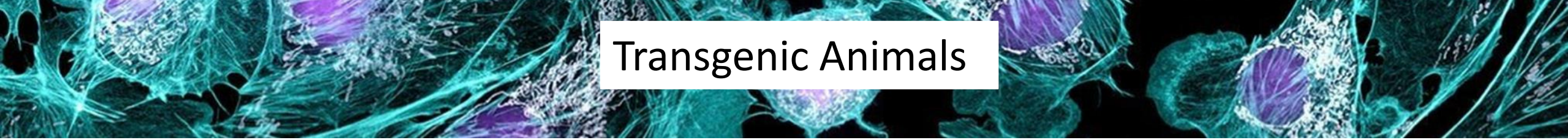
Advanced Cell Biology and Biotechnology

ACBB 2021/22

…the lecture is about to begin…

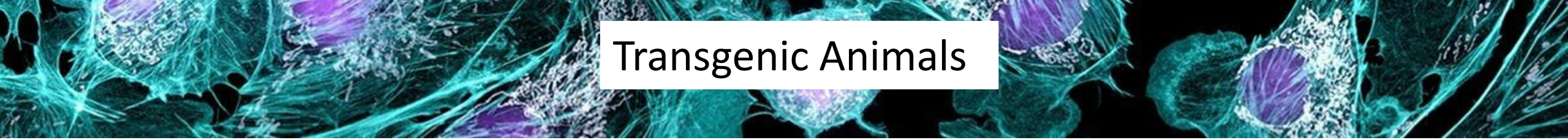


Transgenic Animals

1- DNA microinjection
- random insertion

2- embryonic stem cell-mediated gene transfer
- random insertion
- homologous recombination (double selection)
- Rosa26 locus
- genomic analysis to identify genetically modified animals

- knock-out animals
- knock-in animals
- conditional knock-out (cre-lox technique, inducible systems)
- siRNA
- CRISPR-CAS9



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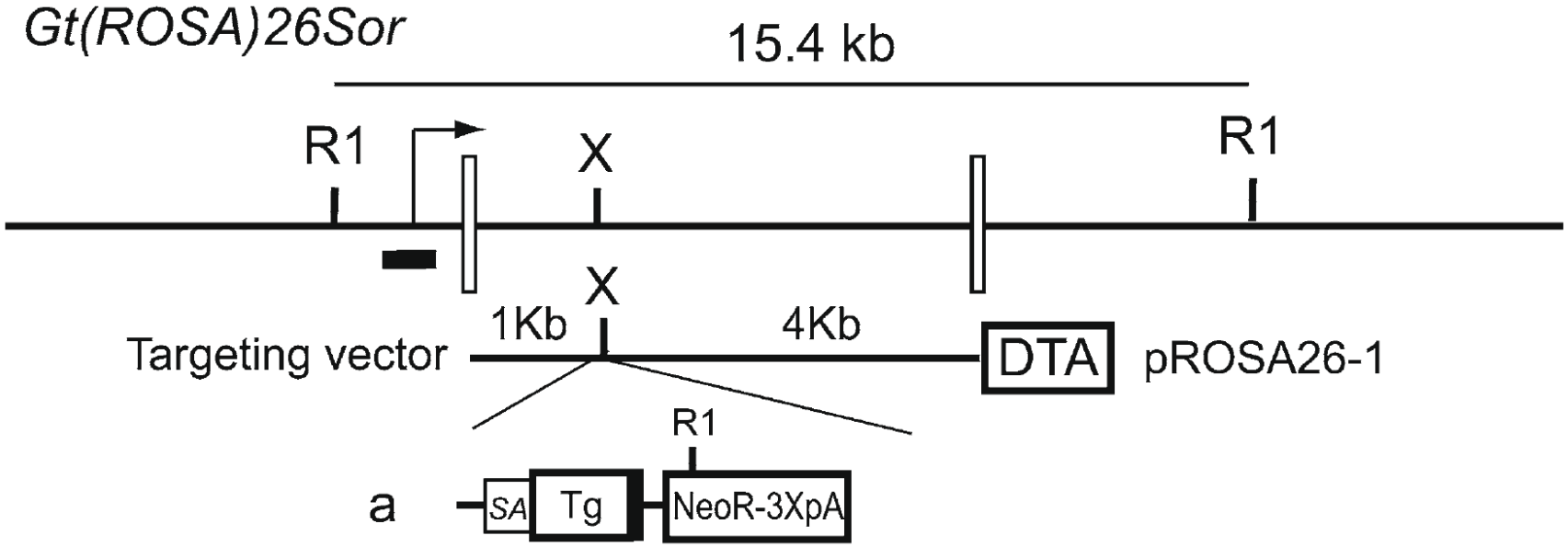
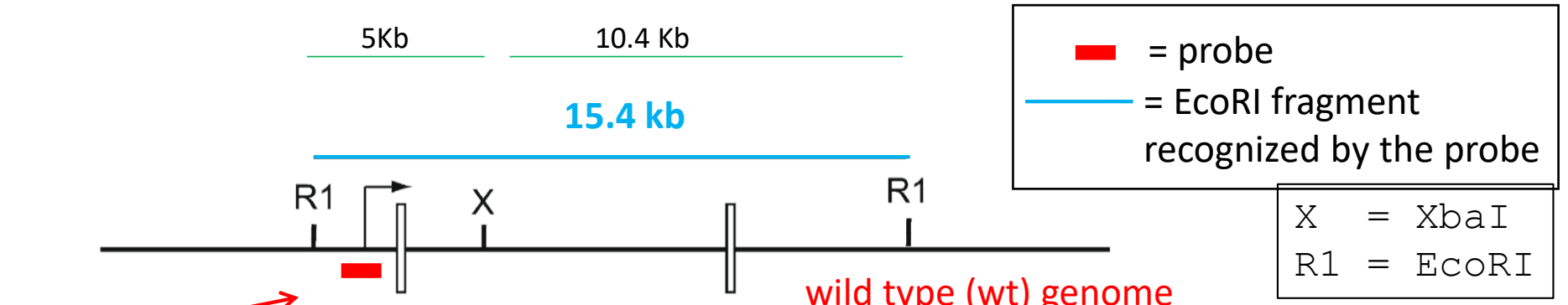
The ROSA26 locus

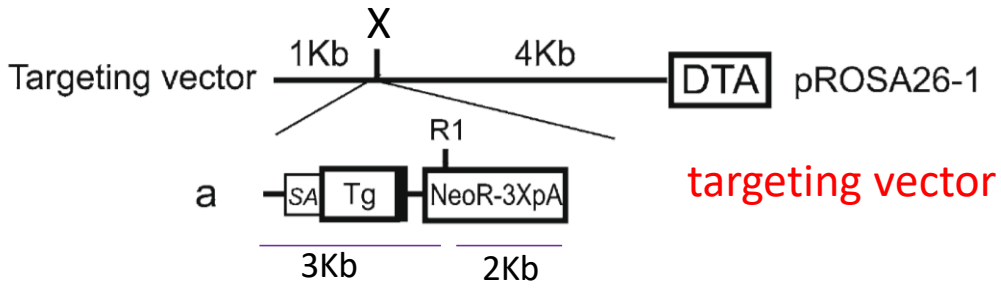
Targeting strategy to insert single-copy transgenes (Tg) into the ROSA26 locus.

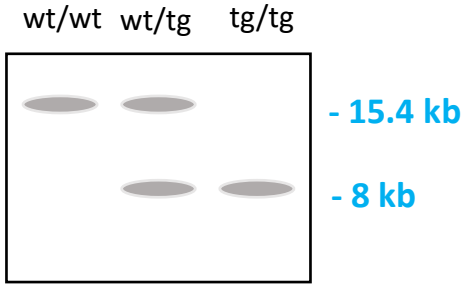


- pROSA26 is an example of targeting vector used to insert transgenes into intron 1 of the ROSA-26 gene
- the basic vector contains 5 kb of ROSA26 genomic sequences to drive homologous recombination and the gene for the **Diphtheria toxin A-subunit (DTA)** as negative selection marker
- the transgene (Tg), in most cases preceded by a splice acceptor (SA) site, is cloned into a unique Xba1 (X) site

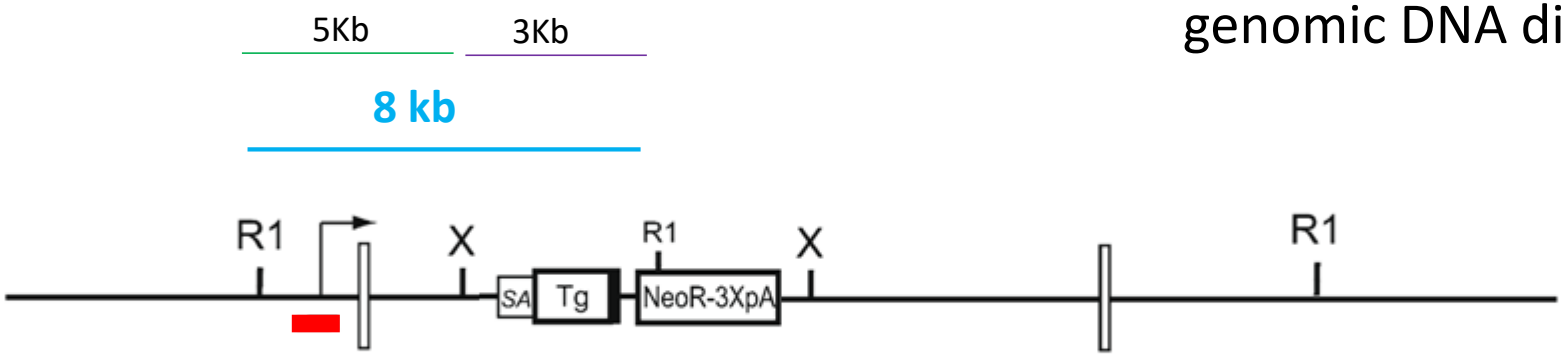
Genomic analysis to identify genetically modified animals



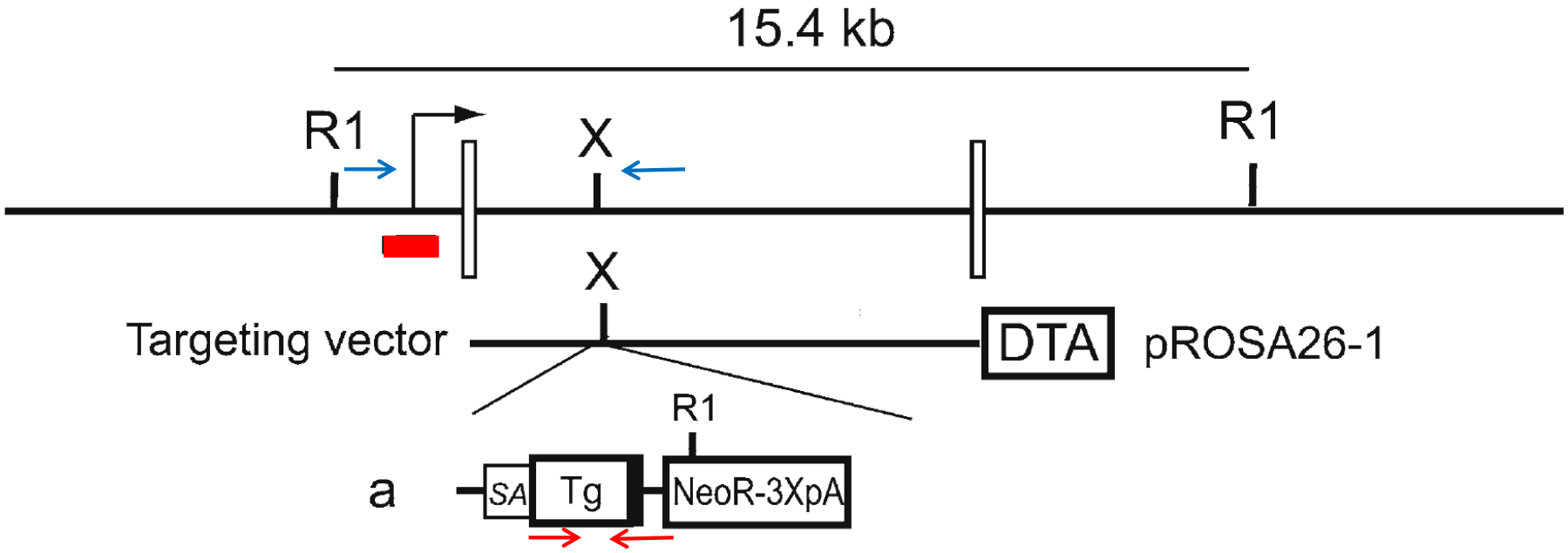




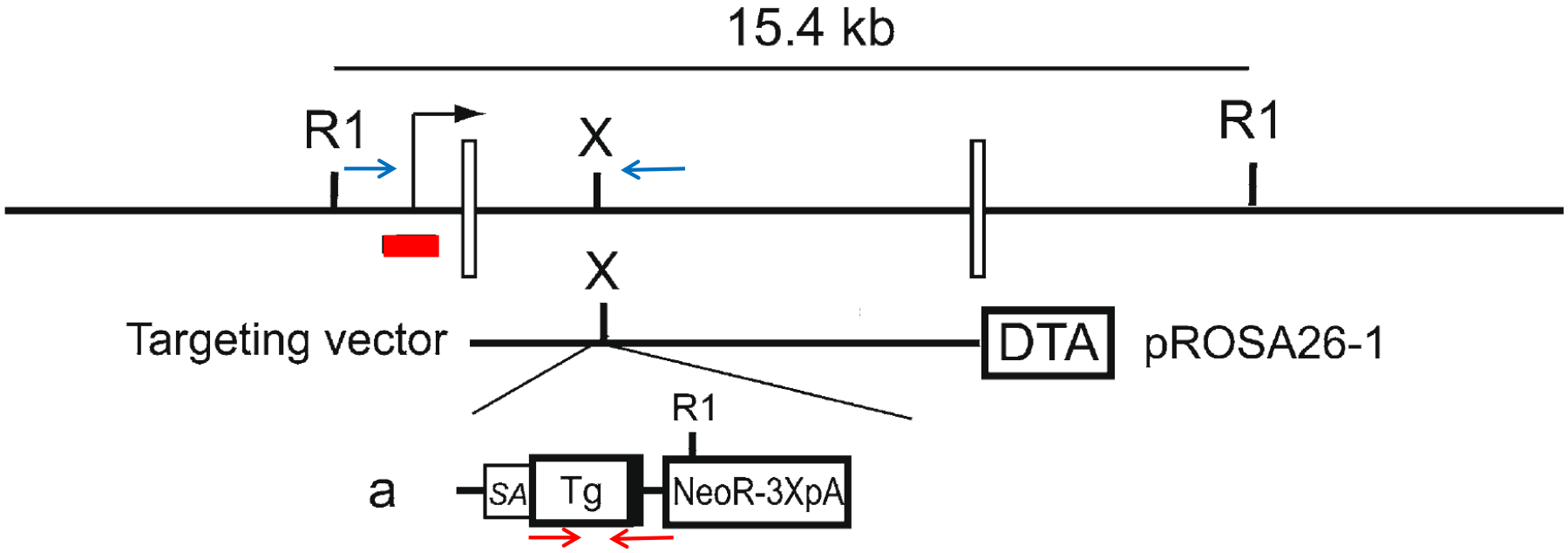
Southern blot with the **probe** on genomic DNA digested with EcoRI



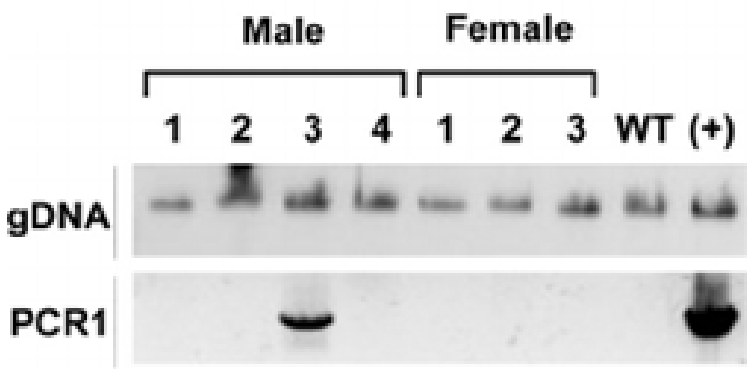
Can PCR be used to identify transgenic animals?



Can PCR be used to identify transgenic animals?



- PCR can be used to **identify transgenic animals**, but often it is not possible to **verify the homologous recombination**, because PCR product would be too long
- to verify homologous recombination Southern blot is necessary



- **PCR** can be used routinely on the offspring of transgenic animals, **to identify transgenic animals**

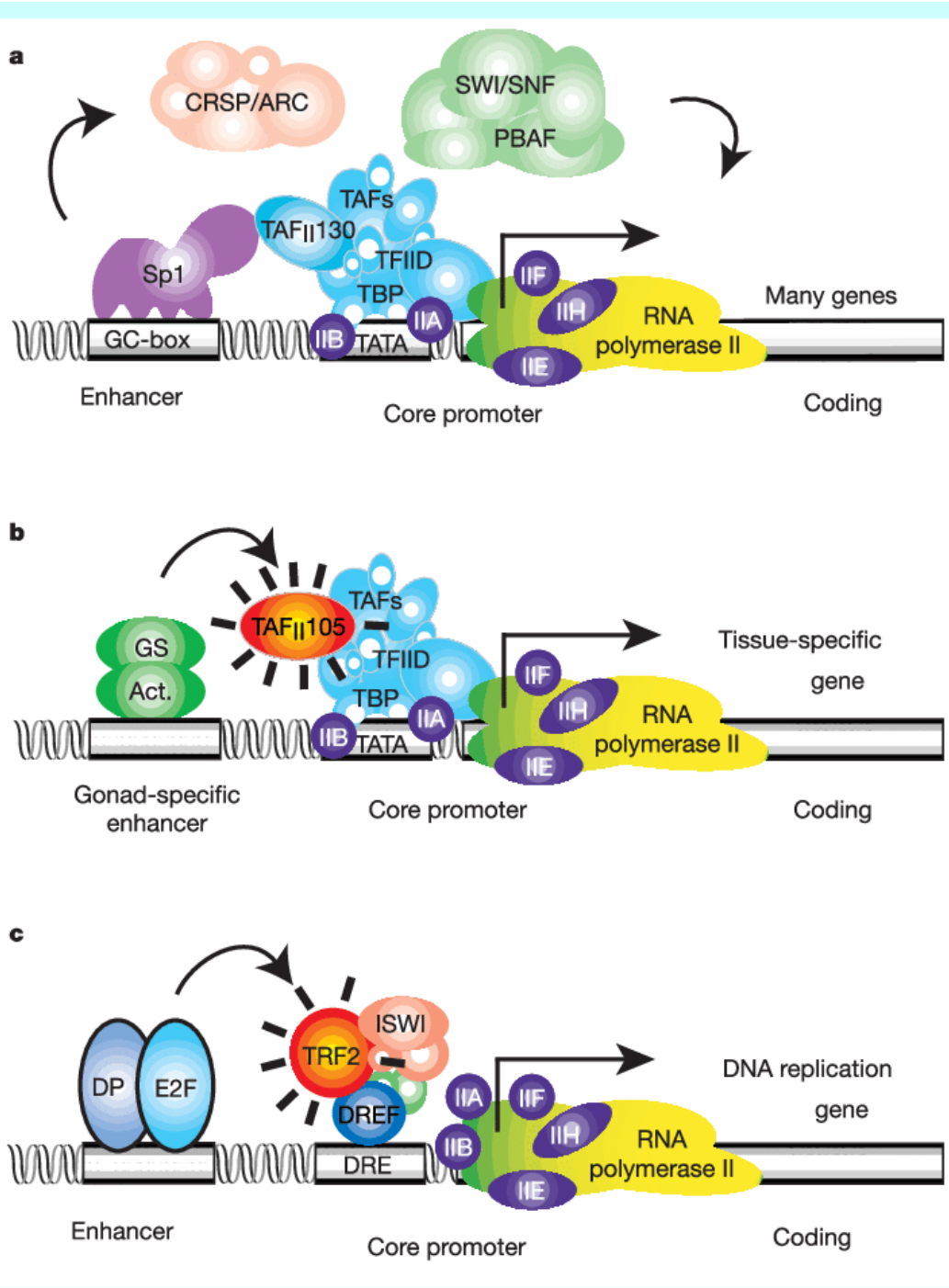
Once you know you have the transgenic mice, how do you verify the correct expression of the exogenous protein?

- western blot
- RT-PCR
- immunohistochemistry

- Which kind of construct do you have to prepare if you want to study:

  - **the over-expression of a protein?**
    (**ubiquitous** / **tissue specific** / inducible)
  - the lack of a protein? (ubiquitous / tissue specific/ inducible/ constitutive)
  - the regulation of a promoter?
  - the localization of a protein?

- How can you discriminate between endogenous and exogenous proteins?
- How can you study the activity of the mutated exogenous protein without the expression of the wild type endogenous protein?


a
CRSP/ARC
SWI/SNF
PBAF
TAFs
TAFII130
TFIID
TBP
Sp1
GC-box
IIB
TATA
IIA
IIF
IIH
IIE
RNA
polymerase II
Many genes
Enhancer
Core promoter
Coding
b
TAFs
TAFII105
TFIID
TBP
GS
Act.
IIB
TATA
IIA
IIF
IIH
IIE
RNA
polymerase II
Tissue-specific
gene
Gonad-specific
enhancer
Core promoter
Coding
c
ISWI
TRF2
DP
E2F
DREF
DRE
IIA
IIB
IIF
IIH
IIE
RNA
polymerase II
DNA replication
gene
Enhancer
Core promoter
Coding

Molecular Systems Biology 3; Article number 73; doi:10.1038/msb4100114
Citation: *Molecular Systems Biology* 3:73

www.molecularsystemsbiology.com

Tissue-specific regulatory elements in mammalian promoters

Andrew D Smith[1,3], Pavel Sumazin[2,3] and Michael Q Zhang[1,*]

Table III Significance of elevated ranks for motifs associated with important factors in liver, skeletal muscle and testis

Tissue	Factors	Motifs	Human *P*-value	Mouse *P*-value
Liver	HNF-1, HNF-3, HNF-4, C/EBP, DBP	68	2.72E−18	4.19E−12
Skeletal muscle	MEF-2, SRF, Myogenin, Sp1	45	1.33E−14	2.29E−5
Testis	SRY, CREM, RFX	30	0.087	1.89E−4

Motifs give the total number of motifs associated with the listed factors.

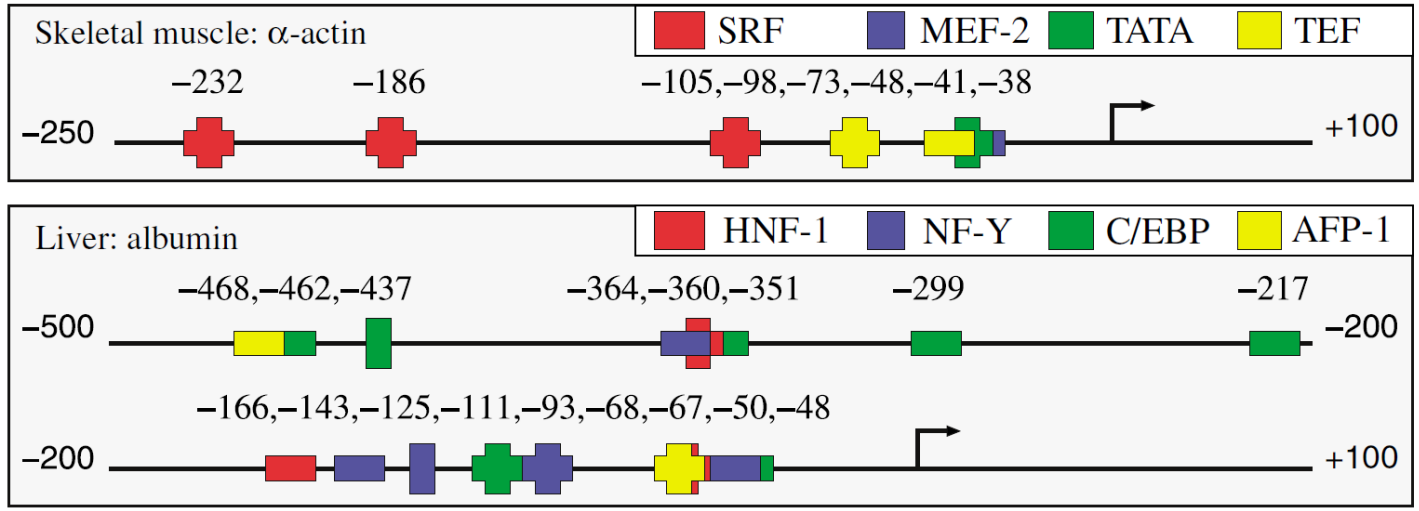


Figure 1 Verified and predicted binding sites in human albumin and skeletal muscle α-actin promoters. Predicted sites are represented by horizontal bars and verified sites by vertical bars. Verified sites for albumin (Paonessa *et al*, 1988; Sawadaishi *et al*, 1988; Frain *et al*, 1990; Li *et al*, 1990) and for α-actin (Boxer *et al*, 1989; MacLellan *et al*, 1994) were mapped to the promoter from CSHLmpd to obtain their correct locations relative to the TSS.

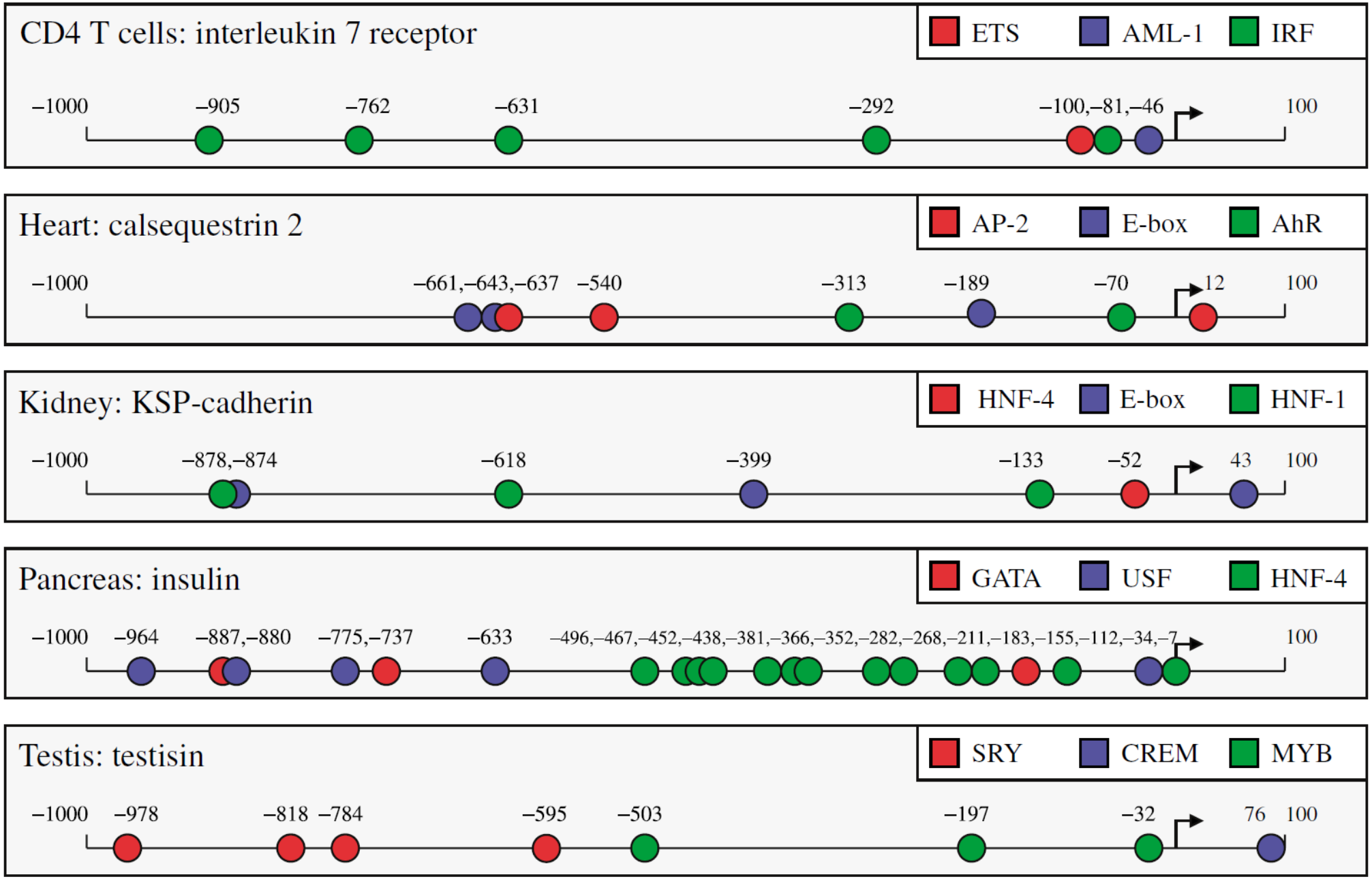


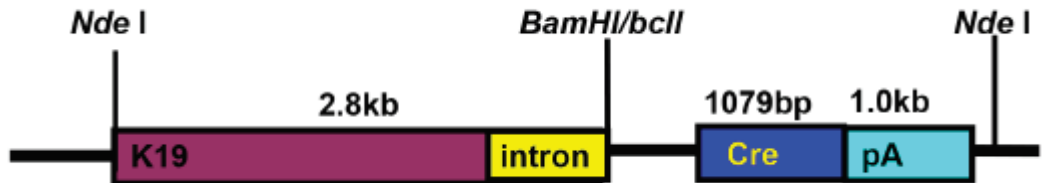
Figure 2 Predicted binding sites for selected factors in promoters from the human tissue-specific sets. The selected factors are among the top ranked in the corresponding tissues.

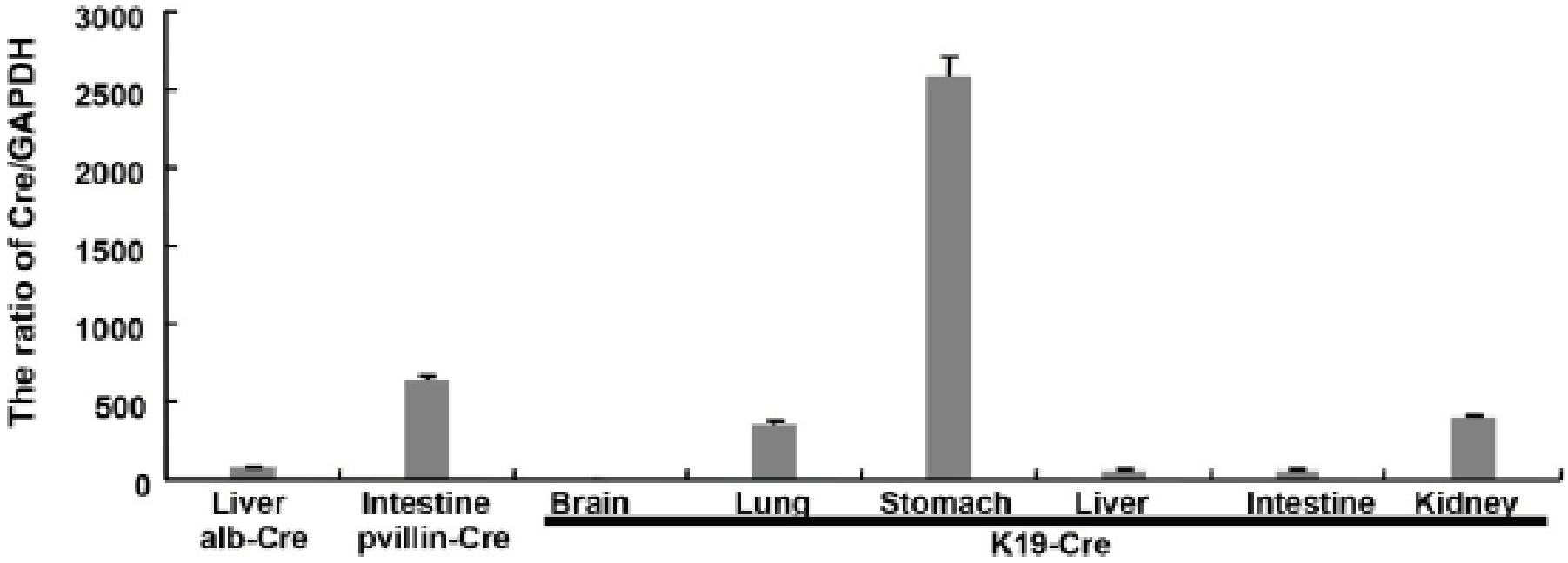
www.impactjournals.com/oncotarget/ Oncotarget, 2017, Vol. 8, (No. 11), pp: 18303-18311

Research Paper

Cytokeratin 19 promoter directs the expression of Cre recombinase in various epithelia of transgenic mice

Gui-Feng Zhao[1], Shuang Zhao[1], Jia-Jie Liu[1], Ji-Cheng Wu[1], Hao-Yu He[1], Xiao-Qing Ding[1], Xue-Wen Yu[2], Ke-Qiang Huang[2], Zhi-Jie Li[1], Hua-Chuan Zheng[1]





- Which kind of construct do you have to prepare if you want to study:

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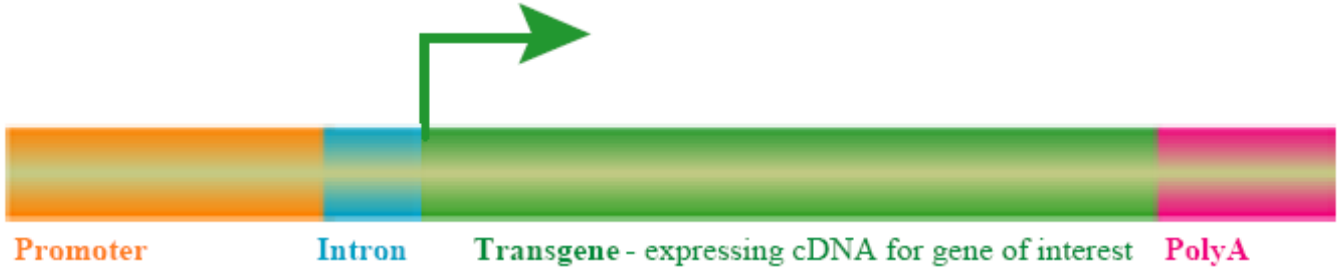
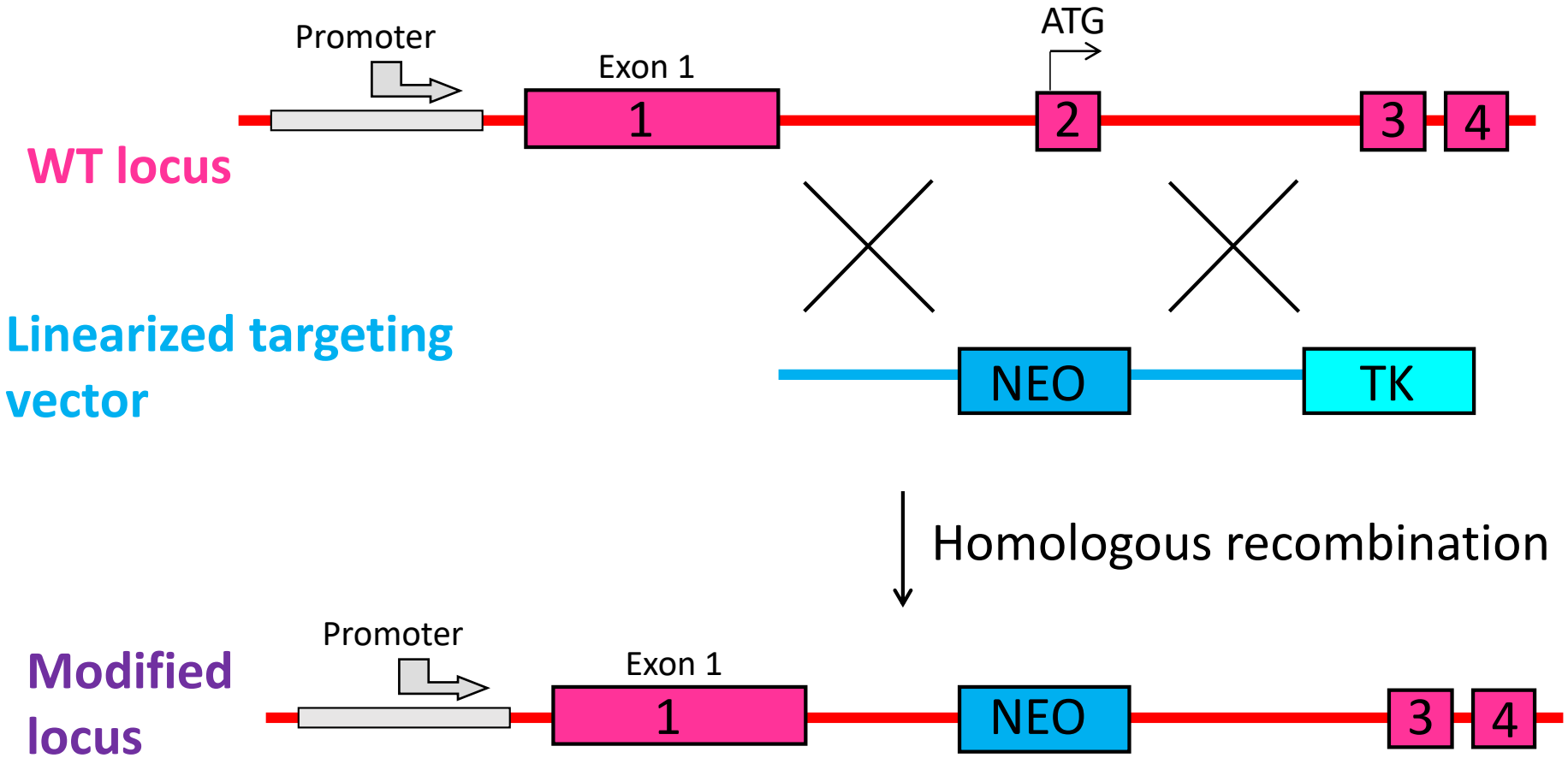
    (ubiquitous / tissue specific / inducible)
  - **the lack of a protein?** (**ubiquitous** / tissue specific/ inducible/**constitutive**)
  - the regulation of a promoter?
  - the localization of a protein?



- How can you discriminate between endogenous and exogenous proteins?
- How can you study the activity of the mutated exogenous protein without the expression of the wild type endogenous protein?

KNOCK-OUT MOUSE

- elimination of a gene through gene targeting to generate a **knock-out mouse**.
- **homologous recombination** allows a researcher to completely remove one or more exons from a gene, which results in the production of a truncated protein or, more often, no protein at all.



ES cells will be resistant to geneticin (G418) and to gancyclovir

Example:

A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye

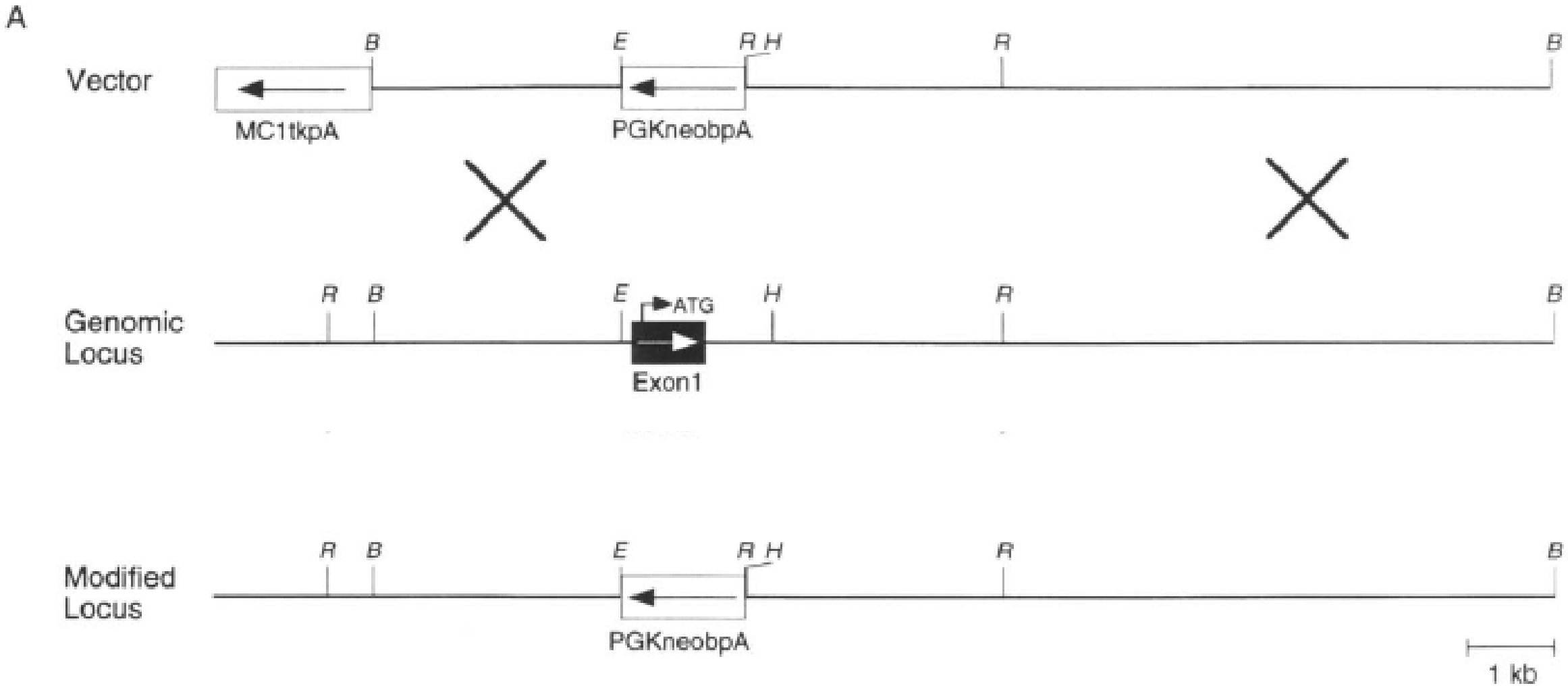
Andrew T. Dudley,[1] Karen M. Lyons,[1,2] and Elizabeth J. Robertson[3]

Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138 USA

Generation of BMP-7 mutant mice

The positive/negative targeting vector used to introduce a null mutation at the BMP-7 locus is shown in Figure 1A. Briefly, an *Eag*I-*Hind*III fragment containing the first coding exon and ~150 bp of upstream sequence was replaced by a PGK–*neo* cassette, in the opposite transcriptional orientation. The *neo* cassette was flanked by ~3.3 and 8.5 kb of 5′ and 3′ homology, respectively. Linearized vector was electroporated into CCE embryonic stem (ES) cells, and individual colonies selected in G418 and GANC were screened by Southern blot analysis using an external probe. Of 840 drug-resistant clones analyzed, we recovered 107 carrying the mutant allele, and 5 of these were injected into blastocysts. Three independent clones, C2, G6, and F9, gave rise to male chimeras that transmitted the mutation to their offspring. We analyzed *BMP-7* homozygous mutant progeny derived from all three clones to confirm that they all exhibit the identical phenotype. This mutation has been designated *BMP-7*^{m1Rob}.

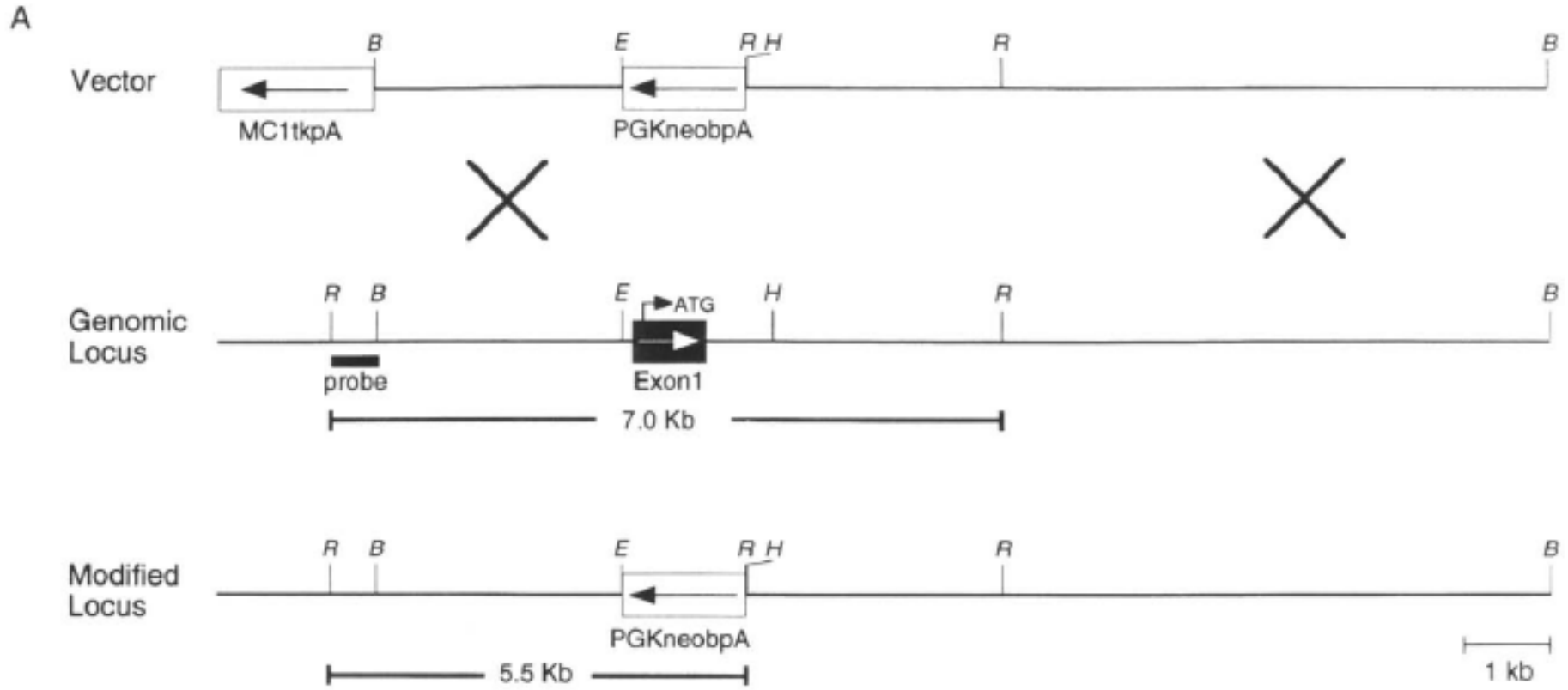
GENERATION OF A NULL ALLELE AT THE BMP-7 LOCUS



- If you analyse by Southern blot the genomic DNA, which restriction enzyme would you use?

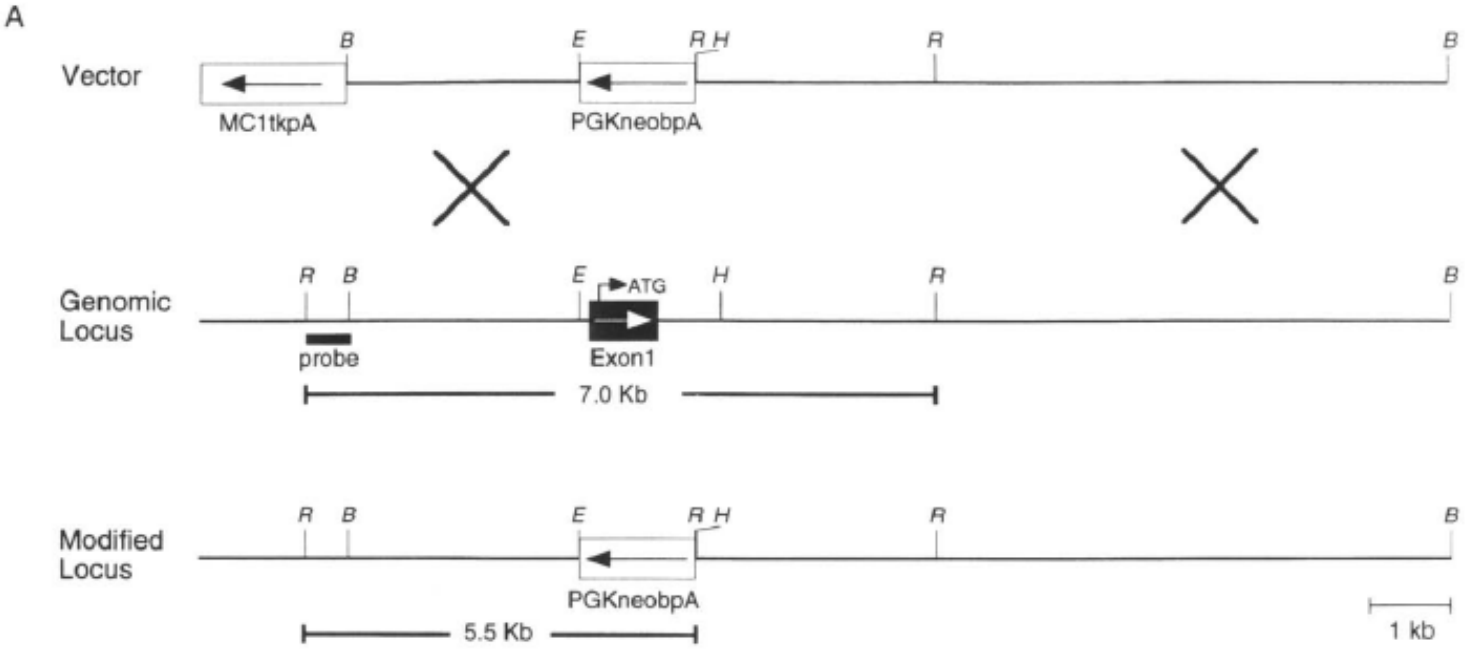
wooclap

GENERATION OF A NULL ALLELE AT THE BMP-7 LOCUS



- If you analyse by Southern blot the genomic DNA digested with EcoRI, which bands do you expect for +/+, m/+ and m/m animals?

GENERATION OF A NULL ALLELE AT THE BMP-7 LOCUS



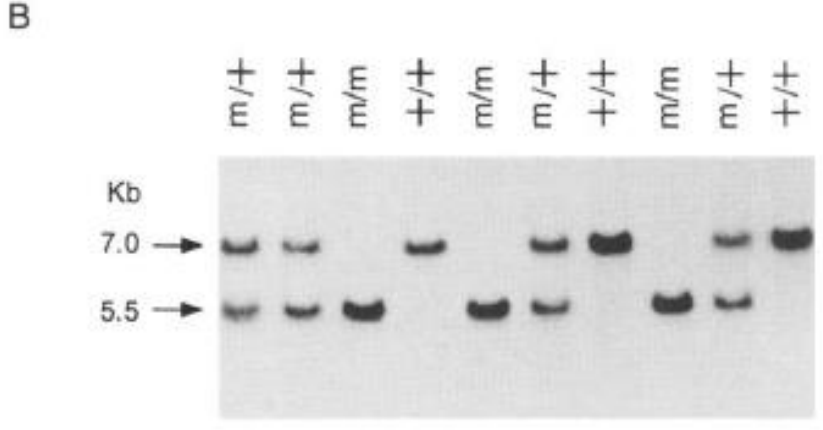


Figure 1. Generation of a null allele at the *BMP-7* locus. (*A*) Schematic representation of the wild-type and mutant alleles and the targeting vector. (B) *Bam*HI; (E) *Eag*I; (H) *Hind*III; (R) *Eco*RI restriction sites. (*B*) Southern blot analysis of mid-gestation stage embryos obtained from heterozygous intercrosses. Genomic DNA was digested with *Eco*RI and hybridized with the 5′ flanking probe. Positions of the 7.0-kb fragment derived from the wild-type allele and 5.5-kb fragment derived from the mutant allele are indicated.

Genotyping procedures

F_1 progeny heterozygous for the mutation were identified by Southern blotting of 10 μg of genomic DNA samples using the procedure described above. Subsequent progeny and embryos were genotyped either by Southern blot analysis or by PCR. The PCR primers were designed to use a common 5′ primer, specific for sequences located 5′ of exon 1 (5′-GCCCGGGCCAGAACTGAGTAAA-3′), in conjunction with a primer specific for the *neo* gene (5′-GGTGCCCACTCCCACTGTCCT-3′) or for *BMP-7* exon 1 sequences (5′-CGTCCACGACCCGAGGTCACTT-3′) to generate 130-bp and 120-bp products specific for the mutant and wild-type alleles, respectively. The PCR conditions were 50 mM KCl, 10 mM Tris-HCl at pH 7.5, 10% glycerol, 0.25 μM each primer, 1 μg of genomic DNA, and 1 unit of Amplitaq (Perkin-Elmer), in a 25-μl total reaction volume. Following an initial denaturation step (94°C for 1 min), samples were subjected to 30 amplification cycles (94°C for 30 sec, 62°C for 30 sec, 72°C for 30 sec). The amplification products were separated on a 1.5% agarose gel and visualized following ethidium bromide staining.

GROSS MORPHOLOGICAL ANALISYS OF BMP-7 MUTANT EMBRYOS

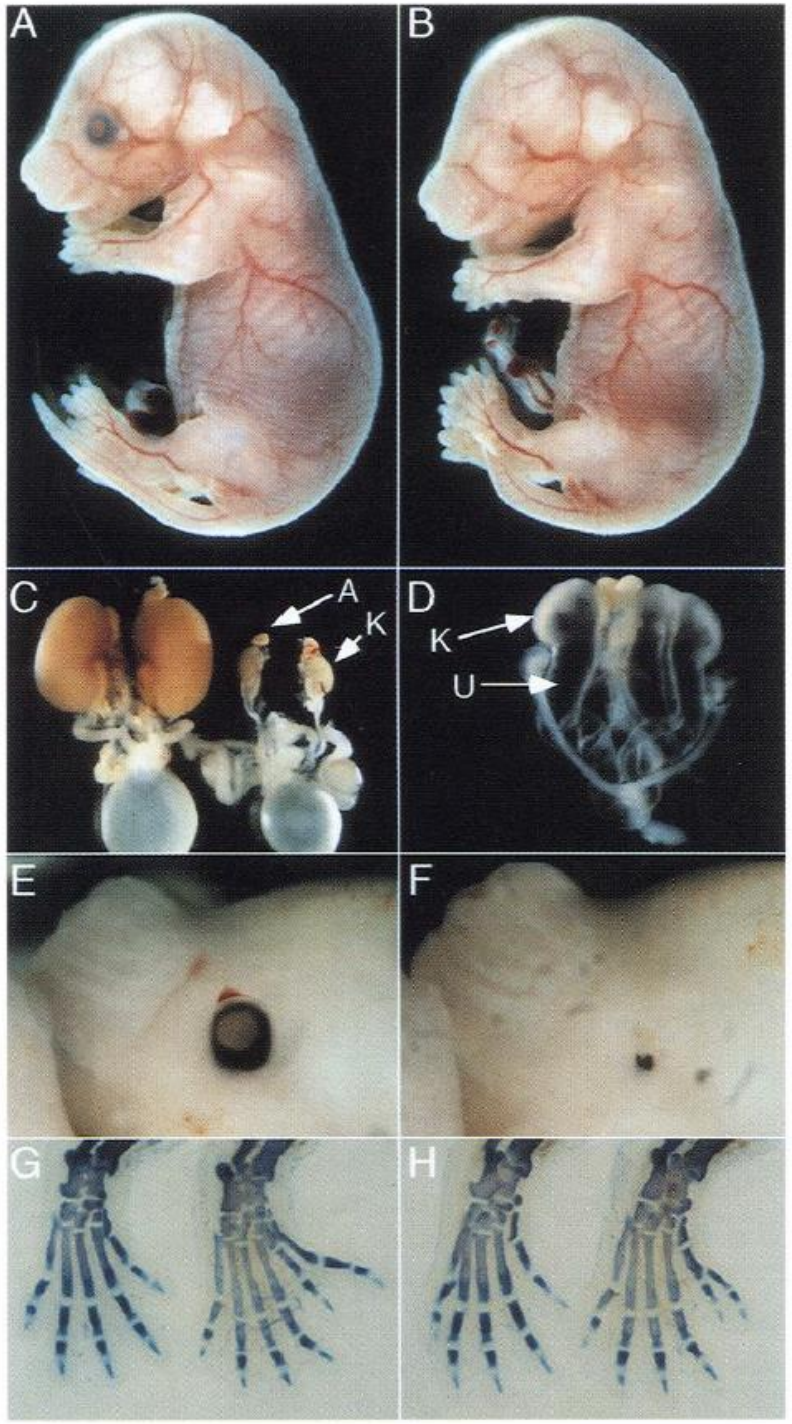


Figure 2. Gross morphological analysis of *BMP-7* mutant embryos. Adjacent panels photographed at the same magnification showing heterozygous (*A*) and homozygous mutant (*B*) littermates at 17 days p.c. (*C*) At 19 days p.c., kidneys (*k*) from *BMP-7* mutant embryos (*right*) are significantly smaller than those from a heterozygous littermate (*left*). The remainder of the urogenital tract and adrenals (K) are morphologically normal. (K) kidney. (*D*) Acute hydroureter phenotype displayed by the majority of BMP-7 mutants at birth. A small mass of kidney tissue remains (K), while the renal pelvis (R) and ureter (U) are extremely distended (*E,F*) High magnification views of eyes at 13.5 days p.c. The wild-type embryo (*E*) shows a well-developed pigmented retinal epithelium, whereas the *BMP-7* mutant embryo (*F*) has only a residual mass of pigmented retinal epithelial cells. (*G,H*) Preaxial polydactyly of the hind limbs. Whole-mount preparations comparing the cartilaginous structures present in wild-type (*left*) and BMP-7 mutant (*right*) limbs are shown.

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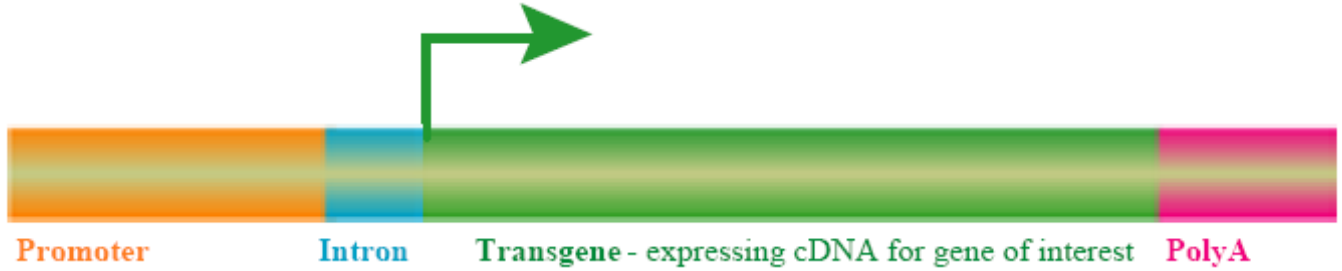
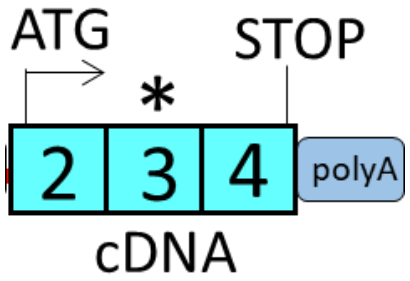
- ..... ?



- How can you discriminate between endogenous and exogenous proteins?

- the activity of a mutated exogenous protein WITHOUT the expression of the wild type endogenous protein?

How is it possible to obtain the expression of a mutated exogenous protein WITHOUT the expression of the wild type endogenous protein?

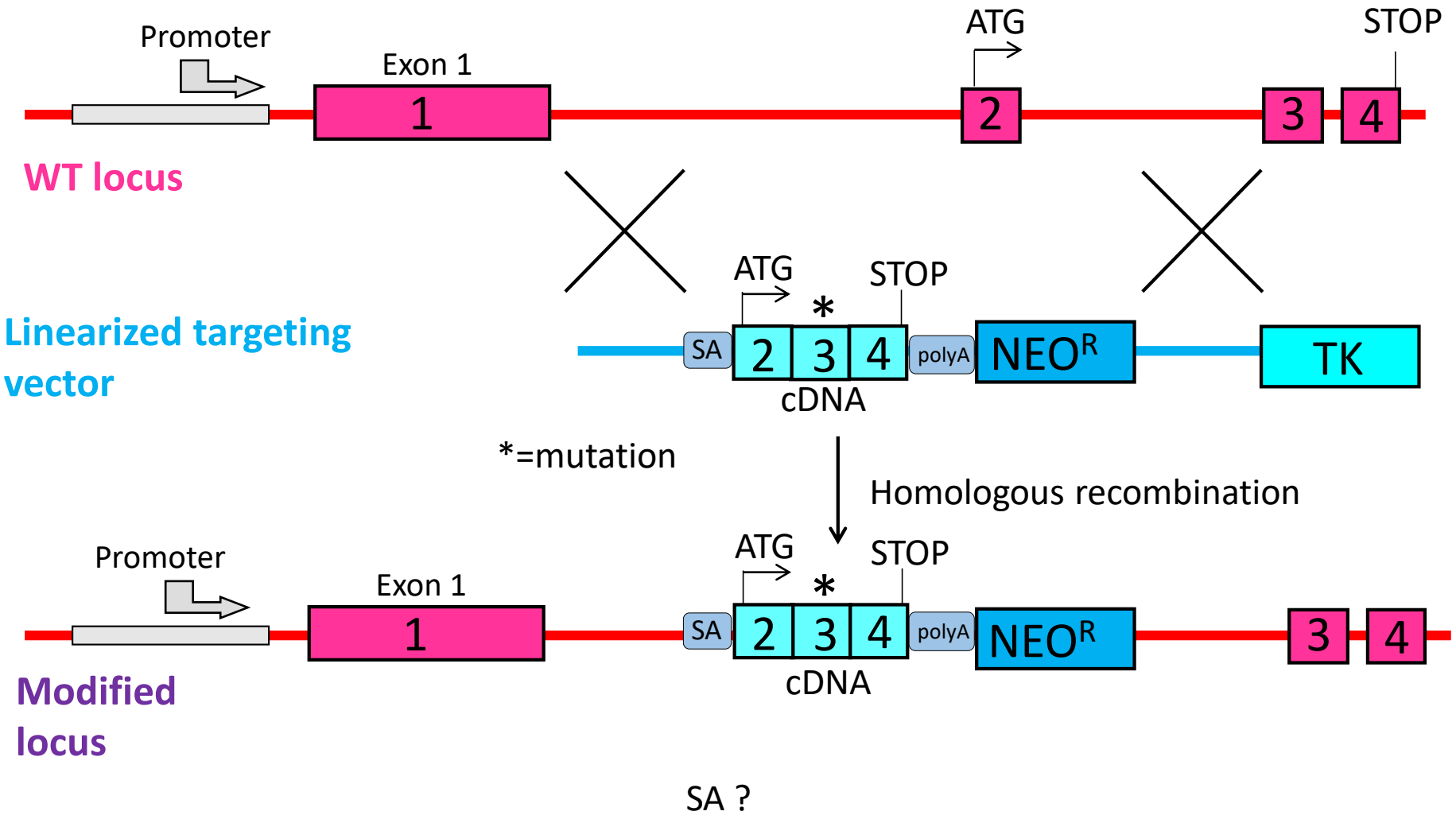


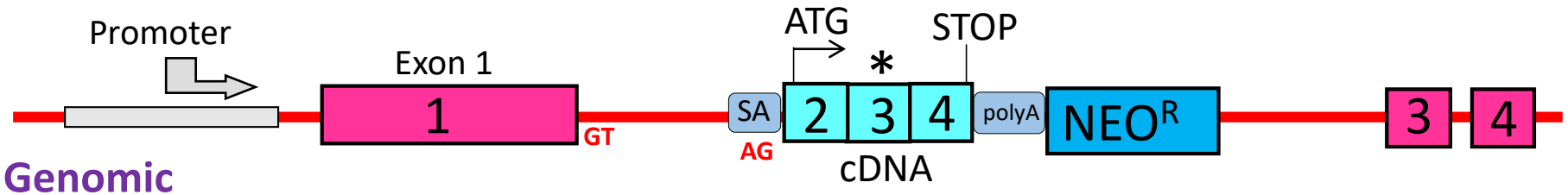
MUTATED FORM

KNOCK-IN MOUSE

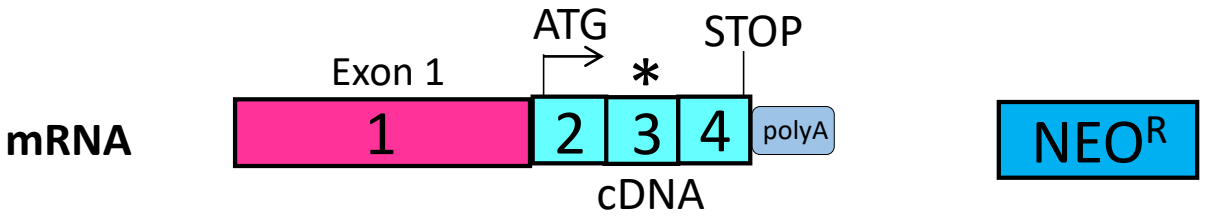
A gene can be eliminated through gene targeting and substituted by a cDNA coding for a mutated form of the gene, to generate a **knock-in mouse**.

Homologous recombination allows to completely remove one or more fundamental exons from a gene, and substitute them with the new cDNA.

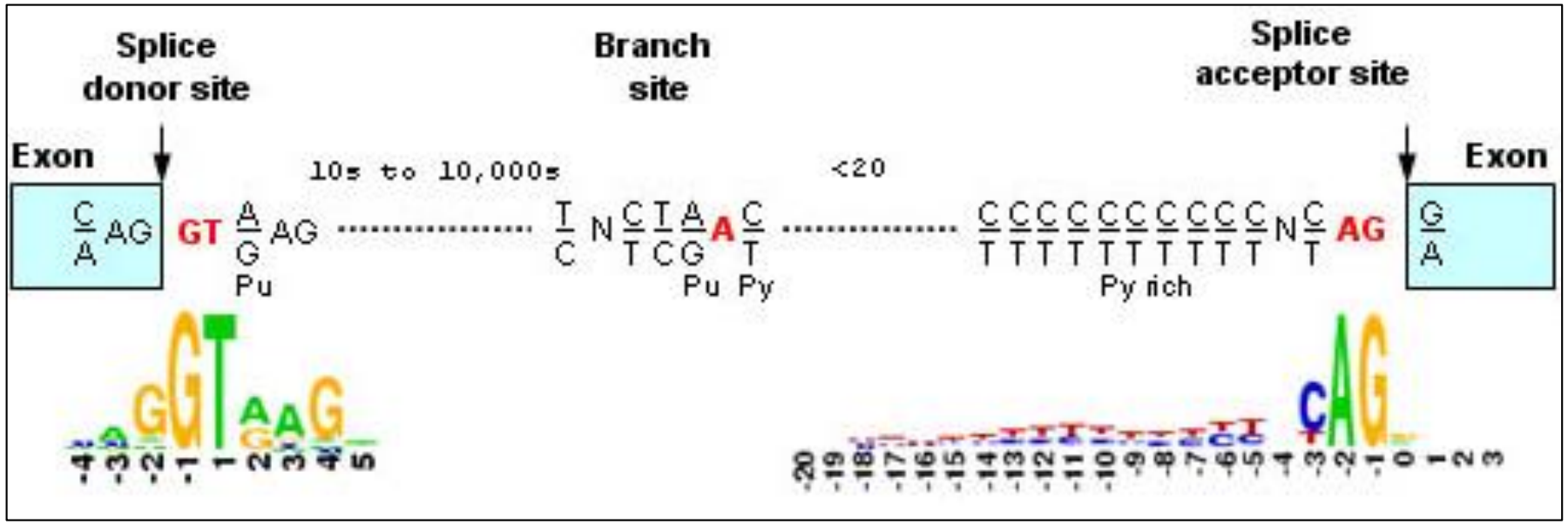




Genomic modified locus



SA=splice acceptor



Uncoupling of Grb2 from the Met Receptor In Vivo Reveals Complex Roles in Muscle Development

Flavio Maina,*† Franca Casagranda,* Enrica Audero,† Antonio Simeone,‡ Paolo M. Comoglio,†§ Rüdiger Klein,* and Carola Ponzetto†

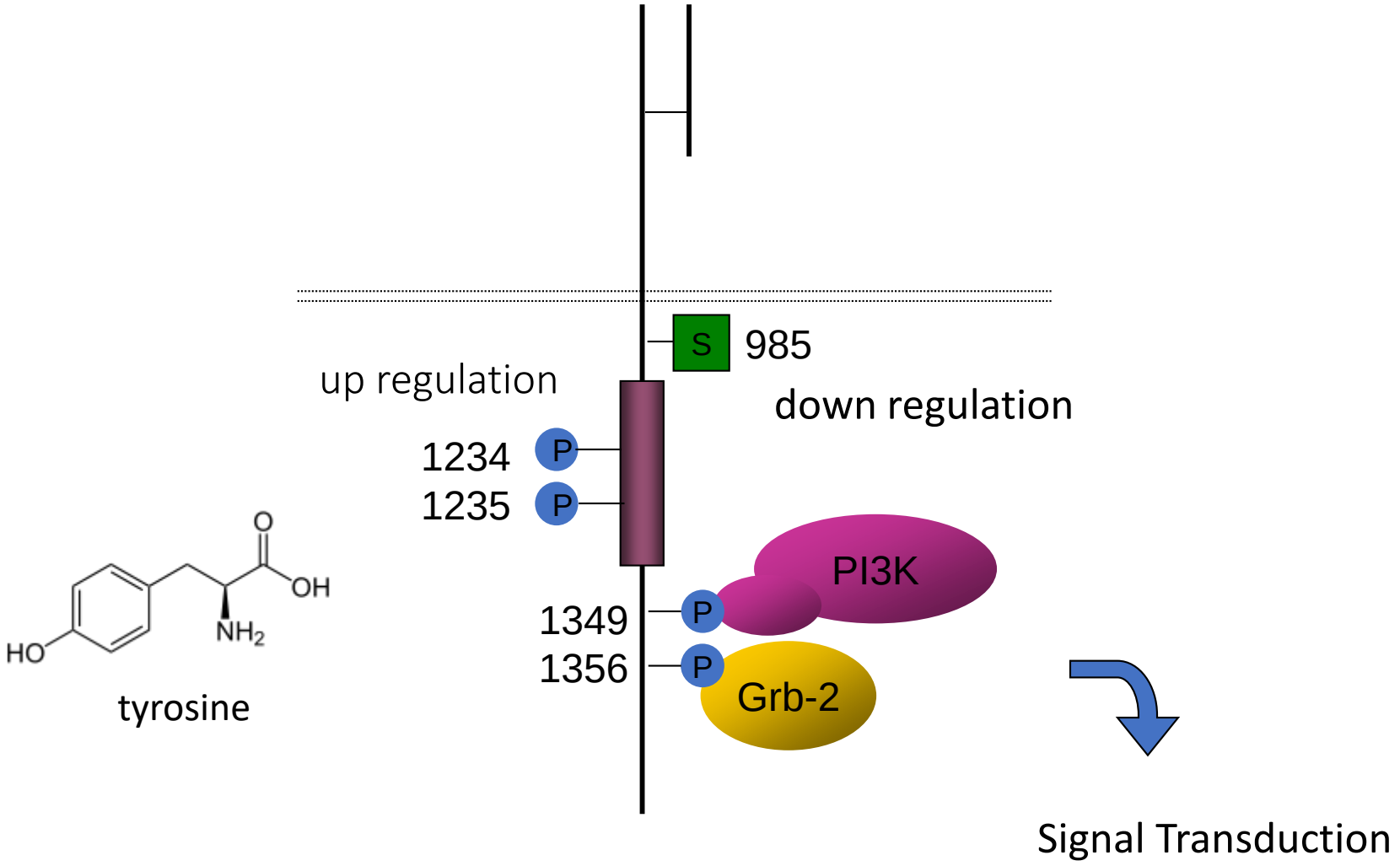
*European Molecular Biology Laboratory
Meyerhofstrasse 1
69117 Heidelberg
Federal Republic of Germany

†Department of Biomedical Sciences and Oncology
University of Torino
10126 Torino
Italy

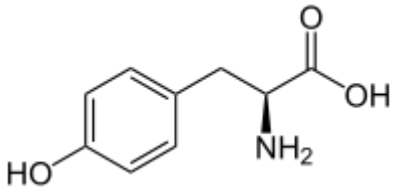
‡International Institute of Genetics and Biophysics
via G. Marconi 12
80125 Napoli
Italy

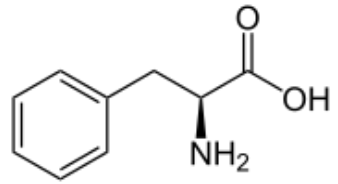
§Institute for Cancer Research
10060 Candiolo (Torino)
Italy

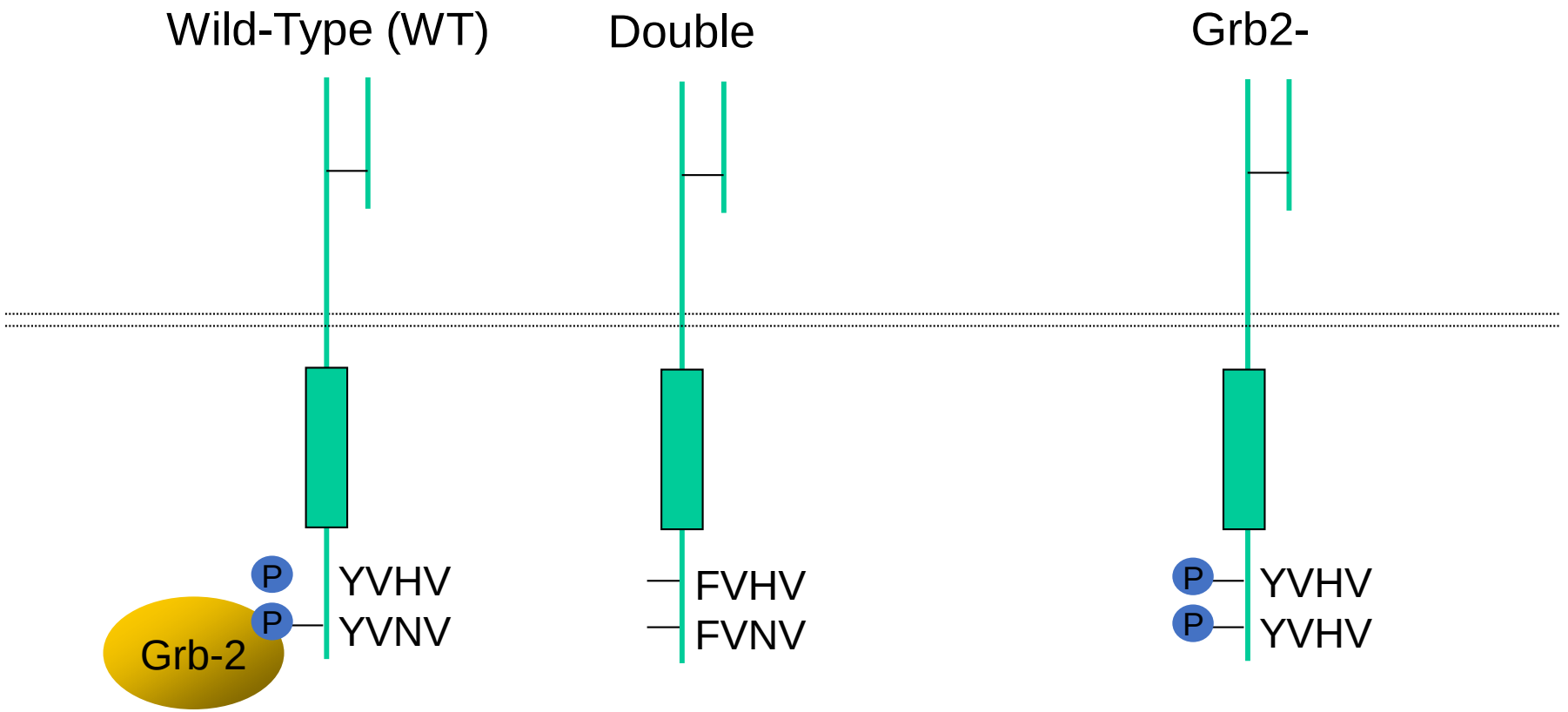
THE HGF/SF RECEPTOR (*MET*)



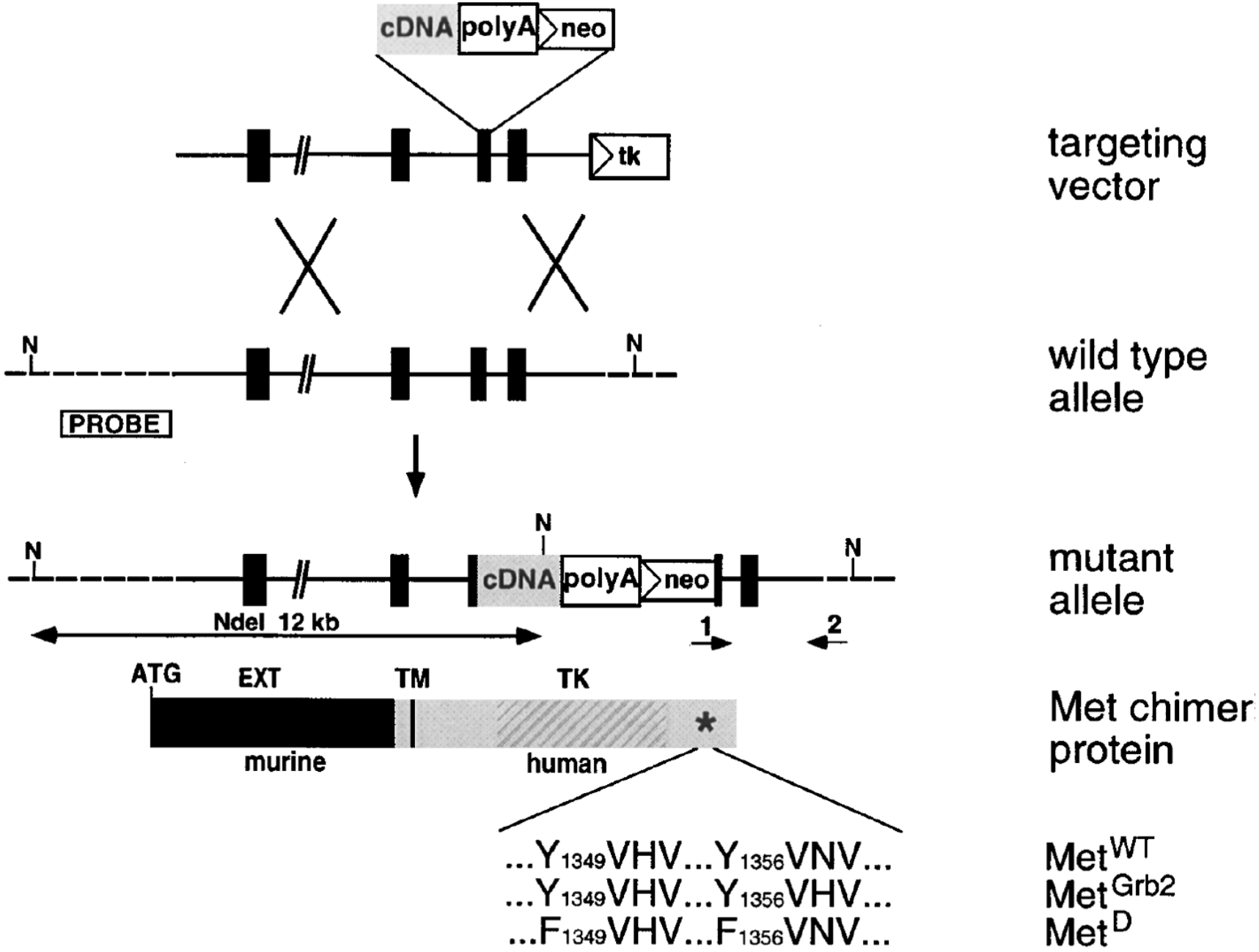
Met^{Wt}	YVHVNATYVNV
Met^{Double}	FVHVNATFVNV
Met^{Grb2-}	YVHVNATYVHV







KNOCK-IN OF POINT MUTATIONS IN THE MET LOCUS



targeting vector

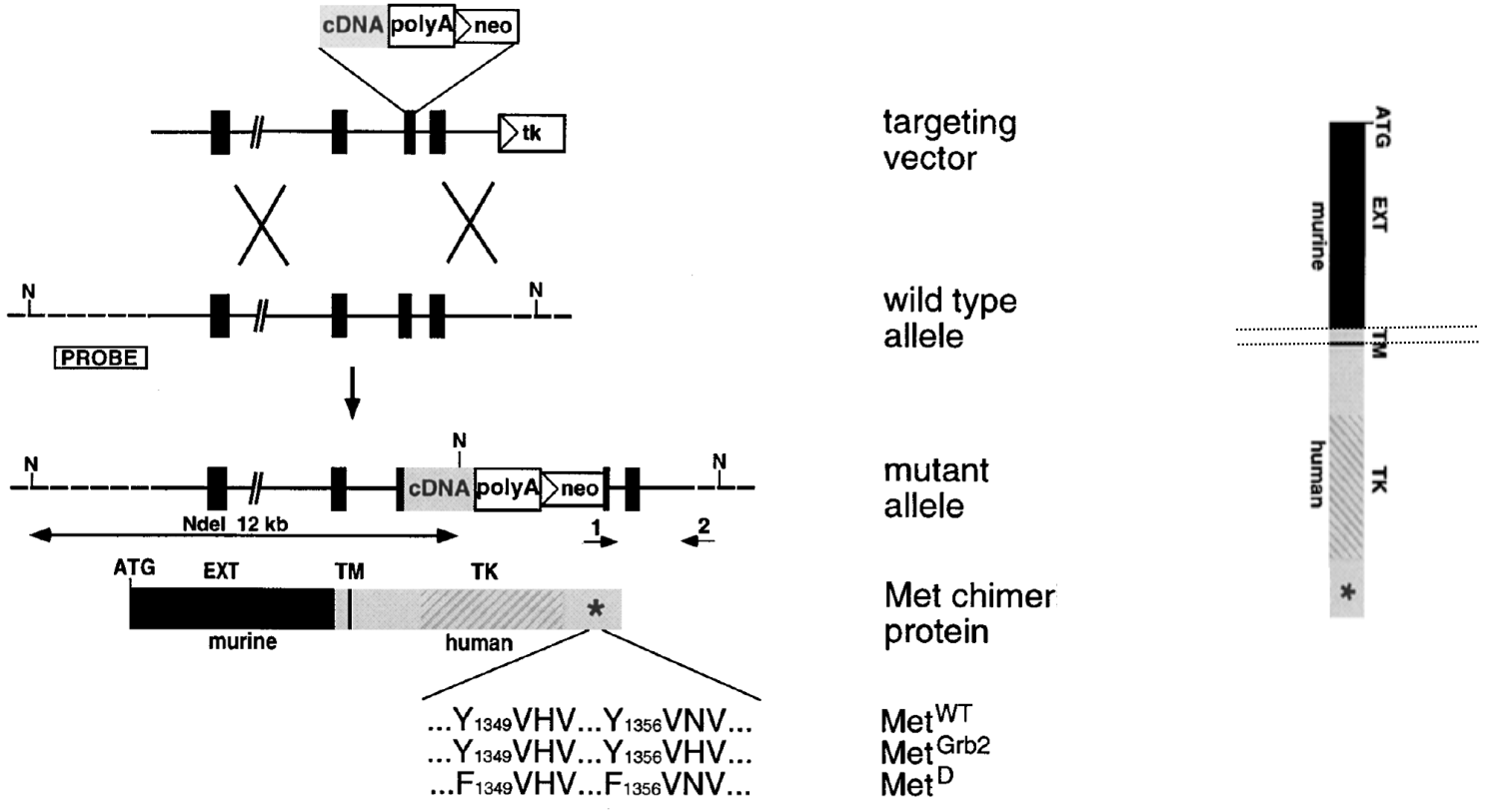
wild type allele

mutant allele

Met chimer protein

Met^{WT}
Met^{Grb2}
Met^{D}

KNOCK-IN OF POINT MUTATIONS IN THE MET LOCUS



The human MET cDNA fragment fused in-frame with the third exon of the genomic clone, codes for the transmembrane and cytoplasmic domain of the Met receptor. N indicates cleavage sites for NdeI. The probe identifies a 16 kb NdeI DNA fragment in the wild-type allele and a 12 kb NdeI DNA fragment in the mutant allele.

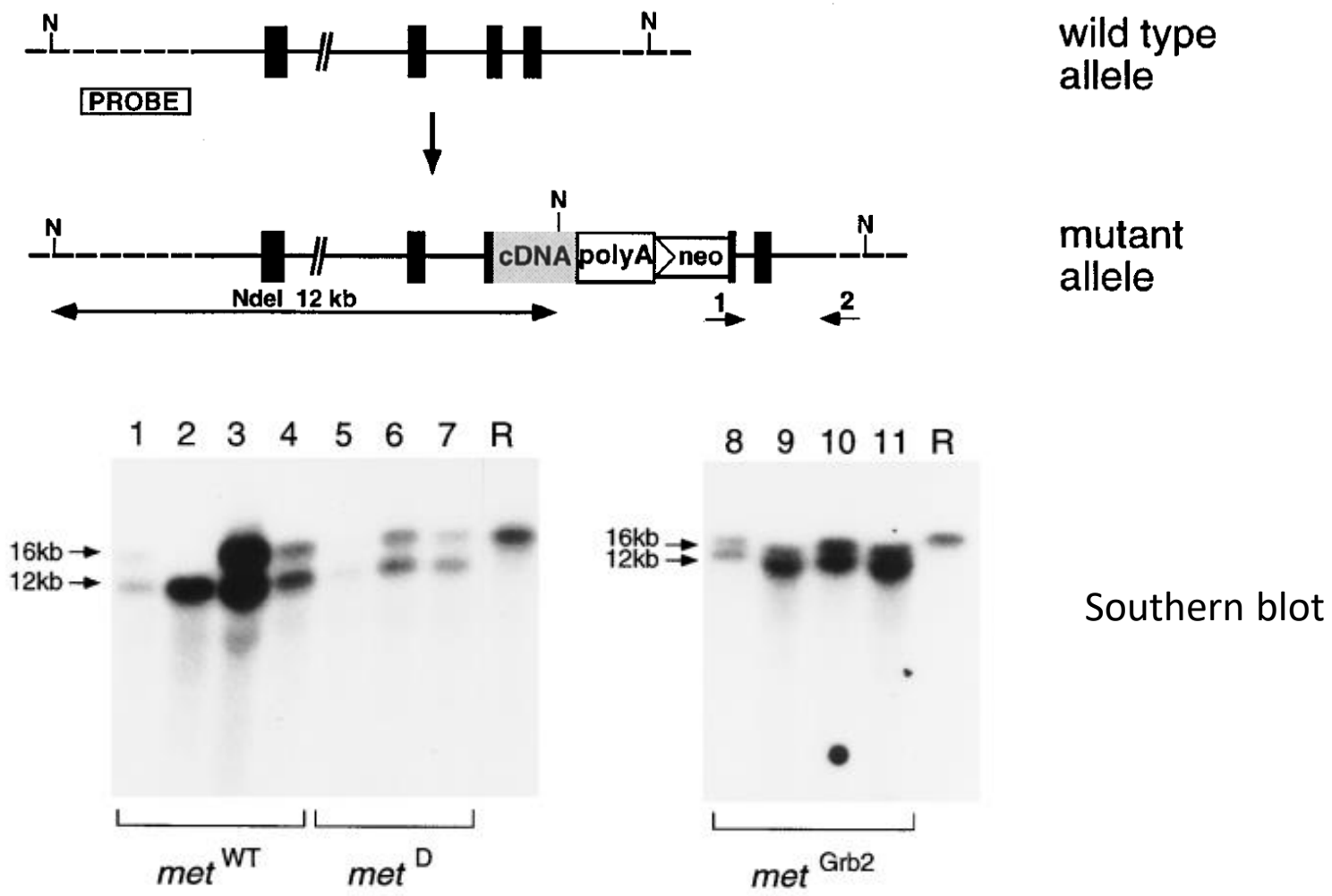
“1” and “2” indicate the location of the oligonucleotides used for PCR screening of the double-selected ES cells.

The chimeric protein can specifically be recognized by anti-human Met antibodies raised against the unique carboxy-terminal peptide. Asterisk indicates the carboxy-terminal SH2 multifunctional docking sites.

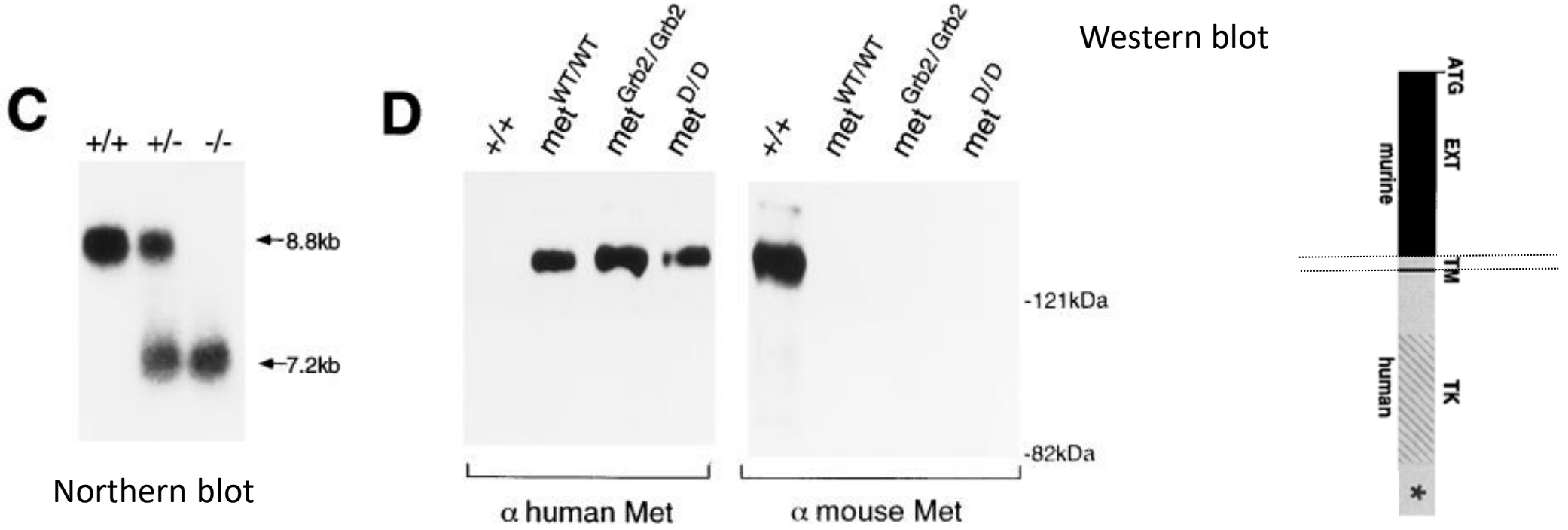
Met^{WT}: chimeric protein with wild-type multifunctional tyrosines.

Met^{Grb2}: chimeric protein with disrupted Grb2 binding site.

Met^{D}: chimeric protein with mutated multifunctional tyrosines.



Southern blots of NdeI digests of genomic DNA isolated from double-selected ES cell clones (lanes 1–11), transfected with the metWT, metD, and metGrb2 constructs.
Lane 2: ES cell clone in which both alleles were targeted.
Lanes R: R1 ES cells control DNA. The probe used identifies a 16 kb NdeI DNA fragment in the wild-type allele and a 12 kb NdeI fragment in the recombinant allele.

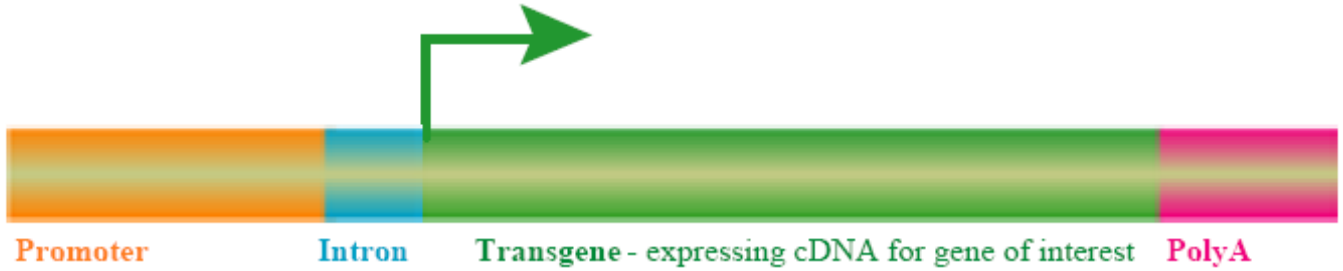


(C) **Northern blot of total RNA isolated from liver of wild-type (+/+), met $^{WT/-}$ (+/-) and met $^{WT/WT}$ (-/-) mice.** The higher 8.8 kb band is the endogenous transcript, and the 7.2 kb band is the recombinant transcript. The difference in size is due to the fact that in the recombinant allele, the untranslated region is substituted by the 1.2 kb β-globin untranslated region and polyadenylation site. The probe used was a mouse Met cDNA fragment located in the extracellular domain common to both alleles.

(D**) Immunoprecipitation and Western blot of extracts of E15.5 homozygous and control (+/+) embryos.**
Left: the antibody used for Western blotting was specific for the human Met protein.
Right: the antibody used for IP and Western blotting was specific for the mouse Met protein.

- Which kind of construct do you have to prepare if you want to study:

  - **the over-expression of a protein**? (ubiquitous **/ tissue specific / inducible**)
  - the regulation of a promoter?
  - **the lack of a protein?** (ubiquitous **/ tissue specific / inducible**)
  - the localization of a protein?
  - ..... ?



- How can you discriminate between endogenous and exogenous proteins?

- How can you study the activity of a mutated exogenous protein without the expression o the wild type endogenous protein?

GENETIC MODIFICATION of CELLS

- transient vs stable transfection of cells

TRANSGENIC ANIMALS

- Two methods to produce transgenic animals:

    1- DNA microinjection
        - random insertion
    2- embryonic stem cell-mediated gene transfer
        - random insertion
        - homologous recombination (double selection)
        - Rosa26 locus
        - genomic analysis to identify genetically modified animals

- knock-out animals
- knock-in animals

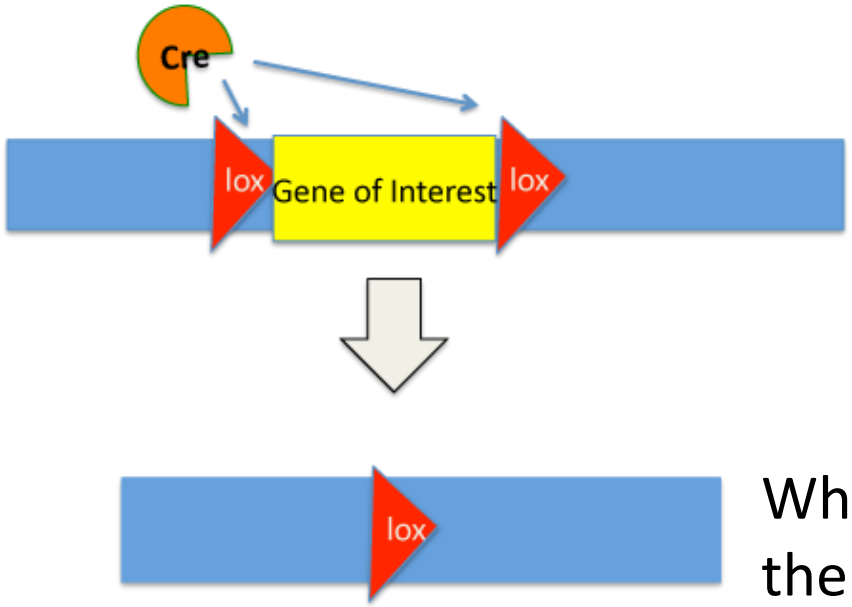
- **conditional knock-out (cre-lox technique, inducible systems)**

- siRNA

- CRISPR-CAS9

CRE-LOX TECHNIQUE

CRE-LOX technique inactivates a gene only in specific tissues and at certain times during development and life.



Your gene of interest is **flanked by 34 bp loxP sites ("floxed")**

Where **CRE recombinase** is expressed at least once, the gene between loxP sites is removed

CRE recombinase expression can be:
- tissue specific (under a tissue specific promoter),
- inducible (under an inducible promoter).

CRE recombinase activity can be:
- regulated (tamoxifen-specific modified oestrogen receptor Cre recombinase fusion protein)

LoxP sequence (34-bp-long)

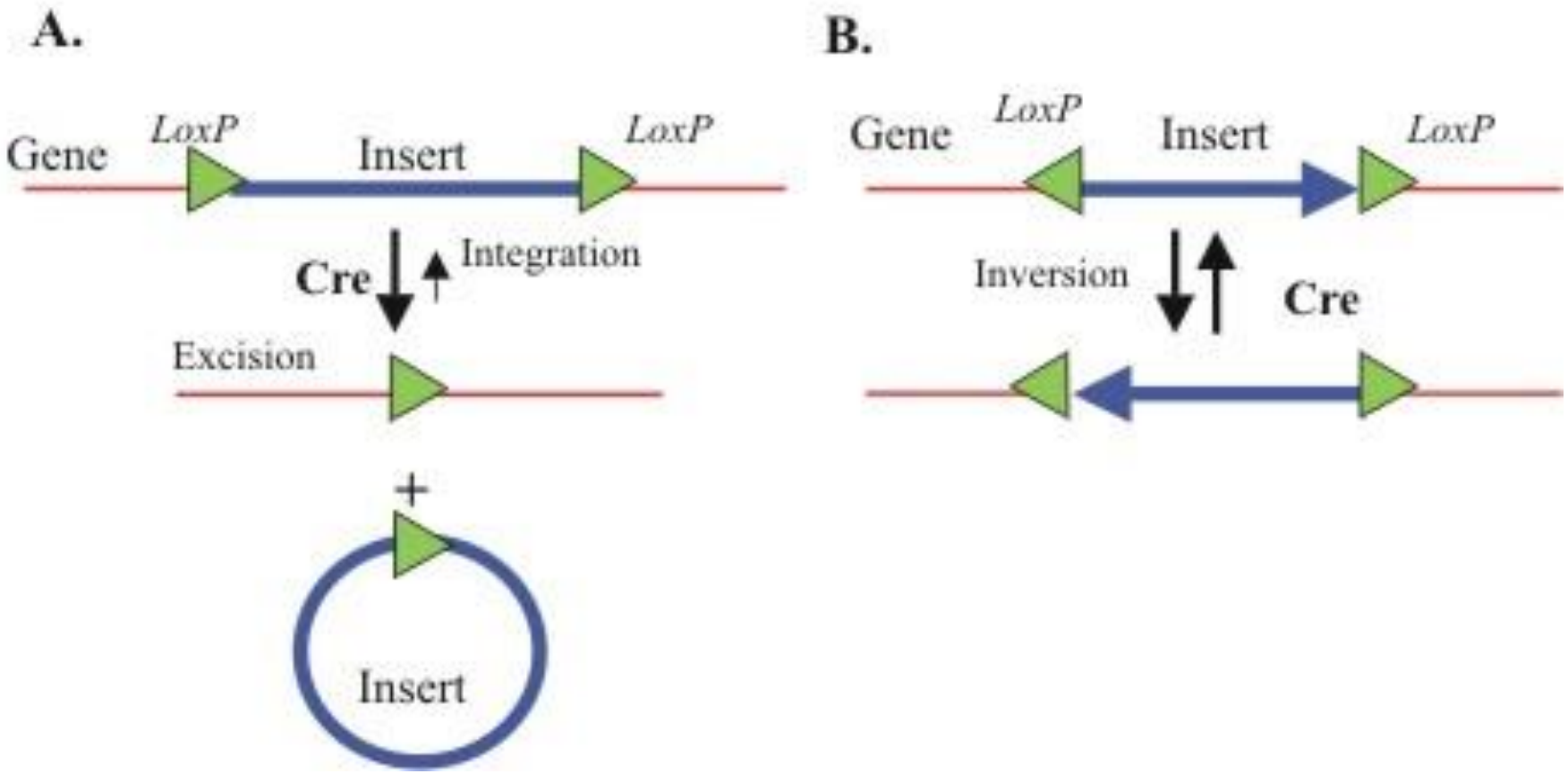
Lox P (**locus of X-over P1**) is a site on the bacteriophage P1 consisting of 34 bp. The site includes an asymmetric 8 bp sequence, variable except for the middle two bases, in between two sets of palindromic, 13 bp sequences.

```
5′-ATAACTTCGTATA NNNTANNN TATACGAAGTTAT-3′
3′-TATTGAAGCATAT NNNATNNN ATATGCTTCAATA-5′
      13bp          8bp         13bp
```

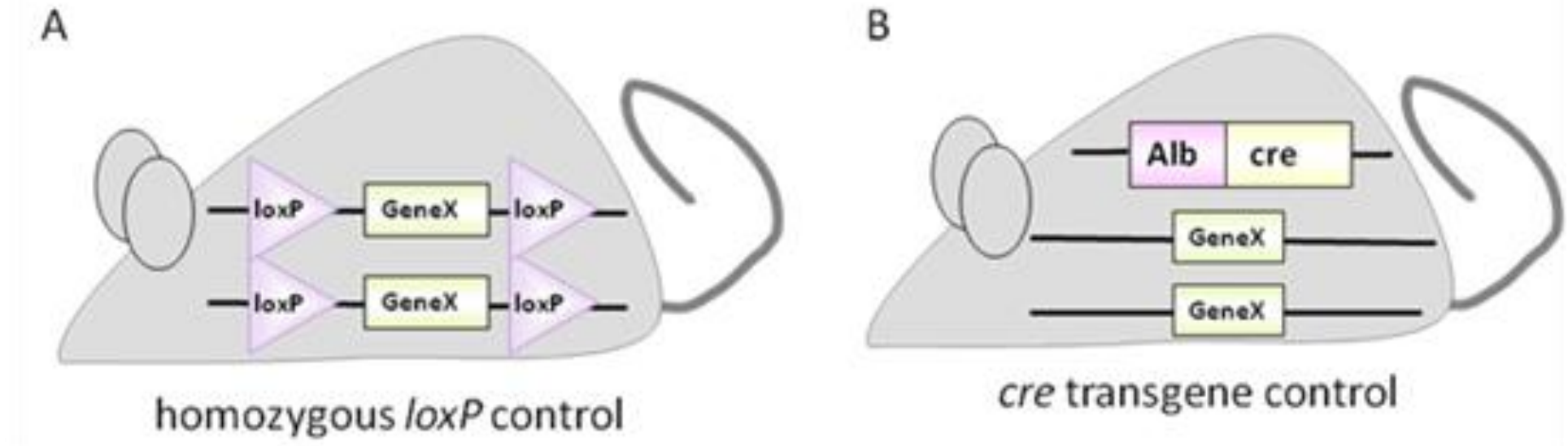
Multiple variants of loxP, in particular lox2272 and loxN, have been used by researchers with the combination of different Cre actions.

Cre protein (from "**C**auses **re**combination" or "**C**yclization **re**combinase") consists of 4 subunits and two domains

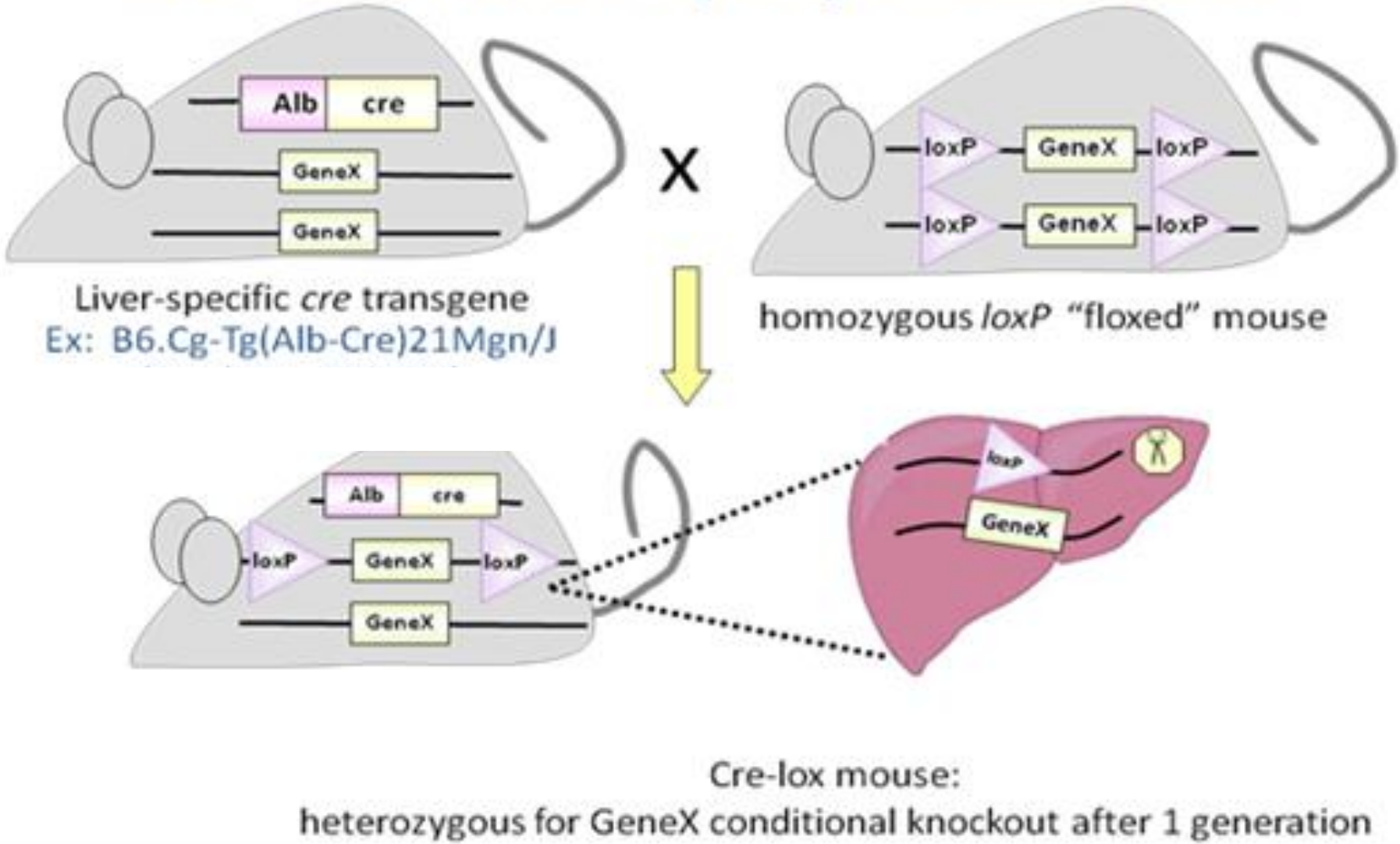
https://www.youtube.com/watch?v=oLPjiwM0G7A



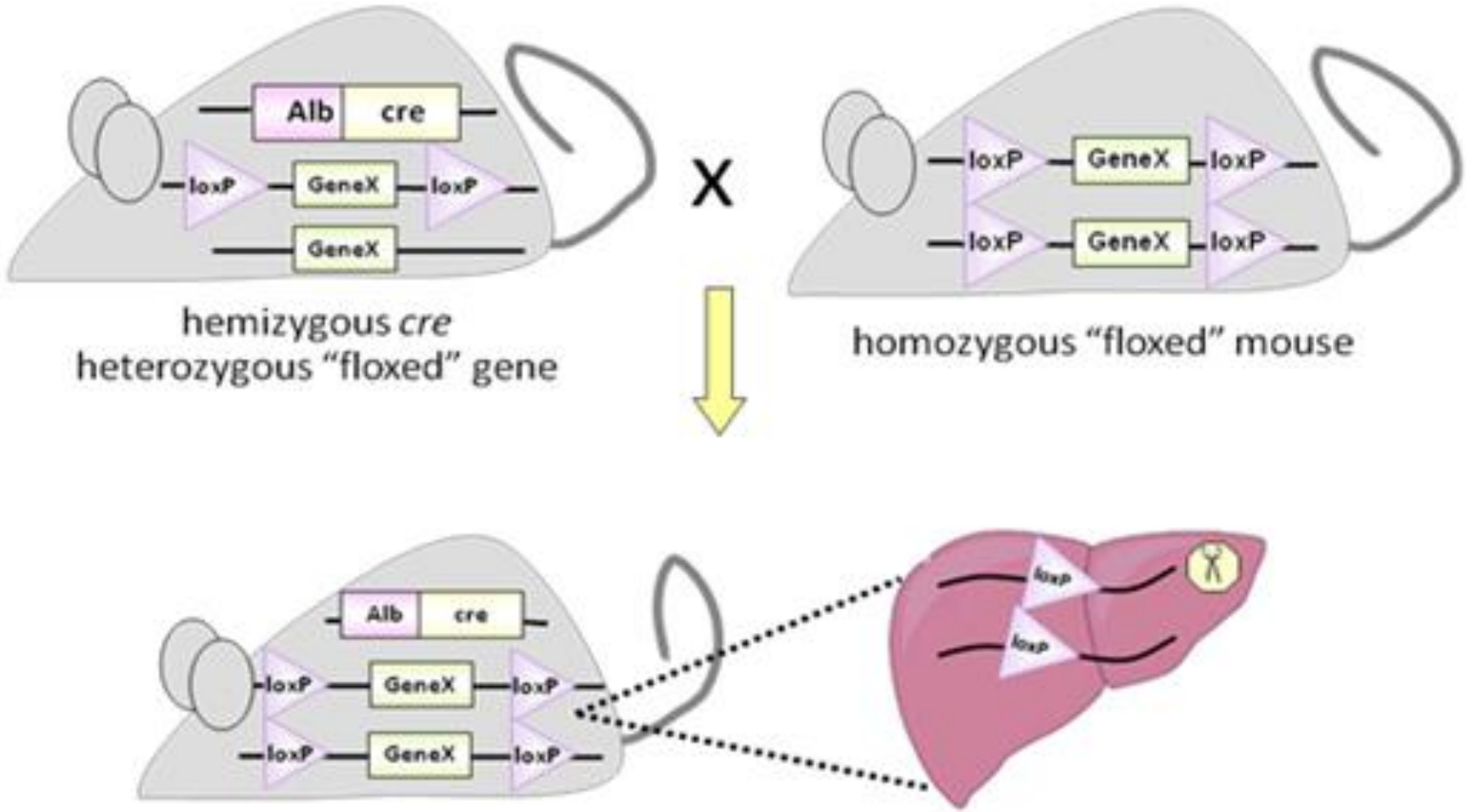
Controls

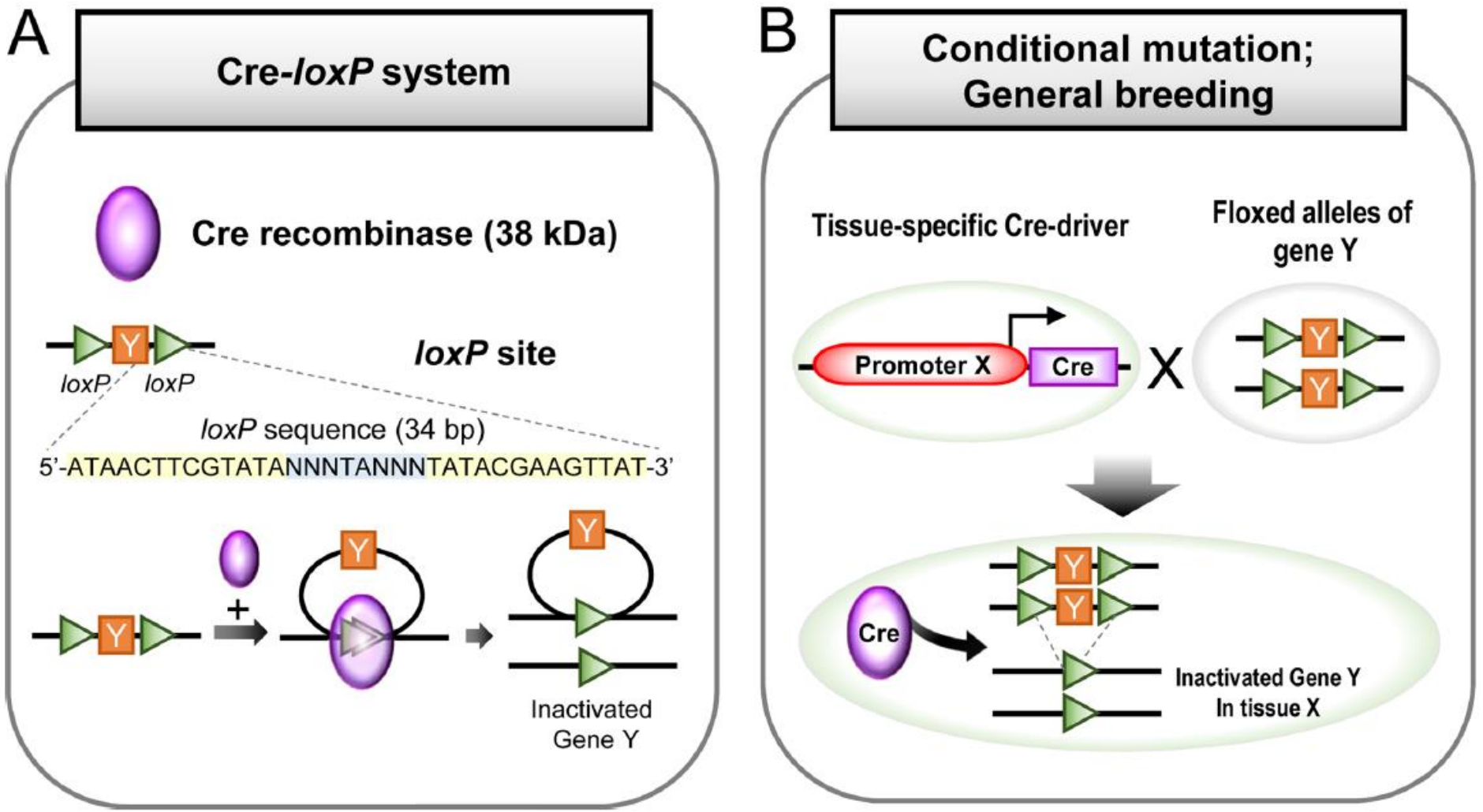

A
loxP
GeneX
loxP
loxP
GeneX
loxP
homozygous *loxP* control
B
Alb
cre
GeneX
GeneX
cre transgene control

Cre - lox *Tissue-Specific* Knockout



Cre - lox *Tissue-Specific* Knockout (cont.)

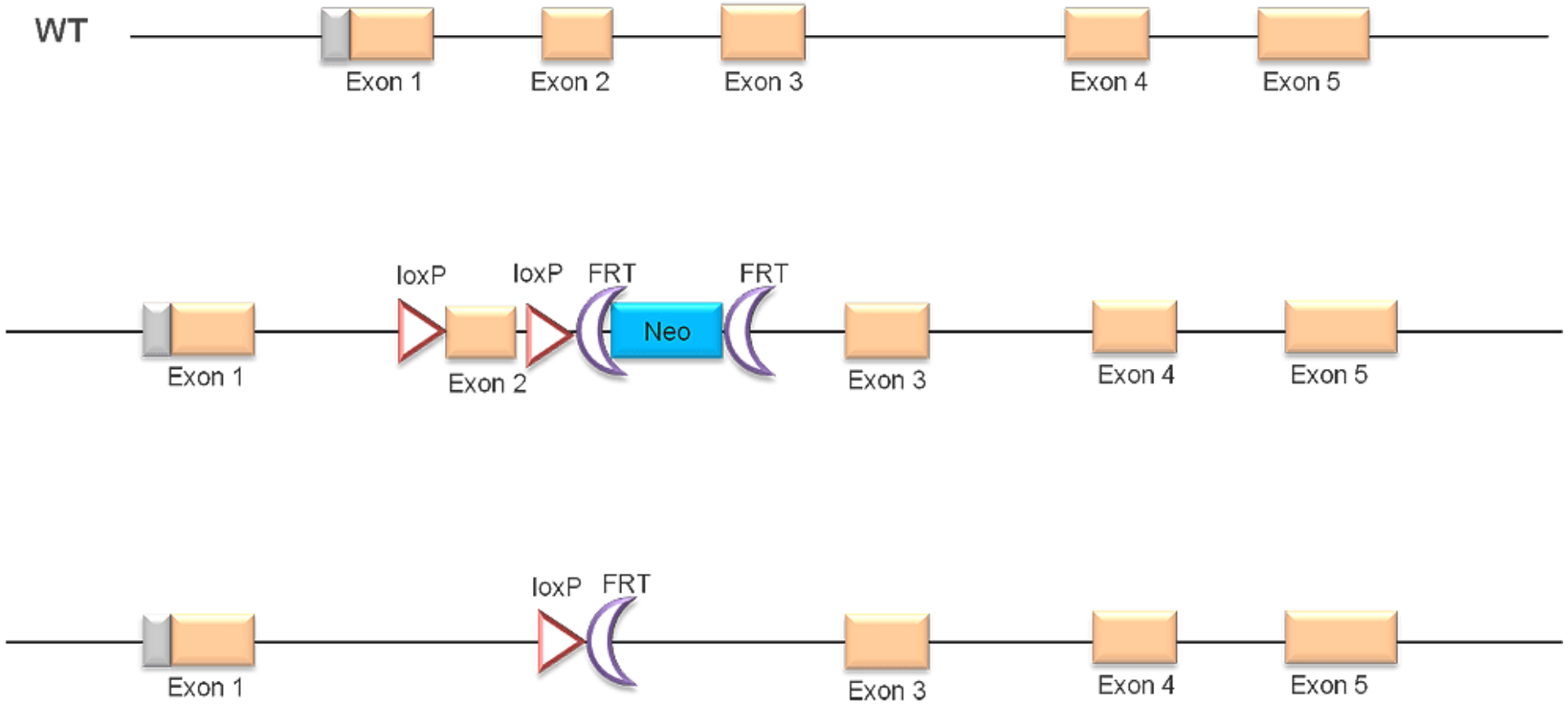




(A) An overview of Cre-loxP system. 38 kDa Cre recombinase recognizes the loxP sites of specific 34 bp DNA sequences.

(B) General breeding strategy for conditional mutation using loxP and Cre driving mouse line. In principle, one mouse must have a tissue-specific driven cre gene and another mouse have loxP flanked (floxed) alleles of interest gene Y. Expression of Cre recombinase excises floxed loci and inactivates the gene Y.

CRE-LOX TO OBTAIN “TRADITIONAL” CONDITIONAL KNOCK-OUT MICE

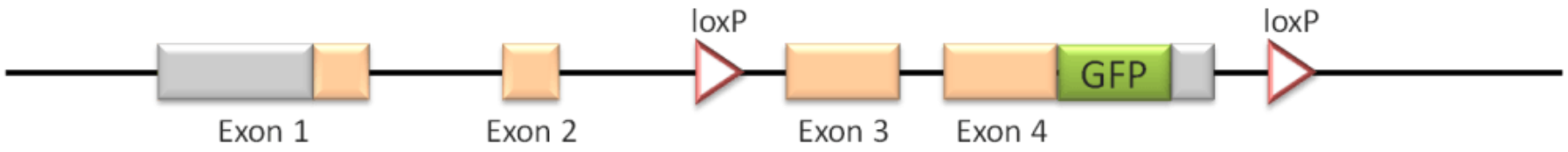


Conditional knockout mouse model in which exon 2 is flanked with loxP sites, Neo cassette is flanked with FRT sites.

Upon flippase (FLP) and Cre recombination, exon 2 is eliminated, and a loxP/FRT footprint remains. The deletion of exon 2 results in a frame shift and early stop codons which can be visualized at the protein level.

CONDITIONAL KNOCK-OUT WITH REPORTER, DRIVEN BY NATIVE PROMOTER

Adding a reporter gene to a conditional knockout model enables the researcher to visualize when and where the gene is expressed, or it can help to show when the gene has been inactivated



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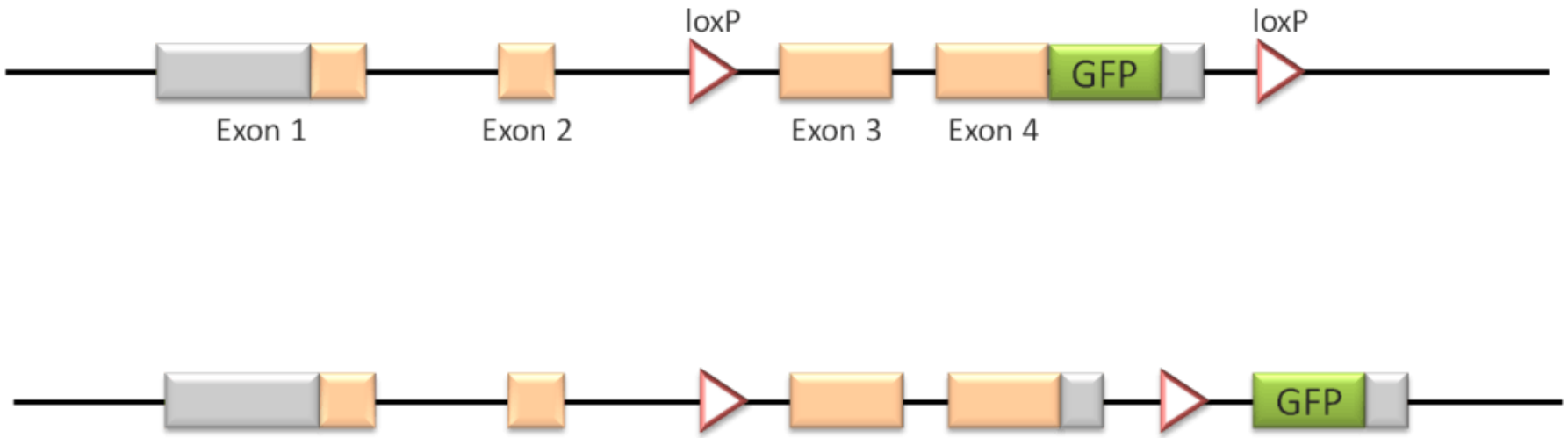

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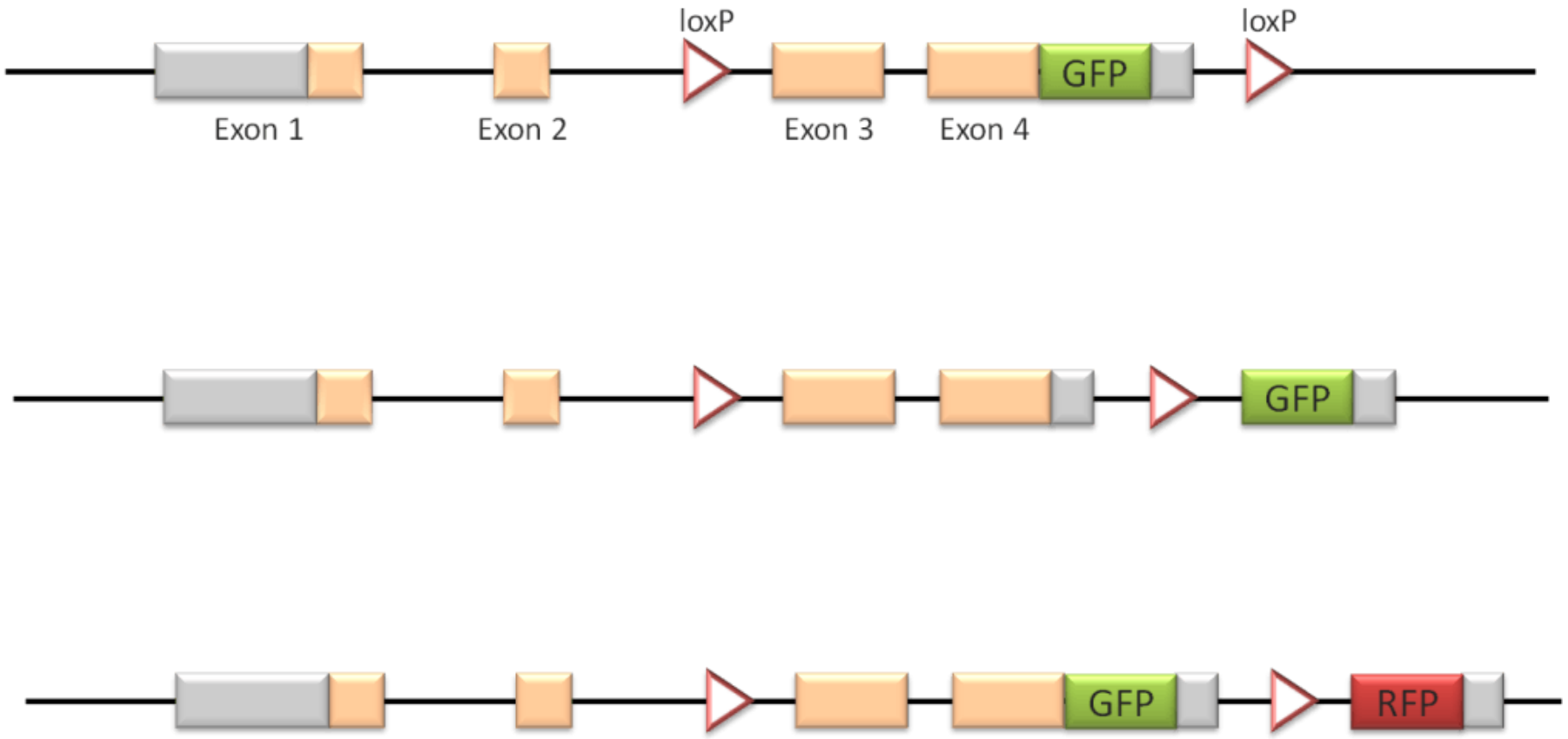

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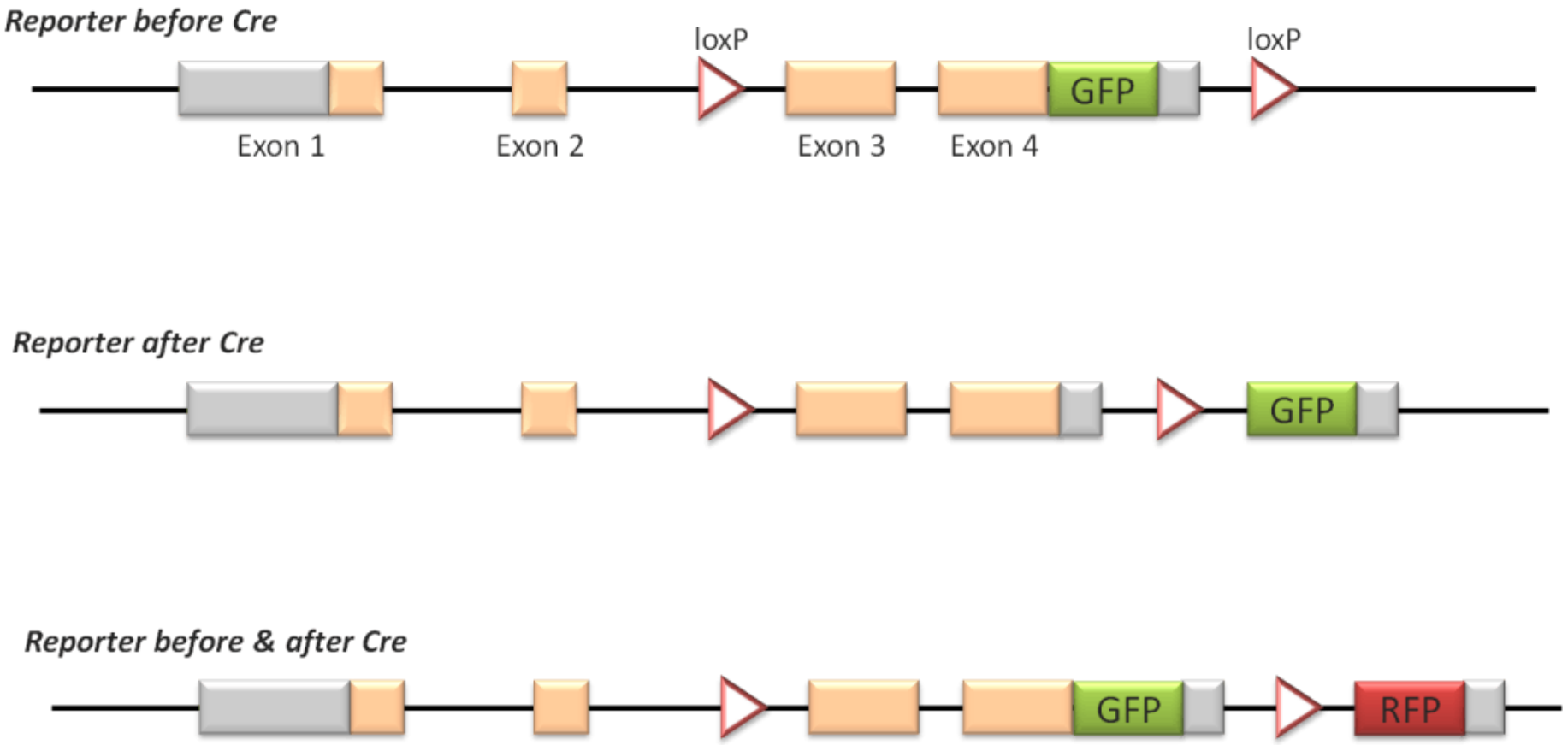
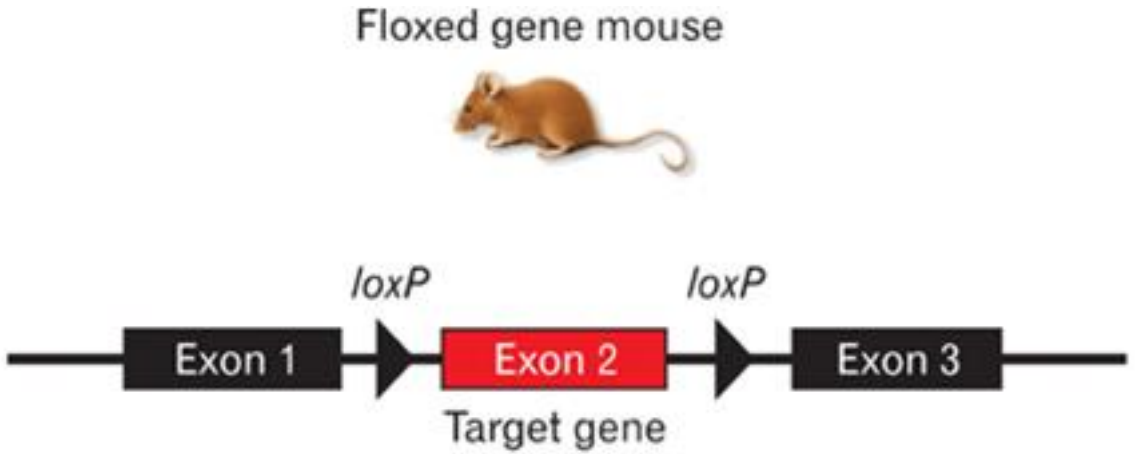
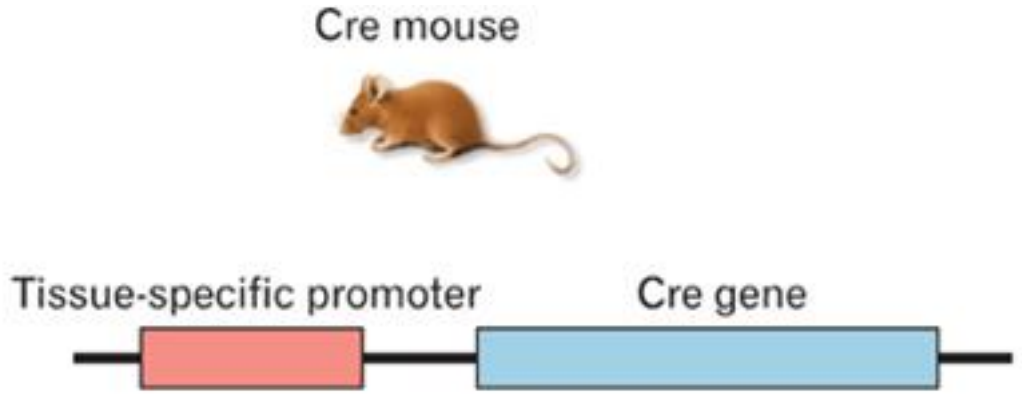


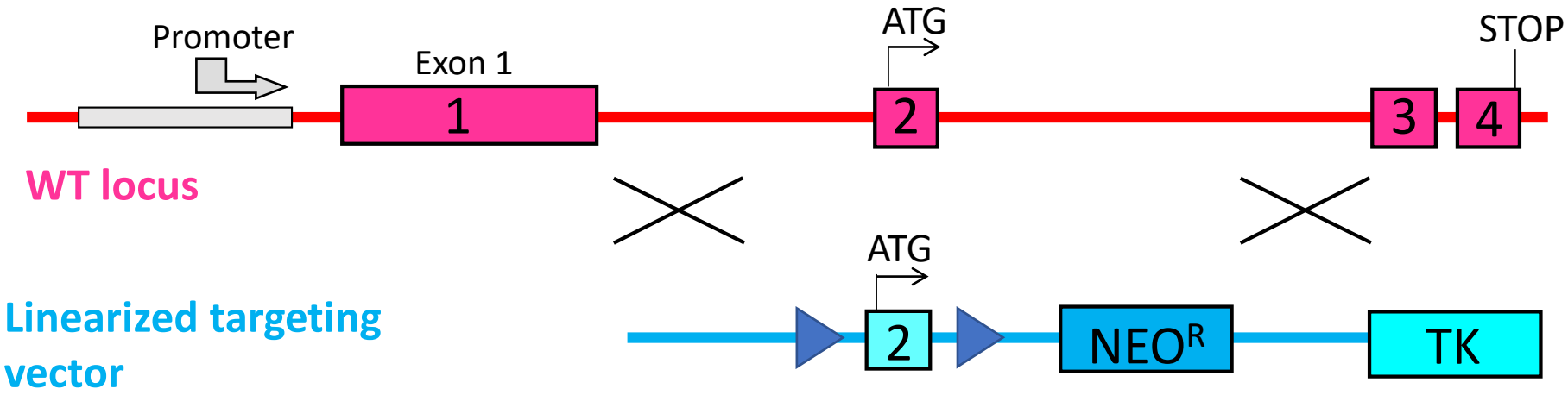


- Which approach should you use to obtain a “floxed“ mouse ?
  Homologous recombination or random insertion?

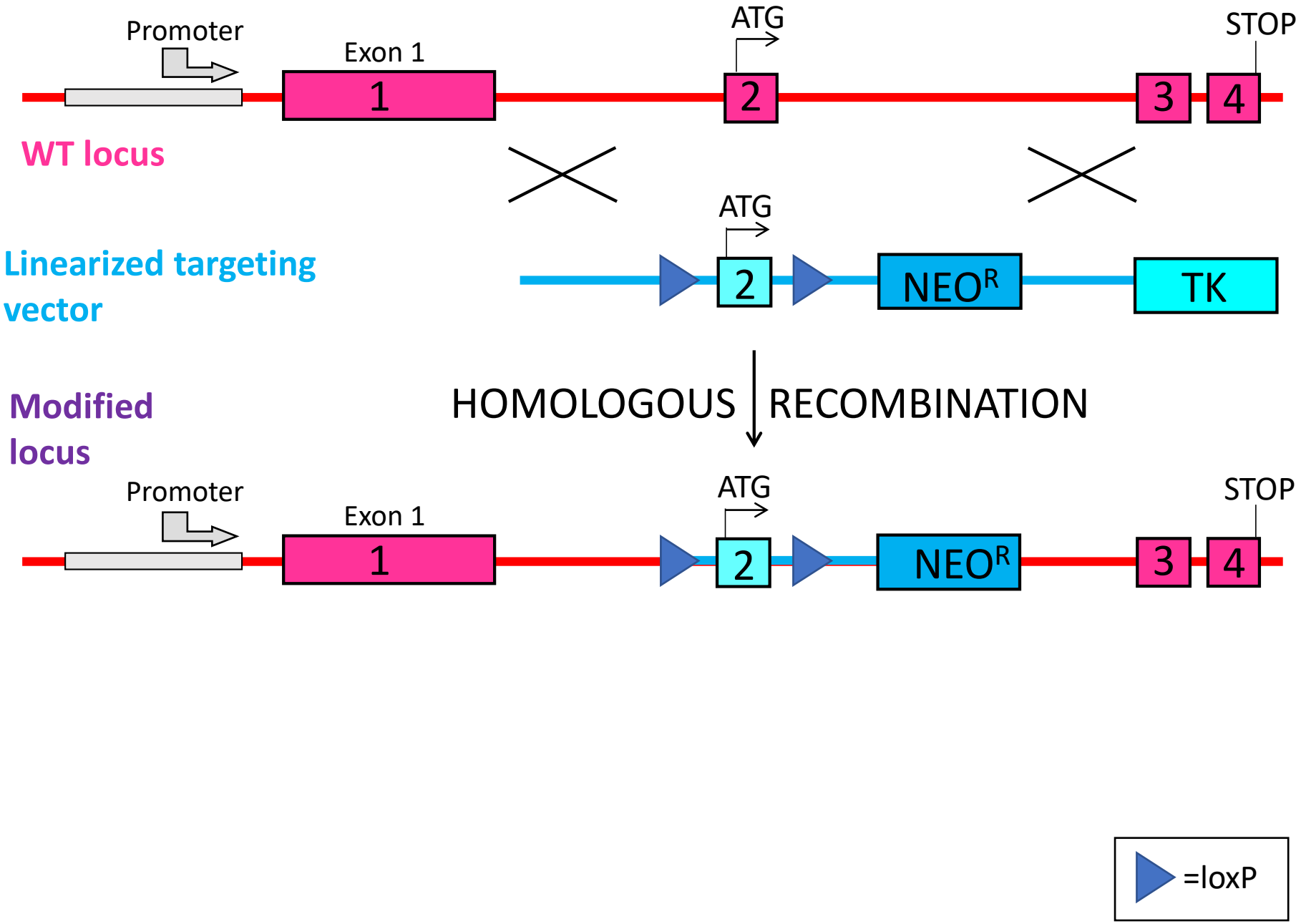


- Which approach should you use to get a mouse with a tissue specific or inducible cre recombinase?
  Homologous recombination or random insertion?

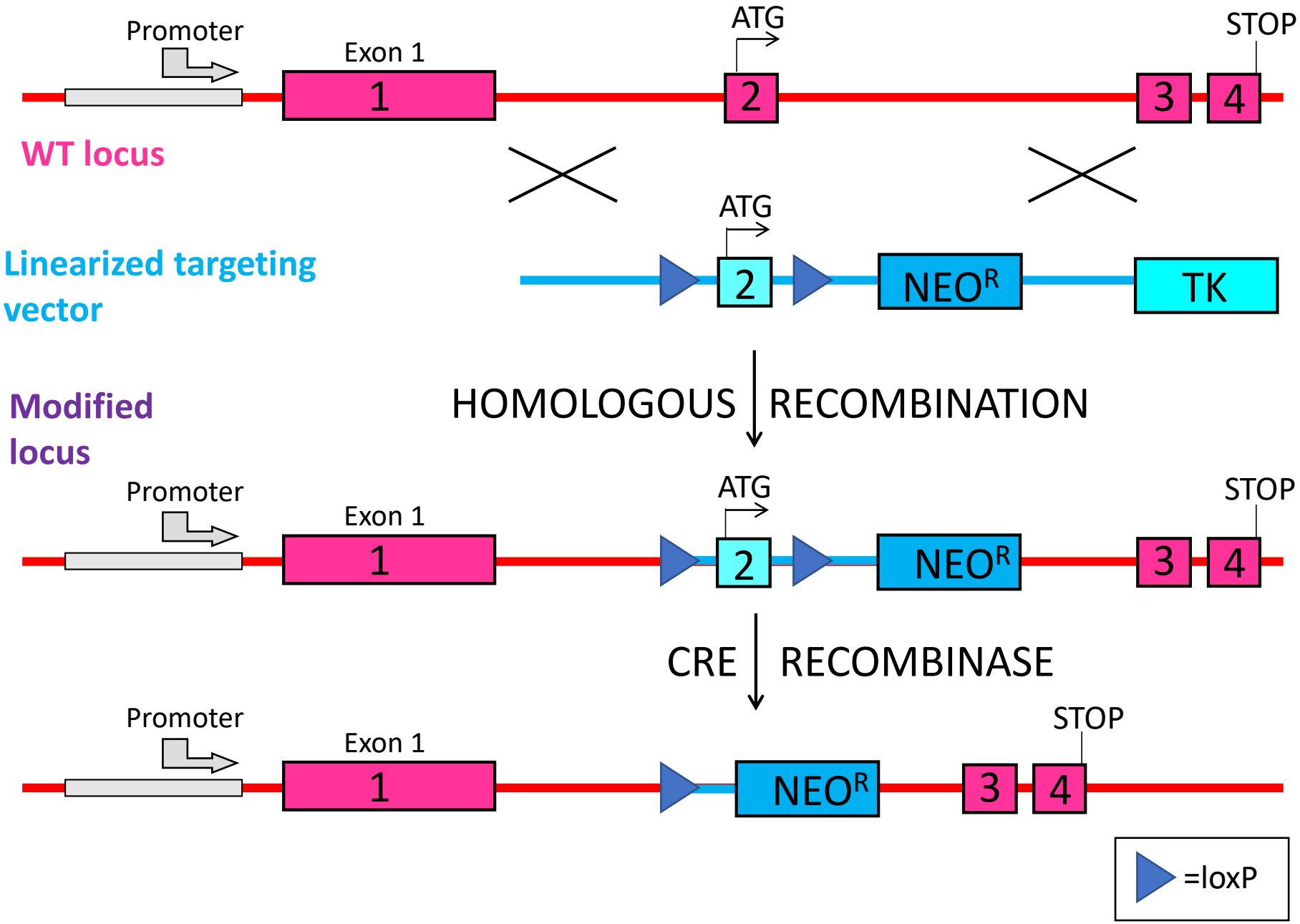
CONSTRUCT TO OBTAIN A FLOXED MOUSE

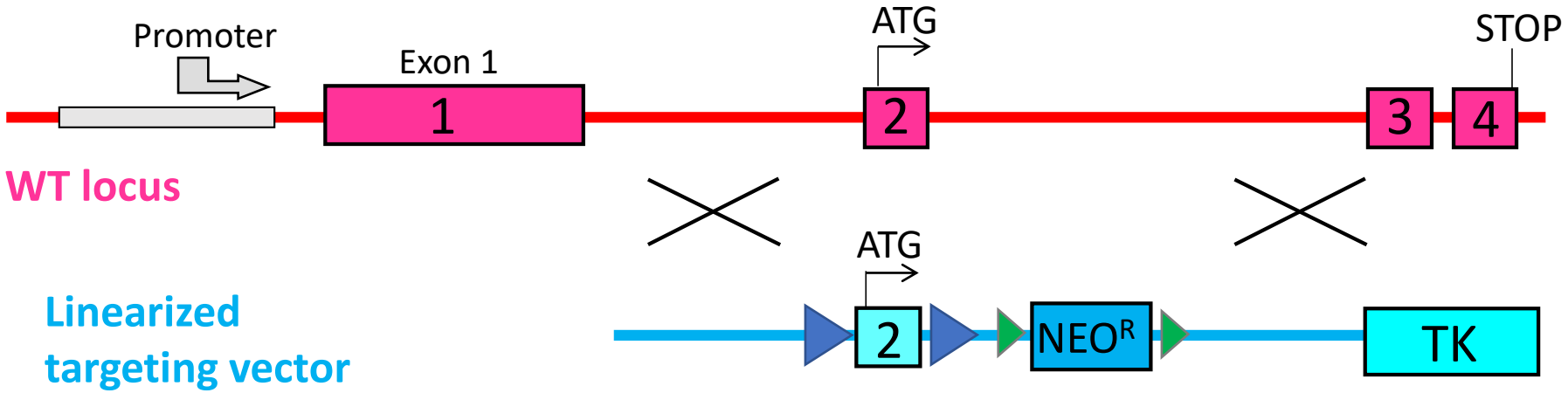


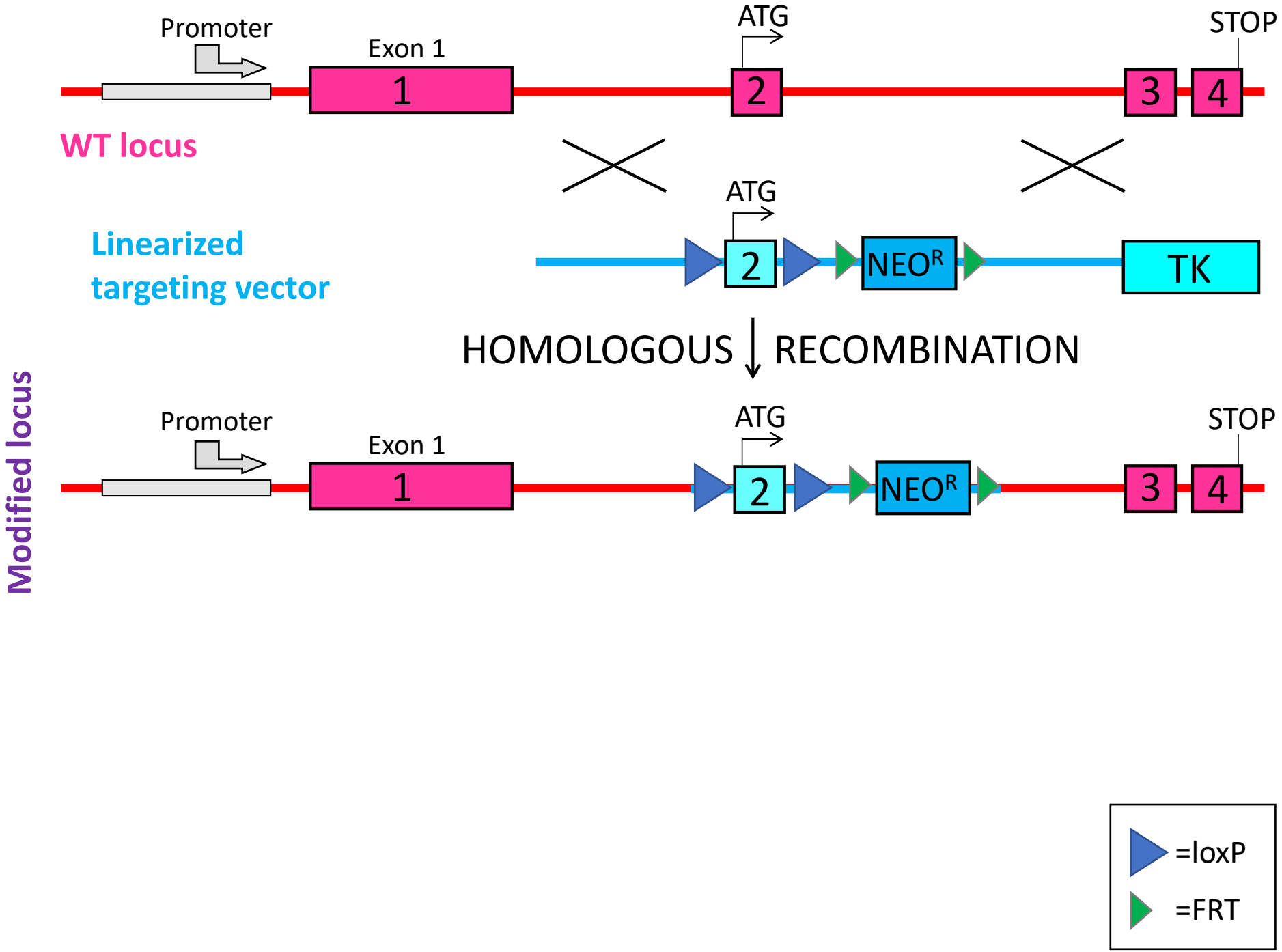
CONSTRUCT TO OBTAIN A FLOXED MOUSE



CONSTRUCT TO OBTAIN A FLOXED MOUSE




Promoter
Exon 1
1
ATG
2
STOP
3
4
WT locus
ATG
Linearized
targeting vector
2
NEOR
TK
=loxP
=FRT


Promoter
Exon 1
ATG
STOP
1
2
3
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WT locus
Linearized
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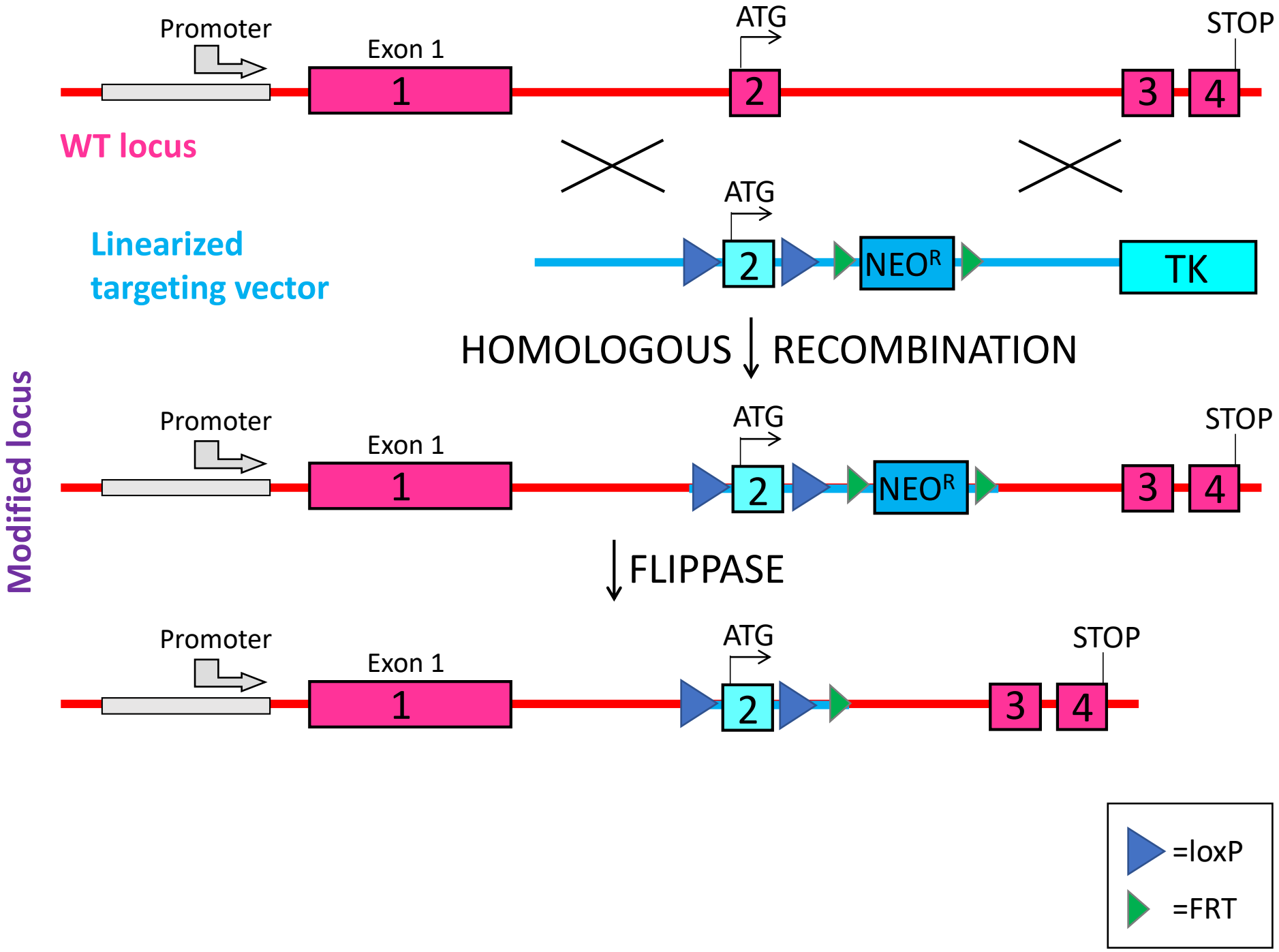
ATG
2
NEOR
TK
HOMOLOGOUS RECOMBINATION
Modified locus
Promoter
Exon 1
ATG
STOP
1
2
NEOR
3
4
=loxP
=FRT


Promoter
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WT locus
Linearized
targeting vector
ATG
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NEOR
TK
HOMOLOGOUS RECOMBINATION
Modified locus
Promoter
Exon 1
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ATG
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NEOR
STOP
3
4
FLIPPASE
Promoter
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ATG
2
STOP
3
4
=loxP
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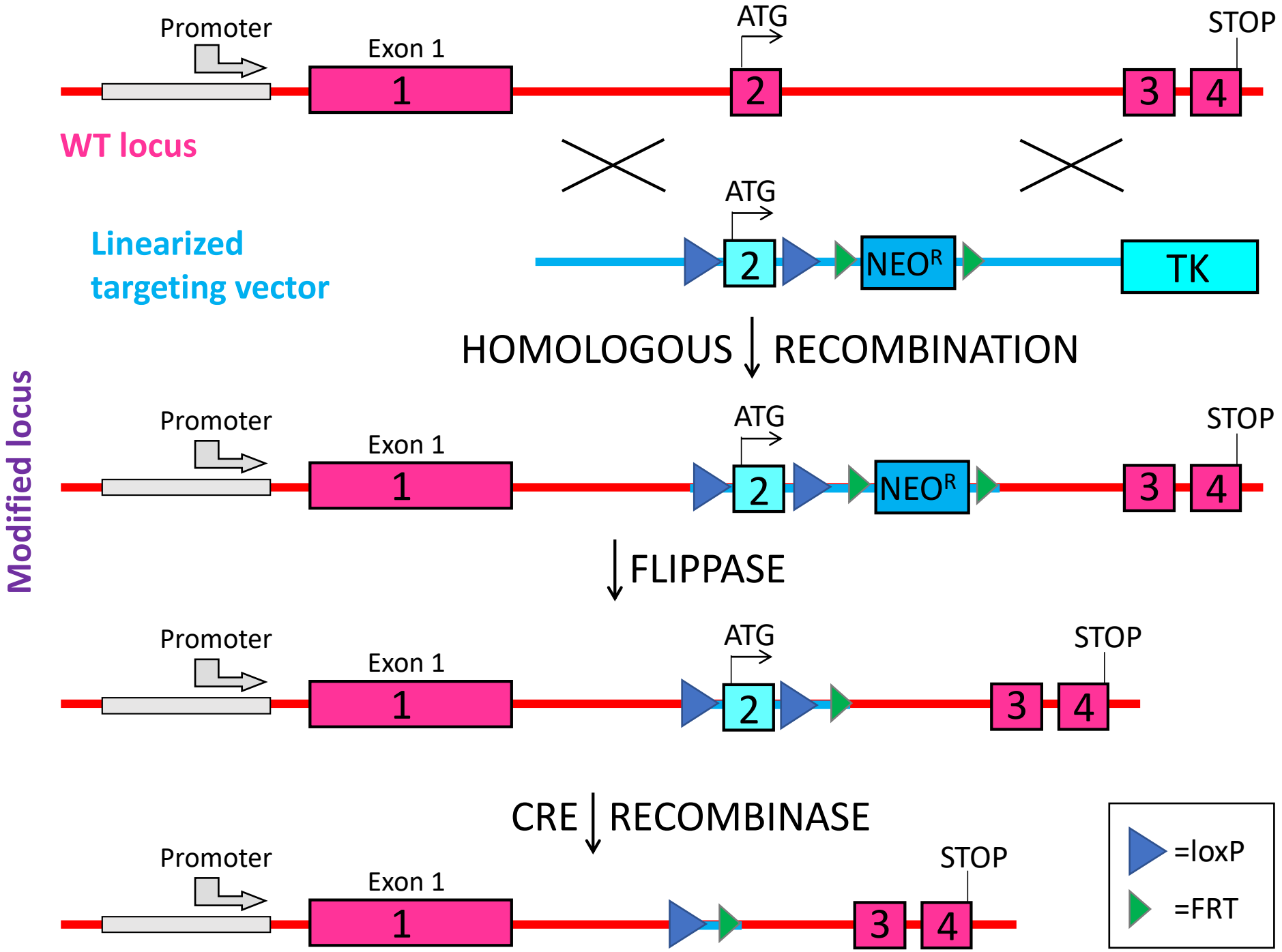
Promoter
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NEOR
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Modified locus
FLIPPASE
CRE RECOMBINASE
=loxP
=FRT