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Transgenic and Knockout Mouse - Approaches

The ability to engineer the mouse genome has proven useful for a variety of applications in research, medicine and biotechnology. Transgenic mice have become powerful reagents for modeling genetic disorders, understanding embryonic development and evaluating therapeutics. These mice and the cell lines derived from them have also accelerated basic research by allowing scientists to assign functions to genes, dissect genetic pathways, and manipulate the cellular or biochemical properties of proteins.

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Mice as Models of Human Disease

Classic genetic analyses are performed by observing a phenotype, designing the necessary cross-pollinations or matings, and using the resulting population to perform statistically significant experiments to find the mutation and to understand the function of the altered gene. Inherited human diseases provide researchers with many phenotypes, and although the human is the mammal we are generally most interested in learning more about, it is also the one animal we cannot use for genetic experiments for obvious ethical reasons. Mice naturally develop conditions that mimic human disease, such as cardiovascular disease, cancer and diabetes, and the inbred laboratory mouse has therefore been used as a model organism to study inherited human diseases for nearly a century. [Lathrop and Loeb \(1916\)](#) published the effects of hormones on the development of tumors in mice in 1916, and the mouse has remained as a favorite model for human disease because it has a relatively low cost of maintenance and a generation time that measures only nine weeks.

Developments in molecular biology and stem cell biology over the last 20 years have allowed researchers to create custom-made mice through gene targeting in mouse embryonic stem (ES) cells. Site-directed mutagenesis in embryonic stem cells and the phenotypic characterization of the corresponding knockout and/or knockin mouse, allows researchers to study gene function as it relates to the entire organism. Now, certain diseases that afflict humans but normally do not strike mice, such as cystic fibrosis

([Snouwaert et al., 1992](#); [Dorin et al., 1992](#)) and Alzheimer's (see [Masliah & Rockenstein, 2000](#) for review), can be induced by manipulating the mouse genome and environment.

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ES Cells and Chimeric Mice

Embryonic stem (ES) cells are [pluripotent](#) cell lines with the capacity of self-renewal and a broad differentiation plasticity. They are derived from pre-implantation embryos and can be propagated as a homogeneous, uncommitted cell population for an almost unlimited period of time without losing their pluripotency and their stable [karyotype](#). Even after extensive genetic manipulation, mouse ES cells are able to reintegrate fully into viable embryos when injected into a host blastocyst or aggregated with a host morula. After these pre-implantation embryos are implanted into a surrogate mother, they develop into mosaic offspring known as chimeras. The tissues of chimeric mice are comprised of a mixture of cells that originated from both the host embryo and the ES cells. The contribution of each originating cell population is seen most visibly in the fur, which is generally striped black (from host cells) and brown (from ES cells). Healthy ES cells can give rise to descendants in all cell types, including functional [gametes](#) to produce more and more mice containing the desired genetic modification ([Thompson et al., 1989](#)). If the proportion of ES cell descendents in the coat of the animal is high, the probability that ES cells are represented in gametes is also high, since ES cells mix thoroughly with host cells early in embryogenesis. ES cells give rise to brown coat color because they are Aw/Aw dominant White-bellied Agouti), and the host cells give rise to black coat color because they are a/a (recessive non-agouti). The ES cells used most commonly are from the 129 strain of mice, while the host embryos are from the C57BL6 strain of mice. If the chimeras are bred to a/a non-agouti mice (for example C57BL6 or Black Swiss), then any brown offspring (Aw/a) must have arisen from ES cell-derived gametes, and 50% of the brown offspring are expected to carry the genetic modification.

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Transgenic Mice

One of the simplest ways to study gene function in a mouse is exogenous expression of a protein in some or all tissues. For this type of genetic modification, a new piece of DNA is introduced into the mouse genome. This piece of DNA includes the structural gene of interest, a strong mouse [gene promoter](#) and [enhancer](#) to allow the gene to be expressed and [vector](#) DNA to enable the transgene to be inserted into the mouse genome. Successful integration of this DNA results in the expression of the transgene in addition to the wild type, basal (endogenous) protein levels in the mouse. Depending on the goal of the experiment, the transgenic mouse will exhibit over-expression of a non-mutated protein, expression of a dominant-negative form of a protein, or expression of a fluorescent-tagged protein. By definition, transgenesis is the introduction of DNA from one species into the genome of another species. Many of the first transgenic mice fit this description well as they were generated to study the overexpression of a human protein, often an oncogene ([Robertson et al., 1986](#)). Currently, the phrase "transgenic mouse" generally refers to any mouse whose genome contains an inserted piece of DNA, originating from the mouse genome or from the genome of another species, and the term includes the standard transgenic mouse as well as a knockin or knockout mouse (see below).

To generate a standard transgenic mouse, a bacterial or viral [vector](#) containing the transgene and any desired markers are injected into a fertilized mouse egg. The DNA usually integrates into one or more loci during the first few cell divisions of preimplantation development. The number of copies of the transgenic fragment can vary from one to several hundred, arranged primarily in head-to-tail arrays, and

the transgenic founder mice are mosaic for the presence of the transgene. Founders are very likely to have germ cells with the integrated transgene, and therefore will be able to vertically transmit the integrated gene, and all cells of the progeny transgenic mouse contain the transgene. This method is relatively quick, but includes the risk that the DNA may insert itself into a critical [locus](#), causing an unexpected, detrimental genetic mutation. Alternatively, the transgene may insert into a locus that is subject to gene silencing. If the protein being expressed from the transgene causes toxicity, excessive overexpression from multiple insertions can be lethal to some tissues or even to the entire mouse. For these reasons, several independent lines mice containing the same transgene must be created and studied to ensure that any resulting phenotype is not due to toxic gene-dosing or to the mutations created at the site of transgene insertion.

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Gene Targeting

Homologous recombination in embryonic stem cells is now a routine method for modifying the mouse genome at a specific locus. The technique was first developed for site-directed mutagenesis in yeast, and has been successfully adapted for mammalian cells ([Smithies et al., 1985](#)). In theory, any deletion, point mutation, inversion or translocation can now be modeled in mice. The DNA construct to be introduced into the genome of the ES cells should contain several kilobases of DNA that are homologous to the mouse genome to provide the best odds of recombination. The vector also contains the modifications to be introduced as well as genes conferring drug resistance or sensitivity so researchers can select the rare recombination events from a large population of ES cells. In yeast, true homologous recombination at the correct locus occurs at a very high rate, so that random integration of the vector into the yeast genome is rare. In mammalian genomes, however, the majority of recombination events do not occur at the desired locus, so ES cells showing resistance to the selective agent must also be screened by Southern blot or by PCR to discover which clones have been correctly targeted.

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Knockins

To avoid the problems of a standard transgenic, many researchers now rely on knockin mice to study the exogenous expression of a protein. A knockin mouse is generated by targeted insertion of the transgene at a selected locus. The insert is flanked by DNA from a non-critical locus, and homologous recombination allows the transgene to be targeted to that specific, non-critical integration site. In this way, a researcher has complete control of the genetic environment surrounding the overexpression cassette and it is likely that the DNA did not incorporate itself into multiple locations. Site-specific knockins result in a more consistent level of expression of the transgene from generation to generation because it is known that the overexpression cassette is present as a single copy. Also, because a targeted transgene is not interfering with a critical locus, the researcher can be more certain that any resulting phenotype is due to the exogenous expression of the protein. Although the generation of a knockin mouse does avoid many of the problems of a traditional transgenic mouse, this procedure requires more time to assemble the vector and to identify ES cells that have undergone homologous recombination.

Knockouts

While traditional transgenic and knockin 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. This can be achieved through random mutation using chemical mutagenesis or a [gene trap](#) approach, or through

gene targeting to generate a knockout mouse. Homologous recombination allows a researcher to completely remove one or more [exons](#) from a gene, (see Figure 1 below) which results in the production of a mutated or truncated protein or, more often, no protein at all. The process and time line for making a knockout mouse with the Cell Migration Consortium Transgenic & Knockout Mouse Initiative has been outlined in the [Time line document](#). The phenotypes of knockout mice can be very complex because all tissues of the mouse are affected, though it is not uncommon for a knockout mouse to display embryonic lethality or to show no phenotype at all.

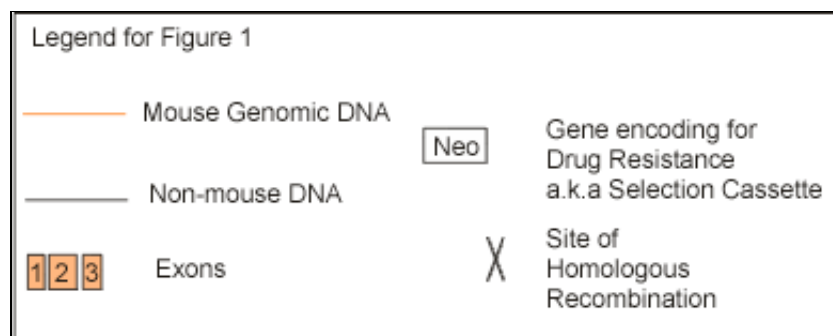
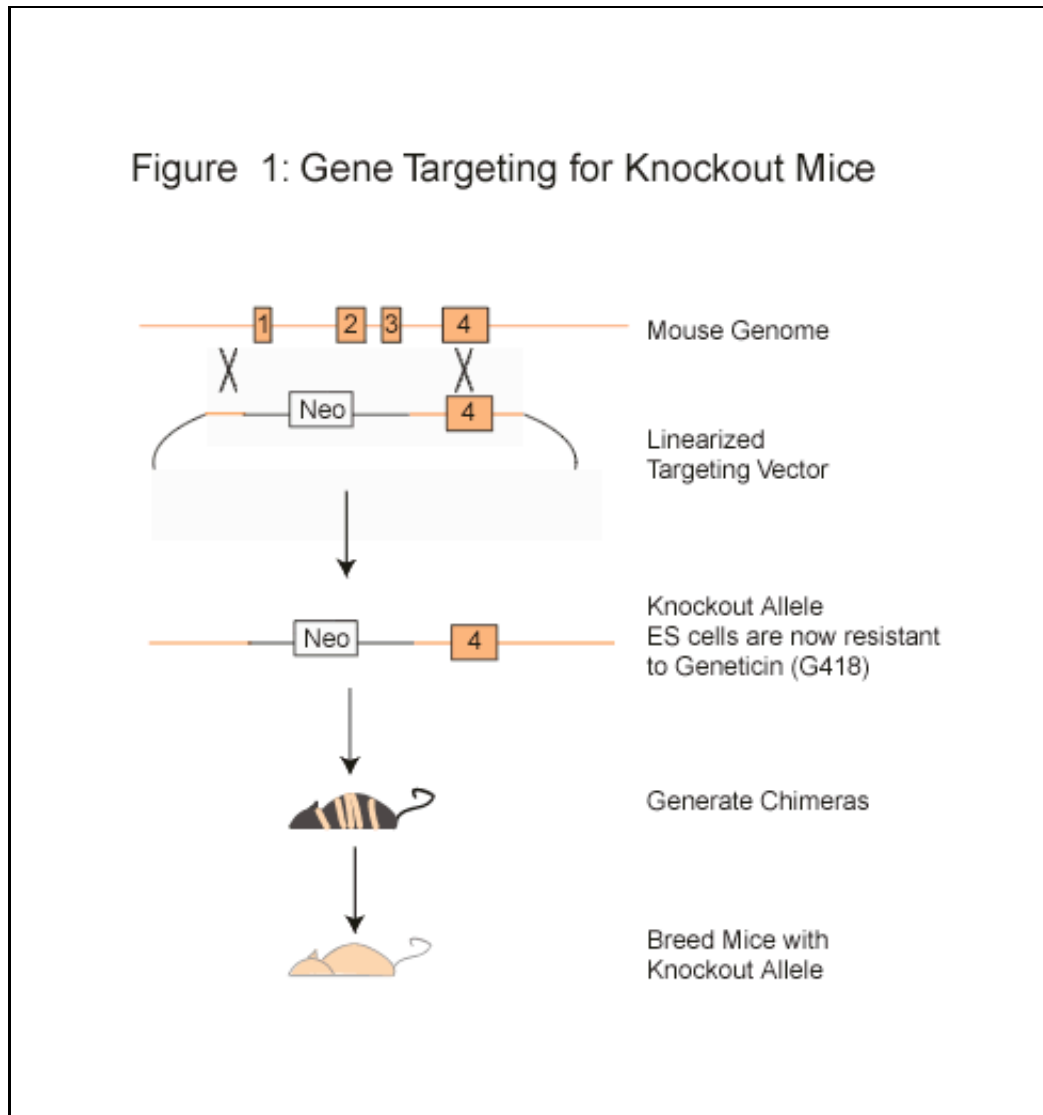


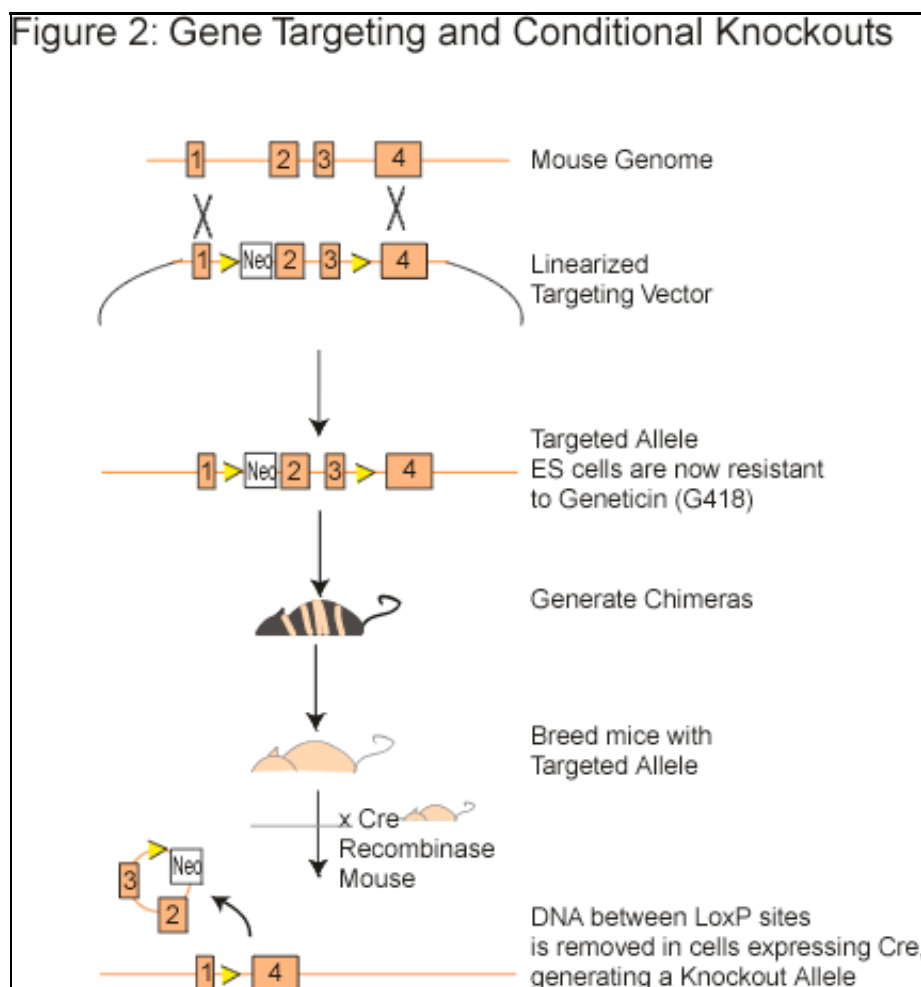
Figure 1: Gene targeting for knockout mice

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Conditional Gene Modifications

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. Conditional gene modification using Cre-lox technology allows the gene of interest to be knocked-out in only a subset of tissues or only at a particular time, circumventing lethality. Because gene targeting can be controlled both spatially and temporally, the function of a given gene can be studied in the desired cell types and at a specific time point. This genetic dissection allows researchers to define gene function in development, physiology or behavior.

Cre recombinase, a site-specific integrase isolated from the P1 bacteriophage, catalyzes recombination between two of its consensus DNA recognition sites ([Sauer & Henderson, 1988](#)). These loxP sites are 34 base pairs in length, consisting of two 13bp palendromic 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. (See Figure 2) 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.



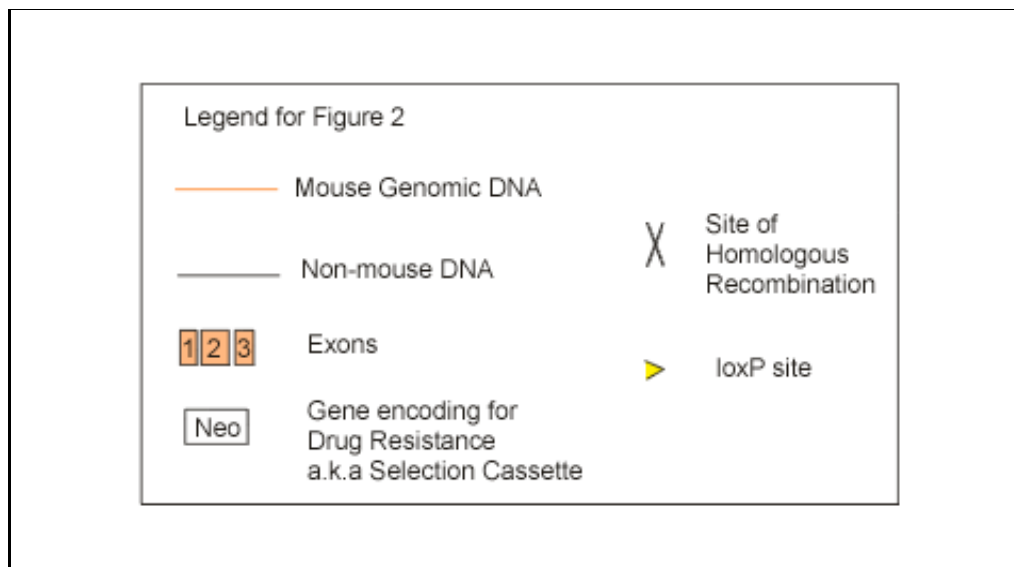
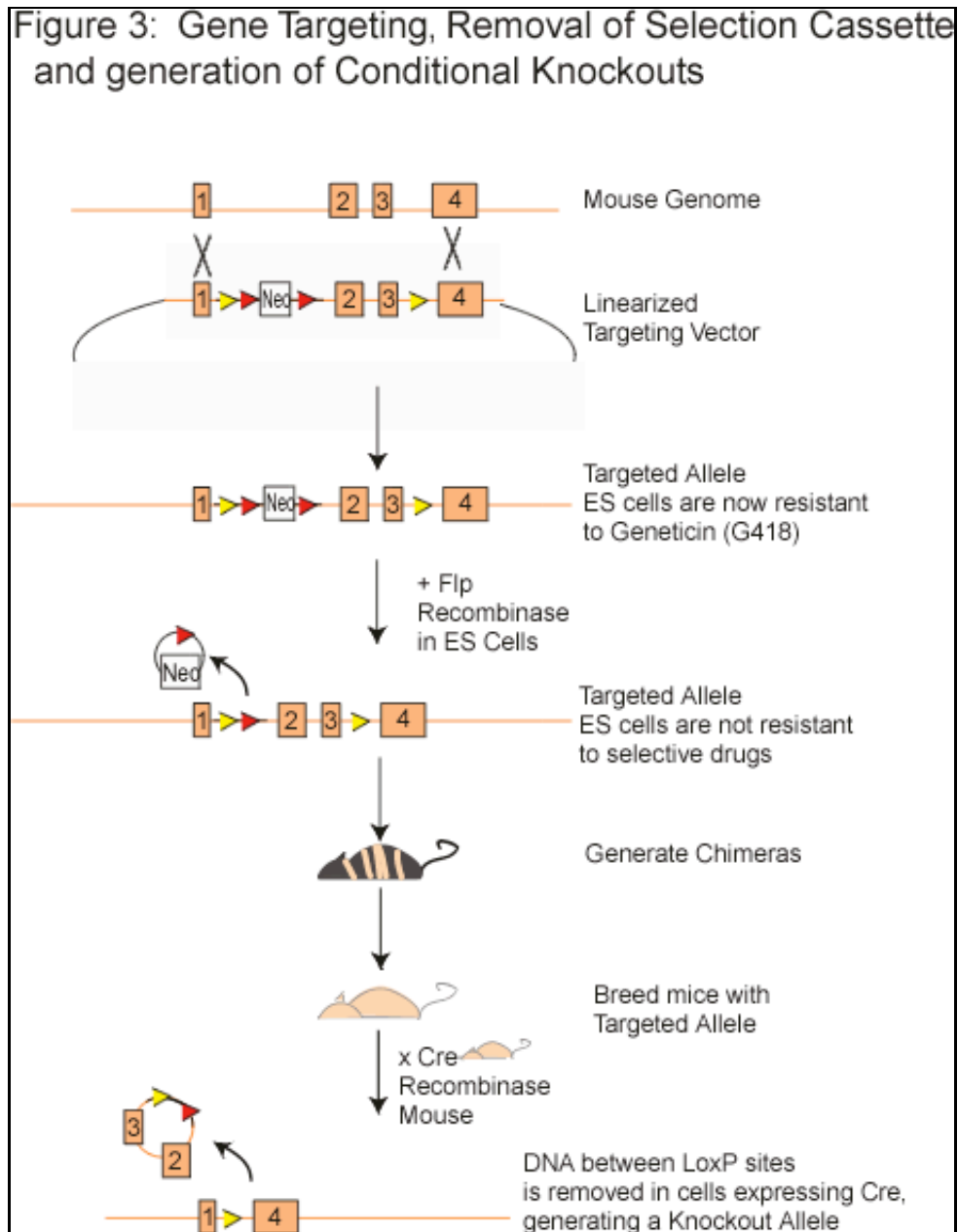


Figure 2: Gene targeting and conditional knockouts

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LoxP sites do not recombine in the absence of Cre recombinase, so regulation of the expression of Cre recombinase also regulates the DNA recombination and the initiation of the genomic alteration. An extensive collection of mice have been generated, each line expressing Cre from a promoter that is either tissue specific, cell specific, developmentally specific or responsive to an exogenous agent like tetracycline. With such a collection available, several promoter-specific mouse models can be studied in parallel. Additionally, researchers have generated an extensive collection of vectors that express Cre recombinase from a reliable promoter, and transient expression of Cre results in high rates of recombination in cultured cells. Thus, recombination can be triggered in ES cells to generate a more traditional knockout mouse in addition to the tissue-specific knockout. Alternatively, the mouse can be bred and grown as a pseudo- wild type with out any recombination, and then a population of cells cultured from this mouse can be transfected with a Cre-expression vector to generate recombined cells.

Recently, Flp recombinase (and its Frt DNA sites) have also proven useful in mouse transgenics ([Vooijs et al., 1998](#); [Dymecki & Tomasiewicz, 1998](#)). Although few lines of mice have been generated to express Flp in vivo, this system is very useful for the removal of the selection gene from the targeted gene at the ES cell stage. The presence of a Neomycin resistance cassette in an intron can result in an alteration of gene function and therefore produce an unwanted or even lethal phenotype ([Scacheri et al., 2001](#)). This problem can be avoided if the investigator utilizes both the Cre and Flp recombination systems. A targeting vector containing both a Flp-flanked neoR marker and a loxP-flanked exon can be introduced into ES cells. After selection, the Neomycin resistance cassette can be removed with Flp recombinase before the ES cells are injected into host blastocysts to make mice. (See Figure 3) With this system, the chimeric offspring contain only a minimal genetic modification (the addition of two loxP sites and one Frt site) in the gene of interest, limiting the likelihood of a complicating phenotype. As with a loxP-only targeting, the regulated expression of Cre results in the regulated alteration of this gene.



Legend for Figure 3

	Mouse Genomic DNA		Site of Homologous Recombination
	Non-mouse DNA		loxP site
	Exons		Frt site
	Gene encoding for Drug Resistance a.k.a Selection Cassette		

Figure 3: Gene targeting, removal of selection cassette, and generation of conditional knockouts

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Glossary of Terms

Bacteriophage

A virus that infects bacteria

Cis orientation

Two LoxP sites are in CIS on a piece of DNA if they are in opposite directionalities. The sequences of CIS LoxP sites mirror one another.

Exogenous expression

Non-typical expression of a gene, usually due to a change in or replacement of the promoter of the gene. Can cause an expression level that is higher, lower or differently regulated for that cell type.

Exon

A portion of a gene that contains sequence that codes for the protein

Floxed

Flanked by loxP sites.

Gamete

In mammals, an egg or a sperm

Gene Enhancer

A region of DNA that is separate from the Gene Promoter that also affects the transcription of the gene. Enhancers have been found within introns or even several kilobases from the 5' or 3' end of the gene.

Gene Promoter

A regulator region of DNA a short distance from the 5' end of a gene that acts as the binding site for RNA polymerase.

Gene trap

A sequence of DNA that is designed with at least (1) a splice acceptor to insert itself into genes and (2) a selection cassette to disrupt transcription. See [The Center for Modeling Human Disease](#) for a great explanation.

Integrase

An enzyme that catalyses the insertion or removal of a piece of DNA into/from a larger piece of DNA.

Intron

A non-coding sequence located between exons of a gene.

Karyotype

The chromosome complement of an individual or cell, as seen during mitotic metaphase. In mice, a normal karyotype has 40 sister chromatid pairs.

Locus/Loci

The specific, physical location(s) on a chromosome (or chromosomes).

Morula

The globular mass of cells formed by the cleavage of the fertilized egg in the first stages of its development.

Oncogene

A gene that contributes to the production of a cancer. Oncogenes are generally mutated forms of normal cellular genes.

PCR

Polymerase chain reaction- a method for amplifying specific DNA segments which exploits certain

features of DNA replication.

Pluripotent

Able to develop and differentiate into any of a large number of cell types. For example, pluripotent cells can become skin, blood, nerves, sperm or muscle in the right conditions.

Southern Blot

Transfer of electrophoretically separated fragments of DNA from the gel to an absorbent sheet such as paper. This sheet is then immersed in a solution containing a labeled probe that will bind to a fragment of interest.

Trans orientation

LoxP sites are in TRANS if they all have the same directionality.

Vector

In cloning, the plasmid or phage chromosome used to carry the cloned DNA segment.

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Additional Reading

Books:

- Manipulating the Mouse Embryo: A Laboratory Manual (Third Edition) By Andras Nagy, Marina Gertsenstein, Kristina Vintersten & Richard Behringer. © 2003 764 pp. (ISBN 0-87969-591-9) Available from Cold Spring Harbor Laboratory.
- Cre/ loxP Recombination System and Gene Targeting: Transgenesis Techniques, Principles and Protocols, Second Edition. Ralf Kühn & Raul M. Torres 2002. pps. 175-204 (ISBN 1-59259-178-7) This is a chapter of an eBook available at [Humana Press](#)
- Knockout Mice fact sheet at the NIH National Human Genome Research Institute at <http://www.genome.gov/12514551#1>

Reviews:

- Muller U. 1999. Ten years of gene targeting: targeted mouse mutants, from vector design to phenotype analysis. Mech Dev. 82:3-21. [PubMed](#)
- Nagy A. 2000. Cre recombinase: the universal reagent for genome tailoring. Genesis. 26:99-09. [PubMed](#)

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