REVIEW

A most formidable arsenal: genetic technologies for building a better mouse

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The mouse is one of the most widely used model organisms for genetic study. The tools available to alter the mouse genome have developed over the preceding decades from forward screens to gene targeting in stem cells to the recent influx of CRISPR approaches. In this review, we first consider the history of mice in genetic study, the development of classic approaches to genome modification, and how such approaches have been used and improved in recent years. We then turn to the recent surge of nuclease-mediated techniques and how they are changing the field of mouse genetics. Finally, we survey common classes of alleles used in mice and discuss how they might be engineered using different methods.

The origins of mice as a genetic system

The house mouse, *Mus musculus*, has been a tool of genetic inquiry for well over a century. A case can be made that the tradition dates back even further to 18th century Japan and China where local mouse and rat fanciers wrote guidebooks on how to raise these animals, including detailed breeding strategies to obtain particular coat patterns (Tokuda 1935; Kuramoto 2011). The hobby of mouse fancying was exported to England and from there to the United States, where fancier stocks formed the basis of many modern inbred strains (Morse 1978). Nevertheless, the first genetic experiment using mice may have been performed in 1887 by August Weismann, who surgically removed the tails of mice, bred them, and found offspring with tails of normal length (Weismann 1889). This result challenged Lamarckian inheritance and led Weismann to propose the germ theory of inheritance, which stated that hereditary information was transmitted only through germ cells: the sperm and egg. The origin of mice as a model system for genetic analysis, with intrinsic variable and heritable traits worthy of study, dates to 1902 when Lucien Cuénot published the first in a series of papers de-

scribing Mendelian inheritance for mouse coat color characteristics (Cuénot 1902). Mendel himself had started down this same road fifty years earlier, only for his bishop to snuff out the nascent breeding program because a monk had no business, frankly, breeding (Paigen 2003).

Following Cuénot's papers (Cuénot 1902), genetic research using the mouse began in earnest, often through the study of spontaneous mouse mutants. One line of research further explored the complicated genetics of pigmentation and the sometimes-surprising coincident phenotypes. The Dominant white spotting allele *W* (an allele of the receptor tyrosine kinase *Kit*) was found to affect not only pigmentation but also hematopoiesis and fertility, for instance. Another line focused on the genetic basis of other variable traits such as histocompatibility, sex determination and X-inactivation, and classic studies of tail length and the *T* locus (Silver 1995). Perhaps the most consequential endeavor, however, was the effort to understand the relationship between genetics and cancer, as this led to the establishment of many of today's inbred mouse strains as well as the founding of mouse genetic research centers such as the Jackson Laboratory in 1929, Oak Ridge in 1943, MRC Harwell in 1947, and the National Institute of Genetics in Japan in 1949 (Silver 1995). Alongside research using laboratory inbred strains, study of wild mouse populations led to important insights in immunity, cancer, and adaptation (Phifer-Rixey and Nachman 2015). Modern genomic techniques now couple wild populations' variations and selective pressures with molecular precision (Harr et al. 2016; Barrett et al. 2019). A full accounting of the history of mouse genetic research is beyond the scope of this article, but has been the subject of several excellent books and reviews (Russell 1985; Silver 1995; Paigen 2003; García-García 2020).

Even with these impressive genetic bona fides, the true potential of mouse genetics lies in the ability of the researcher to modify the mouse genome. This ability, along with the fact that mice are mammals with substantial genetic similarity to humans, has made mice a favorite

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system for understanding the genetic underpinnings of mammalian development and human disease (Rosenthal and Brown 2007). Indeed, as of July 28, 2020, there were 64,204 mutant mouse alleles listed in the Mouse Genome Informatics (MGI) database. The tools available to generate these alleles have been steadily expanded from forward genetic screens, to viral and gene trap-mediated approaches, as well as classic homologous recombination-based targeting in mouse embryonic stem cells (mESCs) that served as the basis for the myriad knockout, knock-in, knockdown, conditional, and lineage-tracing mouse models that now exist. More recently, nuclease-mediated technologies such as CRISPR/Cas have led to a second explosion of techniques. These not only promise an easier, more efficient means to traditional genetic ends, but are also opening the door to ways of modifying the genome that were previously impractical or impossible with classical techniques.

Forward genetic screens

Forward genetic screens involve germline mutagenesis and subsequent breeding of male mice to obtain a collection of novel mutations. Offspring in the first generation (termed G1, the result of crossing a mutagenized mouse with a wild-type female) can be immediately screened for dominant mutations giving rise to a phenotype of interest. For recessive mutations, G1 males can be crossed with a wild-type female and then any G2 females are crossed back to their G1 father to generate homozygous offspring in the third generation (G3). After identifying mutant lines with the desired phenotype, the underlying mutation is identified. This was traditionally achieved by time-consuming genetic mapping but can now be resolved through genome sequencing, greatly accelerating the overall process (Wang et al. 2015; Geister et al. 2018). The advantage of genetic screens is that mutations are generated semi-randomly throughout the genome, so screens are unbiased with respect to the underlying mutation and can reveal unexpected genotype–phenotype connections. One reason for this is that such screens can identify many genes important for a given process that have not yet been investigated or linked to that process. A second is that genetic screens induce not only null alleles, but also hypomorphic, hypermorphic, and neomorphic alleles through point mutations, truncations, insertions, deletions, and chromosomal rearrangements, that can affect coding or regulatory sequences. Of note, chemical mutagenesis and other nontargeted genome modification techniques (e.g., transgene, retrovirus, and transposon insertion events, discussed below) are not completely random but exhibit some bias or unique preference in mutation/insertion site (Takahashi et al. 2007; Li et al. 2013; de Jong et al. 2014). We therefore refer to these events as semi-random throughout the review. Although the prospect can be daunting, with careful planning screens can be performed by individual laboratories (Horner and Caspary 2011) and often reveal exciting, unforeseen biology (Huangfu et al. 2003). Screens have

been used to better understand topics ranging from mouse development (Kasarskis et al. 1998; Nolan et al. 2000), to circadian rhythms (Vitaterna et al. 1994), congenital heart disease (Li et al. 2015), and the genetic basis of sleep (Miyoshi et al. 2019), among many others.

The precursor to mouse genetic screens arose from an interest in cancer and the effects of atomic radiation on mammals. These effects were investigated using mice, principally at the Oak Ridge National Laboratory in Oak Ridge, Tennessee, and the MRC Radiobiological Research Unit in Harwell, England. This research generated numerous mutant mouse strains, but because X-rays induce double-stranded breaks (DSBs) in DNA, the approach often caused large deletions and chromosomal rearrangements that affected multiple genes, making interpretation of the mutant phenotypes difficult (Silver 1995). Radiation has been supplanted by chemical mutagenesis with the DNA alkylating agent N-ethyl-N-nitrosourea (ENU), which is more mutagenic than X-rays (Russell et al. 1979) and more likely to result in point mutations (Popp et al. 1983). Because screening large numbers of mice in the G3 generation is so time- and labor-intensive, there are several modified approaches that allow for recovery of recessive mutations in the G2 generation. Specifically, modifier (enhancer or suppressor) and noncomplementation screens are performed by crossing G1 mutants with an existing mutant strain and assaying for a modified phenotype or failure to complement. This usually identifies new genes that genetically interact with the gene of interest or novel alleles of the same gene, respectively. Deletion screens, in which G1 males are crossed to females carrying a deletion of a chromosomal interval, facilitate rapid identification of mutations in this region. Finally, balancer screens, most famous from the world of fly genetics, can also be performed in mice even though the number of existing balancer chromosomes is limited (Zheng et al. 1999). These involve crossing mutagenized males to mice carrying a balancer chromosome, which has the following features: one or a series of tandem chromosomal inversions that suppress recombination within the region, a dominant visible trait such as coat color, and often a recessive lethal mutation (Zheng et al. 1999; Ye et al. 2016). This combination of factors allows efficient breeding schemes to quickly isolate mutations within the balanced chromosome or region (Kile et al. 2003).

Transgenics, viruses, transposons, and gene trap-mediated transgenesis

Transgenesis is the process of introducing genetic information from one organism into another. The first mouse carrying exogenous DNA was made in 1974 by injecting simian virus 40 (SV40) DNA into the cavity of an E3.5 mouse blastocyst, although the DNA was incorporated variably throughout the animal (Jaenisch and Mintz 1974). A similar study using infection of morula-stage embryos with Moloney leukemia virus subsequently demonstrated that exogenous provirus could integrate into the germline (Jaenisch 1976). The technique was refined to

show that injection of DNA into the pronucleus of a one-cell embryo resulted in incorporation and expression of DNA from a variety of sources (Brinster et al. 1981; Costantini and Lacy 1981; Gordon and Ruddle 1981; Harbers et al. 1981; Wagner et al. 1981a,b). Transgenesis via pronuclear injection is still popular whenever expression of an exogenous gene is desired (Palmiter and Brinster 1985; Jaenisch 1988; Pu et al. 2019). The technique is reliable, although relatively inefficient and the microinjection process itself has the potential to induce cellular and chromosomal damage (Yamauchi et al. 2007). Nevertheless, pups can quickly be screened by PCR to identify transgenics. Due to its semi-random site of integration, the transgene is subject to the effects of local environment (chromatin state, proximity to enhancers, etc.) such that individual founders can exhibit different expression levels and expression patterns, their expression levels can lessen over time due to methylation-induced silencing, and they often have multiple copies of the transgene integrated in tandem (Wall 2001; Davis et al. 2012). These effects can be partially suppressed through the inclusion of insulator elements on both ends of the inserted sequence (Giraldo et al. 2003), though position effects are still sometimes observed (Farzaneh et al. 2019). Furthermore, transgenes can disrupt endogenous genes by insertional mutagenesis so transgenics should be analyzed for unexpected phenotypes. Transgenics are most safely maintained as heterozygotes because homozygotes can be complicated to genotype and are more likely to display undesired phenotypes (Davis et al. 2012). However, transgenics can be bred to homozygosity to check whether an essential gene was disrupted or whether the transgene has unexpected dosage-dependent toxicity (Wagner et al. 1983; Woychik et al. 1985). Multiple individuals should always be screened for expression patterns or levels and founders selected carefully. The insertion site should be mapped whenever possible. Once laborious, this is now facilitated through various PCR techniques or next-generation sequencing. Alternatively, transgenesis can be targeted without resorting to homologous recombination-based techniques by recombinase-mediated insertion, provided the recognition site has been knocked in to the genome (Schilit et al. 2016).

Several techniques that also rely on the random insertion of genetic material were developed in the intervening decades to enable lineage tracing, gene knockout, and gene and enhancer discovery that we only briefly mention here, as they have largely been superseded by other methods. Infection of preimplantation or postimplantation embryos with retroviruses leads to semi-random integration of the viral genome and occasional disruption of mouse genes, which can then be identified using viral sequences as a landmark (Schnieke et al. 1983; Soriano et al. 1987). Similarly, mESCs can be infected with a retrovirus, the cells injected into blastocyst stage embryos, and the mutant lines recovered by breeding the chimeric offspring (Robertson et al. 1986).

Transposons are genetic elements that insert semi-randomly into the genome upon expression of a transposase enzyme (Beckmann and Largaespada 2020). Terminal inverted repeat sequences located at each end of a transpo-

son are bound by the transposase, which mobilizes the element and mediates its insertion at a new locus. Several systems have been used in mice, each with their own efficiencies and insertion biases, but piggyBac and Sleeping Beauty are the most widely used. These systems have been used to drive insertional mutagenesis in both mESCs (Luo et al. 1998) and mice (Collier et al. 2005; Ding et al. 2005; Dupuy et al. 2005). Transposon studies carried out in an embryo or adult mouse are basically a variation of a forward genetic screen. The transposon is typically activated in a subset of cells, mice with a desired phenotype are identified (often a tumor or developmental malformation), and the transposon insertion site is mapped.

Gene trapping has been used widely to generate mutant alleles, rapidly clone them, and assess their expression patterns (Gossler et al. 1989; Friedrich and Soriano 1991; von Melchner et al. 1992; Friedel and Soriano 2010). Typically, a trapping vector is used that contains a splice acceptor attached to a promoterless reporter gene such as *lacZ* or *βgeo*. mESCs are transfected with the vector or infected with a retrovirus transducing a gene trap and selected for expression of the reporter gene, as insertion within an intron of an expressed gene will also result in marker expression. The trapped gene can be cloned using 5' or 3' RACE and sequences from the reporter gene (Chen et al. 2004). One caveat of the approach is that alternative splicing can splice around the trap in some gene trap designs if the splice acceptor is not strong enough, resulting in a hypomorphic allele (McClive et al. 1998). A second is that correctly trapped genes will not be identified if the gene is not expressed in mESCs. Nonexpressed genes can be successfully trapped, however, by using polyadenylation trap vectors. These use a separate promoter to drive expression of the resistance marker, which itself contains a splice donor but no poly(A) sequence, relying instead on the 3' UTR of the trapped gene (Zambrowicz et al. 1998; Stanford et al. 2001; Chen et al. 2004). Multiple large-scale trapping efforts have taken place, sometimes targeting specific classes of genes, many using sophisticated vectors that allow further modification or conditional mutagenesis of the trapped locus (Wurst et al. 1995; Zambrowicz et al. 1998; Mitchell et al. 2001; Stanford et al. 2001; Chen et al. 2004; Skarnes et al. 2004; Schnutgen et al. 2005; Singla et al. 2010). A significant number of genes have also been trapped through the targeted insertion of promoterless trap vectors using homologous recombination rather than random insertion (Friedel et al. 2005). Numerous trapped lines are available that can be used to generate mutant mice (Table 1).

Embryonic stem cells and homologous recombination

Experiments performed in the 1970s demonstrated that tumor-derived embryonal carcinoma cells injected into mouse blastocysts could generate chimeric mice but never successfully colonized the germline (Brinster 1974; Mintz and Illmensee 1975; Papaioannou et al. 1975). In 1981, Evans and Kaufman (1981) and Martin (1981) independently isolated embryo-derived mESCs and

Table 1. Mouse genetic resources for finding mutant mouse strains, ES lines, genome information, and other helpful sites

Mutant mouse resources				
Resource	Acronym	Description	References	Website
Mouse Genome Informatics	MGI	Database of mouse alleles, gene expression, phenotypes, publications, and recombinases	Law and Shaw 2018	http://www.informatics.jax.org
International Mouse Strain Resource	IMSR	Database of mouse strains, stocks, and ES lines and links to the holding institution	Eppig et al. 2015	http://www.findmice.org
Knockout Mouse Project Repository	KOMP	Database for cells and mice generated through the KOMP	Austin et al. 2004	https://www.komp.org
International Mouse Phenotyping Consortium	IMPC	Standardised phenotyping data for mutant mice made by the International Mouse Knockout Consortium.	Dickinson et al. 2016	https://www.mousephenotype.org
Mutagenetix		Database of ENU alleles from the Beutler laboratory and the Australian Phenomics Network	Wang et al. 2018	https://mutagenetix.utsouthwestern.edu/home.cfm
International Gene Trap Consortium	IGTC	Database of gene trap lines from member laboratories and institutions	Nord et al. 2006	https://igtc.org
National Mouse Metabolic Phenotyping Centers	MMPC	USA-based centers providing mouse phenotyping services		https://www.mmpc.org
International Society for Transgenic Technology	ISTT	Scientific society supporting the rapid exchange of transgenic technology and ideas; maintains online resources, listserv, and organizes a meeting		https://www.transtechsociety.org
Mutant mouse repositories ^a				
Repository	Acronym	Strains	Location	Website
Australian Phenome Bank	APB	1619 mouse strains	Australia	http://pb.apf.edu.au
Center for Animal Resources and Deveopment	CARD	1757 mouse strains; 31 mESC lines	Japan	http://cardb.cc.kumamoto-u.ac.jp/transgenic
Canadian Mouse Mutant Repository	CMMR	845 mouse strains; 13,654 mESC lines	Canada	http://www.cmmr.ca
Charles River Laboratories	CRL	56 mouse strains	USA	https://www.criver.com
Envigo (formerly Harlan Sprague Dawley)	HSD	45 mouse strains	USA	https://www.envigo.com
European Mouse Mutant Archive	EMMA	6920 mouse strains.	Europe	https://www.infrafrontier.eu
European Mouse Mutant Cell Repository	EuMMCR	16,828 mESC lines.	Europe	http://www.eummc.org
GemPharmatech	GPT	6910 mouse strains	China	http://www.gempharmatech.com/en
MRC Harwell	HAR	1491 mouse strains	UK	http://www.har.mrc.ac.uk
JAX Mice and Services	JAX	11,109 mouse strains; 2 mESC lines	USA	https://www.jax.org/jax-mice-and-services
Korea Mouse Phenotyping Center	KMPC	157 mouse strains	Korea	http://www.mouseinfo.kr
National Cancer Institute at Frederick	NCIMR	139 mouse strains	USA	http://mouse.ncifcrf.gov
National Institute of Genetics	NIG	142 mouse strains	Japan	http://www.shigen.nig.ac.jp/mouse/nig
Oak Ridge Collection at JAX	ORNL	908 mouse strains	USA	https://www.jax.org/research-and-faculty/tools/oak-ridge-strains
RIKEN BioResource Research Center	RBRC	5357 mouse strains; 1747 mESC lines	Japan	http://www.brc.riken.jp/lab/animal/en
National Applied Research Laboratories	RMRC-NLAC	351 mouse strains	Taiwan	http://www.nlac.org.tw/RMRC/index_e.aspx
Shanghai Model Organisms Center	SMOC	2898 mouse strains	China	https://www.modelorg.com
Taconic Biosciences	TAC	2725 mouse strains	USA	http://www.taconic.com

Continued

Table 1. *Continued*

Mutant mouse repositories ^a				
Repository	Acronym	Strains	Location	Website
Texas A&M Institute for Genomic Medicine	TIGM	195 mouse strains; 142,543 mESC lines	USA	http://www.tigm.org
University of North Carolina, Chapel Hill Systems Genetics Core	UNC	75 mouse strains	USA	http://csbio.unc.edu/CCstatus/index.py
Genome information				
Resource	Acronym	Description	References	Website
University of California, Santa Cruz Genome Browser	UCSC	Genome browser with integration of wide-ranging “omics” data sets as unique tracks, including predicted CRISPR sites	Lee et al. 2020a	https://genome.ucsc.edu
Ensembl	Ensembl	Genome browser and annotation; resources for comparative genomics, regulation, and genome variation	Yates et al. 2020	https://www.ensembl.org/Mus_musculus
National Center for Biotechnology Information	NCBI	National sequence repository with extensive bioinformatic tools		https://www.ncbi.nlm.nih.gov

^aInformation retrieved from the International Mouse Strain Resource (IMSR) at <http://findmice.org> on June 12, 2020, except for Envigo, whose information was retrieved from their website. Several smaller repositories omitted for space can be found at <http://findmice.org>

demonstrated that in chimeras these cells could contribute much more extensively to multiple organs. Several years later it was shown that mESCs were able to colonize the germline (Bradley et al. 1984), even when carrying a transgene (Gossler et al. 1986; Robertson et al. 1986). In 1987, building on work in cancer cells and fibroblasts, homologous recombination (HR) between exogenous and chromosomal DNA was demonstrated in mESCs (Folger et al. 1982; Lin et al. 1984; Smithies et al. 1984, 1985; Thomas et al. 1986; Doetschman et al. 1987; Thomas and Capecchi 1987). By introducing exogenous DNA with a desired genetic alteration between two arms of homology to a region of interest, it became possible to make precise genetic modifications. Because the natural ratio of HR to random insertion is generally low, typically around one to several thousand (Würtele et al. 2003), the first alterations were to genes where correctly targeted cells could be directly selected for, for example, mutation of the gene *Hprt*, which allowed growth in selective media (Doetschman et al. 1987; Hooper et al. 1987; Kuehn et al. 1987; Thomas and Capecchi 1987). HR was soon extended to nonselectable loci, by screening large numbers of clones by PCR (Joyner et al. 1989; Zimmer and Gruss 1989), and demonstrated in nonexpressed genes (Johnson et al. 1989). The process was made more efficient with the introduction of positive-negative selection that selects both for cells that integrate donor DNA into the genome and simultaneously against cells that integrate the DNA randomly (Mansour et al. 1988). This constellation of breakthroughs enabled scientists to manipulate the mouse genome with precision and relative ease. Almost any genetic change one could imagine could now be introduced and studied in a living mammal.

In ES cell gene targeting strategies, the targeting constructs are typically defined by two homology arms (5' and 3') that are several kilobases in length. These flank a selectable marker such as *neoR* and any mutations or genetic sequence to be inserted at the intended locus. The positive selection marker can be flanked by *loxP* or FRT sites to enable its later removal using a site-specific recombinase (SSR) (see “Conditional Mutations” below). This is oftentimes desirable as its presence can interfere with local gene expression (Rijli et al. 1994; Fiering et al. 1995) or can affect gene expression from the locus (Meyers et al. 1998). Outside the homology arms is a negative-selection marker, most commonly herpes simplex virus-1 thymidine kinase (HSV-TK) or diphtheria toxin fragment A (DTA), which selects against random insertion (Mansour et al. 1988; Yagi et al. 1990). Targeting efficiencies for positive-negative HR can range from <1% of clones correctly targeted to near 80% for permissive loci using well-optimized vectors. The ratio of HR to random insertion depends on how the DNA is introduced to cells, electroporation yielding the best ratio for the effort (Vasquez et al. 2001). The efficiency of targeting can be improved by increasing the length of the homology arms (Hasty et al. 1991; Deng and Capecchi 1992). Of particular importance, homology arms constructed from the same strain as the mESC line that will be targeted greatly increase the targeting rate (te Riele et al. 1992). Most early targeting was performed in mESCs derived from the agouti-pigmented 129 mouse strain, as these cells had a high rate of germline transmission (Simpson et al. 1997; Threadgill et al. 1997). Once the targeted mESC clone is verified, the cells can either be injected into the cavity of embryonic day E3.5 blastocyst stage embryos or aggregated with

E2.5 morula stage embryos (Behringer et al. 2014). Because not all chimeras have mESC contribution to the germline, using agouti 129 mESCs and nonagouti host embryos, chimeras with the highest degree of overall mESC contribution can easily be chosen based on coat color. Alternatively, complete germline transmission can be consistently achieved by injecting mESCs into a mutant mouse strain that cannot form male germ cells, a technique that is offered commercially (Koentgen et al. 2016). If the final mouse needs to be a different strain, then at least 10 generations of time-consuming and costly backcrossing are required to be considered congenic, though this can be accelerated by speed congenics (Markel et al. 1997; Wakeland et al. 1997). Even after backcrossing, genes near the targeted locus are more likely to be derived from the mESC line, introducing a variable that could complicate subtle phenotypes such as behavior (Gerlai 1996). Indeed, the strain for an experiment should be carefully considered as different backgrounds can show variable phenotypes from an otherwise identical underlying mutation (Brewer et al. 2015). By using newer culture conditions there are now mESC lines from other inbred strains that show high degrees of germline competency, such as C57BL/6N-derived JM8 or VGB6 cells used in the Knockout Mouse Project (KOMP) (Poueymirou et al. 2007; Pettitt et al. 2009). Additionally, there are protocols for deriving germline-competent mESCs from many strains (Czechanski et al. 2014) and numerous lines are commercially available. Therefore, when possible, researchers should build their targeting constructs and perform the targeting itself in a mESC line derived from the same strain they use in their mouse colony. Alternative sources of germline-competent pluripotent cells include embryonic germ cells and induced pluripotent stem cells, though these are rarely used for generating novel mouse models (Labosky et al. 1994; Stewart et al. 1994; Okita et al. 2007; Wernig et al. 2007).

Alternative techniques to accelerate targeting or boost its efficiency have also been explored. The possibility of HR directly in zygotes was tested, but resulted in a single case of successful targeting among 500 embryos injected, so the technique was considered infeasible (Brinster et al. 1989). Knockdown of *Blm*, a known suppressor of HR, improved targeting by around threefold at multiple loci (Luo et al. 2000; Fukuda et al. 2016). Most portentously, inducing a DSB at the edit site, here by using a rare cutting endonuclease, enhanced HR efficiency as much as 5000-fold (Smith et al. 1995; Donoho et al. 1998). However, until recently, generating such a specific DSB was so laborious as to be impractical.

Programmable nucleases

In 1996, zinc finger nucleases (ZFNs) were introduced, representing a new customizable method for the induction of DSBs at a specific site in the genome (Kim et al. 1996). ZFNs are engineered proteins consisting of multiple ZF motifs and the FokI nuclease domain. The FokI domain induces DSBs in DNA when bound as a dimer, re-

quiring two ZFNs to bind in reverse orientation, one on each strand of DNA (Bitinaite et al. 1998; Smith et al. 2000). Typically, each ZF binds a 3-nt recognition sequence; with concatenated domains, a recognition sequence of nine or more nucleotides can be engineered. However, designing ZFNs in a purely modular fashion is difficult, as the recognition sequence of individual fingers may be altered by the adjacent fingers (Carroll 2011).

Transcription activator-like effector nucleases (TALENs) were the next evolution in targetable nucleases (Christian et al. 2010). Like ZFNs, TALENs use the FokI nuclease domain to facilitate the induction of DSBs. However, rather than relying on ZFs, TALE DNA-binding domains are used to dictate sequence specificity. Each TALE domain recognizes a single nucleotide based on the repeat variable di-residue (RVD), a pair of amino acids that dictate nucleotide interaction (Boch et al. 2009; Moscou and Bogdanove 2009); multiple domains can be strung together to produce a nuclease with a unique binding sequence. TALENs provided the potential to target any sequence of nucleotides within a genome.

While both ZFNs and TALENs provided a valuable resource for genetic manipulation and were successfully used in embryos to make mutant mice (Meyer et al. 2010; Sung et al. 2013), new nucleases need to be constructed for each target. Additionally, if the target does not contain inverted binding sites for each copy of the nuclease, two different nucleases would need to be produced to allow for dimerization of FokI. In the process of testing binding efficiency and specificity, numerous protein constructs would need to be cloned and produced for each locus. Over the years, various tools and databases have been developed to offer improved prediction and design of both ZFNs and TALENs (Heigwer et al. 2013; Joung and Sander 2013; Feng et al. 2014; Sakuma and Yamamoto 2016; Zhao et al. 2016). However, because of this complicated workflow, they were quickly overshadowed by the advent of an RNA-guided endonuclease.

CRISPR/Cas9 editing

The history of CRISPR (clustered regularly interspaced short palindromic repeats) began with the observation of a repeated DNA sequence in *E. coli* (Ishino et al. 1987). The function of these repeated sequences was subsequently identified in adaptive immunity (Mojica et al. 1995; Jansen et al. 2002; Barrangou et al. 2007). The typical locus of a CRISPR system (Fig. 1A) consists of a spaced repeat region accompanied by a protein-coding region. The repeat region acts as a library of previous infections, with unique spacers between each repeat corresponding to previously encountered invading DNA. Each spacer and repeat represent a CRISPR RNA (crRNA). The adjacent protein-coding region encodes multiple CRISPR-associated (Cas) proteins that are involved in the acquisition, processing, and utilization of crRNAs. Mature crRNAs function to guide the Cas endonuclease to target DNA (Fig. 1B). The cleavage target for a given Cas complex is specified by two elements: a recognition sequence

Figure 1. Overview of CRISPR/Cas mechanism. (A) A typical CRISPR/Cas9 locus consists of a repeat-spacer array, transactivating CRISPR RNA (tracrRNA), and a Cas protein-coding region. (B) The repeat-spacer array encodes the CRISPR RNAs (crRNA), which form a heteroduplex with the tracrRNA and guide the Cas9 endonuclease to its target. The crRNA:tracrRNA duplex can be replaced with a single guide RNA (gRNA). (C) Binding of the gRNA is dictated by the protospacer sequence and the protospacer adjacent motif (PAM) site located downstream. The specific PAM site is unique for the various orthologs of Cas9. (R) A/G; (W) A/T; (Y) C/T (D) Cas9 creates DSBs via its RuvC and HNH nuclease domains. Cas9 nickases can be made via a single amino acid substitution in either of the nuclease domains. (E) Cas12a, another single nuclease Cas system, uses a single crRNA, an increased protospacer region, an upstream PAM site, and a single RuvC domain that induces a 5-nt 5' overhang.

(protospacer) encoded in the crRNA that must occur in the target DNA next to a protospacer adjacent motif (PAM) whose identity is specific to individual Cas complexes (Fig. 1C). Once the Cas complex is bound to a target, endonuclease domains induce DSBs in the DNA (for review, see Marraffini and Sontheimer 2010).

Cas9, the endonuclease of the class 2 type II CRISPR system (Makarova et al. 2020), was the first to be explored for gene editing as it only required a single protein (Jinek et al. 2012; Cong et al. 2013; Mali et al. 2013). Type II systems require an additional nonprotein-coding transactivating CRISPR RNA (tracrRNA) that is necessary for the recruitment and activation of Cas9 (Deltcheva et al. 2011). The crRNA:tracrRNA complex can be replaced with a single guide RNA (sgRNA or gRNA) that contains both sequences and a short linker to maintain proper conformation (Fig. 1B). Harnessing this technology, the first mouse models were created, paving the way for the use of CRISPR/Cas9 in mice (Shen et al. 2013; Wang et al. 2013). The efficiency of CRISPR/Cas editing promised to speed up the creation of in vivo mouse models by bypassing mESCs to directly edit zygotes.

Early experiments used *Streptococcus pyogenes* Cas9 (SpCas9), which recognizes an NGG PAM site. While theoretically, NGG, or its complement, NCC, should occur once every 8 bp in a random sequence, the specificity of the protospacer and the requirement of the PAM to occur as close to the editing site as possible left targets of interest without efficient gRNAs. Subsequently, groups began using directed evolution to alter the PAM specificity of Cas9. Additionally, SpCas9 orthologs, such as the *Staphylococcus aureus* SaCas9, proved a valuable resource as they recognize different PAM sequences (Fig. 1C; Kleinstiver et al. 2015a,b). Cas12a, previously known as Cpf1, is an additional class 2 single protein CRISPR/Cas system that recognizes a T-rich PAM sequence (Fig. 1E), supply-

ing new editing possibilities for AT-rich regions of the genome (Zetsche et al. 2015). Additionally, Cas12a is a type V system and does not require the use of a tracrRNA (Makarova et al. 2015). Currently, a plethora of Cas9 and Cas12a variants provide flexibility in PAM and gRNA selection (Table 2; Ma et al. 2019; Chatterjee et al. 2020; Miller et al. 2020; Walton et al. 2020).

Additional mutations have been made in Cas9 to alter the functionality of the enzyme. Mammalian codon optimization was used to improve expression and nuclear localization sequences were added to facilitate proper localization (Cong et al. 2013; Mali et al. 2013). Early alterations also targeted the nuclease domains. Cas9 contains two nuclease domains: RuvC cleaves the targeted strand, while HNH cleaves the nontargeted strand (Fig. 1D). A D10A substitution inactivates the RuvC domain, while an H840A substitution inactivates the HNH domain (Jinek et al. 2012). Inactivating one domain results in a DNA nickase (Cas9n) while inactivating both results in catalytically dead Cas9 (Cas9d). The modified activity has not only aided the development of multiple genome engineering strategies, as discussed below, but also opened the door for various nonediting roles, such as transcriptional regulation and chromosomal mapping (Wang et al. 2016a; Adli 2018), the discussion of which is beyond the scope of this review.

CRISPR/Cas can be used to create two general types of mutations. The first is the formation of small insertions or deletions (indels) through the repair of DSBs via nonhomologous end joining (NHEJ) (for review, see Pannunzio et al. 2018). Indels typically result in frameshift mutations, which often lead to an early stop codon and subsequent nonsense-mediated decay (NMD) of mRNA transcripts. To facilitate large deletions, two distinct gRNAs are used to induce DSBs that flank the region of interest, effectively deleting the internal sequence (Li et al. 2014; Zheng et al. 2014; Kraft et al. 2015; Zhang et al. 2015).

Table 2. CRISPR resources: PAM sites or editing windows for Cas variants, base editors, and prime editors, as well as the systems in which each has been tested

CRISPR/Cas nucleases	PAM	Human cells	Mouse cells	Embryos	References
Cas9 (Note: Cas9 PAM sites are downstream from protospacer region)					
SpCas9	NGG	Y	Y	Y	Jinek et al. 2012; Cong et al. 2013; Mali et al. 2013; Shen et al. 2013
SpCas9-VQR	NGAN	Y	Y	Y	Kleinstiver et al. 2015a; Robertson et al. 2018
SpCas9-VRER	NGCG	Y	Y	Y	Kleinstiver et al. 2015a; Robertson et al. 2018
SpCas9-QQR	NAAG	Y	N	N	Anders et al. 2016
xCas9 3.7	NG, NNG, GAA, GAT, CAA	Y	N	N	Hu et al. 2018
SpCas9-NG	NG	Y	N	N	Nishimasu et al. 2018
SpCas9-NRRH	NRRH	Y	N	N	Miller et al. 2020
SpG	NGN	Y	N	N	Walton et al. 2020
SpRY	NRN > NYN	Y	N	N	Walton et al. 2020
SaCas9	NNGRRT	Y	Y	Y	Ran et al. 2015; Zhang et al. 2016
SaCas9-KKH	NNNRRT	Y	Y	Y	Kleinstiver et al. 2015b; Robertson et al. 2018
St1Cas9	NNAGAAW	Y	Y	N	Müller et al. 2016; Agudelo et al. 2020
St3Cas9	NGGNG	Y	N	N	Müller et al. 2016
Nm1Cas9	NNNNGATT	Y	N	N	Hou et al. 2013
Nm2Cas9	NNNNCC	Y	N	N	Edraki et al. 2019
FnCas9	NGG	Y	Y	Y	Hirano et al. 2016
CjCas9	NNNNRYAC	Y	Y	Y	Kim et al. 2017a; Lee et al. 2020c
Cas12a (Note: Cas12a PAM sites are upstream of protospacer region)					
AsCas12a	TTTV	Y	Y	Y	Zetsche et al. 2015; Kim et al. 2016; Hur et al. 2016
AsCas12a-RR	TYCV	Y	Y	N	Gao et al. 2017
AsCas12a-RVR	TATV	Y	Y	N	Gao et al. 2017
CeCas12a	TTTN	Y	N	N	Chen et al. 2020
ErCas12a	YTTN	Y	Y	Y	Liu et al. 2020c
FnCas12a	TTTV	Y	N	N	Zetsche et al. 2015
LbCas12a	TTTV	Y	Y	Y	Zetsche et al. 2015; Kim et al. 2016
LbCas12a-RVRR	TNTN	Y	N	N	Toth et al. 2020
Mb3Cas12a	TTV	Y	Y	Y	Wang et al. 2020d
Base editors	Window	Human cells	Mouse cells	Embryos	References
ABEs					
ABE7.10	N4-N7	Y	Y	Y	Gaudelli et al. 2017; Liu et al. 2018c
ABEmax	N4-N7	Y	Y	Y	Koblan et al. 2018; Huang et al. 2019a
CP-ABEmax	N4-N12	Y	N	N	Huang et al. 2019b
ABE8e	N4-N8	Y	N	N	Richter et al. 2020
SaABE8e	N3-N14	Y	N	N	Richter et al. 2020
enAsABE8e	N8-N14	Y	N	N	Richter et al. 2020
CBEs					
BE3	N4-N8	Y	Y	Y	Komor et al. 2016; Sasaguri et al. 2018
YEE-BE3	N5-N6	Y	N	N	Kim et al. 2017b
YE1-BE3-FNLS	N5-N7	N	Y	Y	Zuo et al. 2020
Target-AID	N1-N5	Y	Y	Y	Nishida et al. 2016; Sasaguri et al. 2018
BE4	N4-N8	Y	Y	Y	Komor et al. 2017
BE4max	N4-N8	Y	Y	N	Koblan et al. 2018; Yang et al. 2020
St1BE4max	N2-N12	Y	Y	N	Agudelo et al. 2020
CP-CBE4max	N4-N11	Y	N	N	Huang et al. 2019b
hyeA3A-BE4max	N4-N15	Y	Y	Y	Zhang et al. 2020a
BEACON	N7-N12	Y	Y	Y	Wang et al. 2020a
Dual BEs					
A&C-BEmax	N2-N17	Y	N	N	Zhang et al. 2020b
SPACE	A-G: N4-N7 C-T: N2-N7	Y	N	N	Grunewald et al. 2020
Target-ACEmax	A-G: N4-N7 C-T: N1-N5	Y	N	N	Sakata et al. 2020
Prime editors					
PE3		Y	Y	Y	Anzalone et al. 2019; Liu et al. 2020b

Continued

Table 2. *Continued*

Online CRISPR resources	Description	References	Website
Addgene CRISPR Guide	A resource for CRISPR plasmids, experimental guides, and an up-to-date blog		https://www.addgene.org/guides/crispr
CHOPCHOP	Aids in the design of gRNAs, lists predicted efficiency and off-targets, and provides a visual interface for gRNA location; is compatible with numerous genomes and PAM sites for Cas9 and Cas12a orthologs; provides advanced customization for various parameters in the design of gRNAs	Labun et al. 2019	https://chopchop.cbu.uib.no
CRISPOR	Aids in the design of gRNAs, lists predicted specificity, efficiency, and off-targets; is compatible with numerous genomes and PAM sites from Cas9 and Cas12a orthologs	Haeussler et al. 2016	http://crispor.tefor.net/crispor.py
CRISPR RGEN Tools	A suite of CRISPR tools that include Cas-OFFinder, Microhomology, and Cas-Designer to aid in the design of multiple aspects of a CRISPR experiment; s compatible with numerous genomes and PAM sites from Cas9 and Cas12a orthologs	Bae et al. 2014	http://www.rgenome.net
E-CRISP	Aids in the design of gRNAs, lists predicted efficiency and off-targets and provides a visual interface for gRNA location; is compatible with numerous genomes and PAM sites for Cas9 and Cas12a orthologs; provides advanced customization for various parameters in the design of gRNAs	Heigwer et al. 2014	http://www.e-crisp.org/E-CRISP/designcrispr.html
GPP sgRNA Designer	Aids in the design of gRNAs; lists predicted specificity and off-targets; has a limited number of compatible genomes and PAM sites for Cas9 and Cas12a; can be used for the design of gRNAs for CRISPRa or CRISPRi experiments		https://portals.broadinstitute.org/gpp/public/analysis-tools/sgma-design
Off-Spotter	Aids in the design of gRNAs; lists predicted off-targets; has a limited number of compatible genomes and PAM sites for Cas9	Pliatsika et al. 2015	https://cm.jefferson.edu/Off-Spotter
Commercial CRISPR resources	Website		
Horizon Discovery	https://horizondiscovery.com/navigation/gene-editing/gene-editing-reagents		
IDT	https://www.idtdna.com/pages/products/crispr-genome-editing/alt-r-crispr-cas9-system		
Synthego	https://www.synthego.com/products/crispr-kits		
Thermo Fisher Scientific	https://www.thermofisher.com/us/en/home/life-science/genome-editing/geneart-crispr.html		

(M) A/C; (R) A/G; (W) A/T; (S) C/G; Y = C/T, K = G/T, V = A/C/G, H = A/C/T, D = A/G/T, B = C/G/T

The second type of mutation is the introduction of specific edits using donor DNA via homology-directed repair (HDR). HDR is much less efficient than NHEJ, leading to a lower success rate. Various groups have worked along multiple lines to improve the efficiency of CRISPR/Cas-induced HDR. Early experiments typically used plasmid dsDNA as a donor, similar in design as used for HR, with modest efficiency (Cong et al. 2013; Mali et al. 2013). Single-stranded oligodeoxynucleotide (ssODN) donors were also used alongside Cas9 to introduce short tags and *loxP* sites, albeit with variable efficiencies (Yang et al. 2013; Bishop et al. 2016). With both strategies, NHEJ was still the predominant outcome. These limitations spurred researchers to consider alternative approaches and attempt to quantify the numerous parameters that produce high-efficiency donors. Two promising systems have been

developed in recent years, Easi-CRISPR and Tild-CRISPR (Quadros et al. 2017; Miura et al. 2018; Yao et al. 2018). Efficient additions with ssDNA inserts CRISPR (Easi-CRISPR) (Quadros et al. 2017) uses long ssDNA (lssDNA) with short homology arms ranging from ~50 to 100 bp in length flanking the sequence to be inserted. Easi-CRISPR has been used to create floxed alleles and knock-in cassettes up to ~1.5 kb, with efficiency ranging from 8.5% to 100% and 25% to 67%, respectively (Quadros et al. 2017). While the results of this study are promising, using lssDNA limits the length of insertion to ~1–1.5 kb due to errors in production. Using dsDNA can introduce larger segments of exogenous DNA into the targeted locus. Targeted integration with linearized dsDNA CRISPR (Tild-CRISPR) (Yao et al. 2018) uses linear dsDNA with 800 bp of homology flanking the desired sequence edit, with

no other DNA outside the homology arms. One group successfully inserted cassettes ranging from 0.6 to 6 kb via injection into mouse embryos, with efficiencies ranging from 6.9% to 54.4% in pups across six genomic loci (Yao et al. 2018). Alternatively, homology-independent targeted integration (HITI) and related techniques have been used to insert various protein tags onto genes of interest. HITI uses a gRNA target sequence in both the gene of interest and the plasmid containing the insertion. The resulting DSBs are then repaired via NHEJ rather than HDR. Unlike HDR-based Cas9 editing, HITI provides an avenue for editing nonmitotic cells and tissues (Suzuki et al. 2016; Gao et al. 2019; Li et al. 2019).

Another method for improving efficiency has been to actively recruit donor DNA to the targeted locus. One option is to use a modified Cas9 containing a monomeric streptavidin domain and biotinylated donor DNA. The strong affinity of streptavidin-biotin recruits the donor DNA to the site of the DSB. Using this strategy, flanking *loxP* sites were successfully inserted at a rate of 15%–22%, when screened in pups (Ma et al. 2017). A second group inserted larger fluorescent tags, reporting high rates of success, with one locus exhibiting correct tagging in three out of three founder males (Gu et al. 2018). Alternatively, Aird et al. (2018) described the use of Cas9 fused to a porcine circovirus 2 rep (PCV) domain. This PCV domain allows for the covalent linkage of ssDNA to the Cas9 protein, which resulted in increased rates of HDR when attempting to insert fluorescent tags (Aird et al. 2018). However, the investigators only examined the use of this strategy in human cell lines. As such, the translation of this success into mice is unknown, but promising. Overall, recruiting donor DNA to the targeted locus appears to have a significant effect on the success of HDR.

When using CRISPR/Cas there are potential pitfalls to consider. Early emphasis was placed on determining the specificity of Cas9, in an effort to examine the scope of off-target mutations. Wild-type Cas9 can tolerate nucleotide mismatches in the gRNA, especially in the 5' end. Reducing the length of the protospacer sequence from 20 to 17 nt or 18 nt may impart a positive effect on specificity while maintaining on-target activity by increasing the thermodynamic impact of each nucleotide (Hsu et al. 2013; Kescu et al. 2014). Directed mutagenesis has produced multiple high-fidelity enzymes that boast on-target activity comparable with wild type with significantly reduced levels of off-target mutations (Kleinstiver et al. 2016; Slaymaker et al. 2016; Chen et al. 2017; Lee et al. 2018). Multiple webtools exist to aid in the design of gRNAs and prediction of off-target binding, incorporating various modified Cas9 enzymes and thermodynamic models of gRNA binding (Table 2). Efficacy of gRNA designs can be validated in vitro via nuclease-based mismatch cleavage assays, such as Surveyor or T7E1 (Vouillot et al. 2015; Sentmanat et al. 2018). Another strategy to reduce off-target mutations is through the use of Cas9n and two gRNAs. One gRNA targeted to each strand of DNA in conjunction with Cas9n will create two staggered nicks in the DNA, creating a DSB. This technique reduces off-target effects by requiring the annealing of

two separate gRNAs in close proximity (Ran et al. 2013a). Off-target screening should always be performed to characterize the extent of additional genome modifications in each individual experiment. However, when generating mice, off-target mutations can be removed via outcrossing, unless tightly linked.

The majority of analysis has focused on off-target mutations; however, recent studies have reported various on-target rearrangements when using CRISPR/Cas9. One study examined the variability in alleles generated using a single cut site, either in an exon or intron, and found evidence of various forms of genomic damage at three different loci. Most alleles resulting from a cut in an exon exhibited indels <50 bp; however, there were instances of deletions up to 6 kb in length. Various alleles recovered from either exon or intron targeted gRNAs also resulted in large insertions up to 2.5 kb, or displayed evidence of translocation or inversion (Kosicki et al. 2018). A second group performed analysis on 109 different loci using Cas9n with pairs of gRNAs to reduce off-target effects. Southern blot analysis revealed possible genomic damage at 40% of all loci examined, accounting for roughly 8% of all clones (Rezza et al. 2019). Of note, both studies were performed in mESCs; when creating in vivo models, extensive DNA damage may partially account for the low recovery of CRISPR/Cas9 engineered live pups. However, both studies emphasize the need for proper screening of mutations at both off-target and on-target loci, as the full extent of genomic damage may not be readily apparent via short-range PCR and sequencing.

Base editors

In 2016, the Liu laboratory (Komor et al. 2016) published a paper describing a Cas9 fusion protein that could alter a nucleotide without a DSB, referred to as a base editor (BE) (Fig. 2A). The first BEs consisted of Cas9n fused to a cytidine deaminase domain (APOBEC1) and a uracil DNA glycosylase inhibitor (UGI). APOBEC1 converts cytidine to uracil without requiring a break in the dsDNA backbone. The UGI represses base excision of the uracil while the Cas9n nicks the nonedited strand, resulting in more favorable repair of the nicked strand and thus effecting a C-to-T transition mutation (Fig. 2B). The following year, the same group published a new variant, Cas9n fused to an adenine deaminase domain (TadA) able to convert adenine into inosine, which would effectively be repaired as guanine (Gaudelli et al. 2017). Multiple groups have worked on improving the efficiency and specificity of both cytidine base editors (CBEs) and adenine base editors (ABEs) (Komor et al. 2017; Koblan et al. 2018). BEs function as a genetic pickaxe, able to alter a single nucleotide with relative precision and accuracy, and offer a wide variety of uses: correcting or mimicking disease-causing SNPs, altering enzyme function via precise amino acid changes, inducing an early stop codon, altering a binding motif, or even inducing or altering alternative splicing behavior, as was recently reported using a CBE system (Yuan et al. 2018).

Figure 2. CRISPR/Cas base editors and prime editor. (A) Base editor systems can induce nucleotide changes without the need for DSBs in the target DNA. Cas9n (D10A) is used to nick the unedited strand to facilitate incorporation of the deaminated nucleotide. (B) Base editors use a deaminase domain to convert C to T (or A to G). (C) The conversions can occur within the ssDNA deaminase activity window upstream of the PAM site. (D) The width of the activity window is dictated by the base editor system. (E) The prime editor system uses a Mo-MLV RT domain to reverse-transcribe small insertions and deletions into the target DNA. A specialized pegRNA contains a protospacer, scaffold sequence, an RT template, and a primer binding sequence. Cas9n (H840A) nicks the targeted strand of DNA to allow the RT domain to reverse transcribe the template into the DNA strand. (F) The PE3 system uses an additional gRNA to induce a nick in the unedited strand at a nearby locus to facilitate incorporation of the edited DNA.

The deaminase enzymes catalyze ssDNA. When the BE binds to its target, the gRNA forms a heteroduplex with the complementary DNA strand, leaving the noncomplementary strand exposed as ssDNA (Fig. 2C). The targetable nucleotides therefore fall within the protospacer sequence ($N_1 \dots N_{20}$) prior to the PAM site ($N_{21}N_{22}N_{23}$). Particular BEs have varying targeting windows (Fig. 2D); for instance, the recent CBEmax is optimal at nucleotides N_4 through N_8 , while ABEmax is optimal at N_4 through N_7 (Koblan et al. 2018). Altering the Cas9 enzyme also affects the targeting window; saBE3 has an expanded window of editing between N_2 and N_{15} (Kim et al. 2017b). Additionally, the use of novel circularly permuted variants of spCas9 can adjust the targeting window (Oakes et al. 2019). This allows for an increase in the editing window of CBEmax (CP-CBEmax) and ABEmax (CP-ABEmax) to N_4 through N_{11} and N_4 through N_{12} , respectively (Huang et al. 2019b). Careful selection of a BE system can produce an efficient window for the desired mutation.

While base editing certainly has advantages over traditional CRISPR/Cas systems, it is not without its caveats. Currently, only four of 12 possible base edits are achievable (C to T, G to A, A to G, and T to C), thus allowing for only 66 amino acid substitutions out of a possible 420. Selecting an optimal gRNA is more restricted when using base editing. First, as discussed above, the target nucleotide must fall within the window of editing dictated by the BE. Second, the deaminases are not selective and will potentially edit all targets within the window. Early in vitro data suggest that the enzyme works sequentially, moving from upstream nucleotides to downstream ones (Komor et al. 2016). This can be either a detriment or a

strength, depending on the desired edit. Additionally, base editing can result in off-target mutations similarly to traditional CRISPR/Cas systems. Care must be taken to screen predicted off-target sites based on gRNA mismatches and the respective editing windows for potential mutations. However, the rate of indel formation is lower than traditional Cas9 via the use of Cas9n.

Many disease-causing alleles can be traced to deleterious point mutations and the ability to produce and study mammalian models of those mutations is quite valuable. Base editing is seen as a powerful toolbox to create disease models accurately and efficiently. Multiple BEs have been successfully used to create mouse models (Liu et al. 2018c; Yeh et al. 2020; Wang et al. 2020c). However, reports have begun to shed light on the promiscuity of CBE systems in mouse embryos. A recent study used whole genome sequencing to examine the frequency of off-target mutations. The investigators injected sibling embryos at the one-cell stage with either ABE or CBE, both using the same gRNA, and compared de novo SNPs and indels with noninjected sibling embryos. A twofold increase in novel SNPs was observed using CBE, while ABE had levels comparable with those of controls. Surprisingly, only 2% of the SNPs correlated with predicted off-target sites, indicating that screening off-targets based on the gRNA sequence is not sufficient to adequately determine the degree of unintended mutations. The number of indels was comparable between the three groups, indicating that the cytosine deaminase is the likely culprit and not the Cas9n (Lee et al. 2020b). Further optimization and analysis of CBE systems is necessary depending on the experiment.

Prime editor

To overcome the editing limitations of BEs, the Liu group (Anzalone et al. 2019) introduced a system, termed a prime editor (PE) (Fig. 2E). PEs consist of Cas9n fused to a Mo-MLV reverse transcriptase (RT) domain alongside a multi-function prime editing gRNA (pegRNA). The pegRNA consists of a protospacer, a modified scaffold, and an extended 3' sequence containing a template and complementary binding sequence, known as the primer binding sequence (PBS). Cas9n nicks the PAM-adjacent DNA strand, the free DNA strand hybridizes with the PBS of the pegRNA, the RT domain reverse transcribes the template into the DNA strand, and finally the DNA is repaired. In PE3, a second gRNA nicks the nonedited strand to favor repair using the newly edited DNA (Fig. 2F).

Like BEs, PE benefits from the ability to avoid DSBs. Indel formation is much lower than that of normal Cas9 HDR, and is comparable with BE. The second strength of prime editing lies in its specificity. To properly introduce the desired mutation, the target DNA must contain both a compatible protospacer region and complementarity to the PBS. This stringency results in significantly lower off-target mutations, with the investigators reporting <1% at predicted sites. The pegRNA also imparts PE's main restriction: The length of the template has an upper limit. The PE system is suited to small-scale edits, with successful insertions up to 44 bp and deletions up to 80 bp (Anzalone et al. 2019).

PE was recently used in mice, by injecting mRNA encoding the PE3 system into one-cell embryos (Liu et al. 2020b). One locus yielded correct editing in 10%–27% of live pups; however, no successful editing was observed at a second locus. Additionally, indel frequency was low (<0.3%), while targeted deep sequencing and whole-genome sequencing found no off-target editing. The major problem noted by the investigators was the fidelity of the edit in embryos, as unintended conversions at the targeted base were observed. The investigators concluded that prime editing shows promise as a highly specific mechanism for gene editing in mice, however the accuracy of the editing may need improvement alongside substantial screening of pegRNAs (Liu et al. 2020b).

In vivo delivery of CRISPR/Cas systems

Successful editing requires careful consideration on the delivery of CRISPR/Cas machinery. The Cas enzyme and gRNA are delivered in one of three forms: plasmid DNA, RNA, or in complex as ribonucleoproteins (RNPs). A single plasmid containing Cas and gRNA sequences can be used (Cong et al. 2013; Mali et al. 2013), but may result in several potential drawbacks. The delay in editing is on the order of several hours, as the enzyme needs to be transcribed and translated. The window of editing can also be quite long, as the plasmid persists, resulting in prolonged expression and greater off-target risk. Additionally, the plasmid may randomly integrate into the genome. Delivering the components as RNA is less risky as it reduces the potential of prolonged expression

or genomic integration and editing proceeds sooner once the mRNA is translated. A drawback is that the gRNA is exposed to native RNA degradation machinery, which could reduce efficiency. Chemically modified gRNAs can be acquired commercially to improve stability. The method considered to support the highest efficiency, while reducing off-target effects, is the delivery of Cas nuclease and gRNA as an RNP complex (Sung et al. 2014; Liang et al. 2015; Zuris et al. 2015; Gertsenstein and Nutter 2018). The complexes consist of a single Cas9 nuclease and a single gRNA molecule and are precomplexed in vitro before delivery, typically using 1:1.1 molar ratio of Cas9:gRNA (Gertsenstein and Nutter 2018). Editing begins upon nuclear localization, the gRNA is protected from degradation by the Cas nuclease, and the limited supply of RNP reduces the window of editing, resulting in fewer off-target mutations. However, while many Cas variants are commercially available as protein, the majority of engineered Cas9 and Cas12a enzymes are only available as plasmids, adding the necessary steps of protein production, purification, and activity verification.

Once the CRISPR/Cas payload is determined, the system must be efficiently delivered. The means of delivery will differ depending on the goal of the experiment; however, two main strategies are commonly used when attempting to create an in vivo model. The first is electroporation, an efficient method of transferring DNA into a large quantity of cells. Similar to gene targeting, mESCs can be electroporated to deliver the CRISPR/Cas reagents. Individual colonies of mESCs can be screened for proper editing before being injected into blastocysts. Alternatively, electroporation can be used to transfer CRISPR/Cas reagents directly into zygotes. The zygote electroporation of nuclease (ZEN) technique can deliver Cas9 RNP and ssDNA via a high-throughput workflow, yielding knock-in rates of correct mutations in over 40% of living pups (Qin et al. 2015; Wang et al. 2016b). A similar protocol, CRISPR RNP electroporation of zygotes (CRISPR-EZ), also uses RNP and ssDNA, reporting successful HDR in 42% of live pups (Chen et al. 2016). Alternatively, the improved genome-editing via oviductal nucleic acids delivery (i-GONAD) technique uses direct electroporation of the oviduct in pregnant females to induce CRISPR/Cas editing without ex vivo manipulation of zygotes (Takahashi et al. 2015; Ohtsuka et al. 2018; Kobayashi et al. 2020; Sato et al. 2020). These protocols highlight the strength of electroporation: high-throughput processing of embryos without requiring as much technical expertise as direct injection. Of note, both ZEN and CRISPR-EZ use ssDNA; larger mutations requiring a dsDNA donor may not be feasible.

The second delivery strategy is direct physical injection into embryos (Shen et al. 2013; Wang et al. 2013; Yang et al. 2013; Gertsenstein and Nutter 2018). Injection requires technical expertise but can achieve some of the highest rates of successful CRISPR/Cas9 editing. CRISPR/Cas reagents can be injected either into the cytosol or the nucleus. Pronuclear injection can yield the highest efficiency as the reagents are physically delivered directly to the site of editing. This efficiency is

potentiated when used in conjunction with RNPs as editing can begin within minutes. Cytosolic injection can still achieve high rates of successful HDR without the need for Nomarski optics. However, the most opportune time for injection, yielding the greatest efficiencies, is debated.

Timing of delivery is indeed important, as homologous recombination occurs predominantly in the late S and G2 phases. Chemical synchronization of the cell cycle was examined in HEK293T cells and indicated that increased HDR efficiency could be achieved via synchronization up to roughly 33% when nucleofecting RNP at the G2 stage (Lin et al. 2014). While chemical synchronization can be used for cell culture, these techniques cannot be used in embryos, although embryos may be more synchronized if produced by in vitro fertilization. Microinjection during the two-cell stage could increase efficiency, as the G2 phase is prolonged (~12 h) and coincides with zygotic genome activation, providing a more open chromatin configuration. Editing efficiency up to 35% was observed for insertion of a fluorescent tag when injecting at the two-cell stage compared with 6.5% in zygotes (Gu et al. 2018). Conversely, a recent paper compared pronuclear injection at various time points during both the one-cell and two-cell zygote stages and reported the most successful knock-in efficiencies at pronuclear stages 3 and 4 of the one-cell embryo, corresponding to S phase when the paternal and maternal DNA are replicating, prior to the fusion of the pronuclei. Additionally, injecting into a single pronucleus can only result in a heterozygote, while injecting into both pronuclei can result in a homozygote (Abe et al. 2020). This can be particularly useful when attempting to create alleles that may be nonfunctional in essential genes, as homozygous zygotes would not survive development.

Alternatively, lipid- and viral-mediated transfection have both been used for CRISPR/Cas editing. Lipid-mediated transfection is predominantly used in cell culture (Ran et al. 2013b), and can effectively transport DNA, RNA, or RNP across the cell membrane (Zuris et al. 2015). Once optimized, transfection is an efficient method for introducing CRISPR/Cas editing in cell culture and can be used to assess the efficacy of gRNAs prior to embryo editing. Conversely, viral-mediated transfection is mainly used for editing in somatic tissues (Yeh et al. 2020; Zhang et al. 2020c), although it has been used to edit embryos for certain applications (Mizuno et al. 2018; Romeo et al. 2020). Adeno-associated viruses (AAVs) are commonly used to deliver DNA encoding the Cas nuclease and gRNA; however, smaller Cas9 homologs are better suited due to space limitation within the viral envelope (Wang et al. 2020a).

HR, CRISPR, and generating specific alleles

Traditional homologous recombination and newer CRISPR-centric techniques are both capable of generating most common modified mouse alleles, so the questions naturally arise: Which is better and should this be done in mESCs or zygotes? We refrain from providing an out-

right recommendation here, as the desired allele, available laboratory expertise and reagents, and nature of available transgenic facilities will yield many different answers. However, we first evaluate tradeoffs between the approaches, before briefly reviewing common classes of transgenic mouse alleles and relevant points for making them by HR or CRISPR.

A recent study (Rezza et al. 2019) compared traditional HR and CRISPR/Cas9 in mESCs across 128 loci in the mouse genome. The investigators defined two measures to assess targeting: discovery index, describing the ability to produce mutant clones, and validation index, describing the number of correct mutant clones. They found that at every locus tested, CRISPR/Cas had a significantly higher discovery index, albeit a lower validation index compared with traditional HR. HR yielded correct bands in 99.5% of clones, while CRISPR/Cas yielded 92.4%. However, the investigators note that CRISPR/Cas showed higher efficiency when inserting sequences >8 kb.

When using traditional gene targeting, each mESC clone contains a single recombination event on one of two homologous chromosomes that is validated prior to blastocyst injection. On the other hand, CRISPR/Cas has the potential to create two distinct mutant alleles, one on each homologous chromosome, in a single cell or zygote. This is an especially relevant concern for knock-out alleles, as each NHEJ event will be uniquely repaired. Additionally, CRISPR/Cas reagents injected into a zygote can continue to edit for as long as they persist, resulting in more than two different mutant alleles if editing occurs after the one-cell stage (Yen et al. 2014).

However, despite the comforting known quantity aspect of traditional HR, CRISPR has two enormous advantages over its tried and true forebear. First, the ability to perform in embryo editing allows researchers to bypass mESCs altogether, yielding significant time and cost savings (Fig. 3). Second, the increase in editing efficiency means repair sequences do not need to contain a positive selection marker and genome edits can be made to produce a “clean” allele without leaving any exogenous sequence behind (Fig. 4). One caveat is that the relatively lower efficiency of obtaining large insertions by CRISPR may still limit one or both of these advantages for the time being.

Null alleles

One of the most common goals in mouse genetics is to interrogate gene function by generating a complete loss-of-function, or null allele. Indeed, the KOMP was established to generate a null mutant for every mouse gene (Austin et al. 2004). Null alleles can be generated by removal of one or several exons, or creation of short indel mutations that cause a frameshift in the resulting transcript leading to NMD. Such efforts are typically focused at the 5' end of genes to increase the chances of NMD and reduce the chance of making a mutation that results in a truncated protein that retains some function. Prospective null alleles should be verified by RNA and/or protein expression analysis to confirm there is no residual gene product and the allele is therefore a true null. An alternative approach

Figure 3. Typical workflow. The typical workflow required for the creation of a mouse model varies between gene targeting or CRISPR. For gene targeting, mESC clones should be screened via PCR and Southern blot to verify correct integration prior to injection into blastocysts. Chimeras are chosen based on coat color contribution and bred. Their pups are then screened via PCR (and coat color if crossing to a different strain) for germline transmission of the intended mutation. For CRISPR, gRNA cutting efficiency should be verified via PCR and Surveyor (or T7E1) assays. After introducing the CRISPR reagents to embryos, founder pups should be screened for both the correct mutation and any off-target effects. CRISPR has the potential to significantly reduce the time needed to create a mouse model.

is to delete all (or most) exons of a gene and thereby prevent any expression whatsoever. While this method assuredly creates a null allele, the lost DNA may include regulatory regions that affect other genes or encode microRNAs. A recent report described a mechanism of genetic compensation, whereby degraded mRNA transcripts lead to the up-regulation of related genes, blunting the impact of the mutation (El-Brolosy et al. 2019). Conversely, alleles that transcribe no mRNA at all do not exhibit this phenomenon and have comparatively stronger phenotypes. The extent of this effect across existing

mouse strains is not currently known, but will be worth accounting for when designing future knockout mice, particularly if the gene has closely related paralogs. According to MGI, 14,981 genes have mutant mouse alleles and a further 6937 genes are mutated in mESC lines, so assessing existing models is an important first step in any new mouse genetic research project. However, depending on the nature of the allele and the genetic background of the strain it was generated in, researchers may still prefer generating their own knockout, even if one already exists.

Figure 4. Designing alleles via gene targeting or CRISPR/Cas. Various genetic alterations can be constructed using either gene targeting or CRISPR. Gene targeting constructs typically consist of sequence containing the intended edit and a positive selection marker flanked by homology arms to facilitate integration. A negative selection marker is located outside of the homology arms to select against random insertion. For CRISPR, repair constructs are mainly used for conditional, tag, and reporter alleles and do not require the use of selection markers. Base or prime editing systems can be used for smaller point mutations. The conditional reporter is depicted here at the commonly targeted *ROSA26* locus using a splice acceptor (SA) to drive the reporter off the endogenous promoter. Alternatively, an exogenous promoter such as CAG can be used. Orange triangles represent *FRT* sites, red triangles are *loxP* sites, red stars are point mutations, single white triangles are nicks, and double white triangles are DSBs.

For traditional HR gene targeting to induce a frameshift through exon deletion, a repair construct would be used to replace the targeted exon with a selection cassette flanked by SSR sites (Fig. 4). When using CRISPR/Cas, two strategies to induce a frameshift are available. The first involves using a single gRNA to induce a DSB within an exon (or two gRNAs with Cas9n), with the goal of making an indel that causes an early stop codon. The sequence of the exon should be examined for potential stops induced by frameshifts downstream from the DSB, as short genes especially may be unlikely to generate an NMD-causing mutation. The alternative method is to use gRNAs that flank an exon, ideally resulting in the deletion of the exon. When using Cas9n, this approach would require four different gRNAs, which may impose limitations on the ability to design efficient gRNAs while reducing off-target effects, potentially negating the benefit of using Cas9n.

To delete an entire gene, HR repair constructs can be built with homology arms using sequence from either side of a gene and a resistance cassette in between where the gene would otherwise be. Regions up to 40 kb were consistently deleted via HR by Regeneron using a process termed VelociGene, as part of the KOMP, so this approach is accessible by HR for many genes (Valenzuela et al. 2003; Bradley et al. 2012). Larger regions can be deleted by knocking in widely spaced *loxP* sites (see below) and expressing Cre recombinase, but this approach requires multiple rounds of targeting (Ramirez-Solis et al. 1995).

CRISPR/Cas targeting at two distant sites and deletion of the intervening sequence has been demonstrated in mouse embryos at the kilobase and megabase scales (Kato et al. 2017; Dumeau et al. 2019). The repaired allele can be made somewhat more predictable using a short donor that bridges the intended deletion. CRISPR/Cas provides an overall simple approach to creating a knockout allele; however, the alleles recovered in each individual will vary, requiring screening, sequencing, and functional analysis to verify that the modification results in a null. Another consideration is whether the targeted gene is essential, as CRISPR/Cas may edit both copies of the gene, resulting in inviable mice. To overcome this, it may be beneficial to inject only one pronucleus during the single cell stage, injecting one cell at the two-cell stage, or carefully titrating the RNP dose. Conversely, using traditional gene targeting would guarantee that each founder had an identical heterozygous mutation.

Point mutations

It is also possible to engineer precise edits to the mouse genome using HR or CRISPR-based strategies (Fig. 4). In traditional gene targeting, the targeting vector contains the intended mutation in close proximity to the selection cassette, which is flanked by SSR sites for later removal with a recombinase such as Cre. For CRISPR/Cas targeting, the reagents are supplied along with a short donor sequence,

usually a ssODN, harboring the mutation, and if necessary, additional silent mutations in the PAM or recognition sequence to prevent postrepair recutting. If compatible, a base or prime editor system can be used to change the nucleotide without introducing a DSB or repair construct, and would ideally reduce indel formation compared with traditional CRISPR/Cas9. This type of allele is one where CRISPR may have a decided advantage as short ssDNA donors can be produced commercially in just days or weeks. Furthermore, even after removal of the resistance cassette using an SSR such as Cre (see “Conditional Mutations” below), some residual exogenous sequence will remain and even these short sequences can interfere with gene expression (Meier et al. 2010). The ability to introduce targeted point mutations is a powerful complement to the semi-random point mutations obtained in ENU mutagenesis screens, as they allow a researcher to leverage biochemical, structural, and genomic knowledge in an organismal context under the control of endogenous regulatory elements. Point mutations impart a degree of experimental subtlety to reveal biology that may be missed with a simple knockout. Indeed, point mutations have been used to tremendous effect, enabling the introduction and correction of disease mutations (Zeiher et al. 1995; Long et al. 2014), mutation of regulatory binding sites (Arango et al. 1999; Soukup et al. 2019), phospho-mimetic and phospho-dead mutations (Heuchel et al. 1999; Aubin et al. 2004), kinase and other enzymatic-activating or inactivating mutations (Olson and Soriano 2009; Yamagata et al. 2009), and allelic series that systematically test individual binding or regulatory sites on a single protein (Klinghoffer et al. 2002; Tallquist et al. 2003; Brewer et al. 2015).

Knock-ins and tagging

The coding sequence of genes can be similarly extended or replaced using traditional gene targeting or CRISPR-based methods, the only difference being the nature of the targeting vector or donor sequence. This approach can be used to add a tag or fluorescent protein to a gene, generating a fusion protein that can enable biochemical, localization, expression, imaging, and sorting experiments. These are typically added at the 5' or 3' end of a gene along with a short flexible linker to alleviate any steric hindrances; however, with careful planning and detailed knowledge of a gene's protein structure such tags can also be added internally. Another use is to replace a gene with a paralog or functionally related gene (Hanks et al. 1995; Wang et al. 1996), an ortholog from another species (Hanks et al. 1998; Geng et al. 1999), perform domain swapping between genes (Klinghoffer et al. 2001), or insert a human disease allele (Wheeler et al. 1999; Leissring et al. 2000). This is sometimes possible by knocking in one or a few replacement exons, but becomes unwieldy if the entire gene must be replaced and especially so if the endogenous regulation is to be maintained. In these cases, knock-in of a complete or partial cDNA along with a splice acceptor creates a hybrid transcript that expresses the desired protein product. However, this can affect gene expression by bypassing

any splicing-mediated regulation (Hoch and Soriano 2006). Therefore, a control cDNA should always be knocked in in a separate mouse strain to control for this effect (Klinghoffer et al. 2001). Knock-in–knockout strategies replace a gene or the first exon with an alternative gene, such as a reporter, which allows simultaneous study of gene expression and loss-of-function analysis (Le Mouellé et al. 1990; Mansour et al. 1990). Finally, genes can be knocked in to a locus and expressed as a separate protein from a single transcript, thus leaving the original gene product relatively unaffected. This can be achieved by inserting a sequence at the 3' end of a gene in-frame, with a self-cleaving peptide sequence (e.g., P2A) in between, or with a virally derived internal ribosome entry sequence (IRES) that allows independent translation of the two genes from a dicistronic transcript (Mountford et al. 1994; Kim et al. 2011).

When using traditional gene targeting to create a knock-in or tagged allele, the repair construct should contain the modification or tag of choice (fluorescent protein, epitope, etc.) aligned within the same reading frame as the targeted protein, along with a selection cassette flanked by SSR sites (Fig. 4). When using CRISPR/Cas, the size of the knock-in dictates the method used. For smaller tags, the prime editing system can be used to insert up to ~45 bp without inducing a DSB. The prime editor holds promise as an accurate and efficient method to insert small tags. However, if the insert is larger, a lssDNA (~1- to 1.5-kb) or dsDNA (>1.5-kb) repair template can be used alongside traditional CRISPR/Cas9. Large, complex knock-ins may benefit from CRISPR/Cas editing in mESCs to facilitate screening rather than direct injection into zygotes.

Conditional mutations

A common goal of researchers is to achieve spatiotemporal control over gene expression. One approach is to place genes under the control of drugs that can be administered to mice. The tetracycline-controlled *trans*-activator protein (tTA) and related reverse tTA (rtTA) system can be used in mice to repress or activate genes, respectively, in response to the drug doxycycline (Furth et al. 1994; Kistner et al. 1996). The gene of interest is put under control of a tetracycline responsive element and crossed to a mouse expressing tTA/rtTA, sometimes under control of a tissue-specific promoter. This system enables reversible activation or repression of a gene in a time- and dose-dependent manner. Several updates have been made to the original system to enhance its usefulness in mice, making the system both less leaky and more sensitive to doxycycline for greater dynamic range and accessibility in hard-to-drug tissue; for instance, the central nervous system, where crossing the blood-brain barrier is limiting for many small molecules (Dow et al. 2014; Das et al. 2016). Another approach for conditional control of gene expression uses the yeast-derived GAL4-UAS system, where GAL4 activator is expressed by a tissue-specific promoter and drives expression of a gene controlled by GAL4-responsive UAS elements (Ornitz et al. 1991). However, this system did not achieve the same popularity in the

mouse as it did in the fly and there were several reports of GAL4-induced toxicity (Habets et al. 2003; Govindarajan et al. 2005).

More commonly, spatiotemporal control is achieved through the use of SSRs (Brandt and Dymecki 2004). These enzymes mediate recombination between short recognition sequences that can be inserted into specific genetic loci via HR. Recognition sequences have an intrinsic directionality such that the recombinase will loop out and delete the sequence between two recognition sequences oriented in parallel. Antiparallel recognition sites can be used to create inversions and this approach has been used to generate balancer chromosomes, discussed above (Ramirez-Solis et al. 1995).

Conditional knockouts are made by flanking a critical exon or exons of a gene with recognition sites (Fig. 4). Expression of the recombinase deletes the flanked region either creating a truncated protein, or more typically, introducing a frameshift that causes NMD and thus a null allele. Naturally, this approach can also be used to flank entire genes or genomic regions, as well as regulatory sequences, with the caveat that efficiency of recombination generally decreases with increasing distance between recognition sequences (Coppoolse et al. 2005; Liu et al. 2013). Genes can also be conditionally activated by placing a STOP signal, such as tandem polyA motifs or a resistance marker, between the promoter and the coding sequence of the gene to be expressed. Removal of the intervening STOP by the SSR enables expression of the gene. This strategy can be used to overexpress (or express mutant forms of) genes to study their function (Chan et al. 2004), mark cells with expression of LacZ or EGFP for lineage tracing or cell sorting (Soriano 1999; Abe and Fujimori 2013), exert optogenetic control over signaling (Deisseroth 2011), express signaling sensors such as calcium indicators (Chen et al. 2013), or to kill cells by expression of DTA (Voehringer et al. 2008).

Expression of the SSR (or tTA/rtTA) is achieved by creating a separate mouse line and then crossing the two. The SSR line is often made as a transgene that inserts semirandomly into the genome, but whose expression is driven by a promoter and regulatory sequences that restrict expression to a tissue or cell type of interest. As for all transgenes, multiple founders should be screened for proper expression, especially to assure there is no misexpression in the male or female germline (Luo et al. 2020). Alternatively, SSRs can be knocked in to the locus of a gene with the desired expression pattern. This can purposely knock out the endogenous gene in the process or the SSR can be knocked in at the 3' end creating a fusion transcript that codes for a self-cleaving peptide or IRES as discussed above (Michael et al. 1999; Lange et al. 2012). An alternative approach is to knock the SSR into a gene of interest not in the mouse genome, but in a bacterial artificial chromosome (BAC) that is then inserted into the mouse genome as a transgene, thereby retaining the desired regulatory activity without affecting the endogenous gene (Parkitna et al. 2009). Finally, exogenous genes can be targeted to so-called "safe harbor" loci, genomic sites where exogenous DNA can be intro-

duced and stably expressed without being influenced by (or influencing) nearby genetic elements or disrupting a developmentally important gene, thus avoiding undesirable positional effects. The most popular safe harbor in the mouse is the *ROSA26* locus, which disrupts a nonprotein-coding gene of unknown function, though several other safe harbors have been identified (Friedrich and Soriano 1991; Bronson et al. 1996; Zambrowicz et al. 1997; Beard et al. 2006; Hippenmeyer et al. 2010; Liu et al. 2018b). Identification of safe harbor sites will be important for gene therapy approaches and the mouse is currently the best organismal model for this effort (Papapetrou and Schambach 2016). In most cases, the above strategies can also be used to drive expression of any gene of interest, not only SSRs.

Temporal control can be further refined using the ERT/ERT2 system where the SSR is fused to a mutant estrogen receptor that retains it in the cytoplasm until the administration of tamoxifen (Feil et al. 1996; Indra et al. 1999). The degree of SSR-ERT activity can be tuned by the tamoxifen dose, number of doses, and route of administration (Park et al. 2008). High doses of tamoxifen can induce abortions in pregnant mice, an effect that can be offset by coadministration of progesterone (Nakamura et al. 2006). Nevertheless, dosing should be carefully controlled, particularly for embryonic recombination. Similar to tamoxifen-inducible systems, a fusion can be made between an SSR and a mutant progesterone receptor that is activated by administration of the synthetic steroid RU486, though this system is less widely used (Kellendonk et al. 1996). Alternatively, the SSR can be expressed by a promoter that is only active under certain conditions, such as an inflammatory stimulus, which is how the first conditional Cre allele was made (Kuhn et al. 1995). More recently, photoactivatable SSRs have been used successfully in mice (Schindler et al. 2015).

There is now a toolbox of multiple SSRs available for conditional genetics, but by far the most widely used is the Cre-lox system (Tsien et al. 1996). Cre recombinase is a bacteriophage-derived SSR that mediates recombination between 34-bp-long *loxP* sites. There are now over 3000 Cre recombinase-expressing mouse strains or mESC lines listed at MGI driven by hundreds of unique drivers, attesting to its usefulness. Extending Cre's capabilities, mutant *loxP* sites that do not recombine with the WT sequence or are inactivated after undergoing recombination can be used to generate gene expression switches (FLEX switches) or mediate efficient insertion of floxed DNA into a previously targeted genomic *loxP* site, respectively (Fukushige and Sauer 1992; Araki et al. 1997; Schnutgen et al. 2003). These have also been used to generate a lineage reporter that can mark two lineages, depending on the level of Cre activity (Klinger et al. 2010). Complementing Cre, the Flp-FRT (Dymecki 1996; Buchholz et al. 1998; Raymond and Soriano 2007), Dre-Rox (Anastassiadis et al. 2009), Vika-Vox (Karimova et al. 2018), Nigri-Nox (Liu et al. 2018a), and ϕ C31 SSR systems (Buchholz et al. 1998; Raymond and Soriano 2007) have all been validated or optimized for use in mice. These

systems can be used in combination to mark multiple lineages or in intersectional genetic schemes to create sophisticated logic gates governing conditional gene expression (Awatramani et al. 2003; He et al. 2017; Soriano 2018; Liu et al. 2020a; Poulin et al. 2020). An alternative intersectional genetic strategy could use a split-Cre, where two fragments of the gene are expressed by different promoters and the protein is only reconstituted where expression overlaps (Hirrlinger et al. 2009).

To generate a conditional allele, a pair of SSR sites need to be inserted flanking an exon or gene for removal upon expression of the SSR. When using traditional HR gene targeting, a repair construct should contain the WT exon flanked by SSRs along with a selection cassette flanked by distinct SSR sites (e.g., *loxP* sites flanking the exon and FRT sites flanking the selection cassette). If using CRISPR/Cas to create a conditional allele, multiple methods may be considered. Early reports suggested that using two gRNAs alongside two ssODNs could be used to insert flanking SSR sites (Yang et al. 2013); however, correct insertion of both SSR sites on the same allele happens at a low frequency (Gurumurthy et al. 2019). A single lssDNA containing both SSR sites and the flanked exon can be used to ensure that both SSR sites are inserted in *cis*. Alternatively, a single SSR site can be inserted and once that mouse strain is screened and verified, a second SSR site can be inserted through a second round of CRISPR/Cas editing. Although currently untested, the prime editor system may be able to insert flanking SSR sites through the codelivery of two pegRNAs. While this method suffers the same pitfalls as using unmodified Cas9, the decreased prevalence of indels may increase the overall efficiency. CRISPR/Cas has the potential to significantly reduce the time necessary for obtaining a conditional allele, however published efficiencies of simultaneously modifying two regions on the same allele are still quite low and will require future optimization.

Lineage tracing and clonal analysis

To study cell fate and mark distinct lineages, conditional expression of reporter alleles is now commonplace (Legué and Joyner 2010). There are an enormous number of mouse reporter lines that conditionally express an enzyme (e.g., *lacZ*) or fluorescent protein following expression of Cre or another SSR. This irrevocably marks all cells and their progeny and is often used to validate Cre lines, perform lineage analysis, and mark conditional mutant cells when used in conjunction with a conditional mutant allele. However, different reporters and mutant alleles can have varying rates of recombination, depending on both the distance between SSR recognition sequences and other less well-understood effects likely involving the local chromatin environment (Zheng et al. 2000; Vooijs et al. 2001), so such mice should be carefully analyzed to understand the degree of overlap between the marked (e.g., fluorescent) and mutant populations. This is especially important for tamoxifen-inducible systems where the window of recombination is limited or the goal is to mark very few cells for clonal analysis, as the limited

number of events may highlight differences in efficiency that are otherwise overcome by a constitutively active Cre line. For particularly timing-sensitive experiments, it is worth noting that tamoxifen exhibits an ~12-hr delay to be converted to the active metabolite 4-hydroxytamoxifen (4-OHT) and delivered to embryonic tissue, after which it can remain active for hours or even days, depending on the dose, Cre line, and particular recombination (Nakamura et al. 2006). More rapid recombination can be induced by administering 4-OHT directly. Several systems that link reporter expression with loss of a mutant allele, assuring overlap between the two populations. Mosaic analysis with double markers (MADM) relies on Cre-mediated interchromosomal mitotic recombination to generate homozygous mutant and wild-type daughter cells from a heterozygous parent cell, while simultaneously marking mutant cells with a fluorescent protein (Zong et al. 2005). An alternate strategy, mosaic mutant analysis with spatial and temporal control of recombination (MASTR), uses FLP-ER to control expression of Cre-EGFP, which simultaneously marks cells and mediates recombination of any existing floxed conditional allele (Lao et al. 2012). Two recent tools for clonal analysis are worth pointing out. The first is the *Brainbow* and *Confetti* class of reporters, which uses pairs of variant and mutually incompatible *lox* sites to randomly express one or a combination of fluorescent proteins upon Cre expression, ideally using an inducible Cre, allowing multiple adjacent clones to be tracked (Livet et al. 2007; Snippert et al. 2010; Cai et al. 2013; Baggiolini et al. 2015). Second, CRISPR-based lineage tracing uses a CRISPR-induced mutation rather than expression of a marker to track cells. As cells divide during embryogenesis, mutations accumulate so that the lineage relationship and fate of these cells can be reconstructed using single-cell sequencing (Kalhor et al. 2018; Chan et al. 2019; Bowling et al. 2020; Wagner and Klein 2020).

When constructing reporter alleles, traditional gene targeting repair constructs typically contain a ubiquitous promoter driving the expression of a selection cassette flanked by SSR sites (Fig. 4). Further downstream is the reporter itself. When using CRISPR/Cas, due to the size of the reporter, a dsDNA or lssDNA donor will most likely be required. Using two gRNAs in conjunction with Cas9n would ideally yield fewer off-target mutations.

Existing resources

The fastest way to make any kind of mouse is if someone else has already made it for you. The best places to begin searching are at the International Mouse Strain Resource, which compiles strain information from multiple repositories, and the Mouse Genome Informatics database, which also contains a list of most of the existing alleles for a given gene, as well as phenotype and expression data, and a list of publications using each allele. These websites and additional resources, including mutagenesis consortia and mutant mouse repositories, are listed in Table 1.

The future of mouse genetics

Charles Dickens opened *A Tale of Two Cities* by declaring, “It was the best of times, it was the worst of times.” Although admittedly not as consequential as human travails during the French Revolution, the same sentiment could apply to mice during the current CRISPR revolution. In many ways, there has been no better time to make a mouse. Forty years of genetic lessons and tool building have now been supercharged so that new and more sophisticated genetic models can be made with less effort, cost, and time than ever before. However, CRISPR has also opened the door to making these same genetic edits, once the exclusive domain of mice, flies, worms, yeast, and bacteria, in any system that can express exogenous DNA or be injected with CRISPR RNP complexes. In particular, the stunning complexity seen in human ESC-derived organoids and gastruloids will certainly appeal to researchers interested in studying human disease, as this grants them a source of tissue, made of *human* cells, without the need to maintain a mouse colony (for review, see Simunovic and Brivanlou 2017). Traditional model organisms with certain superior embryological properties such as fish, frog, and chick can now be strong genetic systems, as well, not to mention the increasing number of nontraditional organisms that now vie for genetic attention (Reardon 2019). However, between the need to study development and disease in a living mammal, the existing unrivaled genetic toolkit, and its many advantageous characteristics for laboratory use, it is hard to see the mouse going away anytime soon (Lloyd et al. 2016). Rather than a passing of the torch, the age of CRISPR will be more like a sharing of the genetic spotlight. It is going to be quite a show.

Glossary

4-OHT: 4-hydroxytamoxifen
AAV: adeno-associated virus
ABE: adenine base editor
BAC: bacterial artificial chromosome
BE: base editor
Cas: CRISPR-associated protein
Cas9d: catalytically dead Cas9
Cas9n: Cas9 nickase
CBE: cytidine base editor
CRISPR: clustered regularly interspaced short palindromic repeats
CRISPR-EZ: CRISPR RNP electroporation of zygotes
crRNA: CRISPR RNA
DSB: double-stranded breaks
DTA: diphtheria toxin fragment A
Easi-CRISPR: efficient additions with ssDNA inserts–CRISPR
ENU: N-ethyl-N-nitrosourea
G1: generation 1
G2: generation 2 or second growth phase
G3: generation 3
gRNA: guide RNA
HDR: homology-directed repair
HITI: homology-independent targeted integration
HR: homologous recombination
HSV-TK: herpes simplex virus-1 thymidine kinase

i-GONAD: improved genome editing via oviductal nucleic acids delivery
IRES: internal ribosome entry sequence
KOMP: knockout mouse project
lssDNA: long single-stranded DNA
MADM: mosaic analysis with double markers
MASTR: mosaic mutant analysis with spacial and temporal control of recombination
mESCs: murine embryonic stem cells
MGI: mouse genome informatics
NHEJ: nonhomologous end joining
NMD: nonsense-mediated decay
ODN: oligodeoxynucleotide
PAM: protospacer-adjacent motif
PBS: primer-binding sequence
PCR: polymerase chain reaction
PCV: porcine circovirus 2 rep
PE: prime editor
pegRNA: prime-editing gRNA
RNP: ribonucleoprotein
RT: reverse transcriptase
SNP: single nucleotide polymorphism
SSR: site-specific recombinase
SV40: simian virus 40
TALEN: transcription activator-like effector nuclease
Tild-CRISPR: targeted integration with linearized dsDNA–CRISPR
tracrRNA: transactivating CRISPR RNA
tTA: tetracycline-controlled *trans*-activator protein
UAS: upstream activation sequence
UGI: uracil DNA glycosylase inhibitor
UTR: untranslated region
ZEN: zygote electroporation of nuclease
ZFN: zinc finger nuclease

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