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ATELIER SUR LES MODÈLES RONGEURS TRANSGÉNIQUES EN RECHERCHE

Thierry Alquier, Christian Demers, Hélène Héon,
Jean-François Schmouth et Christine Vande Velde.

26 février 2021

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Tips and steps to take before requesting the creation of a new strain of mice ... Animal care committee's point of view.

Christian Demers, M.Sc.
ACC coordinator

3R principle

- Replacement:
  - Could you answer your questions without an animal?
  - Could you answer your questions without a new strain of mice?

SOCIETY FOR HUMANE SCIENCE
ALTERNATIVES TO ANIMALS *

University of Windsor **

Canadian Centre for Alternatives to Animal Methods

* https://www.forhumanescience.org/what_we_do/influencing-science-culture/alternatives-to-animals/
** https://www.uwindsor.ca/ccaam/

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3R principle

- Reduction:
  - The type of mouse created will have an impact on the number of mice produced (lox P, Cre, inducible, etc.) and on the number of controls required.

Photo tirée de: https://phenome.jax.org/

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3R principle

- Refinements:
  - The type of mouse created may require special housing conditions
  - Creating mice for certain mutations may require working only with heterozygous mice or with animals of a very early age.

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Before Creation

- Does the mouse already exist?
  - Here: Ask CIPA
  - In Canada: Ask CIPA: Canadian Animal Ethic Coordinators Network
  - Worldwide: commercial suppliers: Jackson lab., Taconic, etc .:
  - Other resources:
    - International mouse strain resource (IMSR): http://www.findmice.org/
    - Canadian mouse mutant repository (CMMR):
    - http://www.cmmr.ca/
    - Mouse Genome Informatics: http://www.informatics.jax.org/
    - The CHUM Research Centre's transgenesis and animal modelling core facility: https://www.chumontreal.qc.ca/en/crchum/facilities-and-services

Animal Use Protocol

- The committee needs to understand the benefits for science, human or animal health before the creation of this mouse.
- 3R: Explain all taken action.
- Explain steps that have been done to find the mouse and / or stem cells and / or sperm and / or embryos (websites, etc.).

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After Creation

- Phenotyping: Does the obtained model correspond to the expected phenotype?
- Housing: Are the housing and food conditions adequate?
- Welfare evaluation
- Establish a mouse passport: For your team, for the animal care personnel and for other institutions if you share this new strain.

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Mouse and rat passport Formulaire # 8 A

How to improve the reproducibility of studies? *

The importance of properly reporting the animal model in publications.

ARRIVE

The ARRIVE Guidelines Checklist

Animal Research: Reporting In Vivo Experiments

Carol Kilkenny[1], William J Browne[2], Innes C Cuthill[3], Michael Emerson[4] and Douglas G Altman[5]

[1]The National Centre for the Replacement, Refinement and Reduction of Animals in Research, London, UK, [2]School of Veterinary Science, University of Bristol, Bristol, UK, [3]School of Biological Sciences, University of Bristol, Bristol, UK, [4]National Heart and Lung Institute, Imperial College London, UK, [5]Centre for Statistics in Medicine, University of Oxford, Oxford, UK

Experimental procedures	7	For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). b. When (e.g. time of day). c. Where (e.g. home cage, laboratory, water maze). d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).
Experimental animals	8	a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.
Housing and husbandry	9	Provide details of: a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish). b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment). c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.
Sample size	10	a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group. b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used. c. Indicate the number of independent replications of each experiment, if relevant.
Allocating animals to experimental groups	11	a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done. b. Describe the order in which the animals in the different experimental groups were treated and assessed.
Experimental outcomes	12	Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).

* https://www.recherche-animale.org/comment-ameliorer-la-reproductibilite-des-etudes

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Good breeding practices

Hélène Héon DMV, M. Sc.

Inbred breeding

Most transgenic mouse lines have an inbred genetic background

Inbred strain: mice are isogenic, all individuals have the same genotype and are homozygous at all loci.

Permits good experimental reproducibility for genetically influenced traits→ consistent and uniform animal model for study

Inbred line will become a subline after
–3 generations of non-sibling mating
–10 generations of brother-sister mating

Use brother-sister mating

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Limit genetic drift

- Constant tendency of genes to evolve even in the absence of selective forces.
- Spontaneous mutations randomly may disappear or become fixed in a colony → may alter the mice phenotype.
- Small colonies are more affected than large ones

Inexorable, cannot be prevented but can be limited

Use brother-sister mating
Refresh your inbred line periodically
Cryopreserve embryos or sperm periodically

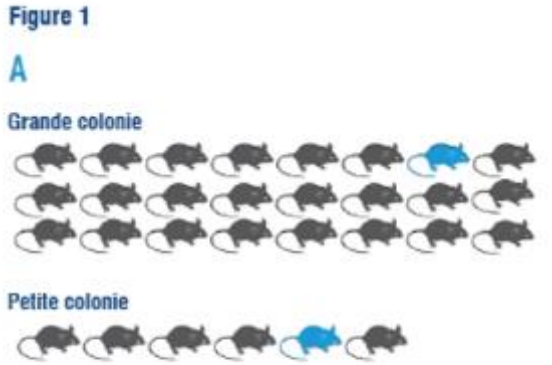

Figure from : Peter Kelmenson, *Comment rafraichir vos lignées de souris mutantes ou transgéniques*, The Jackson Laboratory, 2018 Charles Rivers Laboratories International Inc., www.criver.com

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Limit genetic drift

Backcross to the inbred control strain every 5-10 generations

- After 5 generations: Minimum of 2 backcrosses needed to “refresh” X and Y chromosomes, and mitochondrial genome.
- After 10 generations: 3 backcrosses are recommended.
- Establish a small “refreshed colony” and use these mice to replace old breeders from the existing colony.

See: https://www.jax.org/news-and-insights/jax-blog/2018/april/how-to-refresh-your-mutant-or-transgenic-mouse-strains

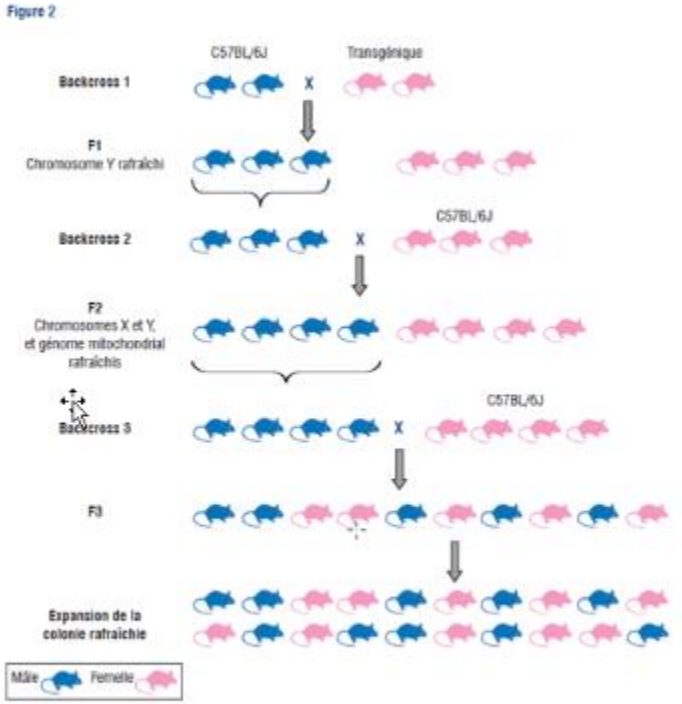

Figure from : Peter Kelmenson, *Comment rafraichir vos lignées de souris mutantes ou transgéniques*, The Jackson Laboratory, 2018 Charles Rivers Laboratories International Inc., www.criver.com

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Genetic contamination

- Mice from one line accidentally crossed with mice from another line.

- Crossing mice of the same strain but from two different suppliers can be considered in some cases as a genetic contamination. Genetic polymorphisms among C57BL/6 strains may change the phenotype.

Separate lines with similar nomenclature
Breeders must be genotyped periodically
May be possible to confirm strain or substrain background with genome scanning

Fergusson et coll. Defective insulin secretory response to intravenous glucose in C57Bl/6J compared to in C57Bl/6N mice. Mol Metab. 2014 Sep 28;3(9):848-54
Zurita et coll. Genetic polymorphisms among C57BL/6 mouse inbred strains. Transgenic Res. 2011 Jun;20(3):481-9.

Common breeding mistakes

Poor breeding management and poor inventories

- Old mice kept in breeding → small number of litters or loss of litters
- Delayed PCR → Late mating of pairs or trios
  - Reduction of breeding performances
  - Mice kept unnecessarily

- Problems are often not reported (no gestation, litter loss, small litters, aggressive females…)
  - Should not be considered normal without investigation
  - May be possible to correct the problem

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Good record keeping is essential

- Weekly inventory is recommended

You should know:

- The percentage of gestations
- The average number of weaned pups/litter
- The number of productive litters a female will have
- The reproductive lifespan of males
- Generation number

So you can predict

- How many breeding pairs or trios are needed
- When breeders should be replaced
- When it’s time to backcross to the inbred control strain

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Pairs or trios ?

Compile breeding statistics to choose the best breeding configuration

PLOS ONE

RESEARCH ARTICLE

Two of a Kind or a Full House? Reproductive Suppression and Alloparenting in Laboratory Mice

Joseph P. Garner[1], Brianna N. Gaskill[2,3], Kathleen R. Pritchett-Corning[2,4]*

PLOS ONE | DOI:10.1371/journal.pone.0154966 May 5, 2016

- Females help each other
- Trios :Suppression of reproduction in one of the 2 females
- Pairs: better nest quality score.
- Reproductive performance/cage not different in duos and trios → no advantage using trios.

Effects of Breeding Configuration on Maternal and Weanling Behavior in Laboratory Mice

Gillian C Braden,[1] Skye Rasmussen,[1,2] Sebastien Monette,[1,3] and Ravi J Tolwani[1,2,*]

Vol 56, No 4
Journal of the American Association for Laboratory Animal Science
July 2017

C57BL/6J

- Trio and harem : Weaning weight higher.
- Less anxiety-related behavior in mice bred in pairs
- Pairs produce more mice than harem breeding (no mention for trios)
- No mention of the effect of harem breeding on male mice health

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Production planning

Besoin	20 souris mâles de 5 semaines, 1 groupe / mois	
Souris	Lignée KO fond C57BL/6	
	sevrage à 21 jours	
	Statistique élevage basées sur souris C57BL/6 type sauvage	
1	**Nombre de souris requises**	20
2	**Âge**	1
	Si elle doivent avoir le même âge entrer : 1	
	Si peuvent avoir 2 semaines âge de différence (ex : 5 et 6 semaines) : 2	
	Si peuvent avoir 4 semaines âge de différence (ex 4 à 9 semaines) : 4	
3	**Fréquence requise**	4
	Si hebdomadaire entrer : 1	
	Au 2 semaines entrer : 2	
	Au 4 semaines entrer : 4	
4	**Diviser ligne 1 par le plus petit chiffre de la ligne 2 ou 3 et arrondir chiffre rond supérieur**	20
5	**Sexe**	2
	Si deux sexes peuvent être utilisés enter : 1	
	Si seulement 1 sexe peut être utilisé enter : 2	
6	**Type élevage**	1
	Homozygote X homozygote , entrer 1	
	Hétérozygote X homozygote , entrer 2	
	Hétérozygote X hétérozygote , entrer 4	
7	**Surplus (tampom)**	1
	Non, entrer 1	
	Oui, entrer un facteur tampon pour assurer une surproduction	
	ex : 10 % de souris requises en surplus , entrer 1,1	
8	**Nombre de souris à naître/semaine**	40
	Multipliez les lignes 4X5X6X7 (arrondir au chiffre rond le plus élevé)	
	Productivité de la colonie- statistiques pour C57BL/6 WT	
9	**Nombre moyen de petits sevrés/portée**	5,6
10	**Nombre moyen de portées/femelles**	5,4
11	**Vie productive (semaines)**	30
12	**Calcul de la productivité de la colonie**	1,01
	Diviser ligne 10 par ligne 11, multiplier par ligne 9 (arrondir au centième le plus près)	
13	**Calcul du nombre de femelles en reproduction requises**	40
	Diviser ligne 8 par ligne 12,(arrondir chiffre rond le plus près)	

- Determine needed genotype (experimental animals and controls)
- Use appropriate breeding scheme
- Use appropriate approach to plan the production

Translated from : Breeding strategies for maintaining colonies of laboratory mice, The Jackson Laboratory Resource Manual, 2009

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Documentation

Nomenclature

Understand nomenclature

Use appropriate abbreviations

Follow nomenclature rules

Use appropriate nomenclature in publications

Mouse Nomenclature Home Page : http://www.informatics.jax.org/mgihome/nomen/

B6.129P2-Apoa1^{tm1Unc}/J

Background

Targeted gene

Targeted mutation

Allele designation

Lab registration code

Lab maintaining the strain

From : The Jackson Laboratory

Mouse passport


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Mouse and rat passport Formulaire # 8 A

GENERAL INFORMATION:

Information pertaining to

Complete nomenclature

Genetic background

Genotyping

Husbandry

Welfare issues

Breeding recommendations

Expected phenotype

From : Wells et coll. Assessing the welfare of genetically altered mice Lab Anim. 2006 Apr;40(2):111-4.

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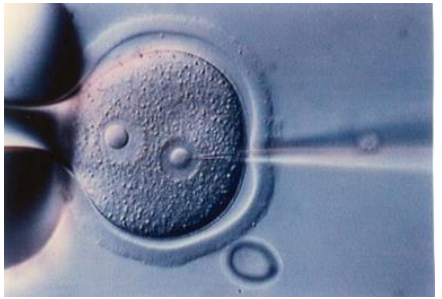
What about the “off-targets”: CRISPR low hanging fruit argument

Jean-François Schmouth, PhD

Rodent model generation approaches; a brief history

- Random integration transgenic
  - Transgene insertion via pronuclear microinjection
  - Relatively easy and rapid
  - No control over the insertion site
  - No control over the copy numbers inserted



- Gene targeting in embryonic stem cells
  - DNA construct integration in embryonic stem cells
  - Embryonic stem cells injection into blastocysts
  - Tedious and labor intensive
  - Construct inserted at a specific locus
  - Control over the copy numbers inserted

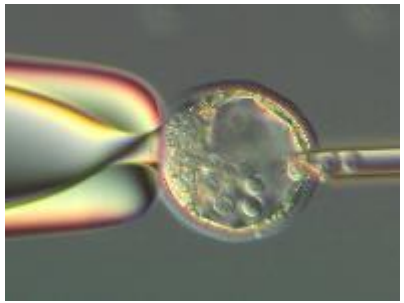


Image courtesy of ISTT and JoVE journal

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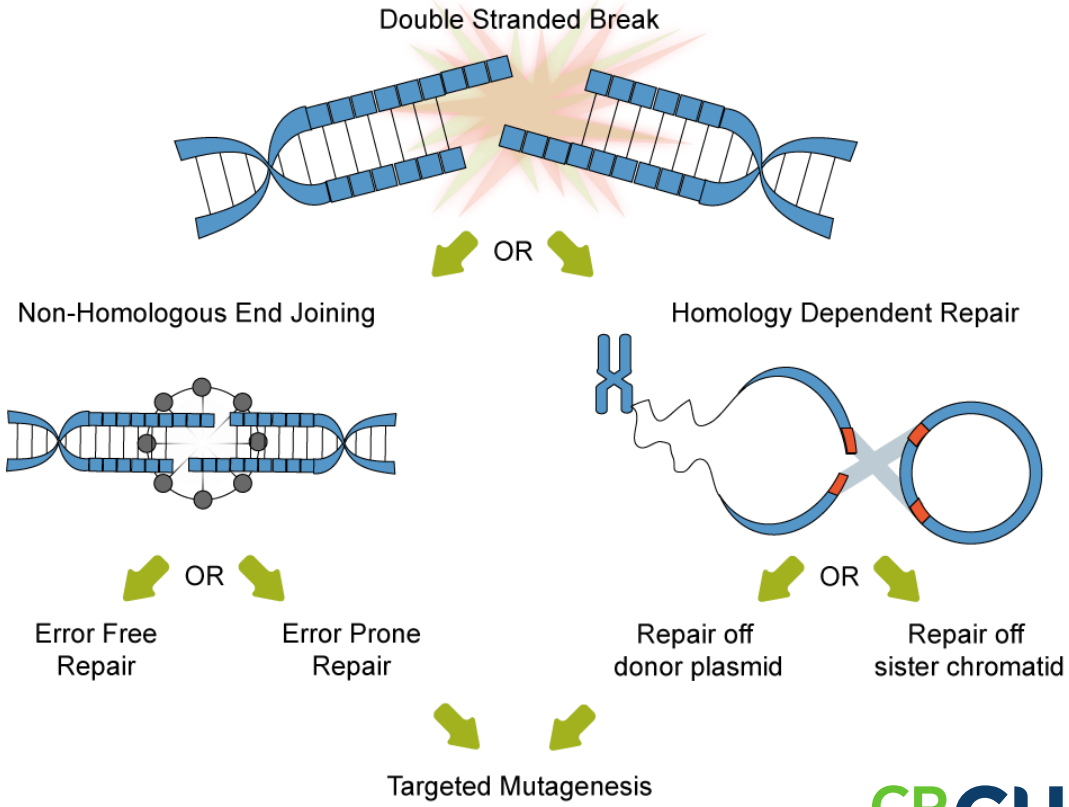
Targeted mutagenesis depends on intrinsic mammalian DNA repair mechanisms

-Highly active
-Active in dividing and non-dividing cells
-Integration occurs frequently



-Only active in dividing cells (S to G2 phase)
-Targeting occurs infrequently

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Customizable DNA/RNA-binding proteins for site-specific DNA modification via double-stranded break

- Meganucleases
- Transcription Activator Like Effector Nucleases (TALEN)
- Zinc Finger (ZF)
- Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 (CRISPR associated proteins)

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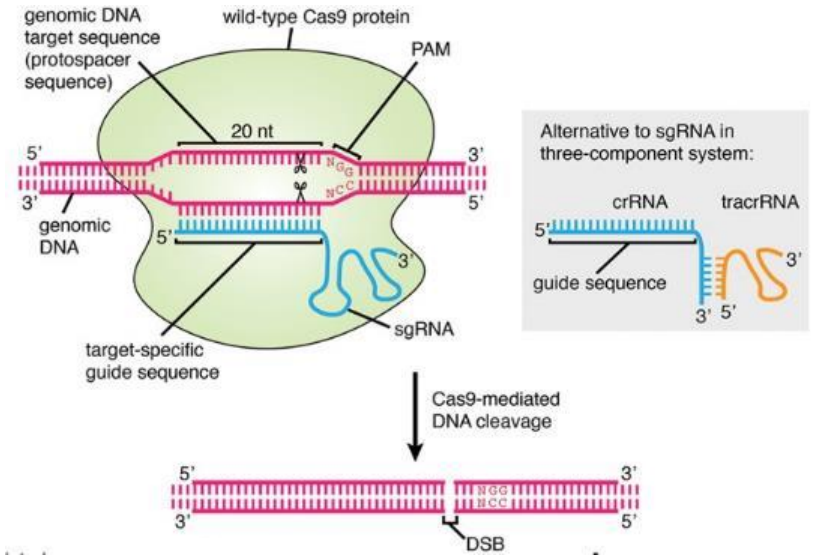
CRISPR/Cas9 functions as a gene editing tool

- Components required:
  - Cas9 protein
  - sgRNA or crRNA + tracrRNA
- Homology dependent (20 nucleotides)
- Protospacer Adjacent Motif (PAM)



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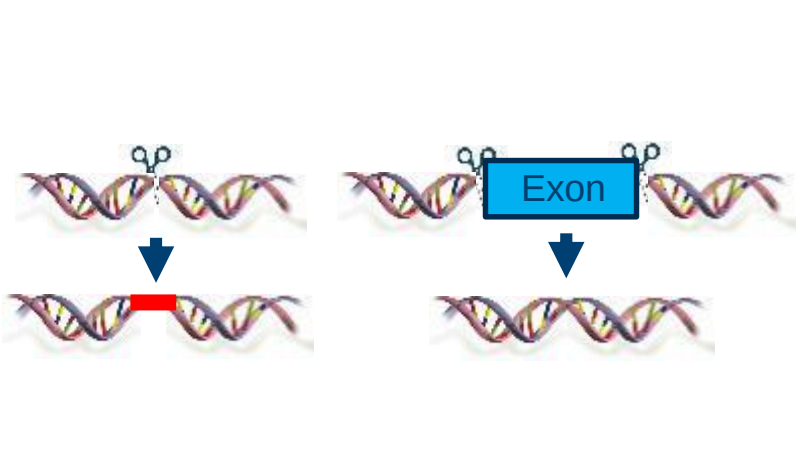
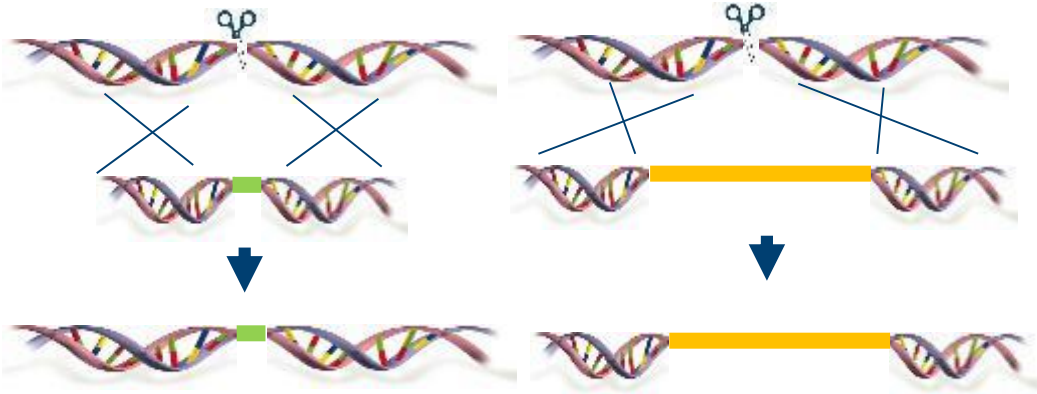
Rodent model generation approaches; the history re-written

NHEJ

HR





Easy

Difficult

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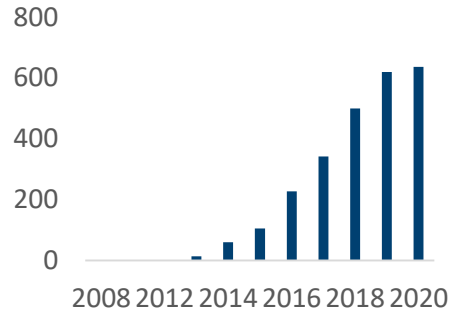
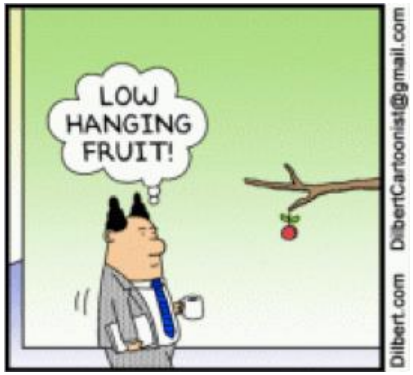
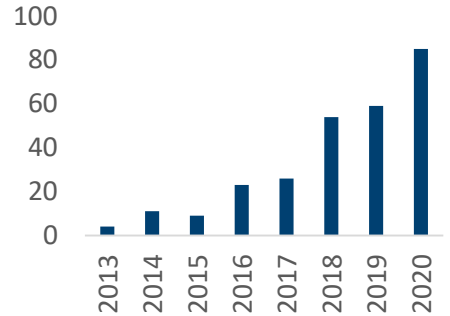
Low hanging fruit; « what about the off-targets »

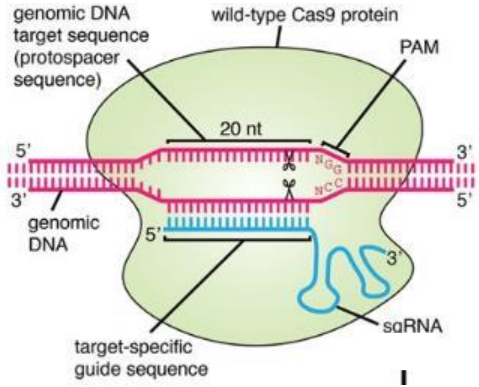
Search query: CRISPR Mouse





Search query: CRISPR Rat





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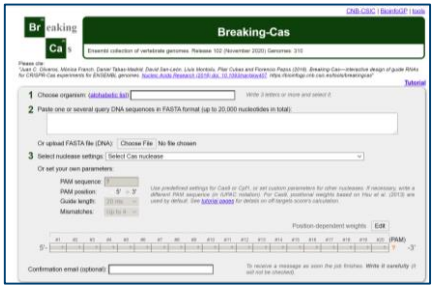
Guides prediction software

- Chop-Chop:
  - https://chopchop.cbu.uib.no/
- CRISPOR
  - http://crispor.tefor.net/crispor.py
- Breaking-Cas
  - https://bioinfogp.cnb.csic.es/tools/breakingcas/



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Guides prediction software

Download as Excel tables: Guides / Guides, all scores / Off-targets / Saturating mutagenesis assistant

Position/ Strand	Guide Sequence + *PAM* + Restriction Enzymes + Variants Only G- Only GG- Only A-	MIT Specificity Score	CFD Spec. score	Predicted Efficiency Show all scores Doench '16	Mor.-Mateos	Outcome Out-of-Frame	Lindel	Off-targets for 0-1-2-3-4 mismatches + next	Genome Browser links to matches sorted by CFD off-target score
35 / fw	CATTATCACAGACGATGTGG *TGG* Enzymes: *Hpy166II* **Cloning / PCR primers**	87	90	63	43	42	77	0 - 0 - 2 0 - 0 - 0 - 0 - 0 91 off-targets	H02Rik 4:intergenic:Slco3a1-Gm22326 4:intron:Ntrk3 4:intron:Galp 3:intron:Th 4:intergenic:Gm10161-Akap13 4:intergenic:Tshz3-Zfp536 **Off-target primers**
45 / fw	GACGATGTGGTGGACATAGC *AGG* **Cloning / PCR primers**	86	93	52	42	46	78	0 - 0 - 0 - 7 - 57 0 - 0 - 0 - 2 - 1 64 off-targets	4:exon:9530053A07Rik 4:intergenic:KCNQ1DN-Cdkn1c 4:intron:Nav2 4:intron:Tead1 **Off-target primers**
14 / rev	CACCACATCGTCTGTGATAA *TGG* **Cloning / PCR primers**	80	92	38	31	56	74	0 - 0 - 2 - 8 - 80 0 - 0 - 1 - 0 - 1 90 off-targets	4:intergenic:Abca16-E130201H02Rik 4:intergenic:Gm10161-Akap13 4:intron:Nav2 **Off-target primers**
57 / rev	CAAACCTCACCAGCACGGGC *AGG* Enzymes: *Bsp1286I, Alw21I* **Cloning / PCR primers**	80	88	55	29	58	65	0 - 0 - 3 - 5 - 93 0 - 0 - 0 - 0 - 1 101 off-targets	4:intron:Btbd16 3:intergenic:Ctbp2-Tex36 4:intron:Stk32c 4:intergenic:Nps-4930544L04Rik 4:exon:Gm15024 **Off-target primers**

```
guide:      CATTATCACAGACGATGTGG TGG
off-target: CAATATCTCAGATGATGGGG GGG
              *    *    *    *
```

CFD Off-target score: 0.071429
MIT Off-target score: 0.12
Position: chr7:78377969-78377991:+
Distance from target: 20.337 Mbp
>20Mbp = unlikely to be in linkage with target

genome.ucsc.edu/cgi-bin/hgTracks?db=mm10&position=chr7:78377969-78...

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Off-targets; the prevalance

Off-target mutations are rare in Cas9-modified mice

Vivek Iyer[1,4], Bin Shen[2,4], Wensheng Zhang[1,3], Alex Hodgkins[1], Thomas Keane[1], Xingxu Huang[2] & William C Skarnes[1]

[1]Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge, UK. [2]Ministry of Education Key Laboratory of Model Animal for Disease Study, Model Animal Research Center of Nanjing University, Nanjing, China. [3]Cambridge-Suda Genome Research Center, Soochow University, Suzhou, China. [4]These authors contributed equally to this work.
e-mail: xingxuhuang@mail.nju.edu.cn or skarnes@sanger.ac.uk

Exome sequencing in the knockin mice generated using the CRISPR/Cas system

Kazuo Nakajima[1], An-a Kazuno[1], John Kelsoe[2], Moe Nakanishi[3], Toru Takumi[3] & Tadafumi Kato[1]

Genome-Wide Off-Target Analysis in CRISPR-Cas9 Modified Mice and Their Offspring

Yan Dong,*[,1] Haimei Li,[†,1] Liang Zhao,[‡] Peter Koopman,[‡] Feng Zhang,[†] and Johnny X. Huang[†,‡,2]
*Gansu Provincial Maternity and Child Care Hospital, 143 North Road, Qilihe District, Lanzhou 730050, China, [†]School of Bioscience and Technology, Weifang Medical University, Weifang, Shandong 261053, China, and [‡]Institute for Molecular Bioscience, The University of Queensland, Brisbane, QLD 4072, Australia
ORCID IDs: 0000-0002-3489-7421 (L.Z.); 0000-0001-6939-0914 (P.K.); 0000-0003-3595-208X (J.X.H.)

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Off-targets; the prevalance

A- A+

PgmNr 277: Whole genome sequencing puts Cas9 off-target mutagenesis into the context of genetic drift.

Authors:
L.M.J. Nutter [1]; S. Khalouei [2]; J.D. Heaney [3]; D.G. Lanza [3]; S.M. Murray [4]; K. Peterson [4]; J.R. Seavitt [3]; J.A. Wood [5]; A. Ramani [2]

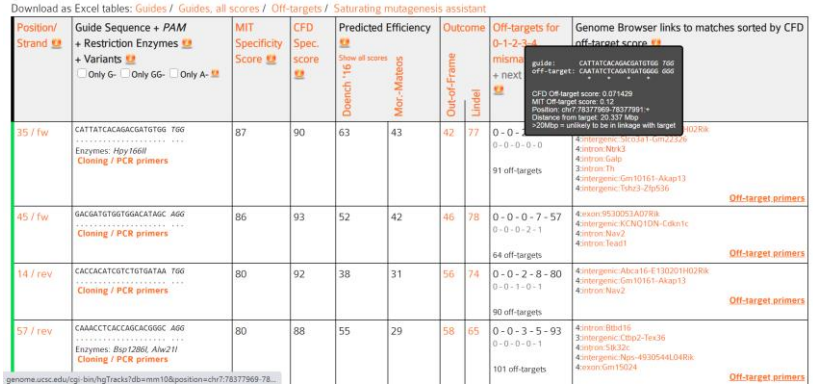
View Session | Add to Schedule

Affiliations:
1) The Centre for Phenogenomics, The Hospital for Sick Chlidren, Toronto, ON, Canada; 2) The Centre for Computational Medicine, The Hospital for Sick Children, Toronto, Canada, M5G 1X8; 3) Baylor College of Medicine, Houston, TX, 77030; 4) The Jackson Laboratory, Bar Harbor, ME, 04609; 5) Mouse Biology Program, University of California Davis, Davis, CA, 04609

There are reports demonstrating that Cas9 introduces off-target mutations and others that off-target mutation rates are low. The majority of reports investigate one or a few guide RNAs, which may result in sequence or chromosome location bias. The Knockout Mouse Phenotyping Project (KOMP2) produces mutant mice in a high-throughput pipeline using Cas9 for mutagenesis in the inbred C57BL/6N strain. This has enabled us to use whole genome sequencing to assess mutations in the genomes of 51 Cas9-derived founder mice representing 162 different gRNAs along with 25 inbred control mice. Illumina paired-end reads provided >35X coverage with ≥90% of bases with >25 reads. Variants (SNPs and indels) were identified using GATK4.0 and structural variants using an intersection of Lumpy, Manta, CNVkit, and Wham, followed by MetaSV. Variants were filtered out when they occurred in two or more mice, indicating the variant likely resulted from genetic drift rather than from Cas9 activity. We used CasOFFinder to identify predicted off-targets with up to 5 mismatches and one DNA or RNA bulge among the variants in the respective founder for each gRNA. There were 20 genes for which one or more Cas9-induced off-target mutations were identified (46 total with a range of 1-10 and average of 2.3 per founder). For 31 genes, no Cas9-induced off-target mutations were identified. Importantly, these analyses demonstrated that there was an average of ~3,500 variants unique to each animal – founder or untreated control. Two important conclusions can be drawn; (1) with appropriately designed Cas9 gRNAs off-target mutagenesis is rare; and (2) genetic drift within a carefully maintained inbred line of mice results in thousands of genetic variants between individuals within that line. These results have implications in the use of mice to model human disease, i.e. that backcrossing or outcrossing mice introduces significantly more variation than the use of Cas9 and the appropriate controls are littermate or line mate wild-type mice for most genetic experiments. These results also raise the question. What is “normal” genetic sequence in the context of model organisms and in humans - patients, controls, tissues and cell lines; for both assessing the specificity of Cas9 for genome editing and assessing the consequences of variants associated with disease?

Off-targets, how to detect them

- Targeted re-sequencing (+/- high-throughput)
  - Relatively quick and easy
  - Inexpensive
  - Depends on *in silico* software prediction specificity
- Whole genome sequencing
  - Expensive
  - Requires computer power, bioinformatics knowledge and a proper analysis pipeline
  - Unbiase results and complete picture



Download as Excel tables: Guides / Guides, all scores / Off-targets / Saturating mutagenesis assistant

Position/ Strand	Guide Sequence + *PAM* + Restriction Enzymes + Variants	MIT Specificity Score	CFD Spec. score	Predicted Efficiency: Doench '16	Predicted Efficiency: Mor.-Mateos	Outcome: Out-of-Frame	Outcome: Lindel	Off-targets for 0-1-2-3-4 mismatches + next	Genome Browser links to matches sorted by CFD off-target score
35 / fw	CATTATCACAGACGATGTGG TGG Enzymes: *Hpy166II* Cloning / PCR primers	87	90	63	43	42	77	0 - 0 - 0 - 0 - 0 91 off-targets	Off-target primers
45 / fw	GACGATGTGGTGGACATAGC AGG Cloning / PCR primers	86	93	52	42	46	78	0 - 0 - 0 - 7 - 57 0 - 0 - 0 - 2 - 1 64 off-targets	Off-target primers
14 / rev	CACCACATCGTCTGTGATAA TGG Cloning / PCR primers	80	92	38	31	56	74	0 - 0 - 2 - 8 - 80 0 - 0 - 1 - 0 - 1 90 off-targets	Off-target primers
57 / rev	CAAACCTCACCAGCACGGGC AGG Enzymes: *Bsp1286I, AlwI1I* Cloning / PCR primers	80	88	55	29	58	65	0 - 0 - 3 - 5 - 93 0 - 0 - 0 - 0 - 1 101 off-targets	Off-target primers

Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR

Maximilian Haeussler[1*], Kai Schönig[2], Hélène Eckert[3], Alexis Eschstruth[4], Joffrey Mianné[5], Jean-Baptiste Renaud[6], Sylvie Schneider-Maunoury[4], Alena Shkumatava[3], Lydia Teboul[5], Jim Kent[1], Jean-Stephane Joly[6] and Jean-Paul Concordet[7*]

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Recommendations/suggestions

1) Sequence verify founder animals (F0)

2) Sequence verify germline transmitted animals (N1)

A) **Backcross** your animals before generating publishable data (up to 5 generation)
B) If possible, work with strains originating from more than one founder
C) If possible, work with two independent strains generated using two different guides
D) Test for predicted off-target mutations (especially the one in linkage)
E) Perform whole genome sequencing

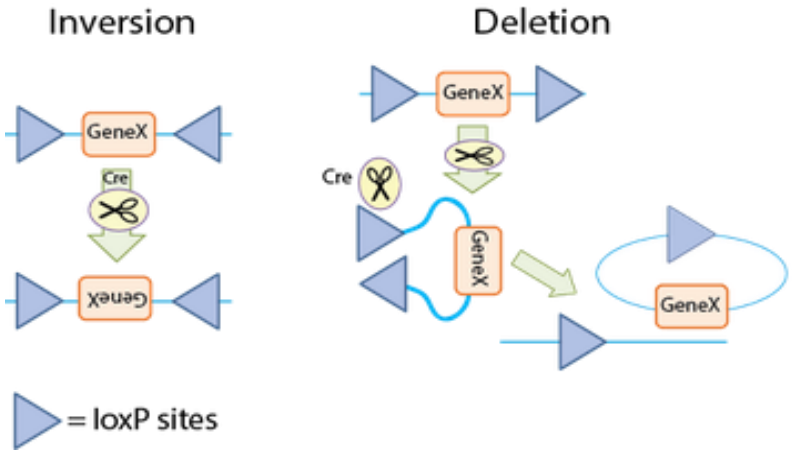
The Cre-Lox system: a cautionary tale

Thierry Alquier, PhD

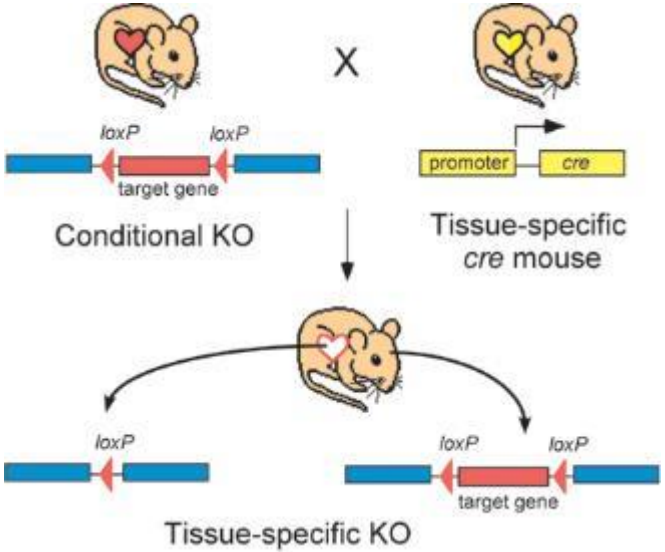
Cre-Lox system principles

- CRE recombinase isolated from bacteriophages based on its ability to recognize/bind loxP DNA sequences and recombine DNA flanked by loxP

13bp 8bp 13bp
ATAACTTCGTATA -NNNTANNN-TATACGAAGTTAT



- CRE expression driven by a tissue/cell-specific promoter allows to generate a tissue-specific gene KO



Timing of the KO is dependent on the temporal expression pattern of the promoter driving CRE expression

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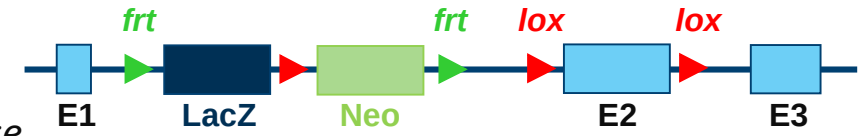
Cre-Lox system principles

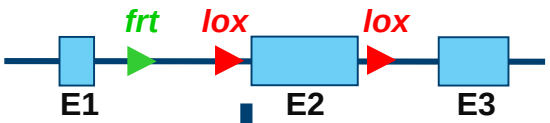
What do you typically get when you generate or purchase a « loxed » mouse ?

Ex: International KO Mouse Consortium (IKMC)



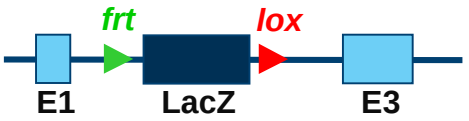
ES cells from C57BL/6N

Crossed with a FLIPASE mouse



Crossed with tissue-specific CRE mouse

Crossed with tissue-specific CRE mouse



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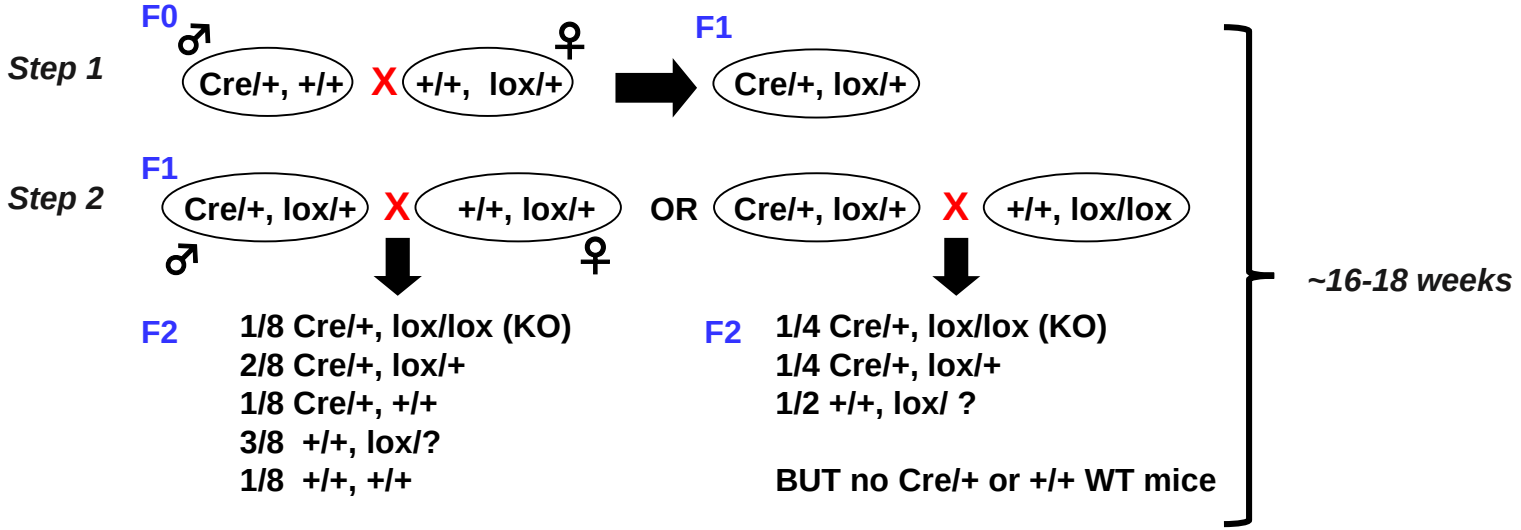
Cre-Lox breeding scheme



Pros : -generation of control littermates
-avoiding genetic background drifts (for mixed background)

Cons : -low % of Cre/+, lox/lox (KO) mice

- **Cre/+** carrier should be a male (to avoid potential phenotype emerging during pregnancy)
- **Cre/+, lox/+** should not be bred together (potential CRE toxicity)
- **Cre/+, lox/+ and +/+, lox/?** from **F2** should not be used for breeding because of potential segregation of a specific genetic background if the mice are on a mixed background.

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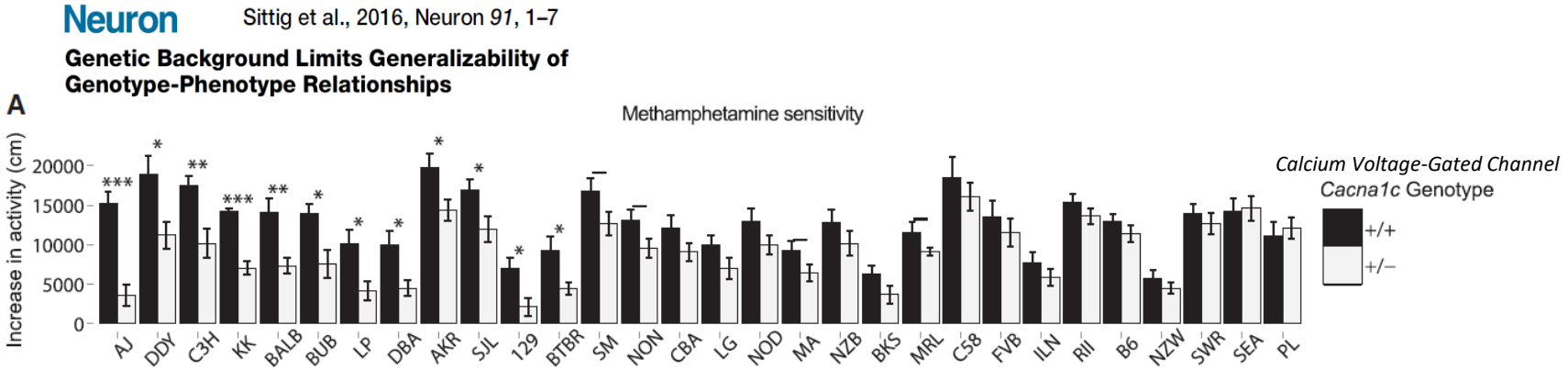
The importance of the genetic background

- The most widely used strains for the generation of transgenic mice harbor major genotypic and phenotypic differences: 129S1, FVB, DBA, C57BL/6
- Most Cre-Lox animals have mixed genetic background
  ex: Lox mice generated using ES cells from 129S1 or BL/6N mice crossed with a CRE strain from Jax (IKMC uses C57BL/6N ES cells to target alleles)
- The phenotype associated with a genetic manipulation will be influenced by the genetic background



The importance of the genetic background

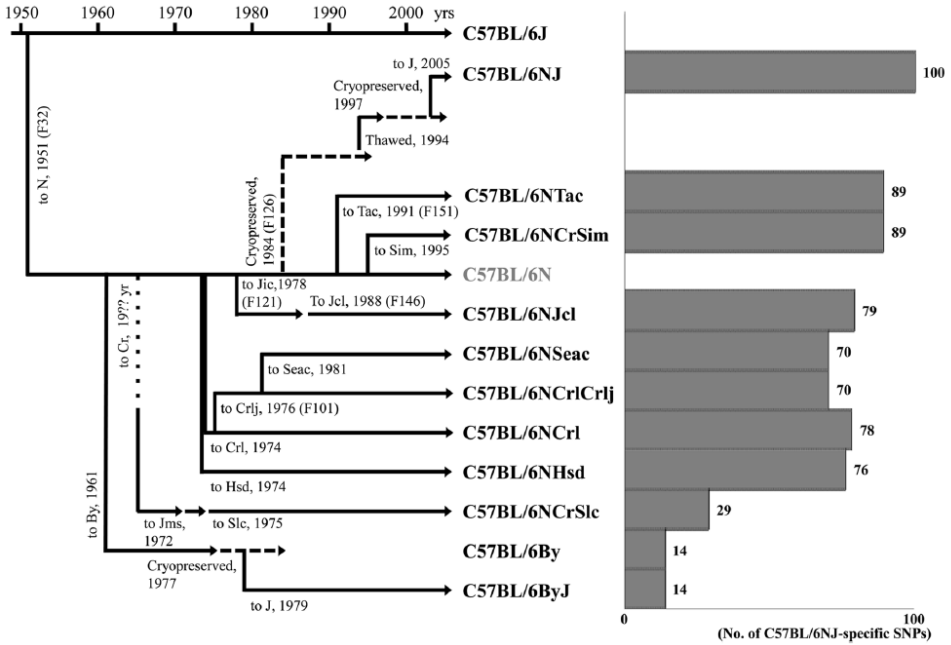
C57BL/6 substrains

Table 1. C57BL/6 substrains investigated in this study

Substrain		Source
C57BL/6N substrains	C57BL/6NJ	The Jackson Laboratory (Bar Harbor, MA, USA)
	C57BL/6NCrSim	Simonsen Laboratories, Inc. (Gilroy, CA, USA)
	C57BL/6NTac	Taconic Farm Inc. (New York, NY, USA)
	C57BL/6NJcl	CLEA Japan Inc. (Tokyo, Japan)
	C57BL/6NSeac	Kyudo Co. Ltd. (Tosu, Japan)
	C57BL/6NCrlCrlj	Charls River Laboratories Japan, Inc. (Yokohama, Japan)
	C57BL/6NCrl	Charls River Laboratories International, Inc. (Wilmington, MA, USA)
	C57BL/6NHsd	Harlan Laboratories, Inc. (Indianapolus, IN, USA)
	C57BL/6NCrSlc	Japan SLC, Inc. (Hamamatsu, Japan)
	C57BL/6By	The Jackson Laboratory (Bar Harbor, MA, USA)
	C57BL/6ByJ	The Jackson Laboratory (Bar Harbor, MA, USA)
C57BL/6J substrains	C57BL/6J	The Jackson Laboratory (Bar Harbor, MA, USA) via Charls River Laboratories Japan, Inc. (Yokohama, Japan)
	C57BL/6JJcl	CLEA Japan Inc. (Tokyo, Japan)
	C57BL/6JJmsSlc	Japan SLC, Inc. (Hamamatsu, Japan)
	C57BL/6JEiJ	The Jackson Laboratory (Bar Harbor, MA, USA)
	C57BL/6JOlaHsd	Harlan Laboratories, Inc. (Indianapolus, IN, USA)
	C57BL/6JRccHsd	Harlan Laboratories, Inc. (Indianapolus, IN, USA)
	C57BL/6JBomTac	Taconic Farm Inc. (New York, NY, USA)

Nomenclatured strain names of each C57BL/6 substrain were in accordance with JAX® NOTES [16].

Mekada et al., Exp. Animals 2014



Deletions (NNT), retrotransposon insertion (Raptor) & copy number variations (IDE, FGFBP3)

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The importance of the genetic background

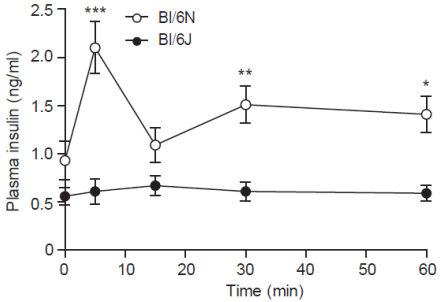
C57BL/6J vs. 6N: the NNT mutation

- C57BL/6J have a mutation in the *nicotinamide nucleotide transhydrogenase* (NNT) gene leading to impaired transfer of hydrogen between NADH and NADP+ in the inner mitochondria membrane, reduced NADPH content and altered mitochondrial function.

Defective insulin secretory response to intravenous glucose in C57Bl/6J compared to C57Bl/6N mice

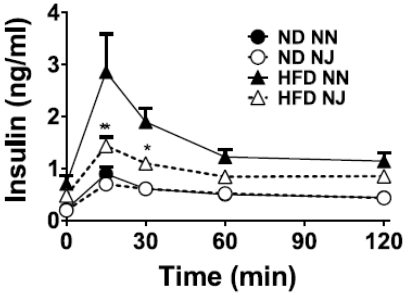
MOLECULAR METABOLISM

Fergusson G., Ethier M. et al. 2014



Differential Insulin Secretion of High-Fat Diet-Fed C57BL/6NN and C57BL/6NJ Mice: Implications of Mixed Genetic Background in Metabolic Studies *Attané C. et al. 2016*

PLOS ONE



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The importance of the genetic background

Solutions:

- Backcrossing (breeding or IVF) on a pure background
  - No perfect strains (e.g. C57BL/6N has a mutation in *Crb1* leading to retinopathy)
  - Do your homework, get informations and chose the background wisely

- Mixed background
  - Limit the genetic drift (c.f. breeding) & always use control littermates
  - Genotype for known mutations (e.g. NNT) & select experimental animals
  - Provide a detailed and complete information on background strain, breeding practice, and control groups

Attention to Background Strain Is Essential for Metabolic Research: C57BL/6 and the International Knockout Mouse Consortium

Diabetes 2016;65:25–33 | DOI: 10.2337/db15-0982

Fontaine D. A. et al. Diabetes 2016

"Overall, we found ~60% of publications in the past 4 years in the journal Diabetes had incomplete explanations of the background substrain".

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The importance of the control groups

Why including CRE littermates matters ?

- CRE transgene site of insertion and number of copies are often unknown = potential mutagenesis and phenotype (most CRE strains have a phenotype).
- The genome contains degenerate loxP sites that are recognized and recombined by CRE leading to off-target recombination of genes in a tissue-specific manner.

Cre expression driven by the α-myosin heavy chain promoter can be cardiotoxic (*Pugach et al. JMCC 2015*)

"We identified 619 loxP-like sites. 227 sites overlapped with annotated genes & 55 of these genes were expressed in cardiac muscle. Expression of ~26% of the 27 genes tested was disrupted in αMyHC-CRE (+/-) mice"

Why including loxP littermates matters ?

- Insertion of loxP sites/Neo in introns can affect the normal expression pattern of the targeted gene

Why including heterozygous KO mice (CRE/+, lox/+) may matter ?

- Important to assess a potential gene-dosage relation with the phenotype
- More representative of pathological conditions with reduced gene expression

What about WT littermates ? They can be phenotypically compared to lox mice and/or CRE & included

The Cre-Lox system is a powerful tool but is not perfect

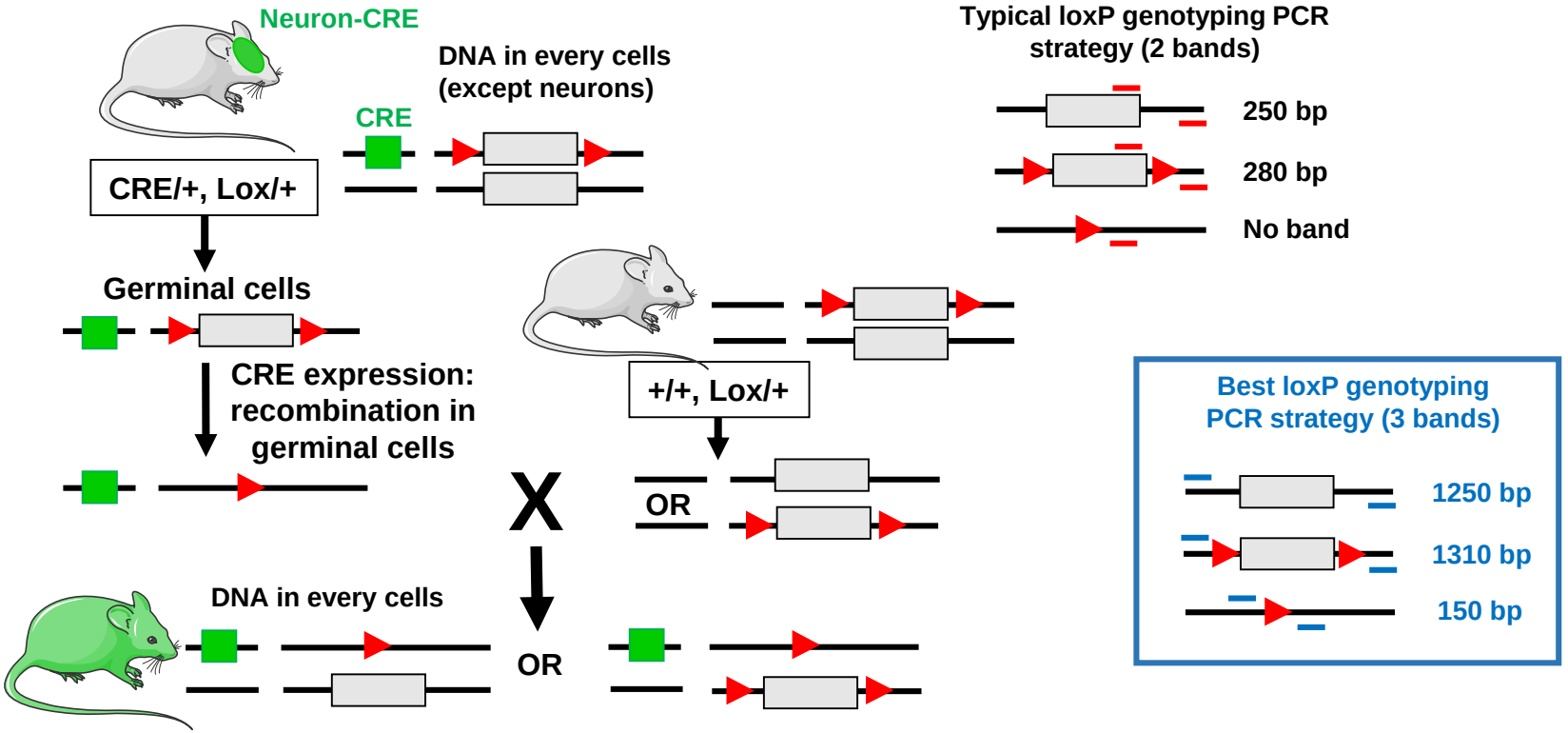
- Mutagenesis induced by CRE insertion & related phenotype
- Expression of the human Growth Hormone minigene from the CRE transgene
- Leakiness of the promoter driving CRE expression
- Ectopic CRE expression during gametogenesis (whatever the promoter)
- Silencing of the promoter after several generations
- Toxicity of CRE (strong promoter and/or targeting of degenerate loxP sites)
- Developmental impact of gene KO when CRE is expressed during embryogenesis

Metabolic Pitfalls of CNS *Cre*-Based Technology
Cell Metabolism *Harno E. et al. 2013*

Pancreas-Specific Cre Driver Lines and Considerations for Their Prudent Use
Cell Metabolism *Magnuson M. A. et al. 2016*

Considerations and guidelines for mouse metabolic phenotyping in diabetes research Diabetologia (2018) 61:526–538 *Alquier & Poitout*

Ectopic CRE expression during gametogenesis



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Ectopic CRE expression during gametogenesis

Neuron

Optimizing Nervous System-Specific Gene Targeting with Cre Driver Lines: Prevalence of Germline Recombination and Influencing Factors

Luo et al., 2020, Neuron *106*, 1–29

Table 1. Prevalence of Germline Recombination in Mouse Cre Driver Lines Designed for Nervous System-Specific Recombination

Cre line Common Name	Full Cre Line Name/ Source	Target Gene/ Reporter	Breeding Strategy[a]	Germline Recombination Efficiency, Cre from Father[b]	Germline Recombination Efficiency, Cre from Mother[b]	Germline Recombindation Efficiency, Parental Sex Effects Unknown[b]	Reference/ Associated Publication[c]	Contributors[d]
CaMKIIα-Cre (T29-1)	Tg(Camk2a-cre) T29-1Stl	*Khdrbs3*$^{tm1.1Schei}$/J	C	31.3% (5/16)	0% (0/7)	–	–	Elisabetta Furlanis, Lisa Traunmüller, Peter Scheiffele
GFAP-Cre	Tg(GFAP-cre)25Mes	*Gja1*^{tm1Kwl}	C	16.7% (7/42) of Cre negative offspring	50% (8/16) of Cre negative offspring	–	Zhang et al., 2013	–
	Tg(GFAP-cre)25Mes	*Epas1*^{tm1Mcs}/J	A or C	50% (9/18)	42.9% (6/14)	–	–	Ariane Pereira, Jeremy N. Kay
Synapsin1-Cre	B6.Cg-Tg(Syn1-cre)671Jxm/J	*Prkar2b*^{tm3Gsm}	F	observed	0 or less than male	–	Zheng et al., 2013	–
	B6.Cg-Tg(Syn1-cre)671Jxm/J	*Hif1a*^{tm1Rsjo}	C	63%	0	–	Zheng et al., 2013	–

Ectopic germline recombination activity of the widely used Foxp3-YFP-Cre mouse: a case report

2019 John Wiley & Sons Ltd, *Immunology*, **159,** 231–241

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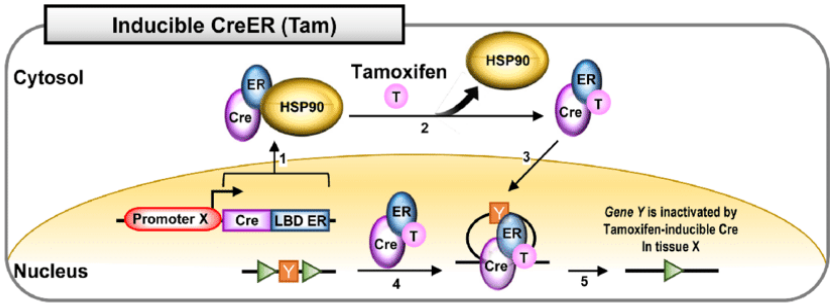
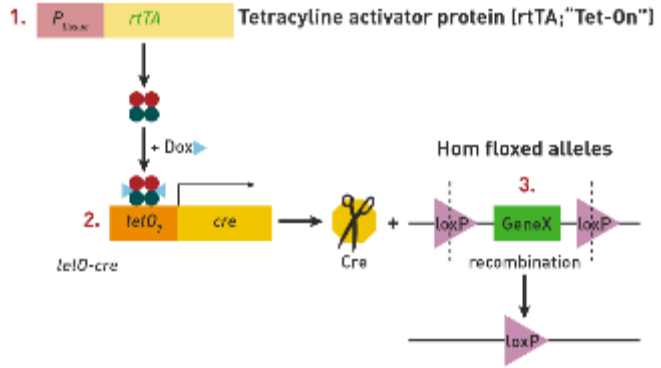
Alternate systems to control CRE temporal expression/activity

1-Tamoxifen-inducible (CreER & CreERT2)



- Tamoxifen (ER agonist): dose & time dependent side effects
- Wash out periods required
- Low recombination efficiency (mosaicism)
- Spontaneous CreER activity
- Novel TAM receptor: Na_V channel

2-Tetracycline-inducible (Tet-ON)



- Tetracycline (antibiotics): dose & time dependent side effects (e.g. mitochondrial dysfunction).
- tTA affects gene expression (β-cells)
- Wash out periods required
- Triple transgenic mouse

3-Photoactivable CRE and self-cleaved inducible CreER (next generation)

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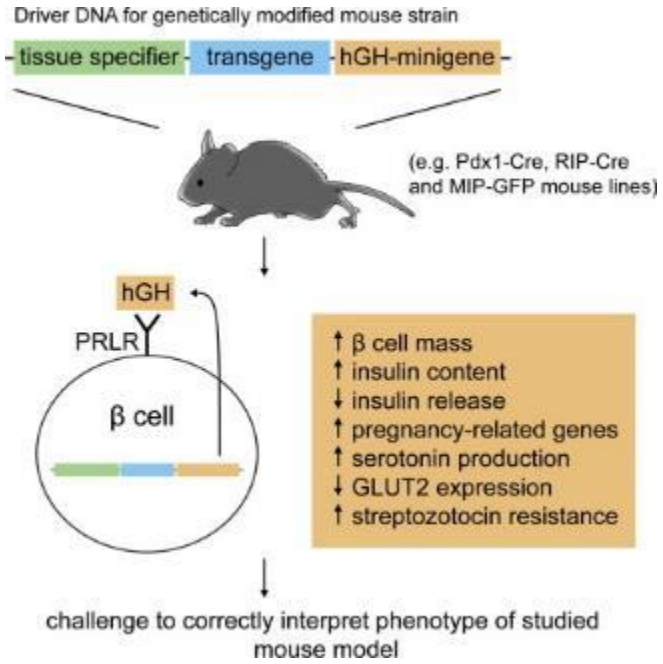
CRE transgene: the human Growth Hormone minigene



Impaired Islet Function in Commonly Used Transgenic Mouse Lines due to Human Growth Hormone Minigene Expression
Cell Metabolism *Brouwers B. et al. 2014*

Phenotypic Characterization of MIP-CreERT^{1Lphi} Mice With Transgene-Driven Islet Expression of Human Growth Hormone
Diabetes *Oropeza D. et al. 2015*

Metabolic and Behavioural Phenotypes in Nestin-Cre Mice Are Caused by Hypothalamic Expression of Human Growth Hormone
PLOS ONE *Declercq J. et al. 2015*

Trends in Endocrinology & Metabolism
2018, Vol. 29, No. 10 *De Faudeur G. et al. 2018*

List of 300 Cre strains with mGH gene
Mouse Lines – Groep Biomedische Wetenschappen KU Leuven

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Guidelines for Cre-Lox strategies

- **Seek for informations/get advices (CIPA & transgenesis core are here to help you!)**
- Use CRE hemizygous breeders
- Check mGH status of your favorite CRE strain
- Optimize genotyping PCR to verify germline CRE expression & recombination
- Use reporter strains to assess CRE specificity (e.g. $ROSA^{mT/mG}$) in multiple tissues
- INCLUDE experimental CRE littermates
- ALWAYS USE control littermates (not from a separate colony or from purchase)
- Consider alternatives/complementary strategies (Cre-expressing viruses)
- **Provide a complete information on background strain, breeding practice and control groups**

Take home messages

Take Home Message #1

CIPA & the veterinary team are your friends!

Before and during your project, CIPA and the veterinary team can provide critical information to help you reach your research goals faster.

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Take Home Message #2

Words matter!

Use the appropriate nomenclature and breeding strategies for your mouse lines.

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Take Home Message #3

Tips, tricks and tech exist here!

The transgenic platform uses cutting-edge approaches and can design and execute a turn-key project for you.

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Take Home Message #4

Know your limits!

All tools have limits.
All models have limits.
No model is perfect.

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Take Home Message #5

Be in the know!

It is the responsibility of the researcher to know the details of their animal model and to report it accurately.

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With great power comes great responsibility

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