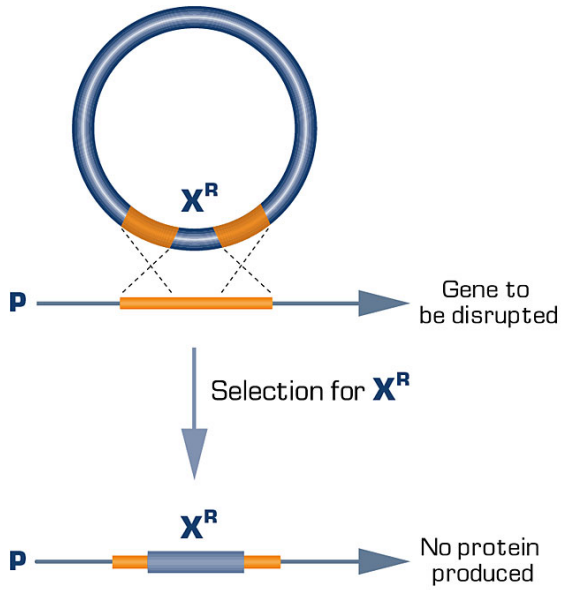
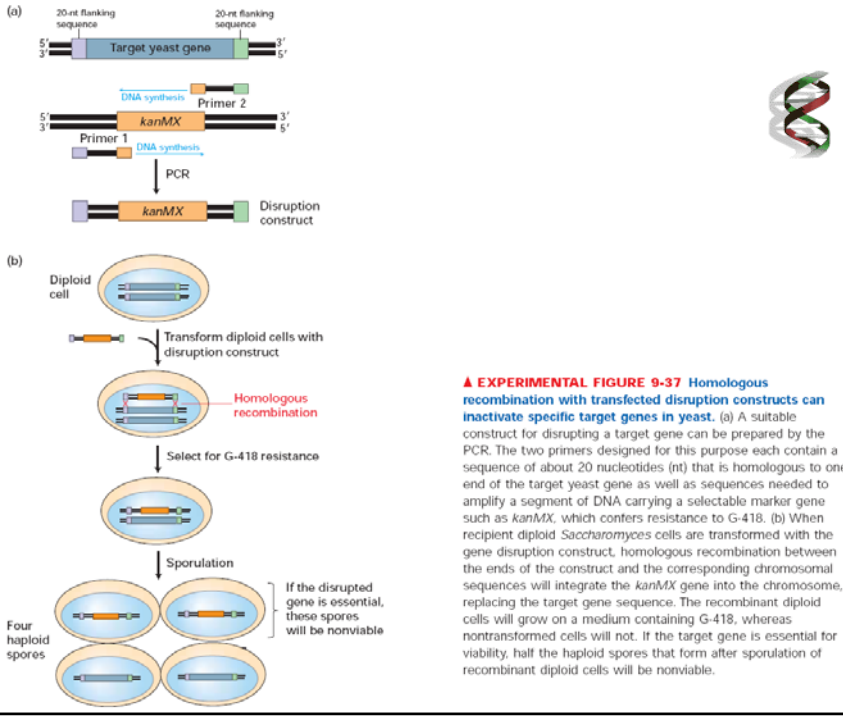
Aplicaciones biotecnológicas de la recombinación homóloga:
"gene targeting"
"gene knock out"





▲ **EXPERIMENTAL FIGURE 9-37 Homologous recombination with transfected disruption constructs can inactivate specific target genes in yeast.** (a) A suitable construct for disrupting a target gene can be prepared by the PCR. The two primers designed for this purpose each contain a sequence of about 20 nucleotides (nt) that is homologous to one end of the target yeast gene as well as sequences needed to amplify a segment of DNA carrying a selectable marker gene such as *kanMX*, which confers resistance to G-418. (b) When recipient diploid *Saccharomyces* cells are transformed with the gene disruption construct, homologous recombination between the ends of the construct and the corresponding chromosomal sequences will integrate the *kanMX* gene into the chromosome, replacing the target gene sequence. The recombinant diploid cells will grow on a medium containing G-418, whereas nontransformed cells will not. If the target gene is essential for viability, half the haploid spores that form after sporulation of recombinant diploid cells will be nonviable.

Aplicaciones de la recombinación homóloga:

"gene targeting"

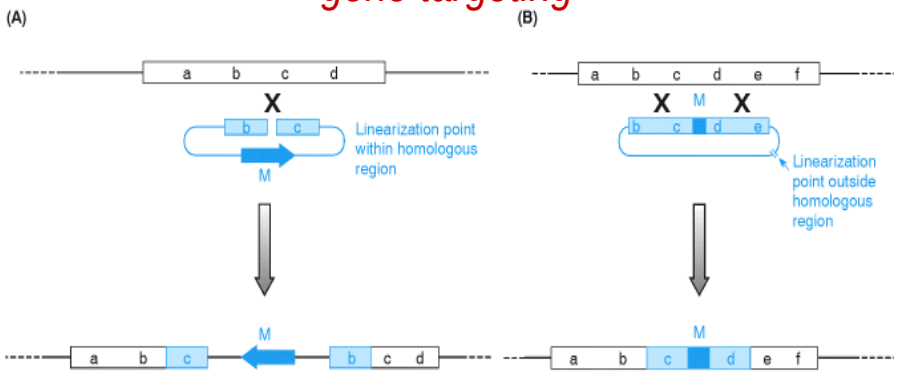


Figure 21.4. Gene targeting by homologous recombination can inactivate a predetermined chromosomal gene within an intact cell. (A) Insertion vector method. The introduced vector DNA (blue) is cut at a unique site within a sequence which is identical or closely related to part of a chromosomal gene (black). Homologous recombination (X) can occur, leading to integration of the entire vector sequence including the marker gene (M). *Note* that the letters do not represent exons but are simply meant to indicate linear order within the gene. (B) Replacement vector method. In this case, the marker gene is contained within the sequence homologous to the endogenous gene, and the vector is cut at a unique location outside the homologous sequence. A double recombination or gene conversion event (X X) can result in replacement of internal sequences within the chromosomal gene by homologous sequences from the vector, including the marker gene.

Doble reemplazo

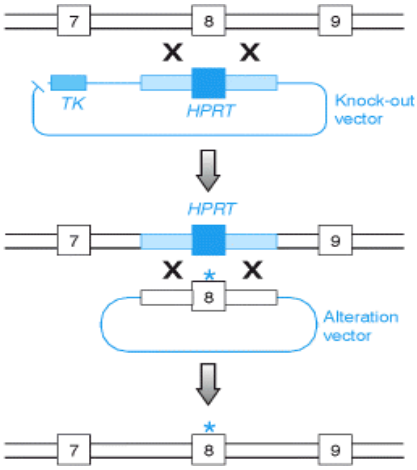


Figure 21.5. Double replacement gene targeting can be used to introduce subtle mutations. Both the methods in *Figure 21.4* result in introduction of a substantial amount of exogenous sequence within the endogenous gene. To introduce a subtle mutation without leaving residual exogenous sequence, a double replacement method with positive and negative selection can be used (Melton, 1994). Exons in the endogenous gene are represented as numbered large boxes, and introns as long thin boxes. In order to introduce a subtle mutation, such as a single nucleotide substitution in exon 8, a replacement knock-out vector is used with a marker gene (e.g. the *HPRT* gene) flanked by homologous sequences from introns 7 and 8, and a second marker, such as the herpes simplex thymidine kinase (*TK*) gene outside the homologous region. Gene conversion, or double crossover within the flanking intron sequences, can lead to replacement of exon 8 by the *HPRT* gene, and can be selected for if a mutant *HPRT*$^-$ ES cell is used. A positive-negative selection system can be used: selection in the first step is for *HPRT*$^+$*TK*$^-$ cells. Cells containing random vector integrations will contain the *TK* gene and can be killed with the thymidine analog *gancyclovir* (see *Figure 22.13*). The second replacement involves introducing an altered exon 8 with a point mutation (*) to replace the *HPRT* gene and can be screened by identifying *HPRT*$^-$ cells. *Note* that mice engineered in this way cannot be described as transgenic because of the lack of foreign sequences in the germline.

Selección positiva-negativa

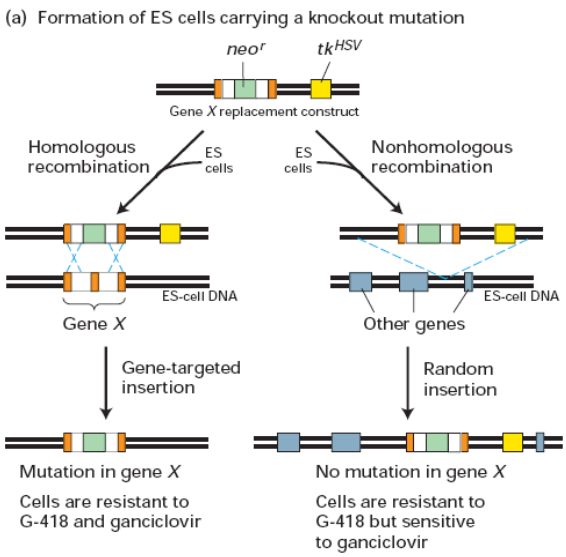
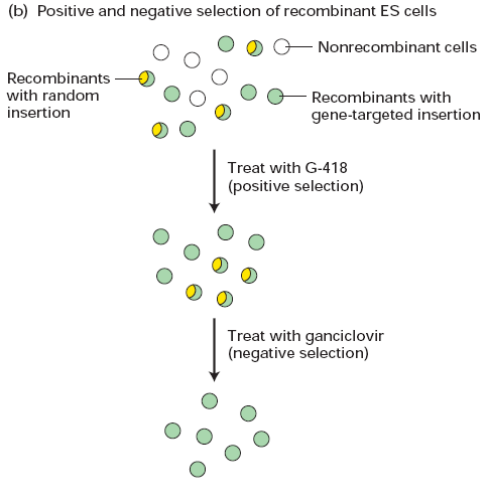
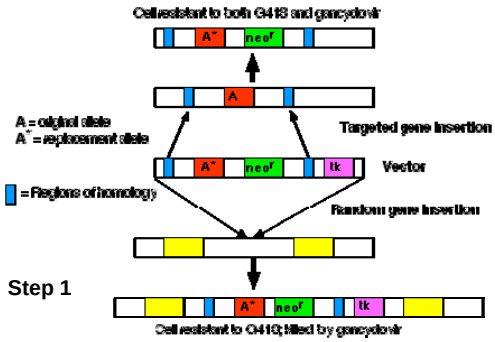


Figure 8-33. Isolation of mouse ES cells with a gene-targeted disruption by positive and negative selection. (a) When exogenous DNA is introduced into ES cells, random insertion via nonhomologous recombination occurs much more frequently than gene-targeted insertion via homologous recombination. Recombinant cells in which one copy of the gene X (orange) is disrupted can be obtained by using a recombinant vector that carries gene X disrupted with neo^r (light red), a neomycin-resistance gene, and, outside the region of homology, tk^{HSV} (purple), the thymidine kinase gene from herpes simplex virus. The viral thymidine kinase, unlike the endogenous mouse enzyme, can convert the nucleotide analog ganciclovir into the monophosphate form; this is then modified to the triphosphate form, which inhibits cellular DNA replication in ES cells. Thus ganciclovir is cytotoxic for recombinant ES cells carrying the tk^{HSV} gene. Nonhomologous insertion includes the tk^{HSV} gene, whereas homologous insertion doesn't; therefore, only cells with nonhomologous insertion are sensitive to ganciclovir. (Lodish)



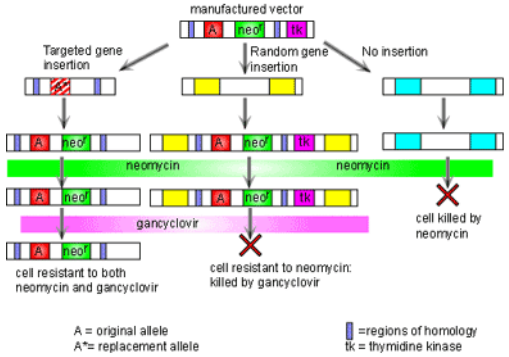
▶ **EXPERIMENTAL FIGURE 9-38 Isolation of mouse ES cells with a gene-targeted disruption is the first stage in production of knockout mice.** (a) When exogenous DNA is introduced into embryonic stem (ES) cells, random insertion via nonhomologous recombination occurs much more frequently than gene-targeted insertion via homologous recombination. Recombinant cells in which one allele of gene *X* (orange and white) is disrupted can be obtained by using a recombinant vector that carries gene *X* disrupted with *neo*^{r} (green), which confers resistance to G-418, and, outside the region of homology, tk^{HSV} (yellow), the thymidine kinase gene from herpes simplex virus. The viral thymidine kinase, unlike the endogenous mouse enzyme, can convert the nucleotide analog ganciclovir into the monophosphate form; this is then modified to the triphosphate form, which inhibits cellular DNA replication in ES cells. Thus ganciclovir is cytotoxic for recombinant ES cells carrying the tk^{HSV} gene. Nonhomologous insertion includes the tk^{HSV} gene, whereas homologous insertion does not; therefore, only cells with nonhomologous insertion are sensitive to ganciclovir. (b) Recombinant cells are selected by treatment with G-418, since cells that fail to pick up DNA or integrate it into their genome are sensitive to this cytotoxic compound. The surviving recombinant cells are treated with ganciclovir. Only cells with a targeted disruption in gene *X*, and therefore lacking the tk^{HSV} gene, will survive. [See S. L. Mansour et al., 1988, *Nature* **336**:348.]

Selección positiva-negativa

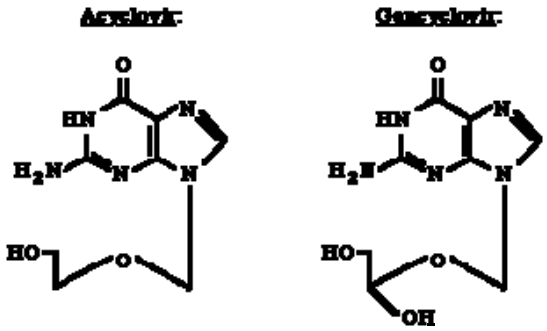


http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/T/TransgenicAnimals.html#Cre_loxP

"gene targeting": selección positiva-negativa

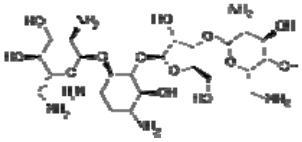


Construction of a vector. The vector consists of two homologous regions which have complementary strands of DNA on either side of the targeted gene in the genome. The disrupted gene along with a gene coding for neomycin resistance are included between the homologous regions. A thymidine kinase gene is included outside the homologous region. Once the vector is introduced into the host cell, it can either be incorporated into the genome by homologous recombination (the desired result), undergo complete insertion into the genome at an undesirable point or not be taken up by the cell at all. Neomycin resistance is used to select for cells in which the vector has been taken up and then gancyclovir sensitivity is used to select for cells which have undergone recombination. http://www.scq.ubc.ca/?p=264
http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/T/TransgenicAnimals.html#Cre_loxP



Nucleoside analogues are in fact **pro-drugs**, since they need to be phosphorylated before becoming effective.
This is the key to their selectivity:
•**Acyclovir** is phosphorylated by **HSV *tk*** 200 times more efficiently than by cellular enzymes. The cell DNA polymerase is less sensitive to it than the viral DNA polymerase.
•**Gancyclovir** is 10 times more effective against CMV than acyclovir since it is specifically phosphorylated by a **CMV-encoded kinase** encoded by gene *UL97* :

Neomycin



neomycin-resistance gene (*neo*^{R})

neomycin phosphotransferase

amino-glycoside phosphotransferase

Geneticin
G418

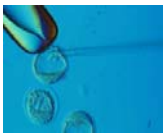
aminoglicósido análogo a la neomicina: inhibe la síntesis de proteínas en eucariotas (es fosforilado por el producto del gen *neo*^{R})

Similar to other **aminoglycosides**, **neomycin** is a broad spectrum antibiotic effective against both Gram positive and Gram negative bacteria. **It interferes with protein synthesis** in sensitive bacterial cells such as species of *Proteus* and *Staphylococcus.* Neomycin is mainly used topically in the treatment of skin and mucous membrane infections, wounds, and burns. Although it is also used systemically, it is highly toxic. Neomycin was first isolated in 1949 by the American microbiologist Selman Waksman from a strain of the bacterial species *Streptomyces fradiae.*

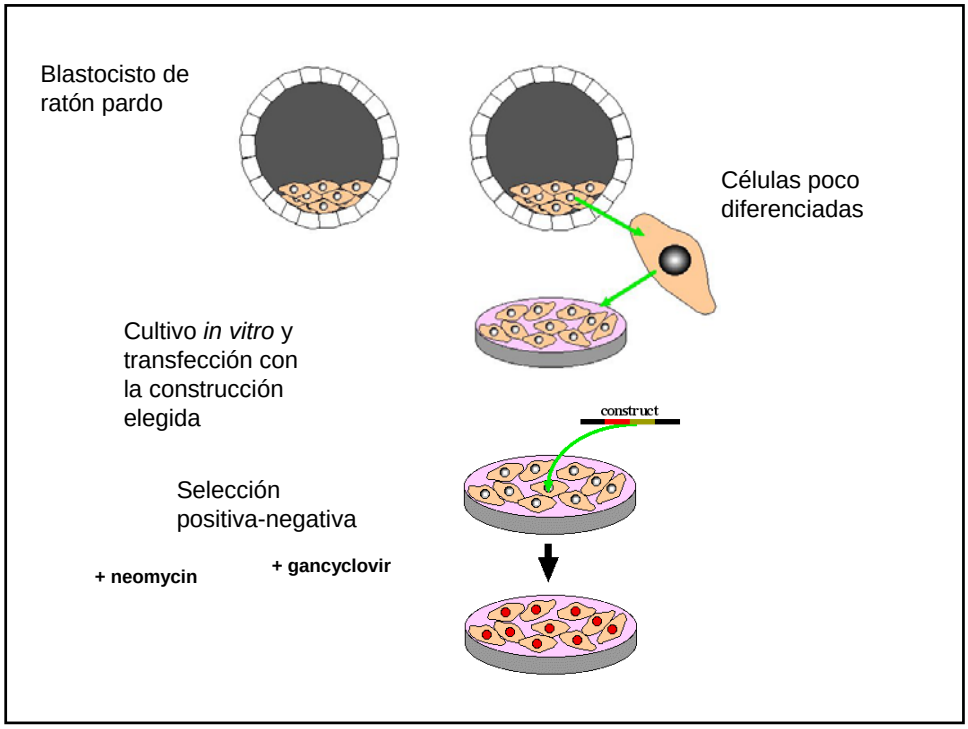
The first knockout mouse

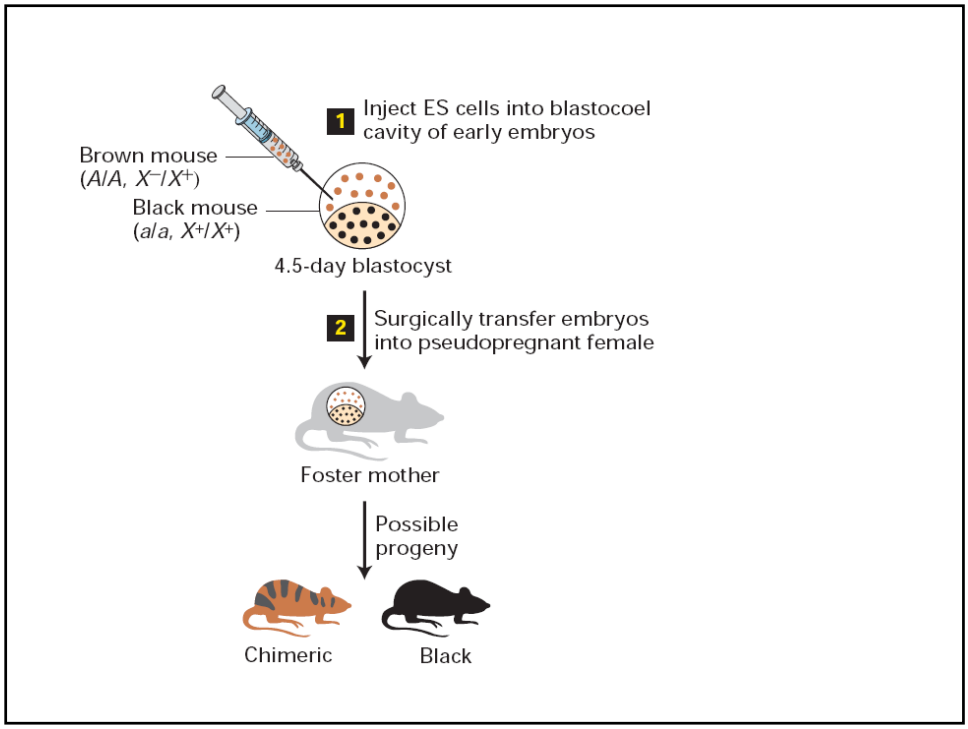
was created by Mario R. Capecchi, Martin Evans and Oliver Smithies in 1989

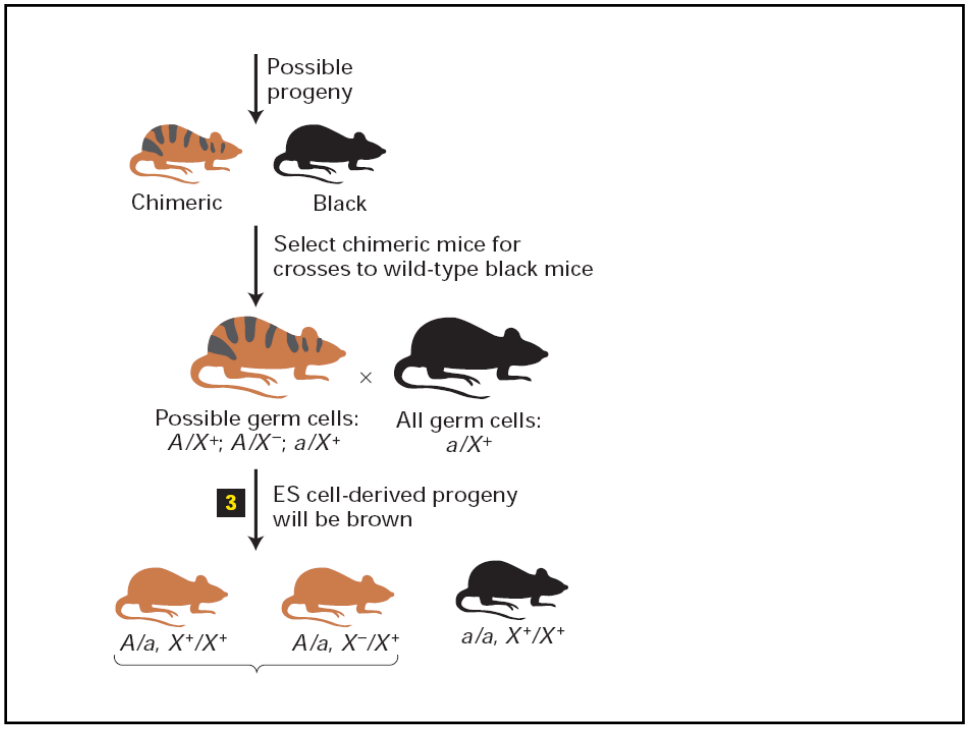
Nobel Prize for Medicine in 2007

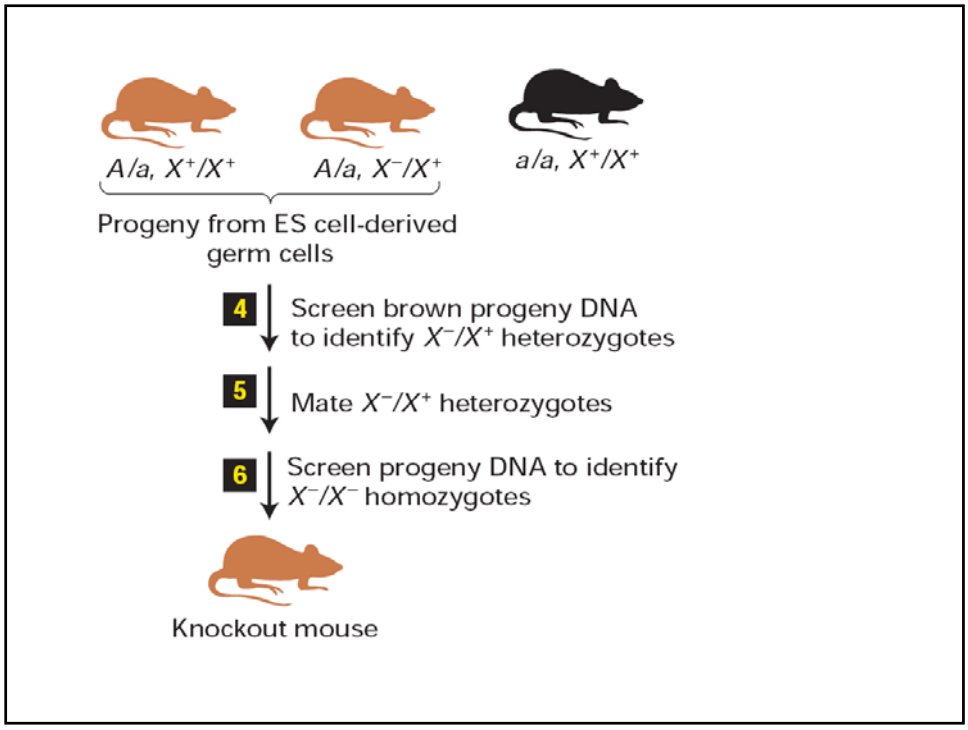


http://cancer.ucsd.edu/Research/Shared/tgm/genetargeting.asp


Blastocisto de ratón pardo
Células poco diferenciadas
Cultivo *in vitro* y transfección con la construcción elegida
construct
Selección positiva-negativa
+ neomycin
+ gancyclovir


1
Inject ES cells into blastocoel cavity of early embryos
Brown mouse (A/A, X⁻/X⁺)
Black mouse (a/a, X⁺/X⁺)
4.5-day blastocyst
2
Surgically transfer embryos into pseudopregnant female
Foster mother
Possible progeny
Chimeric
Black


Possible progeny
Chimeric
Black
Select chimeric mice for crosses to wild-type black mice
Possible germ cells: A/X+; A/X−; a/X+
×
All germ cells: a/X+
3
ES cell-derived progeny will be brown
A/a, X+/X+
A/a, X−/X+
a/a, X+/X+


A/a, X+/X+
A/a, X−/X+
a/a, X+/X+
Progeny from ES cell-derived germ cells
4
Screen brown progeny DNA to identify X−/X+ heterozygotes
5
Mate X−/X+ heterozygotes
6
Screen progeny DNA to identify X−/X− homozygotes
Knockout mouse