ABERRANT mRNA SPLICING IN COAGULATION FACTOR DEFICIENCIES: FROM MOLECULAR MECHANISMS TO RNA-BASED THERAPEUTIC APPROACHES

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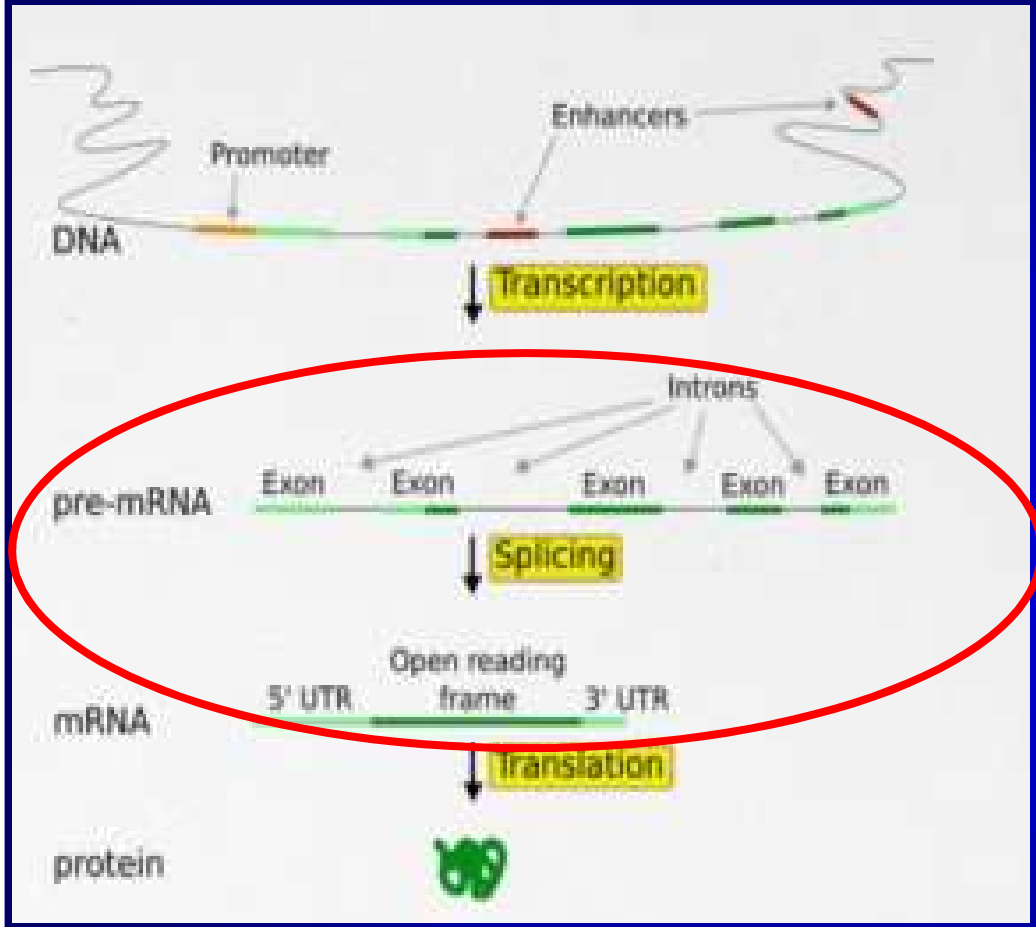
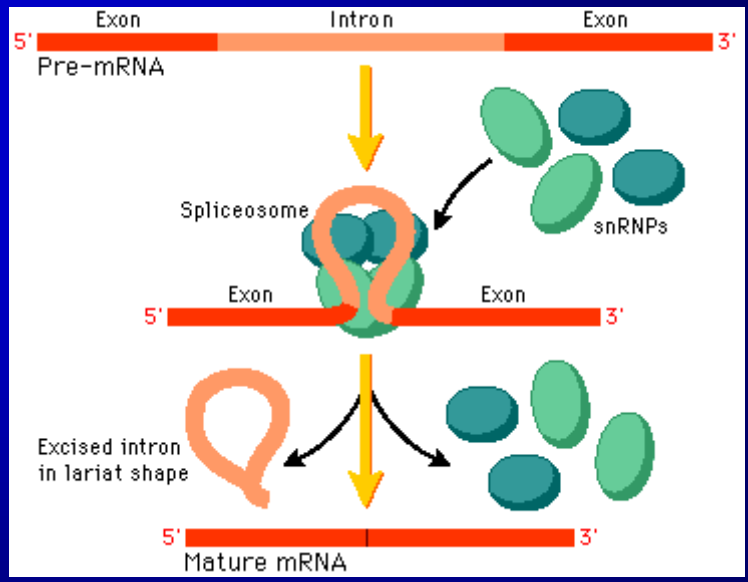
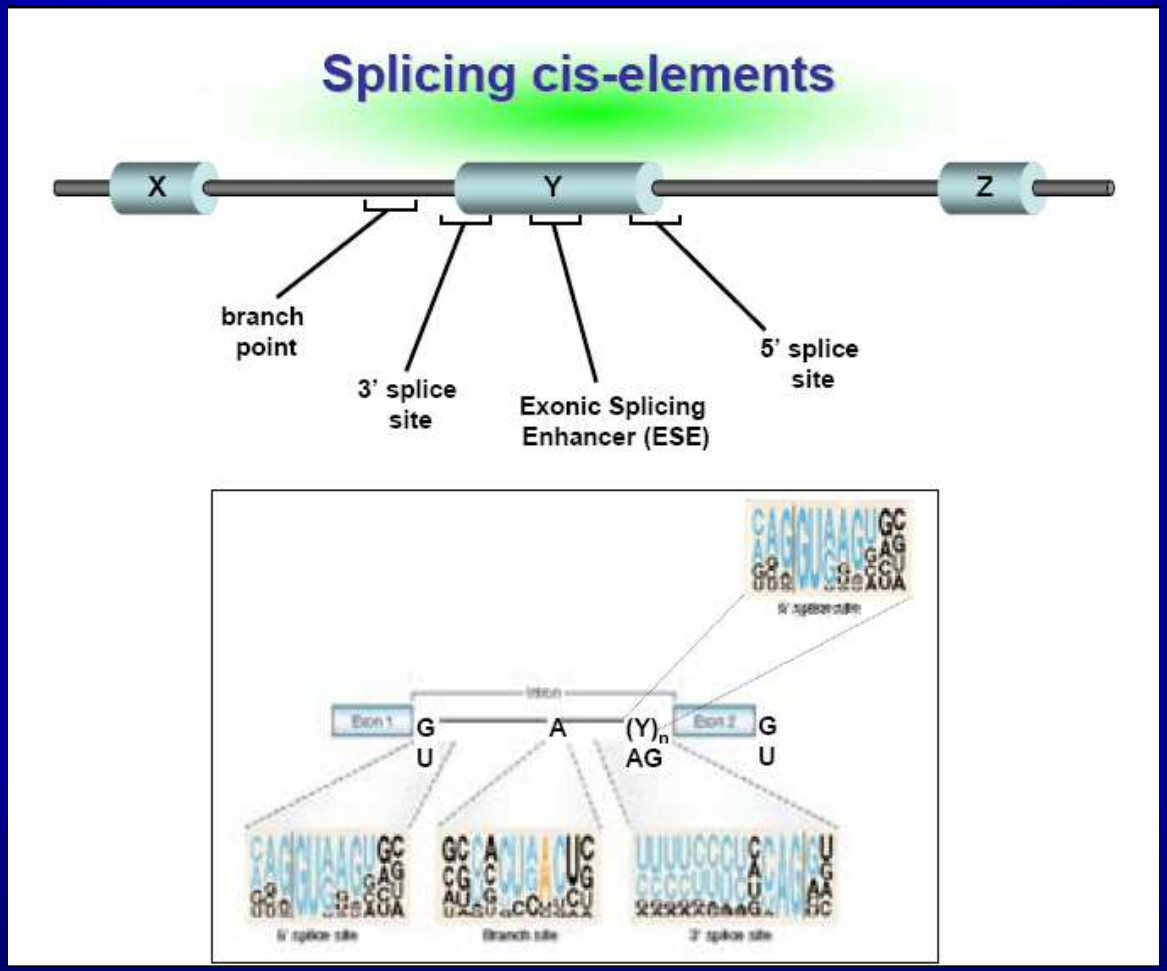
mRNA SPLICING

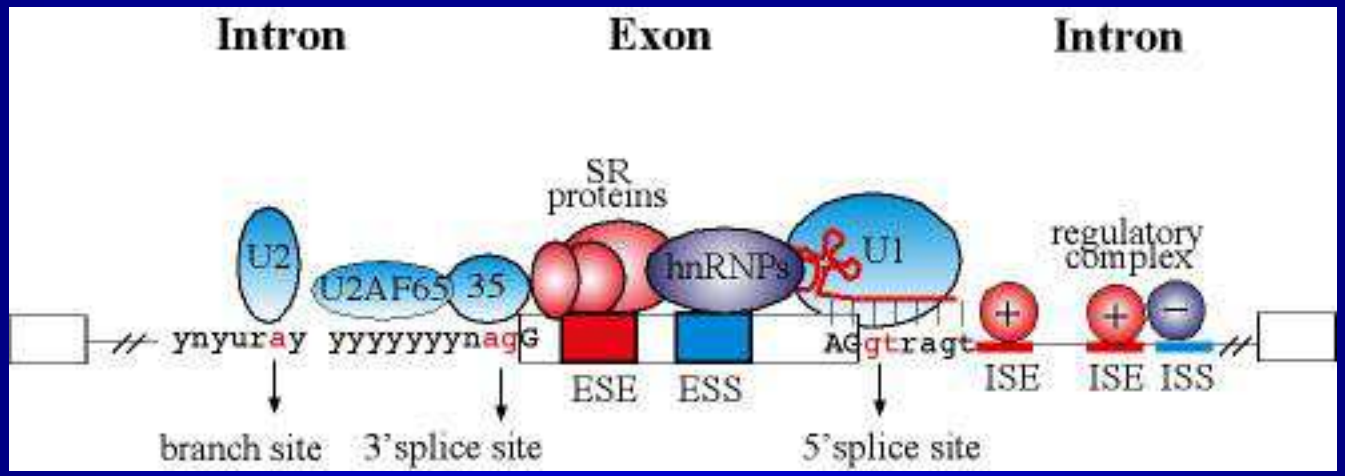


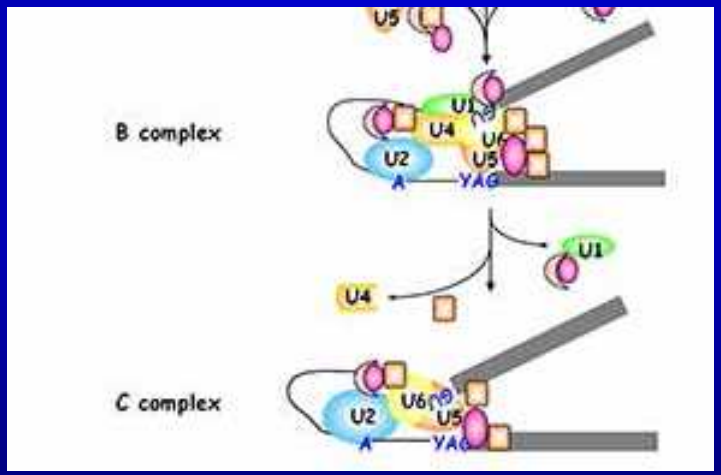


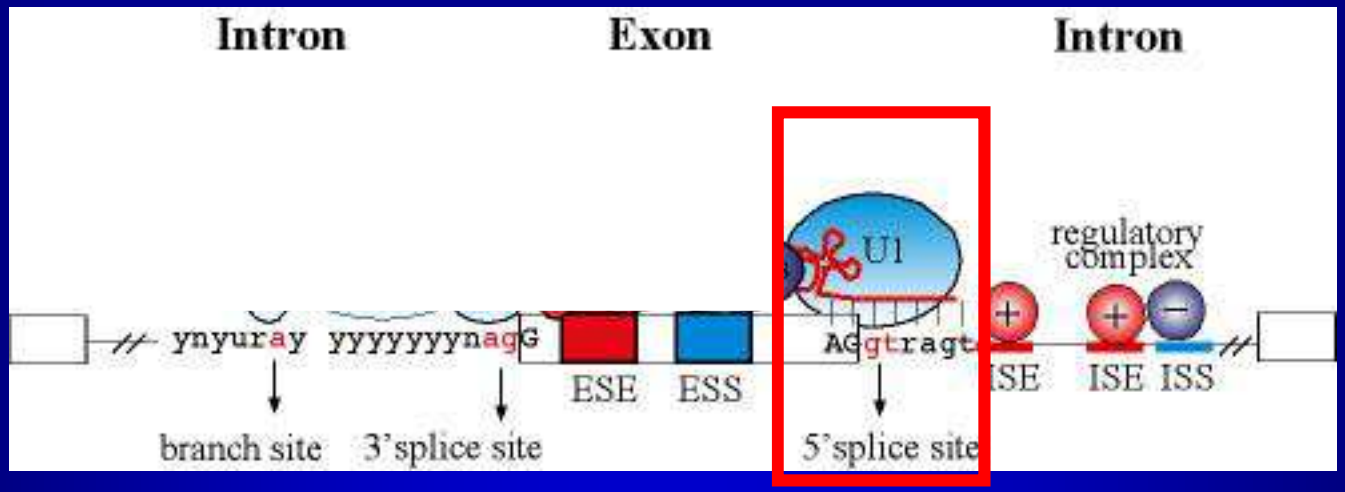
Several sequence elements,
both conserved and exon-specific,
are required for proper definition of the exons.

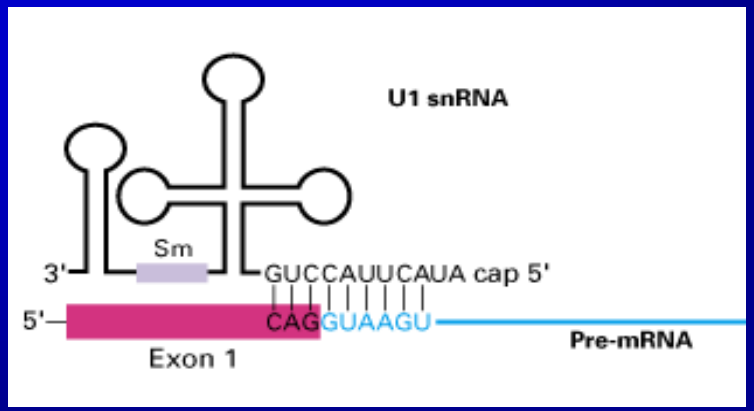


Sequence elements define the proper assembly of the complex Spliceosome machinery



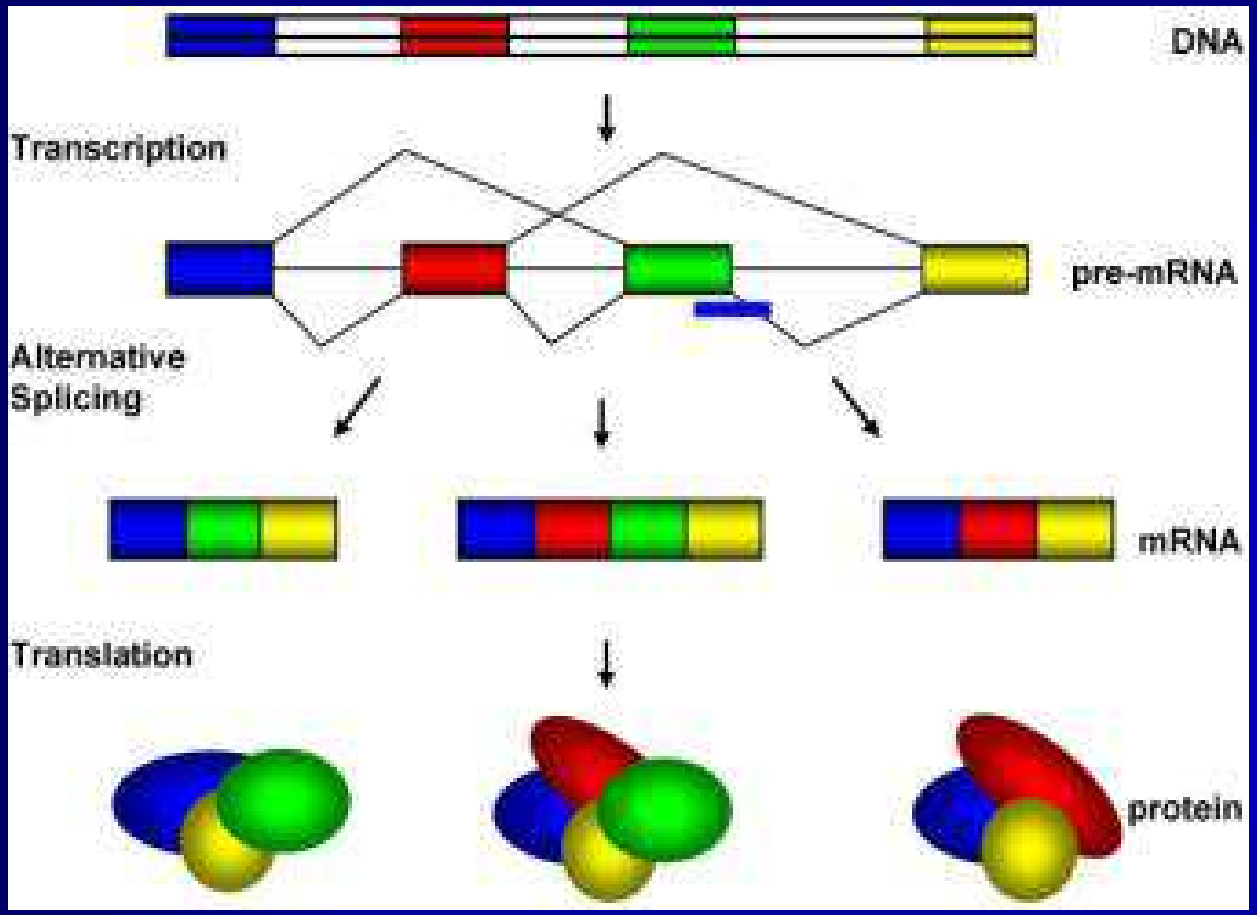






Key role of the U1snRNP in the earliest splicing step, and thus in exon definition

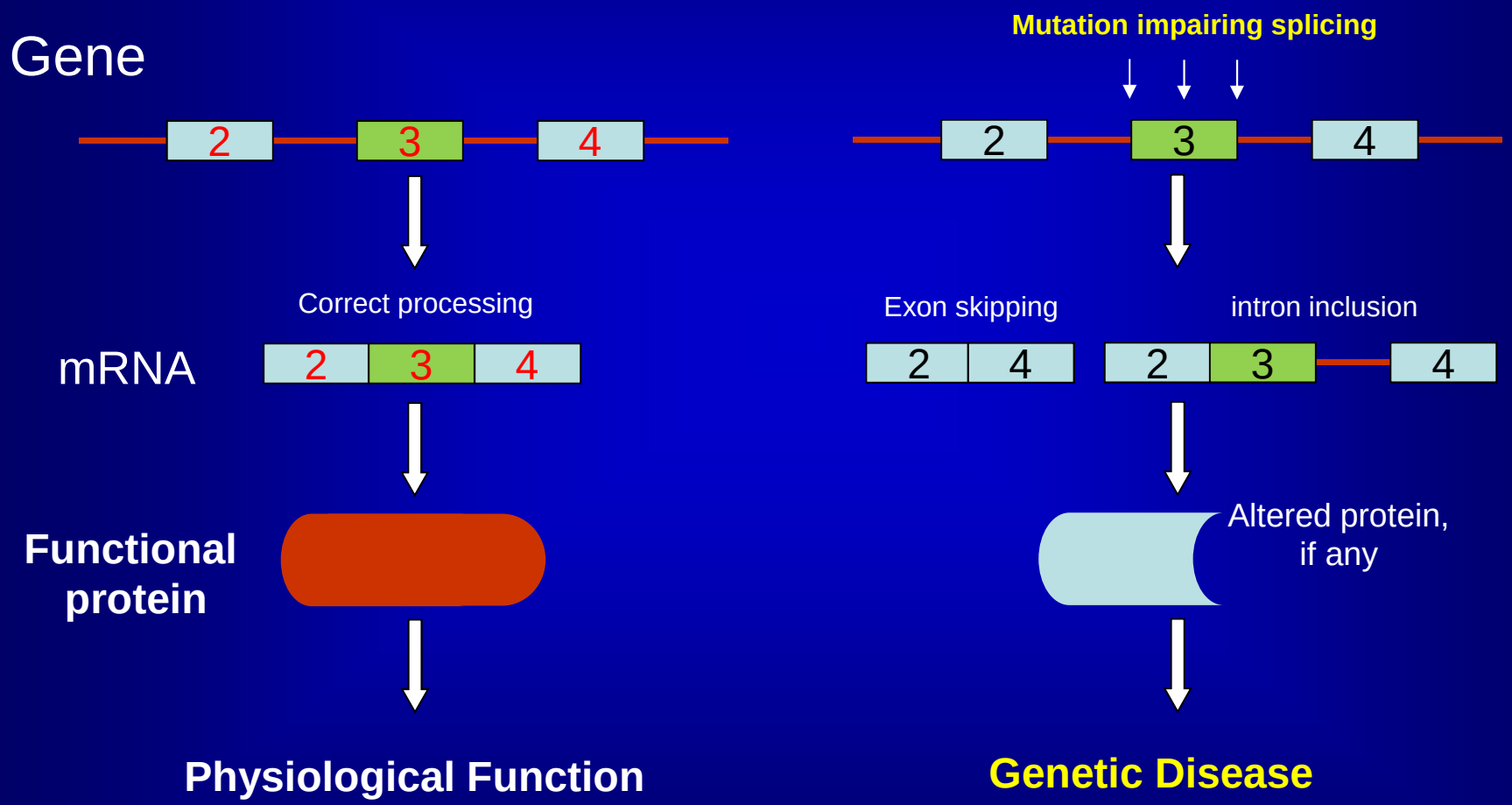
This complexity makes the spliceosome susceptible to derangements



Protein isoforms or unproductive splicing

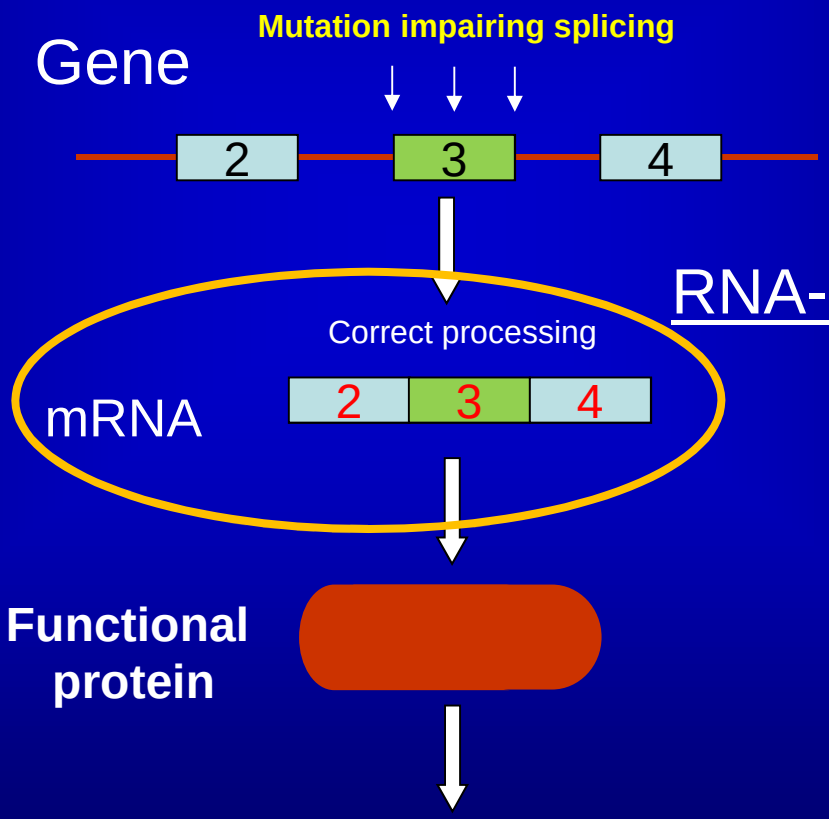
80% of human genes undergo alternative splicing

Mutations affecting mRNA splicing are frequent cause of severe human genetic disease forms (>20-30%)



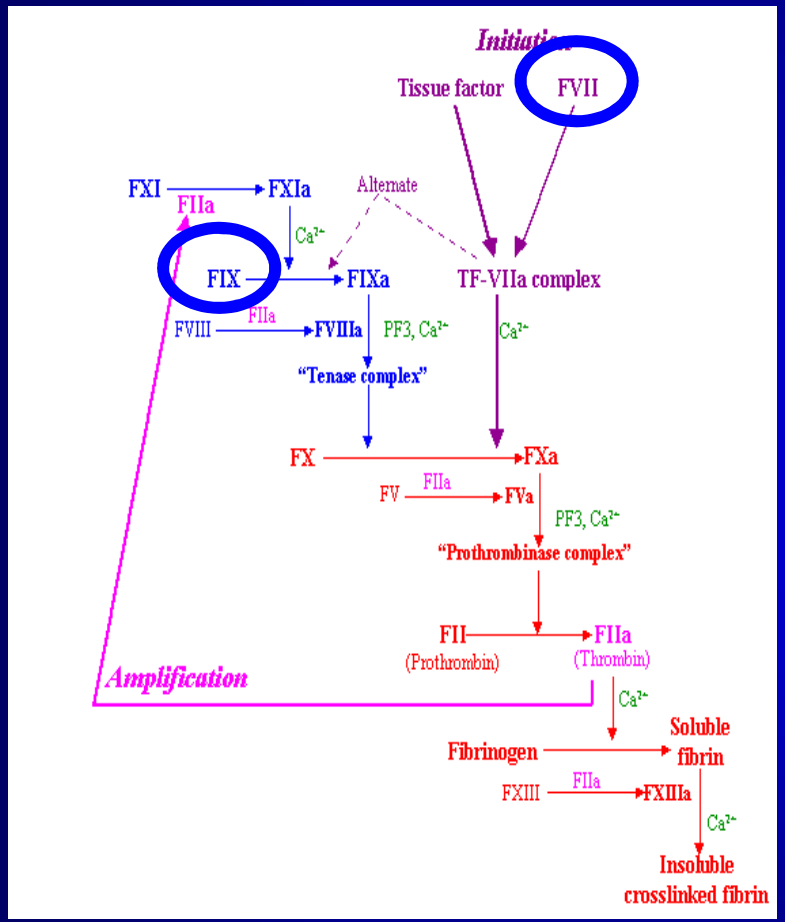
Molecular mechanisms of genetic disorders

Modulation of pre-mRNA splicing



RNA-based Therapeutic Approaches

Rescue of physiological function



Coagulation factor disorders as models

- splicing mutations are relatively frequent in severe coagulation factor deficiencies
- even tiny increase in activity of plasma proteins could significantly increase the coagulation efficiency and thus ameliorate the bleeding phenotype in patients
- protein and activity levels of clotting factors can be evaluated by enzymatic assays (hardly feasible for most of the other human diseases).

IVS7 +5G□A (FVII Lazio)

A

TGG / GTGG**G**TACC

IVS7 5'ss is in the 1st of highly homologous 37bp repeats containing identical **cryptic 5'ss (*)**

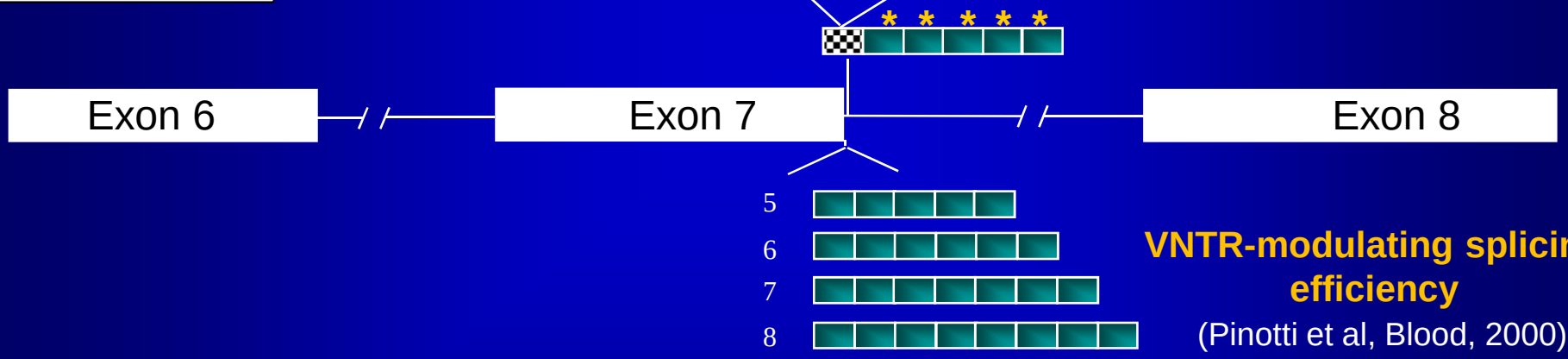
(Pinotti et al, Blood, 1998)

Exon 6 — Exon 7 — Exon 8

IVS7 +5G□A (FVII Lazio)

A

TGG / GTGG**G**TACC

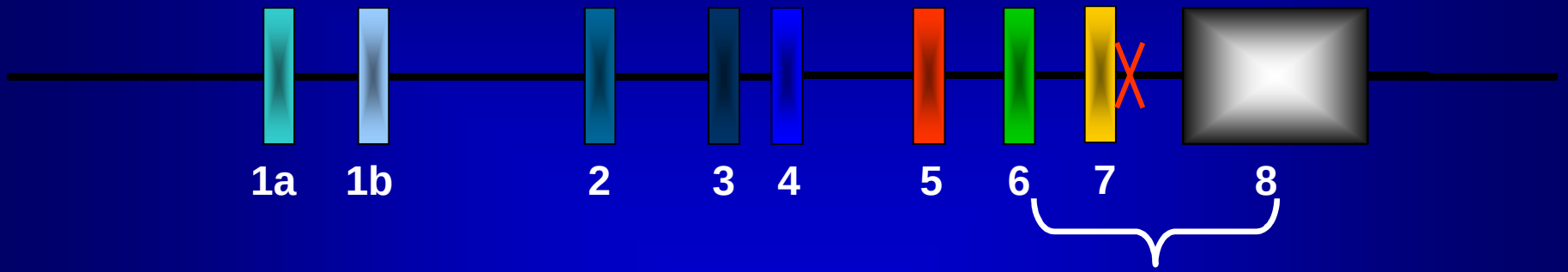


VNTR-modulating splicing efficiency

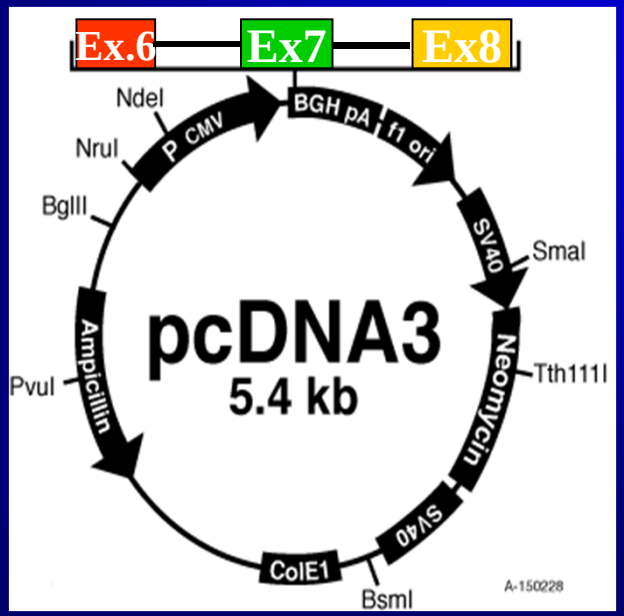
(Pinotti et al, Blood, 2000)

What’s the molecular mechanism of the IVS7+5G/A mutation?

Expression studies in eukaryotic cells: Minigene approach



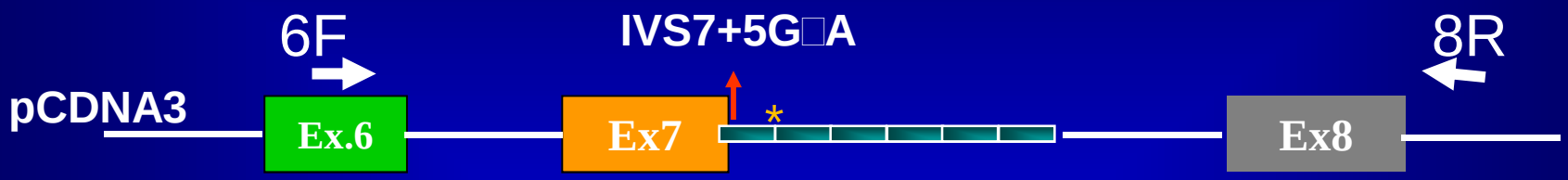
IVS7 Minigene (ex.6-8)

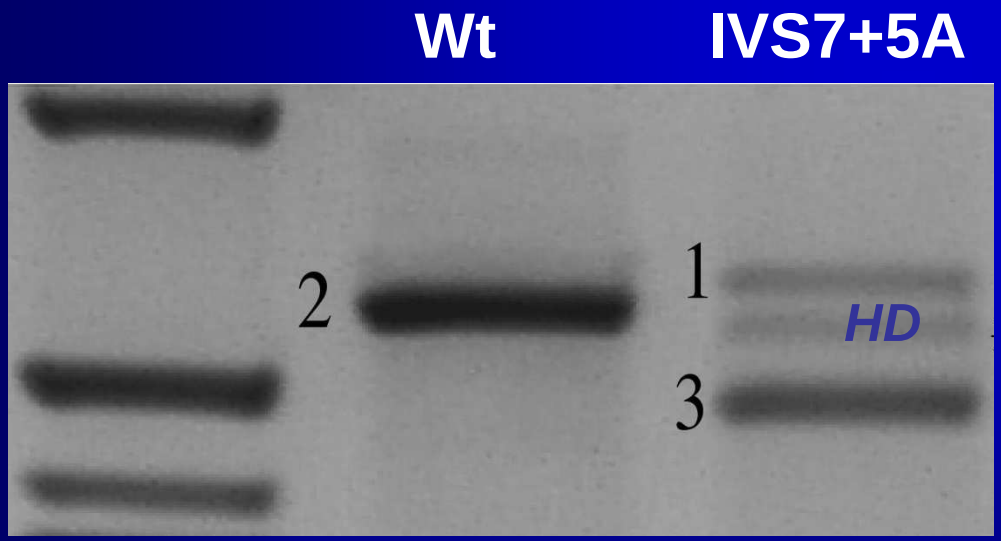


Expression in mammalian cells and studies at the mRNA level

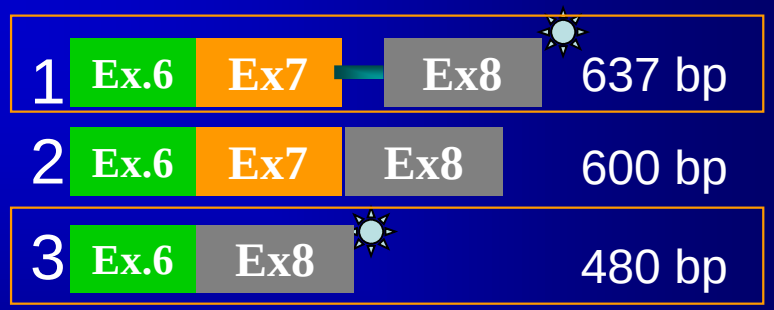
Pinotti et al, Blood 2008

The mutation induces exon skipping or 1° repeat inclusion





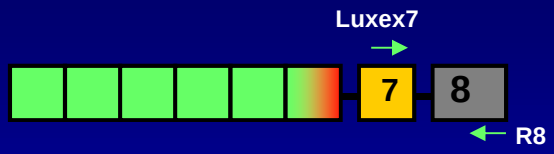
RT-PCR 6F-8R

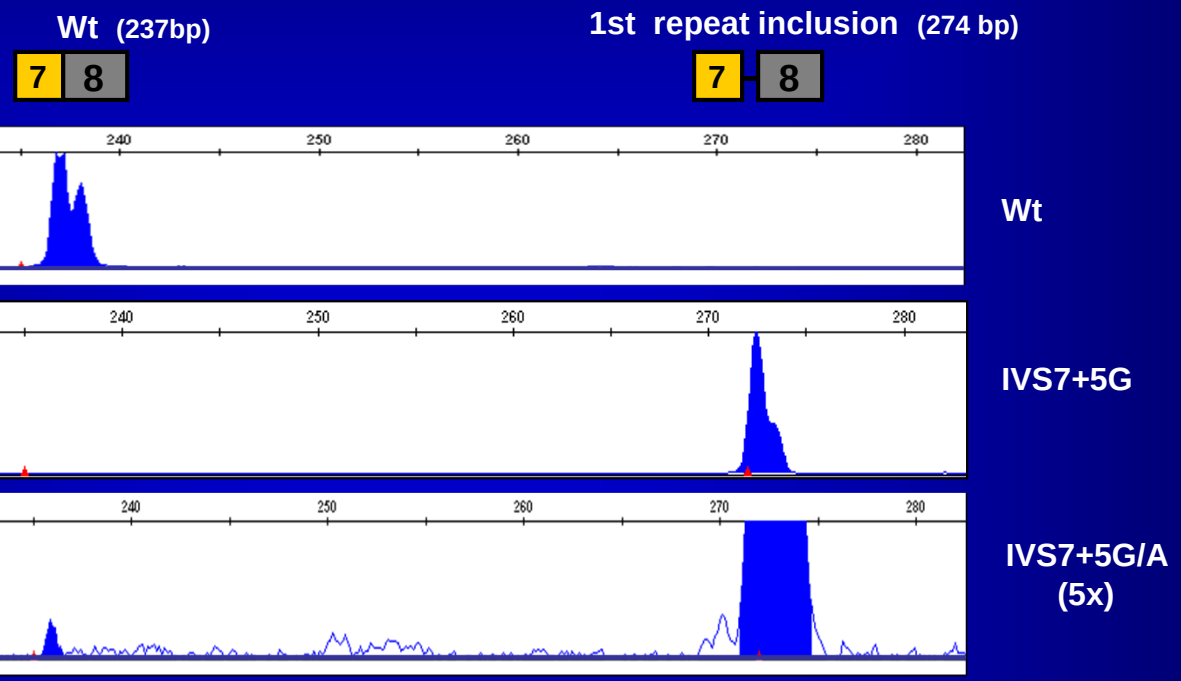


Causing frameshift and premature termination of translation

Pinotti et al, Blood 2008

Fluorescent labeling of RT-PCR products

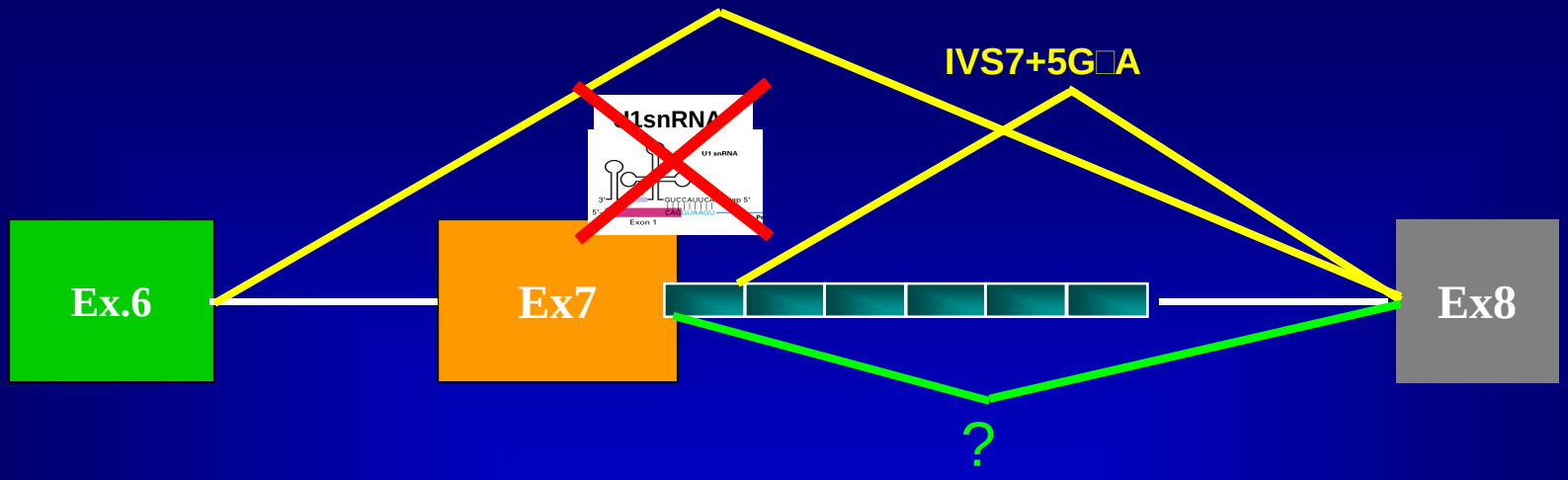




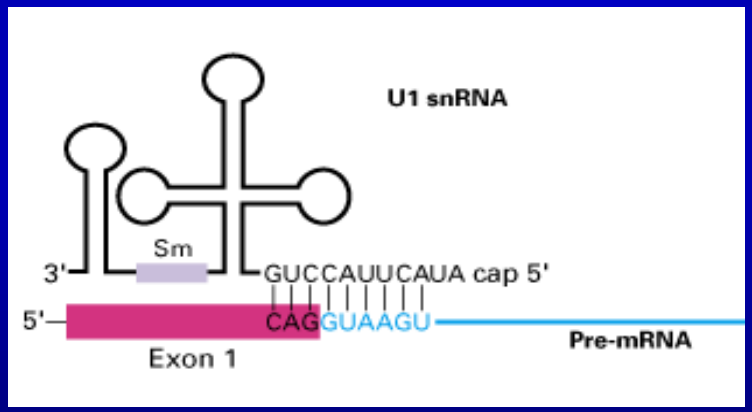
The mutation impairs but not abrogates splicing (0.3±0.1%),

thus accounting for residual FVII levels in patients

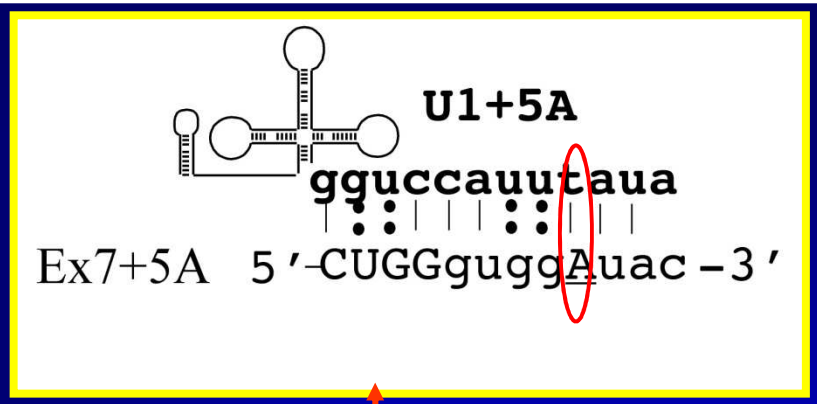
Pinotti et al, Blood 2008

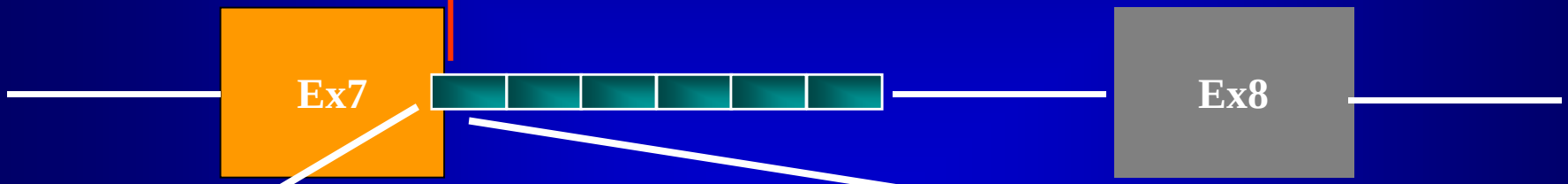


Restore exon definition by compensatory U1 snRNA changes



Could this rescue FVII splicing impaired by the IVS7+5G/A?



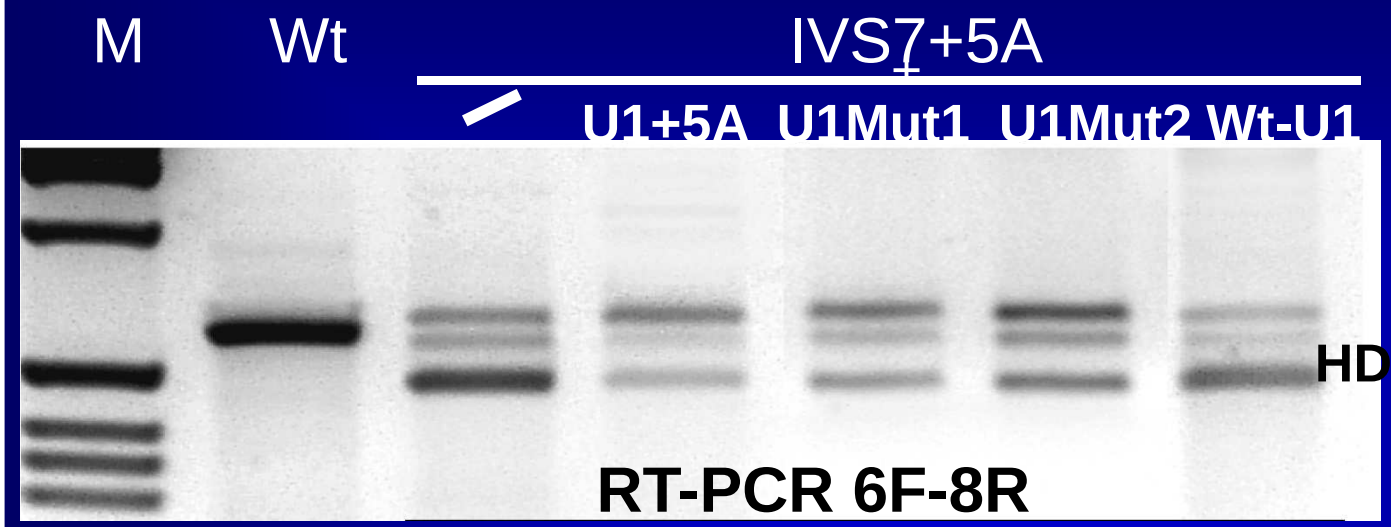


U1 mut1 U1+5A U1 mut2

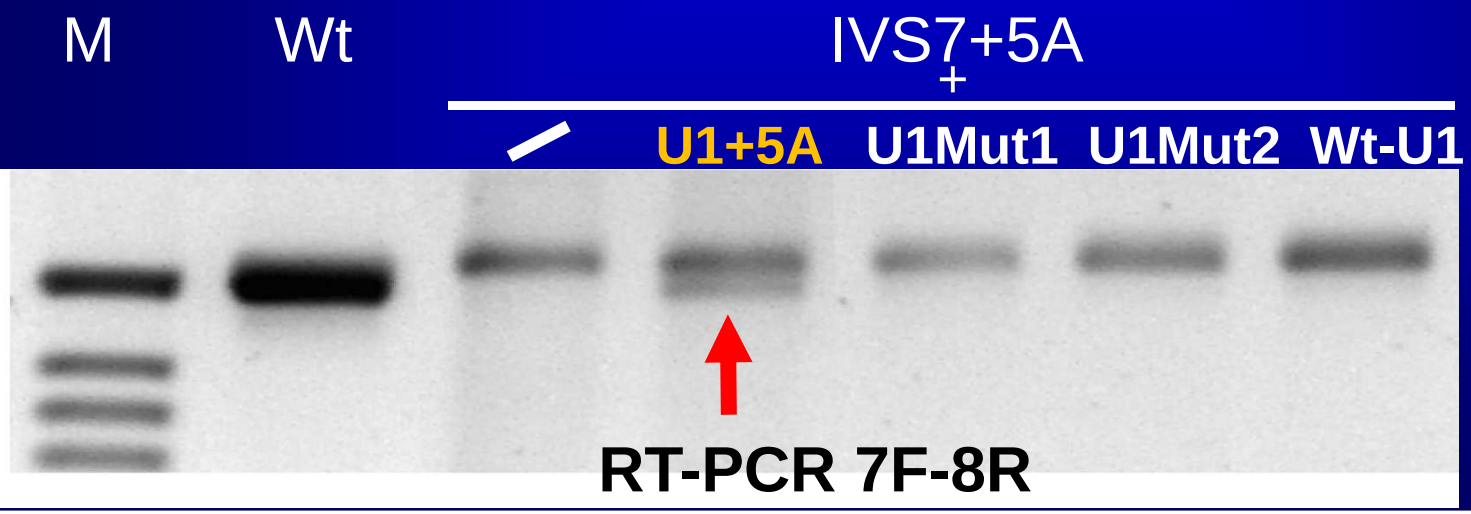
CGCGGTGCTGGgtggAtaccactctccctgtccgac
cgcggtgctggGtgggtgccactcttccctgtccgac
cgcggtgctgggtgggtgccactctccctgtccgac
cgcggtgctgggtgggtgccactctccctgtccgac
cgcggtgctgggtgggtgccactctccgctgtccgac

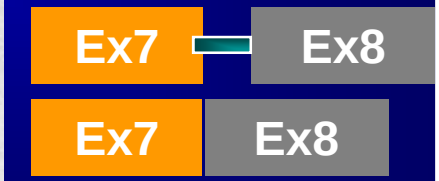
We constructed expression vectors for U1snRNAs, designed to bind the mutated 5'ss or different sequences in repeats

Modified U1snRNAs reduced exon 7 skipping



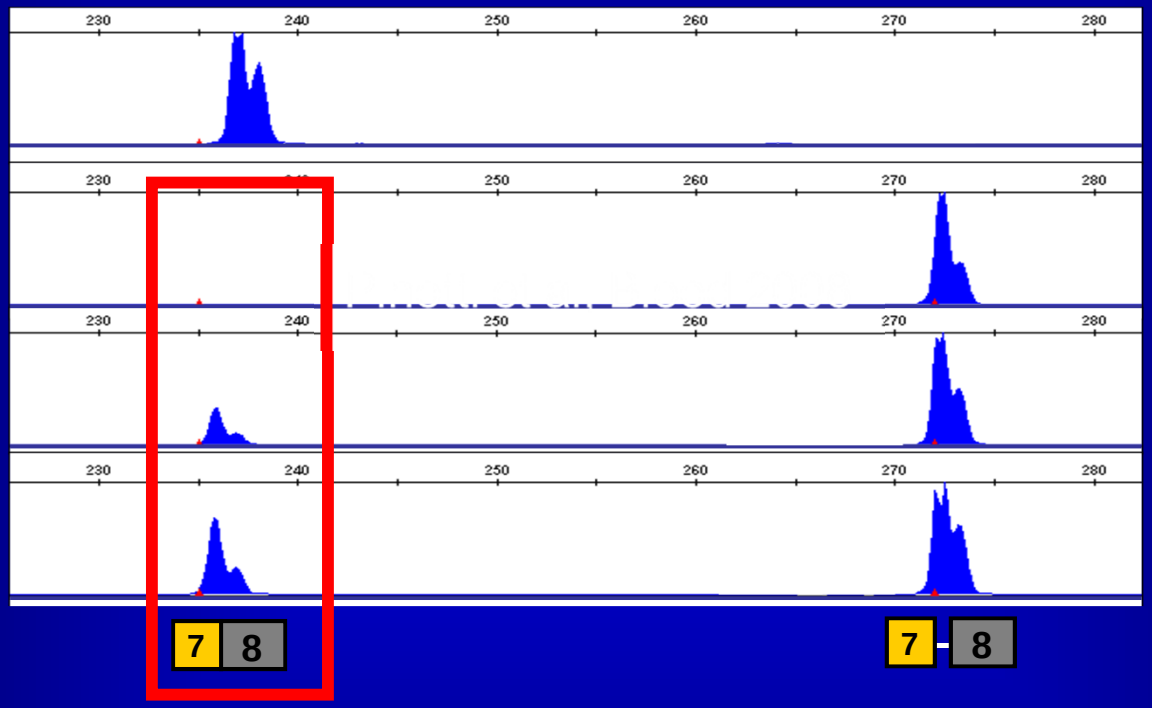
Only U1+5A, binding the mutated 5'ss, partially rescued splicing





Pinotti et al, Blood 2008

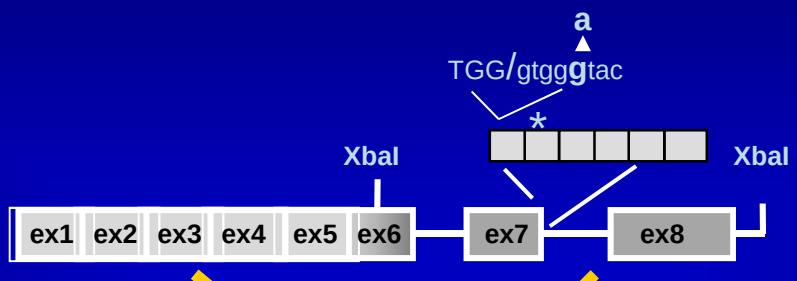
The snU1+5A was able to partially rescue correct splicing ….and in a dose-dependent manner

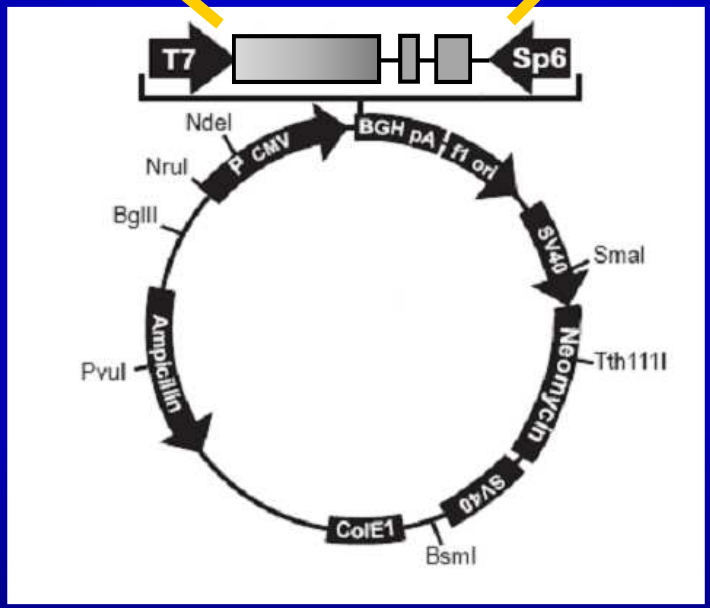


The correctly spliced form corresponded to 15% of the aberrant forms

Pinotti et al, Blood 2008

Does the rescue of correct mRNA produce an increase in FVII protein expression?

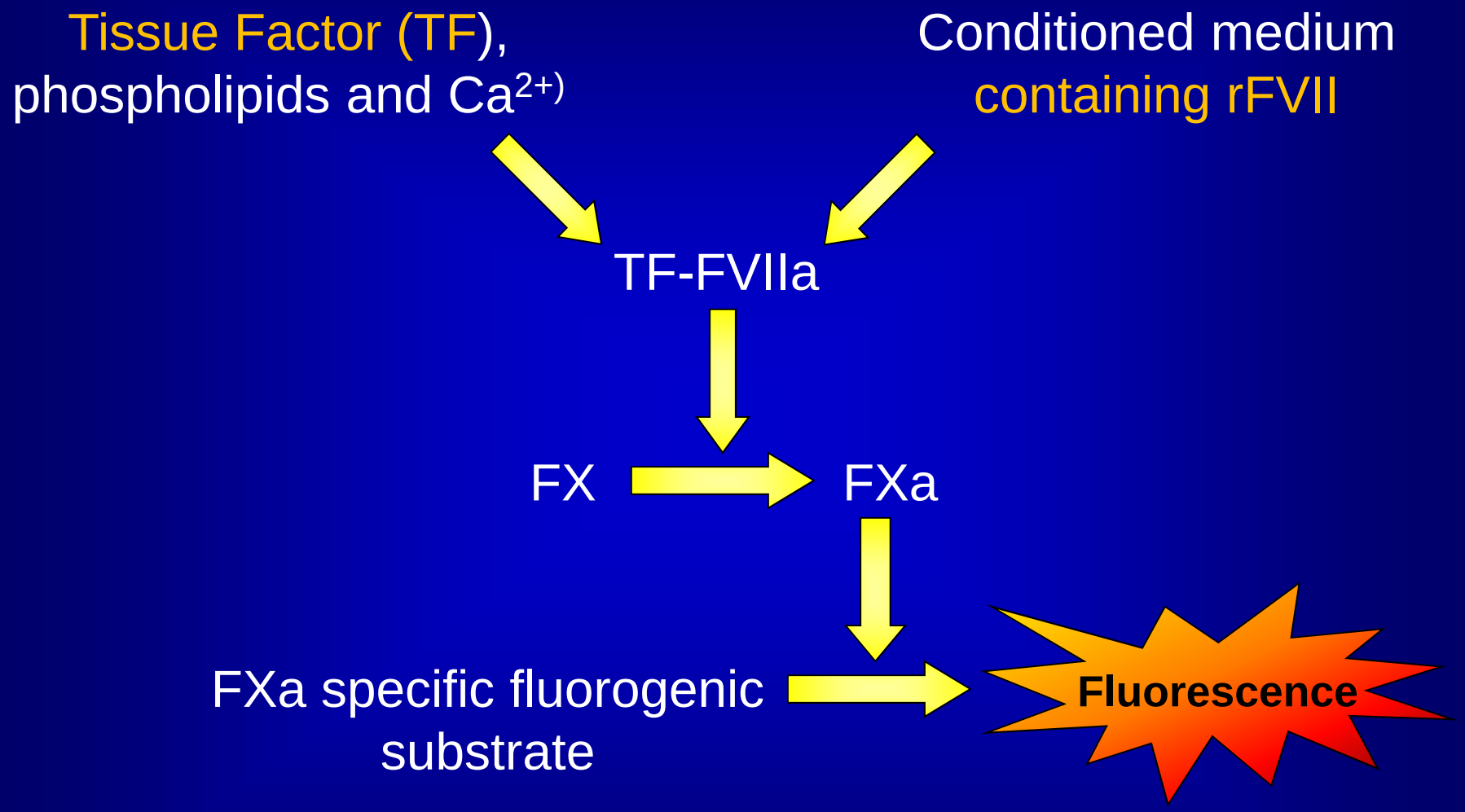




HYBRID cDNA- GENE CONSTRUCT

Expression in Cos-7 cells and studies at the mRNA and protein levels

Fluorogenic Assays



Coagulation Assays

Prothrombin time (PT)

Human ***FVII deficient plasma supplemented with rFVII***

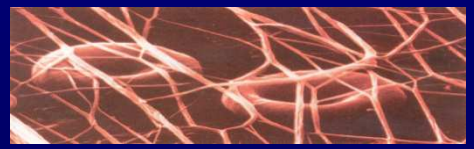
Tissue factor

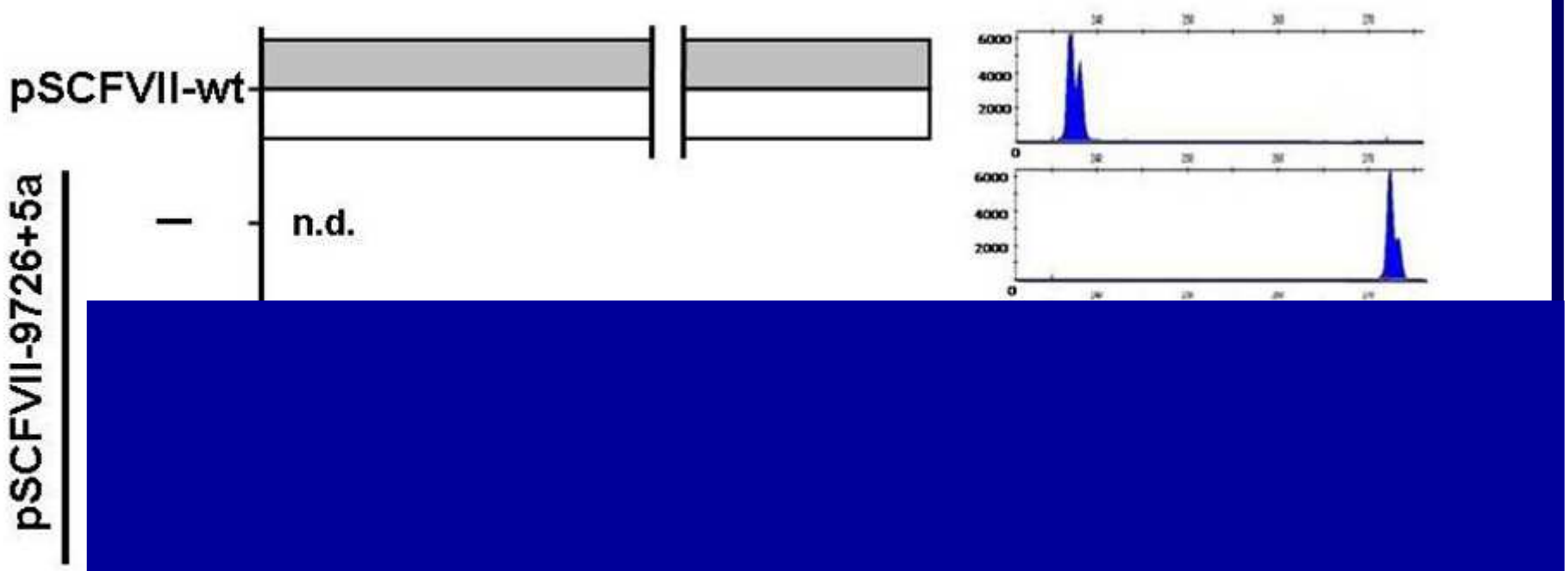
Phospholipid, Calcium

Coagulation pathway

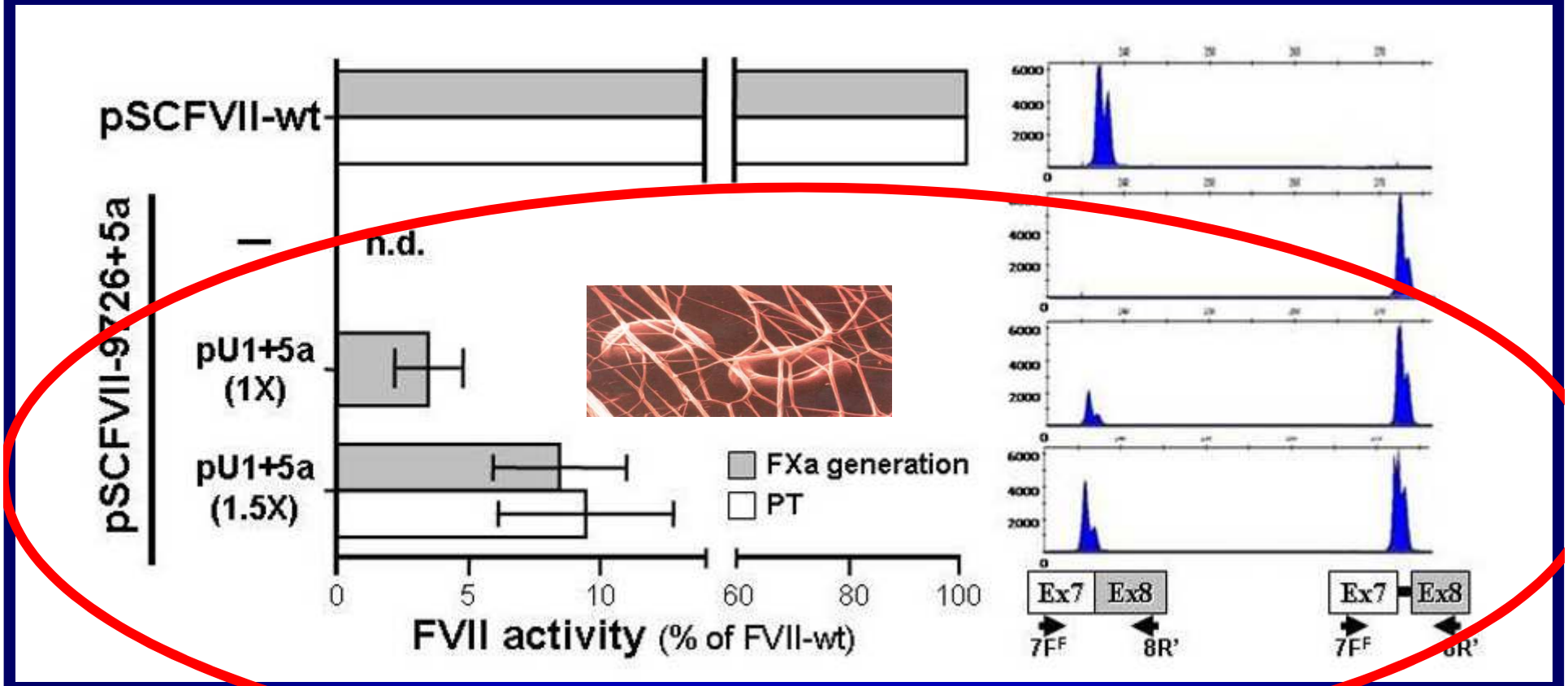
T I M E

Fibrin clot



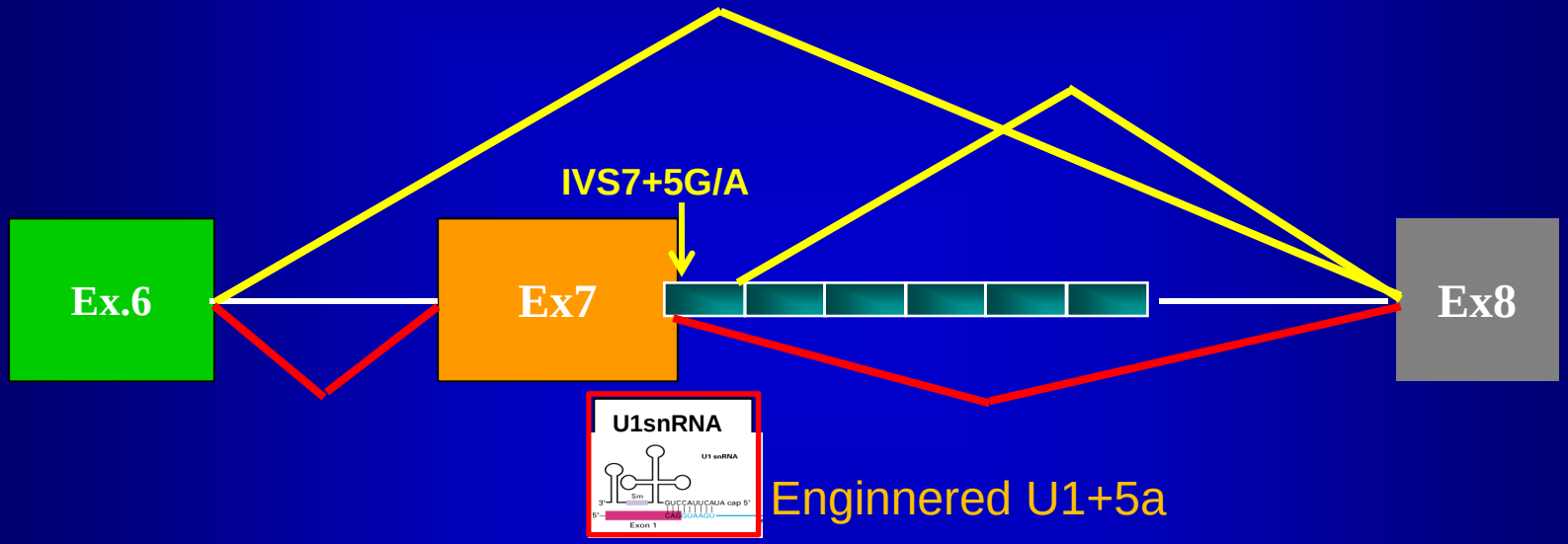


Pinotti et al, Blood 2009



…. to a level that would be, in vivo, well beyond the therapeutic threshold

Modified U1snRNA are able to re-direct the spliceosome assembly and restore exon definition in cellular models

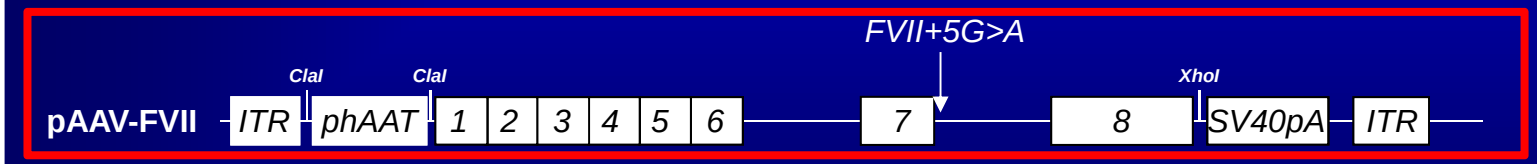


What about correction efficacy in vivo?

CREATION OF THE MOUSE MODEL OF HUMAN FVII DEFICIENCY CAUSED BY SPLICING MUTATION

Liver-restricted expression of the human FVII cassette in wt mice

Evaluation of hFVII in hepatocytes and plasma by species-specific assays

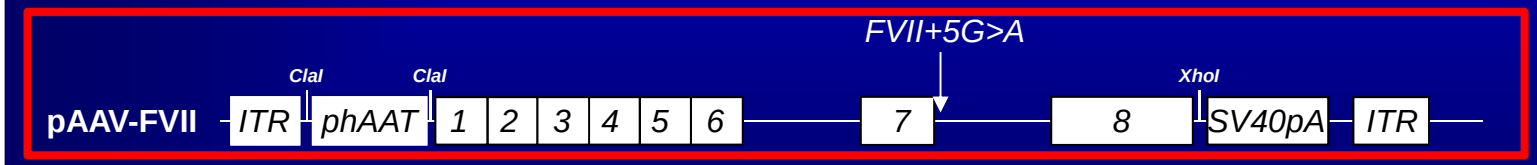


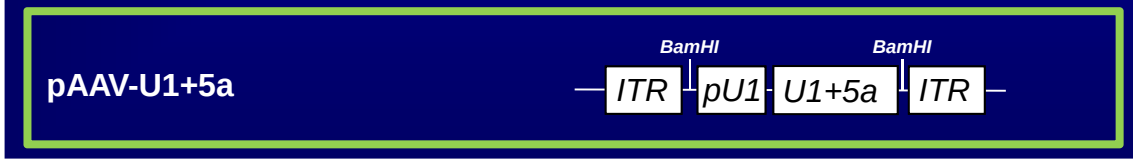
ASSESSMENT OF THE U1+5A-MEDIATED RESCUE

Liver-restricted expression of the human FVII cassette in wt mice

Co-expression of the U1+5a

EVALUATION OF RESCUE



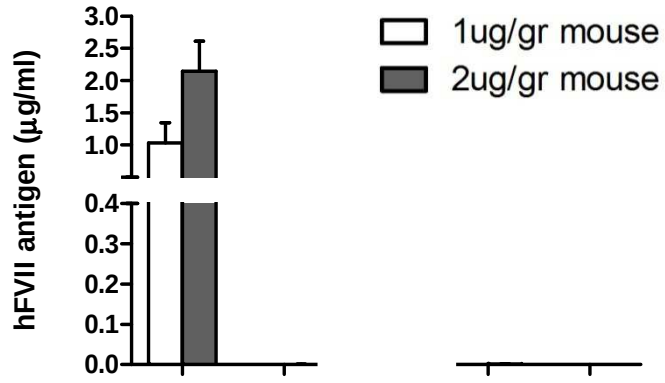


HYDRODYNAMIC INJECTION STUDIES

pAAV2-hAAT-FVII+5A

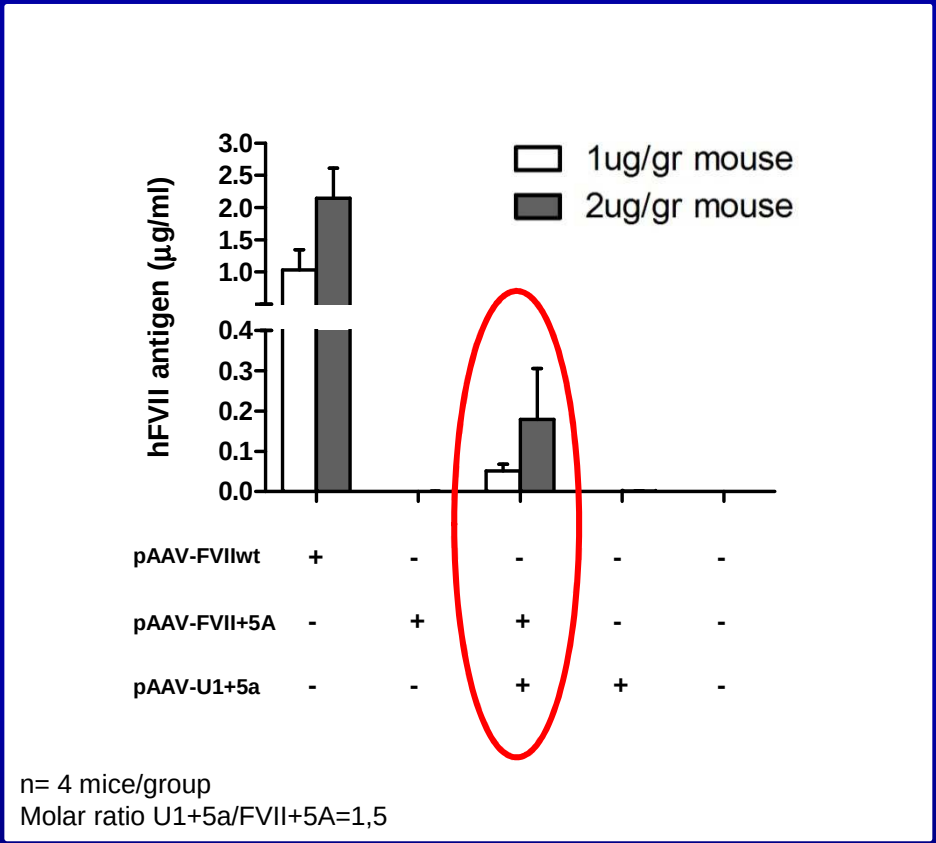
pAAV8-U1+5a

The expression of the FVII+5A variant did not resulted in appreciable plasma hFVII levels



n= 4 mice/group
Molar ratio U1+5a/FVII+5A=1,5

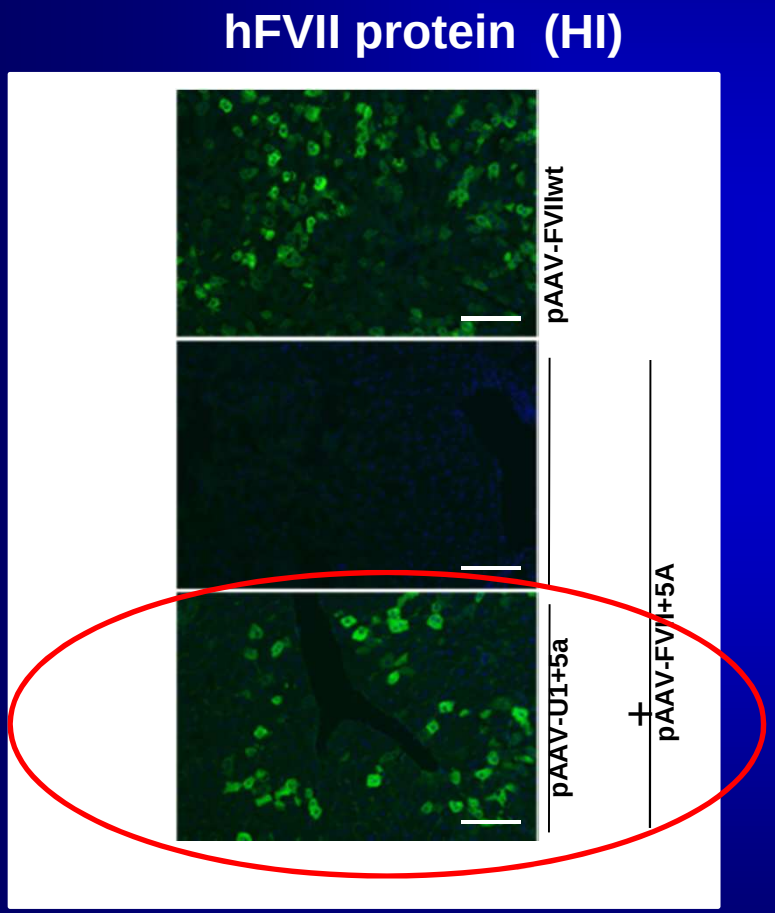
U1+5a rescue of circulating hFVII protein levels



n= 4 mice/group
Molar ratio U1+5a/FVII+5A=1,5

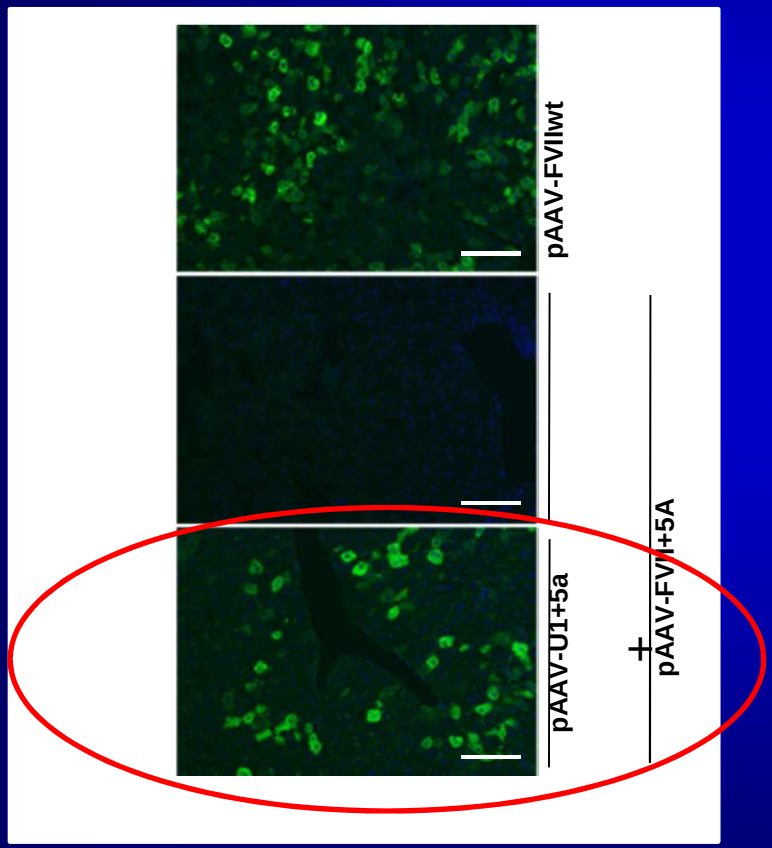
Compared to levels in mice injected with FVII wt plasmid, the correction obtained after injection of U1+5a was ~8,5 %

U1+5a-mediated rescue of hFVII expression in mouse liver

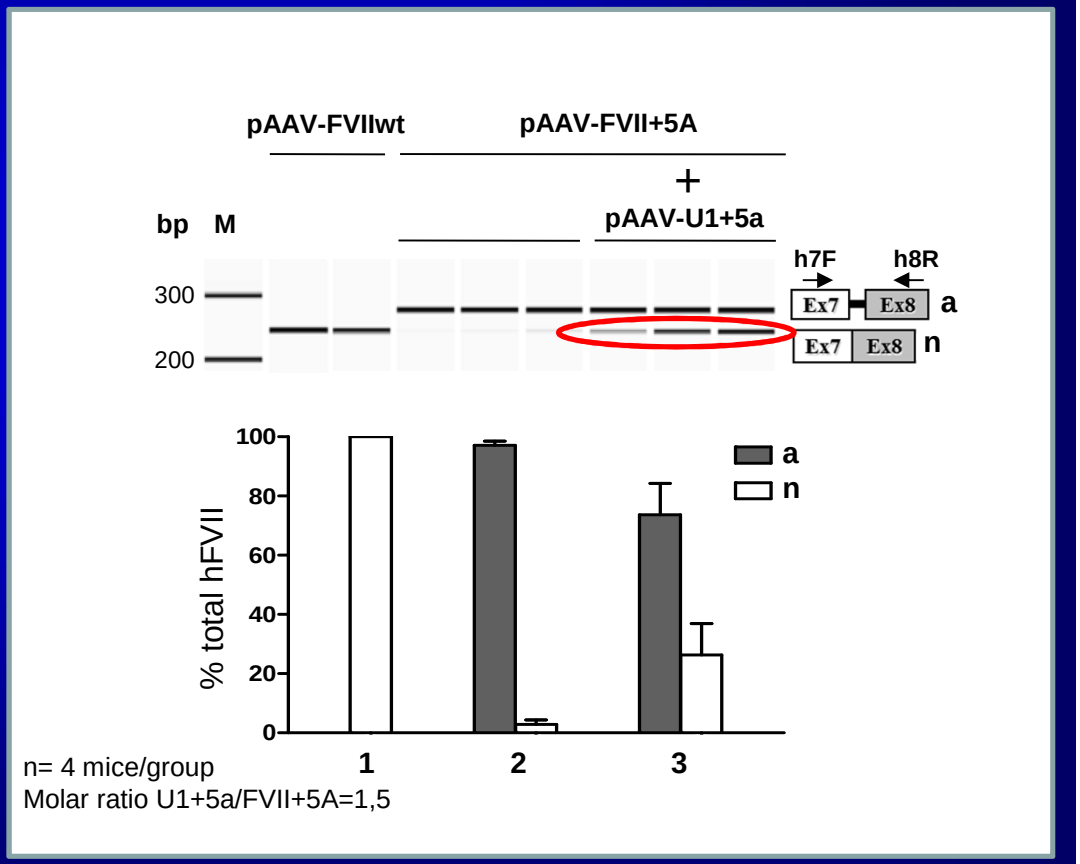


U1+5a-mediated rescue of hFVII expression in mouse liver

hFVII protein (HI)



hFVII mRNA (RT-PCR)

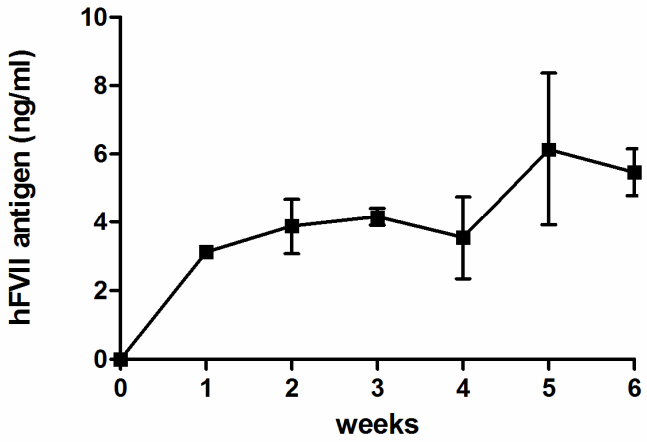


PROLONGED RESCUE BY ADENOASSOCIATED VIRAL VECTORS

AAV2-hAAT-FVII+5A

AAV8-U1+5a

STUDIES WITH ADENOASSOCIATED VIRAL VECTORS



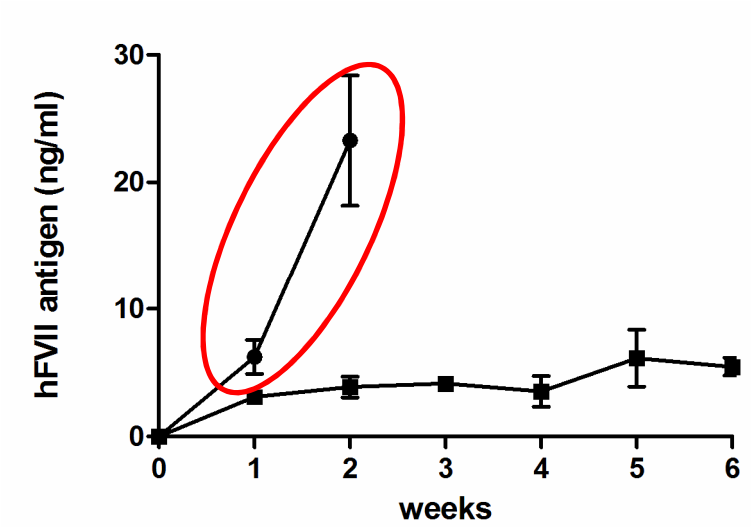
	AAV2-FVII+5A (vg/mouse)	AAV8-U1+5a (vg/mouse)
—■—	$1.2*10^{12}$	$1.2*10^{11}$

U1+5a-mediated rescue of circulating hFVII levels was appreciable and prolonged

STUDIES WITH ADENOASSOCIATED VIRAL VECTORS

AAV2-hAAT-FVII+5A

AAV8-U1+5a

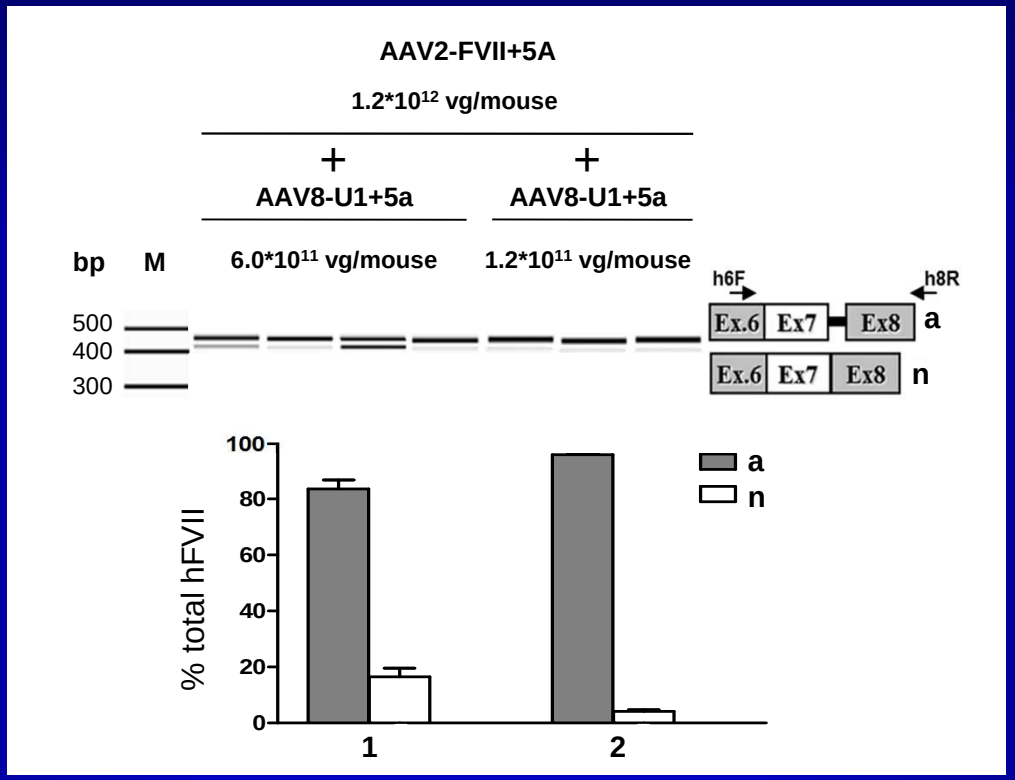


	AAV2-FVII+5A (vg/mouse)	AAV8-U1+5a (vg/mouse)
●	$1.2*10^{12}$	$6.0*10^{11}$
■	$1.2*10^{12}$	$1.2*10^{11}$

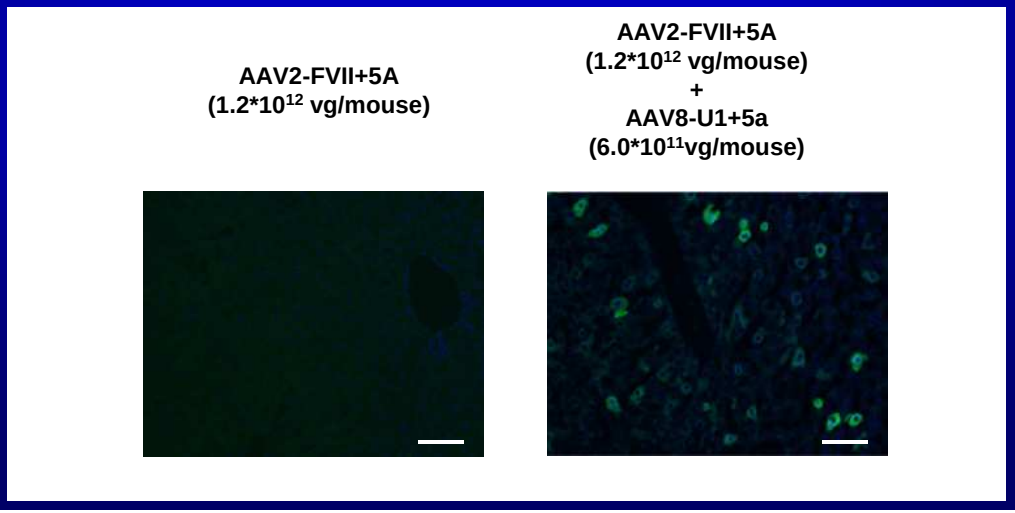
and the correction extent was dose-dependent

U1+5a-MEDIATED RESCUE IN MOUSE LIVER

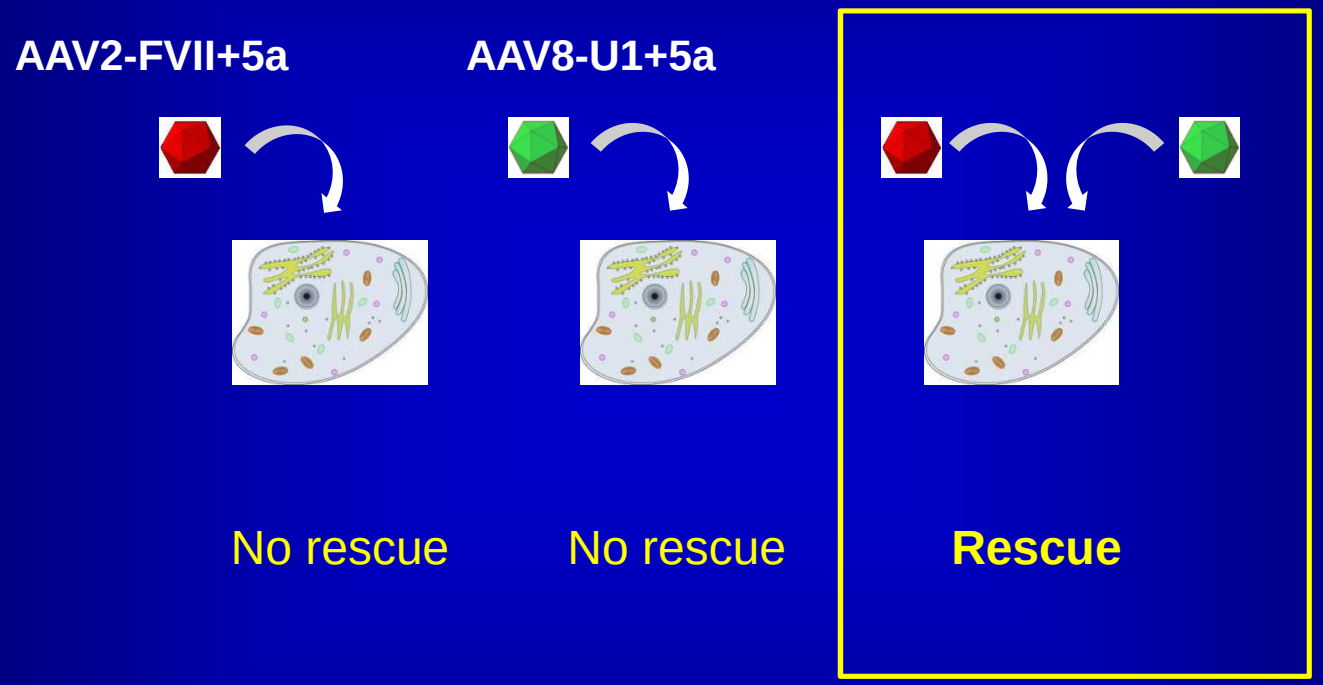
hFVII mRNA (RT-PCR)



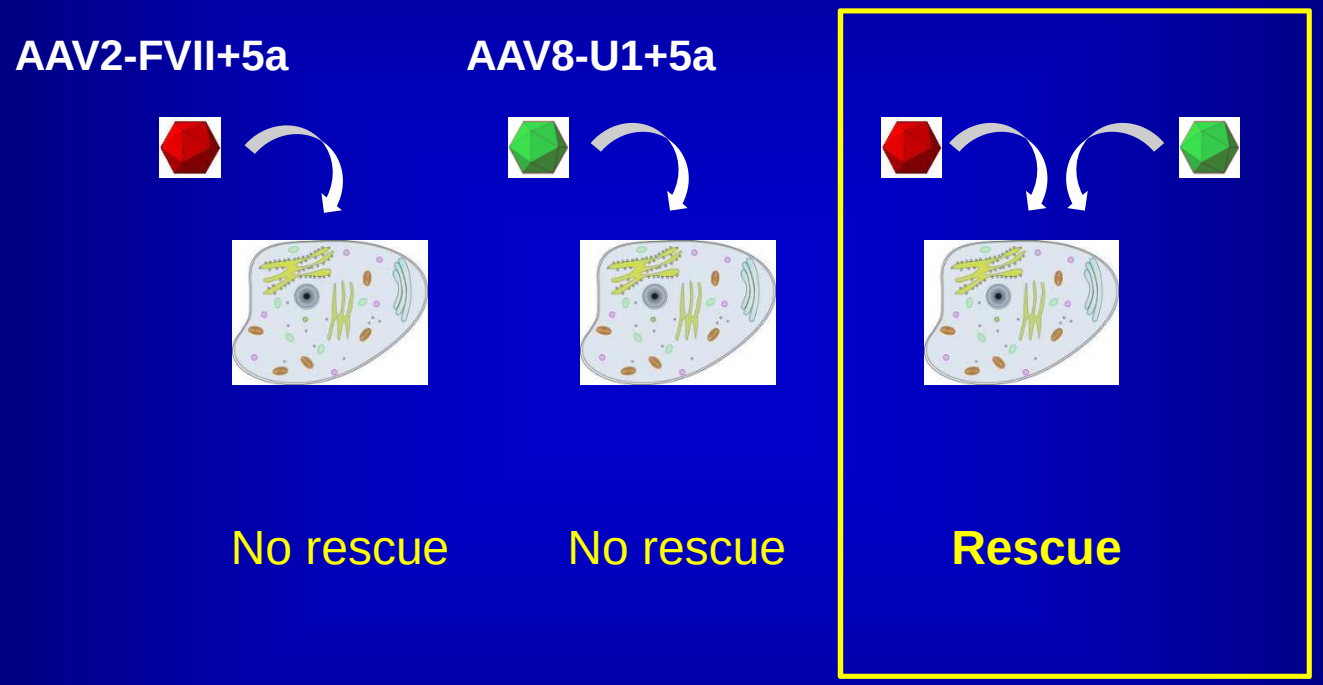
hFVII protein (HI)



LIMITATION OF THE MOUSE MODEL

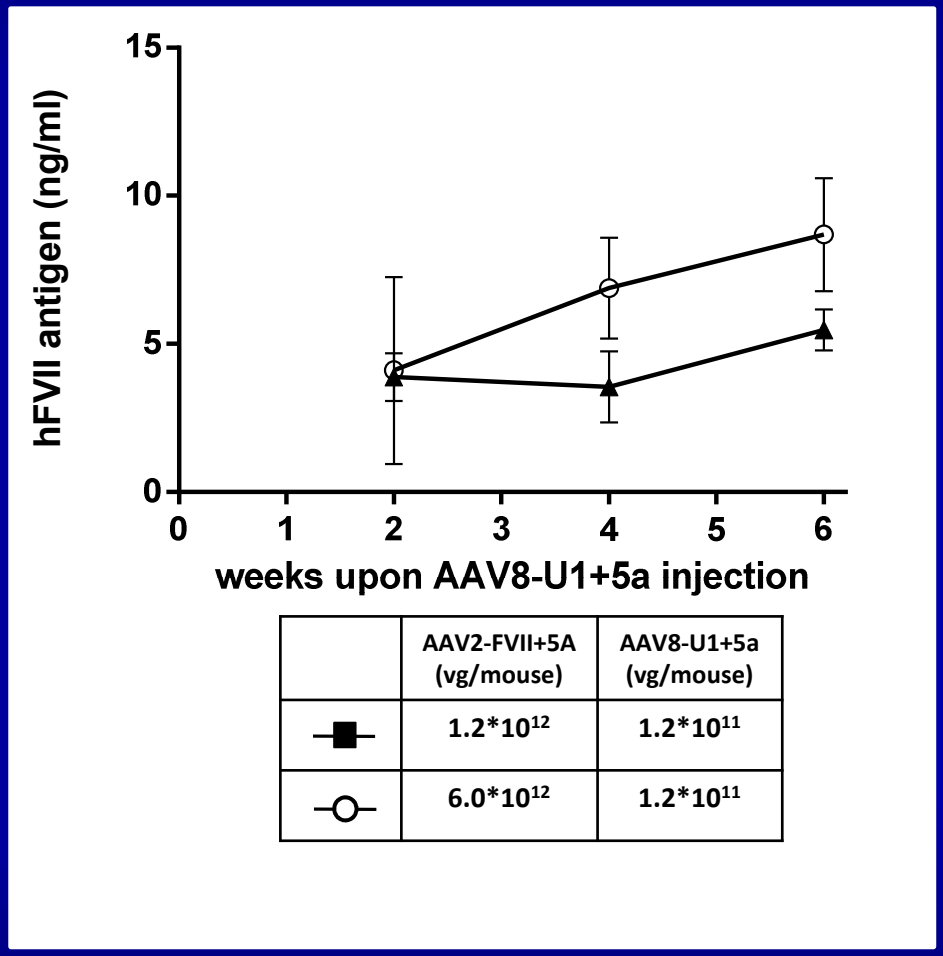


LIMITATION OF THE MOUSE MODEL



IF TRUE, INCREASING THE SUBSTRATE WOULD RESULT IN INCREASED RESCUE

INCREASING THE DOSE OF THE hFVII SUBSTRATE RESULTED IN INCREASED RESCUE BY THE U1+5a



…therefore, rescue efficiency in patients, expressing the FVII mRNA in all hepatocytes, should be much more pronounced

CONCLUSION I

- **Engineered U1snRNA are capable to re-direct the spliceosome assembly to the mutated exon-intron juntion and rescue mRNA processing and secretion of functional FVII;**

- **For the first time, we provide the «proof-of-principle» for the U1-mediated correction *in vivo***

HOWEVER

- **the approach implies one modified U1snRNA for each splicing mutation, thus limiting the potential applicability**

- ***In vivo*, a liver-toxicity has been observed with the highest doses.**

This could be due to

Off-target effect

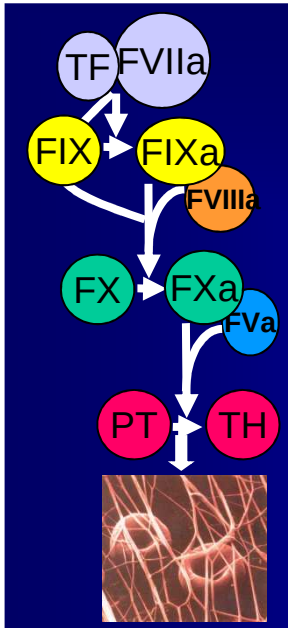
Can we be more sequence specific?

Oversaturation of UsnRNA pathway

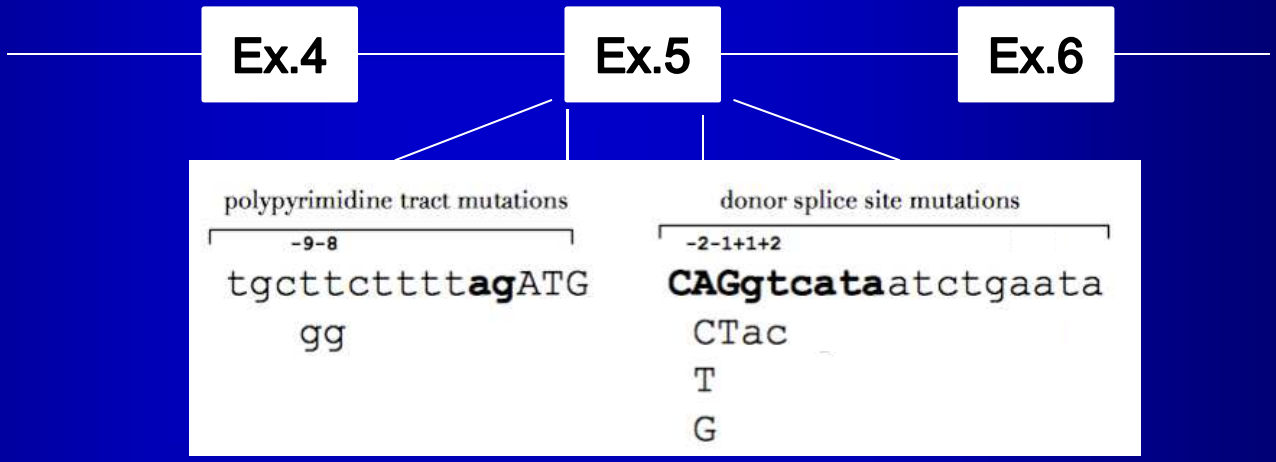
(Grimm, Streetz et al. 2006)

(Vickers, Sabripour et al. 2011)

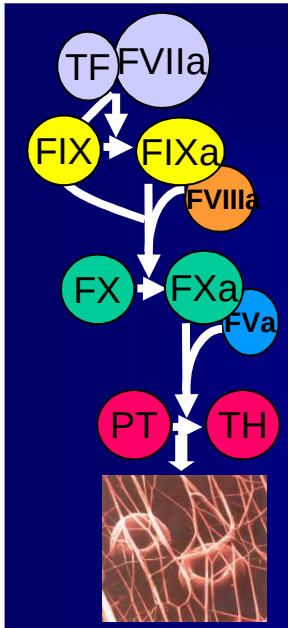
Reduction of amount of modified U1snRNA



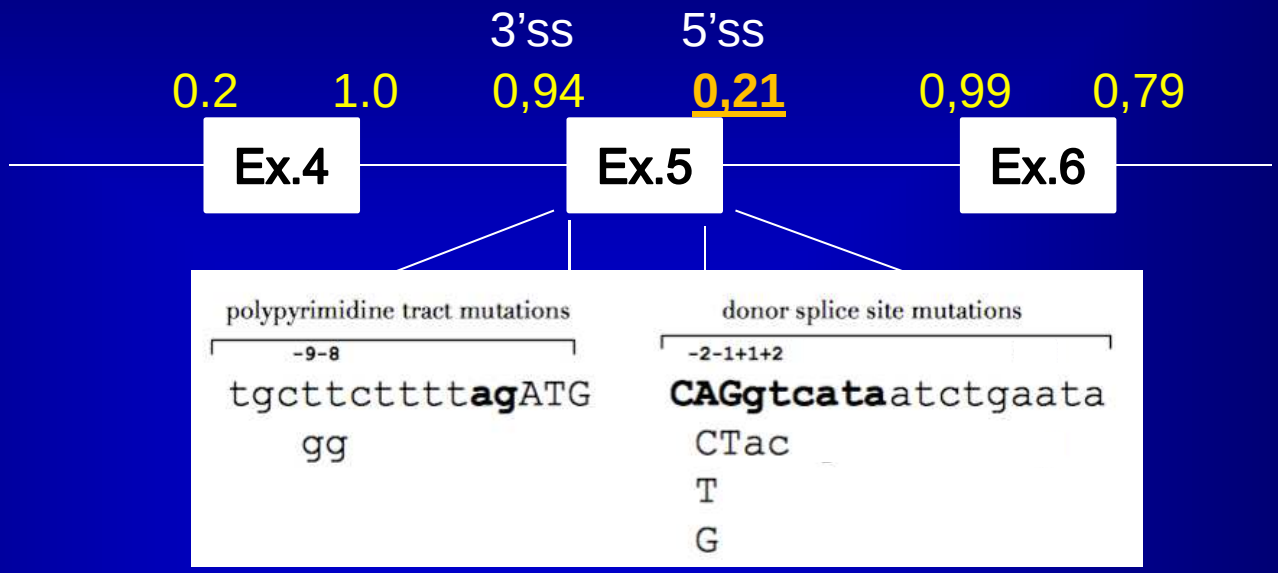
Hemophilia B model



Alanis et al, HMG 2012

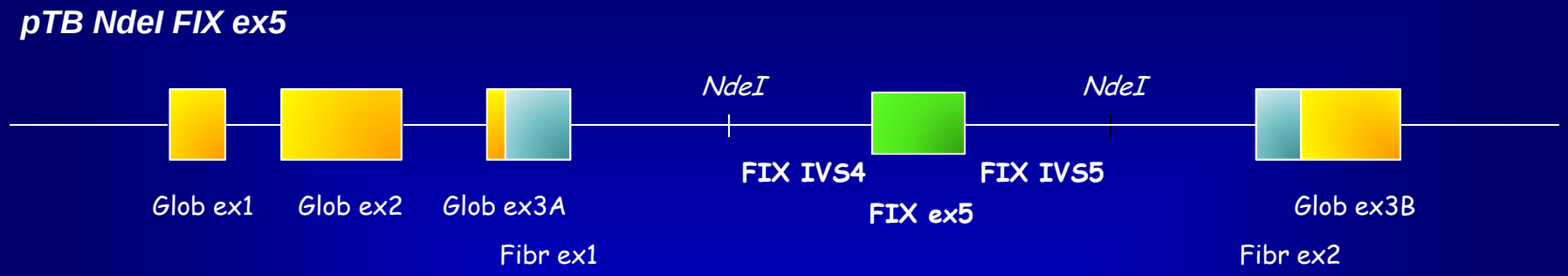


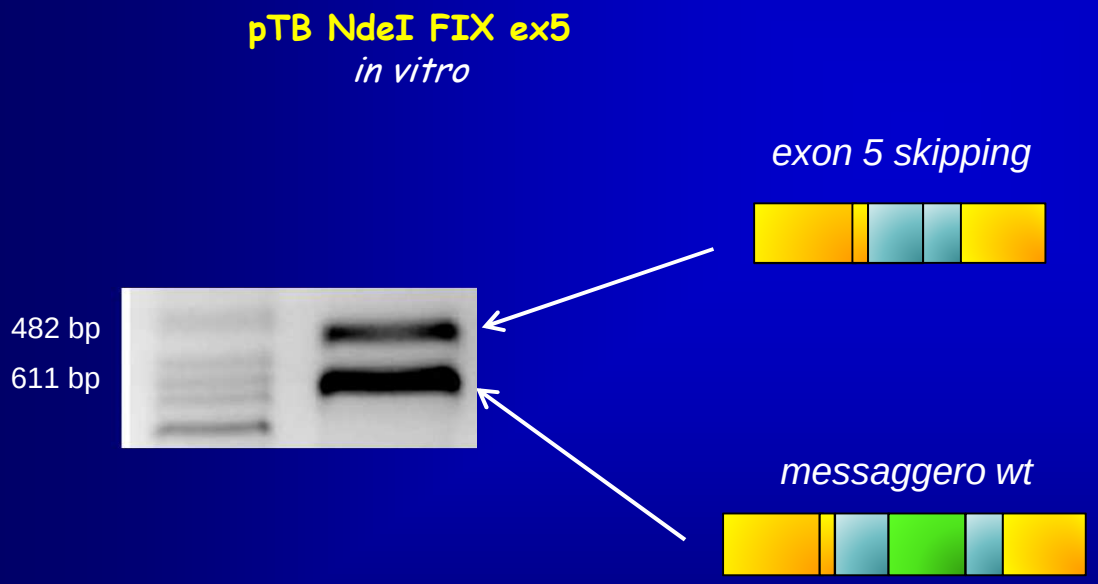
F9 Exon 5 is poorly defined



Alanis et al, HMG 2012

Expression studies with hybrid F9 minigenes



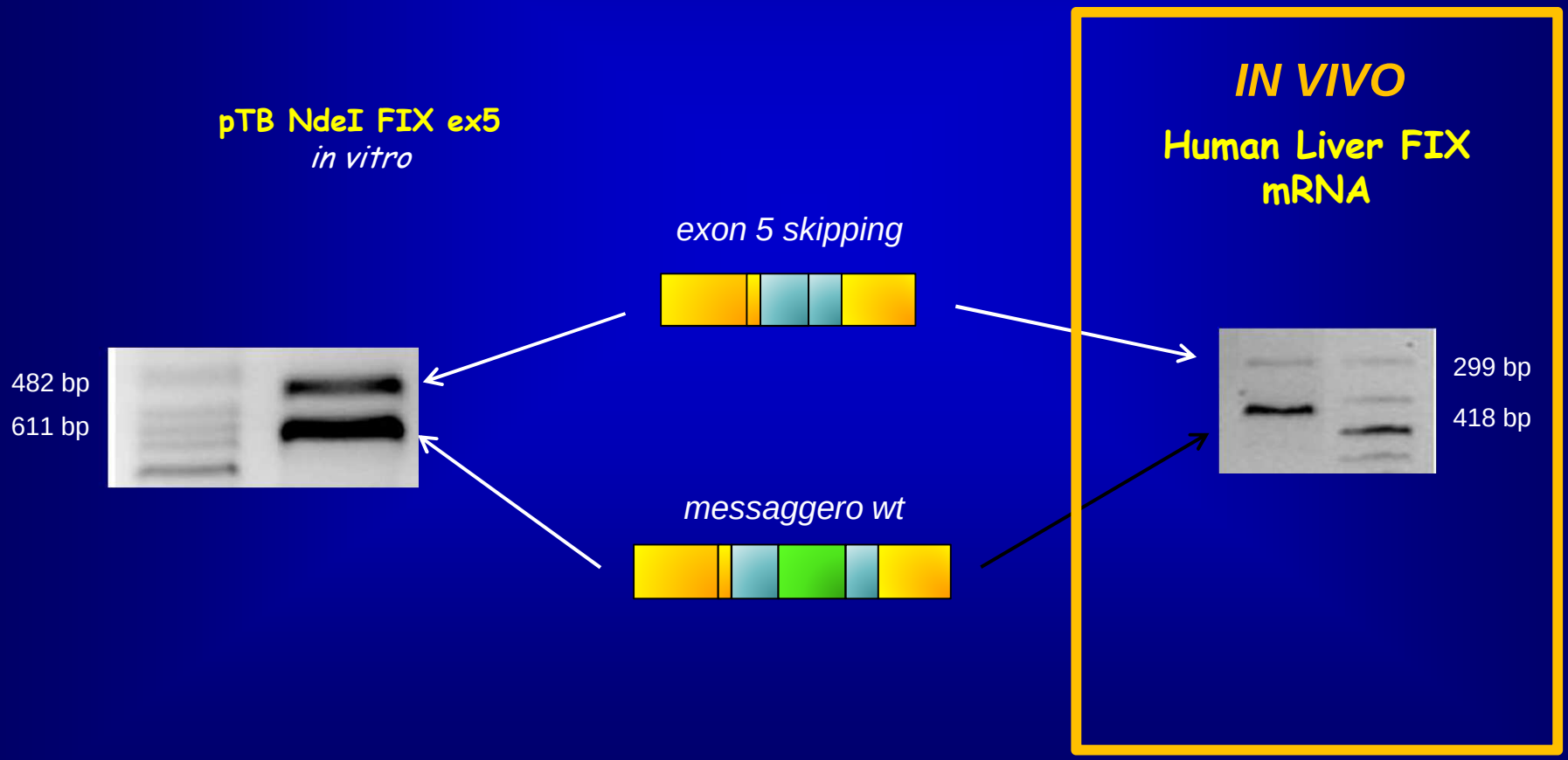


Exon 5 is poorly defined

Alanis et al, HMG 2012

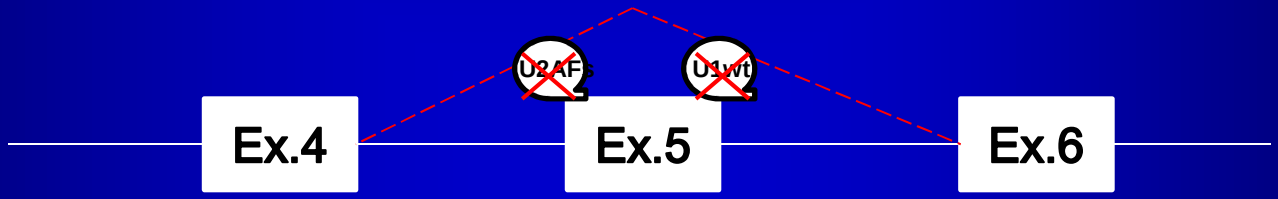
Is this due to the hybrid minigene features?

Exon 5 is poorly defined *in vivo*



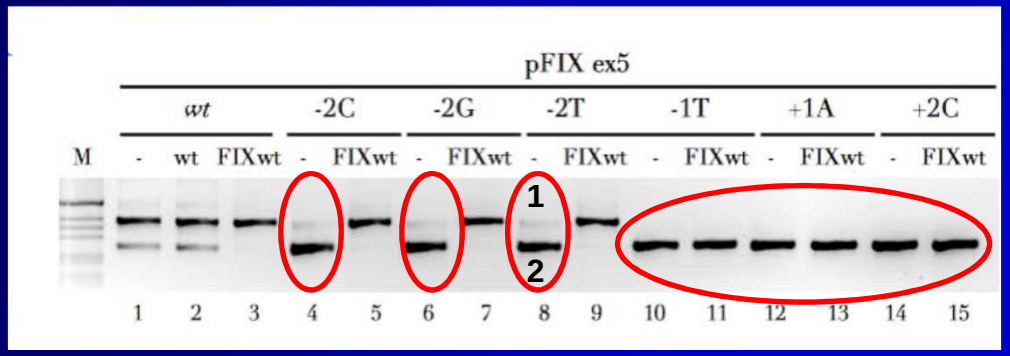
Exon 5 is poorly defined

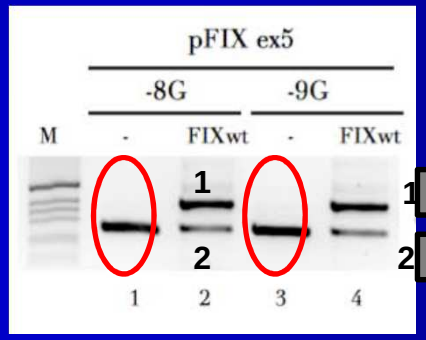
Alanis et al, HMG 2012

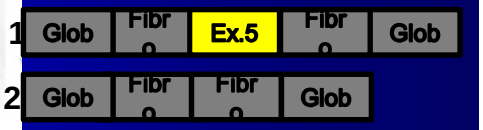


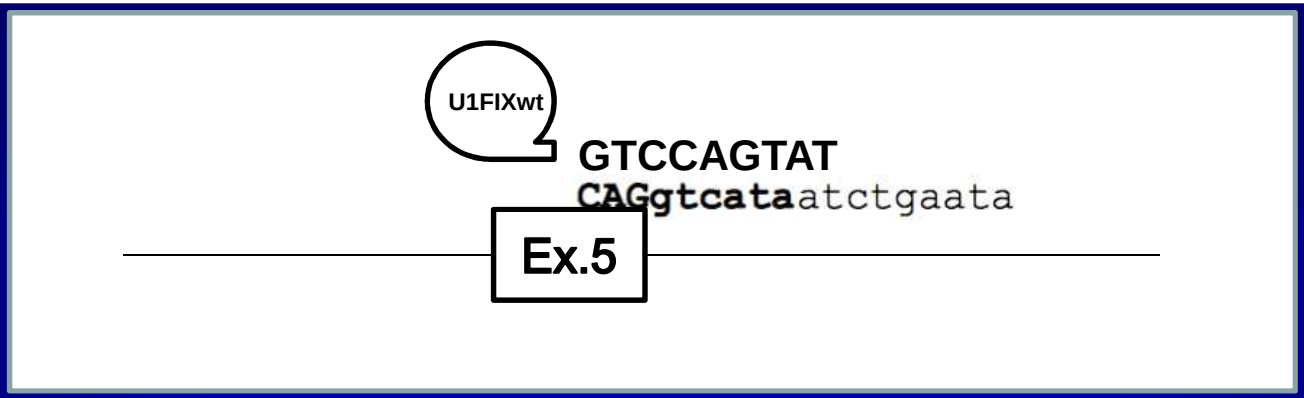
The mutations, reducing exon 5 definition, are candidate to induce exon 5 skipping

All mutations induced exon 5 skipping



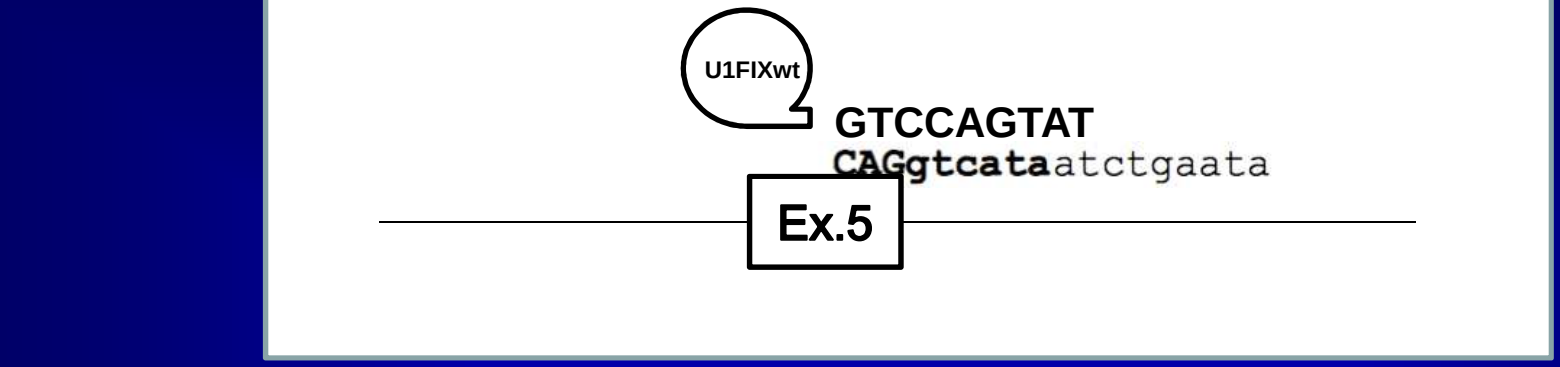


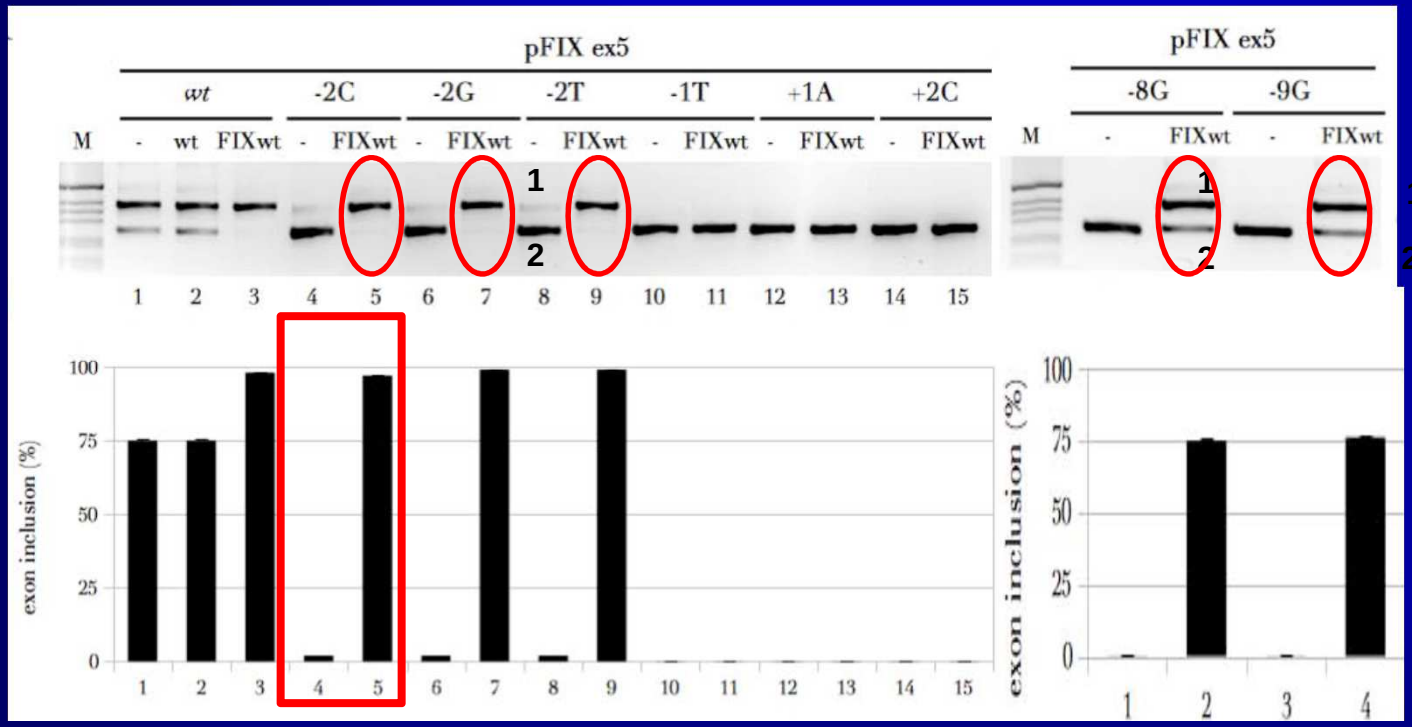


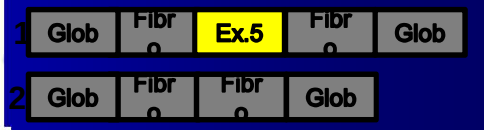


Rescue by U1snRNA targeting the FIX donor splice site

Alanis et al, HMG 2012







A single U1snRNA rescued mutations at either donor or acceptor sites

Alanis et al, HMG 2012

However, U1snRNA targeting 5’ss might not ensure enough sequence specificity

Exon –specific U1snNAs (ExSpeU1) targeting intronic sequences

Design of ExSpeU1

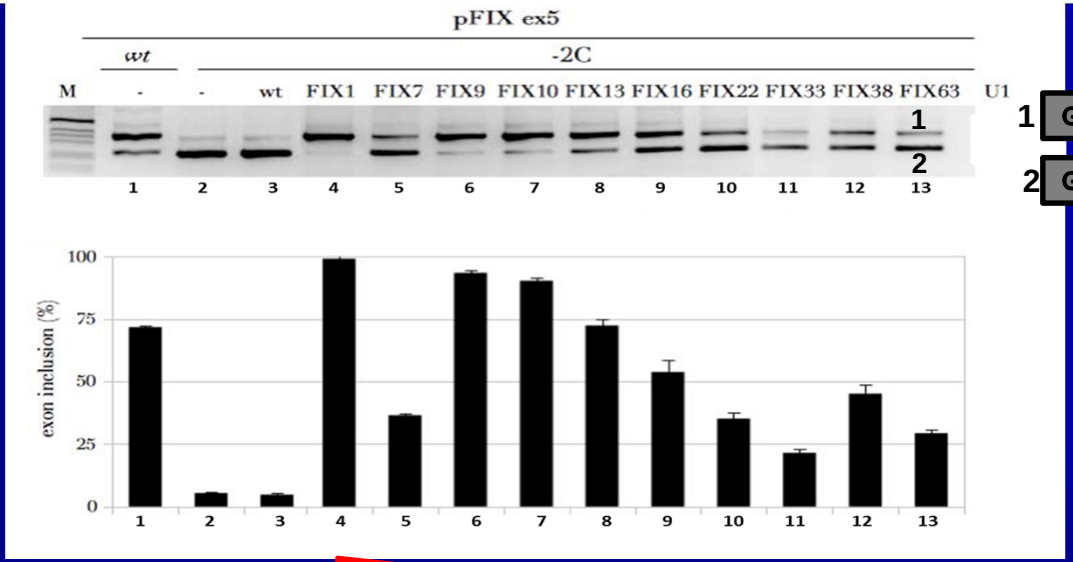
F. Pagani
ICGEB, Trieste

Alanis et al, HMG 2012

Rescue by Exon –specific U1snNAs

Design of ExSpeU1

FIX-IVS5 -2C variant



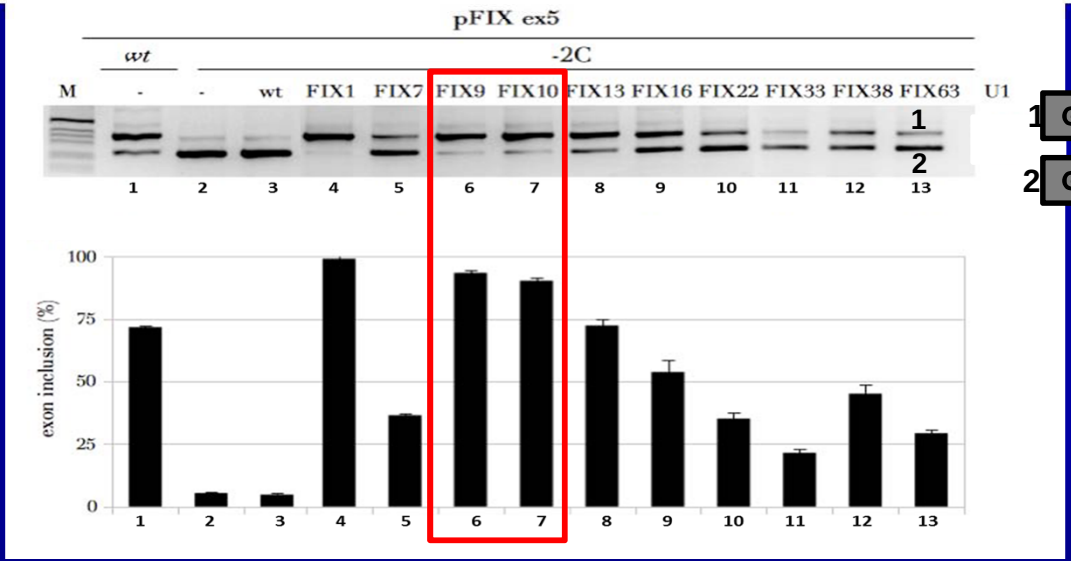
Exon-specific U1 rescued splicing to appreciable levels

Alanis et al, HMG 2012

Rescue by Exon –specific U1snNAs

FIX-IVS5 -2C variant

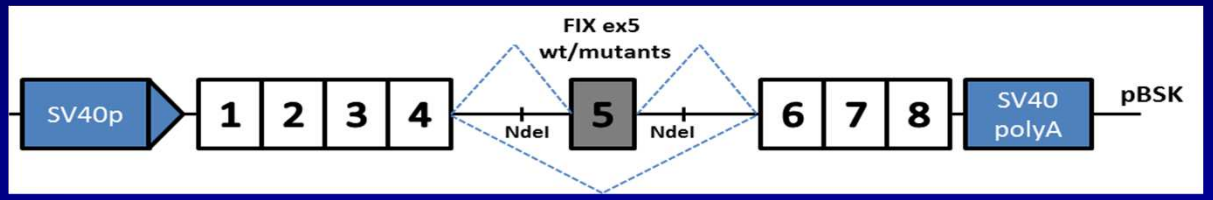
Design of ExSpeU1



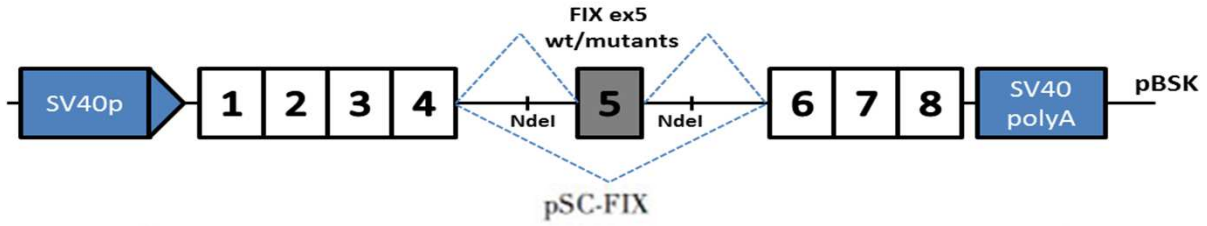
ExSpeU1 FIX9 and FIX10

Alanis et al, HMG 2012

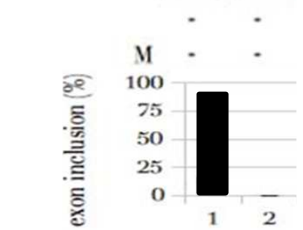
Rescue of FIX biosynthesis and function:



Alanis et al, HMG 2012

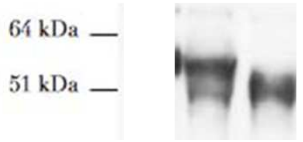


Differential impact on



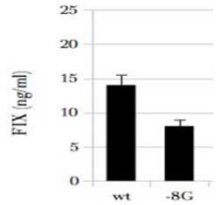
Splicing (exon skipping)

RNA



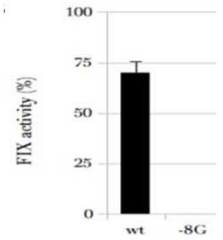
Protein profile (deleted FIX)

Protein Isoforms



Secretion (impaired)

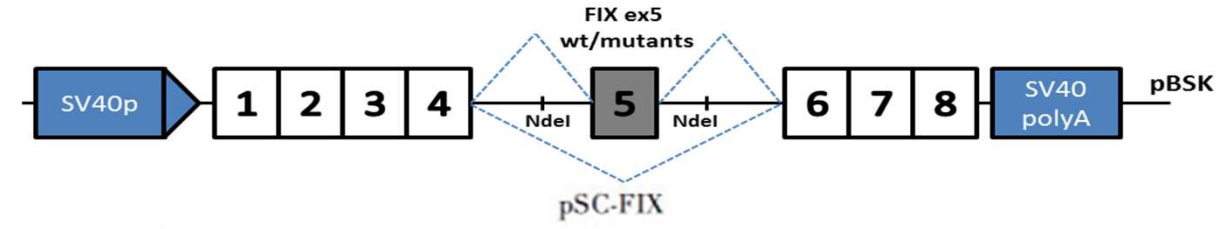
Secreted levels

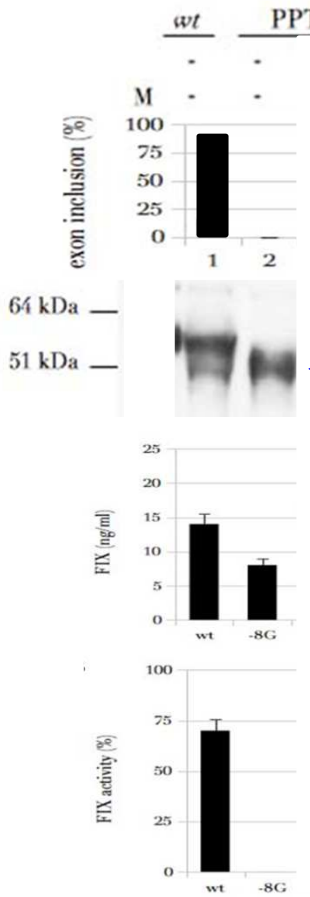


Activity (abolished)

Coagulant Act.

The deleted variant, lacking EGF2, is secreted but inactive





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is secreted

but inactive

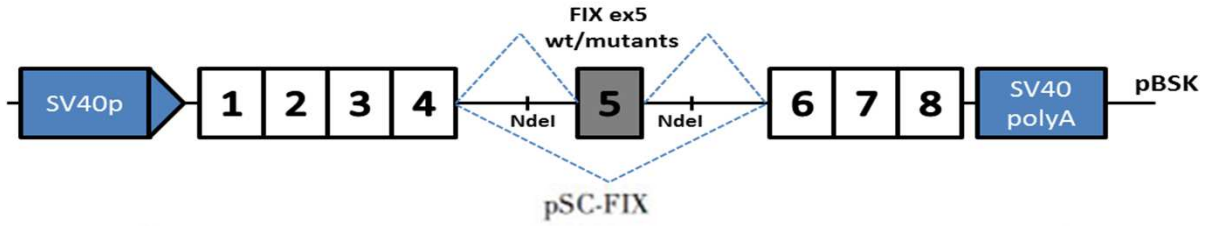
RNA

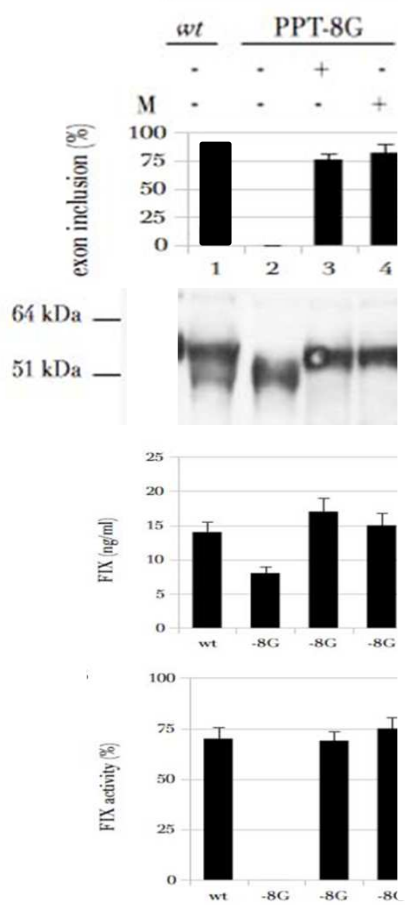
Protein Isoforms

Secreted levels

Coagulant Act.

The deleted variant, lacking EGF2, is secreted but inactive





U1snRNA mediated rescue of:

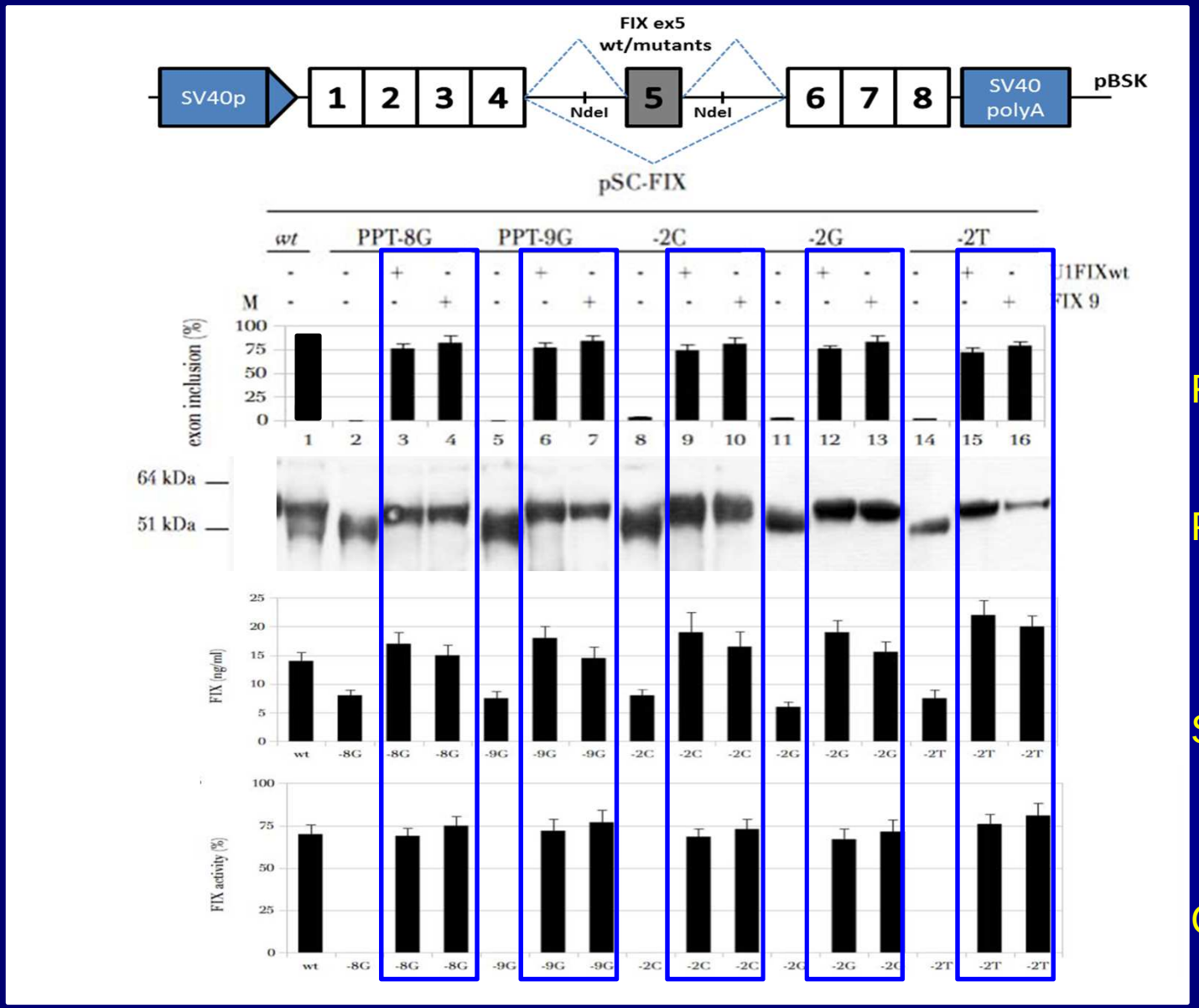
Splicing — RNA

protein pattern — Protein Isoforms

Secretion — Secreted levels

activity — Coagulant Act.

The deleted variant, lacking EGF2, is secreted but inactive



RNA

Protein Isoforms

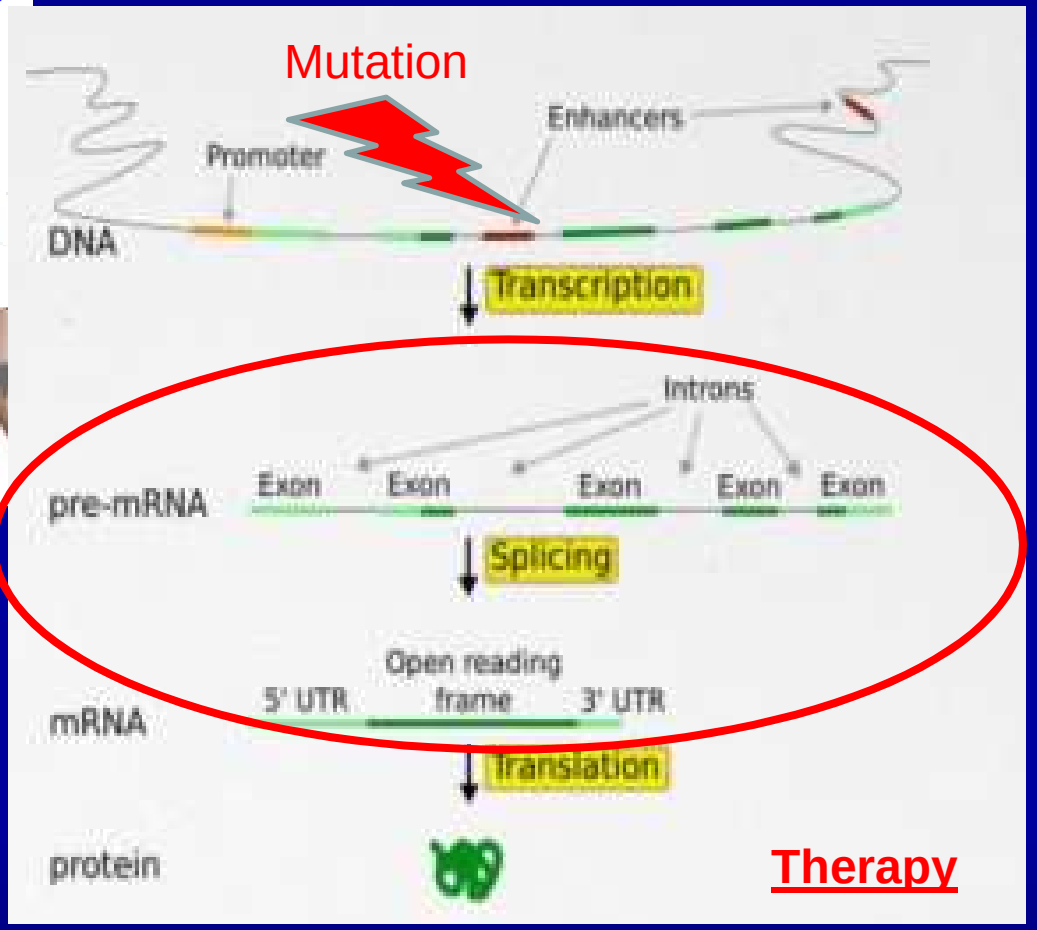
Secreted levels

Coagulant Act.

A unique ExSpeU1 completely rescued FIX function in the presence of different mutations at either the donor or acceptor splice sites

REPLACEMENT GENE THERAPY

ADVANTAGES



INTERVENTION AT pre-mRNA LEVEL

- **Maintainance of the gene regulation**
- **Correction in physiological tissues only**
- **Small size of the therapeutic expression cassette**