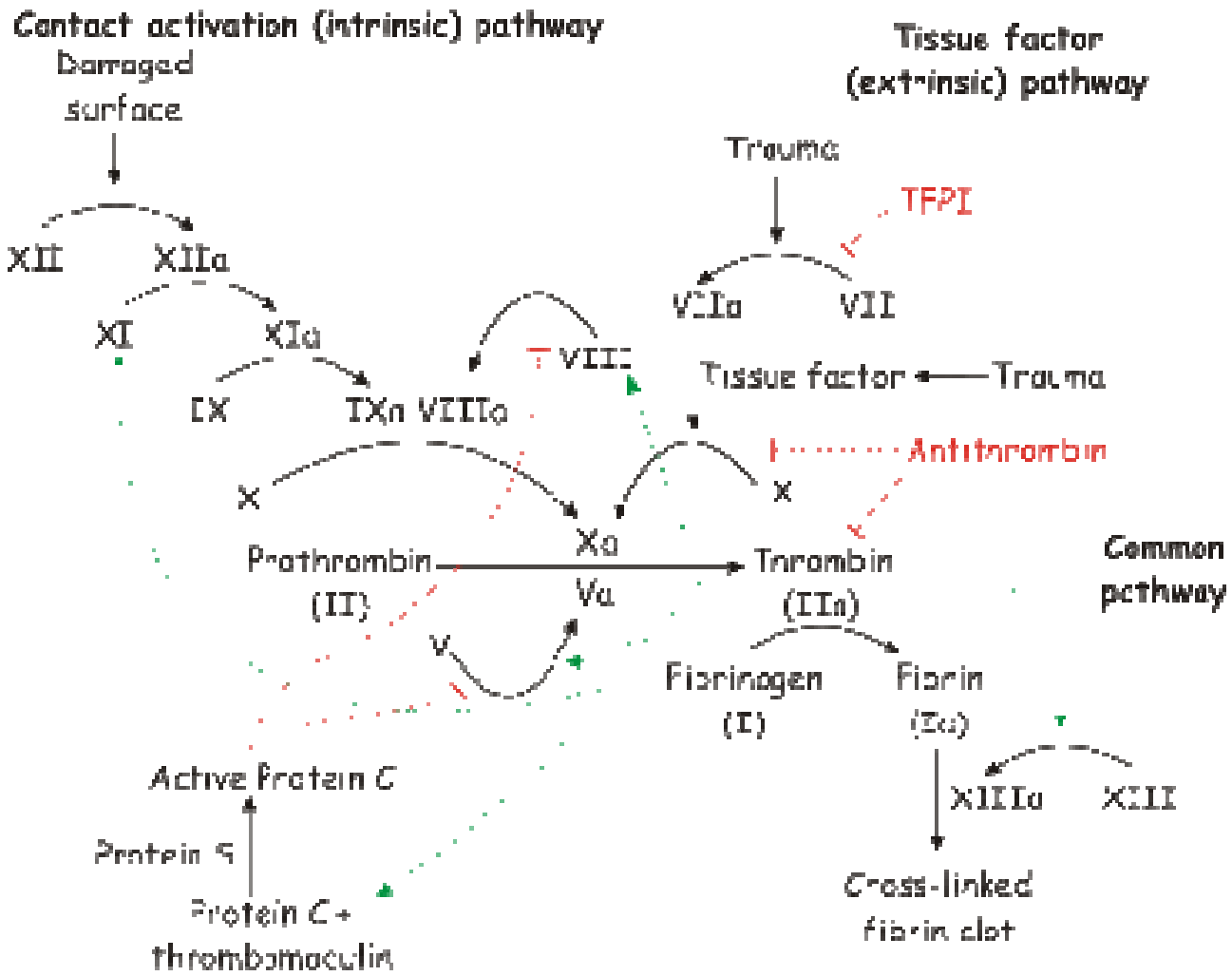
Basi molecolari delle malattie

Nuove strategie terapeutiche nella cura dell’emofilia A: Spliceosome Mediated RNA trans-splicing (SMaRT)

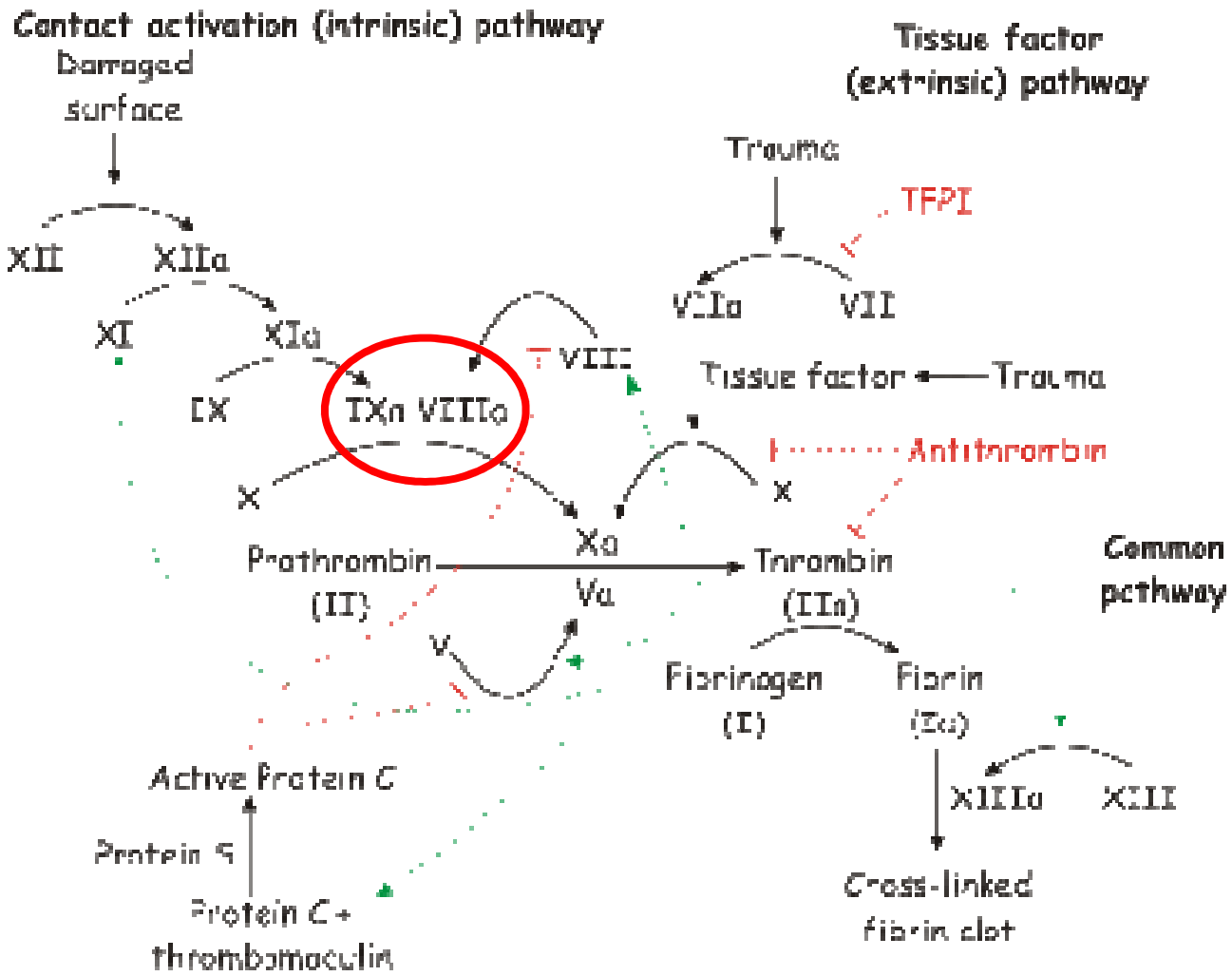
HEMOPHILIA

- An X-linked bleeding disorder caused by mutations in the gene for coagulation Factor VIII (hemophilia A).
- HA affects 1:5000 males worldwide.
- Severe cases are characterized by frequent spontaneous bleeding episodes (joints).
- Hemorrhagic events in untreated severe patients can be fatal.

The coagulation cascade



The coagulation cascade



La diagnosi di HA

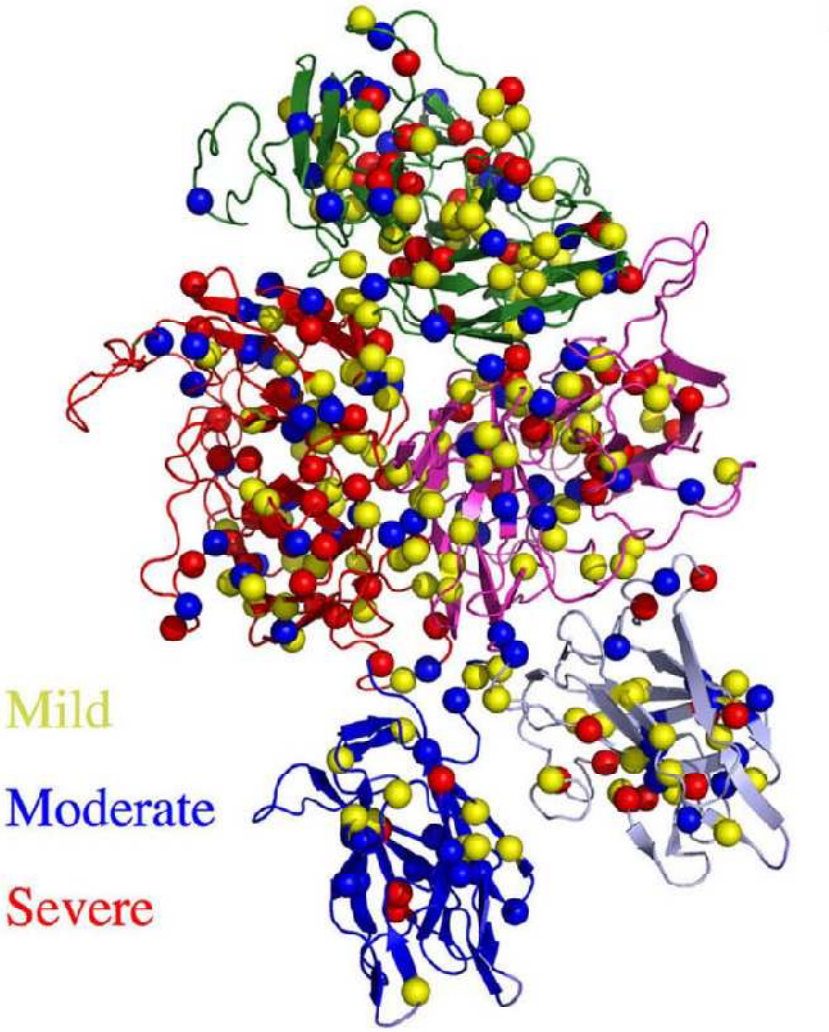
La diagnosi dell'emofilia A è ottenuta, in prima fase, attraverso un semplice test della coagulazione, denominato aPTT (tempo di tromboplastina parziale), che risulta allungato; in seconda fase, il dosaggio diretto del FVIII (test cromogenico) permette di quantificarne il deficit

Clinical classification:
6-30% Mild
1-5% Moderate
< 1% Severe

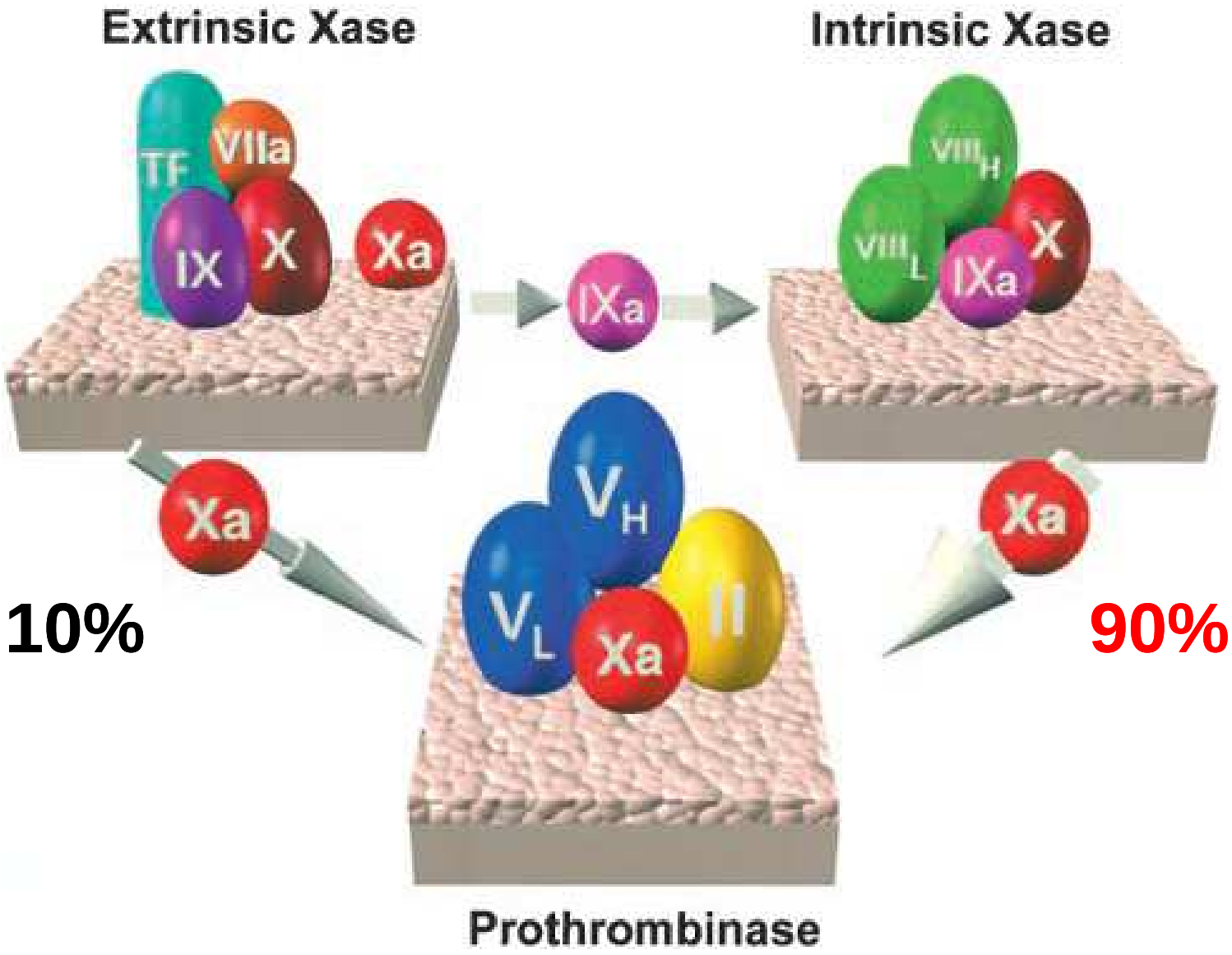
Nelle forme lievi e moderate gli episodi di sanguinamento si verificano dopo traumi importanti o interventi chirurgici; nelle forme più severe, episodi emorragici a livello muscolare o intramuscolare possono insorgere spontaneamente o a seguito di traumi lievi.

La mutazione più frequente, presente nel 40% dei pazienti affetti da emofilia A, è l'**inversione dell'introne 22 (gene A)** del gene per il FVIII per ricombinazioni con sequenze omologhe ripetute situate a 500 Kb di distanza.

I casi che non presentano questa mutazione sono, invece, associati ad un insieme assai eterogeneo di alterazioni genetiche



Intrinsic & Extrinsic Xase Complex



Current Treatment for Hemophilia

- **Infusion of either recombinant or plasma-derived clotting factor concentrates in response to bleeds**

  (Kogenate®, Helixate®, Ricombinase®, Refacto®, NovoSeven®)

- **Disadvantages**
  - Protein has a short half-life
  - Risks of plasma-derived products
  - Ongoing tissue damage because bleeds are treated rather than prevented
  - Expense (> US 100K/year)

Antibody formation to FVIII (Inhibitors, IgG anti-FVIII)

Advantages in gene therapy

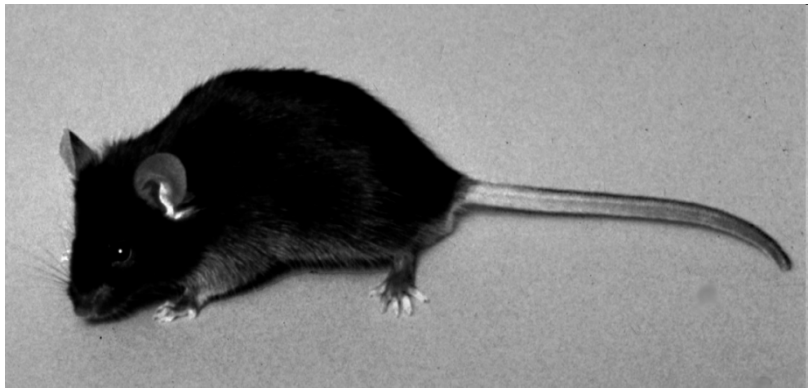
- Minimal elevation in the factor level (>1%) is clinically beneficial
- Successful gene transfer would result in continuous maintenance of clotting factor levels adequate to prevent rather than treat most bleeds after they have occurred
- More convenient (no intravenous injection)
- Risks of blood products avoided

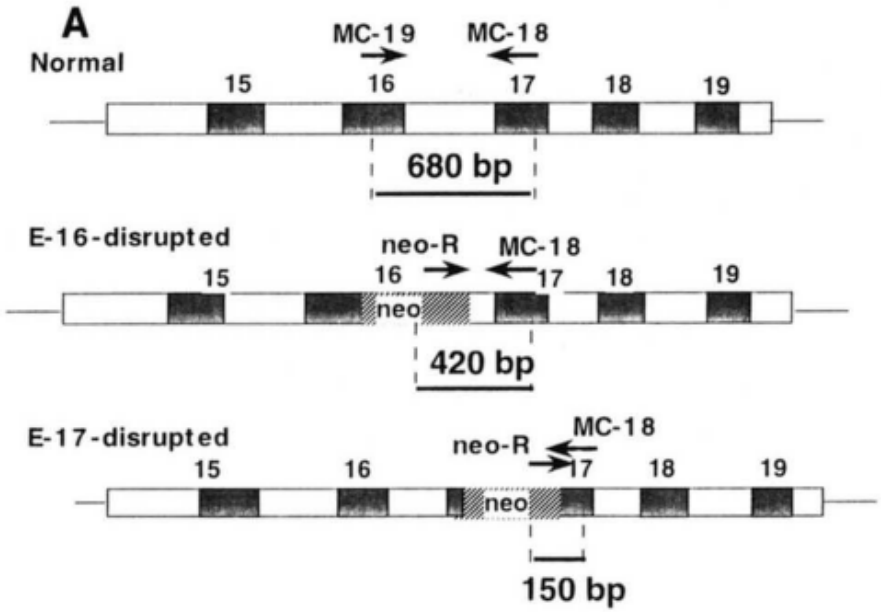
Animal model for HA

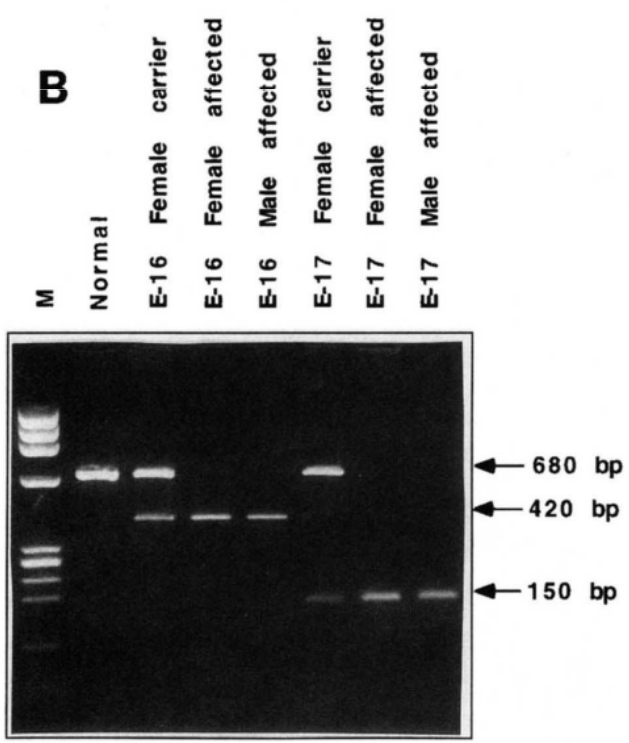
Dogs

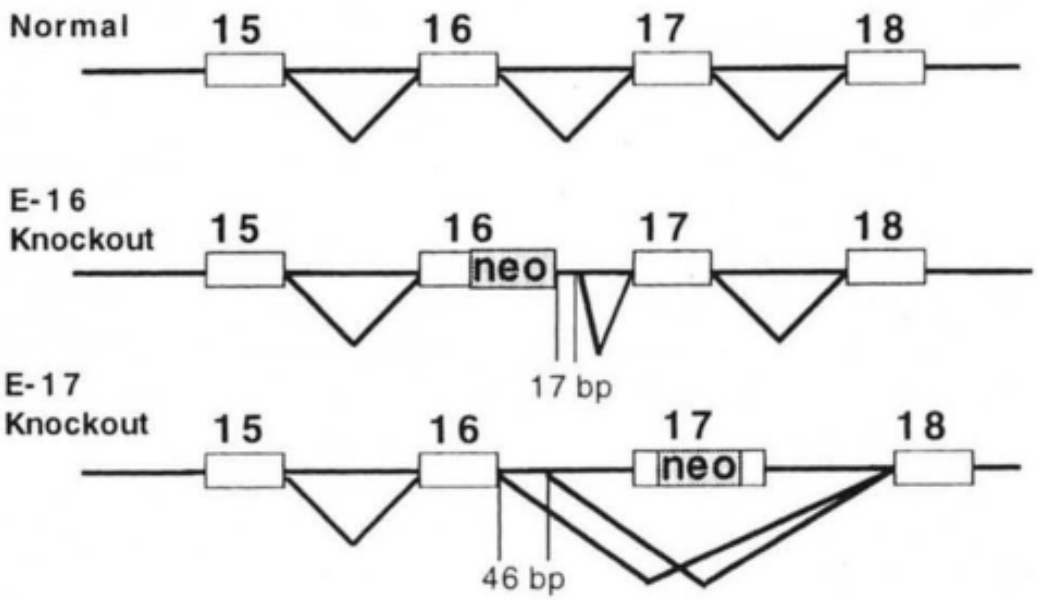
- Expensive
- Long generation time

MICE (E16 – E17)

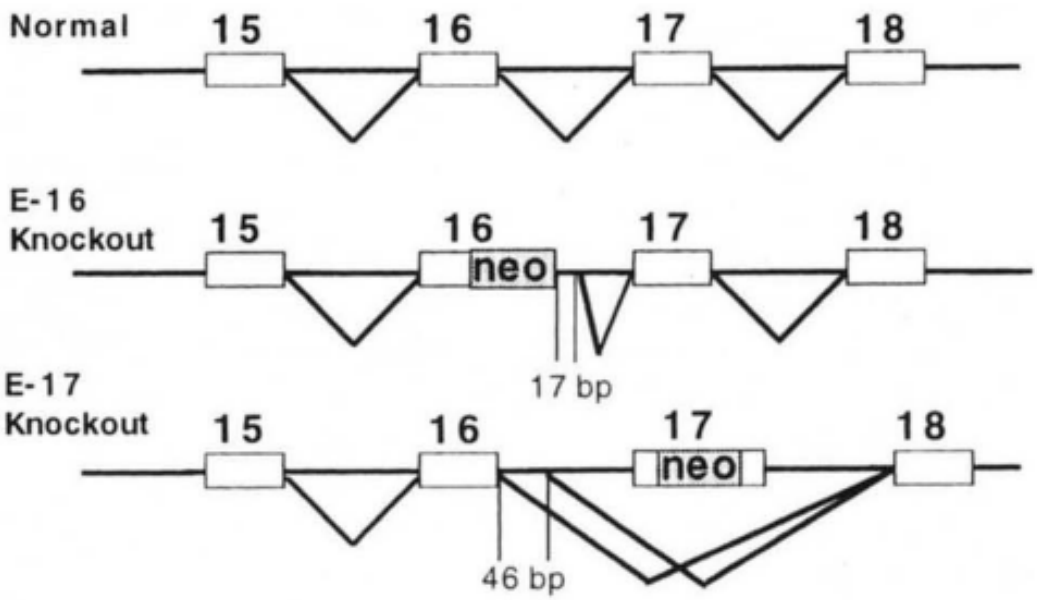


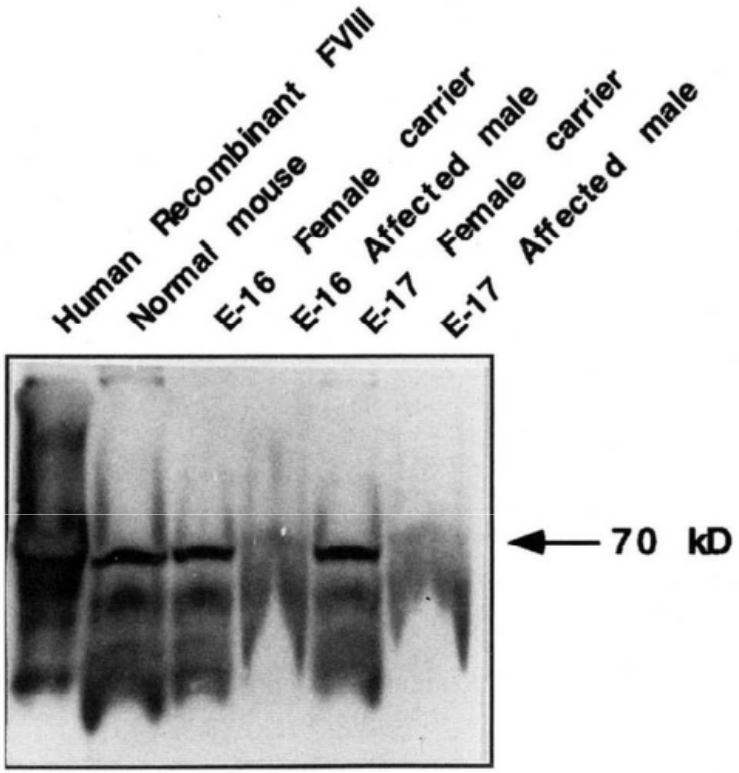






- Ablation of ex.16 and ex.17
- No light chain
- No functional FVIII





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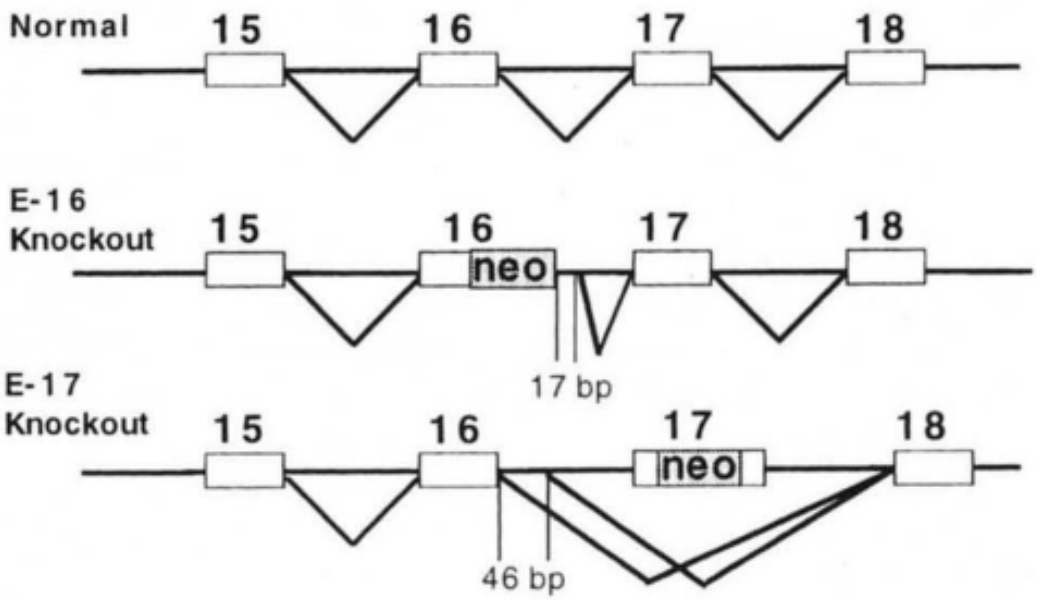
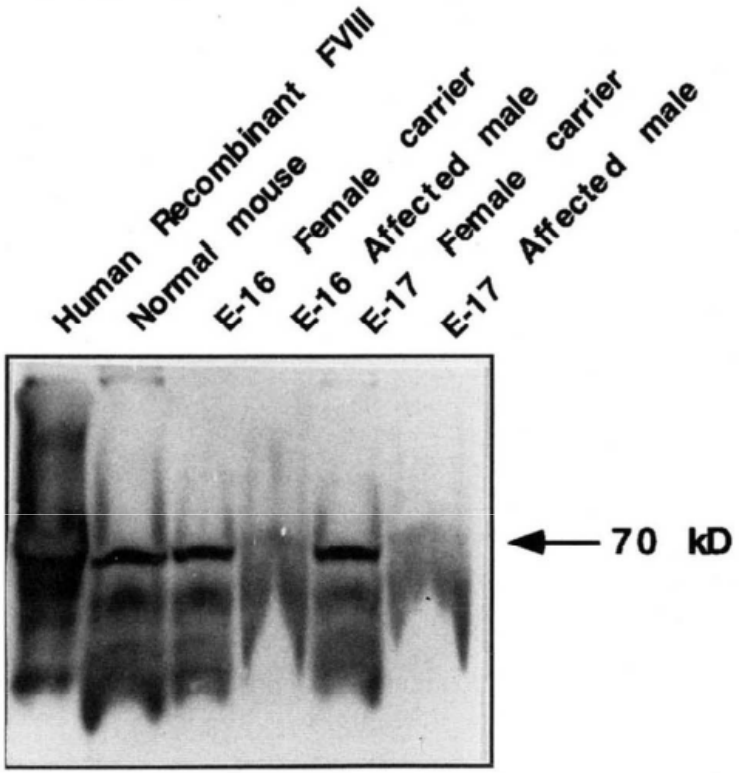
- No light chain
- No functional FVIII

MoAbs to hFVIII





- Ablation of ex.16 and ex.17
- No light chain
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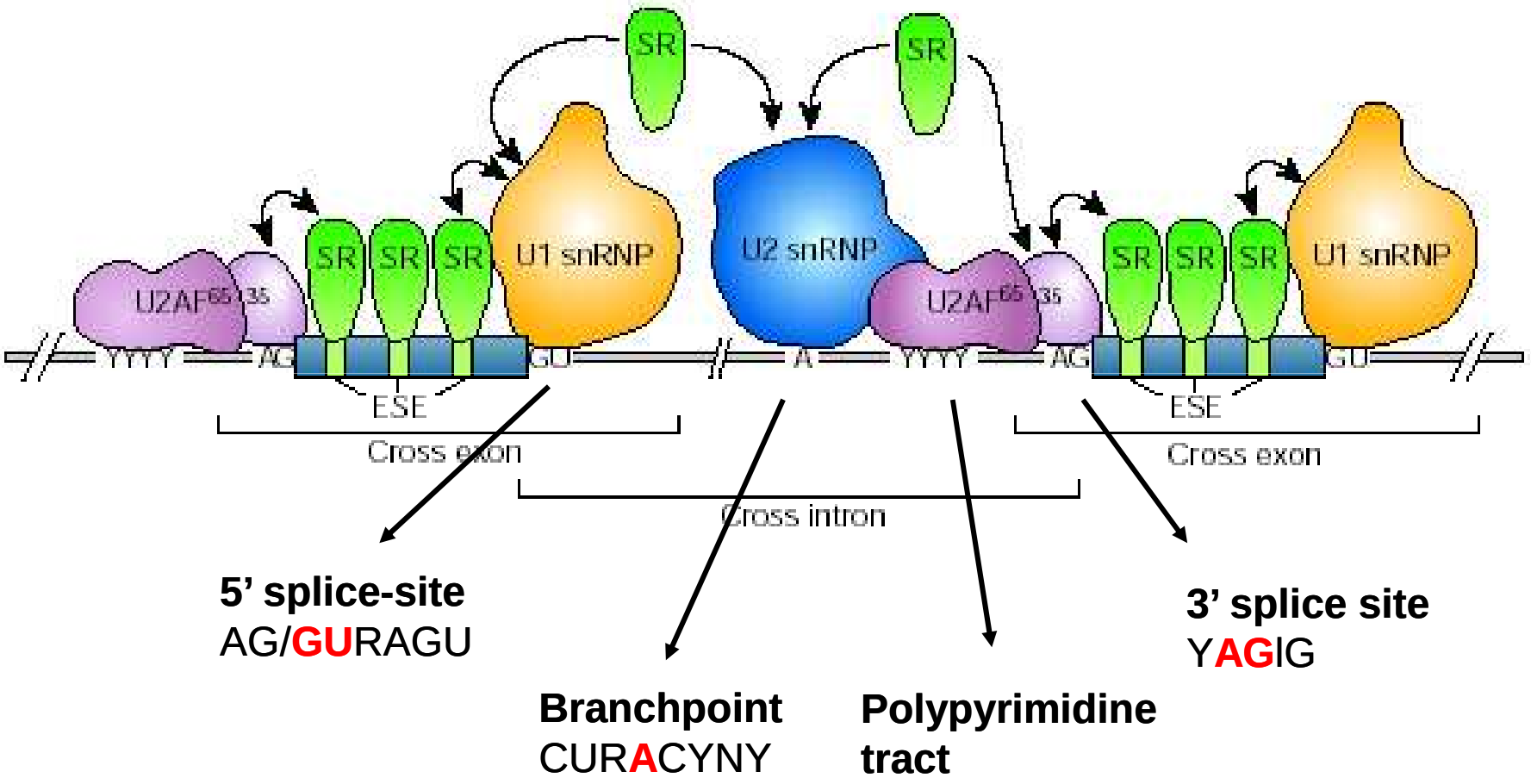
MoAbs to hFVIII

E-16 and E-17 mice have a mild phenotype

Splicing



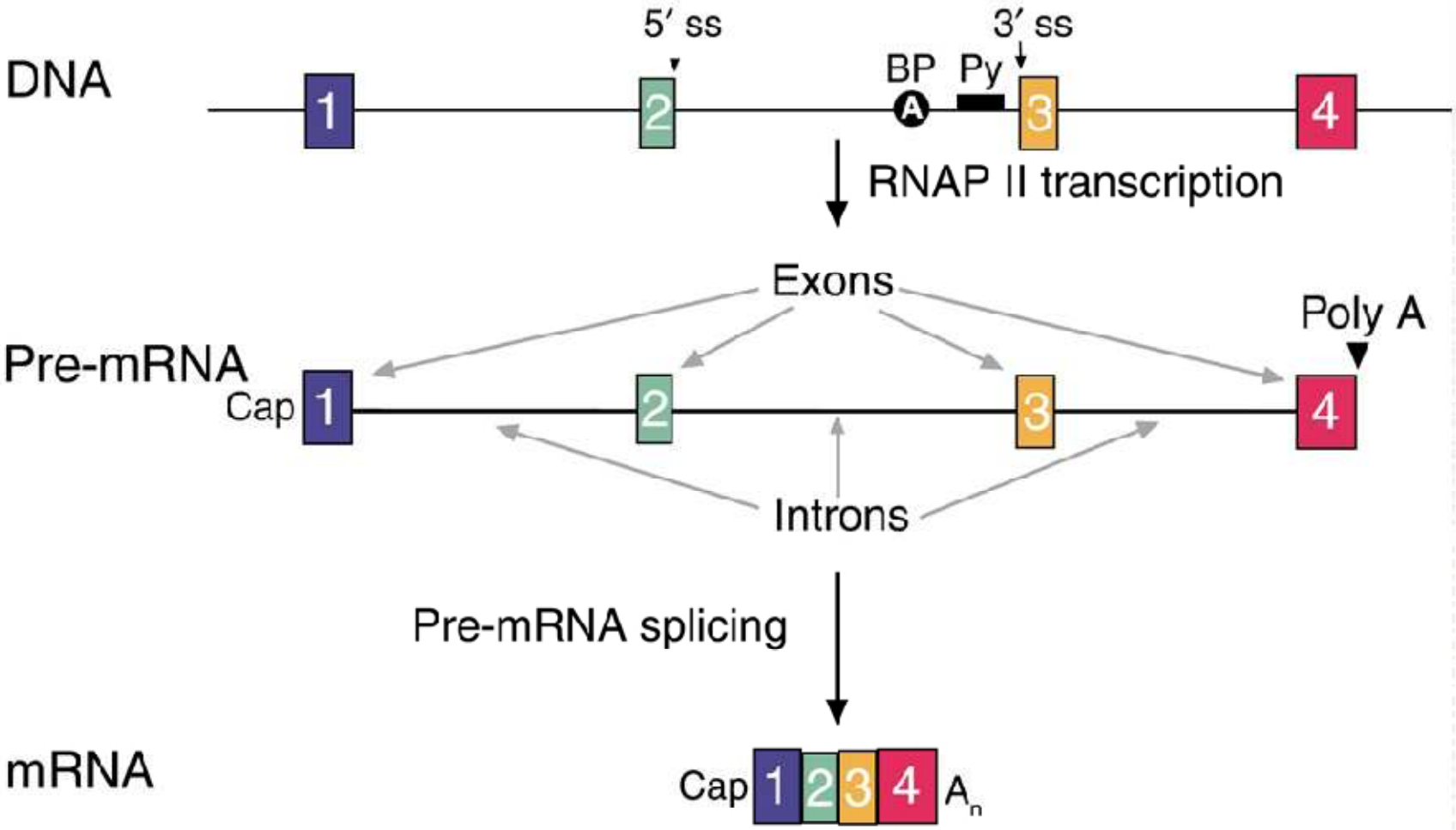
5’ splice-site
AG/**GU**RAGU

Branchpoint
CUR**A**CYNY

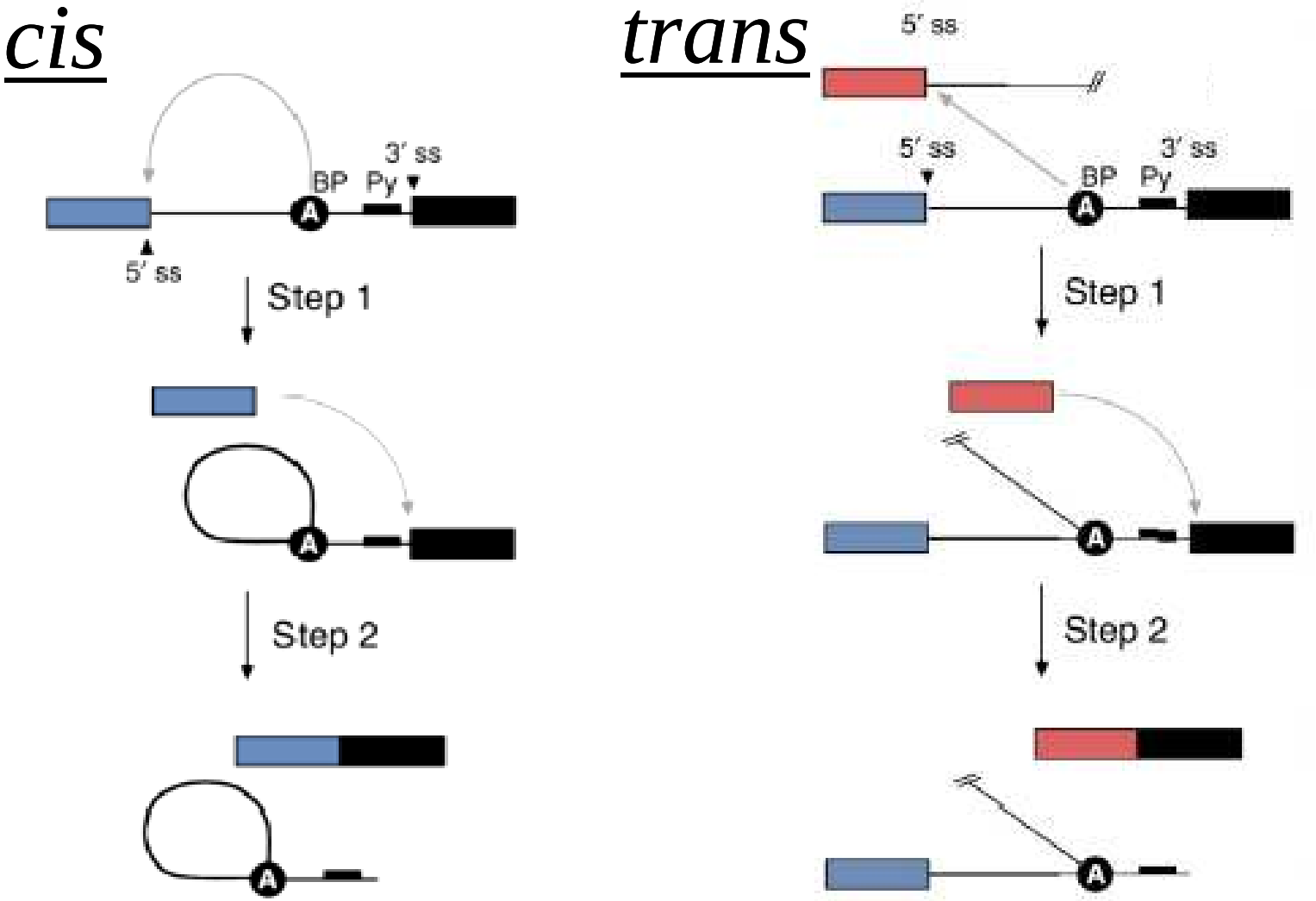
Polypyrimidine tract

3’ splice site
Y**AG**IG

Splicing


DNA
5′ ss
3′ ss
BP
Py
A
1
2
3
4
RNAP II transcription
Exons
Poly A
Pre-mRNA
Cap
1
2
3
4
Introns
Pre-mRNA splicing
mRNA
Cap
1
2
3
4
A
n

Trans-Splicing



Esempi in natura

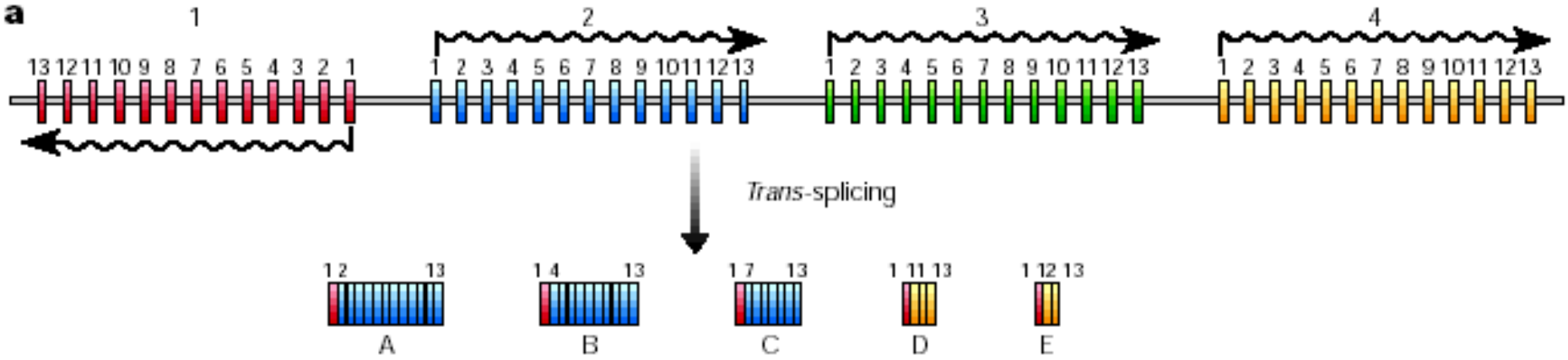


Figure 4 Alternative *trans*-splicing in mammals and flies. **a**, Four cytochrome *P450 3A* genes are arranged in a cluster that spans ~200 kilobases of genomic DNA. For simplicity, the genes have been designated 1–4 (they were designated *CYP3A43*, *CYP3A4*, *CYP3A7*, *CYP3A5*, respectively, in the original reference)[76]. Gene 1 is transcribed from one DNA strand, whereas genes 2–4 are transcribed from the opposite strand. The direction of transcription on each strand is indicated by the wavy arrows. The small coloured boxes are exons, and the pale grey lines are introns. Hybrid intergenic mRNAs are produced by *trans*-splicing between exon 1 of gene 1 and various exons in genes 2 and 4 to generate the mRNAs labelled A–E.

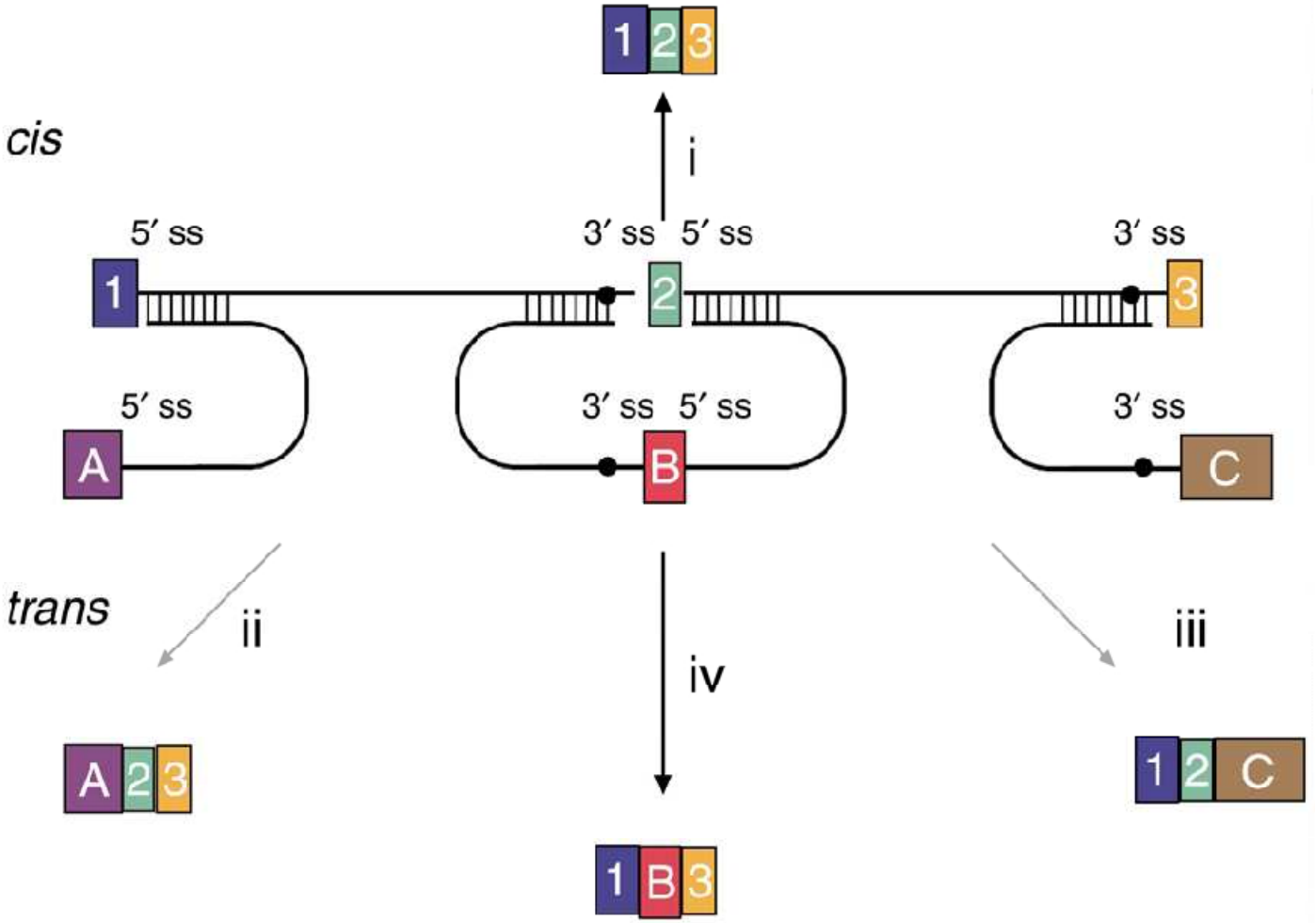
TRANS-SPLICING-
Hybrid coding/non-coding RNAs

- **Ribozymes**
  self-catalytic RNA molecules

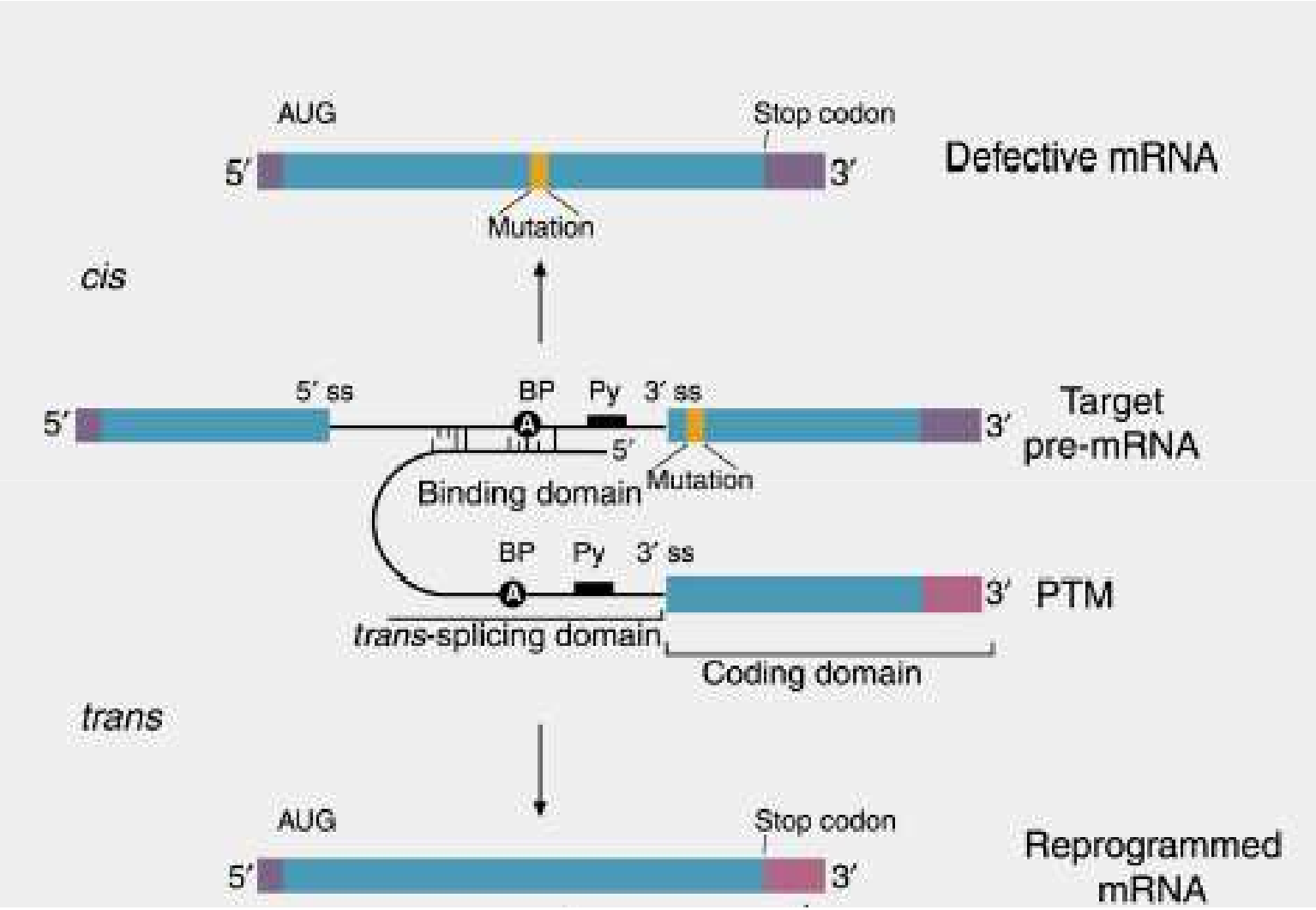
- **tRNA endonuclease** (archaebacterium)

- **SMaRT**
  Spliceosome Mediated RNA Trans-splicing

SMaRT: spliceosome mediated RNA *trans*-splicing

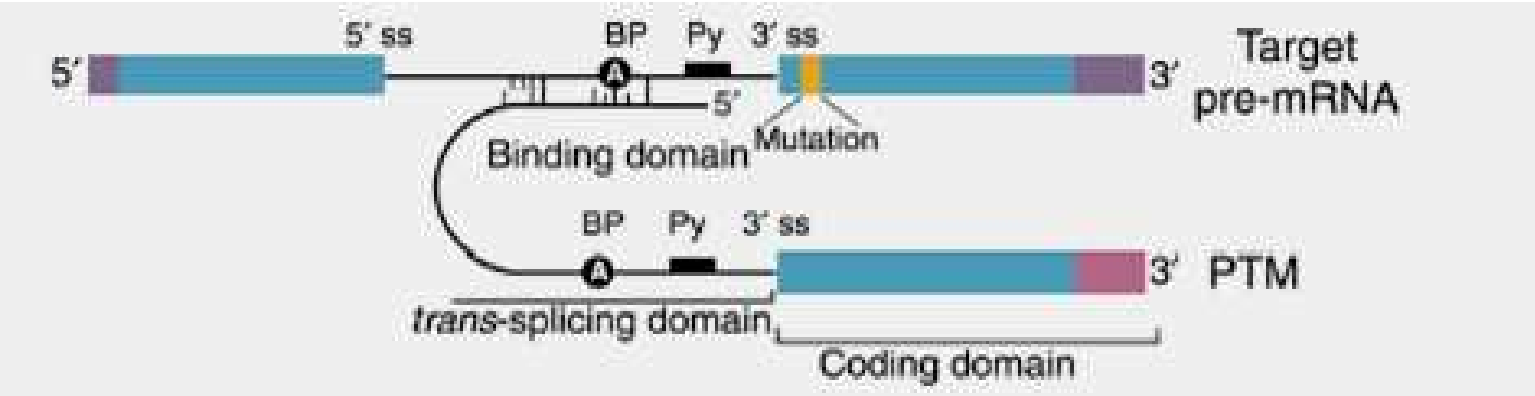


SMaRT: spliceosome mediated RNA *trans*-splicing



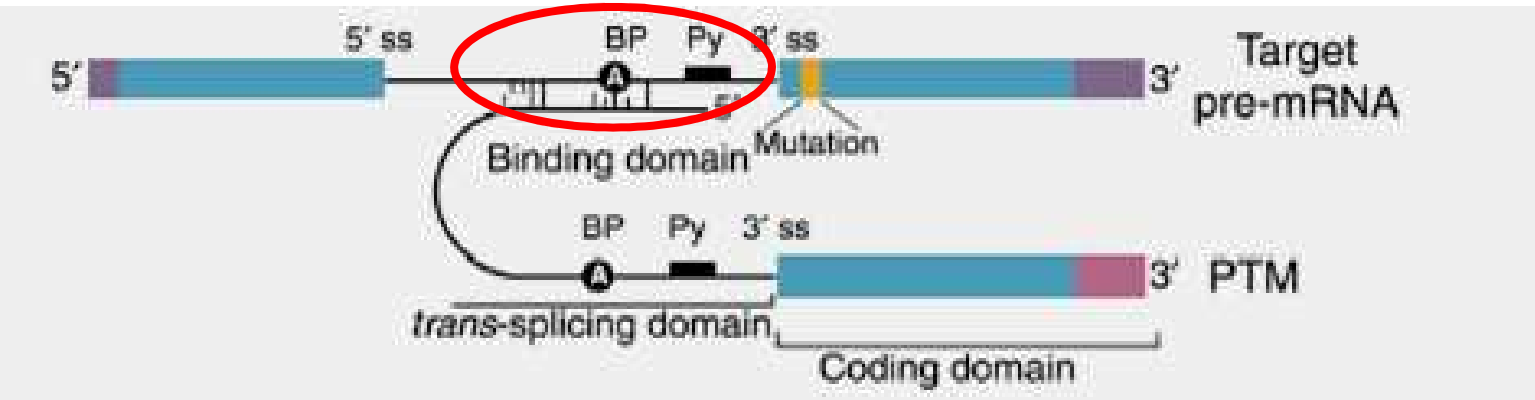
PMT: pre-*trans*-splicing molecule

PTMs structure



Prototype PTMs comprise three domains:

PTMs structure

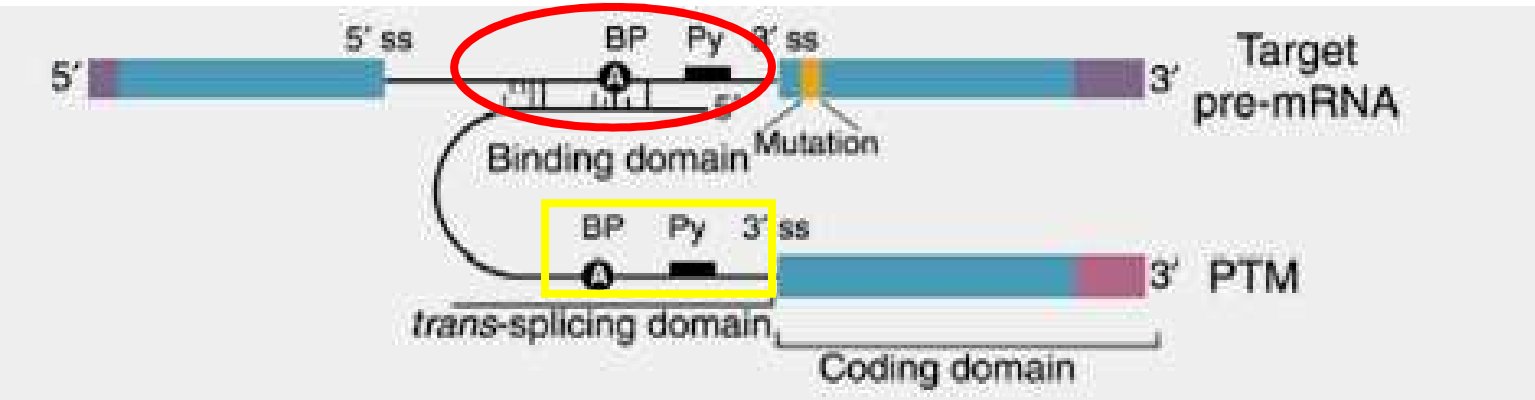


Prototype PTMs comprise three domains:

Binding domain

complementary to the target intron

PTMs structure



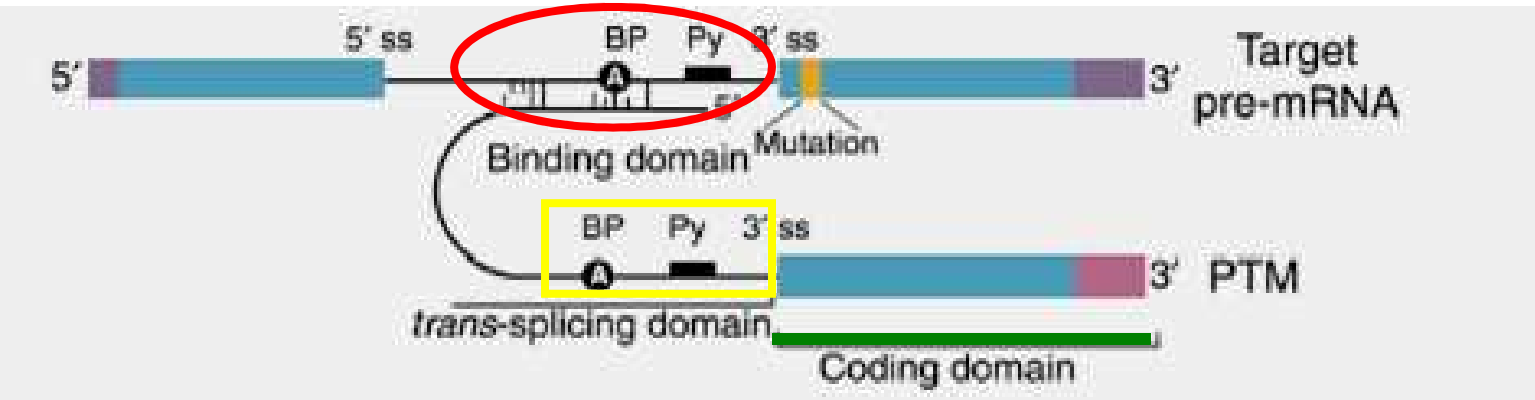
Prototype PTMs comprise three domains:

Binding domain
complementary to the target intron

Splicing domain
containing necessary splicing elements
Donor; Branch point; Polypirimidin tract; Acceptor

} TSD
Trans-splicing domain

PTMs structure



Prototype PTMs comprise three domains:

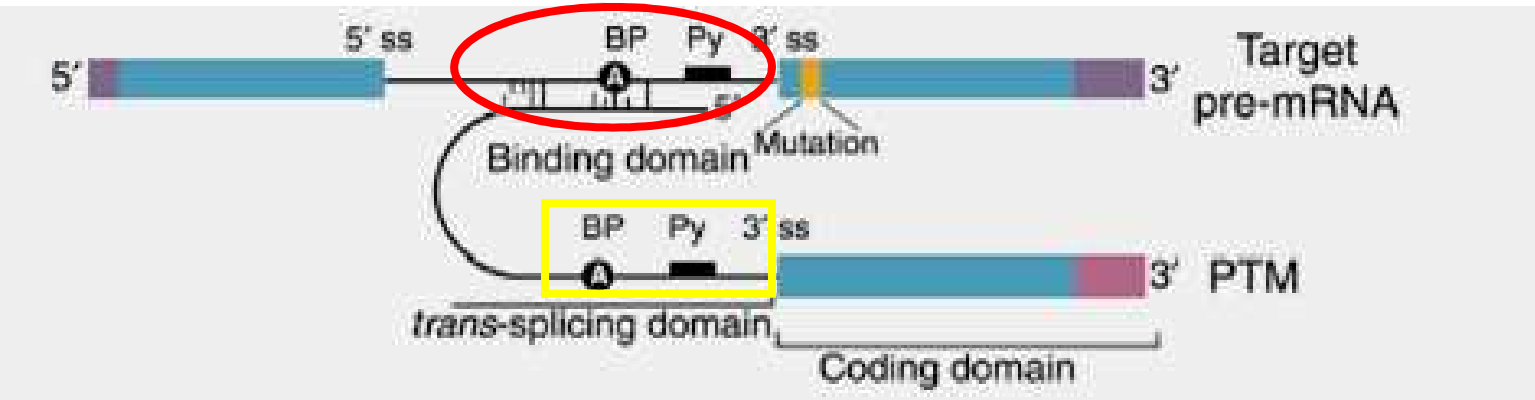
Binding domain
complementary to the target intron

Splicing domain
containing necessary splicing elements
Donor; Branch point; Polypirimidin tract; Acceptor

Coding domain
to be trans-spliced to the target

TSD
Trans-splicing domain

PTMs structure



The length and composition of the binding domains can be modified to alter efficiency, specificity, and targeting location within a pre-mRNA.

The splicing domain is designed to maximize trans-splicing activity and, therefore, typically contains a potent branchpoint sequence (UACUAAC) and a long pyrimidine-rich tract.

SPLICING ELEMENT DEFINITION

Position	Splice site type	Motif	Consensus value (0-100)
+3	Acceptor	CTTCAAGgtgagcg	69.56
+7	Donor	AAGgtgagc	95.71
+31	Acceptor	atctgggtcgaggg	76.4
+34	Donor	tgggtcgag	88.7
+56	Acceptor	ccttcctcgcaggg	94.13
+61	Acceptor	ctcgcagggcagag	80.9
+92	Donor	gaggtgtag	74.18
+94	Acceptor	ggtgtagcgcaggc	81.05
+147	Acceptor	ttctctgcacagCT	89.78

Depending on the Trans-splicing molecule (5',3' or 5'-3') splicing element position had to be defined also for the presence of putative criptic site nearby

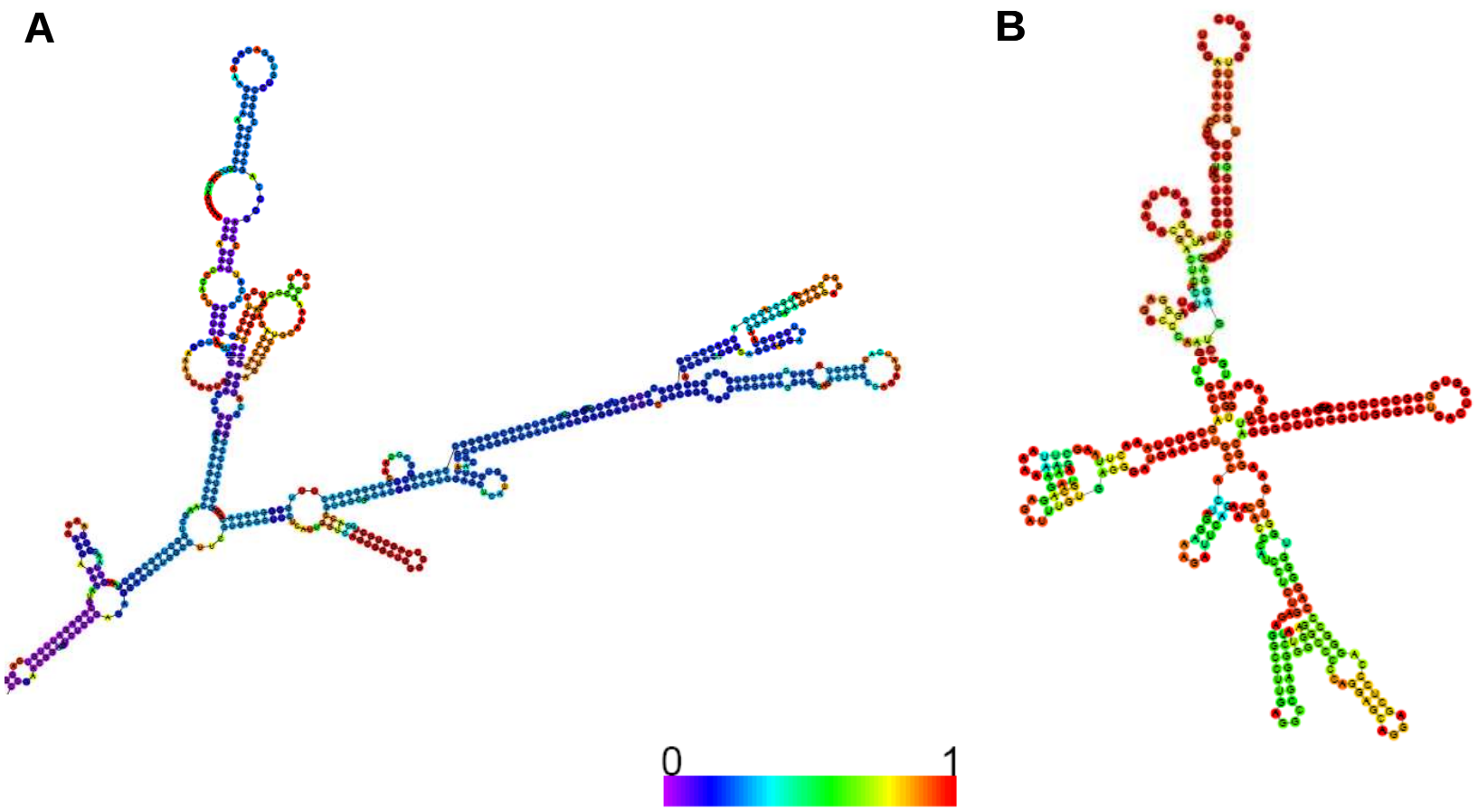
SPLICING ELEMENT DEFINITION

Branch Point position	Branch Point motif	Consensus value (0-100)
61	ccctgAc	95.07
75	tcctcAg	95.75
89	gcctcAa	91.75
94	ctctcAg	93.91
107	ccctcAc	100
120	ttctgAc	90.17

Branch points are normally located within 50bp upstream of the acceptor site of introns

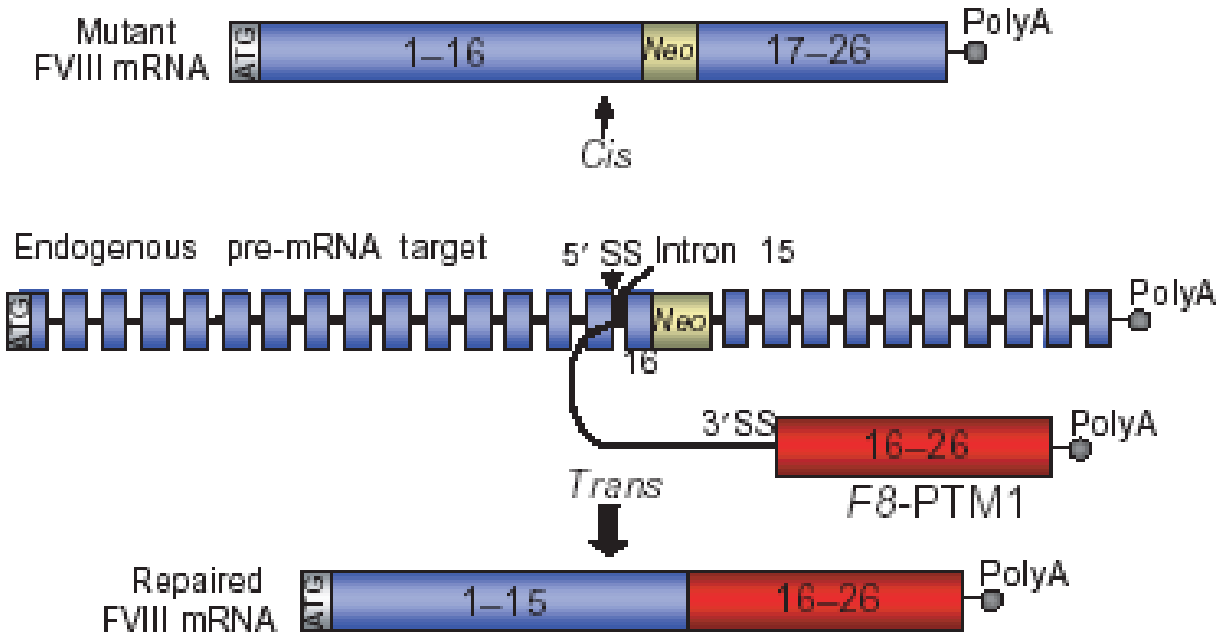
PTMs VALIDATION

RNA secondary structure prediction

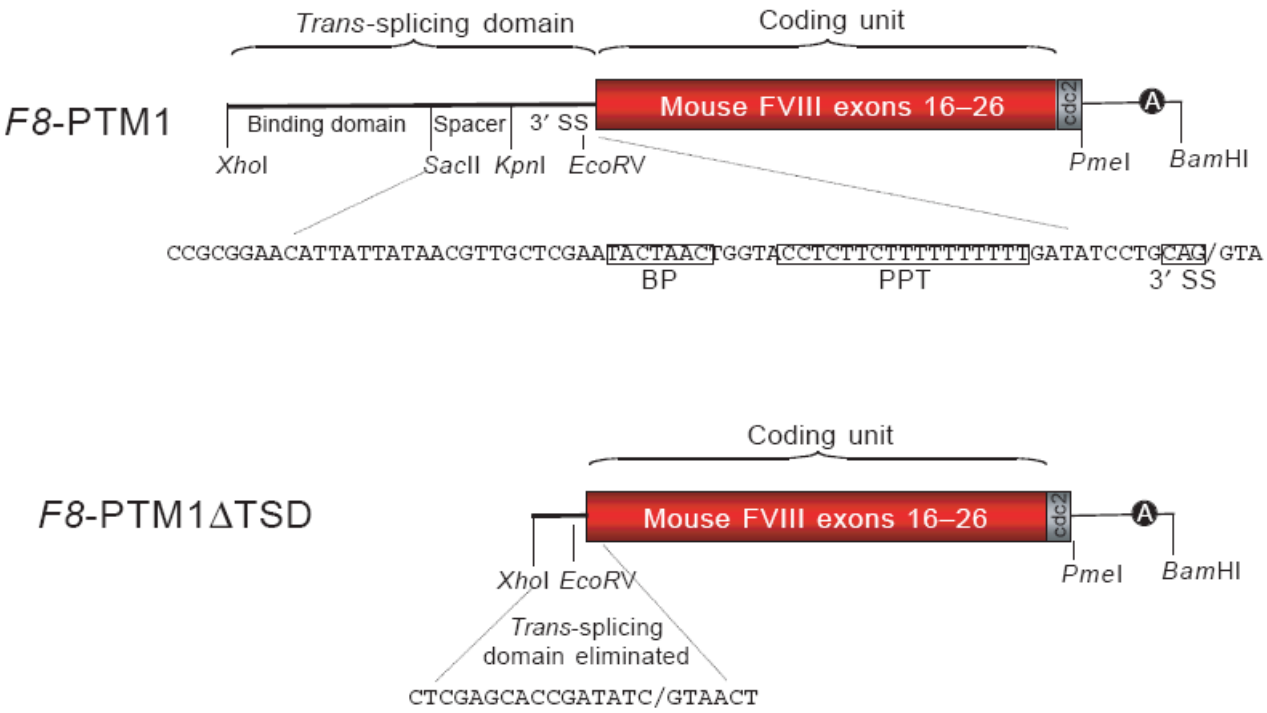


Phenotype correction of hemophilia A mice by spliceosome-mediated RNA *trans*-splicing

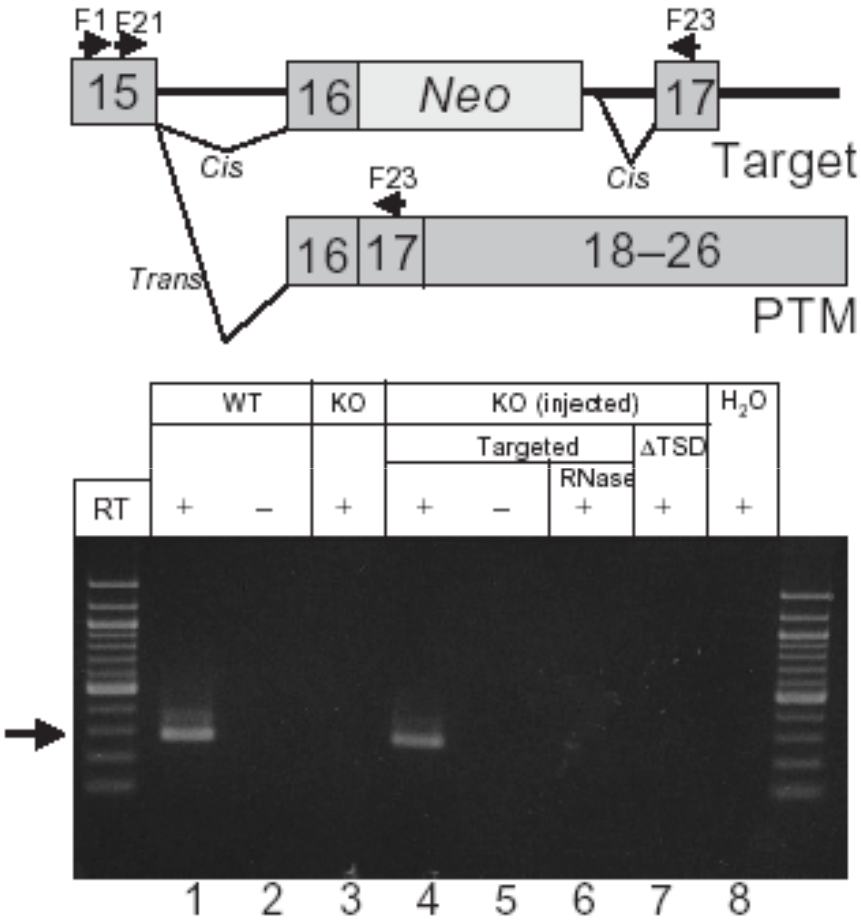
Hengjun Chao[1,3], S Gary Mansfield[2,3], Robert C Bartel[2], Suja Hiriyanna[2], Lloyd G Mitchell[2], Mariano A Garcia-Blanco[2] & Christopher E Walsh[1]



Pre-Trans-splicing Molecules

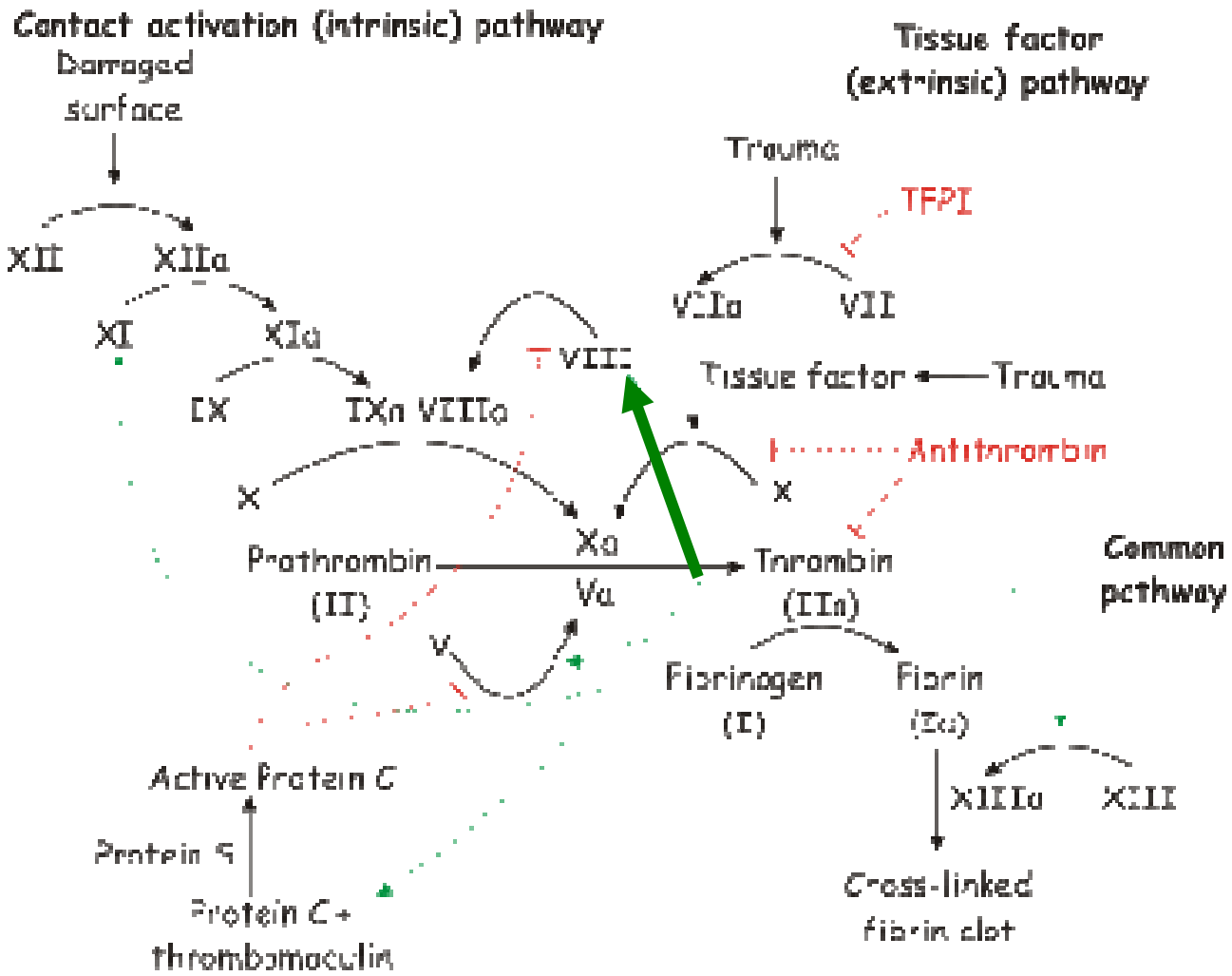


Correction of HA in E-16 mice (Naked DNA injection)



mFVIII mRNA

The coagulation cascade



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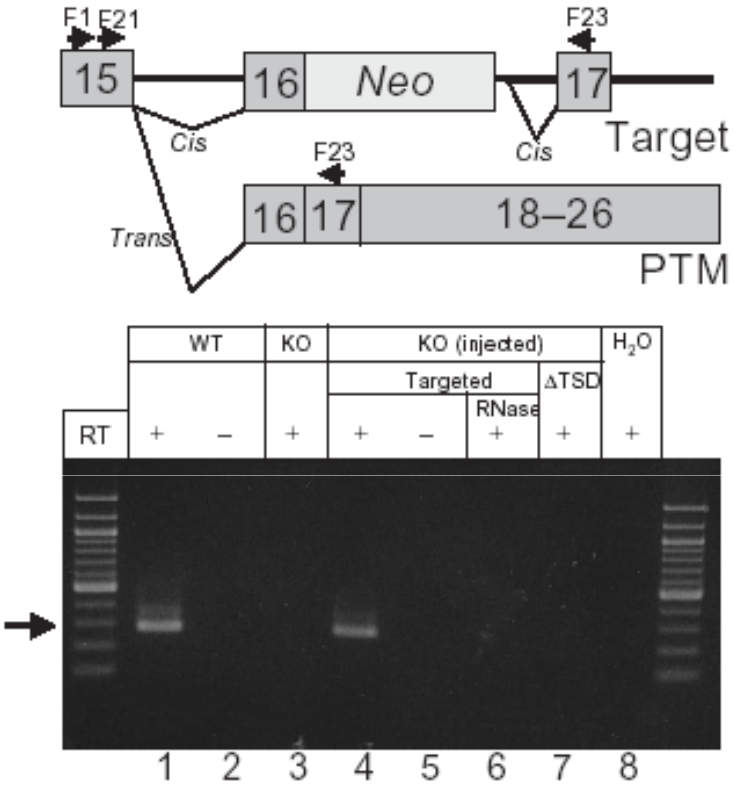
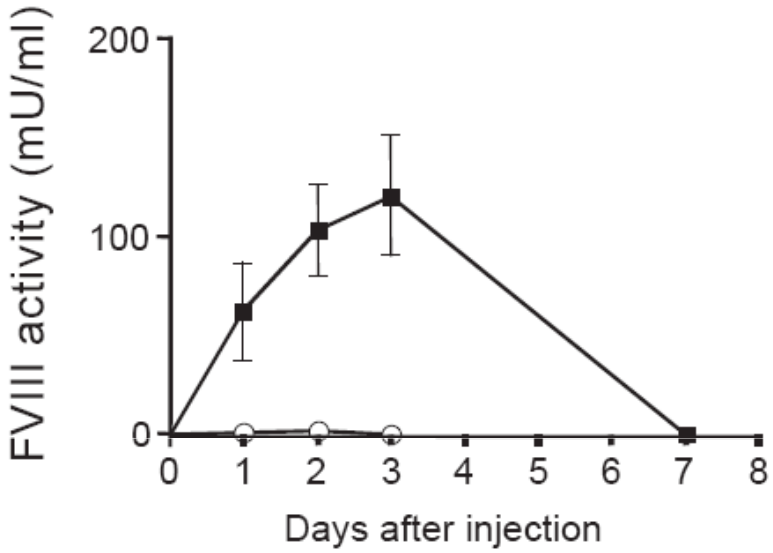


mFVIII mRNA

mFVIII activity



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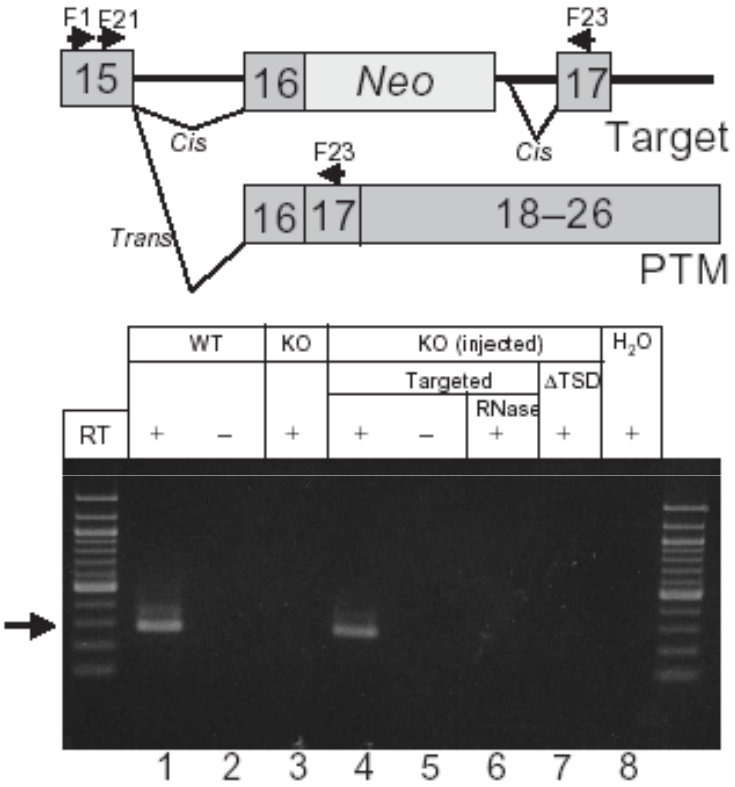

mFVIII mRNA

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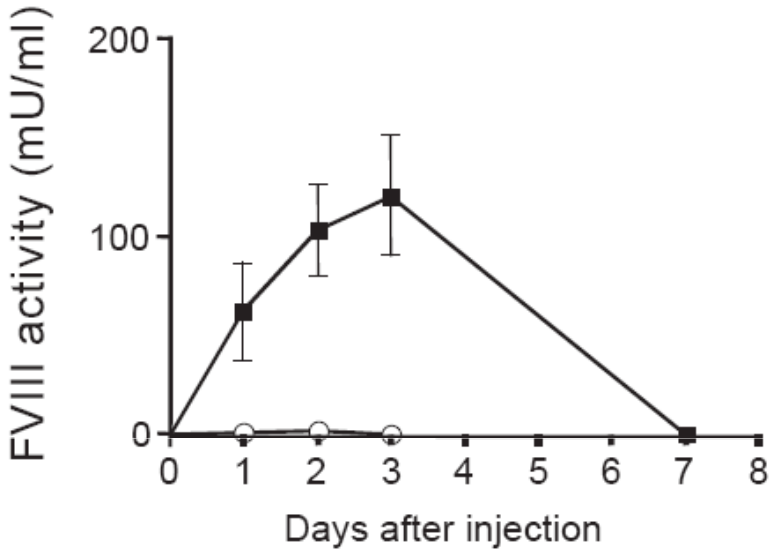
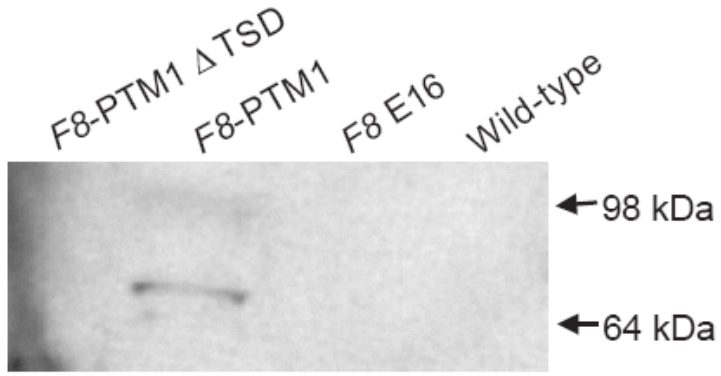
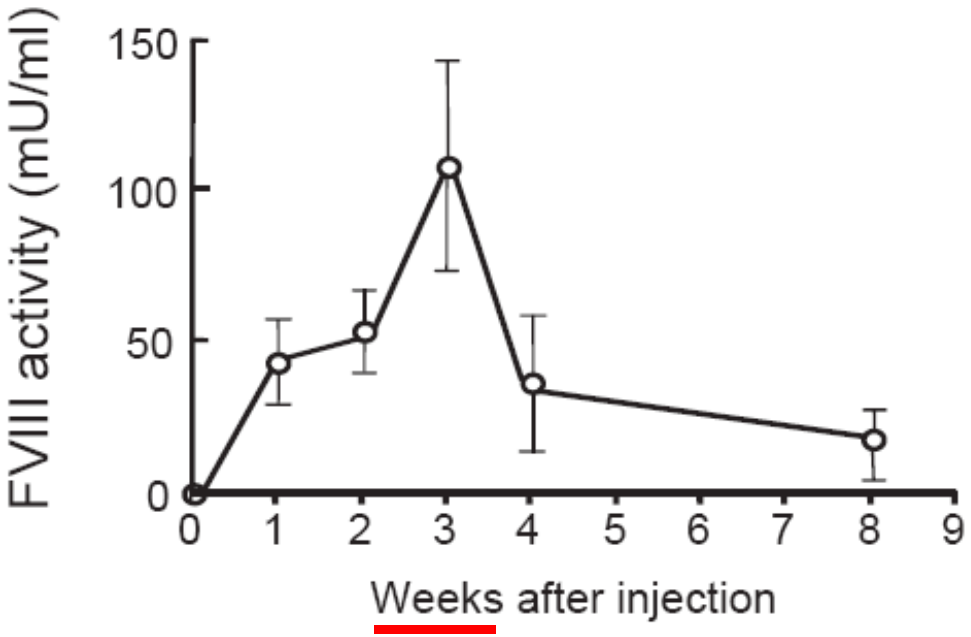
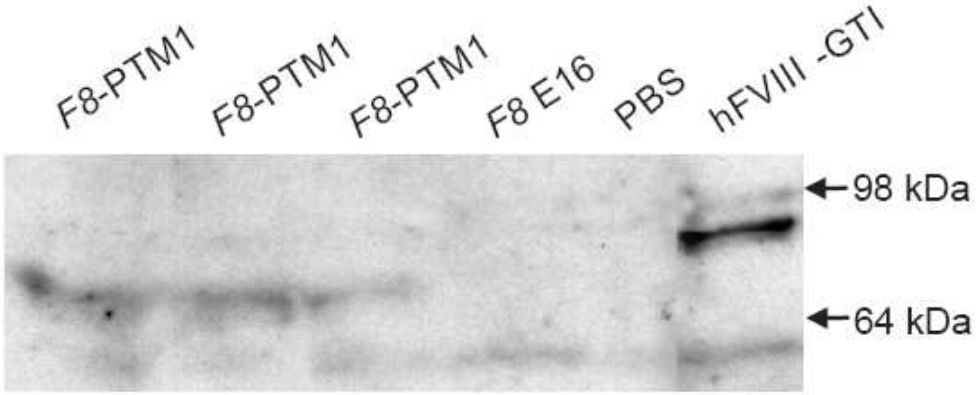




mFVIII protein (light chain)

Correction of HA in E-16 mice (Adenovirus vector)





FVIII protein (light chain)

Trans-splicing advantages.....

- **Reduction of transgene size**
  replacement only of mutated portion

- **Gene expression under the control of endougenous regulatory elements**

- **Tissue specific expression**
  Repaired products are limited by the presence of the endogenous target

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- **Reduction of transgene size**
  replacement only of mutated portion

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- **Tissue specific expression**
  Repaired products are limited by the presence of the endogenous target

.....and disadvantages

- **<u>Low efficiency</u>**

Examples of successful Trans-splicing

- Cystic Fibrosis (CFTR) *in vivo*

-Spinal muscular atrophy (SMA) *in vitro*

- Epidermolysis bullosa simplex with muscular dystrophy (EBS-MD) *in vitro*

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