Eukaryotic Gene Expression: Basics & Benefits

P N RANGARAJAN

Lecture 37

Knockout mice

Lecture 29	Cloning and Expression vectors
Lecture 30	Eukaryotic Protein Expression Systems –I
Lecture 31	Eukaryotic Protein Expression Systems –II
Lecture 32	Eukaryotic Protein Expression Systems –III
Lecture 33	Human Gene Therapy
Lecture 34	DNA Vaccines
Lecture 35	Transgenic Animals
Lecture 36	Transgenic Plants
Lecture 37	Knockout Mice

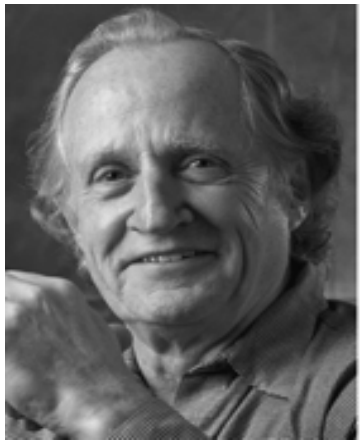
What are knockout mice?

A knockout mouse is a mouse in which a specific gene has been inactivated or “knocked out” by replacing it or disrupting it with an artificial piece of DNA.

The loss of gene activity often causes changes in a mouse's phenotype and thus provides valuable information on the function of the gene.

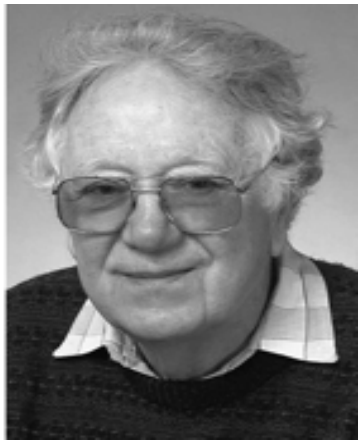
Researchers who developed the technology for the creation of knockout mice won Nobel Prize in the year 2007

The Nobel Prize in Physiology or Medicine 2007 was awarded jointly to Mario R. Capecchi, Sir Martin J. Evans and Oliver Smithies *"for their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells"*.



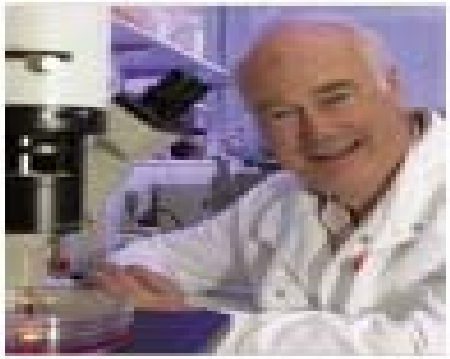
Mario R. Capecchi

Sir Martin J. Evans



Oliver Smithies

The ability to delete or mutate any gene of interest in mice has transformed the landscape of mammalian biology research.



Cultivation of embryonic stem (ES) cells – Martin Evans

• Gene targeting – Oliver Smithies

• Gene knockout – Mario Capecchi

Gene correction by Oliver Smithies

Targeted correction of a mutant HPRT gene in mouse ES cells.

Nature 330:576-8, 1987

This modification of a chosen gene in pluripotent ES cells demonstrates the feasibility of this route to manipulating mammalian genomes in predetermined ways.

Nature. 1985 Sep 19-25;317(6034):230-4.

Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination.

Germ-line transmission of a planned alteration made in a Hprt gene by homologous recombination in embryonic stem cells.

Proc Natl Acad Sci U S A. 86:8927-31, 1989

Gene knockout by Mario Capecchi

Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells.

Cell 51:503-12, 1987

The protocol described herein should be useful for targeting mutations into any gene.

Targeted disruption of the murine int-1 protooncogene resulting in severe abnormalities in midbrain and cerebellar development.

Nature 346:847-50, 1990

Importance of knockout mice in biology & medicine

Knocking out the activity of a gene provides valuable information about the function(s) of that gene. Since many genes are common between humans and mice, knockout mice gives researchers information about the function of that gene in humans.

Knockout mice have been useful in studying and modeling different kinds of cancer, obesity, heart disease, diabetes, arthritis, anxiety, aging and Parkinson disease. Knockout mice also offer a biological context in which drugs and other therapies can be developed and tested.

Transgenic *vs* Knockout mice

A transgenic mouse typically expresses one or more copies of a gene (cDNA) that is integrated into its genome in a random fashion.

A knockout mouse is a mouse in which both alleles of a gene are deleted in a targeted fashion by homologous recombination

While traditional transgenic mice are generated to express a protein, much information can be learned from the elimination of a gene or the deletion of a functional domain of the protein

Transgenic mice *vs* Knockout mice

Creation of transgenic mice involves injection of DNA into fertilized eggs and looking for adult animals that produce their protein of interest.

In transgenic mice, the transgene gets inserted into the genome randomly.

Thus, transgenic mice were limited to gain-of-function mutations, wherein two copies of the normal gene are joined by additional copies, which would overproduce the normal / mutant protein.

Using transgenic mice, one can infer a gene's function by observing what happens when there was too much of the protein or when a mutant copy interfered with the normal one.

Transgenic mice *vs* Knockout mice

The gene knock out technology allowed researchers to delete specific genes.

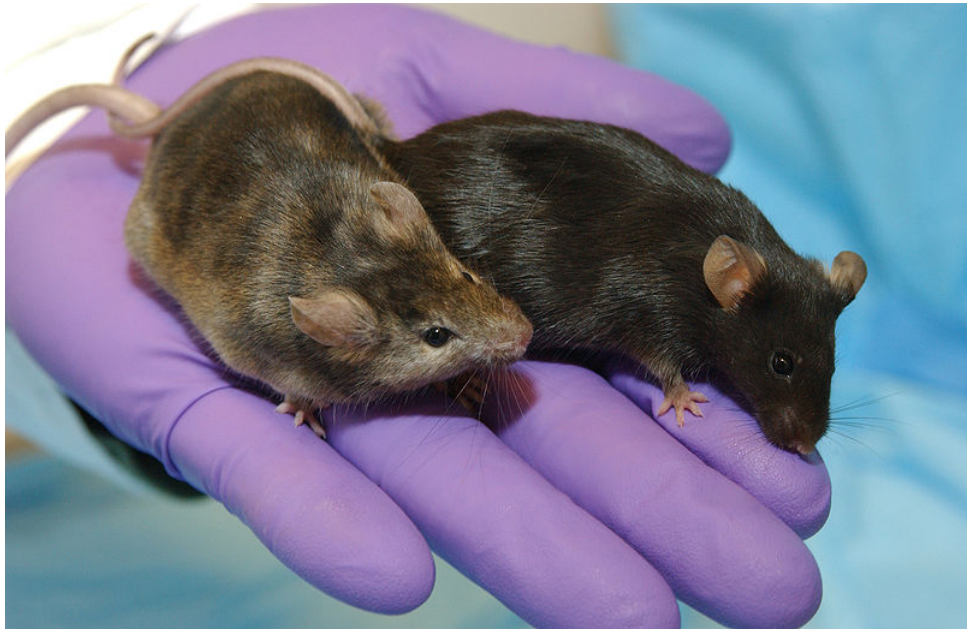
In this case, the transgene which is usually a mutant or deleted version of the normal gene, is introduced into embryonic mouse cells in culture and then allowed to recombine with its sister DNA in the genome.

The recombination cuts out the resident DNA and inserts the mutant DNA, which can then form a damaged protein or no protein at all.

The embryonic stem cells then grow into a whole mouse with a specific mutation exactly where it is supposed to be in the genome.

Thus, the gene knock out technology allowed researchers to study loss-of-function mutations wherein one can infer a gene's function by observing what happens when the gene is absent or when mutant copy of the gene is expressed instead of the normal one.

The first human disease model created with this technology was cystic fibrosis, from the labs of both Smithies and Evans.



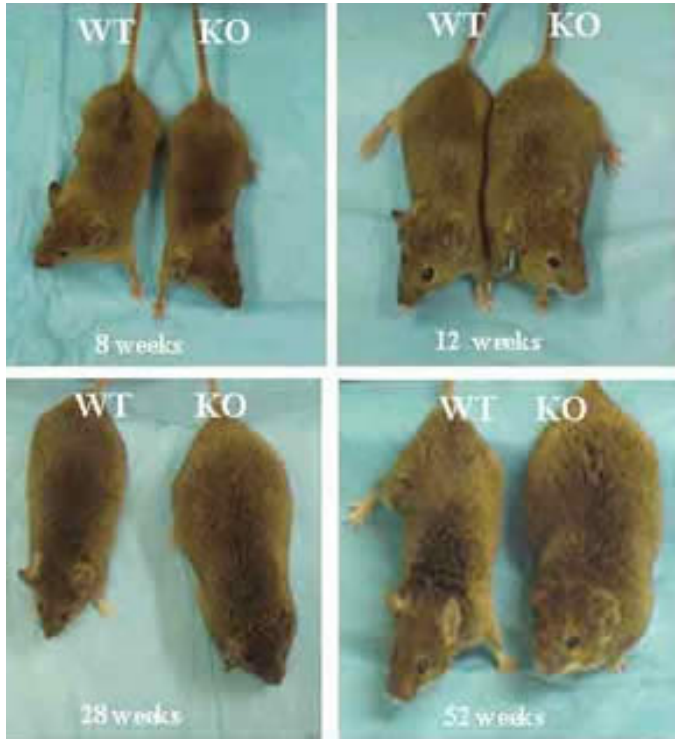
A laboratory mouse in which a gene affecting hair growth has been knocked out (left), is shown next to a normal lab mouse

Source: Maggie Bartlett , National Human Genome Research Institute

http://www.genome.gov/pressDisplay.cfm?photoID=5006

A knockout mouse (left) that is a model of obesity, compared with a normal mouse.

Source:
Lexicon Genetics Incorporated
http://www.genome.gov/pressDisplay.cfm?photoID=80

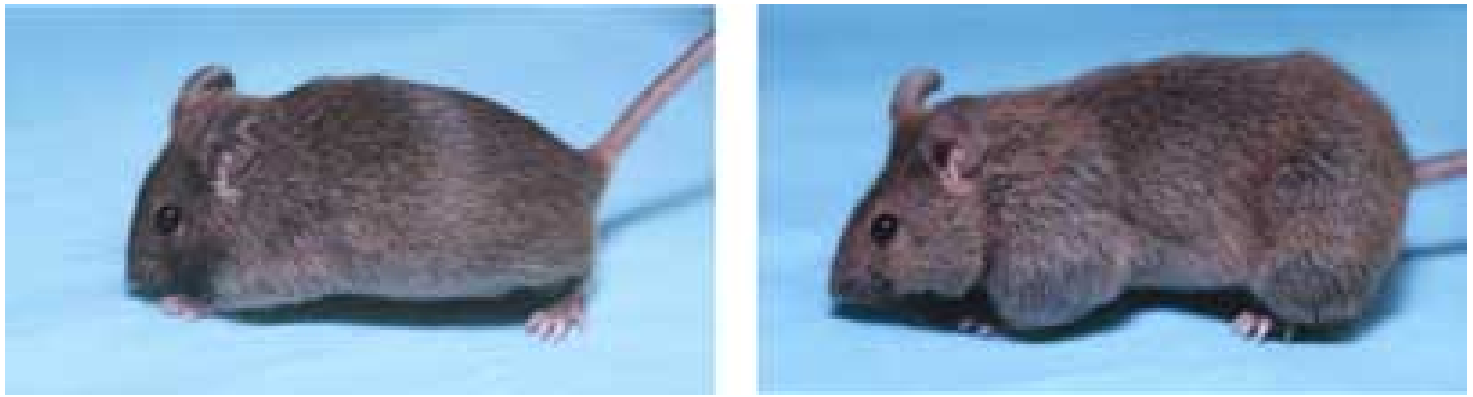


Knocking out expression of Nhlh2, a basic helix-loop helix transcription factor in mice results in adult onset obesity.

Nhlh2 knockout mice at 8 weeks of age are similar to normal mice in weight and body structure, but older Nhlh2 knockout animals have an increase in abdominal fat and body weight.

http://www.umass.edu/vasci/faculty/good/good.html

normal knockout

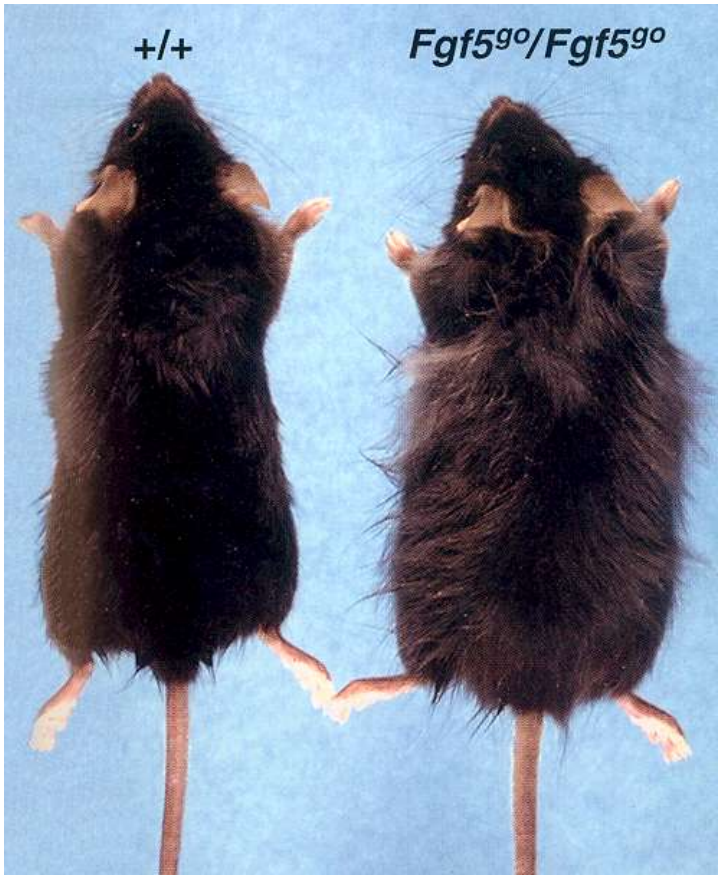


GDF8 (Myostatin) knockout mouse

More than twice the muscle mass of a wildtype mouse

http://www.bbc.co.uk/science/genes/gene_safari/wild_west/bigger_and_better02.shtml

FGF5 knockout mouse has long, angora-like hair



http://www.med.uni-jena.de/ivm/deutsch/method/method_7.htm

Targeted mutation of the DNA methyltransferase gene results in embryonic lethality.

Li E, Bestor TH, Jaenisch R. Cell. 1992 69:915-26.

Mutant ES cells homozygous for the mutation were viable and showed no obvious abnormalities with respect to growth rate or morphology, and had only trace levels of DNA methyltransferase activity.

However, homozygous mutant mouse embryos were stunted, delayed in development, and did not survive past mid-gestation.

These results indicate that while a 3-fold reduction in levels of genomic m5C has no detectable effect on the viability or proliferation of ES cells in culture, a similar reduction of DNA methylation in embryos causes abnormal development and embryonic lethality.

Knocking out p53 gene

It was well known that *p53*, a tumour suppressor gene is deleted or mutated in half of human cancers.

It was therefore of interest to see what happens if you delete *p53* gene in mice.

p53 knockout mice developed normally but developed a variety of cancers including lymphomas when they grew old.

Thus, the knock out model provided the ultimate proof that p53 is indeed a tumour suppressor.

One important result the gene knockout models revealed is that cells need more than one mutant gene to become cancerous. When you knock out p53, it takes many months for cancer to arise. Thus, cells need more genetic alterations than just that one.

Knockout mice and cancer

Similarly, mice engineered to express a mutant Rb gene indeed developed tumors, but not the ones researchers were expecting. Instead of eye tumors, the animals suffered from pituitary and thyroid gland cancers.

It was later found that found that a second gene protected the eye cells from cancer, and both mutations were required for tumors to form.

Knockout mice and cancer

In another study, when the gene encoding telomerase, the protein responsible for keeping chromosome tips long and healthy, was knocked out in mice.

In such mice, cancers arose when chromosome tips shorten over the course of a lifetime of cell division. The failing tips cause chromosomes to break and recombine incorrectly, until finally cells reach the large number of mutations needed for transition to malignancy.

The most important factor that drives cancer is age

The knock out mice models also contributed to understanding the function of genes in development.

Very often oncogenes were found to be critical in normal development.

Generation of knockout mouse by homologous recombination: the methodology

Generation of a knockout mouse by homologous recombination

1. Creating a knockout construct

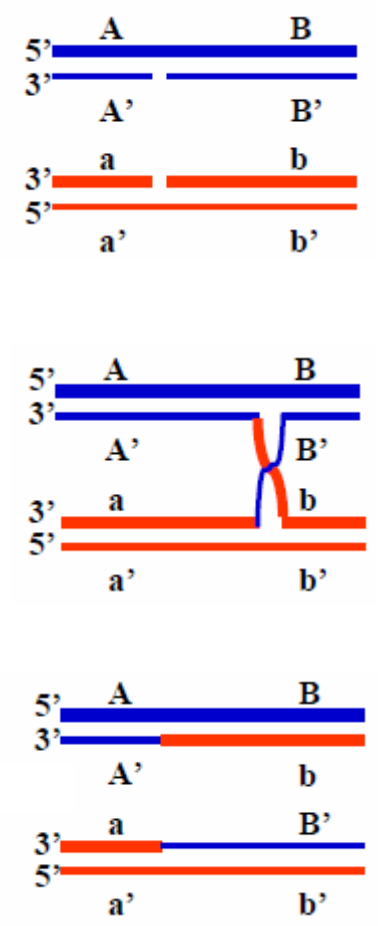
2. Introduce the knockout construct into mouse embryonic stem (ES) cells in culture.
3. Screen ES cells and select those whose DNA includes the new gene.

4. Implant selected cells into normal mouse embryos, making "chimeras“

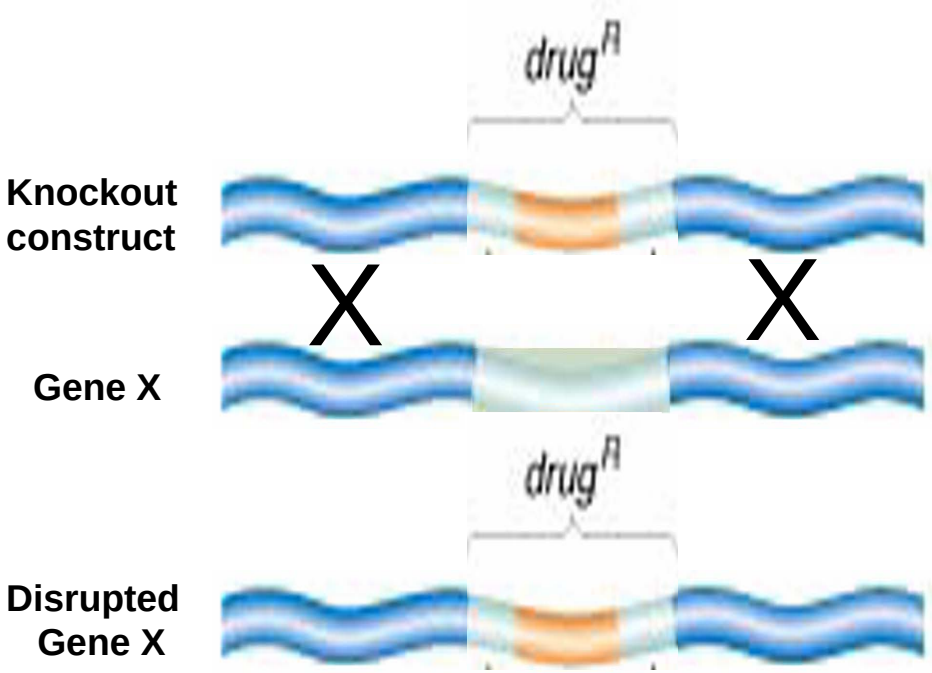
5. Implant chimeric embryos in pseudopregnant females.

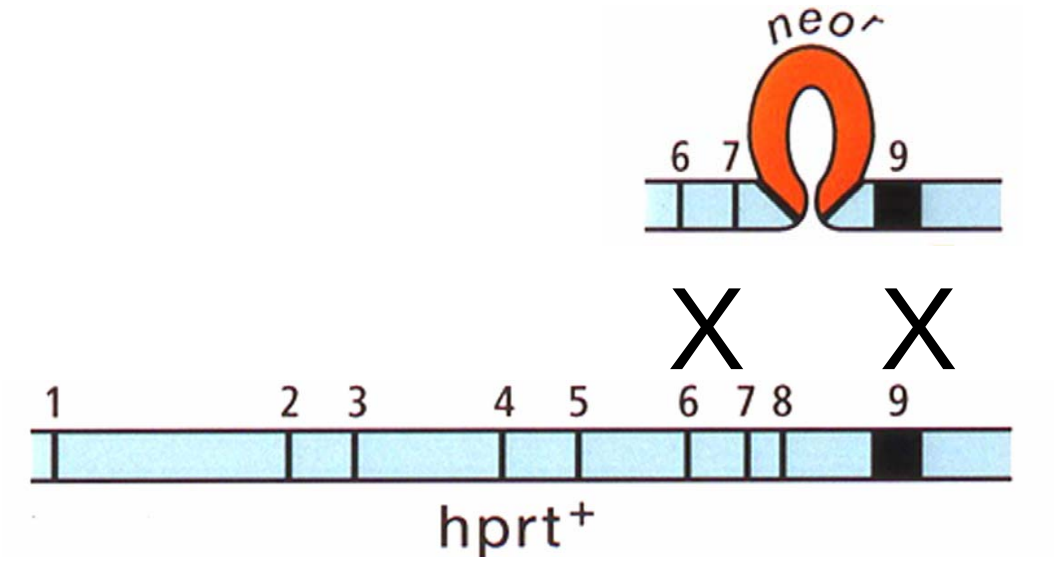
6. Females give birth to chimeric offspring, which are subsequently bred to verify transmission of the new gene, producing a mutant mouse line.

Homologous Recombination (Single Crossover Event)

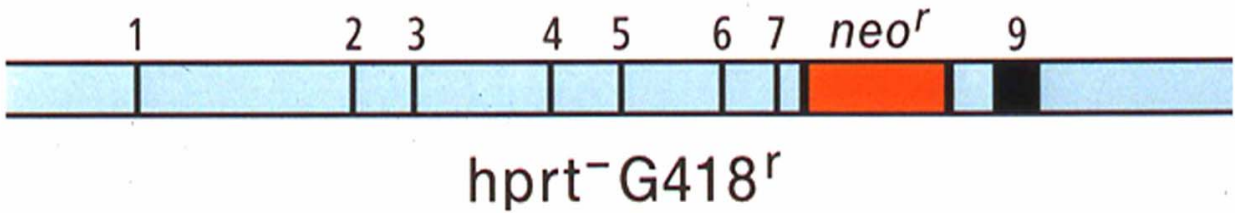


Gene knockout is accomplished by homologous recombination involving a double crossover event





(Hypoxanthine-guanine phosphoribosyltransferase)



DISTINGUISHING BETWEEN

RANDOM INTEGRATION

AND

HOMOLOGOUS RECOMBINATION

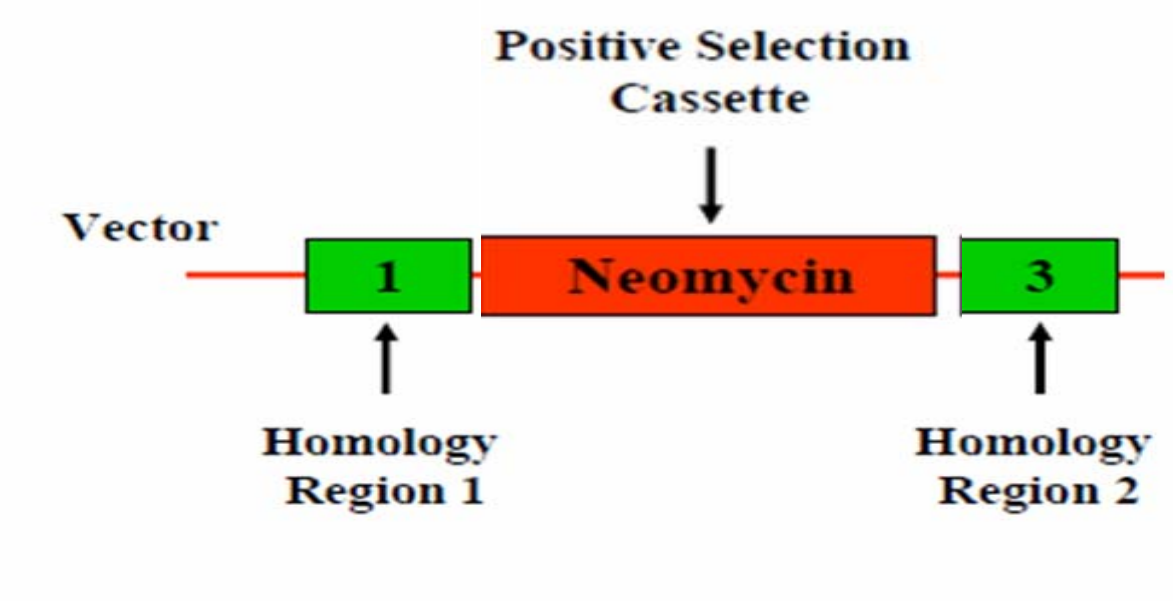
USING POSITIVE AND NEGATIVE

SELECTION MARKERS

Positive Selection Markers

Positive selection markers are used to enrich for recombination events:

Expression cassettes encoding antibiotic resistance genes (Neo^R)

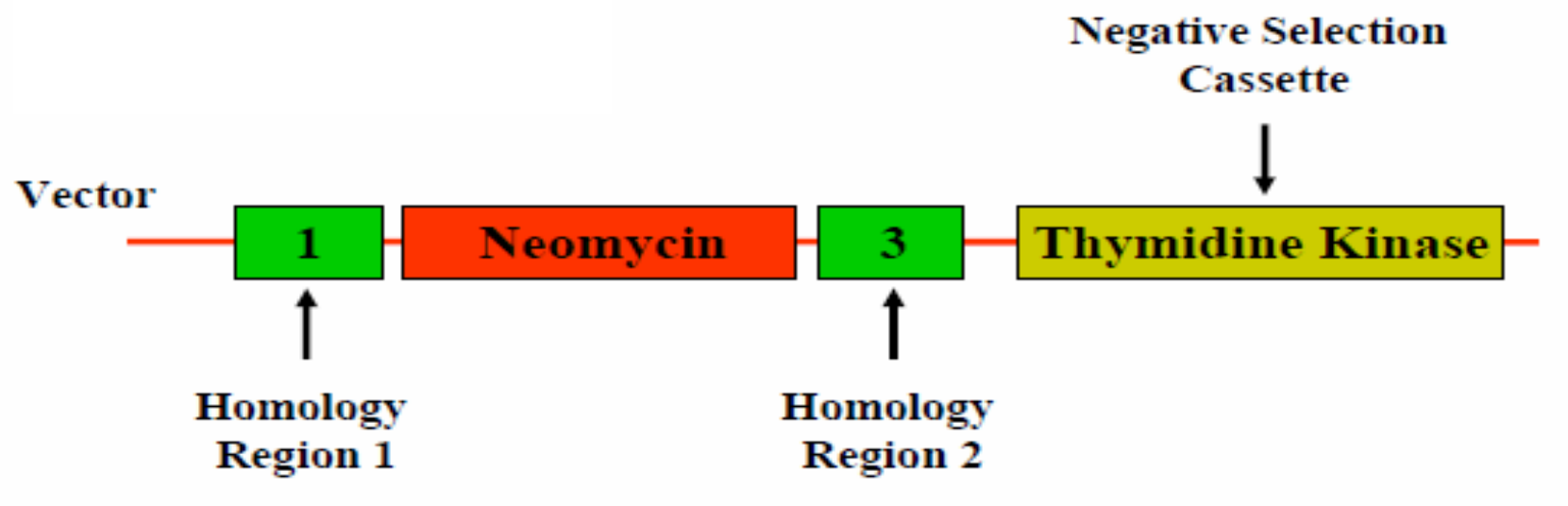


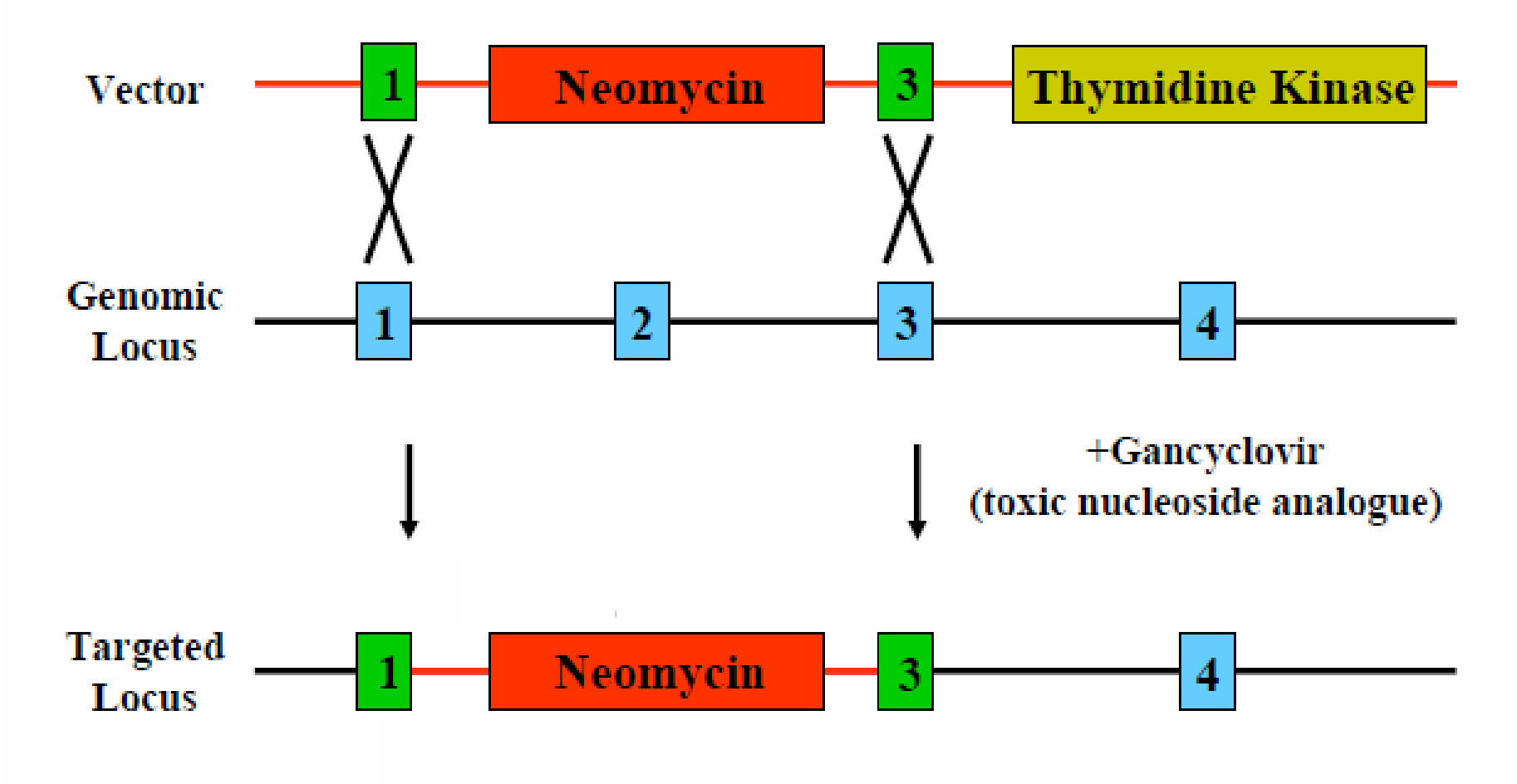
Negative Selection Markers

Negative selection markers used to enrich for homologous recombination events over random insertions:

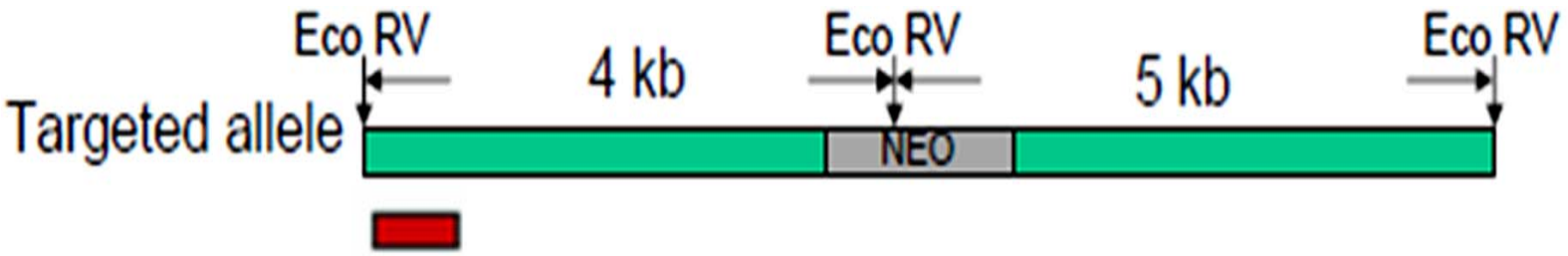
Use of Herpes Simplex Virus (HSV) Thymidine Kinase (TK) gene coupled with gancyclovir treatment.

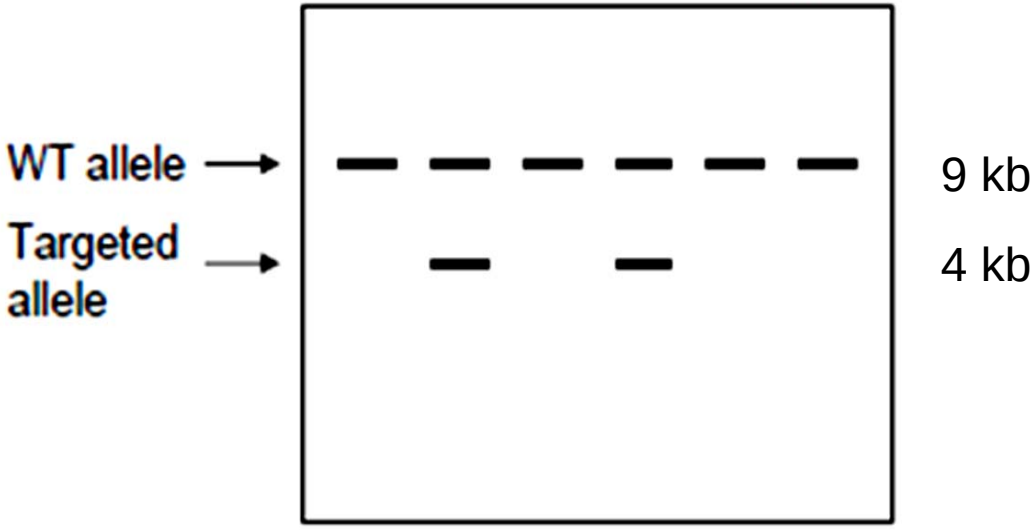
Ganciclovir is a synthetic nucleoside analog of deoxyguanosine, which is phosphorylated by HSV-TK and the metabolite inhibits DNA replication when incorporated into DNA by termination of DNA elongation.

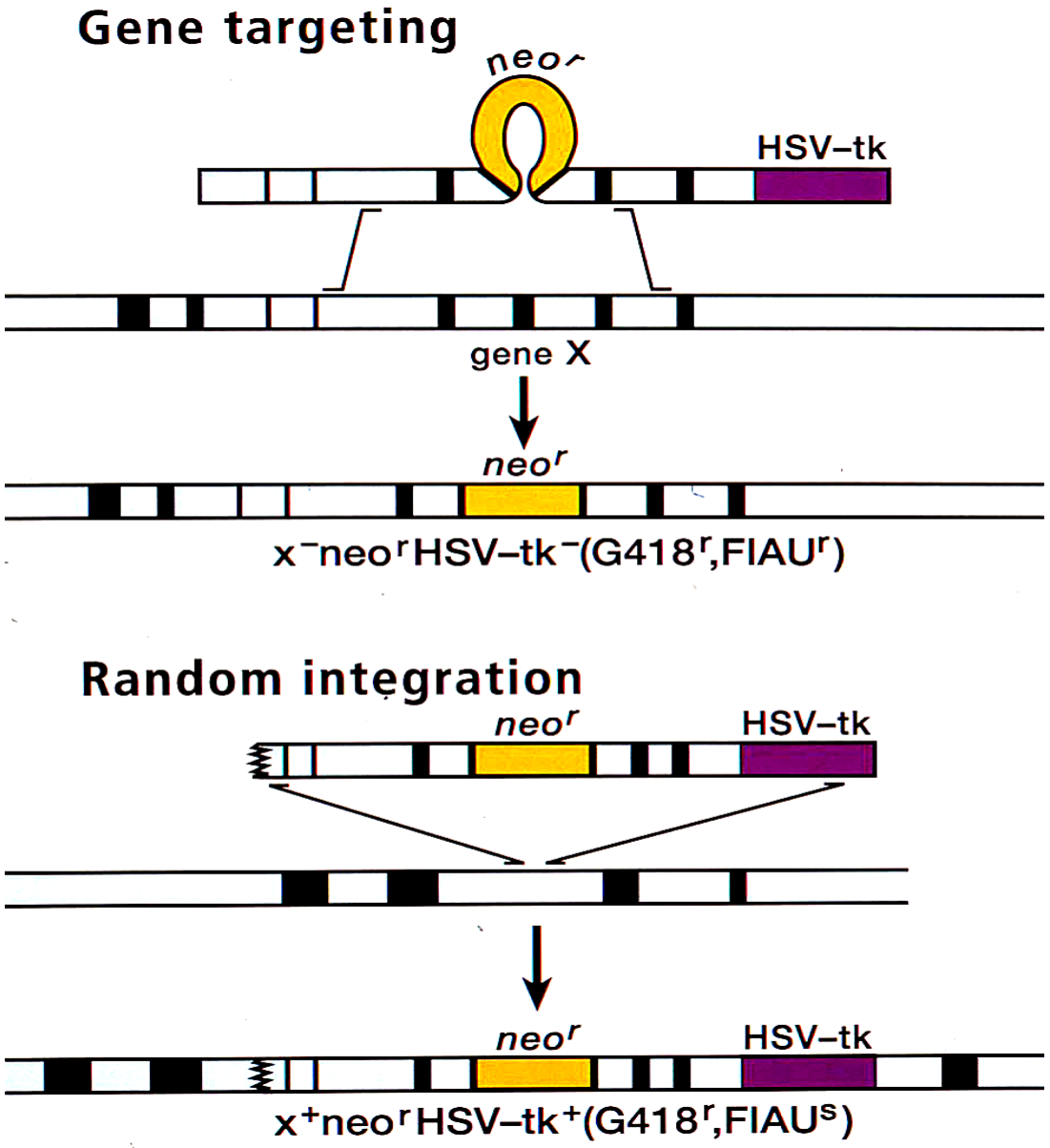




Gene targeting:	Neo^R, $HSV\text{-}TK^-$	$G418^R$, $Gancyclovir^R$
Random integration:	Neo^R, $HSV\text{-}TK^+$	$G418^R$, $Gancyclovir^S$


Eco RV
4 kb
Eco RV
5 kb
Eco RV
Targeted allele
NEO


WT allele
Targeted allele
9 kb
4 kb


Gene targeting
neo
HSV–tk
gene X
neo
x⁻neo HSV–tk⁻(G418 ,FIAU)
Random integration
neo
HSV–tk
neo
HSV–tk
x⁺neo HSV–tk⁺(G418 ,FIAU)

Drawbacks of knockout mice

Many genes that participate in interesting genetic pathways are essential for either mouse development, viability or fertility. Therefore, a traditional knockout of the gene can never lead to the establishment of a knockout mouse strain for analysis.

About fifteen percent of gene knockouts are developmentally lethal and therefore cannot grow into adult mice. Thus, it becomes difficult to determine the gene function in adults.

To overcome these drawbacks, the **conditional knockout approach** was developed which allowed researchers to delete the gene of interest in a time- and space-dependent manner using **site-specific recombinases.**

Using these recombinases, it is possible to knock out the expression of a gene in a specific mouse tissue or at a specific stage of development or in response to an inducer.

The Cre-loxP system

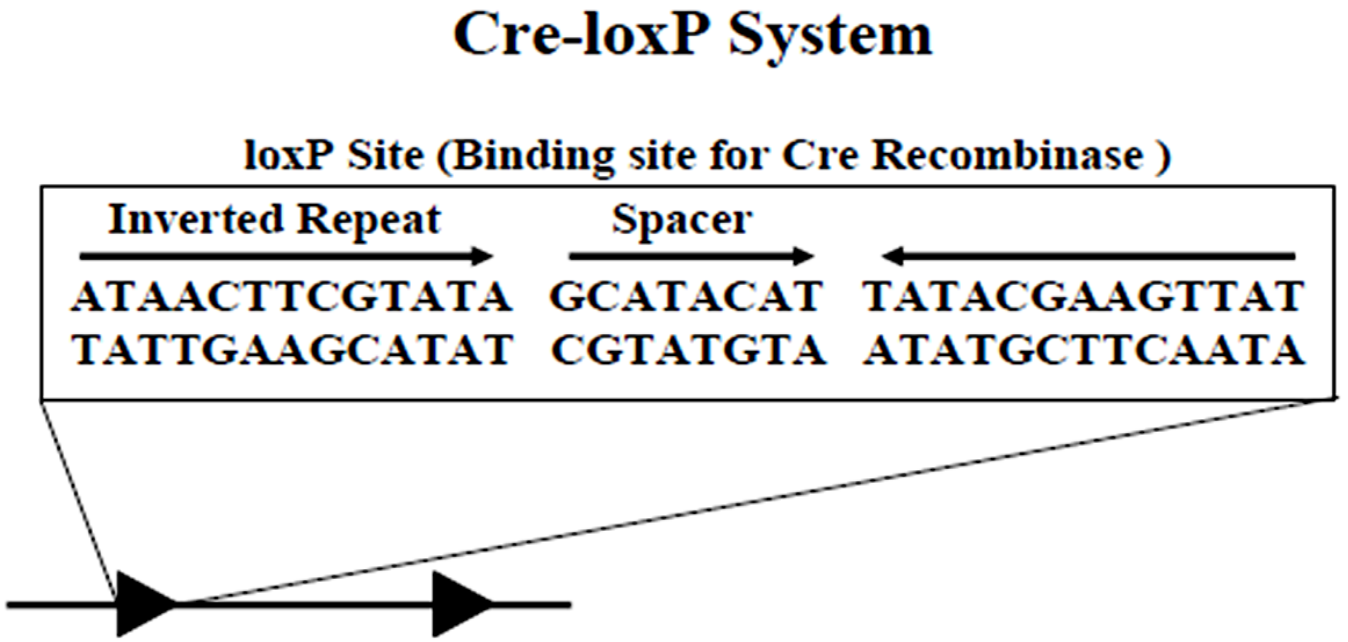
1987 Brian Sauer's introduction of the Cre-loxP system for temporal control of transgenic gene expression.

1995 K Rajewsky demonstrated “Inducible gene targeting in mice” using the Cre-loxP conditional knockout.

Sauer B. Functional expression of the cre-lox site-specific recombination system in the yeast Saccharomyces cerevisiae. Mol Cell Biol. 1987 Jun;7(6):2087-96.

Kuhn R, Schwenk F, Aguet M, Rajewsky K. Inducible gene targeting in mice. Science. 1995 Sep 8;269(5229):1427-9

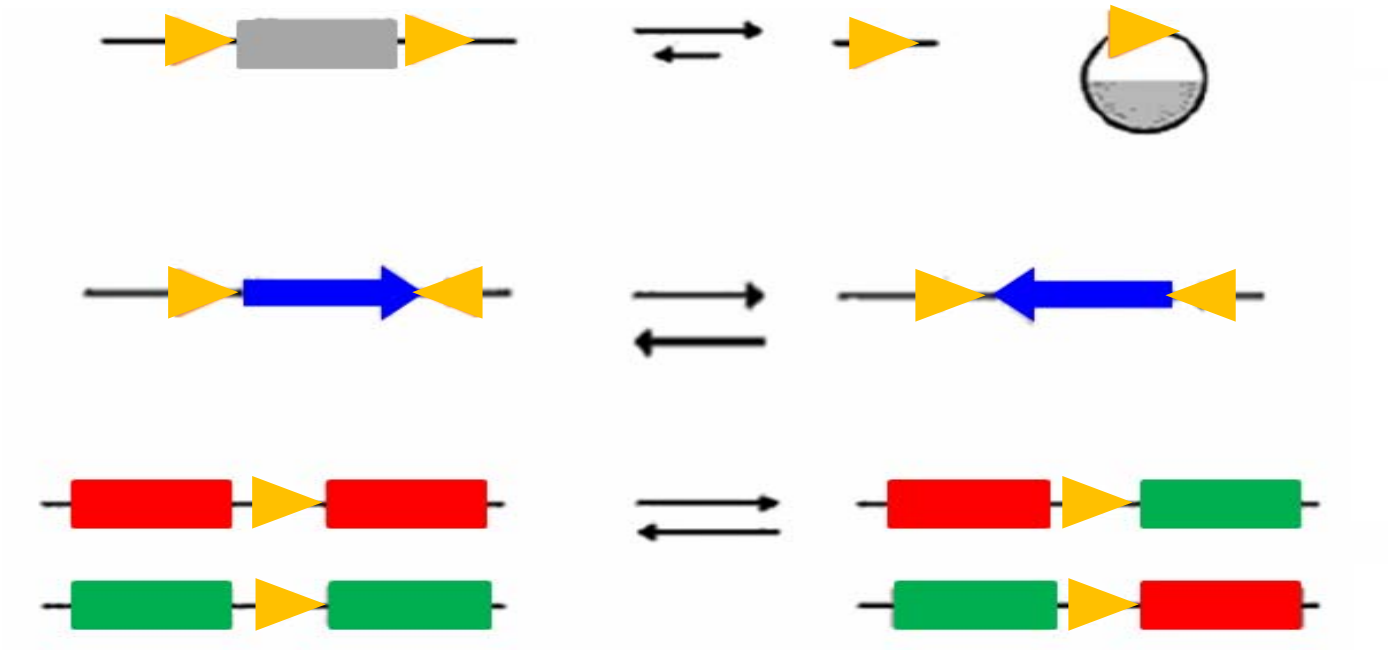
Cre recombinase, a site-specific integrase isolated from the P1 bacteriophage, catalyzes recombination between two of its consensus DNA recognition sites known as the loxP sites which are 34 base pairs in length. They consist of two 13bp palindromic sequences that flank a central sequence of 8bp which determines the directionality of the loxP site.



Two **loxP sites** are most often placed in a trans orientation on either side of an essential, functional part of a gene so that recombination removes that functionality and knocks-out the gene.

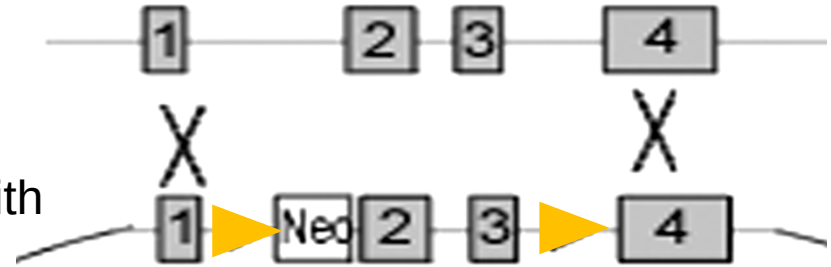
LoxP sites can also be placed in a cis orientation to invert the intervening sequence.

LoxP sites placed on different chromosomes can be used to generate targeted translocations, though this recombination event occurs at a relatively low frequency compared to the highly-efficient intra-gene recombination.

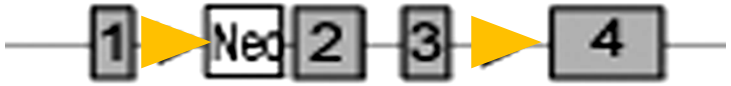


Gene of interest

KO construct with Neo^{R} gene & **Loxp sites**



Recombination between mouse genome and targeting vector with **Loxp sites**



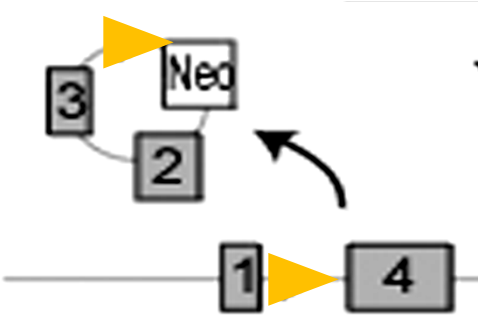
Neomycin-resistant ES cells with targeted allele

Chimeric mouse

Homozygous knockout ready mouse

X

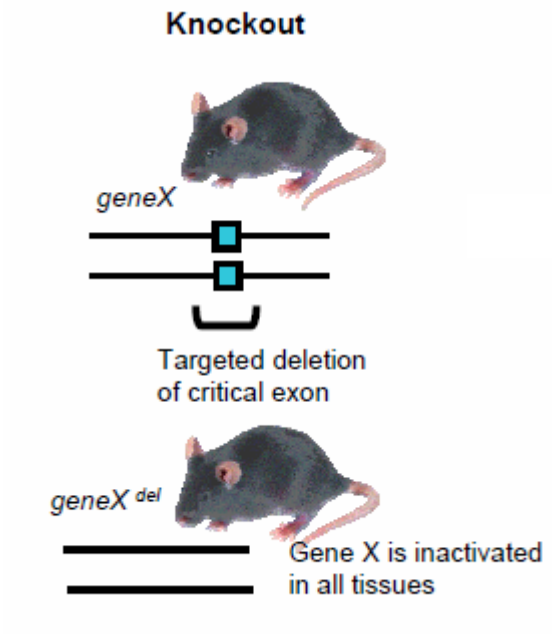
Mouse expressing Cre recombinase

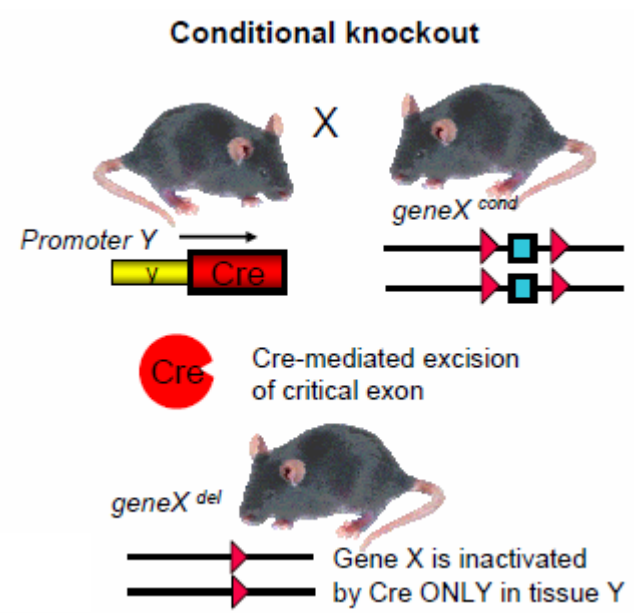


Conditional knock out mouse

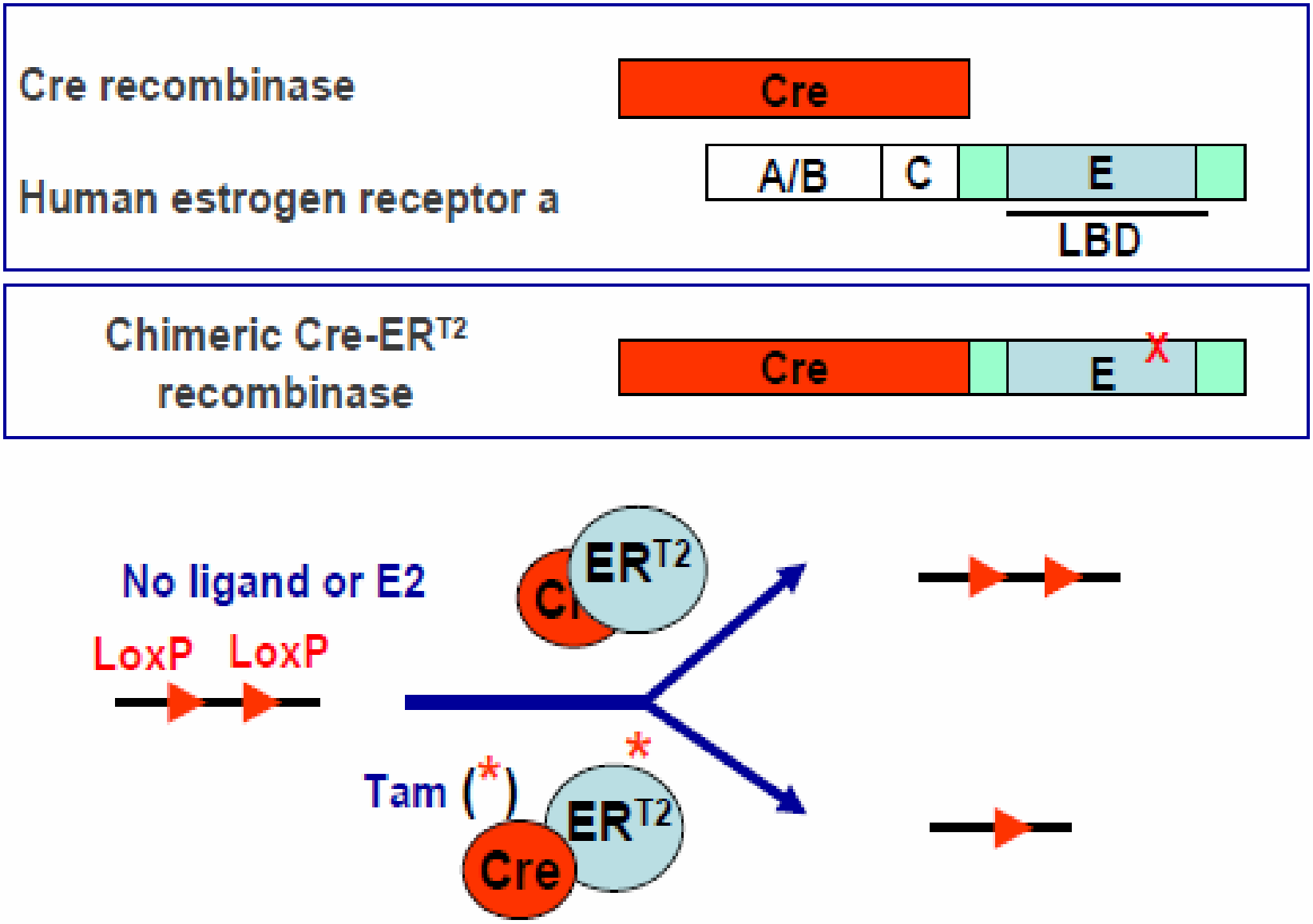
Spatial and temporal knockout is achieved by the choice of promoter used to drive the *Cre* gene expression.

Several mouse lines each expressing Cre from a promoter that is either tissue specific, cell specific, developmentally specific or responsive to an exogenous agent like tetracycline are now available. Thus, several promoter-specific mouse models can be generated.





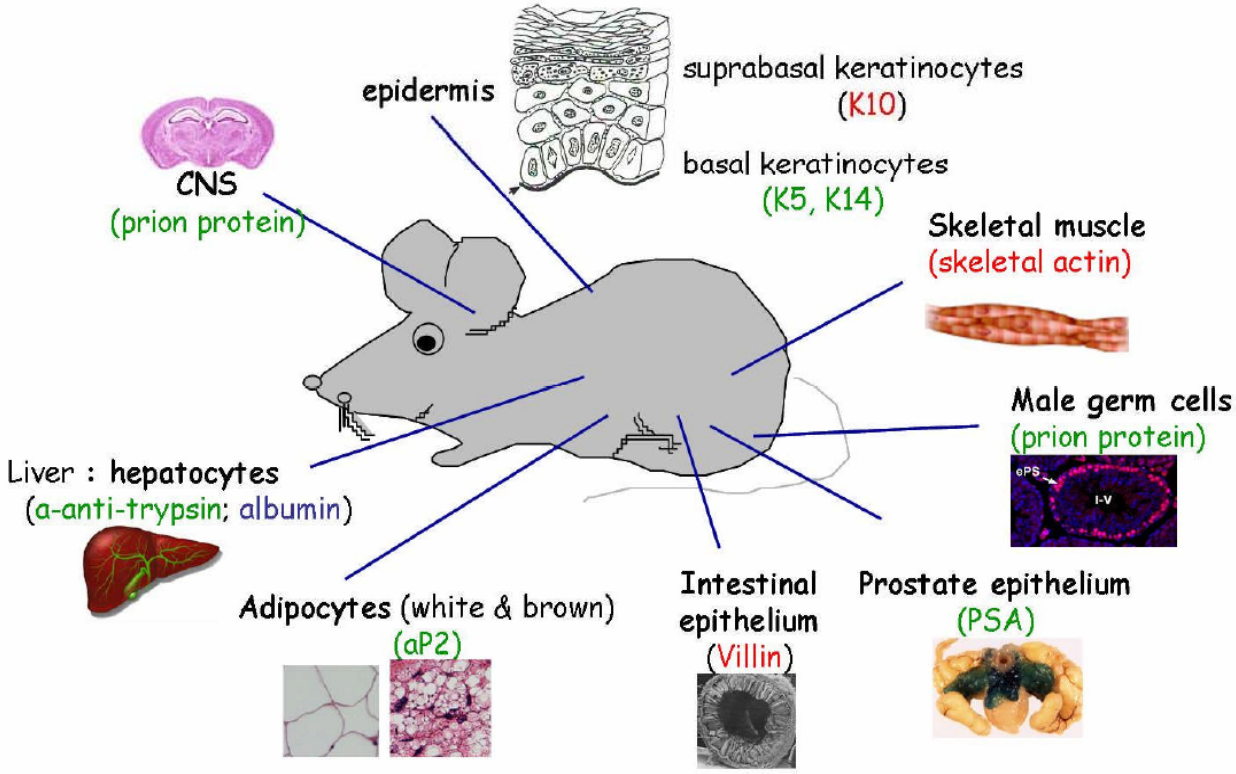
http://www.tecan.com/platform/content/element/3469/AutomatedMouse_SystematicGenerationOfDiseaseModels_NadiaRosenthal.pdf



The Cre-ER/Loxp inducible system

http://www.tecan.com/platform/content/element/3469/AutomatedMouse_SystematicGenerationOfDiseaseModels_NadiaRosenthal.pdf

Validated Cre-ER^T Lines (IGBMC&ICS)



http://www.tecan.com/platform/content/element/3469/AutomatedMouse_SystematicGenerationOfDiseaseModels_NadiaRosenthal.pdf

Similarly, Flp recombinase (and its Frt DNA sites) have also been used for generating conditional knockout mice as well as transgeic mice.

Flp-mediated tissue-specific inactivation of the retinoblastoma tumor suppressor gene in the mouse.
Vooijs M, van der Valk M, te Riele H, Berns A.
Oncogene. 1998 Jul 9;17(1):1-12.

Cre-mediated somatic site-specific recombination in mice.
Akagi K, Sandig V, Vooijs M, Van der Valk M, Giovannini M, Strauss M, Berns A.
Nucleic Acids Res. 1997 May 1;25(9):1766-73.

Using Flp-recombinase to characterize expansion of Wnt1-expressing neural progenitors in the mouse.
Dymecki SM, Tomasiewicz H.
Dev Biol. 1998 Sep 1;201(1):57-65.

Other recombinases: Dre, phiC31

Knockout *vs* Knockin

In contrast to knockout in which a gene or part of a gene is deleted, knockin is the replacement of a gene by mutant version of the same gene using homologous recombination.

Knockin is very useful when establishing a disease model of a specific disease-related mutation in human gene.

Impact on biomedical research

More than 10,000 different genes in mice, approximately half of the genes in the mammalian genome, have been studied with gene targeting.

Roadmap to disrupt each of the ~20,000 genes in the mouse genome before 2010.

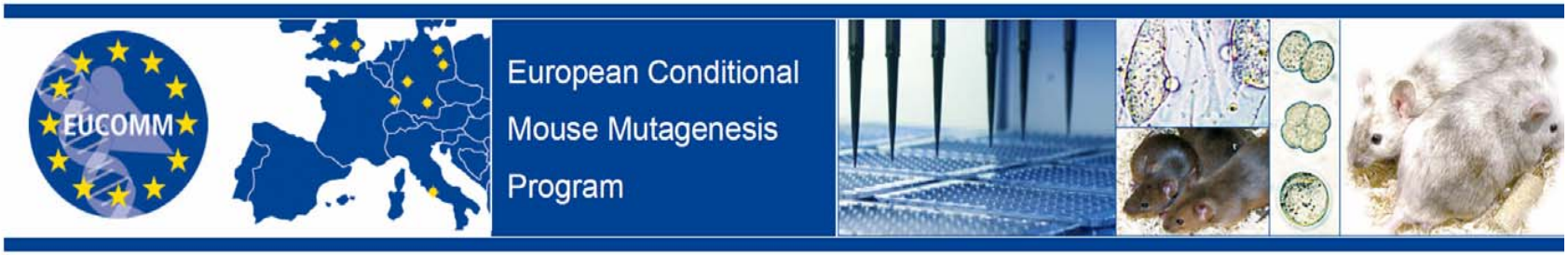
http://www.nih.gov/science/models/mouse/knockout/komp.html

The **Knockout Mouse Project (KOMP)** is a trans-NIH initiative that aims to generate a comprehensive and public resource comprised of mouse embryonic stem (ES) cells containing a null mutation in every gene in the mouse genome.

In June 2007, NIH announced it will provide $4.8 million to establish and support a repository for its Knockout Mouse Project.

http://www.komp.org/

The KOMP Repository is the official archive and distribution center for the Knockout Mouse Project (KOMP), a major 5-year trans-NIH initiative designed to generate null alleles in C57BL/6 embryonic stem (ES) cells for most genes not already available as knockout mice. Nearly 8500 genes are targeted for deletion,



Global Gene Knockout Programs

	Type of Resource	Type of Knockout	2006	2007	2008	2009	2010	Totals
KOMP	ES Cell	Targeted Deletion	175	500	941	942	942	3500
	Mouse	Targeted Deletion	50	50	50	50	50	250
	ES Cell	Targeted Conditional	1000	1000	1000	1000	1000	5000
	Mouse	Targeted Conditional	30	100	40	40	40	250
EUCOMM	ES Cell	Trapped Conditional	3000	6000	3000			12000
	ES Cell	Targeted Conditional	1000	3000	4000			8000
	Mouse	Mixed	20	100	200			320
NorCOMM	ES Cell	Trapped Conditional	1000	4000	3000	2000		10000
	ES Cell	Targeted Conditional	100	400	750	750		2000
	Mouse	Mixed	25	25	25	25		100
Cumulative for All Programs	ES Cell	Trapped (Conditional)	4000	14000	20000	22000		22000*
	ES Cell	Targeted (Deletion)	175	675	1616	2558	3500	3500
	ES Cell	Targeted (Conditional)	2100	6500	12250	14000	15000	15000
	Mouse	Mixed	125	400	715	830	920	920

http://www.tecan.com/platform/content/element/3469/AutomatedMouse_SystematicGenerationOfDiseaseModels_NadiaRosenthal.pdf

Companies that specialize in the generation of transgenic and knockout mice

http://www.polygene.ch/gene-targeting.php

http://www.ozgene.com

http://www.vegabiolab.com

GENE TARGETING

Enter the rat

F. Kent Hamra

Advances in stem-cell technology have broken the barrier to gene targeting in mammals other than mice. A wide array of research opportunities now opens up, especially in studies involving the laboratory rat.

NATURE|Vol 467|9 September 2010 161-163

C. Tong, et al., "Production of p53 gene knockout rats by homologous recombination in embryonic stem cells," *Nature 467,* 211–213 (2010).

Geurts, A. M. *et al. Science 325, 433 (2009).*

Izsvak, Z. *et al. Nature Methods 7, 443–445 (2010).*

Rat models are an alternative to mice that may enable the creation of new gene disruptions that are unavailable in the mouse.

Although mice have been the animal model of choice for most geneticists, the rat has traditionally been favored by physiologists and pathologists.

Rats have a heart rate similar to that of humans, while mice have a heart rate five to ten times as fast.

Rats have been used as important models for human cardiovascular disease, diabetes, arthritis, and many autoimmune and behavioral disorders.

Rat models are superior to mouse models for testing the pharmacodynamics and toxicity of potential therapeutic compounds,

http://www.knockoutrat.org

5978–5990 Nucleic Acids Research, 2005, Vol. 33, No. 18
doi:10.1093/nar/gki912

SURVEY AND SUMMARY

Zinc finger nucleases: custom-designed molecular scissors for genome engineering of plant and mammalian cells

Sundar Durai[1,3], Mala Mani[1], Karthikeyan Kandavelou[1,2], Joy Wu[1], Matthew H. Porteus[4,5] and Srinivasan Chandrasegaran[1,*]

THE ZINC-FINGER NUCLEASES

Trends Genet. 2010 Sep 23. [Epub ahead of print]
Gene targeting in the rat: advances and opportunities.
Jacob HJ, Lazar J, Dwinell MR, Moreno C, Geurts AM.

Clin Sci (Lond). 2010 Jul 6;119(8):303-11.
Zinc-finger nucleases: new strategies to target the rat genome.
Geurts AM, Moreno C.

Gene targeting in the rat: advances and opportunities.
Jacob HJ, Lazar J, Dwinell MR, Moreno C, Geurts AM.
Trends Genet. 2010 Sep 23. [Epub ahead of print]

Zinc-finger nucleases: new strategies to target the rat genome.
Geurts AM, Moreno C.
Clin Sci (Lond). 2010 Jul 6;119(8):303-11.

Zinc finger nuclease-mediated transgene deletion.
Petolino JF, Worden A, Curlee K, Connell J, Strange Moynahan TL, Larsen C, Russell S.
Plant Mol Biol. 2010 Aug;73(6):617-28. Epub 2010 May 8.

Highly efficient endogenous human gene correction using designed zinc-finger nucleases.
Urnov FD, Miller JC, Lee YL, Beausejour CM, Rock JM, Augustus S, Jamieson AC, Porteus MH, Gregory PD, Holmes MC.
Nature. 2005 Jun 2;435(7042):646-51. Epub 2005 Apr 3.

Design, engineering, and characterization of zinc finger nucleases.
Mani M, Kandavelou K, Dy FJ, Durai S, Chandrasegaran S.
Biochem Biophys Res Commun. 2005 Sep 23;335(2):447-57.

Key publications

Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981 Jul 9;292(5819):154-6.

Formation of germ-line chimeras from embryo derived teratocarcinoma cell lines.
Nature 309:255-6, 1984

Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector.
Nature 323:445-8, 1986

Thomas KR, Folger KR, Capecchi MR. High frequency targeting of genes to specific sites in the mammalian genome. *Cell 44:419-28, 1986*

Smithies O, Gregg RG, Boggs SS, Koralewski MA, Kucherlapati RS.
Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. Nature. 1985 Sep 19-25;317(6034):230-4.

Zijlstra M, Bix M, Simister NE, Loring JM, Raulet DH, Jaenisch R.
Beta 2-microglobulin deficient mice lack $CD4^{-}8^{+}$ cytolytic T cells.
Nature. 1990 Apr 19;344(6268):742-6.

Muller U. 1999. Ten years of gene targeting: targeted mouse mutants, from vector design to phenotype analysis. Mech Dev. 82:3-21.

Nagy A. 2000. Cre recombinase: the universal reagent for genome tailoring. Genesis. 26:99-09.

Nobel Lecture

Gene Targeting into the 21st Century

Mario R. Capecchi

Howard Hughes Medical Institute
Department of Human Genetics
University of Utah School of Medicine

December 7, 2007

http://nobelprize.org/nobel_prizes/medicine/laureates/2007/capecchi-slides.pdf

Embryonic Stem Cells: The Mouse Source —vehicle for Mammalian Genetics

Martin Evans

Nobel Lecture

http://nobelprize.org/nobel_prizes/medicine/laureates/2007/evans-slides.pdf

Oliver Smithies

Nobel lecture

http://nobelprize.org/nobel_prizes/medicine/laureates/2007/smithies-slides.pdf

Cell Migration Consortium Transgenic & Knockout Mouse Initiative

http://www.cellmigration.org/resource/komouse/komouse_approaches.shtml

http://learn.genetics.utah.edu/content/tech/transgenic/

http://www.eucomm.org/information/protocols/