Genetic Manipulation of Cells & Animals II

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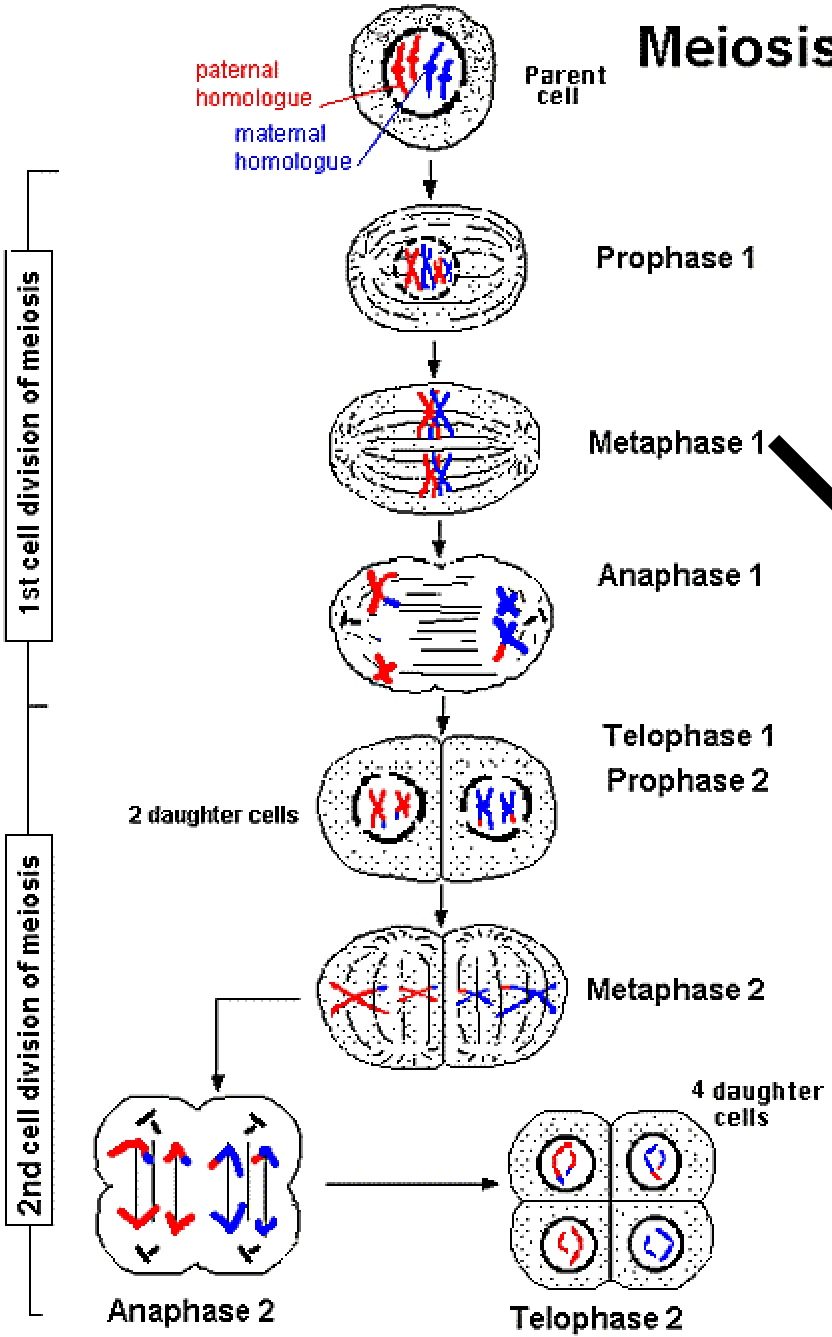
Different methods to modify the function of endogenous gene

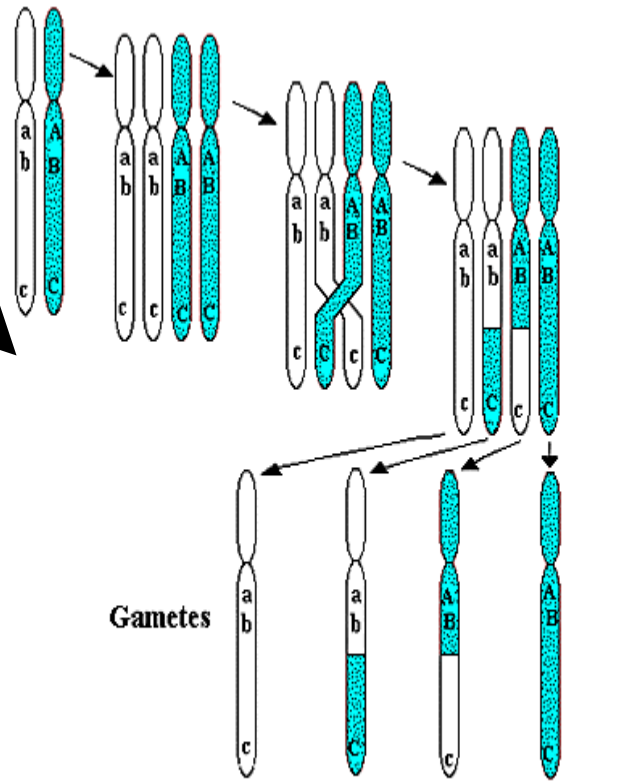
- Gene targeting
  (homologous recombination)
- Inhibition of gene expression
  - miRNA
  - siRNA
  - antibodies
  - dominant negative mutant
  - aptamers
- Insertional mutagenesis
  The transgene integrates randomly into an existing gene & abolishes its function (like gene targeting but random)

Gene Targeting

- It can be used as site directed mutagenesis but in vivo
- Homologous recombination is very rare in mammalian cells occurring 10^4 10^5 less frequently than random integration
- The frequency of homologous recombination depends on
  - Length of the homolog region
  - Degree of similarity with the target
- The strategy used to identify cells in which gene targeting has taken place depends on the construct design**?!!**

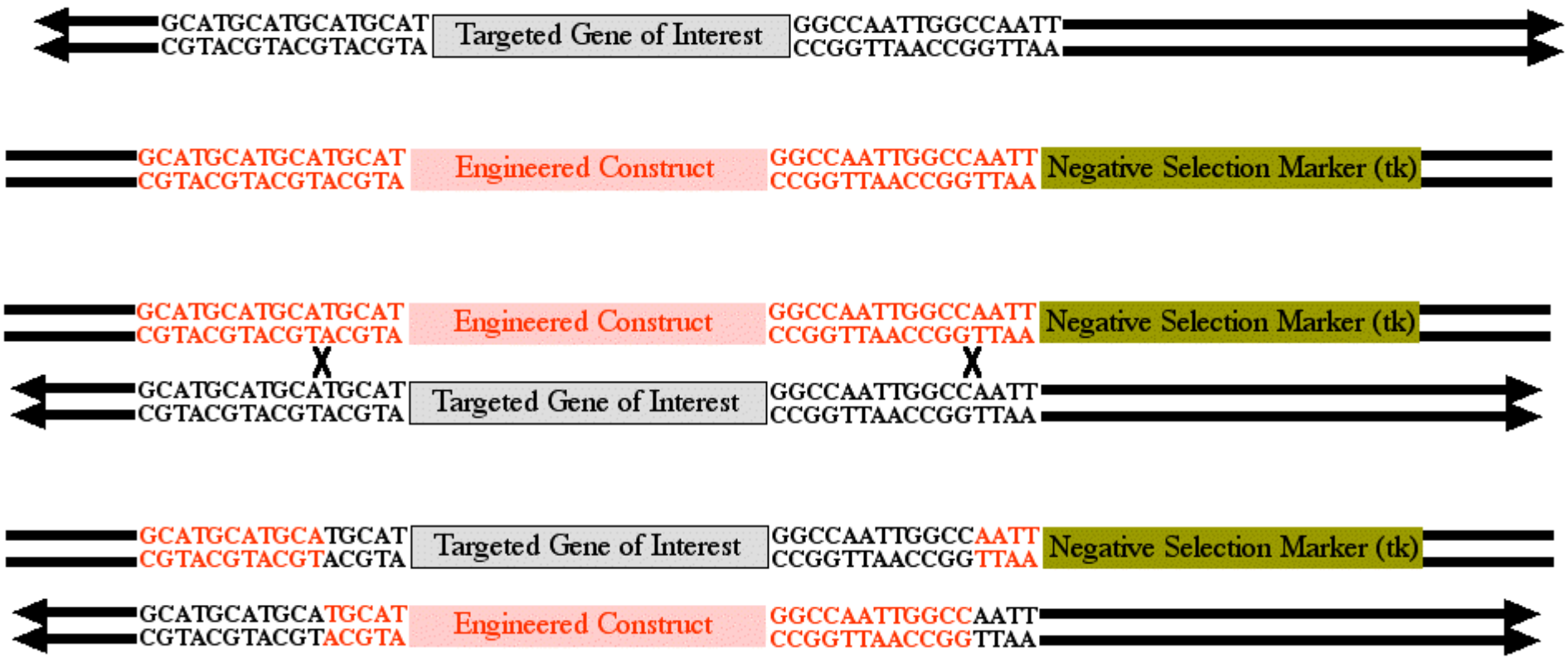
Meiosis





Crossing-over and recombination during meiosis

Homologous Recombination?!!!



Non-homologous Recombination?!

Types of constructs (vectors)

- ## Insertion vector method:

  Target the locus of interest by a single reciprocal recombination event leading to the insertion of the entire vector

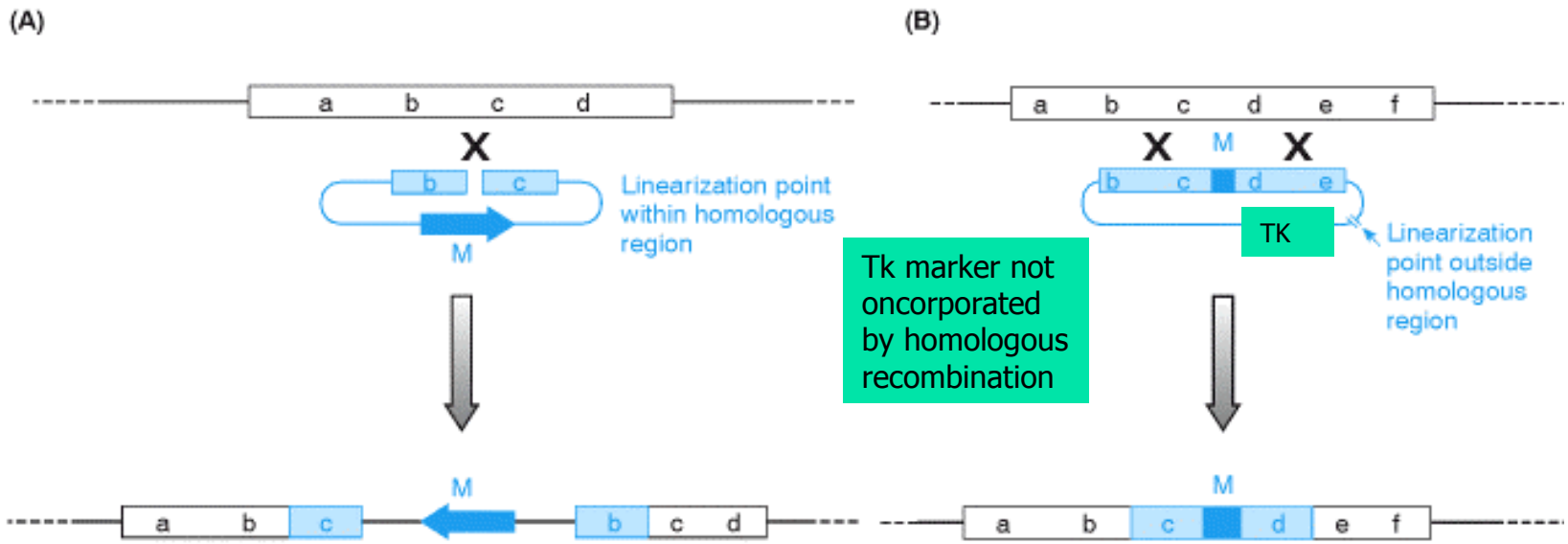
- ## Replacement vector method:

  Are designed to replace some of the sequence in the chromosomal gene with a homologous sequence of the introduced DNA

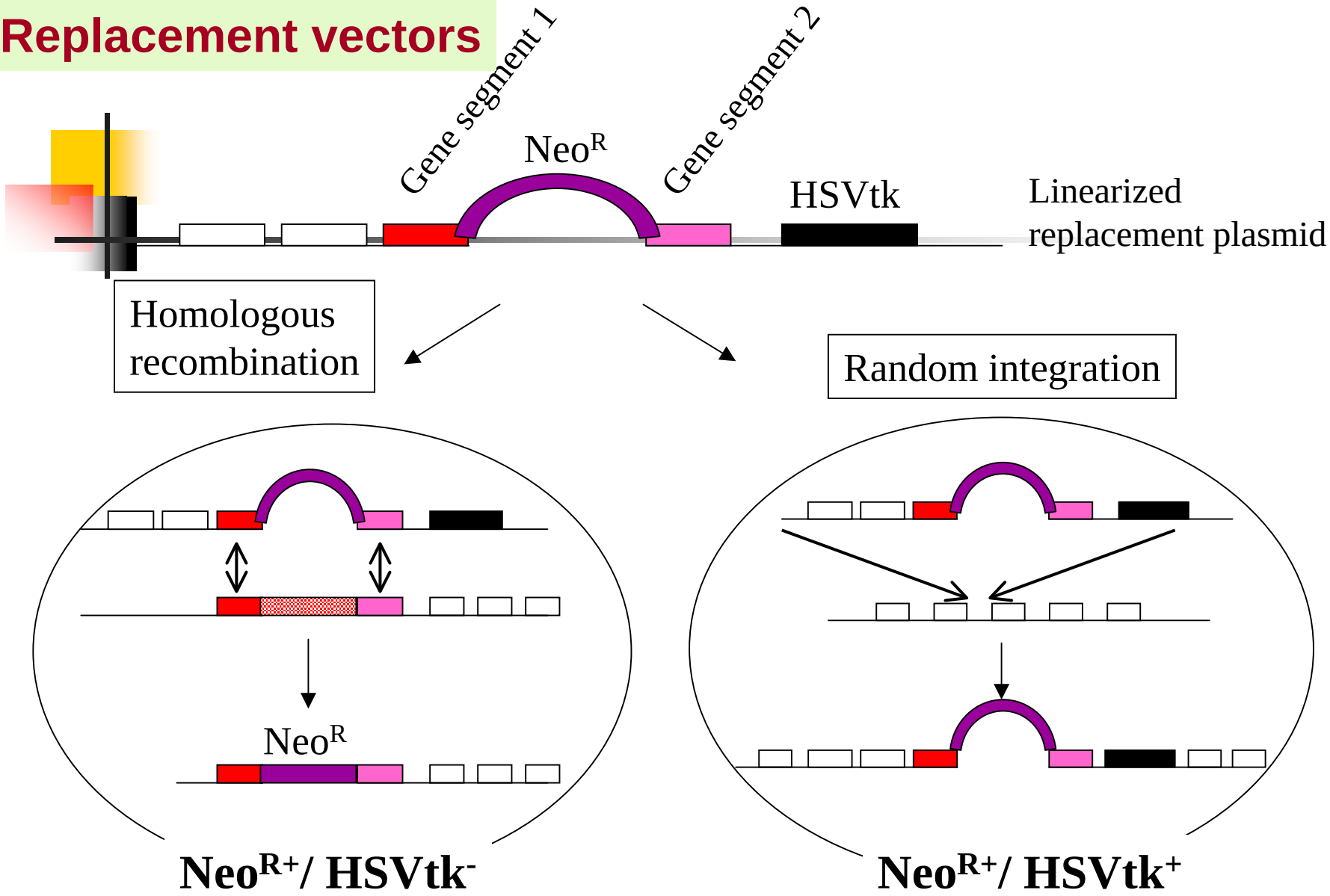
In both strategies, a large segment of the vector DNA containing the neo Marker gene is introduced into the targeted locus causing disruption & the Generation of null allele (gene knock out)

Hit & run strategy

Tag & exchange strategy



Replacement vectors



HSVtk will convert gancyclovir into a toxic drug and kill HSVtk+ cells

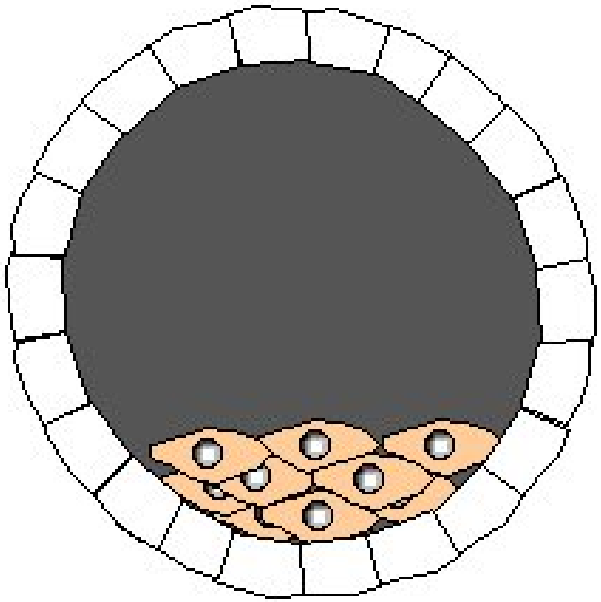
Tips:

- Gene targeting is used in the production of genetically modified animals
- It is used to target ES cells or somatic cells (lower efficiency)
- It is described as targeted gene rather than a transgene

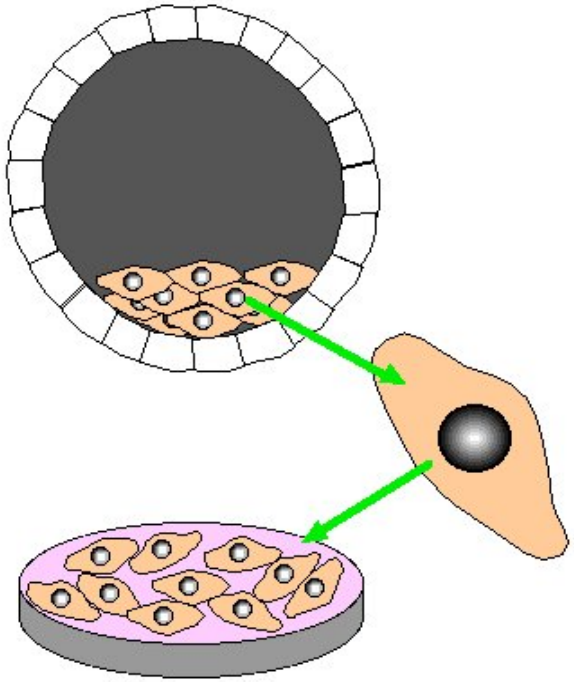
Recently 2 groups have reported the production of pigs with targeted disruption in the gene fo alpha 1,3 galactosyl transferase which encodes a protein with carbohydrate groups (not in humans) and represent one of the major factors responsible for rejection of organs transplanted from pigs to humans

Knockout Mouse

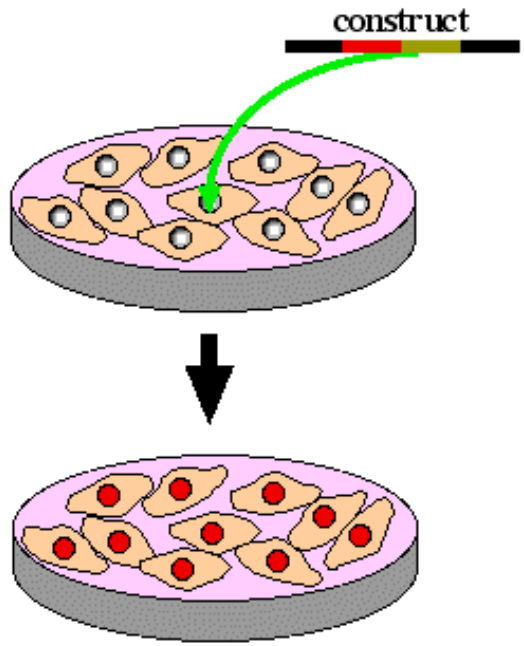
- has both alleles of a particular gene replaced with an inactive allele
- This is usually accomplished by using homologous recombination to replace one allele followed by two or more generations of selective breeding until an breeding pair are isolated that have both alleles of the targeted gene inactivaated or knocked out
- Knock out mice allow investigators determine the role of a particular gene by observing the phenotype of individuals that lack the gene completely



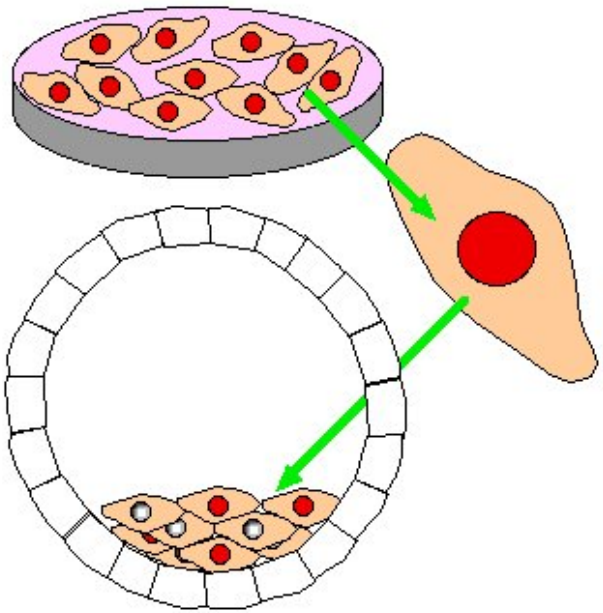
Isolate developing embryo at blastocyst stage. This embryo is from a strain of mice with gray fur



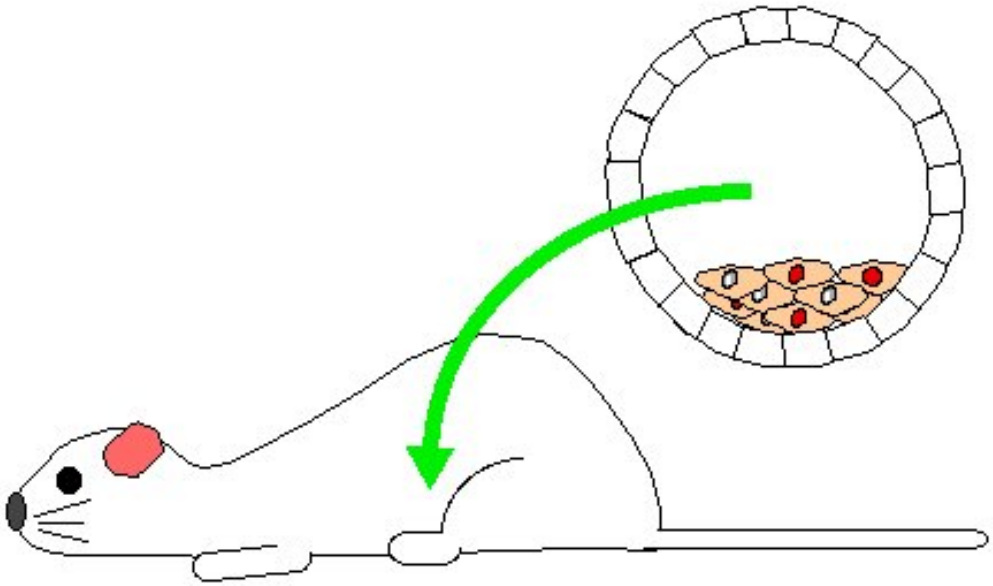
Remove embryonic stem cells from gray-fur blastocyst.Grow stem cells in tissue culture



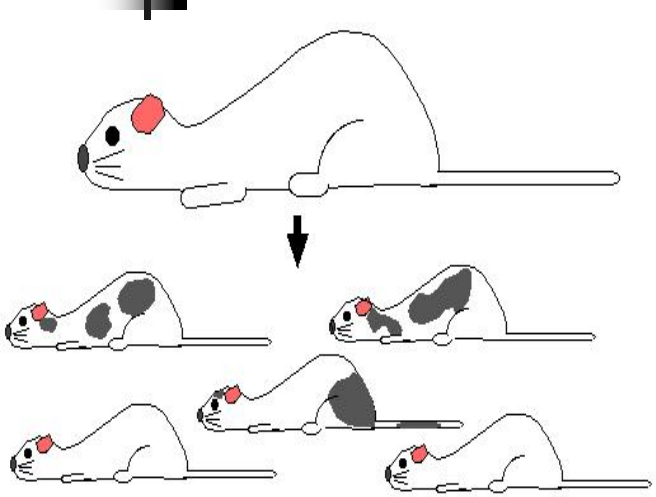
Transfect stem cells with homologous recombination construct. Select for homologous recombination by growing stem cells in neomycin and gancyclvir.



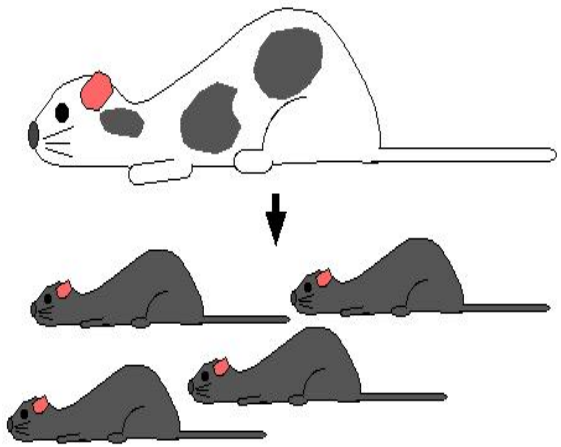
Remove homologously recombined stem cells from petri dish and inject into a new blastocyst that would have only white fur



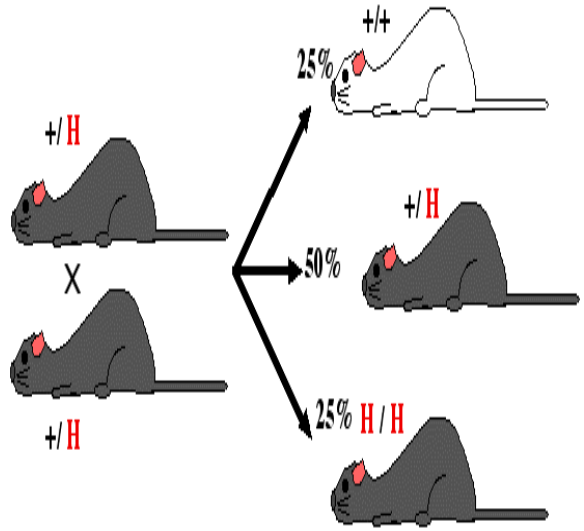
Implant several chimeric blastocysts into pseudo-pregnant, white fur mouse.



Mother will give birth to a range of mice. Some will be normal white fur mice but others will be chimeric mice. Chimeric mice have many of their cells from the original white fur blastocyst but some of their cells will be derived from recombinant stem cells. Fur cells from recombinant stem cells produce gray patches which are easily detected.



Mate the chimeric mice with wild-type white fur mice. If the gonads of the chimeric mice were derived from recombinant stem cells, all the offspring will have gray fur. Every cell in gray mice are heterozygous for the homologous recombination.



Mate heterozygous gray mice (+/ H) and genotpye the gray offspring. Identify homozygous recombinants (H / H) and breed them to produce a strain of mice with both alleles knocked out. The pure breeding mouse strain is a **"knockout mouse".**

Site specific recombination

- These systems are found in several bacteriophage as well as bacteria & yeast

- Each system contains 2 components:

1. Short specific recognition sequence at which recombination occurs
2. Recombinase enzyme that recognizes this sequence & carries out the recombination recombination reaction when two copies of the sequence are present

- It allows conditional gene inactivation
- It allows chromosome engeneering

The Cre-loxP recombination system

- The Cre-loxP recombination system for bacteriophage P1 has been most widely used
- The cre recombinase (causes recombination) is to mediate recombination between two loxP sequences
- LoxP sequences are 34bp long and comprise 2 inverted 13-bp repeats separated by a central assymetric 8-bp spacer
- If the 2 loxP sites are in the same orientation= intervening sequence between them is excised
- If the loxP sites are in opposite orientation= the intervening sequence is inverted

lox P recognition sequece

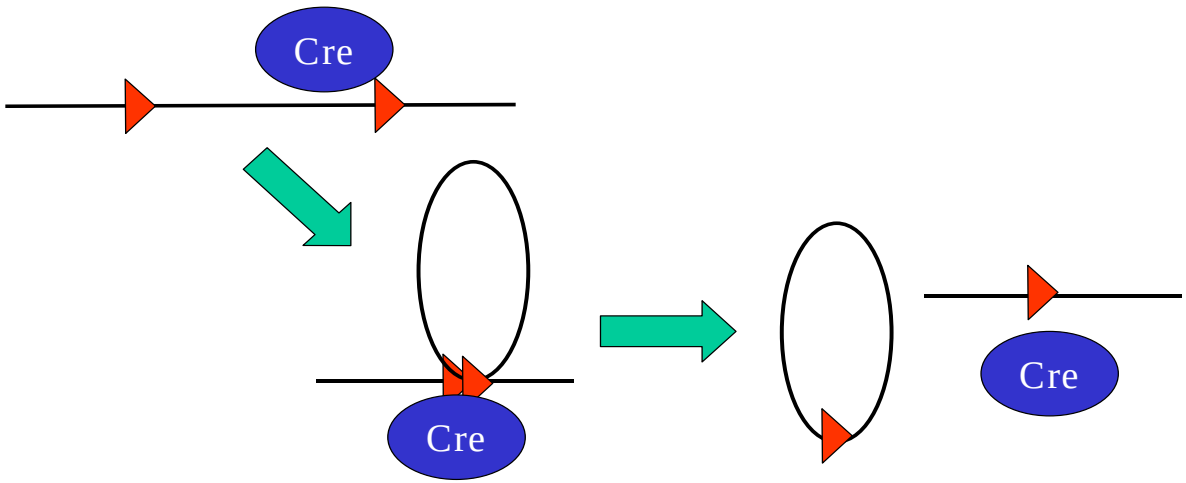
Cre-lox technology

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

Cre-lox technology

Cre – a site-specific recombinase enzyme from the P1 phage.

Recognises a 34bp DNA sequence *loxP* =



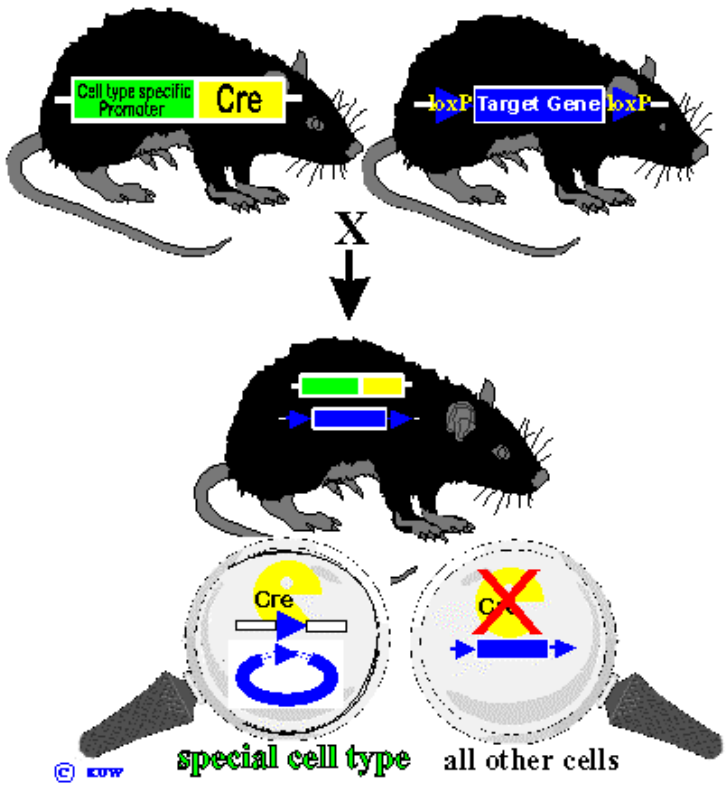
If CRE recombinase expressed

Gene between loxP sites is removed

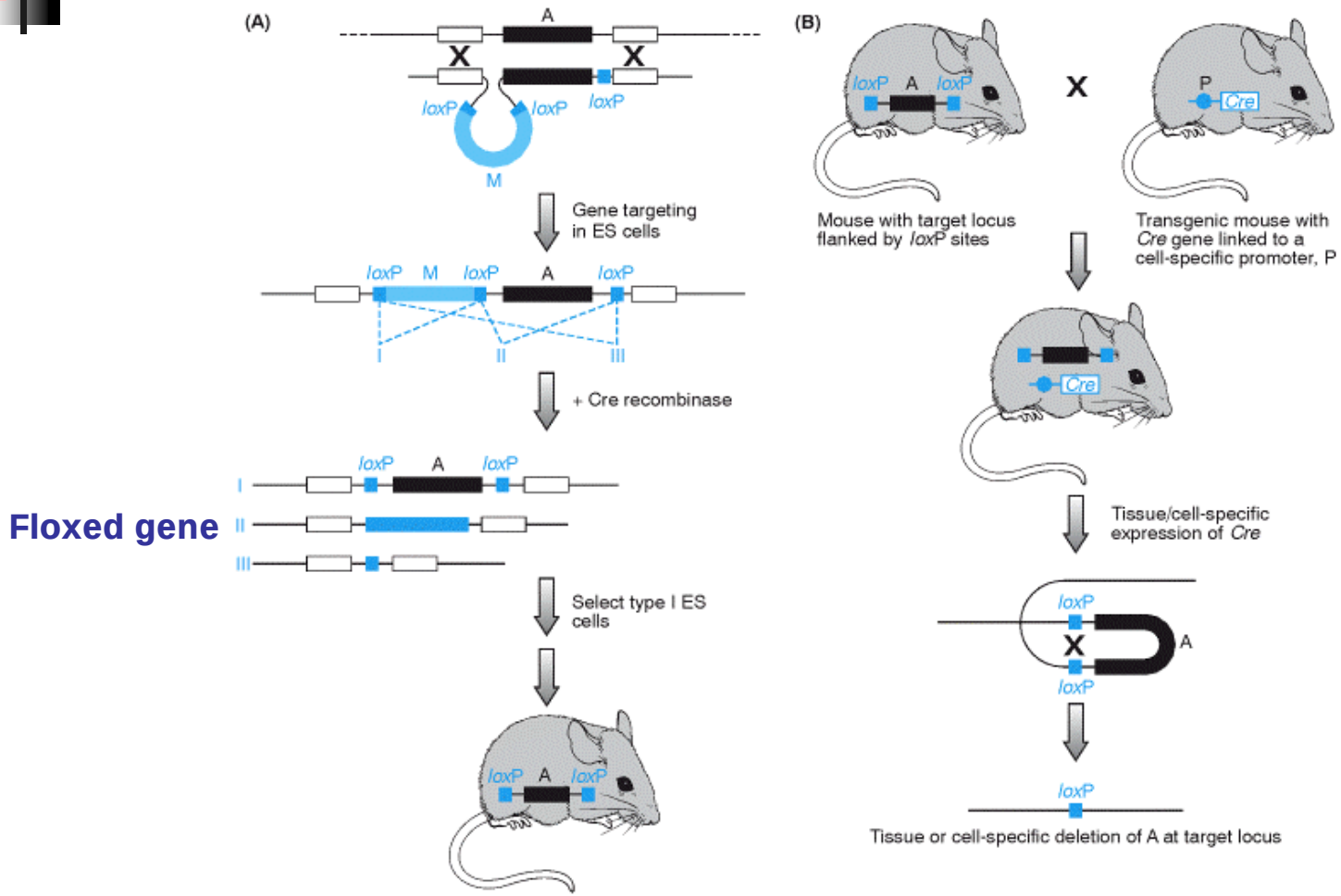
Conditional gene activation

inactivate a gene only in specific tissues
and at certain times during development and life

- Some genes are critical in the early development, so simple knockout are not preferred as they are lethal in the embryonic state
- To overcome this problem: conditional knockout is used to inactivate the target gene in only the selected cells (animal will survive)
- **An example**
- Conditional knockout of DNA polymerase β (enzyme essential for embryonic development)
- Gene targeting (homologous recombination) to replace an essential gene segment **flanked by loxP sequences**
- Mice carrying this targeted mutation were mated with a starin of mice carrying a **Cre transgene** under the control of **T lymphocyte specific promotor**
- Offspring of the cross breeding were identified & survived till adulthood
- The cre product was only expressed in the T cells deleting the essential exon & inactivating the gene


Cell type specific Promoter
Cre
loxP
Target Gene
loxP
X
Cre
special cell type
all other cells

How to FLOX a gene



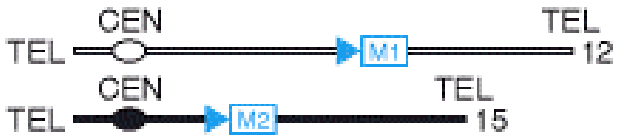
Chromosome engineering

Gene Targeting + Cre-loxP recombination

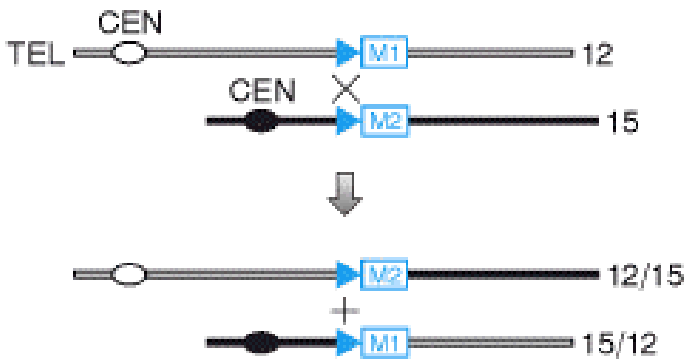
- Gene targeting is used to integrate loxP sites at the desired chromosomal location & subsequently transient expression of Cre recombinase is used to mediate a selected chromosome rearrangement

- That will lead to the creation of mouse models with specific chromosomal abnomalities

(A) 1. Use sequential gene targeting to introduce *loxP* site (▶) plus a marker gene (M) into two desired locations on different chromosomes

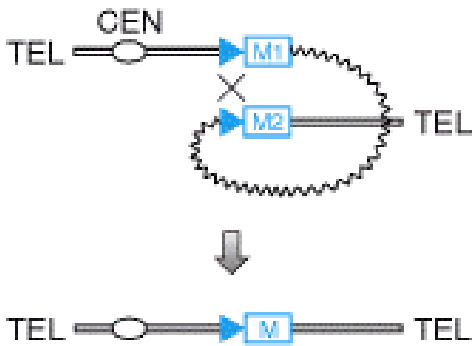


2. Expose *loxP*-containing chromosomes to Cre recombinase



(B) 1. Use sequential gene targeting to introduce *loxP* site plus a marker gene into two desired locations flanking chromosomal region to be deleted (〰)

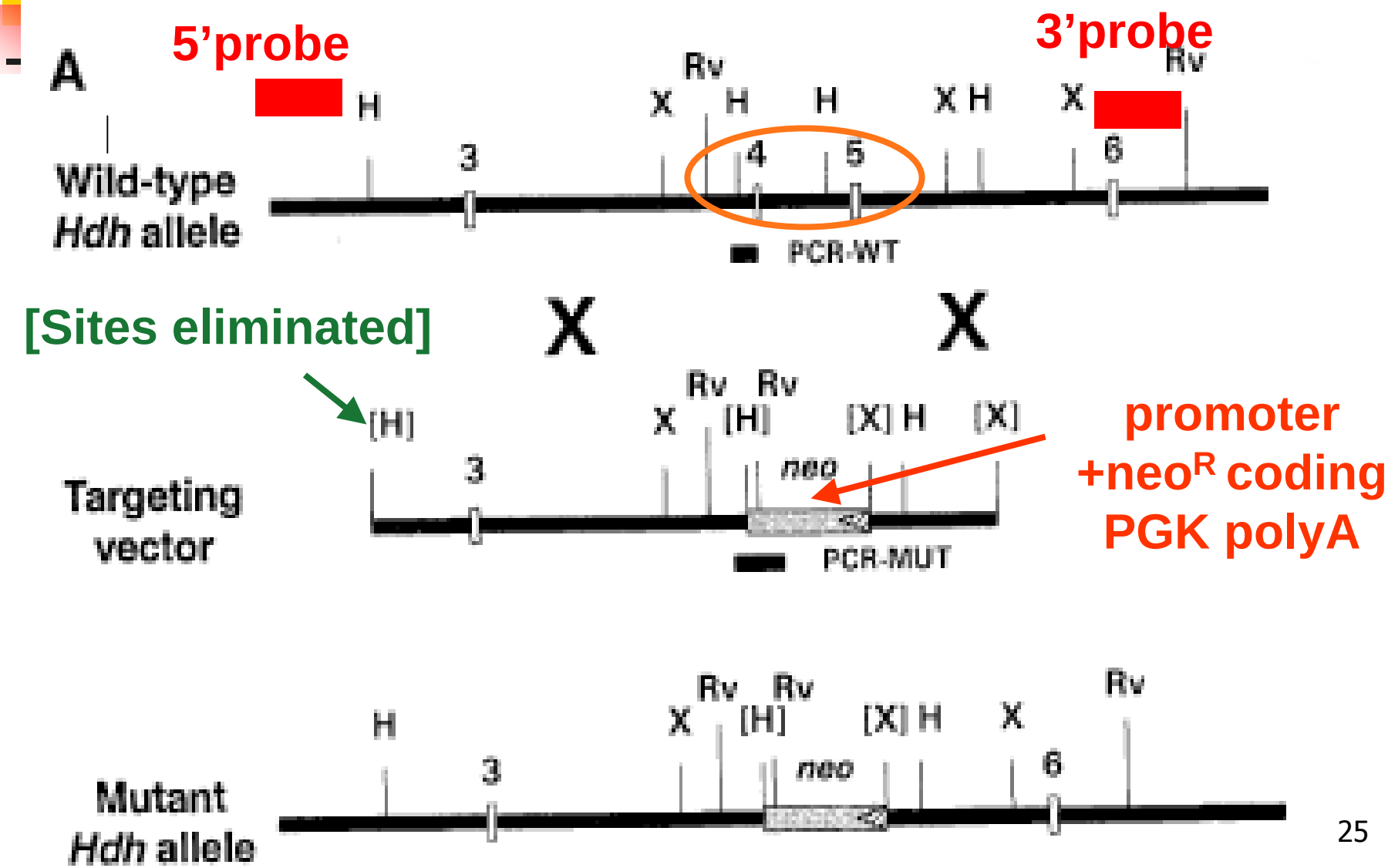
2. Allow to undergo intrachromosomal recombination in presence of Cre recombinase



Gene targeting example: Inactivation of *Hdh*

- *Hdh* is the mouse homolog of the human Huntington's Disease gene
- HD is an autosomal dominant disease the results from a CAG expansion in the *HD* gene
- Question: Does this expansion disrupt the normal function of HD or is it a gain of function mutation?
- What is the normal function of HD?

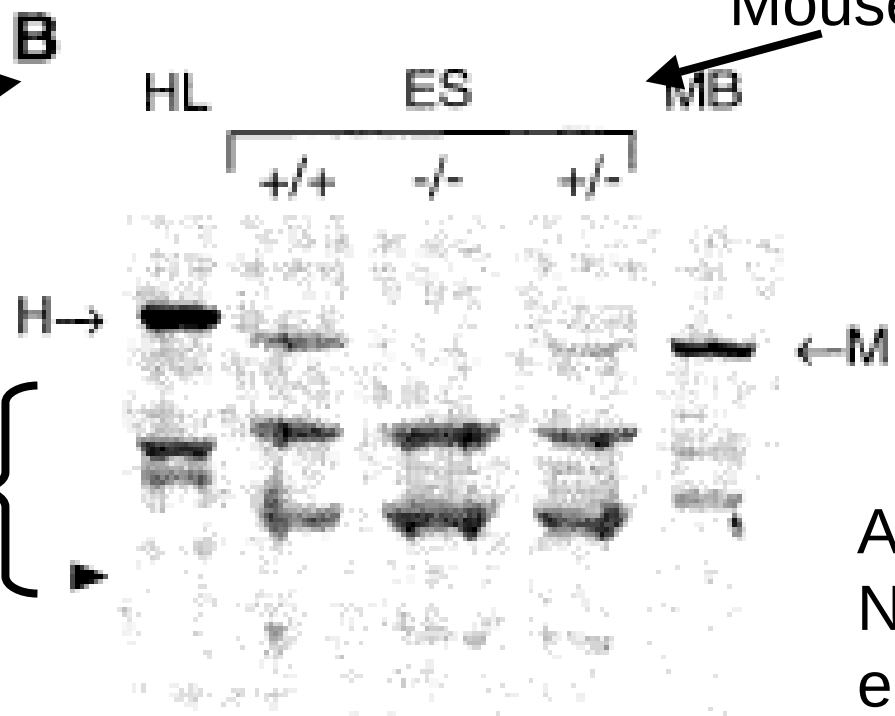
Design a targeting vector for *Hdh*



Proof protein is gone

Human Lymphoblast cells

Mouse brain



nonspecific

Antibody N-terminal epitope

-Western blot to detect huntingtin protein

Good Luck