Cutting-Edge Review

A CRISPR Path to Engineering New Genetic Mouse Models for Cardiovascular Research

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Abstract—Previous efforts to target the mouse genome for the addition, subtraction, or substitution of biologically informative sequences required complex vector design and a series of arduous steps only a handful of laboratories could master. The facile and inexpensive clustered regularly interspaced short palindromic repeats (CRISPR) method has now superseded traditional means of genome modification such that virtually any laboratory can quickly assemble reagents for developing new mouse models for cardiovascular research. Here, we briefly review the history of CRISPR in prokaryotes, highlighting major discoveries leading to its formulation for genome modification in the animal kingdom. Core components of CRISPR technology are reviewed and updated. Practical pointers for 2-component and 3-component CRISPR editing are summarized with many applications in mice including frameshift mutations, deletion of enhancers and noncoding genes, nucleotide substitution of protein-coding and gene regulatory sequences, incorporation of *loxP* sites for conditional gene inactivation, and epitope tag integration. Genotyping strategies are presented and topics of genetic mosaicism and inadvertent targeting discussed. Finally, clinical applications and ethical considerations are addressed as the biomedical community eagerly embraces this astonishing innovation in genome editing to tackle previously intractable questions. (*Arterioscler Thromb Vasc Biol*. 2016;36:1058-1075. DOI: 10.1161/ATVBAHA.116.304790.)

Key Words: epitope ■ frameshift mutation ■ genetics ■ genome editing ■ genotype ■ nucleotide

The 2007 Nobel Prize in Physiology or Medicine was awarded for pioneering work in embryonic stem cells (ESC), which developed techniques to modify the mouse genome leading to the creation of thousands of knockout mouse strains, including hundreds that exhibit cardiovascular phenotypes (ftp://ftp.informatics.jax.org/pub/reports/MGI_Knockout_Full.html). Traditionally, the generation of knockin or knockout mouse models for cardiovascular study required intricate design and construction of a targeting vector, electroporation of the vector into a limited number of ESC lines, selection and enrichment of targeted ESC clones, screening of clones with validated probes by Southern blotting, and expansion of correctly targeted clones for injection into the blastocyst of a mouse embryo.[1] If ensuing chimeric mice were able to pass the targeted allele through the germline, and if phenotypic haploinsufficiency leading to premature debilitating disease or death did not occur, heterozygous offspring could be intercrossed to create mice homozygous for the targeted allele. The time and effort involved in creating a mouse in this manner is considerable (typically more than 1 year) with no guarantee of success. The advent of nuclease-directed genome editing (NDGE) in animals with zinc finger nucleases[2] and transcription activator–like effector nucleases[3] simplified the labor-intensive task of generating genetically

modified mice while reducing the time to completion. These technologies also augmented the range of animals that could successfully undergo genome modifications.[4] However, the more recent clustered regularly interspaced short palindromic repeats (CRISPR) system has rapidly ascended as the *méthode de choix* for genome editing (Figure 1A) because of its incredible simplicity and high efficiency. Indeed, more than half of the entire literature pertaining to CRISPR through 2015 was reported in that year (Figure 1B).

Although many reviews have been published on the subject of CRISPR,[5–7] the field has moved at such a ferocious pace, with many pearls of knowledge only recently shared and debated through a discussion group started in early 2013 (https://groups.google.com/forum/#!forum/crispr), that an update is timely, particularly in cardiovascular biology where only a few papers have been published using CRISPR genome editing in mice. Here, we briefly summarize the historical roots of CRISPR and the key elements of the technology as they apply to the generation of new mouse models. We provide an overview of 2-component (2c) and 3-component (3c) CRISPR, highlighting the strengths and weaknesses of each with specific applications. We address important issues related to the design and testing of crucial components of CRISPR as well as genetic mosaicism, robust genotyping,

Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org   DOI: 10.1161/ATVBAHA.116.304790

| Nonstandard Abbreviations and Acronyms	
2c CRISPR	2-component CRISPR
3c CRISPR	3-component CRISPR
CRISPR	clustered regularly interspaced short palindromic repeats
Cas9	CRISPR-associated protein 9
crRNA	CRISPR RNA
DSB	double-strand break
DSD	double-strand DNA
ESC	embryonic stem cell
HDR	homology-directed repair
indel	insertion or deletion
lncRNA	long noncoding RNA
NDGE	nuclease-directed genome editing
NHEJ	nonhomologous end joining
PAM	protospacer adjacent motif
PTC	premature termination codon
sgRNA	single-guide RNA
SNP	single-nucleotide polymorphism
SSO	single-strand oligonucleotide
tracrRNA	transactivating crRNA
TFBS	transcription factor–binding site

and inadvertent (off) targeting, all of which must be carefully considered for unambiguous evaluation of CRISPR-derived mice. Finally, we provide some clinical perspective for future applications and challenges that must be overcome for CRISPR to have tangible impact in the treatment of cardiovascular disease.

Origins of CRISPR and Its Transition to the Animal Kingdom

In 1987, a peculiar arrangement of homologous direct repeats having dyad symmetry and unknown function was discovered in *Escherichia coli*.[8] Similarly arrayed sequences were subsequently found in archaea and other species of bacteria.[9] These repeat sequences were named CRISPR.[10] No CRISPR sequences were identified in eukaryotes or viruses, suggesting a unique function of CRISPR to species of prokaryotes.[10] The first

clue as to the function of CRISPR sequences emerged with the discovery of similar sized CRISPR spacers between CRISPRs as well as flanking CRISPR-associated (*Cas*) protein-coding genes, implying inter-related functional gene loci.[10] An important breakthrough occurred in 2005 when the CRISPR spacers were found to be of bacteriophage and plasmid origin.[11,12] These findings sparked the prescient hypothesis that CRISPRs endow bacteria with genetic immunity against foreign DNA.[12] Then, in 2007, studies proved CRISPR and adjacent *Cas* operons confer acquired resistance in bacteria to invading viruses.[13] The mechanism underlying such adaptive immunity occurs via endonuclease-mediated cleavage of pre-CRISPR transcripts by Cas proteins and subsequent processing into smaller CRISPR RNA (crRNA) that are complementary to viral DNA, leading to silencing of the alien invasion.[14] Extended CRISPR sequences, reflecting sequential infections of bacteria, imparted greater resistance to bacteriophage assault.[12,13] Thus, many prokaryotes acquire immunity that is hard wired into the host genome. Importantly, in the type II CRISPR-Cas system, the procurement of CRISPR spacers (or protospacers) from the initial invasion of a bacteriophage or plasmid requires a protospacer adjacent motif (PAM) immediately 3′ of the CRISPR spacer sequence in invading DNA.[15] This orientation with respect to CRISPR spacer and PAM is also critical for CRISPR-mediated NDGE in the mouse genome. Although the CRISPR acronym is more aptly applied to prokaryotic immunity, it has become engrained in the lexicon of eukaryotic biologists and the public as a powerful means of altering genomes. For a more in depth study of CRISPR in prokaryotic biology, the reader is referred to several outstanding reviews from pioneers in the field.[16–18]

Two key breakthroughs precipitated the "CRISPR Craze."[19] First, a transactivating crRNA (tracrRNA) was established as essential for crRNA processing in *Streptococcus pyogenes*.[20] The second important discovery occurred 1 year later when crRNA, tracrRNA, and Cas9 were combined in vitro and shown to cleave the complementary and noncomplementary strands of target plasmid DNA 3 base pairs upstream of a PAM.[21,22] Importantly, the crRNA and tracrRNA could be combined into 1 hybrid transcript (so-called single-guide RNA [sgRNA]) and still direct Cas9 cutting of target DNA.[21] Over the ensuing months, an avalanche of papers
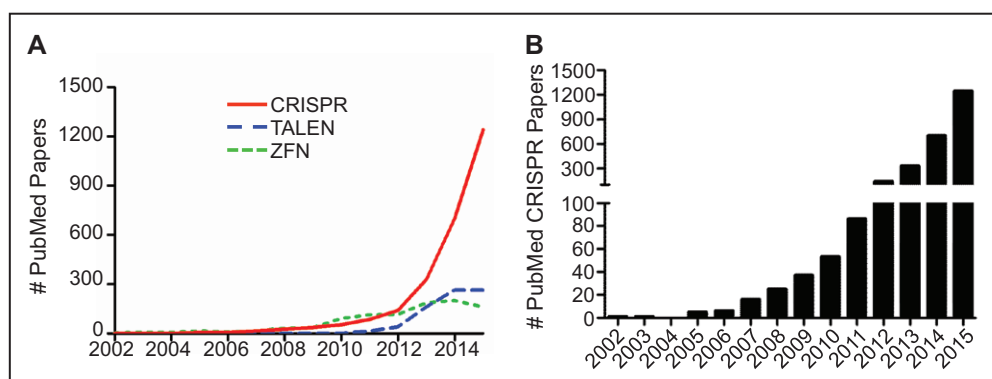
Figure 1. Explosive rise in clustered regularly interspaced short palindromic repeats (CRISPR)–related publications. **A**, Comparative publication track record for the 3 major methods of nuclease-directed genome editing during the past 14 years. **B**, Annual number of CRISPR publications based on PubMed search. After a relatively inactive period (2002–2006), a first wave of papers was published based on the prokaryote adaptive immune function of CRISPR (2007–2011) followed by a second wave relating to CRISPR genome editing (2012–present). Data for both graphs obtained on December 30, 2015.

demonstrated NDGE in human and mouse cell lines[23–25] as well as various animal models[26–34] using the main components comprising CRISPR genome editing, each of which is considered next.

Components of CRISPR Genome Editing

Component 1: Cas9 Endonuclease

The most common endonuclease used in CRISPR genome editing is the class II effector protein, Cas9, from *S pyogenes* (Spycas9).[35] The *Spycas9* gene is transcribed as a ≈4.2-kb transcript encoding a 1368 amino acid protein of ≈160 kDa. The transcript has been codon optimized for efficient translation in mammals and engineered to carry nuclear localization signals for proper targeting to the nucleus as well as epitope tags for easy detection.[23–25] The *SpyCas9* cDNA is found in several plasmids, most notably pX330 from addgene (https://www.addgene.org/42230/).[23] The pX330 plasmid serves as a template for in vitro transcription of *Spycas9*. However, it is much easier to purchase ready-made SpyCas9 protein (PNA Bio, http://pnabio.com) or more frequently, *Spycas9* mRNA (TriLink BioTechnologies, http://www.trilinkbiotech.com) for immediate microinjection in mouse zygotes. The longer 4.5-kb *Spycas9* mRNA from TriLink includes a capped, 5′ synthetic untranslated region harboring a strong Kozak sequence for enhanced translation, an α-globin 3′ untranslated region, and a 120-bp polyadenylated tail to augment stability (Tiffany Teng of TriLink BioTechnologies, personal communication).

It is possible to inject mouse zygotes directly with a plasmid containing both *Spycas9* and sgRNA.[36] However, this approach is complicated by the reduced chance of transmitting an edited allele through the germline because of embryonic cell divisions preceding Cas9/sgRNA transcription and Cas9 translation. Furthermore, there are unpredictable effects of a randomly integrated plasmid in the mouse genome and unknown consequences of continuous expression of Cas9 protein over the life of the mouse. Nevertheless, a recent study showed that mice carrying a *Cas9* transgene under control of the cardiac-specific *Myh6* promoter exhibited no observable toxicity. These mice allow for rapid assessment of gene function in the adult heart after a single injection of adeno-associated virus carrying an sgRNA of interest, thus circumventing potential embryonic lethality that otherwise occurs with conventional gene targeting strategies.[37] Additional mouse models will likely emerge that further limit expression of Cas9 to heart or blood vessels using drug-responsive, cell-specific promoters driving Cas9 directly or indirectly via Cre recombinase in the context of the *Rosa26* floxed stop *Cas9* transgenic mouse (https://www.jax.org/strain/024857).[38]

The SpyCas9 protein has a unique bilobed structure.[39,40] The α-helical target recognition lobe makes important contacts with the sgRNA bound to its target genomic DNA. The nuclease lobe contains the HNH and RuvC-like nuclease domains, each of which mediates an individual nick in the complementary (via HNH) or noncomplementary (via RuvC-like) strand of the double helix, thus creating a blunt double-strand break (DSB). In addition, a PAM-interacting domain within the nuclease lobe binds PAM sequences (NGG or NAG) that are necessary for igniting SpyCas9 nuclease activity, leading to target DNA cleavage[41] 3 base pairs upstream of the PAM sequence.[42] The PAM-interacting domain may also function to facilitate unwinding of the double helix in a 3′ to 5′ direction from the PAM site to allow for strand invasion of the sgRNA, Watson–Crick base pairing between the sgRNA and its complementary target DNA sequence, and formation of a so-called R loop structure.[40,43] PAM sequences may be diversified through alterations in SpyCas9[44] or the utilization of other RNA-guided endonucleases.[45–47] This increases the sequence space that may be efficiently targeted such that virtually every nucleotide in the mouse genome may undergo genome editing.

Additional derivations of SpyCas9 have been engineered that expand its functionality. For example, either of the nuclease domains may be mutated (D10A or H840A) to create a nickase (Cas9n), which increases the specificity of genome editing.[21,48] We do not advocate the use of Cas9n for generating cardiovascular mouse models because of the inherent complexity in experimental design as well as the lower efficiency of SpyCas9n versus wild-type SpyCas9 in mediating genome edits,[49] although editing genomes is highly locus dependent and there may be instances where Cas9n is desirable.[50] However, newer generations of SpyCas9 carrying mutations that do not alter the 2 nuclease domains have been developed that also reduce off-targeting events. These include enhanced specificity SpyCas9 (https://www.addgene.org/71814/)[51] and a high fidelity SpyCas9 (https://www.addgene.org/72247/).[52] It will be important to see whether these newer versions of SpyCas9 are offered as ready-made mRNA or protein and how well they perform in editing the mouse genome when compared with wild-type SpyCas9.

Another modification of SpyCas9 involves mutation of both nuclease domains that effectively yields a dead or deactivated version of the enzyme (dCas9). This alteration in SpyCas9 preserves its sgRNA-directed binding to target DNA; however, dCas9 is unable to cut DNA rendering the protein a suitable platform for many applications, most notably activation or repression of genes through the conjugation of transcriptional activators or repressors.[53] This approach offers a potentially powerful means of rescuing mouse phenotypes resulting from CRISPR-mediated NDGE of distal enhancer sequences by directly activating the endogenous promoter. Another application of dCas9 involves joining the protein with a fluorophore for purposes of tracking Cas9 binding across the genome.[54] A recent study utilizing such an approach revealed Cas9 targeting both euchromatic and heterochromatic sequences through a diffusion-like mechanism, suggesting most of the mouse genome may be targeted for editing regardless of epigenetic state.[55] This finding is consistent with Cas9-mediated cleavage of methylated DNA.[56] Recently, however, an in vitro study showed that nucleosomes represent a barrier for Cas9 binding and cleavage, suggesting the state of chromatin around a gene of interest in the mouse zygote may be an important determinant of CRISPR-mediated NDGE.[57] The latter point underscores the need to test each sgRNA in advance of experimentation (below).

Endonuclease activity of SpyCas9 requires a conformational change in its structure, which is mediated by the sgRNA/target DNA sequence.[40] We know little, however, about endonuclease-independent activity of this protein in the mouse. Preliminary physiological data suggest no overt phenotypes arising from constitutive expression of Cas9 in mice,[37,38] and RNA-seq studies have been done in cultured cells transiently[58] or stably[59] expressing targeted Cas9 with little changes in gene expression beyond those expected. It must be stressed, however, that no studies have yet to interrogate the transcriptome or proteome of tissues in mice with chronic expression of Cas9. It is possible that Cas9 binds other proteins in the cytosol or nucleus affecting subtle perturbations in cell physiology. Furthermore, as a bacterial protein, Cas9 may elicit an immune response. Thus, until more thorough studies are carried out, it is premature to conclude that constitutive expression of Cas9 is not without side effects. Efforts should therefore be made to minimize the window of time in which Cas9 is expressed in mice. This is best achieved by microinjecting Cas9 mRNA or protein into the mouse zygote in combination with the next component of CRISPR technology.

Component 2: sgRNA

The Cas9 endonuclease is inactive until it is bound and ushered to a specific genomic address by the sgRNA. The sgRNA serves as a dual function of both chaperone and activator of Cas9 endonuclease activity through the union of 2 separable sequences transcribed as a chimeric RNA.[21] The chaperone (or guiding) function of the sgRNA is achieved by a user-defined 20-nucleotide CRISPR RNA (crRNA or protospacer sequence in recognition of similarly functioning sequences in bacteria and archaea), whereas the activator function is served by the invariant 80-nucleotide tracrRNA (Figure 2A). The tracrRNA, derived from the *S pyogenes* genome, comprises many structural modules that serve critical roles for Cas9 binding and activation.[60] The crRNA sequence is complementary to the target sequence to be edited in the mouse genome and although sequences longer than 20 nucleotides might intuitively confer greater specificity, they are cleaved to ≈20 nucleotides during processing.[48]

The 20-nucleotide crRNA sequence must precede a PAM recognition site (NGG or NAG for SpyCas9) located immediately 3′ of the last nucleotide of the crRNA sequence. Binding and cleavage of DNA by Cas9 requires the PAM
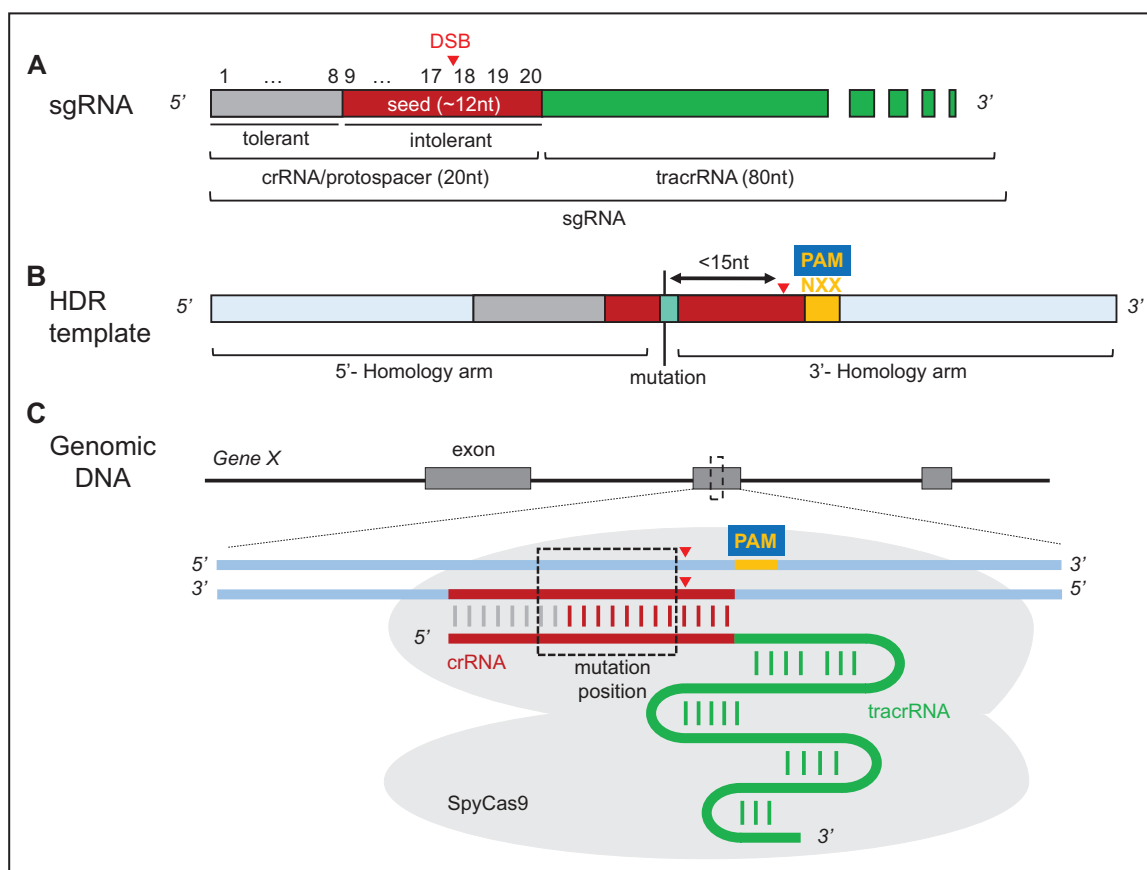
Figure 2. Characteristics of single-guide RNA (sgRNA) and homology-directed repair (HDR) repair template. **A**, The sgRNA is a chimeric molecule comprising a user-defined 20 nucleotide crRNA containing mismatch-tolerant (gray) and intolerant (red) nucleotides and an invariant 80-nucleotide transactivating crRNA (tracrRNA, green). The red triangle here and below denotes the double-strand break (DSB) that occurs after Cas9 binding (**C**). **B**, The HDR template is generally a single-strand oligonucleotide with homology arms of 50 to 70 nucleotides on each side. Note position of sequence edit (labeled mutation) at center of HDR template within 15 nucleotides of the DSB. The XX below protospacer adjacent motif (PAM) denotes silent nucleotide substitutions that prevent secondary nonhomologous end joining (NHEJ). Intolerant sequence changes in the crRNA are important when considering unintended edits and making substitutions for prevention of secondary NHEJ. **C**, Schematic representation of sgRNA and bilobed SpyCas9 over a genomic target site. Note the absence of PAM in sgRNA sequence.

sequence.[21,22] Importantly, the PAM sequence must not be included in the design of the 20-nucleotide crRNA sequence because it would compete for Cas9 binding or perturb normal structural changes that must occur for Cas9 to be activated (Rodolphe Barrangou, personal communication).[60] The crRNA sequence encompasses a PAM proximal seed sequence of ≈10 to 12 nucleotides that is relatively intolerant to mutations[61]; that is, nucleotide substitutions in the seed sequence tend to mitigate Cas9 binding and cleavage of the target DNA sequence. However, a PAM distal sequence of approximate equal length is more tolerant to mutations and thus changes here tend to have less impact on Cas9 binding and activity (Figure 2A). These sequence characteristics of the crRNA have been incorporated in many programs that predict potential off-target sites in the mouse genome (below). As a final point, chemical modifications to the crRNA sequence that enhance stability have been shown to exhibit superior activity over unmodified crRNA sequences,[62] providing a means of targeting Cas9 to potentially intractable genomic sites, although this has yet to be formally demonstrated in the mouse.

Numerous online tools are available for the identification of optimal crRNA sequences.[63] The most commonly used algorithm is the CRISPR Design Tool from the Feng Zhang Laboratory (http://crispr.mit.edu).[56] The algorithm is built on several important sequence characteristics such as a PAM (NGG or NAG) after the crRNA sequence, absence of polyU at the 3′ end of the crRNA sequence, and as little sequence identity to other potential off-target sites in genome as possible, especially in the seed region of the crRNA. The output display is a rank-ordered list of optimal crRNA sequences for a user to select based on the number of theoretical off-target sites (higher scored crRNAs are best).

Essentially all of the front-end work of CRISPR-mediated NDGE involves preparation of the sgRNA. Once a crRNA sequence is selected, a pair of complementary oligonucleotides should be ordered for cloning into the pX330 vector. After verification of the cloned crRNA sequence, the plasmid is tested for sgRNA activity and then processed for in vitro transcription, purification, and visual analysis for integrity with a BioAnalyzer. A distilled version of a detailed protocol for cloning the crRNA into the pX330 vector and in vitro transcription/purification of the sgRNA[64] is available online (Method I in the online-only Data Supplement). Some commercial companies now offer custom synthesis of crRNA and tracrRNA sequences, which minimizes the hands-on workload; however, companies do not typically test synthetic crRNAs for Cas9-dependent cleavage activity.

Most (>80% in our experience) sgRNAs will direct a Cas9-induced DSB. However, a recent report indicated that nucleosomes may obstruct Cas9 binding and cleavage.[57] Given the cost in time and money to engineer a genetically modified mouse, we advise careful attention to sgRNA design and activity testing before its synthesis and purification for microinjection. We typically design and test at least 2 sgRNAs for activity before injecting into a mouse zygote. There are at least 2 ways of testing the activity of an sgRNA. One method measures DSB cleavage directly at the intended genomic site using a nuclease such as Surveyor, which digests heteroduplex DNA after the annealing of wild-type sequence with related sequence containing insertions or deletions (indels) that result during the repair of a DSB.[65] Another approach assesses DSB cleavage indirectly through the reconstitution of a split EGxxFP reporter plasmid (http://www.addgene.org/50716/).[36] Both methods involve transfecting cells with a plasmid carrying a Cas9 and an sgRNA (eg, pX330) or individual store-bought components. The Surveyor assay is slightly less labor intensive but more temperamental than the split reporter assay, which we routinely use in testing sgRNAs. An advantage of the split reporter is that the same highly transfectable cell line (eg, HEK-293) may be used, regardless of the species' genome to be edited. Typical results using the split reporter assay are presented online (Figure I in the online-only Data Supplement). In addition, we provide some key tips for testing sgRNAs using the split reporter assay (Method I in the online-only Data Supplement).

Component 3: Homology-Directed Repair Templates

A third component of CRISPR is essential for precision-guided genome editing and involves introduction of a donor DNA template that is incorporated into the host genome during homology-directed repair (HDR). The donor DNA template may either be a single-strand oligonucleotide (SSO) or a double-strand DNA (DSD) molecule, both of which contain a centrally positioned sequence substitution or addition flanked by homology arms that overlap the Cas9–sgRNA targeted region of the genome, including the PAM sequence (Figure 2B). The centrally located sequence may represent nucleotide substitutions, short sequence inserts (eg, restriction site, *loxP* site, and small epitope tag) or long sequence inserts (eg, Cre recombinase and reporter gene). The function of the SSO or DSD template is to incorporate a subtle (eg, single-nucleotide substitution) or more substantial (eg, transgene) edit to the targeted genomic site during repair of a DSB, thereby generating a predictable and inheritable modification of the mouse genome.

The decision to use an SSO or DSD repair template is dependent on the size of the sequence change to be merged into the mouse genome. Commercial synthesis of SSO is limited to a length of 200 nucleotides, although recent studies in mice have used longer SSO of nearly 1 kb.[66] We suggest the use of SSO HDR templates for integrating single- or multiple-nucleotide substitutions, restriction sites, *loxP* sites, or small epitope tags, such as 3xFLAG. It is recommended that SSO be purified, generally by polyacrylamide gel electrophoresis (eg, PAGE Ultramers from Integrated DNA technologies, https://www.idtdna.com). The length of an SSO is dictated by the size of the centrally located sequence change. For example, if a disease-associated single-nucleotide polymorphism (SNP) is to be integrated into the mouse genome, the SSO could be as short as 100 nucleotides, but more generally is 120 to 140 nucleotides in length with the SNP situated near the middle of the SSO and close to the PAM sequence. Sequence inserts such as *loxP* (34 nucleotides) or 3xFLAG (63 nucleotides) necessitate longer SSO, typically in the range of 165 to 185

nucleotides. We do not recommend using SSO of length >185 nucleotides because of poor coupling efficiencies and the increased risk of sequence errors during synthesis (search *loxP* under the MIT CRISPR Discussion Forum for further details, https://groups.google.com/forum/#!forum/crispr).   Recently, improved integration of inserts ≤100 nucleotides was reported with the use of phosphorothioate-modified SSO with shorter homology arms.[67]

In addition to the length and central sequence to be recombined into the mouse genome, there are additional points to consider in the design of an SSO. First, we recommend the SSO be of the same strand as the sgRNA so as to avoid competitive hybridization with sgRNA and its complementary genomic sequence. Further consideration should be taken when using multiple SSO templates to avoid template hybridization, such as using 2 SSO templates for *loxp* insertion. In the latter instance, the palindromic sequence of *loxp*, when represented by SSO templates on opposite strands, may lead to unintended SSO complementary binding, thus compromising HDR efficiency and accuracy. Second, the HDR repair mechanism after a DSB competes with another repair mechanism known as nonhomologous end joining (NHEJ) that can occur after incorporation of the SSO into the mouse genome. To avoid the latter undesirable circumstance, many investigators mutate the PAM sequence (eg, NGG>NXX) within the SSO (Figure 2B). If the PAM is within a protein-coding region, then the change must preserve the amino acid sequence. Alternatively, silent mutations could be introduced in the SSO at wobble positions of codons along the PAM proximal seed region of the corresponding crRNA sequence. Mutations in the seed region of the SSO HDR template can also reduce secondary NHEJ by rendering the sgRNA ineffective in creating a DSB because of poor binding of the sgRNA to the newly modified genome. Irrespective of the SSO alteration, the goal is to minimize secondary genome editing via NHEJ and subsequent loss of the desired sequence edit.

Another important consideration in the design of an SSO (or DSD) is the position of the sequence substitution or insert relative to the DSB 3 base pairs upstream of the PAM. The most successful HDR templates will have the position of the substitution or inserted sequence no >10 to 15 nucleotides from the DSB (Figure 2B) as the efficiency of incorporating a sequence change or insert falls dramatically as the distance of the edit increases from the DSB.[68,69] We have noted a recombination of PAM mutation without the flanking desired edit, suggesting a short sequence length is sufficient for homologous recombination. Given the close association of Cas9 with the PAM sequence, we routinely place the sequence edits 5′ of the DSB. Newer endonucleases such as Cpf1 with broader PAM sequence recognition and unique cutting attributes,[46] increase targeted sequence space for genome editing thus potentially allowing more flexibility in getting desired edits as close to the DSB as possible.

A primary limitation of CRISPR genome editing is the unfavorable recombination of larger insertions requiring DSD templates than those that can be incorporated with SSO templates. Nevertheless, there are reports of DSD templates injected into the mouse zygote for the integration of reporter transgenes[70,71] or a conditional allele.[72] These studies used conventional homology arms of variable lengths (3–9 kb) in a circular DSD template; linearization of the DSD donor is not done so as to reduce random integration in the mouse genome. Recently, DSD templates with shorter homology arms (0.3 kb) were used in mouse ESC to generate a reporter mouse line.[73] This finding is noteworthy because of the emergence of gBlocks and GeneArt strings technology where a DSD template (≤3kb) can be synthesized and sequence confirmed for direct zygote injections without the need for labor-intensive PCR cloning of homology arms around the sequence of interest. These synthetic DSD donor templates have yet to be used on a large scale as of this date,[74] although we have noted some success when using gBlocks DNA in the mouse. Although there has been reported success with DSD templates in vivo, use of SSO is more prevalent and efficient, and we therefore recommend SSO for most CRISPR editing applications in the mouse. Now that each component of CRISPR editing has been discussed, we turn to their combined use for versatile genome editing in mice.

Two-Component CRISPR

The most basic form of CRISPR-mediated NDGE in mice uses 2 components, Cas9 and sgRNA. In 2c CRISPR, a DSB is repaired through the error-prone NHEJ mechanism (Figure 3A). During NHEJ repair, host DNA sequences are most frequently deleted; however, inserted sequences and substitutions may also occur.[75,76] NHEJ is the dominant mode of DSB repair in the mouse zygote with upward of 80% of pups showing some form of nucleotide change in targeted sequences.[28] Importantly, the multiplex nature of 2c CRISPR[23] allows for multiple genes to be targeted simultaneously in the mouse.[28] Furthermore, unlike ESC-based knockouts, where only 1 allele generally recombines the mutation following electroporation, 2c CRISPR often results in biallelic targeting in the mouse zygote.[28] Thus, multiplex 2c CRISPR affords the unprecedented opportunity to study multiple inactivated alleles in founder mice.[28] Of course, on breeding founders to the Filial 1 generation targeted alleles will segregate out and the same complex breeding scheme required in traditional compound knockouts will apply.

Targeting Protein-Coding Genes

Several outcomes are possible after NHEJ repair of a DSB within a protein-coding sequence. The most desirable result would be disruption of the reading frame and introduction of a premature termination codon (PTC), leading to nonsense-mediated mRNA decay and loss of protein expression. This would most likely yield a true knockout, especially if the first coding exon is targeted with sgRNA and no internal promoters driving expression of an alternative isoform are present. Such an approach was used to target exon 1 of the *Pcsk9* gene in mice using adenovirus delivery to the liver with results showing a decrease in plasma levels of both PCSK9 protein and cholesterol.[77] A second consequence of NHEJ repair of a coding exon 1 is deletion of promoter elements leading to complete silencing of gene expression. A third scenario is indels near splice donor or acceptor sites leading to aberrant splicing with loss of critical amino acids or the generation of
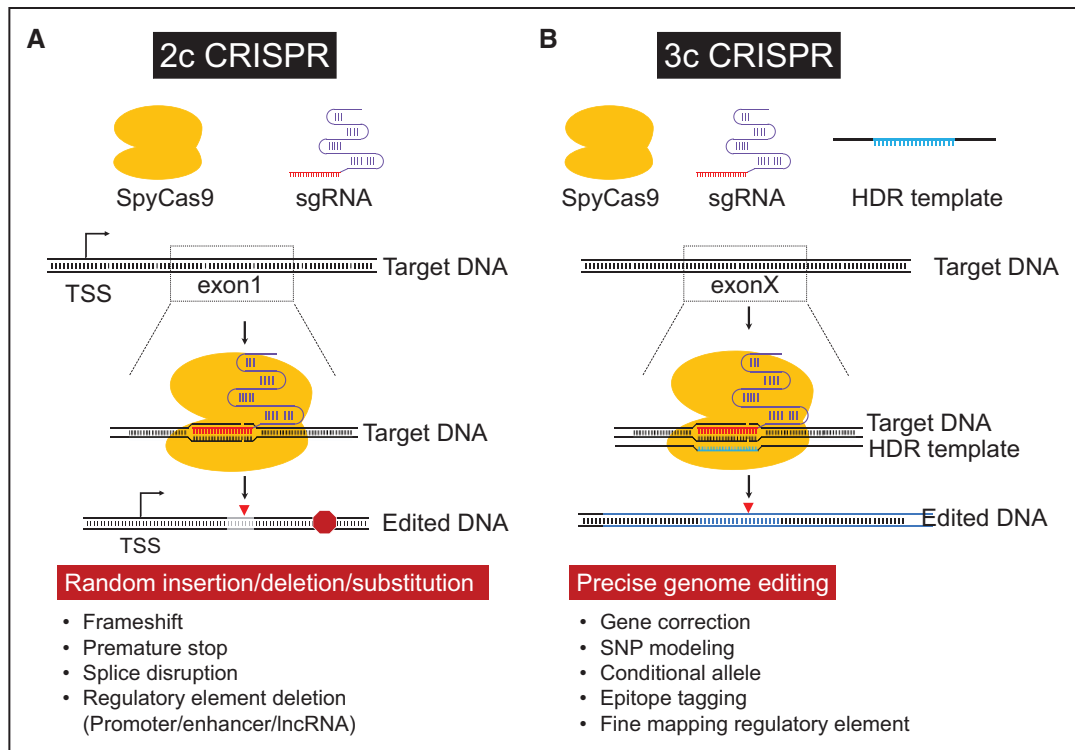
Figure 3. Versatility of clustered regularly interspaced short palindromic repeats (CRISPR) genome editing. **A**, In 2-component CRISPR (2c CRISPR), the SpyCas9 and single-guide RNA (sgRNA) mediate unpredictable insertions, deletions, or substitutions close to the double-strand break (DSB, red triangle) resulting in an altered reading frame, sometimes with an attending premature termination codon (red octagon). Two sgRNAs can also lead to deletions of genomic DNA segments between 2 DSBs (not shown). **B**, In 3-component CRISPR (3c CRISPR), introduction of a homology-directed repair (HDR) repair template facilitates precision-guided genome editing over nonhomologous end joining (NHEJ), resulting in the incorporation of subtle sequence edits or small sequence tags such as *loxP* sites or 3xFLAG (blue). See text for more details. TSS indicates transcription start site.

a frameshift mutant. A fourth effect of NHEJ repair is an in-frame mutation with loss or gain of ≥1 contiguous amino acid or, in the case of single-nucleotide substitutions, introduction of a missense mutation. The latter genetic modifications are less likely to result in a true null phenotype, but could inform the role of key amino acids in the biology of a protein (eg, stability, trafficking, and activity). Finally, a single-nucleotide substitution may also result in the introduction of a nonsense codon with loss of normal protein expression. These examples highlight the major limitation of 2c CRISPR, namely the unpredictability in how NHEJ repair will occur. Some laboratories, including our own, use >1 sgRNA to exon 1 of a protein-coding gene to increase the likelihood of a null allele.[78] It should be noted, however, that with every additional sgRNA used in 2c CRISPR, there is an increased risk of unintended edits and an expanded number of different alleles, which can complicate analysis. Given the unpredictability in 2c CRISPR of coding exons, extensive sequence analysis around the targeted site and breeding of mice to homozygosity are essential to determine whether a null or partial null allele is generated.

A limitation of targeting coding genes with 2c CRISPR relates to pervasive transcription and the disproportionate number of 5′ antisense long noncoding RNA (lncRNA) genes that are adjacent to or overlap the first exon of many protein-coding genes (Figure II in the online-only Data Supplement). It may be necessary in such instances to target an alternate downstream exon coding for a critical functional domain so as to avoid confounding interpretation of data with the simultaneous inactivation of both coding and noncoding genes. Of course, targeting downstream exons with sgRNA requires that disruption of the exon will lead to loss in protein expression. As with any approach that removes or disrupts an internal exon, there is the chance for hypomorphic alleles (ie, incomplete knockout of the gene). Thus, the choice of downstream exon to be targeted with 2c CRISPR must be weighed carefully.

In some instances, it may be prudent to use a pair of sgRNAs flanking a faulty coding exon to remove the exon and restore expression of the defective protein. A classic example is the *dystrophin* gene in the *mdx* mouse model of muscular dystrophy, where a nonsense codon in exon 23 results in a truncated dystrophin protein.[79] Recently, 2c CRISPR was applied via adeno-associated virus to adult *mdx* mice with encouraging results: a significant number of skeletal, cardiac, and smooth muscle cells showed restored expression of dystrophin on deletion of exon 23 and restoration of the reading frame.[80,81] Furthermore, skeletal myopathy was partially rescued with this therapeutic intervention.[80,81] This highlights the power of 2c CRISPR-mediated NDGE in potentially curing a disease of the heart and blood vessels. However, 2c CRISPR may be used to model human diseases where truncation of a protein exists. For example, PTCs in the *ALPK3* gene were recently found in neonates who succumbed to precocious cardiomyopathy.[82] It may prove informative to generate similar

alleles of *Alpk3* in mice to understand the nature of this new genetic form of human cardiomyopathy.

As with any gene inactivation study, it is important to know about the protein-coding gene of interest before designing sgRNA(s) for genome modifications. This can best be discerned with the UCSC Genome Browser (https://genome.ucsc.edu/)[83] and inspecting gene structure for the first coding exon as well as the presence of any downstream isoforms that may not be inactivated by a frameshift mutation. For example, some genes harbor multiple transcript variants arising from independent promoters, thus requiring judicious design of an sgRNA to ensure all isoforms are targeted with 2c CRISPR.[84] It is also advisable to evaluate multiple gene prediction tracks for less well-annotated transcripts as well as ENCODE-derived chromatin remodeling tracks (eg, H3K27Ac), DNase I hypersensitivity tracks, and ChIP-seq tracks on the UCSC Genome Browser to determine the presence of any functional regulatory regions that could be lost with 2c CRISPR deletion of protein-coding sequence. The latter is of importance given the presence of regulatory elements within protein-coding exons.[85]

Targeting Noncoding Sequences

In addition to targeting protein-coding genes, 2c CRISPR may be used for knocking out noncoding genes. For example, a 23-kb segment of the *Rian* lncRNA was deleted from the mouse using 2 pairs of sgRNAs flanking the region of interest.[86] In addition to loss in expression of *Rian*, the deletion also resulted in reduced expression of adjacent genes suggesting *Rian*, a nuclear lncRNA, functions in *cis* to regulate gene expression. A limitation of the latter approach is the use of so many sgRNAs, which heightens the risk of unintended editing. Inasmuch as lncRNAs exhibit diverse functions in maintaining cellular homeostasis, 2c CRISPR will find increasing use in deciphering the function of this class of noncoding RNA in the vascular system.[87] In addition to lncRNA genes, microRNAs are also amenable to 2c CRISPR deletion, though none have yet to be edited in the mouse. Interestingly, microRNAs may be found within a host lncRNA so it will be important to try and distinguish the biological significance of each using carefully designed 2c CRISPR editing.

Most sequence variants occur in noncoding sequence space and some are associated with human diseases.[88] Furthermore, increasing evidence indicates some noncoding sequence variants overlap with binding sites for transcription factors or microRNAs that regulate gene expression.[89–91] Many gene regulatory sites are found in larger sequence blocks known as enhancers. Thus, genome editing of enhancers in mice will be a critical approach toward defining the functionality of sequence variants implicated in human disease. Historically, elucidating the function of regulatory sequences or enhancers via genetic inactivation in mice lagged far behind that of protein-coding genes. This stems from the large ratio of enhancers (>1 million) to protein-coding genes (≈20 000) and the inherent difficulty in pinpointing dominant-acting enhancers in vivo. However, many papers have shown the functional activity of cardiovascular-restricted enhancers in mice with reporter genes, such as lacZ.[92] Such studies have yielded insight into target gene regulation

and the development of cell-specific Cre-driver mice for gene inactivation studies, but they can be limited by copy number variations, random integration events in the genome, and the out-of-genomic context in which they are evaluated.[93]

Now, with 2c CRISPR, the deletion of enhancers in their native genomic milieu is as easy as inactivating a protein-coding sequence. Indeed, in the last year, several enhancers have been deleted in mice using 2c CRISPR including those associated with expression of *Bcl11a*,[94] *Blimp1*,[95] *Mef2c*,[96] *Stat5*,[97] *Pcdh*,[98] *Mmp13*,[99] *Gata3*,[100] and *Cebpa*.[101] In each case, deletion of the enhancer compromised expression of the associated target gene. In addition to enhancers, boundary elements such as locus control regions or insulators may be interrogated with 2c CRISPR. In this context, an insulator located within repeat sequences, and hence intractable for removal through conventional gene targeting, was deleted with 2c CRISPR; mice lacking the regulatory region exhibited reduced expression of the *Tyr* gene.[102]

The deletion of regulatory regions is best achieved with paired sgRNAs that flank the enhancer or boundary element (Figure III in the online-only Data Supplement). It should be emphasized that deleting regulatory regions in this manner has some limitations. For example, there is the risk of segmental inversion or (less likely) duplication.[103] Furthermore, deletions result in a genomic cavity with excision of hundreds or, in the case of superenhancers,[104] thousands of nucleotides. This presents a challenge when attempting to attribute differences in target gene expression to a dominant transcription factor–binding site (TFBS). Furthermore, deleting large segments of DNA may directly perturb expression of genes outside the immediate target gene of interest. However, when combined with chromosome conformation capture and RNA-seq data, the deletion of enhancers or boundary elements will often provide strong evidence for a regulatory region enhancing or reducing the expression of a specific gene locus. The approach of deleting regulatory regions also provides a foundation for finer resolution studies in mice that carry more precise edits of key TFBS as well as specific SNPs associated with human disease.[105]

The trove of ENCODE data (eg, histone modifications of chromatin and gene expression) on the UCSC Genome Browser could inform the potential function of an enhancer and seems a reasonable starting point for testing the in vivo function of a putative regulatory region (Figure III in the online-only Data Supplement). However, recent transgenic mouse work found that enhancer activity was more a function of transcription factor occupancy over an enhancer region than specific chromatin marks.[106] Moreover, changes in gene expression after 2c CRISPR deletion of a putative distal regulatory domain in the human genome could not easily be predicted by the presence of a chromatin mark.[107] These studies underscore our primitive knowledge of functional TFBS within enhancers and their activity in an in vivo setting. The multiplexed editing regulatory assay was recently reported as a large scale in vitro 2c CRISPR screen for functional regulatory elements in the mouse genome.[108] This assay also uncovered unmarked regulatory elements indicating the full complement of functional DNA sequences in the human and mouse genomes is larger than previously thought. Accordingly, there

will be an increasing need for elucidating functional regulatory elements across the genome. One approach to prioritizing putative functional elements is through 2c CRISPR–mediated deletion of enhancer regions carrying variants linked to disease followed by the use of precision-guided CRISPR editing of the specific variant (below).

To summarize, 2c CRISPR is an easy and widely applied method of CRISPR-mediated NDGE in the mouse. All an investigator needs is a validated sgRNA (or pair when deleting an exon, microRNA, lncRNA, or enhancer) and store-bought Cas9 mRNA or Cas9 protein for submission to an academic transgenic mouse core (a list of many such cores within the continental United States is available on request). 2c CRISPR is highly efficient given that NHEJ occurs throughout much of the cell cycle, making it especially attractive for in vivo editing of postmitotic cells, such as cardiomyocytes. 2c CRISPR may be multiplexed allowing an investigator to target multiple gene loci, particularly gene paralogs in which inactivation of one may be compensated for by another. The primary weaknesses with 2c CRISPR are the unpredictable nature of editing and the elevated risk of off targeting when using multiple sgRNAs.

Three-Component CRISPR

3c CRISPR combines a donor DNA template with Cas9 and sgRNA to promote HDR over NHEJ after a DSB (Figure 3B). The primary strength of 3c CRISPR is precision editing of the genome through user-defined substitutions or additions of sequences during synthesis or construction of the donor template. Given the high frequency of SpyCas9 PAM sequences in the mouse genome (1 in every ≈12 base pairs), and attainable edits within the HDR template recombining when placed within 1 to 15 base pairs from the DSB, it is theoretically possible to perform precision-guided 3c CRISPR editing at virtually every nucleotide in the mouse genome. Newer generation endonucleases[45–47] that recognize distinct PAM sequences from those of SpyCas9 further strengthen this notion.

3c CRISPR facilitates subtle edits to the genome, such as single-nucleotide replacements. This approach has extraordinary power in modeling human disease as well as elucidating the function of specific amino acids or regulatory sequences. Similar to 2c CRISPR, 3c CRISPR may be multiplexed for the editing of >1 sequence.[28] Furthermore, biallelic targeting is often observed allowing for immediate investigative work in founder mice.[70] The primary weakness of 3c CRISPR is its lower efficiency than NHEJ repair in mice,[109] probably because HDR is confined to the replication phase of the cell cycle when sister chromatid pairs are made available for normal HDR to proceed. There is a report of enhanced HDR in mice after coinjection of an inhibitor of NHEJ (eg, Scr7)[110]; however, our own experience and that of others[111] is that use of such an inhibitor has little apparent benefit in elevating HDR in the mouse zygote. Recently, the application of a small molecule agonist of RAD51 (promotes homologous recombination) increased the efficiency of HDR in rabbits.[111] There has yet to be a consensus as to the use of this approach in the mouse. Other interventions to increase efficiency of 3c CRISPR in mice include electroporation of zygotes[112] and phosphorothioate modification of the ends of SSO.[67] Because the injected

mouse zygote undergoes rapid cell divisions, enhancement of 3c CRISPR in this context is less critical than, for example, the targeting of postmitotic cardiomyocytes. Below, we summarize several applications of 3c CRISPR in mice.

Targeting Protein-Coding Genes

A popular application of 3c CRISPR is the introduction of subtle edits into coding sequences that fix or model an experimental human disease. In fact, one of the first applications of 3c CRISPR in mice involved the correction of cataracts by repairing a disrupted reading frame in the *Crygc* gene.[113] A similar remedial intervention was achieved in the *mdx* mouse model of muscular dystrophy where a PTC yielding a truncated *Dmd* gene was corrected with base pair changes engineered within the SSO repair template.[114] With thousands of Mendelian traits now cataloged (http://www.omim.org/), including many associated with cardiovascular disease,[115] 3c CRISPR will have profound use in the biological study of point mutations in protein-coding genes strongly linked to human diseases. In addition, exome sequencing will continue to disclose rare mutations associated with defects in the cardiovascular system that will be amenable for study in the mouse.[82] Because the desired edit will generally involve only 1 nucleotide substitution, SSO donors of 120 to 140 nucleotides length will be sufficient as HDR templates. A challenge in correcting or modeling a human variant in protein-coding sequence is the constrained sequence space for designing an optimal sgRNA; if the desired edit is too far removed from the DSB, integration of the edit may not occur or will do so inefficiently. New-generation sgRNAs and SSO repair templates with chemical or spatial modifications[116] as well as alternative endonucleases with different PAM requirements (above) could circumvent these challenges. A new method of genome editing offers a potentially exciting alternative wherein point mutations are achieved in the absence of a DSB and donor repair template.[116a]

There are many protein-coding genes in the mouse genome whose function is completely unknown. Rather than use 2c CRISPR to inactivate such a gene in an unpredictable manner, one could use 3c CRISPR with an SSO targeting the first coding exon and carrying a nucleotide substitution that would generate a PTC and nonsense-mediated decay. One important consideration is the position of the precision-guided PTC relative to a downstream exon–exon junction. In general, nonsense-mediated decay will occur if the PTC is at least 50 to 55 nucleotides upstream of an exon–exon junction.[117] 3c CRISPR has distinct advantages in this regard because not all PTC generated through 2c CRISPR will fulfill this important criterion. Moreover, it is much easier to genotype a precision-guided mutation than variable indels and substitutions stemming from 2c CRISPR (see Genotyping section).

In addition to modeling human diseases or exploring the function of uncharacterized protein-coding genes, 3c CRISPR will be increasingly used to interrogate the functional significance of individual amino acids. For example, definitive function of such post-translational modifications as phosphorylation, ubiquitinylation, sumoylation, and acetylation will necessitate the introduction of missense mutations to show loss in modification has biological consequences in the mouse.

Critical amino acids involved in nucleocytoplasmic shuttling, secretion, and interactions with other proteins, DNA, or RNA will also be subject to 3c CRISPR in mice. Studies conducted in this manner may reveal what was true in cultured cells is untrue in a living animal.

In this context, CRISPR-mediated edits in cell culture models may be undermined by frequent contamination with other cell types or species of mycoplasma as well as genomic instability.[118] Of course, some cell types such as ESC or human inducible pluripotent stem cells will be essential for experimental therapeutic gene editing. In addition, if a human protein-coding gene is not represented in the mouse genome, CRISPR editing would need to be carried out in a suitable human cell line. In general, however, we suggest CRISPR editing be done in the mouse over cultured cells. Not only will results be obtained in a more physiological setting, CRISPR editing of a diploid genome (mouse) is easier than a polyploid genome (many cell lines). Furthermore, unintended edits in mice are less problematic than those in cell culture where off targeting is permanent. Published protocols are available in those instances where CRISPR editing of cultured cells is necessary,[119,120] although we advise using one of the newer generation Cas9 endonucleases exhibiting low off-target activity.[51,52]

Targeting Noncoding Sequences

3c CRISPR genome editing of lncRNA genes in mice is somewhat problematic. One challenge relates to the relatively low sequence conservation of lncRNAs when compared with protein-coding genes. For example, a human-specific lncRNA involved in smooth muscle cell[121] and endothelial[122] cell differentiation cannot be modeled in a mouse and thus would require 3c CRISPR editing in a human vascular cell line. However, *Braveheart*, a mouse-specific lncRNA involved in cardiomyocyte differentiation,[123] represents an attractive lncRNA for CRISPR editing in the mouse. Given our primitive understanding of the functionality of transcribed noncoding sequences, the approach to 3c CRISPR may not be as intuitive as with protein-coding genes. One idea would be injecting an HDR template carrying a subtle edit at a splice donor/acceptor site to disrupt normal splicing. Alternatively, one could introduce a polyadenylation signal cassette in the first exon of the lncRNA, thereby generating a truncated transcript because of premature termination of transcription. The latter would require a DSD HDR template.

Historically, evaluating loss of a regulatory region (eg, enhancer) on target gene expression in mice used *Cre*/loxP technology leaving a genomic cavity. More subtle approaches that generated mice in which the phasing of the DNA sequence was preserved through base pair substitutions showed the importance of regulatory elements in target gene expression; however, these studies are few in number and still had the issue of removing a selectable marker with a retained *loxP* site.[124–126] On the surface, the confounding effects of Cre-mediated deletions or retention of a 34 bp *loxP* site may seem of trivial consequence. However, given the pervasive transcription of the mouse genome with many thousands of lncRNAs and the known effects of even small sequence additions to the genome after genetic modifications,[127] it is best to make as subtle a nucleotide change as possible for optimal interpretations. Interestingly, out of a survey of 646 mutant mice in which subtle mutations were introduced, only 16 involved regulatory regions[128] and only one of these mutant mice carried the desired mutation without added or subtracted sequences.[129] The latter was achieved with the labor intensive hit and run approach to genetic modification of the mouse genome, a technology that is rarely used today.[128]

Engineering mice with subtle edits to regulatory elements such as TFBS or microRNA-binding sites requires advanced knowledge as to the functionality of the relevant sequence. At a minimum, traditional methods of analyzing regulatory sequences (eg, mutagenesis in luciferase reporter and gel shift) should be performed before the generation of a CRISPR mouse. It may be wise to delete a larger segment of DNA (eg, enhancer) first in the mouse with 2c CRISPR and then perform secondary experiments with 3c CRISPR that disrupt individual TFBS within the DNA segment. An obvious challenge exists with enhancers that have multiple TFBS[100]; ENCODE and SNP data will be a useful guide for prioritizing those elements of most interest. In addition, it is possible to engineer >1 edit in closely juxtaposed TFBS in the mouse. For example, we have recently generated a mouse derived from an SSO repair template in which 2 separable regulatory elements were mutated (J.M. Miano, unpublished data, 2016). As of this writing, only one published study has reported the effects of subtle nucleotide substitutions in a TFBS using 3c CRISPR in mice. A serum response factor-binding CArG box located in the first intron of the *Cnn1* gene was altered at 3 positions (CCT>GAC) and shown to nearly abolish expression of the mRNA and protein as well as in vivo serum response factor binding to the mutated element.[130] This result is somewhat surprising given the large number of regulatory sequences over target gene loci in the mouse genome. Finally, a number of SNPs found in microRNAs or their respective binding sites will also be important to model in mice. 3c CRISPR studies that assess the role of single regulatory elements in the mouse offer definitive proof of function and will inform the nature of regulatory SNPs associated with human disease.

Generating Conditional Alleles in Mice

A limitation of CRISPR editing in mice is that all cells will carry the sequence change. However, if the edit is a heritable SNP associated with human disease, the mouse would more closely model the human condition. However, from an experimental perspective, elucidating the underlying cause of a phenotype will be challenging. Fortunately, it is possible to generate conditional alleles with *loxP* sites using 3c CRISPR and subsequently excise the intervening DNA with a suitable Cre mouse line.[70] As with ESC-based gene targeting, it is crucial to carefully deliberate over where exactly the 34 base pair *loxP* sequences should be inserted using ENCODE and other experimental data.[1] If possible, we find it best to flox the promoter region and first coding exon to generate a true null allele after Cre-mediated excision. However, if an adjacent or overlapping antisense lncRNA is present near the first coding exon or an internal promoter exists, alternative plans will be necessary. We also recommend a floxed interval of DNA in the range of 1 to 2 kb to minimize the size of the Cre-mediated deletion and for ease in sequence validation of the edited mouse.

Several approaches exist for making a conditional mouse using 3c CRISPR. One method combines an sgRNA with Cas9n and a DSD donor template carrying both *loxP* sites.[72] A second method involves the synthesis of 2 SSO of length no >180 nucleotides with the 34 base pair *loxP* sequence placed at the center of the SSO within 10 base pairs of the DSB (Figure IV in the online-only Data Supplement). This approach is essentially identical to one previously published.[70] Because 2 *loxP* sequences must integrate on the same strand, the efficiency of generating a conditional allele with 3c CRISPR is low (MIT Discussion Forum). The key to success is screening many pups (at least 40–50) with primers flanking each *loxP* site (Figure IV in the online-only Data Supplement). Both of the aforementioned methods have low success rates. A third approach, one we are currently evaluating, involves a 2-step process wherein a mouse zygote is injected with sgRNA, Cas9, and 1 SSO containing one of the *loxP* sites. Pups are then PCR screened for proper integration of the *loxP* site (Figure IV in the online-only Data Supplement) and Sanger sequenced for *loxP* nucleotide integrity followed by breeding for germ line transmission of the first *loxP* site. Positive mice are then used for the expansion of zygotes and another round of microinjections using a second sgRNA and SSO carrying the other *loxP* sequence.

We wish to point out that while conditional mice can be generated using 3c CRISPR, we have noted several mutations and truncations of one or both *loxP* sequences after integration in the mouse. Whether these sequence changes stem from the initial commercial synthesis of the SSO or during HDR is unknown. Of note, several tolerable substitutions exist across the *loxP* sequence,[131] and recent testing in our laboratory has confirmed Cre-mediated excision of a floxed segment of DNA in a mouse, despite the presence of a single base pair deletion in position 3 of the *loxP* sequence. A comprehensive summary of all tolerable changes to a *loxP* sequence would be a helpful resource for the mouse genetics community.

Finally, it is possible to limit gene inactivation to specific cardiovascular cell types without expending the time and effort in generating a floxed protein-coding or noncoding gene. For example, the *Rosa26* floxed stop *Cas9* transgenic mouse (https://www.jax.org/strain/024857)[38] could be crossed with a cell-specific and inducible Cre-driver mouse resulting in restricted Cas9 availability to the cardiovascular cell of interest. Delivery of sgRNA and HDR template to the heart or vasculature may be achieved using emerging viral and nonviral modes of transfer.[132,133] The approach here, however, is complicated by the lack of Cas9 expression in all cells of interest because of inefficient Cre activity; persistent Cas9 expression in those cells where Cre excised the floxed stop cassette; ineffective delivery of sgRNA/HDR template to vascular cells with adeno-associated virus[134]; and possible inflammatory response with the use of adenoviral vectors.

Generating Epitope-Tagged Mice

A persistent problem in biomedical research is the ineffective or inconsistent performance of an antibody for the detection of a protein in cultured cells, a Western blot, or a tissue section. Indeed, the recent National Institutes of Health notice on Scientific Rigor and Transparency (NOT-OD-16-011) will require assurances that a specific antibody will perform in the anticipated manner. An exciting application of 3c CRISPR is integrating a synthetic peptide (eg, 3xFLAG) to which a well-characterized antibody exists into an endogenous protein-coding gene. As with *loxP* sequences, these small peptides are small enough that they may be engineered within an SSO template as close to the DSB as possible. The most important considerations in epitope tagging an endogenous protein-coding gene are the nature of the tag and whether the tag should be placed at the 5′ or 3′ end of the gene. We find that the 3xFLAG and HA tags yield the most specific results, at least in cultured cells.

The generation of an epitope-tagged protein with 3c CRISPR has several useful applications beyond simple expression analysis under normal and pathological conditions. For example, if the tagged protein is a transcription factor, in vivo chromatin immunoprecipitation-sequence analysis may be done in the heart or vasculature to assess genome-wide in vivo occupancy of a TFBS. This can be profitable when the TFBS is well characterized.[89] In addition, pulldown assays may be done to define interacting partners of the protein or to elucidate post-translational modifications. If the tagged protein undergoes nucleocytoplasmic shuttling or is secreted out of the cell, studying the biology of these and other processes may be done in an unambiguous fashion. Furthermore, tagged proteins can be directly purified from primary cells by immunoprecipitation and used for functional characterization (eg, enzymatic activity).

In summary, 3c CRISPR is the most versatile mode of genome editing with wide applications in the mouse. Although the efficiency (≈10%) is much lower than that observed with 2c CRISPR (≈50%), the strength of precision-guided genome editing afforded by 3c CRISPR makes it an attractive approach for modeling human diseases and elucidating the function of coding and noncoding sequences in the mouse. During the past 2 years, we have generated 10 mouse models carrying a variety of edits in protein-coding and noncoding sequences as well as the incorporation of *loxP* sites and an epitope tag. The formula we follow for 3c CRISPR in mice is the one first described by the Jaenisch Laboratory.[28] Recently, a cloning-free method of preassembled Cas9 protein and commercially synthesized crRNA and tracrRNA along with SSO repair template was reported to exhibit superior genome editing rates.[135] Time will tell whether this approach to CRISPR-mediated NDGE will supplant original methods of injecting Cas9 mRNA, sgRNA, and HDR donor template. A practical summary of the key steps to follow for 2c CRISPR and 3c CRISPR in mice (Figure 4) is provided in the supplement (Method II in the online-only Data Supplement). The work associated with CRISPR genome editing does not end with the birth of live-born mice. There are 3 important issues to be carefully considered once founder mice are obtained and these are summarized next.

Genetic Mosaicism

Genetic mosaicism represents cell–cell variability in genotype wherein some cells exhibit no genome modification (wild-type), and others show expected or unexpected genome modifications. In ESC-based genome modification, there are
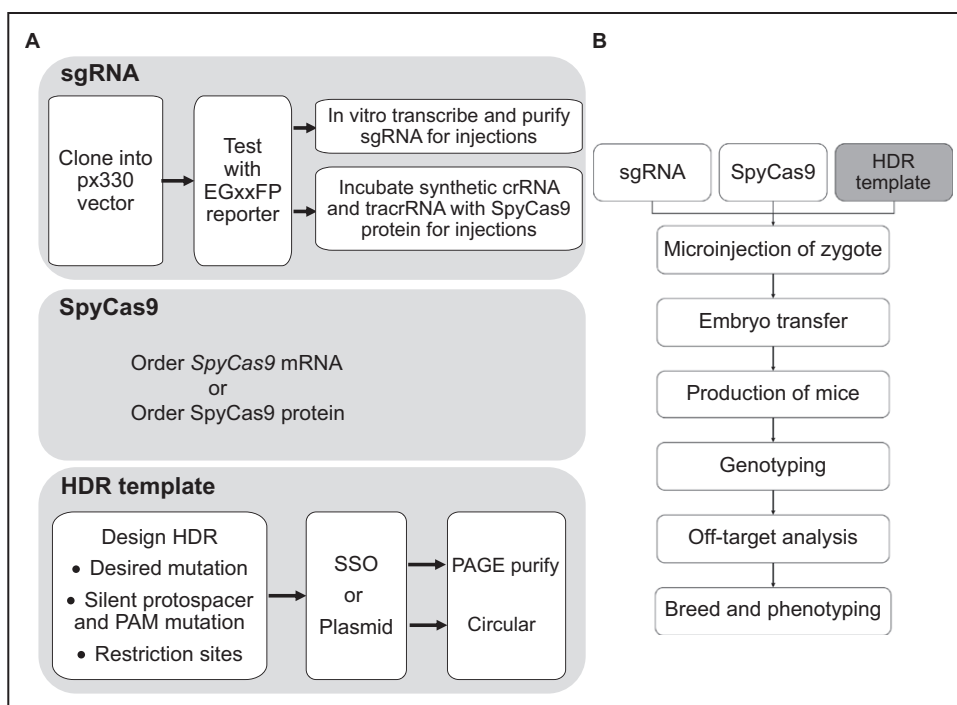
Figure 4. Work flow for clustered regularly interspaced short palindromic repeats (CRISPR) genome editing in mice. **A**, Each CRISPR component is indicated in a gray rectangle. The single-guide RNA (sgRNA) requires careful design and cloning into the pX330 vector followed by testing in the EGxxFP split reporter to validate its activity (see Method I in the online-only Data Supplement). Verification of activity is followed either by in vitro transcription of chimeric sgRNA with T7 polymerase and purification of the sgRNA for injection with SpyCas9 mRNA or, less frequently, SpyCas9 protein (present approach), or independent synthesis of the crRNA and tracrRNA and preincubation with SpyCas9 protein (emerging approach). **B**, General workflow of operations in generating a CRISPR-edited mouse line. The gray box indicating HDR template signifies an optional component. See text for more details. HDR indicates homology-directed repair; PAM, protospacer adjacent motif; and SSO, single-strand oligonucleotide.

generally only 2 possible outcomes across a population of cells in a newborn (founder) mouse: cells that carry only wild-type alleles or cells carrying an allele with a specific genomic modification derived from the engineered ESC. The relative distribution of each cell type is unpredictable because ESC carrying a genome modification are microinjected into the blastocyst of a mouse embryo (≈3.5 days of development) and admix with existing (wild-type) stem cells comprising the inner cell mass. Thus, it is not unusual in this scenario to have a modified allele in a founder mouse incapable of being transmitted through the germline to the Filial 1 generation. Moreover, even if the modification is passed on to the next generation, the resulting mouse is considered to be a chimera because of frequent differences in the strain of microinjected ESC versus the ESC of the inner cell mass in the recipient mouse. The latter often necessitates considerable back-cross breeding to generate a near homogenous strain of mouse for unambiguous assessment of the genome modification.

Genetic heterogeneity in CRISPR-derived mice is different and has its own advantages and challenges. On the one hand, chimerism is a nonissue because there is no admixing of different strains of ESC. Moreover, microinjections may be done in C57BL/6J zygotes, a common strain among knockout mouse models. In this case, extensive back-crossing is avoided. Furthermore, as editing occurs early in embryonic development, there is a high likelihood of an edited allele being transmitted to the next generation; indeed, we have yet to experience an edited allele not undergoing germline transmission. On the other hand, because of the competition between the unpredictable genome editing of 2c CRISPR and the more precision-guided editing of 3c CRISPR, as well as the prolonged editing that presumably occurs after zygotic division in either 2c CRISPR or 3c CRISPR, the number of different alleles even in a single founder mouse can be considerable (Figure V in the online-only Data Supplement).[78,136] In fact, there is diversity of alleles both within and between founder pups (Figures IV and VIB in the online-only Data Supplement). This presents a challenge in sorting through the different alleles across cells in tail snips or other tissues prepared for genotyping to clearly define whether the desirable genome modification exists. Fortunately, there are discriminating methods of genotyping CRISPR-derived mice and we turn to this subject next.

Genotyping of CRISPR Mice

Most methods of genotyping CRISPR mice are based on the judicious design of PCR primers and the validation of genome edits via Sanger sequencing of cloned PCR amplicons. Recently, a digital PCR-based assay was reported for high resolution determination of specific edits in various cell culture systems.[50] In some cases, such as the integration of donor templates with long homology arms, Southern blotting may be needed to ensure proper genome editing. Below, we describe simple PCR-based assays, including one that often identifies single base pair edits introduced with 3c CRISPR.

Genotyping 2c CRISPR Mice

The genotyping of 2c CRISPR founder mice derived from ≥1 closely spaced sgRNAs across a coding exon requires PCR primers flanking the DSB(s) by ≈200 nucleotides. Note that this approach will not detect large deletions.[137] However, indels in the range of 35 to 100 nucleotides can easily be resolved in a 2% agarose gel (Figure VIIa and VIIb in the online-only Data Supplement). The PCR products may then be excised from the gel, column purified (Qiagen), and cloned directly into a plasmid (TOPO PCR cloning kit) for Sanger sequence analysis. As there likely will be multiple alleles because of mosaicism, we recommend sequencing at least 4 to 6 clones per founder mouse. Once a desired allele is defined, the founder mouse should be bred for germline transmission of the edited allele in accordance with Mendel's first law of allelic segregation during gamete formation. Depending on the nature of the edit, a new and more discriminating PCR primer may be synthesized to differentiate wild-type from heterozygous and homozygous mutant pups (ie, making the 3′ most nucleotides of primer specific to the indel or substitution).

The genotyping of deleted exons, enhancers or other noncoding sequences with 2 sgRNAs is somewhat easier because the deleted sequence can be distinguished from the wild-type sequence on a 1% agarose gel (Figure VIIB in the online-only Data Supplement). The PCR primers should be designed 100 to 200 nucleotides outside the 2 DSBs flanking the segment to be deleted. Once germ line transmission occurs, it may be necessary to design a third primer to the deleted sequence to differentiate the different genotypes arising from heterozygous interbreeding (Figure VIIB in the online-only Data Supplement).

Genotyping 3c CRISPR Mice

There are 2 general approaches to genotyping founder mice derived from 3c CRISPR genome editing. One involves the design of flanking primers outside a precision-guided insertion (eg, *loxP* or 3xFLAG) followed by resolution on a 2% agarose gel (Figures IV and VIa in the online-only Data Supplement). However, this approach is inadequate for subtle genome edits, such as point mutations. Although introducing a novel restriction site in the SSO repair template may be done for genotyping slight edits, this approach requires PCR amplification and restriction digestion. Thus, we developed a novel multiplex PCR assay to differentiate wild-type from mutant genotypes in mice carrying minor substitutions in DNA, including single base pair changes[130] (unpublished data). In this scenario, 2 forward primers are used: one forward primer of ≈50 nucleotides contains a 5′ extension of ≈30 nucleotides of sequence unrelated to anything in the mouse genome, followed by 20 nucleotides matching the wild-type genome; and another forward primer that does not have an extended 5′ end but is identical to the 20 nucleotide core sequence save the desired substitution(s) located at the terminal 3′ end of the primer. The 2 forward primers are combined with a common reverse primer in a PCR reaction and the products are run on a 2% to 2.5% agarose gel (Figure VIIC in the online-only Data Supplement).[130] This PCR approach requires some front-end work, including the design of both wild-type and mutant

templates in a plasmid for pretesting of the primers and optimization of the PCR parameters (Method III in the online-only Data Supplement). We recommend following this procedure as we have had much success in using this robust genotyping strategy, including detection of an SNP. It is also possible to design a forward primer that anneals specifically to an inserted sequence (eg, 3xFLAG). We recently found this approach to be helpful in filtering down a high number of edited mice (Figure VIB and VIC in the online-only Data Supplement). As with 2c CRISPR, Sanger sequencing amplified DNA around the edited region should be performed, especially with inserts such as *loxP* and epitope tags where initial PCR genotyping may suggest successful integration, but unacceptable mutations may be present (see sequencing data in Figure VID in the online-only Data Supplement).

Inadvertent Targeting

A limitation of CRISPR genome editing with wild-type Cas9 is inadvertent targeting of distal sites sharing sequence complementarity to the crRNA, especially in the seed sequence of the PAM proximal region (Figure 2A). This is of major concern when using primary or stable cell lines where such off targeting is indelible. For this and other reasons (above), we do not advocate the generation of CRISPR-edited cell culture models unless it is absolutely necessary (eg, human inducible pluripotent stem cells or mouse ESC, both of which exhibit low levels of inadvertent targeting[138]). Inadvertent targeting is less problematic in the mouse genome because of Mendel's second law of independent assortment of alleles during gametogenesis, assuming the off-target edit is not in linkage disequilibrium with the intended target. Nonetheless, there are several ways to mitigate CRISPR off targeting in the mouse. For example, select a crRNA with little to no homology to coding exons or functional noncoding sequences. If point mutations are planned using 3c CRISPR, then the reduced sequence landscape may limit options for choosing an ideal crRNA. In addition, shortening the crRNA sequence at the 5′ end from 20 to 17 to 18 nucleotides may reduce inadvertent targeting that otherwise would occur with full-length crRNA.[139] In addition, several derivatives of Cas9 exist that exhibit low off targeting, including Cas9n,[48] esCas9,[51] and Cas9-HF1.[52] Cas9n is cumbersome and less efficient than its wild-type counterpart; however, the latter 2 Cas9 nucleases are likely more efficient because mutations are in the PAM interacting domain and not the nuclease domains. Neither esCas9 nor Cas9-HF1 has yet been tested for generating CRISPR mice. It may also be desirable to inject Cas9 protein with sgRNA and HDR template to limit inadvertent targeting. Some cores inject lower concentrations of each CRISPR component; however, there is no consensus as to the efficacy of this approach in lowering inadvertent targeting.

Whichever measures are taken to minimize off-targeting at the front-end, because each sgRNA and its intended target site will be unique and have variable efficiencies in terms of chromatin accessibility, assessing inadvertent genome editing at the back-end will be essential and journal/grant reviewers will request that this issue be addressed in some manner. A minimum requirement is to identify related sequences in

the mouse genome, particularly those occurring in exons or noncoding sequences with potential function, using many computational tools.[7] Potential inadvertent target sequences (≈10 based on computer analysis) are then PCR-cloned and subjected to Sanger sequencing to ascertain any indels/substitutions. Several sophisticated methods exist for interrogating the genome for inadvertent edits (eg, whole-genome sequencing),[7] but these are impractical for animal studies and are mainly used in cell culture models that demand a more thorough analysis for off-targeting events.

Clinical Perspectives for CRISPR Genome Editing

Genome editing has already made its way into the clinic,[140] so the application of CRISPR editing to prevent or treat some aspects of cardiovascular disease may soon be realized. The most direct approach will be 2c CRISPR inactivation of a gene expressed in the liver whose product exacerbates a cardiovascular disease. One such therapeutic target is proprotein convertase subtilisin/kexin type 9 (*PCSK9*), a protease that cleaves the low-density lipoprotein receptor, thereby decreasing low-density lipoprotein receptor surface density and increasing plasma low-density lipoprotein levels, which can lead to atherosclerosis and myocardial infarction.[141] As proof of principle, delivery of Cas9 and a sgRNA targeting *PCSK9* to a humanized mouse liver chimera effectively reduced circulating levels of human PCSK9 protein without observable toxicity.[142] Similar targeting of mouse *Pcsk9* with adeno-associated virus carrying a smaller version of Cas9 and a sgRNA has been reported.[45]

An equally exciting approach will be using 3c CRISPR to correct individual mutations in genes that cause inherited cardiovascular diseases. For example, genetic disorders associated with life-threatening arrhythmias such as the Long QT Syndrome could be treated by 3c CRISPR genome correction of such mutated genes as *KCNQ1*, *KCNH2*, and *SCN5A*.[143] Inherited cardiomyopathies caused by mutations in titin or myosin-binding protein c may also be amenable to 3c CRISPR genome editing.[115] In the clinical realm of hemostasis and thrombosis, 3c CRISPR could be used to treat patients with hemophilia A caused by mutations in the *F8* gene encoding factor VIII or hemophilia B caused by mutations in *F9* encoding Factor IX.[144]

CRISPR genome editing offers great advantages over other forms of genetic therapy: CRISPR is rapid, specific, and permanent. However, extensive technical, financial, and ethical challenges remain before CRISPR-based therapies enter the clinic. For example, CRISPR components must be effectively delivered to the correct organ and cell, perhaps using emerging nanotechnologies.[133] There will also be a lot of genome editing work done in patient-derived inducible pluripotent stem cells that could be engineered to differentiate to correct cell types and then deployed on biocompatible grafts. Of course, CRISPR genome editing must be efficient and off-target effects must be minimized with the latest generation of Cas9 endonucleases.[51,52] Given these constraints, the first cardiovascular diseases to be treated with CRISPR-Cas9 may be autosomal diseases caused by genes such as *PCSK9* or *F9* that are expressed in the liver.

Finally, enormous practical and ethical challenges must be overcome before CRISPR genome editing is used to correct heritable cardiovascular diseases in human embryos generated by in vitro fertilization. A highly publicized and controversial paper used 3c CRISPR in tripronuclear human zygotes to edit the *HBB* locus that encodes β-globin, a gene with multiple mutations underlying β-thalassemias.[145] The team found that genome targeting was inefficient and not specific. This article sparked much debate over the ethics of genome editing in the human germline,[146,147] and an international meeting that included the National Academy of Sciences in the United States recommended a moratorium on genome editing of human embryos until the risks are assessed and society agrees on the appropriate use of CRISPR and other powerful genome editing techniques.[148] Recently, however, regulators in China, Sweden, and the United Kingdom granted scientists permission to edit genomes of human embryos.[149]

The path of CRISPR genome editing is paved well for exciting discoveries, innovative technologies, and new hopes for treating some forms of human disease. To date, only a small handful of CRISPR-mediated NDGE studies related to cardiovascular-related genes have been conducted in mice.[37,77,114,130,142] However, the CRISPR Craze will continue to inspire more and more investigators to engineer new mouse strains for a variety of scientific endeavors, from elucidating functional coding and noncoding sequences and modeling human cardiac and vascular disorders to assessing the safety and efficacy of novel CRISPR-based therapies[80,142] for the prevention and treatment of cardiovascular diseases.

Acknowledgments

Sources of Funding

Disclosures

None.

References

1. Davis J, Maillet M, Miano JM, Molkentin JD. Lost in transgenesis: a user's guide for genetically manipulating the mouse in cardiac research. *Circ Res*. 2012;111:761–777. doi: 10.1161/CIRCRESAHA.111.262717.
2. Bibikova M, Golic M, Golic KG, Carroll D. Targeted chromosomal cleavage and mutagenesis in Drosophila using zinc-finger nucleases. *Genetics*. 2002;161:1169–1175.
3. Wood AJ, Lo TW, Zeitler B, et al. Targeted genome editing across species using ZFNs and TALENs. *Science*. 2011;333:307. doi: 10.1126/science.1207773.
4. Gaj T, Gersbach CA, Barbas CF III. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol*. 2013;31:397–405. doi: 10.1016/j.tibtech.2013.04.004.

5. Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. *Cell*. 2014;157:1262–1278. doi: 10.1016/j.cell.2014.05.010.

6. Doudna JA, Charpentier E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science*. 2014;346:1258096. doi: 10.1126/science.1258096.

7. Bolukbasi MF, Gupta A, Wolfe SA. Creating and evaluating accurate CRISPR-Cas9 scalpels for genomic surgery. *Nat Methods*. 2015;13:41–50. doi: 10.1038/nmeth.3684.

8. Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. *J Bacteriol*. 1987;169:5429–5433.

9. Mojica FJ, Díez-Villaseñor C, Soria E, Juez G. Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. *Mol Microbiol*. 2000;36:244–246.

10. Jansen R, Embden JD, Gaastra W, Schouls LM. Identification of genes that are associated with DNA repeats in prokaryotes. *Mol Microbiol*. 2002;43:1565–1575.

11. Mojica FJ, Díez-Villaseñor C, García-Martínez J, Soria E. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *J Mol Evol*. 2005;60:174–182. doi: 10.1007/s00239-004-0046-3.

12. Bolotin A, Quinquis B, Sorokin A, Ehrlich SD. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology*. 2005;151(pt 8):2551–2561. doi: 10.1099/mic.0.28048-0.

13. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, Horvath P. CRISPR provides acquired resistance against viruses in prokaryotes. *Science*. 2007;315:1709–1712. doi: 10.1126/science.1138140.

14. Brouns SJ, Jore MM, Lundgren M, Westra ER, Slijkhuis RJ, Snijders AP, Dickman MJ, Makarova KS, Koonin EV, van der Oost J. Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science*. 2008;321:960–964. doi: 10.1126/science.1159689.

15. Mojica FJ, Díez-Villaseñor C, García-Martínez J, Almendros C. Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology*. 2009;155 (pt 3):733–740. doi: 10.1099/mic.0.023960-0.

16. van der Oost J, Westra ER, Jackson RN, Wiedenheft B. Unravelling the structural and mechanistic basis of CRISPR-Cas systems. *Nat Rev Microbiol*. 2014;12:479–492. doi: 10.1038/nrmicro3279.

17. Marraffini LA. CRISPR-Cas immunity in prokaryotes. *Nature*. 2015;526:55–61. doi: 10.1038/nature15386.

18. Sontheimer EJ, Barrangou R. The bacterial origins of the CRISPR genome-editing revolution. *Hum Gene Ther*. 2015;26:413–424. doi: 10.1089/hum.2015.091.

19. Pennisi E. The CRISPR craze. *Science*. 2013;341:833–836. doi: 10.1126/science.341.6148.833

20. Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, Eckert MR, Vogel J, Charpentier E. CRISPR RNA maturation by transencoded small RNA and host factor RNase III. *Nature*. 2011;471:602–607. doi: 10.1038/nature09886.

21. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*. 2012;337:816–821. doi: 10.1126/science.1225829.

22. Gasiunas G, Barrangou R, Horvath P, Siksnys V. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc Natl Acad Sci U S A*. 2012;109:E2579–E2586. doi: 10.1073/pnas.1208507109.

23. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F. Multiplex genome engineering using CRISPR/Cas systems. *Science*. 2013;339:819–823. doi: 10.1126/science.1231143.

24. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM. RNA-guided human genome engineering via Cas9. *Science*. 2013;339:823–826. doi: 10.1126/science.1232033.

25. Jinek M, East A, Cheng A, Lin S, Ma E, Doudna J. RNA-programmed genome editing in human cells. *Elife*. 2013;2:e00471. doi: 10.7554/eLife.00471.

26. Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh JR, Joung JK. Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol*. 2013;31:227–229. doi: 10.1038/nbt.2501.

27. Shen B, Zhang J, Wu H, Wang J, Ma K, Li Z, Zhang X, Zhang P, Huang X. Generation of gene-modified mice via Cas9/RNA-mediated gene targeting. *Cell Res*. 2013;23:720–723. doi: 10.1038/cr.2013.46.

28. Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F, Jaenisch R. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell*. 2013;153:910–918. doi: 10.1016/j.cell.2013.04.025.

29. Gratz SJ, Cummings AM, Nguyen JN, Hamm DC, Donohue LK, Harrison MM, Wildonger J, O'Connor-Giles KM. Genome engineering of Drosophila with the CRISPR RNA-guided Cas9 nuclease. *Genetics*. 2013;194:1029–1035. doi: 10.1534/genetics.113.152710.

30. Friedland AE, Tzur YB, Esvelt KM, Colaiácovo MP, Church GM, Calarco JA. Heritable genome editing in C. elegans via a CRISPR-Cas9 system. *Nat Methods*. 2013;10:741–743. doi: 10.1038/nmeth.2532.

31. Li W, Teng F, Li T, Zhou Q. Simultaneous generation and germline transmission of multiple gene mutations in rat using CRISPR-Cas systems. *Nat Biotechnol*. 2013;31:684–686. doi: 10.1038/nbt.2652.

32. Yang D, Xu J, Zhu T, Fan J, Lai L, Zhang J, Chen YE. Effective gene targeting in rabbits using RNA-guided Cas9 nucleases. *J Mol Cell Biol*. 2014;6:97–99. doi: 10.1093/jmcb/mjt047

33. Niu Y, Shen B, Cui Y, et al. Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. *Cell*. 2014;156:836–843. doi: 10.1016/j.cell.2014.01.027.

34. Hai T, Teng F, Guo R, Li W, Zhou Q. One-step generation of knockout pigs by zygote injection of CRISPR/Cas system. *Cell Res*. 2014;24:372–375. doi: 10.1038/cr.2014.11.

35. Shmakov S, Abudayyeh OO, Makarova KS, Wolf YI, Gootenberg JS, Semenova E, Minakhin L, Joung J, Konermann S, Severinov K, Zhang F, Koonin EV. Discovery and Functional Characterization of Diverse Class 2 CRISPR-Cas Systems. *Mol Cell*. 2015;60:385–397. doi: 10.1016/j.molcel.2015.10.008.

36. Mashiko D, Fujihara Y, Satouh Y, Miyata H, Isotani A, Ikawa M. Generation of mutant mice by pronuclear injection of circular plasmid expressing Cas9 and single guided RNA. *Sci Rep*. 2013;3:3355. doi: 10.1038/srep03355.

37. Carroll KJ, Makarewich CA, McAnally J, Anderson DM, Zentilin L, Liu N, Giacca M, Bassel-Duby R, Olson EN. A mouse model for adult cardiac-specific gene deletion with CRISPR/Cas9. *Proc Natl Acad Sci U S A*. 2016;113:338–343. doi: 10.1073/pnas.1523918113.

38. Platt RJ, Chen S, Zhou Y, et al. CRISPR-Cas9 knockin mice for genome editing and cancer modeling. *Cell*. 2014;159:440–455. doi: 10.1016/j.cell.2014.09.014.

39. Nishimasu H, Ran FA, Hsu PD, Konermann S, Shehata SI, Dohmae N, Ishitani R, Zhang F, Nureki O. Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell*. 2014;156:935–949. doi: 10.1016/j.cell.2014.02.001.

40. Jinek M, Jiang F, Taylor DW, Sternberg SH, Kaya E, Ma E, Anders C, Hauer M, Zhou K, Lin S, Kaplan M, Iavarone AT, Charpentier E, Nogales E, Doudna JA. Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. *Science*. 2014;343:1247997. doi: 10.1126/science.1247997.

41. Sternberg SH, Redding S, Jinek M, Greene EC, Doudna JA. DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature*. 2014;507:62–67. doi: 10.1038/nature13011.

42. Garneau JE, Dupuis MÈ, Villion M, Romero DA, Barrangou R, Boyaval P, Fremaux C, Horvath P, Magadán AH, Moineau S. The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature*. 2010;468:67–71. doi: 10.1038/nature09523.

43. Jiang F, Doudna JA. The structural biology of CRISPR-Cas systems. *Curr Opin Struct Biol*. 2015;30:100–111. doi: 10.1016/j.sbi.2015.02.002.

44. Kleinstiver BP, Prew MS, Tsai SQ, Topkar VV, Nguyen NT, Zheng Z, Gonzales AP, Li Z, Peterson RT, Yeh JJ, Aryee MJ, Joung JK. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature*. 2015;523:481–485. doi: 10.1038/nature14592

45. Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, Zetsche B, Shalem O, Wu X, Makarova KS, Koonin EV, Sharp PA, Zhang F. In vivo genome editing using Staphylococcus aureus Cas9. *Nature*. 2015;520:186–191. doi: 10.1038/nature14299.

46. Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, van der Oost J, Regev A, Koonin EV, Zhang F. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell*. 2015;163:759–771. doi: 10.1016/j.cell.2015.09.038.

47. Kleinstiver BP, Prew MS, Tsai SQ, Nguyen NT, Topkar VV, Zheng Z, Joung JK. Broadening the targeting range of Staphylococcus aureus

CRISPR-Cas9 by modifying PAM recognition. *Nat Biotechnol*. 2015;33:1293–1298. doi: 10.1038/nbt.3404.

48. Ran FA, Hsu PD, Lin CY, Gootenberg JS, Konermann S, Trevino AE, Scott DA, Inoue A, Matoba S, Zhang Y, Zhang F. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell*. 2013;154:1380–1389. doi: 10.1016/j.cell.2013.08.021.

49. Merkle FT, Neuhausser WM, Santos D, Valen E, Gagnon JA, Maas K, Sandoe J, Schier AF, Eggan K. Efficient CRISPR-Cas9-mediated generation of knockin human pluripotent stem cells lacking undesired mutations at the targeted locus. *Cell Rep*. 2015;11:875–883. doi: 10.1016/j.celrep.2015.04.007.

50. Miyaoka Y, Berman JR, Cooper SB, Mayerl SJ, Chan AH, Zhang B, Karlin-Neumann GA, Conklin BR. Systematic quantification of HDR and NHEJ reveals effects of locus, nuclease, and cell type on genome-editing. *Sci Rep*. 2016;6:23549. doi: 10.1038/srep23549.

51. Slaymaker IM, Gao L, Zetsche B, Scott DA, Yan WX, Zhang F. Rationally engineered Cas9 nucleases with improved specificity. *Science*. 2016;351:84–88. doi: 10.1126/science.aad5227.

52. Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z, Joung JK. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature*. 2016;529:490–495. doi: 10.1038/nature16526.

53. La Russa MF, Qi LS. The New State of the Art: Cas9 for Gene Activation and Repression. *Mol Cell Biol*. 2015;35:3800–3809. doi: 10.1128/MCB.00512-15.

54. Ma H, Naseri A, Reyes-Gutierrez P, Wolfe SA, Zhang S, Pederson T. Multicolor CRISPR labeling of chromosomal loci in human cells. *Proc Natl Acad Sci U S A*. 2015;112:3002–3007. doi: 10.1073/pnas.1420024112.

55. Knight SC, Xie L, Deng W, Guglielmi B, Witkowsky LB, Bosanac L, Zhang ET, El Beheiry M, Masson JB, Dahan M, Liu Z, Doudna JA, Tjian R. Dynamics of CRISPR-Cas9 genome interrogation in living cells. *Science*. 2015;350:823–826. doi: 10.1126/science.aac6572.

56. Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O, Cradick TJ, Marraffini LA, Bao G, Zhang F. DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol*. 2013;31:827–832. doi: 10.1038/nbt.2647.

57. Horlbeck MA, Witkowsky LB, Guglielmi B, Replogle JM, Gilbert LA, Villalta JE, Torigoe SE, Tjian R, Weissman JS. Nucleosomes impede Cas9 access to DNA *in vivo* and *in vitro*. *Elife*. 2016;5:. doi: 10.7554/eLife.12677.

58. Essletzbichler P, Konopka T, Santoro F, Chen D, Gapp BV, Kralovics R, Brummelkamp TR, Nijman SM, Bürckstümmer T. Megabase-scale deletion using CRISPR/Cas9 to generate a fully haploid human cell line. *Genome Res*. 2014;24:2059–2065. doi: 10.1101/gr.177220.114.

59. Wang P, Lin M, Pedrosa E, Hrabovsky A, Zhang Z, Guo W, Lachman HM, Zheng D. CRISPR/Cas9-mediated heterozygous knockout of the autism gene CHD8 and characterization of its transcriptional networks in neurodevelopment. *Mol Autism*. 2015;6:55. doi: 10.1186/s13229-015-0048-6.

60. Briner AE, Donohoue PD, Gomaa AA, Selle K, Slorach EM, Nye CH, Haurwitz RE, Beisel CL, May AP, Barrangou R. Guide RNA functional modules direct Cas9 activity and orthogonality. *Mol Cell*. 2014;56:333–339. doi: 10.1016/j.molcel.2014.09.019.

61. Wu X, Scott DA, Kriz AJ, Chiu AC, Hsu PD, Dadon DB, Cheng AW, Trevino AE, Konermann S, Chen S, Jaenisch R, Zhang F, Sharp PA. Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells. *Nat Biotechnol*. 2014;32:670–676. doi: 10.1038/nbt.2889.

62. Hendel A, Bak RO, Clark JT, et al. Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. *Nat Biotechnol*. 2015;33:985–989. doi: 10.1038/nbt.3290.

63. Graham DB, Root DE. Resources for the design of CRISPR gene editing experiments. *Genome Biol*. 2015;16:260. doi: 10.1186/s13059-015-0823-x.

64. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc*. 2013;8:2281–2308. doi: 10.1038/nprot.2013.143.

65. Guschin DY, Waite AJ, Katibah GE, Miller JC, Holmes MC, Rebar EJ. A rapid and general assay for monitoring endogenous gene modification. *Methods Mol Biol*. 2010;649:247–256. doi: 10.1007/978-1-60761-753-2_15.

66. Yoshimi K, Kunihiro Y, Kaneko T, Nagahora H, Voigt B, Mashimo T. ssODN-mediated knock-in with CRISPR-Cas for large genomic regions in zygotes. *Nat Commun*. 2016;7:10431. doi: 10.1038/ncomms10431.

67. Renaud JB, Boix C, Charpentier M, et al. Improved genome editing efficiency and flexibility using modified oligonucleotides with TALEN and

CRISPR-Cas9 Nucleases. *Cell Rep*. 2016;14:2263–2272. doi: 10.1016/j.celrep.2016.02.018.

68. Inui M, Miyado M, Igarashi M, Tamano M, Kubo A, Yamashita S, Asahara H, Fukami M, Takada S. Rapid generation of mouse models with defined point mutations by the CRISPR/Cas9 system. *Sci Rep*. 2014;4:5396. doi: 10.1038/srep05396.

69. Bialk P, Rivera-Torres N, Strouse B, Kmiec EB. Regulation of gene editing activity directed by single-stranded oligonucleotides and CRISPR/Cas9 systems. *PLoS One*. 2015;10:e0129308. doi: 10.1371/journal.pone.0129308.

70. Yang H, Wang H, Shivalila CS, Cheng AW, Shi L, Jaenisch R. One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. *Cell*. 2013;154:1370–1379. doi: 10.1016/j.cell.2013.08.022.

71. Wang B, Li K, Wang A, Reiser M, Saunders T, Lockey RF, Wang JW. Highly efficient CRISPR/HDR-mediated knock-in for mouse embryonic stem cells and zygotes. *Biotechniques*. 2015;59:201–2, 204, 206. doi: 10.2144/000114339.

72. Lee AY, Lloyd KC. Conditional targeting of Ispd using paired Cas9 nickase and a single DNA template in mice. *FEBS Open Bio*. 2014;4:637–642. doi: 10.1016/j.fob.2014.06.007.

73. Petrezselyova S, Kinsky S, Truban D, Sedlacek R, Burtscher I, Lickert H. Homology arms of targeting vectors for gene insertions and CRISPR/Cas9 technology: size does not matter; quality control of targeted clones does. *Cell Mol Biol Lett*. 2015;20:773–787. doi: 10.1515/cmble-2015-0047.

74. Perry KJ, Henry JQ. CRISPR/Cas9-mediated genome modification in the mollusc, Crepidula fornicata. *Genesis*. 2015;53:237–244. doi: 10.1002/dvg.22843.

75. Wang T, Wei JJ, Sabatini DM, Lander ES. Genetic screens in human cells using the CRISPR-Cas9 system. *Science*. 2014;343:80–84. doi: 10.1126/science.1246981.

76. Canver MC, Bauer DE, Dass A, Yien YY, Chung J, Masuda T, Maeda T, Paw BH, Orkin SH. Characterization of genomic deletion efficiency mediated by clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 nuclease system in mammalian cells. *J Biol Chem*. 2014;289:21312–21324. doi: 10.1074/jbc.M114.564625.

77. Ding Q, Strong A, Patel KM, Ng SL, Gosis BS, Regan SN, Cowan CA, Rader DJ, Musunuru K. Permanent alteration of PCSK9 with *in vivo* CRISPR-Cas9 genome editing. *Circ Res*. 2014;115:488–492. doi: 10.1161/CIRCRESAHA.115.304351.

78. Oliver D, Yuan S, McSwiggin H, Yan W. Pervasive genotypic mosaicism in founder mice derived from genome editing through pronuclear injection. *PLoS One*. 2015;10:e0129457. doi: 10.1371/journal.pone.0129457.

79. Sicinski P, Geng Y, Ryder-Cook AS, Barnard EA, Darlison MG, Barnard PJ. The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. *Science*. 1989;244:1578–1580.

80. Long C, Amoasii L, Mireault AA, McAnally JR, Li H, Sanchez-Ortiz E, Bhattacharyya S, Shelton JM, Bassel-Duby R, Olson EN. Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy. *Science*. 2016;351:400–403. doi: 10.1126/science.aad5725.

81. Nelson CE, Hakim CH, Ousterout DG, Thakore PI, Moreb EA, Castellanos Rivera RM, Madhavan S, Pan X, Ran FA, Yan WX, Asokan A, Zhang F, Duan D, Gersbach CA. *In vivo* genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. *Science*. 2016;351:403–407. doi: 10.1126/science.aad5143.

82. Almomani R, Verhagen JM, Herkert JC, et al. Biallelic truncating mutations in ALPK3 cause severe pediatric cardiomyopathy. *J Am Coll Cardiol*. 2016;67:515–525. doi: 10.1016/j.jacc.2015.10.093.

83. Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, Haussler D. The human genome browser at UCSC. *Genome Res*. 2002;12:996–1006. doi: 10.1101/gr.229102..

84. Streb JW, Kitchen CM, Gelman IH, Miano JM. Multiple promoters direct expression of three AKAP12 isoforms with distinct subcellular and tissue distribution profiles. *J Biol Chem*. 2004;279:56014–56023. doi: 10.1074/jbc.M408828200.

85. Neznanov N, Umezawa A, Oshima RG. A regulatory element within a coding exon modulates keratin 18 gene expression in transgenic mice. *J Biol Chem*. 1997;272:27549–27557.

86. Han J, Zhang J, Chen L, Shen B, Zhou J, Hu B, Du Y, Tate PH, Huang X, Zhang W. Efficient *in vivo* deletion of a large imprinted lncRNA by CRISPR/Cas9. *RNA Biol*. 2014;11:829–835. doi: 10.4161/rna.29624.

87. Miano JM, Long X. The short and long of noncoding sequences in the control of vascular cell phenotypes. *Cell Mol Life Sci*. 2015;72:3457–3488. doi: 10.1007/s00018-015-1936-9.

88. Maurano MT, Humbert R, Rynes E, et al. Systematic localization of common disease-associated variation in regulatory DNA. *Science*. 2012;337:1190–1195. doi: 10.1126/science.1222794.

89. Benson CC, Zhou Q, Long X, Miano JM. Identifying functional single nucleotide polymorphisms in the human CArGome. *Physiol Genomics*. 2011;43:1038–1048. doi: 10.1152/physiolgenomics.00098.2011.

90. Miller CL, Haas U, Diaz R, Leeper NJ, Kundu RK, Patolla B, Assimes TL, Kaiser FJ, Perisic L, Hedin U, Maegdefessel L, Schunkert H, Erdmann J, Quertermous T, Sczakiel G. Coronary heart disease-associated variation in TCF21 disrupts a miR-224 binding site and miRNA-mediated regulation. *PLoS Genet*. 2014;10:e1004263. doi: 10.1371/journal.pgen.1004263.

91. Johnson R, Richter N, Bogu GK, Bhinge A, Teng SW, Choo SH, Andrieux LO, de Benedictis C, Jauch R, Stanton LW. A genome-wide screen for genetic variants that modify the recruitment of REST to its target genes. *PLoS Genet*. 2012;8:e1002624. doi: 10.1371/journal.pgen.1002624.

92. Pennacchio LA, Ahituv N, Moses AM, et al. *In vivo* enhancer analysis of human conserved non-coding sequences. *Nature*. 2006;444:499–502. doi: 10.1038/nature05295.

93. Long X, Miano JM. Remote control of gene expression. *J Biol Chem*. 2007;282:15941–15945. doi: 10.1074/jbc.R700010200.

94. Canver MC, Smith EC, Sher F, et al. BCL11A enhancer dissection by Cas9-mediated in situ saturating mutagenesis. *Nature*. 2015;527:192–197. doi: 10.1038/nature15521.

95. Wang S, Sengel C, Emerson MM, Cepko CL. A gene regulatory network controls the binary fate decision of rod and bipolar cells in the vertebrate retina. *Dev Cell*. 2014;30:513–527. doi: 10.1016/j.devcel.2014.07.018.

96. Hu J, Verzi MP, Robinson AS, Tang PL, Hua LL, Xu SM, Kwok PY, Black BL. Endothelin signaling activates Mef2c expression in the neural crest through a MEF2C-dependent positive-feedback transcriptional pathway. *Development*. 2015;142:2775–2780. doi: 10.1242/dev.126391.

97. Metser G, Shin HY, Wang C, Yoo KH, Oh S, Villarino AV, O'Shea JJ, Kang K, Hennighausen L. An autoregulatory enhancer controls mammary-specific STAT5 functions. *Nucleic Acids Res*. 2016;44:1052–1063. doi: 10.1093/nar/gkv999.

98. Li J, Shou J, Guo Y, Tang Y, Wu Y, Jia Z, Zhai Y, Chen Z, Xu Q, Wu Q. Efficient inversions and duplications of mammalian regulatory DNA elements and gene clusters by CRISPR/Cas9. *J Mol Cell Biol*. 2015;7:284–298. doi: 10.1093/jmcb/mjv016.

99. Meyer MB, Benkusky NA, Onal M, Pike JW. Selective regulation of Mmp13 by 1,25(oh)2d3, PTH, and Osterix through distal enhancers. *J. Steroid Biochem. Mol. Biol*. 2015 doi: 10.1016/j.jsbmb.2015.09.001

100. Ohmura S, Mizuno S, Oishi H, Ku CJ, Hermann M, Hosoya T, Takahashi S, Engel JD. Lineage-affiliated transcription factors bind the Gata3 Tce1 enhancer to mediate lineage-specific programs. *J Clin Invest*. 2016;126:865–878. doi: 10.1172/JCI83894.

101. Avellino R, Havermans M, Erpelinck C, et al. An autonomous CEBPA enhancer specific for myeloid-lineage priming and neutrophilic differentiation. *Blood*. 2016 doi: 10.1182/blood-2016-01-695759

102. Seruggia D, Fernández A, Cantero M, Pelczar P, Montoliu L. Functional validation of mouse tyrosinase non-coding regulatory DNA elements by CRISPR-Cas9-mediated mutagenesis. *Nucleic Acids Res*. 2015;43:4855–4867. doi: 10.1093/nar/gkv375.

103. Kraft K, Geuer S, Will AJ, et al. Deletions, inversions, duplications: Engineering of structural variants using CRISPR/Cas in mice. *Cell Rep*. 2015;10:833–839. doi: 10.1016/j.celrep.2015.01.016.

104. Li Y, Rivera CM, Ishii H, Jin F, Selvaraj S, Lee AY, Dixon JR, Ren B. CRISPR reveals a distal super-enhancer required for Sox2 expression in mouse embryonic stem cells. *PLoS One*. 2014;9:e114485. doi: 10.1371/journal.pone.0114485.

105. Yao L, Tak YG, Berman BP, Farnham PJ. Functional annotation of colon cancer risk SNPs. *Nat Commun*. 2014;5:5114. doi: 10.1038/ncomms6114.

106. Dogan N, Wu W, Morrissey CS, Chen KB, Stonestrom A, Long M, Keller CA, Cheng Y, Jain D, Visel A, Pennacchio LA, Weiss MJ, Blobel GA, Hardison RC. Occupancy by key transcription factors is a moreaccurate predictor of enhancer activity than histone modifications or chromatin accessibility. *Epigenetics Chromatin*. 2015;8:16. doi: 10.1186/s13072-015-0009-5.

107. Tak YG, Hung Y, Yao L, Grimmer MR, Do A, Bhakta MS, O'Geen H, Segal DJ, Farnham PJ. Effects on the transcriptome upon deletion of a distal element cannot be predicted by the size of the H3K27Ac peak in human cells. *Nucleic Acids Res*. 2016 doi: 10.1093/nar/gkv1530

108. Rajagopal N, Srinivasan S, Kooshesh K, Guo Y, Edwards MD, Banerjee B, Syed T, Emons BJ, Gifford DK, Sherwood RI. High-throughput mapping of regulatory DNA. *Nat Biotechnol*. 2016;34:167–174. doi: 10.1038/nbt.3468.

109. Singh P, Schimenti JC, Bolcun-Filas E. A mouse geneticist's practical guide to CRISPR applications. *Genetics*. 2015;199:1–15. doi: 10.1534/genetics.114.169771.

110. Maruyama T, Dougan SK, Truttmann MC, Bilate AM, Ingram JR, Ploegh HL. Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. *Nat Biotechnol*. 2015;33:538–542. doi: 10.1038/nbt.3190.

111. Song J, Yang D, Xu J, Zhu T, Chen YE, Zhang J. RS-1 enhances CRISPR/Cas9- and TALEN-mediated knock-in efficiency. *Nat Commun*. 2016;7:10548. doi: 10.1038/ncomms10548.

112. Qin W, Dion SL, Kutny PM, Zhang Y, Cheng AW, Jillette NL, Malhotra A, Geurts AM, Chen YG, Wang H. Efficient CRISPR/Cas9-Mediated Genome Editing in Mice by Zygote Electroporation of Nuclease. *Genetics*. 2015;200:423–430. doi: 10.1534/genetics.115.176594.

113. Wu Y, Liang D, Wang Y, Bai M, Tang W, Bao S, Yan Z, Li D, Li J. Correction of a genetic disease in mouse via use of CRISPR-Cas9. *Cell Stem Cell*. 2013;13:659–662. doi: 10.1016/j.stem.2013.10.016.

114. Long C, McAnally JR, Shelton JM, Mireault AA, Bassel-Duby R, Olson EN. Prevention of muscular dystrophy in mice by CRISPR/Cas9-mediated editing of germline DNA. *Science*. 2014;345:1184–1188. doi: 10.1126/science.1254445.

115. McNally EM, Barefield DY, Puckelwartz MJ. The genetic landscape of cardiomyopathy and its role in heart failure. *Cell Metab*. 2015;21:174–182. doi: 10.1016/j.cmet.2015.01.013.

116. Richardson CD, Ray GJ, DeWitt MA, Curie GL, Corn JE. Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. *Nat Biotechnol*. 2016;34:339–344. doi: 10.1038/nbt.3481.

116a. Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR. Programmable editing of a target base in genomic DNA without double-strand DNA cleavage [published online ahead of print April 20, 2016]. *Nature*. doi:10.1038/nature17946. http://www.nature.com/nature/journal/vaop/ncurrent/full/nature17946.html.

117. Maquat LE. Nonsense-mediated mRNA decay: splicing, translation and mRNP dynamics. *Nat Rev Mol Cell Biol*. 2004;5:89–99. doi: 10.1038/nrm1310.

118. Geraghty RJ, Capes-Davis A, Davis JM, Downward J, Freshney RI, Knezevic I, Lovell-Badge R, Masters JR, Meredith J, Stacey GN, Thraves P, Vias M; Cancer Research UK. Guidelines for the use of cell lines in biomedical research. *Br J Cancer*. 2014;111:1021–1046. doi: 10.1038/bjc.2014.166.

119. Yang L, Yang JL, Byrne S, Pan J, Church GM. CRISPR/Cas9-Directed Genome Editing of Cultured Cells. *Curr Protoc Mol Biol*. 2014;107:31.1.1–31.1.17. doi: 10.1002/0471142727.mb3101s107.

120. Abrahimi P, Chang WG, Kluger MS, Qyang Y, Tellides G, Saltzman WM, Pober JS. Efficient gene disruption in cultured primary human endothelial cells by CRISPR/Cas9. *Circ Res*. 2015;117:121–128. doi: 10.1161/CIRCRESAHA.117.306290.

121. Bell RD, Long X, Lin M, Bergmann JH, Nanda V, Cowan SL, Zhou Q, Han Y, Spector DL, Zheng D, Miano JM. Identification and initial functional characterization of a human vascular cell-enriched long noncoding RNA. *Arterioscler Thromb Vasc Biol*. 2014;34:1249–1259. doi: 10.1161/ATVBAHA.114.303240.

122. Boulberdaa M, Scott E, Ballantyne M, et al. A role for the long noncoding RNA SENCR in commitment and function of endothelial cells. *Mol. Ther*. 2016 doi: 10.1038/mt.2016.41

123. Klattenhoff CA, Scheuermann JC, Surface LE, Bradley RK, Fields PA, Steinhauser ML, Ding H, Butty VL, Torrey L, Haas S, Abo R, Tabebordbar M, Lee RT, Burge CB, Boyer LA. Braveheart, a long noncoding RNA required for cardiovascular lineage commitment. *Cell*. 2013;152:570–583. doi: 10.1016/j.cell.2013.01.003.

124. Tavner F, Frampton J, Watson RJ. Targeting an E2F site in the mouse genome prevents promoter silencing in quiescent and post-mitotic cells. *Oncogene*. 2007;26:2727–2735. doi: 10.1038/sj.onc.1210087.

125. Boulende Sab A, Bouchard MF, Béland M, Prud'homme B, Souchkova O, Viger RS, Pilon N. An Ebox element in the proximal Gata4 promoter is required for Gata4 expression in vivo. *PLoS One*. 2011;6:e29038. doi: 10.1371/journal.pone.0029038.

126. Arango NA, Lovell-Badge R, Behringer RR. Targeted mutagenesis of the targeted mouse Mis gene promoter: *in vivo* definition of genetic pathways of vertebrate sexual development. *Cell*. 1999;99:409–419.

127. Meier ID, Bernreuther C, Tilling T, Neidhardt J, Wong YW, Schulze C, Streichert T, Schachner M. Short DNA sequences inserted for gene targeting can accidentally interfere with off-target gene expression. *FASEB J*. 2010;24:1714–1724. doi: 10.1096/fj.09-140749.

128. Menke DB. Engineering subtle targeted mutations into the mouse genome. *Genesis*. 2013;51:605–618. doi: 10.1002/dvg.22422.

129. Studer M, Gavalas A, Marshall H, Ariza-McNaughton L, Rijli FM, Chambon P, Krumlauf R. Genetic interactions between Hoxa1 and Hoxb1 reveal new roles in regulation of early hindbrain patterning. *Development*. 1998;125:1025–1036.

130. Han Y, Slivano OJ, Christie CK, Cheng AW, Miano JM. CRISPR-Cas9 genome editing of a single regulatory element nearly abolishes target gene expression in mice. *Arterioscler Thromb Vasc Biol*. 2015;35:312–315. doi: 10.1161/ATVBAHA.114.305017.

131. Santoro SW, Schultz PG. Directed evolution of the site specificity of Cre recombinase. *Proc Natl Acad Sci U S A*. 2002;99:4185–4190. doi: 10.1073/pnas.022039799.

132. Li L, He ZY, Wei XW, Gao GP, Wei YQ. Challenges in CRISPR/CAS9 Delivery: Potential Roles of Nonviral Vectors. *Hum Gene Ther*. 2015;26:452–462. doi: 10.1089/hum.2015.069.

133. Yin H, Song CQ, Dorkin JR, et al. Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components in vivo. *Nat Biotechnol*. 2016;34:328–333. doi: 10.1038/nbt.3471.

134. Denby L, Nicklin SA, Baker AH. Adeno-associated virus (AAV)-7 and -8 poorly transduce vascular endothelial cells and are sensitive to proteasomal degradation. *Gene Ther*. 2005;12:1534–1538. doi: 10.1038/sj.gt.3302564.

135. Aida T, Chiyo K, Usami T, Ishikubo H, Imahashi R, Wada Y, Tanaka KF, Sakuma T, Yamamoto T, Tanaka K. Cloning-free CRISPR/Cas system facilitates functional cassette knock-in in mice. *Genome Biol*. 2015;16:87. doi: 10.1186/s13059-015-0653-x.

136. Yen ST, Zhang M, Deng JM, Usman SJ, Smith CN, Parker-Thornburg J, Swinton PG, Martin JF, Behringer RR. Somatic mosaicism and allele complexity induced by CRISPR/Cas9 RNA injections in mouse zygotes. *Dev Biol*. 2014;393:3–9. doi: 10.1016/j.ydbio.2014.06.017.

137. Parikh BA, Beckman DL, Patel SJ, White JM, Yokoyama WM. Detailed phenotypic and molecular analyses of genetically modified mice generated by CRISPR-Cas9-mediated editing. *PLoS One*. 2015;10:e0116484. doi: 10.1371/journal.pone.0116484.

138. Tan EP, Li Y, Velasco-Herrera Mdel C, Yusa K, Bradley A. Off-target assessment of CRISPR-Cas9 guiding RNAs in human iPS and mouse ES cells. *Genesis*. 2015;53:225–236. doi: 10.1002/dvg.22835.

139. Fu Y, Sander JD, Reyon D, Cascio VM, Joung JK. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat Biotechnol*. 2014;32:279–284. doi: 10.1038/nbt.2808.

140. Tebas P, Stein D, Tang WW, et al. Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. *N Engl J Med*. 2014;370:901–910. doi: 10.1056/NEJMoa1300662.

141. Cohen JC, Boerwinkle E, Mosley TH Jr, Hobbs HH. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. *N Engl J Med*. 2006;354:1264–1272. doi: 10.1056/NEJMoa054013.

142. Wang X, Raghavan A, Chen T, Qiao L, Zhang Y, Ding Q, Musunuru K. CRISPR-Cas9 targeting of PCSK9 in human hepatocytes in vivo. *Arterioscler Thromb Vasc Biol*. 2016 doi: 10.1161/ATVBAHA.116.307227

143. Moss AJ, Kass RS. Long QT syndrome: from channels to cardiac arrhythmias. *J Clin Invest*. 2005;115:2018–2024. doi: 10.1172/JCI25537.

144. Park CY, Kim DH, Son JS, Sung JJ, Lee J, Bae S, Kim JH, Kim DW, Kim JS. Functional Correction of Large Factor VIII Gene Chromosomal Inversions in Hemophilia A Patient-Derived iPSCs Using CRISPR-Cas9. *Cell Stem Cell*. 2015;17:213–220. doi: 10.1016/j.stem.2015.07.001.

145. Liang P, Xu Y, Zhang X, et al. CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes. *Protein Cell*. 2015;6:363–372. doi: 10.1007/s13238-015-0153-5.

146. Church G. Perspective: Encourage the innovators. *Nature*. 2015;528:S7. doi: 10.1038/528S7a.

147. Lanphier E, Urnov F, Haecker SE, Werner M, Smolenski J. Don't edit the human germ line. *Nature*. 2015;519:410–411. doi: 10.1038/519410a.

148. Olson S, ed. *International Summit on Human Gene Editing: A Global Discussion*. National Academy of Sciences: Washington, DC: 2016.

149. Callaway E. Embryo-editing research gathers momentum. *Nature*. 2016;532:289–290.

Highlights

- Clustered regularly interspaced short palindromic repeats (CRISPR) is a facile and highly efficient genome editing technology that essentially supplants traditional embryonic stem cell–based approaches to genome modification in the mouse.
- The 3 components to CRISPR technology are the Cas9 endonuclease, a single (chimeric)–guide RNA, and a homology-directed repair template for precision-guided genome editing.
- CRISPR technology offers an investigator a wide range of genome editing options, from deletions and frameshift mutations (using 2-component CRISPR) to nucleotide replacement editing and integration of small sequence tags (using 3-component CRISPR).
- Robust genotyping assays can discriminate desired edits among multiple alleles stemming from genetic mosaicism while inadvertent (off) targeting is addressed with carefully planned front-end strategies in CRISPR design followed by back-end Sanger sequencing of possible off-target sites and proper back-crossing of mice to segregate out any unintended sequence edits.
- CRISPR genome editing presents some exciting opportunities to combat cardiovascular diseases with a genetic basis.

Arteriosclerosis, Thrombosis, and Vascular Biology

A CRISPR Path to Engineering New Genetic Mouse Models for Cardiovascular Research

Joseph M. Miano, Qiuyu Martin Zhu and Charles J. Lowenstein

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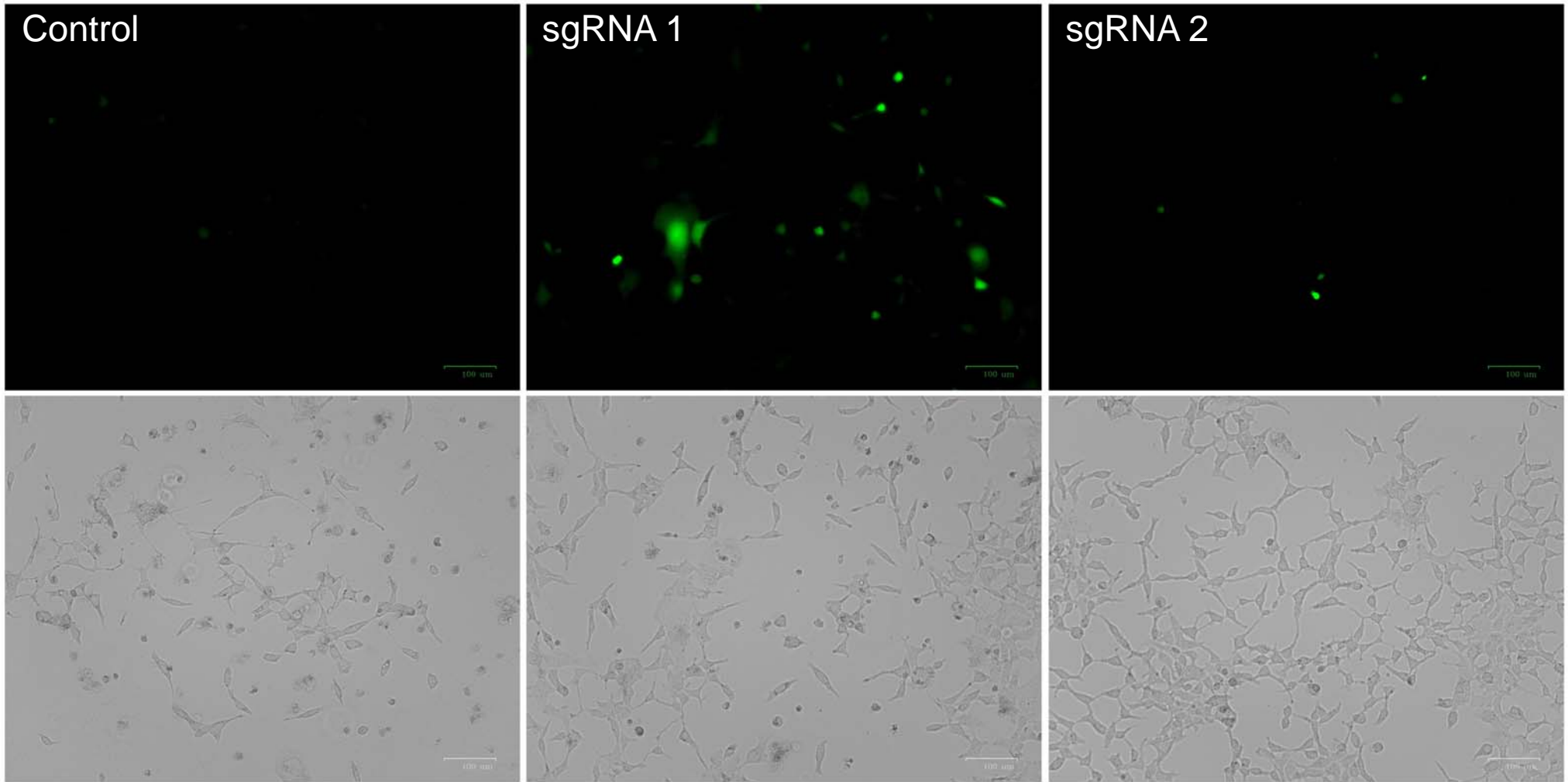World Wide Web at:
http://atvb.ahajournals.org/content/36/6/1058

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http://atvb.ahajournals.org/content/suppl/2016/04/20/ATVBAHA.116.304790.DC1.html
http://atvb.ahajournals.org/content/suppl/2016/04/20/ATVBAHA.116.304790.DC2.html

Supplemental Figure I. **Testing sgRNAs in vitro**. Immunofluorescence microscopy for GFP in HEK-293 cells co-transfected with empty EGxxFP split reporter plasmid and sgRNA (Control) or EGxxFP slit reporter carrying one of two target sequences and either sgRNA 1 or sgRNA 2. Variable GFP signal presumably reflects level of sgRNA activity. Please note, the empty control EGxxFP reporter occasionally exhibits some GFP fluorescence. This "background" signal is uniformly reduced across all conditions. Below are phase contrast images of the same fields as above. Note that results from this assay and Surveyor may not reflect actual activity in a mouse zygote. Some labs find it useful to test sgRNAs in early embryos; this however, is not practical for the vast majority of labs.
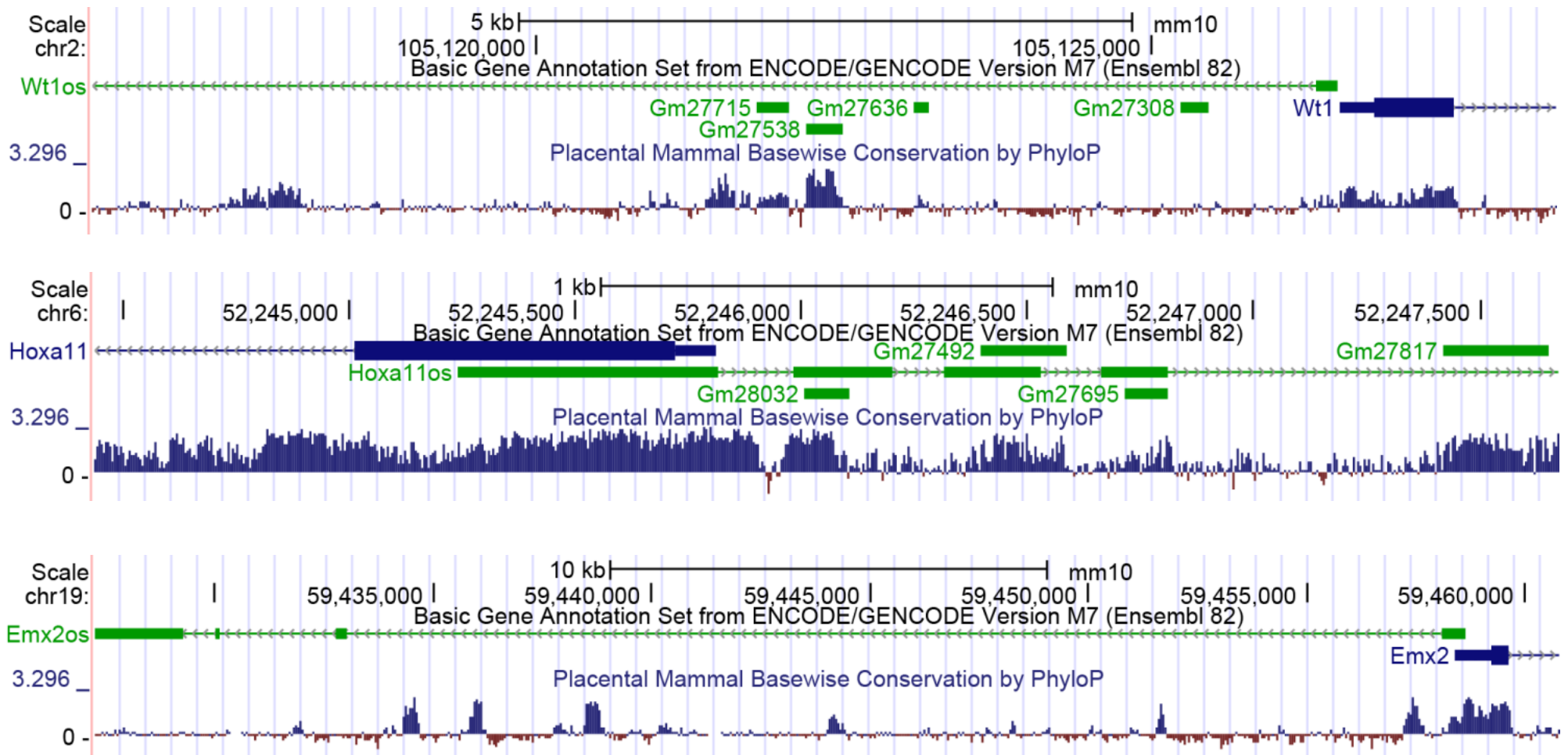
Supplemental Figure II. Sense-antisense gene pairs. UCSC Genome Browser tracks depicting several protein-coding genes (blue labeled Wt1, Hoxa11, and Emx2) that overlap or are in close proximity to adjacent 5' antisense long non-coding RNA genes (labeled in green). These three examples are representative of a large number of similarly arrayed coding/non-coding gene pairs in the mouse and human genomes. Targeting the first exon of either coding or non-coding gene with 2c CRISPR or 3c CRISPR using *loxP* sequences could complicate interpretation of phenotypes. Thus alternative strategies are needed to differentiate effects of inactivating a protein-coding (or non-coding) gene from the neighboring gene. The suffix "os" stands for opposite strand.
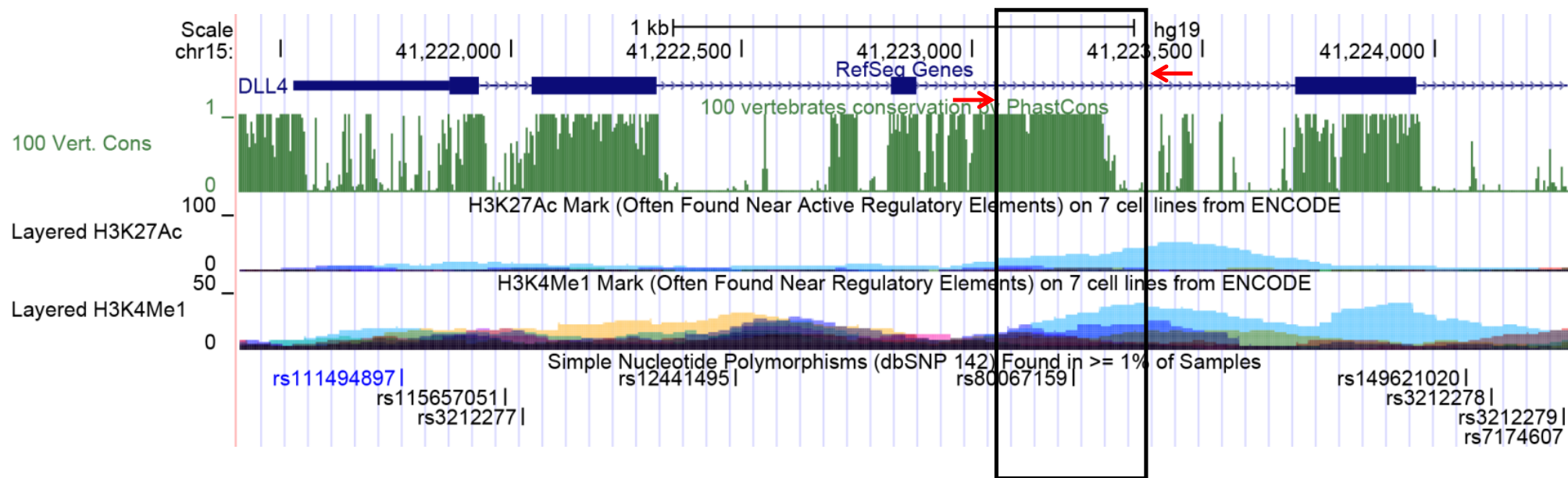
Supplemental Figure III. **Strategy for deleting enhancer using ENCODE data**. UCSC Genome Browser tracks showing the human *DLL4* gene, an endothelial restricted gene, with a highly conserved intron 3 region containing an experimentally-defined enhancer (black rectangle) that would be an excellent target for 2c CRISPR-mediated deletion in the orthologous position of the mouse genome. ENCODE track data below intron 3 depict chromatin marks (H3K27Ac and H3K4Me1) supportive of an enhancer. The teal color in both chromatin mark tracks reflects the binding activity of each chromatin factor in HUVEC, consistent with the EC-restricted expression of *DLL4*. Small red arrows at top represent hypothetical positions for sgRNAs, the remote position of which from exon-intron boundaries would not likely have an effect on splicing of the *DLL4* gene. Note presence of common SNP within enhancer region at bottom. There are thousands of similar genome snapshots that can be generated to assist investigators in the design of 2c CRISPR-mediated deletion of enhancer regions in the mouse genome as a first approximation of important regulatory sites (and variants therein) controlling target gene expression.
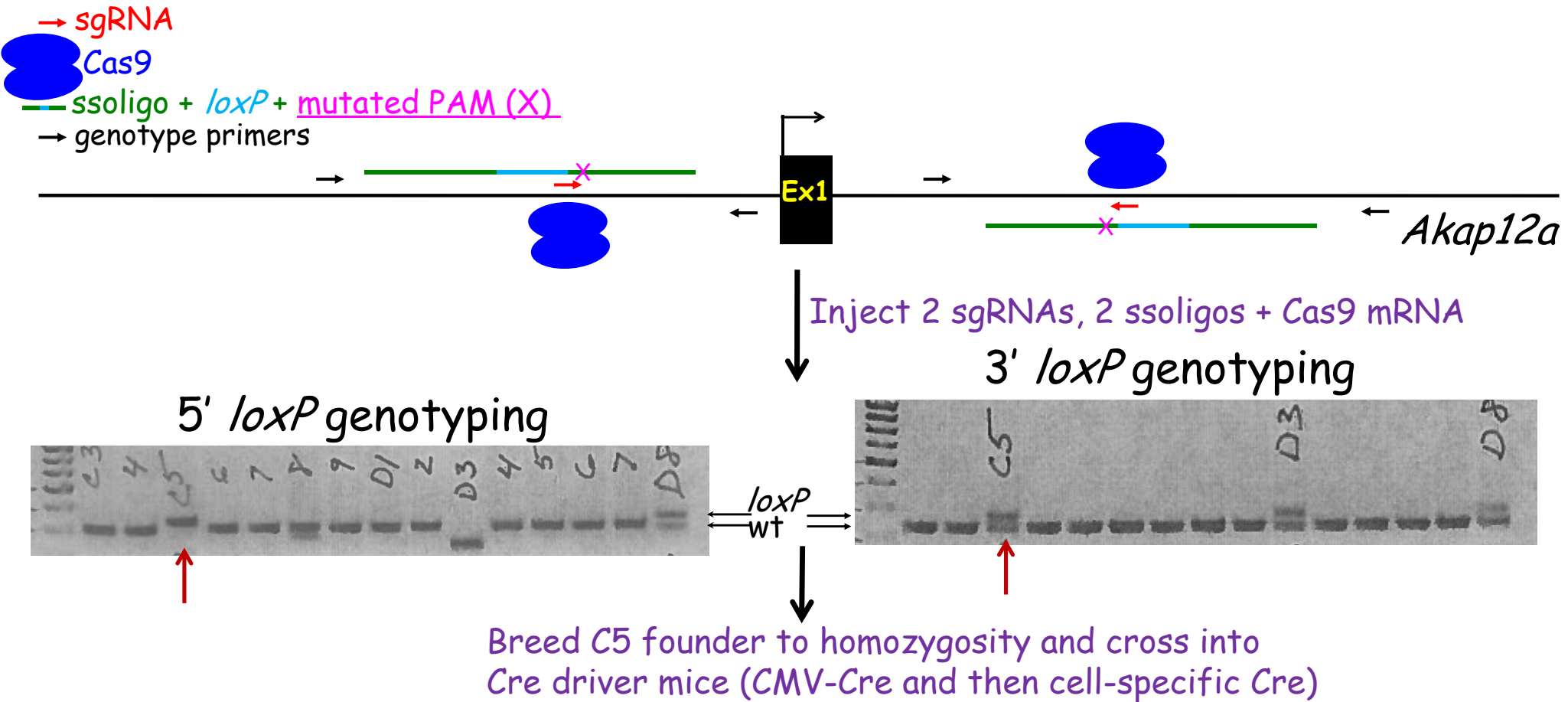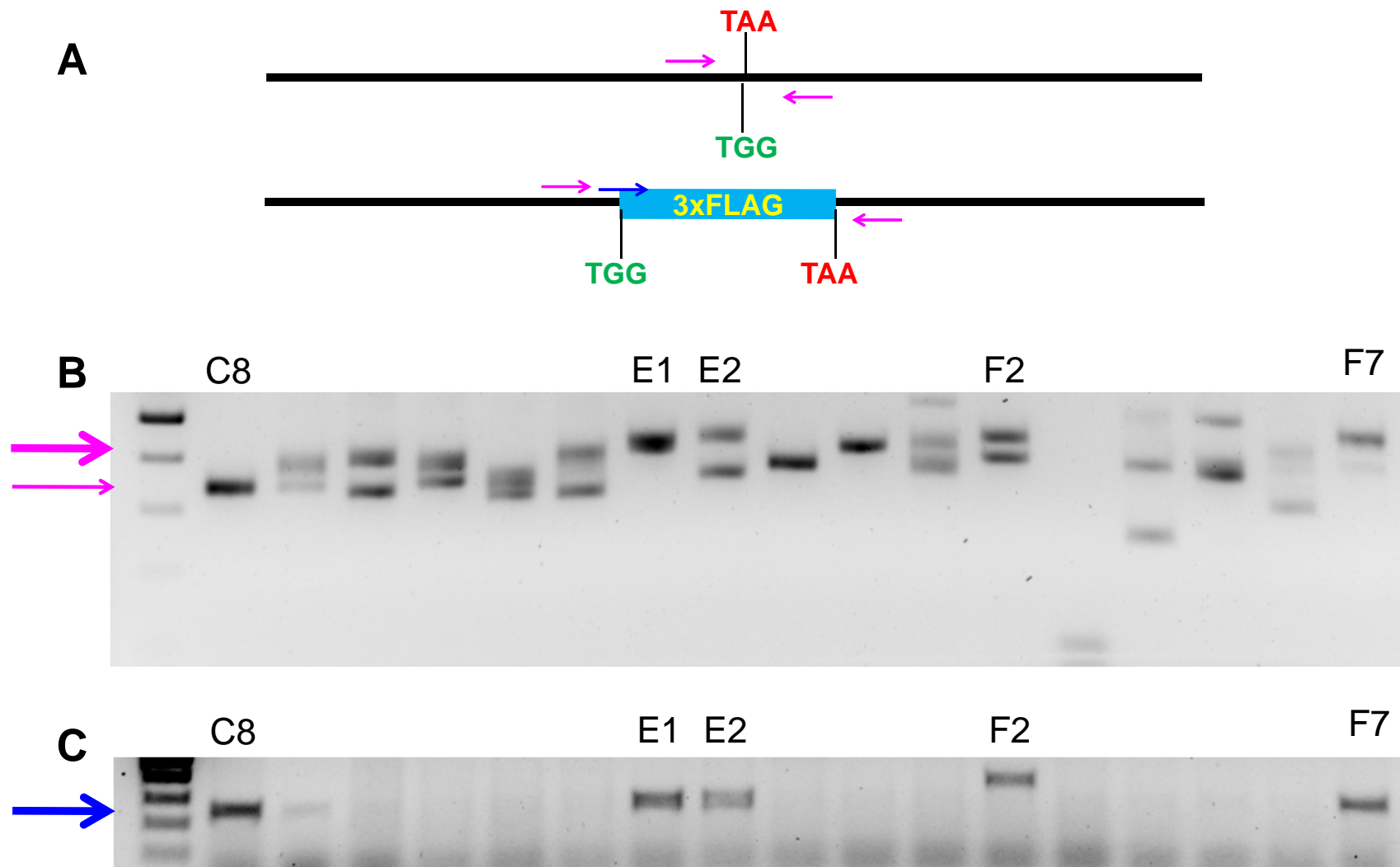
Supplemental Figure IV. 3c CRISPR targeting of the *Akap12a* locus for integrating *loxP* sequences. Schematic of 5' *Akap12a* locus and two sgRNAs (red arrows) with attending Cas9 protein (blue) and HDR template containing centrally-positioned *loxP* site (green-teal lines). These components were injected into mouse zygotes for the generation of several founder pups that were genotyped with primers (black arrows) flanking the 5' and 3' *loxP* sequences. Founder C5 (vertical red arrows) had bi-allelic targeting of the 5' *loxP* site and mono-allelic targeting of the 3' *loxP* site (confirmed by Sanger sequencing). Breeding these mice to homozygosity followed by crossing into the CMV-Cre mouse allowed us to confirm expected excision as determined by Sanger sequencing. This was the only founder (of 40) that yielded both *loxP* sites on same allele, though the 5' *loxP* site had a single base deletion. See text for more details and compare with genotyping strategies in Supplemental Figures VIa and VII. Note, the field is changing rapidly and this successful targeting appears to be a rare event; most attempts using the above approach yield only one *loxP* site integrated and/or show mutations in the *loxP* sequence. Consequently, other approaches are emerging such as sequential targeting of each *loxP* site in two rounds of microinjection (i.e., screening for correctly targeted 5' *loxP* site followed by injection of zygotes carrying the 5' targeting event with ssoligo carrying the 3' *loxP* site). Some labs continue to use traditional ESC-based methods for making a conditional mouse.
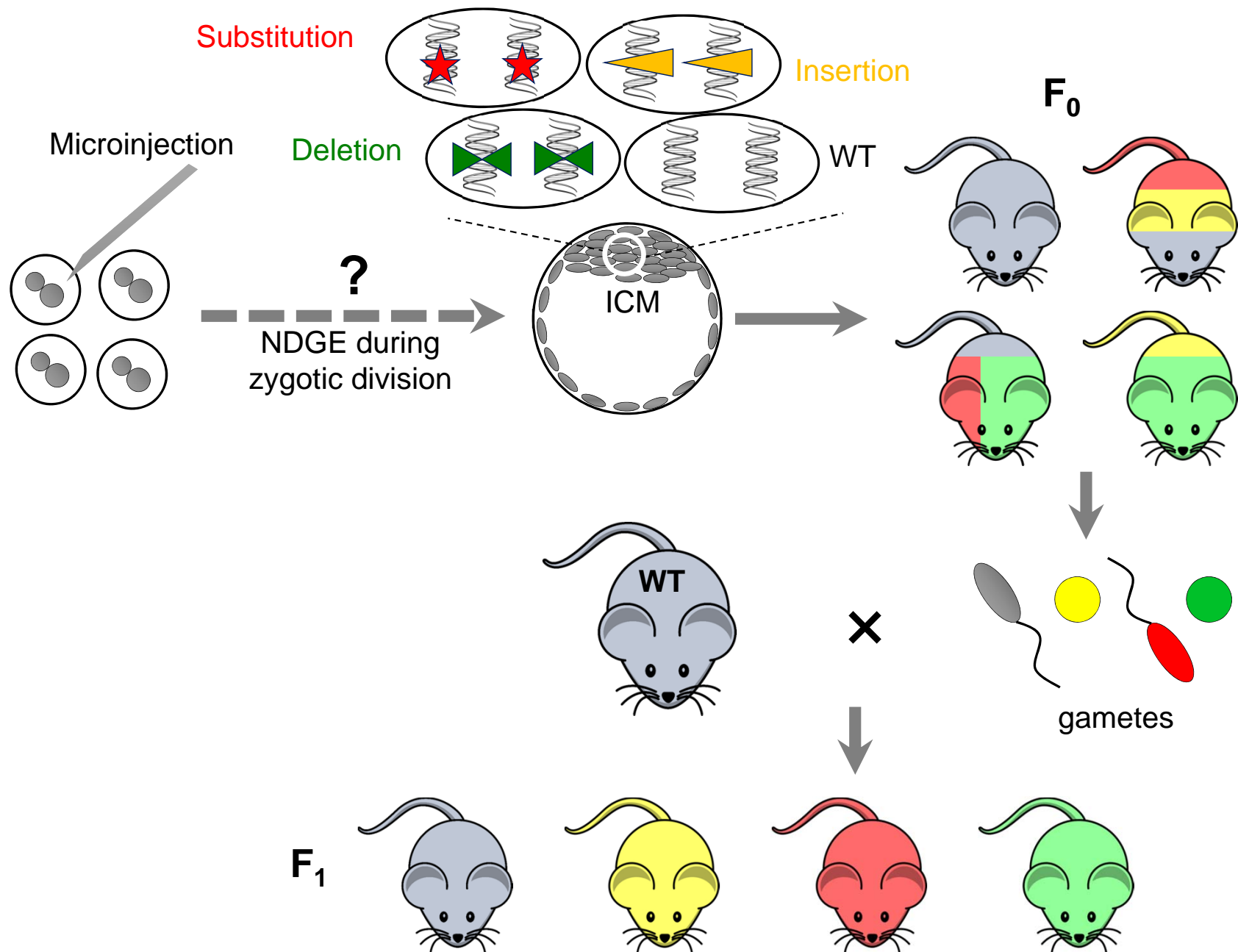
Supplemental Figure V. Genetic mosaicism in CRISPR mice. The dotted line indicates the period leading up to day 3.5 (blastocyst stage with inner cell mass [ICM] indicated). The question mark indicates our uncertainty as to how long CRISPR components persist following initial injection. The mosaicism schematized in cells of the inner mass reflects a number of events, many of which are unpredictable, but clearly demonstrated in Supplemental Figures IV, VI, and VII, that reflect the ongoing editing following zygotic divisions, leading to mosaic genotypes in F_0 mice. Allelic segregation during gametogenesis allows one to screen F_1 pups with robust genotyping strategies (see text and figures below).

Supplemental Figure VI. **3c CRISPR targeting of *Myocd* locus with 3xFLAG epitope**. (**A**) Schematic of 3xFLAG knockin and genotyping strategy. The pink primers amplify a wild type band of ~330 bp (lower thin arrow in **B**) whereas the presence of 3xFLAG shifts the PCR product up to ~393 bp (upper thick arrow in **B**). Note diversity in alleles across founders with more than two alleles in some individual founder pups, consistent with mosaicism. (**C**) Higher specificity genotyping using a hybrid forward primer (blue in **A**) of genomic/3xFLAG sequence with reverse pink primer. Only 5 pups show positive results (blue arrow), though C8 would not have been predicted based on the genotyping results in panel **B**. See Supplemental Figure VID for sequencing data derived from careful excision of upper bands in E1, E2, F2, and F7) and TA cloning.

D

CAGCAGTGGTAAACACCCG crRNA
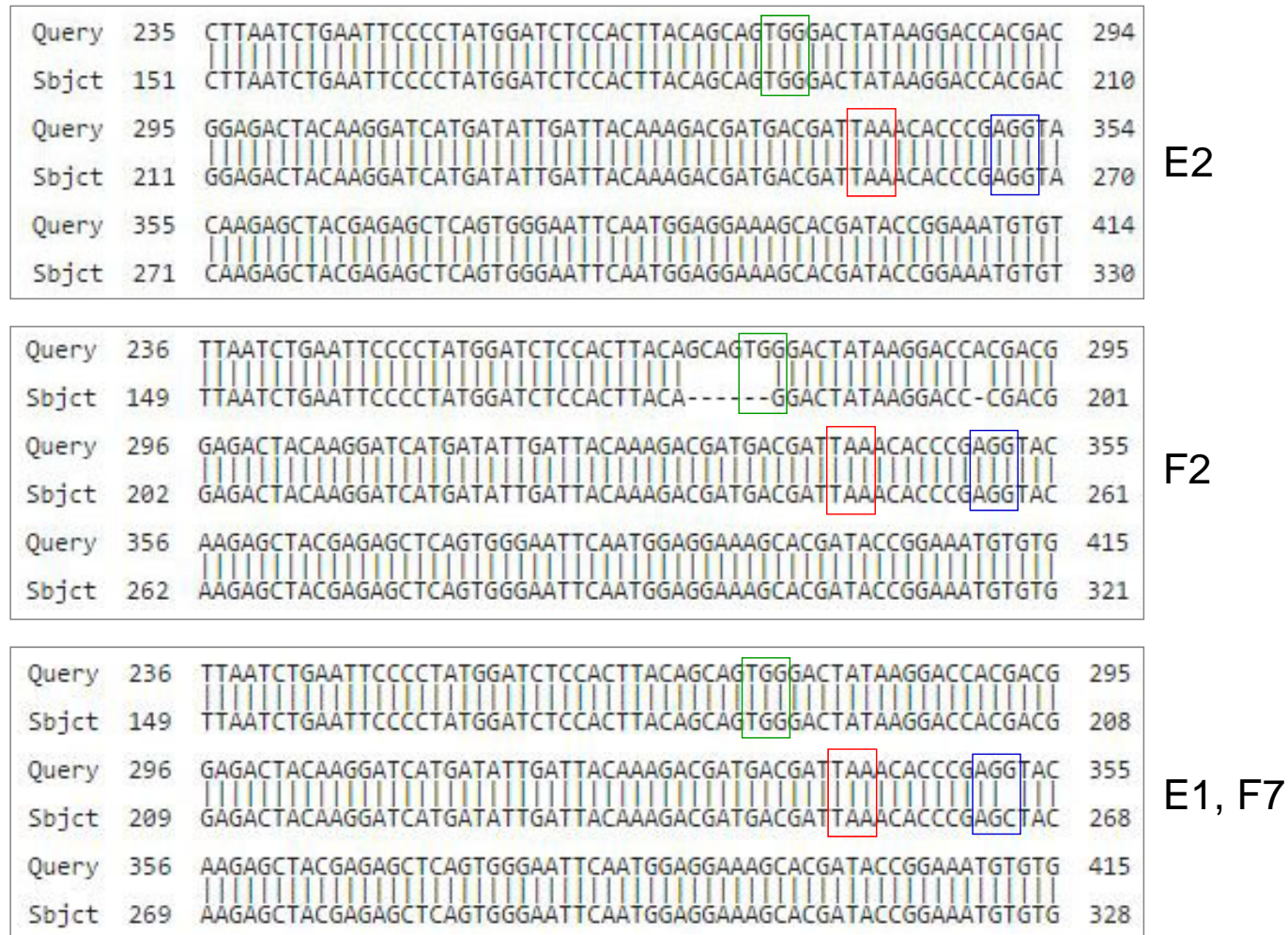
Supplemental Figure VI continued. (**D**) Sanger sequence analysis of four founders (E1, E2, F2, and F7) that genotyped positive with the 5' FLAG-specific forward primer (see Suppl. Fig VIC). The green boxed sequence is the last amino acid; the red boxed sequence is the stop codon and the intervening sequence between the TGG and TAA represents the 63nt 3xFLAG. The blue boxed sequence is the PAM. The crRNA of the sgRNA used for editing is shown at top. Although each of these founders yielded seemingly positive results in both genotyping assays (supportive of 3xFLAG), only E1, E2, and F7 showed correct sequence. Note that mutation in the PAM (AGG>AGC) is not seen in E2 and F2. We surmise this occurs via repair or incomplete recombination. Also note that in F2, while 3xFLAG recombined in, subsequent NHEJ (due to absence of PAM mutation?) resulted in deletions. In addition, there appears to be some discrepancy in the size of the PCR product of F2 and the sequencing result here. We emphasize the need to analyze at least two sequence-confirmed lines of mice to address this and other variables.
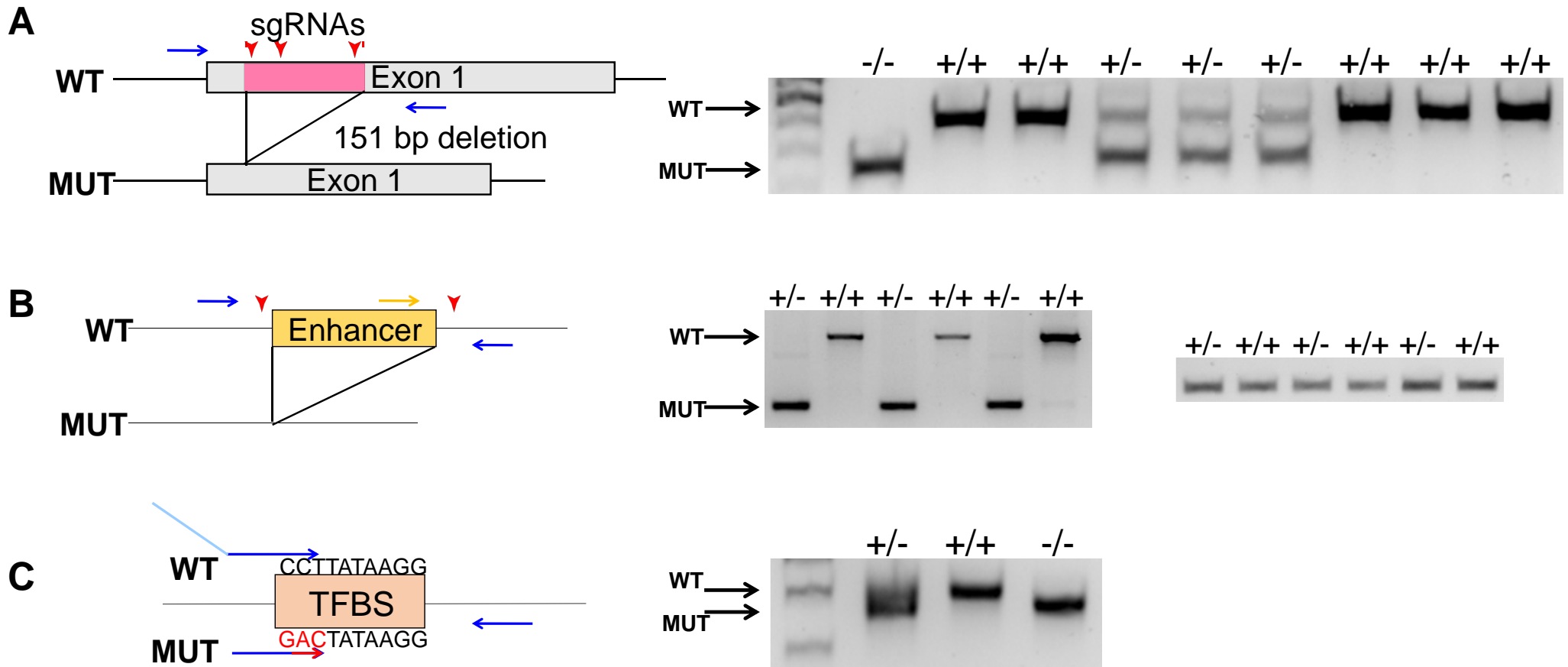
Supplemental Figure VII. **Strategies for genotyping CRISPR mice**. (**A**) 2c CRISPR deletion in exon 1 of a gene. Three sgRNAs (red arrowheads) used to create a 151 bp deletion resulting in a frameshift and premature termination codon (not shown). Primers (in blue) flanking the deletion yield the corresponding PCR products in the gel at right. (**B**) 2c CRISPR deletion of enhancer. Here, primers yield only one band in each lane of gel at left, requiring a separate PCR with a primer to the enhancer (orange) to verify the heterozygous pups in gel at right. It may be possible to combine all 3 primers and resolve the bands in a 2% agarose gel. (**C**) 3c CRISPR genotyping strategy depicting two forward primers of different length (30 nt) that specifically anneal to either wild type (upper) or mutant (lower) sequence (edits shown in red). Combining these forward primers with a common reverse primer yields expected bands in 2% gel at right. This assay requires optimization during the generation of CRISPR mice (see Supplemental Method 3 for more details). Note that we have successfully genotyped mice with only 1 base difference at the 3' end of the mutant primer. Schematics are not drawn to scale.

Supplemental Method #1: Preparation and activity testing of sgRNA

1. Define the top scoring 1-3 crRNA sequences that overlap or are as close as possible (ideally < 15bp) to the genomic sequence to be edited with the Design Tool from Feng Zhang's lab (http://crispr.mit.edu). Be sure first nucleotide is a guanine; if it is not, you may substitute the first nucleotide with guanine or simply add a guanine in the first position followed by your 20 nucleotide crRNA sequence.

2. Order Watson (5'$CACC$GN_{19}) and Crick ($AAAC$$N_{19}$C) complementary crRNAs for cloning into pX330 vector. **Do not include the adjacent PAM in your ordered sequence**.

3. Combine two oligonucleotides (1:1, v/v in nuclease-free H_2O), heat for 5 min at 95°C in a heat block, and slowly cool for 45 min with tubes remaining in the block; the block is removed from heat source and placed on bench for optimal annealing of oligonucleotides.

4. Linearize pX330 with *BbsI* (no need to dephosphorylate vector) and ligate the annealed oligonucleotides (no need to phosphorylate) o/n at 16°C.

5. Transform ligation reaction into suitable bacteria, pick colonies, and prepare DNA for Sanger sequencing to confirm fidelity of cloned crRNA.

6. Meanwhile, PCR clone a ~500bp amplicon around the genomic region to be edited and clone into the *BamHI/SalI* sites of the EGxxFP split reporter (addgene, plasmid #50716). This plasmid should then be co-transfected along with each individual pX330 plasmid carrying a sequence-verified sgRNA into HEK293 cells. Be sure to include an empty EGxxFP reporter without a targeted insert as a control. Two days later, assess reconstituted EGFP positivity as a qualitative measure of the DSB cleavage efficiency of each sgRNA (see Supplemental Figure I).

7. Design a forward primer with T7 promoter (5'-TAATACGACTCACTATAGG-3') followed by the crRNA sequence (for a total length of ~39 nucleotides) and combine with the universal reverse primer (corresponding to the 3' end of the tracrRNA in pX330, 5'AAAAGCACCGACTCGGTGCC-3') for PCR amplification of the entire sgRNA in pX330 (use AccuStart II Gel Track PCR Supermix (Cat. #84228)). Perform PCR in all 8 wells of a strip with minimum volume of 20μl/tube to increase PCR product yield.

8. Clean up pooled PCR reactions with appropriate PCR purification kit and aim to achieve at least 500 ng of PCR product (~100 nucleotides) for the in vitro transcription.

9. Perform in vitro transcription on PCR product with the MEGAshortscript T7 Transcription Kit (Thermo Fisher Scientific, Cat. #AM1354) to achieve at least 25-30 μg of in vitro transcribed sgRNA. Reaction time is 4-5 hours, but overnight incubation often yields higher concentrations of sgRNA.

10. Purify in vitro transcribed sgRNA with RNeasy mini kit (part2) or MEGAclear Transcription Clean-up Kit (Thermo Fisher Scientific, Cat. #AM1908) and aim for a concentration of 0.5-1 μg/μl.

11. Assess integrity of sgRNA before injection into mouse zygote with small RNA (6-150 nucleotide) chip of the Agilent 2100 BioAnalyzer. Use ~100ng of sgRNA for this analysis.
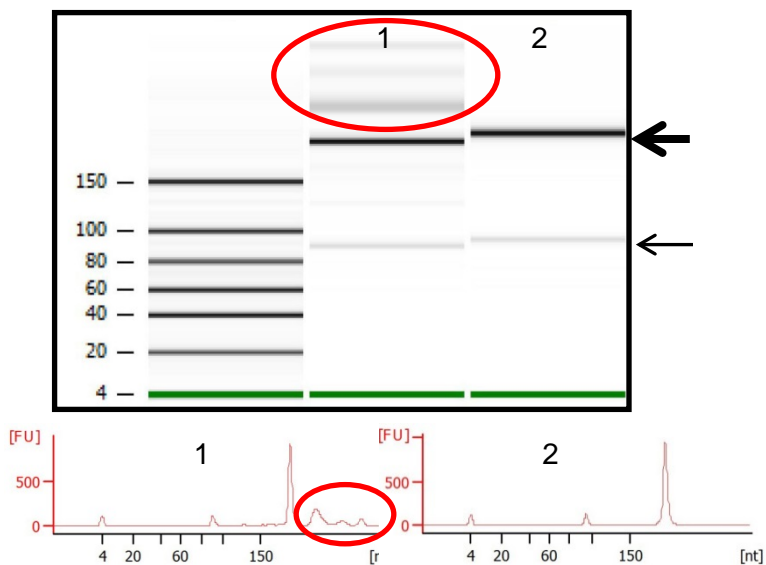
BioAnalyzer data of sgRNAs. It is absolutely essential to verify integrity of sgRNA with a BioAnalyzer before submitting to a core for microinjection into a mouse zygote. At left is a typical small RNA gel of two sgRNAs that were processed as above. Bands represent a sgRNA (90-100 nucleotide range, small arrow; note that a sgRNA may often migrate a little faster than its actual size of 100 nucleotides). A dominant higher molecular weight species, likely represents some dimeric structure of the sgRNA (large arrow) and some higher molecular weight species (red circle in sgRNA #1) represent some unknown RNA. The electropherogram traces below illustrate a cleaner sgRNA #2 as compared to the contaminated (red circle) species in sgRNA#1. These samples demonstrate the utility in testing 2 sgRNAs as it is difficult to predict which sgRNA will work best and provide the cleanest final sample preparation for generating genome-edited mice.

Supplemental Method #2: Quick Summary of CRISPR Path to Engineering Genetic Mouse Model

1. Study the genomic region of interest on the UCSC Genome Browser (https://genome.ucsc.edu) and select optimal region(s) to be edited based on ENCODE and other experimental data.
2. Select crRNA(s) either manually or with assistance of any number of search algorithms (see text).
    a. Follow procedures in Supplemental Method 1 for cloning crRNA into plasmid.
    b. Purchase Cas9 mRNA (TriLink BioTechnologies) or Cas9 protein (PNA Bio or New England Biolabs) and, if doing 3c CRISPR, HDR template (single-strand oligonucleotide as PAGE purified Ultramer from Integrated DNA Technologies).
    c. Do not resuspend SSO until just before microinjections.
3. Test activity of sgRNA by transfecting cells with pX330 plasmid carrying Cas9 and sgRNA in HEK-293 cells with an EGxxFP split reporter containing PCR amplified ~500 bp target genomic DNA.
4. Purify sgRNA per Supplemental Method 1 and submit with Cas9 mRNA or protein and, if necessary, HDR template to an academic Transgenic Core for microinjections.
5. Cores vary slightly, but we typically have components mixed just before microinjection at these concentrations: Cas9 mRNA (100 ng/μl), sgRNA(s) (25-50 ng/μl), and single-strand oligonucleotide repair template (100 ng/μl; dissolved and mixed just before injecting). Components are mixed in 10 mM Tris-HCl, pH 7.5 + 0.1 mM EDTA.
6. Components are injected into cytoplasm of C57BL/6J zygotes. Note, pup yields may be higher with hybrid zygotes such as FVB/C57BL/6J, but these mice will need to be back-crossed.
7. Pups may be genotyped as early as day 7 by toe clip or day 20 by ear punch with pre-optimized PCR genotyping assays (see Supplemental Method 3).
8. Putative positive founder DNA is further analyzed by gel purification of band of interest (*e.g.*, shifted band with 3xFLAG or *loxP*, deletion, or precision PCR of 3c CRISPR with discriminating primers, see Supplemental Figures IV, VI-VII); cloned in suitable vector (TOPO), and Sanger sequenced.
9. Same genomic DNA from positive founder should be interrogated for unintended edits based on computer-predicted off-target sites. DNA is amplified with primers flanking putative off-target site, TOPO cloned, and Sanger sequenced. Several individual colonies from TOPO cloning here and in Step 8 should be sequenced since multiple alleles may be present in founders (see Supplemental Figure V and text for more details on mosaicism).
10. Sequence-confirmed founders should be bred for germ line transmission of intended edits. Once transmission of allele occurs in the F_1 generation, founders may be studied if biallelic targeting occurs (*e.g.*, tissue expression of target gene associated with a deleted enhancer).
11. If using genome editing in a hybrid strain, back-cross to the appropriate strain to generate homozygous mice.
12. If off targeting events were noted in the founder mouse, repeat analysis in the F_1 or later generation to determine whether the off target edit was lost during breeding.
13. Test two independent founder-derived F_1 lines to validate phenotypes.

Supplemental Method #3: Pre-Optimization of Multiplex PCR Assay for Genotyping 3c CRISPR Mice

1. Define the subtle edit and test activity of sgRNA according to Supplemental Method #1.
2. Download ~1 kb of genomic sequence with the subtle edit placed in center of sequence.
3. Design primers (with suitable restriction sites) that flank the edited region by ~200-300 nt on each side. Use the PrimerQuest tool under the Integrated DNA Technologies Tool bar; http://www.idtdna.com/site). To save time, we typically order two pairs of primers to test each in case there is PCR failure.
4. PCR clone the genomic fragment into a host plasmid and sequence validate.
5. Perform site-directed mutagenesis (QuikChange kit) on the validated plasmid with oligos that contain the same mutations that will be created in the single-strand oligonucleotide HDR repair template. The goal is to simulate the genomic edit that will exist in the mouse. Sequence validate the mutant plasmid.
6. Design two forward primers that terminate at the edited nucleotides with one (wild type) containing a ~30-35 nt 5' extension of irrelevant sequence to the mouse genome and the other (mutant) with the same core sequence but without the 5' extension and ending with the SNP or multi-base mutations Design reverse primer ~150 bp downstream of the sgRNA (see Supplemental Figure VIIC and below).
7. Create a word document (below) summarizing overall strategy with sgRNAs, PCR primers etc.
8. Spike mouse genomic DNA with about one genomic equivalent of either the wild type or mutant plasmid generated in steps 4-5 and optimize PCR conditions (varying the annealing step first and then cycle number) that will result in the wild type and mutant primers only amplifying their respective wild type and mutant test plasmids. Then try to multiplex with both primers and the common reverse primer in a reaction with equal parts of wild type and mutant test plasmid.
   a. Use AccuStart II Gel Track PCR Supermix (Cat. #84228) for all PCR reactions.
9. Run PCR reactions on at least a 2% agarose gel until the dye front has moved at least ¾ length of a 14 cm gel. We sometimes find it necessary to increase the agarose concentration to 2.5%.
10. Because the forward primers are 30-35 nt different in size, a mutant and wild type allele should be resolved (see image at right and Supplemental Figure VIIC).
11. This approach has been published (reference 127 in the text) and we since have developed similar assays for several new projects, including those with single base pair mutations.


```
        GTGCACACGGCTGTGTGTTAAAGCTCCAGTTGCCTACCAGGCTTGATGCTTTAGTTATGG
181     ---------+---------+---------+---------+---------+---------+ 240
        CACGTGTGCCGACACACAATTTCGAGGTCAACGGATGGTCCGAACTACGAAATCAATACC


        GGAAGAAGCCTCACCTTGCTTTGTAGTGTTGATCTTTACGCTTTGAAGCCTGTTTGCAGT
241     ---------+---------+---------+---------+---------+---------+ 300
        CCTTCTTCGGAGTGGAACGAAACATCACAACTAGAAATGCGAAACTTCGGACAAACGTCA


        ATATATTTCAACTTCCACAGACAAAGTTAACACACTATTCTTGACGTTTTGCATGAAGAA
301     ---------+---------+---------+---------+---------+---------+ 360
        TATATAAAGTTGAAGGTGTCTGTTTCAATTGTGTGATAAGAACTGCAAAACGTACTTCTT


        CACAACATATTGAAAAGAAATCTGAGCAAATTAATATAGGATGACCGTTTTTTGATAAGA
361     ---------+---------+---------+---------+---------+---------+ 420
        GTGTTGTATAACTTTTCTTTAGACTCGTTTAATTATATCCTACTGGCAAAAAACTATTCT
                                                        WT-For →
                crRNA₁                                  MUT-For →
        CCTCCTGTGTACTCTCTGGCAGGTATCTCCACTCAGCCATGATTATTTACCGTCACCTGA
421     ---------+---------+---------+---------+---------+---------+ 480
        GGAGGACACATGAGAGACCGTCCATAGAGGTGAGTCGGTACTAATAAATGGCACTGGACT
                                          crRNA₂
                      Y   L   H   S   A   M   I   I   Y   R   D   L   K -


        AGCCCCACAATGTGCTGCTTTTTACCCTGTATCCCAATGCTGCCATCATTGCGAAGATTG
481     ---------+---------+---------+---------+---------+---------+ 540
        TCGGGGTGTTACACGACGAAAAATGGGACATAGGGTTACGACGGTAGTAACGCTTCTAAC
                       crRNA₃
          P   H   N   V   L   L   F   T   L   Y   P   N   A   A   I   I   A   K   I   A -


        CGGACTACGGGATCGCACAGTACTGCTGCAGGATGGGAATAAAGACATCAGAGGGCACCC
541     ---------+---------+---------+---------+---------+---------+ 600
        GCCTGATGCCCTAGCGTGTCATGACGACGTCCTACCCTTATTTCTGTAGTCTCCCGTGGG
                                                    Rev
```

Key
crRNA sequence to clone into pX330 plasmid for DSB efficiency

PAM sequence

Oligos for making templates to optimize genotyping strategy

SNP substitution site (GA>TC)

3c CRISPR

SpyCas9

sgRNA

HDR template

Target DNA

exonX

Target DNA
HDR template

Edited DNA
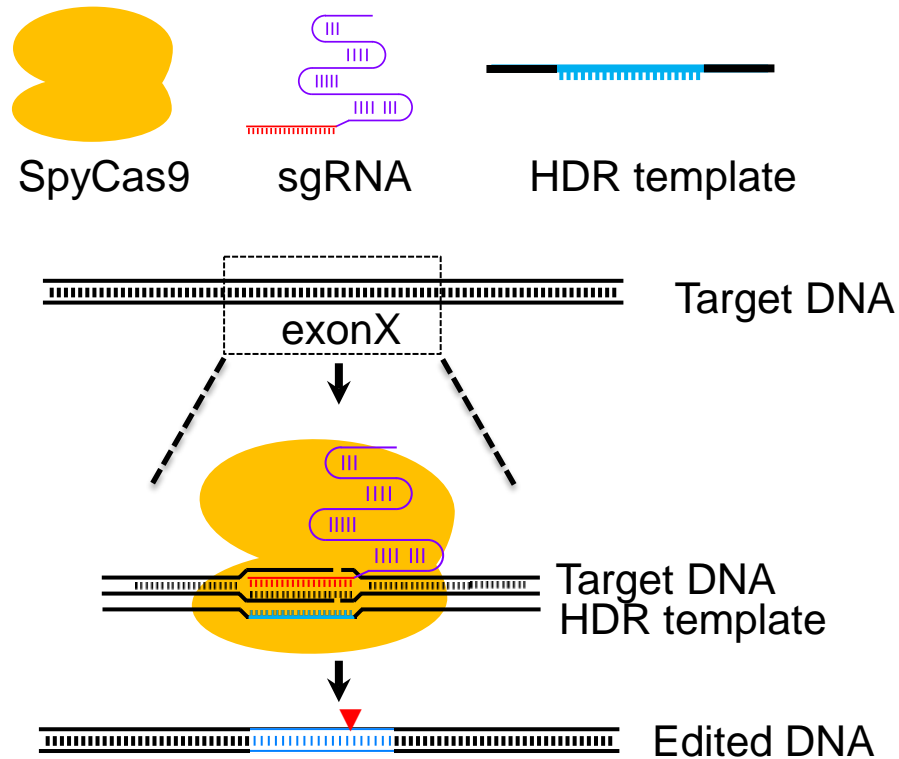
Precise genome editing

- Gene correction
- SNP modeling
- Conditional allele
- Epitope tagging
- Fine mapping regulatory element