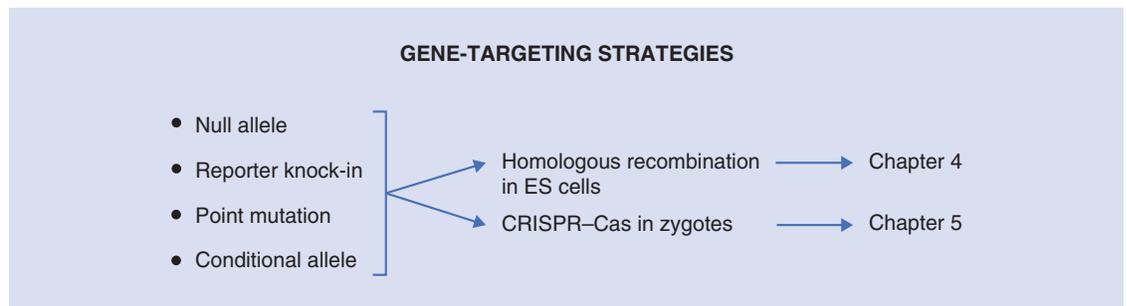


## CHAPTER 3

# Mouse Gene-Targeting Strategies for Maximum Ease and Versatility

Well-planned strategies are an essential prerequisite for any mutational analysis involving gene targeting. Consideration of the advantages or disadvantages of different methods will aid in the production of a final product that is both technically feasible and versatile. Strategies for gene-targeting experiments in the mouse are discussed, including the rationale behind some of the common elements of gene-targeting vectors, such as homologous DNA and the use of different site-specific recombinases. We detail positive and negative selection as well as screening strategies for homologous recombination events in embryonic stem (ES) cells. For the planning stages of making different types of alleles, we first consider general strategies and then provide details specific to either homologous recombination in ES cells or making alleles by gene editing with CRISPR–Cas in preimplantation embryos. The types of alleles considered are null or knockout alleles, reporter gene knock-in alleles, point mutations, and conditional null alleles.

## INTRODUCTION



The molecular techniques used to build a gene-targeting construct (or vector) are standard (see Chapter 10 in Behringer et al. 2014), and the goal of producing a mutation in a specific gene is conceptually straightforward. However, many aspects of the design of a simple knockout strategy or a more complex knock-in strategy involving a gene-targeting construct will have a bearing on the success of the procedure and on the versatility of the resulting mutation. With little additional work, you may be able to build features into an initial gene-targeting vector that will save you from having to construct a second vector as the project unfolds. Whether you are using homologous recombination in

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### BOX 1. GENE CHECKLIST BEFORE CONSTRUCTING TARGETED MUTANT ALLELES

Answers to the following fundamental questions about the locus in question will facilitate the best design for your desired mutation.

- How many coding and noncoding exons are present?
- Where is the start of transcription?
- Which exon contains the translation start codon?
- Which exon contains the translation stop codon?
- How many kilobases does the genomic region encoding the open reading frame span?
- Is there evidence for alternative splicing? If so, which exons are alternatively spliced?
- Are protein isoforms generated by the locus?
- Can you easily delete the entire locus, that is, can the entire locus be deleted by removing <20 kb?
- If you delete a specific exon(s), can the remaining exons splice together in-frame?
- If the gene (genomic region) is relatively large (>20 kb), is there a group of contiguous exons that reside within a small (<20-kb) genomic region that could be deleted?
- Do you have the sequence of the locus from the strain of mice to be used or from which the ES cells were derived?
- Have you performed a sequence comparison of nonexonic sequences among human, mouse, and other mammals to identify the location of conserved sequences that might regulate expression?

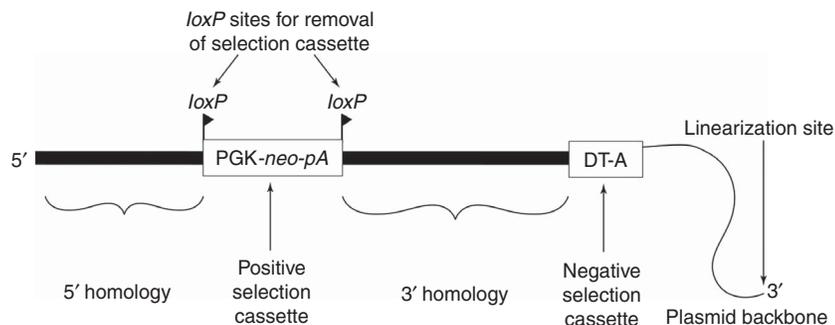
embryonic stem (ES) cells or using CRISPR–Cas technology to create a mutation either directly in preimplantation embryos or in ES cells, the strategies discussed in this overview will help you anticipate future needs and provide you with information to evaluate the trade-offs inherent in designing different kinds of alleles or using alternative technologies.

Before you begin, make sure that you have thorough and detailed information about the locus you intend to target (Box 1) to facilitate the best design for the mutation. For homologous recombination in ES cells, the first two steps for gene targeting (see Chapter 2: Obtaining or Generating Gene Mutations in Mice [Papaioannou and Behringer 2023a]) are to build a gene-targeting construct specific to your gene of interest and to produce a mutation in ES cells in vitro. For CRISPR–Cas manipulations, the first step is to identify a sequence to generate a guide RNA to target the gene and then produce a null mutation directly in preimplantation embryos. In this chapter, we will present the rationale for different aspects of gene-targeting vector design and discuss strategies for making different types of mutant alleles. Once a targeting strategy and technology has been chosen, you can move on to either Chapter 4: Embryonic Stem Cell Gene Targeting and Chimera Production in Mice (Papaioannou and Behringer 2023b) or Chapter 5: Recovering a Targeted Mutation in Mice from Embryonic Stem Cell Chimeras or CRISPR–Cas Founders (Papaioannou and Behringer 2023c).

## TOOLKIT FOR THE DESIGN OF GENE-TARGETING VECTORS

### Homologous DNA

Certain details should be considered when generating any type of gene-targeting vector. One of the most important is to use genomic DNA that is isogenic with the ES cell line that will be used for the gene-targeting experiments. In other words, both the genomic DNA used to generate the gene-targeting vector and the ES cells should be derived from the same inbred strain/substrain of mouse. This is also true for CRISPR–Cas knock-in manipulations in embryos: Genomic DNA sequences used in the gene-targeting vector must be identical to the strain of preimplantation mouse embryo manipulated. A complete sequence match between the targeting vector homology and the



**FIGURE 1.** Elements of an idealized targeting construct for homologous recombination in embryonic stem (ES) cells. The targeting constructs used for different targeting strategies vary in detail, but the usual components necessary for successful gene targeting include (1) homologous, isogenic DNA totaling 5–8 kb and evenly split between upstream and downstream arms of the targeting construct; (2) a positive selectable marker, such as *neo*, driven by a promoter active in ES cells, such as PGK, and surrounded by recombinase sites, such as *loxP* sites, to facilitate its eventual removal from the targeted allele; (3) a negative selectable marker, such as DT-A, to screen against random integration events, located outside the region of homology; and (4) a linearization site in the plasmid backbone located some distance from the negative selectable marker to protect it from degradation of the ends after linearization. The other essential elements (not shown) are 5' and 3' probes to the endogenous locus that are external to the regions of homology in the construct. These are used in Southern analysis of restriction enzyme digests to diagnose 5' and 3' crossover events to recognize a correctly targeted allele. Alternatively, a polymerase chain reaction (PCR)-based strategy can be used.

locus to be mutated (i.e., isogenic DNA) facilitates obtaining the maximum frequency of homologous recombination or homology-directed repair. Conversely, potential sequence mismatches resulting from the use of DNA from a different mouse strain can reduce the frequency of homologous recombination or homology-directed repair or even prevent it altogether. Therefore, it is essential to match the specific strain and substrain genomic library used to generate your gene-targeting construct with the ES cell line or embryos you plan to target or, alternatively, to amplify the arms of homology from the ES cells or mouse strain by polymerase chain reaction (PCR) using high-fidelity polymerases.

For homologous recombination in ES cells, the total amount of sequence homology to be used in the gene-targeting vector should be between 5 and 8 kb, which represents a balance between increased chances of homologous recombination and ease of vector construction: Smaller amounts of homology can reduce the targeting frequency and larger amounts can complicate vector construction as well as the identification of homologous recombinants. In general, the homologous sequence should be split between the upstream and downstream arms of homology in the vector (Fig. 1). One arm of homology can be as short as 1 kb in length, with the other arm making up the balance of the total recommended homology of 5–8 kb. The arms of homology in the targeting vector should flank any genomic sequences you wish to delete or modify. If you are using CRISPR–Cas in ES cells, the length of homology used in a knock-in targeting vector depends upon the molecular strategy used (see below).

### Site-Specific Recombinases

DNA recombinases that recognize specific sequences are routinely used in genetic strategies to generate tissue-specific and/or conditional mutations and other types of alleles. The primary system currently used is the *Cre/loxP* system, but the *Flp/FRT* and *Dre/rox* systems, which work similarly, are also used (Anastassiadis et al. 2009; Han et al. 2021).

*Cre* is a DNA recombinase that specifically recognizes 34-base pair (bp) sequences called *loxP* sites. The central 8-bp core of the *loxP* site is flanked by inverted repeats and specifies an orientation to the 34-bp sequence. *loxP* sites can be placed on either side of a segment of DNA. The length of the intervening DNA can be relatively small (<1 kb) or very large (in the range of cMs). The intervening DNA is called “flox” (flanked by *loxP* sites). Because the *loxP* sites have an orientation, they can

## BOX 2. REMOVING A SELECTION CASSETTE FROM ES CELLS IN VITRO

Removing the selection cassette from your targeted ES cell clones in vitro involves transiently expressing the appropriate recombinase (usually Cre or Flp), and then isolating clones and genotyping for the removal of the cassette (Anastassiadis et al. 2013). Electroporate circular recombinase expression plasmids into the ES cell clones, plate at clonal density, and culture without selection. Pick and then genotype the ES cell clones. Usually, ~10%–20% of the clones will have had the cassette removed by the transient recombinase expression. In the past, some investigators have used a floxed *neo*-HSV *tk* dual cassette so that the loss of *tk* can be selected for by culture in gancyclovir or FIAU. This results in nearly all of the surviving ES cell clones having the floxed cassette removed. If you do this, however, remember that HSV *tk* expression causes male sterility (see Box 3) and male chimeras made with ES cells that retain a *neo*-HSV *tk* will likely be sterile.

Triple *loxP* strategies have been used in the past to generate conditional null alleles. In this strategy, two *loxP* sites flank a selection cassette (e.g., *neo*) and the third *loxP* site is added to flank important exons. However, depending on the efficiency and timing of Cre excision following transient exposure to Cre recombinase, three different alleles could be produced requiring some effort to sort out. Nowadays, most labs use a dual Cre/Flp strategy to flox important exons and flrt the selection cassette. Thus, expression of Flp will remove the selection cassette, leaving the important exons floxed.

One caveat of the in vitro approach for removing a selection cassette is that it requires a second electroporation and cell cloning step that could compromise obtaining germline transmission of the targeted allele. In addition, it is necessary to choose the targeted clone(s) to electroporate with the recombinase vector. Often, only one clone is chosen for this purpose, which can be a big mistake because that clone has probably not been screened for germline transmission ability after the initial targeting. An alternative is to remove the selection cassette in vivo, after mice have been produced (see Deletion of Selection Cassettes In Vivo in Chapter 6: Strategies for Maintaining Mouse Mutations [Papaioannou and Behringer 2023d]).

flank a piece of intervening DNA in either direct orientation or inverted orientation. Cre will recognize *loxP* sites flanking a piece of DNA and catalyze the deletion of the intervening DNA if the flanking *loxP* sites are in direct orientation or it will invert the intervening DNA if the *loxP* sites are in inverted orientation. A similar situation occurs with Flp recombinase and *FRT* sites, and Dre recombinase and *rox* sites. DNA sequences flanked by *FRT* sites have been called “flrted.”

For ES cell manipulations, selectable marker expression cassettes are floxed or flrted (Fig. 1) so that they can be removed from the targeted locus by Cre or Flp, respectively. In this way, extraneous DNA sequences that might complicate the analysis of the mutant are removed from the targeted locus either in vitro, before chimera production (Box 2), or in vivo, once mutant mice have been produced (see below).

### Positive Selection for Vector Incorporation in ES Cells

When using ES cells for gene targeting, positive selection of ES cell clones that have integrated the gene-targeting vector is usually achieved using a neomycin phosphotransferase (*neo*) gene expression cassette that confers resistance to the drug G418, although other selectable markers can be used. However, the presence of a selectable marker gene with its associated promoter (e.g., the *Pgk* promoter) at targeted loci has been shown in some cases to influence the expression of neighboring genes (e.g., *Myf5*; Kaul et al. 2000). Therefore, it is a good idea to flank the positive selectable marker with *loxP* sites to provide the option of removing the selectable marker later with Cre recombinase (Fig. 1).

Once homologous recombination has been achieved, the floxed selectable marker can be deleted either in the targeted ES cells or later in mice. Removal is accomplished in ES cells by transient in vitro Cre expression before advancing to the next step of making chimeras (Box 2). If mice carrying the targeted allele have been produced, the floxed selectable marker can be deleted by pronuclear injection of a Cre expression plasmid or RNA into zygotes carrying the floxed gene, by culturing zygotes in membrane-permeable CRE protein (Ryder et al. 2014), or by breeding mice carrying the floxed gene with Cre transgenic mice (see Chapter 6: Strategies for Maintaining Mouse Mutations [Papaioannou and Behringer 2023d]). In any case, using a floxed allele strategy provides a simple way to generate two different alleles for the targeted gene, one with the selectable marker and one without. Having two

different alleles could be useful for chimera studies later in the analysis of a phenotype (see Chapter 17: Getting around an Early Lethal Phenotype in Mice with Chimeras [Papaioannou and Behringer 2023e]).

An added benefit of the floxed selectable marker strategy is that it provides the opportunity to make homozygous mutant ES cell lines without ever making a mouse. By deleting the selectable marker from the targeted locus by Cre expression in heterozygous ES cells, you can reuse the original targeting vector and the same selective drug a second time to target the remaining wild-type allele, a strategy called “marker recycling” (Abuin and Bradley 1996). Other recombinase/recombinase target site combinations (e.g., *Flp/FRT*, *Dre/rox*) can also be used to remove selectable markers.

### Negative Selection against Random Integration Events in ES Cells

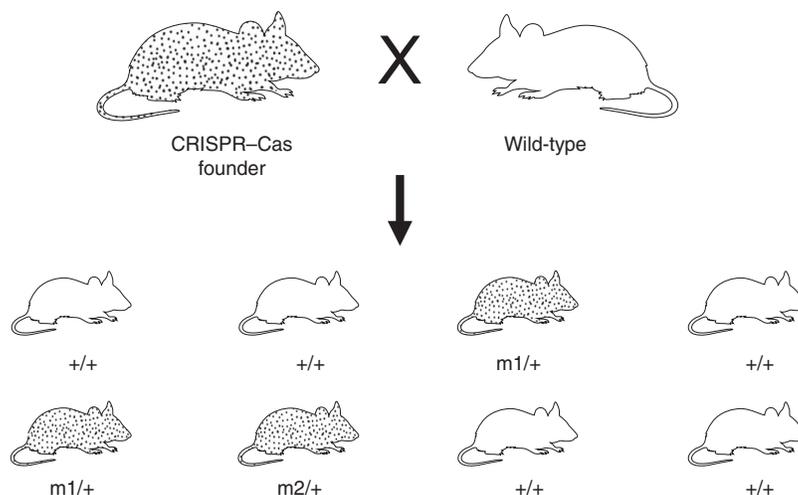
Negative selection against ES cell clones that have incorporated the gene-targeting construct by random insertion can be accomplished using a herpes simplex virus (HSV) thymidine kinase (*tk*) gene outside the region of homology in the construct. Electroporated ES cells are then cultured with ganciclovir or FIAU (1,2-deoxy-2-fluoro-1- $\beta$ -D-arabinofuranosyl-5-iodouracil). After homologous recombination, the ends of the gene-targeting vector are lost, including the plasmid backbone and *tk* expression cassette. Thus, cells that contain the *tk* gene resulting from random integration of the vector will be killed by the drug selection. Note, however that retention of *tk* can result in male sterility (Box 3). Negative selection can also be achieved using a diphtheria toxin A-chain (DT-A) gene (Fig. 1) that does not require the addition of a drug for selection, but rather kills all cells in which it is incorporated and expressed.

### Screening for Homologous Recombination Events in ES Cells or Mice

Even after positive-negative selection, many of the ES cell colonies that survive drug selection will not be homologous recombinants but instead will contain random integration events in which the negative selectable marker was fortuitously lost or damaged. Therefore, all resistant colonies must still be screened using PCR or Southern blot analysis to identify ES cell clones with correct gene targeting. Southern blot analysis requires the use of a diagnostic restriction enzyme digest with a probe, called the external probe, that is not contained within the regions of homology included in the gene-targeting vector. A unique restriction enzyme site is usually introduced along with the positive selection marker during construction of the gene-targeting vector for ease of screening by Southern blotting. The diagnostic restriction enzyme site should be located outside of any floxed selectable marker so that, after Cre expression and deletion of the marker gene, the diagnostic restriction enzyme site remains. Using this strategy, a diagnostic digest and hybridization with an external probe will yield a smaller DNA fragment for the mutant allele compared to the wild-type allele. This is desirable for technical reasons because if the mutant band were larger, it could be hard to distinguish from a partial restriction enzyme digest on a Southern blot.

#### BOX 3. HSV *tk* AND MALE STERILITY

HSV *tk* expression is toxic to the germ cells of male transgenic mice and causes dominant sterility. This was observed in transgenic mice expressing HSV *tk* in the kidney (Al-Shawi et al. 1988) and in transgenic mice that fortuitously carried an HSV *tk* sequence as part of a larger transgene construct (Braun et al. 1990). Subsequent studies revealed that the coding region of HSV *tk* contains a promoter that is transcriptionally active in the differentiating germ cells of male transgenic mice. Fortunately, HSV *tk* is not active during female gametogenesis, and HSV *tk*-containing transgenes can be transmitted through the female germline. HSV *tk* is widely used in *in vitro* gene-targeting strategies, and if it is retained at the targeted locus, it can cause unanticipated difficulties in achieving germline transmission of the mutant allele from male chimeras. To get around this problem, the male germ cell promoter of HSV *tk* has been mutated to express a *tk* protein that retains enzyme activity for negative selection but is not expressed in male germ cells and is therefore compatible with male fertility (Salomon et al. 1995).



**FIGURE 2.** Segregation of CRISPR–Cas-induced alleles from founders. A CRISPR–Cas mosaic founder male or female (stippled) is bred to wild-type mice. The progeny that are heterozygous for different mutant alleles (mutation 1, m1 and mutation 2, m2, *stippled*) are identified by polymerase chain reaction (PCR) followed by DNA sequencing and can then be used to generate individual pedigrees.

To characterize the targeted allele fully—and this is essential—both 5' and 3' ends of the altered allele must be examined thoroughly to determine the structure of the gene-targeting events that have occurred through both the 5' and 3' arms of homology. When using Southern blot analysis, if for some reason both 5' and 3' external probes cannot be identified, the targeting event can be identified initially using one external probe, and the allele can be further investigated by additional analysis using a probe within the region of targeting vector homology (e.g., an internal probe such as *neo*).

For CRISPR–Cas manipulations of preimplantation embryos, the resulting mice are screened to identify founders that carry modifications in the locus of interest. Because of variation in the activity of CRISPR–Cas, these founders could carry multiple different alleles. In addition, the founders could be mosaic depending on the timing of CRISPR–Cas activity relative to cleavage (Mizuno et al. 2014; Yen et al. 2014). Once founders are identified they are bred to wild-type mice of the strain appropriate for your studies to segregate alleles in their progeny and to segregate away any off-target events (Fig. 2). The progeny are analyzed to molecularly define the various alleles generated, and individuals carrying the desired alleles are then used to create pedigrees/lines for study.

## DIFFERENT TYPES OF ALLELES

### General Strategy for Making a Null Allele

The first mutation most investigators aim to make when they initiate the study of a particular gene is a complete loss of function—that is, a null allele. Null alleles are very useful because the phenotype of the homozygous null mouse provides information about the earliest essential role of the product(s) expressed from the locus during development. In addition, null alleles can be used in combination with other alleles for more complex studies. Fortunately, a null allele is one of the simplest mutations to generate, both conceptually and technically, by gene targeting in ES cells or CRISPR–Cas manipulations in preimplantation embryos.

For small genes (<20 kb), the most straightforward strategy is to delete all protein-coding exons. This guarantees the generation of a null allele because protein products cannot be synthesized from the targeted locus. Thus, once the deletion of the DNA sequences has been verified, it is not necessary to analyze mRNA or protein for the targeted gene in homozygous mutants, because there will be none expressed. If the coding sequences are not removed in their entirety, mRNA and protein could still

potentially be expressed from the targeted locus, and characterization of variant transcripts generated from the targeted allele will be necessary. This characterization can be time-consuming and difficult to interpret. Partial protein products that could potentially be synthesized from a targeted locus may be problematic if they retain some function. Antibodies for the gene product may not be available to determine if a partial protein product is being expressed, further complicating the interpretation.

An argument against designing or selecting a deletion that removes all coding exons would be the presence of regulatory elements for neighboring genes located within the introns (e.g., *Shh*; Lettice et al. 2003; West et al. 2016). This possibility should be kept in mind, especially if other genes are located nearby. Sequence comparisons of genomic DNA between mouse and other mammals will reveal if there are conserved sequences within the introns that might indicate the presence of such regulatory elements, but they will not necessarily reveal the identity of the gene being regulated.

Generating null alleles for a larger gene requires a little more thought because standard gene-targeting strategies are not as efficient for generating large (>20-kb) deletions; thus, more complex strategies are necessary. With the deletion of all protein-coding exons of a large gene, it is even more likely that regulatory elements of adjacent genes, or even all or part of another gene, could be located within the introns of the gene being deleted. Sequence comparisons of genomic DNA between mouse and other vertebrates might reveal conserved sequences within introns, and searches of transcriptome databases could indicate if another gene is within or overlaps with the gene to be deleted.

With larger genes, the strategy most likely to result in a null allele is the creation of a deletion of up to 20 kb that removes the initial protein-coding exons, including the exon containing the translation initiation site. Alternatively, the translation initiation exon can be maintained but downstream exons deleted such that abnormal transcripts produced are removed by nonsense-mediated decay. Another possibility is that the transcription initiation site(s), if known, could be deleted. If a deletion is designed to remove a subset of internal protein-coding exons, it is important to check that the exons that remain after the deletion cannot splice in-frame to generate a partial protein product. If they can do so, reconsider the position of the deletion or introduce a frameshift mutation. In fact, null alleles produced by any method should be thoroughly evaluated to ensure the elimination of residual proteins produced through such mechanisms as alternative splicing, alternative promoter usage, or translation initiation from non-AUG start codons, all of which have been documented in gene targeting (Hosur et al. 2020).

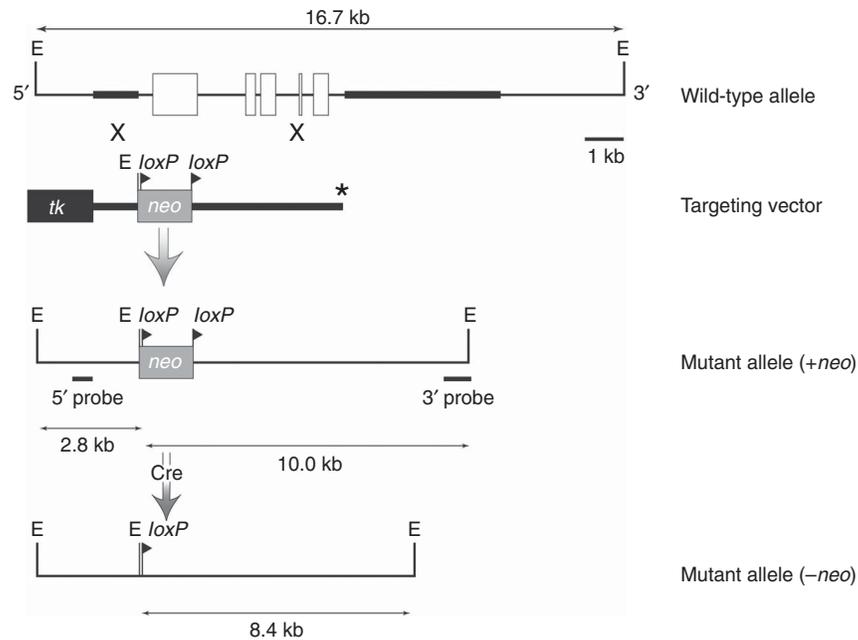
### *Making Null Alleles by Homologous Recombination in ES Cells*

The foregoing considerations about the final mutation design are relevant regardless of the method used to produce the mutation. Homologous recombination in ES cells allows good control for producing the correct allele before making mutant mice. Deletions for mutating either small (Fig. 3) or large genes (Fig. 4) by homologous recombination in ES cells are created most simply by using a replacement gene-targeting strategy: A selectable marker replaces coding exons and a positive–negative selection scheme is used to enrich for ES cell clones that have undergone homologous recombination. The primary features of a replacement vector are a plasmid backbone containing a positive selection marker positioned between two regions of homology and a negative selectable marker next to one of the arms of homology (see Fig. 1). It is not essential to use a negative selection marker, although its inclusion will increase the ratio of targeted to randomly integrated clones, thus reducing the workload during screening of drug-resistant ES cell clones. Linearized targeting vectors are usually introduced into ES cells by electroporation, followed by culture in selective medium, and subsequent identification of homologous recombinant ES cell clones (see Chapter 11 in Behringer et al. 2014).

### *Making Null Alleles with CRISPR–Cas*

Null mutations made by CRISPR–Cas manipulations of preimplantation embryos are usually generated by creating small insertions/deletions (indels) (Fig. 5). The size of these indels varies depending on the amount of DNA inserted or removed by nonhomologous end joining repair after the CRISPR–Cas-induced double-strand break. If CRISPR–Cas acts in the zygote, two different alleles

## Chapter 3



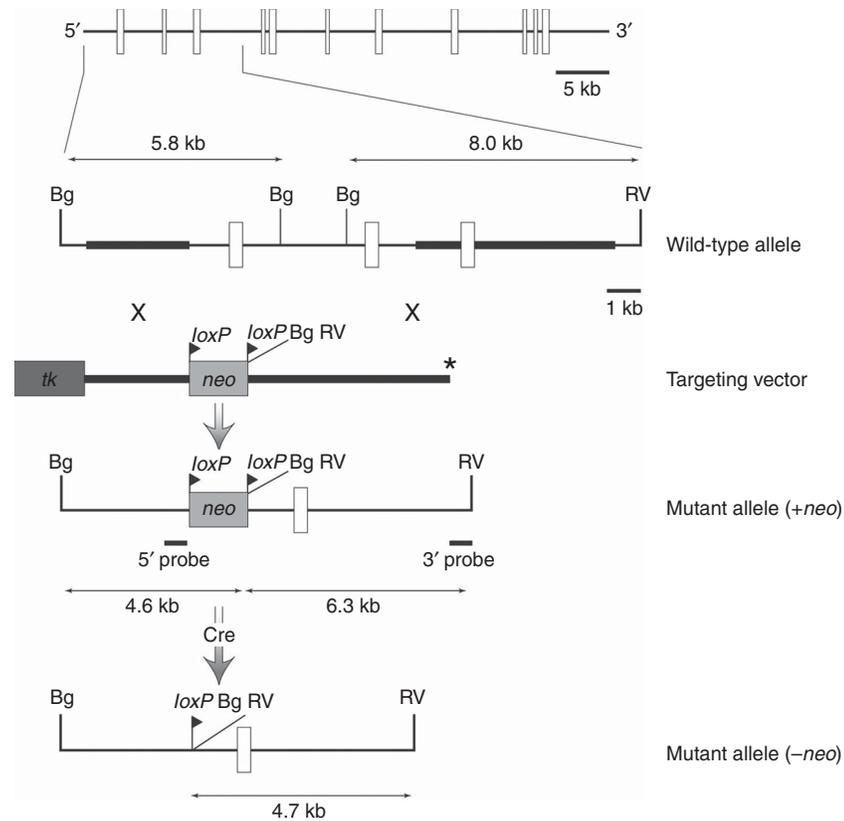
**FIGURE 3.** Deletion of all protein-coding exons for a small gene by homologous recombination in ES cells. All of the protein-coding exons of the wild-type allele (*open boxes*) are replaced by a floxed neomycin (*neo*) cassette to ensure the generation of a null allele. In this case, the total amount of homology in the targeting vector (5.5 kb) is divided as a 1.2-kb 5' arm and a 4.3-kb 3' arm (*thick lines*). The *neo* cassette includes an *EcoRI* (E) site to identify homologous recombinants by Southern analysis. An HSV thymidine kinase (*tk*) cassette is placed adjacent to the 5' arm of homology for negative selection. The site of vector linearization is indicated by the asterisk (\*). In this case, 5' and 3' external probes are used to identify the targeted mutant allele. The sizes of the wild-type and mutant bands for Southern analysis are indicated. The floxed *neo* cassette can be removed by Cre. (+*neo*) Allele retaining the *neo* cassette; (-*neo*) allele in which the *neo* cassette has been deleted.

might be generated; four may be generated if the cut/repair occurs after DNA replication. If the CRISPR–Cas components act on the targeted allele after cell division, then each blastomere could have different alleles such that the resulting founder mouse would be mosaic for two or more alleles (Yen et al. 2014; Clark et al. 2020).

A guide RNA is identified to target a specific sequence within the target gene, typically an exon (Chapter 2: Obtaining or Generating Gene Mutations in Mice [Papaioannou and Behringer 2023a]). It is also possible to use CRISPR–Cas to create larger defined deletions by introducing two guide RNAs that flank the region to be deleted (Fujii et al. 2013; Boroviak et al. 2016). The guide RNA(s) and Cas (RNA, plasmid, or protein) are introduced into preimplantation embryos by microinjection or electroporation (see Chapter 7 in Behringer et al. 2014; Hashimoto et al. 2016). The resulting pups born from these manipulations are then screened by PCR for mutations in the locus of interest. It is likely the founders carrying the various CRISPR–Cas-induced mutations will be mosaic and can carry multiple different mutant alleles in their germline that are isolated by breeding (Fig. 2).

### Reporter Gene Knock-Ins

On many occasions during phenotypic analysis of a mutant allele, it is useful to have a simple cellular reporter to follow the expression of the gene or to track mutant cells. Introducing a reporter into a specific locus by gene targeting (a so-called knock-in strategy) can be used to achieve the goal of faithful recapitulation of the expression of an endogenous gene by a readily detectable reporter. This method exploits the intact regulatory elements of the endogenous chromosomal locus, at the same time rendering the gene nonfunctional by deletion or alteration of crucial protein-coding exons. Knock-in strategies are also possible that preserve the function of the gene of interest. One of the important steps in designing a knock-in allele is to determine the position within the gene to introduce



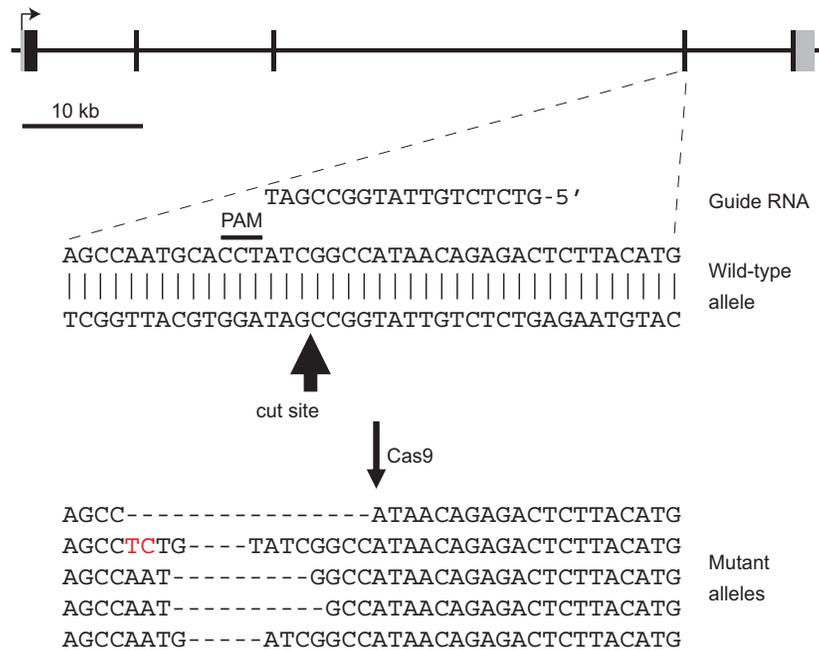
**FIGURE 4.** Deletion of the initial protein-coding exons for a large gene by homologous recombination in ES cells. The coding exons (*open boxes*) of this gene span 38 kb (*top*). Therefore, the first two coding exons are deleted. This region (6.3 kb) is replaced by a floxed *neo* cassette. A total of 9.0 kb of homology is divided into a 3.0-kb 5' arm and a 6.0-kb 3' arm (*thick lines*). The *neo* cassette includes *Bgl*I (Bg) and *Eco*RV (RV) sites to identify homologous recombinants by Southern analysis. An HSV *tk* cassette is placed on the 5' arm of homology. The site of vector linearization is indicated by the asterisk (\*). A 3' external probe is used for the initial identification of homologous recombinants. Once these clones are identified, they are expanded and can be analyzed with an internal probe to characterize the structure of the 5' recombination event. The sizes of the wild-type and mutant bands for Southern analysis are indicated. The floxed *neo* cassette can be removed by Cre. (+*neo*) Allele retaining the *neo* cassette; (*neo*) allele in which the *neo* cassette has been deleted.

the reporter. The reporter gene can be engineered (1) as an ATG fusion with the endogenous transcript, (2) as a fusion transcript by generating a bicistronic transcript using an internal ribosome entry site (IRES) sequence or T2A peptide cleavage sequence, (3) by creating a chimeric transcript using a splice acceptor sequence, or (4) by a combination of both an IRES and splice acceptor.

Depending on the strategy, insertion of the reporter gene sequence can be within protein coding or untranslated exons or within introns. A conservative approach to obtain a faithful reporter is to make a simple insertion without deleting any sequence. This will avoid unintentional removal of regulatory elements in a deleted sequence. However, you might consider a small deletion of the protein-coding sequences within an exon to increase the chances of generating a null allele. Introduction of a reporter into untranslated sequences could potentially yield wild-type, hypomorphic (destabilization of the transcript), hypermorphic (stabilization of the transcript), or null alleles, depending on the specific situation (e.g., *Vegfa*; Miquerol et al. 2000).

There are some notes of caution.

- In spite of your best intentions and design efforts, knock-ins will not necessarily reflect the endogenous expression pattern of the targeted gene. This is because the inserted reporter could act as a dominant insertional mutation by altering gene transcription. We just do not know enough about the regulation of genes to be able to avoid this in all cases. Thus, validation of the reporter



**FIGURE 5.** Generation of null alleles using CRISPR–Cas. The genomic structure of the *Tyr* locus and sequence from exon 4 is shown. A guide RNA adjacent to a PAM (protospacer-adjacent motif) sequence is identified to make a double-strand cut at a predicted site in exon 4 (thick arrow). Guide RNA and Cas9 (DNA, RNA, or protein) are introduced into zygotes by injection or electroporation. Various possible insertion or deletion (indel) alleles created by CRISPR–Cas are shown below. (Red sequence) insertion; (dashes) deletions; (black boxes) protein coding exons; (gray boxes) untranslated regions; (arrow on exon 1) transcription start site. (Adapted from Fig. 1 in Yen et al. 2014.)

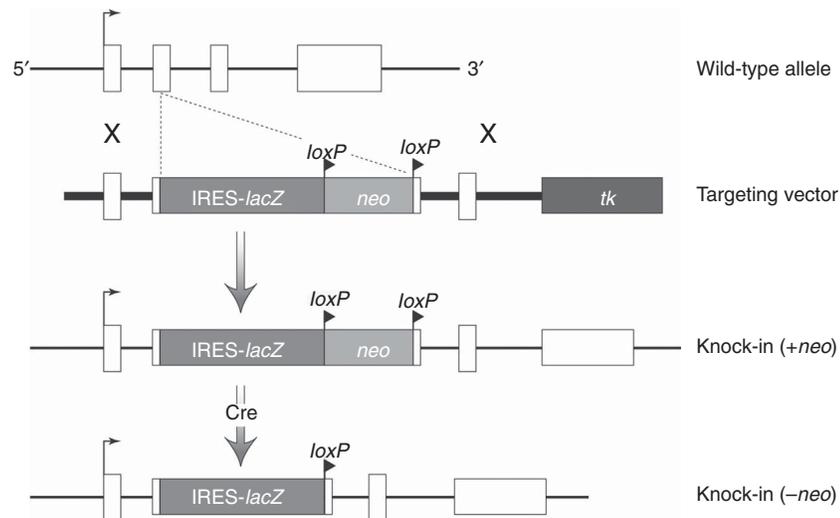
knock-in requires comparisons between endogenous and reporter expression. Once validated for specific stages and tissues, the knock-in can be used with confidence to report the expression of the targeted locus during mutant analysis.

- The activity of a reporter protein or its relative stability may result in a slightly different expression pattern compared with that of the endogenous mRNA or protein expression pattern. For example, X-Gal staining reveals the enzymatic activity of the protein  $\beta$ -galactosidase, which may have different degradation kinetics from the endogenous protein of the mutated gene. If antibodies to the endogenous protein and the marker proteins are available, the extent of this discrepancy can be determined by comparing the two.

Knock-in reporters are useful for following the mutant cells because they mark cells that transcribe the targeted locus. This can be very helpful for learning whether the expressing cells are still present in a mutant animal and, if so, how their behavior differs from wild-type cells. In addition, knock-in reporters can be used in combination with null alleles to determine whether the locus is regulated by negative feedback mechanisms. Assuming that the knock-in allele is also a null allele, the expression of the reporter in knock-in/wild-type heterozygotes is compared with expression in knock-in/null heterozygotes, both of which have a single knock-in reporter allele. If expression of the reporter is more extensive in the knock-in/null heterozygotes, this would suggest a negative feedback regulation of the gene by its own gene product.

### *Knock-In Alleles by Homologous Recombination in ES Cells*

For homologous recombination in ES cells, knock-in alleles are usually generated using a replacement gene-targeting strategy (Fig. 6). The reporter is followed directly by a floxed positive selection marker gene, such as *neo*, that can be removed later by Cre recombinase, either in vitro or in vivo. As mentioned above, alternative recombinase systems could also be used. The 5' and 3' arms of homology



**FIGURE 6.** Reporter knock-in by homologous recombination in ES cells. In this example, a *lacZ* reporter with a poly(A) is introduced into the second exon of a gene followed by a floxed *neo* expression cassette that can subsequently be removed either in targeted cells or in animals by Cre recombinase. An internal ribosome entry site (IRES) is used to bypass the requirement of having *lacZ* in frame with the coding region of the endogenous gene. An HSV thymidine kinase (*tk*) cassette is placed adjacent to the 3' arm of homology for negative selection. The floxed *neo* cassette can be removed by Cre. Gene sequences can be deleted when making a knock-in, but one must be aware that regulatory sequences might be removed. Instead of a reporter, the coding region of any gene of interest can be knocked in. Many times, knock-ins also knock out endogenous gene function. (*Thick lines*) Regions of homology; (+*neo*) allele retaining the *neo* cassette; (-*neo*) allele in which the *neo* cassette has been deleted.

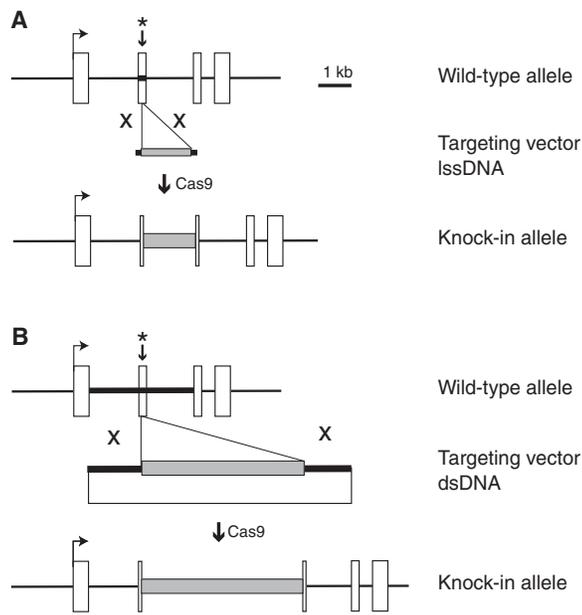
flank the reporter-*neo* sequences and a negative selection marker can also be added outside the region of homology. Strategies to identify homologous recombinants by Southern analysis or PCR are the same as for generating a standard null allele.

### Knock-In Alleles with CRISPR-Cas

Multiple methods to generate knock-in alleles using CRISPR-Cas have been developed (Gu et al. 2018; Miura et al. 2018; Abe et al. 2020). The feasibility and efficiency of these methods depends on the length of DNA to be introduced into the target locus and perhaps the target locus itself. Relatively new approaches to introduce exogenous sequences into a target locus have exploited the stages of preimplantation development that have higher recombination activity. One fundamental difference for generating knock-in alleles by CRISPR-Cas manipulations in preimplantation embryos versus using ES cells is that there is no selectable marker within the gene-targeting vector.

In one approach, used for introducing shorter sequences, long single-stranded DNA (lssDNA) with ~50–100 nt of homology for a target locus on each end, can be used to knock in up to ~1.5–2.0 kb of heterologous sequence (Fig. 7A; Quadros et al. 2017). Conveniently, lssDNA of this length can be synthesized commercially. Advances in high-fidelity synthesis of lssDNA should facilitate the introduction of longer sequences into a target locus by this approach.

A second approach, used to knock in longer sequences, is to generate a standard double-stranded DNA (dsDNA) gene-targeting vector with 0.5- to 2.0-kb homology flanking the sequence to be introduced into a target locus (Fig. 7B). In one study, two-cell-stage embryos were injected with dsDNA gene-targeting vectors when chromatin is relatively open and there is likely higher homologous recombination activity during an extended G<sub>2</sub> phase of the cell cycle (Gu et al. 2018). In addition, targeting efficiencies were further increased when the dsDNA gene-targeting vectors were biotinylated and a streptavidin Cas9 fusion protein was used to localize the repair template to the target locus. In another study, zygotes were injected, coinciding with the S phase of the cell cycle (Abe et al. 2020). Both methods result in the generation of knock-in alleles at reasonably high frequencies.



**FIGURE 7.** Strategies to generate CRISPR–Cas knock-in alleles. Guide RNAs with associated PAM sequences are identified to generate a double-strand break in the exon of choice. After knock-in, the guide RNA and PAM sequences are separated by the insert, preventing recutting of the edited locus by CRISPR–Cas. Asterisks (\*) indicate location of guide RNA and associated PAM sequence. (A) Knock-in of an insert (gray) up to ~2.0 kb. A long single-stranded (lss) DNA vector with 50–100 nt arms of homology (thick lines, 5' and 3' of the insert) is used. (B) Knock-in of an insert (gray) >2.0 kb. A double-stranded (ds) DNA vector with 0.5- to 2.0-kb arms of homology (thick lines, 5' and 3' of the insert) is used.

### Producing Point Mutations or Small Changes

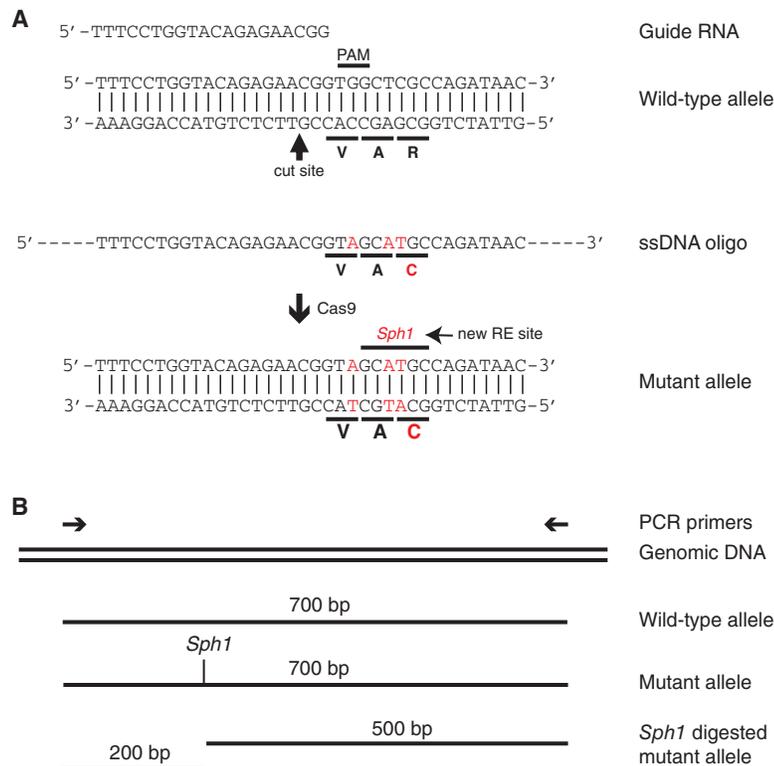
Another popular and versatile type of mutation to engineer is a point mutation. These can be used to create a stop codon that will truncate a protein product, alter transcription factor binding sites for transcriptional regulation studies, alter a specific amino acid by a missense mutation to create a variant protein, or perhaps mimic a mutation identified by human genetic studies as a disease-causing mutation. Many other types of alterations, such as splice mutations and untranslated region (UTR) modifications, are also possible with point mutations or other small changes.

#### *Strategies for Point Mutations by Homologous Recombination in ES Cells*

There are three general methods for creating point mutations or small changes by gene targeting in ES cells, each with advantages and potential disadvantages. These include (1) the *Cre/loxP* strategy (Arango et al. 1999), (2) the double-replacement strategy (Moore et al. 1995), and (3) the hit-and-run strategy (Hasty et al. 1991). Currently, however, the more straightforward and preferred way to generate this type of mutation is by CRISPR–Cas manipulations of preimplantation embryos.

#### *Strategies for Point Mutations Using CRISPR–Cas*

CRISPR–Cas manipulations in preimplantation embryos can be used to generate point mutations or to insert small sequence modifications (e.g., epitope tags, *loxP* sites). A guide RNA is identified to target a specific sequence within your favorite gene. The cut site should preferably be within 15 bp of the intended point mutation/epitope tag for highest efficiency of targeting. An oligonucleotide ~200 nt in length with 60–100 nt of homology flanking the intended point mutation/epitope tag is synthesized. This oligonucleotide also includes silent mutations in the PAM (protospacer-adjacent motif) site (or the spacer sequence) that prevent recutting of the edited locus by CRISPR–Cas. The oligonucleotide can also be designed to create a new restriction enzyme site to aid in the genotyping of the mutant mice (Fig. 8). Alternatively, there are PCR methods that will directly detect point mutations (e.g., Hindson et al. 2011). The guide RNA, the oligonucleotide that contains your desired mutation in a segment of homology, and Cas (RNA, plasmid, or protein) are introduced into preimplantation embryos by microinjection or electroporation. The resulting pups born from these manipulations are then screened by PCR for mutations in the locus of interest to identify founders. As with CRISPR-induced indels, it is likely the founders will carry various CRISPR–Cas-induced



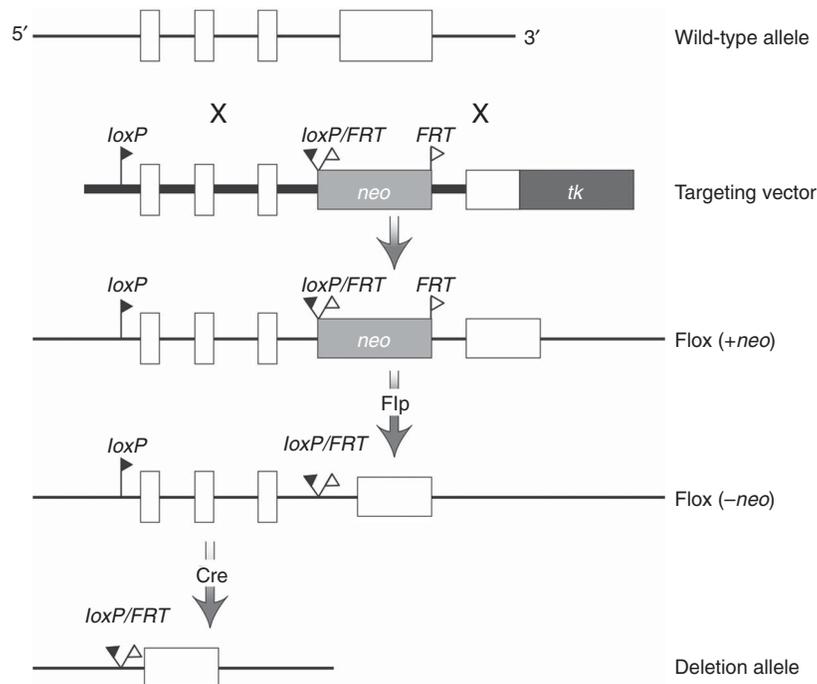
**FIGURE 8.** (A) Strategy to generate a CRISPR–Cas-induced point mutation. An arginine (R) to cysteine (C) missense mutation is introduced using a ~100-nt single-stranded DNA oligonucleotide (ssDNA oligo) with the desired sequence changes (red text). Publicly available programs are used to identify candidate guide RNAs with associated PAM sequences to the target sequence. CRISPR–Cas cuts 3–4 bp 5' from the PAM site. A nucleotide change in the ssDNA oligo will mutate the PAM site to prevent recutting of the allele after the point mutation has been created. In this example, another nucleotide change in the ssDNA oligo creates a new restriction enzyme (RE) site that is exploited for PCR genotyping. (B) PCR genotyping strategy to identify mice carrying the point mutation. Primers will amplify a wild-type sequence (~700 bp). If the mutations described in A are present, then digestion with *Sph1* will cut the mutant allele into ~200- and ~500-bp DNA fragments that can be visualized on an agarose gel. (A) alanine, (V) valine.

mutations in their germline, including indels as well as the desired point mutation. Once progeny from the founders are generated, it is important to confirm the sequence of the intended point mutation and surrounding sequences. The same CRISPR–Cas strategy can be used in ES cells where screening for the desired mutation can be done in ES cell clones before making chimeras.

### Conditional Null Alleles

Many genes are expressed during embryogenesis and in adult tissues and are therefore likely to have multiple roles in different tissues at different stages of development. Targeted mutations such as null alleles may cause early embryonic lethality, precluding the study of gene function at later stages of development or after birth. Conditional genetic strategies provide methods to bypass early lethality to study complex gene function. If you anticipate that your gene might fall into this category, and especially if you are mainly interested in the role of the gene in a specific tissue or organ, consider making a conditional null allele or obtaining one from the International Mouse Phenotyping Consortium (IMPC) (<https://www.mousephenotype.org/>).

Conditional null alleles are usually generated by targeting two *loxP* sites in the same orientation into noncoding regions (e.g., into introns or gene flanking regions) that flank critical protein-coding exons (Fig. 9). Once mice with a conditional allele have been produced, you may then use Cre recombinase at the time and in the tissue that you choose to act on these *loxP* sites to cause the time- or tissue-specific deletion of the floxed exons. Conditional alleles must be thoroughly charac-



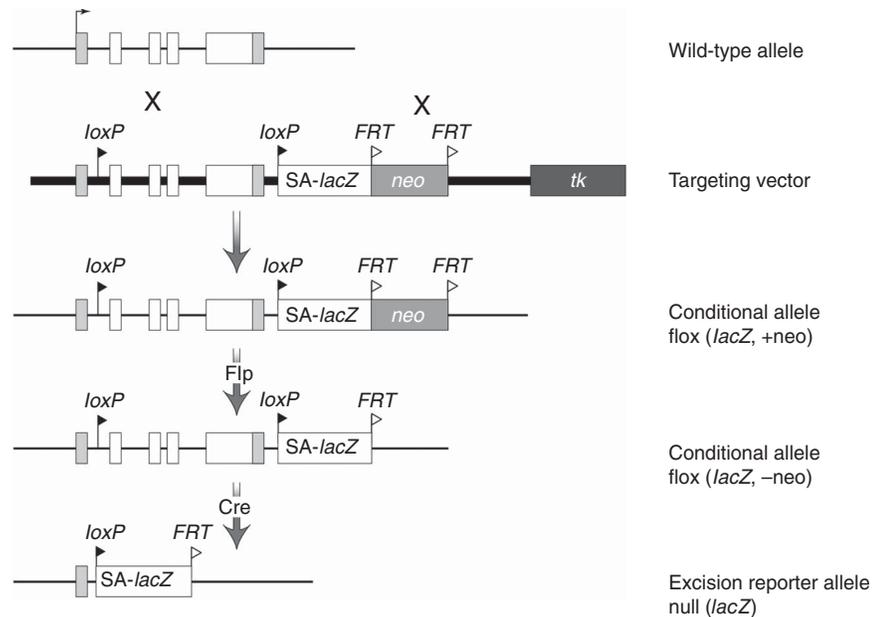
**FIGURE 9.** Generation of a conditional null allele by homologous recombination in ES cells. In this strategy, a replacement vector is generated in which an *FRT*-flanked *neo* expression cassette is introduced into an intron. *loxP* sites are engineered to flank exons. It is very useful to engineer a restriction site 5' of the 5' *loxP* site to identify targeting events by Southern analysis that have included both *loxP* sites. An HSV *tk* cassette is placed adjacent to the 3' arm of homology. After homologous recombination, a conditional allele is generated that has *neo* in an intron. This allele may behave as wild-type, a hypomorph, or even a null. *neo* can be removed by Flp expression, yielding a conditional allele that will most likely behave as wild-type unless the *loxP* sites have unintentionally disrupted an important regulatory sequence. Cre expression will delete the intervening exons to generate a loss-of-function allele. (Thick lines) Regions of homology; (Flox [+*neo*]) floxed allele that contains the *neo* cassette; (Flox [-*neo*]) floxed allele without the *neo* cassette.

terized to determine that they function as predicted (see Chapter 18: Tissue- and/or Temporal-Specific Mutations in Mice Using Conditional Alleles [Papaioannou and Behringer 2023f]).

A conditional null allele with a linked reporter can also be engineered to monitor the excision of floxed exons by Cre recombinase using a reporter gene such as *lacZ* or *GFP* (Fig. 10). This type of allele allows you to visualize recombination events (i.e., knockout of the gene of interest) with cellular resolution. This can be particularly useful if you do not have an antibody to the protein encoded by the gene of interest or if the cells that express the gene are scattered throughout organs, thus making RNA in situ analysis at the cellular level challenging.

There are a number of ways to design a conditional allele with a linked reporter. Basically, critical exons are flanked by *loxP* sites, a promoterless reporter gene is inserted downstream from the coding region of the gene of interest, and, if using ES cells, a selectable marker that subsequently can be excised in vitro or in vivo is incorporated for gene targeting. Ideally, the conditional allele should behave like a wild-type allele and not express the reporter. After Cre-mediated excision of the floxed exons, the allele should become null and express the reporter under the control of the endogenous regulatory sequences. In contrast to the “standard” conditional alleles in which only *loxP* sites remain in the locus after removal of the selection cassette, there is a greater likelihood of an introduced reporter altering the transcriptional regulation of the gene in question. In addition, just like any other knock-in, the introduced reporter may or may not mimic the expression of the endogenous locus.

It is thus important to characterize thoroughly conditional alleles that use reporters, assaying for normal gene activity before Cre-mediated excision and validating the reporter expression pattern after Cre excision. To validate the reporter expression pattern, generate mice heterozygous for the recom-



**FIGURE 10.** Generation of a conditional null allele with a linked reporter by homologous recombination in ES cells. In this strategy, a replacement vector is generated in which a splice acceptor (*SA-lacZ*) pA cassette followed by an *FRT*-flanked *neo* expression cassette is introduced 3' of the gene of interest. *loxP* sites are engineered to flank exons. It is very useful to engineer a restriction site 5' of the 5' *loxP* site to identify targeting events by Southern analysis that have included both *loxP* sites. An HSV *tk* cassette has been placed adjacent to the 3' arm of homology. After homologous recombination, a conditional allele is generated that has the *lacZ* and *neo* expression cassettes 3' of the locus. This allele may behave as wild-type, a hypomorph, or even a null. The *lacZ* reporter should be silent because it is not transcribed and does not have a basal promoter. *neo* can be removed by Flp expression in vitro or in vivo, yielding a conditional null allele with a silent reporter. Cre expression will delete the intervening exons to generate a loss-of-function allele and a chimeric transcript containing exon 1 and *lacZ*, leading to  $\beta$ -galactosidase expression. (Boxes) Exons; (shaded boxes) untranslated regions; (Flox [*lacZ*, +*neo*]) floxed allele that contains the *SA-lacZ* and *neo* cassettes; (Flox [*lacZ*, -*neo*]) floxed allele that contains the *SA-lacZ* cassette without the *neo* cassette; (Null [*lacZ*]) recombined allele with the *SA-lacZ* cassette.

bined allele and compare the reporter expression pattern with that of the endogenous gene during development. This information will define the potential limitations of your reporter. In a number of instances, this type of conditional allele with a linked reporter of excision has acted as a hypomorphic allele before Cre-mediated excision (e.g., mice homozygous for the conditional allele or mice heterozygous for the conditional allele and a null allele of the gene show a phenotype) (e.g., *Fgf8*; Meyers et al. 1998). This would limit the usefulness of the conditional allele, but many experiments are still possible depending on the stage that you want to analyze and when the mice are compromised. The utility of this type of allele in contrast to standard conditional alleles is that recombination can be measured directly and with cellular resolution. So, consider your experiments and decide on the type of allele that best suits your needs.

### Conditional Null Alleles by Homologous Recombination in ES Cells

There are many gene-targeting strategies to make conditional alleles by homologous recombination in ES cells, but all require the use of a positive selectable marker, which may either remain in the conditional allele or be removed, for example, by using a second type of recombinase system, such as Flp recombinase (Figs. 9 and 10). In many cases, a conditional allele with the selectable marker in place can function as a wild-type allele. However, this will have to be determined for each individual case because the presence of the selectable marker and its promoter could potentially generate a null or hypomorphic allele as described in the previous section. If the presence of the selectable marker results

in a decrease in the level of gene expression, this hypomorphic allele could provide a useful genetic alteration for further analysis.

To ensure that the recombinase sites will function as planned in the targeted allele, it is worth testing for excision of the floxed and flrtd exons in vitro in ES cells before mice are made. Demonstrate that the specific recombinase sites flanking the selection cassette function to delete the selection cassette in vitro (Box 2). In addition, show that the recombinase sites flanking critical exons function to delete those exons in vitro. If you are waiting until later to remove the selection cassette in vivo by breeding, take the time now to express the appropriate recombinase(s) in vitro to ensure the deletion can be produced. If one of the recombinase sites is damaged, then you will be generating mice with an allele that will not function properly, wasting a lot of time and effort.

Mice with the conditional allele should be phenotypically identical to wild-type mice and thus easily maintained as homozygous stocks. If this is not the case, you have a hypomorphic or null allele, which was not intended but could still be useful. Once you have a conditional allele in mice, introduction of Cre recombinase by breeding with *cre*-transgenic mice will result in Cre-mediated excision of the floxed coding exons, producing a deletion allele at a time and tissue of your choosing. For breeding strategies to make use of a conditional allele for time- or tissue-specific gene ablation, see Chapter 18: Tissue- and/or Temporal-Specific Mutations in Mice Using Conditional Alleles (Papaioannou and Behringer 2023f).

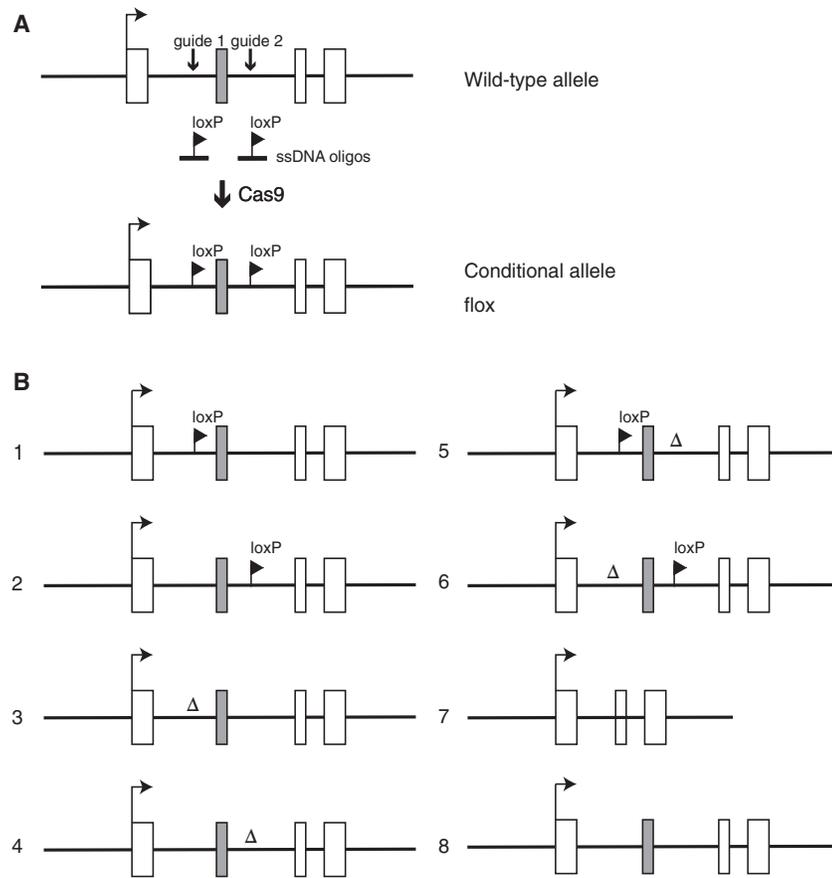
### *Conditional Null Alleles Using CRISPR–Cas*

CRISPR–Cas can be used to generate conditional null alleles by directly modifying zygotes (Clark et al. 2020). Multiple approaches have been developed. One method is independent of the distance between the placement of the two *loxP* sites and the others are limited by this distance.

The introduction of two *loxP* sites using single-stranded oligonucleotides is independent of the distance between the two sites (Fig. 11A). *LoxP* sites are 34 bp in length. Thus, they can be introduced into the genome using a guide RNA, Cas9, and an oligonucleotide that includes the *loxP* sequence surrounded by target gene homology (see Producing Point Mutations or Small Changes, above). You can attempt to place both *loxP* sites directly into zygotes simultaneously, but obtaining both *loxP* sites flanking the intended exon(s) on the same chromosome can be inefficient (Yang et al. 2013; Gurusurthy et al. 2019). It is more likely that you will obtain mice containing one or the other of the *loxP* sites targeted in the correct position (Fig. 11B). If male founders with a single correctly targeted *loxP* site are generated, then you can wait until they are sexually mature, isolate and cryopreserve their sperm, and subsequently perform in vitro fertilization (IVF) of oocytes obtained by superovulation of wild-type females (Behringer et al. 2018; Takeo and Nakagata 2018a,b), and repeat the CRISPR–Cas injection or electroporation to insert the second *loxP* site. You could do this through natural matings to preserve the male founder, but the oocyte yield from plugged females even if superovulated will be lower and the process could take much longer. Although you must go through an extra generation to obtain the floxed allele using this sequential targeting approach, it appears to work relatively efficiently to generate the desired conditional null allele. If you perform this sequential targeting approach, there are a few caveats to consider.

First, the founder male with one *loxP* site correctly inserted at the target locus should be genotyped very carefully. It is possible that CRISPR–Cas induced an indel at the introduction site for the second *loxP* site, which would prevent its insertion. Thus, you must select founder males that have a wild-type sequence at the location for insertion of the second *loxP* site or redesign the guide to target the indel sequence. Second, you can use oligonucleotides to introduce both *loxP* sites during the second round. This increases the chances of obtaining *loxP* sites in *cis* on the same chromosome, but genotyping will be required to determine if the *loxP* sites are in *cis* or *trans*.

There are two methods of making a conditional allele that are dependent on the distance between the two *loxP* sites.

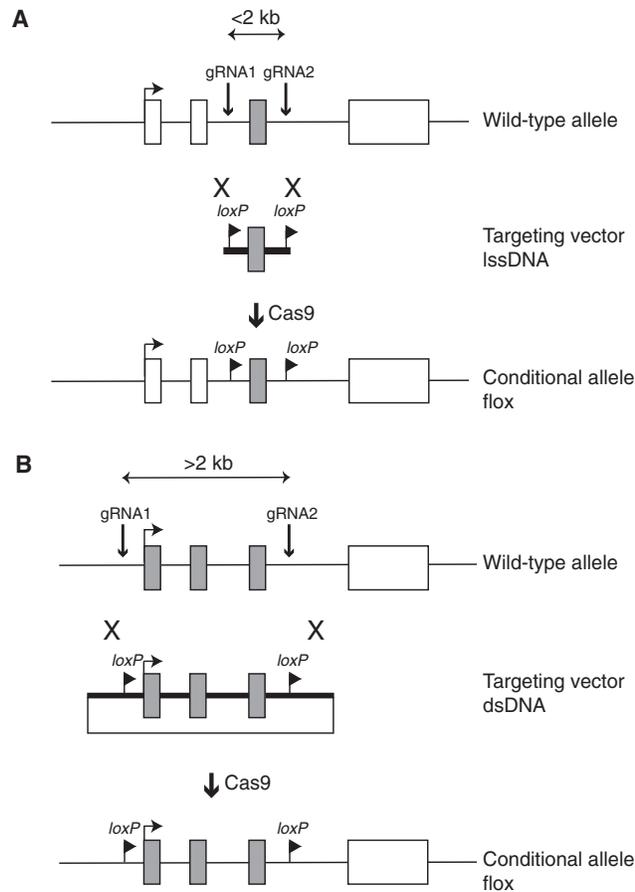


**FIGURE 11.** Generation of a conditional null allele using CRISPR–Cas and oligonucleotides. (A) Wild-type and conditional (floxed) alleles are shown. (Open boxes) exons; (arrow on exon 1) start of transcription. Two guide RNAs recognize the locations for introduction of *loxP* sites to flank a critical exon (shaded). Single-stranded DNA oligonucleotides of ~100 nt with the *loxP* sites centrally located are synthesized. Guide RNAs, ssDNA oligos, and Cas9 (DNA, RNA, or protein) are introduced into zygotes by injection or electroporation. If successful, the exon will be flanked by *loxP* sites. (B) Other possible outcomes from the experiment in A: (1 and 2) only one *loxP* site is introduced; these alleles could be used for a subsequent round of targeting to insert the second *loxP* site; (3 and 4) indels ( $\Delta$ ) are created at one site or the other; (5 and 6) a *loxP* site is correctly inserted at one site but the other site has an indel ( $\Delta$ ); (7) exon 2 is deleted; (8) the locus remains wild-type.

1. If the two sites are to be placed within 2.0 kb of each other, then a ssDNA with ~50–100 bp of homology for the target sequence on each end can be used to introduce both *loxP* sites flanking the desired exon(s) (Fig. 12A; Miura et al. 2018).
2. If the two *loxP* sites are to be placed farther than 2.0 kb apart, then a dsDNA targeting vector can be used to knock in both *loxP* sites at once (Fig. 12B; Yao et al. 2018). When you generate the dsDNA targeting vector, the *loxP* sites are placed such that the PAM site and guide RNA target sequence are split so that CRISPR–Cas does not cut the targeting vector or recut the edited locus.

It is likely that new methods or variations of current methods will yield ever more efficient approaches to generate conditional null alleles by directly modifying zygotes using CRISPR–Cas. So, keep an eye on the literature! Once a targeting strategy and technology has been chosen, you can move on to either Chapter 4: Embryonic Stem Cell Gene Targeting and Chimera Production in Mice (Papaioannou and Behringer 2023b) or Chapter 5: Recovering a Targeted Mutation in Mice from Embryonic Stem Cell Chimeras or CRISPR–Cas Founders (Papaioannou and Behringer 2023c).

## Chapter 3



**FIGURE 12.** Generation of a conditional null allele using CRISPR–Cas and long single-stranded DNA (lssDNA) or double-stranded DNA (dsDNA) targeting vectors. Wild-type and conditional alleles (floxed) are shown. (Open boxes) exons; (arrow on exon 1) start of transcription. (A) If the placement of *loxP* sites is <math>< 2\text{ kb}</math> apart, then a lssDNA is synthesized incorporating *loxP* sites that will flank a critical exon (shaded); 50–100 nt of homology is included at each end of the lssDNA. Guide RNA, lssDNA, and Cas9 (DNA, RNA, or protein) are introduced into zygotes by injection or electroporation. (B) If the placement of *loxP* sites is >2 kb apart, then a dsDNA gene-targeting vector is created incorporating *loxP* sites that will flank critical exons (shaded); 0.5–2.0 kb of homology is included at each end of the targeting vector. The PAM sequence is mutated in the dsDNA target vector so that it will not be cut by CRISPR–Cas before or after gene editing. Guide RNA, dsDNA targeting vector, and Cas9 (DNA, RNA, or protein) are introduced into zygotes by injection or electroporation.

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