# Genetic modification of the mouse

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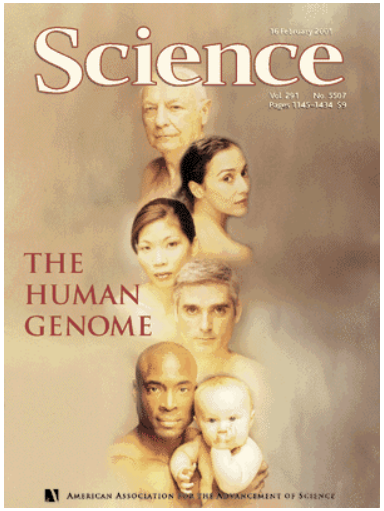
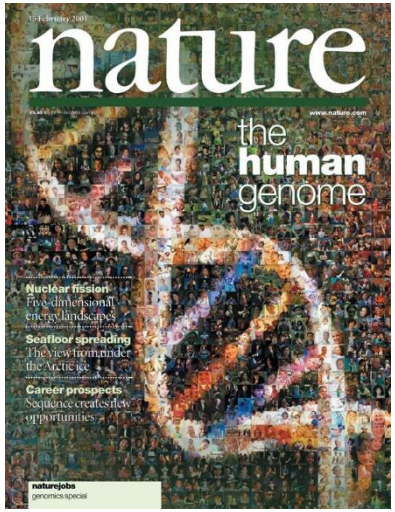
# Genomic routes to understanding biology

- We already knew of the role of certain genes within the genome due to effects of disruptions in these genes
  - Human genetic disease
  - Studies on Knock-out animal models



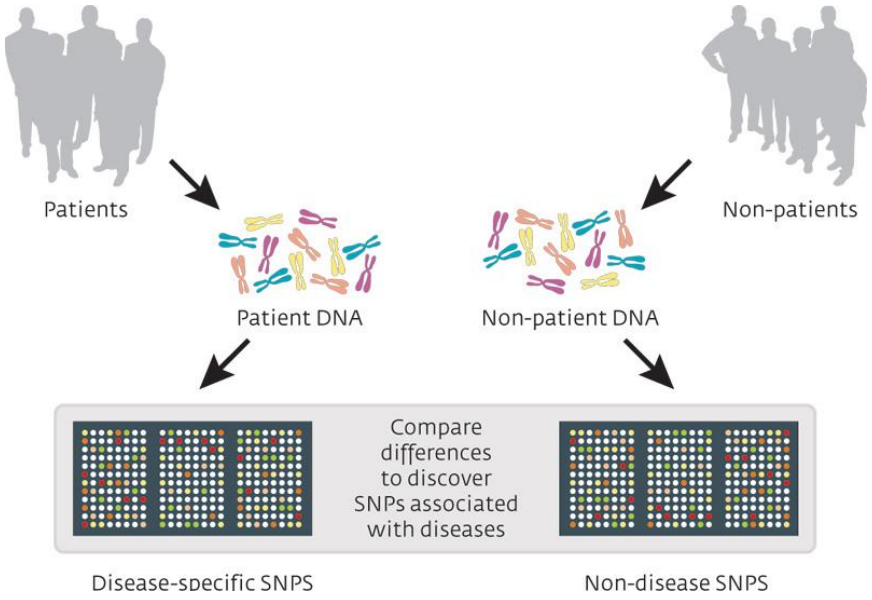




- In 2001 we were presented with a catalogue of genes in our genome
- The vast majority were/are of unknown function

- Suddenly, we were presented with a huge task of attributing function to these genes
- Which genes could be contributing to genetic components of disease ?
- Which gene products could be future targets for therapeutics ?

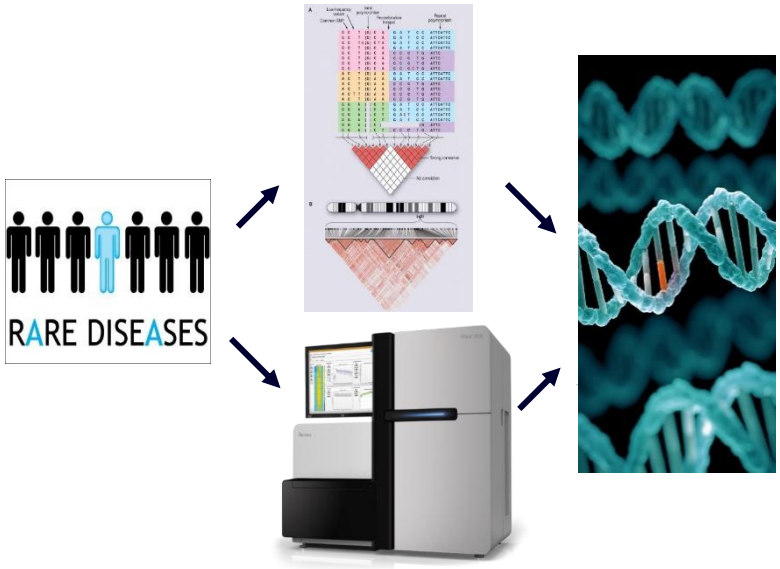
# Genetic Association studies

- In many complex diseases (cancer, diabetes, cardiovascular disease, neurodegeneration), there are many factors which contribute to the disease
- Environment and lifestyle are critical  issues, but there is often a strong genetic component
- Why is that certain individuals are more susceptible to a particular diseases?
- Genetic variation and the prevalence of sequence variants must provide the explanation




- Using simple genotyping by looking at common genome variants (SNPs) and comparing patients vs controls, allows statistical methods to be applied which highlight particular loci or even particular SNPs as being criticial

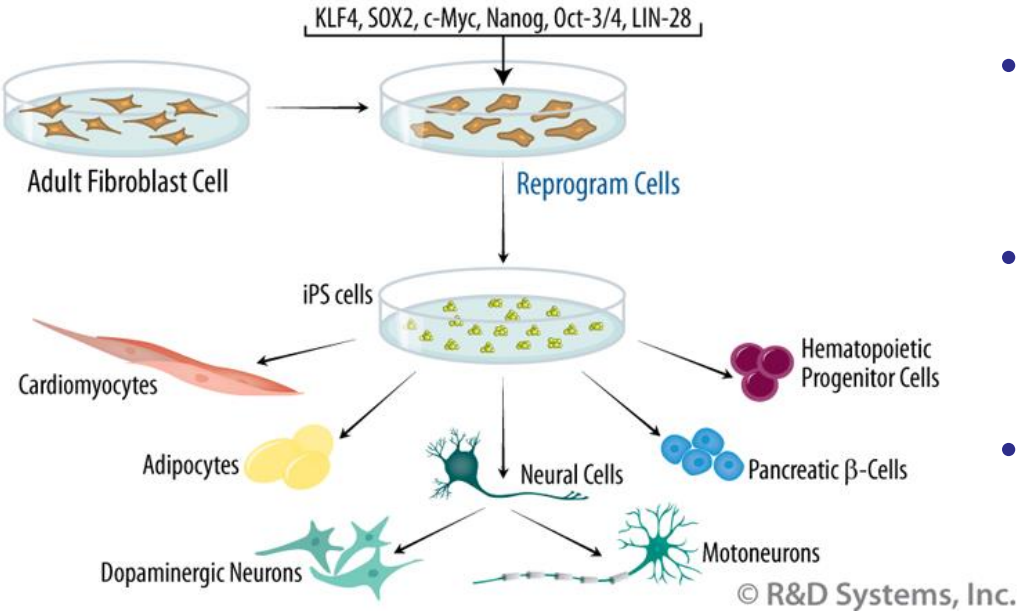
# Rare diseases




- Rare disease provide us with a phenotype
- Sequencing technologies can locate the causative mutation
- We then need a model system to explore the underlying biology

# How to study gene function – human patients?

- Patients with gene mutations can help us understand gene function
- Human's don't make particularly willing experimental organisms
- An observational science and not an experimental one
- Genetic make-up of humans is highly variable
- Difficult to pin-point the gene responsible for the disease in the first place



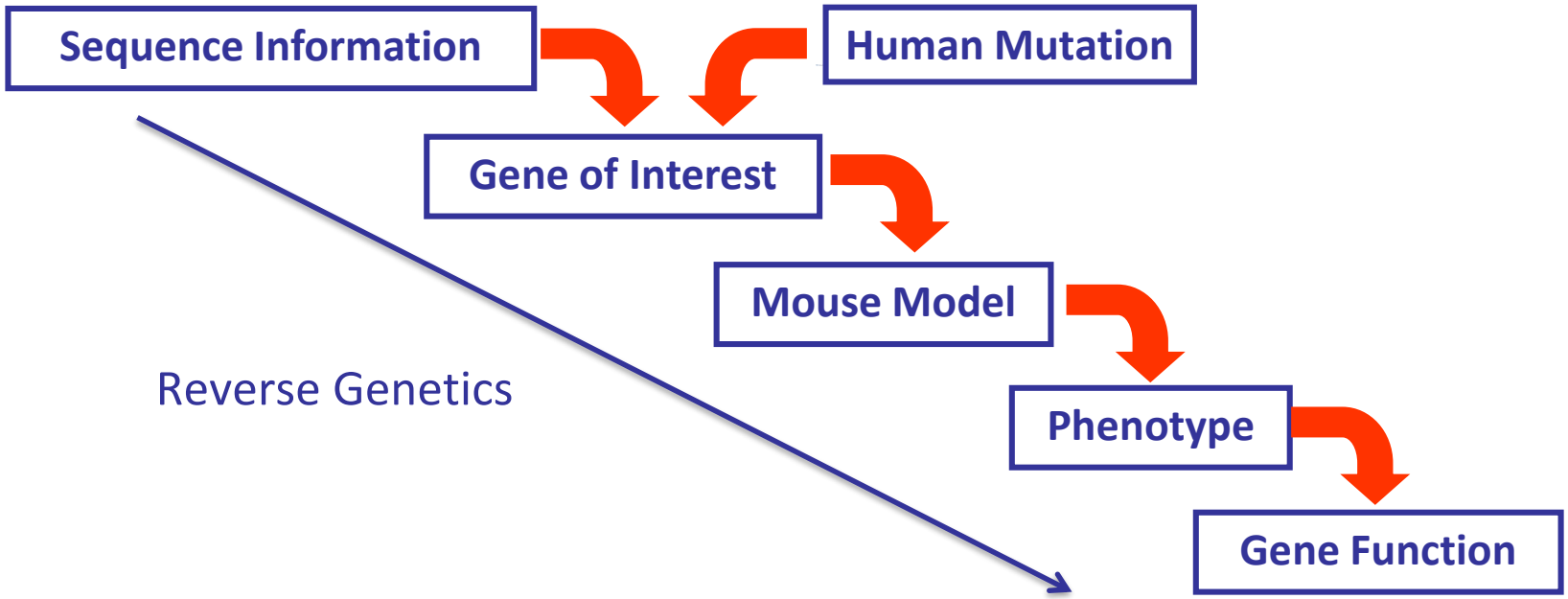

- Stem cell biology provides an experimental route using human material
- The in vitro cell types that can be generated are frequently more embryonic than adult
- But this is an exciting and emerging field.....

# Mouse patients

- Full ability to manipulate the genome experimentally
- Easy to maintain in the laboratory – breeding cycle is approximately 2 months
- Mouse and human genomes are similar in size, structure and gene complement
- Most human genes have murine counterparts
- Mutations that cause disease in human gene, generally produce comparable phentoypes when mutated in mouse
- Mice have genes that are not represented in other model organisms e.g. *C. elegans*, *Drosophila* – genes of the immune system

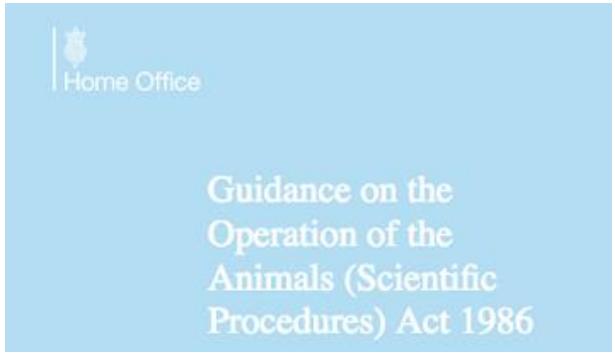
# Genetically modified mouse models

- We can manipulate the genome of a mouse to enable us to “reverse” the normal genetics of mapping function to genes
- We now have an experimental system to enable genes to be manipulated and then to observe the outcome in a whole organism



# Animal experimentation needs careful justification

- A cost-benefit analysis is essential before commencing an in vivo study
- The potential benefits must outweigh the impacts for the mouse




- Project license – defines the programme of work and performs the cost-benefit analysis, justifying any pain, harm or lasting suffering to the animals
- Personal license – defines the authority to perform animal experiments, outlining the training and competence

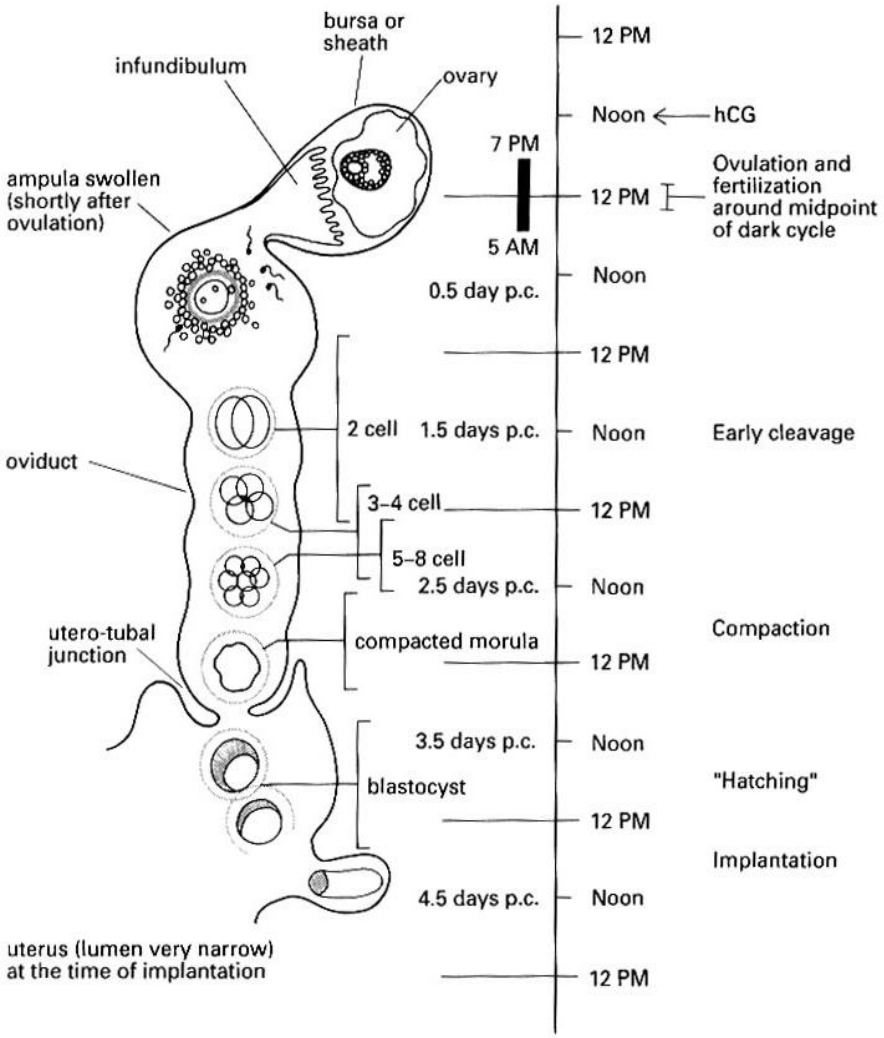
| | **Standard** | **Contemporary** |
|---|---|---|
| **Replacement** | Methods which avoid or replace the use of animals | Accelerating the development and use of models and tools, based on the latest science and technologies, to address important scientific questions without the use of animals |
| **Reduction** | Methods which minimise the number of animals used per experiment | Appropriately designed and analysed animal experiments that are robust and reproducible, and truly add to the knowledge base |
| **Refinement** | Methods which minimise animal suffering and improve welfare | Advancing animal welfare by exploiting the latest *in vivo* technologies and by improving understanding of the impact of welfare on scientific outcomes |

# What can I do with my gene of interest

- Gain of function
  - Overexpression of a gene of interest ➡ “Transgenic mouse”
  - Insertion of a gene of interest ➡ “Knockin mouse”

- Loss of function
  - Mutation of a specific gene of interest ➡ “Knockout mouse”
  - Overexpression of a mutant gene ➡ “Transgenic mouse”
  - RNA interference ➡ “Knockin mouse”

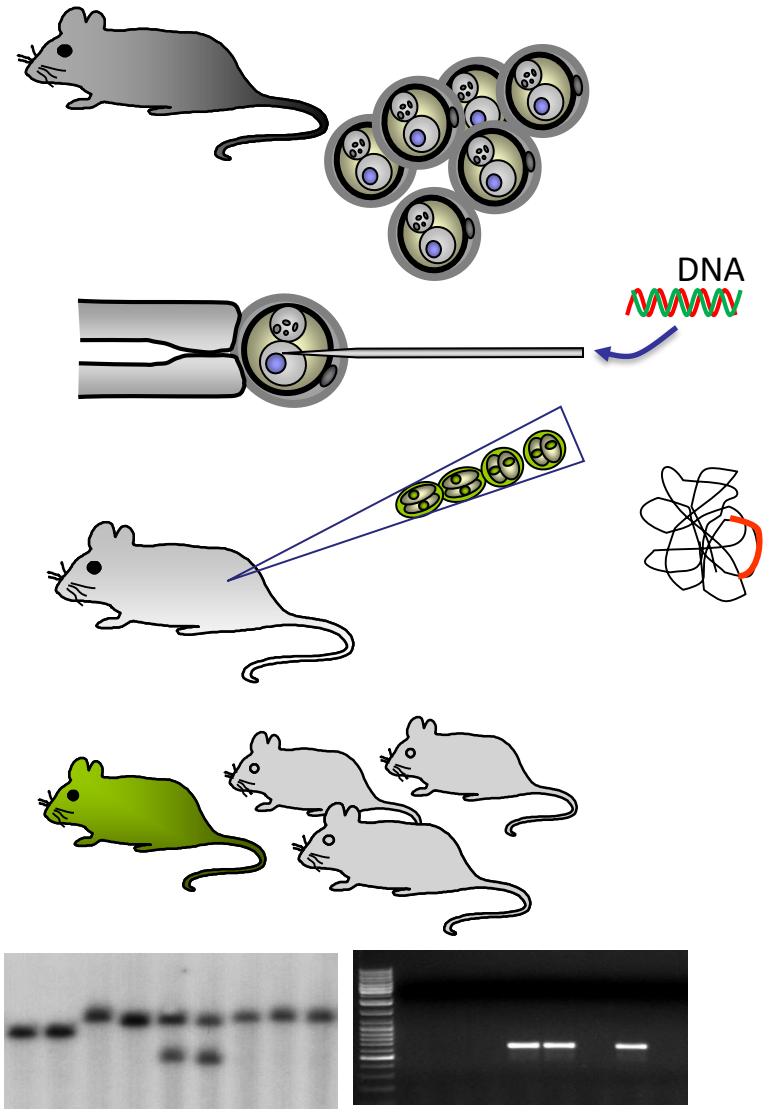
# Mouse pre-implantation Development




- The preimplantation development of the mouse provides us a window of opportunity to genetically manipulate the mouse

- Two stages of development are frequently used
  - Fertilized oocyte
  - Blastocyst

# Pronuclear Injection




Recovery of mouse zygotes

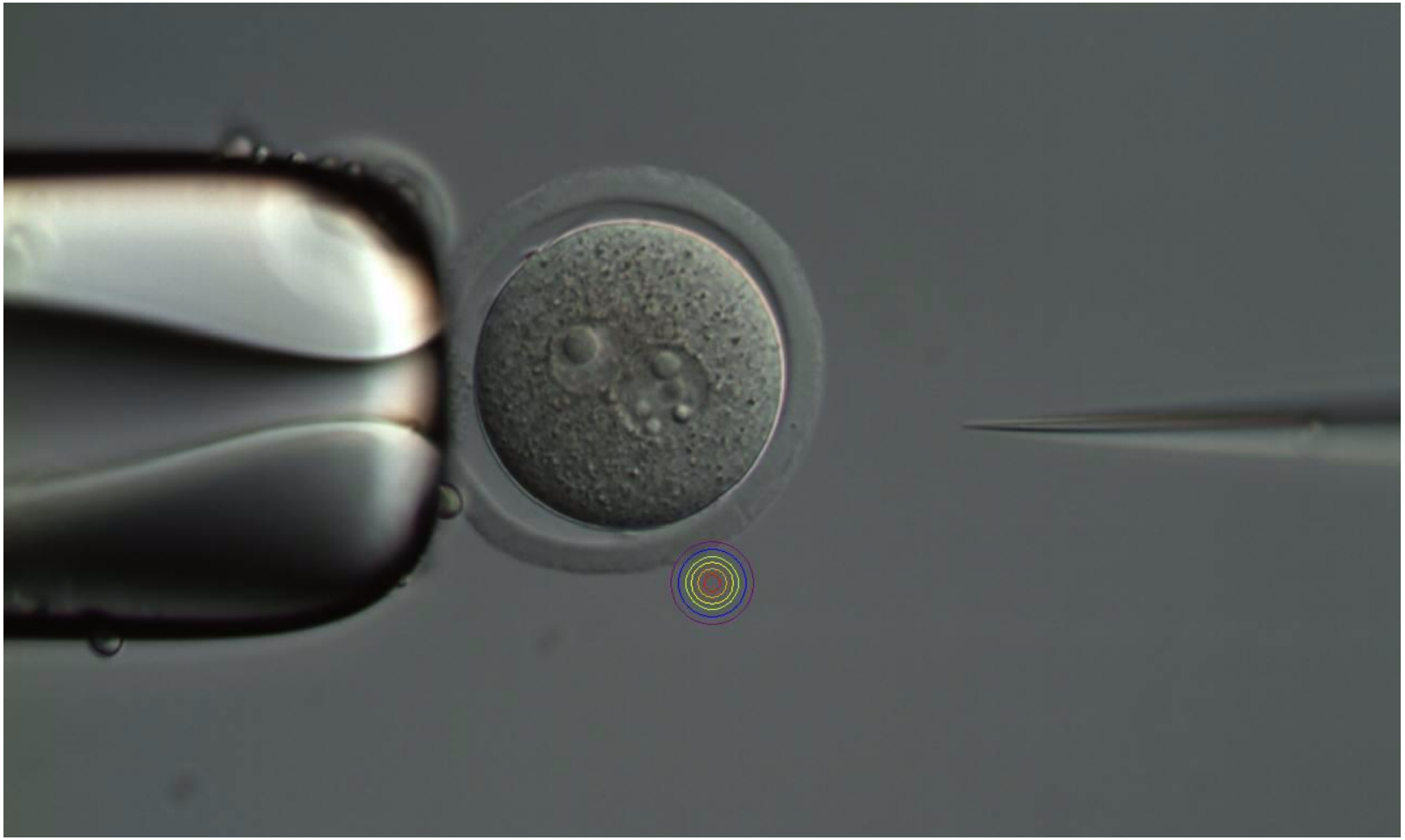
Pronuclear injection of transgene

Random integration of DNA construct into the genome

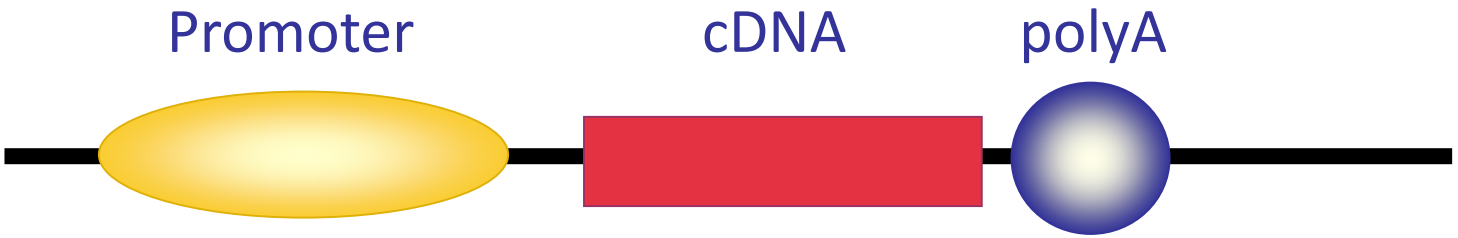
Reimplantation of injected zygotes into foster mice

Potential founders for integrated transgene by PCR and Southern blot analysis

# The Microinjection process



# Classical transgenic constructs




Promoter:

Well characterized
Ubiquitous (e.g. CMV, SV40)
Tissue-specific (e.g. αCAMKII)
Inducible (e.g. tetracycline system)

+ Doxicycline ➡ Expression ON

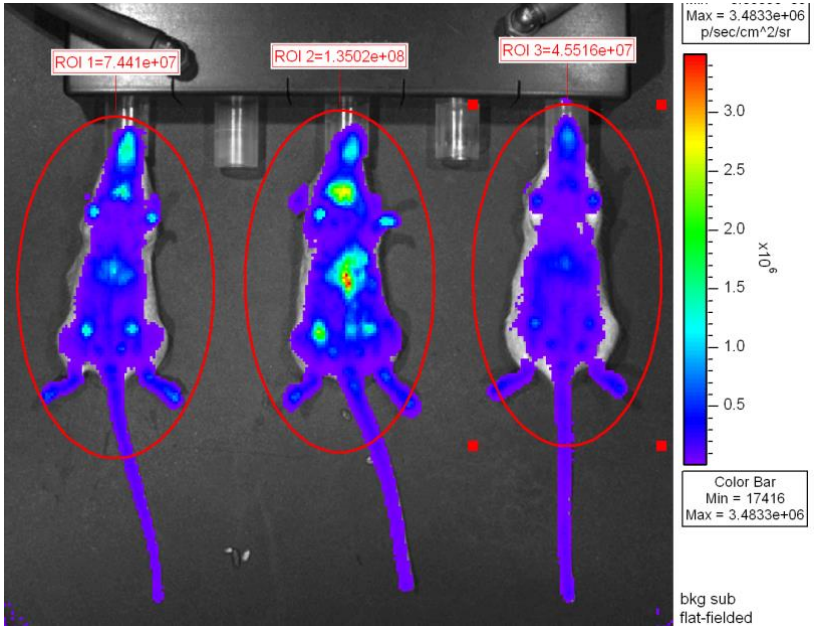
- Doxicycline ➡ Expression OFF

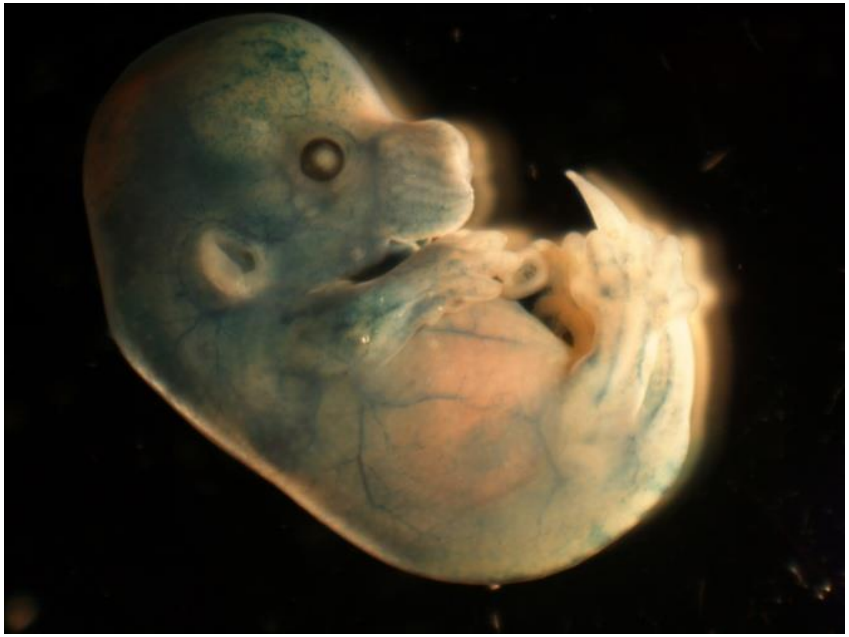
cDNA

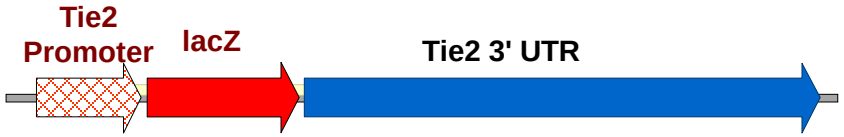
Transgene:

cDNA or mini gene
Epitope-Tag (*myc*, FLAG)
Reporter gene (GFP, *lacZ*)

# Transgenic models

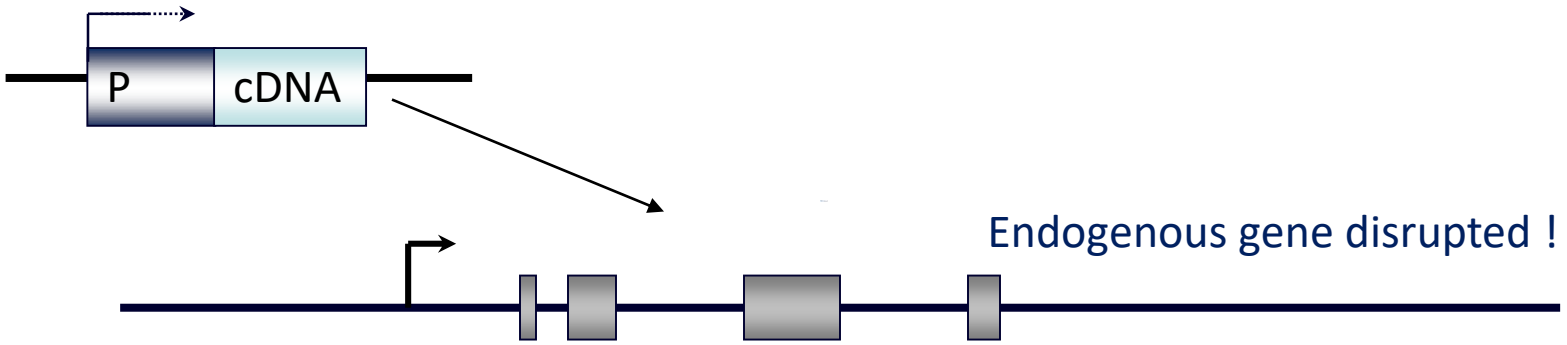


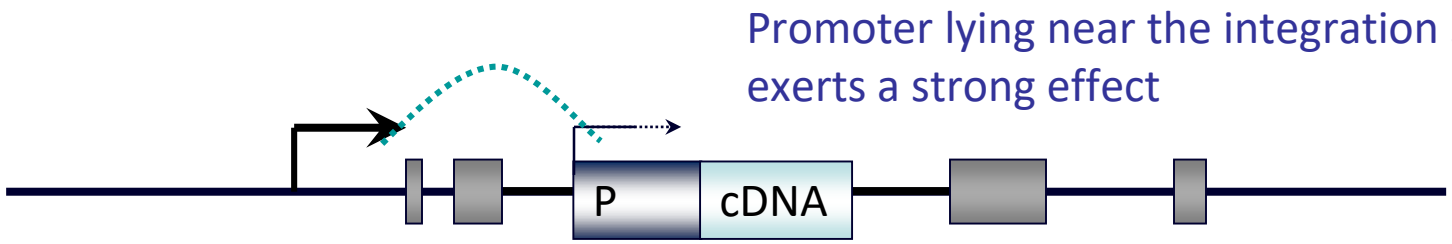



# The problems of random integration I

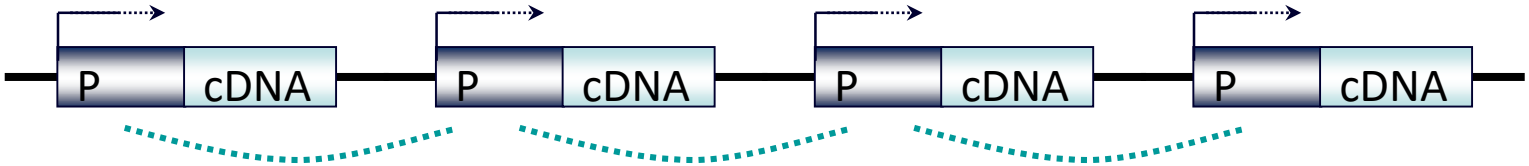
- Transgenic constructs integrate randomly into the genome.
- Random insertion can cause mutagenesis.



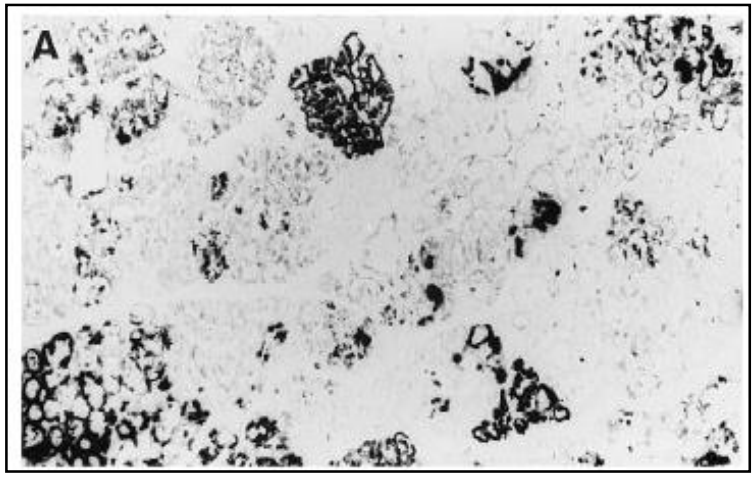

- Position effects can alter or silence the expression pattern



# The problems of random integration II



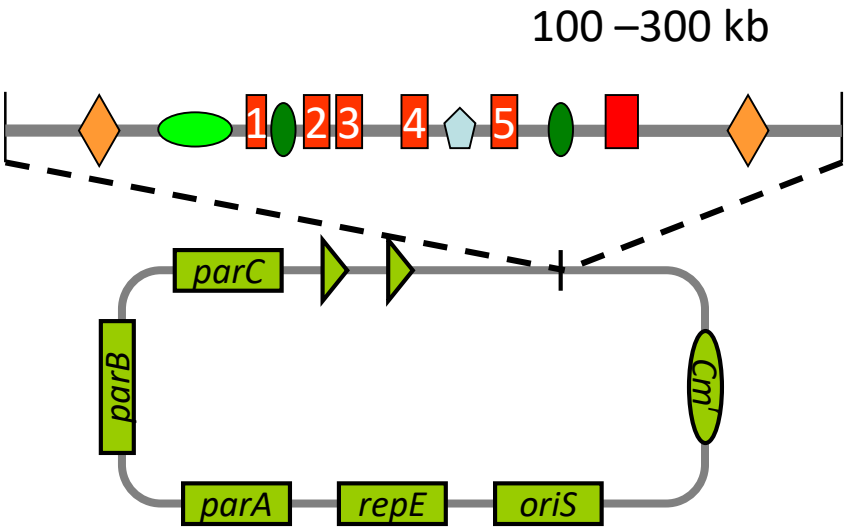

- The integration of transgenes as tandem repeats is associated with unstable expression
- An array of repetitive promoters is thought to unsettle the expression
- The resulting transgenic expression is often only present in a subset of the cells
- This phenomenon can even lead to an extinction of expression




Not every cell expresses transgene

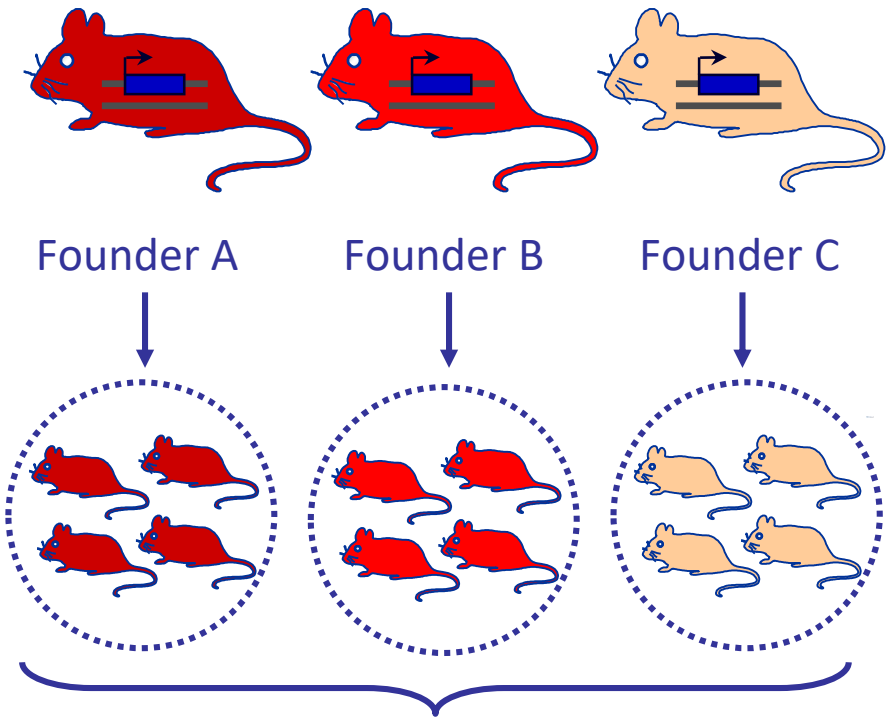
Individual transgenic mice are not directly comparable

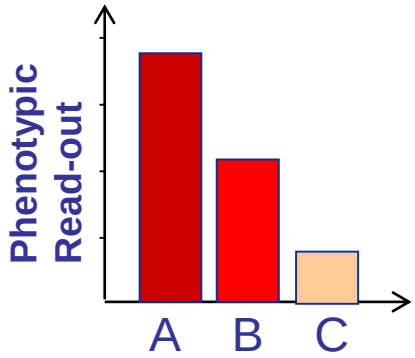
# BAC transgenes




- BAC Transgenics
  - All regulatory regions are present
  - Copy number is low
  - Large amount of insulating buffer sequence is present
- Same insertion dangers apply
  - Mapping the transgene insertion is very challenging as BACs are rarely linearised prior to injection i.e. you don't know the ends
- BACs may carry extra genes

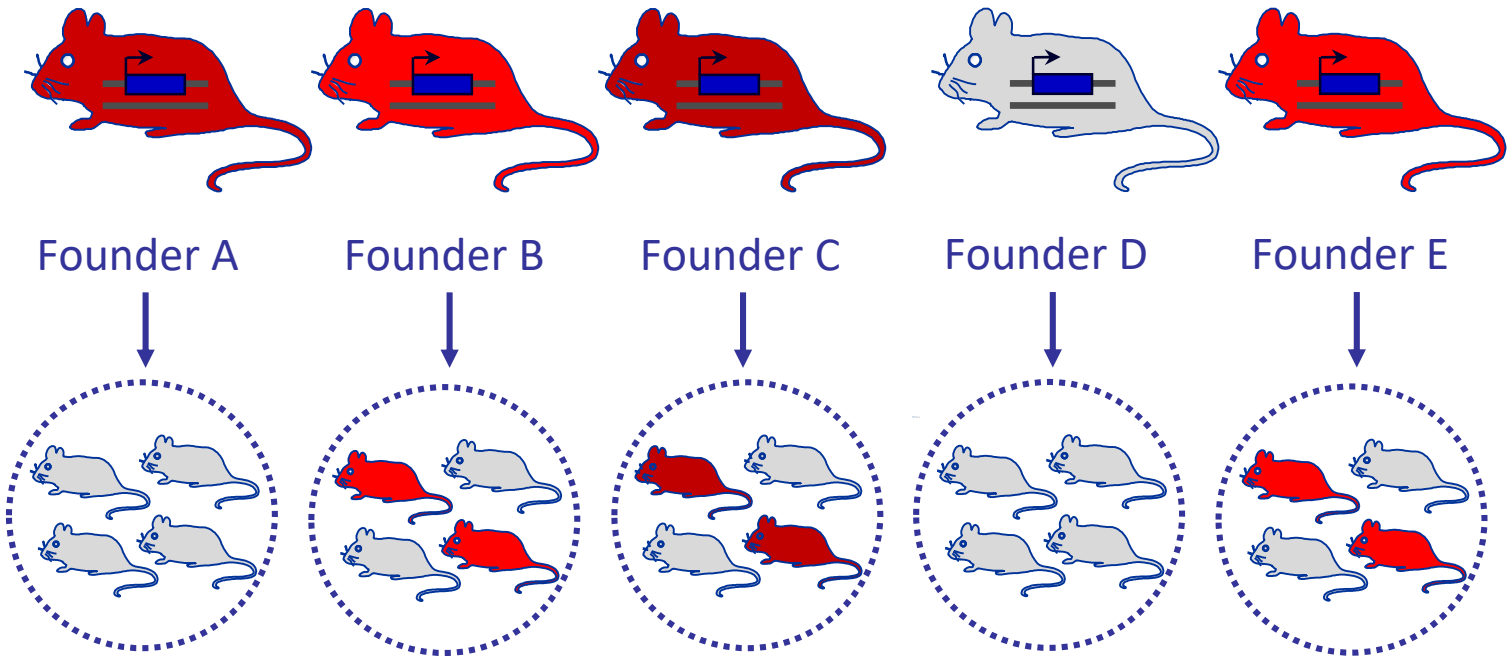
# Lines of transgenic mice



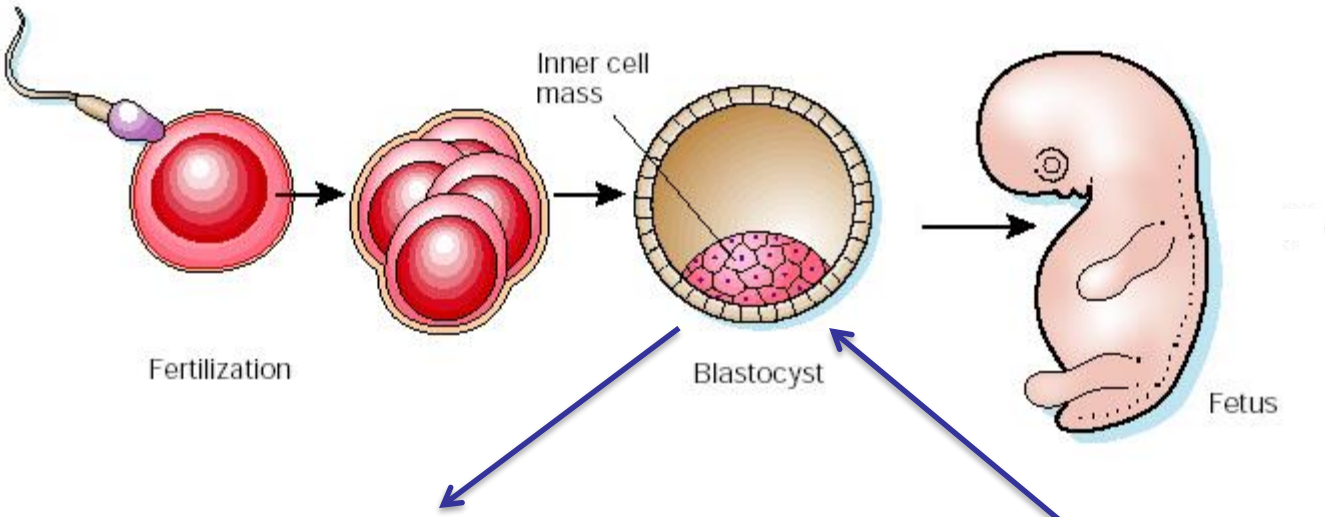

- Due to the variation in expression, independent lines of transgenic mice must be generated, bred and phenotyped independently before conclusions can be drawn
- In an ideal situation, the variability of the strength of transgene expression can be correlated with variability in phenotype

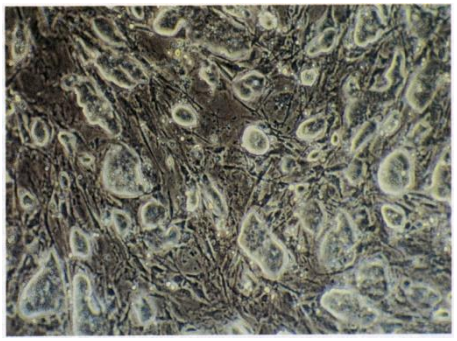
# A typical experiment with transgenic founders




- This variability means that a number of independent mouse lines must be maintained per construct
- Large animal cost of transgenic research
- Targeted manipulations via ES cells or via direct microinjection of nucleases are tackling this problem

# Embryonic Stem Cells



Embryonic Stem cells

Proliferation

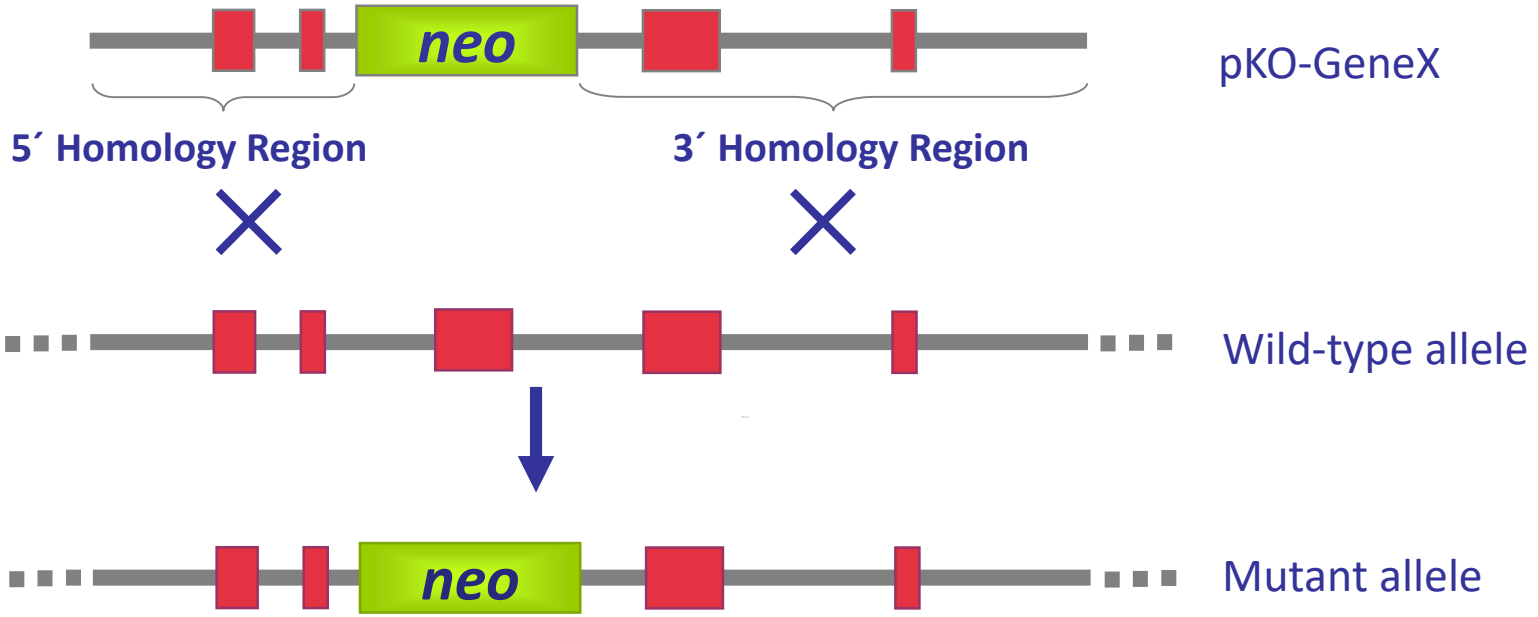
Genetic Manipulation

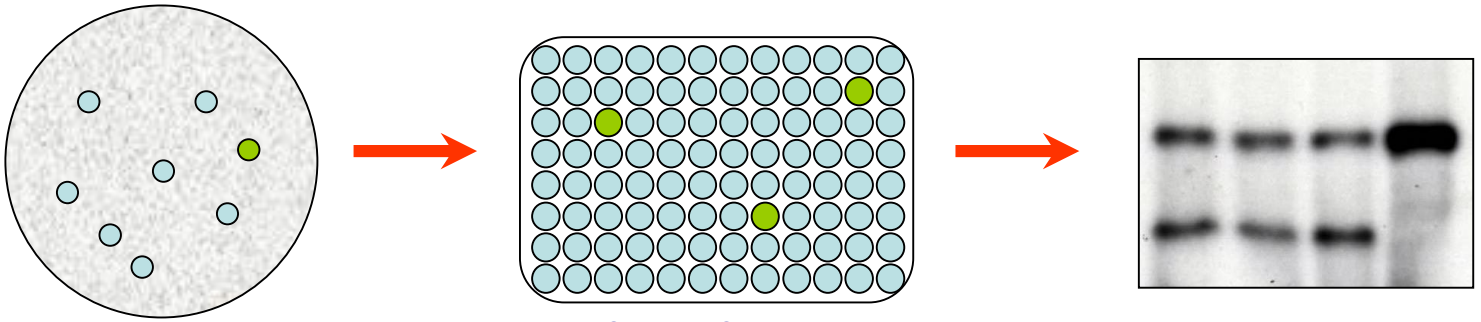
Differentiation

# Genetic Manipulation of ES cells

- Homologous recombination occurs at usable frequencies
- By building constructs which contain the required manipulation, these can be introduced into cells with selectable markers and cells with the required manipulation can be isolated
  - Gene Knock-out
  - Conditional (e.g. Tissue Specific Knock-out)
  - Humanization
  - Point mutation Knock-in

# Homologous recombination – the traditional route to gene modification



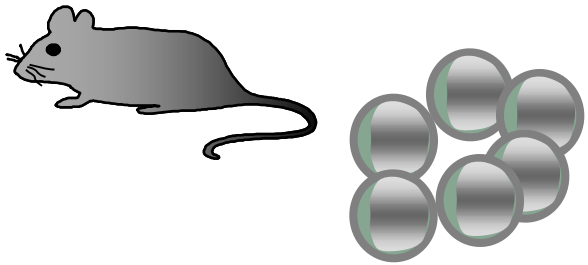



ES cell transfection

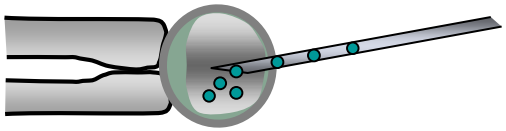
Isolation of transfected ES cell clones

Screening

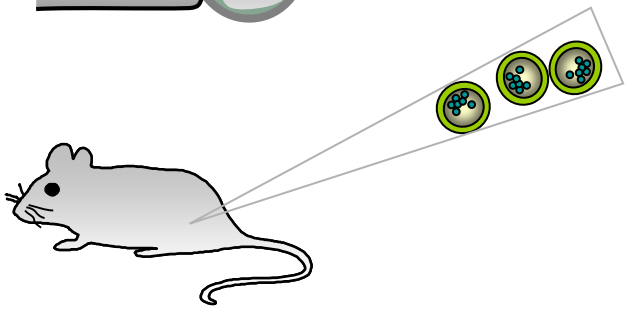
# Blastocyst Injection



Recovery of mouse blastocysts



Blastocyst injection with targeted ES cells



Reimplantation of injected blastocysts into foster mice

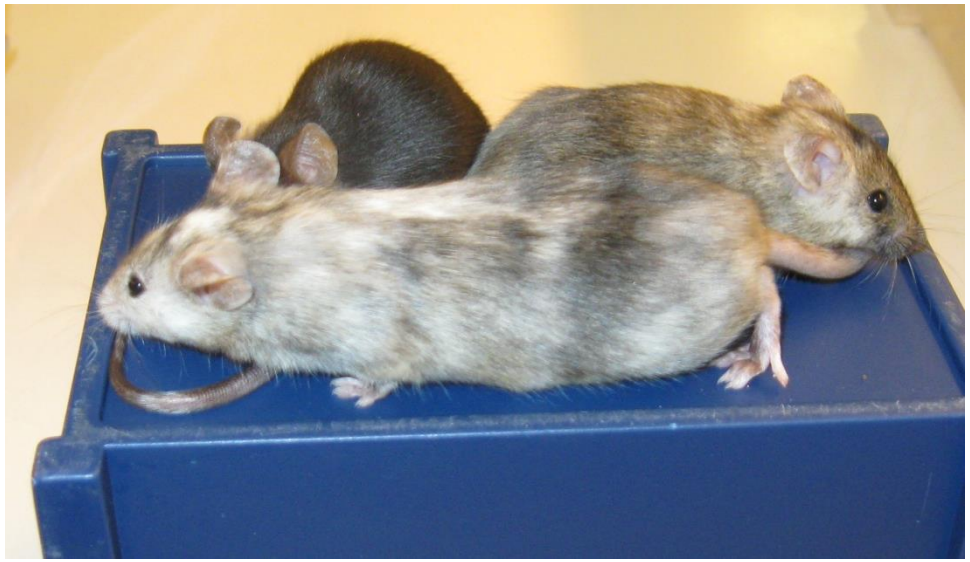
Pups born are “chimeras” of the host blastocysts and the injected ES cells

# The Microinjection process

# Chimeras

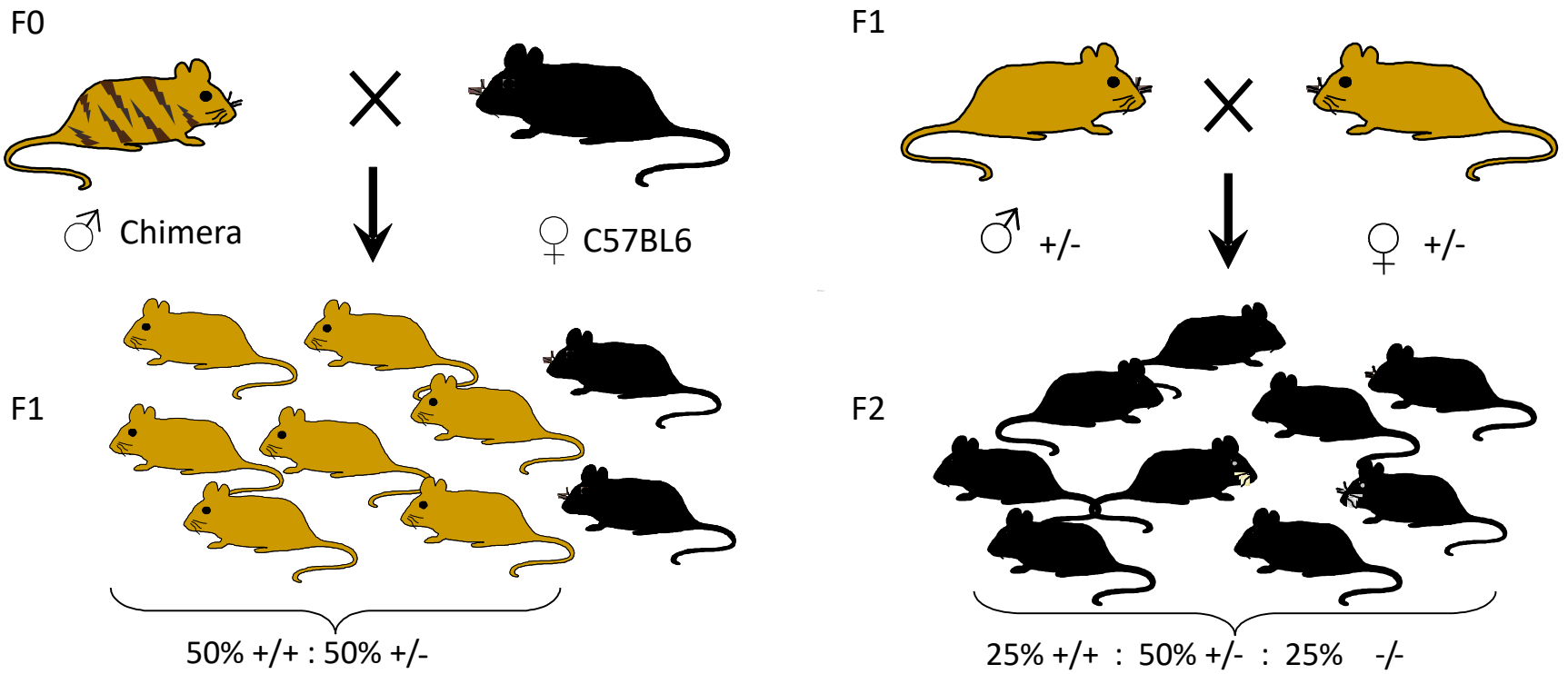
- Different coat colour makers in the injected cells and the host blastocysts allow us to distinguish the contribution of the injected stem cells
- The aim is for the stem cells to contribute to the germ cells of the chimera so that the engineered mutation is passed onto subsequent generations

"Brown" (129) ES cells injected into "black" (C57Bl/6) embryos

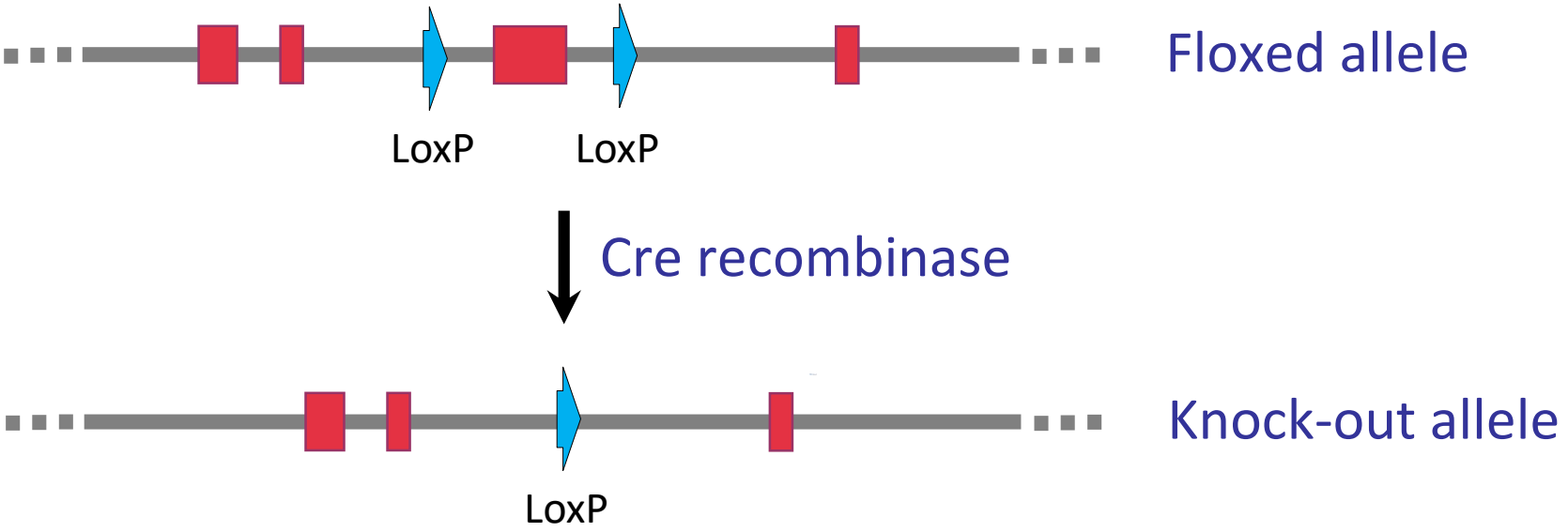


"Black" (C57BL/6) ES cells injected into "white" (FVB) embryos

# Breeding Scheme

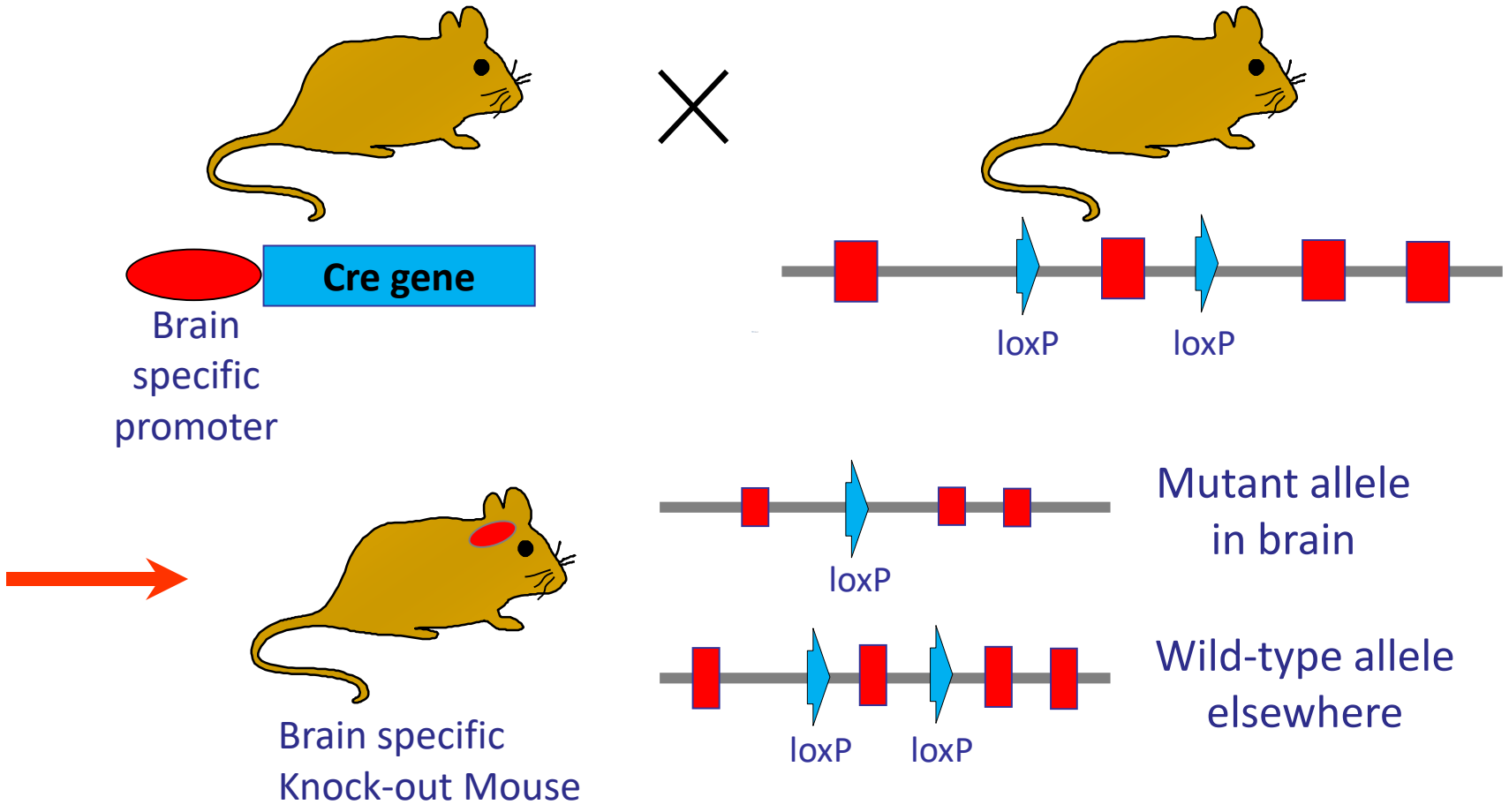


# Conditional Manipulations




- Cre recombinase from P1 Bacteriophage catalyses the recombination between specific 34 bp sequences known as loxP sites
- If positioned appropriately within the genome, introducing Cre recombinase can lead to chromosomal deletions
- The activity of the Cre recombinase can be restricted both spatially and temporarily to fine tune the genetic ablation

# Tissue specific knockouts

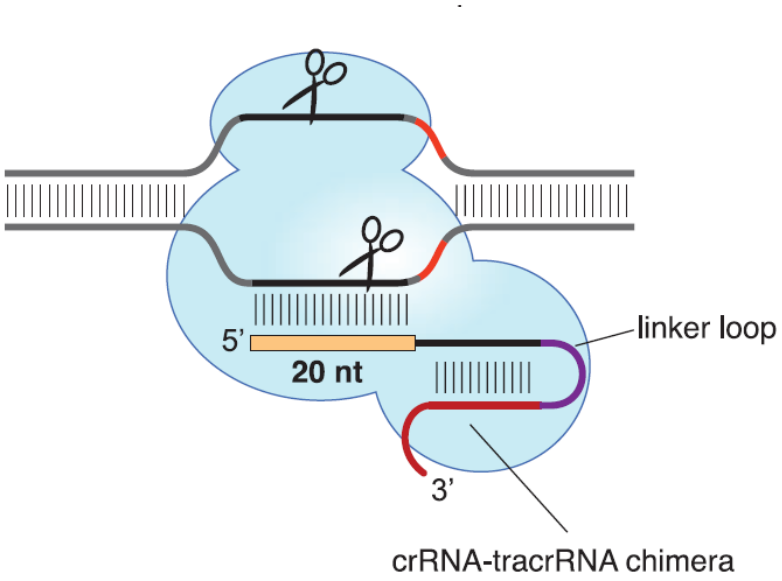


# Molecular scissors

- Over the last 10 years, there has been a revolution in our ability to manipulate the genome in a precise and sequence dependent manner

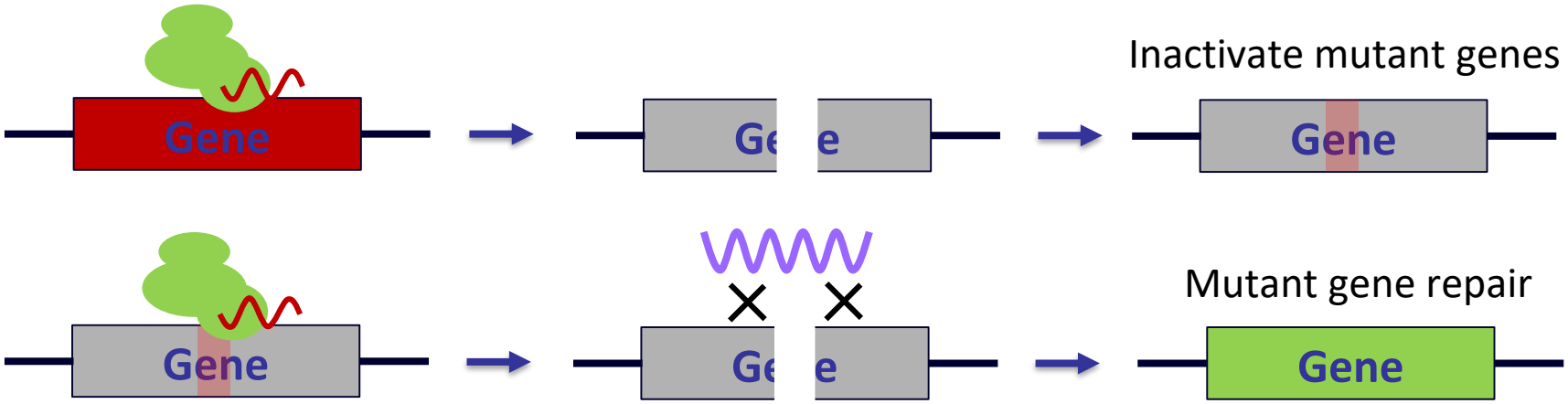
# CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)

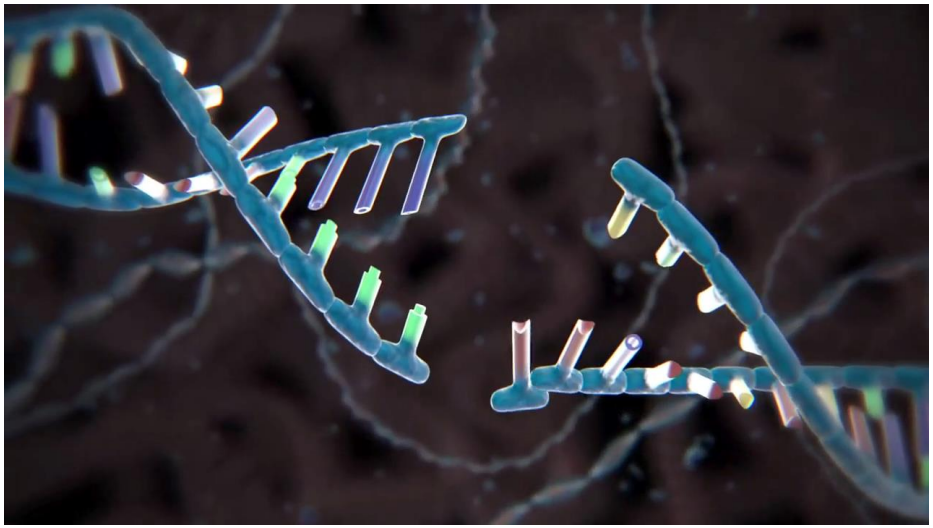
- Prokaryotes have evolved a nuclease system involved in defence against invading phages and plasmids.
- Central to this system are DNA repeats called CRISPRs
- CRISPR loci consist of CRISPR-associated (Cas) genes as well as non-coding RNA elements capable of programming the specificity of the CRISPR-mediated nucleic acid cleavage.

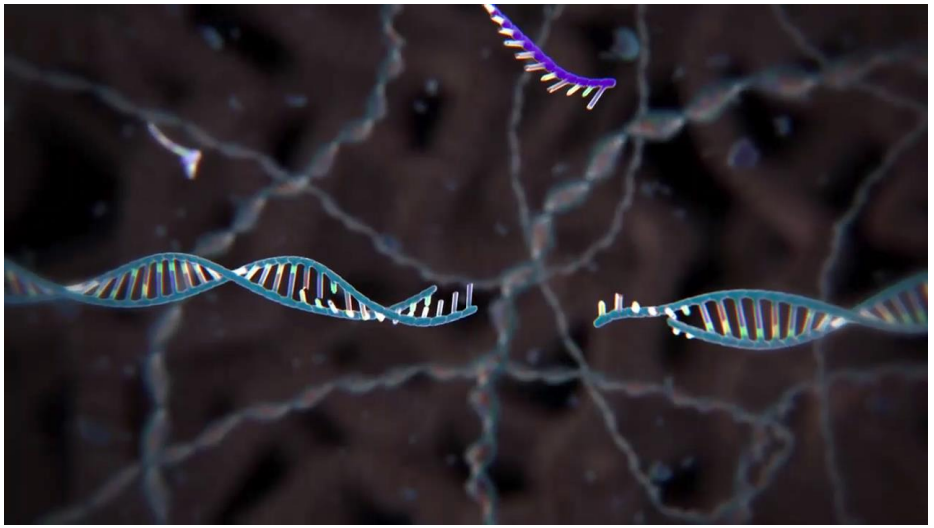



- Cas9 is a nuclease which is directed to its cleavage site by a guide RNA
- This guide RNA contains a 20 nt sequence which defines where the nuclease cleaves
- The cleavage site can be any 20 nt sequence, but the cleavage requires an NGG motif downstream

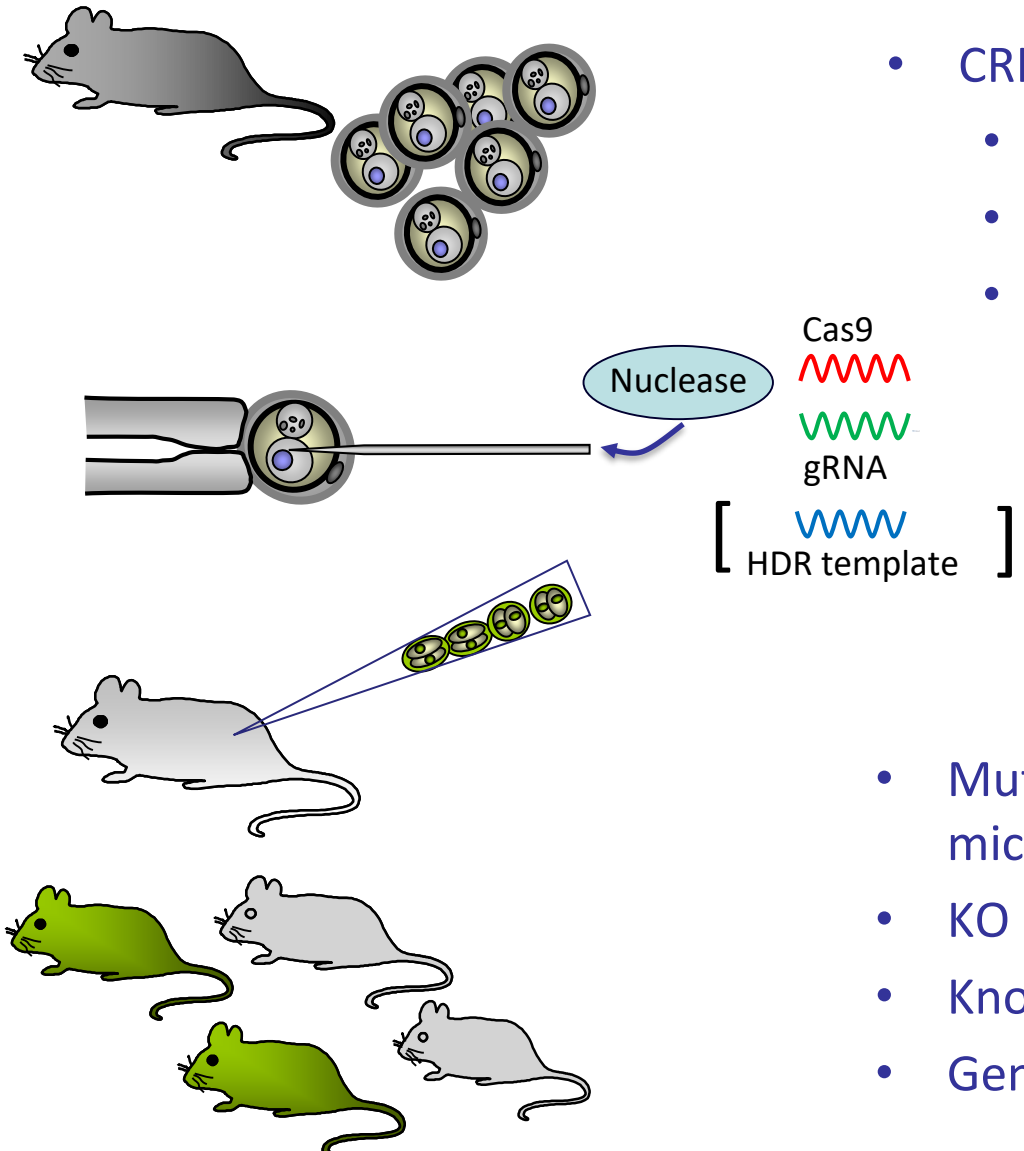
# How cutting DNA can be used to modify the genome





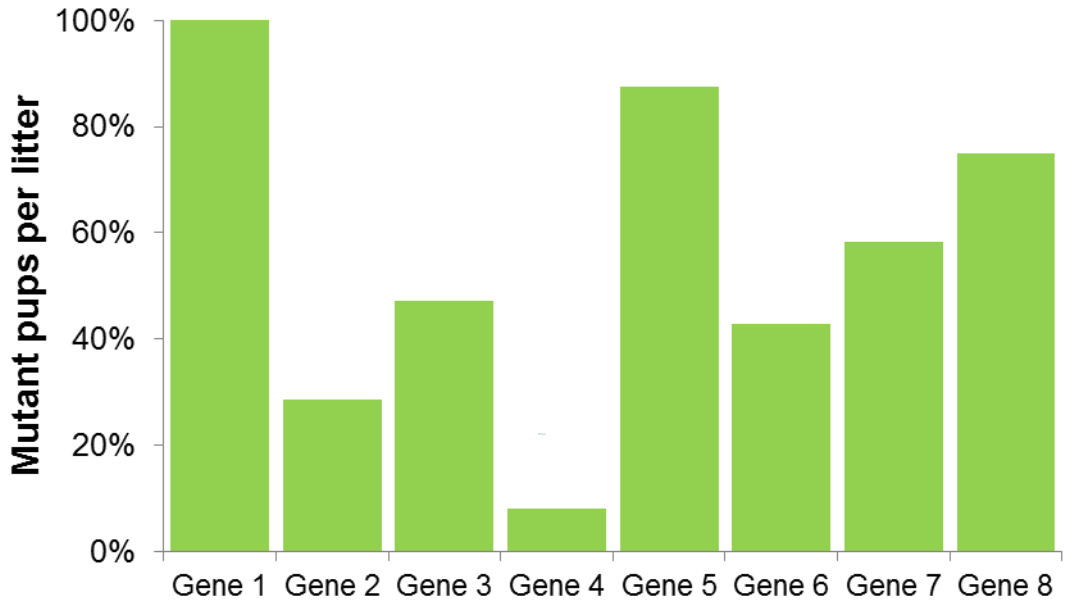
# Microinjection of nucleases into mouse oocytes




- CRISPR machinery can be supplied as
  - Plasmid DNA
  - Cas9 mRNA / gRNA
  - Cas9 protein / gRNA

- Mutant offspring generated in a simple microinjection experiment (3 months)
- KO by indel mutation
- Knock-in by Homology Dependent Repair
- Genomic deletion

# Simple Knock-out by indel mutation

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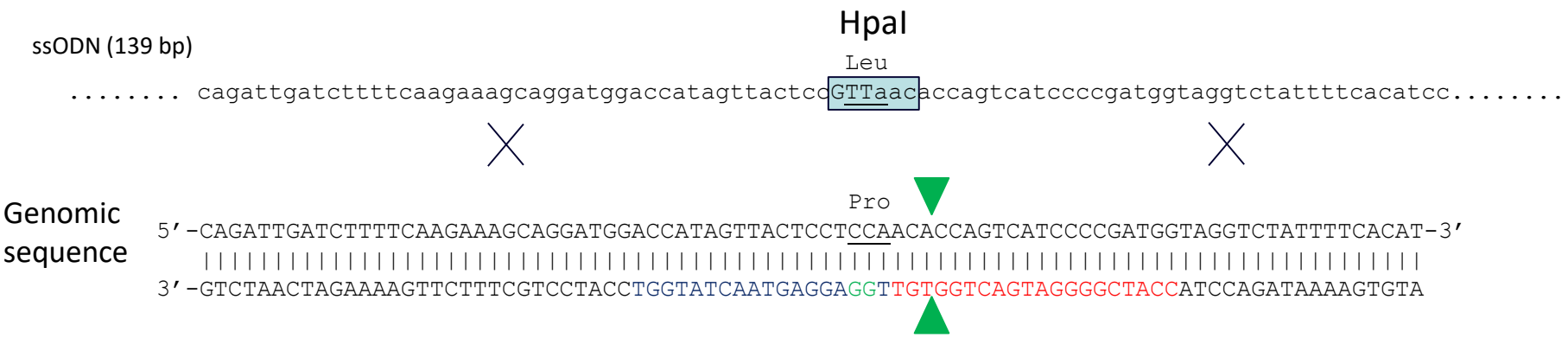
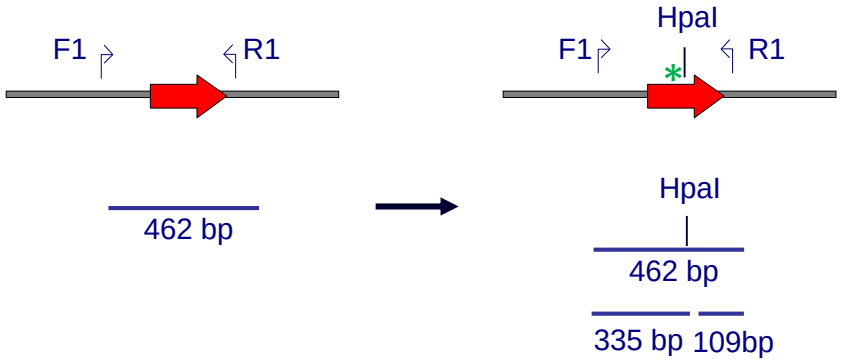
WT    GCCTCATGGATTCAAAGCCACTGAGGCCTGGCCACTGGGGGCGCCATTCGCCATTAAAAGGTCCTGCTGGGCTTTT
592a  GCCTCATGGATTCAAAGCCACTGAGGCCTGGCCACTGGGGGCGCCATTCGCCATTAAAAGGTCCTGCTGGGCTTTT
592b  GCCTCATGGATTCAAAGCCA-------------------------TTCGCCATTAAAAGGTCCTGCTGGGCTTTT
594a  GCCTCATGGATTCAAAGCCACTGAGG--------------GCGCCATTCGCCATTAAAAGGTCCTGCTGGGCTTTT
594b  GCCTCATGGATTCAAAGCCA-------TTACAGAATGGGGGCGCCATTCGCCATTAAAAGGTCCTGCTGGGCTTTT
595   GCCTCATGGATTCAAA-----------------ACTGGGGGCGCCATTCGCCATTAAAAGGTCCTGCTGGGCTTTT
597a  GCCTCATGGATTCAAAGCCACTGAGGCCTGGCCACTGGGGGCGCCATTCGCCATTAAAAGGTCCTGCTGGGCTTTT
597b  GCCTCATGGATTCAAAGCCACTG--------------GGGGCGCCATTCGCCATTAAAAGGTCCTGCTGGGCTTTT
598   GCCTCATGGATTCAAAGCCACTG--------------GGGGCGCCATTCGCCATTAAAAGGTCCTGCTGGGCTTTT
599   GCCTCA---------------------------CTGGGGGCGCCATTCGCCATTAAAAGGTCCTGCTGGGCTTTT
```

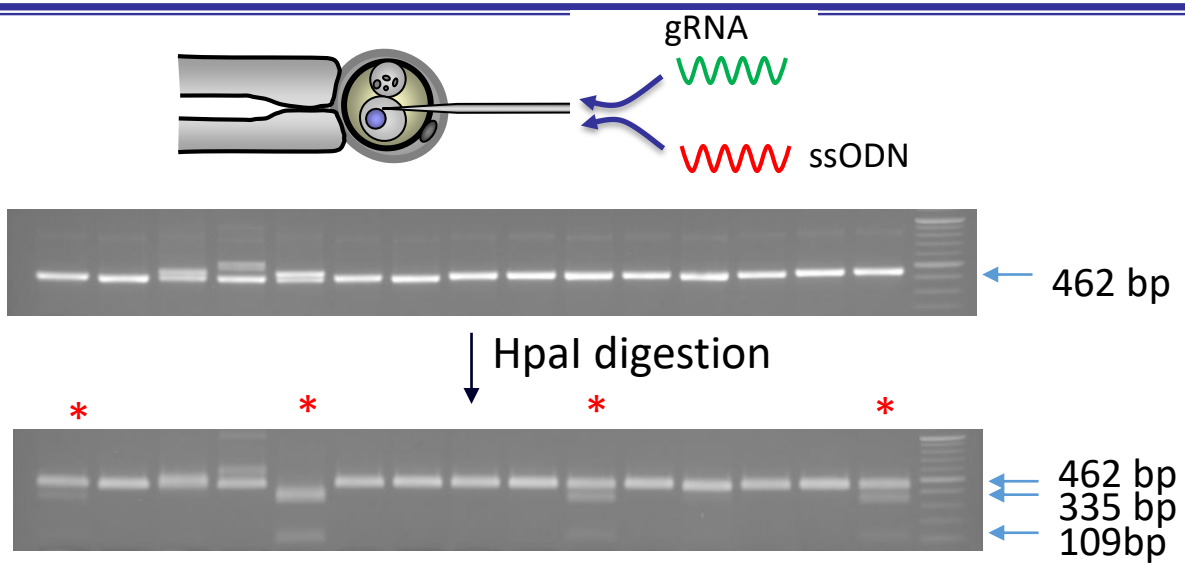
# Point mutation changes




- Codon CCA (Proline) is converted into TTA (Leucine)
- The previous codon is also mutated (synonymous) to destroy the PAM
- This manipulation also adds a novel HpaI restriction site, allowing easy genotyping

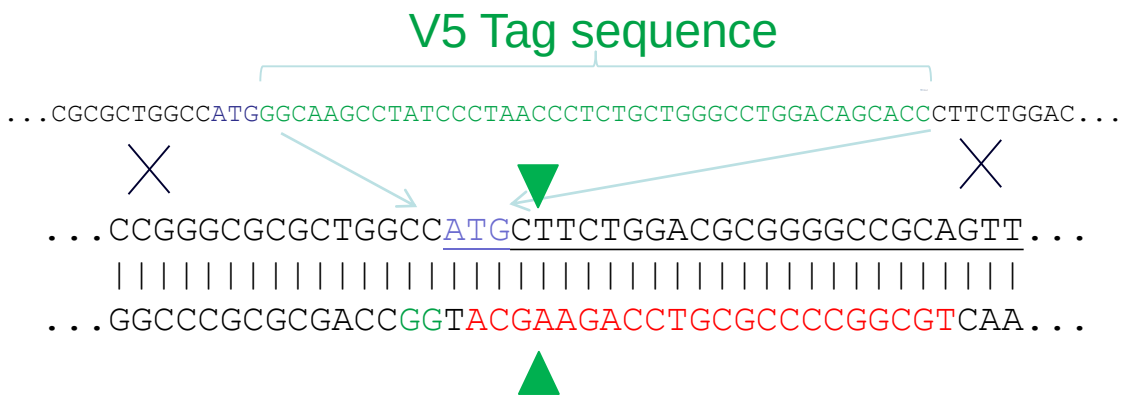


# Homology Directed Repair




```
WT        ATGGACCATAGTTACTCCTCCAACACC-AGTCATCCCC
KI        ATGGACCATAGTTACTCCgttAACACC-AGTCATCCCC
863-4     ATGGACCATAGTTACTCCTCCAACACCCAGTCATCCCC
863-7     ATGGACCATAGTTACTCCGTTAACACC-AGTCATCCCC
867-1     ATGGACCATAGTTACTCCGTTAACACC-AGTCATCCCC
867-2     ATGGACCATAGTTACTCCTCCAACACC-AGTCATCCCC
872-3     ATGGACCATAGTTACTCCGTTAACACC-AGTCATCCCC
872-4     ATGGACCATAGTTACTCC--------------TCCCC
877-2     ATGGACCATAGTTACTCCGTTAACACC-AGTCATCCCC
877-3     ATGGACCATAGTTACTCCGTTAACACC-AGTCATCCCC
877-4     ATGGACCATAGTTACTCCTCCAACA---AGTCATCCCC
879-1     ATGGACCATAGTTACTCCGTTAACACC-AGTCATCCCC
879-7     ATGGACCATAGTTACTCCTCCAACACC-AGTCATCCCC
879-6     ATGGACCATAGTTACTCCGTTAAC—C--AGTCATCCCC
```

# Larger modifications peptide tagging

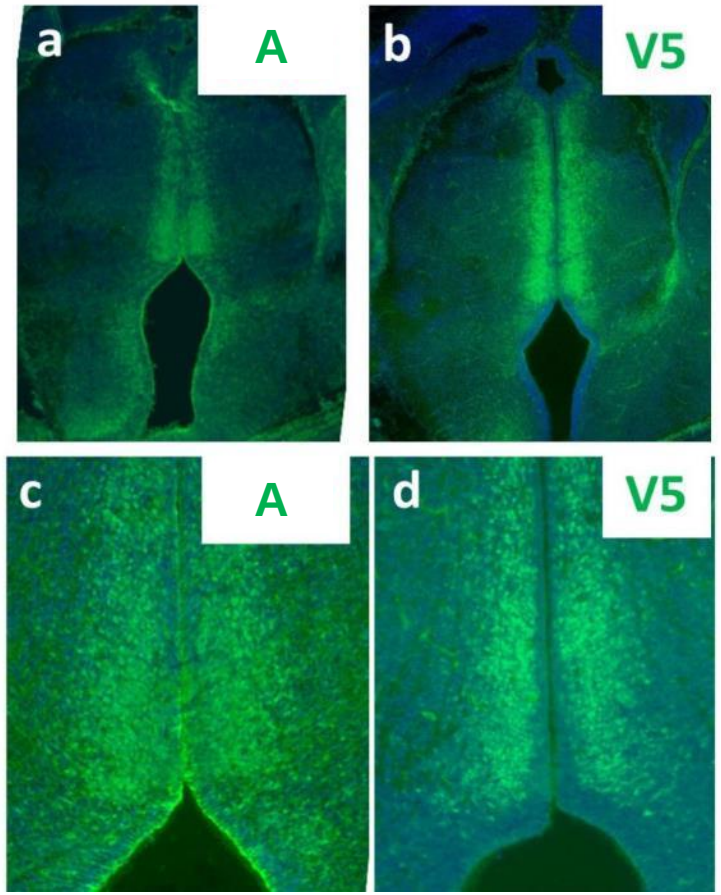
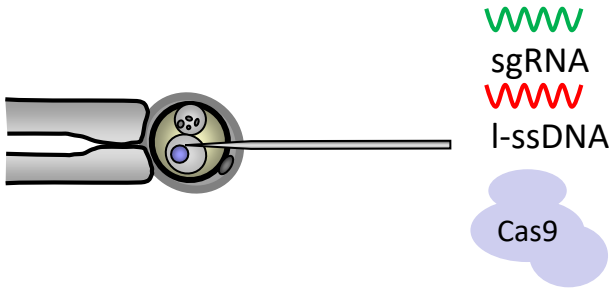



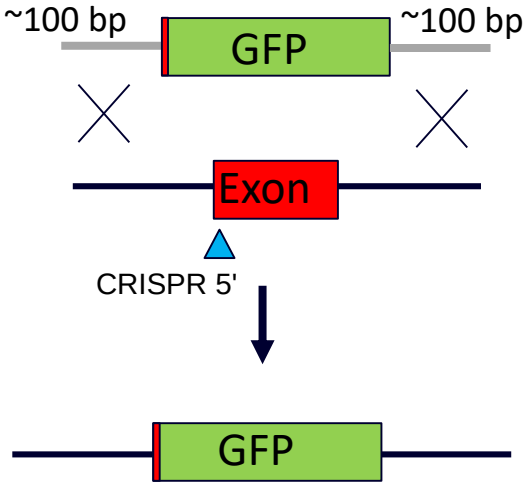

Image courtesy of Ian Dykes

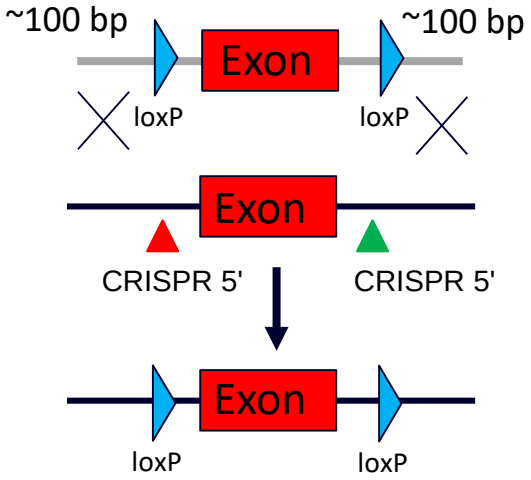
- A reliable strategy for introducing peptide tags to follow endogenous protein expression
- Very useful where there is no reliable antibody or where an existing antibody is unsuitable for e.g. ChIPseq applications

# *Larger insertions – long single-stranded DNA*

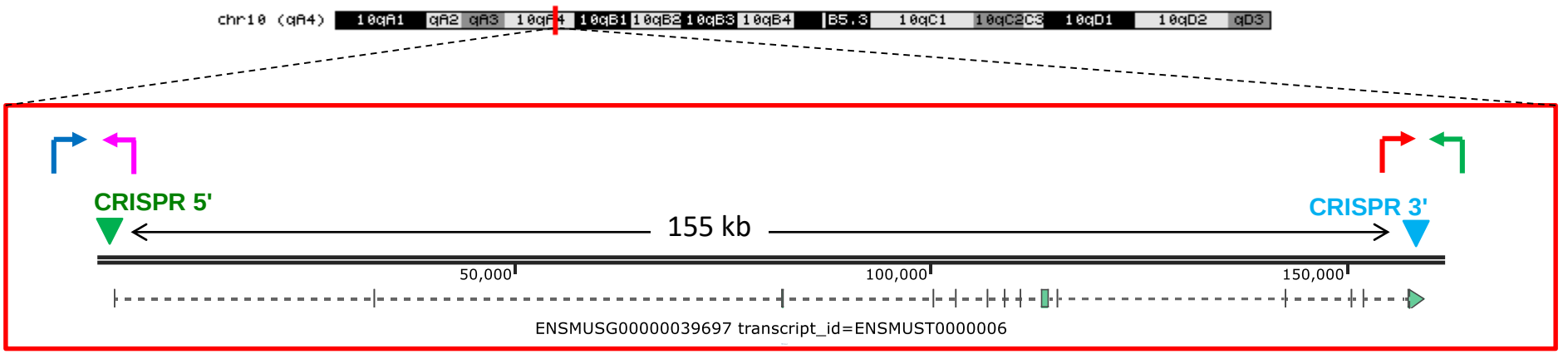
- Larger insertions
  - GFP Knock-in
  - loxP flanking a criticial exon
- ssDNA can be made in the lab and used as a template following the CRISPR cut

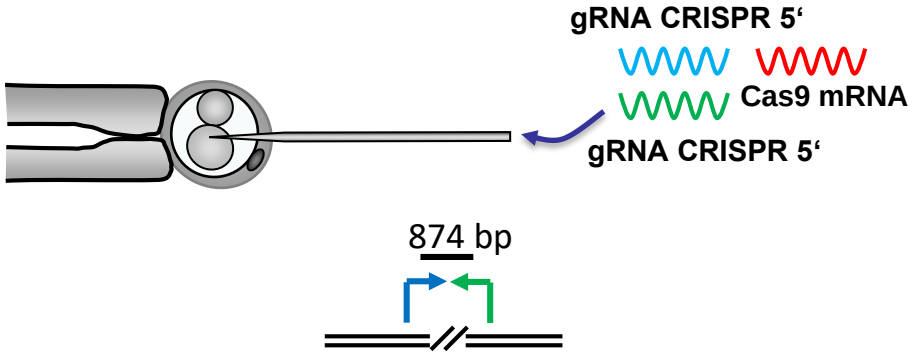


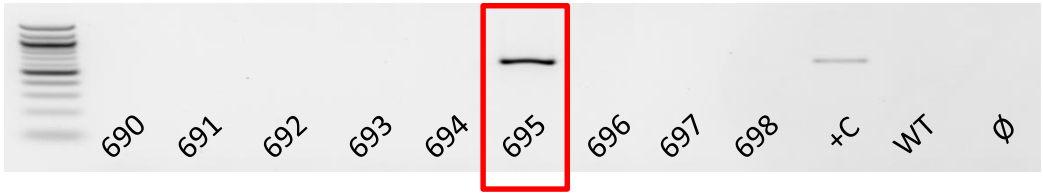



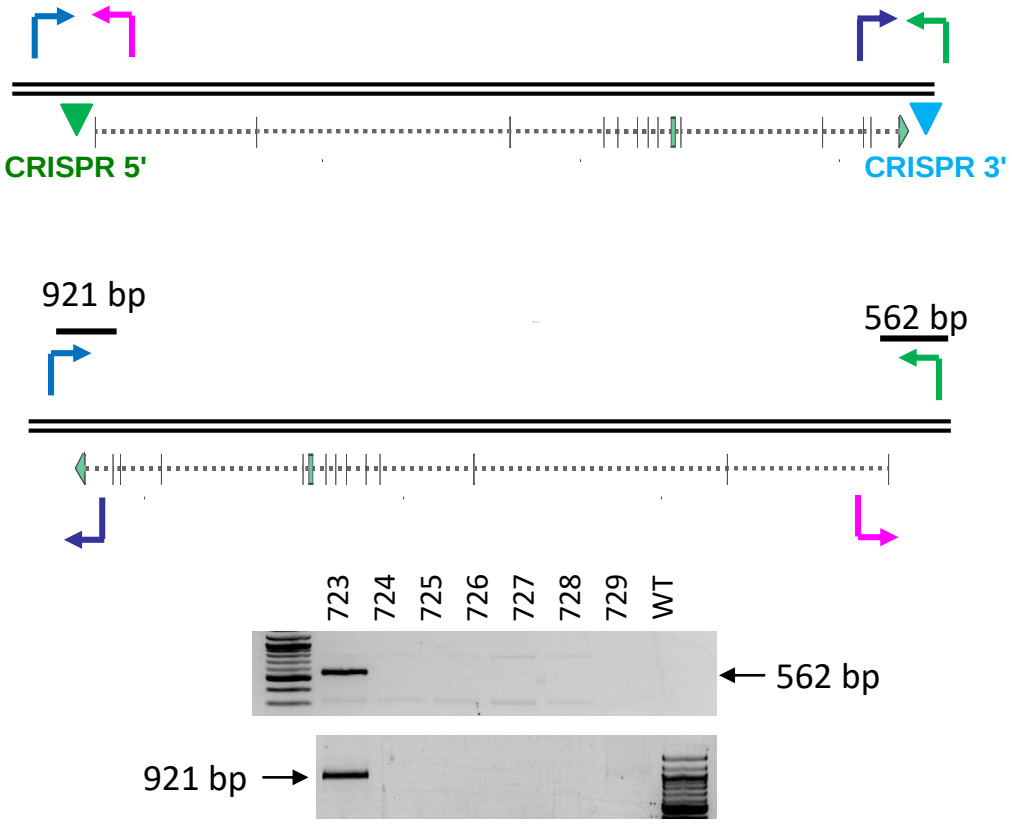
# Large chromosomal deletions



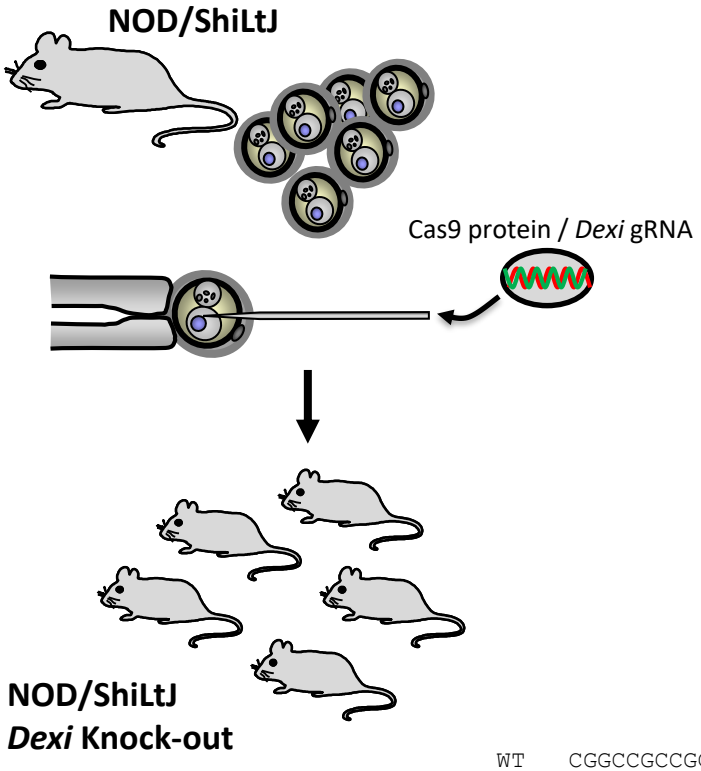



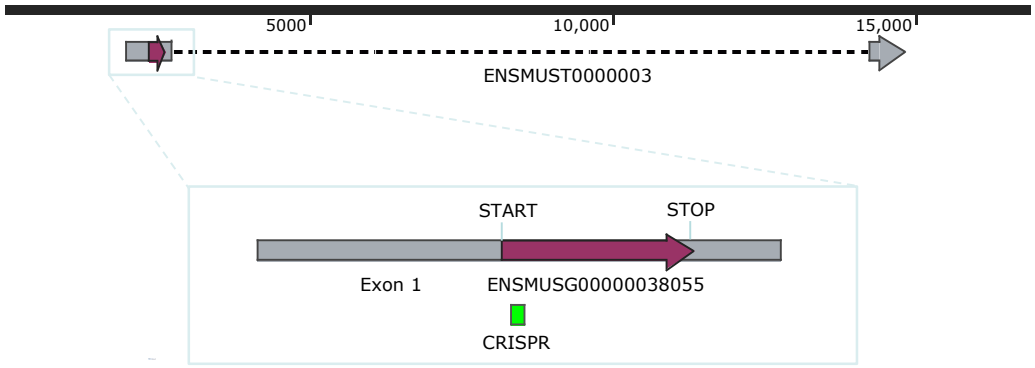



# ……..and inversions



# CRISPR mutagenesis on a disease model background



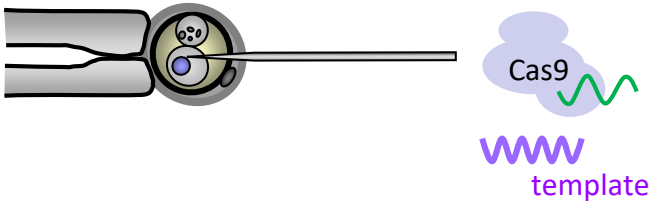




| | |
|---|---|
| Embryos injected | 79 |
| Embryos transferred | 47 |
| No. of pups born | 19 |
| No. of pups harbouring *Dexi* mutations | 4 |

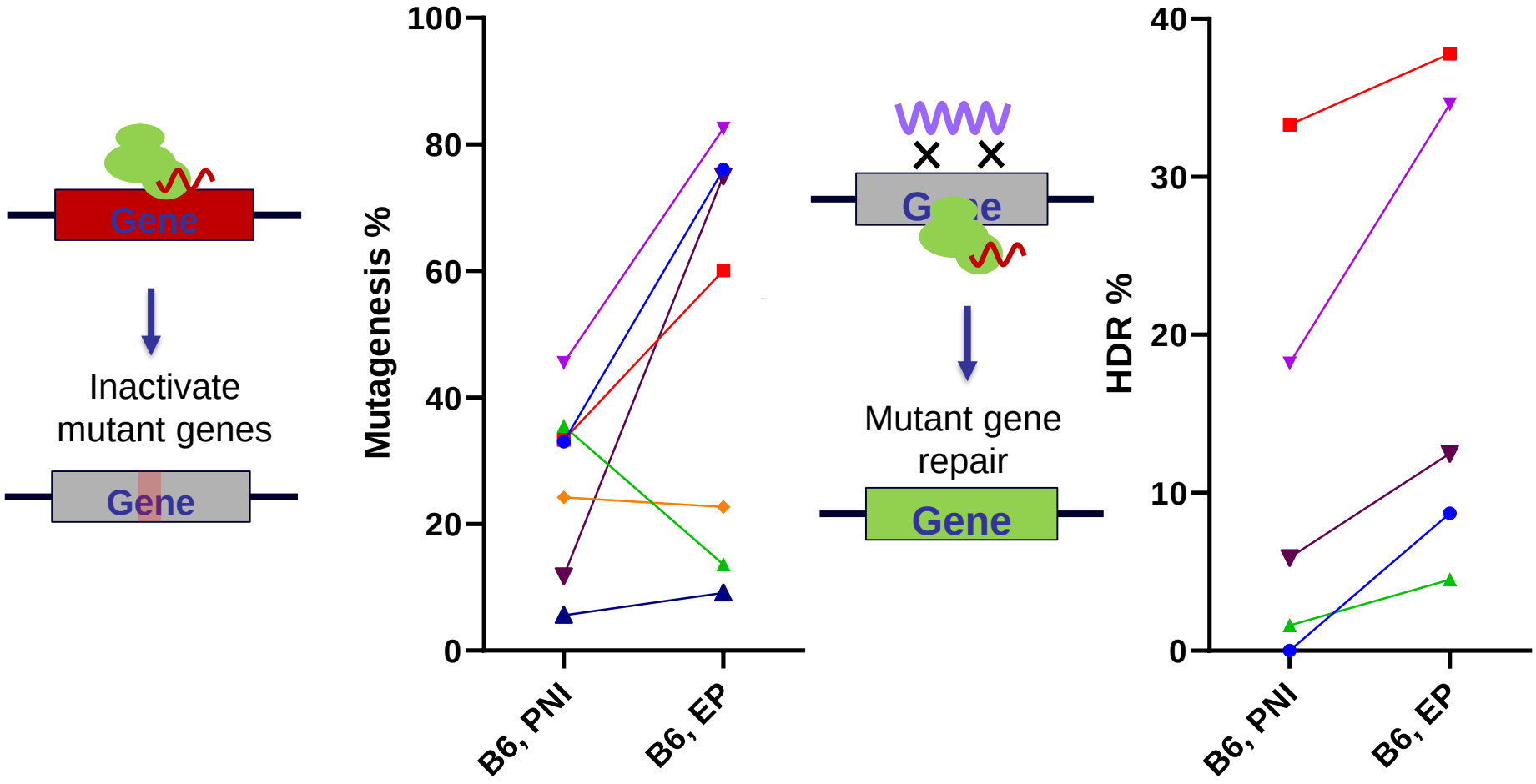
```
WT    CGGCCGCCGCCCCCATCGCCGGCCCCTGCATGCCCGGCGCCCGGGTCGCAGCCCACCTGGACGCTCTGGGCCCCCTGGTCT
#92   CGGCCGCCGCCCCCATCGCCGGCCCCTGCATGCCC----------------------GGACGCTCTGGGCCCCCTGGTCT
#95   CGGCCGCCGCCCCCATCGCCGGCCCCTGCATGCCCGGCGCCCGGGTCGCAGCCCANNNNNNNNNNNNNNNNNNNNNNNNNN
#97   CGGCCGCCGCCCCCAT----------------------------------------ACGCTCTGGGCCCCCTGGTCT
#103  CGGCCGCCGCCCCCATCGCCGGCCCCTGCATGCCCGGCGCCCGGGTCGCCAGCCCACCTGGACGCTCTGGGCCCCCTGGTCT
```

# New methods make it even easier to make a mouse



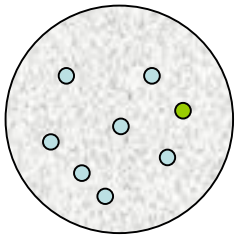



# And efficiencies seem improved

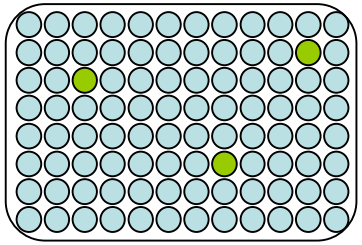


# “Game changing” technology

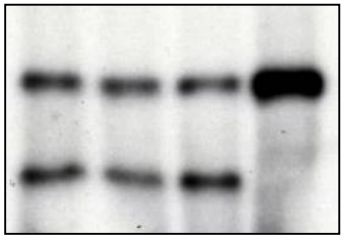
Targeting vector



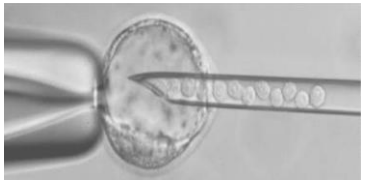
ES cell transfection



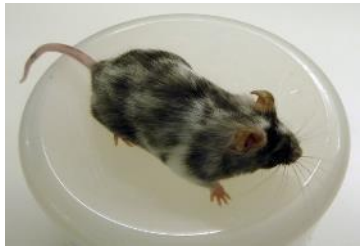
Isolation of transfected ES cell clones



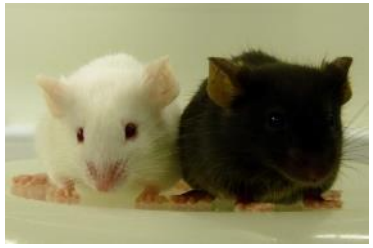
Screening



Blastocyst injection

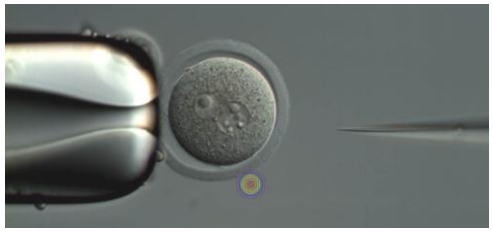


Chimera production

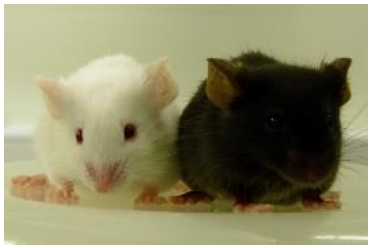


Germline transmission

Breeding of experimental cohorts



Nuclease microinjection



Founders

Breeding of experimental cohorts

# EU/NIH Knock-out projects

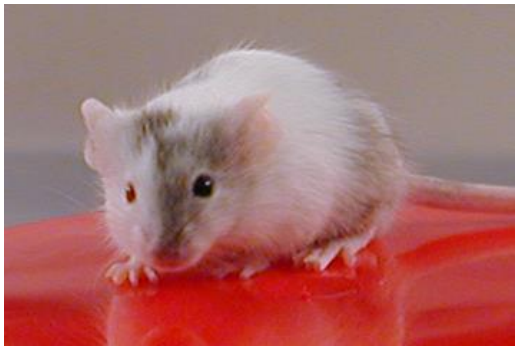
KOMP Repository

Knock Out Mouse Project

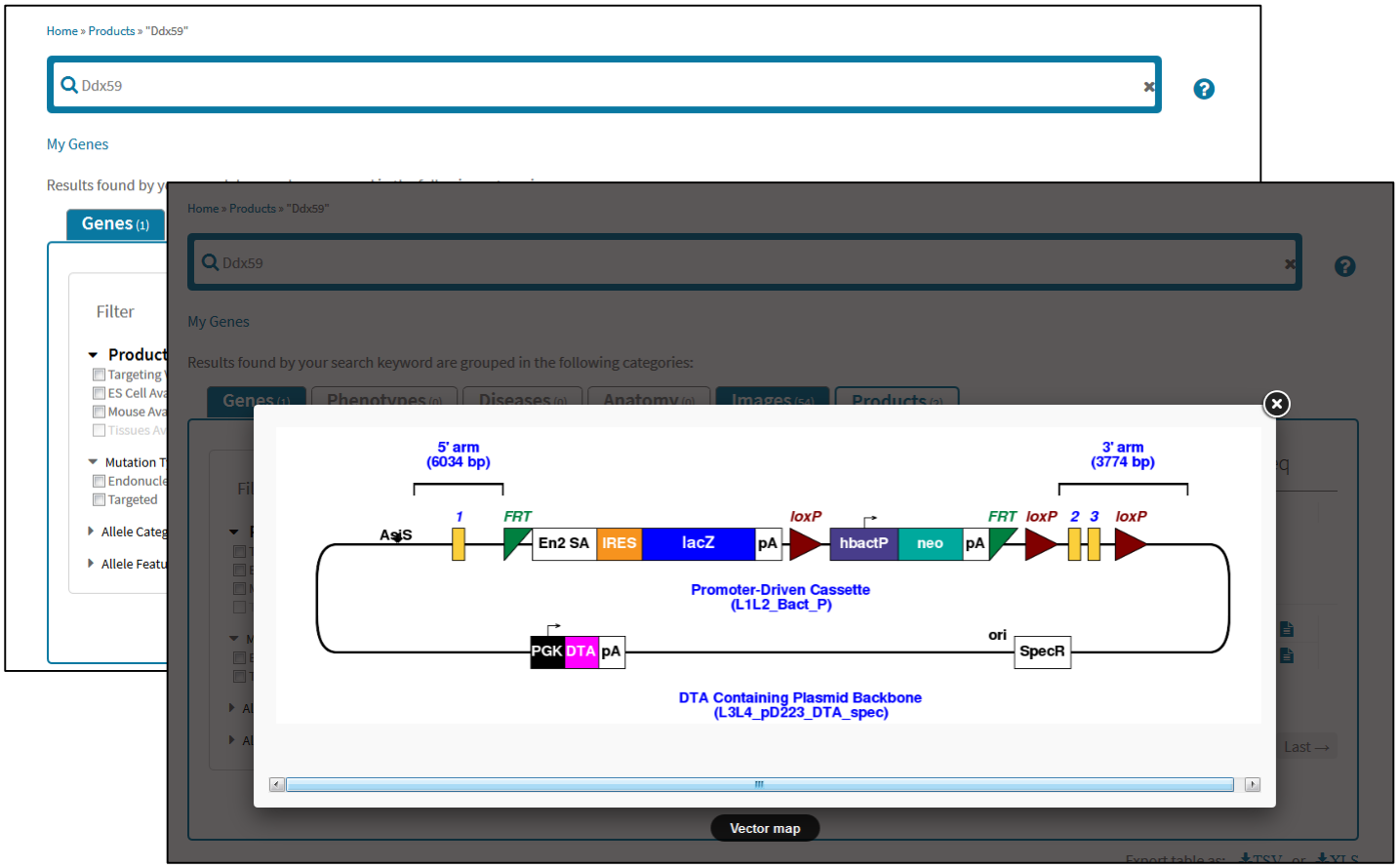

AIM: A Knock-out C57BL/6 ES cell line for every annotated gene in the genome

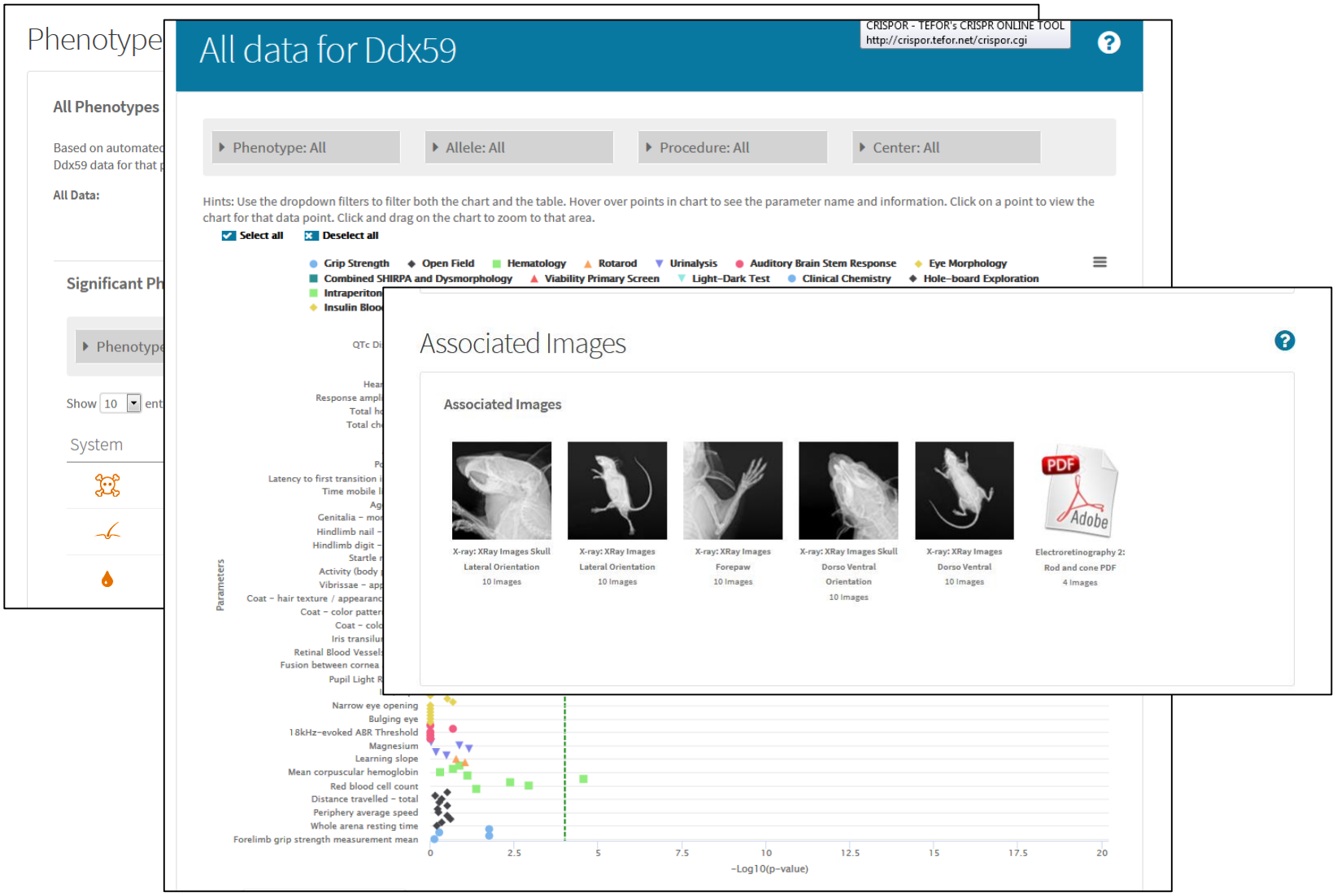
**Gene Search** → **Cell line available?** → **Blastocyst Injection**

→



# www.mousephenotype.com


Home » Products » "Ddx59"
Ddx59
My Genes
Genes (1)
Filter
Home » Products » "Ddx59"
Ddx59
My Genes
Results found by your search keyword are grouped in the following categories:
Genes
Phenotypes
Diseases
Anatomy
Images
Products
5' arm
(6034 bp)
3' arm
(3774 bp)
1
FRT
AsiS
En2 SA
IRES
lacZ
pA
loxP
hbactP
neo
pA
FRT
loxP
2
3
loxP
Promoter-Driven Cassette
(L1L2_Bact_P)
PGK
DTA
pA
ori
SpecR
DTA Containing Plasmid Backbone
(L3L4_pD223_DTA_spec)
Vector map


Phenotype
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System
All data for Ddx59
CRISPOR - TEFOR's CRISPR ONLINE TOOL
http://crispor.tefor.net/crispor.cgi
Phenotype: All
Allele: All
Procedure: All
Center: All
Hints: Use the dropdown filters to filter both the chart and the table. Hover over points in chart to see the parameter name and information. Click on a point to view the chart for that data point. Click and drag on the chart to zoom to that area.
Select all
Deselect all
Grip Strength
Open Field
Hematology
Rotarod
Urinalysis
Auditory Brain Stem Response
Eye Morphology
Combined SHIRPA and Dysmorphology
Viability Primary Screen
Light–Dark Test
Clinical Chemistry
Hole–board Exploration
Associated Images
Associated Images
X-ray: XRay Images Skull Lateral Orientation 10 Images
X-ray: XRay Images Lateral Orientation 10 Images
X-ray: XRay Images Forepaw 10 Images
X-ray: XRay Images Skull Dorso Ventral Orientation 10 Images
X-ray: XRay Images Dorso Ventral 10 Images
Electroretinography 2: Rod and cone PDF 4 Images
Parameters
Narrow eye opening
Bulging eye
18kHz–evoked ABR Threshold
Magnesium
Learning slope
Mean corpuscular hemoglobin
Red blood cell count
Distance travelled – total
Periphery average speed
Whole arena resting time
Forelimb grip strength measurement mean
0
2.5
5
7.5
10
12.5
15
17.5
20
−Log10(p−value)

# What’s wrong with my mouse ?