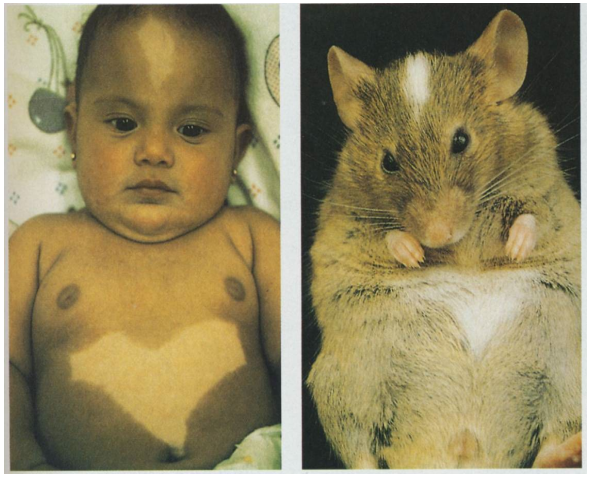
University of Basel
Center for Transgenic Models

Euthanasia of laboratory animals.
(What animals are euthanized and why?)

Pawel Pelczar 31.03.2023

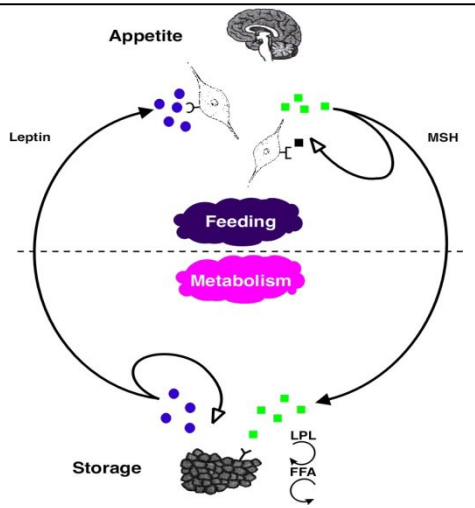
Why use an animal (mouse) as a research model?

- **Very close to humans**



Piebaldism
Mutation in the KIT gene that affects melanocyte development

- **Sufficiently complex**



Integrated Control of Appetite and Fat Metabolism by the **Leptin-POMC Pathway**

- **Extensive transgenic methods**

Including classical (DNA microinjection, ES cells) and new transgenic methods
ZFNs, TALENs, CRISPR/Cas9, Base editors, Prime editors

Applications of transgenic/GMO models

Study the function of a specific gene.

Study mechanisms that control gene expression.

Model human biological systems in a genetically control environment.

Replicate specific characteristics, symptoms of a disease.

Develop an animal model to test therapeutic strategies safer and faster.

Consequently – most “laboratory mice” are GMOs

Species

Genetically Modified Species

Over 97% of all genetically modified (GM) animals were **mice,** with relatively small numbers of fish, rats, rabbits and pigs.

Further more, in the mouse transgenic population, over 91% were used with the purpose of **biological basic research.**

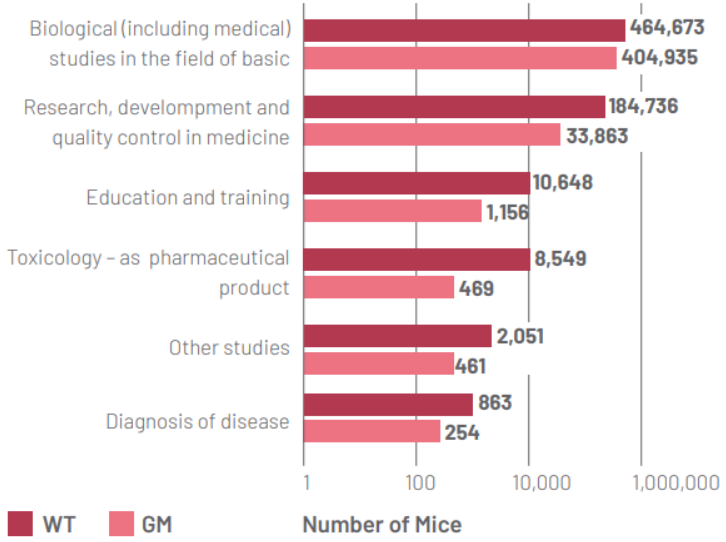
Studies of the **immune system** used the most GM mice (117’919), accounting for~40% of the total mice used. The nervous system (96’257) and oncology (63’223) followed.

Note that "wild-type mice" (WT) simply indicates that no specific modifications were made to the genome of the animal

Animal Numbers of Genetically Modified Animals for each Species

Species	Wild-Type	Genetically Modified
Mice	671,531	441,138
Fish	138,853	7,003
Rats	222,390	3491
Rabbits	3,113	8
Pigs	11,512	2
Grand Total	**1,047,399**	**451,642**

Number of Genetically Modified Mice split by Study Purpose



3 R C C

Case example: tg-mouse model for Cov19 studies

1st Covid-19 cases were reported at the end of **2019** in China.

Covid-19 susceptible species – **bats**, humans, primates and ferrets.

Wild-type mice are resistant to Cov-19 infection due to receptor (ACE2) incompatibility.

Transgenic mice expressing human ACE2 (hACE2) are susceptible to Cov-19 infection.

May 2020

Article

The pathogenicity of SARS-CoV-2 in hACE2 transgenic mice

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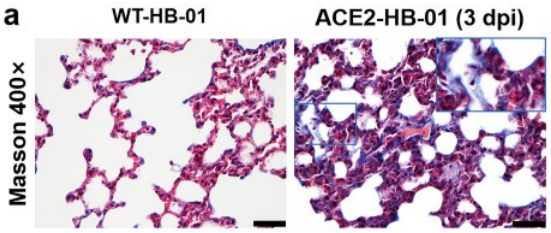
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the cause of coronavirus disease 2019 (COVID-19), which has become a public health emergency of international concern[1]. Angiotensin-converting enzyme 2 (ACE2) is the cell-entry receptor for severe acute respiratory syndrome coronavirus (SARS-CoV)[2]. Here we infected transgenic mice that express human ACE2 (hereafter, hACE2 mice) with SARS-CoV-2 and studied the pathogenicity of the virus. We observed weight loss as well as virus replication in the lungs of hACE2 mice infected with SARS-CoV-2. The typical histopathology was interstitial pneumonia with infiltration of considerable numbers of macrophages and lymphocytes into the alveolar interstitium, and the accumulation of macrophages in alveolar cavities. We observed viral antigens in bronchial epithelial cells, macrophages and alveolar epithelia. These phenomena were not found in wild-type mice infected with SARS-CoV-2. Notably, we have confirmed the pathogenicity of SARS-CoV-2 in hACE2 mice. This mouse model of SARS-CoV-2 infection will be valuable for evaluating antiviral therapeutic agents and vaccines, as well as understanding the pathogenesis of COVID-19.



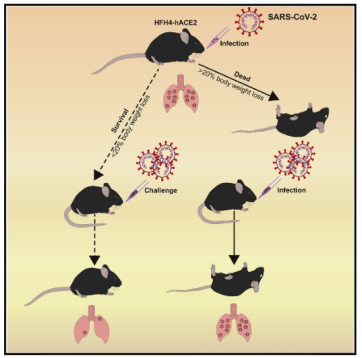
July 2020

Cell

Article

Pathogenesis of SARS-CoV-2 in Transgenic Mice Expressing Human Angiotensin-Converting Enzyme 2

Graphical Abstract



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In Brief

A SARS-CoV-2 hACE2 transgenic mouse infection model recapitulates a number of infection symptoms and pathology in COVID-19 patients. Pre-exposure to SARS-CoV-2 was able to protect mice from severe pneumonia.

Highlights

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- SARS-CoV-2 infection localizes to lungs of mice and causes typical interstitial pneumonia
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All Covid-19 vaccines currently in use were developed with the help of GM mouse models.

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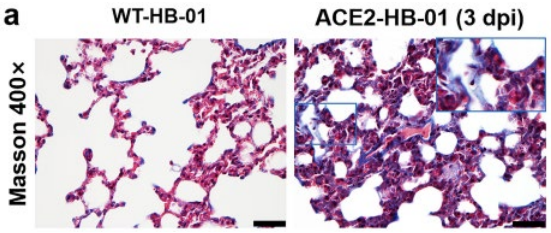

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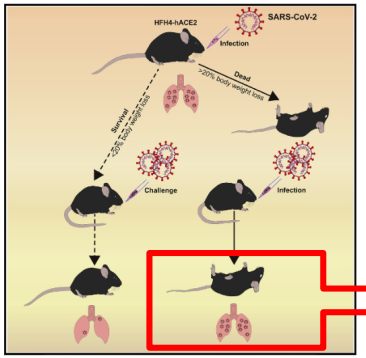

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Final fate of most laboratory animals

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All Covid-19 vaccines currently in use were developed with the help of GM mouse models.

Two main classes of euthanized laboratory animals

1. Laboratory animals **in experiment**

Predetermined number of animals used (and euthanized) under an animal experiment license

License application granted only if:

- No viable alternative exists to animal experiments
- Balance of interest evaluation shows scientific gains outweigh the burden placed on animals
- Experimental design is sound - statistically meaningful data obtained with lowest number of animals
- Effort is made to alleviate pain and stress (anesthesia/analgesia, social groups, humane end points)
- Annual and final reports account for all animal used and euthanized during the experiment

2. **Surplus** laboratory animals

Animals bred (and euthanized) to generate experimentall animals

- Its estimated that surplus mice outweigh the experimental mice by ~3-4:1
- Most surplus mice are a consequence of breeding and using GM mice

Why can't we avoid surplus animals in GM breeding?

How can we reduce animal surplus?

What are surplus laboratory animals?

Laboratory animals that can’t be used but are unavoidably generated

What are laboratory animals?

A correctly designed experiments aims
to eliminate all variables except for the one being tested.
This also includes genetic variability animals (mice).

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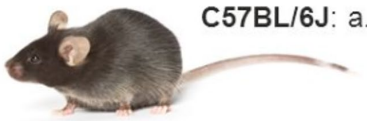
A lab mouse is a model organism that is very different form its wild ancestor.

Inbred laboratory mouse strains

all mice within a strain are genetically identical
homozygous across the genome
reproducible models of disease

A/J: high incidence of spontaneous lung adenomas

C3H/HeJ: high incidence of spontaneous hepatomas



C57BL/6J: a.k.a. "The Mouse"

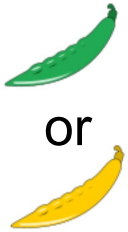
The Jackson Laboratory

Natural laws apply also to laboratory mice.

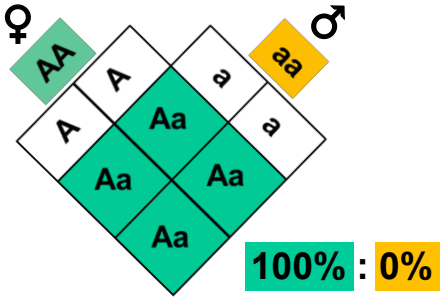
Some key rules of genetics.

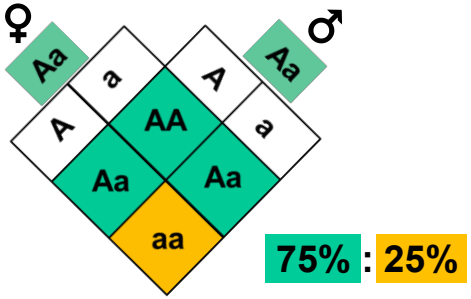
Mendelian Inheritance

Gregor Mendel



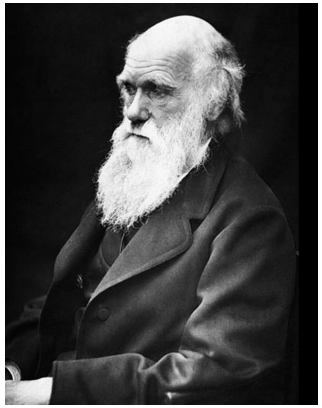
Each trait (gene) is present as two alleles but only dominant trait (**A**) is manifested.
Only one of these traits is passed to the offspring in a random manner.





Evolution

Organisms change and species evolve trough natural selection of traits.



Charles Darwin

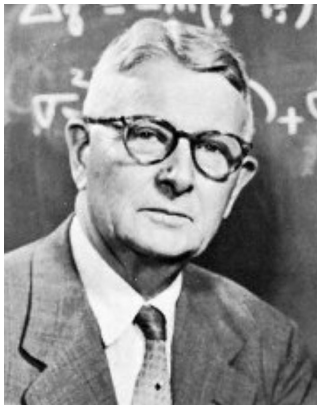
Mutations

Novel traits can arise by mutation.

Hugo de Vries

Genetic Drift

New mutation can spread rapidly in small populations.



Seawall Wright

These rules apply to peas, humans and laboratory mice…

What does this mean in the laboratory?

typical experiment

One genetic variable (**AA** vs **aa**).

Same sex, same age, same environment, same test agent (drug?).

How to generate GM experimental groups?

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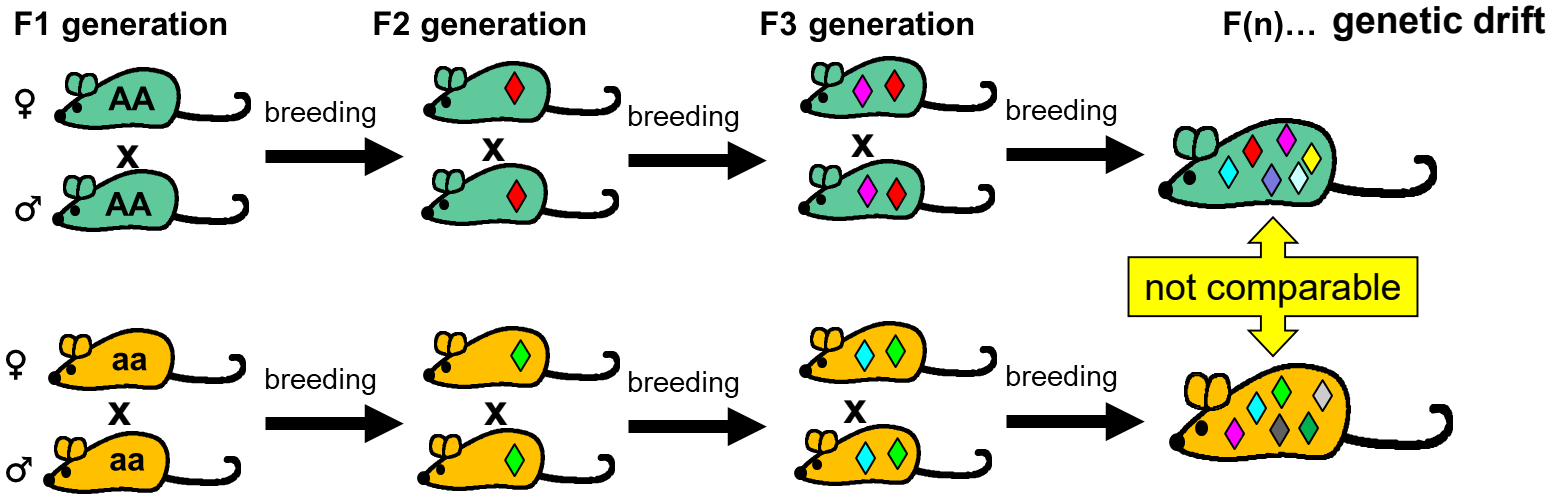


genetic drift

Every generation of breeding introduces 30-50 random, **heritable** mutations.
Statistically, at least one ♦ mutation/generation will have a deleterious effect.

Pure breeding GM lines diverge rapidly due to mutations and genetic drift typical for small populations.

For most applications, we can't design meaningful experiments that compare pure breeding GM lines.

Genetic drift is not an imaginary problem

Even the expert like **NIH** and **Jackson labs** could not manage to prevent their **C57BL/6** strains from genetically diverging.

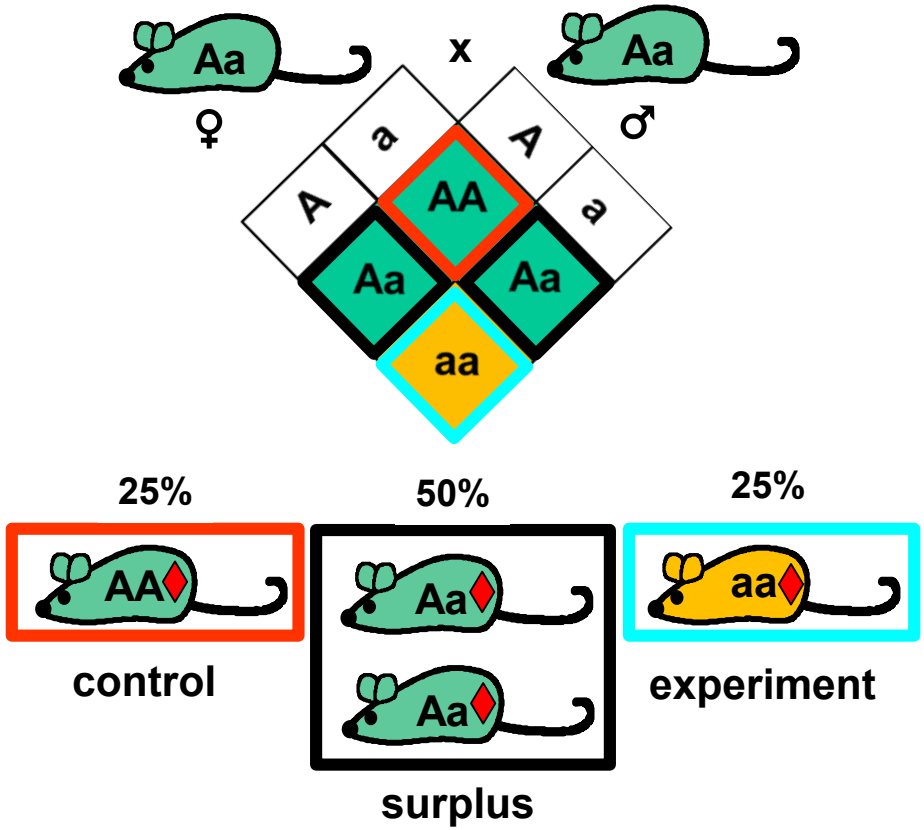
1. Mice on the C57BL/6N genetic background carry the *Crb1<rd8>* mutation.

Photoreceptor degeneration is observed in spots, caused by retinal folds and pseudorosettes, found in the fundus of the eye of all vendor lines of the C57BL/6N line (Mattapallil et al. 2012). The C57BL/6J strain does not carry the *rd8* mutation.

2. C57BL/6J mice have a mutation in the Nnt gene that may affect metabolism.

Nicotinamide nucleotide transhydrogenase (*Nnt*) gene, located on mouse chromosome 13, encodes a mitochondrial protein involved in β-cell mitochondrial metabolism. C57BL/6J mice have a naturally occurring in-frame five-exon deletion that removes exons 7–11 and results in **inappropriate glucose homeostasis** in male B6J mice. B6J mice have a normal life span and actually have a robust weight gain **and develop obesity and insulin resistance on a high fat diet** (Nicholson, et al. 2010). C57BL/6N lines do not have this mutation.

Generation of genetically identical groups has a cost.

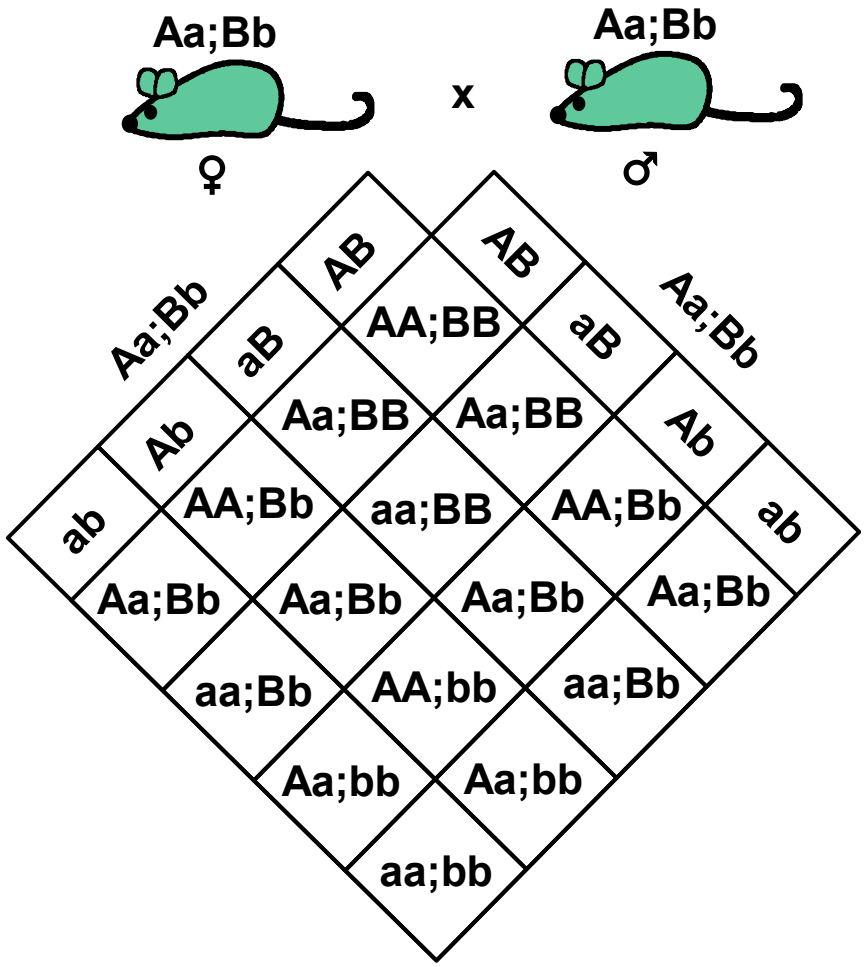


Advantage – Generation of experimental cohorts by Aa x Aa intercross
ensures genetic homogeneity and identical age of the animals.
Any genetic variation♦ that results form the breeding will be found in all the offspring.
These are key factors in the experimental design.

Disadvantage – this breeding scheme inadvertently produces
animals that can't be used in an experiment.

Additional alleles increase breeding complexity

Example – (**Aa;Bc x Aa;Bb**) intercross of a two-transgene GMO line



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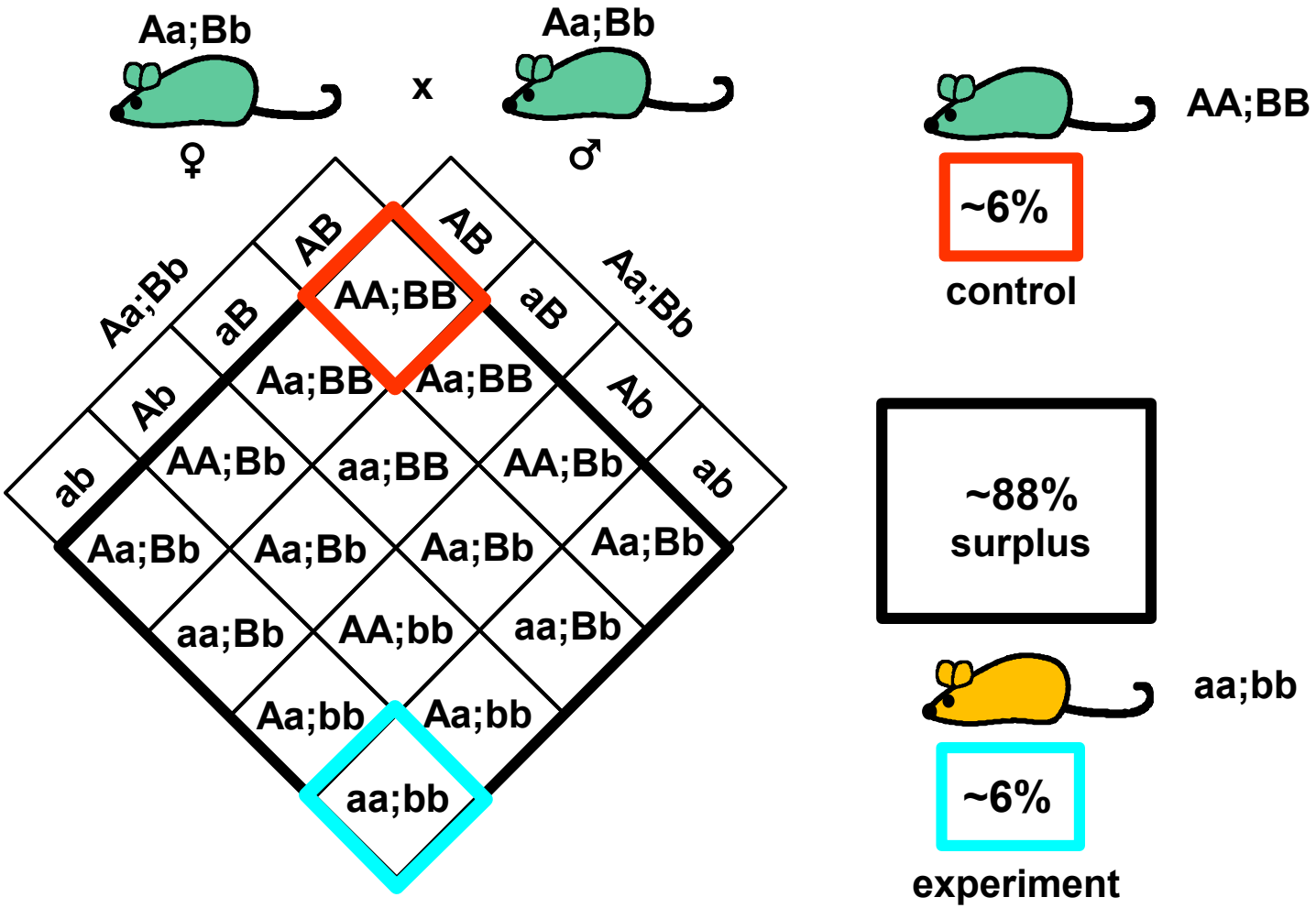

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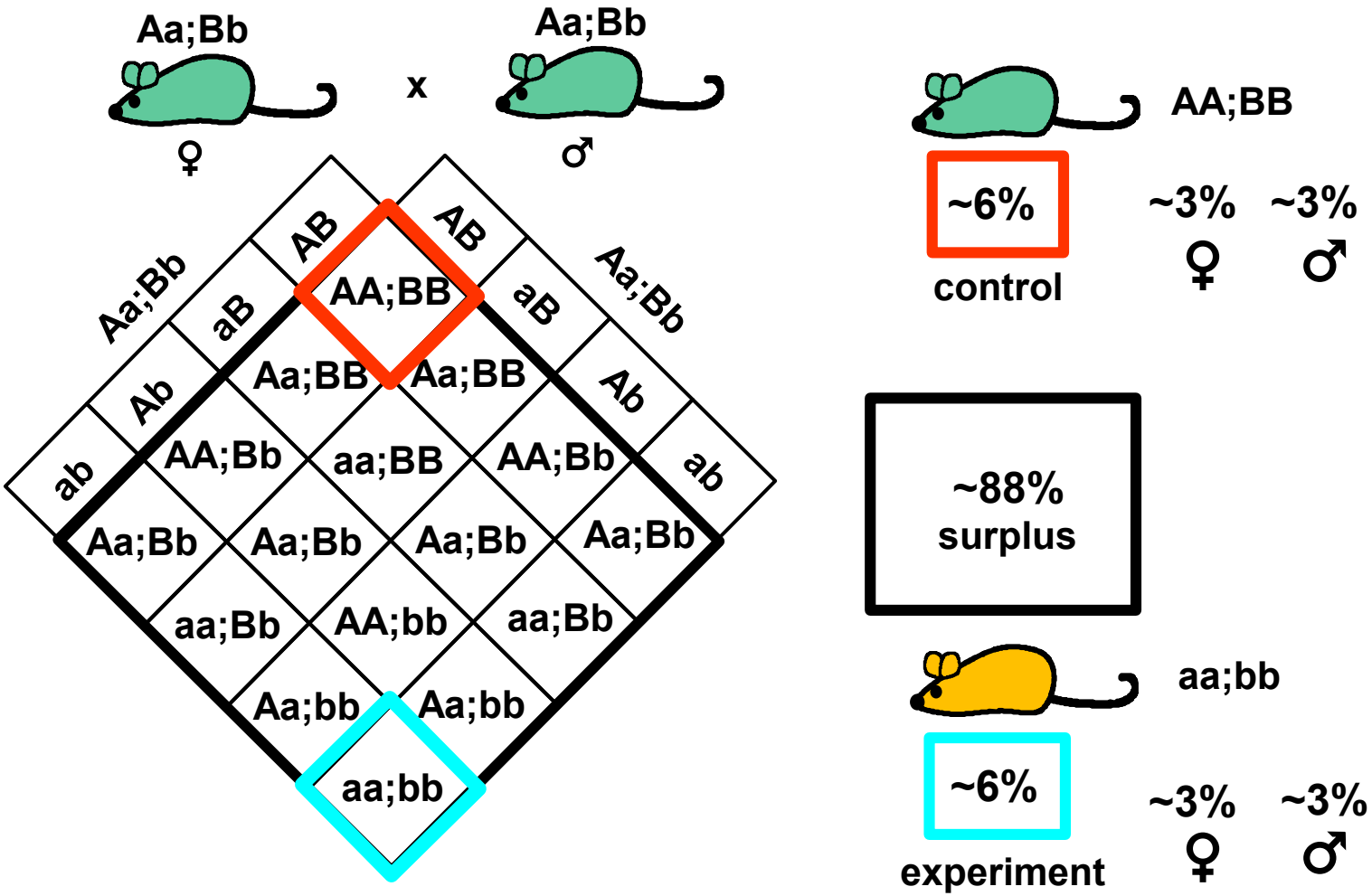

What can be done?

What can be done?

1. Animal experiments are always the last resort, they need to be licensed and therefore well justified.
2. Routine application of 3R (reduce, refine, replace) principles in the experimental design.
3. Optimal (centralized?) planning of breeding schemes for dominant and recessive traits that minimizes the generation of unwanted genotypes but still allows the generation of meaningful data (statistical power!).
4. Experimental design that uses both sexes in the experimental groups.
5. Sharing of unwanted animals with other researchers, zoo donations, rehoming.
6. Cryopreservation of lines to reduce unnecessary “maintenance breeding”. This approach also prevents mutations and genetic drift.
7. Use of new genome engineering technologies (**CRISPR/Cas9**) to add additional genetic traits on preexisting transgenic background without additional breeding.
8. Adaptation of new genome engineering technologies (**CRISPR/Cas9**) to generate experimental groups without breeding.

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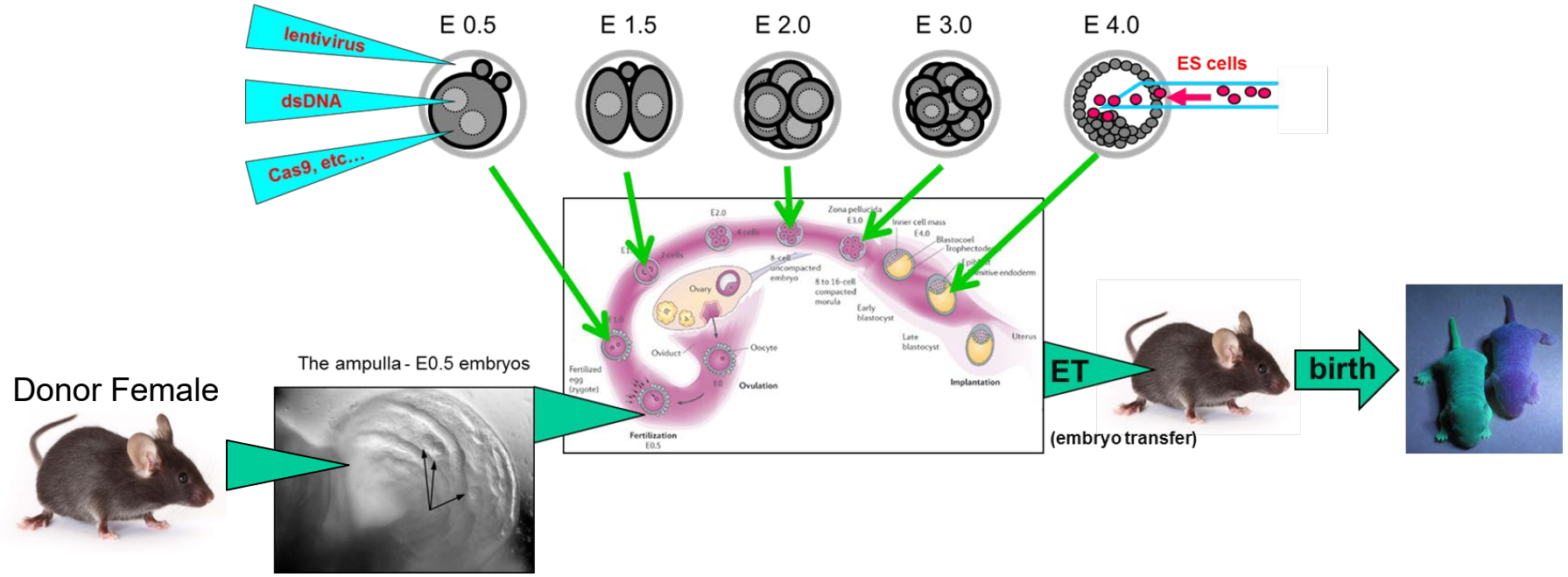
How to make a transgenic mouse ?

Target cell – the preimplantation embryo



The technical aspects were developed in the 1980s and remain largely unchanged.

Definitions

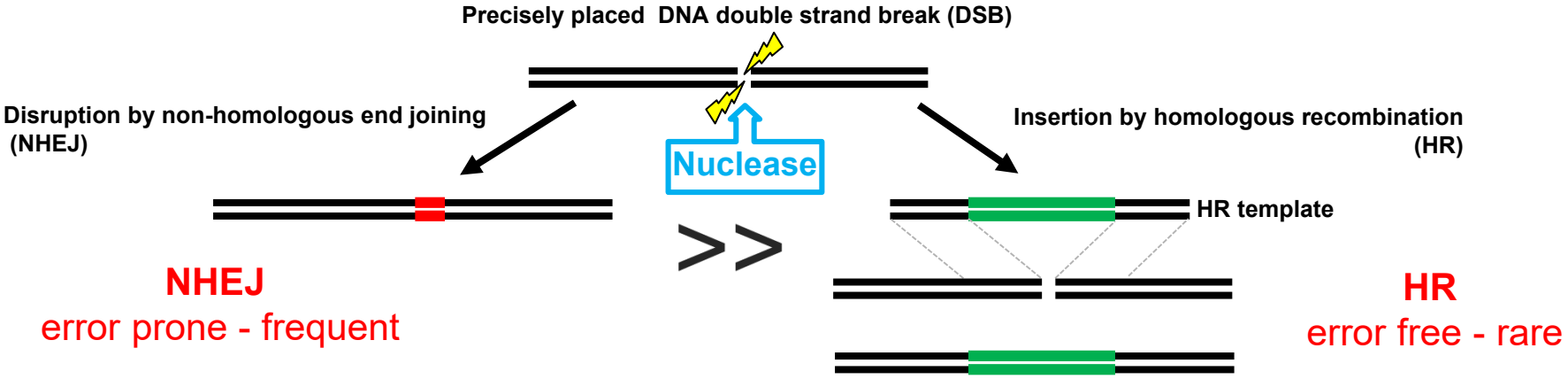
Transgenic organism

contains a gene which has been artificially inserted instead of acquiring it through reproduction

Genetically modified organism (GMO)

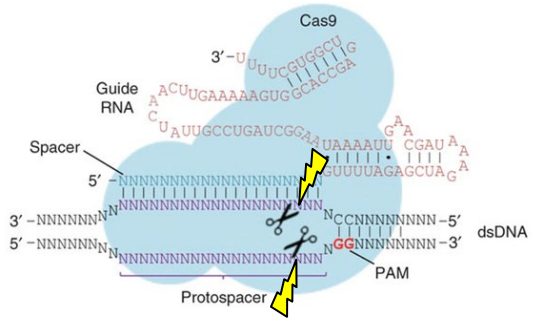
an organism whose genetic material has been altered using genetic engineering techniques

CRISPS/Ca9 systems for genome engineering



Cas9 creates the DSB – host repair does "genome engineering"

Cas9/CRISPR

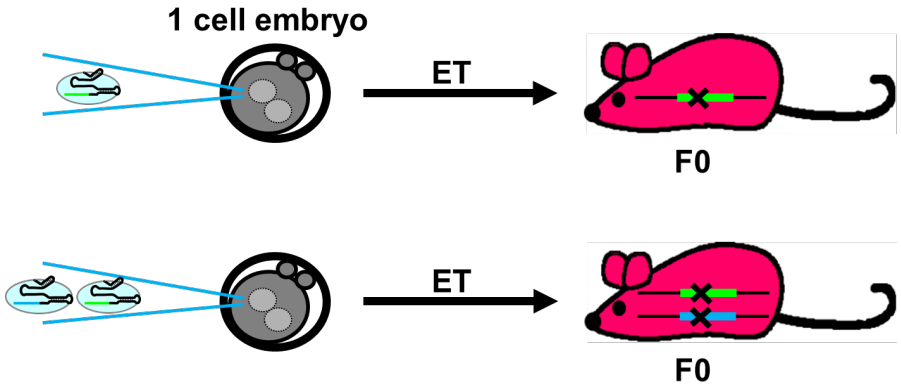


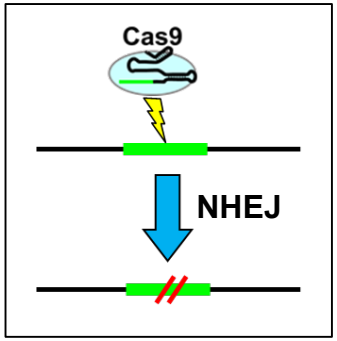
Cas9 RNPs are easy to generate and use.

Multiplexing with multiple gRNAs possible.

Gersbach et al., 2014

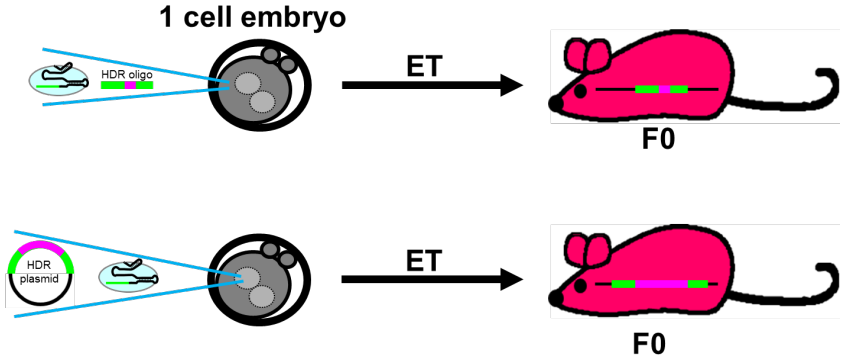
Generating knock-outs with Cas9/CRISPR

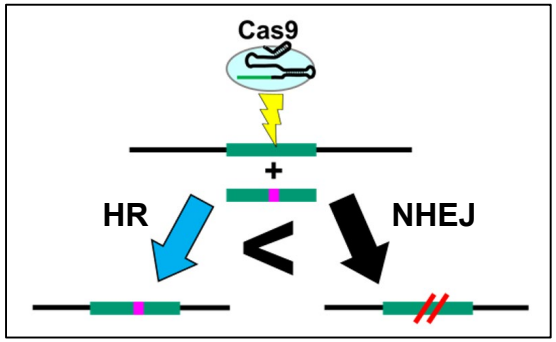




targeting efficiency up to 90% *Wang et al., 2013*

Generating knock-ins with Cas9/CRISPR



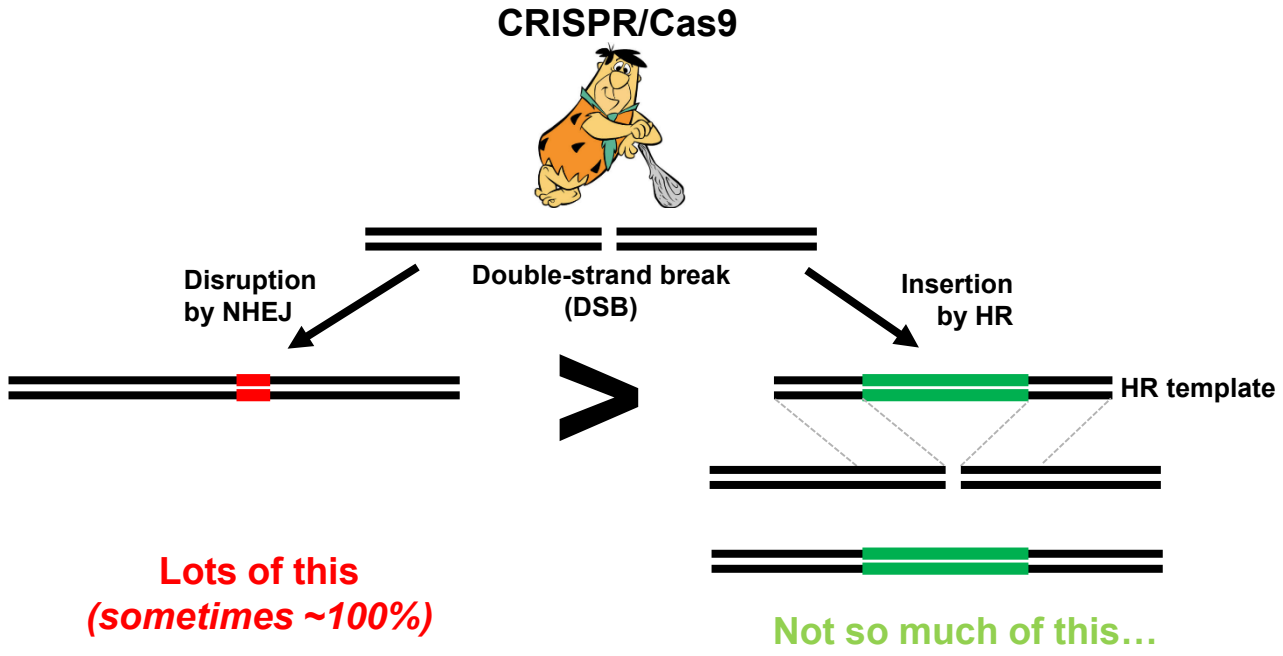


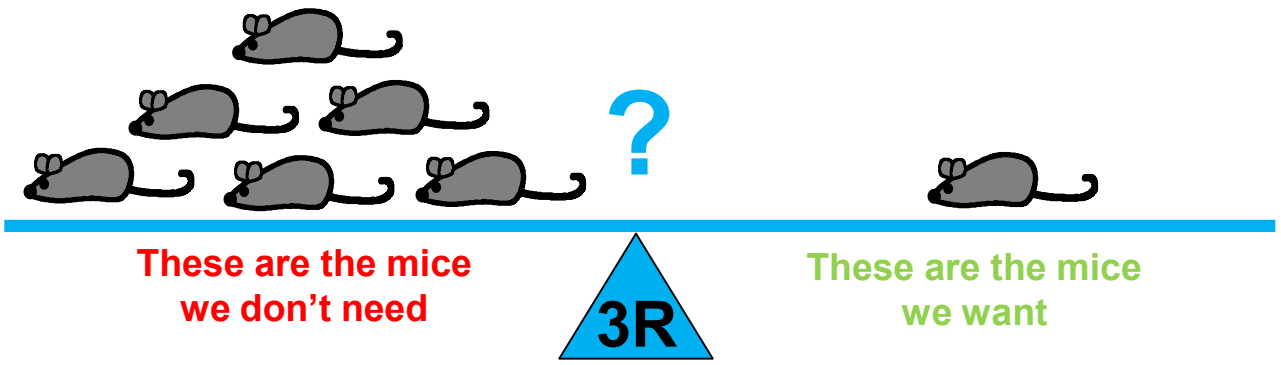
knock-in efficiency (~0-50%) *Wang et al., 2013*

Main challenge of genome engineering

Genome Editing vs. “genome destruction”

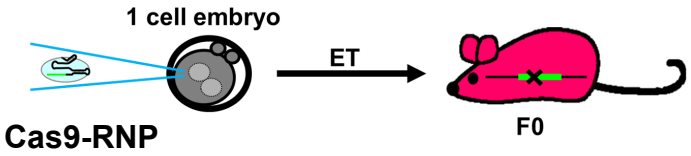
Much of what currently passes for “genome editing” should really be called “genome vandalism” (*G.Church)*

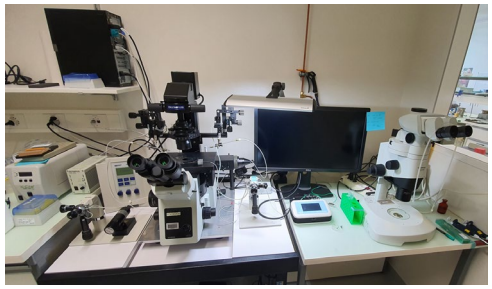




Solution 1 – make lots of KO mice (they are easy to make)

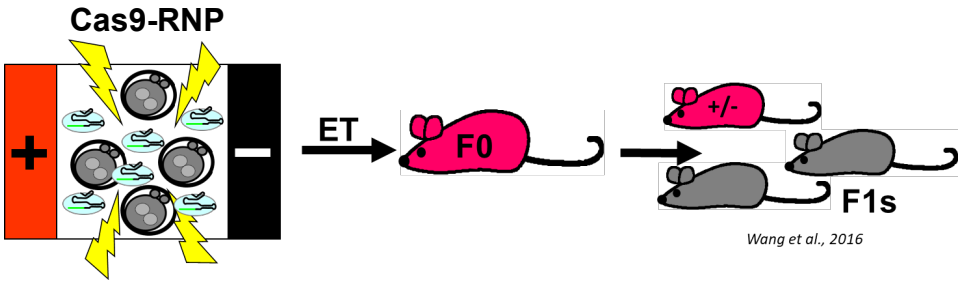
zygote microinjection

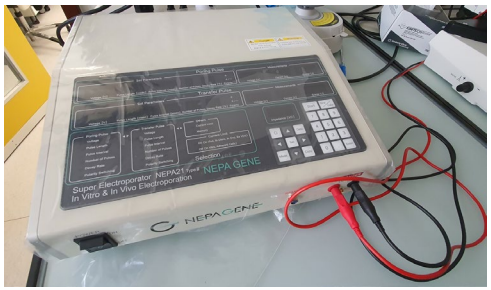




OR

zygote electroporation





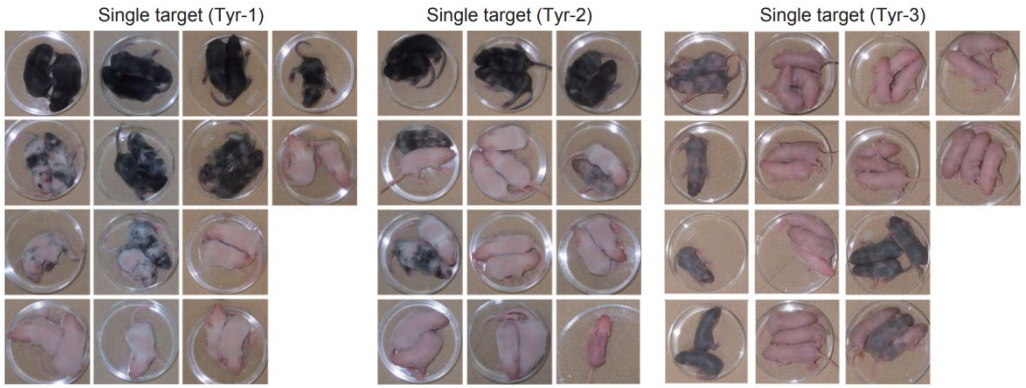
Do we really need to generate and breed KO mouse lines?

Can we increase KO efficiency so no lines need to be established and bred?

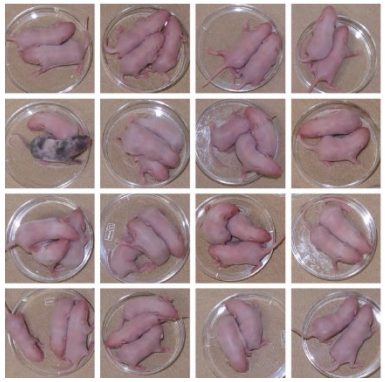
Triple-CRISPR - F0 mice as experimental cohorts

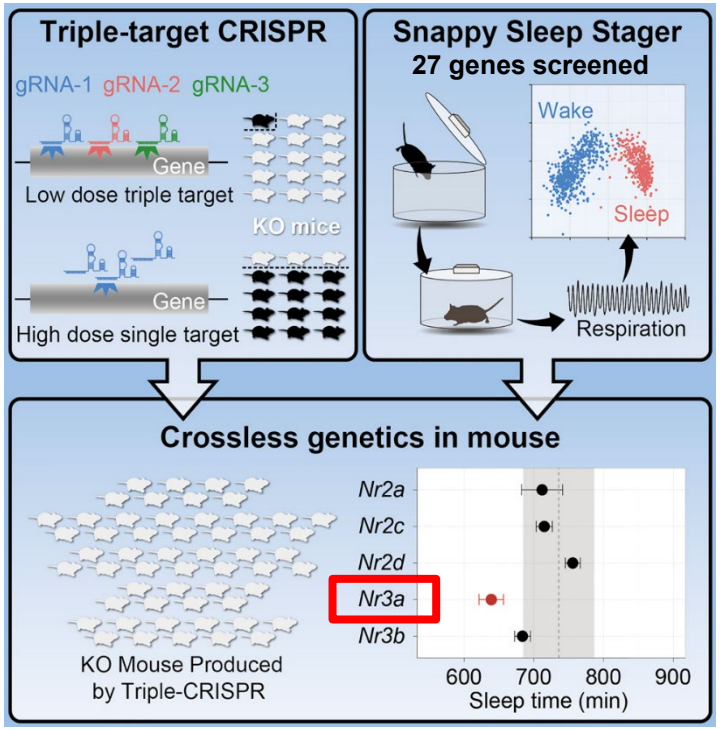
Sunagawa et al., 2016

Tyrosinase (Tyr) – coat color gene



Triple target (Tyr-1, 2, 3)





At least 95% of mice are deficient in both alleles.
Up to 3 genes can be targeted simultaneously.

Works only with non-essential genes.
No tissue specificity.

Well suited for screening and exploratory approaches

Additional alleles increase breeding complexity

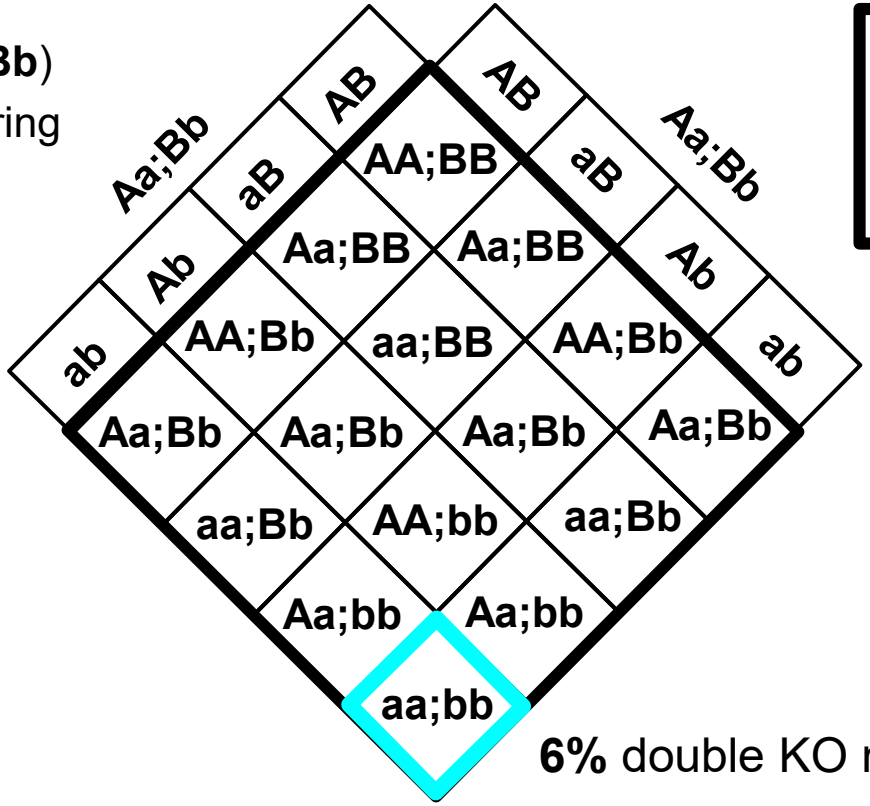
These rules also apply for generating complex GMO lines

Example – Intercross of intercross two GMO lines (**aa;BB x AA;bb**)

Aim – generation of an (**aa;bb**) double KO line

1st cross – (**aa;BB x AA;bb**)
All offspring (**Aa;Bb**) **–** all useful

2nd cross – (**Aa;Bb x Aa;Bb**)
6% of useful (**aa;bb**) offspring

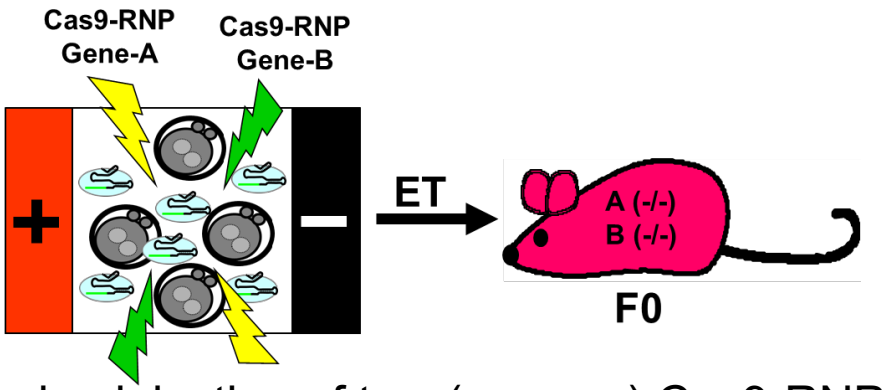


~94% surplus

6% double KO mice

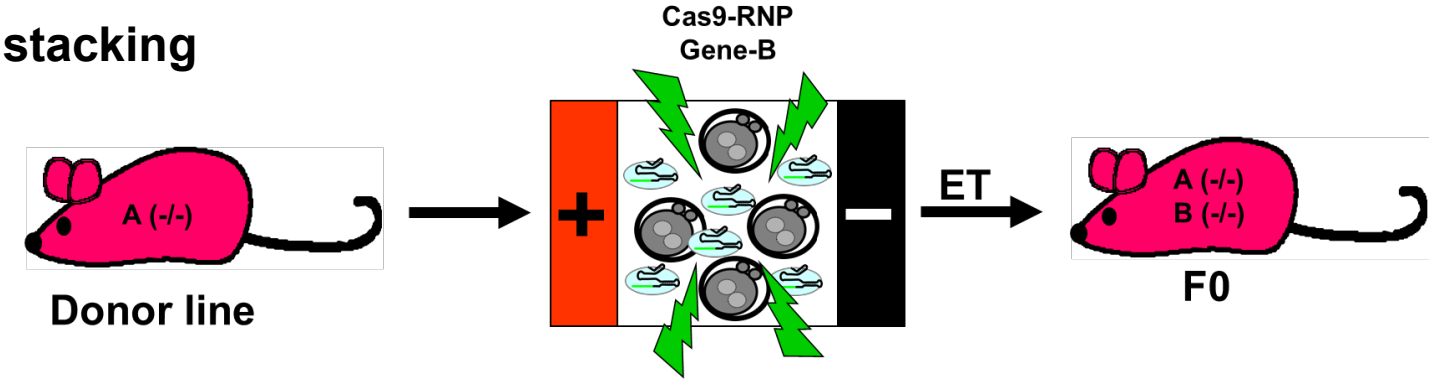
CRISPR/Cas9 can reduce surplus breeding of complex GMO lines

multiplexing



Co-electroporation or co-microinjection of two (or more) Cas9-RNPs allows targeting multiple genes simultaneously

locus stacking



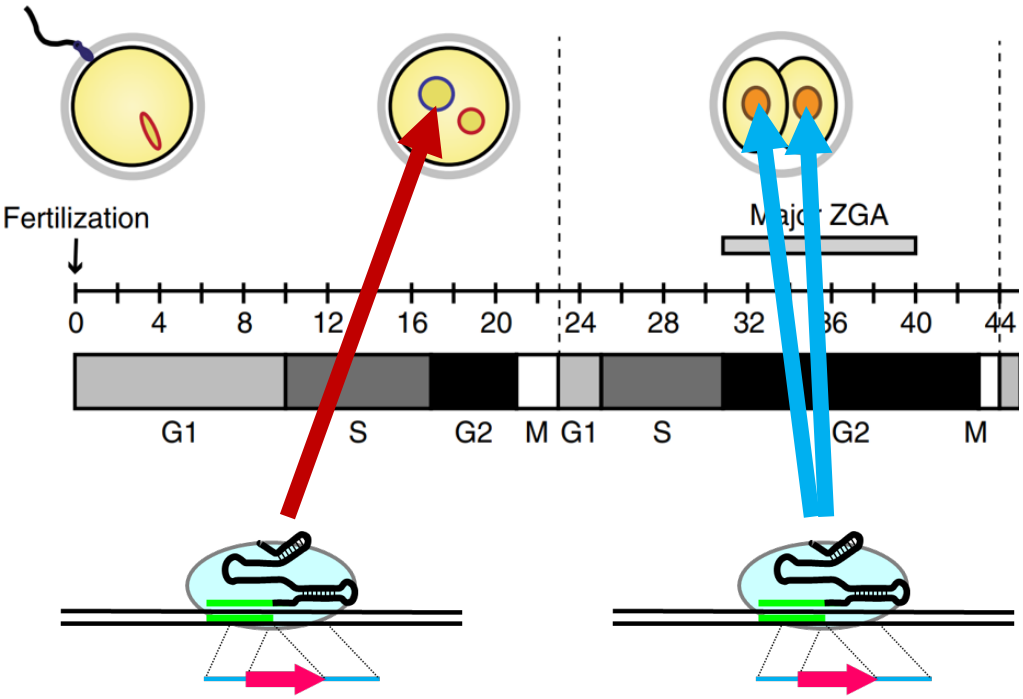
Co-electroporation or co-microinjection of Cas9-RNPs into embryos from an established GMO “Donor” line allows addition of new GMO traits without (or with less) breeding

Solution 2 – make knock-in mice more efficiently

1. Reduce animal numbers by using fewer mice to establish a KI-line.
2. Increase KI efficiency so no lines need to be established and bred.

Increasing the precision of genome engineering

Increasing HR recombination rates



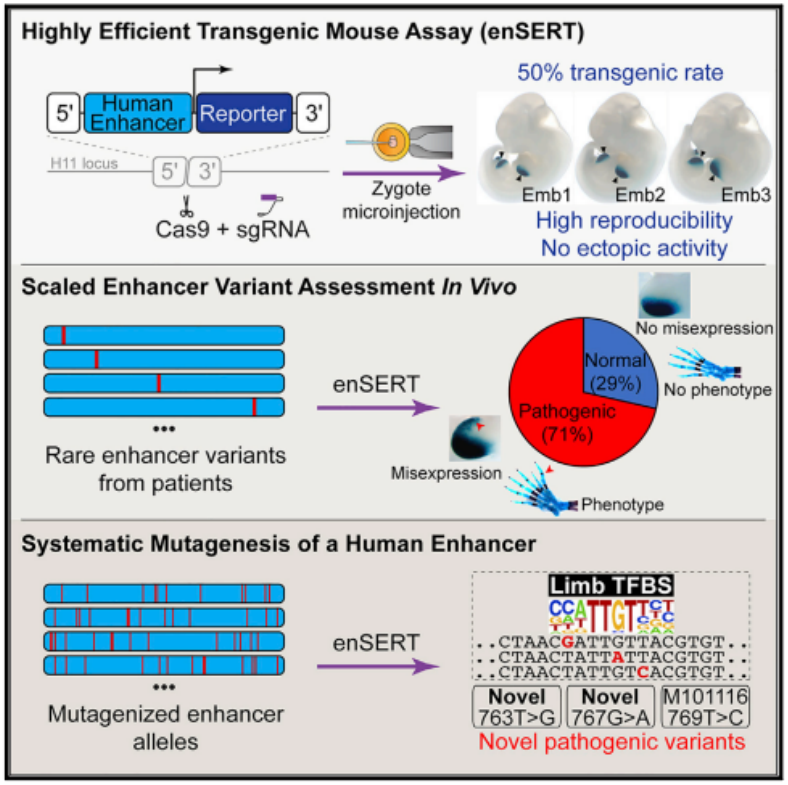
	Cas9 Zygote injections	Cas9 2-cell injections
HR efficiency	**0.7 - 6.5%**	**11 - 53%**
	+	+++

Gu et al., 2018

NHEJ-mediated in/dels are still a possible (major) outcome

Do we need to generate stable KI mouse lines ?

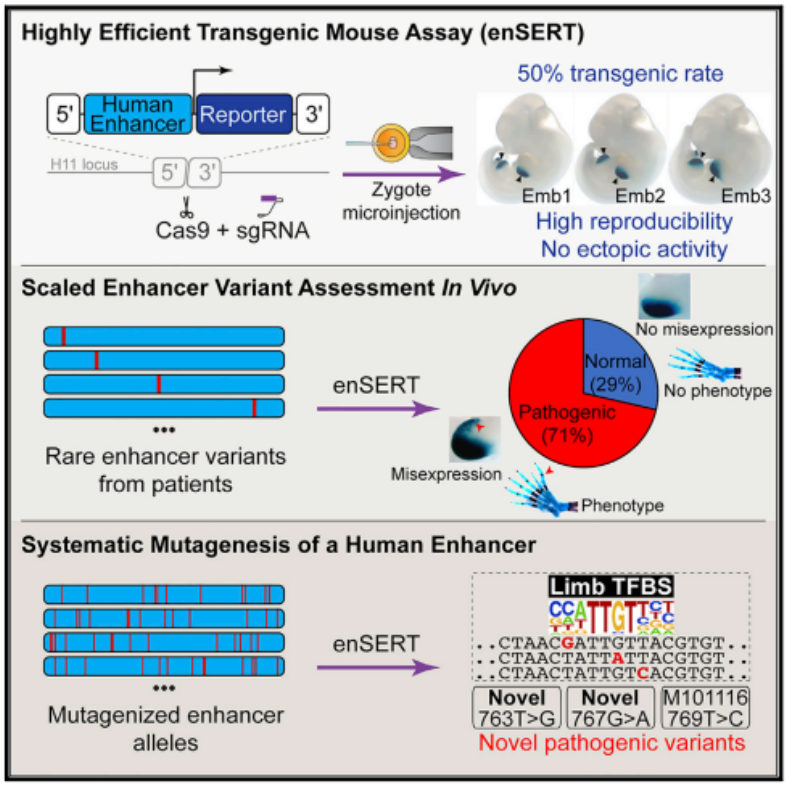
enSERT — a highly efficient CRISPR/Cas9-mediated site-specific transgenic mouse assay



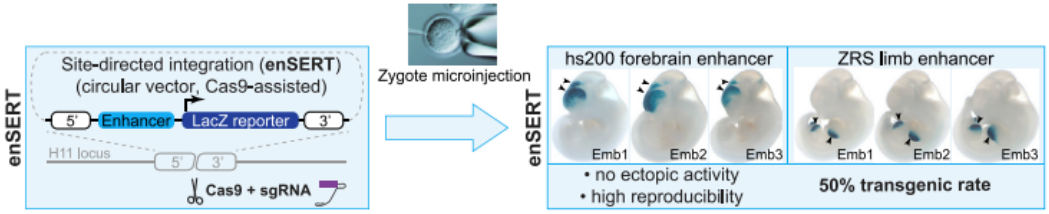
Kvon et al., 2020

Do we need to generate stable KI mouse lines ?

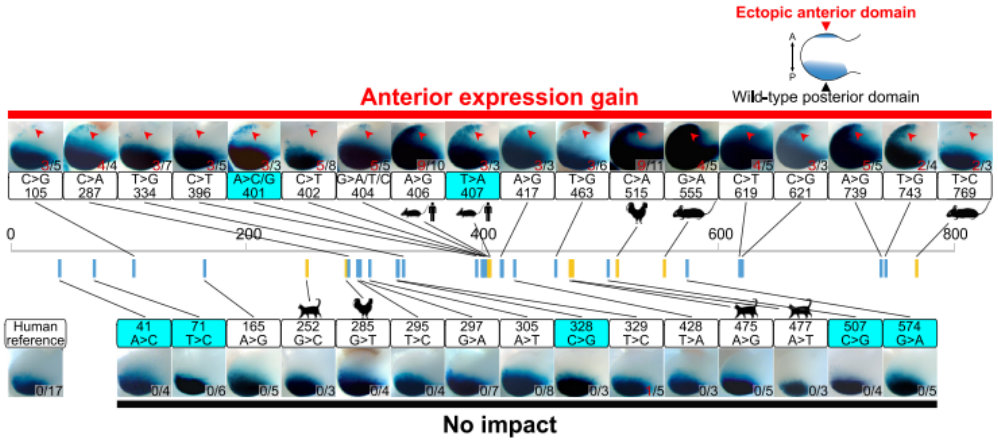
enSERT — a highly efficient CRISPR/Cas9-mediated site-specific transgenic mouse assay



Kvon et al., 2020



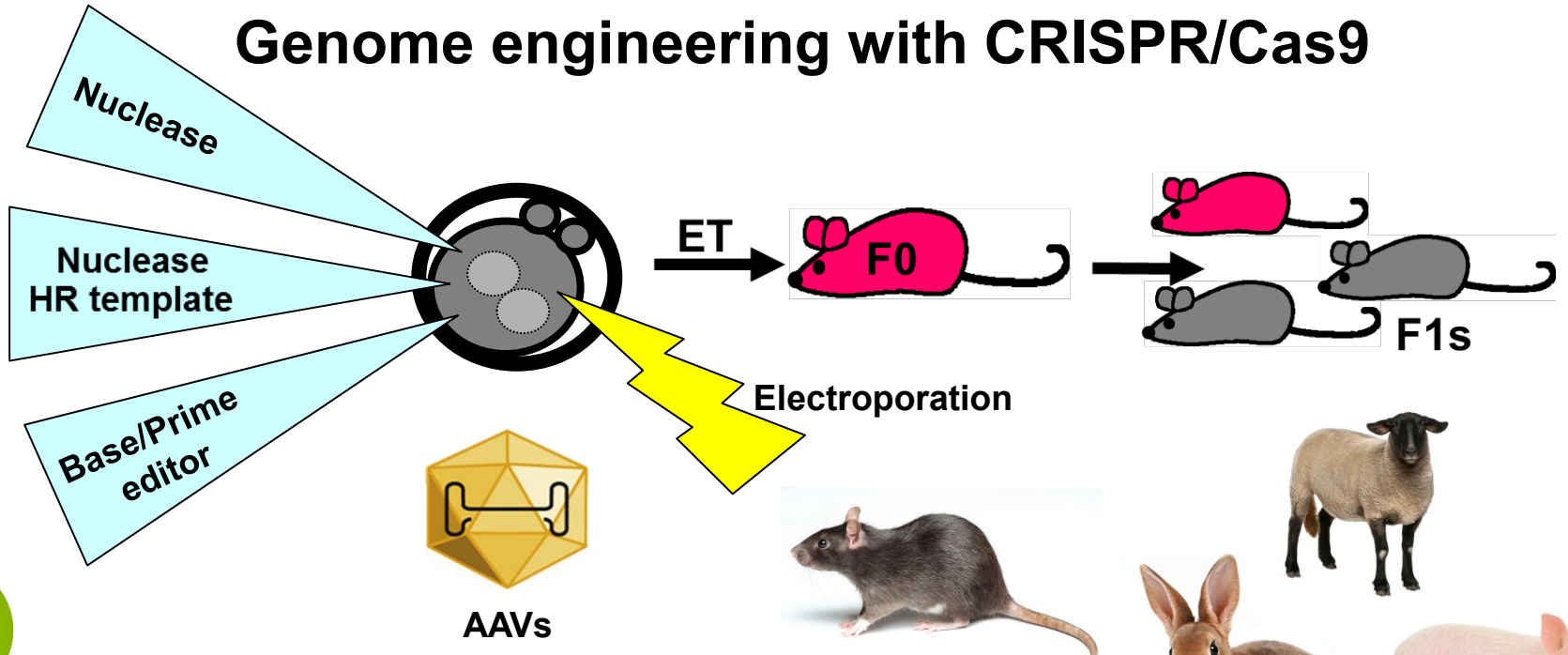
enSERT has a higher insertion rate and does not show position effects.



When applicable, enSERT facilitates testing of multiple reporter variants **without generating stable mouse lines.**

Conclusions

Genome engineering with CRISPR/Cas9



- Fast and Simple
- Targeted gene disruption possible
- Targeted transgene integration possible (no need for selectable markers)
- Precise mutations possible (no need for selectable markers)
- Works in many species and strain backgrounds
- Reagents are easy to use and readily available

Efficiency and absolute control of modification remain a challenge

Final thoughts on CRISPR/Cas9 genome editing

New genome engineering methods allow for virtually unlimited and rapid manipulation of genomes enabling generation of highly customized transgenic and GMO animal models (not only mice). Constant improvements strive to increase efficiency and specificity of these methods.

Potential 3R impact

Will more animal models be used?

accessibility – lower barrier to entry will increase the demand for new models.

genotyping – gene modification is carried out in mouse embryos, hence initial genotyping is performed directly on pups (not ES clones). Desired modification are sometimes rare.

Will less animal models be used?

sophistication – better models, fewer founders animals needed, fewer lines per construct needed, smaller experimental groups. Use of F0 cohorts (Triple-CRISPR, enSERT) can make excessive breeding redundant.

rapidity – on demand model generation in desired genetic background, less backcrossing and maintenance breeding needed.

How do we define surplus animals?

Animals that can't be used but are unavoidably generated.

This definition scientifically justified. It is also easy to defend as an integral part of good experimental design.

Animals that could have been used but were generated and not used.

Breeding of mice is not an exact science. Even in best case, one needs to over calculate to account for unforeseen events that will affect mouse breeding. There is also an issue of "preemptive breeding" to meet deadlines and anticipate reviewers comments (publications, grants). This issue is an intrinsic aspect of current research culture.

"all the animals used/euthanized" **minus** "animals used under license".

This definition accounts for mice from the two definitions above plus all the mice that are bred solely as "backups" or for the purpose of "maintaining the line". Not clear how widespread this practice is but it is also the one that can be easily reduced by eliminating redundancies. For example - the same mouse line does not need to be maintained by multiple labs, and the same transgenes/mutations do not need to be maintained in multiple mouse lines. Lines can also be cryopreserved as embryos or sperm for long term storage to minimize unnecessary breeding. Thy can also be rapidly generated *de novo* using new genome engineering techniques (tripleCRISPR, enSERT, AAVs, etc…)