

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](#).

Genetically modified mice

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



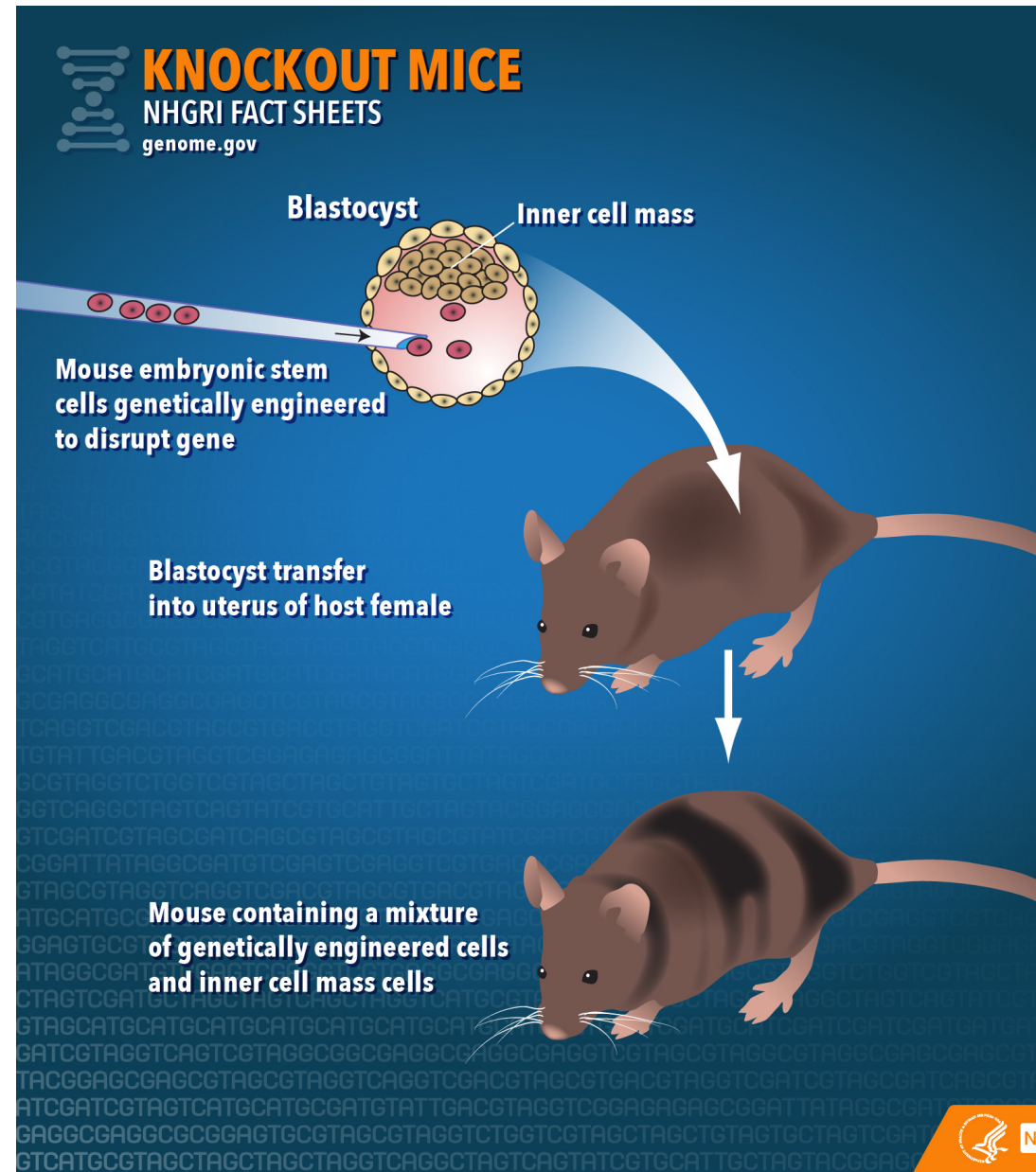
Knockouts

- 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 known (not a 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



Generation of gene targeted mice



Targeted ES cells
are injected
into blastocysts...



...which are
implanted into
foster mothers



...which give birth
to chimeric mice



Mating between
chimeric mouse
and normal
mouse.



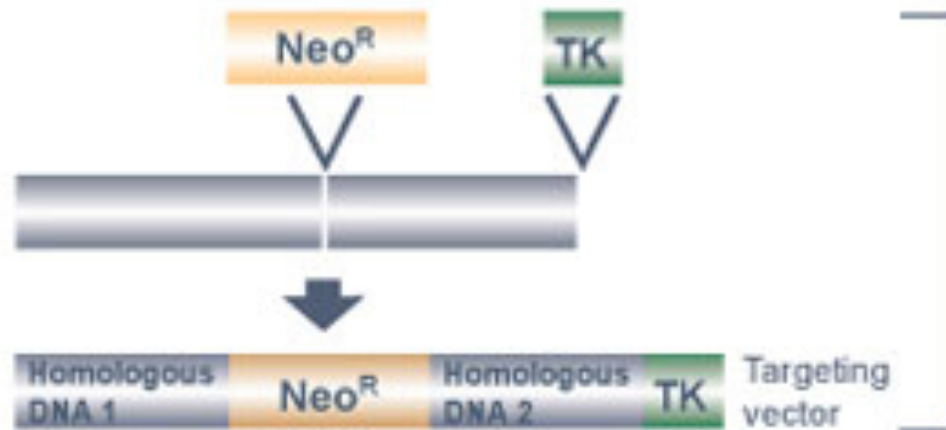
Gene targeted mice



Normal mice

Step 1: Designing the targeting vector

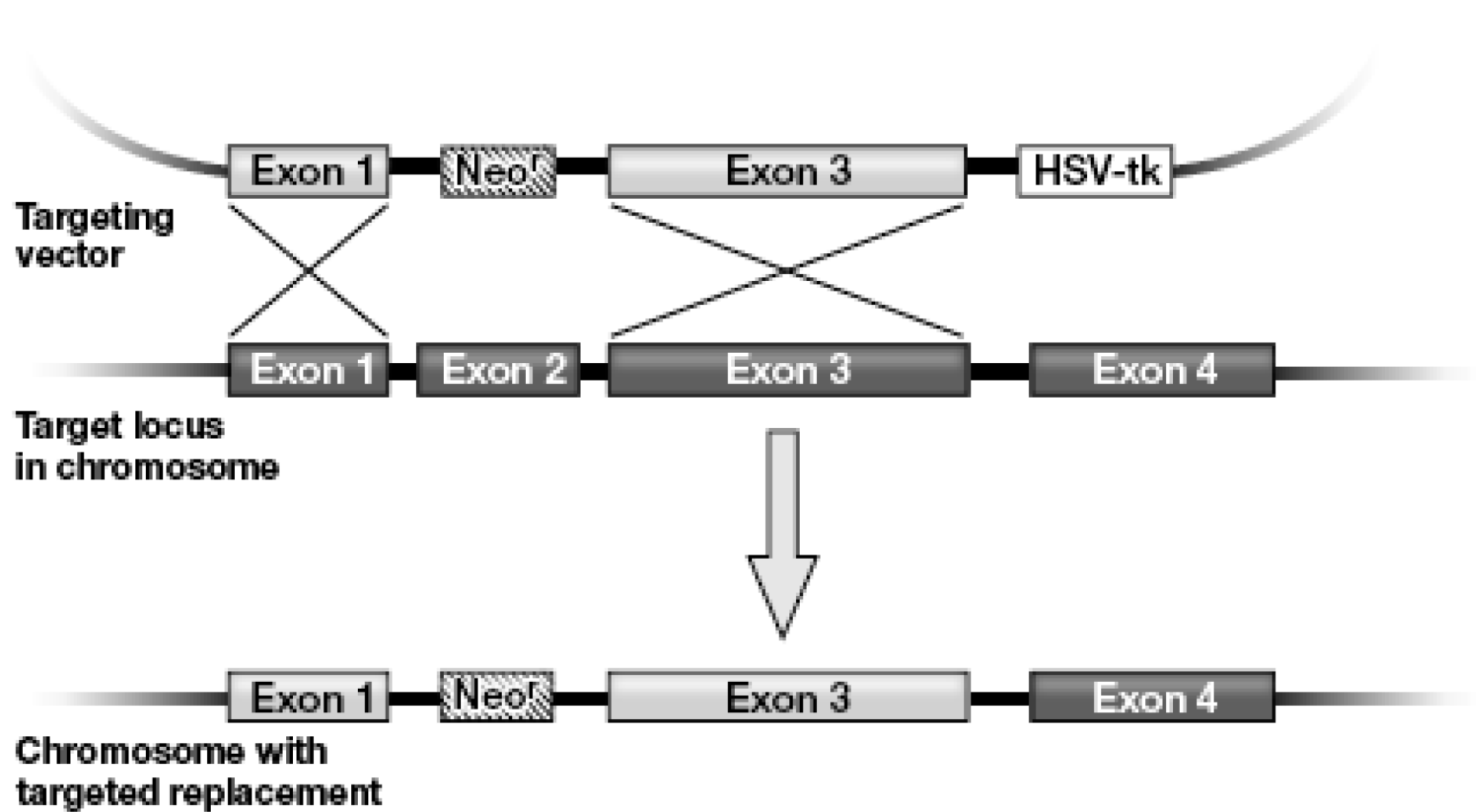
Homologous DNA 1 Target gene Homologous DNA 2 Target gene & flanking sequences

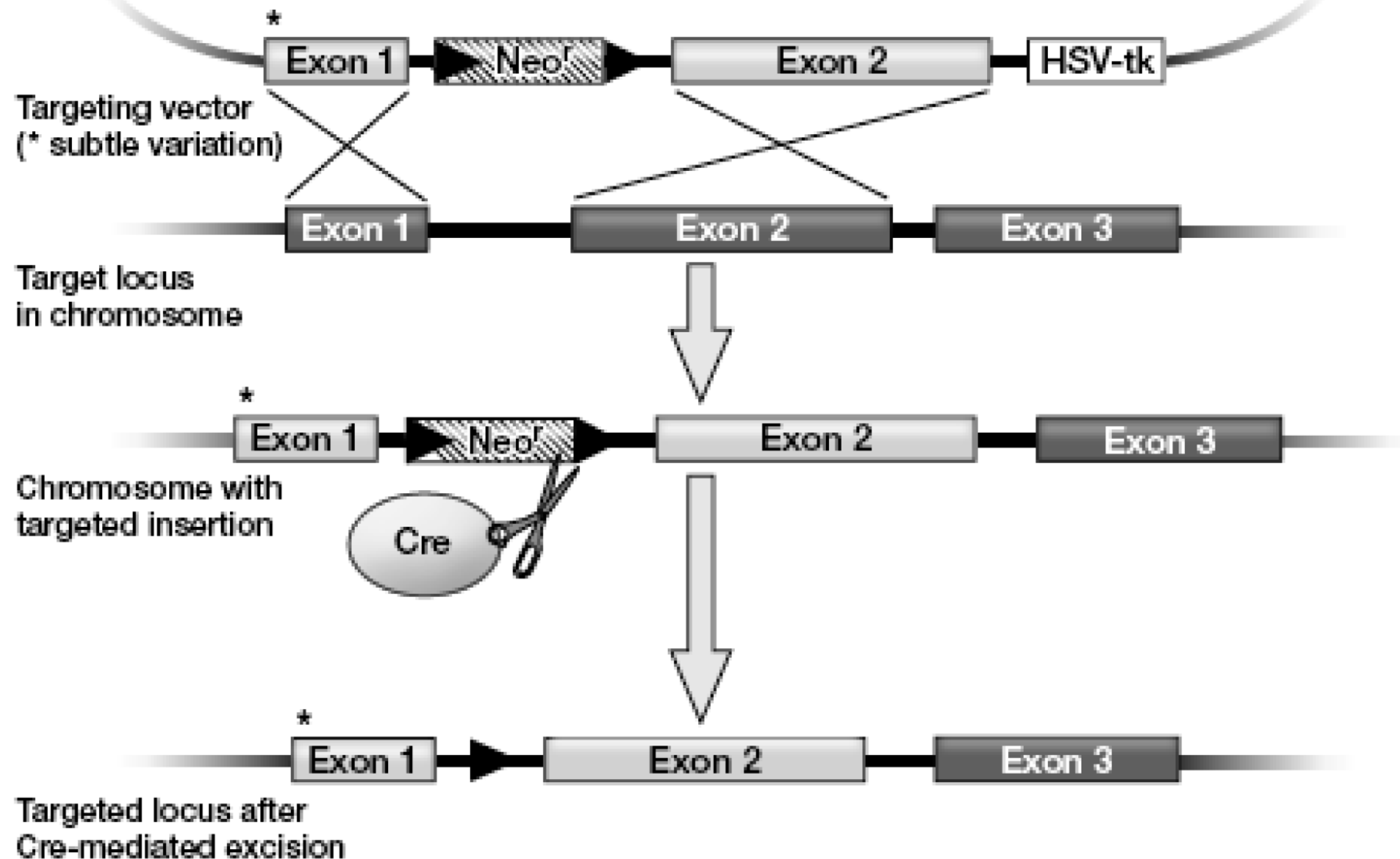


The markers Neo^R and TK are inserted into the target gene sequence to make a targeting vector sequence.

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



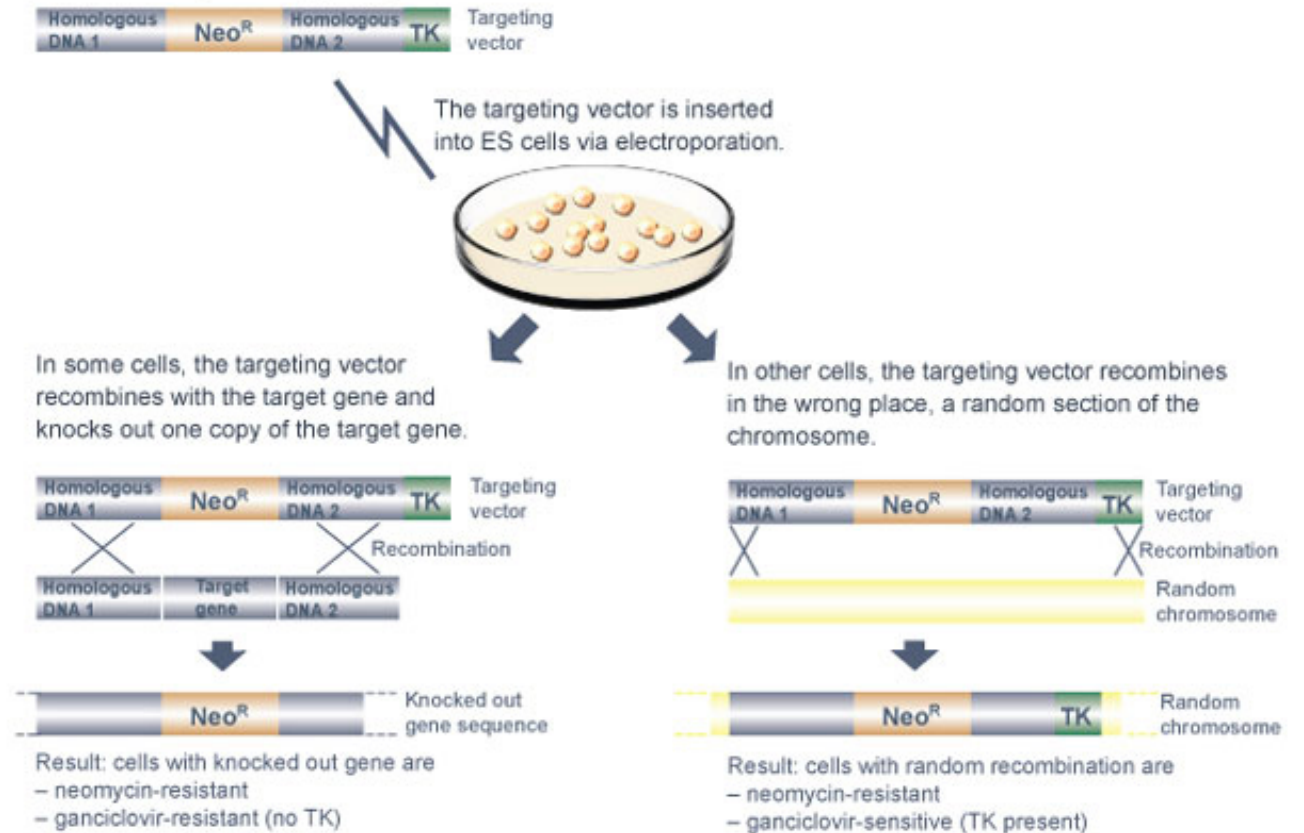


Inserting the vector into Embryonic Stem (ES) cells. ES cells are isolated from early stage mouse embryos (4 days after fertilization).

ES cells can still be used even 10 years after harvesting

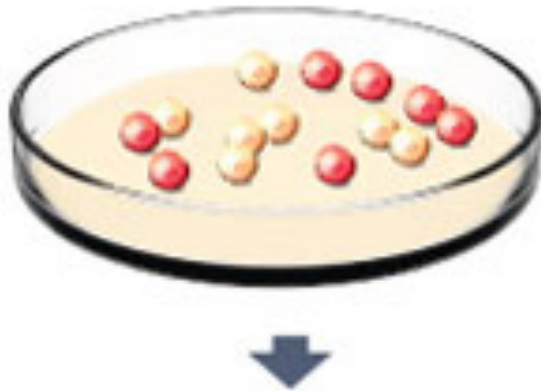
<https://www.nature.com/scitable/content/the-steps-involved-in-making-a-knockout-7334330>

Step 2: Inserting the targeting vector into ES cells



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

Step 3: Selecting cells

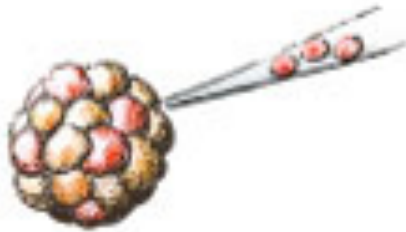


Only the cells that have successfully incorporated the targeting vector into the target gene survive in the presence of neomycin and ganciclovir (shown in red).

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

Inject the cells with one copy knockout into normal 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>

Step 5: Breeding

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



The resulting chimeric (spotted) mouse contains a mix of its own cells and the heterozygous knockout cells. This mouse is bred with a normal (white) mouse.

Among their offspring are mice that are capable of passing the knocked-out gene to their own offspring.

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/>














Mouse Genome Informatics

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Getting Started:

- [Introduction to mouse genetics](#)
- [How to use MGI \(Text & Video\)](#)
- [Cre Portal Tutorial](#)



MGI is the international database resource for the laboratory mouse, providing integrated genetic, genomic, and biological data to facilitate the study of human health and disease.

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New:
Strains, Genomes, TSSs, SNPs
and more...



What's new at MGI

updated May 6, 2019

- We retired the MouseCyc Pathways resource. [Read more...](#)
- New: Strain Detail pages with SNP Profile Heat Maps, more genome sequences, Transcription Start Site coordinates, and SNP Query enhancements. [Read more...](#)
- A new Multiple Genome Viewer allows you to compare chromosomal regions and synteny blocks between 19 inbred strains. [Read more...](#)
- MouseMine now includes sequence data for 19 mouse inbred strains. [Read more...](#)
- New matrices allow the anatomical comparison of expression, phenotype, and Cre reporter patterns. New Search tools let you find tissue-specific genes and recombinase (cre) reporters. [Read more...](#)

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Community Interest

MGI Workshops

- If you would like to host an MGI workshop at your institution, [contact User Support](#) to discuss the details.

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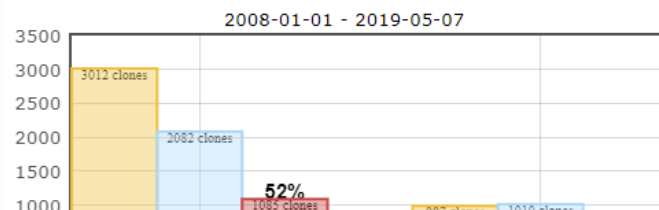
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KOMP Repository GLT Rates as of May 7, 2019



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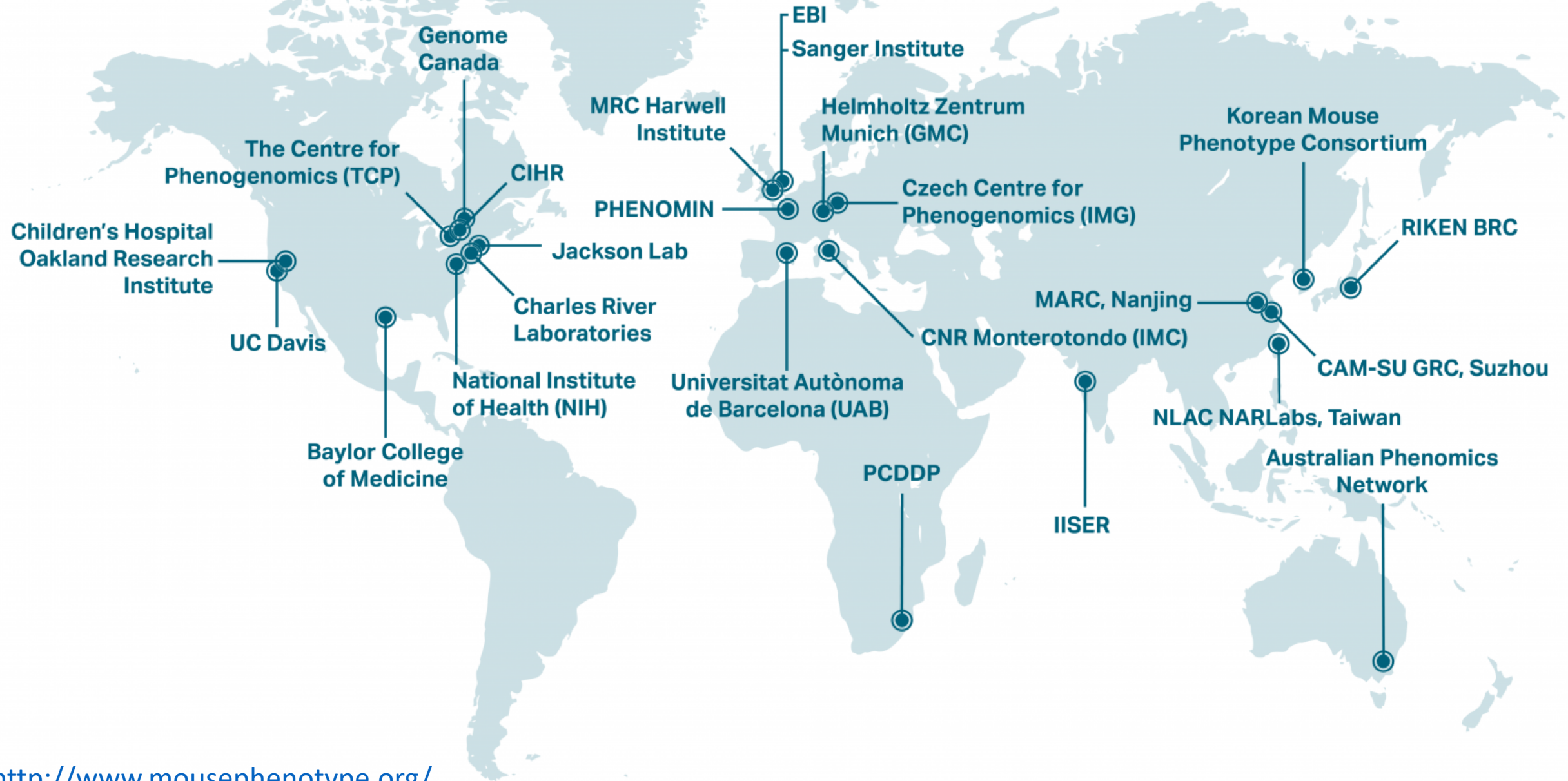
News

Posted on February 26, 2019.

February 20, 2019 -

**MMRRC Announces Availability of KOMP Repository
Mice & ES Cells**

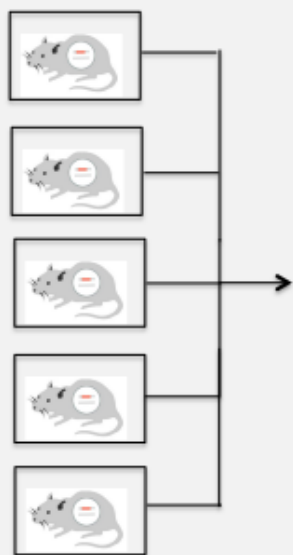
International Mouse Phenotyping Consortium (IMPC)



What does IMPC do?

Mouse production and phenotyping

Standardized allele production and phenotyping pipelines



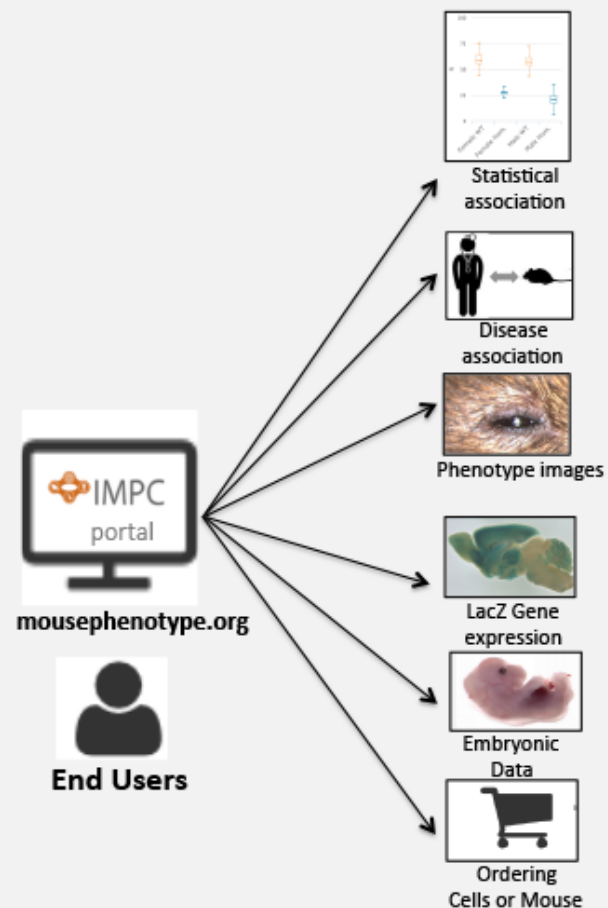
Data analysis

- Quality control
- Statistical analysis
- Disease association



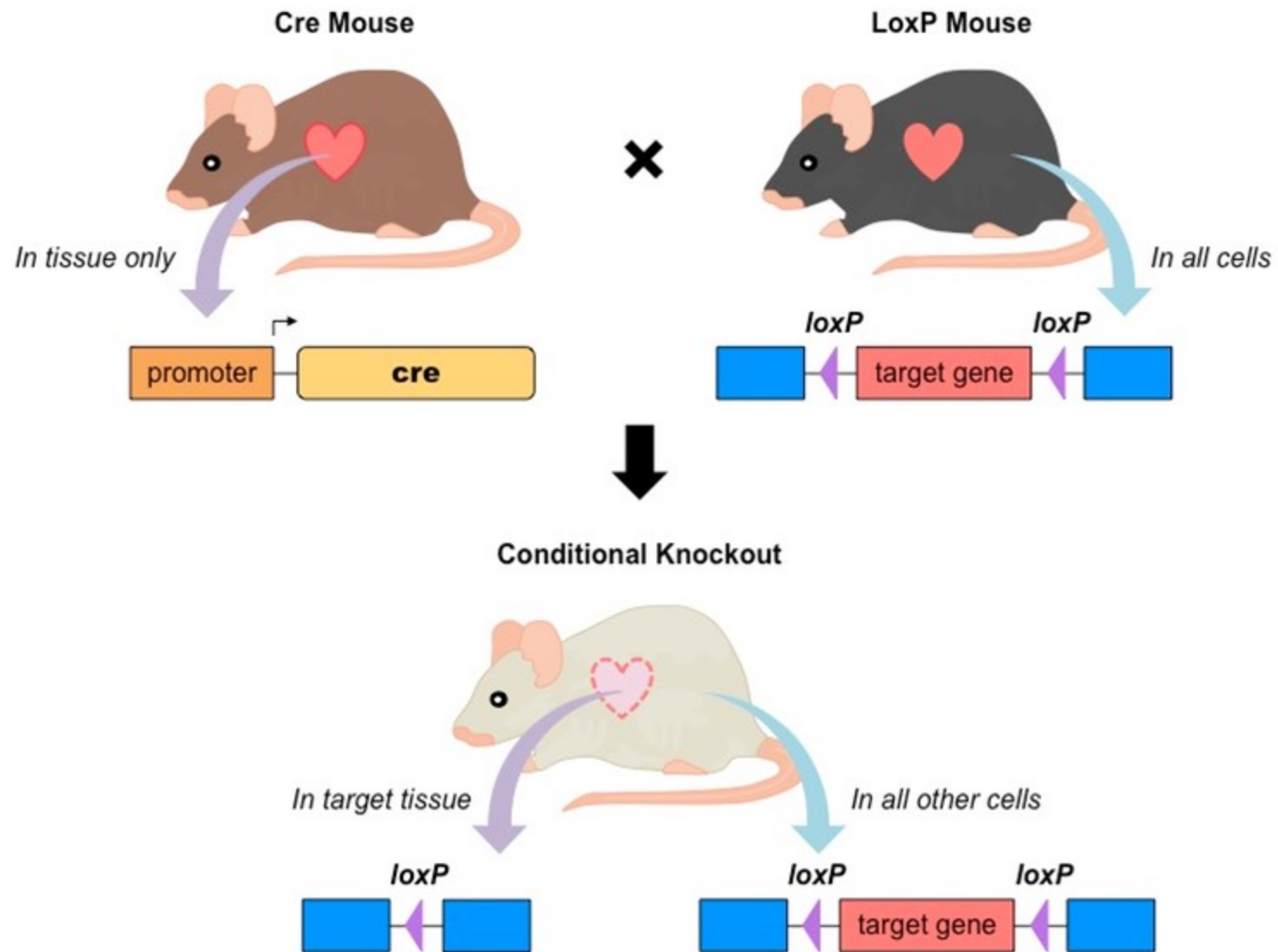
Data distribution

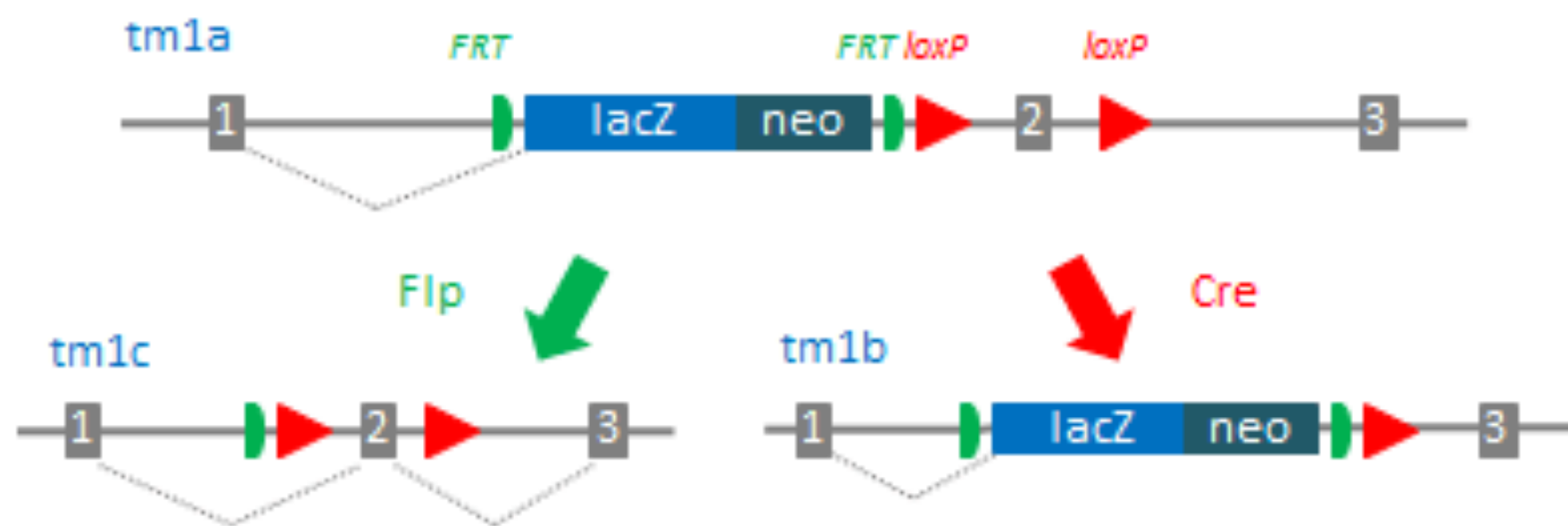
- Free data access and visualization
- Embryonic and adult data
- Human disease association
- ES cells and mouse ordering



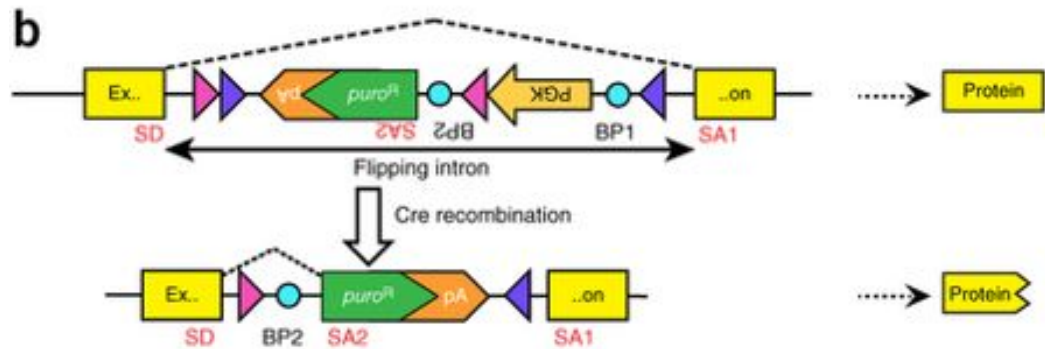
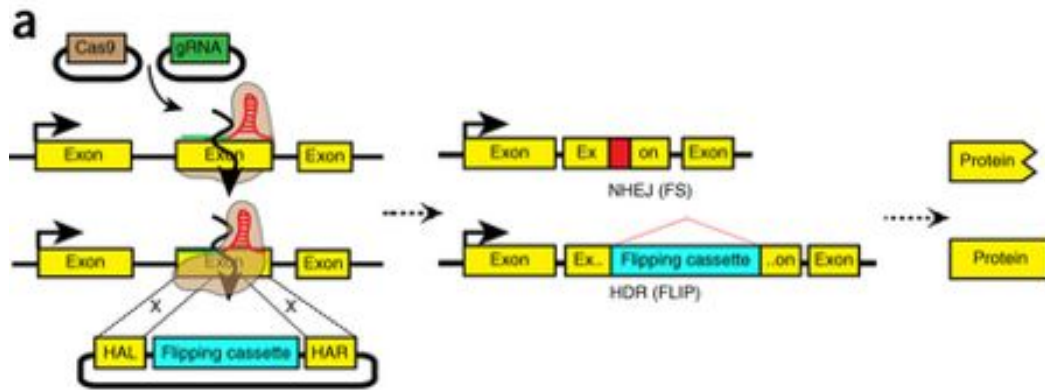
Conditional knockouts

- 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. cerevisiae*
 - Recognizes the 34 bp long FRT sites

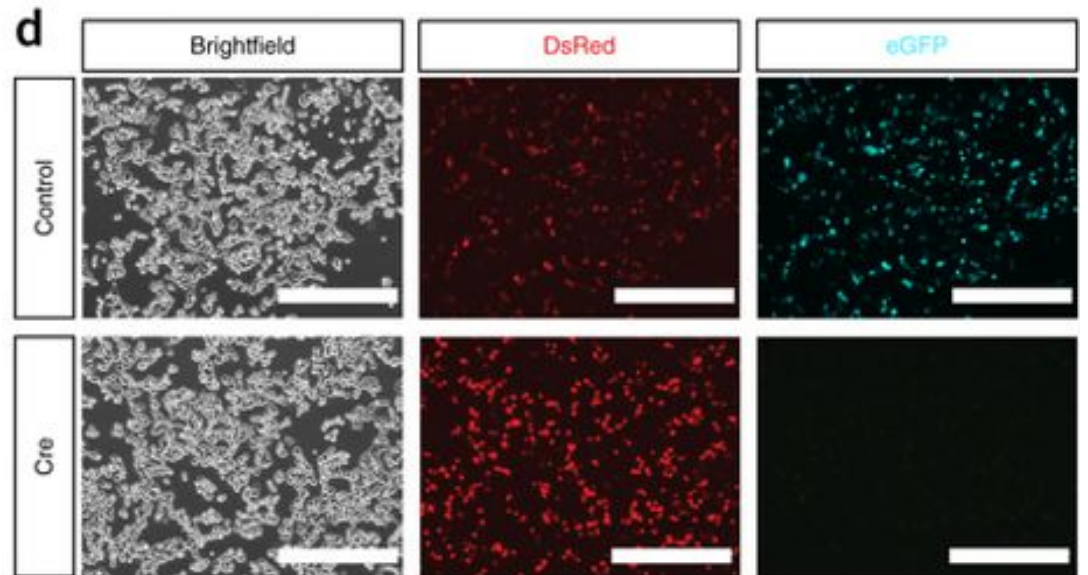
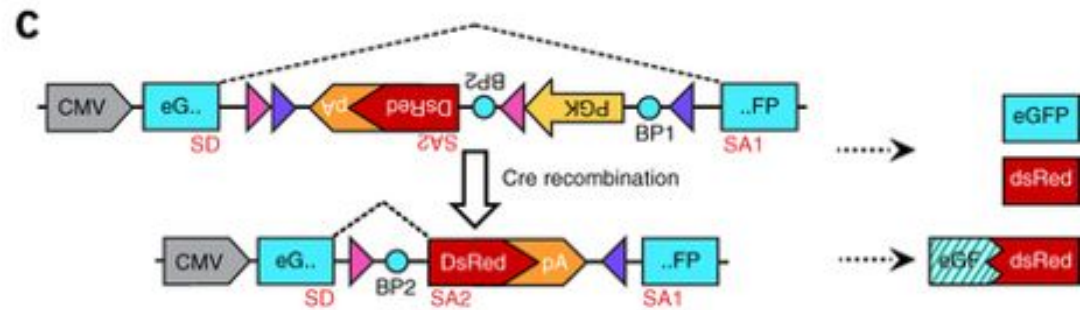




People can get very creative on the system they design



1. Disruption of the original splice site (BP1 removed)
2. Polyadenylation signal (pA) to terminate transcription
3. New splice acceptor (BP2-SA2)



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

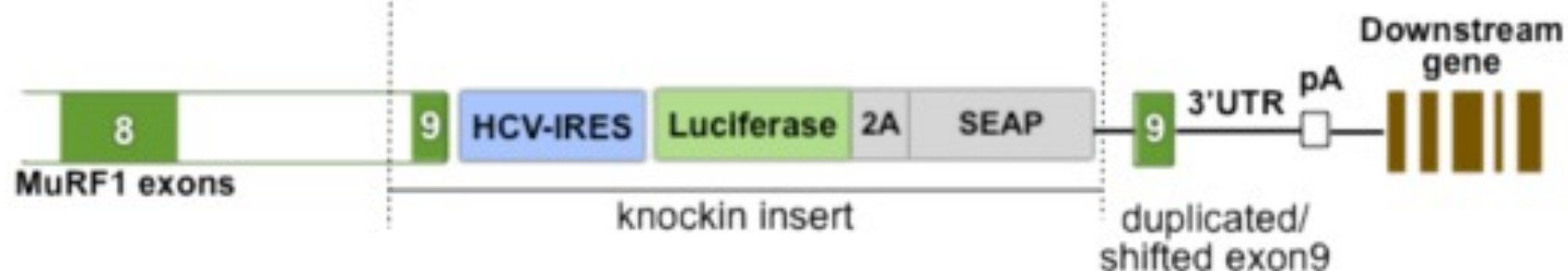


ISOFORM EXPRESSION

MuRF1-hiLUCs knockin strategy



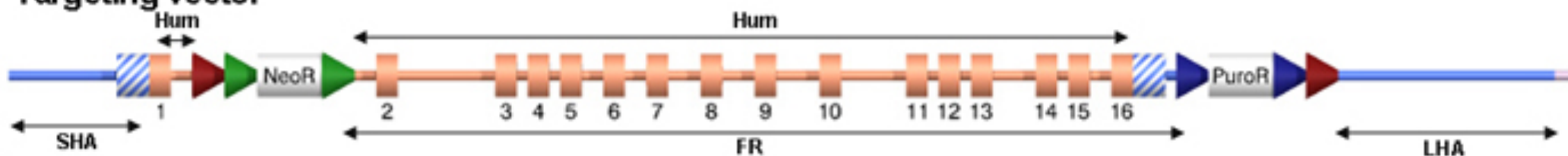
Final targeted allele configuration



Mouse genomic locus



Targeting vector



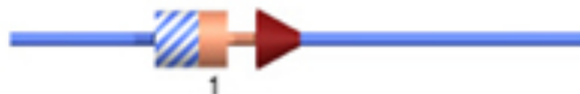
Targeted allele (after homologous recombination)








Humanised / Conditional KO allele (after Flp recombination)



Constitutive KO allele (after Cre recombination)



 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)

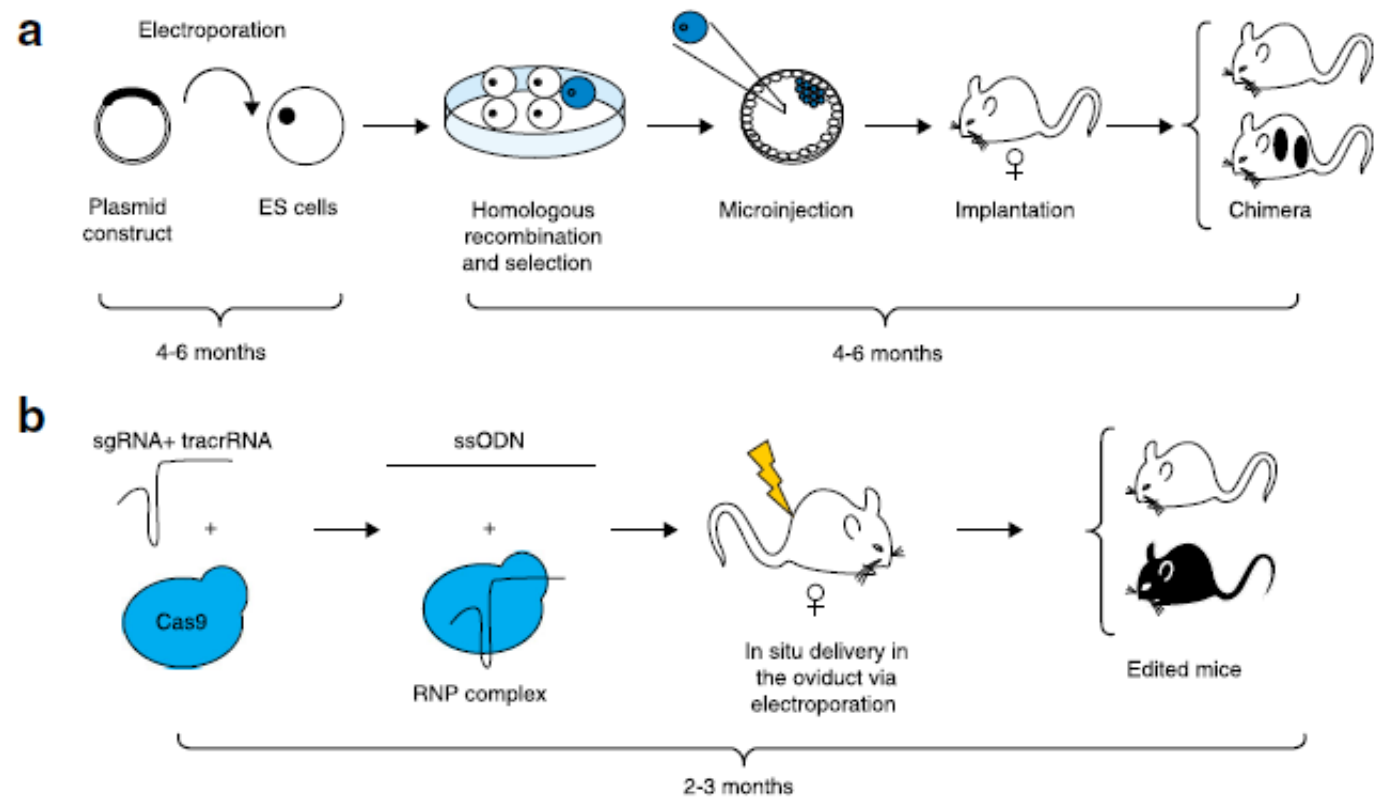


Fig. 1 a Generation of knockout and knockin alleles using embryonic stem (ES) cell technology in mice. A cloning procedure is undertaken to insert the construct into a plasmid vector as a template to replace the endogenous locus. This template could be a drug-selection cassette only (knockout) or an 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

