Dai meccanismi molecolari allo sviluppo di strategie terapeutiche innovative per i difetti genetici della coagulazione

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SISET 2016

Molecular mechanisms for new therapeutic approaches (1)

Substitutive therapy (protein)

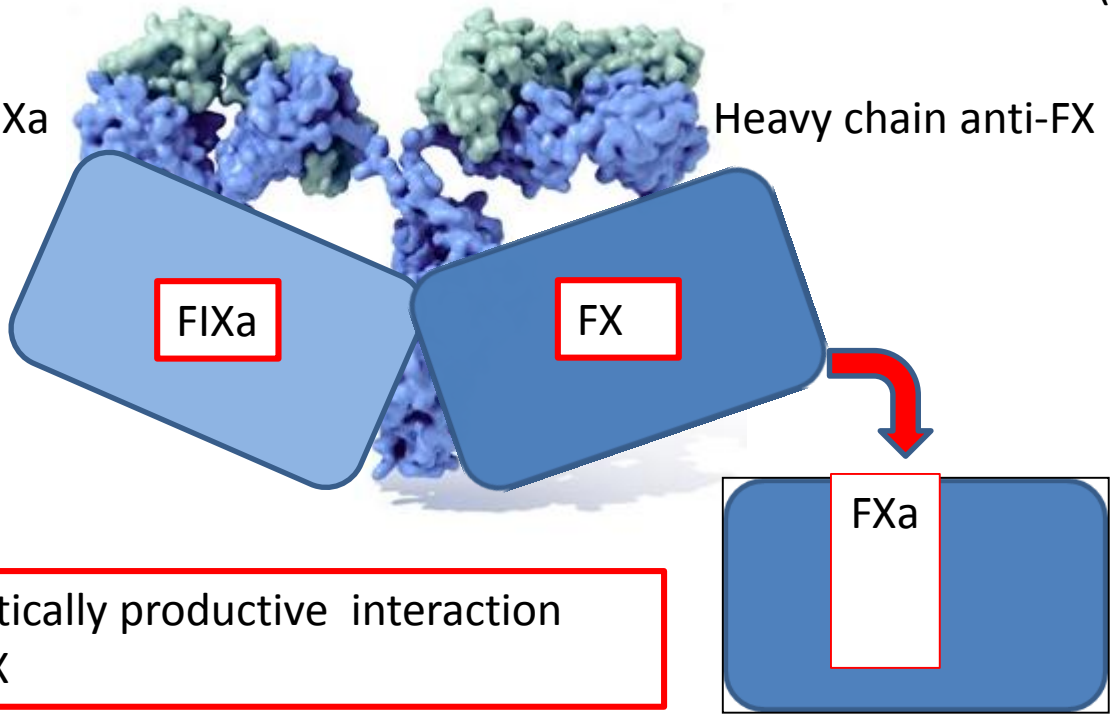
- extending the half-life/bio-distribution of circulating exogenous factor protein
- mimicking the coagulation function of factor VIII by bispecific antibody technology

Disrupting anticoagulant proteins (antibodies, aptamers, RNAi)

- tissue factor pathway inhibitor (TFPI)
- antithrombin (AT3)

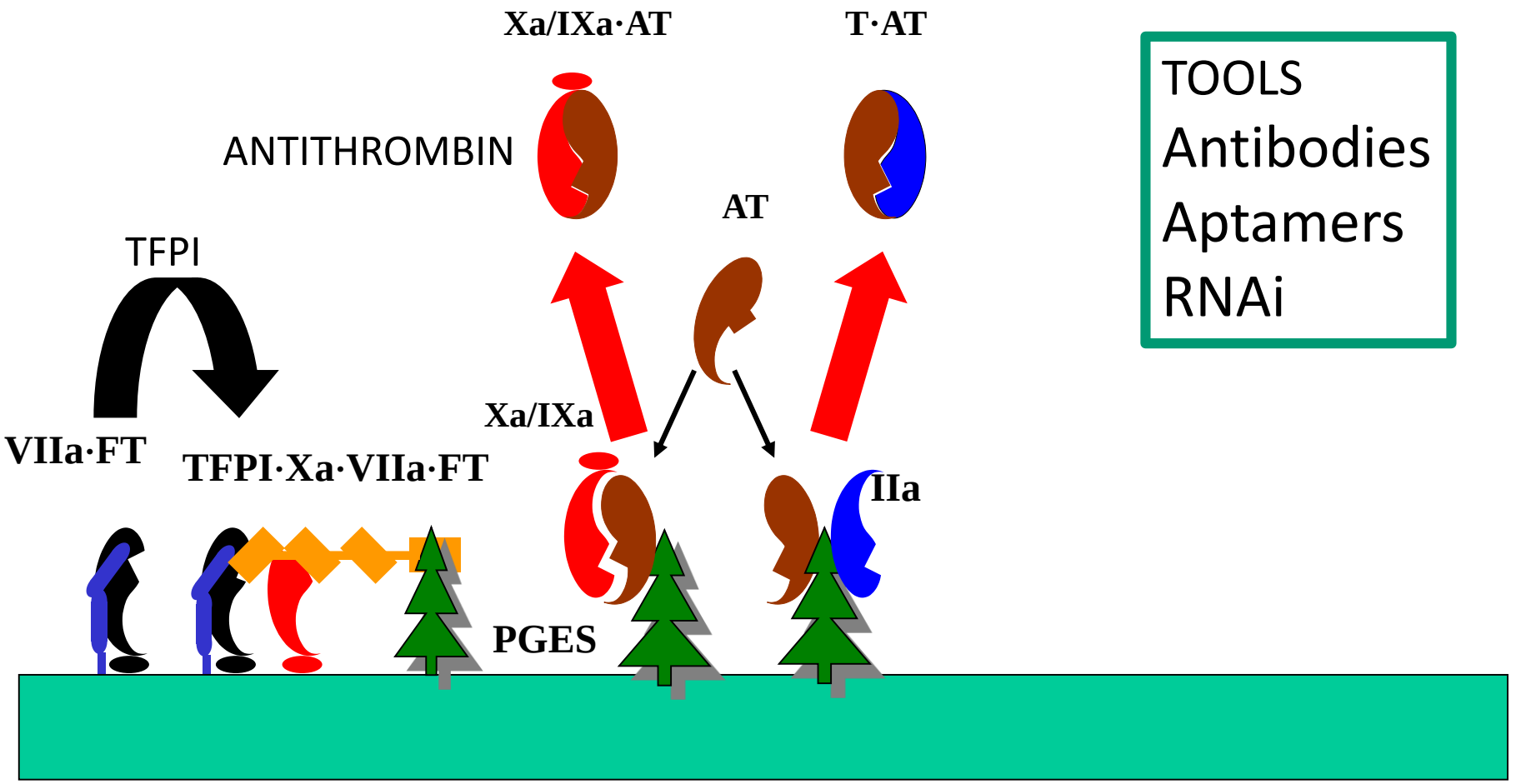
Humanized asymmetric antibody mimicking FVIIIa function

Emicizumab (ACE910)



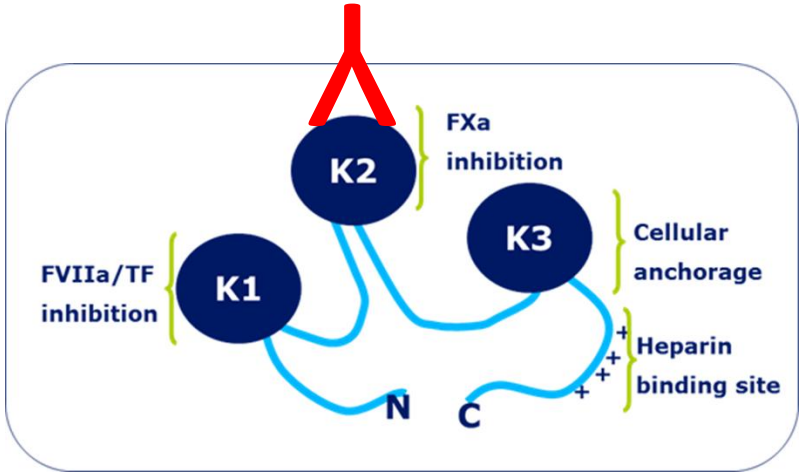
Maintaining a catalytically productive interaction between FIXa and FX

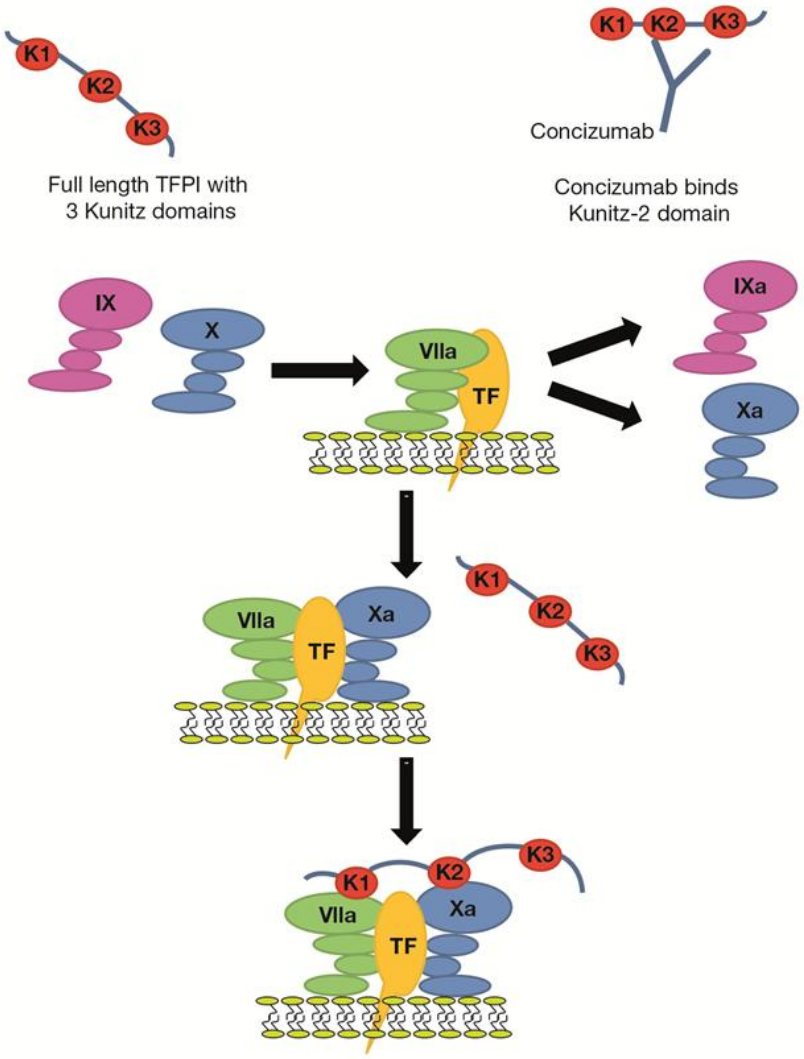
Decreasing anticoagulant function: Inhibition of inhibitors



TOOLS
Antibodies
Aptamers
RNAi

Inhibition of TFPI by specific antibodies (Concizumab)





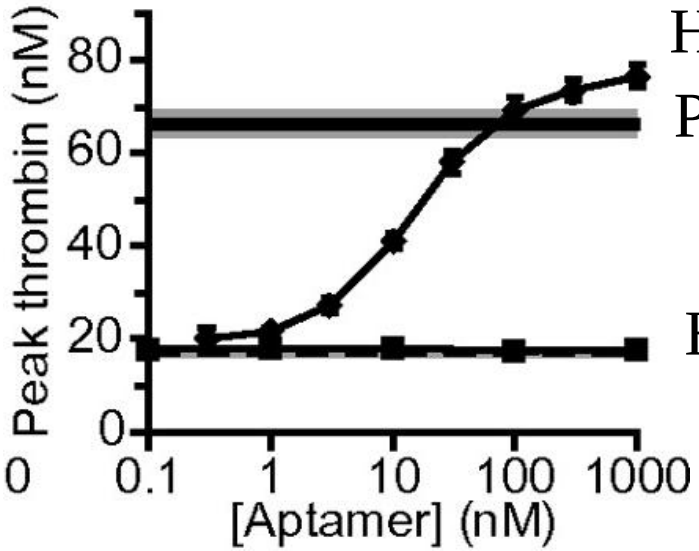
Blockage of the KPI-2 domain prevents TFPI binding to both FXa and FVIIa/TF

Downregulation of TFPI inhibition of the coagulation cascade

Further FXa generation in response to tissue injury.

blood
JOURNAL OF THE AMERICAN SOCIETY OF HEMATOLOGY

Aptamer anti TFPI ARC19499: increased thrombin generation in Hemophilia plasma



HA + aptamer

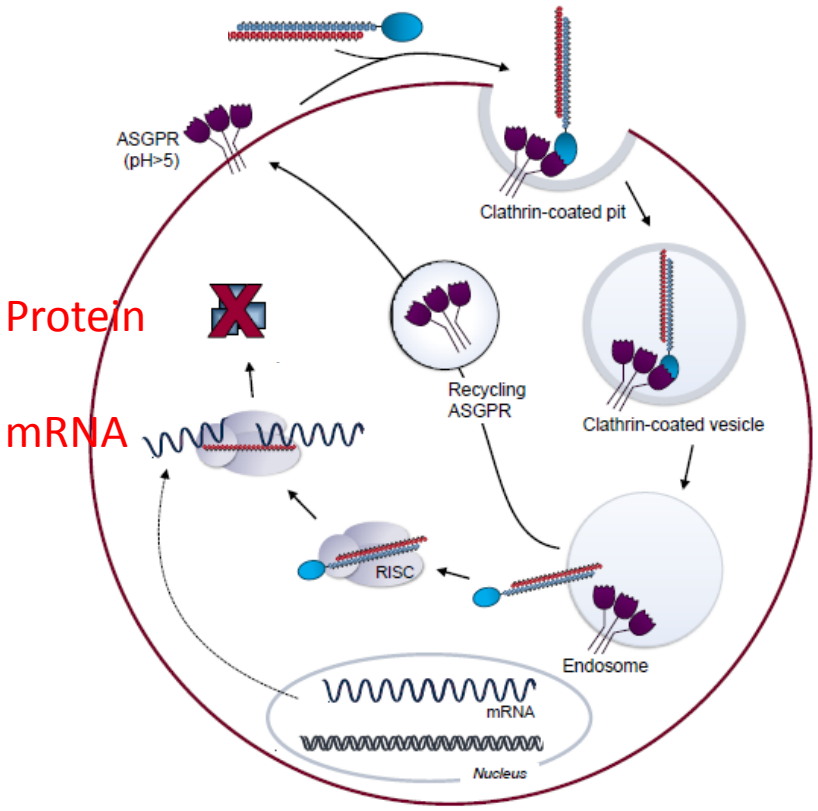
PNP

HemophiliaA (HA)

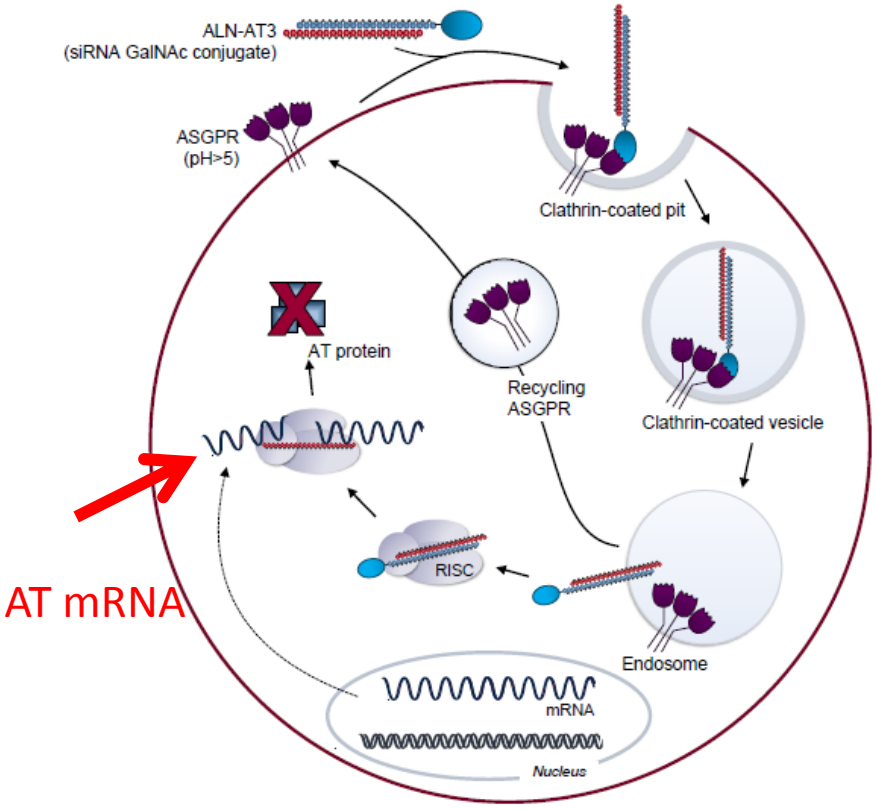
ARC19499 (♦)
negative control oligonucleotide (■).

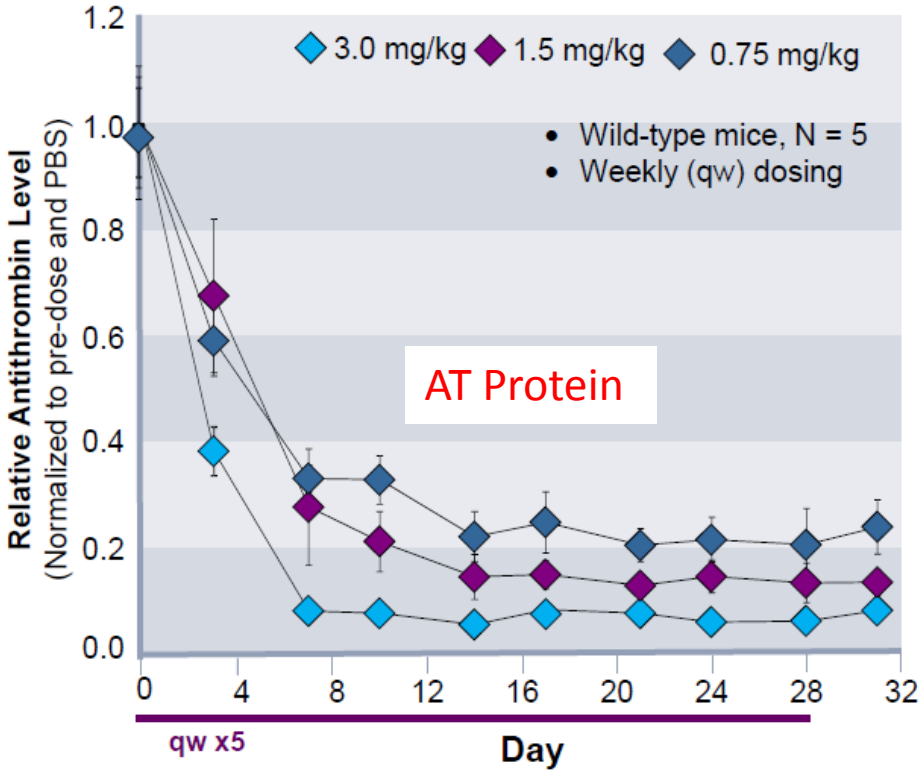
Waters E K et al. Blood 2011;117:5514-5522

RNA interference (RNAi)



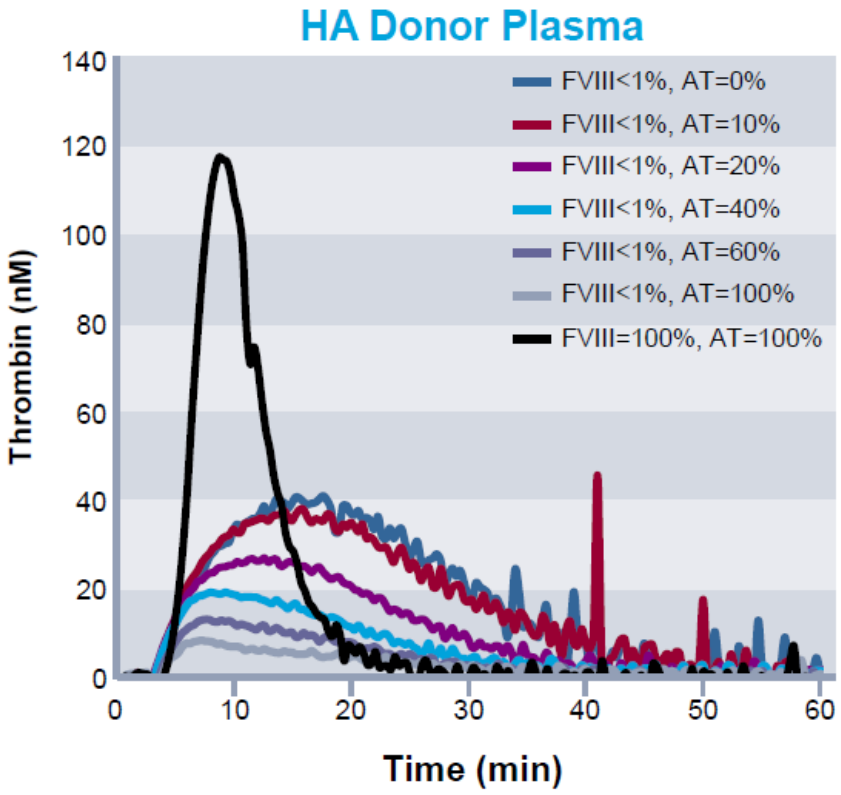
RNAi Targeting Antithrombin

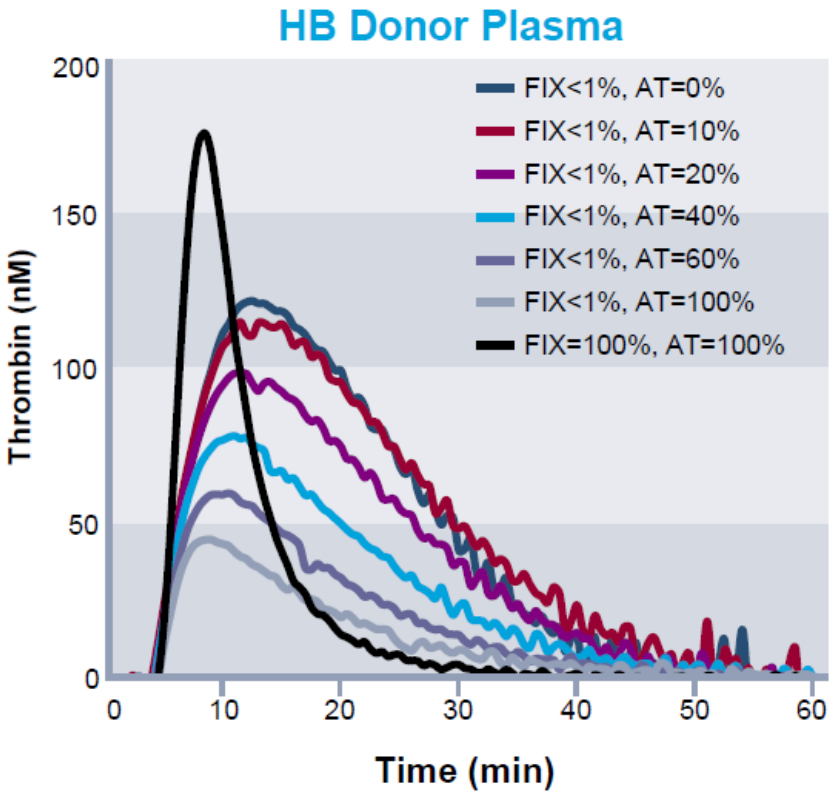




Antithrombin Depletion Increased Thrombin Generation in Hemophilia Plasma

ALN-AT3





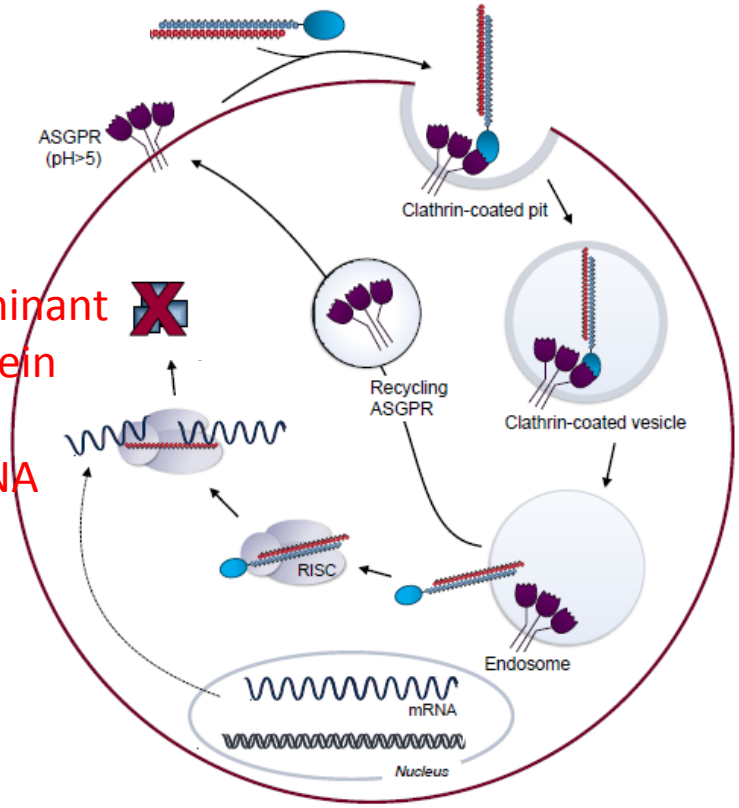
- Antithrombin depletion increases peak height and delays inhibition of thrombin

Molecular mechanisms for new therapeutic approaches (2)

RNA directed/based therapy

- Engineered small RNA: RNAi, U1snRNA
- Drug-induced translational readthrough of stop codons
- Transcription activation TALE-TF

Counteracting the dominant negative effect in VWD type 2 by RNAi



Dominant Protein

mRNA

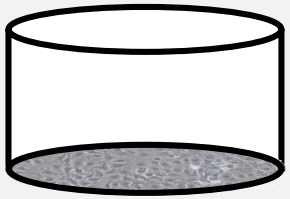
Caterina Casari

Counteracting the dominant-negative effect

in vitro

Plasmids
pSV-hVWF-DEL
pSV-hVWF-wt

Co-transfection

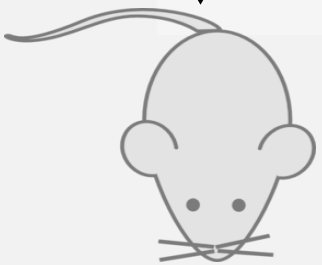


COS-1 cells

in vivo

Plasmids
pLIVE-mVWF-DEL
pLIVE-mVWF-wt

Co-injection via hydrodynamic gene transfer



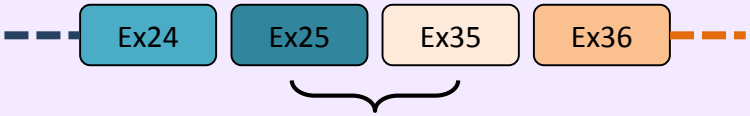
Vwf $^{-/-}$ mice

Heterozygous state expression

VWF antigen levels & VWF multimer profile were assessed in conditioned media and in plasma.
Bleeding phenotype was assessed in vivo

Counteracting the dominant-negative effect: allele-specific RNAi -*in vitro*-

- Use RNAi to specifically block expression of the dominant negative allele



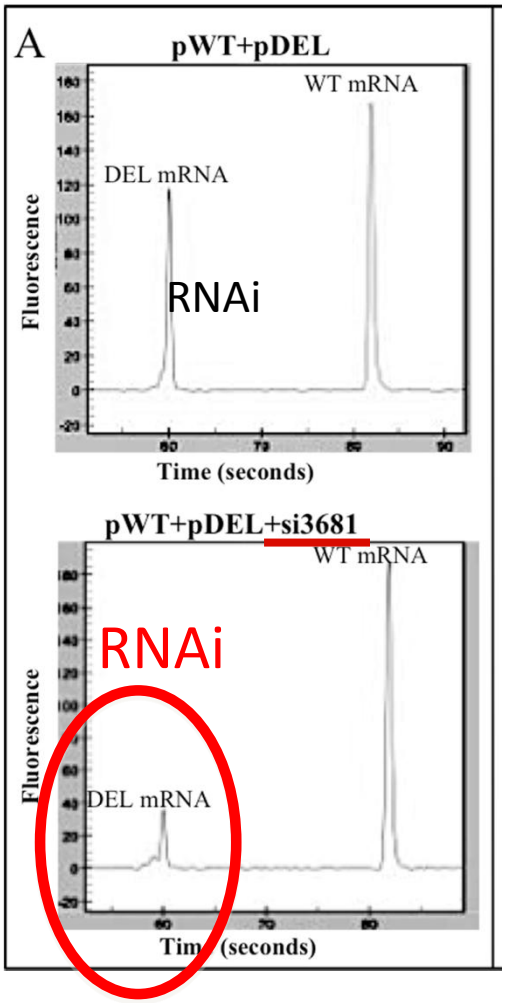
Mutant junction sequence connecting exons 25-35 is targeted by RNAi

WT-VWF not affected

Casari C et al. Blood 2010

Counteracting the dominant-negative effect: allele-specific RNAi -*in vitro*-

- Use RNAi to specifically block expression of the dominant negative allele



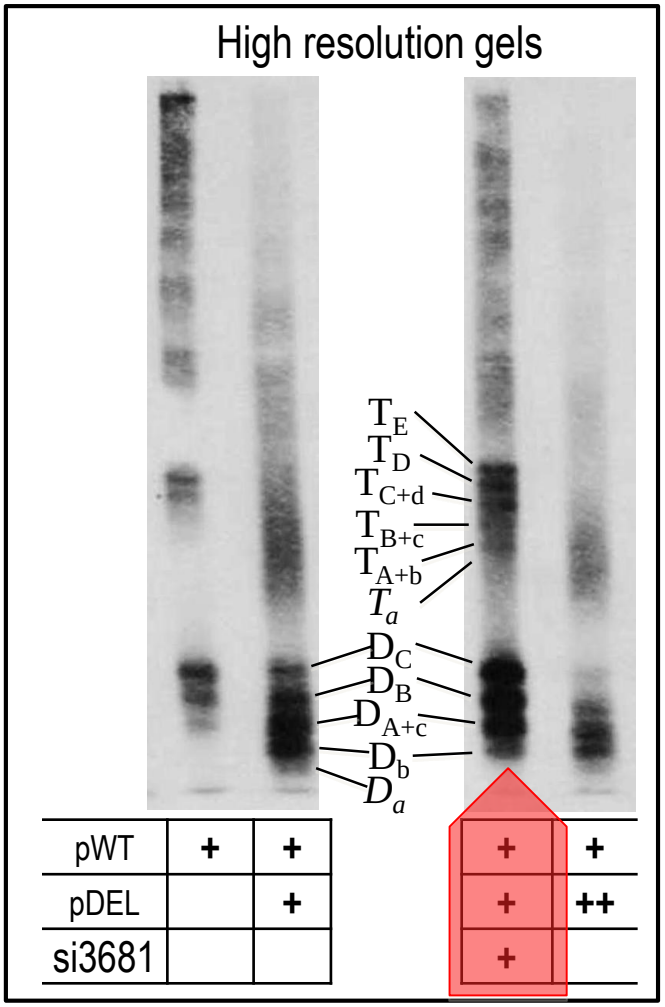
Capillary electrophoresis of RT- PCR products from transfected cells

si3681 decreased DEL-VWF mRNA & did not affect WT-VWF mRNA

Selective decrease of the altered mRNA and thus of the dominant negative VWF

Casari C et al. Blood 2010

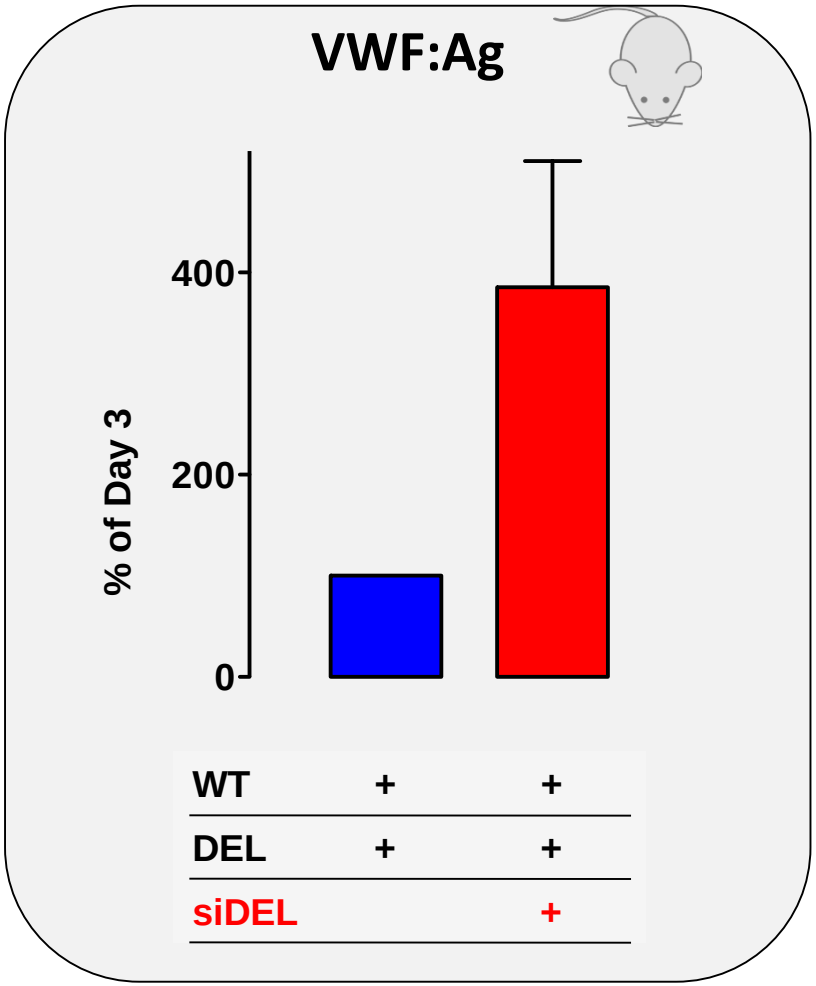
Counteracting the dominant-negative effect: Partial rescue of VWF



Partial rescue of HMWMs

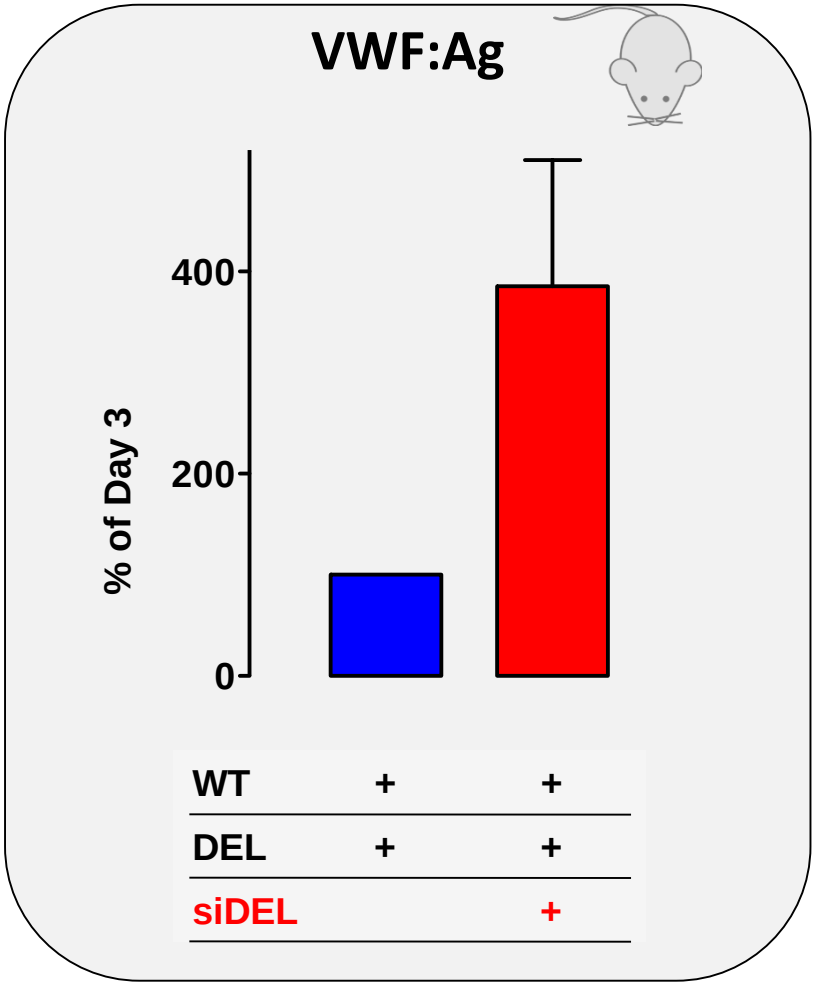
Casari C et al. Blood 2010

in vivo RNA *interference*

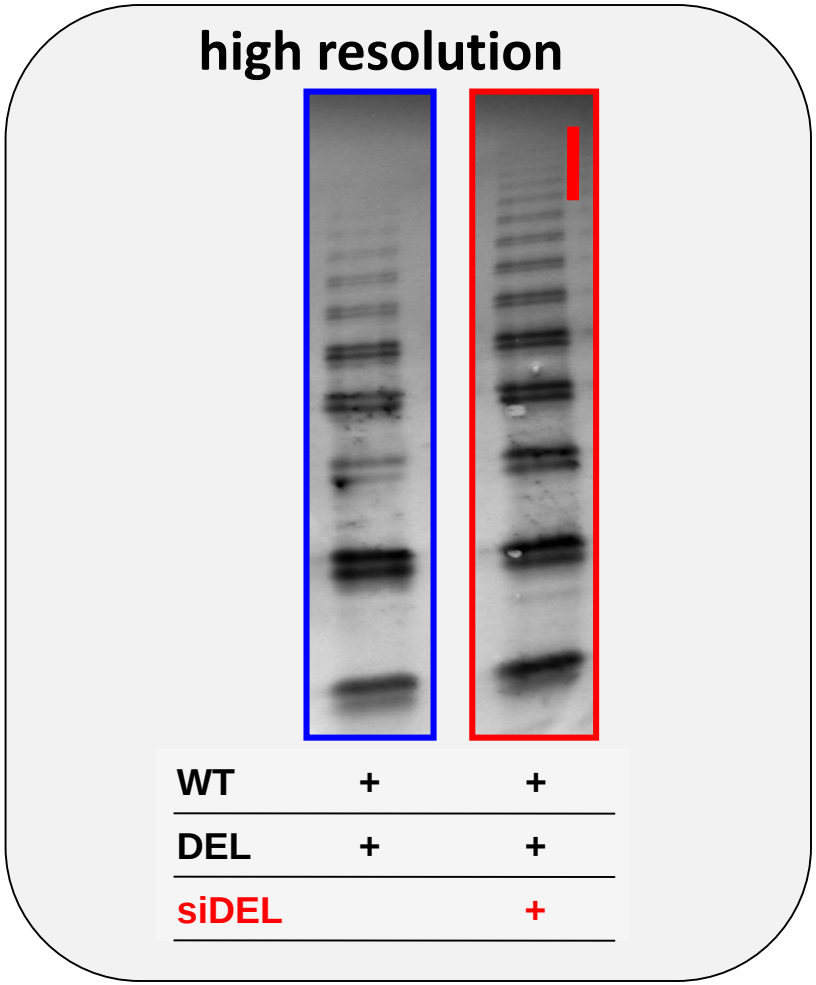


3.8 fold increase of the antigen levels by siDEL administration

in vivo RNA *interference*



3.8 fold increase of the antigen levels by siDEL administration



improvement of the multimer profile

Counteracting the dominant-negative effect in VWD type 2 by RNAi

- The effects of mutations with dominant-negative effect were reproduced for the first time *in vivo* in *Vwf*-/- mice

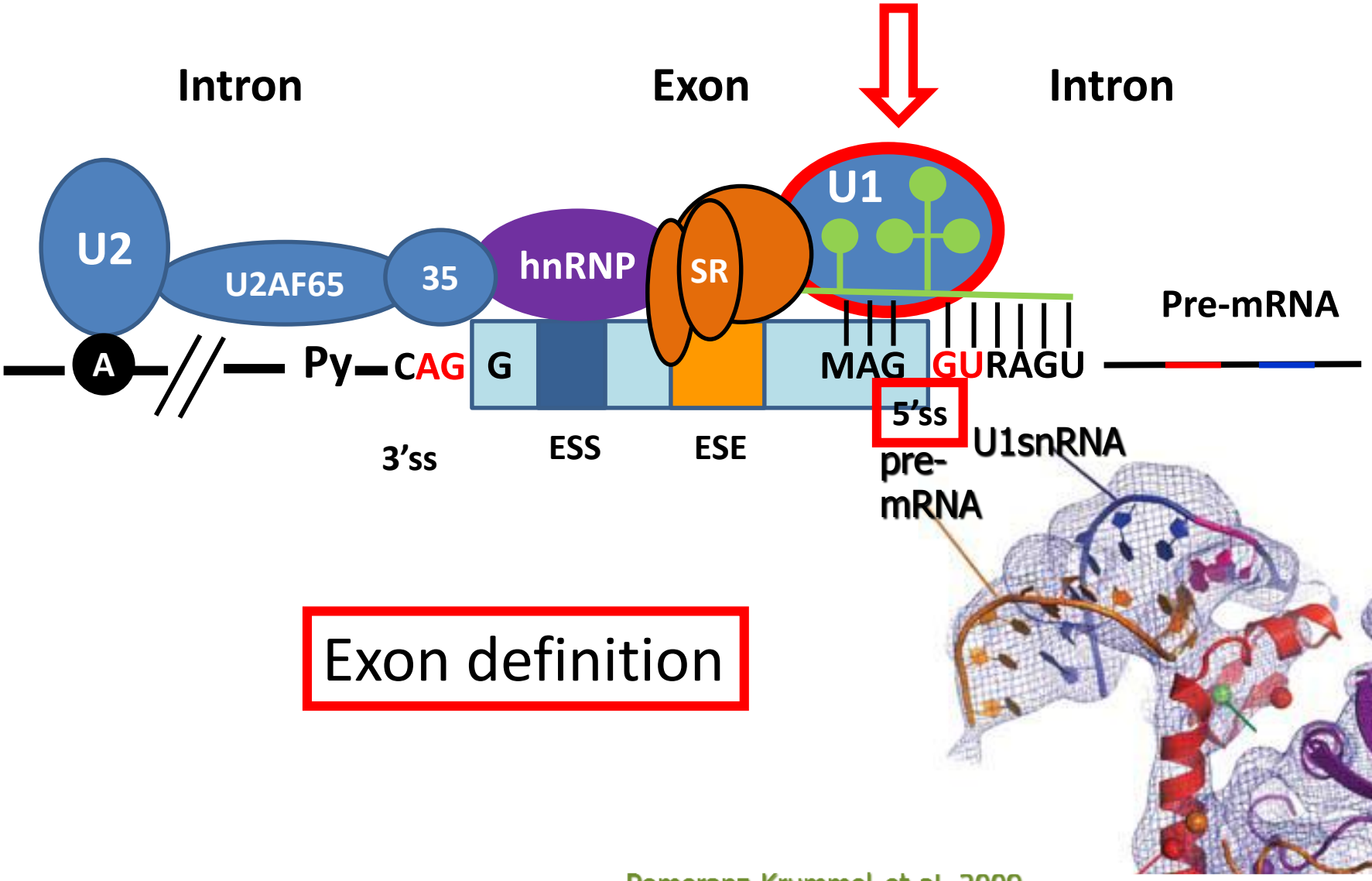
- In our mouse model for the heterozygous expression of mutations with dominant-negative effects on VWF, we were able to counteract the deleterious effects of the mutations by the *in vivo* administration of siRNAs, selectively targeting the mutant mRNA molecules
  → *siRNA was administered for the first time in a mouse model of VWD and could represent a therapeutic approach*

Molecular mechanisms for new therapeutic approaches (2)

RNA directed/based therapy

- Engineered small RNA: RNAi, **U1snRNA**
- Drug-induced translational readthrough of stop codons
- Transcription activation TALE-TF

Splicing steps: focus on U1snRNA



Exon definition

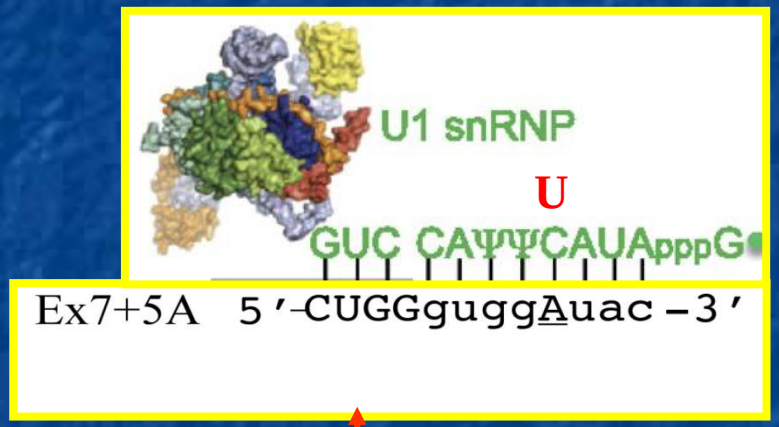
Pomeranz-Krummel et al. 2009

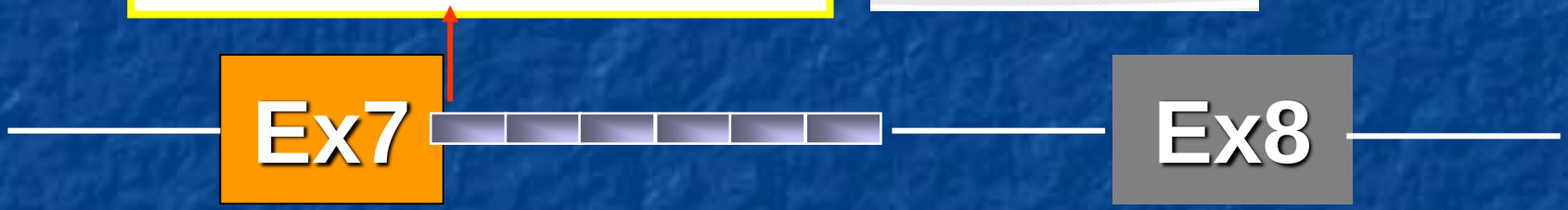
Recognition of 5′ss by base-pairing to the 5′ end of U1 snRNA: Consensus sequence of 5′ss

Adapted from Roca X et al. Genes Dev. 2013;27:129-144

Affinity and position

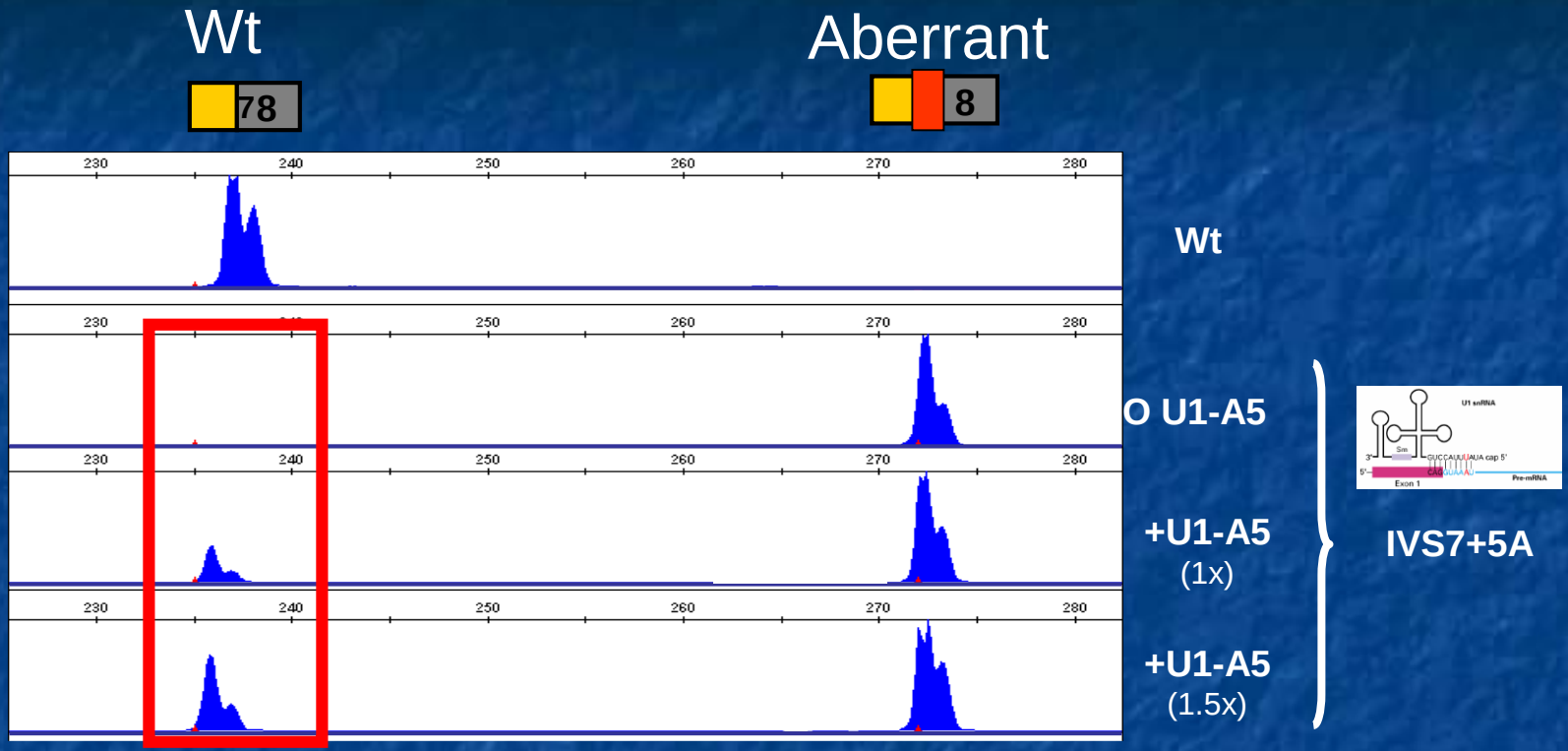
Splicing Modulation by modified U1 as tools for therapeutic approach





Rescue by Engineered U1snRNA with improved complementarity –increased affinity - to the mutated donor splice site?

Dose dependent induction of correct splicing

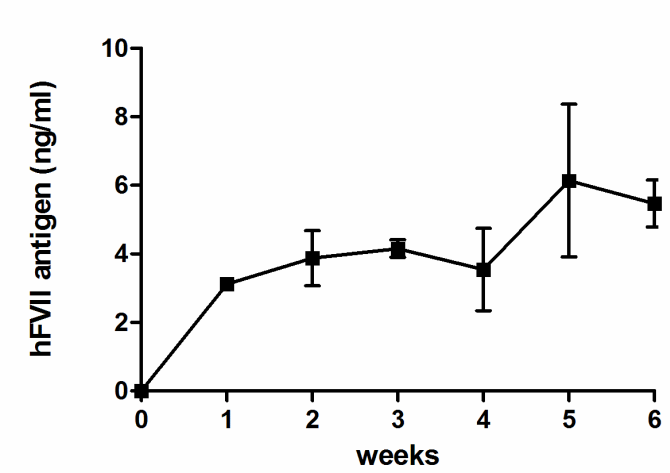


The correctly spliced form is 15±5% of the aberrant form

Pinotti Blood 2008

The U1+5a-mediated rescue of circulating hFVII levels was prolonged overtime by AAV delivery

Human FVII



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

Penn
University of Pennsylvania

Balestra et al JTH 2014

Splicing Modulation by modified U1

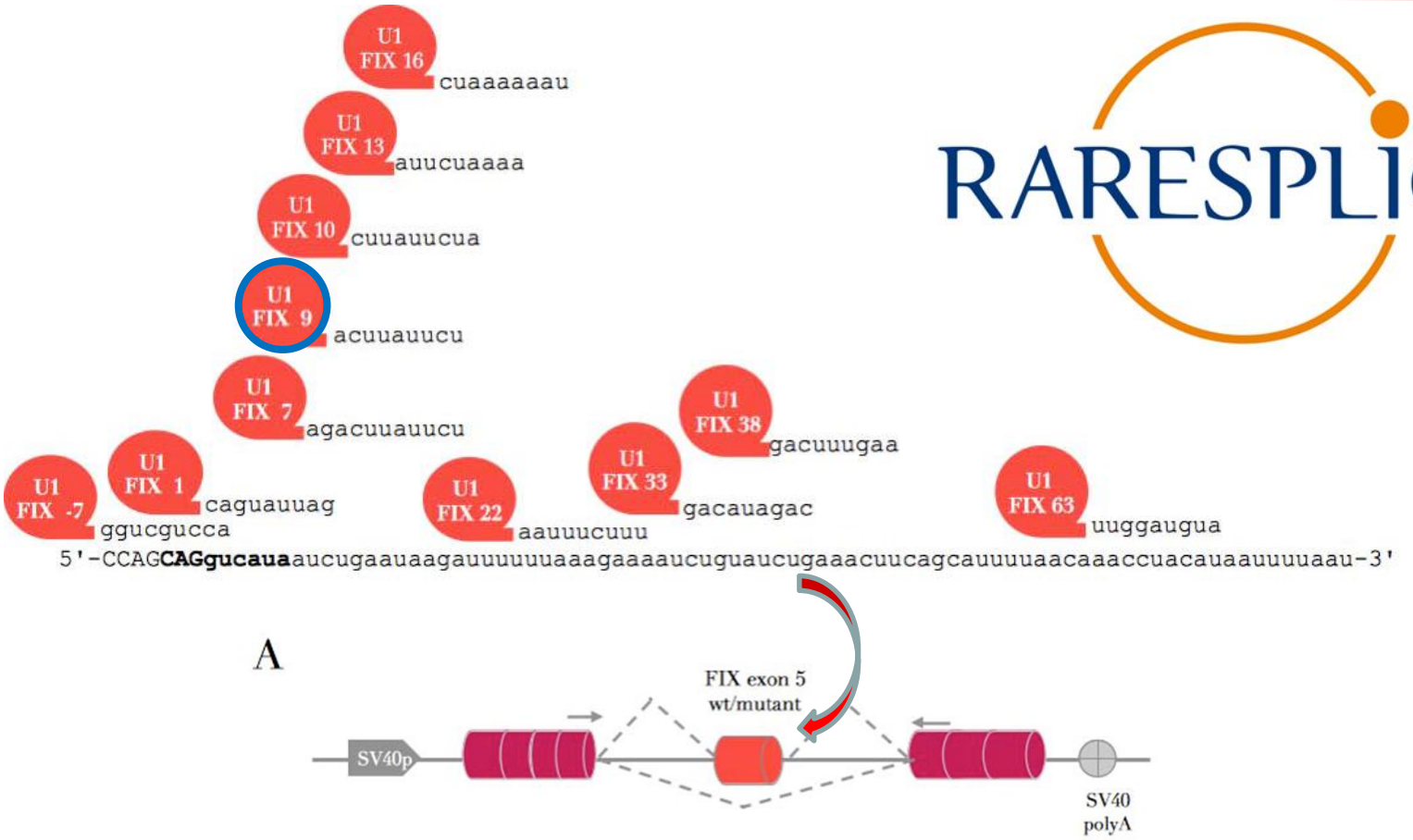
Mutation specific U1snRNAs
Able to correct a single 5'ss change

?

Exon specific U1 snRNAs
able to correct multiple splicing mutations

RARESPLICE

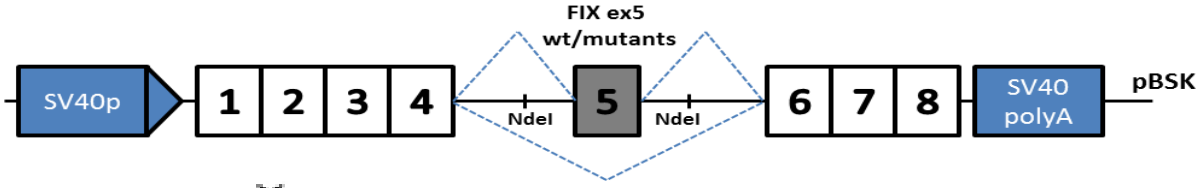
Engineered U1s (ExonSpecificU1s) for splicing correction of FIX exon 5 natural mutations



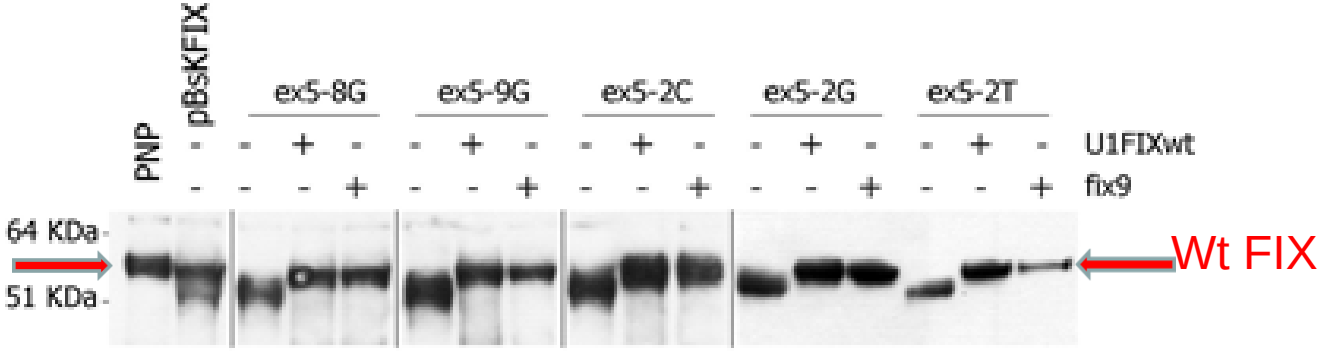
- Targeting U1 snRNAs to specific intronic regions located downstream DDSS to improve exon recognition and inclusion?

Alanis et al HMG 2012

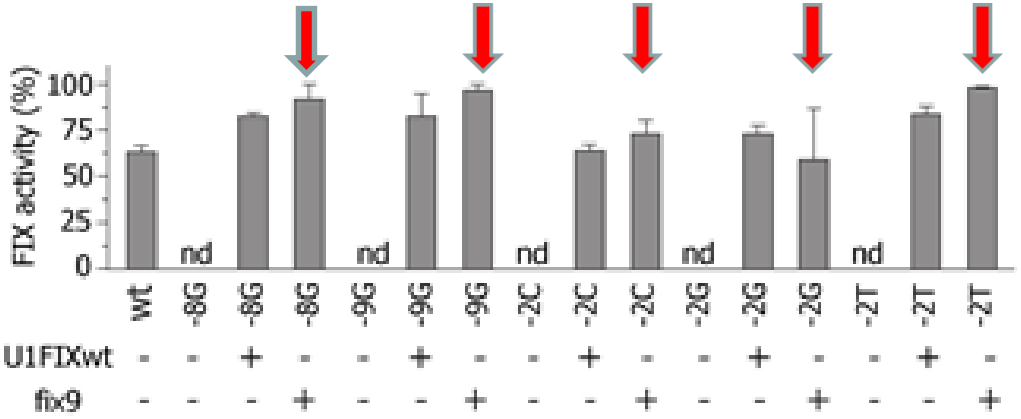
Rescue of FIX protein and FIX function by ExonSpecificU1



Protein



Function

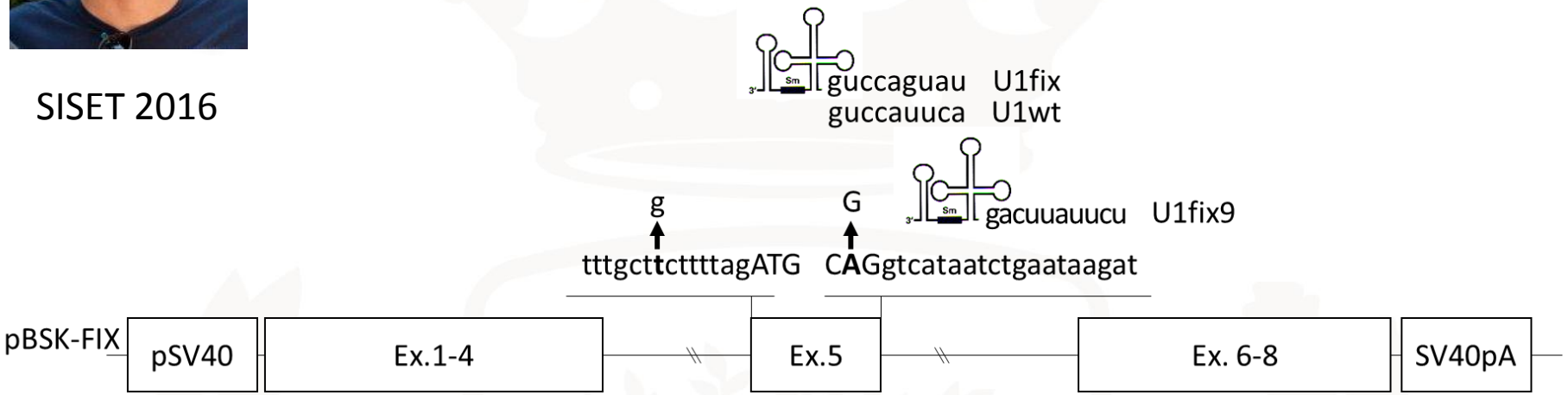


Alanis et al HMG 2012

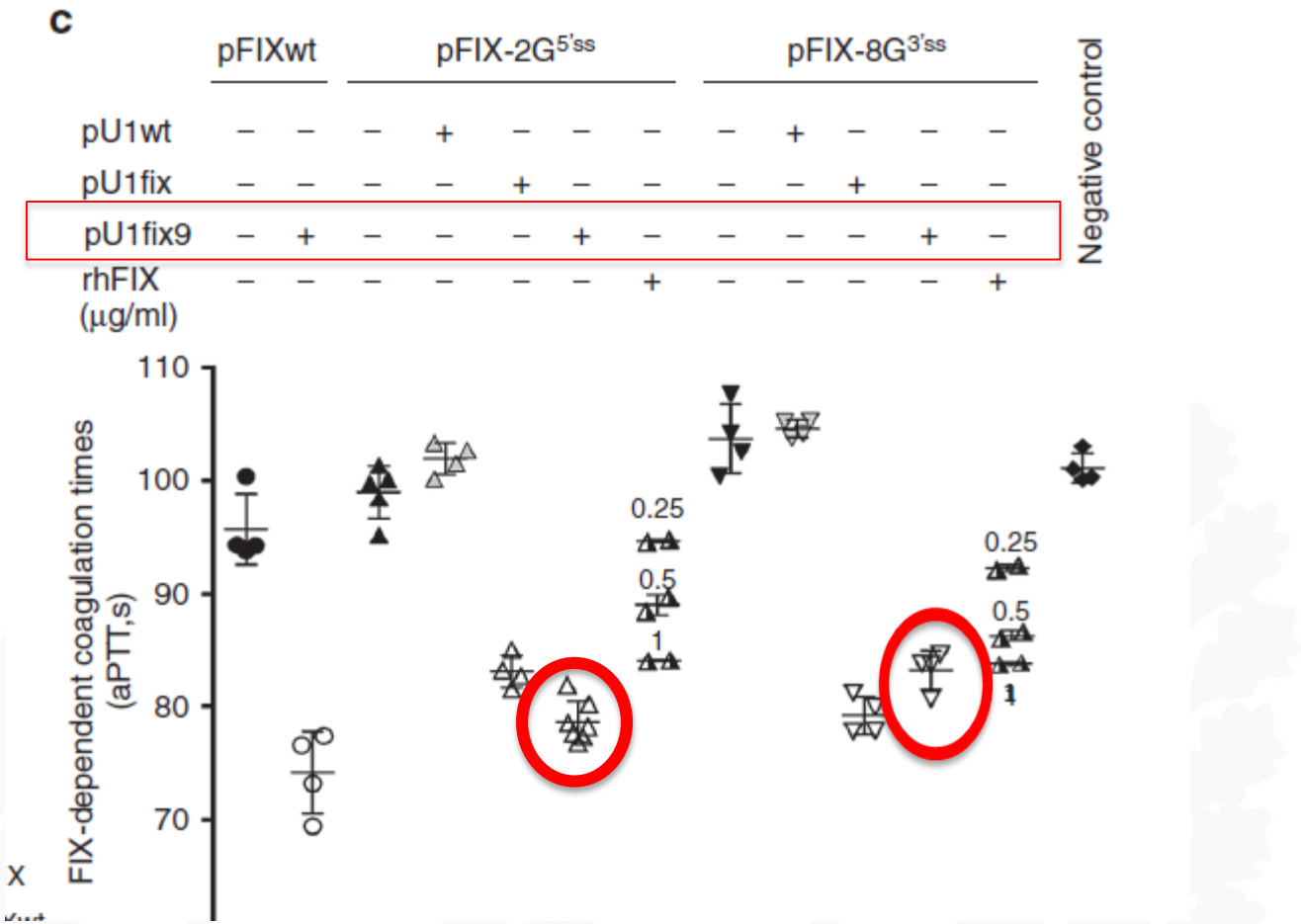
Dario Balestra

SISET 2016

Evaluation of ExSpeU1-mediated rescue



ExSpeU1-mediated rescue of hFIX expression: coagulation time



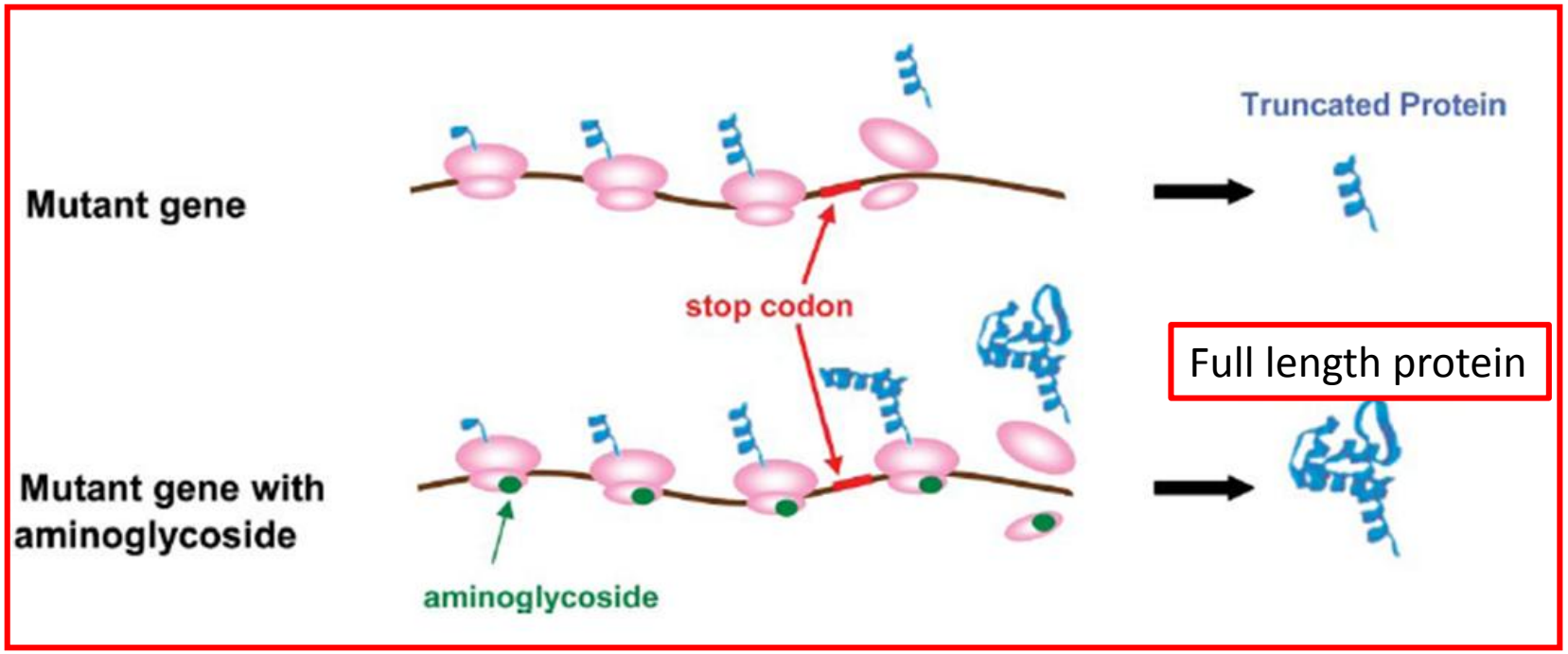
Shortening (~20sec) of coagulation time

Molecular mechanisms for new therapeutic approaches (2)

RNA directed/based therapy

- Engineered small RNA: RNAi, U1snRNA
- **Drug-induced translational readthrough of stop codons**
- Transcription activation TALE-TF

mRNA-based approaches:
Drug-induced translational readthrough of stop codons



Aminoglycosides bind the decoding site within the A-site in the ribosomal small subunit

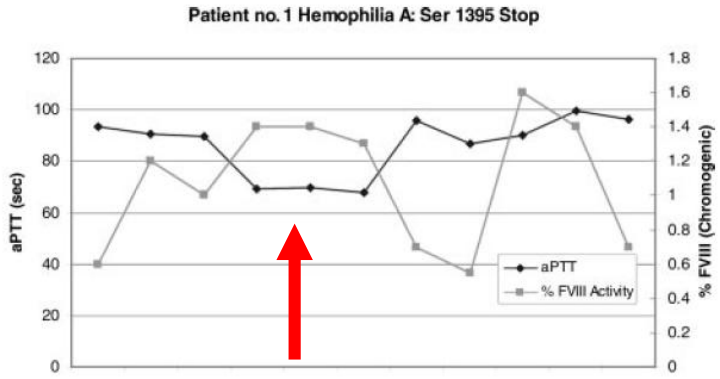
Aminoglycoside suppression of nonsense mutations in severe hemophilia

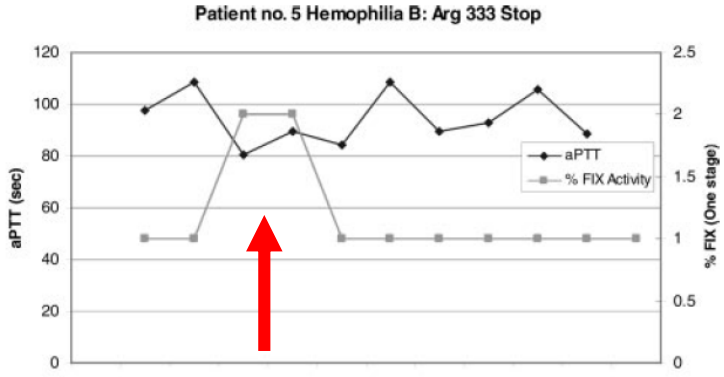
Paula D. James, Sanj Raut, Georges E. Rivard, Man-Chiu Poon, Margaret Warner, Susan McKenna, Jayne Leggo, and David Lillicrap

BLOOD, 1 NOVEMBER 2005 • VOLUME 106, NUMBER 9

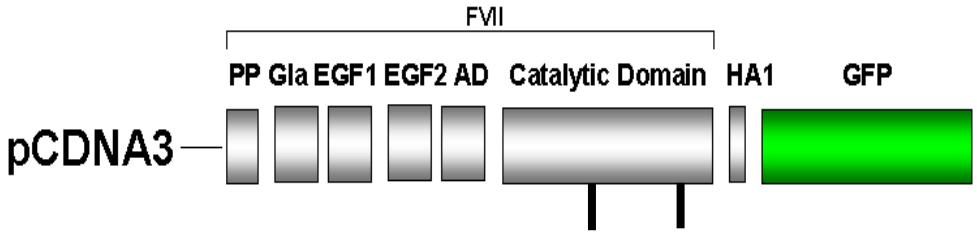
Table 1. Patients with severe hemophilia with known nonsense mutations treated with gentamicin

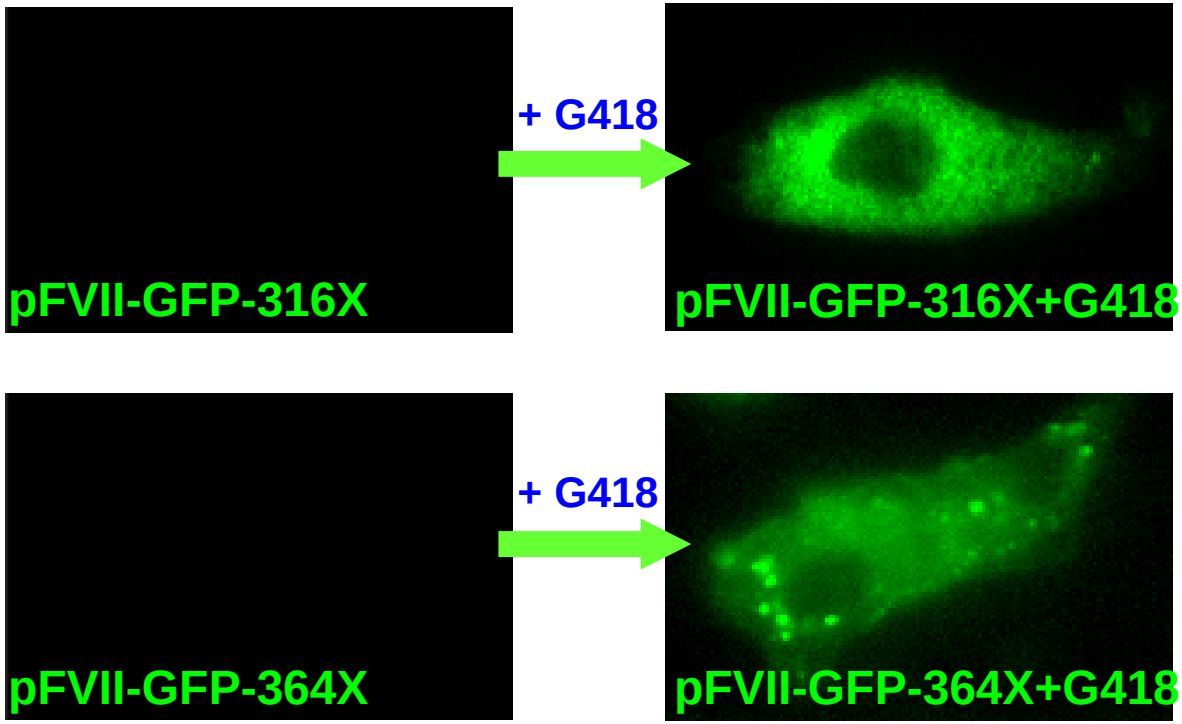
Patient no.	Hemophilia	Nucleotide	Mutation	Conserved amino acid
1	A	4241C > A	Ser1395Stop	No
2	B	30875C > T	Arg252Stop	Yes
3	A	6403C > T	Arg2116Stop	Yes
4	A	1536C > T	Arg427Stop	Yes
5	B	31118C > T	Arg333Stop	Yes





FVII Deficiency: cellular models

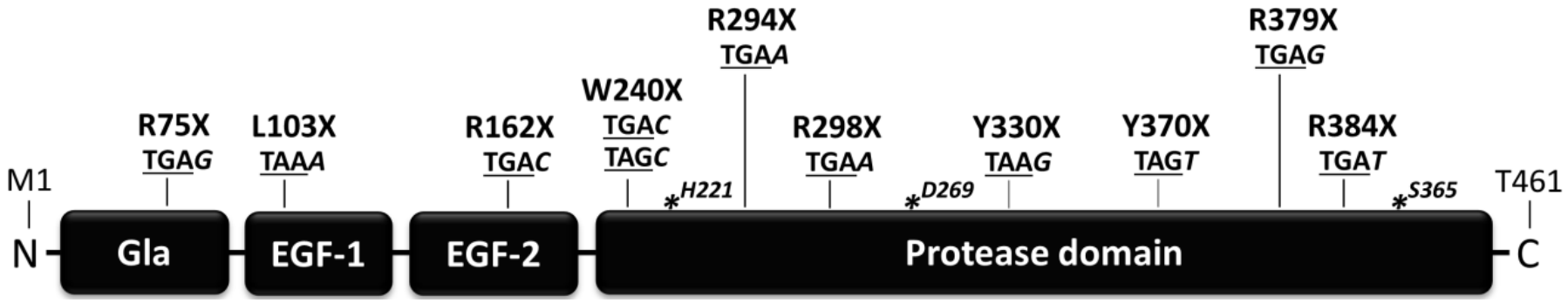




fluorescence supports partial rescue of full-length FVII biosynthesis

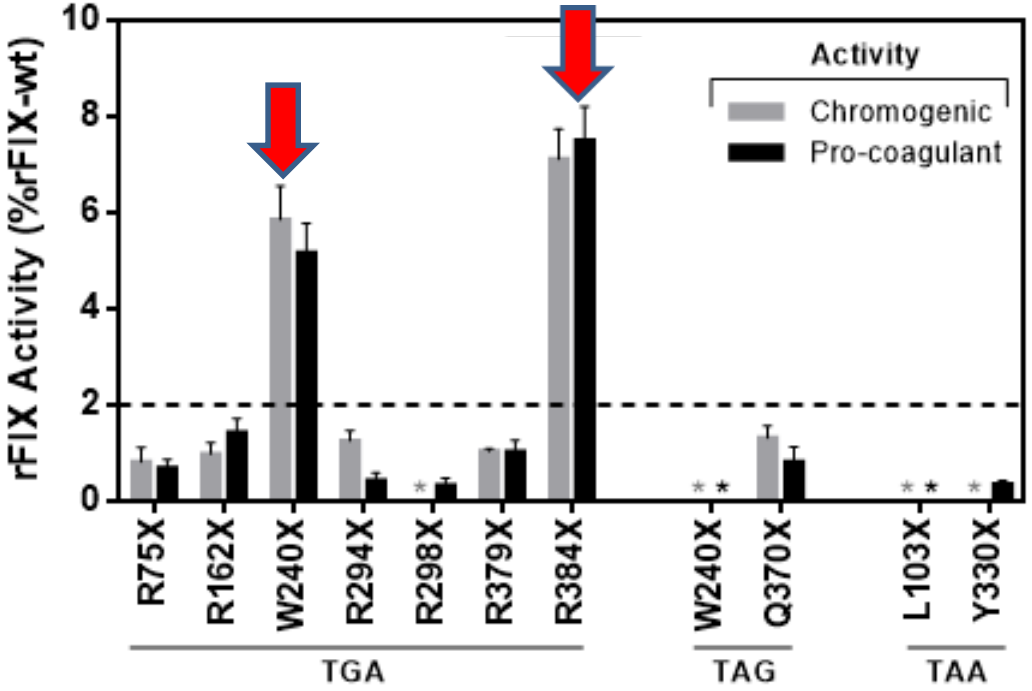
Pinotti et al, JTH 2006

Drug-induced translational readthrough investigated by expression of nonsense variants present in 70 % of HB patients with premature stop codons



Mattia Ferrarese

only two variants displayed a remarkable rescue of activity



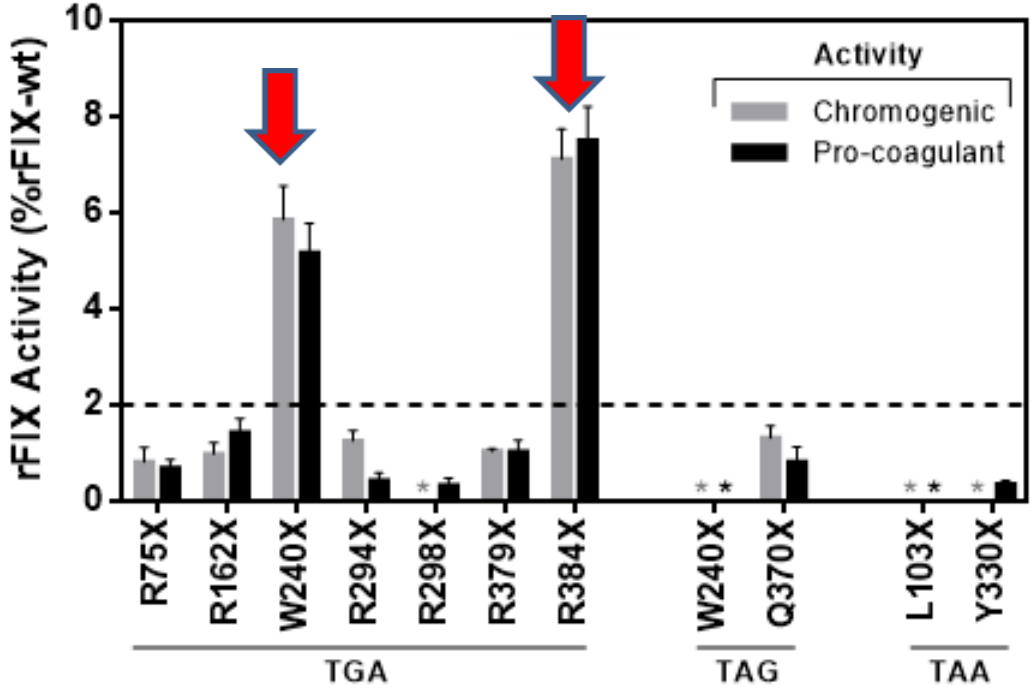
Expression of the **most probable missense variants** arising from readthrough

only two variants displayed a remarkable rescue of activity

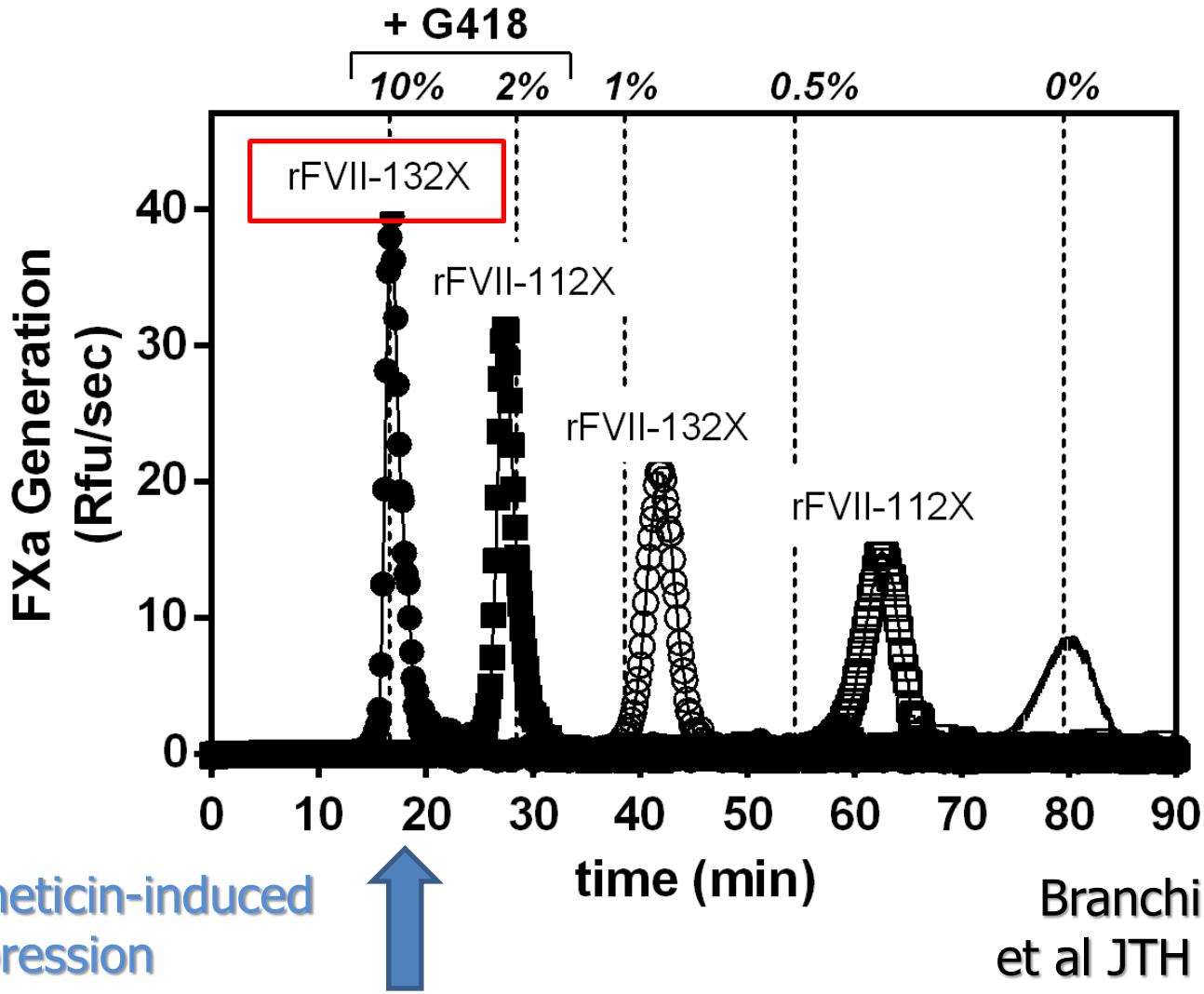
Re-insertion of **W** wild-type residue

R384W

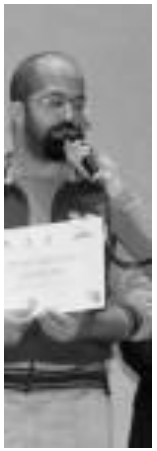
gain-of-function



Stop codons in FVII deficiency: the appreciable rescue of the p.Cys132X is driven by re-insertion of the wild-type residue!



Branchini et al JTH 2016



Drug-induced translational readthrough of stop codons

- Only a few nonsense mutations in coagulation factor genes are remarkably responsive to drug-induced readthrough due to specific nucleotide sequence and protein structure constraints.

- The recombinant expression of nonsense variants

  helps interpreting the poor response reported in the few investigated patients

  Helps selecting candidate patients eligible for treatment

Molecular mechanisms for new therapeutic approaches (2)

RNA directed/based therapy

- Engineered small RNA: RNAi, U1snRNA
- Drug-induced translational readthrough of stop codons
- **Transcription activation - TALE-TF**

AN ENGINEERED TALE-TRANSCRIPTION FACTOR RESCUES F7 PROMOTER ACTIVITY IMPAIRED BY MUTATIONS CAUSING SEVERE FACTOR VII DEFICIENCY

RNA

Silvia Pignani
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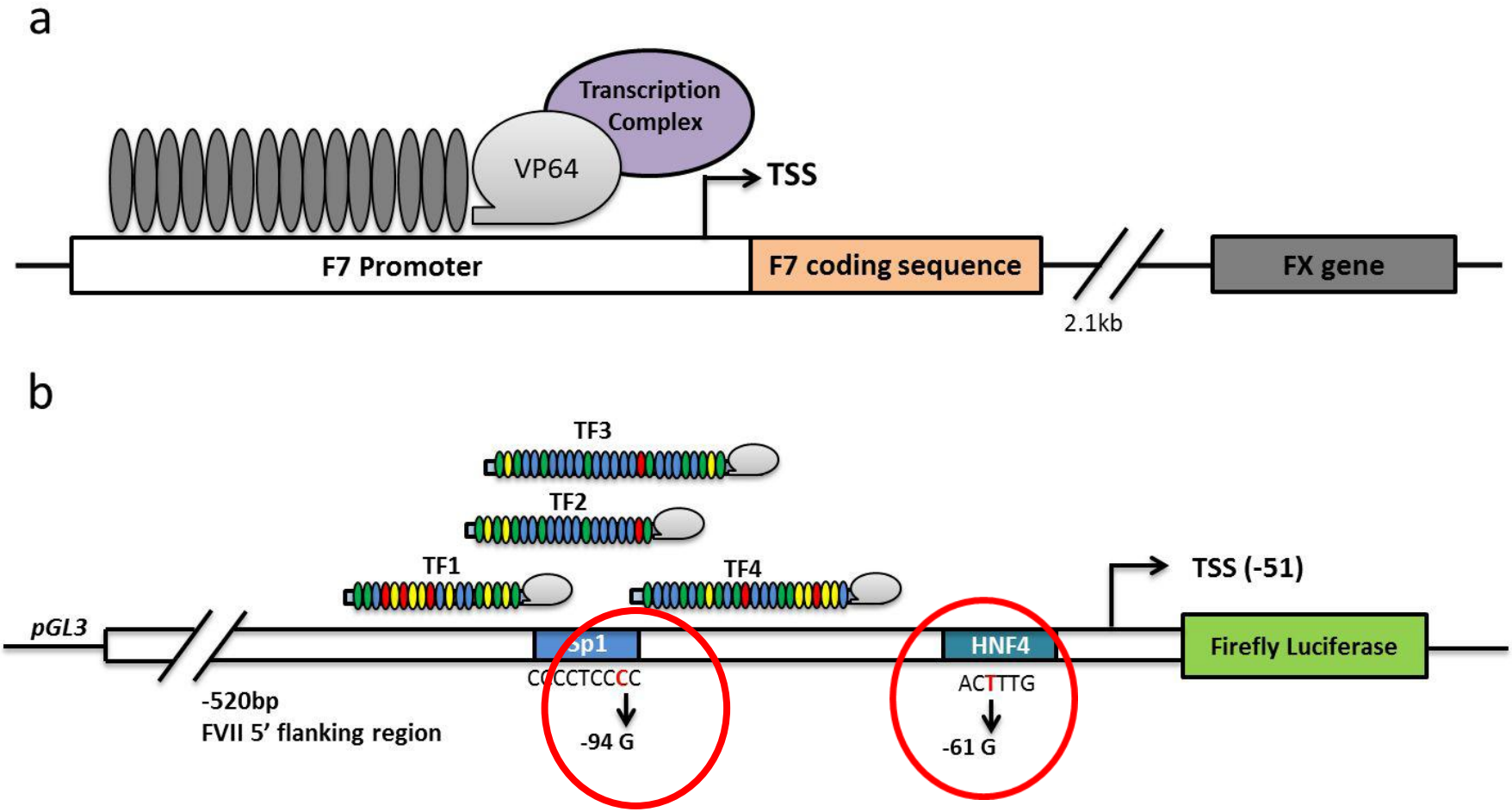
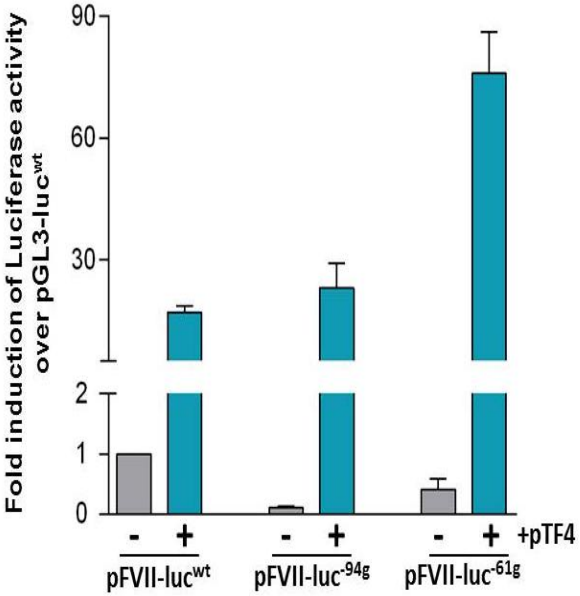
pgnslv@unife.it

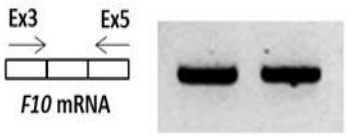
ENGINEERED TALE-TRANSCRIPTION FACTORS TO RESCUE TRANSCRIPTION OF FACTOR VII IMPAIRED BY PROMOTER MUTATIONS

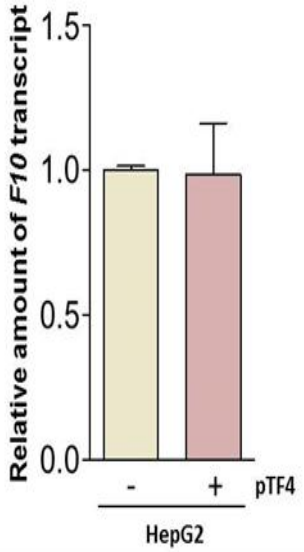


TALE-TF4 enhances activity of the *F7* promoter in HepG2 cells



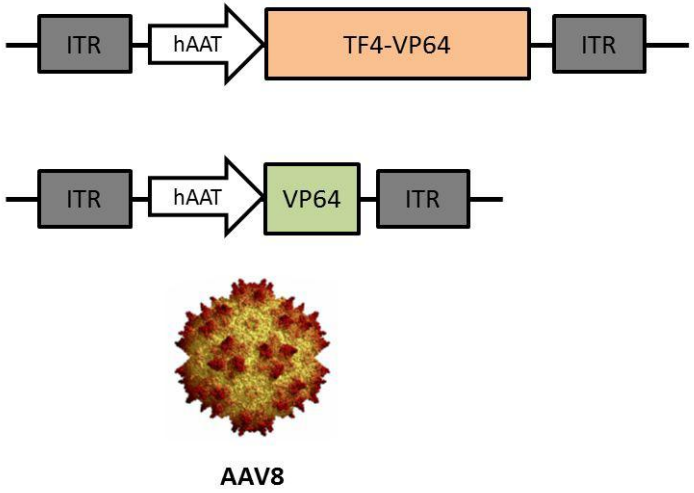
wild type mutants

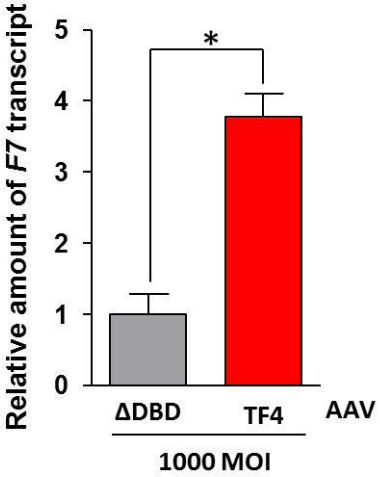


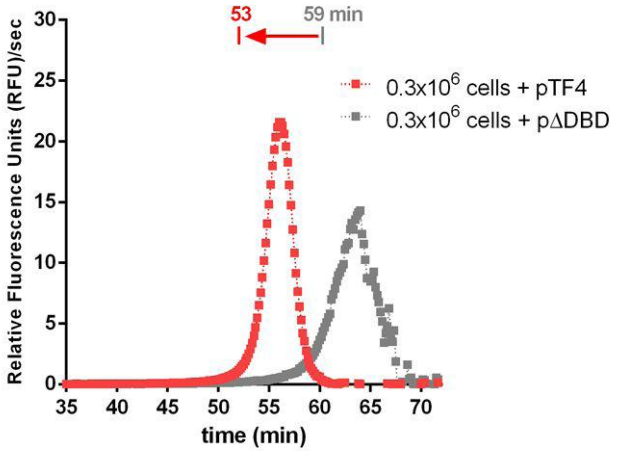


TALE-TF4 does not induce trascription of *F10*

TF4 increased F7 mRNA and FVII procoagulant levels in hepatocytes transduced by AAV







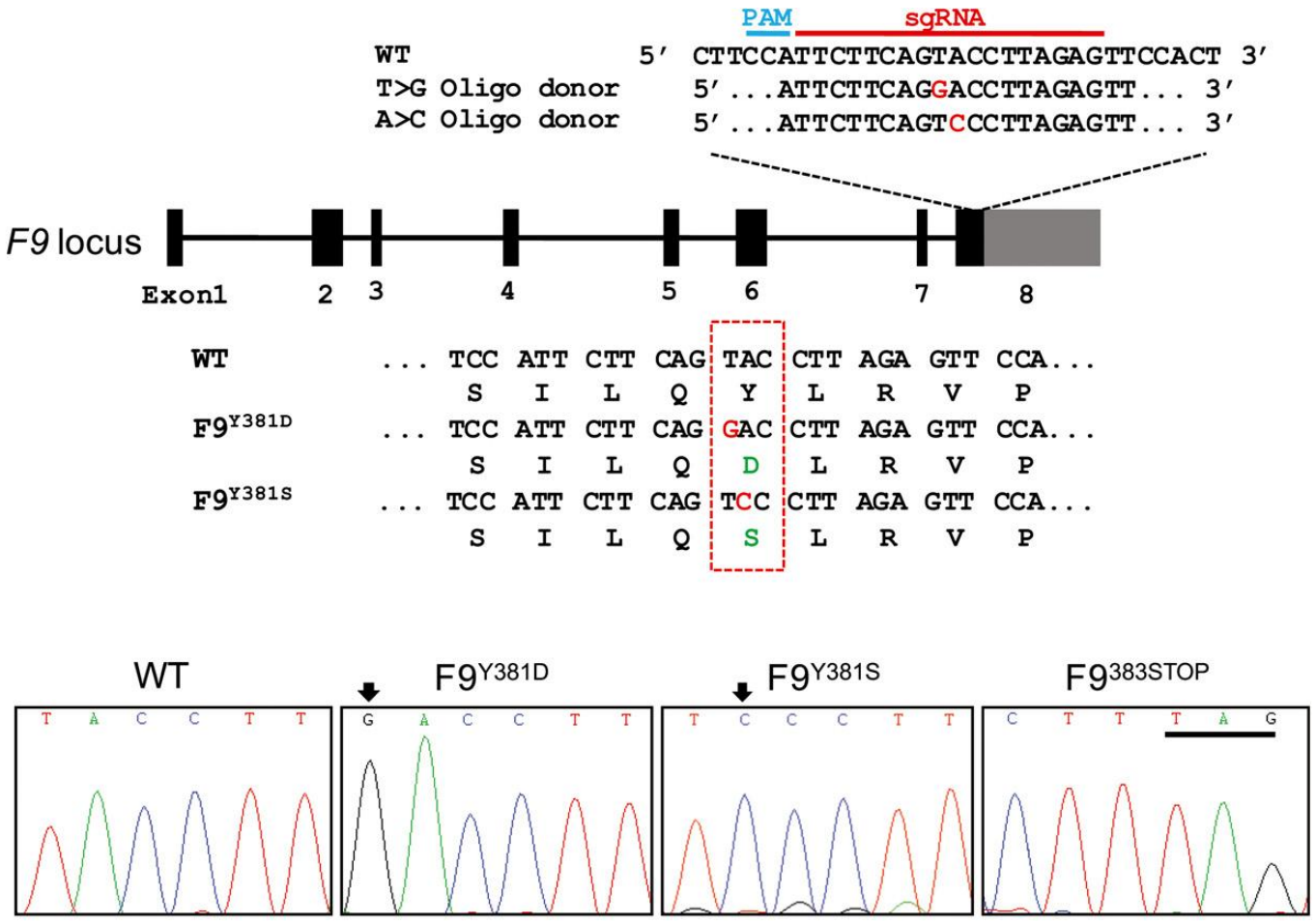
Experimental evidence for TALE-TFs as gene-specific tools useful to counteract disease-causing promoter mutations

Molecular mechanisms for new therapeutic approaches (3)

Gene Editing (Correction/Insertion):

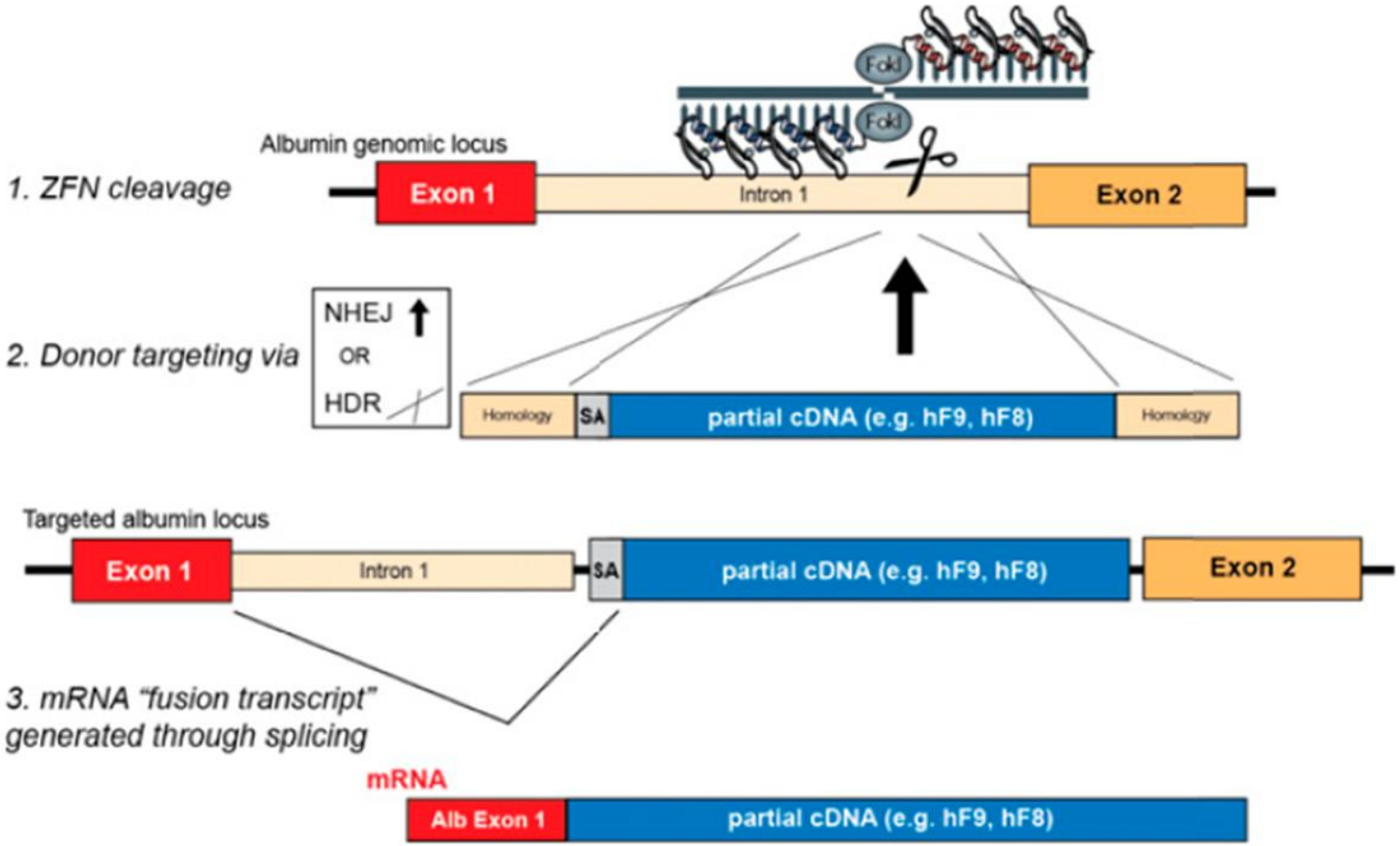
- Zinc Finger Nuclease
- TALE Nuclease
- CRISPR/Cas9
- Without nuclease

Genome editing in situ by CRISPR/Cas9 restores hemostasis in F9 mutant mice



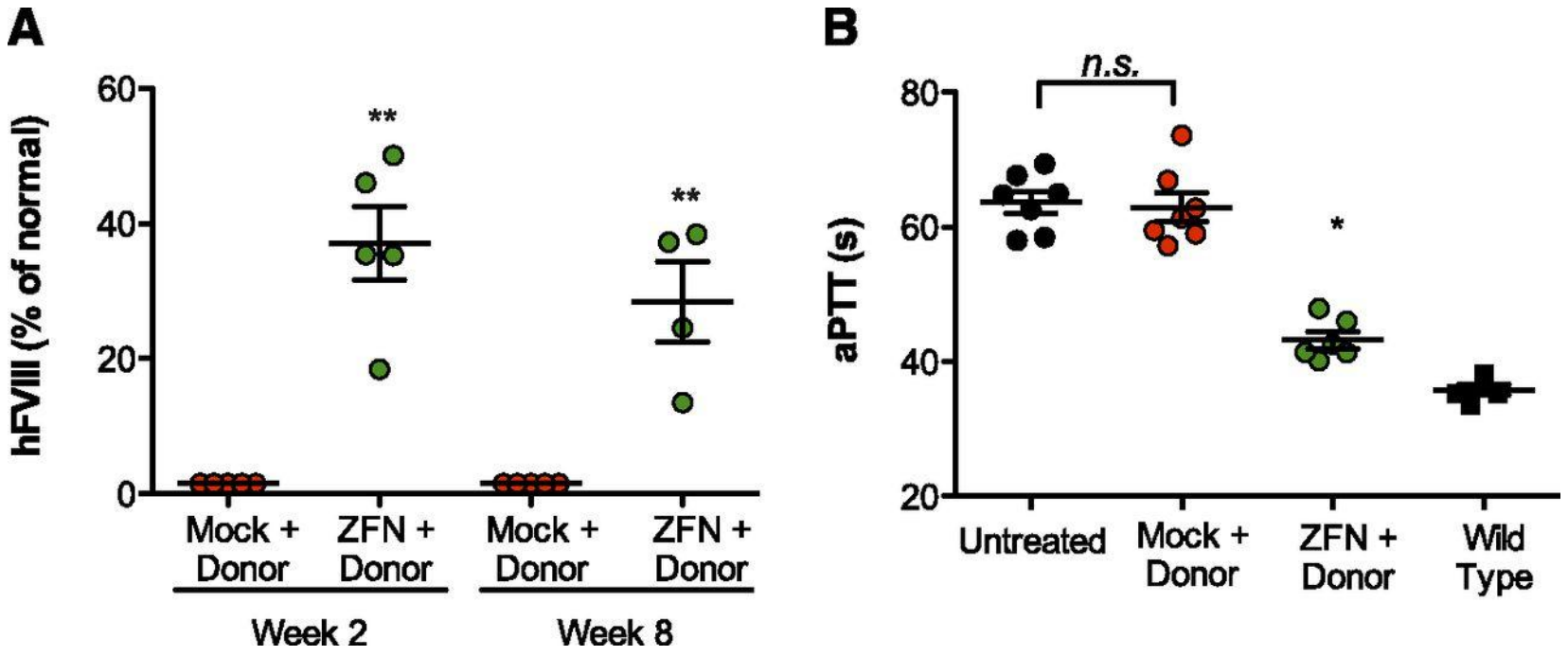
Yuting Guan et al EMBO Mol Med 2016

Targeting albumin supports production of therapeutic levels of FVIII and functional correction of hemophilia A phenotype.



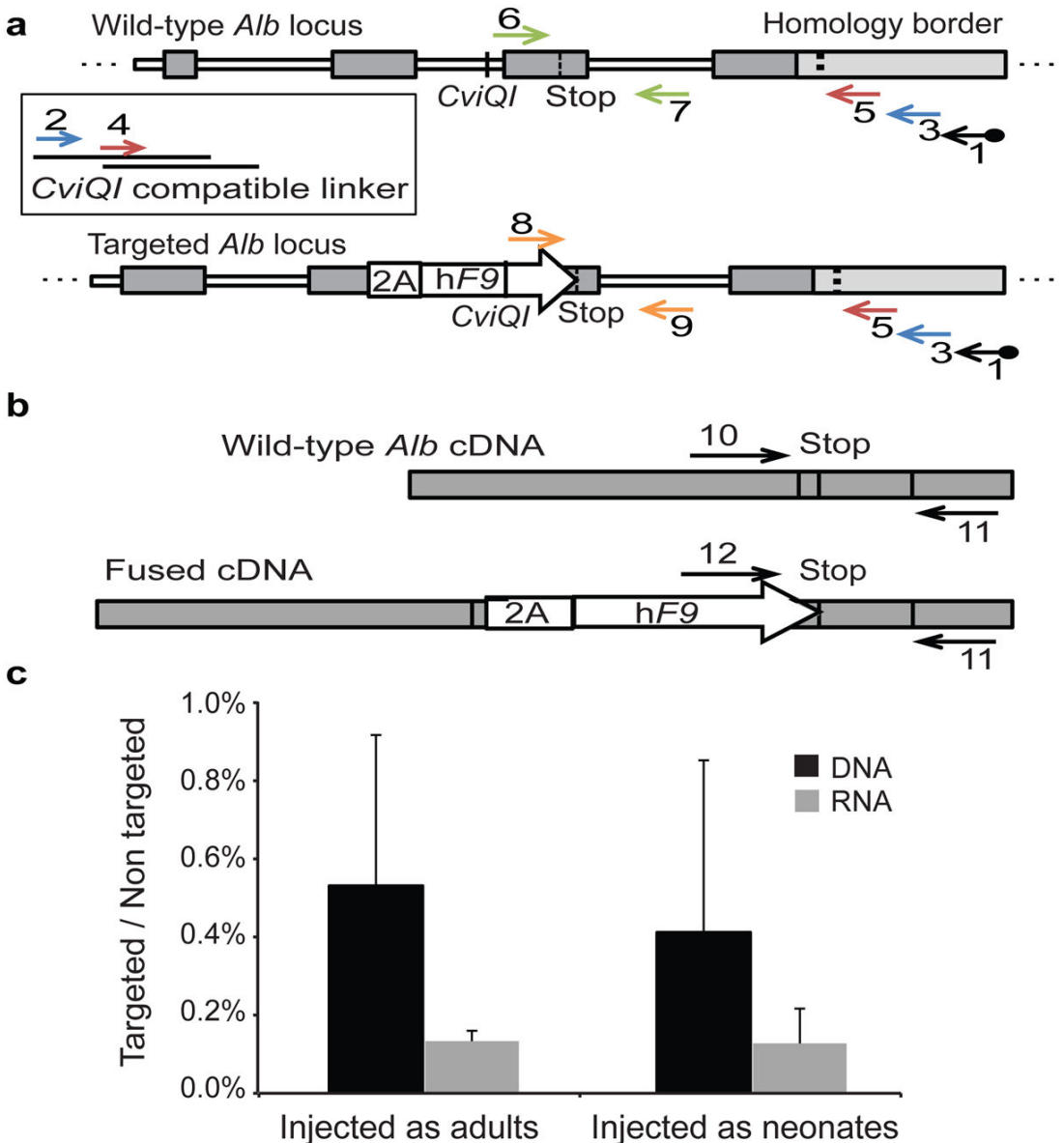
Sharma et al. Blood 2015

Targeting albumin supports production of therapeutic levels of FVIII and functional correction of hemophilia A phenotype.



Rajiv Sharma et al. Blood 2015;126:1777-1784

Promoterless gene targeting without nucleases ameliorates haemophilia B in mice



Barzel et al
Nature 2015

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