Overview

Obtaining or Generating Gene Mutations in Mice

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The starting point in a mutational analysis of gene function is obtaining or producing a mutant. Here different methods of obtaining mouse mutants are discussed, including screening for spontaneous mutants, screening for mutants following chemical or X-ray mutagenesis, and producing mutations through targeted manipulation of the genome. Manipulation of the genome can be random, as in different types of insertional mutagenesis. Alternatively, targeted manipulation such as gene targeting using homologous recombination in embryonic stem (ES) cells or gene editing by CRISPR–Cas can be used to produce custom mutations in a specific gene. The basic methods are outlined, and the advantages and disadvantages of homologous recombination and CRISPR–Cas gene editing are discussed. Resources for obtaining mutations that already exist are provided. If, for your planned study, no suitable mutations are available, there is advice about what you should know about your gene of interest before embarking on a gene targeting experiment.

INTRODUCTION

OBTAIN OR GENERATE A MOUSE MUTATION

- EXISTING MUTATION → Papaioannou and Behringer 2023d
- HR OR CRISPR–Cas IN ES CELLS → Papaioannou and Behringer 2023a
- CRISPR–Cas IN ZYGOTES → Papaioannou and Behringer 2023a

A variety of ways are available to obtain mutations in mice. One way is simply to wait for spontaneous mutations to arise. But this can be a tedious pastime, and the mutations are unpredictable to say the least. Thus, various other methods have been devised either to speed up the process or to make it more predictable. The early articles in this collection (see http://cshprotocols.cshlp.org/site/mousephenotypes) concentrate on mutations produced by gene targeting either (1) by homologous recombination in embryonic stem (ES) cells followed by germline transmission from ES cell chimeras, or (2) by CRISPR–Cas-mediated gene editing directly in preimplantation embryos. We will discuss the advantages and disadvantages of each approach. However, the method of phenotypic analysis pre-

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From the Mouse Phenotypes collection by Virginia E. Papaioannou and Richard R. Behringer.

Cite this overview as *Cold Spring Harb Protoc*; doi:10.1101/pdb.over107956

sented in later articles in this collection is by no means limited to this category of mutations created by targeted mutagenesis, but is equally applicable to mutants generated or obtained in other ways.

VARIOUS WAYS TO OBTAIN OR GENERATE MUTANTS

Spontaneous Mutations

The spontaneous mutation rate in mice is one gamete in approximately 100,000 for a specific gene and, of course, the identification of spontaneous mutations is biased toward mutant phenotypes that can be detected visually (e.g., coat color, gross morphology, or behavior). Attempting a genetic screen for spontaneous mutations in mice is thus logistically impractical. However, in addition to targeted mutagenesis, there are many other ways to induce mutations in mice. These methods can be grouped into broad categories, including chemically induced mutations, radiation-induced deletions, insertional mutations, and transgene expression. With these methods, mutations in specific genes of interest can be either selected or directed.

Chemical Mutagenesis Using Ethylnitrosourea

Chemical mutagens can greatly increase the mutation frequency, making genetic screens in mice highly feasible. Numerous groups around the world have performed small- and large-scale chemical mutagenesis screens in mice to isolate dominant and recessive mutations (e.g., Kasarskis et al. 1998; Hrabe de Angelis et al. 2000; Kile et al. 2003). The most powerful chemical mutagen in mice is ethylnitrosourea (ENU). ENU induces an average per-locus mutation frequency of 1.5×10^{-3}. A mutation in a single gene of choice can be recovered on average in 1 in 600–700 gametes screened. To date, every ENU-induced mutation that has been sequenced in the mouse is a point mutation, and, because ENU causes point mutations, it defines single-gene function. ENU creates nonsense, missense, splice, and regulatory mutations that can affect gene, RNA, and protein function; thus, null, neomorph, antimorph, hypomorph, and hypermorph mutations can be isolated. The advantages of ENU mutagenesis are the rich variety of alleles generated, the possibility of identifying mutations by phenotype during the screen, and the fact that many mutations can be generated and isolated using simple methods. The primary disadvantages are the labor and mouse space required to perform a screen and the effort required to map and identify the molecular lesions.

Typically, adult males of a particular inbred strain are injected intraperitoneally with a precise dose of ENU to mutagenize spermatogonial stem cells. ENU treatment causes a depletion of mature germ cells, resulting in transient sterility. The spermatogonial stem cells are spared but their DNA acquires numerous point mutations throughout the genome depending on the dose of ENU administered. The spermatogonial stem cells then undergo spermatogenesis to produce sperm. The ENU-injected males usually regain fertility 11–17 wk post-ENU treatment and are then bred using different breeding schemes designed to generate pedigrees for phenotypic analysis to isolate mutations. A well-designed phenotypic screen of the progeny will identify mutations affecting specific structures or processes of interest (Cordes 2005). Mice with fully penetrant dominant mutations that are viable and fertile are easy to genotype because the dominant phenotype can be used to identify the mutants. However, mice heterozygous for recessive ENU-induced mutations can initially be difficult to genotype because the gene and chromosomal location are usually unknown. In these cases, one must perform test crosses to identify carriers (Fig. 1). Genetic mapping eventually leads to the identification of the chromosomal location of the mutation. Subsequently, linked polymorphic markers can be used to track the mutation.

Advances in genome sequencing and bioinformatic tools (e.g., exome sequencing) have greatly facilitated the identification of ENU-induced mutations (Buchovecky et al. 2013; Geister et al. 2018). Ultimately, the ENU mutation is identified, and, if you are fortunate, it may cause a restriction enzyme polymorphism that can be exploited in a polymerase chain reaction (PCR) genotyping strategy. Alternatively, if there is no restriction enzyme polymorphism, single-stranded conformation polymorphism or other strategies can be used for genotyping.

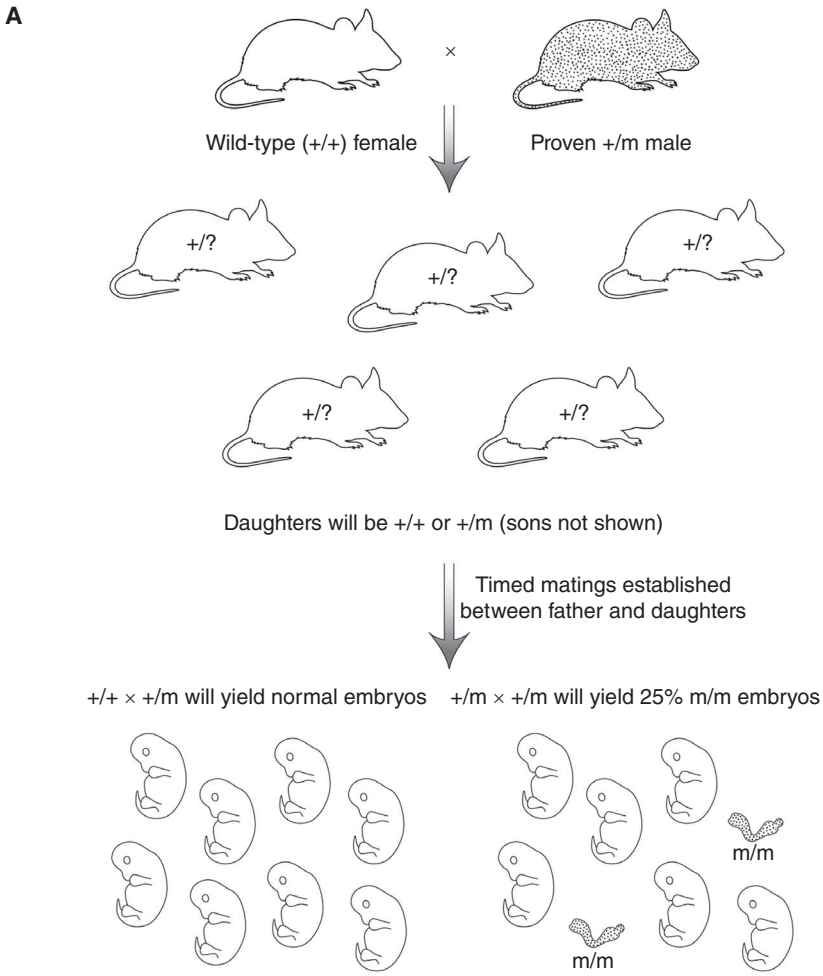

FIGURE 1. Test crosses for recessive ethylnitrosourea (ENU)-induced mutations. Spontaneous and ENU-induced mutations may not be molecularly defined. Therefore, genetic crosses must be performed to identify mice that carry the mutation. Two situations are shown. (*A*) A male known to be heterozygous for a recessive embryonic lethal mutation is bred with wild-type females to generate progeny. The daughters from this cross will be +/+ or +/m. To obtain homozygous mutant embryos for analysis, timed matings are established between the male carrier and his daughters for dissection at time points chosen to yield the mutant phenotype. Approximately 50% of the time the cross will be between a +/+ daughter and the +/m father that will yield +/+ and +/m embryos that will have a wild-type phenotype, and 50% of the time the cross will be between a +/m daughter and the +/m father that will yield +/+, +/m, and m/m embryos. Therefore, ~25% of the embryos from this cross should show the mutant phenotype. If new heterozygous males are needed, then the sons from a known carrier are test crossed with their siblings to distinguish +/+ and +/m males. (*B*) A male known to be heterozygous for a recessive viable but sterile mutation is bred with wild-type females to generate progeny. The offspring from this cross will be +/+ or +/m. To obtain homozygous mutants for analysis, matings are established at random between the siblings. There is a 1 in 4 chance that you have set up a mating between two +/m mice. Once progeny are born they can be examined for the mutant phenotype. Those matings yielding homozygous mutants provide the genetic evidence that both of the parents are heterozygous carriers. (Modified, with permission, from Papaioannou and Behringer 2005, © Cold Spring Harbor Laboratory Press.) (*Figure continued on following page.*)

As a community resource, ENU-mutagenized sperm and corresponding genomic DNA have been cryopreserved from mice (Sakuraba et al. 2005) and a database of random ENU-induced point mutations in specific genes with mutation–phenotype associations has been created (https://mutagenetix.utsouthwestern.edu) (Wang et al. 2018). In vitro fertilization using the cryopreserved sperm can be used to recover the allele of interest in mice. ES cells have also been treated with ENU to create random mutations (Chen et al. 2000). ES cells containing desired mutations can be used to generate mutant mice via germline transmission through chimeras (Vivian et al. 2002).

Cite this overview as *Cold Spring Harb Protoc*; doi:10.1101/pdb.over107956

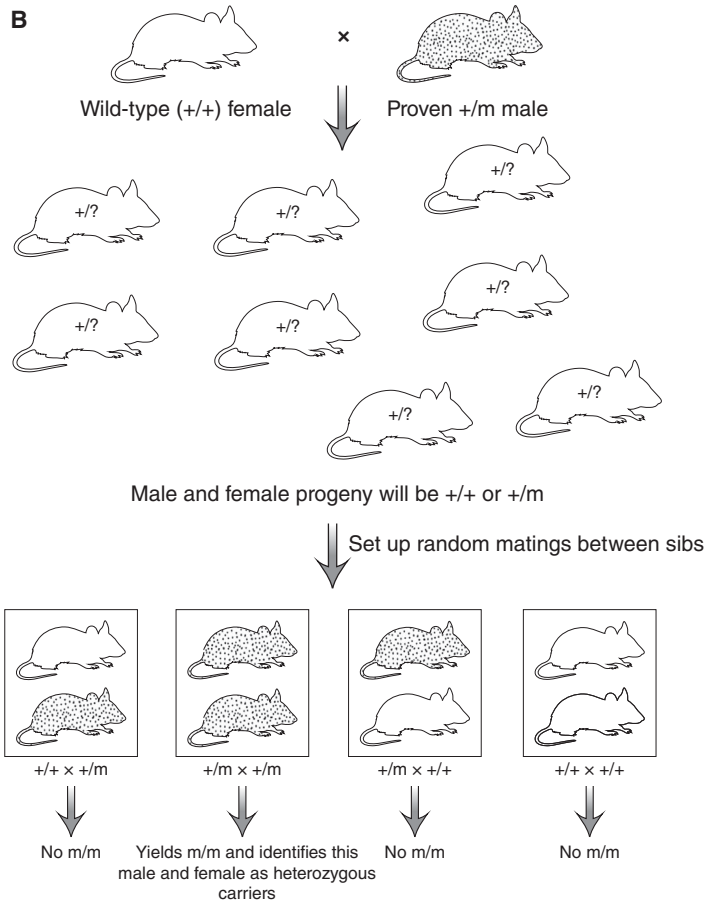

FIGURE 1. *Continued from previous page.*

X-Ray Mutagenesis

Exposing mice to X-rays causes large genetic deletions in germ cells that can be transmitted to progeny. The mutagenized mice are bred to mice with appropriate genetic markers to identify the deletion alleles. Classically, this approach has been used to generate deletion complexes, which usually include multiple loci. One well-characterized deletion complex is the albino deletion complex, a series of overlapping deletions that include the albino (*Tyr*) locus (Holdener and Magnuson 1994). These deletions were identified by test crosses with mice that are homozygous for the recessive albino allele (Tyr^c). The presence of white mice in the progeny indicated that the albino locus was deleted in the germ cells of the X-ray-treated parent. By combining various deletions, one can define loci that affect diverse developmental processes. These genetic resources tend to be used primarily for gene discovery rather than as primary tools for understanding the function of one particular gene.

X-rays have also been used in ES cells to create a series of cM-sized deletions at a specific locus (Thomas et al. 1998). This approach can be used to assess large regions of the genome and also regions of haploinsufficiency.

Insertional Mutagenesis

When a piece of exogenous DNA integrates into the genome, it can physically disrupt an endogenous gene or alter its expression at the level of transcription or RNA splicing. This is called an insertional mutation. Insertional mutations can result in various types of alleles but most frequently result in a

loss of function. The exogenous DNA can be delivered into cells by viruses or as purified DNA molecules. Below we discuss various types of insertional mutations in the mouse.

Viruses

Viruses can be used to infect preimplantation-stage mouse embryos or ES cells to cause insertional mutations (Jaenisch et al. 1983; Robertson et al. 1986). The viral sequence can then be used as a molecular tag to follow proviral genome inserts in pedigrees and ultimately to clone the mutated genes. Viruses can also be modified for specific mutagenesis screens (e.g., gene traps; Friedrich and Soriano 1991).

Transgenes

Insertional mutagenesis is one of the by-products of generating transgenic mice by pronuclear injection of DNA into zygotes. It is estimated that ~5%–10% of random integrations of foreign DNA into the genome fortuitously disrupt an endogenous gene. The transgene can serve as a molecular tag to clone the disrupted gene(s). One of the problems with transgene insertional mutations is that they can be associated with large deletions, duplications, inversions, and translocations. It is thus not always clear if the mutant phenotype is caused by one or multiple gene disruptions. If chromosomal rearrangements are present at the site of transgene integration, molecular cloning of the gene responsible for the mutant phenotype can be challenging. Another potential complication is that the expression of the transgene itself may confuse the analysis of the insertional mutant phenotype.

Gene Traps

Gene traps are DNA or viral vectors typically introduced into ES cells by electroporation or retroviral infection to generate insertional mutations that disrupt the function of endogenous genes (Robertson et al. 1986; Gossler et al. 1989; Friedrich and Soriano 1991; Stanford et al. 2001). Gene trap vectors are engineered to include reporters like *lacZ*, which in principle allow you to follow the expression pattern of the trapped locus. ES cell lines with gene trap insertions can be prescreened for reporter expression patterns or to identify the trapped genes. Ultimately, mice generated from ES cell lines with gene traps can then be screened for mutant phenotypes. Because gene traps are essentially random insertional mutations, the resulting mutant alleles can have diverse activity. It is easy to genotype heterozygous mice for gene trap insertions by virtue of the reporter activity, but identification of homozygous mutant mice is challenging until the locus is cloned. Libraries of ES cell lines with gene trap insertions and associated sequence tags have been generated and are available from the International Gene Trap Consortium (https://igtc.org/), which has integrated the publicly available gene trap lines from a variety of different large-scale screens (Nord et al. 2006; Guan et al. 2010). These resources can facilitate the generation of a mutant for a specific gene by allowing you to acquire the ES cell clone and generate mice from ES cell chimeras.

Transposons

Transposons are another class of insertional mutagens that can be used in mice to generate mutant phenotypes (Horie et al. 2003). *Sleeping Beauty* (*SB*) is a Tc1/mariner-type transposon resurrected from salmonid fish and developed as a transposon mutagenesis system in mice. The SB transposase specifically recognizes *SB* transposon inverted repeats. The *SB* transposon is initially introduced into the mouse genome as a transgene either by pronuclear injection or through transfection of ES cells. Transgenic male mice carrying the *SB* transgene are then bred with transgenic mice that express the SB transposase in the germline. The resulting double transgenic mice are then bred with wild-type mice, and mobilization of the transposable element is monitored in the next generation. The SB transposase causes the transposition of the *SB* transposon through a precise cut-and-paste mechanism to insert at TA dinucleotides. This transposition can occur *in cis* locally on the same chromosome or *in trans* to other chromosomes.

Cite this overview as *Cold Spring Harb Protoc*; doi:10.1101/pdb.over107956

The *piggyBac* (*PB*) transposon system has also been adapted for use in mice (Ding et al. 2005). *PB* transposons insert at TTAA tetranucleotides, which are duplicated upon insertion. One advantage of transposon mutagenesis systems is that the mice create additional mutations simply by breeding.

Transgenic Expression

Finally, transgenic mice generated by various methods are frequently created to express a foreign gene product to elicit a mutant phenotype (e.g., *Gh*; Palmiter et al. 1982; Schilit et al. 2016). These are typically gain-of-function experiments for the intentional misexpression or overexpression of a gene in a specific tissue or many tissues and are different from transgene-induced insertional mutations, mentioned in the previous section, that randomly disrupt endogenous genes. By design, a tissue-specific or broadly expressed promoter may have been used to direct the expression of the gene. If a tissue-specific regulatory sequence was used, then one already knows which tissues to examine first for a phenotype in the transgenic mouse. However, sometimes the resulting phenotypes are not as simple as expected and a more comprehensive analysis will be necessary.

OBTAINING AN EXISTING MUTANT

There are numerous repositories that archive mutations either as live mice or cryopreserved preimplantation embryos or sperm. Therefore, there may already be a mutation in your gene of interest and no need to generate a new mutant. One of the first places to check is Mouse Genome Informatics (MGI; www.informatics.jax.org). Search for your gene using the official gene name, then on the "Gene Detail" page check under "Mutations, Alleles and Phenotypes" to learn what alleles have been reported. Then look under "Find Mice (IMSR)," which takes you to the International Mouse Strain Resource (www.findmice.org). There you will see if live mice, embryos, and/or sperm are available from specific repositories located around the world with links to references and ordering information.

In addition to the gene-trap screens in ES cells mentioned in the previous section, there have also been extensive international efforts to produce mutations in every gene in the mouse genome, making them available through publicly funded repositories. The National Institutes of Health (NIH)-funded Knockout Mouse Project (KOMP) initiated this ambitious mutagenesis project using ES cell technologies to produce knockout or conditional mutations. The European Conditional Mouse Mutagenesis Program (EUCOMM) and the North American Conditional Mouse Mutagenesis project (NorCOMM), using a combination of gene targeting and gene trapping in ES cells, also had the goal of mutating large numbers of genes. In 2007 these three programs, along with the Texas A&M Institute for Genomic Medicine (TIGM), joined forces to minimize overlap and share resources, creating the International Knockout Mouse Consortium (IKMC). By 2012, more than 17,000 different genes had been mutated and multiple mouse lines had been produced (Lloyd 2011; Bradley et al. 2012).

Subsequently, a high-throughput functional genomics project coordinated by the International Mouse Phenotyping Consortium (IMPC; https://mousephenotype.org) has validated and phenotyped a large number of mouse lines (Brown and Moore 2012). By 2021, the number of targeted knockout (null) mutations in mice made by the scientific community and the IMPC totaled more than 11,000 unique genes, over half the mouse genome. Many of these mutations include reporters and a large number are conditional-ready alleles. Through the IMPC, more than 5000 mutant mouse lines, mostly on the C57BL/6N strain background, have been expanded for phenotyping using standardized protocols (Birling et al. 2021). The adult phenotyping pipeline includes a battery of behavioral, metabolic, physiological, and morphometric tests, both live and terminal. For embryonic lethal mutations, which account for more than one-third of mutations, an embryonic pipeline includes assessment of the time of death and micro-computed tomography (microCT) 3D imaging at key developmental stages. Overall, 75% of lines tested have at least one phenotype and many show pleiotropy. Nearly 20% show sexually dimorphic phenotypes. Thus, you may find mutant ES cells, live mice, and/or cryopreserved preimplantation embryos and sperm available from public repositories, many with extensive phenotypic data.

It is very costly for a repository to keep mutant mice "on the shelf" as live animals. Thus, most strains are cryopreserved as preimplantation embryos or sperm. Once you purchase a mutant from a repository, the mutant will likely have to be recovered from the cryoarchive. This is costly and time-consuming; typically, you will receive mice in 2–4 mo. Alternatively, you or your institution's mouse core facility could purchase frozen preimplantation embryos for thawing and embryo transfer or frozen sperm for in vitro fertilization (IVF) and embryo transfer (Protocol: **Oviduct Transfer** [Nagy et al. 2006]; Protocol: **In Vitro Fertilization in Mice** [Takeo and Nakagata 2018]). This can also be costly but mice could be born after ~3 wk.

Another option to obtain a mutant mouse if it has been produced but is not archived in a repository is to do a literature search and contact the laboratory that generated the mutant. That information is also available in MGI. If they no longer have the mutant, they may have provided it to other laboratories that can be contacted. You can also search for recent papers that used the mutant and contact those laboratories. Another approach would be to use social media to ask if others have the mice. If a mutation is not available in your gene of interest or an existing mutation is not of the type that you want, you have the option of making a custom mutation.

GENE TARGETING FOR A CUSTOM MUTATION

Homologous Recombination in ES Cells or CRISPR–Cas Gene Editing?

Over the past three decades, the methods used to produce specific mutations in mice through homologous recombination in ES cells have been honed and improved such that they have become highly accurate, efficient, and almost routine, well within the reach of many laboratories or done as a service through companies or institutional core facilities. The pitfalls and limitations of the technology are well-researched and well-understood. In the past few years, CRISPR–Cas technology has exploded onto the scene, promising faster, simpler, and virtually unlimited possibilities for gene manipulation (Doudna and Mali 2016) directly in preimplantation embryos (Williams et al. 2016), although the technology can also be applied to stem cells in vitro (Smith et al. 2016). The technology is rapidly developing and improvements continue to refine the procedures.

So which method should you choose for your project? The answer to that will depend on your having a full understanding of the capabilities and limitations of the different methods and recognizing your own requirements and resources (Table 1). You may even find that a combination of the methods is the most suitable for your needs. For example, with CRISPR–Cas zygote gene editing, what you gain in time by directly editing the zygotic genome must be counterbalanced by the limitations on the size of inserts for knock-in alleles and the need to outcross founders because of mosaicism. If you choose to do the gene editing in ES cells in vitro, you will have the opportunity to select the desired allele whether you use homologous recombination or CRISPR–Cas editing. The trade-off here is that CRISPR–Cas produces more targeted clones than homologous recombination but homologous recombination has a higher fidelity, resulting in greater efficiency (Rezza et al. 2019). Your own resources may also be an important factor. Cost, availability of services, technical support, and efficiency all need to be considered. If your laboratory or institution has one or the other technology up and running with high efficiency, that may be what you should go with.

Gene Targeting by Homologous Recombination in ES Cells—the Basics

The complex procedures used for targeted gene mutation in the mouse by homologous recombination in ES cells can be broken down into five basic gene targeting steps.

1. Build a targeting construct specific to your favorite gene (Overview: **Mouse Gene-Targeting Strategies for Maximum Ease and Versatility** [Papaioannou and Behringer 2023a]).
2. Produce a mutation in ES cells by electroporating the construct that will target a change to one allele of this gene following homologous recombination (Overview: **Embryonic Stem Cell Gene Targeting and Chimera Production in Mice** [Papaioannou and Behringer 2023b]).

TABLE 1. Comparison of different gene-editing technologies

Gene manipulation method	Requires ES cell culture	Positive selection used	Negative selection used	Requires chimera production	Type of alleles generated	Knock-ins with small (<2 kb) inserts	Knock-ins with large (>2 kb) inserts	# alleles targeted per manipulation	# alleles transmitted from founder
Homologous recombination in ES cells[a]	Yes	Yes	Yes	Yes	Almost any	Yes	Yes	1	1
CRISPR–Cas editing in zygotes	No	No	No	No	Almost any	Yes	Currently challenging	1 or 2	Multiple

(ES) Embryonic stem.
[a]Includes CRISPR–Cas gene editing in ES cells.

3. Isolate clonal lines of correctly targeted ES cells and grow up a sufficient number for further experiments, freezing some for future use (Overview: **Embryonic Stem Cell Gene Targeting and Chimera Production in Mice** [Papaioannou and Behringer 2023b]).
4. Produce ES cell chimeras by injecting the correctly targeted ES cells into mouse blastocysts or by doing morula aggregation (Overview: **Embryonic Stem Cell Gene Targeting and Chimera Production in Mice** [Papaioannou and Behringer 2023b]).
5. Breed the male chimeras to recover offspring carrying the targeted mutation through germline transmission of the targeted allele (Overview: **Recovering a Targeted Mutation in Mice from Embryonic Stem Cell Chimeras or CRISPR–Cas Founders** [Papaioannou and Behringer 2023c]) and breed them to establish a mutant line (Overview: **Strategies for Maintaining Mouse Mutations** [Papaioannou and Behringer 2023d]).

CRISPR–Cas Gene Editing—the Basics

The procedures to generate targeted gene mutations in the mouse by gene editing depend on the type of allele you want to generate (null, point mutant, or knock-in). This approach can be used to generate most commonly desired mutations directly in preimplantation embryos. However, complex alleles could be generated first in ES cells by CRISPR–Cas gene editing because you could then screen for the desired allele before generating mice using chimeras. The following steps refer to gene editing in preimplantation embryos.

Steps to Make a CRISPR–Cas Null Allele

1. Choose the exon(s) you want to mutate and use online CRISPR–Cas design tool software (https://en.wikipedia.org/wiki/CRISPR/Cas_Tools) to identify a sequence to design an appropriate guide RNA (Overview: **Mouse Gene-Targeting Strategies for Maximum Ease and Versatility** [Papaioannou and Behringer 2023a]).
2. Inject zygotes in vitro or electroporate zygotes in vitro or in vivo with a single-guide RNA and Cas9 RNA or protein.
3. Transfer in vitro manipulated embryos into foster mothers.
4. Identify founders with insertions or deletions (indels) in your gene of interest.
5. Cross founders to segregate unique indel alleles (Overview: **Recovering a Targeted Mutation in Mice from Embryonic Stem Cell Chimeras or CRISPR–Cas Founders** [Papaioannou and Behringer 2023c]) and breed them to establish a mutant line (Overview: **Strategies for Maintaining Mouse Mutations** [Papaioannou and Behringer 2023d]).

Steps to Make a CRISPR–Cas Point Mutation Allele

1. Choose the exon in which you want to introduce a point mutation and use online CRISPR–Cas design tool software (https://en.wikipedia.org/wiki/CRISPR/Cas_Tools) to identify a sequence to design an appropriate guide RNA (Overview: **Mouse Gene-Targeting Strategies for Maximum Ease and Versatility** [Papaioannou and Behringer 2023a]).
2. Inject zygotes in vitro or electroporate zygotes in vitro or in vivo with a single-guide RNA, Cas9 RNA or protein, and an oligonucleotide homologous to your target sequence containing the desired point mutation.
3. Transfer in vitro manipulated embryos into foster mothers.
4. Identify founders with the desired point mutation.
5. Cross founders to segregate the desired point mutation from other targeted alleles (indels) (Overview: **Recovering a Targeted Mutation in Mice from Embryonic Stem Cell Chimeras or CRISPR–Cas Founders** [Papaioannou and Behringer 2023c]) and breed them to establish a mutant line (Overview: **Strategies for Maintaining Mouse Mutations** [Papaioannou and Behringer 2023d]).

Steps to Make a CRISPR–Cas Knock-In Allele

1. Use online CRISPR–Cas design tool software (https://en.wikipedia.org/wiki/CRISPR/Cas_Tools) to identify a sequence to design a guide RNA to induce a double-strand break in the region of the desired knock-in.
2. Build a targeting construct specific to your favorite gene (Overview: **Mouse Gene-Targeting Strategies for Maximum Ease and Versatility** [Papaioannou and Behringer 2023a]).
3. Inject zygotes in vitro or electroporate zygotes in vitro or in vivo with a single-guide RNA (or two guide RNAs to decrease off-target events), Cas9 RNA or protein, and the targeting vector DNA.
4. Transfer in vitro manipulated embryos into foster mothers.
5. Identify founders with the desired knock-in or conditional allele.
6. Cross founders to segregate the desired mutation from other targeted alleles (indels) (Overview: **Recovering a Targeted Mutation in Mice from Embryonic Stem Cell Chimeras or CRISPR–Cas Founders** [Papaioannou and Behringer 2023c]) and breed them to establish a mutant line (Overview: **Strategies for Maintaining Mouse Mutations** [Papaioannou and Behringer 2023d]).

PHENOTYPIC ANALYSIS

Whether you start with an existing mutation or have made one by gene targeting or editing, the final step, and ultimate goal, is the phenotypic analysis in heterozygous and/or homozygous mutant mice. The following articles in the collection deal with breeding mice to maintain the mutation and produce offspring for phenotypic analysis (Overview: **Strategies for Maintaining Mouse Mutations** [Papaioannou and Behringer 2023d]), and special techniques to use in phenotypic analysis (Overview: **Special Breeding Techniques for Use in Mouse Mutation Analysis** [Papaioannou and Behringer 2023e]). From there, the branching decision pathway (see Fig. 1 in Overview: **Strategies for the Production and Phenotypic Analysis of Mutations in the Mouse** [Papaioannou and Behringer 2023f]) leads you through a phenotypic analysis by first determining the earliest time of action of the mutation and then applying strategies and techniques suitable for analysis of different stages of embryonic development, postnatal development

or adulthood, including dominant and recessive mutations (see http://cshprotocols.cshlp.org/site/mousephenotypes).

Using chimeric mice as a phenotyping tool is discussed (Overview: **Getting around an Early Lethal Phenotype in Mice with Chimeras** [Papaioannou and Behringer 2023g]), and finally the pathway leads to the use of conditional alleles (Overview: **Tissue- and/or Temporal-Specific Mutations in Mice Using Conditional Alleles** [Papaioannou and Behringer 2023h]).

BEFORE YOU BEGIN

Armchair Biology

What Do You Want and What Do You Expect?

Before you embark on generating or obtaining a mutant, it is worth taking a moment to reflect on exactly what you expect from your gene-targeting experiment. Ideally, this goal was determined long before the desired allele was obtained or designed, but it is never too late to reconsider your approach. The decision to do gene targeting should not be taken lightly because it is an expensive and time-consuming procedure. Even if everything goes well and works the first time, it will be about 6 months from the time you have the finished targeting construct ready for electroporation into ES cells until you could possibly see a homozygous mutant animal. In our experience, a year is a realistic estimate and 2 years is not unusual. CRISPR–Cas gene editing should be quicker because it bypasses the ES cell tissue culture steps but still requires considerable time and effort. That being the case, a lot of thought and a few preliminary experiments could save considerable time and trouble.

First, plan the type of mutation that will be most informative and/or versatile. This will depend on the gene and its products, and how it functions. Overview: **Embryonic Stem Cell Gene Targeting and Chimera Production in Mice** (Papaioannou and Behringer 2023b) provides some pointers for making the most of the information that you have.

Second, try to predict the mutant phenotype. Although vast experience indicates that the only surefire way to determine the effect of a mutation is to make the mutation, this is not a thinking scientist's approach. There are prognosticators that can take some of the guesswork out of the prediction, thus guiding the experimental strategy. Foremost among these is the expression pattern of the gene. Stories circulate about expression patterns that seem to have nothing to do with the mutant phenotype, and mutations that have no overt phenotype despite extensive gene expression. But we maintain that these situations are the exceptions, if they exist at all, and that the absence of a mutant phenotype in an expressing tissue is more likely a reflection of our ignorance about how to look for it.

BOX 1. IMPRINTING AND X INACTIVATION: EFFECT ON EMBRYOS DUE TO PREFERENTIAL X INACTIVATION OF PATERNAL X CHROMOSOME IN EXTRAEMBRYONIC MEMBRANES

As a rule, X inactivation results from random inactivation of one or the other X chromosome so that females end up with a random mix of cells with either the paternally derived X or the maternally derived X inactive. However, there is an exception to the random inactivation rule in the embryo: There is preferential inactivation of the paternally derived X chromosome in the trophectoderm and primitive endoderm, and consequently in their cellular derivatives. This peculiarity of the embryo, which is a form of imprinting, usually has little effect on experimental work. However, if you have produced a mutation in an X-linked gene that is expressed in either of these tissue lineages (i.e., placental structures or yolk sac endoderm), there will be two classes of heterozygous females with respect to gene expression: one class that will be functionally null in the trophectoderm and primitive endoderm lineages because it inherits the mutant allele from the mother and the paternal chromosome is inactivated, and the other class that will be functionally wild-type because it inherits the mutant allele from the father and the maternal chromosome is active (see Fig. 2). For an example of analysis of this type of mutation, see *Esx1* (Li and Behringer 1998).

Get to Know Your Gene

Armchair biology can get you into the right frame of mind for dealing with the unexpected by anticipating some of the possibilities of a gene disruption. With the current amount of genome information available, it is possible to learn a lot about your gene that might bear on its function. The following is a checklist of what you should know about the gene to help you prepare before you begin.

- *What is the chromosomal location of your gene?* This is an important piece of information for many reasons. Is the gene X-linked? If so, males will be hemizygous for the mutation and effects might show up in XY ES cells, in chimeras, or in males, where there is only a single X chromosome. Heterozygous females, which will have one wild-type allele active in half their cells because of X inactivation, might escape this problem, unless the gene is expressed in the extraembryonic tissues that are subject to imprinting effects (see Box 1; Fig. 2). Alternatively, they might show mosaic

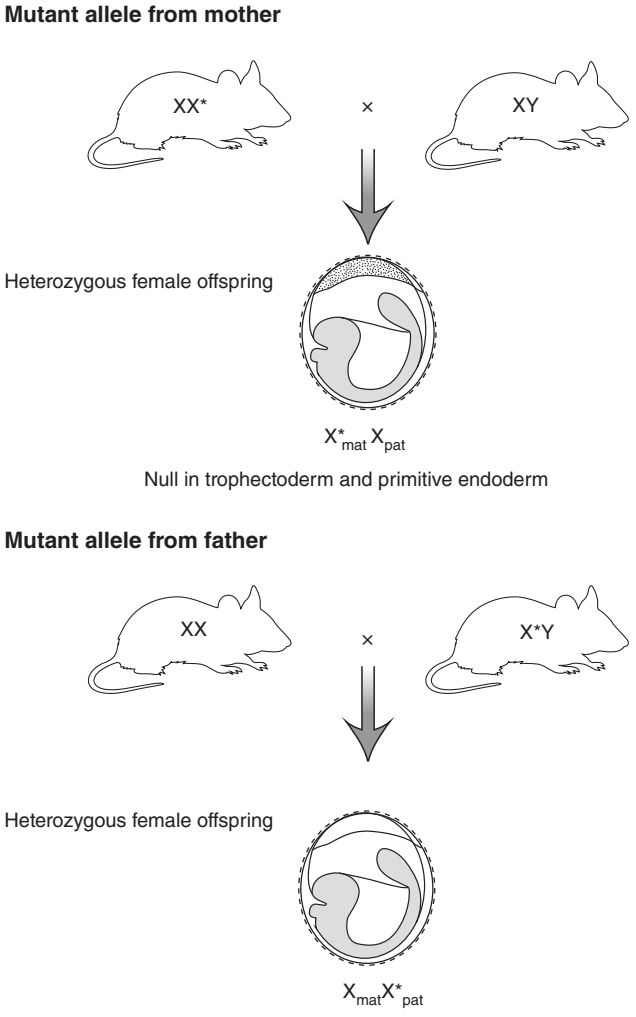

FIGURE 2. Imprinting of an X-linked mutant gene. If an X-linked mutant allele (*) is inherited from the mother (*top cross*), heterozygous female offspring are effectively null for the gene product in the trophectoderm and primitive endoderm lineages because the paternally derived X chromosome (X_{pat}) is preferentially inactivated and the maternally derived chromosome (X^*_{mat}) is active but carries the mutation. If the mutant allele is inherited from the father (*bottom cross*), heterozygous females are wild type for the gene in the extraembryonic tissues because the maternally derived allele is the only one active in the trophectoderm and primitive endoderm lineages. (Modified, with permission, from Papaioannou and Behringer 2005, © Cold Spring Harbor Laboratory Press; embryo reproduced, with permission, from Papaioannou and Hadjantonakis 2003.)

Cite this overview as *Cold Spring Harb Protoc*; doi:10.1101/pdb.over107956

effects reflecting the mosaicism of cells with the mutant allele on the active X chromosome. Is the gene Y chromosome–linked? Similar to X linkage, there will be only one copy of the gene in XY cells.

- *Does the gene fall within an autosomal region subject to imprinting effects?* If so, heterozygous offspring may be differently affected depending on the parent of origin of the mutant allele (Fig. 3).
- *Are there known mutations that map to the chromosomal region where your gene resides?* Check these carefully because if they have not yet been molecularly characterized, they could turn out to be alleles of your gene. To see if this is the case, you might obtain DNA from the known mutants or obtain the mutants themselves.
- *Is your favorite gene a member of a gene family?* If family members overlap in their expression pattern, some overlap of function could lead to redundancy. In this case the areas of unique expression of your gene might indicate areas where a mutant effect is most likely.

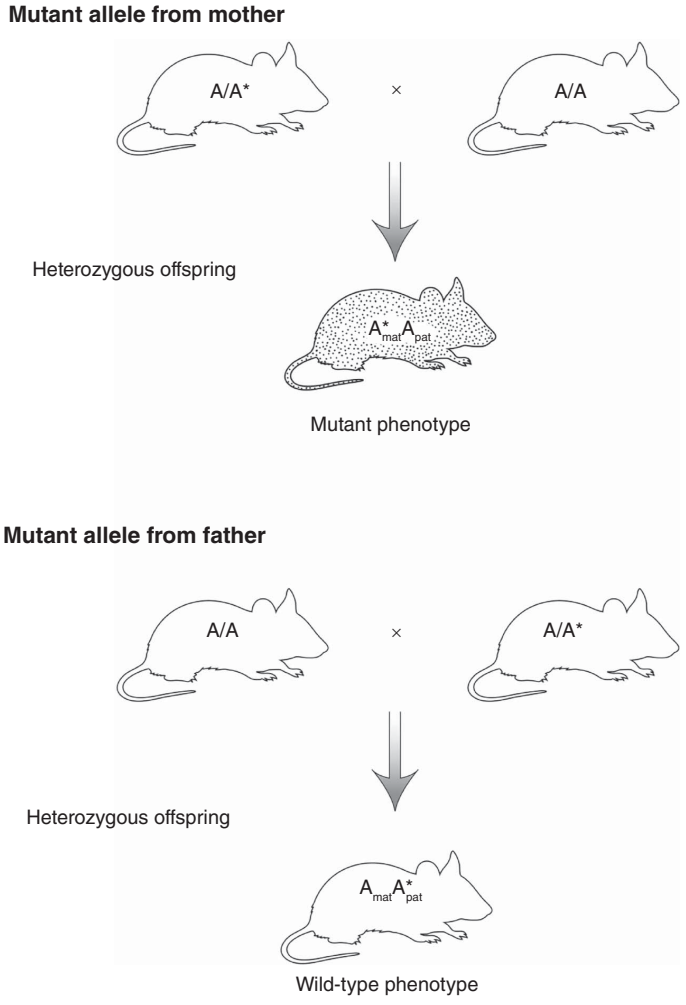

FIGURE 3. Inheritance of an imprinted, autosomal mutant gene. In this example, the locus is only expressed from the maternal allele. When a mutation (*A**) is inherited from the mother (*top cross*), heterozygous offspring will have one mutant maternal allele (A^*_{mat}) and one inactive paternal allele (A_{pat}) and will thus show a mutant phenotype. When the mutation is inherited from the father (*bottom cross*), the normal, maternally inherited allele will be the active copy and the heterozygous offspring will be phenotypically wild type. It should be noted that the situation would be reversed if the imprinted locus is expressed from the paternal allele. (Reprinted, with permission, from Papaioannou and Behringer 2005, © Cold Spring Harbor Laboratory Press.)

- *Is anything known about orthologous or homologous genes in other species?* Look for any existing mutations in your gene in other species or any chromosomal deletions in humans that include this gene. Determine if any human disease loci are mapped to the chromosomal region (www.omim.org). Any of this information could provide a clue as to the expected phenotype.
- *Can you make predictions based on the expression pattern and the nature of the protein?* Expression patterns tell you when and where the action of a mutation could have its origins. Proteins can act within the cells in which they are produced (cell-autonomous) and/or they can act on other cells (cell-non-autonomous).
- *Is anything known about interactions of the gene product with other gene products?* This information may be useful for placing the gene product into known biochemical pathways.

All of these considerations, which can lead to fruitful lines of investigation, are better thought through in advance before embarking on gene targeting. In addition, consider that the mutation may result in a mouse that looks perfectly normal on the surface. Prior knowledge about the gene and gene product could help reveal a subtle or cryptic phenotype.

What Is the Gene Expression Pattern?

A starting point in gene targeting experiments is to define the expression pattern of your chosen gene. It may be your favorite because of its expression in an adult organ of interest or its complicity in a disease process. But remember that the same gene may be used during development or in other tissues and could thus have an effect at an unanticipated time and place. Search the literature for reports of the gene's expression and consider doing a survey of expression in a panel of adult organs and embryonic stages, even if only at the level of reverse transcriptase polymerase chain reaction (RT-PCR) or northern blot analysis (commercial blots are available). Although a detailed expression analysis may not be what you choose to do at this stage, expression in embryos will at least alert you to the possibility of an embryonic phenotype. If you are using a mutation from the KOMP project, there may be detailed expression data available (West et al. 2015). The Gene Expression Database in MGI (GXD; http://www.informatics.jax.org/expression.shtml) is an excellent resource that collates expression data from published papers with emphasis on endogenous gene expression during development. You can check the expressed sequence tag (EST) databases to identify tissues in which transcripts for your favorite gene have been isolated as a guide to expression. There are also numerous transcriptomes available in public databases (e.g., Gene Expression Omnibus [GEO]; www.ncbi.nlm.nih.gov/geo) that can be examined for the expression of your gene of interest.

Equipped with a good understanding of your gene of interest and the different possibilities for making mutations, you are ready to move on.

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Obtaining or Generating Gene Mutations in Mice

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Cold Spring Harb Protoc; doi: 10.1101/pdb.over107956 originally published online November 6, 2023