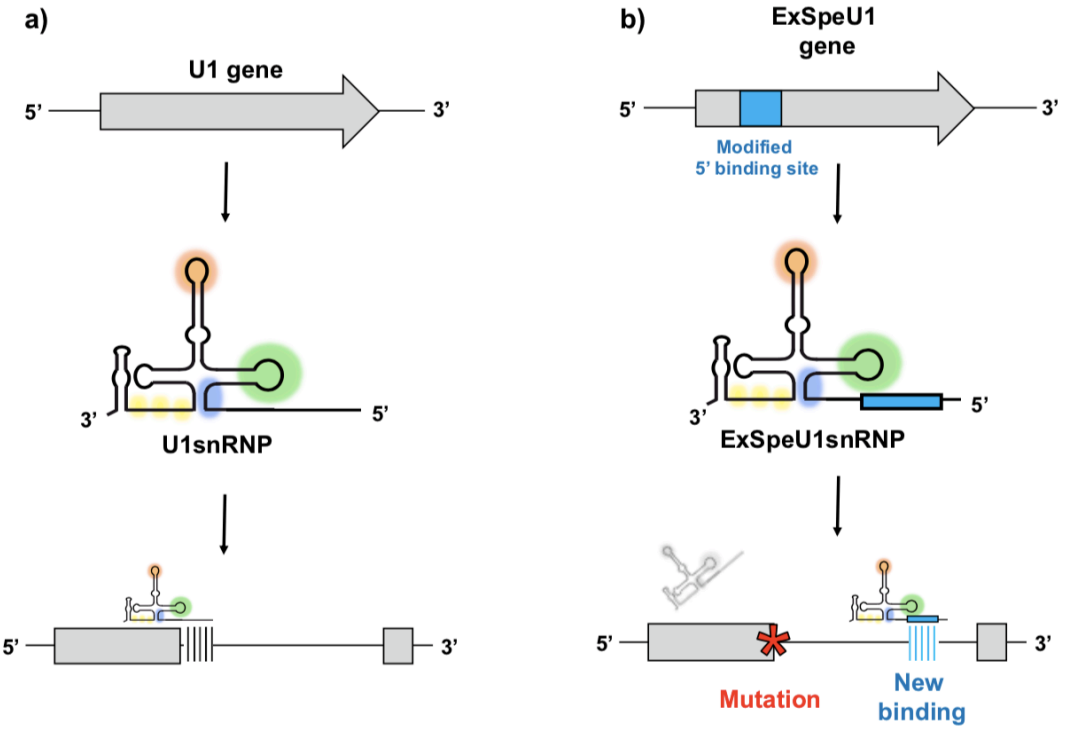

Figure 12: ExSpeU1 design. a) WT. b) ExSpeU1 strategy in mutated sequence at 5'ss. 5'-3 line represent introns and gray box the exon. Colours represents the ring of Sm proteins (yellow), U1-70K (green), U1-A (orange) and U1-C (blue)

1.6. **Cystic fibrosis**

Cystic fibrosis (CF, MIM #219700) is the most usual autosomal recessive genetic disease in European descents which affects about 1:2000-4000 new-borns in the US having higher morbidity in some European countries (Burgel et al., 2015; Farrell et al., 2017; Kosorok et al., 1996; Palomaki et al., 2004).

CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, important for the regulation of ion and fluid homeostasis across epithelial barriers (Collawn & Matalon, 2014). CFTR regulates epithelial sodium

channels (ENaCs) with several functions including cAMP-dependent chloride and bicarbonate secretion (Griesenbach et al., 2015).

Chronic CF lung disease is leaded by CFTR-mediated chloride and bicarbonate transport loss. In CF context, airway infection and neutrophilic inflammation leads to mucus-obstructed airways, in the advanced case characterized by irreversible and progressive bronchiectasis (Stoltz et al., 2015). These pathological appearances are direct consequence of the primary lung deficiency or a relatively severe derived complication. In CF, the airway surface layer (ASL) of lung cells becomes dehydrated since Cl- is not correctly secreted, leading to unrestrained Na+ absorption causing originating defective mucous clearance (Fig. 13, Haq et al., 2016). Accumulation of adhesive persistent mucus in relation to epithelial surfaces not only in lungs, but in many organs such as pancreas, gastrointestinal tract, hepatobiliary system, sweat glands and reproductive tract is the result of ion transport defect (Sheppard & Nicholson, 2002). Lung association is the major cause of mortality, dominated by chronic infections and airway obstruction, irreversibly leading to respiratory failure (Marcorelles et al., 2014).

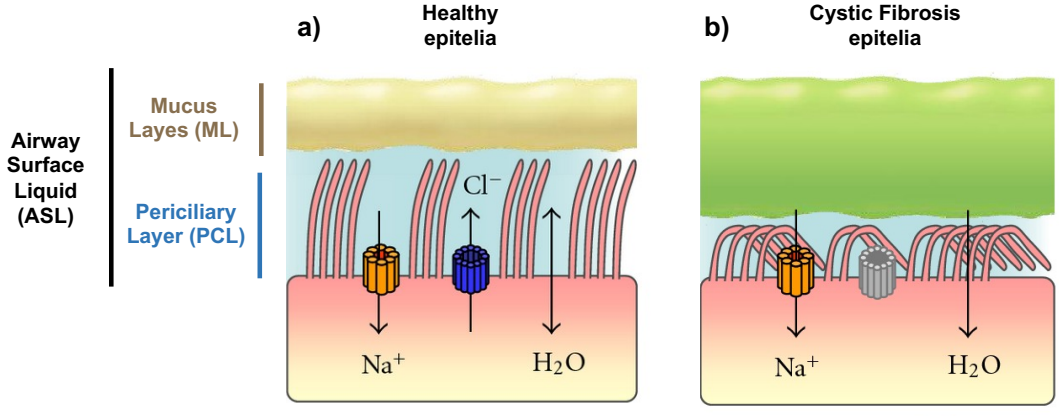

Figure 13: Airway surface liquid of lung epithelia in a) Healthy and b) Cystic Fibrosis patient. Modified and edited from (Reeves et al., 2012)

1.6.1. **CF symptoms and disorder spectrum**

CF has huge phenotypic variability as supported by the wide-ranging spectrum of disorder severity observed in patients with the same genotype (Ratjen et al., 2015). In fact, distinctive symptoms of the disease range from classical severe CF to an "Atypical" milder form (Fig. 14).

In add, CFTR patients who initially have normal pancreatic function may develop pancreatic insufficiency (PI) as they age, and pancreatic vitamin supplements with enzyme replacements may be taken life-long (Li & Somerset, 2014). Gastrointestinal, pancreatic and/or hepatic manifestations during the evolution of the disease happen in the majority of affected individuals, which negatively influence life quality and survival rate (Gelfond & Borowitz, 2013).

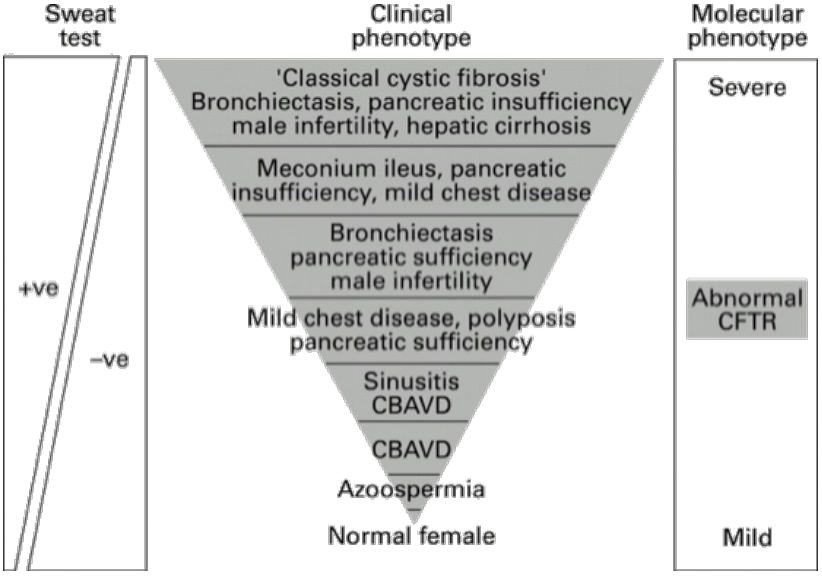

Figure 14: Phenotypic manifestations in CF homozygotes From (Wallis, 1997)

Atypical CF is characterized by a very different disorder affecting diverse organ systems to varying degrees with fluctuations over time CF and represents approximately 2% of affected individuals (Paranjape & Zeitlin, 2008). It is often diagnosed only in adult age, since the disorder does not necessitate hospitalization during childhood (Schram, 2012). In some cases, this CF form does not manifest elevated sweat chloride levels as it happens in the sever form of CF (Table 1).

System	Symptoms
Respiratory	Chronic sinusitis, nasal polyposis, poorly controlled obstructive lung disease, recurrent pneumonia, digital clubbing
Gastrointestinal	Meconium ileus, rectal prolapse, atypical acute pancreatitis or chronic pancreatitis, diarrhea, constipation, weight loss or poor weight gain, nutritional deficiency
Endocrine and metabolic	Diabetes mellitus, hypochloremia, hypokalemia, metabolic alkalosis
Genitourinary	Azoospermia in men, reduced fertility in women
Other	Dermatitis secondary to nutritional deficiencies, unexplained anemia, early aqueous wrinkling

Table 1: Affected systems and symptoms in Atypical CF. From (Schram, 2012)

1.6.2. **CFTR gene**

CFTR gene is located on human chromosome 7. It contains 27 exons and generates a 6.4 kb mRNA (Gregory et al., 1990) developing in a phospho-regulated chloride channel found in the apical membranes of epithelial cells (Fig. 15b and 15c, Ontalus et al. 1996; Choi et al. 2001).

CFTR architecture entails two membrane spanning domains (MSD1 and MSD 2), two nucleotide binding domains (NBD1 and NBD2), and a regulatory (R) domain (K. Du & Lukacs, 2009). The residues involved in the binding suggests that the interactions

between cytosolic nucleotide-binding domain NBDs and the R domain likely occur at the interface between NBD1 and NBD2 sequences (Hwang et al., 2018). Phosphorylation of the R domain is essential for the activation of CFTR proteins, but a complete mechanism understanding of how the R domain regulates the channel still remains unclear (Hwang et al., 2018; Ostedgaard et al., 2001; Seibert et al., 1999).

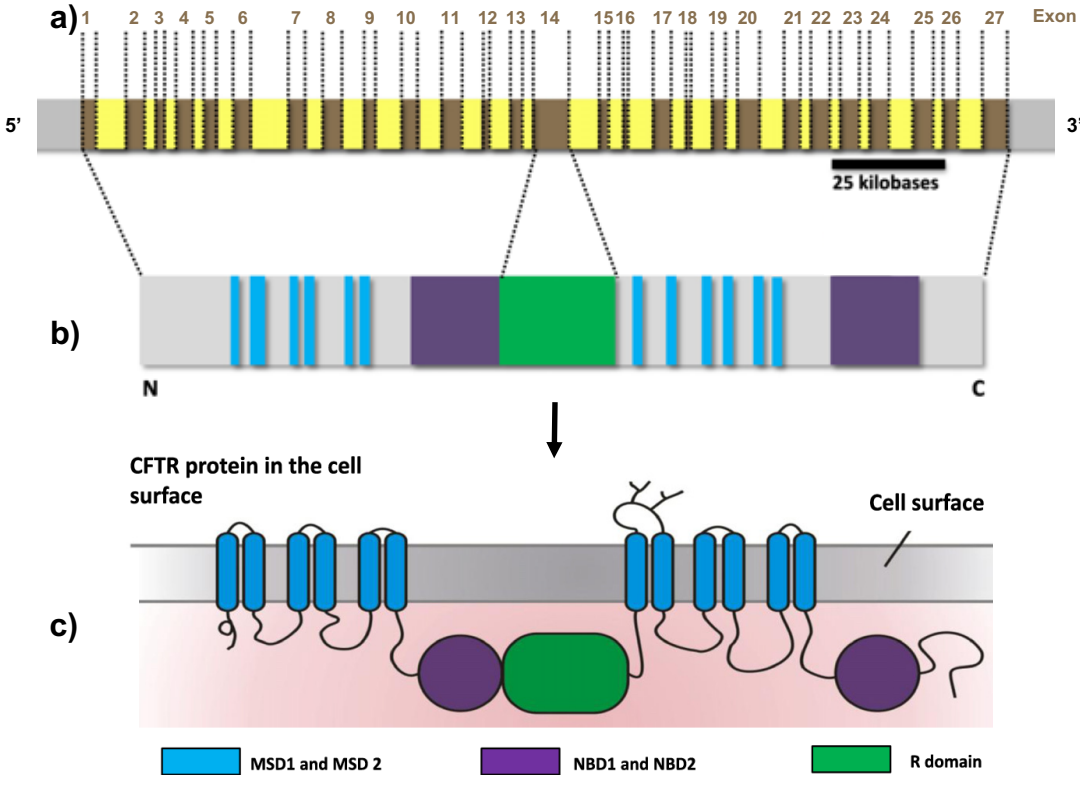

Figure 15: CFTR gene and protein structure. a) CFTR gene containing introns (yellow) and exons (brown). b) Scheme of protein primary structure sequence. c) CFTR protein in the membrane surface. Modified and edited from (Bonadia et al., 2014)

According to the primary biological defect in cell phenotype, six disease classes were proposed as landmark reference, not considering the severity of the disease, as reported in Figure 16 (Welsh & Smith, 1993). The first class is characterised by the absence of CFTR protein. In class II the pathology is linked to misfolded protein retention at the endoplasmic reticulum, with consequent proteasome-mediated degradation. Phe508del (ΔF508) is the most prevalent CFTR mutation belonging to this class, accounting for ~70% of CF alleles. Alteration of channel regulation which results in channel opening impairment characterizes occurs in class III mutations, while a reduced conduction resulting in a decreased ion flow strikes class IV. A significant decrease in mRNA or protein characterized Class V, while class VI mutations cause considerable plasma membrane instability (Reeves et al., 2012).

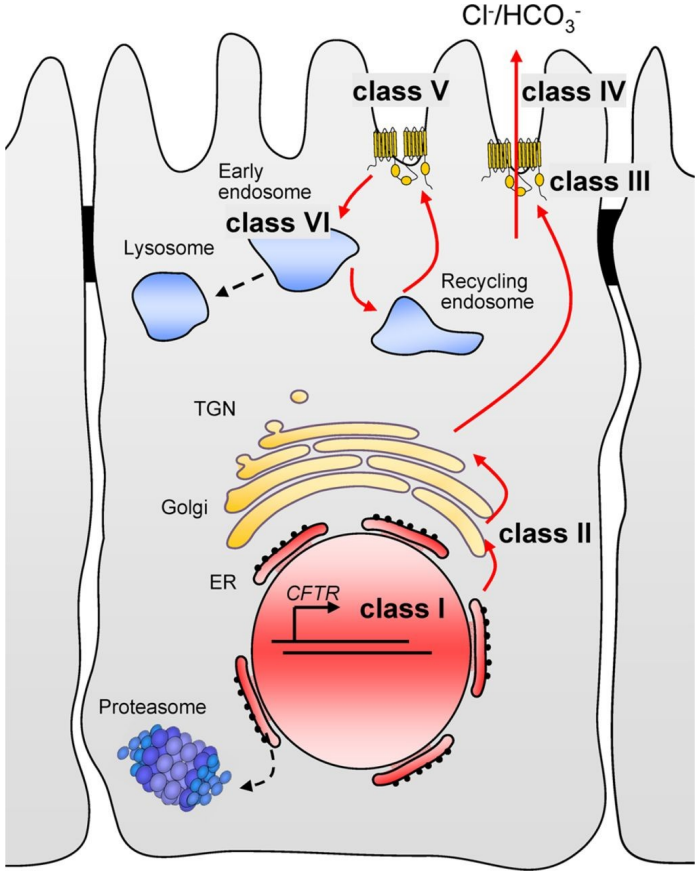

Figure 16: Classes of CFTR mutations. From (Veit et al., 2016)

1.6.3. **Available therapies**

CF patient survival increased in line with scientific development. This was due to the discovery of new insights in the pathology, as well as possible therapies and mainly antibiotics, as shown in figure 17. When in the 50's anti pseudomonal and ant staphylococcal antibiotics were produced, together with sweat chloride test and high fat diet, survival increased by reaching 20 years. Historically, in the last 7 decades, life expectancy augmented from 2-3 years to more than 50 years (Fig. 17). The main therapies have been antibiotics and the application of recombinant human DNase (rhDNase, Chernick et al., 1961). The subsequent amelioration was linked to the discovery of specific drugs/molecules such as the enteric-coated pancreatic enzymes, aztreonam for inhalation solution (AZLI) and the tobramycin inhalation solution (TIP). Recent technologies such as high-throughput screening (HTS) and innovative drugs improved longevity and life quality in several ways but they are not expected to address the systemic pathology of CF (Clancy, 2018).

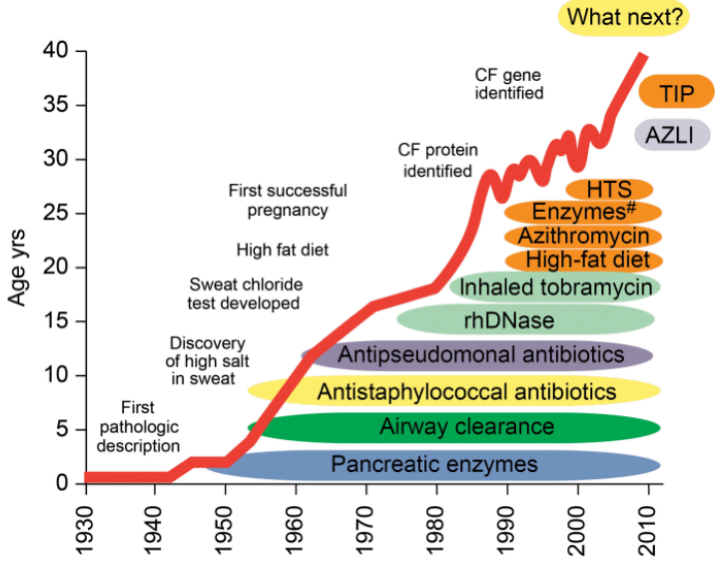

Figure 17: Schematic illustration of CFTR therapies in relation to patient survival over the decades. From (European Respiratory Society, 2009)

In the last years CF research started to adopt all possible strategies for new therapies, from stem cells, to CRISPR/Cas9 technology, gene therapy and so on, in conjugation with specific drugs, antibiotics and molecular inhibitors, as displayed in function of CF class in figure 18. Particularly, several studies focusing on how to recover pharmacologically the function of mutated CFTR proteins have identified new drugs that have been approved for the treatment of CF (Hoy, 2019; Strug et al., 2018). These drugs, working as modulators, such as potentiators and correctors, recover the defective proteins acting on different CFTR processing steps with a mutation-specific efficacy. Precisely, CFTR potentiators are able to induce an improvement in the functionality of such CFTR channels, with amelioration in previously described class III or IV defects (Fakioğlu & Altun, 2020). In a parallel way, correctors improve both CFTR intracellular processing, facilitating the capability to move in the correct location on the cell surface and the production itself of CFTR protein (Rubin, 2018). Innovative approach like this were successfully used for developing mutation-oriented strategies for personalized medicine.

Unfortunately, last life-long drugs have a tremendously high price and this creates significant barrier to patient access in many countries (Hollin & Robinson, 2016). Central in CF research is the investigation on how to restore muco-ciliary clearance to eliminate excessive viscid secretions.

With this goal, ENaC inhibitors are being considered as stand-alone therapy, especially in combination with accessible hydrators - for example hypertonic saline (De Boeck & Amaral, 2016). Inhibition of ENaC, known to cause hyperactivity in the lung leading to airway surface dehydration and mucus accumulation in cystic fibrosis (CF) patients, was the result of a specific antisense oligonucleotides (ASOs) approach in mouse model (Crosby et al., 2017). This strategy down-regulated mucus marker expression and ameliorated goblet cell metaplasia, inflammation, and airway hyper-responsiveness as well. A similar RNA-based antisense oligonucleotide (AON) recovered exon skipping caused by a single mutation in exon 16 (Igreja et al. 2016).

Association between CF and inflammation is another main subject in CFTR therapeutical research. In fact, CFTR expression does not involve only epithelial cells

and neutrophils, but many other structural cells present in the lung, including macrophages, lymphocytes, and airway smooth muscle cells. These actors play a central role in triggering CF airway inflammatory responses. Locally, *Pseudomonas, Burkholderia cepacian, Achromobacter* species have developed in the last years strong antibiotic resistance for the classic drugs. The same resistance was found in atypical mycobacteria and fungi, more and more difficult to treat (De Boeck & Amaral, 2016).

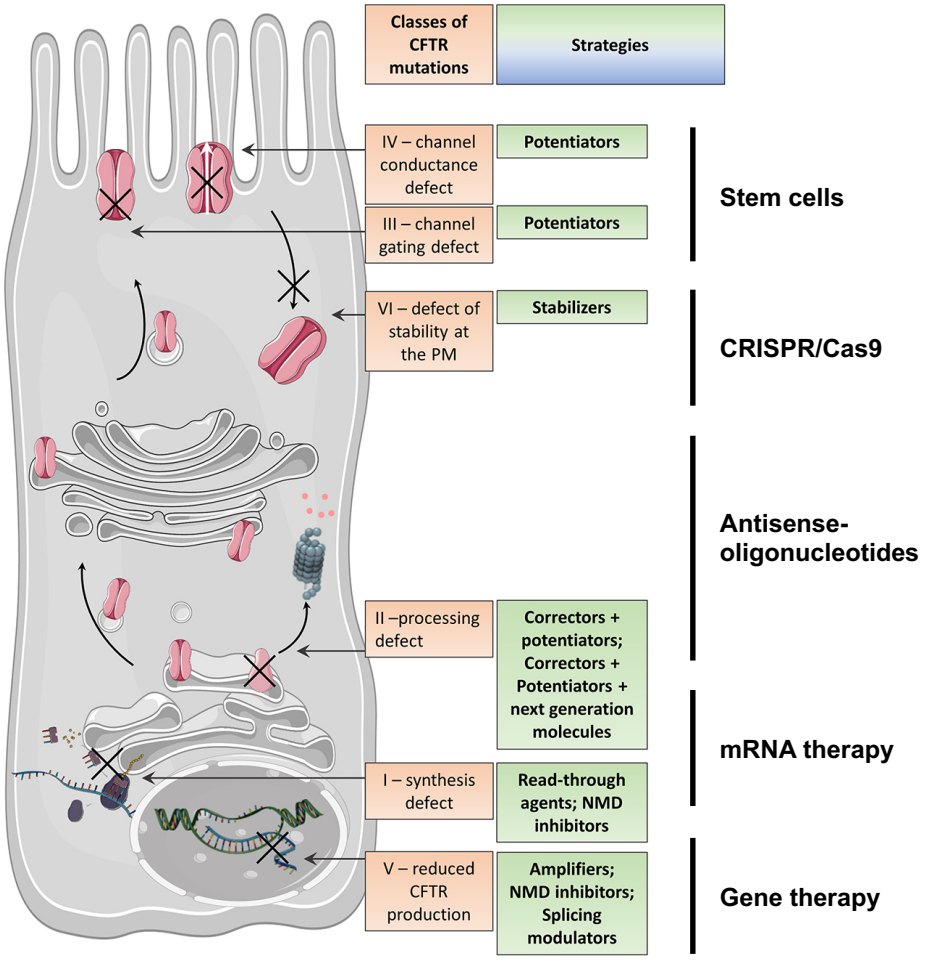

Figure 18: CFTR protein defects and corresponding therapeutical strategies. In the right part, the main strategies emerging from CF research to contrast pathology due to CFTR mutations. From (Pranke et al., 2019)

1.7. **Aim of the thesis**

1.7.1. **Investigation on the role of exonic elements in splicing rescue**

Exonic regulatory elements may be the direct or indirect target of disease-causing mutations, and ExSpeU1 strategy could be an efficient molecular tool to rescue the splicing pattern and the protein through RNA-based gene therapy.

Dense splicing regulatory element presence in *Factor IX* exon 5 was found being extremely susceptible to mutation-induced splicing derangement. In addition, exon skipping in the mutants was suggested to be partially influenced by the formation of novel binding sites for two splicing factors: hnRNPA1 and DAZAP1. In a few disease-causing splicing exonic mutations in coagulation factor IX (FIX) exon 5, splicing pattern was totally rescued by a modified U1snRNP particle, through an SRSF2-dependent enhancement mechanism (Tajnik et al., 2016).

Inclusion of a cryptic exon may act through the creation of a new ESE motif recognized by splicing factors, as in a pathological deep intronic mutation (FGB, afibrinogenemia, c.115–600A>G). Even in this case, researchers demonstrated to impair recognition of the cryptic ESE by morpholino oligonucleotide which efficacy accounted for >50% (Davis et al., 2009). From two independent studies, it was confirmed that ESE (but not always ESS) is functional in different exonic contexts, suggesting that recognition motifs are not only linear nucleotide sequences, but there may be structural features whose conservation is essential for optimal exon recognition (Muro et al., 1999; Staffa & Cochrane, 1995).

In this scenario, intense and descriptive studies on the architectural structure of ExSpeU1-based splicing rescue with focus on regulatory elements has not been done yet. With this goal I focussed on two systems: *Factor IX* exon 5 and *FANCA* exon 8 that differ in their ExspeU1 rescue efficiency, through the analysis of a series of chimeric *FIX-FANCA* minigenes.

1.7.2. **Application of Exon Specific U1 strategy in Cystic Fibrosis**

Approximately 13% of CFTR variants reported in CFTR database (https://www.cftr2.org/) affect pre-mRNA splicing and the most frequent effect on the mRNA is skipping of the exon from the mature transcript. Exon skipping mutations are mechanistically heterogeneous as they can affect the 5'ss consensus site, the 3'ss (including the polypyrimidine tract) or exonic regulatory elements. Thus, gene variants are expected to avoid proper synthesis of the full-length, normal CFTR protein because of deletions, nonsense mutations, frameshifts, or aberrant mRNA splicing (Marson et al., 2016).

As different molecular mechanisms are involved in aberrant splicing of *CFTR*, a common strategy that works with different skipped exons and splicing mutations would represent an important therapeutic improvement. In fact, research on CF is challenging new strategies to develop innovative therapeutic molecules through basic research, therapy discovery and implementation. For this goal, I focussed on ten relatively frequent splicing mutations that cause skipping of corresponding exons 5, 10, 13, 16 and 18. Splicing mutations 711+3A>C/G and 711+5G>A are located in the 5'ss consensus of exon 5; 1863C>T (p.Y577Y) and 1898+3A>G in an exonic regulatory element and in the 5'ss consensus of exon 13, respectively; 2789+5G>A and 3120G>A are located at the 5'ss consensus of exon 16 and 18 respectively, whereas TG13T3, TG13T5, TG12T5 are variants at the polypyrimidine tract of exon 10. With a series of ExSpeU1s panel, I investigated this strategy in their splicing rescue, not only at the RNA level but also, at the protein level. Through part of data shown in this thesis, a research article was published (Donegà et al., 2020).

2. Material and methods

2.1. **Purchased items**

Chemical reagents used for the experiments included in this thesis are listed below:

- Standard solutions: NaCl, Na_2HPO_4, KCl, KH_2PO_4 and KOH from Riedel-de Haën™. HEPES, EDTA and boric acid from Sigma Aldrich. Tris from Invitrogen. Agarose from Euroclone;
- Bacterial cells: DMSO and $MgCl_2$ from Riedel-de Haën™. PEG_{4000} from Serva;
- Eukariotic cells: DMEM and FBS from Gibco Life technologies. Antibiotic antimycotic and DMSO from Sigma Aldrich. Effectene from QIAGEN. Lipofectamine 2000 from Thermo Fischer Scientific;
- RNA: Tri-Reagent from Ambion. Chloroform from Sigma Aldrich. 2-Propanol for Riedel-de Haën™. Ethanol from Merck. Dnase I from Promega. dNTPs from Rovalab. Reverse transcription kit and Random Primer (RP) from Invitrogen;
- DNA: Restriction endonucleases and CIP from New England Biolabs (NEB). T4 DNA Ligase from Promega. NucleoSpin® Plasmid kit and NucleoBond® Xtra midi kit from Machery-Nagel. QIAquick® Gel Extraction Kit from QIAGEN;
- PCR: Taq DNA polymerase from NEB, dNTPs from Rovalab
- Western Blot (WB): Methanol fromMerck.

Enzymes used (following manufacturer's instructions) for the experiments included in this thesis are listed below:

- Restriction enzymes: from New England Biolabs (NEB);

- DNA modifying enzymes, including DNase I and T4 DNA ligase from New England Biolabs (NEB). Calf intestinal phosphatase (CIP) from Promega;
- *Taq* DNA polymerase from Roche.

2.2. **Solutions**

Standard solutions used in this thesis are listed below:

- PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HP0_4, 1.8 mM KH_2PO_4, pH 7.4;
- Luria-Bertani medium (LB): 1% w/v Difco Bactotyptone, 0.5% w/v Oxoid yeast extract, 1% w/v NaCl in dH2O, pH 7.5;
- TSS: 10% w/v PEG 4000, 5% v/v DMSO, 35 mM $MgCl_2$ in sterile LB medium, pH 6.5;
- 10x TBE: 108 g/L Tris, 55 g/L boric acid, 9.5 g/L EDTA;
- PBST: 0.1% v/v Tween 20 in 1x PBS.

Solutions used for Western blot (WB) are listed below:

- 10x running buffer: 250 mM Tris, 1.92 M glycine, 1% w/v SDS in dH_20, pH ~8.3;
- 10x transfer buffer: 250 mM Tris, 1.92 M glycine in dH_2O, pH ~8.3. When diluted to 1x, methanol was added to 20% v/v;
- Upper Tris buffer: 0.5 M Tris-HCl pH 6.8;
- Lower Tris buffer: 1.5 mM Tris pH 8.8;
- 2x lysis buffer: 30 mM HEPES pH 7.5, 0.5 mM NaCl, 1% v/v NP-40, 20% v/v glycerol in dH_2O;
- 5x SDS sample loading buffer: 250 mM Tris-HCl pH 6.8, 10% w/v SDS, 30% v/v glycerol, 5% β-mercaptoethanol, 0.02% w/v bromophenol blue in dH2O.

2.3. **Plasmid DNA modifications**

2.3.1. **Enzymatic reactions**

Restriction enzymes were purchased from NEB, including buffers supplied by the same company. For analytical experimental purposes, 300 ng of plasmids were digested in a volume of 20 µl containing the appropriate units (Us) of the restriction enzyme per µg of DNA. Vector and insert preparative digestions were made using 3 µg DNA in 50 µl reaction volume; next, heat inactivation was used to terminate enzymatic activity.

To drop the vector background in cloning strategies, I took advantage of CIP, an enzyme catalysing the removal of 5'-phosphate groups from DNA and RNA. CIP-treated fragments lack the 5'-phosphoryl termini required by ligases; thus, they are not able to self-ligate.

Standard reaction was done in a final volume of 50 µl by means of 0.5 U / 1 µg DNA at 37°C for 30 minutes, following heat inactivation at 85°C for 15 minutes.

To create a compatible end for ligation, I used Klenow enzyme, the large fragment of DNA Polymerase I, which acts by retaining polymerization and 3' -> 5' exonuclease activity but lacks 5' -> 3' exonuclease activity.

A 20 µl mix of 1 U of Klenow per µg DNA, 1x NEBuffer 2.1 and supplemented with 25 µM dNTPs was incubated 10 minutes at room temperature, then inactivated for 20 minutes at 70°C.

To allow subsequent ligation by catalysing the exchange reaction of a 5'-phosphate from ATP to the 5'-hydroxyl-terminus of ds/ssDNA and RNA, I used T4 Polynucleotide Kinase.

A mix of T4 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; next, enzyme heat inactivation by incubating at 65°C for 20 minutes.

2.3.2. **Elution and purification from agarose gel**

Agarose gels are common tool for DNA restriction enzyme digestions fast analysis, DNA concentration valuation and classic DNA fragment separation prior to elution from the gel. Samples including 1x DNA Loading Buffer (0.4% bromophenol blue, 60% glycerol, water) were loaded into submerged wells in agarose gels in concentration from 0.8% w/v (large fragments/RNA quality control) to 3% w/v (small fragments <50 nt).

Gels contained ethidium bromide (0.5 μg/ml) in 1x TBE solution.

Gel electrophoresis was performed at 50-100 mA (depending on the needs) in 1x TBE running buffer for a temporal range of 20-60 minutes, depending on the time necessary to separate the bands.

Then, DNA visualization with UV light made visible the fragment of interest, which was excised using QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instruction.

Finally, amount of gel-extracted DNA was estimated by UV fluorescence of intercalated ethidium bromide in agarose gel electrophoresis as previously described.

2.4. **Competent cells**

2.4.1. **Preparation of E. Coli DH5α**

One-step procedure for the preparation of competent E.Coli DH5α was used (Chung et al., 1989). *E. coli* DH5α strain was grown overnight at 37°C with dynamic shaking in 10 mL of Luria- Bertani (LB) medium.

The day after, 140 mL of fresh medium were added, and cells were grown in the shaker at 37°C until the 600 nm optical density (OD_{600}) was measured between 0.3 and 0.4.

Then, cells were located in pre-cooled 50 mL tubes, and centrifuged at 180 x g for 10 min at 4°C in the centrifuge (Eppendorf 5804/5804 R).
Pellet was re-suspended in ice-cold TSS solution in 10% of the initial volume, aliquoted and rapidly frozen in liquid nitrogen.
Competent cells were stored at -80°C, and their competence was determined by transforming with 100 pg of pUC19 in 100 µL of freshly thawed cells.
If obtained number of colonies was exciding 10^2, the competency was considered to be satisfactory.

2.4.2. **Bacteria transformation and plasmid DNA purification**

In bacterial transformation experiments I used 70 µL of freshly thawed competent cells, while for re-transformation 30 µL.
In both cases, I supplied with 1ng of plasmid DNA (for transformation, from previous inactivated ligation reaction).
Then I incubated in ice for 30min and next heat shock carried out at 42°C for 1 min 15 sec.
The mix containing cells, after 30min in ice, was then plated on LB agar containing 100 µg/mL ampicillin or 7 µg/mL Kanamycin, depending on the antibiotic resistance of the vector.
Afterward, over-night incubation at 37°C and to be able to pick single colonies to expand the day after, in 10/50mL liquid medium LB (the same used in the first step) for small/medium scale DNA purification, respectively.
Thus, dynamic incubation in shaker overnight at 37°C.
The expanded LB was centrifuged at 4500 x g (Eppendorf centrifuge 5804/5804 R) and resulting pellet was used in plasmid purification kits according to the manufacturer's instructions. NucleoSpin® Plasmid kit (Macherey-Nagel, Germany) was used for small scale purification, while for medium scale purification I used NucleoBond® Xtra midi (Machery-Nagel, Germany).

Final elution was performed with water; thus, plasmid DNA was stored in aqueous solution at -20°C. Spectrophotometer used to measure DNA concentration **was** Nanodrop 1000 (Thermo Scientific).
Ultimately, quality check was performed in 2% agarose gel containing 0.5 μg/mL of ethidium bromide in 1x TBE buffer and ran at 90 mA until fully separation of the band level.
Gel visualization was done using UV transillumination; Gel Logic 100 imaging system (Kodak) was used to record photos.

2.5. **Plasmids design**

In engineered minigenes a region of interest is introduced in a basic minigene to create a novel hybrid construct from which, by amplifying a specific segment it is possible to analyse splicing pattern.
pTB minigene is a modified Bluescript KS+ vector comprehending at 5'end: I) SV40 enhancer sequence at the 5' end with II) α-globin gene promoter to allow polymerase II transcription in the transfected cell lines, while at 3' end a poly-A segment from α-globin gene (Pagani, Stuani, et al., 2003).
Within this described sequence, α-globin gene promoter was followed by a series of exonic and intronic sequences composed by α-globin and fibronectin EDB exons.
A single NdeI restriction enzyme site is used to insert the region of interest to test (Fig. 19).
pFAN is an analogous plasmid, derived from pTB, in which the genetic architecture is enriched by FANCA context, with the unique NdeI site flanked by intronic FANCA region bordered by FANCA exon 7-9 (Mattioli et al., 2014).

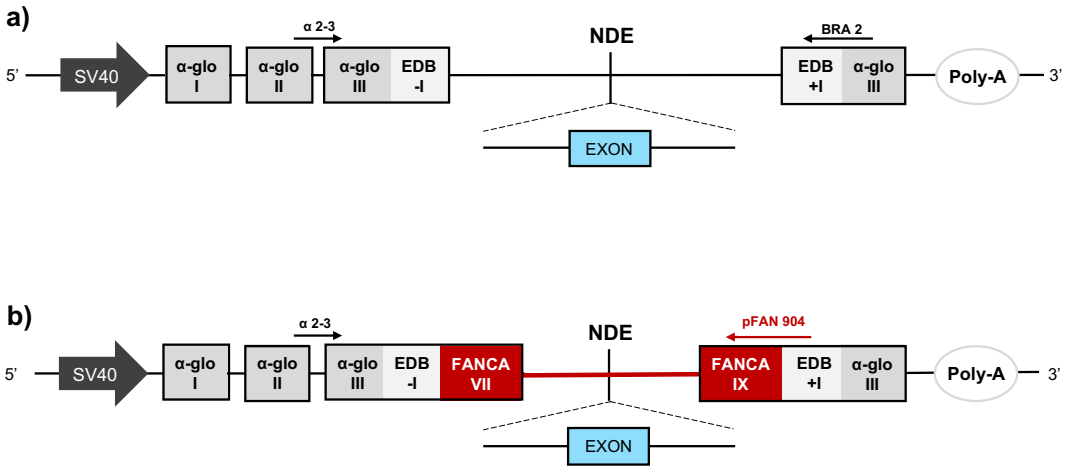

Figure 19: Minigenes representation. a) pTB minigene. Unique NdeI site is represented as insert of the region of interest to test in light blue. b) pFAN minigene. In red, FANCA exVII and Fanca ExIX representation. Arrows indicate primers.

2.5.1. ***Factor IX - FANCA* hybrid minigenes**

To study exonic regulatory element interaction with ExSpeU1, I used Factor IX (FIX) exon 5 and FANCA (FAN) exon 8 models.

Both of them were characterised by mutants at 5'ss and at 3'ss: -2C at 5'ss and -9G at 3'ss for FIX (Alanis et al., 2012), while -5T at 5'ss and -5C at 3'ss for FAN, respectively (Mattioli et al., 2014).

Using Factor IX Exon V (129bp) and FANCA Exon VIII (83bp), both flanked by their intronic sequence, modular hybrid minigenes were constructed.

All hybrid minigenes for FANCA and Factor IX were constructed in pFAN minigene as a vector, thus in this thesis they are named without the vector-prefix (e.g. pFAN-name).

Minigene sequence is available in Supplementary Material.

Once identified specific regulatory sequences, these were cloned in hybrid context for experimental reasons. In the Result session, all the mutants are listed.

In both contexts I used corresponding ExSpeU1s :shift 9 for FIX, shift 10 for FANCA (Alanis et al., 2012; Mattioli et al., 2014)

As a control, the resulting plasmid DNA was sequenced with GATC Biotech (Eurofins): 5 µl of DNA samples (100 ng/µl) in a 1.5 ml tubes with 5 µl of primer at concentration of 5 pmol/µl for each sample were shipped to company.

Primers were specific either for the plasmid vector (universal) or for the minigene. Sequencing results were then confirmed by alignments using Serial Cloner 2.6.1 (freeware online) and analysed using the SnapGene software (GSL Biotech).

2.5.2. *CFTR* minigenes

pTB-CFex10 and pTB-CFex13 minigenes have been used in previous publications (Pagani et al., 2002; Pagani, Buratti, et al., 2003). They contain *CFTR* exons 10 and 13 (historical names exons 9 and 12, respectively) flanked by ~150 bp of intronic sequences in the pTB construct.

pTB-CFex5 and pFAN-CFex18 were created by cloning the corresponding exons along with ~150 bp of intronic sequences in pTB or pFAN minigenes, respectively (Mattioli et al., 2014).

Mutations were introduced by site-directed mutagenesis using the Quick-Change Site-directed Mutagenesis Kit II (Agilent, Santa Clara, USA).

Splicing competent cDNA exon 13 WT and mutants minigenes (pcDNA3.1-CFex13) contain the full *CFTR* cDNA from exon 1 to exon 12, the proximal part of intron 12 (~400bp), the distal part of intron 12 (~300bp), exon 13 , the proximal part of intron 13 (~260bp) , the distal part of intron 13 (~350bp) and the last part of *CFTR* cDNA (exons 14 -27) in pcDNA3.1. *CFTR* cDNA(ex13-) is a derivative of pcDNA3.1-CFex13 without the exon 13 cassette.

Splicing competent cDNA exon 16 WT and 2789+5G>A mutant minigenes (pcDNA5-CFex16) were previously described (Igreja et al., 2015).

ExSpeU1s used for Factor IX and Fanca contexts were created by replacing the sequence between the BclI and BglII sites with oligonucleotides as previously reported (Alanis et al., 2012; Mattioli et al., 2014; Pagani et al., 2002). CFTR target sequences together with the corresponding relative sequences of Exon Specific U1s used in this study are reported in the Supplementary material Table S1.
The identity of minigene constructs was confirmed through sequencing analysis as previously described.

2.6. **mRNA functional splicing analysis**

Minigene approach is an advantageous genetic tool for several reasons:

- to study *cis*- and *trans*-acting elements affecting splicing process;
- to determine whether a specific mutation can compromise the splicing;
- to establish the role of the splice sites in the exon recognition.

Splicing assay is made by transient transfection of minigenes with/without other molecules such as splicing factors, followed by RNA analysis processes, which allows us to study the splicing outcome.

2.6.1. **Cell model**

HeLa cells were grown in Dulbecco's Modified Eagle Medium (DMEM, EuroClone) with glutamine, sodium pyruvate, pyriodoxine and 4.5 g/l glucose supplemented with 10% heat inactivated fetal bovine serum (FBS, EuroClone) and antibiotic antimycotic (Sigma-Aldrich) according to the manufacturer's instruction.
When cells reached confluency, to remove all the medium residues as well as the dead cells, the confluent monolayer of cells from a standard 100 mm dish was washed twice

with 1x PBS solution, then treated with 2 ml Trypsin (PBS containing 0.045 mM EDTA and 0,1% trypsin) and incubated at 37°C for 5 minutes.
After adding 5 ml of DMEM medium to block trypsin, cells were precipitated in dedicated centrifuge at 1000 rpm for 5 minutes and resuspended in pre-warmed medium.
Finally, with a sub-cultivation ratio of 1:5 to 1:10, I plated in a new 100 mm dish 10ml final volume of resuspended cells.

2.6.2. **Transient co-transfection**

To reach a final confluency percentage of 40-70% the day of transfection, 24h before transfection $3x10^5$ HeLa cells were plated as described above into 6-well cell culture dishes. Effectene transfection reagents (QIAGEN) was used according to manufacturer's instructions.
In case of classic transfection, I used 500ng minigene (1unit), while in case of co-transfection with ExSpeU1s or Splicing factors, I added 500ng more for a total of 1ug (2unit, with relative quantities of transfection reagents).
To allow condensation of previously prepared plasmid DNA, 1unit was mixed with 150 µl of EC buffer and 4 µl of Enhancer, then incubated at room temperature for 5 minutes.
5 µl of Effectene Reagent were then added to the mixture and incubated for 10 minutes to allow Effectene-DNA complexes to form.
Then, to the mix 500 µl of complete growth medium were added and the resulting solution was added to cells for 24h at 37°C.
After one day, cells were harvested and subjected to further investigations.

2.6.3. **RNA extraction, cDNA synthesis and PCR**

24h after transfection, 1x PBS was used twice to wash transfected cells and 750 µl of RNA TRI Reagent (Thermo Fisher Scientific) was added in the 6-well cell culture dishes.

TRIzol-added cells were collected in 1.5mL tubes and vortexed for 15 seconds with 200 µl Chloroform. Chloroform is miscible with phenol by having higher density: it forces a sharper separation of the organic and aqueous phases assisting in the removal of the aqueous phase.

Thus, after a 15 minutes 12000rpm 4°C centrifuge, ca 400 µl of aqueous phase (containing RNA) was recovered and 400 µl isopropanol were added.

The mix was centrifuged precipitated at 12,000 g for 30 minutes, then the pellet was rinsed in ethanol 70%, finally resuspended in H_20 and stored at -80°C.

Following manufacturer's instructions, M-MLV Reverse Transcriptase Kit (Invitrogen) was used to synthetise first-strand cDNA.

To denature the RNA at 94°C for 2 minutes, a first mix including 2 µl of random primers (RP, 100 ng/µl Thermo Fisher Scientific), 1µg of total RNA extracted from cells was diluted in water to the final volume of 12 µl.

After denaturation, a second mix was prepared to be incubated at 37°C for 90 minutes: 6 µl of 5x First-Strand Buffer (250 mM Tris-HCl (pH 8.3 RT), 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.

Polymerase chain reaction (PCR) was performed cDNA as template and following the basic protocol of Taq DNA polymerase; 25 µl reaction included: 1x Taq buffer, dNTPs mix (100 µM each), oligonucleotide primers (100 nM each, Sigma-Aldrich), Taq DNA polymerase (2.5 U) and 0.1 ng of DNA template.

Amplification of pTB minigene was characterized by initial denaturation at 94°C for 5 minutes, then 28 cycles with denaturation at 94°C for 45 seconds, annealing at 56°C for 45 seconds, then extension at 72°C for 45 seconds with a final extension step at 72°C for 10 minutes. pFAN amplification differed with annealing temperature of 58°C during 30 cycles amplification.

Gene Amp PCR System (Applied Biosystem) was used to amplify cDNA.
Finally, as previously described, electrophoresis gel visualization and digital photo acquisition.

2.6.4. **Protein extraction and Western Blot**

Cells were harvested 48 hours post-transfection, washed twice in 1x phosphate-buffered saline (PBS), and lysed RIPA buffer with protease inhibitor cocktail (Sigma-Aldrich), in order to inhibit enzymes from degrading proteins for 10 min at 4°C.
To measure the concentration of total proteins, Bradford assay (Bio-Rad Laboratories) was performed using Bradford Protein Assay kit (Bio-Rad).
Bovine serum albumin (BSA) was used as a reference in standard curve for spectrophotometer analysis, (595 nm wavelengths setting).
Before separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), 40 µg of proteins (20µl volume) were boiled in Laemmli buffer for 5 min. SDS-PAGE gel was composed by: 5% stacking gel (pH 6.8) and 10% running gel (pH 8.8).
The gels were run at 35 mA in 1x Running buffer, until the desired bands are well separated.
Proteins were then electroblotted onto polyvinyl difluoride membranes (PVDF, Bio-Rad, Ivry-sur-Seine, France).
The PVDF membrane was activated few seconds in methanol and washed twice with water and again put in Blotting buffer.
The “WB sandwich” composed by papers, gel, activated membrane was assembled. Then, it was placed in the support at 200-250 mA for 75-150 minutes depending on the proteins that have to be transferred.
Then nonspecific binding sites were blocked for 2h at room temperature by 6% (w/v) fat-free milk before an overnight incubation at 4°C with specific primary antibodies

for CFTR 570 (1:1000, CFTR Folding Consortium, University of North Carolina, USA) and Tubulin (1:5000).

Primary antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgGs (1:10,000; Jackson ImmunoResearch Laboratories, Villepinte, France) or rabbit anti-goat IgGs (1:5,000; Dako, Glosturp, Denmark).

Blots were revealed using an Enhanced Chemioluminescence detection kit (Amersham, Les Ulis, France).

Band intensity quantification was performed using ImageJ.

2.7. **Online tools and statistical analysis**

Splice Aid, was used for exact motifs prediction of Splicing related RNA-binding proteins (Piva et al., 2009). The database-tool is freely available at: http://www.introni.it/splicing.html.

Neural Network method was used for splice site prediction using Berkeley Drosophila Genome Project online tool (Reese, 1997). It is freely available at: https://www.fruitfly.org/seq_tools/splice.html.

The statistical analysis using Student's t-test was performed with Prism (Graph- Pad, USA) version 7.0. A standard confidence interval of 95% was used for all statistical analysis and the significance P-value were indicated by asterisks (*P < 0.05; **P < 0.01; ***P < 0.001).

3. Results

3.1. Identification of determinants involved in ExSpeU1 rescue

3.1.1. ExSpeU1 rescues exon skipping defects in Factor IX exon 5 but not in FANCA exon 8

To investigate the role of *cis* acting elements that define the strength of an exon in the ExSpeU1-mediated splicing rescue I focused on Factor IX exon 5 and on FANCA exon 8. Minigene splicing assay for the WT version of Factor IX (FIX) and FANCA (FAN) showed that the WT exons are efficiently included in the final transcripts, according to previous studies (Fig. 20a, b and c lane 1).

In factor IX, I have evaluated two disease causing mutations at the donor (FIX-2A>C, Mut 5') or at the acceptor sites (FIX-9T>G, Mut 3'). These two mutants resulted in complete exon skipping (Fig. 20b lane 2 and 5, respectively). These mutations are not located at the invariant AG and GT dinucleotides that constitute the splice sites and accordingly they do not fully disrupt the splice sites consensus. Co-transfection of two engineered U1s that bind in position 1-10 and 9-21 downstream the 5'ss (FIX-ExSpeU1 1-10 and FIX-ExSpeU1 9-21), completely rescued the splicing pattern in both mutants (Fig. 20b, lane 3-4, 6-7) as previously reported (Alanis et al., 2012).

In FANCA, similarly I evaluated two mutants at the 5'ss (83FAN-3C>T Mut 5') or at 3'(83FAN-5T>C) and they showed total exon skipping (Fig. 20c, lane 2,5). However, unexpectedly in the FANCA context the ExSpeU1s that bind in position 3-10 and 10-27 were not able to fully recover the aberrant splicing with a minor effect (Fig. 20c, lane 3-4, 6-7).

As the mutations that affect the 3' or 5' ss in these two systems are very similar (they are not located in the invariant AG and GT dinucleotides) my results suggest that peculiar architecture of the defective exons is involved in the response to the ExSpeU1.

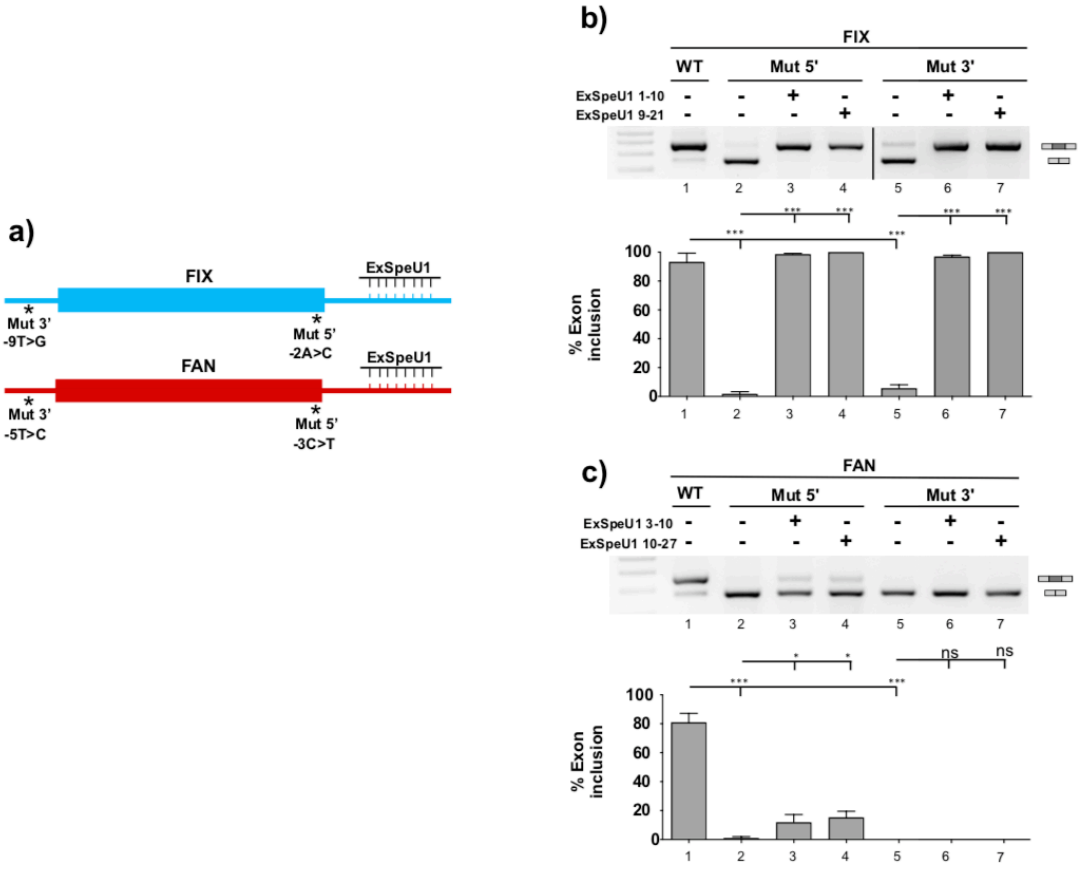

*Figure 20: ExSpeU1s do not rescue aberrant splicing with the same efficiency. a) Schematic representation of FIX (Factor IX Exon 5), FAN (FANCA Exon 8) minigenes, FIX-ExSpeU1 1-10, FIX-ExSpeU1 9-21, FAN-ExSpeU1 3-10, FAN-ExSpeU1 10-27. Exons (boxes), intronic sequences (lines), mutants (*) and ExSpeU1s are indicated. b) FIX and c) FAN hybrid minigenes: upper figures represent the agarose gel and the lower graph the percentage of exon inclusion. Identity of the exon inclusion and skipping bands is indicated. Data are expressed as means ± SD of three independent experiments done in duplicate. Statistical analysis was performed using Student's t-test (***: P < 0.001; **: P < 0.01; *: P < 0.05; ns: not significant).*

3.1.2. **Analysis of the splicing regulatory elements that modulate the ExSpeU1 response to the 5'ss FANCA mutation**

As the ExSpeU1 strategy was very efficient on the 5'ss mutations in Factor IX but not in FANCA, I initially investigated whether this different ExSpeU1 response to the donor site defect present in FANCA was linked with *cis*-acting elements present in upstream sequences, relative to the FANCA 5'ss. For this reason, I created a panel of hybrid minigenes, in which the FANCA ex8 (83bp) sequences, with or without the 5'ss mutation, were progressively substituted with the FIX sequences (129bp, Fig. 21a).

The 6FIX-77FAN construct contains the upstream FIX intron with 6 bases of FIX exon followed by the exonic FANCA sequences and FANCA downstream intron. The 63FIX-41FAN minigene contains the upstream FIX intron with half of FIX exon, ~ half of FANCA exon and FANCA downstream intron. Lastly, 126FIX-3FAN minigene contains the upstream FIX intron, most of FIX exon, 3 bases of FANCA exon and the FANCA downstream intron. I evaluated the splicing pattern of the minigenes with the 5' mutation and the rescue effect of the ExSpeU1 that binds in the FANCA intron (FAN-ExSpeU1 10-27). The results in Fig. 21 show that the progressive substitution of the 3' portions of the FANCA minigene with the FIX sequences gradually improves the splicing response to the ExSpeU1. In fact, while ExSpeU1 did not improve FAN Mut 5' splicing, it rescued splicing in 6FIX-77 FAN Mut 5' and 63FIX-41FAN Mut 5' minigenes. These two minigenes in basal condition showed total skipping and were rescued by the ExSpeU1 inducing an increase to~50% and ~65% of exon inclusion, respectively (Fig. 21b lane 3-6). The 126FIX-3FAN Mut5', that include most of the FIX exon and has only the 5'ss and the downstream FANCA intron, showed in basal condition ~40% of exon inclusion, and was fully rescued by ExSpeU1 (Fig.21b lane 7-8). In all WT minigenes (without the 5'ss mutation), I detected full inclusion and co-transfection of ExSpeU1 did not affect the inclusion levels (Fig.21 c lane 1-7). This result indicates that either enhancing elements present in the FIX context and/or silencing elements in the FANCA context

are involved in the ExSpeU1 rescue efficiency. In addition, my data excludes that the lack of ExSpeU1 rescue in FAN minigene is due to the composition of the mutated FAN 5'ss or of the downstream intron.

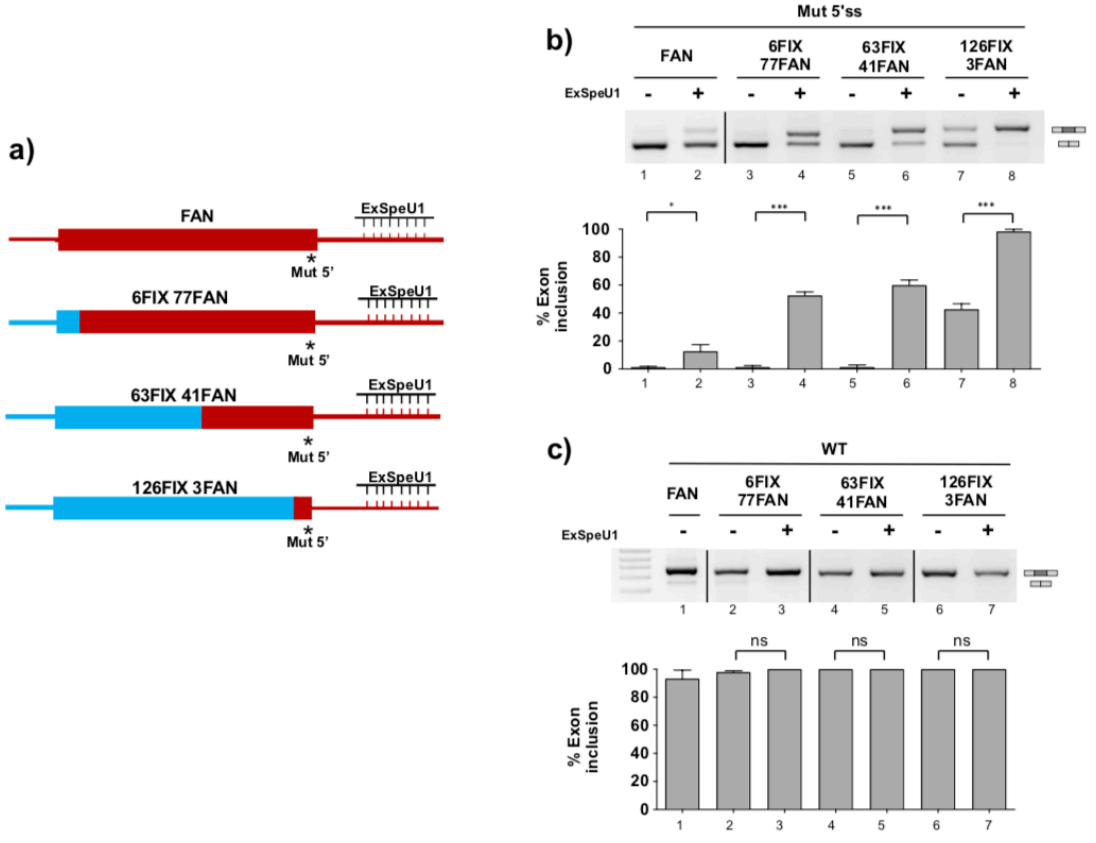

*Figure 21: The composition of upstream intronic and exonic sequences modulates the ExSpeU1 effect on the 5'ss FANCA mutation. a) Schematic representation of FAN, 6FIX-77FAN, 63FIX-41FAN and 126FIX-3FAN s 5'ss and WT minigenes, FAN-ExSpeU1 10-27. Exons (boxes), intronic sequences (lines), mutants (*) and ExSpeU1s are indicated. b) Mut 5'ss and c) WT hybrid minigenes: upper figures represent the agarose gel and the lower graph the percentage of exon inclusion. Identity of the exon inclusion and skipping bands is indicated. Data are expressed as means ± SD of three independent experiments done in duplicate. Statistical analysis was performed using Student's t-test (***: P < 0.001; **: P < 0.01; *: P < 0.05; ns: not significant).*

3.1.3. **Identification of an ExSpeU1-Inhibitory Element in FANCA**

To further investigate the role of *cis*-acting sequences in modulating the ExSpeU1 response in the presence of donor site defects, I created a panel of hybrid minigenes in which the FIX cassette was progressively substituted from the 3' with the FANCA sequences (Fig. 22a).

The resulting minigenes, with or without the 5'ss mutation present in FIX, were transfected alone or co-transfected with the ExSpeU1 that binds in the FIX intron (FIX-ExSpeU1 9-21) followed by analysis of the splicing pattern.

In the presence of the 5FIX 'ss mutation, substitution of the FIX most upstream intronic/exonic parts with the corresponding FANCA sequences (up to 15 bases of FANCA exon) did not affect the response to FIX-ExSpeU1. In fact, the completely skipped exons from FIX, 6FAN-123FIX and 15FAN-108FIX minigenes, were completely recovered by ExSpeU1 (Fig 22b lane 1-6). Interestingly, in 24FAN-93FIX further inclusion of 24 bases in position 15- 24 from the FANCA exon (associated to a reduction of FIX exonic sequences from 108 to 93) completely abolished the ExSpeU1-mediated rescue (Fig. 22b lane 7-8). This lack of effect is maintained in the subsequent 33FAN-81FIX and 42FAN-66FIX minigenes, where again ExSpeU1 was not effective (Fig. 22b lane 9-12).

I tested also the same constructs without the 5'ss mutation. Compared to the normal FIX in which the exon is largely included in basal condition (~85%), 6FAN-123FIX and 15FAN-108FIX showed exon skipping (Fig. 22c lane 1,2,4).

Co-transfection of ExSpeU1 fully rescued splicing (Fig. 22c lane 2-5) suggesting that potential negative elements present in FANCA upstream intron and in the first 15 bases of the FANCA exon are not sufficient to prevent the ExSpeU1 activity. In addition, as found with the corresponding 5'ss mutation construct, the 24FAN-93FIX, 33FAN-81FIX and 42FAN-66FIX minigenes did not respond to ExSpeU1 (Fig. 22c lane 6-11). The striking different effect of ExSpeU1 on 15FAN-108FIX and 24FAN-93FIX in both the WT and 5'ss mut minigenes points to the 15- 24 bases of FANCA exon as important sequences that affect the ExSpeU1 rescue efficiency.

This potential regulatory element present in the FANCA exon in position 15- 24 was named ExSpeU1- Inhibitory Element (ExSpeU1-IE).

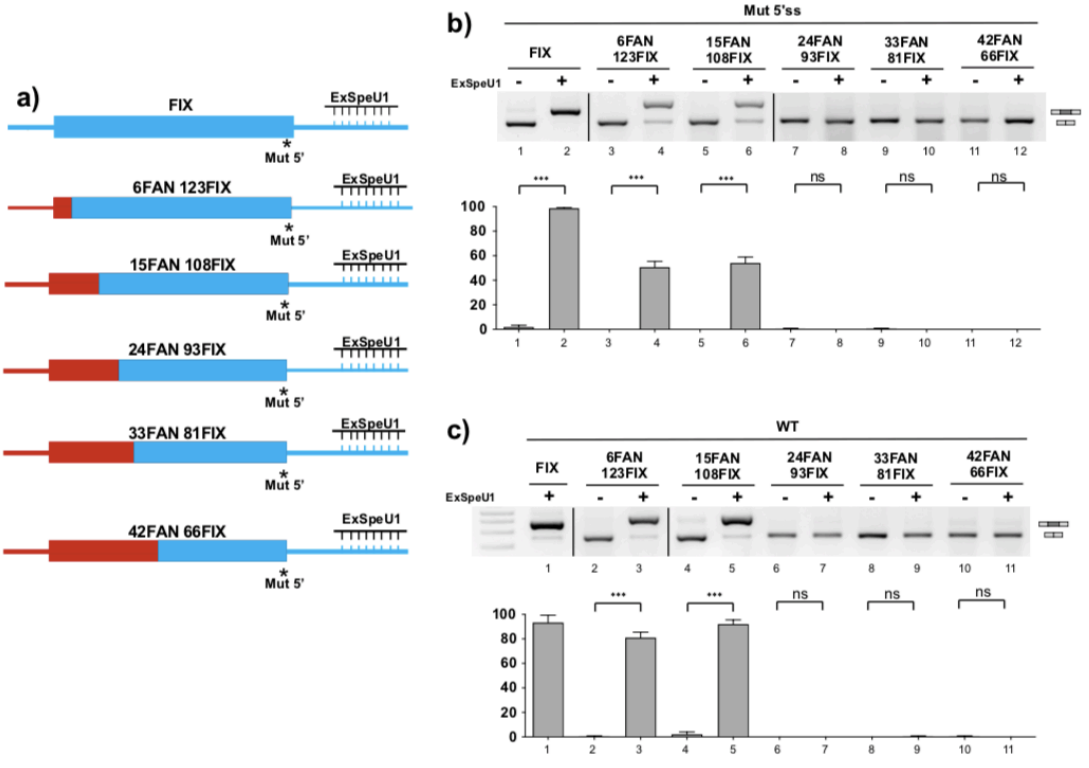

*Figure 22: Identification of an ExSpeU1-Inhibitory Element (ExSpeU1-IE). a) Schematic representation of FIX, 6FAN-123FIX, 15FAN-108FIX, 24FAN-93FIX, 33FAN-81FIX, 42FAN-66FIX 5'ss Mut and WT minigenes and FIX-ExSpeU1 9-21. Exons (boxes), intronic sequences (lines), mutants (*) and ExSpeU1s are indicated. b) Mut 5'ss and c) WT hybrid minigenes: upper figures represent the agarose gel and the lower graph the percentage of exon inclusion. Identity of the exon inclusion and skipping bands is indicated. Data are expressed as means ± SD of three independent experiments done in duplicate. Statistical analysis was performed using Student's t-test (***: P < 0.001; **: P < 0.01; *: P < 0.05; ns: not significant).*

To better understand the function of the ExSpeU1-IE I also performed site-directed mutagenesis experiments. The UG-rich sequence TTTGTTTG in position 15- 24 of FANCA was mutated to TccacTcTG in both the WT and 5' mutant 24FAN-93FIX contexts and the resulting minigenes transfected alone or with ExSpeU1 (Fig. 23a). Co-transfection of ExSpeU1 rescued the complete exon skipping defects in the Mut ExSpeU1-IE minigene (Fig. 23b lane 5-8), but not in the not-mutated counterparts (Fig. 23b lane 1-4). Particularly in the Mut ExSpeU1-IE minigene, ExSpeU1 co-transfection induced a significant splicing rescue both in WT (~75%) and in the 5'ss mut (~45%, Fig. 23c lanes 6,8). Since the site-directed mutagenesis restored the response to the ExSpeU1 in the 24FAN-93FIX contexts, these results suggest that the ExSpeU1-IE is a strong exonic splicing silencer (ESS).

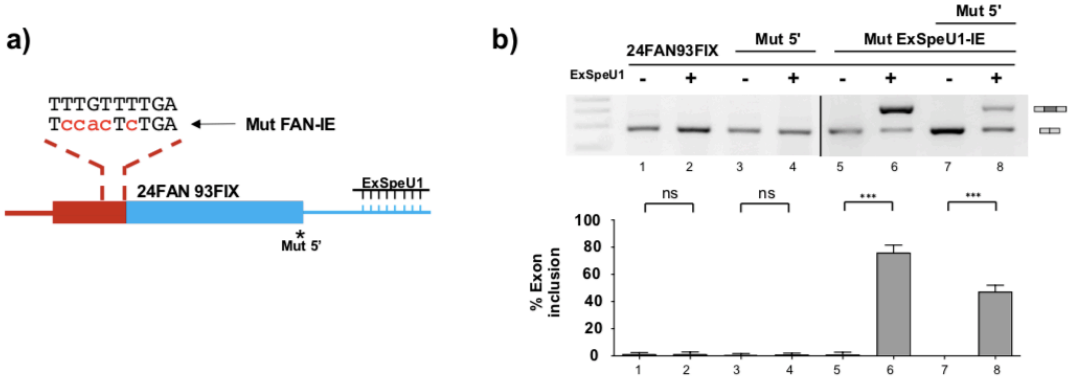

*Figure 23: Site-directed mutagenesis disrupting ExSpeU1-IE in hybrid context. a) Schematic representation of 24FAN-93FIX, 24FAN-93FIX Mut ExSpeU1-IE minigenes, the corresponding same hybrid constructs mutated at 5'ss (FIX-2A>C Mut5'), FIX-ExSpeU1 9-21. Exons (boxes), intronic sequences (lines), mutants and ExSpeU1s are indicated. b) Upper figures represent the agarose gel and the lower graph the percentage of exon inclusion. Identity of the exon inclusion and skipping bands is indicated. Data are expressed as means ± SD of three independent experiments done in duplicate. Statistical analysis was performed using Student's t-test (***: P < 0.001; **: P < 0.01; *: P < 0.05; ns: not significant).*

3.1.4. **The ExSpeU1-IE effect is modulated by the strength of the 5'ss**

As the strength of the donor sites contribute to exon definition, I decided to evaluate their role in relation to the ExSpeU1-IE.

For this reason, I prepared 24FAN-93FIX minigene derivatives with different 5'ss sequences. Based on standard bioinformatic tool that measures the donor site score (Reese, 1997), I created additional 24FAN-93FIX minigenes. In WT, the 5'ss score was 0.21 and in the 5'ss mutant it was 0, thus I created one with an improved score of 0.61 (Improved) and one with a perfect consensus sequence with a score of 1 (Canonical, Fig.24a). The splicing pattern of these minigenes was analysed in basal conditions or after co-transfection with ExSpeU1.

As detected before, the 24FAN-93FIX WT and Mut 5'ss minigenes did not respond to ExSpeU1. Improvement of the score to 0.61 did not rescue splicing in basal conditions but after co-transfection of ExSpeU1 inclusion it increased to 40% (Fig. 24b, lane 6). The minigene with the canonical 5'ss showed full inclusion even in the absence of the ExSpeU1 (Fig.24b lane 7-8).

This result clearly indicates that the strength of the 5'ss an important element involved in the ExSpeU1 rescue and that the negative effect of the ExSpeU1-IE element can be counteracted by improving the strength of the donor site. In the presence of a consensus 5'ss, the strong silencing activity of ExSpeU1-IE is completely abolished.

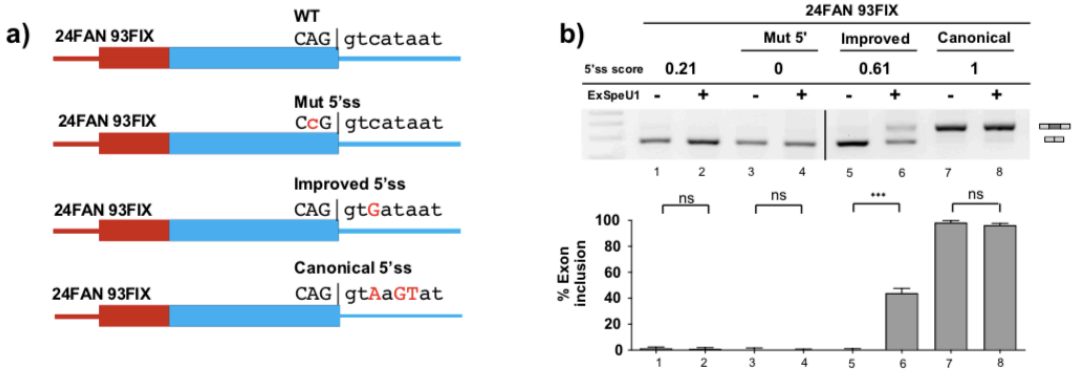

*Figure 24: Donor site contribution in modulating ExSpeU1 response. a) Schematic representation of 24FAN-93FIX, 24FAN-93FIX Mut5',24FAN-93FIX 5' Improved, 24FAN-93FIX 5' Canonical minigenes and ExSpeU1-FIXsh9. Exons (grey boxes), intronic sequences (lines), mutants and ExSpeU1s are indicated. b) Upper figures represent the agarose gel and the lower graph the percentage of exon inclusion. Identity of the exon inclusion and skipping bands is indicated. Data are expressed as means ± SD of three independent experiments done in duplicate. Statistical analysis was performed using Student's t-test (***: P < 0.001; **: P < 0.01; *: P < 0.05; ns: not significant).*

3.1.5. **Deletion of the ExSpeU1-IE in FANCA restores the ExSpeU1 response in both donor and acceptor sites mutants**

To further evaluate the inhibitory role of the ExSpeU1-IE on ExSpeU1, I performed its deletion in the original FANCA contexts. I created 83FAN minigene derivatives with no mutations (WT) or with mutations at the 5'ss (Mut 5'ss) or at the 3'ss (Mut 3'ss, Fig. 25a). The resulting minigenes were transfected alone or with the ExSpeU1. In the WT context, the ExSpeU1-IE deletion induced complete exon inclusion improving from ~80% to 100%, and this was not affected by the ExSpeU1 (Fig. 25b). Interestingly in both the 5'ss and 3'ss mutants the ExSpeU1-IE deletion improved significantly the exon inclusion (Fig. 25 c-d, lanes 3-4). More specifically, mutant in the 3' acceptor site showed total skipping that was not rescued by ExSpeU1-mediated strategy (previous Fig. 20c lane 5-6 and Fig. 25d lane 1-2). Here, ExSpeU1-IE deletion increased only 50% inclusion (Fig. 25d lane 3), but in this context the co-transfection of ExSpeU1 triggered full splicing rescue (Fig. 25d, lane 4).

Taken together, these results indicate that ExSpeU1-IE is a strong ESS regulatory element also in the native FANCA context. Its presence strongly influences the FANCA exon definition and the ExSpeU1 response in both mutants at the donor and acceptor sites.

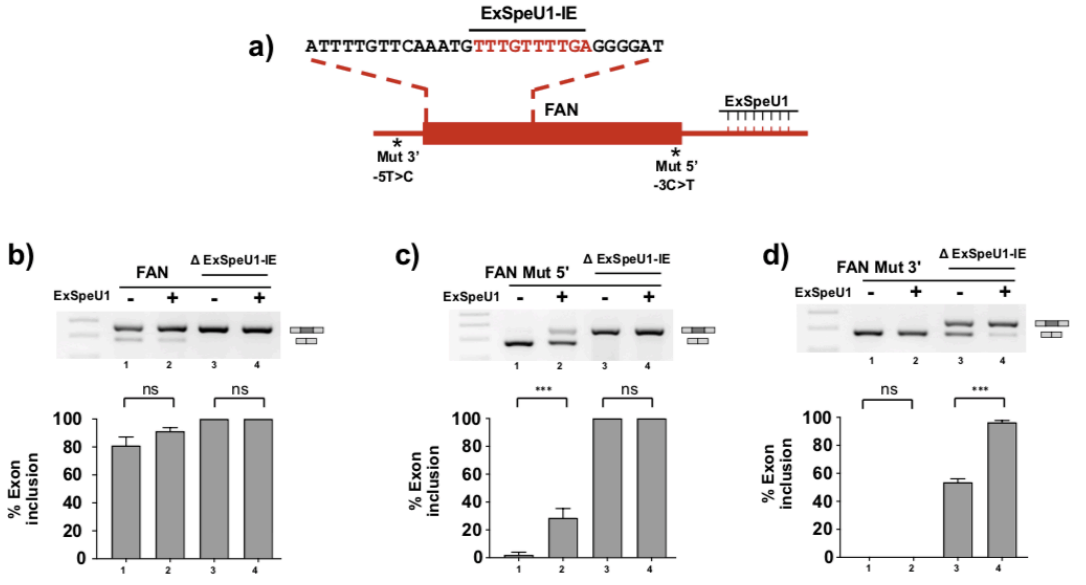

*Figure 25: ExSpeU1-IE as a strong ESS reduces ExSpeU1 activity in FANCA. a) Schematic representation of FAN, FAN Δ ExSpeU1-IE minigenes, the corresponding same mutants at 5'ss and at 3'ss and FAN-ExSpeU1 10-27. Exons (boxes), intronic sequences (lines), mutants (*) and ExSpeU1s are indicated. b), c), d) Upper figures represent the agarose gel and the lower graph the percentage of exon inclusion. Identity of the exon inclusion and skipping bands is indicated. Data are expressed as means ± SD of three independent experiments done in duplicate. Statistical analysis was performed using Student's t-test (***: P < 0.001; **: P < 0.01; *: P < 0.05; ns: not significant).*

3.1.6. **ExSpeU1-IE insertion in Factor IX inhibits the ExSpeU1 rescue**

To further understand the role of the ExSpeU1-IE I inserted this element in the context of FIX minigene. The ExSpeU1-IE was inserted in proximity of the acceptor site, in a position similar to that present in the FANCA exon in the WT context, in the 5'ss

mutant on in the 3'ss mutant (Fig.26a). The insertion of the ExSpeU1-IE in the FIX context had a strong inhibitory effect on the ExSpeU1.

In the WT minigene, the insertion induced complete exon skipping (Fig. 26b, compare lanes 1 and 6) and ExSpeU1 transfection had a modest effect increasing the percentage of exon inclusion to ~45% (Fig. 26b lane 7). In the mutant minigenes, the insertion of ExSpeU1-IE blocked the response to the ExSpeU1 (Fig.26b lane 8-11).

This result indicates that the inhibitory effect of the ExSpeU1-IE can be transferred to other exons and acts on mutations that affect both splice sites.

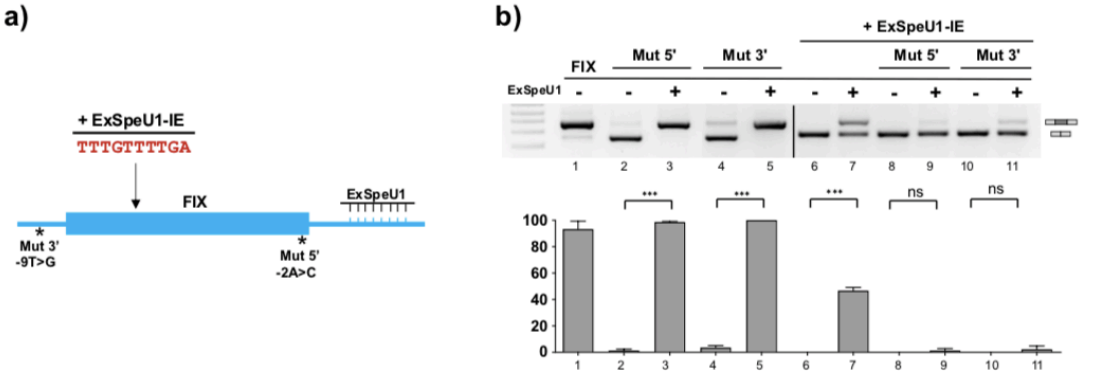

*Figure 26: ExSpeU1-IE modulate ExSpeU1 rescue in Factor IX. a) Schematic representation of FIX and FIX+ExSpeU1-IE minigenes, the corresponding same mutants at 5'ss and at 3'ss and FIX-ExSpeU1 9-21. Exons (boxes), intronic sequences (lines), mutants (*) and ExSpeU1s are indicated. b) Upper figures represent the agarose gel and the lower graph the percentage of exon inclusion. Identity of the exon inclusion and skipping bands is indicated. Data are expressed as means ± SD of three independent experiments done in duplicate. Statistical analysis was performed using Student's t-test (***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$; ns: not significant).*

3.1.7. **Splicing regulation mediated by splicing factors is sensitive to ExSpeU1-Inhibitory Element**

To identify splicing factors potentially involved in the lack of ExSpeU1 rescue activity mediated by the ExSpeU1-IE, I performed over-expression experiments with a panel of splicing factors.

Previous studies on FIX exon 5 have shown that most of the ExSpeU1 rescue activity is dependent on SC35 (Tajnik et al., 2016). Indeed, transfection of SC35 induced a significant increase in the percentage of exon 5 inclusion in the Mut 5'ss minigene (Fig.27 a lane 5/17). As previously reported PTB1, PTB4, TIA1 were also involved even with a less pronounced effect in the 5'ss mutant context, together with SRP55, SRP75 and TRA2B (Fig.27 a). On the other hand, SRP20, HNRPA1 and ETR-3 has a negative impact on splicing inclusion in WT (Fig.27 a, lane 2, 9, 12).

Thus, I tested these splicing factors on two hybrid minigenes that, due to the presence of the ExSpeU1-IE have a different response to ExSpeU1: 15FAN-108FIX that does not have the ExSpeU1-IE and 24FAN 93FIX that contains the ExSpeU1-IE.

In the WT contexts, 15FAN-108FIX, but not 24FAN-93FIX (Fig. 27c), responded to SC35, with minor effect by PTB4 (Fig. 27 b, lane 5,10,11).

However, in the 5'ss mutated context, no splicing factor was able to increase the percentage of exon inclusion (Fig. 27b, lane 14-24).

Taken together, these results suggest that in the particular context of the FANCA-FIX hybrid minigenes, the ExSpeU1-IE might negatively affect the ExSpeU1 rescue through SC35.

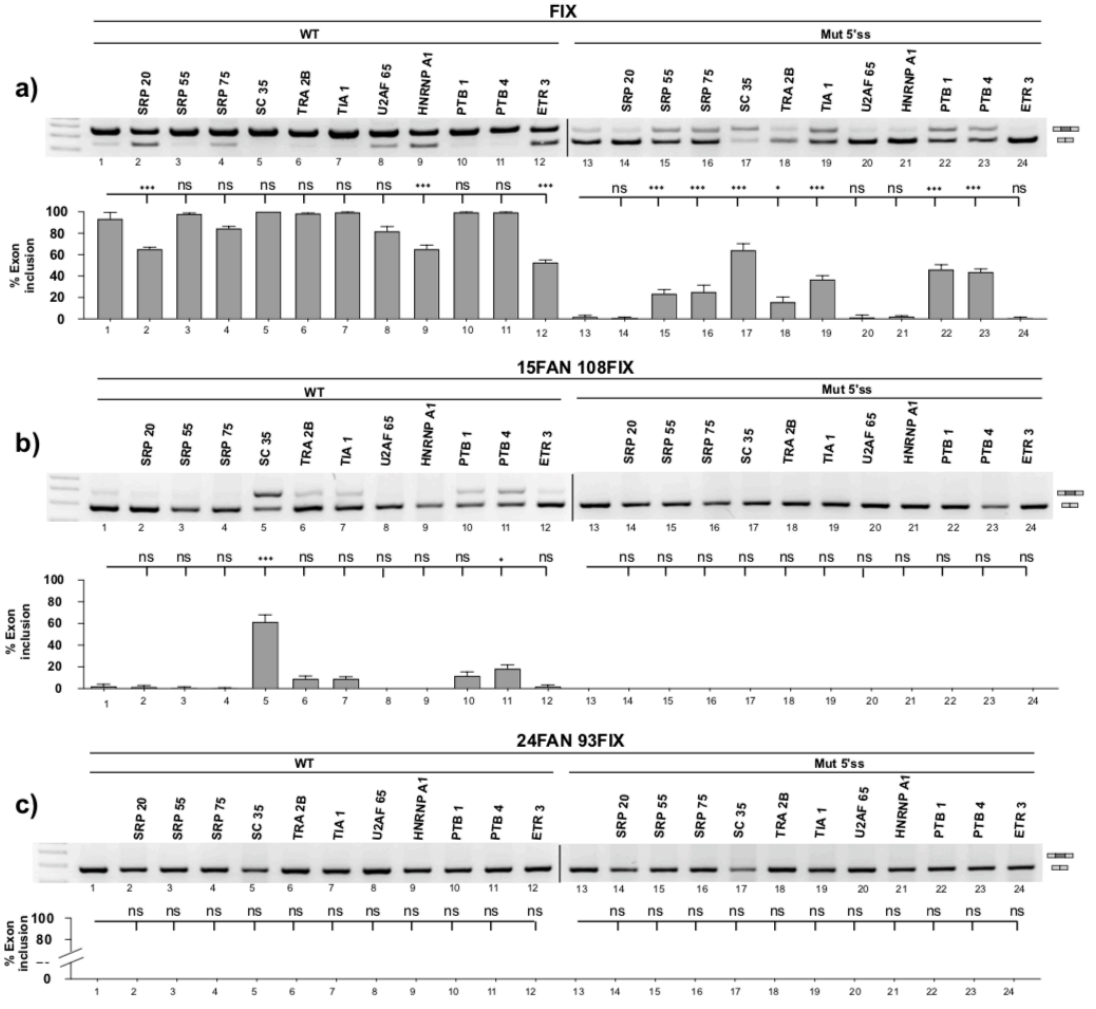

*Figure 27: Splicing factors overexpression in hybrid minigenes. a) FIX, b) 15FAN 108FIX, c) 24FAN 93FIX, both WT and 5'ss Mut minigenes were used. a) and b) Upper figures represent the agarose gel and the lower graph the percentage of exon inclusion. Identity of the exon inclusion and skipping bands is indicated. c), d) , e) agarose gel. Data are expressed as means ± SD of three independent experiments done in duplicate. Statistical analysis was performed using Student's t-test (***: P < 0.001; **: P < 0.01; *: P < 0.05; ns: not significant).*

3.1.8. **Splicing factors overexpression in presence and absence of ExSpeU1-IE in the FANCA context**

To further clarify the potential effect of the ExSpeU1-IE on the ExSpeU1 response and the involvement of splicing factors in a different context I performed co-transfection experiments of splicing factors in the FANCA minigenes. I considered the WT and 5'ss mutant with or without deletion of the ExSpeU1-IE (Fig. 28).

In the WT condition (~80% inclusion), TRA2B was the most active splicing factor to promote exon inclusion (Fig.28a, lane 6). On the other hand, SRP20, SRP55, SRP75, SC35, U2AF65 and hnRNPA1 showed instead a negative effect reducing the percentage of exon inclusion (ranging from 35% to 55%, Fig.28a lane 2, 3, 4, 5, 8, 9). In 5'ss mutant, TRA2B was to only SF to display a trend for enhancing effect increasing the percentage of exon inclusion to ~10% (Fig.28a, lane 18).

The ExSpeU1-IE deletion in the WT context induced complete exon inclusion that was not affected by co-transfection of the splicing factors (Fig.28b, lanes 1-12). Deletion of ExSpeU1-IE in the 5'ss mutant induced nearly complete exon inclusion in basal condition whereas SRP20, SRP55, SRP75, SC35, U2AF65 and hnRNPA1 co-transfection reduced the percentage of exon inclusion (Fig. 28b, lane 14, 15, 16, 17, 20, 21).

Taken these results together, I hypothesize that in the FANCA context, the ExSpeU1-IE might inhibit the activity of TRA2B and/or reinforce the activity of silencing splicing factors just described.

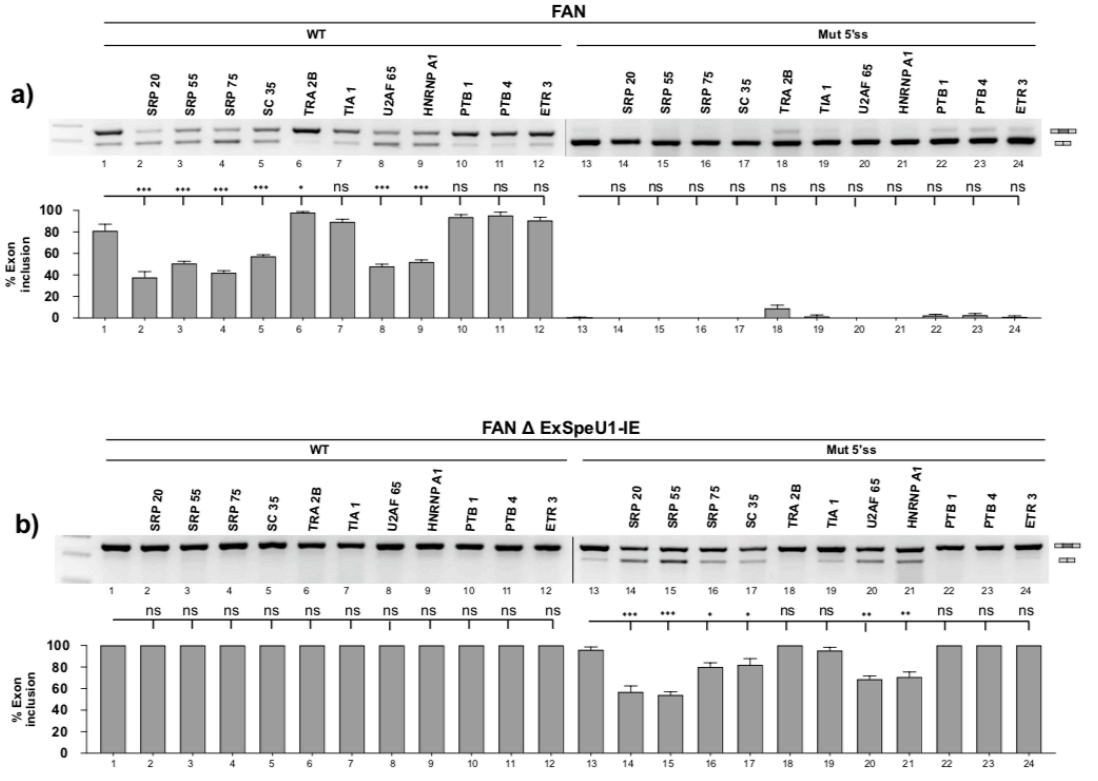

*Figure 28: Splicing factors overexpression in FANCA context. a) FAN and b) FAN ΔExSpeU1-IE minigenes and the corresponding constructs mutated at 5'ss (FIX-Mut 5'). Exons (grey boxes), intronic sequences (lines), mutants and ExSpeU1s are indicated. b) and c) Upper figures represent the agarose gel and the lower graph the percentage of exon inclusion. Identity of the exon inclusion and skipping bands is indicated. Data are expressed as means ± SD of three independent experiments done in duplicate. Statistical analysis was performed using Student's t-test (***: P < 0.001; **: P < 0.01; *: P < 0.05; ns: not significant).*

3.2. **Applications of ExSpeU1 strategy in Cystic Fibrosis**

To assess the therapeutic potential of ExSpeU1s in a specific disease, I focussed on Cystic Fibrosis (CF). I analysed 10 different pathological mutations along the CFTR gene. These splicing mutations were selected based on their relative high frequency in CF, on exon coverage and on the splicing regulatory elements affected (Table 2). Considering the allele frequency in CF reported in the CFTR2 database (www.cftr2.org), 2789+5G>A is the most recurrent mutation (1027 alleles reported), followed by 3120G>A (p.Gln996=), 711+3A>G and 711+5G>A (85, 63 and 60 alleles, respectively) and 1898+3A>G (27 alleles) while 711+3A>C and 1863C>T (p.Y577Y) are rare mutations. Furthermore, I considered TG12T5 and TG13T5 associated with atypical CF, and TG13T3 linked to classical CF.

Exon	Mutant legacy name	cDNA name	Splicing regulatory element	Number of Alleles
5	711+3A>C	c.579+3A>C	5' splice site	Rare
	711+3A>G	c.579+3A>G	5' splice site	63
	711+5G>A	c.579+5G>A	5' splice site	60
10	TG13T3	-	Polypyrimidine tract	Rare
	TG13T5	-	Polypyrimidine tract	Common in CBAVD
	TG12T5	-	Polypyrimidine tract	Common in CBAVD
13	1863C>T	c.1731C>T	Exonic	Rare
	1898+3A>G	c.1766+3A>G	5' splice site	27
16	2789+5G>A	c.2657+5G>A	5' splice site	1027
18	3120G>A	c.2988G>A	5' splice site	85

Table 2: CFTR splicing mutations investigated, relative exon and number of alleles reported in CFTR2 database. CBAVD: Congenital Absence of Vas deference. Number of total alleles from CFTR2 database: 142'036.

3.2.1. **Rescue of 711+5G>A, 711+3A>G and 711+3A>C mutations that cause CFTR Exon 5 skipping**

In CFTR exon 5, I considered three intronic mutations that affect the 5'ss, 711+5G>A, 711+3A>G and 711+3A>C.

To study these mutations, I synthesised a WT minigene with CFTR exon 5 along with the corresponding variants. In parallel, I created a panel of ExSpeU1s that bind in intron 5 at different intronic positions downstream the donor site, U1ex5-9, U1ex5-13, U1ex5-21 and U1ex5-37 bp. These ExSpeU1s have a 9bp tail and span the intron 5 from position 9 to 37. Splicing assay showed that the WT minigene was completely included (Fig. 29, lane 1), the two mutations, 711+3A>C and 711+5G>A, induced complete exon 5 skipping (Fig. 29, lanes 2 and 4) whereas 711+3A>G maintained a small amount of exon inclusion (~18%, Fig. 29, lane 3).

Co-transfection of the four ExSpeU1s induced complete rescue of the three exon 5 splicing defects (Fig. 29, lane 5-16). The only exception was observed for U1ex5-21 and U1ex5-37 on 711+3A>C: these two ExSpeU1 showed a partial recover of splicing on this variant (~50%) (Fig. 29 lane 7, 8).

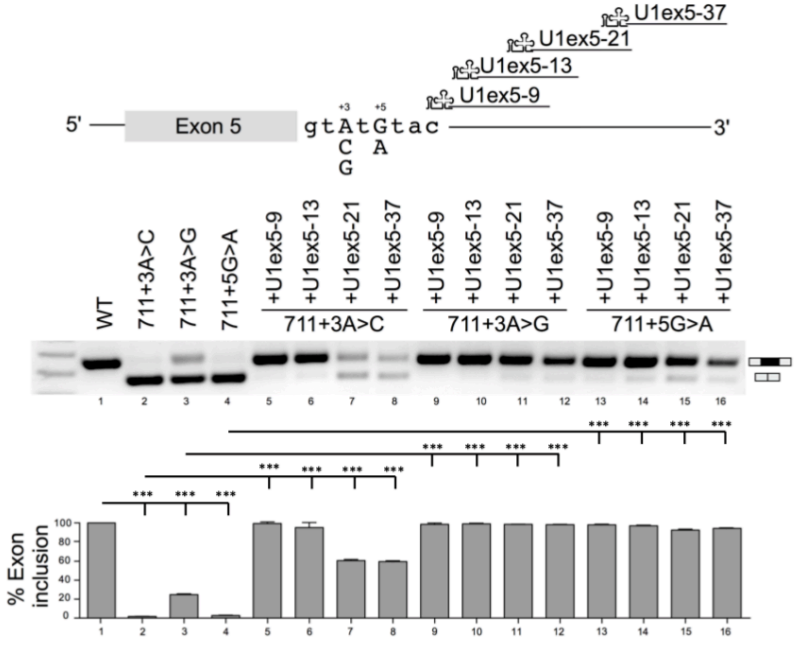

*Figure 29: Rescue of 711+3A>C/G, 711+5G>A mutants in CFTR Exon 5 donor site. Upper panel represents: I) CFTR exon 5 minigenes (pTB-CFex5): WT, 711+3A>C/G (c.579+3A>C/G) , 711+5G>A (c.579+5G>A) and II) ExspeU1s: U1ex5-9, U1ex5-13, U1ex5-21, U1ex5-37. CFTR exons (grey boxes), intronic sequences (lines), mutants and ExSpeU1s are indicated. Intermediate figure represent the agarose gel and the lower graph the percentage of exon inclusion. Identity of the exon inclusion and skipping bands is indicated. Data are expressed as means ± SD of three independent experiments done in duplicate. Statistical analysis was performed using Student's t-test (***: P < 0.001; **: P < 0.01; *: P < 0.05; ns: not significant).*

3.2.2. **ExSpeU1 strategy applied to 3'ss variants of *CFTR* Exon 10**

A short region upstream the acceptor site of CFTR exon 10 is characterised by a polypyrimidine tract in which longer TG repeats - 12 or 13 - are associated with disease phenotypes (Groman et al., 2004; Zuccato et al., 2004). I investigated the rescue of the following TG(m)T(n) pathological variants: TG12T5, TG13T5 and TG13T3. To rescue pathological aberrant splicing of CFTR exon 10, I have synthetized four ExSpeU1 that bind in intron 10 in position 12, 26 and 34 and one complementary to the donor site, called U1ex10(-3).

As previously reported, (Groman et al., 2004; Zuccato et al., 2004) in basal condition, the minigene TG11T7 showed 98% of exon inclusion and the three variants were characterised by a different exon inclusion range: TG12T5 with ~61%, TG13T5 ~54% and TG13T3 ~6% (Fig. 30, lane 2-4). In TG12T5 and TG13T5, co-transfection of U1ex10(-3), U1ex10-12, U1ex10-26 and U1ex10-34 totally rescued aberrant splicing (Fig. 30, lane 5-12). In TG13T3 context U1ex10(-3), U1ex10-12, U1ex10-26 rescued splicing up to ~72-84% of exon inclusion., U1ex10-34 had a lower effect displaying ~51% of exon inclusion (Fig. 30, lane 16).

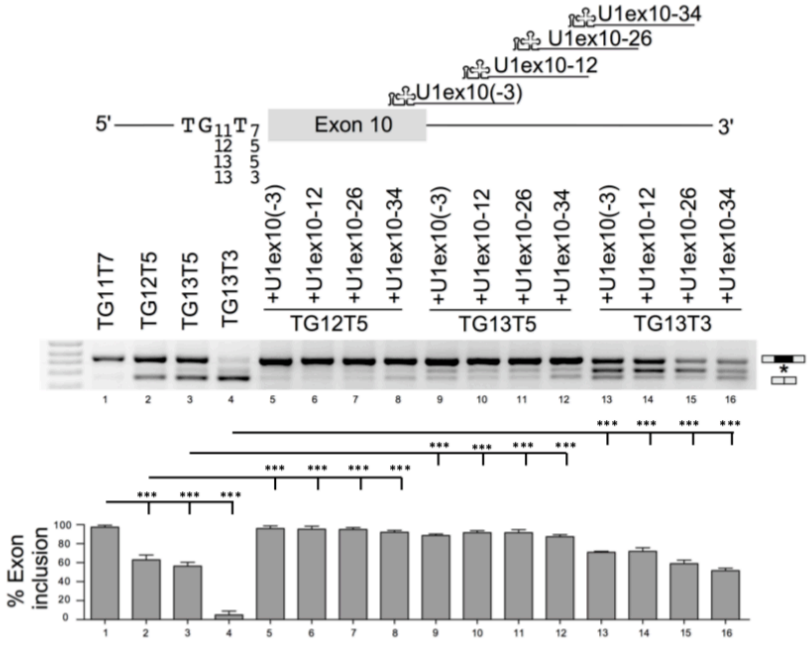

Figure 30: ExSpeU1 strategy applied to the polypyrimidine tract of CFTR Exon 10. Upper panel represents: I) CFTR exon 10 minigenes (pTB-CFex10): TG11T7, TG13T3, TG13T5 and TG12T5 variants and II) ExspeU1s: U1ex10(-3), U1ex10-12, U1ex10-26, U1ex10-34. CFTR exons (grey boxes), intronic sequences (lines), mutants and ExSpeU1s are indicated. Intermediate figure represent the agarose gel and the lower graph the percentage of exon inclusion. Identity of the exon inclusion and skipping bands is indicated. The band indicated with an asterisk corresponds to the activation of a previously described cryptic splice site (Buratti et al., 2001). *Data are expressed as means ± SD of three independent experiments done in duplicate. Statistical analysis was performed using Student's t-test (***: P < 0.001; **: P < 0.01; *: P < 0.05; ns: not significant).*

3.2.3. **U1ex13-11 is able to rescue the exonic and 5'ss exon 13 mutants**

In the context of CFTR exon 13, I investigated the exonic variant 1863C>T (synonymous Y577Y) and the 1898+3A>G mutation. To rescue splicing, I tested five modified U1s: U1ex13-1, U1ex13-9, U1ex13-11, U1ex13-15 and U1ex13-33. Whereas the WT minigene showed ~ 80% of exon inclusion, these two mutants induced a severe splicing defect (Fig. 31, lane 2, 3). U1ex13-1 and U1ex13-9 partially increased to 25-30% exon inclusion in the 1863C>T (Fig. 31 lane 4, 5), and had no effect on 1898+3A>G (Fig. 31 lane 9, 10). Similarly, U1ex13-15 and U1ex13-33 showed no effect on both mutants (Fig. 31 lane 7, 8, 12, 13). The only ExSpeU1 that was able to rescue the splicing defects was U1ex13-11 reaching ~ 85% of exon 13 inclusion (Fig. 31 lane 6, 11).

These results suggest that *CFTR* exon 13 context has some peculiarity that restricts the ExSpeU1 splicing capacity.

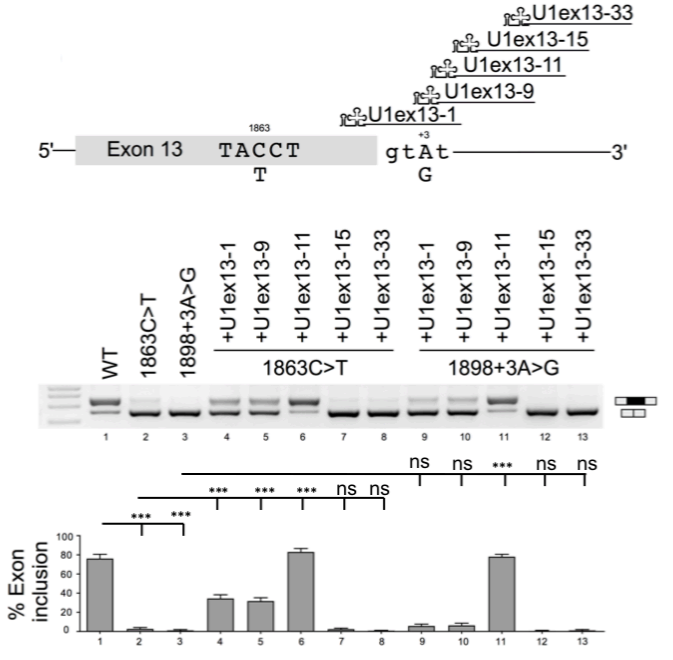

*Figure 31: U1ex13-11 is able to rescue the exonic 1863C>T and 5'ss 1898+3A>G mutants to WT level. Upper panel represents: I) CFTR exon 13 minigenes (pTB-CFex13): WT, 1863C>T (p.Y577Y or c.1731C>T), 1898+3A>G (c.1766+3A>G) and II) ExspeU1s: U1ex13-1, U1ex13-9, U1ex13-11, U1ex13-15 and U1ex13-33. CFTR exons (grey boxes), intronic sequences (lines), mutants and ExSpeU1s are indicated. Intermediate figure represent the agarose gel and the lower graph the percentage of exon inclusion. Identity of the exon inclusion and skipping bands is indicated. Data are expressed as means ± SD of three independent experiments done in duplicate. Statistical analysis was performed using Student's t-test (***: P < 0.001; **: P < 0.01; *: P < 0.05; ns: not significant).*

3.2.4. **ExSpeU1s fully corrects the CFTR Exon 16 donor site mutation**

Located at the donor site of exon 16, the 2789+5G>A is a type V mutation that alters chloride channel functional proteins at the cell surface, associated by a mild phenotype and aberrant splicing.

I investigated its splicing rescue by ExSpeU1s, by inserting Exon 16 and relative flanking intronic regions in a minigene system.

Compared to the WT construct that showed complete exon inclusion, 2789+5G>A induced ~ 50 % of exon inclusion. A total of five ExSpeU1s were tested, spanning from position +7 to +17, including an engineered U1 designed to bind complementary o the donor site, called U1ex16(-3).

In this context, the entire panel of engineered U1s were able to completely correct the splicing defect (Fig. 32 lane 1-7).

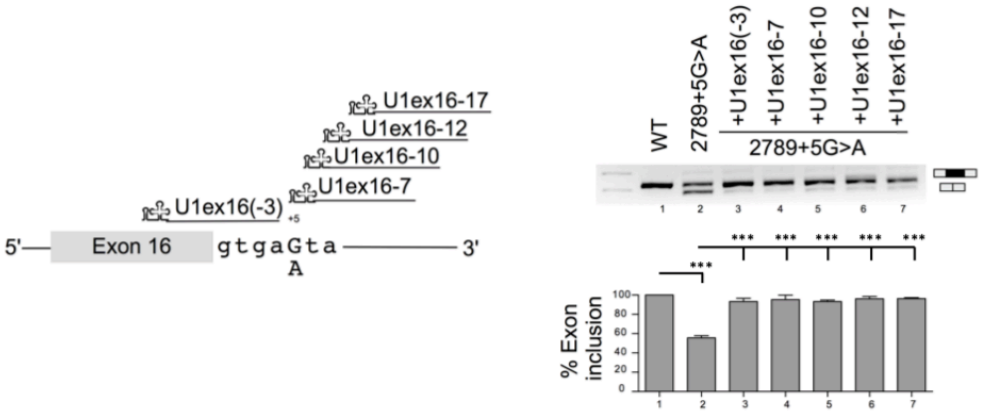

*Figure 32: A panel of ExSpeU1s fully corrects 2789+5G>A mutant at the CFTR Exon 16 donor site. Upper panel represents: I) CFTR exon 16 minigenes (pcDNA5-CFex16): WT, 2789+5G>A (c.2657+5G>A) and II) ExspeU1s: U1ex16-7, U1ex16-10, U1ex16-12, U1ex16-17. CFTR exons (grey boxes), intronic sequences (lines), mutants and ExSpeU1s are indicated. Intermediate figure represent the agarose gel and the lower graph the percentage of exon inclusion. Identity of the exon inclusion and skipping bands is indicated. Data are expressed as means ± SD of three independent experiments done in duplicate. Statistical analysis was performed using Student's t-test (***: P < 0.001; **: P < 0.01; *: P < 0.05; ns: not significant).*

3.2.5. **3120G>A in CFTR exon 18 is nearly fully rescued by ExSpeU1s**

Among the CF mutations identified in exon 18, one of these involves its donor site, with a substitution in the last nucleotide of the corresponding exon (3120G>A), resulting in a mild CF form.

I studied this variant in a minigene system detecting that it induces complete exon 18 skipping compared to WT full inclusion (Fig. 33, lane 2).

I designed four ExSpeU1s binding in intron 18, downstream of the donor site, in position 4, 10, 13 and 20. My results show an efficient splicing rescue to WT. Particularly, U1ex18-4 with U1ex18-20 induced ~85% of exon inclusion and U1ex18-10 with U1ex18-13 resulting in total inclusion (Fig. 33, lanes 3, 4, 5, 6).

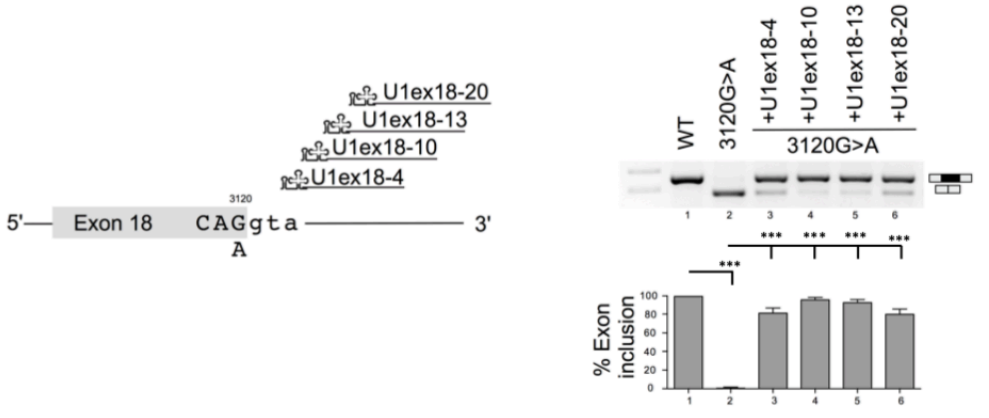

*Figure 33: Splicing pattern of 3120G>A mutant in the last bp of CFTR Exon 18 is almost fully rescued by a panel of ExSpeU1s. Left panel represents: I) CFTR exon 18 minigenes (pFAN-CFex18): WT and 3120 G>A (c.2988G>A) and II) ExspeU1s: U1ex18-4, U1ex18-10, U1ex18-13, U1ex18-20. The indicated WT and mutant minigenes were transfected in HeLa cells alone or co-transfected with the ExSpeU1s. CFTR exons (grey boxes), intronic sequences (lines), mutants and ExSpeU1s are indicated. Right panel figure represents the agarose gel and the graph with the percentage of exon inclusion. Identity of the exon inclusion and skipping bands is indicated. Data are expressed as means ± SD of three independent experiments done in duplicate. Statistical analysis was performed using Student's t-test (***: P < 0.001; **: P < 0.01; *: P < 0.05; ns: not significant).*

3.2.6. **ExSpeU1-mediated recover of CFTR protein levels in exon 13 and exon 16**

To establish a more direct link between the splicing and the CFTR protein rescue I focussed on CFTR exon 13 and 16. WT and mutant *CFTR* exons 13 minigenes were inserted into cDNA splicing competent minigenes that code for the entire *CFTR* protein (pcDNA3.1-CFex13). In these constructs, exon 13 was flanked by a short intronic regions (see Material and Methods for details). As well, positive CFTR cDNA control and negative control constructs lacking exon 13 were designed (Fig. 34).

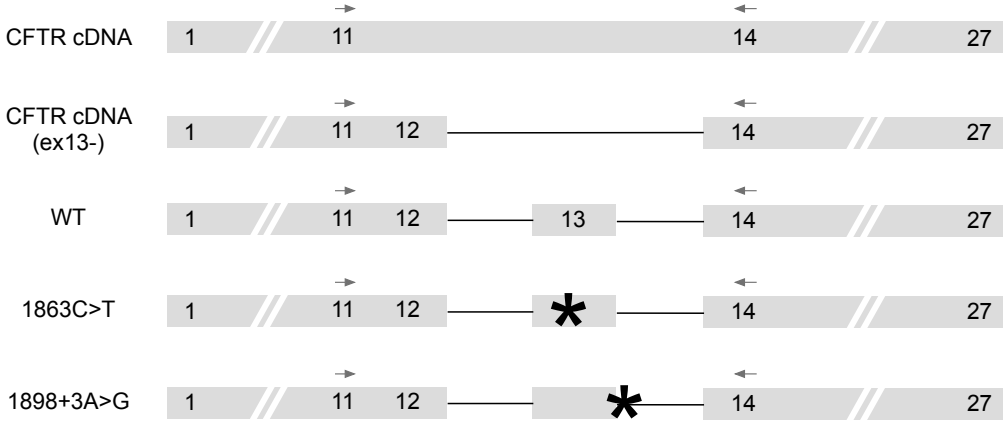

*Figure 34: Scheme of pcDNA3.1-CFex13 minigenes: CFTR cDNA, CFTR cDNA(-ex13), WT, 1863C>T and 1898+3A>G. * : mutations. Arrows: PCR primers.*

The minigenes were transfected alone or with the corresponding active ExSpeU1s followed by analysis of the splicing pattern and evaluation of the *CFTR* protein abundance by Western blotting (Fig. 35). Splicing analysis experiments of the pcDNA3.1-CFex13 constructs showed that two 1863C>T and 1898+3A>G mutants induced a severe splicing defect (Fig. 35b, lane 3, 4) as I have previously observed in the pTB-CFex13 minigene (Fig. 31 lane 2,3). Co-transfection of the ExSpeU1 rescued both mutants efficiently inducing in 1863C>T and 1898+3A>G with 40 % and 70%

of exon inclusion, respectively (Fig. 35b, lane 5, 6). In parallel, I also evaluated the CFTR protein levels by Western Blotting on the cellular extracts. The WT minigene, as expected, showed the mature CFTR form of 160 kDa (Fig. 35c lane 3). The size of this band corresponds to the band present in control full-length *CFTR* cDNA (Fig. 35c lane 1). Transfection of a minigene without the exon 13 sequences (CFTR cDNA ex13-) and of the two exon 13 mutants 1863C>T and 1898+3A>G did not present any detectable mature *CFTR* protein (Fig. 35c lanes 2, 4, 5). Co-transfection of U1ex13-11 produced the full-length fully glycosylated 160KD form of *CFTR* protein (Fig. 35c, lane 6, 7). Compared to the WT, CFTR protein was rescued at ~25% and 75% in 1898+3A>G and 1863C>T mutants, respectively. Thus, the exonic mutant showed a more pronounced increase in *CFTR* protein compared to the 5'ss variant mutants, which is consistent with the observed different splicing rescue efficiency.

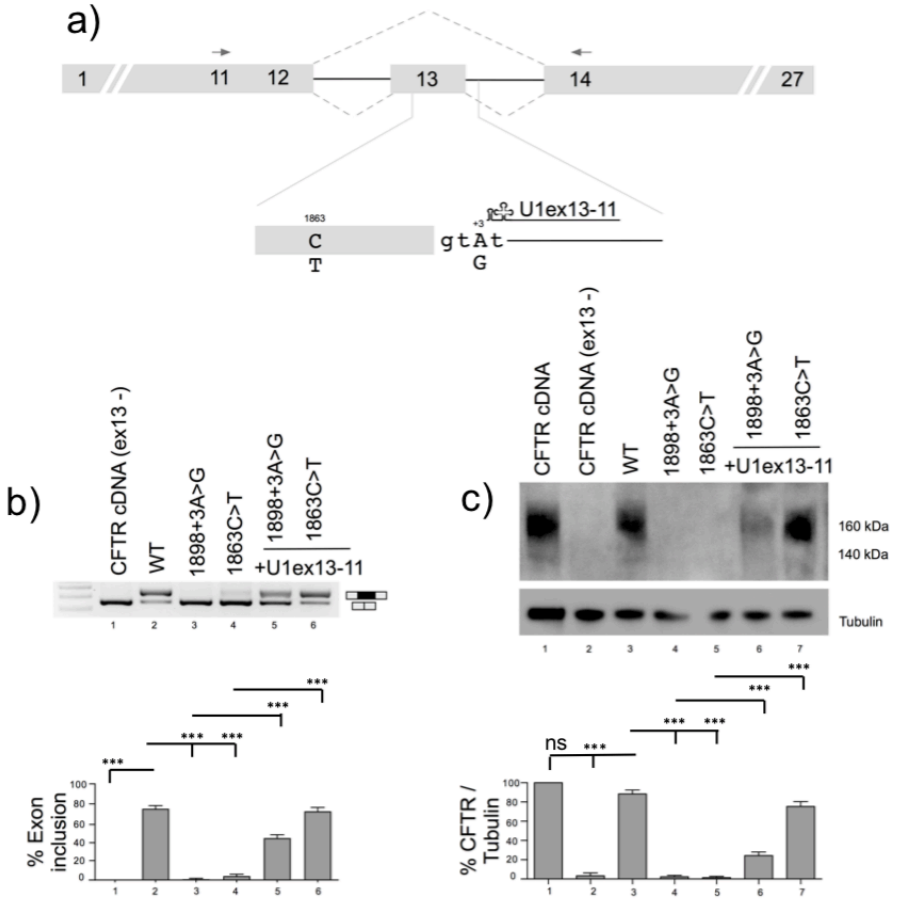

*Figure 35: Exon Specific U1s rescue CFTR protein levels in exon 13 cDNA mutant minigenes. 1863C>T corresponds to c.1731C>T or p.Y577Y mutant and 1898+3A>G corresponds to c.1766+3A>G mutant. CFTR cDNA (ex13-) is a pcDNA3.1-CFex13 minigene without exon 13. a) Schematic representation of pcDNA5-CFex13 minigene: exons (grey box), intronic sequences (lines), splicing pattern (dashed lines), primers (arrows) and ExSpeU1s are indicated. b) Minigene splicing assay: the upper figure shows the agarose gel and the lower panel the percentage of exon inclusion. Identity of the exon inclusion and skipping bands are indicated. c) Western Blot analysis: the graph represents the percentage of CFTR protein normalized over Tubulin. Data are expressed as means ± SD of two independent experiments. Statistical analysis was performed using Student's t-test (***: P < 0.001; **: P < 0.01; *: P < 0.05; ns: not significant).*

Analogously, to investigate the effect of ExSpeU1s on the CFTR protein in the presence of the 2789+5G>A mutation, WT and mutant CFTR exons 16 minigenes were inserted into cDNA splicing competent minigenes that code for the entire CFTR protein (pcDNA5-CFex16).

In this case, I have evaluated U1ex16(-3), U1ex16-7 and U1ex16-12, three active ExSpeU1s shown in Figure 36. These ExSpeU1s fully rescued to WT levels the mutated minigene in the splicing analysis experiments (Fig. 36b).

Consistent with a residual splicing activity in splicing assay, WB analysis detected a reduced amount of CFTR mature form in the mutated minigene, compared to the WT minigene (Fig. 36c, compare lanes 1 and 2).

The results showed that the complete recover of the splicing defect (Fig. 36b, lane 3-5) induced a two-fold increase in the amount of mature CFTR protein of 160kDa (Fig. 36c, lanes 3, 4 and 5).

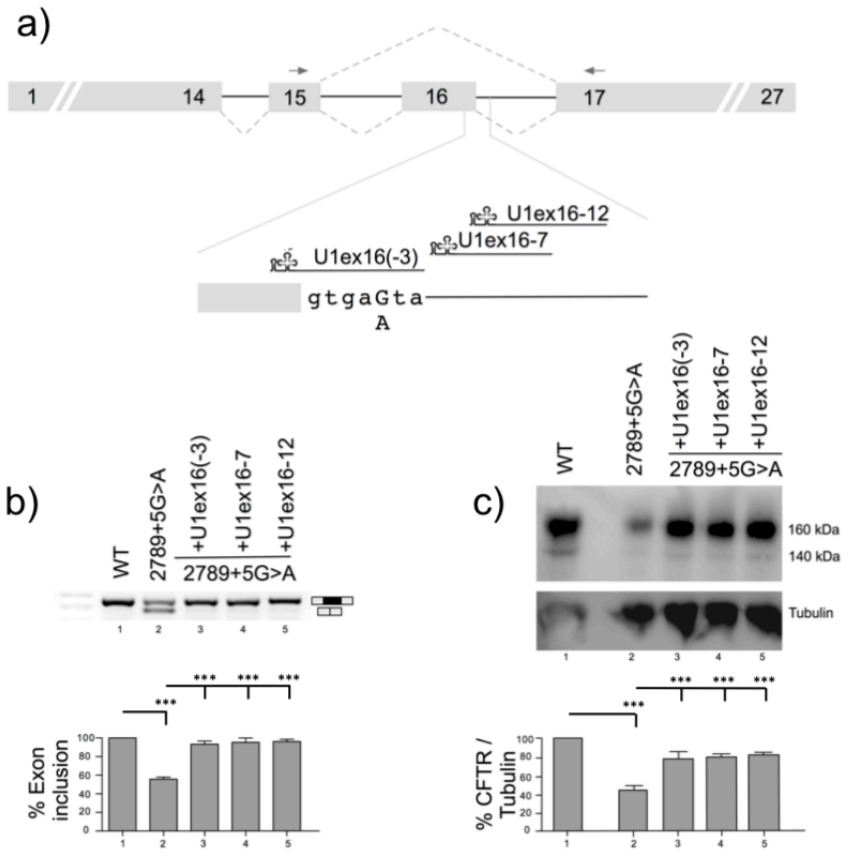

*Figure 36: Exon Specific U1s rescue CFTR protein levels in exon 16 cDNA mutant minigenes. a) Schematic representation of pcDNA5-CFex16 minigene: exons (grey boxes), intronic sequences (lines), splicing pattern (dashed lines), primers (arrows) and position of the ExSpeU1s is indicated. b) Minigene splicing assay: the upper figure shows the agarose gel and the lower panel the percentage of exon inclusion. Identity of the exon inclusion and skipping band are indicated. Data are expressed as means ± SD of two independent experiments. c) Western Blot analysis: the graph represents the percentage of CFTR protein normalized over Tubulin. 2789+5G>A corresponds to c.2657+5G>A mutant. Statistical analysis was performed using Student's t-test (***: P < 0.001; **: P < 0.01; *: P < 0.05; ns: not significant).*

4. Discussion

4.1. Identification of an ExSpeU1 Inhibitory Element (ExSpeU1-IE) that affects exon definition

In the so called "exon definition" process, as originally proposed (Robberson et al., 1990), the spliceosome identifies the correct sequences on pre-mRNA that will be included in the final transcript through a series of complex interactions that occur co-transcriptionally. Typically, the strength of an exon and thus its probability of being included in mature mRNA, depends on the composition of the splice sites (how they conform to consensus sequences) and on a series of less defined, but functionally relevant, exonic and intronic enhancer and silencer *cis*-acting elements. In the presence of disease-causing splicing mutations that reduce, but not completely abolish the exon definition (i.e., defective exons), one interesting and novel therapeutic strategy is the use of ExSpeU1s. ExSpeU1s bind in proximity of the 5'ss and promote exon definition mainly through its specific 70K SR-like protein. This protein promotes the formation of the network of interactions among splicing factors between the splice sites that define the exon (Fig. 37).

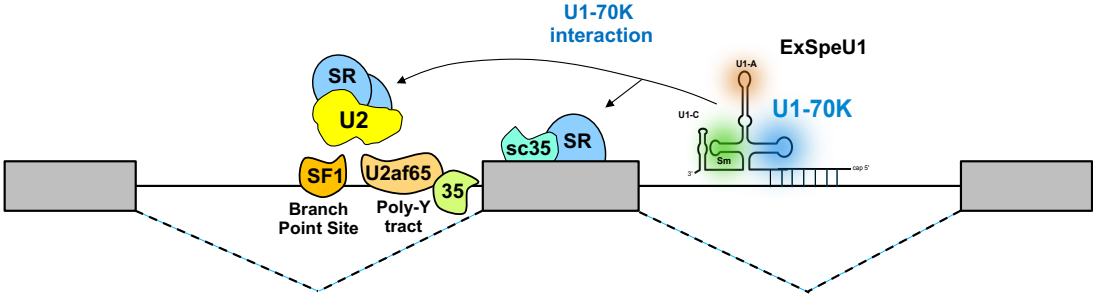

Fig. 37: Scheme of interactive network among splice sites and splicing factors promoting the definition of the exon though interaction of ExSpeU1 and U1-70K protein. Colours represent different SFs; Grey boxes represent exons.

In the development of these novel class of small RNAs an important aspect that have to be considered is to clarify the contribution of the different *cis*-acting elements involved and most important to establish the determinants that transform a “defective exon” in a "not-rescuable" one. To clarify this aspect, I focussed on two clear cases characterised by no response or good response to ExSpeU1, *FANCA* exon 8 and *Factor IX* exon 5, respectively. Disease causing mutations at the donor or acceptor sites induce exon skipping in both cases, but ExSpeU1s are active only on *Factor IX* (Fig. 20b). To clarify this different behaviour, I decided, using the minigene approach, to shuffle *FANCA* exon 8 and *Factor IX* exon 5 sequences creating a series of chimeric constructs that I tested for their response to ExSpeU1. My analysis identified a critical UG-rich sequence in position 15- 24 of FANCA exon, the ExSpeU1 Inhibitory Element (ExSpeU1-IE), that has a strong inhibitory effect on ExSpeU1 activity. This inhibitory function was initially identified in the shuffling experiments when I observed that the 15FAN-108FIX and 24FAN-93FIX showed a striking different response to ExSpeU1 for both the WT and 5’ss mut constructs (Fig. 22). In 24FAN-93FIX minigene, the ExSpeU1-IE function was also evident in site-directed mutagenesis experiments, thus excluding that the deletion present in the chimeric minigenes might have fortuitously created a novel regulatory element or that the size of the exon might have a role (Fig. 23). The important role of ExSpeU1-IE in mediating the activation of the defective exons was also observed when this element was deleted in the native context: indeed, in the FANCA context its deletion was sufficient to correct the aberrant splicing (Fig. 25a). A further evidence comes from the experiments when I introduced the ExSpeU1-IE in the FIX context (Fig. 26): the lack of response to ExSpeU1 in the FIX exon with the ExSpeU1-IE sequences indicates that the inhibitory effect can be transferred to other contexts and is not FANCA exon 8-specific.

The ExSpeU1-IE element is strictly dependent on the strength of the donor site. In fact, improvement in the 5'ss score by site-directed mutagenesis progressively reduce the silencing activity of the ExSpeU1-IE: indeed, the presence of a canonical 5’ss consensus sequence completely abolishes its inhibitory function (Fig. 24). These

results are consistent with previous evidence in other contexts like the SMN2 exon 7 where the exon skipping phenotype is completely recovered by 5'ss improvement (N. N. Singh et al., 2017; R. N. Singh & Singh, 2019).

In an attempt to identify the possible mechanism for ExSpeU1-IE I tested the response of different minigenes with or without the ExSpeU1-IE element to key splicing factors. Overexpression experiment in the defective FIX minigene showed that SC35, also known as SRSF2 or SRp30c is the main splicing factor involved in promoting exon inclusion (Fig. 27a). It belongs to serine/arginine (SR)-rich family, interacting during spliceosome catalytic reaction with its RNA recognition motif (RRM) for binding RNA and an RS domain for binding other proteins. SC35 was also the most relevant enhancing splicing factor in the FAN/FIX hybrid minigene (15FAN108FIX) that does not have the ExSpeU1-IE element. Indeed, SC35 was previously suggested to mediate on the WT FIX exon the ExSpeU1 rescue (Tajnik et al., 2016). Interestingly, in 24FAN93FIX minigene that contains the ExSpeU1-IE element, SC35 was no more able to promote exon inclusion (Fig. 27c, lane 5). Thus, I speculate that part of the ExSpeU1-IE inhibitory activity might be related to this splicing factor. ExSpeU1-IE might block the interaction of SC35 with the 70K protein present on ExpSpeU1. However, probably this mechanism might not be involved in FANCA where SC35 does not play as an enhancing factor. Co-transfection experiments, in normal FANCA, suggest that TRA2B has a major role in promoting exon inclusion (Fig 28). Thus, it is possible that the SC35 mechanism present in FIX is substituted by TRA2B in FANCA. On the other hand, as in the FANCA minigene several splicing factors (including SC35) displayed a negative role, I cannot exclude a direct effect of the ExSpeU1-IE on these splicing factors (Fig. 28).

Future studies are required to better clarify how the exonic context modulate the interactions between the ExSpeU1-IE cis-acting element and the ExSpeU1 snRNP at the molecular level.

4.2. **Therapeutic approach based on ExSpeU1s to correct a panel of CF-causing splicing mutations**

Correction of local CF basic defects has been the main goal in the last years for CF research in developing personalised medicine based on specific mutations belonging to well-known CF classes (De Boeck & Amaral, 2016; Fajac & Wainwright, 2017; Farinha & Matos, 2016; Paranjape & Mogayzel, 2018; Pranke et al., 2019; Roomans, 2014). Research developed several target drugs for the most common mutations, such as ΔF508 leading to two essential classes of modulators: potentiators and correctors (Boyle et al., 2014; Chaudary, 2018; Clancy, 2018; Gees et al., 2018; Gentzsch & Mall, 2018; Guimbellot et al., 2017; Phuan et al., 2018; Van Goor et al., 2009; Wu et al., 2019; Yeh et al., 2017). Nevertheless, CF individuals with mutations that disrupt *CFTR* RNA processing and other basic mechanisms will not benefit of these personalized therapies.

In this thesis, I show that the strategy based on ExSpeU1s can be successfully applied to ten representative CFTR splicing mutations. To study the effect of the mutation on splicing and evaluate the potential splicing rescue activity of ExSpeU1s I created several minigenes with the different exons and flanking intronic regions. To provide a suitable rescuing approach acting at the RNA level, I focussed on a group of splicing mutations that induce exon skipping and are directly associated to CF. Overall, five CFTR exons (5, 10, 13, 16 and 18) were covered by my investigation. Considering the type of mutation, six are located in the 5'ss (donor) consensus, (711+3A>C/G, 711+5G>A, 1898+3A>G, 2789+5G>A, 3120G>A), one affects an exonic regulatory element (1863C>T) and three variants involve the polypyrimidine tract (TG12T5, TG13T5 and TG13T3). Particularly, 711+5G>A is one of the 12 more frequent CF mutations relative to the Italian distribution (Rendine et al., 1997) while 711+3A>G mutation was firstly detected in association with the most common ΔF508, connected to severe gastroparesis (Mandaliya et al., 2016). Located in exon 16 donor site, 2789+5G>A is characterized by sweat glands salt loss and progressive pulmonary disease (Duguépéroux & De Braekeleer, 2005) while 3120G>A in exon 18 is linked

to abnormal sweat chloride (LaMarche Heaney et al., 2006). Variants examined in exon 10 are three polymorphic repeat regions in the 3′- splice site of human *CFTR* exon 10 affecting the polypyrimidine tract interfering with the maturation process of the CFTR pre-mRNA (Cuppens et al. 1998; Pagani et al. 2000; Pagani et al. 2003; Groman et al. 2004; Du et al. 2014; Boussaroque et al. 2020). This study used minigene splicing assays as proof of principle of the ExSpeU1 efficacy on different CFTR defective exons. The efficacy of ExSpeU1s was further analysed at the protein level in two cases using minigenes where the splicing correction produced a full length CFTR mRNA and a normal-sized CFTR protein (Fig. 35 and Fig. 36). My results on different *CFTR* mutations confirm the applicability and translatability of the ExSpeU1 approach in rescuing different exon skipping defects, as previously reported in other cellular and mouse models for different diseases (Balestra et al., 2020; Dal Mas, Fortugno, et al., 2015; Donadon et al., 2018; Tajnik et al., 2016) .

Since this approach is based on the engineered 5' tail that targets the ExSpeU1 on desired intronic sequences, it can be easily applicable to other CFTR mutations. Indeed, I estimate that 40 additional splicing mutations out of the 352 CF-causing variants listed in CFTR2 database (updated to December 2020) might also take advantage of this ExSpeU1 strategy. These variants do not affect the invariant GU and AG dinucleotides at the splice sites but the consensus sequences at the donor sites or poly-pyrimidine tracts close to the acceptor sites.

Interestingly, none of the CFTR defective exons analysed proved to be completely insensitive to the ExSpeU1-mediated rescue, suggesting that they do not contain any potential ExSpeU1-IE like *cis* –acting element. However, among the variants I have evaluated, exon 13 was the most challenging to rescue. In fact, in this case, only one ExSpeU1 (U1ex13-11) among those tested, was able to rescue the splicing defects (Fig. 31, lanes 6 and 11). This apparent low efficiency is probably related to an Intronic Splicing Silencer (ISS) that overlaps with the active U1ex13-11 binding site (Donegà et al., 2020). Since this ISS is located in a particular position of RNA secondary structure, it could limit the availability of the U1snRNP to the 5'ss, as previously suggested (Buratti & Baralle, 2004).

4.3. **RNA-based gene therapy in lung targeted-disease**

Taken together, the minigene experiments I have performed on the CFTR mutations represent the classical first step in the development of therapeutic compounds that act on splicing. Approaches to correct exon skipping defects include and chemical compounds (Suñé-Pou et al., 2020) and Anti Sense Oligonucleotides (ASO) that was tested in CF only in the 2789+5G>A splicing mutation (Igreja et al., 2015). ASO is based on the identification of appropriate splicing regulatory elements and thus more time consuming and expensive, and not easy testable on several splicing defects. Additionally, pharmacological approaches to correct splicing requires high-throughput screening platforms and may lack of specificity (A. Berg et al., 2019; Giuliano et al., 2018; Liang et al., 2017; Merkert et al., 2019; Pereira et al., 2019). Compared to other strategies, ExSpeU1 strategy does not alter the physiological expression of the target gene. Moreover, it is easy to accommodate the short ExSpeU1 coding gene (~ 650 bp) into Adeno Associated Viruses or Lentiviral Vectors for *in vivo* gene therapy (Rogalska *et al.*, 2016; Donadon *et al.*, 2018) or *ex vivo* cellular delivery (Dal Mas, Fortugno, et al., 2015; Dal Mas, Rogalska, et al., 2015; Nizzardo et al., 2015). In CF or lung diseases, splicing defective epithelial progenitor cells either autologous induced pluripotent stem cells or basal stem cells (Ghosh et al., 2017; Gui et al., 2015; Rosen et al., 2015) could be transduced with lentiviral vectors expressing ExSpeU1s and then engrafted into the CF lung. Alternatively, other delivery approaches such as de novo chemical RNA synthesis of modified U1s could also be tested (Ohkubo et al., 2013).

As CF is a recessive disease, the rescue in one allele will be sufficient to rescue the CFTR protein function and the phenotype. Despite the rescue potential of the ExSpeU1 approach, it is important to underline that its beneficial frame is limited to those splicing defects that do not affect the AG and GT dinucleotides of the splice sites. In these cases, the mutation completely disrupts the splicing process and ExSpeU1 are ineffective. As well as for other splicing correction strategies (ASO and chemical compounds) an important aspect that have to be considered is related to

potential off-target effects. However, previous studies in cellular and animal models on Spinal Muscular Atrophy have identified a very limited number of off-targets by RNA-Seq analysis (Dal Mas, Rogalska, et al., 2015; Donadon et al., 2019).

The observation of an increase of CFTR protein levels in our selected cases are undoubtedly extremely important, but this effect must be considered within the patient's genetic background, thus next experiments will have to focus on more "physiological" systems.

In the future, a direct assessment of the ExSpeU1s strategy on the *CFTR* functionality and the evaluation of the ExSpeU1 efficacy and safety profile in patient-derived target cells will be required, taking advantage of patient-derived cellular models with the splicing mutations such as nasal epithelial cells (Pranke et al., 2017) or organoids (Boj et al., 2017; Dekkers et al., 2013, 2016; Sato et al., 2009, 2011).

5. Final notes

5.1. **Patent**

Franco Pagani is listed as inventor of the US patent n. 9669109: ‘A modified human U1snRNA molecule, a gene encoding for the modified human U1snRNA molecule, an expression vector including the gene, and the use thereof in gene therapy of familial dysautonomia and spinal muscular atrophy’.
As such the inventors could potentially benefit from any future commercial exploitation of patent rights, including the use of ExspeU1s in CF.

6. References

Abramowicz, A., & Gos, M. (2018). Splicing mutations in human genetic disorders: examples, detection, and confirmation. In *Journal of Applied Genetics* (Vol. 59, Issue 3, pp. 253–268). Springer Verlag. https://doi.org/10.1007/s13353-018-0444-7

Achsel, T., Stark, H., & Lührmann, R. (2001). The Sm domain is an ancient RNA-binding motif with oligo(U) specificity. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(7), 3685–3689. https://doi.org/10.1073/pnas.071033998

Alanis, E. F., Pinotti, M., Mas, A. D., Balestra, D., Cavallari, N., Rogalska, M. E., Bernardi, F., & Pagani, F. (2012). An exon-specific U1 small nuclear RNA (snRNA) strategy to correct splicing defects. *Human Molecular Genetics*, *21*(11), 2389–2398. https://doi.org/10.1093/hmg/dds045

Baeza-Centurion, P., Miñana, B., Schmiedel, J. M., Valcárcel, J., & Lehner, B. (2019). Combinatorial Genetics Reveals a Scaling Law for the Effects of Mutations on Splicing. *Cell*, *176*(3), 549-563.e23. https://doi.org/10.1016/j.cell.2018.12.010

Balestra, D., Faella, A., Margaritis, P., Cavallari, N., Pagani, F., Bernardi, F., Arruda, V. R., & Pinotti, M. (2014). An engineered U1 small nuclear RNA rescues splicing-defective coagulation F7 gene expression in mice. *Journal of Thrombosis and Haemostasis*, *12*(2), 177–185. https://doi.org/10.1111/jth.12471

Balestra, Dario, Scalet, D., Ferrarese, M., Lombardi, S., Ziliotto, N., Croes, C. C., Petersen, N., Bosma, P., Riccardi, F., Pagani, F., Pinotti, M., & van de Graaf, S. F. J. (2020). A compensatory U1snRNA partially rescues FAH splicing and protein expression in a splicing-defective mouse model of tyrosinemia type I. *International Journal of Molecular Sciences*, *21*(6). https://doi.org/10.3390/ijms21062136

Balestra, Dario, Scalet, D., Pagani, F., Rogalska, M. E., Mari, R., Bernardi, F., & Pinotti, M. (2016). An exon-specific U1snRNA induces a robust factor IX activity in mice expressing multiple human FIX splicing mutants. *Molecular*

Therapy - Nucleic Acids, *5*(10), e370. https://doi.org/10.1038/mtna.2016.77

Banerjee, A. K. (1980). 5'-Terminal cap structure in eucaryotic messenger ribonucleic acids. In *Microbiological Reviews* (Vol. 44, Issue 2, pp. 175–205). https://doi.org/10.1128/mmbr.44.2.175-205.1980

Baralle, M. (2003). Identification of a mutation that perturbs NF1 agene splicing using genomic DNA samples and a minigene assay. *Journal of Medical Genetics*, *40*(3), 220–222. https://doi.org/10.1136/jmg.40.3.220

Benne, R., Van Den Burg, J., Brakenhoff, J. P. J., Sloof, P., Van Boom, J. H., & Tromp, M. C. (1986). Major transcript of the frameshifted coxll gene from trypanosome mitochondria contains four nucleotides that are not encoded in the DNA. *Cell*, *46*(6), 819–826. https://doi.org/10.1016/0092-8674(86)90063-2

Berg, A., Hallowell, S., Tibbetts, M., Beasley, C., Brown-Phillips, T., Healy, A., Pustilnik, L., Doyonnas, R., & Pregel, M. (2019). High-Throughput Surface Liquid Absorption and Secretion Assays to Identify F508del CFTR Correctors Using Patient Primary Airway Epithelial Cultures. *SLAS Discovery*, *24*(7), 724–737. https://doi.org/10.1177/2472555219849375

Berg, J. (2012). *Biochemistry*. W.H. Freeman and Company.

Black, D. L. (2003). Mechanisms of Alternative Pre-Messenger RNA Splicing. *Annual Review of Biochemistry*, *72*(1), 291–336. https://doi.org/10.1146/annurev.biochem.72.121801.161720

Blue, R. E., Curry, E. G., Engels, N. M., Lee, E. Y., & Giudice, J. (2018). How alternative splicing affects membrane-trafficking dynamics. In *Journal of Cell Science* (Vol. 131, Issue 10). Company of Biologists Ltd. https://doi.org/10.1242/jcs.216465

Boj, S. F., Vonk, A. M., Statia, M., Su, J., Vries, R. R. G., Beekman, J. M., & Clevers, H. (2017). Forskolin-induced swelling in intestinal organoids: An in vitro assay for assessing drug response in cystic fibrosis patients. *Journal of Visualized Experiments*, *2017*(120). https://doi.org/10.3791/55159

Bonadia, L. C., De Lima Marson, F. A., Ribeiro, J. D., Paschoal, I. A., Pereira, M. C., Ribeiro, A. F., & Bertuzzo, C. S. (2014). CFTR genotype and clinical outcomes

of adult patients carried as cystic fibrosis disease. *Gene*, *540*(2), 183–190. https://doi.org/10.1016/j.gene.2014.02.040

Boussaroque, A., Audrézet, M. P., Raynal, C., Sermet-Gaudelus, I., Bienvenu, T., Férec, C., Bergougnoux, A., Lopez, M., Scotet, V., Munck, A., & Girodon, E. (2020). Penetrance is a critical parameter for assessing the disease liability of CFTR variants. *Journal of Cystic Fibrosis*, *0*(0). https://doi.org/10.1016/j.jcf.2020.03.019

Boyle, M. P., Bell, S. C., Konstan, M. W., McColley, S. A., Rowe, S. M., Rietschel, E., Huang, X., Waltz, D., Patel, N. R., & Rodman, D. (2014). A CFTR corrector (lumacaftor) and a CFTR potentiator (ivacaftor) for treatment of patients with cystic fibrosis who have a phe508del CFTR mutation: A phase 2 randomised controlled trial. *The Lancet Respiratory Medicine*, *2*(7), 527–538. https://doi.org/10.1016/S2213-2600(14)70132-8

Branlant, C., Krol, A., Ebel, J. P., Lazar, E., Haendler, B., & Jacob, M. (1982). U2 RNA shares a structural domain with U1, U4, and U5 RNAs. *The EMBO Journal*. https://doi.org/10.1002/j.1460-2075.1982.tb00022.x

Buratti, E., & Baralle, F. E. (2004). Influence of RNA Secondary Structure on the Pre-mRNA Splicing Process. *Molecular and Cellular Biology*, *24*(24), 10505–10514. https://doi.org/10.1128/mcb.24.24.10505-10514.2004

Buratti, E., Dörk, T., Zuccato, E., Pagani, F., Romano, M., & Baralle, F. E. (2001). Nuclear factor TDP-43 and SR proteins promote in vitro and in vivo CFTR exon 9 skipping. *EMBO Journal*, *20*(7), 1774–1784. https://doi.org/10.1093/emboj/20.7.1774

Burgel, P. R., Bellis, G., Olesen, H. V., Viviani, L., Zolin, A., Blasi, F., Elborn, J. S., Bell, S., Castellani, C., Dembski, B., Drevinek, P., Heijerman, H., Innes, A., Lindblad, A., Madge, S., De Rijcke, K., & Solé, A. (2015). Future trends in cystic fibrosis demography in 34 European countries. *European Respiratory Journal*, *46*(1), 133–141. https://doi.org/10.1183/09031936.00196314

Burkard, K. T. D., & Butler, J. S. (2000). A Nuclear 3'-5' Exonuclease Involved in mRNA Degradation Interacts with Poly(A) Polymerase and the hnRNA Protein

Npl3p. *Molecular and Cellular Biology*, *20*(2), 604–616. https://doi.org/10.1128/mcb.20.2.604-616.2000

Chandler, D. S. (2011). Pre-mRNA Splicing. In *Encyclopedia of Cancer* (pp. 2972–2977). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-16483-5_4711

Chaudary, N. (2018). Triplet CFTR modulators: Future prospects for treatment of cystic fibrosis. In *Therapeutics and Clinical Risk Management* (Vol. 14, pp. 2375–2383). Dove Medical Press Ltd. https://doi.org/10.2147/TCRM.S147164

Chaudhury, A., Chander, P., & Howe, P. H. (2010). Heterogeneous nuclear ribonucleoproteins (hnRNPs) in cellular processes: Focus on hnRNP E1's multifunctional regulatory roles. In *RNA* (Vol. 16, Issue 8, pp. 1449–1462). Cold Spring Harbor Laboratory Press. https://doi.org/10.1261/rna.2254110

Chernick, W. S., Barbero, G. J., & Eichel, H. J. (1961). In-vitro evaluation of effect of enzymes on tracheobronchial secretions from patients withcystic fibrosis. *Pediatrics*, *27*(4), 589–596.

Choi, J. Y., Muallem, D., Kiselyov, K., Lee, M. G., Thomas, P. J., & Muallem, S. (2001). Aberrant CFTR-dependent HCO3- transport in mutations associated with cystic fibrosis. *Nature*, *410*(6824), 94–97. https://doi.org/10.1038/35065099

Chung, C. T., Niemela, S. L., & Miller, R. H. (1989). One-step preparation of competent Escherichia coli: transformation and storage of bacterial cells in the same solution. *Proceedings of the National Academy of Sciences of the United States of America*, *86*(7), 2172–2175. https://doi.org/10.1073/pnas.86.7.2172

Clancy, J. P. (2018). Rapid therapeutic advances in CFTR modulator science. *Pediatric Pulmonology*, *53*(S3), S4–S11. https://doi.org/10.1002/ppul.24157

Collawn, J. F., & Matalon, S. (2014). CFTR and lung homeostasis. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, *307*(12), L917–L923. https://doi.org/10.1152/ajplung.00326.2014

Coller, J. M., Gray, N. K., & Wickens, M. P. (1998). mRNA stabilization by poly(A) binding protein is independent of poly(A) and requires translation. *Genes and Development*, *12*(20), 3226–3235. https://doi.org/10.1101/gad.12.20.3226

Collins, C. A., & Guthrie, C. (2000). The question remains: Is the spliceosome a ribozyme? In *Nature Structural Biology* (Vol. 7, Issue 10, pp. 850–854). Nature Publishing Group. https://doi.org/10.1038/79598

Crosby, J. R., Zhao, C., Jiang, C., Bai, D., Katz, M., Greenlee, S., Kawabe, H., McCaleb, M., Rotin, D., Guo, S., & Monia, B. P. (2017). Inhaled ENaC antisense oligonucleotide ameliorates cystic fibrosis-like lung disease in mice. *Journal of Cystic Fibrosis*, *16*(6), 671–680. https://doi.org/10.1016/j.jcf.2017.05.003

Cuppens, H., Lin, W., Jaspers, M., Costes, B., Teng, H., Vankeerberghen, A., Jorissen, M., Droogmans, G., Reynaert, I., Goosens, M., Nilius, B., & Cassiman, J. J. (1998). Polyvariant mutant cystic fibrosis transmembrane conductance regulator genes: The polymorphic (TG)m locus explains the partial penetrance of the T5 polymorphism as a disease mutation. *Journal of Clinical Investigation*, *101*(2), 487–496. https://doi.org/10.1172/JCI639

Dal Mas, A., Fortugno, P., Donadon, I., Levati, L., Castiglia, D., & Pagani, F. (2015). Exon-specific U1s correct SPINK5 exon 11 skipping caused by a synonymous substitution that affects a bifunctional splicing regulatory element. *Human Mutation*, *36*(5), 504–512. https://doi.org/10.1002/humu.22762

Dal Mas, A., Rogalska, M. E., Bussani, E., & Pagani, F. (2015). Improvement of SMN2 pre-mRNA processing mediated by exon-specific U1 small nuclear RNA. *American Journal of Human Genetics*, *96*(1), 93–103. https://doi.org/10.1016/j.ajhg.2014.12.009

Davis, R. L., Homer, V. M., George, P. M., & Brennan, S. O. (2009). A deep intronic mutation in FGB creates a consensus exonic splicing enhancer motif that results in afibrinogenemia caused by aberrant mRNA splicing, which can be corrected in vitro with antisense oligonucleotide treatment. *Human Mutation*, *30*(2), 221–227. https://doi.org/10.1002/humu.20839

De Boeck, K., & Amaral, M. D. (2016). Progress in therapies for cystic fibrosis. In *The Lancet Respiratory Medicine* (Vol. 4, Issue 8, pp. 662–674). Lancet Publishing Group. https://doi.org/10.1016/S2213-2600(16)00023-0

Decroly, E., Ferron, F., Lescar, J., & Canard, B. (2012). Conventional and

unconventional mechanisms for capping viral mRNA. In *Nature Reviews Microbiology* (Vol. 10, Issue 1, pp. 51–65). Nature Publishing Group. https://doi.org/10.1038/nrmicro2675

Dekkers, J. F., Berkers, G., Kruisselbrink, E., Vonk, A., De Jonge, H. R., Janssens, H. M., Bronsveld, I., Van De Graaf, E. A., Nieuwenhuis, E. E. S., Houwen, R. H. J., Vleggaar, F. P., Escher, J. C., De Rijke, Y. B., Majoor, C. J., Heijerman, H. G. M., De Winter-De Groot, K. M., Clevers, H., Van Der Ent, C. K., & Beekman, J. M. (2016). Characterizing responses to CFTR-modulating drugs using rectal organoids derived from subjects with cystic fibrosis. *Science Translational Medicine*, *8*(344), 344ra84-344ra84. https://doi.org/10.1126/scitranslmed.aad8278

Dekkers, J. F., Wiegerinck, C. L., De Jonge, H. R., Bronsveld, I., Janssens, H. M., De Winter-De Groot, K. M., Brandsma, A. M., De Jong, N. W. M., Bijvelds, M. J. C., Scholte, B. J., Nieuwenhuis, E. E. S., Van Den Brink, S., Clevers, H., Van Der Ent, C. K., Middendorp, S., & Beekman, J. M. (2013). A functional CFTR assay using primary cystic fibrosis intestinal organoids. *Nature Medicine*, *19*(7), 939–945. https://doi.org/10.1038/nm.3201

Donadon, I., Bussani, E., Riccardi, F., Licastro, D., Romano, G., Pianigiani, G., Pinotti, M., Konstantinova, P., Evers, M., Lin, S., Rüegg, M. A., & Pagani, F. (2019). Rescue of spinal muscular atrophy mouse models with AAV9-Exon-specific U1 snRNA. *Nucleic Acids Research*, *47*(14), 7618–7632. https://doi.org/10.1093/nar/gkz469

Donadon, I., Pinotti, M., Rajkowska, K., Pianigiani, G., Barbon, E., Morini, E., Motaln, H., Rogelj, B., Mingozzi, F., Slaugenhaupt, S. A., & Pagani, F. (2018). Exon-specific U1 snRNAs improve ELP1 exon 20 definition and rescue ELP1 protein expression in a familial dysautonomia mouse model. *Human Molecular Genetics*, *27*(14), 2466–2476. https://doi.org/10.1093/hmg/ddy151

Donegà, S., Rogalska, M. E., Pianigiani, G., Igreja, S., Amaral, M. D., & Pagani, F. (2020). Rescue of common exon-skipping mutations in cystic fibrosis with modified U1 snRNAs. *Human Mutation*, 1– 12.

https://doi.org/10.1002/humu.24116

Du, H., & Rosbash, M. (2002). The U1 snRNP protein U1C recognizes the 5′ splice site in the absence of base pairing. *Nature*, *419*(6902), 86–90. https://doi.org/10.1038/nature00947

Du, K., & Lukacs, G. L. (2009). Cooperative assembly and misfolding of CFTR domains in vivo. *Molecular Biology of the Cell*, *20*(7), 1903–1915. https://doi.org/10.1091/mbc.E08-09-0950

Du, Q., Li, Z., Pan, Y., Liu, X., Pan, B., & Wu, B. (2014). The CFTR M470V, intron 8 poly-T, and 8 TG-repeats detection in Chinese males with congenital bilateral absence of the vas deferens. *BioMed Research International*, *2014*, 689185. https://doi.org/10.1155/2014/689185

Duguépéroux, I., & De Braekeleer, M. (2005). The CFTR 3849+10kbC->T and 2789+5G->A alleles are associated with a mild CF phenotype. *European Respiratory Journal*, *25*(3), 468–473. https://doi.org/10.1183/09031936.05.10100004

Elkon, R., Ugalde, A. P., & Agami, R. (2013). Alternative cleavage and polyadenylation: Extent, regulation and function. In *Nature Reviews Genetics* (Vol. 14, Issue 7, pp. 496–506). Nat Rev Genet. https://doi.org/10.1038/nrg3482

European Respiratory Society. (2009). *ERS - Respiratory health and disease in Europe*. European Lung White Book. https://www.erswhitebook.org/

Fajac, I., & Wainwright, C. E. (2017). New treatments targeting the basic defects in cystic fibrosis. In *Presse Medicale* (Vol. 46, Issue 6P2, pp. e165–e175). Elsevier Masson SAS. https://doi.org/10.1016/j.lpm.2017.01.024

Fakioğlu, D. M., & Altun, B. (2020). New therapeutic approaches in cystic fibrosis. *Turkish Journal of Pharmaceutical Sciences*, *17*(6), 686–697. https://doi.org/10.4274/tjps.galenos.2020.76401

Farinha, C. M., & Matos, P. (2016). Repairing the basic defect in cystic fibrosis - One approach is not enough. In *FEBS Journal* (Vol. 283, Issue 2, pp. 246–264). Blackwell Publishing Ltd. https://doi.org/10.1111/febs.13531

Farrell, P. M., White, T. B., Ren, C. L., Hempstead, S. E., Accurso, F., Derichs, N.,

Howenstine, M., McColley, S. A., Rock, M., Rosenfeld, M., Sermet-Gaudelus, I., Southern, K. W., Marshall, B. C., & Sosnay, P. R. (2017). Diagnosis of Cystic Fibrosis: Consensus Guidelines from the Cystic Fibrosis Foundation. *Journal of Pediatrics*, *181*, S4-S15.e1. https://doi.org/10.1016/j.jpeds.2016.09.064

Frankenstein, Z., Sperling, J., Sperling, R., & Eisenstein, M. (2012). A unique spatial arrangement of the snRNPs within the native spliceosome emerges from in silico studies. *Structure*, *20*(6), 1097–1106. https://doi.org/10.1016/j.str.2012.03.022

Gees, M., Musch, S., Van Der Plas, S., Wesse, A. S., Vandevelde, A., Verdonck, K., Mammoliti, O., Hwang, T. C., Sonck, K., Stouten, P., Swensen, A. M., Jans, M., Van Der Schueren, J., Nelles, L., Andrews, M., & Conrath, K. (2018). Identification and characterization of novel CFTR potentiators. *Frontiers in Pharmacology*, *9*(OCT). https://doi.org/10.3389/fphar.2018.01221

Gelfond, D., & Borowitz, D. (2013). Gastrointestinal complications of cystic fibrosis. *Clinical Gastroenterology and Hepatology*. https://doi.org/10.1016/j.cgh.2012.11.006

Gentzsch, M., & Mall, M. A. (2018). Ion Channel Modulators in Cystic Fibrosis. In *Chest* (Vol. 154, Issue 2, pp. 383–393). Elsevier Inc. https://doi.org/10.1016/j.chest.2018.04.036

Geuens, T., Bouhy, D., & Timmerman, V. (2016). The hnRNP family: insights into their role in health and disease. In *Human Genetics* (Vol. 135, Issue 8, pp. 851–867). Springer Verlag. https://doi.org/10.1007/s00439-016-1683-5

Ghosh, M., Ahmad, S., White, C. W., & Reynolds, S. D. (2017). Transplantation of airway epithelial stem/progenitor cells: A future for cell-based therapy. *American Journal of Respiratory Cell and Molecular Biology*, *56*(1), 1–10. https://doi.org/10.1165/rcmb.2016-0181MA

Giuliano, K. A., Wachi, S., Drew, L., Dukovski, D., Green, O., Bastos, C., Cullen, M. D., Hauck, S., Tait, B. D., Munoz, B., Lee, P. S., & Miller, J. P. (2018). Use of a High-Throughput Phenotypic Screening Strategy to Identify Amplifiers, a Novel Pharmacological Class of Small Molecules That Exhibit Functional Synergy with Potentiators and Correctors. *SLAS Discovery*, *23*(2), 111–121.

https://doi.org/10.1177/2472555217729790

Gregory, R. J., Cheng, S. H., Rich, D. P., Marshall, J., Paul, S., Hehir, K., Ostedgaard, L., Klinger, K. W., Welsh, M. J., & Smith, A. E. (1990). Expression and characterization of the cystic fibrosis transmembrane conductance regulator. *Nature*, *347*(6291), 382–386. https://doi.org/10.1038/347382a0

Griesenbach, U., Pytel, K. M., & Alton, E. W. F. W. (2015). Cystic Fibrosis Gene Therapy in the UK and Elsewhere. *Human Gene Therapy*, *26*(5), 266–275. https://doi.org/10.1089/hum.2015.027

Groman, J. D., Hefferon, T. W., Casals, T., Bassas, L., Estivill, X., Des Georges, M., Guittard, C., Koudova, M., Fallin, M. D., Nemeth, K., Fekete, G., Kadasi, L., Friedman, K., Schwarz, M., Bombieri, C., Pignatti, P. F., Kanavakis, E., Tzetis, M., Schwartz, M., … Cutting, G. R. (2004). Variation in a Repeat Sequence Determines Whether a Common Variant of the Cystic Fibrosis Transmembrane Conductance Regulator Gene Is Pathogenic or Benign. *American Journal of Human Genetics*, *74*(1), 176–179. https://doi.org/10.1086/381001

Grosso, A. R., Gomes, A. Q., Barbosa-Morais, N. L., Caldeira, S., Thorne, N. P., Grech, G., von Lindern, M., & Carmo-Fonseca, M. (2008). Tissue-specific splicing factor gene expression signatures. *Nucleic Acids Research*, *36*(15), 4823–4832. https://doi.org/10.1093/nar/gkn463

Grummt, I. (2003). Life on a planet of its own: Regulation of RNA polymerase I transcription in the nucleolus. In *Genes and Development* (Vol. 17, Issue 14, pp. 1691–1702). Genes Dev. https://doi.org/10.1101/gad.1098503R

Gui, L., Qian, H., Rocco, K. A., Grecu, L., & Niklason, L. E. (2015). Efficient intratracheal delivery of airway epithelial cells in mice and pigs. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, *308*(2), L221–L228. https://doi.org/10.1152/ajplung.00147.2014

Guimbellot, J., Sharma, J., & Rowe, S. M. (2017). Toward inclusive therapy with CFTR modulators: Progress and challenges. In *Pediatric Pulmonology* (Vol. 52, Issue S48, pp. S4–S14). https://doi.org/10.1002/ppul.23773

Haq, I. J., Gray, M. A., Garnett, J. P., Ward, C., & Brodlie, M. (2016). Airway surface

liquid homeostasis in cystic fibrosis: Pathophysiology and therapeutic targets. In *Thorax* (Vol. 71, Issue 3, pp. 284–287). https://doi.org/10.1136/thoraxjnl-2015-207588

Heinrichs, V., Bach, M., Winkelmann, G., & Luhrmann, R. (2006). U1-specific protein C needed for efficient complex formation of U1 snRNP with a 5' splice site. *Science*, *247*(4938), 69–72. https://doi.org/10.1126/science.2136774

Hollin, I. L., & Robinson, K. A. (2016). A Scoping Review of Healthcare Costs for Patients with Cystic Fibrosis. In *Applied Health Economics and Health Policy* (Vol. 14, Issue 2, pp. 151–159). https://doi.org/10.1007/s40258-015-0211-4

Hoy, S. M. (2019). Elexacaftor/Ivacaftor/Tezacaftor: First Approval. In *Drugs* (Vol. 79, Issue 18, pp. 2001–2007). Adis. https://doi.org/10.1007/s40265-019-01233-7

Hwang, T. C., Yeh, J. T., Zhang, J., Yu, Y. C., Yeh, H. I., & Destefano, S. (2018). Structural mechanisms of CFTR function and dysfunction. In *Journal of General Physiology* (Vol. 150, Issue 4, pp. 539–570). Rockefeller University Press. https://doi.org/10.1085/jgp.201711946

Igreja, S., Clarke, L. A., Botelho, H. M., Marques, L., & Amaral, M. D. (2015). Correction of a cystic fibrosis splicing mutation by antisense oligonucleotides. In *Human Mutation* (Vol. 37, Issue 2, pp. 209–215). https://doi.org/10.1002/humu.22931

Irimia, M., Penny, D., & Roy, S. W. (2007). Coevolution of genomic intron number and splice sites. In *Trends in Genetics* (Vol. 23, Issue 7, pp. 321–325). https://doi.org/10.1016/j.tig.2007.04.001

Julien, P., Miñana, B., Baeza-Centurion, P., Valcárcel, J., & Lehner, B. (2016). The complete local genotype-phenotype landscape for the alternative splicing of a human exon. *Nature Communications*, *7*(1), 11558. https://doi.org/10.1038/ncomms11558

Kalsotra, A., Xiao, X., Ward, A. J., Castle, J. C., Johnson, J. M., Burge, C. B., & Cooper, T. A. (2008). A postnatal switch of CELF and MBNL proteins reprograms alternative splicing in the developing heart. *Proceedings of the*

National Academy of Sciences of the United States of America, *105*(51), 20333–20338. https://doi.org/10.1073/pnas.0809045105

Kanadia, R. N., Clark, V. E., Punzo, C., Trimarchi, J. M., & Cepko, C. L. (2008). Temporal requirement of the alternative-splicing factor Sfrs1 for the survival of retinal neurons. *Development*, *135*(23), 3923–3933. https://doi.org/10.1242/dev.024620

Kashima, T., & Manley, J. L. (2003). A negative element in SMN2 exon 7 inhibits splicing in spinal muscular atrophy. *Nature Genetics*, *34*(4), 460–463. https://doi.org/10.1038/ng1207

Konarska, M. M., Padgett, R. A., & Sharp, P. A. (1984). Recognition of cap structure in splicing in vitro of mRNA precursors. *Cell*, *38*(3), 731–736. https://doi.org/10.1016/0092-8674(84)90268-X

Kosorok, M. R., Wei, W. H., & Farrell, P. M. (1996). The incidence of cystic fibrosis. *Statistics in Medicine*, *15*(5), 449–462. https://doi.org/10.1002/(SICI)1097-0258(19960315)15:5<449::AID-SIM173>3.0.CO;2-X

LaMarche Heaney, D., Flume, P., Hamilton, L., Lyon, E., & Wolff, D. J. (2006). Detection of an apparent homozygous 3120G>A cystic fibrosis mutation on a routine carrier screen. *Journal of Molecular Diagnostics*, *8*(1), 137–140. https://doi.org/10.2353/jmoldx.2006.050065

Lewis, J. D., & Izaurralde, E. (1997). The role of the cap structure in RNA processing and nuclear export. In *European Journal of Biochemistry* (Vol. 247, Issue 2, pp. 461–469). https://doi.org/10.1111/j.1432-1033.1997.00461.x

Li, L., & Somerset, S. (2014). Digestive system dysfunction in cystic fibrosis: Challenges for nutrition therapy. In *Digestive and Liver Disease* (Vol. 46, Issue 10, pp. 865–874). https://doi.org/10.1016/j.dld.2014.06.011

Liang, F., Shang, H., Jordan, N. J., Wong, E., Mercadante, D., Saltz, J., Mahiou, J., Bihler, H. J., & Mense, M. (2017). High-Throughput Screening for Readthrough Modulators of CFTR PTC Mutations. *SLAS Technology*, *22*(3), 315–324. https://doi.org/10.1177/2472630317692561

Long, J. C., & Caceres, J. F. (2009). The SR protein family of splicing factors: Master

regulators of gene expression. In *Biochemical Journal* (Vol. 417, Issue 1, pp. 15–27). Portland Press Limited. https://doi.org/10.1042/BJ20081501

Mandaliya, R., Hadjiliadis, D., & Cohen, S. (2016). Gastroparesis Concurrent with Adult Cystic Fibrosis; Are They Related? *American Journal of Medicine*, *129*(5), e21–e22. https://doi.org/10.1016/j.amjmed.2016.01.017

Maniatis, T., & Reed, R. (2002). An extensive network of coupling among gene expression machines. *Nature*, *416*(6880), 499–506. https://doi.org/10.1038/416499a

Marcorelles, P., Friocourt, G., Uguen, A., Ledé, F., Férec, C., & Laquerrière, A. (2014). Cystic Fibrosis Transmembrane Conductance Regulator Protein (CFTR) Expression in the Developing Human Brain: Comparative Immunohistochemical Study between Patients with Normal and Mutated CFTR. *Journal of Histochemistry and Cytochemistry*, *62*(11), 791–801. https://doi.org/10.1369/0022155414546190

Marcotrigiano, J., Gingras, A. C., Sonenberg, N., & Burley, S. K. (1997). Cocrystal structure of the messenger RNA 5' cap-binding protein (elF4E) bound to 7-methyl-GDP. *Cell*, *89*(6), 951–961. https://doi.org/10.1016/S0092-8674(00)80280-9

Marson, F. A. L., Bertuzzo, C. S., & Ribeiro, J. D. (2016). Classification of CFTR mutation classes. *The Lancet Respiratory Medicine*, *4*(8), e37–e38. https://doi.org/10.1016/S2213-2600(16)30188-6

Mattioli, C., Pianigiani, G., De Rocco, D., Bianco, A. M. R., Cappelli, E., Savoia, A., & Pagani, F. (2014). Unusual splice site mutations disrupt FANCA exon 8 definition. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, *1842*(7), 1052–1058. https://doi.org/10.1016/j.bbadis.2014.03.014

Meister, G., Eggert, C., & Fischer, U. (2002). SMN-mediated assembly of RNPs: A complex story. In *Trends in Cell Biology* (Vol. 12, Issue 10, pp. 472–478). https://doi.org/10.1016/S0962-8924(02)02371-1

Merkert, S., Schubert, M., Olmer, R., Engels, L., Radetzki, S., Veltman, M., Scholte, B. J., Zöllner, J., Pedemonte, N., Galietta, L. J. V., von Kries, J. P., & Martin, U.

(2019). High-Throughput Screening for Modulators of CFTR Activity Based on Genetically Engineered Cystic Fibrosis Disease-Specific iPSCs. *Stem Cell Reports*, *12*(6), 1389–1403. https://doi.org/10.1016/j.stemcr.2019.04.014

Muro, A. F., Caputi, M., Pariyarath, R., Pagani, F., Buratti, E., & Baralle, F. E. (1999). Regulation of Fibronectin EDA Exon Alternative Splicing: Possible Role of RNA Secondary Structure for Enhancer Display. *Molecular and Cellular Biology*, *19*(4), 2657–2671. https://doi.org/10.1128/mcb.19.4.2657

Nakayama, K., & Kataoka, N. (2019). Regulation of gene expression under hypoxic conditions. In *International Journal of Molecular Sciences* (Vol. 20, Issue 13, p. 3278). MDPI AG. https://doi.org/10.3390/ijms20133278

Naylor, S. L., Zabel, B. U., Manser, T., Gesteland, R., & Sakaguchi, A. Y. (1984). Localization of human U1 small nuclear RNA genes to band p36.3 of chromosome 1 by in situ hybridization. *Somatic Cell and Molecular Genetics*, *10*(3), 307–313. http://www.ncbi.nlm.nih.gov/pubmed/6202011

Nguyen, T. H. D., Galej, W. P., Fica, S. M., Lin, P. C., Newman, A. J., & Nagai, K. (2016). CryoEM structures of two spliceosomal complexes: Starter and dessert at the spliceosome feast. In *Current Opinion in Structural Biology* (Vol. 36, pp. 48–57). Elsevier Ltd. https://doi.org/10.1016/j.sbi.2015.12.005

Nilsen, T. W., & Graveley, B. R. (2010). Expansion of the eukaryotic proteome by alternative splicing. *Nature*, *463*(7280), 457–463. https://doi.org/10.1038/nature08909

Nizzardo, M., Simone, C., Dametti, S., Salani, S., Ulzi, G., Pagliarani, S., Rizzo, F., Frattini, E., Pagani, F., Bresolin, N., Comi, G., & Corti, S. (2015). Spinal muscular atrophy phenotype is ameliorated in human motor neurons by SMN increase via different novel RNA therapeutic approaches. *Scientific Reports*, *5*(1), 11746. https://doi.org/10.1038/srep11746

Norris, A. D., & Calarco, J. A. (2012). Emerging roles of alternative pre-mRNA splicing regulation in neuronal development and function. *Frontiers in Neuroscience*, *6*(AUG). https://doi.org/10.3389/fnins.2012.00122

Ohkubo, A., Kondo, Y., Suzuki, M., Kobayashi, H., Kanamori, T., Masaki, Y., Seio,

K., Nagai, K., & Sekine, M. (2013). Chemical synthesis of U1 snRNA derivatives. *Organic Letters*, *15*(17), 4386–4389. https://doi.org/10.1021/ol401917r

Ohno, M., Segref, A., Bachi, A., Wilm, M., & Mattaj, I. W. (2000). PHAX, a mediator of U snRNA nuclear export whose activity is regulated by phosphorylation. *Cell*, *101*(2), 187–198. https://doi.org/10.1016/S0092-8674(00)80829-6

Ontalus, V., Cobianu, C., Vasiliu, F., & Parlog, C. (1996). Crystallization behaviour and phase coexistence at morphotrophic phase boundaries in PZT thin films prepared by sol-gel processing. *Journal of Materials Science*, *31*(14), 3639–3642. https://doi.org/10.1007/BF00352771

Ostedgaard, L. S., Baldursson, O., & Welsh, M. J. (2001). Regulation of the Cystic Fibrosis Transmembrane Conductance Regulator Cl- Channel by Its R Domain. In *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.R100001200

Padgett, R. A. (2012). New connections between splicing and human disease. In *Trends in Genetics* (Vol. 28, Issue 4, pp. 147–154). Elsevier. https://doi.org/10.1016/j.tig.2012.01.001

Pagani, F., Buratti, E., Stuani, C., & Baralle, F. E. (2003). Missense, nonsense, and neutral mutations define juxtaposed regulatory elements of splicing in cystic fibrosis transmembrane regulator exon 9. *Journal of Biological Chemistry*, *278*(29), 26580–26588. https://doi.org/10.1074/jbc.M212813200

Pagani, F., Buratti, E., Stuani, C., Bendix, R., Dörk, T., & Baralle, F. E. (2002). A new type of mutation causes a splicing defect in ATM. *Nature Genetics*, *30*(4), 426–429. https://doi.org/10.1038/ng858

Pagani, F., Buratti, E., Stuani, C., Romano, M., Zuccato, E., Niksic, M., Giglio, L., Faraguna, D., & Baralle, F. E. (2000). Splicing factors induce cystic fibrosis transmembrane regulator exon 9 skipping through a nonevolutionary conserved intronic element. *Journal of Biological Chemistry*, *275*(28), 21041–21047. https://doi.org/10.1074/jbc.M910165199

Pagani, F., Stuani, C., Tzetis, M., Kanavakis, E., Efthymiadou, A., Doudounakis, S., Casals, T., & Baralle, F. E. (2003). New type of disease causing mutations: The

example of the composite exonic regulatory elements of splicing in CFTR exon 12. *Human Molecular Genetics*, *12*(10), 1111–1120. https://doi.org/10.1093/hmg/ddg131

Palomaki, G. E., FitzSimmons, S. C., & Haddow, J. E. (2004). Clinical sensitivity of prenatal screening for cystic fibrosis via CFTR carrier testing in a United States panethnic population. *Genetics in Medicine*, *6*(5), 405–414. https://doi.org/10.1097/01.GIM.0000139505.06194.39

Pan, Q., Shai, O., Lee, L. J., Frey, B. J., & Blencowe, B. J. (2008). Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nature Genetics*, *40*(12), 1413–1415. https://doi.org/10.1038/ng.259

Parada, G. E., Munita, R., Cerda, C. A., & Gysling, K. (2014). A comprehensive survey of non-canonical splice sites in the human transcriptome. In *Nucleic Acids Research* (Vol. 42, Issue 16, pp. 10564–10578). Oxford University Press. https://doi.org/10.1093/nar/gku744

Paranjape, S. M., & Mogayzel, P. J. (2018). Cystic fibrosis in the era of precision medicine. In *Paediatric Respiratory Reviews* (Vol. 25, pp. 64–72). W.B. Saunders Ltd. https://doi.org/10.1016/j.prrv.2017.03.001

Paranjape, S. M., & Zeitlin, P. L. (2008). Atypical cystic fibrosis and CFTR-related diseases. In *Clinical Reviews in Allergy and Immunology* (Vol. 35, Issue 3, pp. 116–123). https://doi.org/10.1007/s12016-008-8083-0

Pereira, S. V. N., Ribeiro, J. D., Ribeiro, A. F., Bertuzzo, C. S., & Marson, F. A. L. (2019). Novel, rare and common pathogenic variants in the CFTR gene screened by high-throughput sequencing technology and predicted by in silico tools. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-019-42404-6

Phuan, P. W., Son, J. H., Tan, J. A., Li, C., Musante, I., Zlock, L., Nielson, D. W., Finkbeiner, W. E., Kurth, M. J., Galietta, L. J., Haggie, P. M., & Verkman, A. S. (2018). Combination potentiator (‘co-potentiator’) therapy for CF caused by CFTR mutants, including N1303K, that are poorly responsive to single potentiators. *Journal of Cystic Fibrosis*, *17*(5), 595–606.

https://doi.org/10.1016/j.jcf.2018.05.010

Pinotti, M., Balestra, D., Rizzotto, L., Maestri, I., Pagani, F., & Bernardi, F. (2009). Rescue of coagulation factor VII function by the U1+5A snRNA. *Blood*, *113*(25), 6461–6464. https://doi.org/10.1182/blood-2009-03-207613

Pinotti, M., Rizzotto, L., Balestra, D., Lewandowska, M. A., Cavallari, N., Marchetti, G., Bernardi, F., & Pagani, F. (2008). U1-snRNA mediated rescue of mRNA processing in severe factor VII deficiency. *Blood*, *111*(5), 2681–2684. https://doi.org/10.1182/blood-2007-10-117440

Pistoni, M., Ghigna, C., & Gabellini, D. (2010). Alternative splicing and muscular dystrophy. In *RNA Biology* (Vol. 7, Issue 4, pp. 441–452). Taylor and Francis Inc. https://doi.org/10.4161/rna.7.4.12258

Piva, F., Giulietti, M., Nocchi, L., & Principato, G. (2009). SpliceAid: A database of experimental RNA target motifs bound by splicing proteins in humans. *Bioinformatics*, *25*(9), 1211–1213. https://doi.org/10.1093/bioinformatics/btp124

Pradella, D., Naro, C., Sette, C., & Ghigna, C. (2017). EMT and stemness: Flexible processes tuned by alternative splicing in development and cancer progression. In *Molecular Cancer* (Vol. 16, Issue 1, p. 8). BioMed Central. https://doi.org/10.1186/s12943-016-0579-2

Pranke, I. M., Golec, A., Hinzpeter, A., Edelman, A., & Sermet-Gaudelus, I. (2019). Emerging therapeutic approaches for cystic fibrosis. From gene editing to personalized medicine. In *Frontiers in Pharmacology* (Vol. 10, Issue FEB). Frontiers Media S.A. https://doi.org/10.3389/fphar.2019.00121

Pranke, I. M., Hatton, A., Simonin, J., Jais, J. P., Le Pimpec-Barthes, F., Carsin, A., Bonnette, P., Fayon, M., Stremler-Le Bel, N., Grenet, D., Thumerel, M., Mazenq, J., Urbach, V., Mesbahi, M., Girodon-Boulandet, E., Hinzpeter, A., Edelman, A., & Sermet-Gaudelus, I. (2017). Correction of CFTR function in nasal epithelial cells from cystic fibrosis patients predicts improvement of respiratory function by CFTR modulators. *Scientific Reports*, *7*(1), 1–11. https://doi.org/10.1038/s41598-017-07504-1

Ratjen, F., Bell, S. C., Rowe, S. M., Goss, C. H., Quittner, A. L., & Bush, A. (2015). Cystic fibrosis. In *Nature reviews. Disease primers* (Vol. 1, p. 15010). NIH Public Access. https://doi.org/10.1038/nrdp.2015.10

Reese, M. G. (1997). Improved splice site detection in Genie. *Journal of Computational Biology*, *4*(3), 311–323. https://doi.org/10.1089/cmb.1997.4.311

Reeves, E. P., Molloy, K., Pohl, K., & McElvaney, N. G. (2012). Hypertonic saline in treatment of pulmonary disease in cystic fibrosis. In *The Scientific World Journal* (Vol. 2012). https://doi.org/10.1100/2012/465230

Rendine, S., Calafell, F., Cappello, N., Gagliardini, R., Caramia, G., Rigillo, N., Silvetti, M., Zanda, M., Miano, A., Battistini, F., Marianelli, L., Taccetti, G., Diana, M. C., Romano, L., Romano, C., Giunta, A., Padoan, R., Pianaroli, A., Raia, V., … Piazza, A. (1997). Genetic history of cystic fibrosis mutations in Italy. I. Regional distribution. *Annals of Human Genetics*, *61*(5), 411–424. https://doi.org/10.1046/j.1469-1809.1997.6150411.x

Robberson, B. L., Cote, G. J., & Berget, S. M. (1990). Exon definition may facilitate splice site selection in RNAs with multiple exons. *Molecular and Cellular Biology*, *10*(1), 84–94. https://doi.org/10.1128/mcb.10.1.84

Rogalska, M. E., Tajnik, M., Licastro, D., Bussani, E., Camparini, L., Mattioli, C., & Pagani, F. (2016). Therapeutic activity of modified U1 core spliceosomal particles. *Nature Communications*, *7*, 1–13. https://doi.org/10.1038/ncomms11168

Roomans, G. M. (2014). Pharmacological treatment of the basic defect in cystic fibrosis. *Cell Biology International*, *38*(11), 1244–1246. https://doi.org/10.1002/cbin.10312

Rosen, C., Shezen, E., Aronovich, A., Klionsky, Y. Z., Yaakov, Y., Assayag, M., Biton, I. E., Tal, O., Shakhar, G., Ben-Hur, H., Shneider, D., Vaknin, Z., Sadan, O., Evron, S., Freud, E., Shoseyov, D., Wilschanski, M., Berkman, N., Fibbe, W. E., … Reisner, Y. (2015). Preconditioning allows engraftment of mouse and human embryonic lung cells, enabling lung repair in mice. *Nature Medicine*, *21*(8), 869–879. https://doi.org/10.1038/nm.3889

Rossbach, O., Hung, L. H., Khrameeva, E., Schreiner, S., Koñig, J., Curk, T., Zupan, B., Ule, J., Gelfand, M. S., & Bindereif, A. (2014). Crosslinking-immunoprecipitation (iCLIP) analysis reveals global regulatory roles of hnRNP L. *RNA Biology*, *11*(2), 146–155. https://doi.org/10.4161/rna.27991

Rubin, B. K. (2018). Unmet needs in cystic fibrosis. In *Expert Opinion on Biological Therapy* (Vol. 18, Issue sup1, pp. 49–52). Taylor and Francis Ltd. https://doi.org/10.1080/14712598.2018.1484101

Salgado-Garrido, J., Bragado-Nilsson, E., Kandels-Lewis, S., & Séraphin, B. (1999). Sm and Sm-like proteins assemble in two related complexes of deep evolutionary origin. *EMBO Journal*, *18*(12), 3451–3462. https://doi.org/10.1093/emboj/18.12.3451

Sato, T., Stange, D. E., Ferrante, M., Vries, R. G. J., Van Es, J. H., Van Den Brink, S., Van Houdt, W. J., Pronk, A., Van Gorp, J., Siersema, P. D., & Clevers, H. (2011). Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology*, *141*(5), 1762–1772. https://doi.org/10.1053/j.gastro.2011.07.050

Sato, T., Vries, R. G., Snippert, H. J., Van De Wetering, M., Barker, N., Stange, D. E., Van Es, J. H., Abo, A., Kujala, P., Peters, P. J., & Clevers, H. (2009). Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature*, *459*(7244), 262–265. https://doi.org/10.1038/nature07935

Schmid, M., & Jensen, T. H. (2008). Quality control of mRNP in the nucleus. In *Chromosoma* (Vol. 117, Issue 5, pp. 419–429). Springer. https://doi.org/10.1007/s00412-008-0166-4

Schram, C. A. (2012). Atypical cystic fibrosis: identification in the primary care setting. *Canadian Family Physician Medecin de Famille Canadien*, *58*(12), 1341–1345, e699-704. http://www.ncbi.nlm.nih.gov/pubmed/23242890

Schramm, L., & Hernandez, N. (2002). Recruitment of RNA polymerase III to its target promoters. In *Genes and Development* (Vol. 16, Issue 20, pp. 2593–2620). Genes Dev. https://doi.org/10.1101/gad.1018902

Scorilas, A. (2002). Polyadenylate polymerase (PAP) and 3′ end pre-mRNA

processing: Function, assays, and association with disease. In *Critical Reviews in Clinical Laboratory Sciences* (Vol. 39, Issue 3, pp. 193–224). https://doi.org/10.1080/10408360290795510

Seibert, F. S., Chang, X. B., Aleksandrov, A. A., Clarke, D. M., Hanrahan, J. W., & Riordan, J. R. (1999). Influence of phosphorylation by protein kinase A on CFTR at the cell surface and endoplasmic reticulum. In *Biochimica et Biophysica Acta - Biomembranes*. https://doi.org/10.1016/S0005-2736(99)00163-7

Sheppard, M. N., & Nicholson, A. G. (2002). The pathology of cystic fibrosis. In *Current Diagnostic Pathology* (Vol. 8, Issue 1, pp. 50–59). Churchill Livingstone. https://doi.org/10.1054/cdip.2001.0088

Sheth, N., Roca, X., Hastings, M. L., Roeder, T., Krainer, A. R., & Sachidanandam, R. (2006). Comprehensive splice-site analysis using comparative genomics. *Nucleic Acids Research*, *34*(14), 3955–3967. https://doi.org/10.1093/nar/gkl556

Sillekens, P. T. G., Beijer, R. P., Habets, W. J., & van Venrooij, W. J. (1988). Human UL snRNP-specific C protein: Complete cDNA and protein sequence and identification of a multigene family in mammals. *Nucleic Acids Research*, *16*(17), 8307–8321. https://doi.org/10.1093/nar/16.17.8307

Singh, N. N., Del Rio-Malewski, J. B., Luo, D., Ottesen, E. W., Howell, M. D., & Singh, R. N. (2017). Activation of a cryptic 5 splice site reverses the impact of pathogenic splice site mutations in the spinal muscular atrophy gene. *Nucleic Acids Research*, *45*(21), 12214–12240. https://doi.org/10.1093/nar/gkx824

Singh, R. N., & Singh, N. N. (2019). A novel role of U1 snRNP: Splice site selection from a distance. In *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms* (Vol. 1862, Issue 6, pp. 634–642). Elsevier B.V. https://doi.org/10.1016/j.bbagrm.2019.04.004

Spritz, R. A., Strunk, K., Surowy, C. S., & Mohrenweiser, H. W. (1990). Human U1-70K ribonucleoprotein antigen gene: Organization, nucleotide sequence, and mapping to locus 19q13.3. *Genomics*, *8*(2), 371–379. https://doi.org/10.1016/0888-7543(90)90295-6

Staffa, A., & Cochrane, A. (1995). Identification of positive and negative splicing

regulatory elements within the terminal tat-rev exon of human immunodeficiency virus type 1. *Molecular and Cellular Biology*. https://doi.org/10.1128/mcb.15.8.4597

Stoltz, D. A., Meyerholz, D. K., & Welsh, M. J. (2015). Origins of Cystic Fibrosis Lung Disease. *New England Journal of Medicine*, *372*(4), 351–362. https://doi.org/10.1056/nejmra1300109

Strug, L. J., Stephenson, A. L., Panjwani, N., & Harris, A. (2018). Recent advances in developing therapeutics for cystic fibrosis. *Human Molecular Genetics*, *27*(R2), R173–R186. https://doi.org/10.1093/hmg/ddy188

Su, C. H., Dhananjaya, D., & Tarn, W. Y. (2018). Alternative splicing in neurogenesis and brain development. In *Frontiers in Molecular Biosciences* (Vol. 5, Issue FEB, p. 12). Frontiers Media S.A. https://doi.org/10.3389/fmolb.2018.00012

Suñé-Pou, M., Limeres, M. J., Moreno-Castro, C., Hernández-Munain, C., Suñé-Negre, J. M., Cuestas, M. L., & Suñé, C. (2020). Innovative Therapeutic and Delivery Approaches Using Nanotechnology to Correct Splicing Defects Underlying Disease. In *Frontiers in Genetics* (Vol. 11, p. 731). Frontiers Media S.A. https://doi.org/10.3389/fgene.2020.00731

Susani, L., Pangrazio, A., Sobacchi, C., Taranta, A., Mortier, G., Savarirayan, R., Villa, A., Orchard, P., Vezzoni, P., Albertini, A., Frattini, A., & Pagani, F. (2004). TCIRG1-dependent recessive osteopetrosis: Mutation analysis, functional identification of the splicing defects, and in vitro rescue by U1 snRNA. *Human Mutation*, *24*(3), 225–235. https://doi.org/10.1002/humu.20076

Tajnik, M., Rogalska, M. E., Bussani, E., Barbon, E., Balestra, D., Pinotti, M., & Pagani, F. (2016). Molecular Basis and Therapeutic Strategies to Rescue Factor IX Variants That Affect Splicing and Protein Function. *PLoS Genetics*, *12*(5), 1–16. https://doi.org/10.1371/journal.pgen.1006082

Tang, Y.-Y., Hölzel, B. K., & Posner, M. I. (2015). The neuroscience of mindfulness meditation. *Nature Reviews Neuroscience*, *16*(4), 213–225. https://doi.org/10.1038/nrn3916

Tanner, G., Glaus, E., Barthelmes, D., Ader, M., Fleischhauer, J., Pagani, F., Berger,

W., & Neidhardt, J. (2009). Therapeutic strategy to rescue mutation-induced exon skipping in rhodopsin by adaptation of U1 snRNA. *Human Mutation*, *30*(2), 255–263. https://doi.org/10.1002/humu.20861

Terns, M. P., Dahlberg, J. E., & Lund, E. (1993). Multiple cis-acting signals for export of pre-U1 snRNA from the nucleus. *Genes and Development*, *7*(10), 1898–1908. https://doi.org/10.1101/gad.7.10.1898

Valerio, L. G. (2014). Tenth anniversary of Expert Opinion on Drug Metabolism & Toxicology. *Expert Opinion on Drug Metabolism & Toxicology*, *10*(6), 767–768. https://doi.org/10.1517/17425255.2014.920007

Van Goor, F., Hadida, S., Grootenhuis, P. D. J., Burton, B., Cao, D., Neuberger, T., Turnbull, A., Singh, A., Joubran, J., Hazlewood, A., Zhou, J., McCartney, J., Arumugam, V., Decker, C., Yang, J., Young, C., Olson, E. R., Wine, J. J., Frizzell, R. A., … Negulescu, P. (2009). Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(44), 18825–18830. https://doi.org/10.1073/pnas.0904709106

Veit, G., Avramescu, R. G., Chiang, A. N., Houck, S. A., Cai, Z., Peters, K. W., Hong, J. S., Pollard, H. B., Guggino, W. B., Balch, W. E., Skach, W. R., Cutting, G. R., Frizzell, R. A., Sheppard, D. N., Cyr, D. M., Sorscher, E. J., Brodsky, J. L., & Lukacs, G. L. (2016). From CFTR biology toward combinatorial pharmacotherapy: Expanded classification of cystic fibrosis mutations. *Molecular Biology of the Cell*, *27*(3), 424–433. https://doi.org/10.1091/mbc.E14-04-0935

Vithana, E. N., Abu-Safieh, L., Allen, M. J., Carey, A., Papaioannou, M., Chakarova, C., Al-Maghtheh, M., Ebenezer, N. D., Willis, C., Moore, A. T., Bird, A. C., Hunt, D. M., & Bhattacharya, S. S. (2001). A human homolog of yeast pre-mRNA splicing gene, PRP31, underlies autosomal dominant retinitis pigmentosa on chromosome 19q13.4 (RP11). *Molecular Cell*, *8*(2), 375–381. http://www.ncbi.nlm.nih.gov/pubmed/11545739

Wahle, E., & Keller, W. (1992). The Biochemistry of 3′-End Cleavage and

Polyadenylation of Messenger RNA Precursors. *Annual Review of Biochemistry*, *61*(1), 419–438. https://doi.org/10.1146/annurev.bi.61.070192.002223

Wallis, C. (1997). Diagnosing cystic fibrosis: Blood, sweat, and tears. In *Archives of Disease in Childhood* (Vol. 76, Issue 2, pp. 85–88). BMJ Publishing Group. https://doi.org/10.1136/adc.76.2.85

Wang, H. Y., Lin, W., Dyck, J. A., Yeakley, J. M., Songyang, Z., Cantley, L. C., & Fu, X. D. (1998). SRPK2: A differentially expressed SR protein-specific kinase involved in mediating the interaction and localization of pre-mRNA splicing factors in mammalian cells. *Journal of Cell Biology*, *140*(4), 737–750. https://doi.org/10.1083/jcb.140.4.737

Wang, Y., Liu, J., Huang, B., Xu, Y.-M., Li, J., Huang, L.-F., Lin, J., Zhang, J., Qing-Hua, M., Yang, W.-M., & Wang, X.-Z. (2015). Mechanism of alternative splicing and its regulation. *Biomedical Reports*, *3*(2), 152–158. https://doi.org/10.3892/br.2014.407

Welsh, M. J., & Smith, A. E. (1993). Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. In *Cell* (Vol. 73, Issue 7, pp. 1251–1254). Cell. https://doi.org/10.1016/0092-8674(93)90353-R

Will, C. L., & Lührmann, R. (2011). Spliceosome structure and function. *Cold Spring Harbor Perspectives in Biology*, *3*(7), 1–2. https://doi.org/10.1101/cshperspect.a003707

Wimmer, K., Roca, X., Beiglböck, H., Callens, T., Etzler, J., Rao, A. R., Krainer, A. R., Fonatsch, C., & Messiaen, L. (2007). Extensive in silico analysis of NF1 splicing defects uncovers determinants for splicing outcome upon 5′ splice-site disruption. *Human Mutation*, *28*(6), 599–612. https://doi.org/10.1002/humu.20493

Woychik, N. A., & Hampsey, M. (2002). The RNA polymerase II machinery: Structure illuminates function. In *Cell* (Vol. 108, Issue 4, pp. 453–463). Cell Press. https://doi.org/10.1016/S0092-8674(02)00646-3

Wu, H. xia, Zhu, M., Xiong, X. feng, Wei, J., Zhuo, K. quan, & Cheng, D. yun. (2019). Efficacy and Safety of CFTR Corrector and Potentiator Combination Therapy in

Patients with Cystic Fibrosis for the F508del-CFTR Homozygous Mutation: A Systematic Review and Meta-analysis. *Advances in Therapy*, *36*(2), 451–461. https://doi.org/10.1007/s12325-018-0860-4

Xiao, S. H., & Manley, J. L. (1998). Phosphorylation-dephosphorylation differentially affects activities of splicing factor ASF/SF2. *EMBO Journal*, *17*(21), 6359–6367. https://doi.org/10.1093/emboj/17.21.6359

Yeh, H. I., Sohma, Y., Conrath, K., & Hwang, T. C. (2017). A common mechanism for CFTR potentiators. *Journal of General Physiology*, *149*(12), 1105–1118. https://doi.org/10.1085/jgp.201711886

Zheng, S., & Black, D. L. (2013). Alternative pre-mRNA splicing in neurons: Growing up and extending its reach. In *Trends in Genetics* (Vol. 29, Issue 8, pp. 442–448). Trends Genet. https://doi.org/10.1016/j.tig.2013.04.003

Zuccato, E., Buratti, E., Stuani, C., Baralle, F. E., & Pagani, F. (2004). An Intronic Polypyrimidine-rich Element Downstream of the Donor Site Modulates Cystic Fibrosis Transmembrane Conductance Regulator Exon 9 Alternative Splicing. *Journal of Biological Chemistry*, *279*(17), 16980–16988. https://doi.org/10.1074/jbc.M313439200

7. Supplementary material

7.1. FANCA and Factor IX original sequences

7.1.1. FANCA exon VIII

CATATGctgcagcacccgcccctctgaagtggatggtctgtgccattcacagttgc
ttggagaccatgatgtccataaatAactttggttttatggtttttcagATTTTGTT
CAAATGTTTGTTTTGAGGGGATTTCAGAAAAACTCAGATCTGAGAAGAACTGTGGA
GCCTGAAAAAATGCCGTAGgtaggaggaaaagtcaagtgaagagtttagcggaagg
agccgtccttattccaagtgttgaaatgtatttacgtgtctctctattgaaacgtg
ctgtttgtacctatttacggggtacatgtgatacttgttttgtgcatagagtgtaa
tgatcaagtcagggtgtttagagtctccgtcacctccagtatgtatcatttctgtg
tgttggcgaattCATATG

7.1.2. Factor IX exon V

CATATGttatacattaataaatagggtttttaaacctgtagttcataatttagtga
aagtagaatatccaaacatttagttttaaaccaatcaattatagtgctaccatcat
ttttatgcattattgagaagtttattttacctttctttccactcttatttcaaggc
tccaaaatttctctccccaacgtatattgggggcaacatgaatgcccccaatgtat
atttgacccatacatgagtcagtagttccatgtacttttagaaatgcatgttaaa
tgatgctgttactgtctattttgcttcttttagATGTAACATGTAACATTAAGAAT
GGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATAACAAGGTGGTTTGCTCCTG
TACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTGAACCAGCAGgtcata
atctgaataagatttttaaagaaaatctgtatctgaaacttcagcattttaacaa
acctacataattttaattcctacttgaatctgcttccttttgaaatcatagaaaat
atcagtagcttgaattagaccaattaatttCATATG

7.1.3. **Hybrid minigenes and ExSpeU1s**

Hybrid minigenes were all composed by a FANCA sequence segment conjoint with a Factor IX sequence segment, according to the experimental needs. Details in the composition were previously designated. As described before, ExSpeU1s were used from previous studies, thus their sequences are available online (Alanis et al., 2012; Mattioli et al., 2014).

7.2. **CFTR sequences with ExSpeU1s**

7.2.1. **CFTR Exon 5**

CATATGctgttgaaagaaacatttatgaacctgagaagatagtaagctagatgaat
agaatataattttcattacctttacttaataatgaatgcataataactgaattagt
catattataattttacttataatatatttgtattttgtttgttgaaattatctaac
tttccatttttcttttagACTTTAAAGCTGTCAAGCCGTGTTCTAGATAAAATAAG
TATTGGACAACTTGTTAGTCTCCTTTCCAACAACCTGAACAAATTTGATGAAgtat
gtacctattgatttaatcttttaggcactattgttataaattatacaactggaaag
gcggagtttcctgggtcagataatagtaattagtggttaagtcttgctcagctct
agcttccctattctggaaactaagaaaggtcaattgtatagcagagcaccattctg
gggtctggCATATG

7.2.2. **CFTR exon 10**

CATATGgggccgctctaggActtgataatgggcaaatatcttagtttagatcatgt
cctctagaaaccgtatgctatataatatgtactataaagtaataatgtatacagtg
taatggatcatgggccatgtgcttttcaaactaattgtacataaaacaagcatcta

ttgaaaatatctgacaaactcatctttatttttgatgtgtgtgtgtgtgtgtgtg
tgtgtttttttaacagGGATTTGGGGAATTCTTTGAGAAAGCAAAACAAAACAATA
ACAATAGAAAAACTTCTAATGGTGATGACAGCCTCTTCTTCAGTAATTTCTCACTT
CTTGGTACTCCTGTCCTGAAAGATATTAATTTCAAGATAGAAAGAGGACAGTTGTT
GGCGGTTGCTGGATCCACTGGAGCAGGCAAGgtagttcttttgttcttcactatta
agaacttaatttggtgtccatgtctctttttttttctagtttgtagtgctggaagg
tatttttggagaaattcttacatgagcattaggagaatgtatgggtgtagtgtctt
gtataatagaaattgttccactgataatttactctagtttttttatttcctcatatt
attttcagtggctttttcttccacatctttatattttgcaccacattcaacactgt
atcttgcacatggcgagCATATG

7.2.3. **CFTR exon 13**

CATATGctatggtacagttcagtttgtgacttttgctagtttatgccacttacagt
tagcaaaatcacttcagcagttcttggaatgttgtgaaaagtgataaaaatcttct
gcaacttattccttttattcctcatttaaaataatctaccatagtaaaaacatgtat
aaaagtgctacttctgcaccacttttgagaatagtgttatttcagtgaatcgatgt
ggtgaccatattgtaatgcatgtagtgaactgtttaaggcaaatcatctacactag
atgaccaggaaatagagaggaaatgtaatttaatttccattttctttttagAGCAG
TATACAAAGATGCTGATTTGTATTTATTAGACTCTCCTTTTGGATACCTAGATGTT
TTAACAGAAAAAGAAATATTTGAAAGgtatgttctttgaatTccttacttataatg
ctcatgctaaaataaaagaaagacagactgtcccatcatagattgcatttacctc
ttgagaaatatgttcaccattgttggtatggcagaatgtagcatggtattaactca
aatctgatctgccctactgggccaggattcaagattacttccattaaaacctttc
tcaccgcctcatgctaaaccagtttctctcattgctatactgttatagcaattgct
atctatgtagCATATG

7.2.4. **CFTR exon 16**

Splicing competent cDNA exon 16 WT and 2789+5G>A mutant minigenes (pcDNA5-CFex16) were previously described (Igreja et al., 2015).

7.2.5. **CFTR exon 18**

CATATGtaaaagacaataacttgaacacataattatttagaatgtttggaaagaaa
caaaaatttctaagtctatctgattctatttgctaattcttatttgggttctgaat
gcgtctactgtgatccaaacttagtattgaatatattgatatatctttaaaaaatt
agtgtttttgaggaatttgtcatcttgtatattatagGTGGGATTCTTAATAGAT
TCTCCAAAGATATAGCAATTTTGGATGACCTTCTGCCTCTTACCATATTTGACTTC
ATCCAGgtatgtaaaaataagtaccgttaagtatgtctgtattattaaaaaaacaa
taacaaaagcaaatgtgattttgttttcatttttatttgattgagggttgaagtc
ctgtctattgcattaattttgtaattatccaaagccttcaaaatagacataagttt
agtaaattcaataataagtcagaactgcttacctggccCATATG

7.2.6. **CFTR ExSpeU1s**

Exon	ExSpeU1	ExSpeU1 sequence (5'-3')	*CFTR* target sequence (5'-3')
5	U1ex5-9	GATTAAATCAATAG	CTATTGATTTAATC
	U1ex5-13	AAAAGATTAAATCA	TGATTTAATCTTTT
	U1ex5-21	TAGTGCCTAAAAGA	TCTTTTAGGCACTA
	U1ex5-37	GTATAATTTATAAC	TTATAAATTATACA
10	U1ex10(-3)	AGAACTACCTT	AAGGTAGTTCT
	U1ex10-12	ATAGTGAAGAAC	GTTCTTCACTAT
	U1ex10-26	CCAAATTAAGTTC	GAACTTAATTTGG
	U1ex10-34	CATGGACACCAAAT	ATTTGGTGTCCATG
13	U1ex13-1	TCAAAGAACATAC	GTATGTTCTTTGA
	U1ex13-9	TAAGGTATTCAAA	TTTGAATACCTTA
	U1ex13-11	ATAAGTAAGGTATTCA	TGAATACCTTACTTAT
	U1ex13-15	TTATAAGTAAGGTA	TACCTTACTTATAA
	U1ex13-33	CTCATGCTAAAATA	TATTTTAGCATGAG
16	U1ex16(-3)	ATTCACTTT	AAAGTGAaT
	U1ex16-7	ACATGGAAT	ATTCCATGT
	U1ex16-10	TCTACACAATAGGACATGG	CCATGTCCTATTGTGTAGA
	U1ex16-12	ATAGGACAT	ATGTCCTAT
	U1ex16-17	ACACAATAG	CTATTGTGT
18	U1ex18-4	CTTATTTTTACA	TGTAAAAATAAG
	U1ex18-10	ACGGTACTTATT	AATAAGTACCGT
	U1ex18-13	TTAACGGTACTT	AAGTACCGTTAA
	U1ex18-20	GACATACTTAAC	GTTAAGTATGTC

Supplementary table S1: Sequences of Exon Specific U1s used in this study together with the corresponding CFTR target sequences. From (Donegà et al., 2020)