Best laid plans of men and mice*: Knock-out (KO) and Knock-in mice in research

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***The best-laid plans of mice and men often go awry**

No matter how carefully a project is planned, something may still go wrong with it. The saying is adapted from a line in “To a Mouse,” by Robert Burns.

Geneticly modified mice

- Knock-out or sometimes targeted mutations
- Conditional knock-out
- Knock-in
- CRISPR/Cas9 genome editing technology in mice

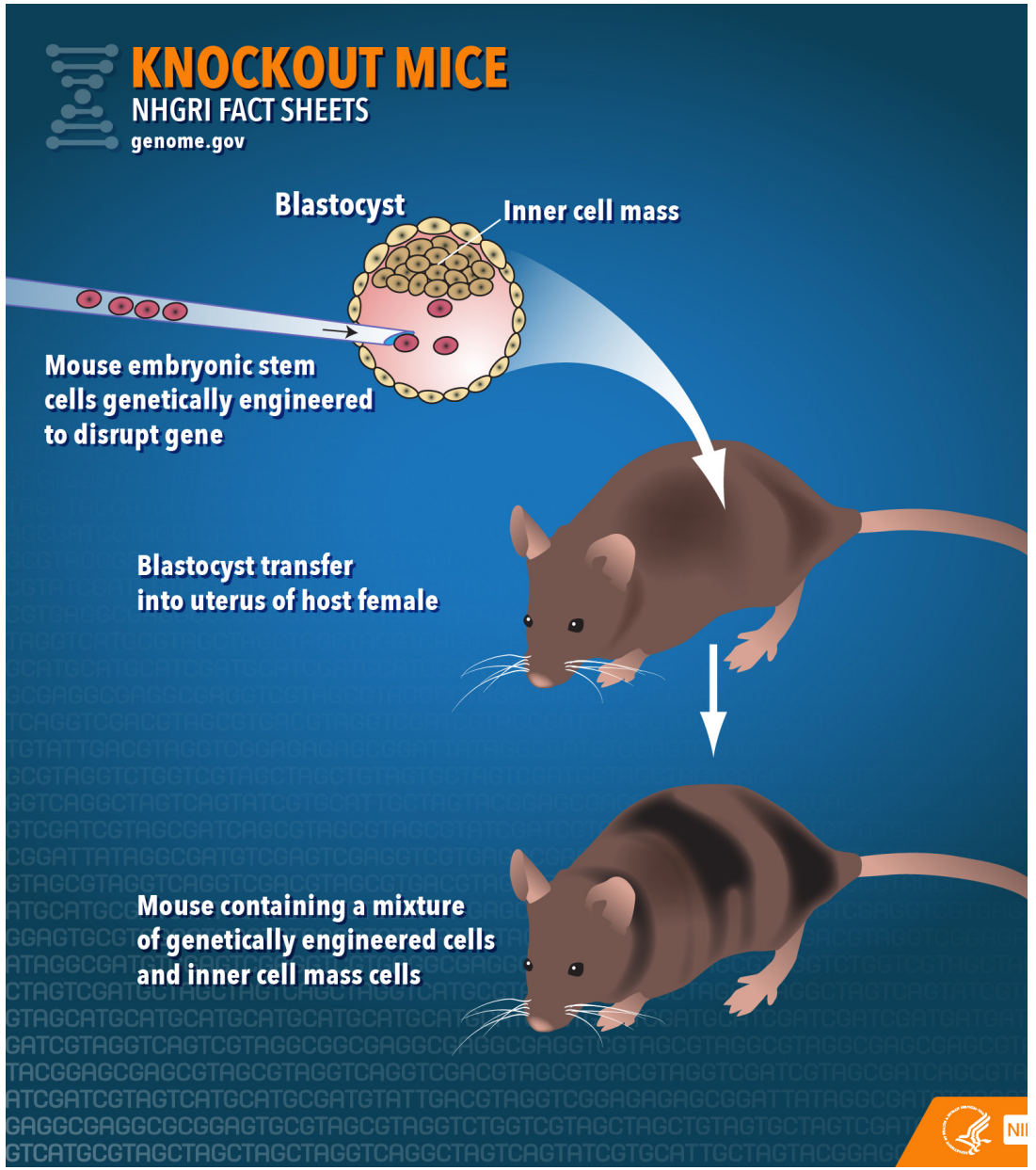
Knockouts

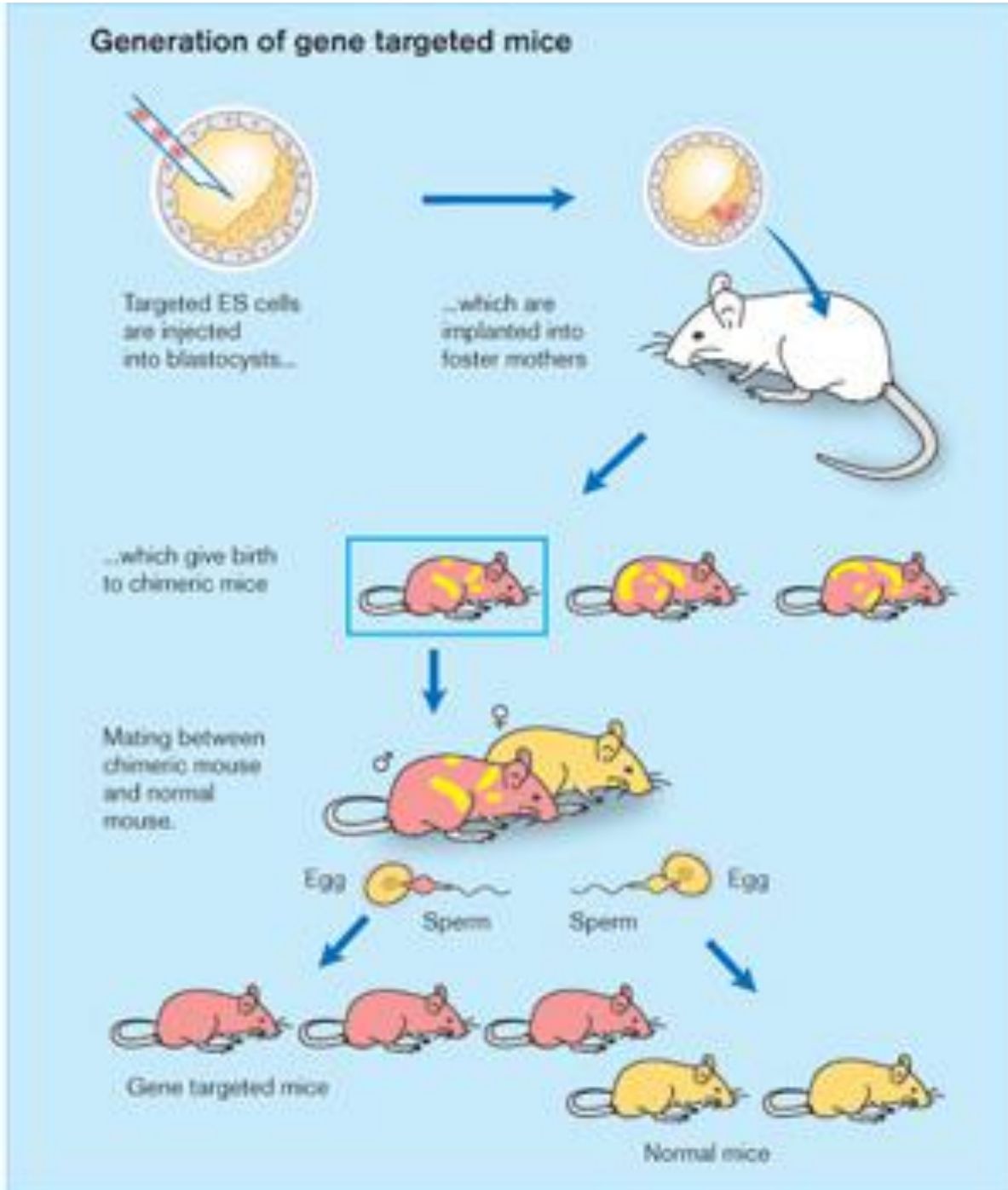
- Knockouts give us clues about what a gene normally does.
- Knockouts will help us understand how a gene contributes to a disease
  - modeling different kinds of cancer, obesity, heart disease, diabetes, arthritis, substance abuse, anxiety, aging and Parkinson disease.
- What we create are loss of function mutations.
- P53 knockout mice; "Methuselah" is a knockout mouse model noted for longevity; while "Frantic" is a model useful for studying anxiety disorders.
- 15% of KOs are embryonic lethal.
  - Cre/loxP system to do conditional knockouts

Two main methods for creating KO mice

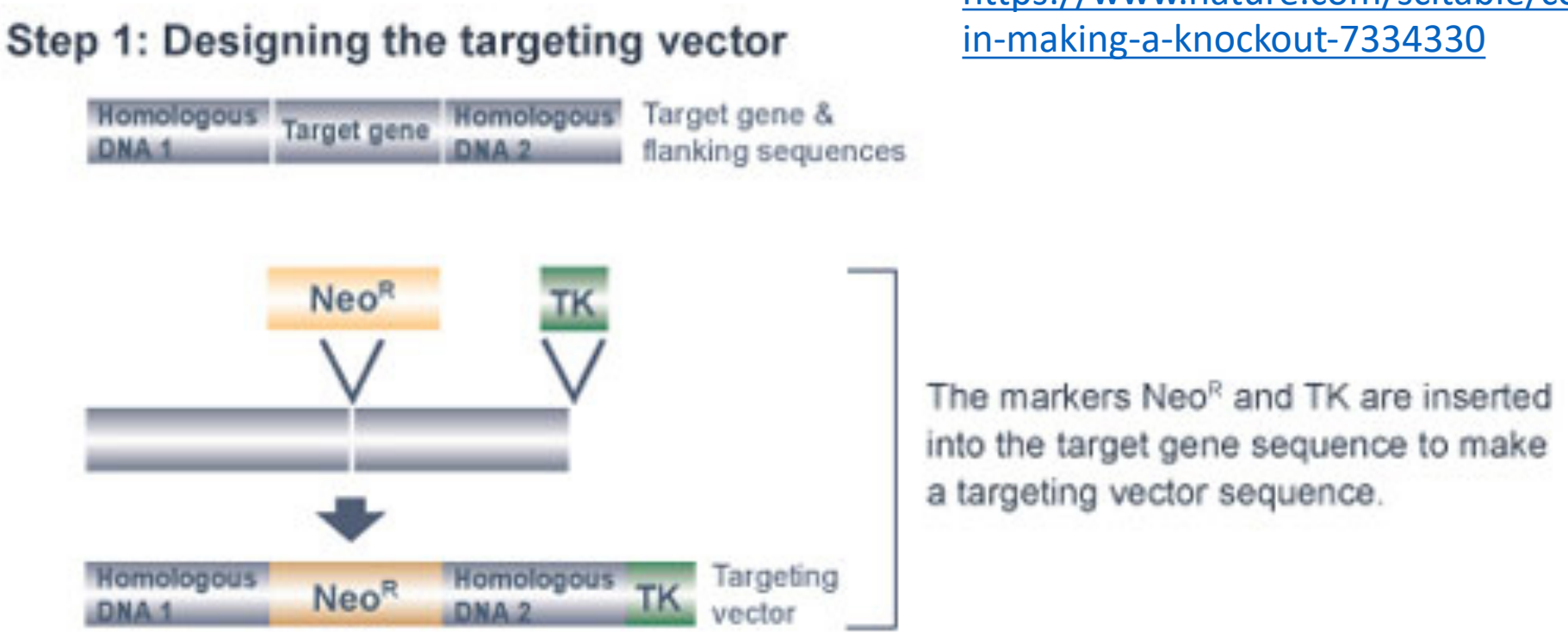
- Homologous recombination
  - The sequence must be know (not problem these days)
  - Uses cell's own DNA repair machinery. At least 2kb homology is required (6-14 kb typical)
  - Only 10^{-2} to 10^{-3} integrations are homologous so check with Southern blot and PCR (recombination peaks are mid-S phase) not dependent on input conc.
- Gene trapping
  - Not very efficient because it is random
  - More time consuming
  - May not insert into a gene
  - May not cause a loss of function

Basic Overview of knock-out mice



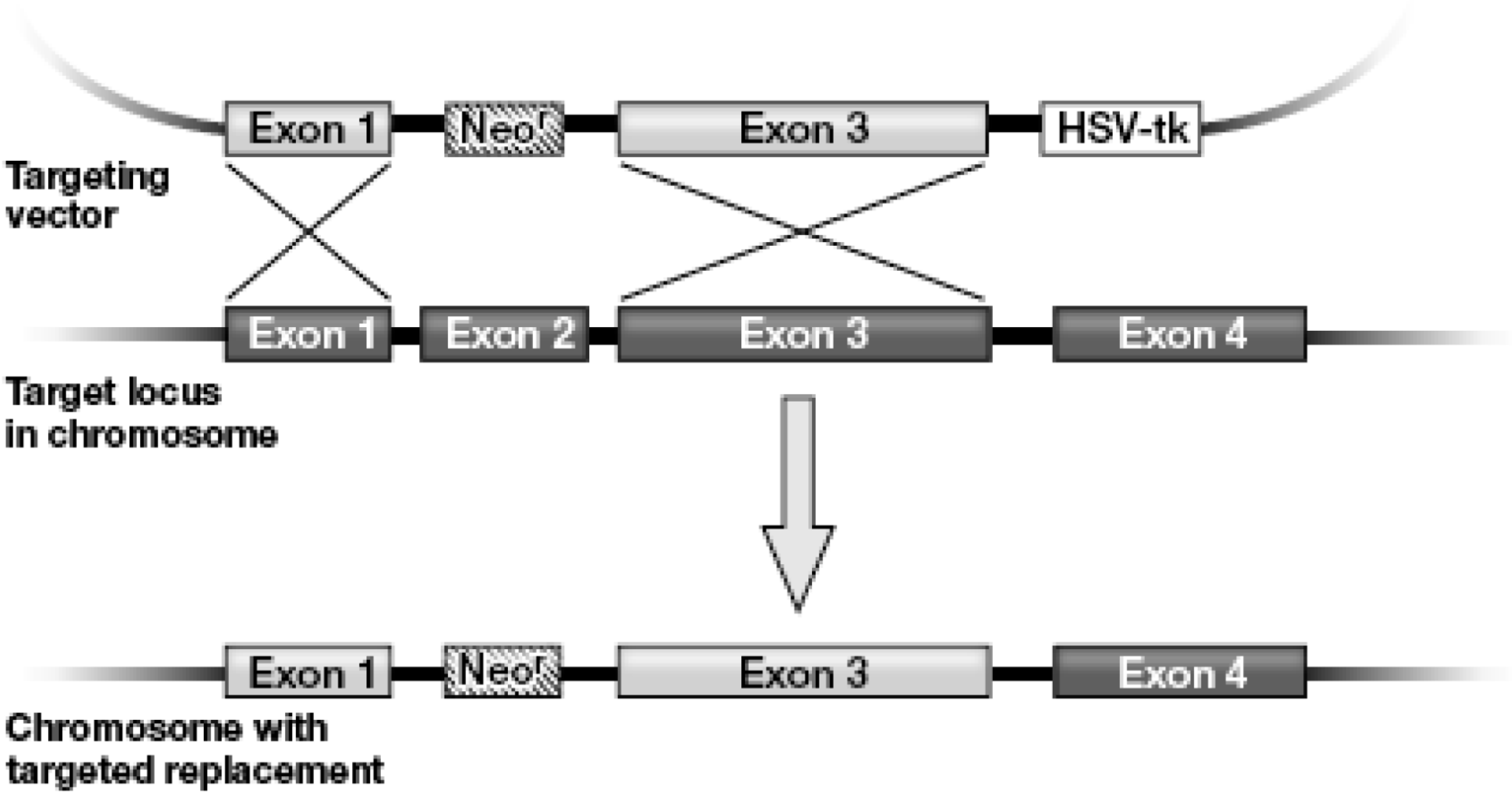


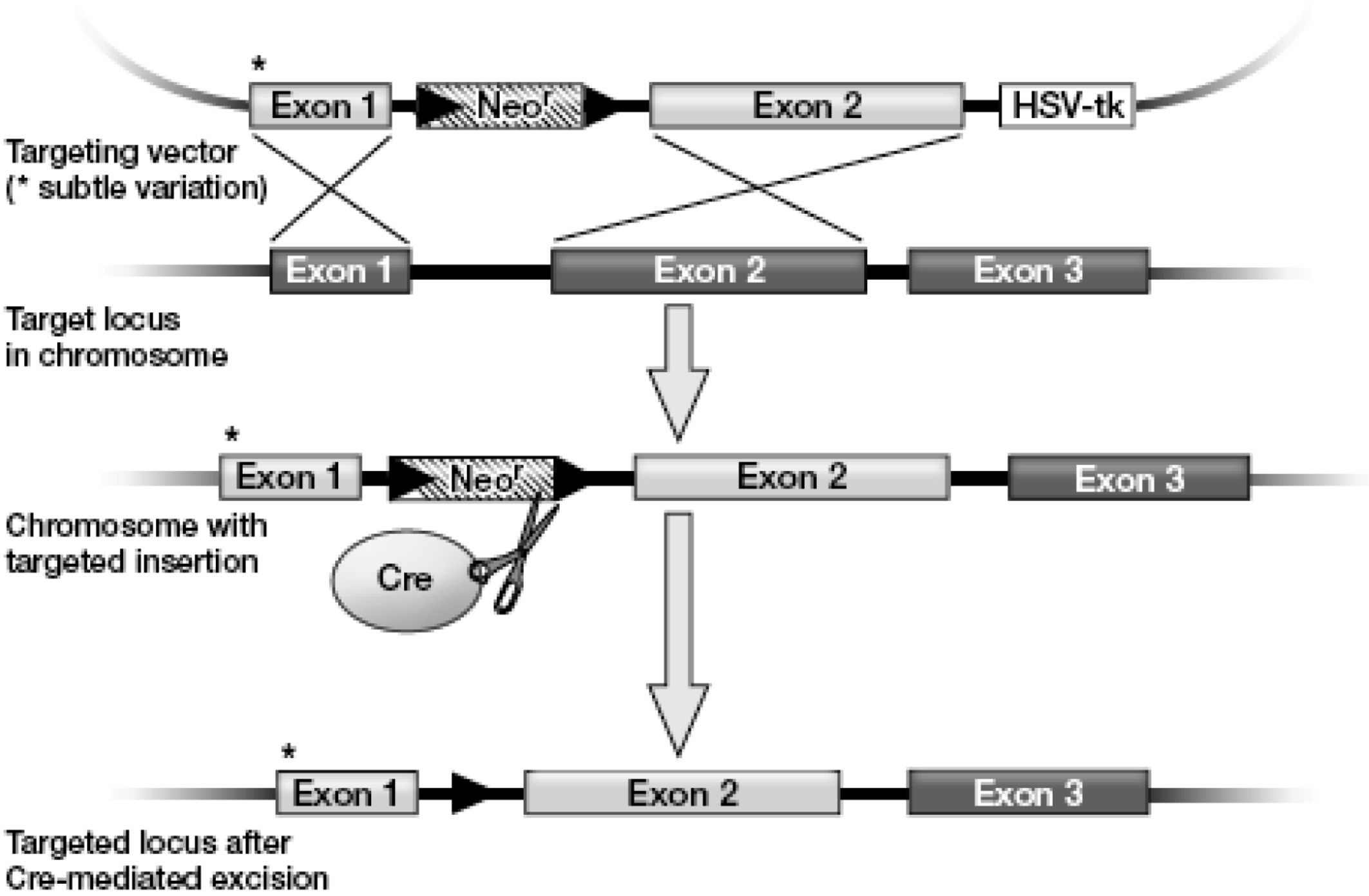
https://www.nature.com/scitable/content/the-steps-involved-in-making-a-knockout-7334330



Preparation of the targeting vector for your favorite gene

Neo^R will be for positive selection and the TK will be used for negative selection

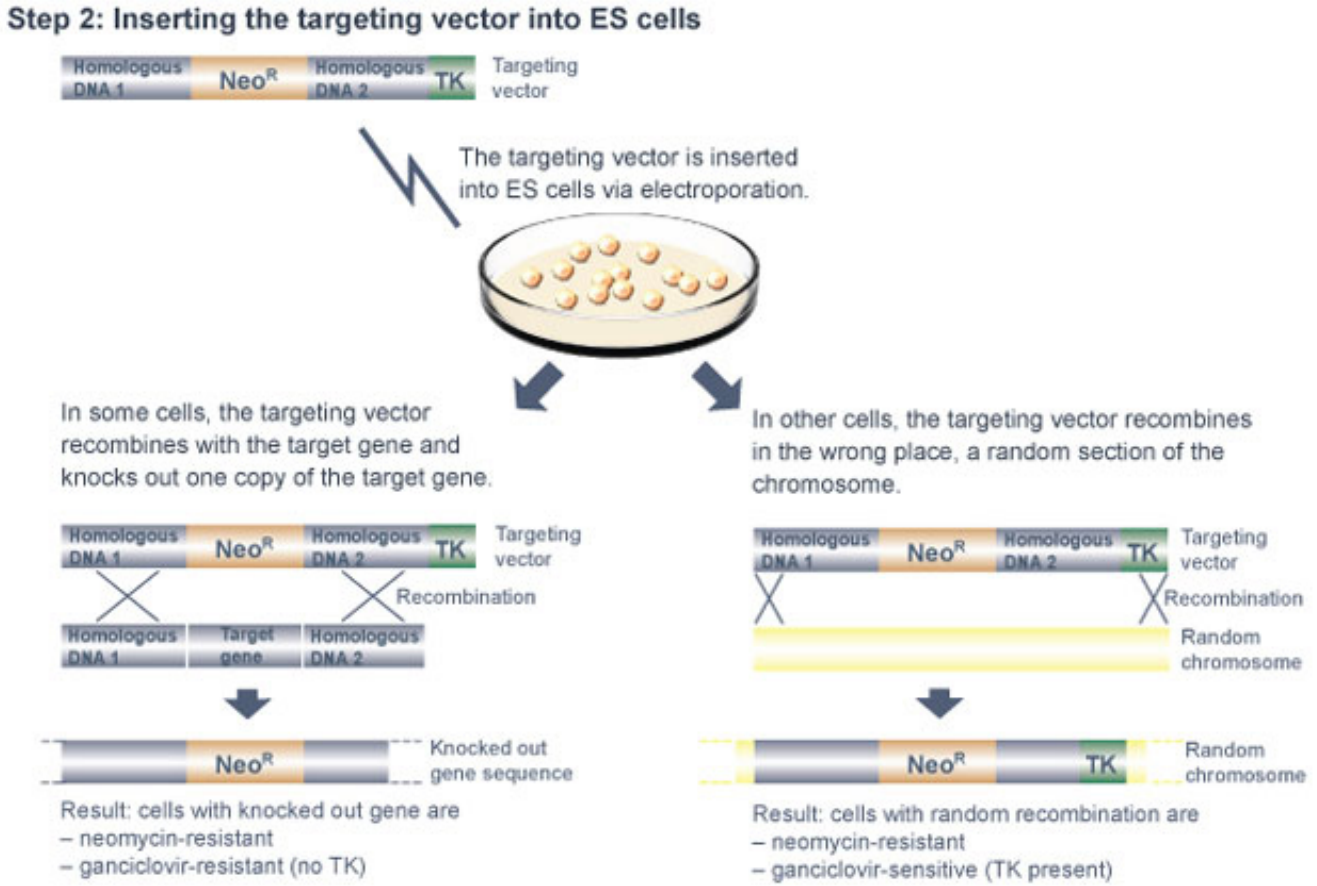

Exon 1
Neo
Exon 3
HSV-tk
Targeting vector
Exon 1
Exon 2
Exon 3
Exon 4
Target locus in chromosome
Exon 1
Neo
Exon 3
Exon 4
Chromosome with targeted replacement


*
Exon 1
Neo^r
Exon 2
HSV-tk
Targeting vector
(* subtle variation)
Exon 1
Exon 2
Exon 3
Target locus
in chromosome
*
Exon 1
Neo^r
Exon 2
Exon 3
Chromosome with
targeted insertion
Cre
*
Exon 1
Exon 2
Exon 3
Targeted locus after
Cre-mediated excision

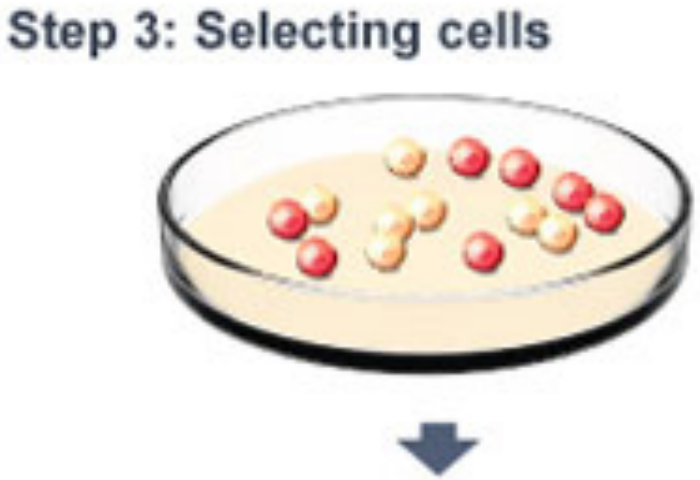
Inserting the vector into Embryonic Stem (ES) cells. ES cells are isolated from early stage mouse embryos (4 days after fertilization).

ES cells can still be used even 10 years after harvesting

https://www.nature.com/scitable/content/the-steps-involved-in-making-a-knockout-7334330



Pick the cells that will survive in the presence of both neomycin and ganciclovir

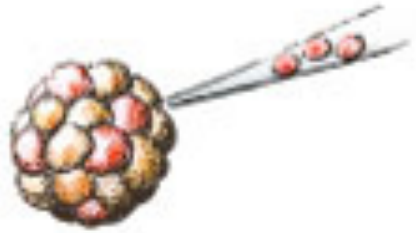


Only the cells that have successfully incorporated the targeting vector into the target gene survive in the presence of neomycin and ganciclovir (shown in red).

https://www.nature.com/scitable/content/the-steps-involved-in-making-a-knockout-7334330

Inject the cells with one copy knockout intonormal mouse blastocysts. We usually pick a mouse strain with a different coat color

Step 4: Injecting cells into a new embryo



Cells containing the targeting vector are then selected and injected into a normal developing mouse embryo.

https://www.nature.com/scitable/content/the-steps-involved-in-making-a-knockout-7334330

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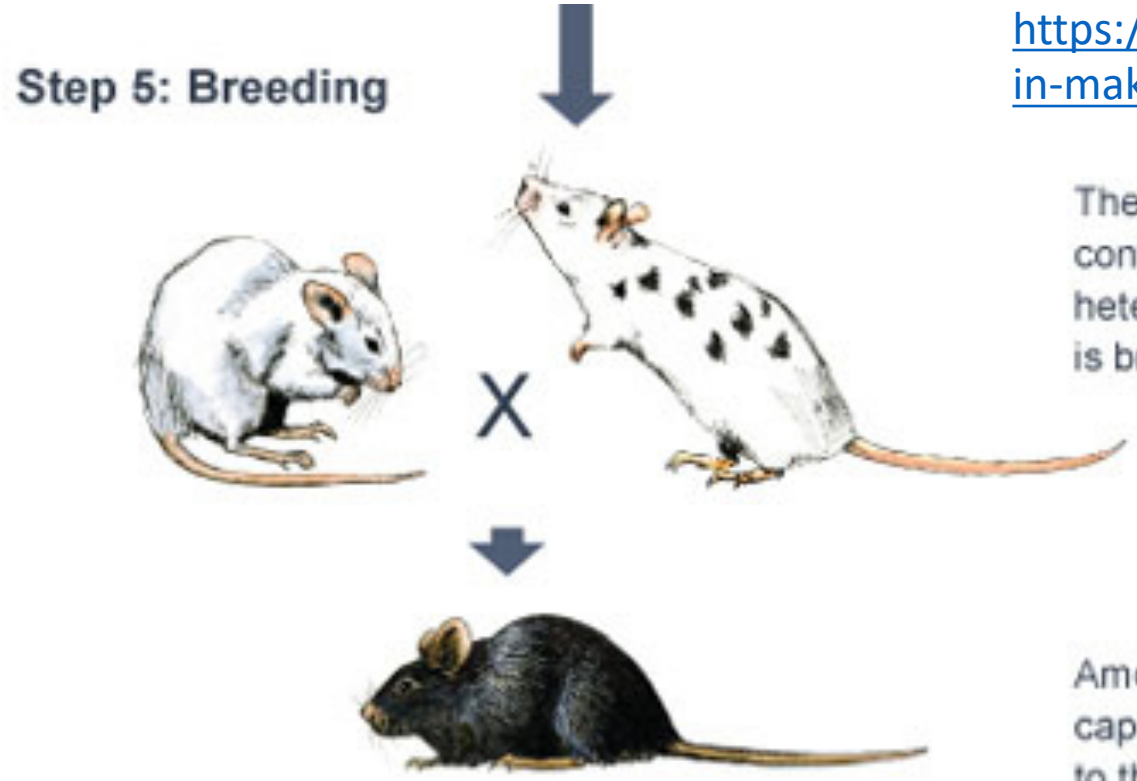
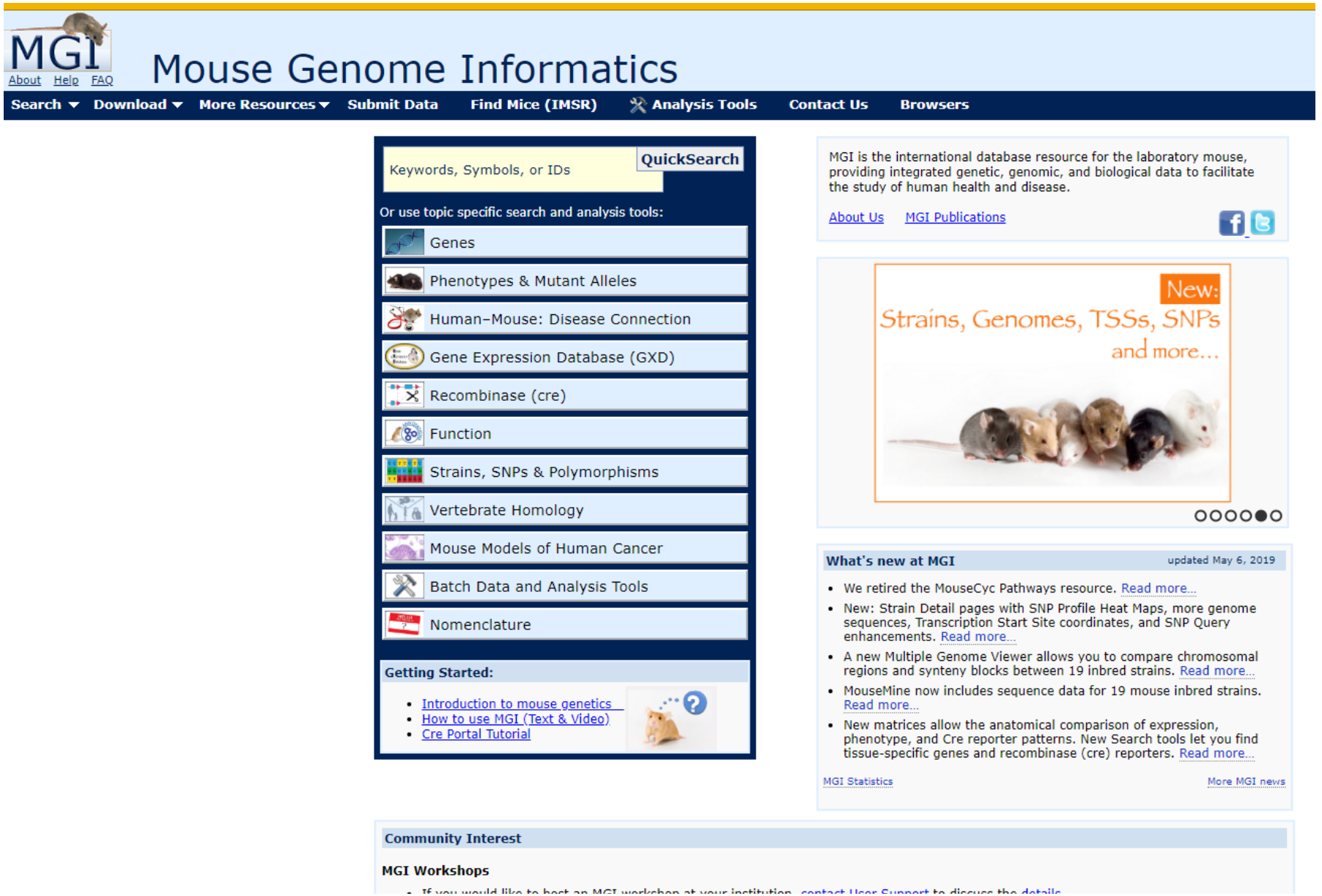
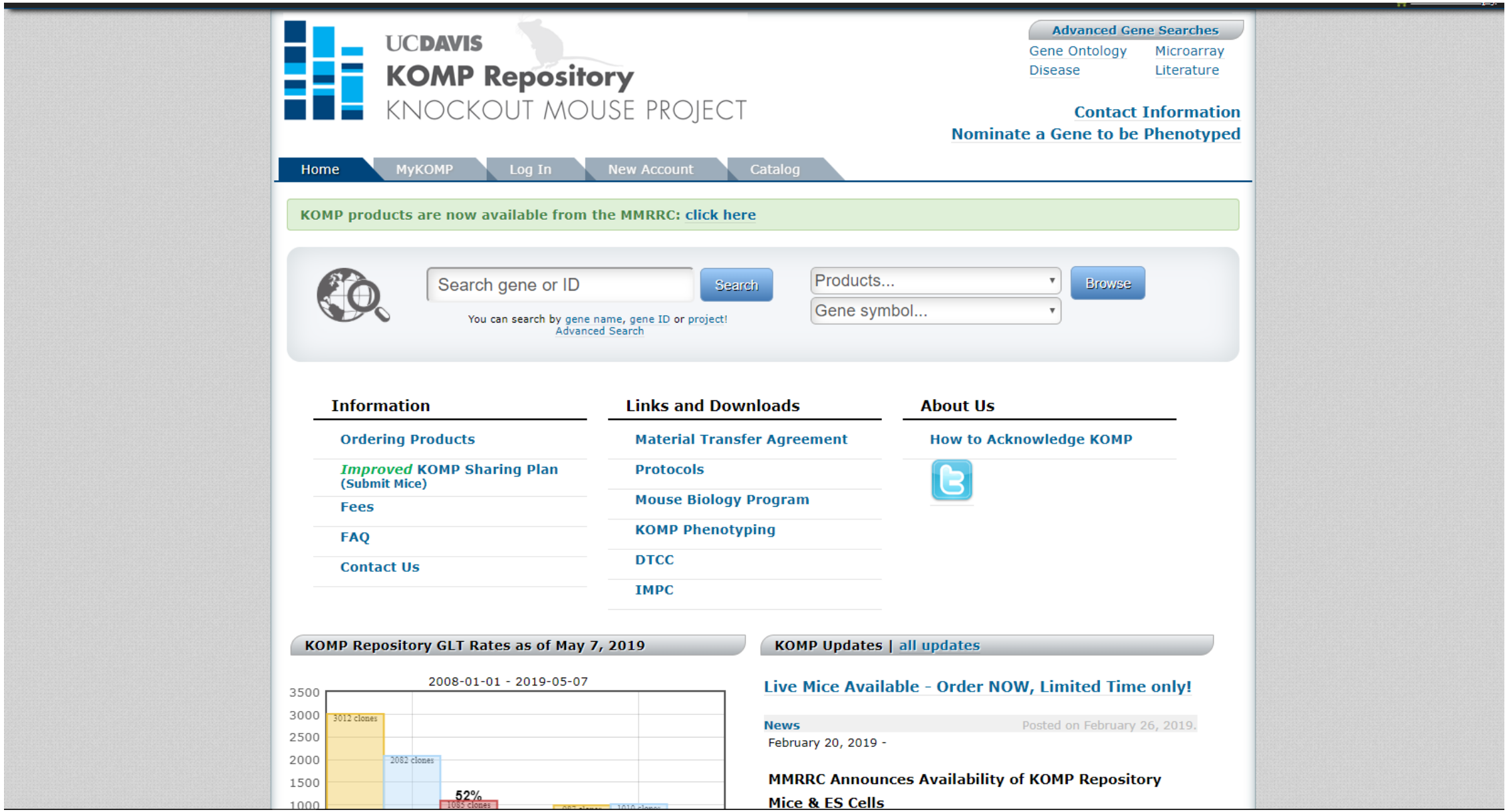


Perform the necessary breeding steps to get the heterozygotes and from the heterozygotes you get start to build your colony

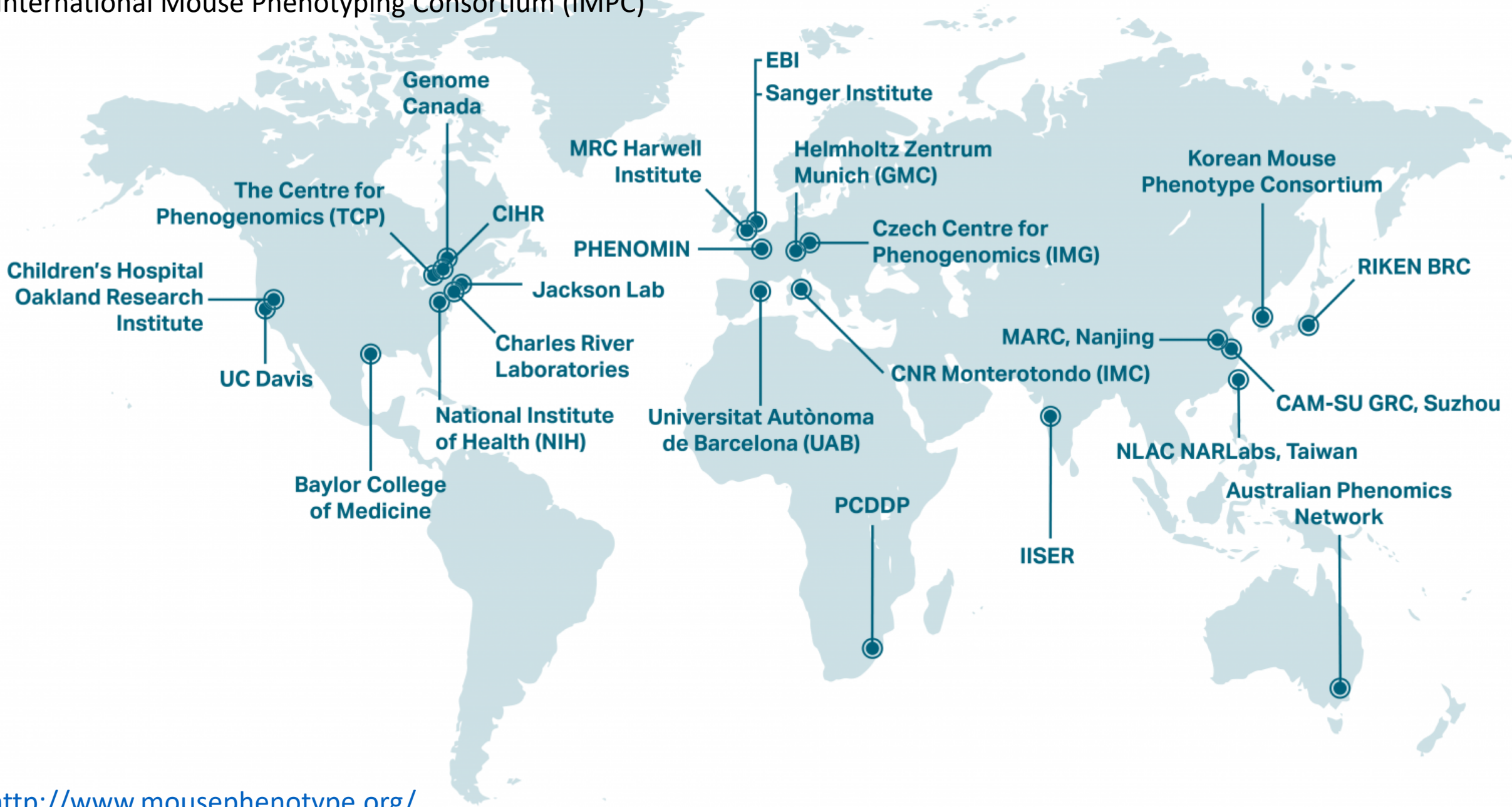
http://www.informatics.jax.org/



https://www.komp.org/



International Mouse Phenotyping Consortium (IMPC)

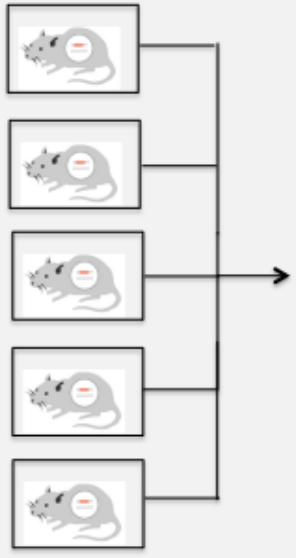


http://www.mousephenotype.org/

What does IMPC do?

Mouse production and phenotyping

Standardized allele production and phenotyping pipelines

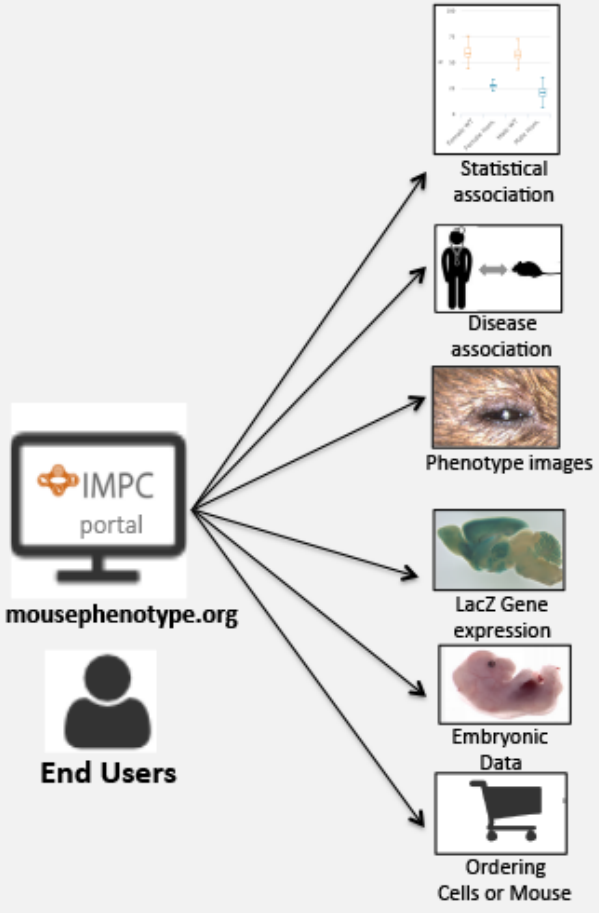


Data analysis

- Quality control
- Statistical analysis
- Disease association

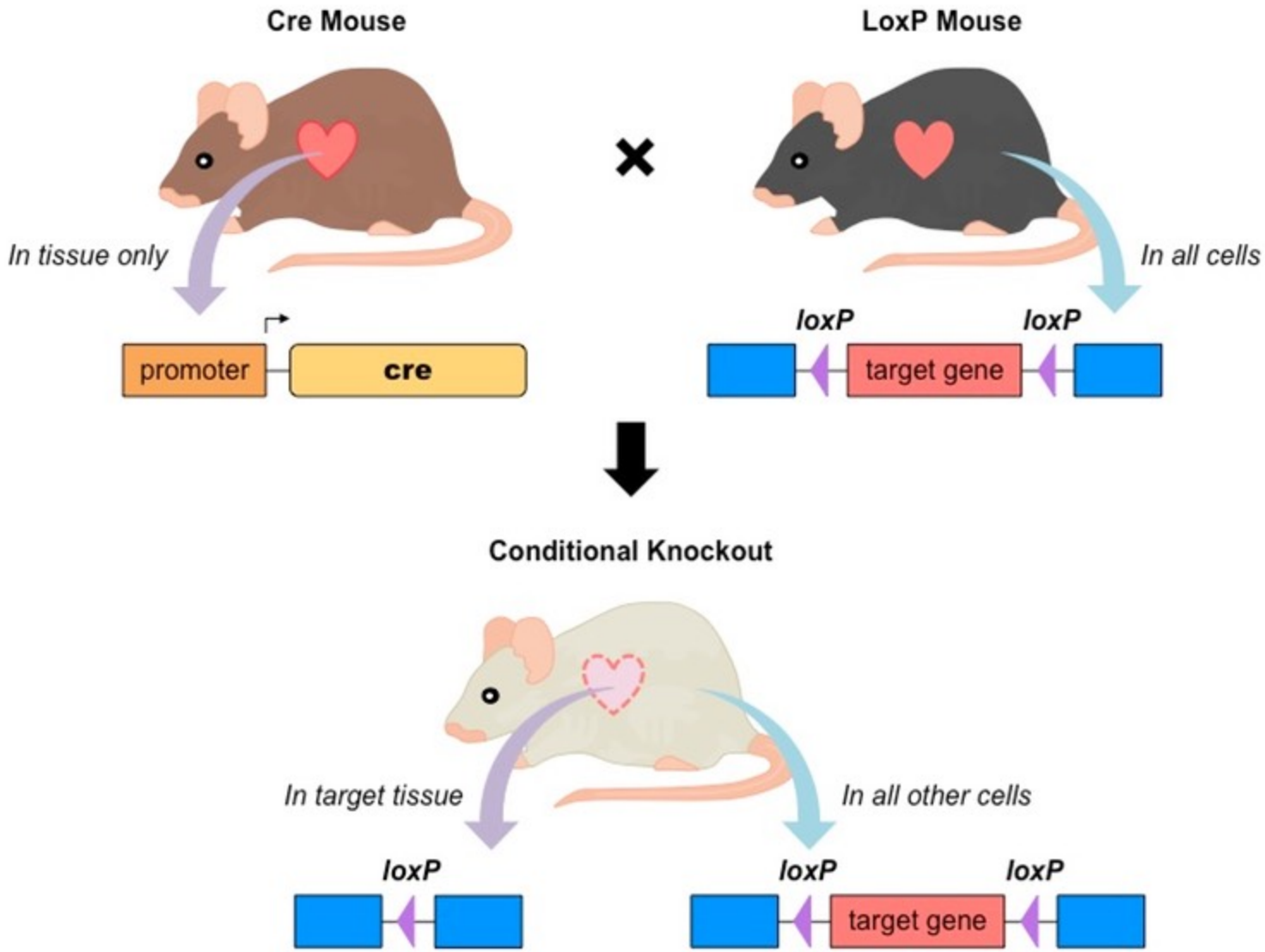
Data distribution

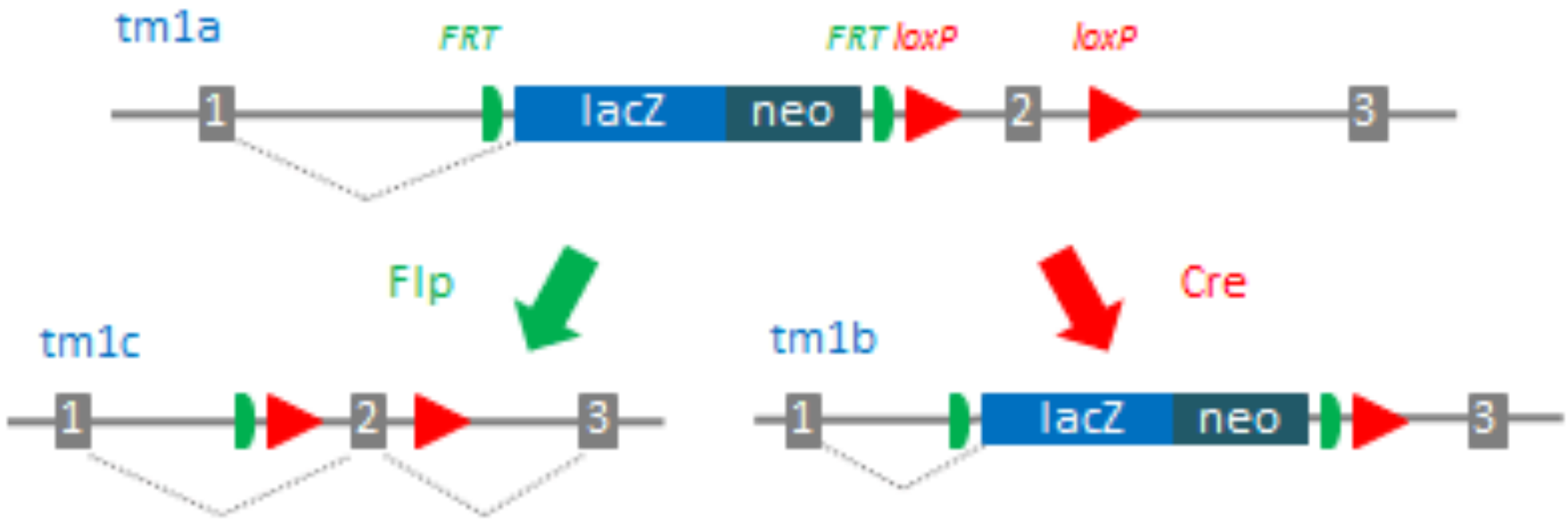
- Free data access and visualizationl
- Embryonic and adult data
- Human disease association
- ES cells and mouse ordering



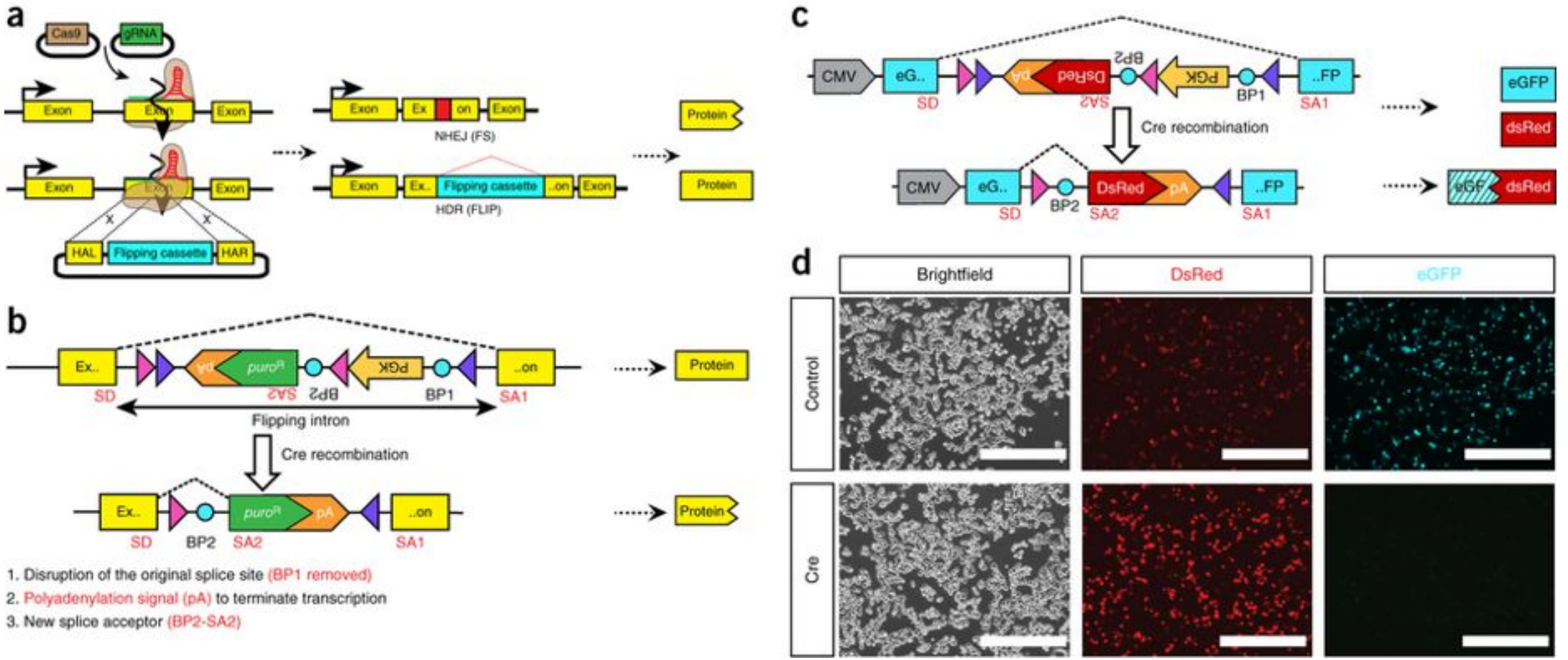
Conditional knockouts

- In both systems, we control the gene expression in a spatial and temporal manner and therefore overcome the embryonic lethal issue
- Chemically induced promoter for Cre
- with Cre/LoxP
  - Derived from the P1 bacteriophage
  - Uses Cre recombinase and loxP sites
  - loxP are 34 bp long
  - The recombinase will excise any region placed between two lox P sites
  - When an exon is flanked by loxP sites it is said to be “floxed”.
  - The floxed allele containing mice are normal. They are then crossed with a Cre expressing mice. The promoter that controls the Cre expression will determine which tissue or cell type the gene will be knockedout in
- With Flp/FRT
  - Less commonly used then cre/LoxP
  - Uses the flippase (Flp recombinase) isolated from S. cervisiae
  - Recognizes the 34 bp long FRT sites


Cre Mouse
LoxP Mouse
In tissue only
In all cells
promoter
cre
loxP
loxP
target gene
Conditional Knockout
In target tissue
In all other cells
loxP
loxP
loxP
target gene


tm1a
FRT
FRT loxP
loxP
1
lacZ
neo
2
3
Flp
Cre
tm1c
tm1b
1
2
3
1
lacZ
neo
3

People can get very creative on the system they design



Knock-in technology

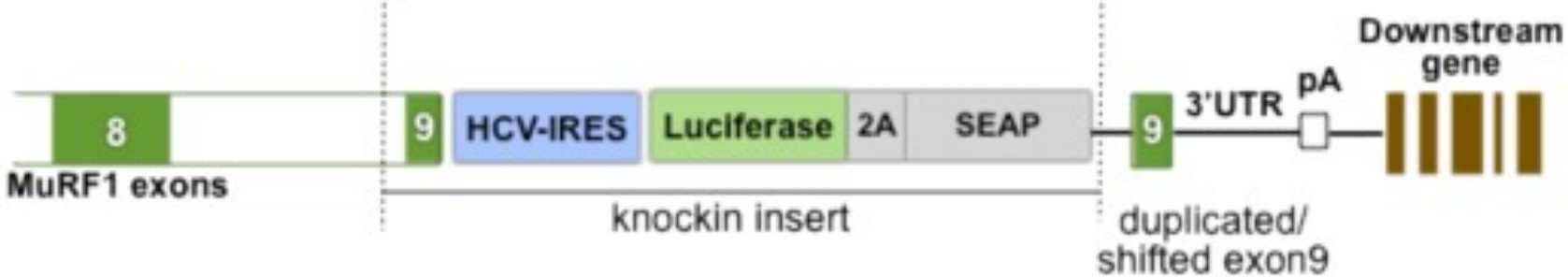
ONE GENE CAN BE PLACED UNDER THE REGULATORY CONTROL OF A CIS-ACTING REGULATORY ELEMENT OF ANOTHER

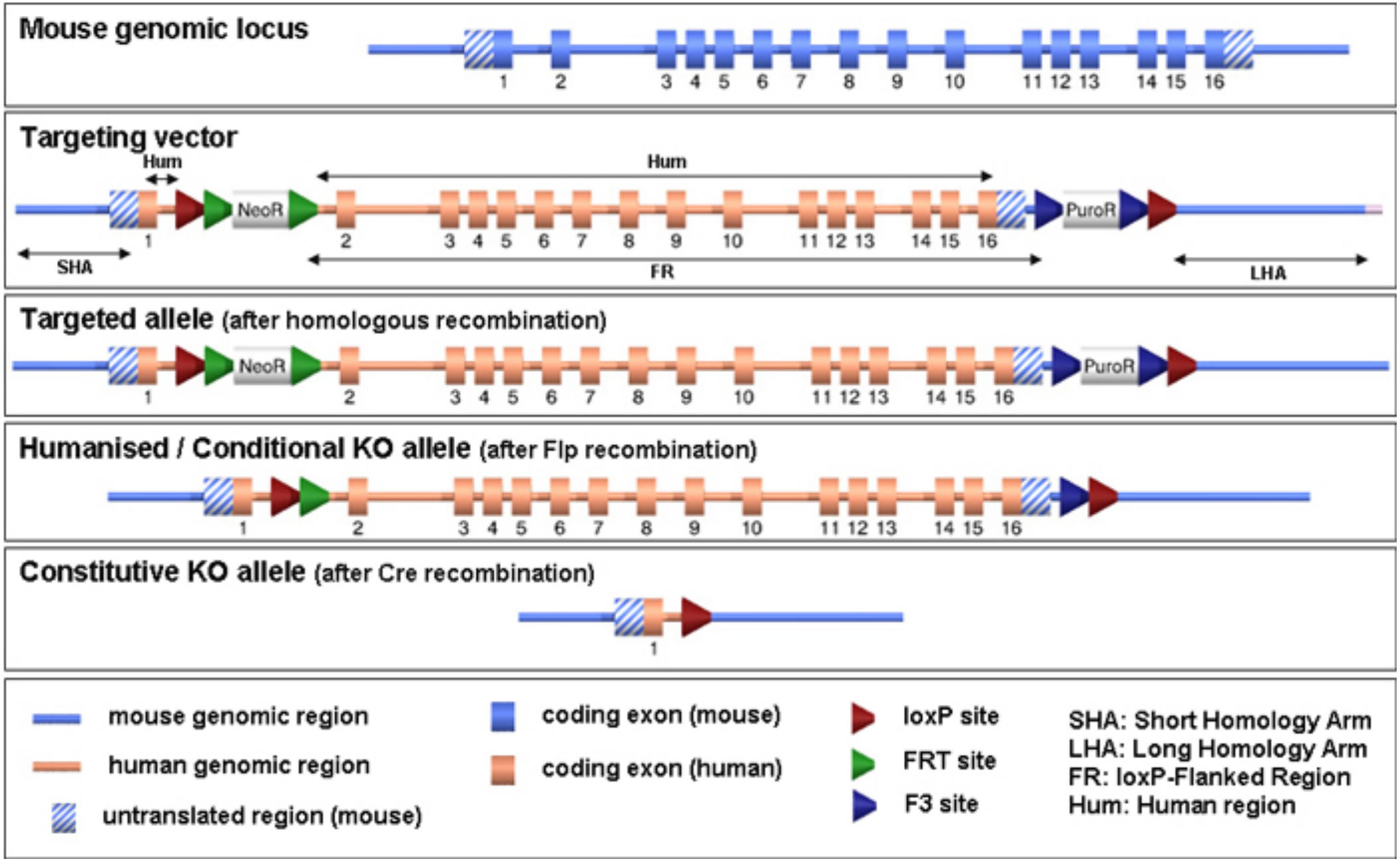
REPORTER GENES SUCH AS LACZ

ISOFORM EXPRESSION

MuRF1-hiLUCs knockin strategy

Final targeted allele configuration




Mouse genomic locus
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Targeting vector
Hum
Hum
NeoR
PuroR
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
SHA
FR
LHA
Targeted allele (after homologous recombination)
NeoR
PuroR
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Humanised / Conditional KO allele (after Flp recombination)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Constitutive KO allele (after Cre recombination)
1
mouse genomic region
human genomic region
untranslated region (mouse)
coding exon (mouse)
coding exon (human)
loxP site
FRT site
F3 site
SHA: Short Homology Arm
LHA: Long Homology Arm
FR: loxP-Flanked Region
Hum: Human region

CRISPR/Cas9 editing in mice

- Generation of knockouts and knock-ins could be a very labor intensive process that requires highly skilled individuals and expensive equipment
- 1-2 year average time for creation
- CRISPR/Cas9 can hopefully simplify the process
- Create single nucleotide mutations that can mimic human mutations.
- For Kos; single guide RNA injected into zygotes to create indels in exons causing a frameshift
- For conditional KOs; two sgRNA and a repair template with loxP sites (less efficient)
- If we use longer repair template, higher success rates, but still very expensive and limited to specialized facilities
- i-GONAD technology; transfer the reagents into the oviduct by electroporation (at 0.7 dpc)

Burgio Genome Biology (2018) 19:27
https://doi.org/10.1186/s13059-018-1409-1

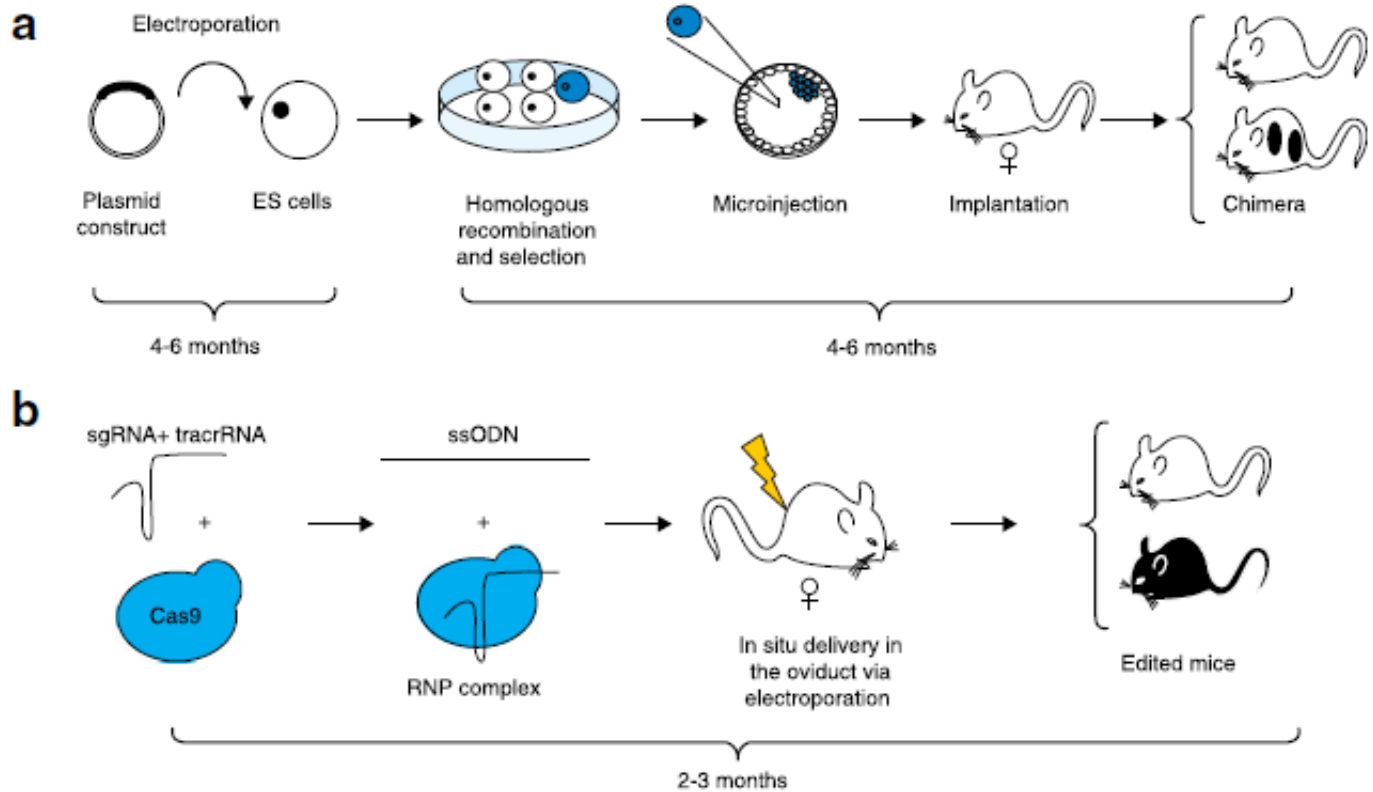


Fig. 1 a Generation of knockout and knockin alleles using embryonic stem (*ES*) cell technology in mice. A cloning procedure is undertaken to insert the construct into a plasmid vector as a template to replace the endogenous locus. This template could be a drug-selection cassette only (knockout) or an exon flanked with two loxP sites, or a more complex feature (knockin). These vectors contain a positive and negative selection cassette. The plasmid is then electroporated into the ES cells and then drug selected in vitro. After verification that the sequence is correctly inserted, the cells are microinjected into a blastocyst, before being surgically transferred into pseudopregnant females. The chimeric progenies will be genotyped to ensure the expected construct is correctly inserted into the genome by homologous recombination. **b** Generation of complex alleles using improved-genome editing via oviductal nucleic acid delivery (i-GONAD) technology. One or two single guide RNAs (*sgRNA*) are designed to either disrupt a critical exon (knockout) or remove an entire exon for replacement with a repair template (knockin). The sgRNAs are synthesized, or in vitro transcribed, and then complexed with the tracrRNA and then Cas9 protein to form a ribonucleoprotein (*RNP*) complex. The RNPs are in situ electroporated with a long single-stranded oligonucleotide repair template (*ssODN*) into the oviduct of a pregnant female. The progenies are genotyped to ascertain successful editing of the gene of interest

Crispr/Cas9 allele design at the IMPC