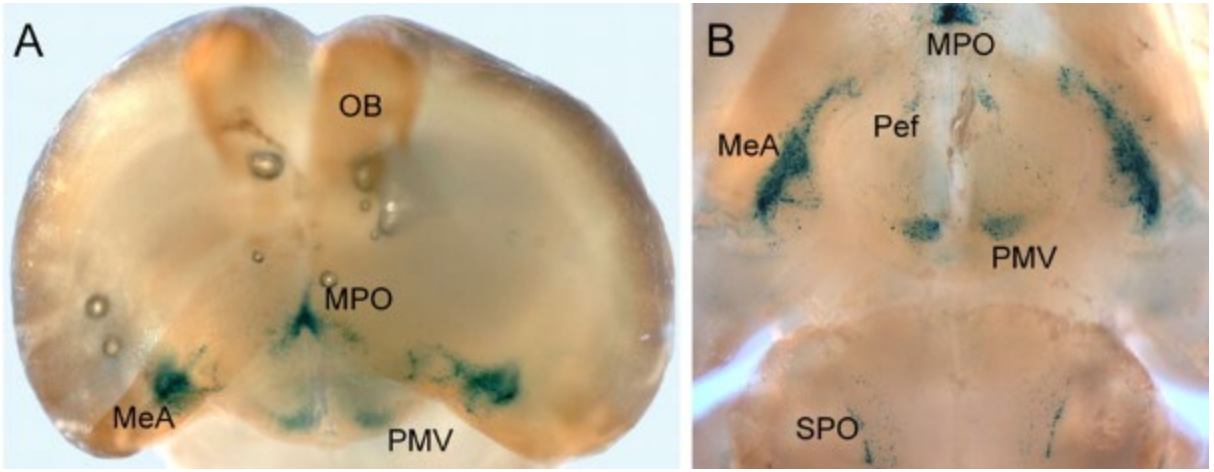
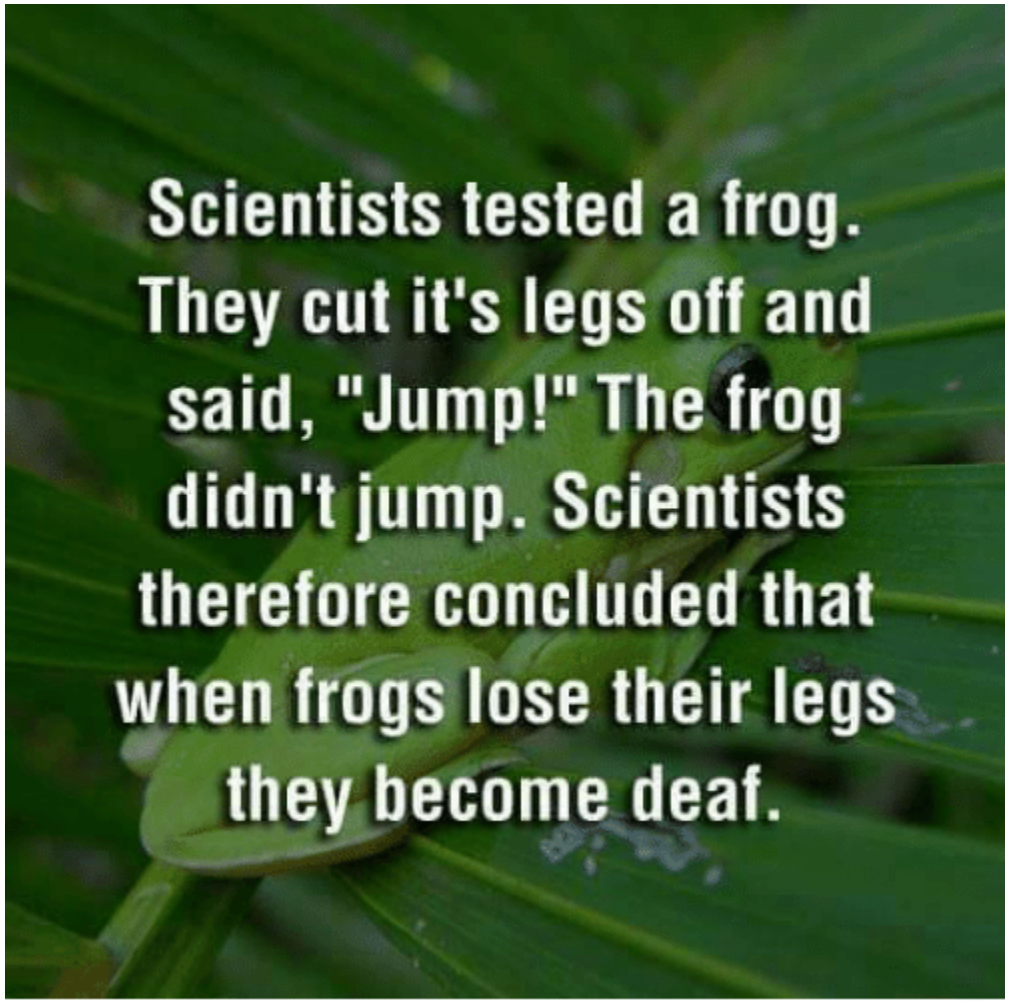
# Lesions, pharmacology, and genetic manipulations




Deussing et al., J. Neurosci., 2010.

Lecture 9

Anita Autry, Ph.D.



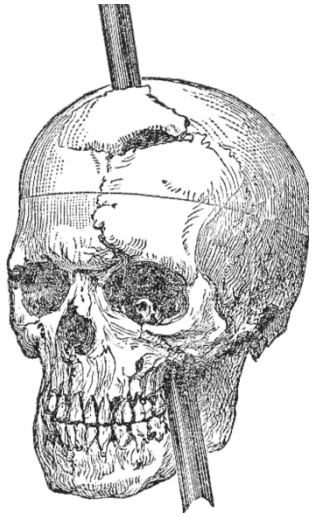

- Reviewer 1: Overinterpreting results. Please provide supplemental discussion up to 5 pages. Cite my work.
- Reviewer 2: Should use more refined manipulations. Can't you use CRISPR for this?
- Reviewer 3: Lack of appropriate controls. Frogs are a terrible model organism for humans. Reject.

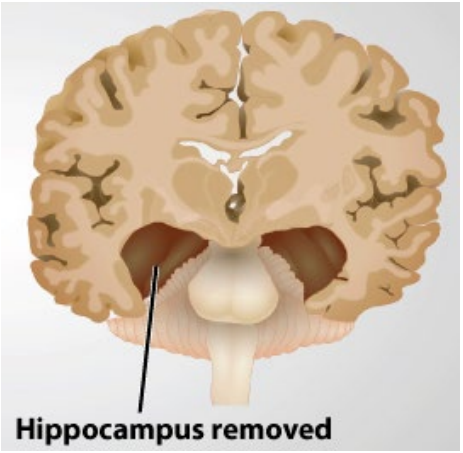
# Manipulations

- Lesions
- Pharmacology
- Knockout
- Knockin
- Conditional Expression
- Inducible expression*
- CRISPR-based expression systems

# Lesions

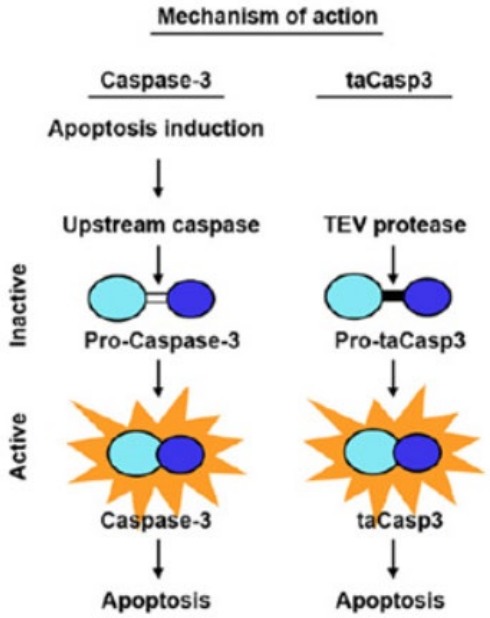
- Physical
  - Knife cuts
  - aspiration
- Electrolytic
- Chemical
  - NMDA
  - Kainic acid
  - Ibotenic acid
  - NBQX
  - Tetanus toxin
  - (6-OHDA)
- Viral
  - DTA
  - Casp3





# Lesions: practical concerns

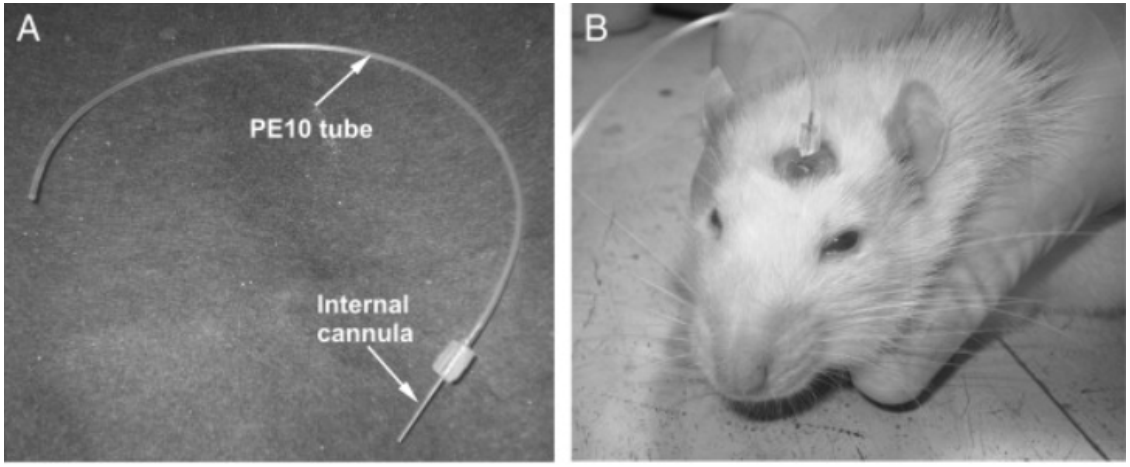
- Interpretation of function
- Physical and electrical lesions destroy cell bodies and fibers of passage
- Chemicals: may be nasty to work with
- Viral lesions take 1 month




Yang et al., Cell, 2013.

# Pharmacology

- Reversible inhibition
  - Muscimol
  - Lidocaine
- Agonists
- Antagonists



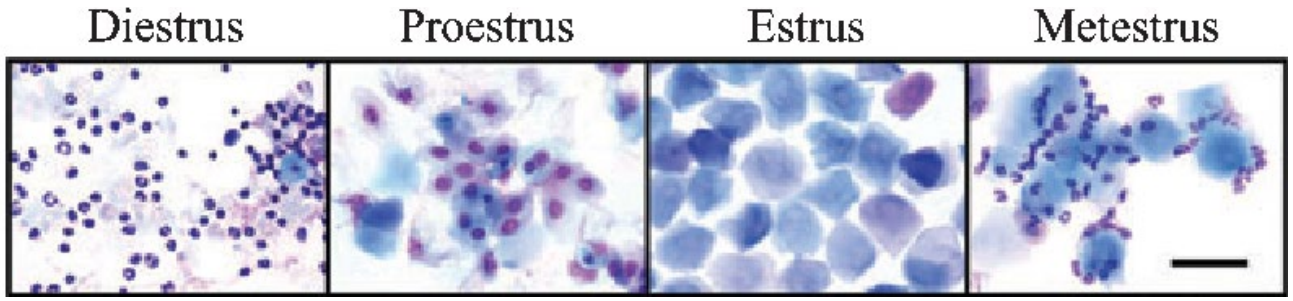

Kokare et al, J. Pharm. Tox. Methods, 2011.

# Pharmacology: practical concerns

- Hard to tell how far drug diffuses from injection site and at what concentration
- Half-life of drug

# Genetic manipulations: mice

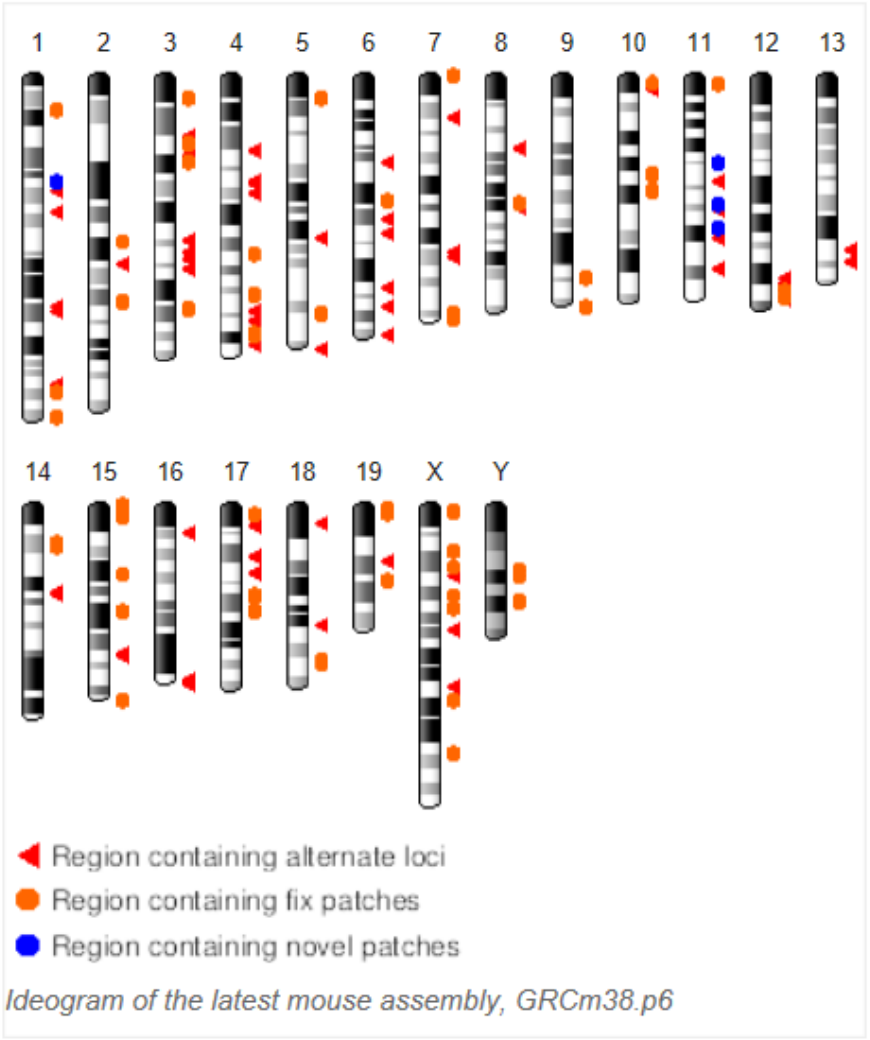
- Fast generation time (19-21 days gestation)
- System for ES cell culturing
- Lots of inbred strains
- Litter size 5-10
- Female estrus 4-5 days
- Puberty around 28 days



# Genome Projects

- First complete human genome assembly released in 2003
  - Current v. 16c
- First draft mouse assembly 2001
  - https://www.ncbi.nlm.nih.gov/grc
  - www.ensembl.org

# Mouse genome

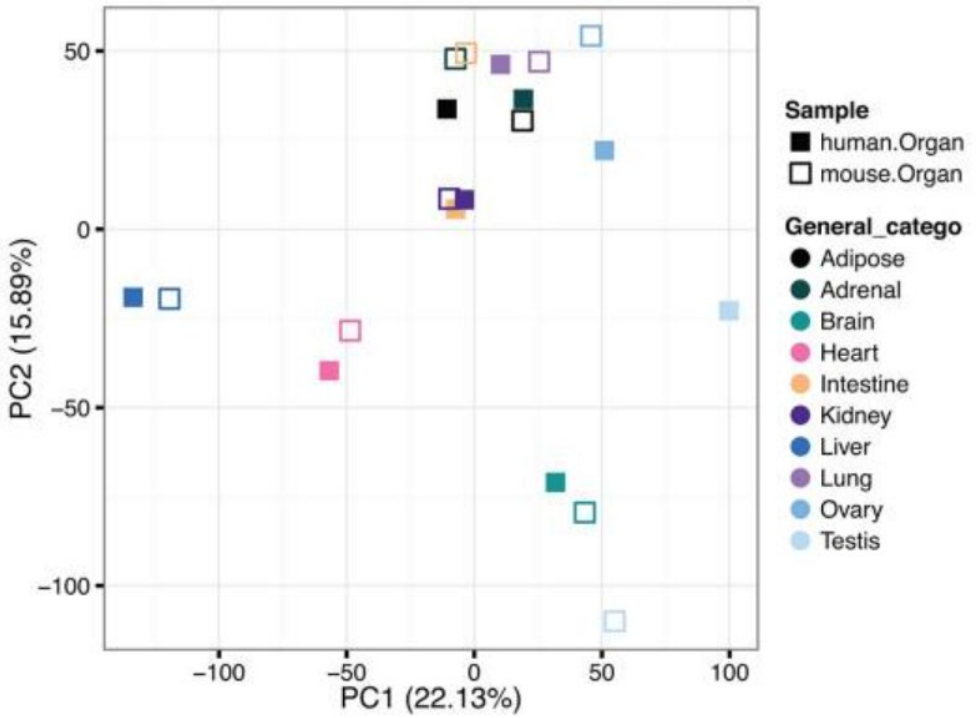

*Ideogram of the latest mouse assembly, GRCm38.p6*

- Coding genes: 22,515
- Non-coding genes: 16,074
- Pseudogenes: 201
- Transcripts: 142,647

- m39 coming soon (2020)

# Mouse ENCODE

- Mapping conserved elements between mouse and human
  - mouseencode.org
  - encodeproject.org
- Comparisons:
  - TF binding sites
  - Chromatin organization
  - Replication timing




Yue et al., Nature, 2015.

# Genetic manipulation resources


IMPC
International Mouse Phenotyping Consortium

MMRRC
Mutant Mouse Resource & Research Centers supported by NIH

UCDAVIS
KOMP Repository
KNOCKOUT MOUSE PROJECT

The Jackson Laboratory

IGTC International Gene Trap Consortium

# Knockout: constructs

- Homologous recombination

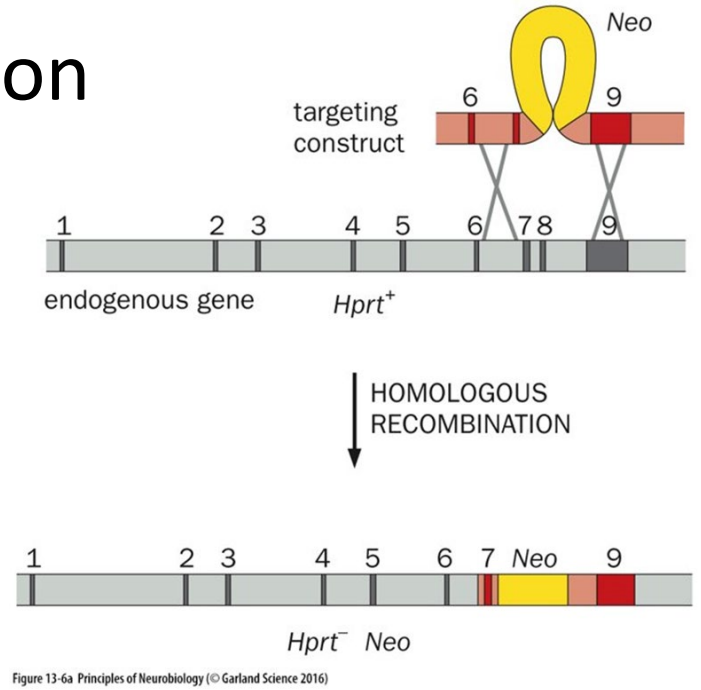
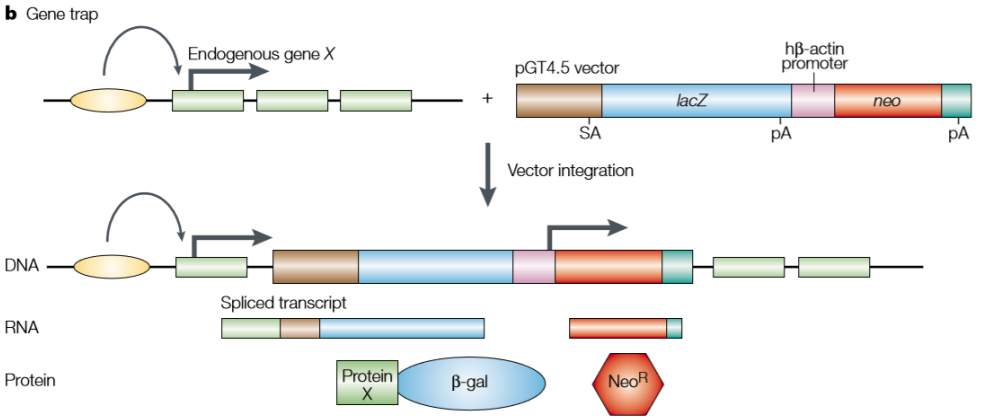



Figure 13-6a Principles of Neurobiology (© Garland Science 2016)

- Gene trapping



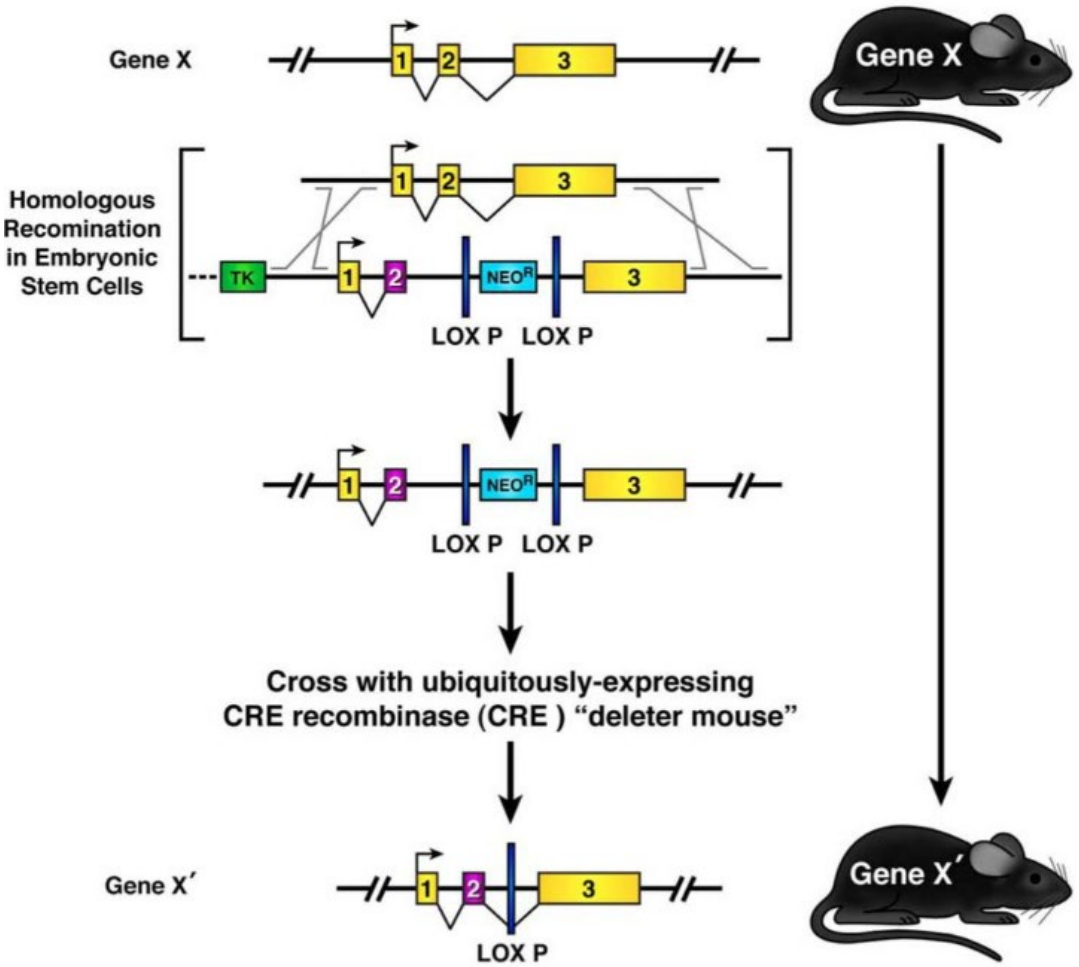

Stanford et al., Nat. Rev. Genetics, 2001.

# Issues with knockouts

- 15% of knockouts are embryonic lethal
- May be genetic redundancy or compensation that masks effects of knockout
- May not recapitulate a human disorder

# Knockin: constructs

- Targets endogenous gene locus for insertion of genetic material




Doyle et al., Transgenic Res., 2013.

# Issues with knockins

- May not recapitulate a human disorder

# Transgene: constructs

- **B**acterial **A**rtificial **C**hromosome: Plasmid containing around 150-350kb

## Objective

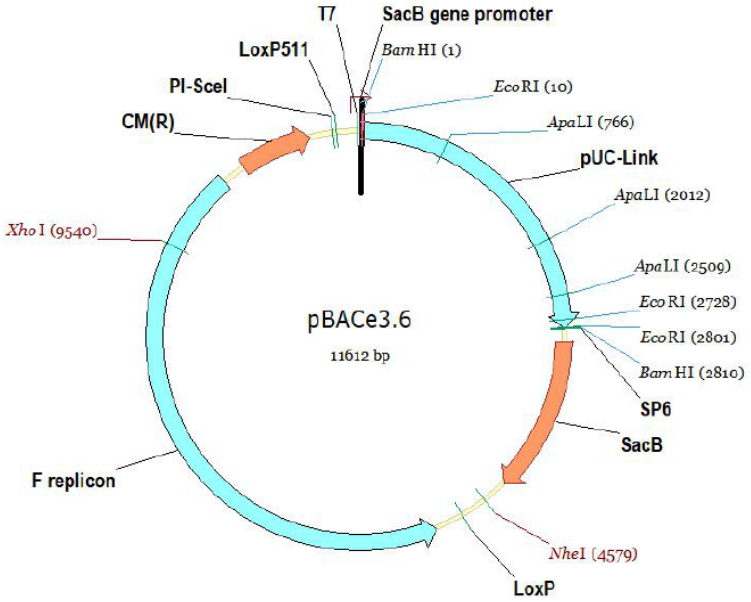
Modify BAC clone RP23-332L13 to insert Flpo-P2A-TagBFP2 cassette at the start codon of mouse Ucn3 gene.

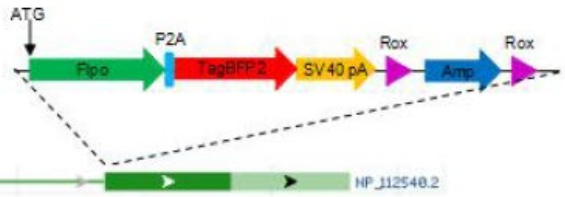
**Tg(Ucn3-cre)KF31Gsat**

Transgene Detail

Mutation details: A cre-expression cassette, followed by a polyadenylation sequence, was inserted into BAC clone RP23-332L13 at the initiating ATG codon of the first coding exon of the Ucn3 gene so that cre expression is driven by the regulatory sequences of the BAC gene. The resulting modified BAC (BX2061) was used to generate this transgene. (*J:100256*)

c. Cloning vector map



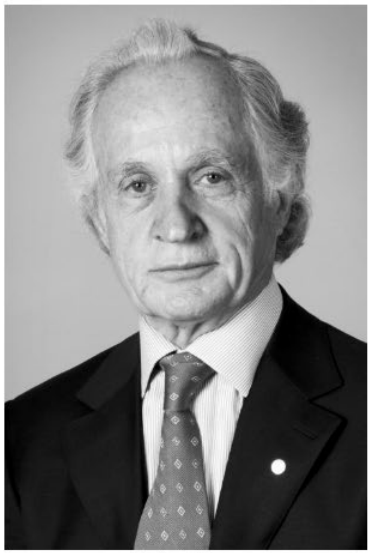



# Issues with transgenes

- Can’t control where transgene integrates
- Can’t control how many times transgene integrates

# Genetic modification of embryonic stem cells

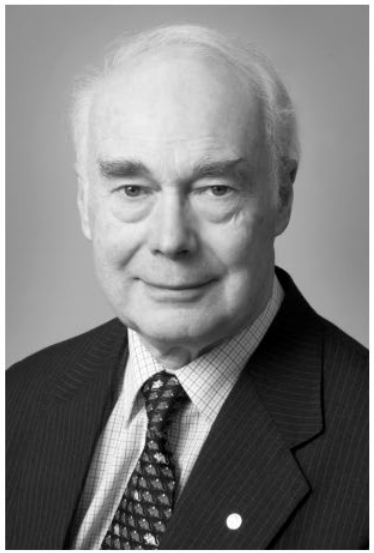
## The Nobel Prize in Physiology or Medicine 2007





Mario R. Capecchi

Prize share: 1/3





Sir Martin J. Evans

Prize share: 1/3



Oliver Smithies

Prize share: 1/3

# Mutant mouse generation

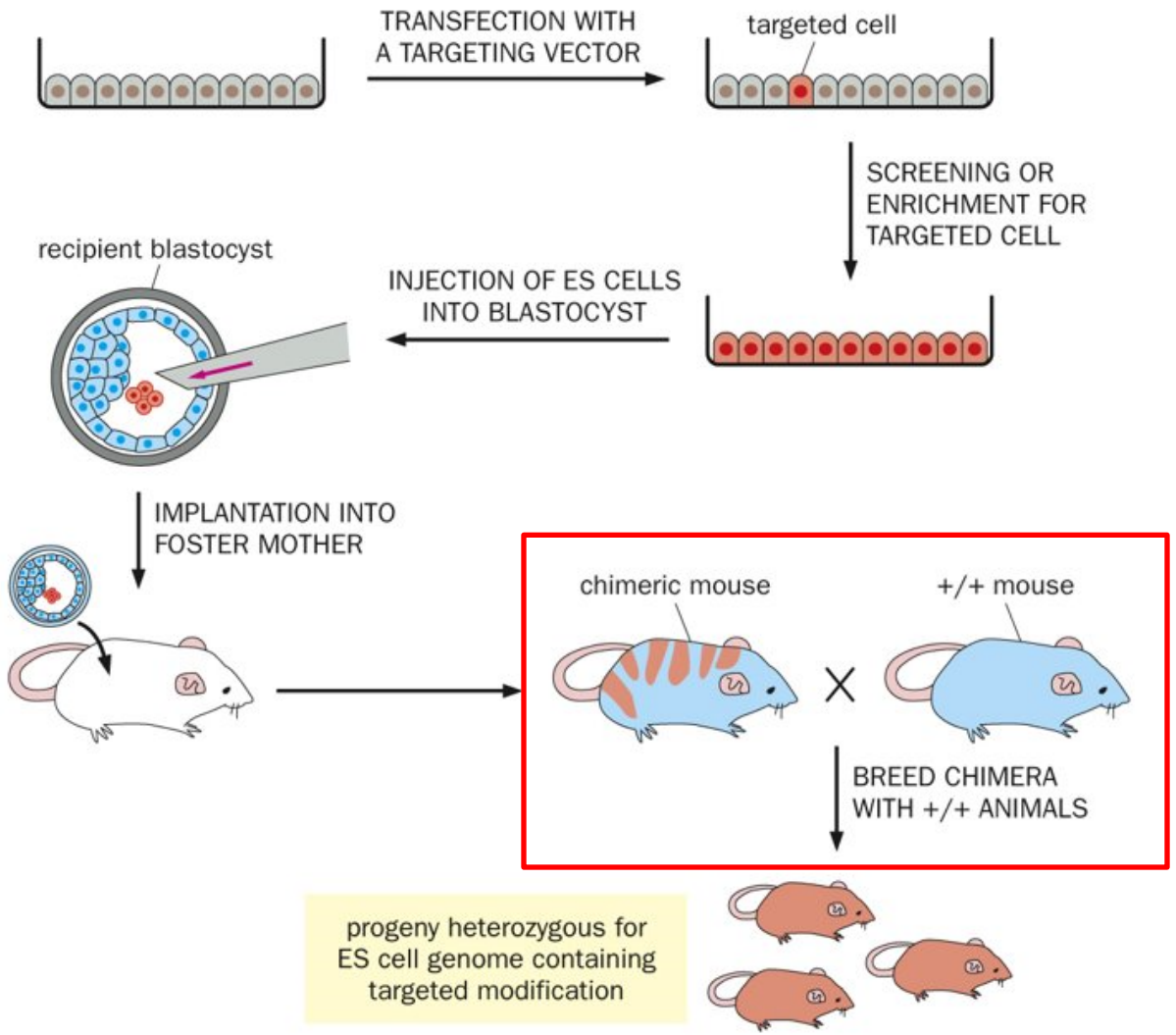
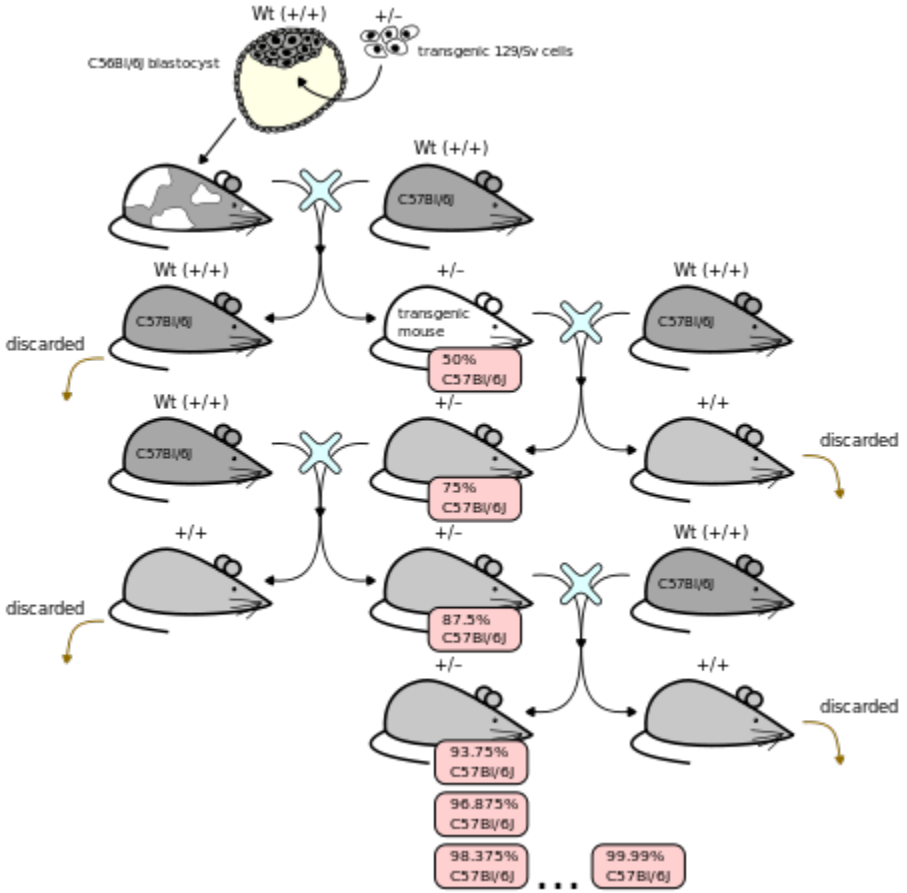



Figure 13-6b Principles of Neurobiology (© Garland Science 2016)

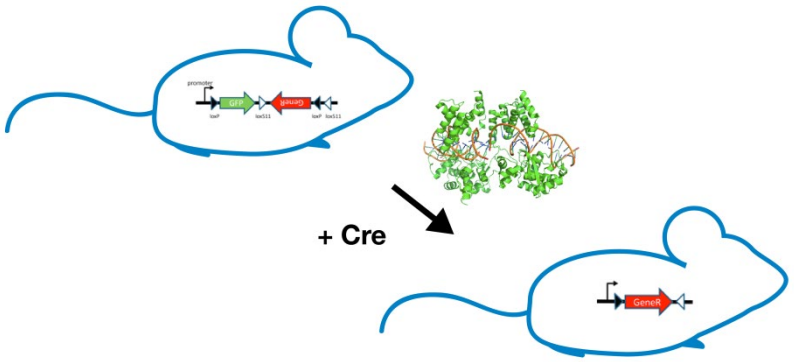
# Backcrossing your mutant

- All mutants will be hybrid strains
- With BAC transgenics, you also have added complication of random insertions
- Balancing selection
- 5-10 generations

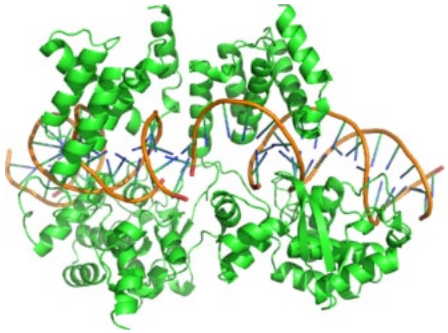


# Conditional expression

- Use genetic tricks to restrict gene from being constitutively expressed
- Combining transgenes and knockins
- Combining conditional alleles with virus
- Combining transgenes with virus




Addgene.org

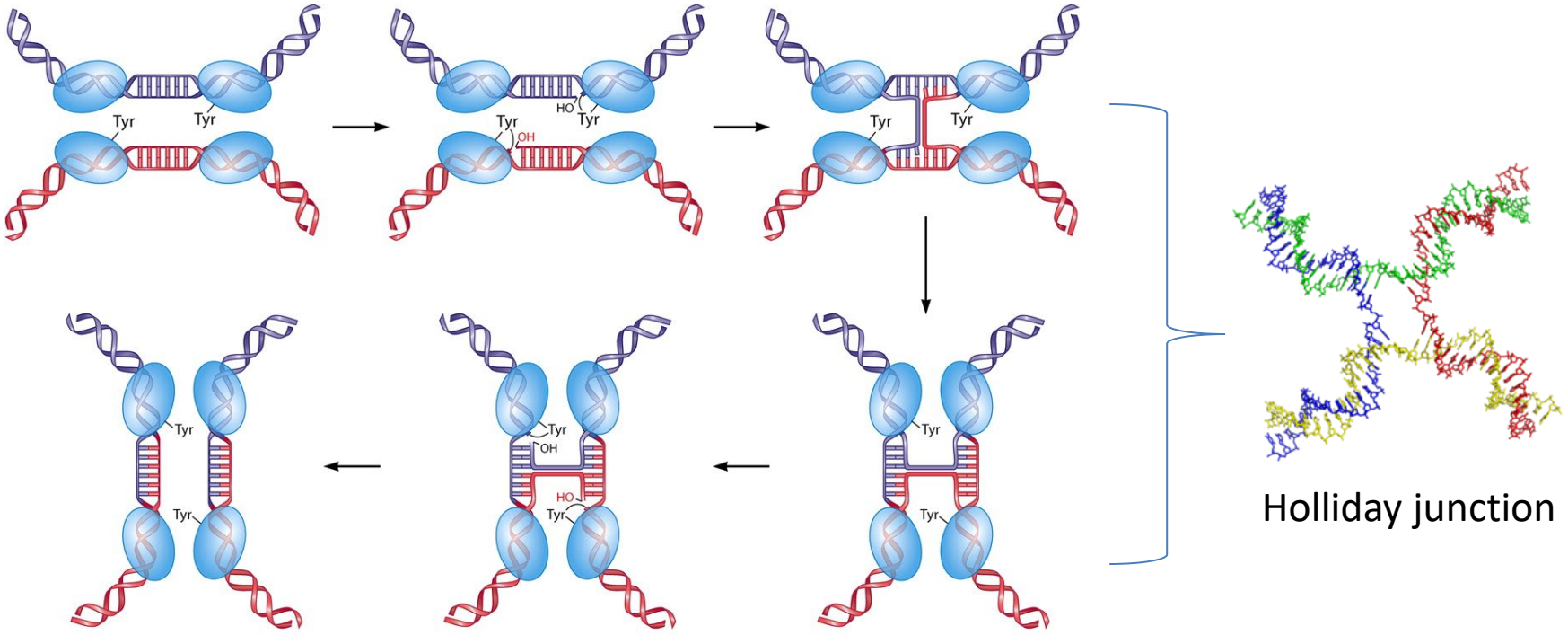


# Cre/lox system

- Derived from P1 bacteriophage; discovered in 1981 by Sternberg and Hamilton
- Cre= "**C**auses **re**combination" (aka cyclization recombination?)
- loxP= "**lo**cus of crossing over (**x**), **P**1"
- "Floxed" = flanked by loxP sites
- Cre recombinase:  tyrosine site-specific DNA recombination enzyme
- loxP: 13 bp palindromic region flanking a 8 bp spacer sequence

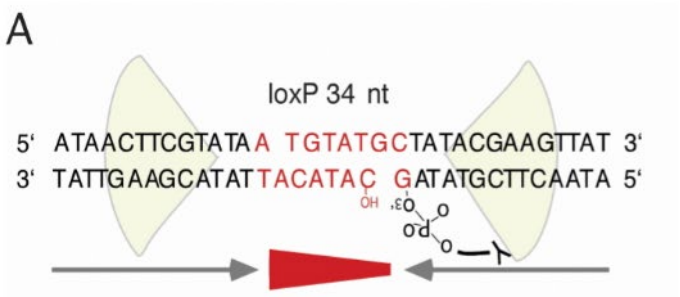
Sternberg and Hamilton, J. Mol. Biol., 1981.

# Tyrosine recombinase reaction



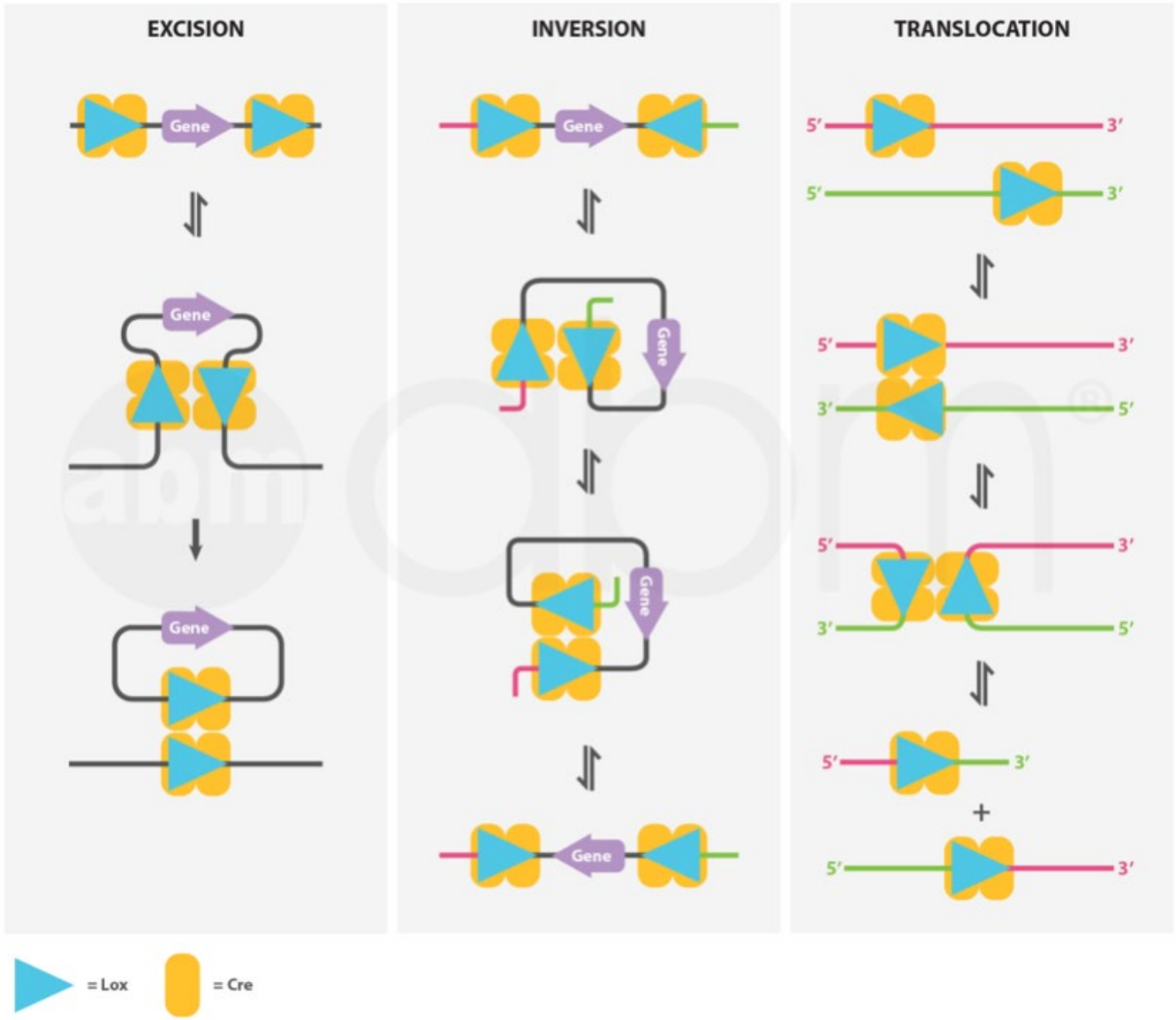

Rajeev et al., Micro. and Mol. Bio. Reviews, 2009.

# Cre recombinase reaction



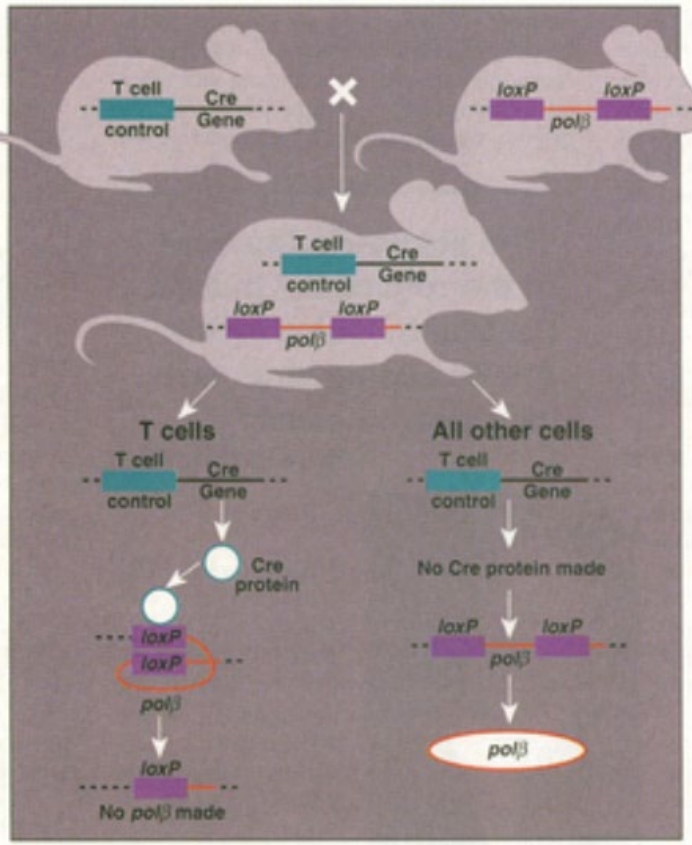

Tronche et al., FEBS Letters, 2002.

# Using pairs of loxP sites



# Tissue specific knockout

olymerase β the gene is needed early in development. Standar




**Cutting out.** The Cre enzyme, which clips out the polymerase β gene segment between the *loxP* sites, is made only in T cells, and thus inactivates the gene only in those cells.

Barinaga, Science, 1994.

## Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting

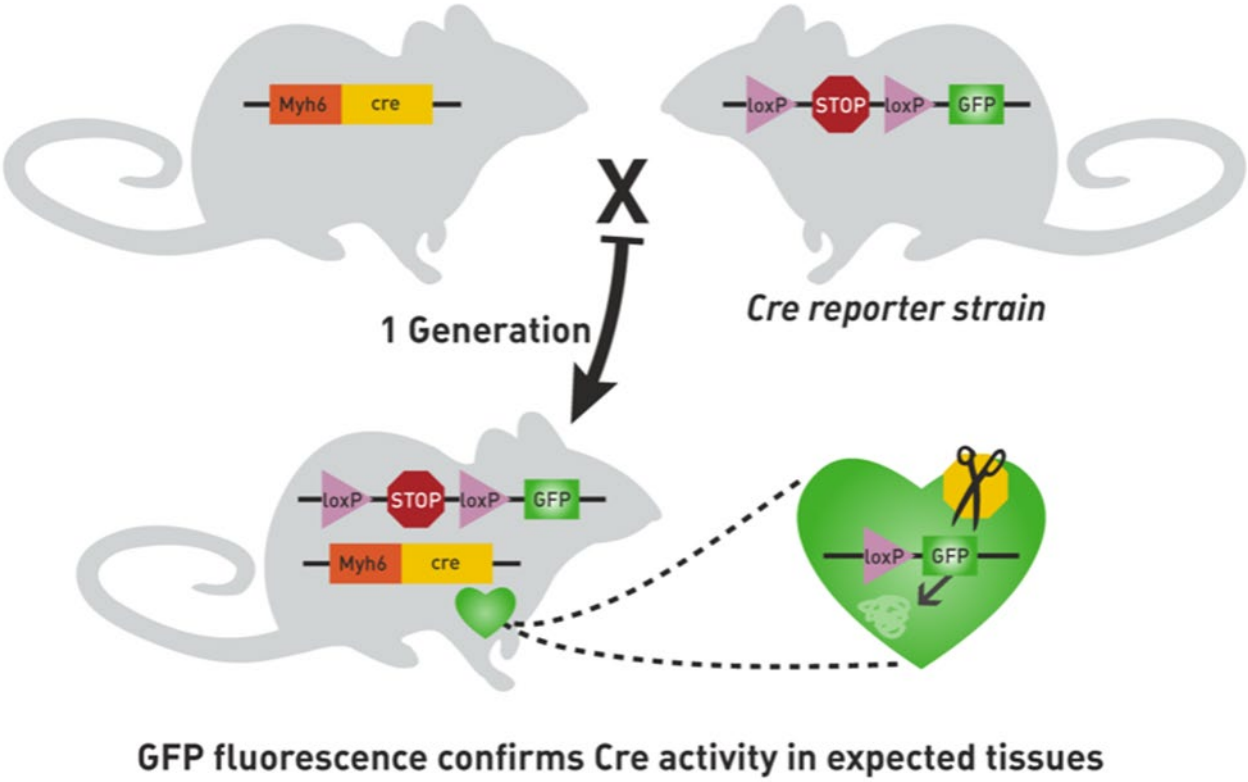
H Gu, JD Marth, PC Orban, H Mossmann, K Rajewsky
+ See all authors and affiliations

*Science* 01 Jul 1994:
Vol. 265, Issue 5168, pp. 103-106
DOI: 10.1126/science.8016642

Although Gu and Rajewsky are the first into print with a tissue-specific knockout of a normal mouse gene, their team is just one of many moving toward the same goal of conditional gene knockouts. "I would guess there are minimally about 100 labs working on this," says Mario Capecchi of the University of Utah, one of the developers of knockout

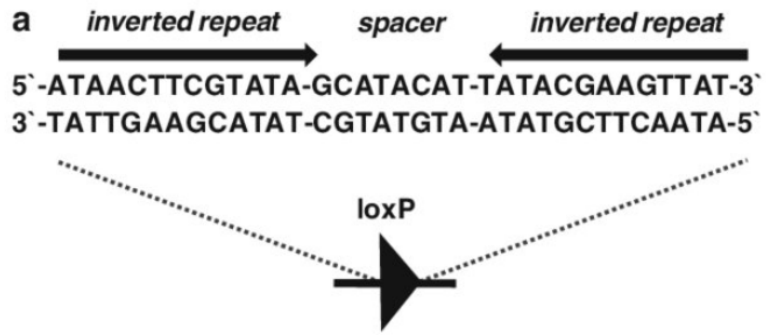
Gu et al., Science, 1994.

# Tissue specific gene expression

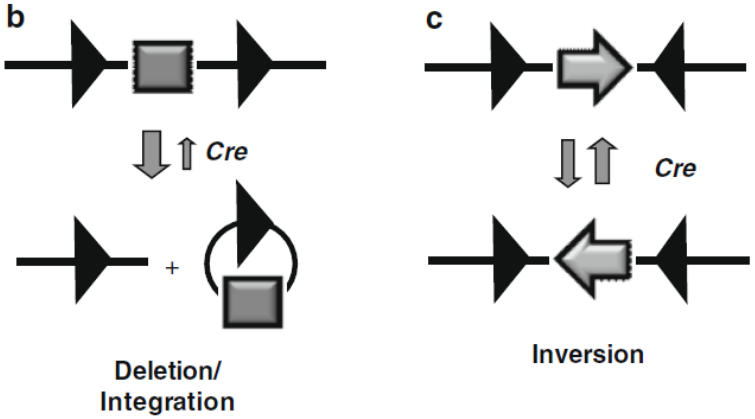



Jax.org

# loxP recognition and recombination




Spacer sequence determines directionality




Recombination by enzyme depends on:
-Location of sites
-Orientation of sites

PERMANENT

REVERSIBLE*

# Spacer region

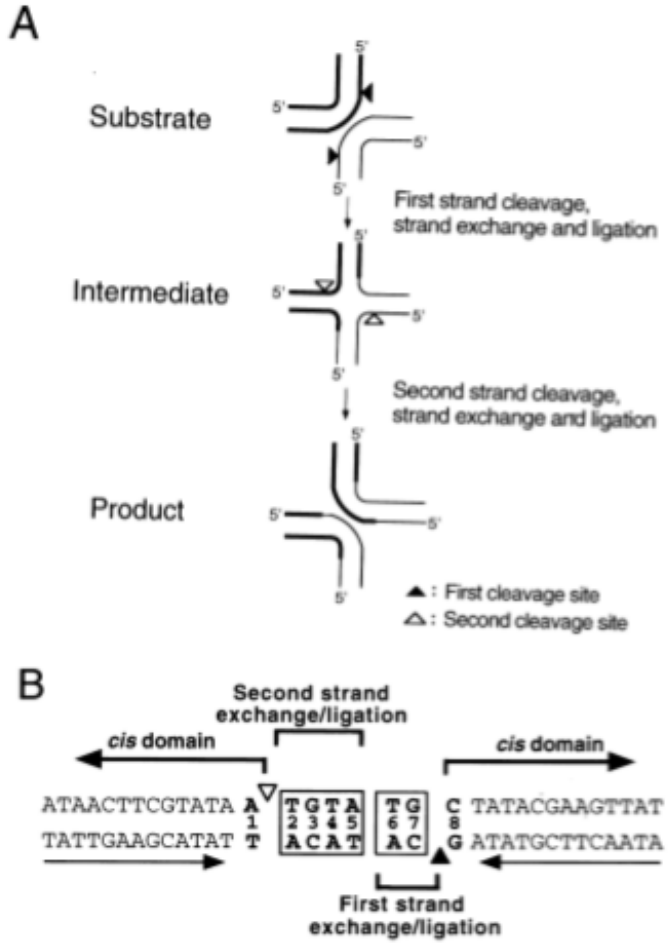


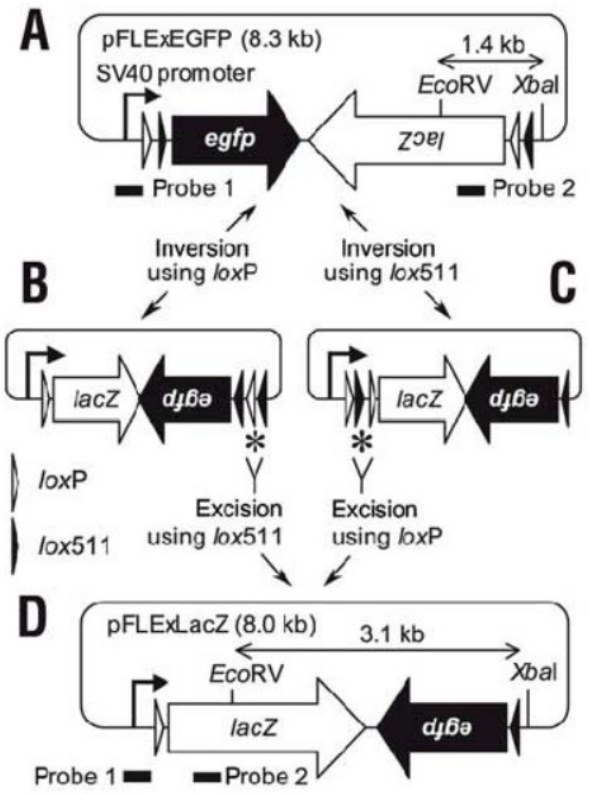

Fig. 7. Schema of Cre-mediated recombination. **(A)** The recombination process of a substrate containing two *loxP* sequences. **(B)** Possible functional domains of the *loxP* spacer region. Thin arrows show a pair of 13-bp inverted repeats.

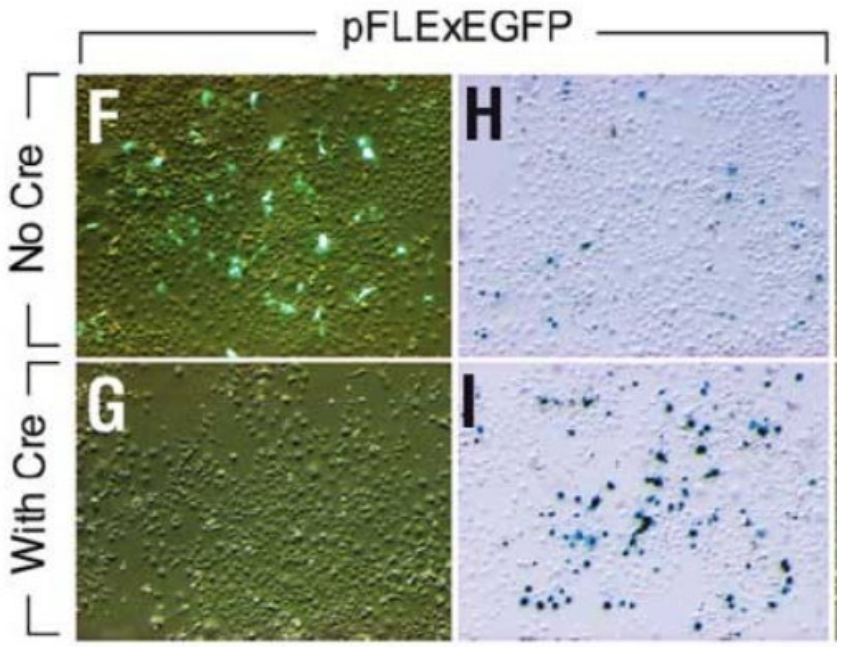
- Assymetric, containing 2 subdomains nt 2-5 and nt 6-7
- Identified synthetic loxP sequence 2272 which is incompatible with wild type loxP

Lee and Saito, Gene, 1998.

# FLEx Switches

- **FL**ip **Ex**cision
- Cre recombinase only recombines 2 sites if the spacer sequence is the same




Schnutgen et al., Nat. Biotech., 2003.

# Lox library

| Name | Left Recognition Region | Spacer | Right Recognition Region |
|---|---|---|---|
| loxP | ATAACTTCGTATA | ATGTATGC | TATACGAAGTTAT |
| lox511 | ATAACTTCGTATA | ATGTATAC | TATACGAAGTTAT |
| lox2272 | ATAACTTCGTATA | AAGTATCC | TATACGAAGTTAT |
| lox5171 | ATAACTTCGTATA | ATGTGTAC | TATACGAAGTTAT |
| m2 | ATAACTTCGTATA | AGAAACCA | TATACGAAGTTAT |
| m3 | ATAACTTCGTATA | TAATACCA | TATACGAAGTTAT |
| m7 | ATAACTTCGTATA | AGATAGAA | TATACGAAGTTAT |

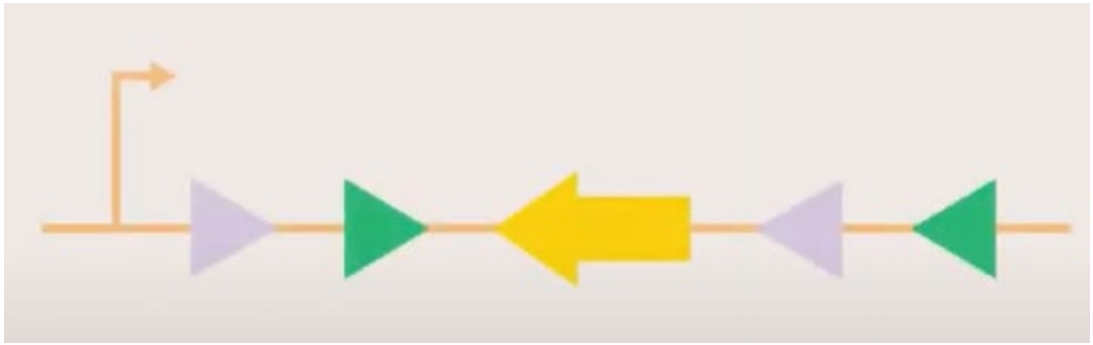
**Source:** Adapted from Missirlis, et al. (3).

# FLEx applications

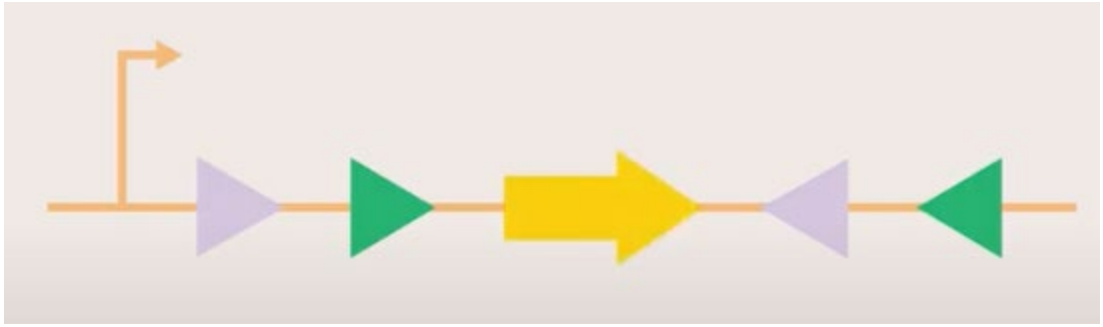
- Tissue-specific gene expression
- Inducible gene expression
- Control of multiple genes
- Gene knock-in
- Combine with AAV (last lecture)

# FLExing

- DIO= **D**ouble-floxed with **I**nverted **O**rientation "Cre-On"



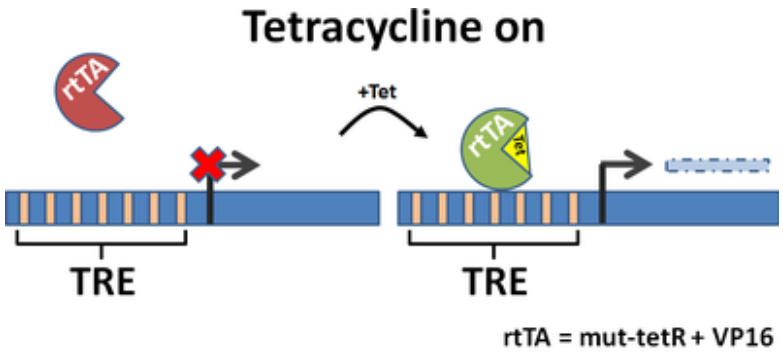
- DO= **D**ouble-floxed in **O**rientation "Cre-Off"



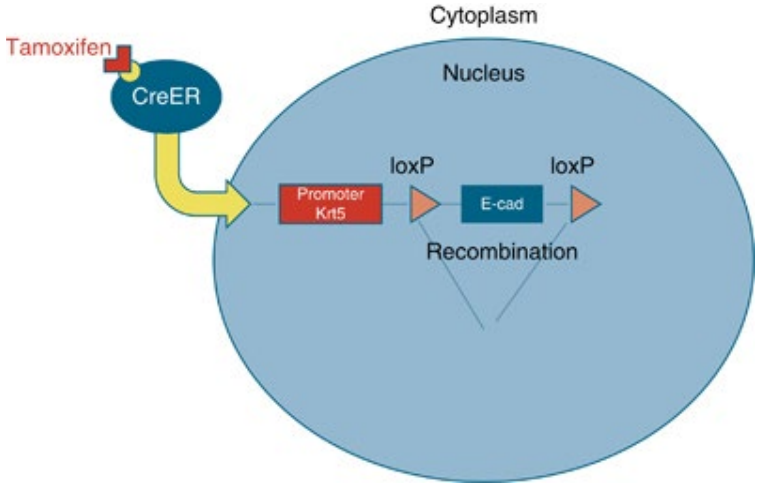
schematic: abm

# Inducible Cre Expression

- Tetracycline

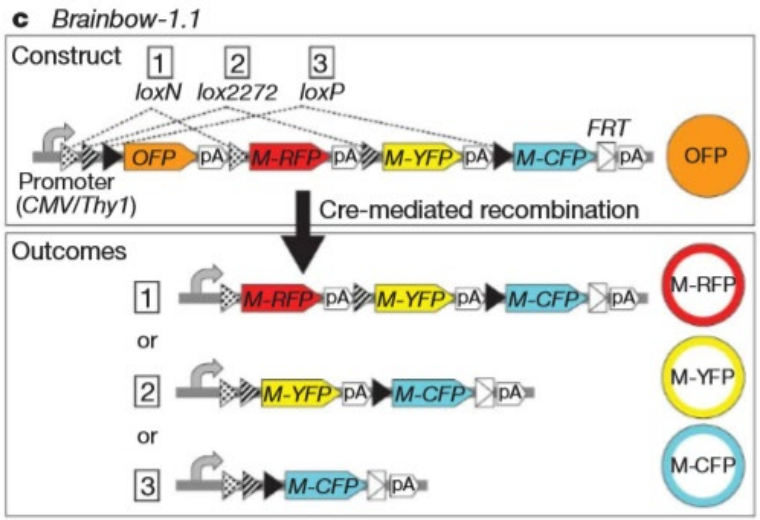


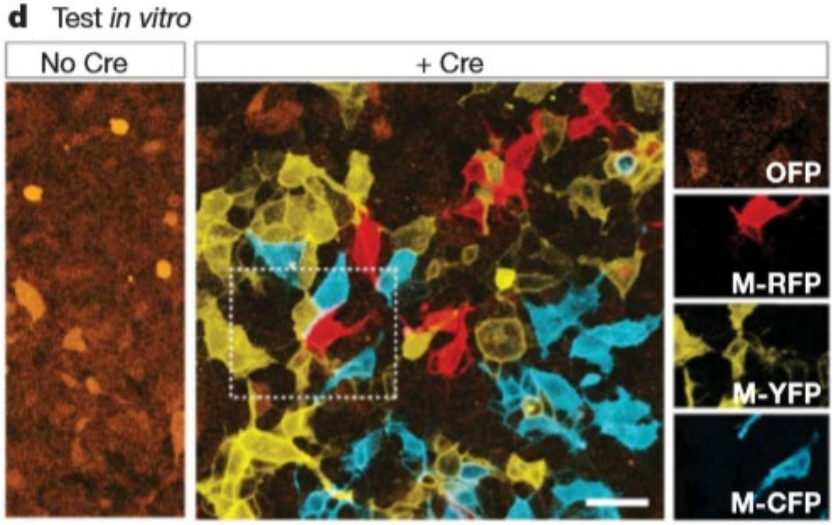

- Tamoxifen




  - Go into greater detail in Lecture 10

# Control of multiple genes: Brainbow



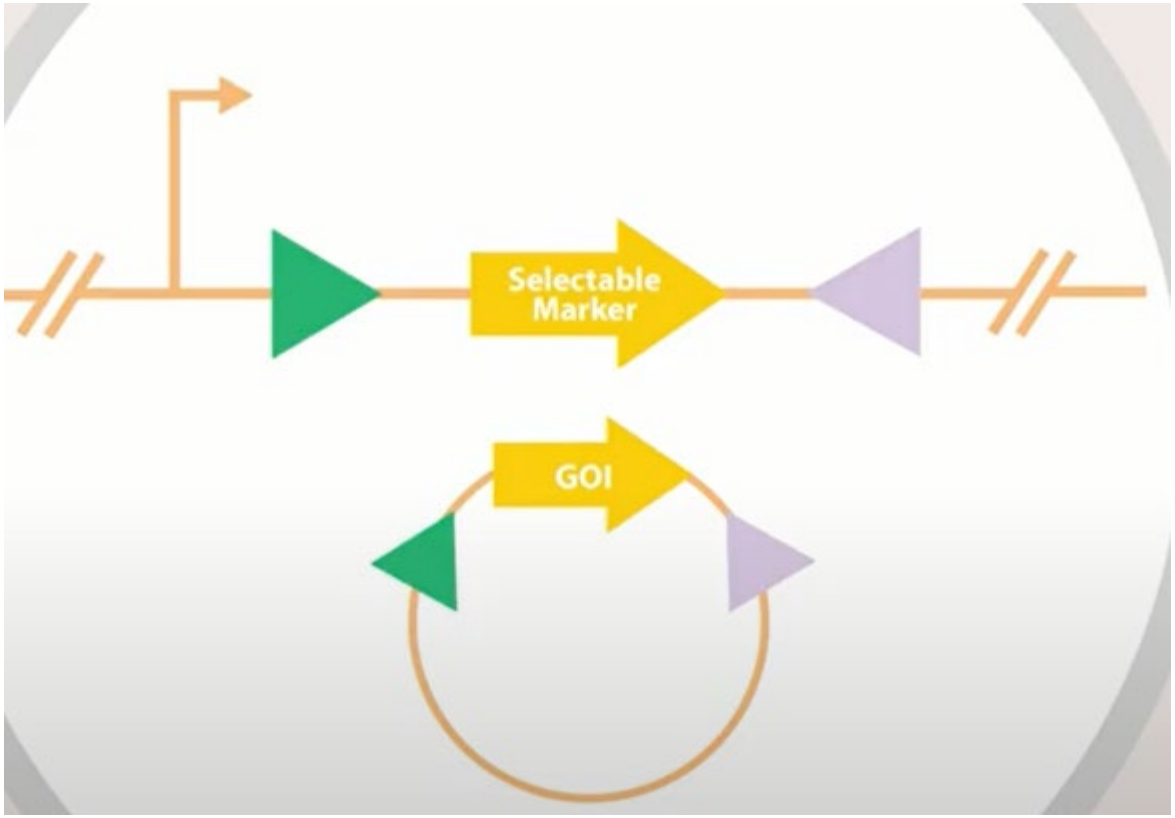





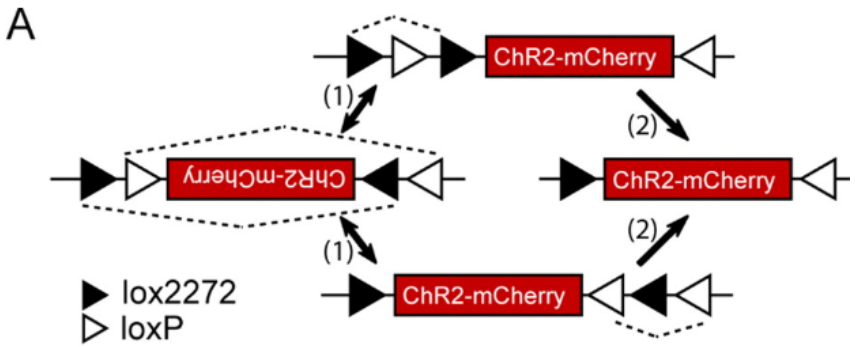


Livet et al., Nature, 2007.

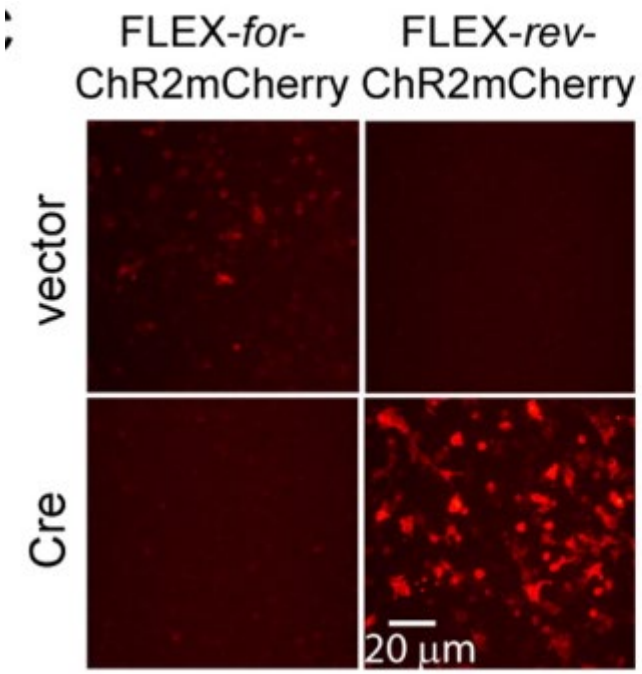
# Gene knock-in

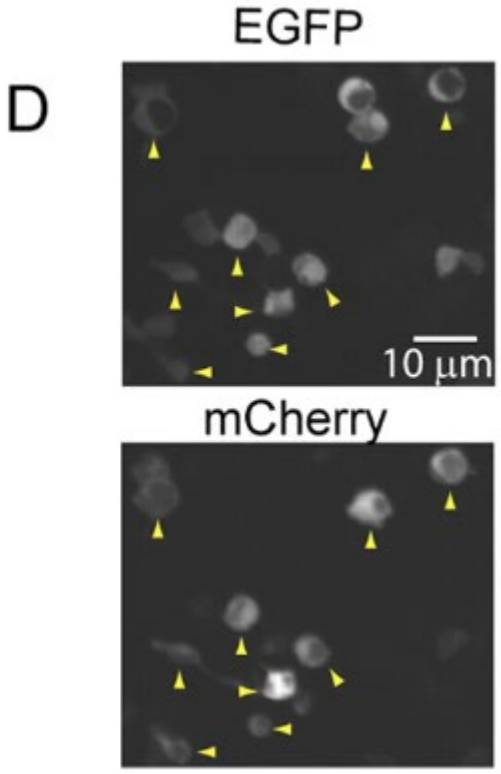


# Combine with AAV







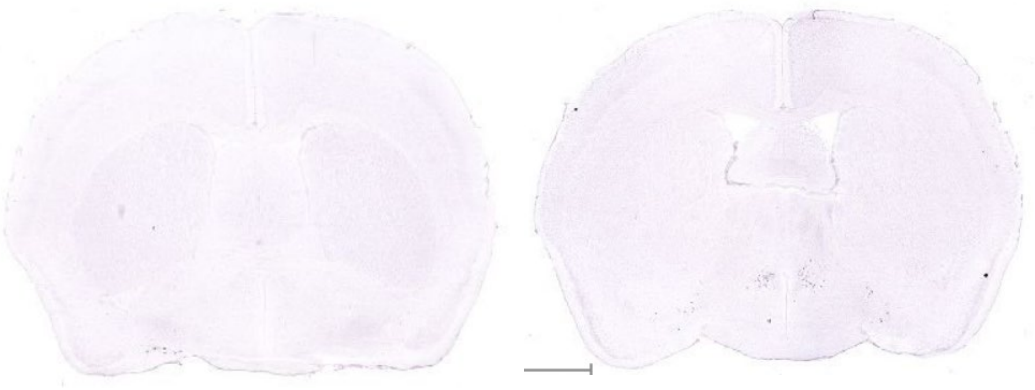




Atasoy et al., J. Neurosci., 2008.

# Cre/lox caveats

- Transgenic Cre insertion may lead to a phenotype
- Cre may be expressed in unexpected sites
- Mosaicism
  - Incomplete expression
  - Incomplete recombination

# Caveat: permanent recombination



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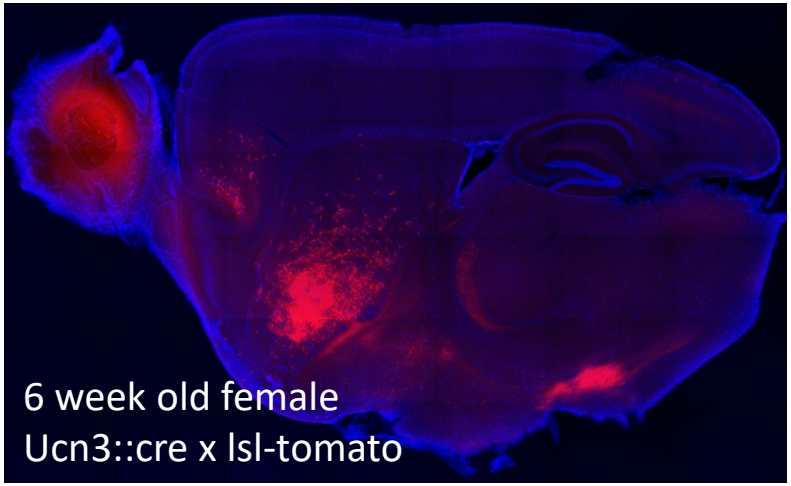
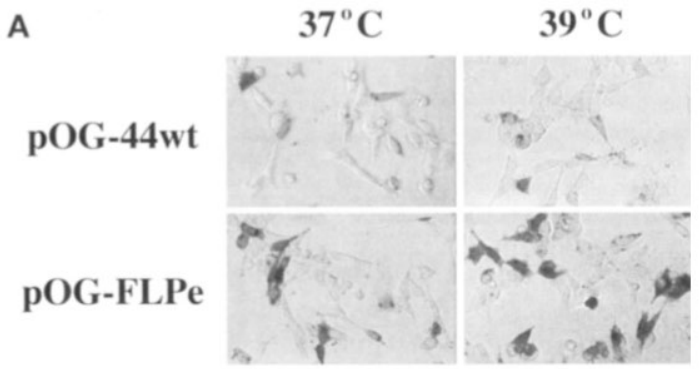


# FLP/Frt system

- Derived from S. cerevisiae, recombinase described in 1980 by Broach and Hicks CSHL, FRT sites in 1986 by Sadowski group
- Like Cre recombinase, FLP recombinase is a conserved site-specific tyrosine recombinase
- FRT site: 48 bp sequence including 2 13 bp inverted repeats, 8 bp spacer, and an upstream 13 bp direct repeat and 1 isolated bp (though only the 34 bp region is functional)

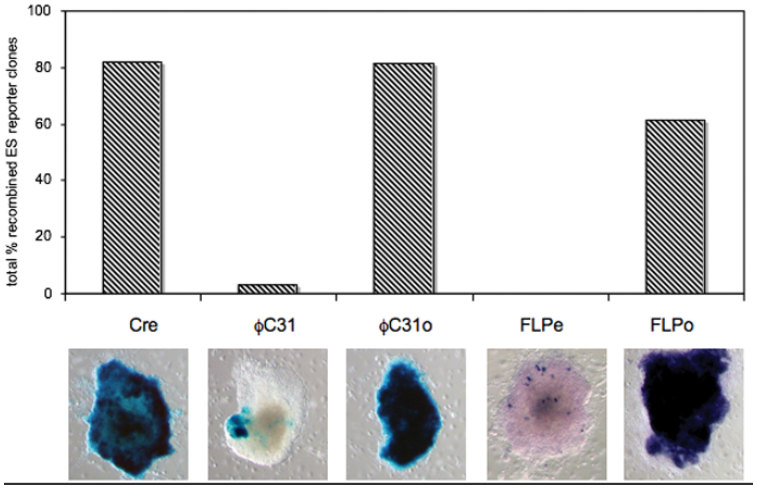
# FLP recombinase improvements

- FLP optimally active at 30°C
- FLPe




Buchholz et al., Nat. Biotechn., 1998.

- FLPo

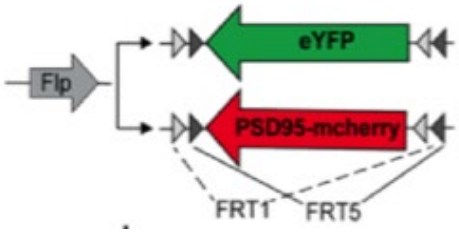



Raymond and Soriano, PLoS One, 2007.

# FRT sites are FLEx-able

| RT | Inverted repeat | Spacer | Inverted repeat | Reference |
|---|---|---|---|---|
| loxP | ATAACTTCGTATAG | CATACA | TTATACGAAGTTAT | Hoess *et al.* (1986) |
| lox5171 | ATAACTTCGTATAG | TAGACA | TTATACGAAGTTAT | Lee and Saito (1998) |
| lox2272 | ATAACTTCGTATAG | GATACT | TTATACGAAGTTAT | Lee and Saito (1998) |
| lox71 | TACCGTTCGTATAG | CATACA | TTATACGAAGTTAT | Albert *et al.* (1995) |
| lox66 | ATAACTTCGTATAG | CATACA | TTATACGAACCGTA | Albert *et al.* (1995) |
| loxJTZ17 | ATAACTTCGTATAG | CATACA | TTATAGCAATTTAT | Thomson *et al.* (2003) |
| rox | TAACTTTAAATAA | TTGGCA | TTATTTAAAGTTA | Sauer and McDermott (2004) |
| FRT | GAAGTTCCTATTC | TCTAGAAA | GTATAGGAACTTC | Senecoff *et al.* (1988) |
| F3 | GAAGTTCCTATTC | TTTAAATA | GTATAGGAACTTC | Schlake and Bode (1994) |
| F5 | GAAGTTCCTATTC | TTCAAAAG | GTATAGGAACTTC | Schlake and Bode (1994) |

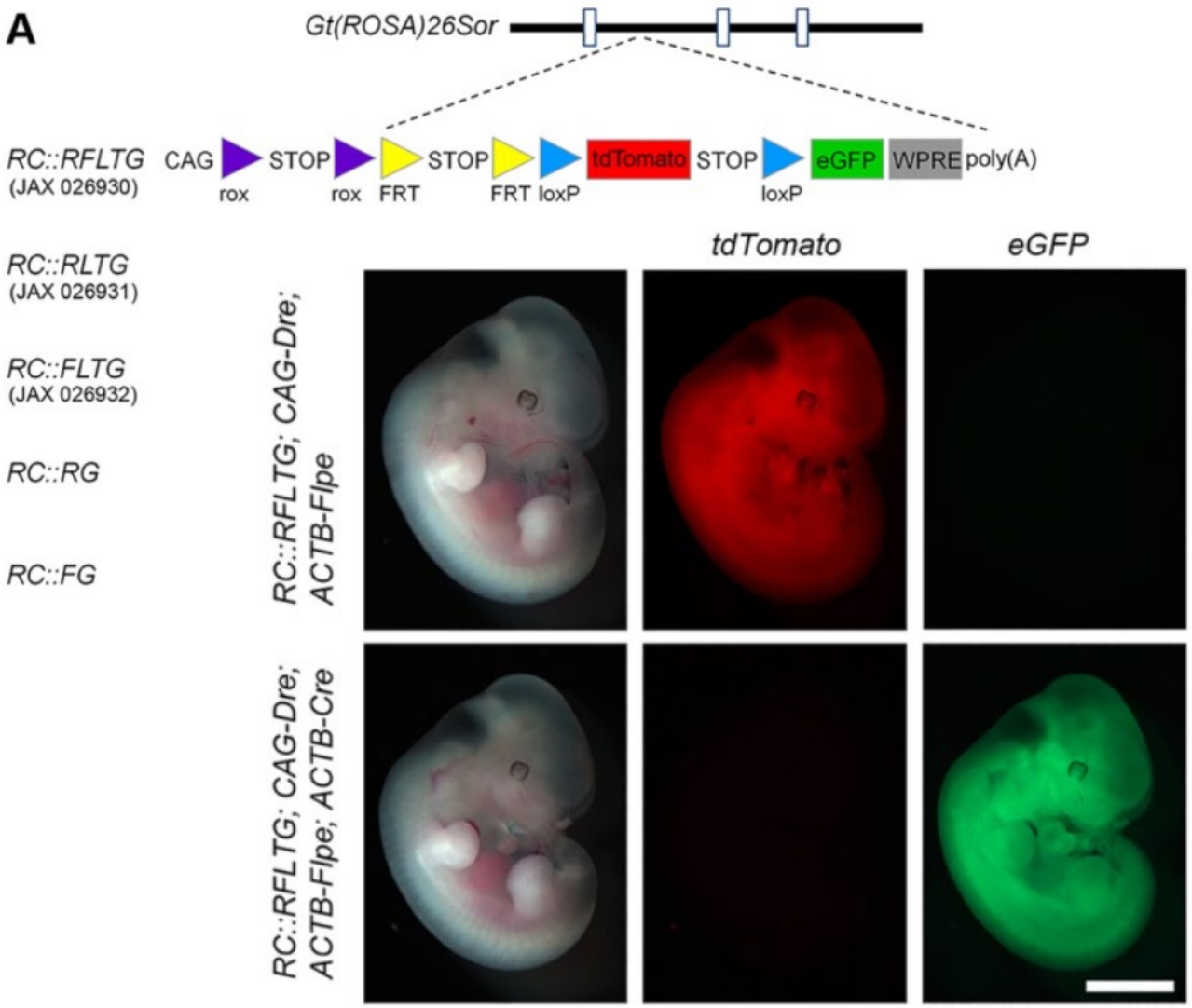
Anastassiadis et al., Methods in Enzymol., 2010.



# Dre/rox

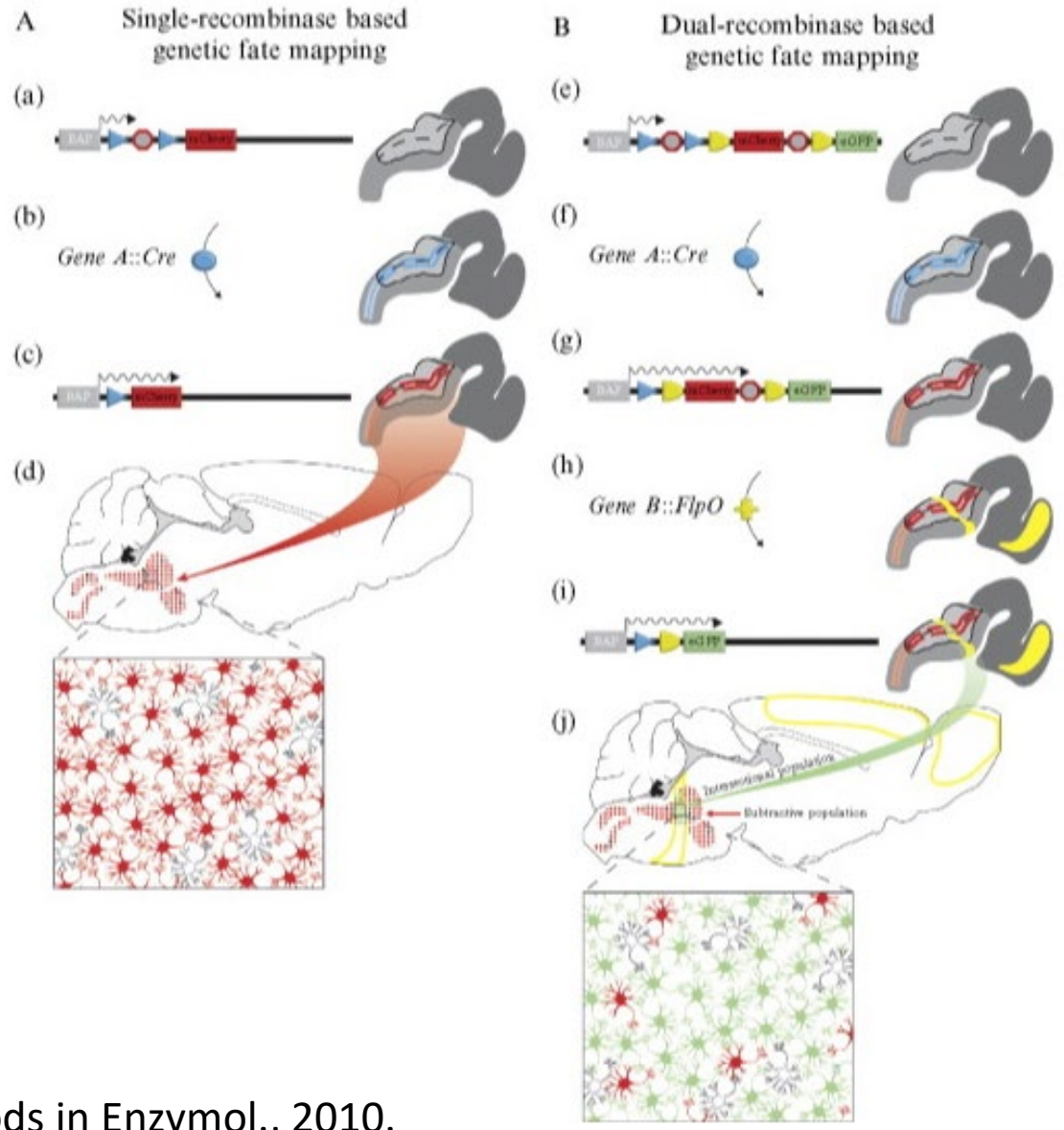
- Dre:  Phage **D**6 site specific **re**combinase
- Rox (**r**egion **o**f x-over): 32 bp region; appears to have a 6bp spacers as opposed to the 8 bp spacer of loxP
- Discovered by Sauer and McDermott 2004
- Shown to not recombine with Cre/loxP systems
- Synthetic rox sites reported (Chuang et al., G3, 2016)

# Intersectional expression




Plummer et al., Development, 2015.

# Sophisticated fate mapping




Dymecki et al., Methods in Enzymol., 2010.

# CRISPR-based expression systems

- **C**lustered **r**egularly **i**nterspaced **s**hort **p**alindromic **r**epeats (CRISPR)
- Sites bind to **C**RISPR **as**sociated protein **9** (Cas9)
- Site specific
- Can be multiplexed
- Next-gen CRISPR technology allowing knockout or knockin

# CRISPR

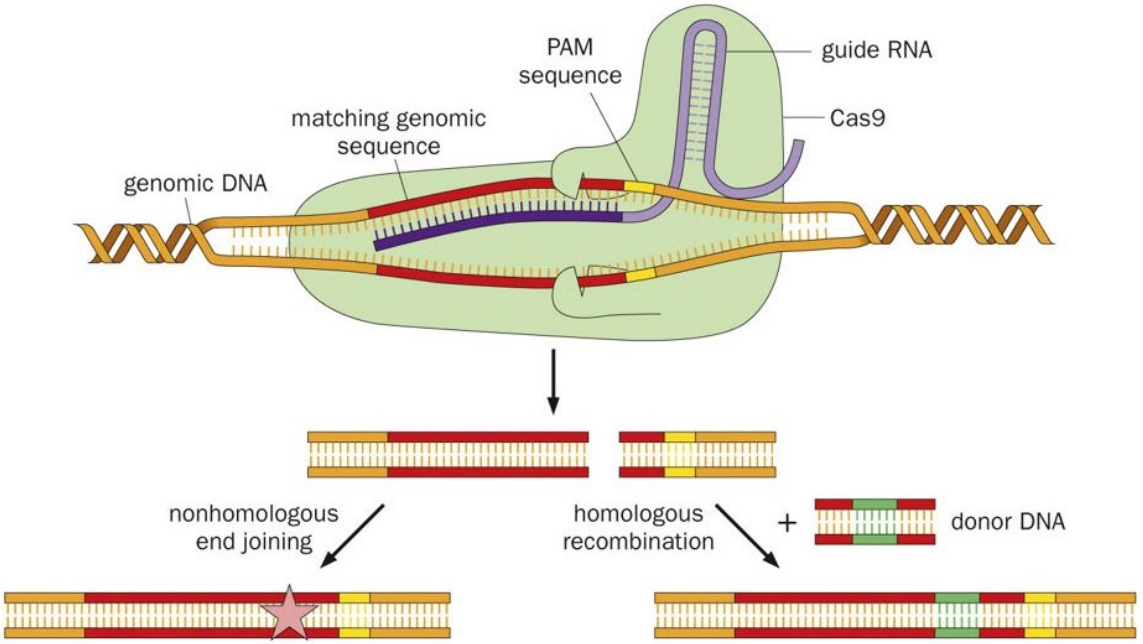



Figure 13-8 Principles of Neurobiology (© Garland Science 2016)