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Modeling disease *in vivo* with CRISPR/Cas9

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Abstract

The recent advent of CRISPR/Cas9-mediated genome editing has created a wave of excitement across the scientific research community, carrying the promise of simple and effective genomic manipulation of nearly any cell type. CRISPR has quickly become the preferred tool for genetic manipulation, and shows incredible promise as a platform for studying gene function *in vivo*.

Here, I discuss the current application of CRISPR technology to create new *in vivo* disease models, with a particular focus on how these tools, derived from an adaptive bacterial immune system, are helping us better model the complexity of human cancer.

Keywords

CRISPR; Cas9; *in vivo*; cancer

Just CRISPR it

Few discoveries truly transform the way we study biology and medicine. In a few short years, CRISPR/Cas9 has done just that. Offering the potential for simple and precise genomic manipulation, CRISPR has taken the scientific world by storm. Identified more than 10 years ago as clustered regularly interspaced short palindromic repeat (CRISPR) sequences and CRISPR-associated (Cas) proteins [1, 2], CRISPR garnered little attention at first. Then, in 2013, shortly after the first demonstration that Cas protein could be programmed to cleave specific DNA sequences by short synthetic guide RNAs [3], the labs of Church, Doudna and Zhang quickly revealed the power of CRISPR-mediated genome modification in mammalian cells [4–6]. In the short period since, the genome editing revolution has taken on a life of its own, introducing a new scientific verb and giving rise to the now commonplace phrase: “CRISPR it!” As labs across the world drive new technological innovations to mutate, silence, induce, and replace genomic elements, we are presented with an expanding array of new tools to interrogate gene function and the biology of disease (Table 1, Key Table).

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The ability to generate targeted mutations in the mammalian genome has formed the backbone of genetic research since the creation of the first knockout mouse by Capecchi and colleagues [7]. So, while the concept of genome editing is not new, methodologies to manipulate the genome have become increasingly sophisticated, targeted, and efficient [8]. The development of engineered zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and most recently CRISPR has promised a new era of genetic engineering. Though they engage the genome by distinct mechanisms, each of these technologies function as genomic scissors that can be directed to induce double-strand breaks in specific DNA sequences [9–11]. In fact, the efficient induction of targeted DNA damage and subsequent DNA repair, by either non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Figure 1) is the core of all targeted genome editing approaches, old and new. HDR uses similar DNA sequences to drive high-fidelity repair, and thus, can be co-opted to allow the integration of exogenous DNA, which acts as the repair template. It is noteworthy, and often overlooked, that all traditional vector-based gene knockout and knock-in strategies utilize HDR mechanisms, although they rely on the sporadic and low-frequency incidence of DNA damage in the target site, to initiate template-directed repair.

In contrast to HDR, double-strand break repair by NHEJ often results in the introduction of random insertions or deletions (indels) that create frameshift alterations in protein coding regions, disrupting gene function [10]. Thus, genome editing by ZFNs, TALENs and CRISPR offers the ability to generate random, but targeted, gene disruption by NHEJ or increase the efficiency of HDR-mediated targeting by catalyzing DNA breaks at target sites. While the particular recruitment of DNA repair machinery is somewhat cell-type dependent, a number of groups have recently identified small molecule inhibitors that can bias DNA repair toward either NHEJ or HDR [12–14], providing another level of control over genomic modification (Figure 1).

The benefits and limitations of ZFN and TALEN-based approaches have been well reviewed elsewhere [15], but it is clear that despite the promise of fast and precise genome editing of virtually any organism [16–19] neither technology has achieved the impact that was initially touted. This is due in large part to the practical limitations of generating active ZFN or TALEN reagents. ZFN engineering is notoriously difficult and the intellectual property surrounding it well guarded. The more modular TALEN system offers a viable ‘open-source’ alternative [9, 20], however the production of effective TALEN tools still requires multistep cloning and significant expertise. In contrast to ZFNs and TALENs, engineering targeted DNA breaks using CRISPR/Cas9 is remarkably simple. Here, genomic targeting is mediated by Cas9 protein complexed with two small RNA species (a CRISPR RNA and *trans*-activating crRNA), or more commonly, a synthetic single guide RNA (sgRNA). DNA recognition is specified by a short (17–20bp) region of the sgRNA sequence complementary to the genomic target, and protospacer adjacent motif (PAM) sequence (usually NGG) present immediately downstream of the target site (Figure 2). Because target specification is defined by an interchangeable 17–20bp region, embedded in a common RNA scaffold, generating tailored CRISPR reagents is straightforward and rapid. The ease and speed with which CRISPR/Cas9 technology has been adopted by the research community is staggering,

as reflected in the recent scientific literature. In 2014, CRISPR-related publications outnumbered those of ZFNs and TALENs almost three to one, and this number continues to climb steeply in 2015 (Figure 3). Moreover, demand for CRISPR tools is sky high. In 2013 (the same year the breakthrough CRISPR papers were published), US-based non-profit plasmid repository Addgene, received more than 4 times the number of requests for CRISPR reagents than TALEN and ZFN combined. Accordingly Addgene's CRISPR repository grew dramatically (<http://www.addgene.org/crispr/>) and in 2014 the number of reagent requests nearly doubled again, and with an ever-increasing number of labs adopting the new technology, this is likely just the beginning. Here I review the latest developments in the application of CRISPR/Cas9-based genome editing for investigating gene function *in vivo*, with a particular focus on how these tools are enabling us to better model the complexity of human cancer.

CRISP 'n' clean mouse models

The mouse as a model organism has been at the forefront of genetic engineering since its inception and has, perhaps not surprisingly, led much of the research on nuclease-directed genome editing. Accordingly, CRISPR has been quickly embraced by the mouse modeling community, bringing genomic manipulation to a new level of flexibility.

Germline

Jaenisch and colleagues provided the first demonstration of the speed at which novel animal models can be produced using CRISPR, producing mutations in up to 8 alleles from a single embryonic stem cell (ESC) transfection [21]. Further, they revealed a surprisingly high efficiency for producing single (95%) or double mutant (70–80%) mice using direct injection of Cas9 mRNA and sgRNAs into fertilized zygotes. The simplicity of this approach enables labs with minimal experience in mouse model production to create customized tools for their gene or genes of interest. Consequently, CRISPR-derived mutant mice have become a popular and relatively affordable offering from a number of commercial entities, as well as many 'in-house' transgenic core facilities. The microinjection platform is fast and requires little lead time to generate new models, however it carries the limitation that such models are constitutive germline events and cannot be used to study homozygous disruption of essential genes, or to generate non-synonymous mutations whose expression compromises embryonic development. It is also important to consider that compound mutant mice will show allelic segregation in the F1 generation. Further, given that the frequency of multiplexed targeted knock-in mutations is low, and most alleles show random indels, it is likely that the particular mutation combinations and, thus phenotypes, produced in each founder will be different [22, 23]. Such mosaicism will complicate the analysis of F0 mice produced by this method. Conversely, the ability to target many genes in one step is advantageous for the production and analysis of multiple mutant alleles that are closely linked genetically, and would be difficult to combine by breeding independent strains.

In part circumventing the issues of deleterious germline mutations, follow-up studies have demonstrated the use of CRISPR zygote injections to produce larger knock-in alleles, either LoxP-flanked 'floxed' genes or endogenous transcriptional reporters [24–26]. The efficiency

of generating these targeted alleles is lower than that reported for generating NHEJ-repaired indels, but sufficiently high that the approach will be broadly useful for generating novel animal tools for research. It is important to note, however, that as the rate of NHEJ-mediated mutagenesis generally outweighs the homology-driven incorporation of exogenous DNA, in most cases, cells that have correctly incorporated the new allele will carry CRISPR-induced mutations in the second allele. As methods to enhance HDR improve [12–14], this may become less of a concern, but until then the generation of animal models by this method will require some careful planning to ensure minimal unwanted genetic disruption.

Transplantation

One alternative to creating germline mutations for disease models is the *ex vivo* modification of stem and progenitor cells that can be transplanted into a syngeneic recipient. Early studies validated the efficiency of CRISPR-induced gene deletion in this setting, with sgRNAs targeting p53 proving as potent as well-validated short hairpin RNA (shRNAs), and able to drive the development of Eμ-Myc lymphomas *in vivo* at a rate similar to cells from p53 null animals [27]. Taking the approach beyond proof-of-concept, Heckl *et al.*, used pooled CRISPR lentivirus to target 8 genes recurrently mutated in myeloid cancers, identifying multiple potential genetic combinations that drive disease, and in many cases creating biallelic mutations in up to 5 genes in the same tumor [28].

The application of CRISPR in transplantation-based *in vivo* models is not limited to gene-by-gene analysis. Sharp and colleagues took a genome-wide screening approach to identify genes that promote tumor growth and metastasis [29]. Such screens are an exciting application of CRISPR methodology, and in a setting of positive selection, will likely yield many interesting candidates. Initial *in vitro* reports also suggest that CRISPR can be effectively applied for genome-scale negative selection [30, 31], though given the importance of how and where each target gene is modified [32], it remains to be seen whether these approaches will outperform similar screens using shRNAs. It is clear however, that in the setting of oncology research, where validating individual or combinatorial cancer drivers from the hundreds of tumor-associated mutations has been a near impossible, but important task, CRISPR provides a feasible and high-throughput means to tackle the problem.

Exogenous delivery

Rather than transplanting CRISPR-modified cells, numerous groups have adapted CRISPR/Cas9 technology for direct *in vivo* delivery, enabling specific and multiplexed genetic modification in select tissues of adult mice. Swiech *et al.* induced targeted gene disruption by microinjection of CRISPR adeno-associated virus into the mouse hippocampus, and demonstrated the potential for tissue-specific multiplexed genome editing by disrupting a family of demethyltransferases (DNMT1–3) [33]. In theory the same methodology could be applied to multiple nodes within a signalling network, or indeed, multiple networks. For modeling oncogenic lesions, exogenous and mosaic delivery CRISPR is often beneficial, enabling the disruption of target genes in a subset of cells surrounded by normal tissue, better reflecting the sporadic nature of tumor initiation. In this regard, Jacks and colleagues adapted a platform used extensively for Cre and Flp recombinase systems, to deliver Cas9/

sgRNA containing lentivirus to lung epithelium, allowing rapid generation of tumors harboring inactivating mutations in tumor suppressor genes [34]. Similar efforts using traditional targeting could take many years to develop. Others have shown efficient gene deletion following viral delivery to the mouse liver [35], and used this approach to develop proof-of-concept models for therapeutic application of CRISPR [36, 37]. Further, Xue *et al.* described the induction of genetically complex hepatocellular carcinomas by simple delivery of naked plasmid DNA through hydrodynamic transfection [38]. Importantly, in addition to disabling gene function through frameshift truncation, they showed it is possible to generate oncogenic point mutations in CTNNB1 (frequently observed in human hepatocellular carcinoma), by co-delivery of a homology repair template. Direct gene editing using this hydrodynamic approach has also enabled functional gene correction *in vivo* [39]. The modification of hepatocytes in these experiments is so far the best example of targeted, tissue specific gene editing *in vivo*, perhaps made possible by the ease of DNA delivery to the liver. In fact there have been surprisingly few examples of efficient and specific gene replacement *in vivo* in adult tissues. This perhaps reflects the challenge of engaging HDR enzymes in post-mitotic tissues, as they are most abundant during S and G2 phases of the cell cycle [40, 41]. Recent work shows that Cas9/sgRNA (protein/RNA) complexes can be effectively released *in vivo* through cationic lipid formulations and drive high-fidelity target mutagenesis [42]. While yet to be fully tested *in vivo*, co-delivery of homologous repair templates in this setting may provide an avenue to the efficient induction of targeted point mutations, which has thus far, proved challenging in most tissues of adult mice.

Inducible models

Not all tissues or cell types in the mouse are easily amenable to delivery of viral elements or naked DNA. Yet, given the flexibility of CRISPR-mediated genome editing there is enormous potential in using the system to model complex cancer-associated genetic events in a wide range of tissue types. Conditional transgenic approaches have been developed to enable inducible and tissue specific Cas9-dependent mutagenesis in the adult mouse, based on the timed induction of Cas9 expression, sgRNA expression or both [43, 44].

Platt *et al.* developed a Cre-dependent CAGs-LSL-Cas9 knock-in transgene [44], while ‘all-in-one’, doxycycline (dox)-inducible constructs were generated to provide both sgRNAs and Cas9 in the germline of the animal [43]. The Cre-dependent model allows simple incorporation of CRISPR-mediated targeting into existing Cre-driven systems, and provides robust and widespread Cas9 expression downstream of the strong CAGs promoter. The dox-inducible model enables targeting in either individual or multiple tissues, is not restricted by the ability to delivery exogenous sgRNAs, and provides a means to extinguish Cas9 expression following gene modification. Both approaches show extremely high efficiency of single or multiplexed gene modification in multiple tissues, and recapitulate the phenotypic consequences seen in traditional genetic knockouts. Each is amenable to the delivery of sgRNAs exogenously or through the germline of the animal, although importantly, the stable integration of Cas9 in the genome, avoids the complication of packaging a large Cas9 cDNA into size restricted viral cassettes.

Together, these studies clearly show that CRISPR/Cas9 transgenic models are an attractive option for studying gene function *in vivo*, however there remain some limitations to overcome. We have noted that induction from the TRE3G promoter is somewhat mosaic in the adult mouse, while strong and constitutive Cas9 expression (post-Cre) in the LSL-Cas9 mouse may carry unintended cellular consequences [45]. While Platt *et al* noted that constitutive Cas9 expression in mice is not toxic [44], in sensitive *in vitro* cell competition assays, we have observed that high levels of sustained Cas9 expression can impair cell proliferation (Han *et al.*, unpublished data). Though not yet tested *in vivo*, the recent description of Cas9 derivatives that can be inducibly and transiently activated by small molecules [46, 47] provides an appealing alternative for inducible genome editing, leaving Cre and dox-regulated systems available to direct other genetic changes.

Modeling the complex genome

Genome editing has broad utility in animal models, beyond the deletion of individual genes. The ability to induce targeted double-strand breaks enables the creation of complex, disease-associated genomic aberrations, such as large chromosomal deletions, inversions, and translocations [48–51]. Multiple groups recently demonstrated the application of CRISPR/Cas9 tools to recreate the *EML4-ALK* intrachromosomal inversion that accounts for approximately 5% of lung tumors. Simultaneously, two labs reported that intratracheal delivery of adenoviral or lentiviral constructs targeting both *Eml4* and *Alk* introns, results in the generation of the *Eml4-Alk* fusion and subsequent development of lung adenocarcinoma [48, 50]. Most importantly, CRISPR-induced *Eml4-Alk* tumors show dramatic response to the *Alk* targeted agent Crizotinib, mirroring the known clinical efficacy of this genotype-drug combination. Similar engineering strategies can be used to functionally probe possible causal relationships between genomic structural variations and human syndromes and diseases. One very recent, and elegant, example defined the importance of a recurrently altered genomic locus, showing that CRISPR-driven disruption of chromatin topology in mice by large deletion or inversion of chromosomal segments, mimics limb malformation observed in humans with corresponding alterations [52]. In some cases, reciprocal translocations observed in human disease are not easily created in the mouse due to differences in respective gene orientation and the consequent production of potentially unstable dicentric chromosomes. To circumvent this limitation, Lagutina *et al.*, took a clever two-step approach, first inverting a large syntenic region on mouse chromosome 3 through a Cre-LoxP strategy, then using CRISPR to catalyse the translocation of two transcription factors, Pax3 and Foxo1, frequently observed in rhabdomyosarcoma [53]. These studies are impressive examples of how CRISPR/Cas9 tools can enable the interrogation of structural genomic variants and the rapid production of tailored animal models for pre-clinical testing.

Perhaps what is most surprising from these reports is the frequency of CRISPR-induced chromosomal rearrangement. In theory, expression of two sgRNAs targeting the same chromosome arm can result in indels at either or both loci (events which prevent further cleavage), deletion of the intervening DNA (large deletion), or segmental inversion. Even in cultured fibroblasts, without significant positive selective pressure, Maddalo *et al.* demonstrated inversion rates of up to 4% [50]. This should give us optimism for the generation of many and varied models of chromosome aberrations, but also reason to be

cautious. The high frequency of large chromosomal events implies that the introduction of any set of multiple sgRNAs (or a single sgRNA with significant off-target activity) could drive the production of unwanted genomic disruptions [54]. These issues are likely to get worse as more and more regions are targeted simultaneously. Thus, the continued development of effective algorithms to generate potent, high fidelity sgRNAs, as well as effective, affordable, and non-biased methods to assess genomic disruption will be key to the ultimate success of CRISPR-based models.

One tool to rule them all

Non-mammalian models

CRISPR technology is not only the domain of the mouse. Even genetically pliable models such as zebrafish, *Caenorhabditis elegans*, and *Drosophila* have benefited from CRISPR-driven genome modification. To date, most effort has focused on adapting and optimizing tools for efficient genome modification in these non-mammalian systems, and there is a large catalogue of successful examples. The details of CRISPR application in non-mammalian models is not the focus of this review, and have been covered elsewhere [55–57], but it is impressive to note that various groups have reported: heritable germline modification, high efficiency introduction of specific mutations, and transgenic, tissue-specific, inducible editing in flies [58–62], fish [39, 63–67] and worms [68–74]. Given these early successes, there is no doubt that CRISPR-based approaches will gain popularity among the community, but with a variety of flexible genetic tools already available to those studying fish, worms, and flies, it will be telling to see whether CRISPR becomes a major and standard component of the invertebrate toolbox for genetic analysis.

Larger mammals

Where genome editing tools, in particular CRISPR technology, show promise to be truly revolutionary, is in larger vertebrate model systems. This includes mice, where CRISPR is changing the landscape of genetic analysis (see above), but also rats, pigs, and non-human primates, where traditional genetic tools developed in mouse have been difficult to adapt. Targeted genomic manipulation in the rat was transformed by the advent of ZFN and TALEN technology, which provided a means to create mutant strains without the need for ESC cultures. And, though ZFNs and TALENs have proven effective, the field has been quick to adapt the newest CRISPR tools [75–79] to produce a range of germline mutant strains, as well as knock-in fluorescent reporters and Cre and LoxP conditional mutants that have proven so effective in the mouse [80]. As is already clear in cell culture models, the simplicity of CRISPR will likely see it become the dominant genome-engineering tool in the rat, and this will translate to an expanded array of rat disease models [81]. While it is unlikely the rat will ever eclipse the mouse as the leading model in disease research, particularly for cancer, the availability of CRISPR-derived customised mutant strains from both commercial and academic centers will significantly increase the spectrum of genetic questions that can be addressed in the larger rodent.

Pigs are a highly relevant model for human disease as they closely reflect many aspects of human physiology. For instance, while existing mouse models of monogenic disorders like

cystic fibrosis (CF) and familial adenomatous polyposis (FAP) do not accurately reflect clinical symptoms, counterpart pig mutants show the classic disease progression observed in humans [82, 83], implying they are superior models for investigating certain diseases. Pigs have not traditionally been a popular choice for research due to size, cost, and limited ability to develop targeted mutants, however, the implementation of simple, one-step CRISPR-mediated genetic engineering [84] could provide a gateway for more routine use of pig models in preclinical research. Indeed, it will be interesting to see whether those that already use pig models for preclinical evaluation of wound-healing, and cardiovascular and metabolic treatments, will adopt the new tools to more closely model genetic-based disease.

In non-human primates, arguably the best research model for learning and cognition, traditional methods of genomic manipulation have largely failed. In 2014, two groups reported successful gene-modification of cynomolgus and rhesus monkeys using CRISPR/Cas9 [85] and TALEN-based [86] gene editing. While these groundbreaking studies open the door to a wide range of research applications, founder animals in both studies showed mosaic gene disruption that could confound phenotypic analysis. Given that the long breeding and gestation cycle of these mammals precludes complex intercrossing strategies adopted in rodents, gene editing in non-human primates will significantly benefit from the development of even more efficient CRISPR methodologies.

The evolution of Cas9 tools for genome manipulation

Though a young field, CRISPR tools and techniques have, and are, evolving at a rapid pace. Initially offering a way to create DNA double-strand breaks and targeted genome modification, the development and modification of Cas9 variants has led to a single platform technology with capabilities far beyond DNA mutagenesis (Table 1, Key Table). Adding to the original toolbox are a recently described array of Cas9 proteins from different bacteria species, each with their own unique target sequence bias, expanding the number of genomic sites that can be modified by CRISPR [37]. In addition, nuclease inactive 'dead' Cas9 (dCas9) has been adapted to enable suppression of gene transcription (CRISPRi) or activation of endogenous gene expression (CRISPRa) [87–92]. It is not yet clear whether CRISPRi holds any advantage over well-tested RNAi technologies, but CRISPRa provides a feasible alternative to cDNA overexpression platforms, particularly for the induced expression of non-coding RNAs that act in *cis*, where transgene-based expression is likely to be ineffective [93]. Further, recent work showed that specific dCas9 fusions could be used to manipulate the epigenome of cells [94]. While early in the evolution of this approach, if broadly effective it will provide an unprecedented tool for dissecting the contribution of disrupted epigenetic regulation on cell behaviour and tumorigenic transformation.

Getting off target

Despite significant effort to design better sgRNAs [95] the potential for off-target DNA cleavage, or in the case of dCas9 variants, undesired changes to gene expression or chromatin patterns, is an ongoing concern. Indeed, we and others have identified bone fide off-target effects with some specific sgRNAs in mouse and human cells [43, 96, 97]. The good news is that a number of recent studies using whole genome sequencing have reported

minimal recurrent off-target mutation events in iPS cells and mice [98–101]. Importantly, while we may not be able to prevent all off-target cleavage, there are now multiple non-biased, genome-wide methods to isolate and track CRISPR-induced DNA breaks [54, 102, 103]. In addition to providing a broad survey of the activity of CRISPR/Cas9 complexes across the genome, these studies have revealed significant variability in the degree of off-target cleavage between individual sgRNAs, implying that it may be difficult to accurately predict off-target potential *a priori*. Thus, it is likely that we will never completely eliminate off-target effects, but for DNA cleavage, the use of paired sgRNAs in conjunction with a Cas9^{D10A} (nickase) variant is effective, and can eliminate many of these unwanted events *in vitro* and *in vivo* [43, 54, 97, 104]. For clinical application (Box 1), where off-target events are a serious concern, extensive empirical testing will be critical. In a research environment, however, it should be simple to properly control for spurious, and likely rare, functional consequences of CRISPR-mediated off-target events, and this should not significantly curtail the use of an otherwise revolutionary technology.

BOX 1

Human models and regenerative medicine

Studying human cells is arguably the most direct path to understanding the genetics of human disease. Genome editing in the form of ZFNs, TALENs, and CRISPR/Cas9 has been a tremendous leap forward for the direct manipulation of the human genome, and has already changed the way many labs understand gene function in normal and transformed cultures. For disease modeling, two recent examples from the Sato and Clevers labs perfectly showcase the unique and effective application of CRISPR-mediated editing in patient-derived 3D colon organoid cultures [105, 106]. Both groups used CRISPR/Cas9 to drive truncating mutations in tumor suppressors *APC*, *TP53*, and *SMAD4* and HDR-mediated activating mutations in proto-oncogenes *KRAS* and *PIK3CA*, to recapitulate the proposed sequence of oncogenic events in colorectal cancer. These studies not only illustrate the ability of CRISPR to generate multiple complex genomic alterations in human tissue, but that such alterations can be temporally controlled, enabling the direct examination of mutational order. In some instances, these and similar organoid models might even provide a suitable surrogate for *in vivo* mouse experiments. In contrast to studying the progression of disease, is the possibility of correcting disease-causing genes for regenerative medicine - perhaps one of the most exciting clinical applications of genome editing. Indeed, many studies have already reported successful ‘gene repair’ with CRISPR tools in patient-derived induced pluripotent stem (iPS) cells and organoid cultures [107–110]. Of course, with the apparent ease of manipulating the human genome, come serious ethical concerns that have been raised publically by many who were integral to the development of CRISPR [111, 112]. Although the first example of CRISPR-mediated modification of human embryos has already surfaced [113], significant backlash from the scientific community and funding bodies suggests that we will not see a flurry of similar publications. The standoff between ethical and moral concerns, and scientific exploration and curiosity will be tense and constantly evolving.

Concluding Remarks

At the current rapid pace of technological development, it is difficult to predict exactly what the future holds for CRISPR-based *in vivo* models. In the coming years there will, undoubtedly, be many more examples of how CRISPR-mediated genome manipulation can be implemented in new ways to advance disease research. What will be most critical is capitalizing on the strengths and unique capabilities of CRISPR (Outstanding Questions). So, while we continue to “CRISPR it”, we should also focus on integrating our new genome editing tools with well-established technologies to provide the most flexible and powerful models. For example, combining CRISPR, RNAi, and/or Cre-based approaches so that multiple genetic events can be controlled independently. Ultimately, with this wide variety of tools now in hand, the challenge that remains is developing and implementing the most accurate and predictive disease models to better understand and treat human disease.

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References

1. Bolotin A, et al. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology* (Reading, England). 2005; 151(Pt 8):2551–2561.
2. Jansen R, et al. Identification of genes that are associated with DNA repeats in prokaryotes. *Molecular microbiology*. 2002; 43(6):1565–1575. [PubMed: 11952905]
3. Jinek M, et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*. 2012; 337(6096):816–821. [PubMed: 22745249]
4. Cong L, et al. Multiplex genome engineering using CRISPR/Cas systems. *Science*. 2013; 339(6121):819–23. [PubMed: 23287718]
5. Jinek M, et al. RNA-programmed genome editing in human cells. *eLife*. 2013; 2:e00471. [PubMed: 23386978]
6. Mali P, et al. RNA-guided human genome engineering via Cas9. *Science*. 2013; 339(6121):823–6. [PubMed: 23287722]
7. Thomas KR, Capecchi MR. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell*. 1987; 51(3):503–12. [PubMed: 2822260]
8. Dow LE, Lowe SW. Life in the fast lane: mammalian disease models in the genomics era. *Cell*. 2012; 148(6):1099–109. [PubMed: 22424222]
9. Cermak T, et al. Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic acids research*. 2011; 39(12):e82. [PubMed: 21493687]
10. Kim H, Kim JS. A guide to genome engineering with programmable nucleases. *Nat Rev Genet*. 2014; 15(5):321–34. [PubMed: 24690881]
11. Urnov FD, et al. Genome editing with engineered zinc finger nucleases. *Nature reviews Genetics*. 2010; 11(9):636–646.
12. Chu VT, et al. Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. *Nat Biotechnol*. 2015; 33(5):543–8. [PubMed: 25803306]
13. Maruyama T, et al. Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. *Nat Biotechnol*. 2015; 33(5):538–42. [PubMed: 25798939]
14. Yu C, et al. Small molecules enhance CRISPR genome editing in pluripotent stem cells. *Cell stem cell*. 2015; 16(2):142–147. [PubMed: 25658371]

15. Gaj T, Gersbach CA, Barbas CF 3rd. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends in biotechnology*. 2013; 31(7):397–405. [PubMed: 23664777]
16. Hockemeyer D, et al. Genetic engineering of human pluripotent cells using TALE nucleases. *Nat Biotechnol*. 2011; 29(8):731–734. [PubMed: 21738127]
17. Miller JC, et al. A TALE nuclease architecture for efficient genome editing. *Nat Biotechnol*. 2010; 29(2):143–148. [PubMed: 21179091]
18. Townsend JA, et al. High-frequency modification of plant genes using engineered zinc-finger nucleases. *Nature*. 2009; 459(7245):442–445. [PubMed: 19404258]
19. Wood AJ, et al. Targeted genome editing across species using ZFNs and TALENs. *Science*. 2011; 333(6040):307. [PubMed: 21700836]
20. Sanjana NE, et al. A transcription activator-like effector toolbox for genome engineering. *Nat Protoc*. 2012; 7(1):171–92. [PubMed: 22222791]
21. Wang H, et al. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell*. 2013; 153(4):910–8. [PubMed: 23643243]
22. Long C, et al. Prevention of muscular dystrophy in mice by CRISPR/Cas9-mediated editing of germline DNA. *Science*. 2014; 345(6201):1184–8. [PubMed: 25123483]
23. Yen ST, et al. Somatic mosaicism and allele complexity induced by CRISPR/Cas9 RNA injections in mouse zygotes. *Dev Biol*. 2014; 393(1):3–9. [PubMed: 24984260]
24. Aida T, et al. Cloning-free CRISPR/Cas system facilitates functional cassette knock-in in mice. *Genome Biol*. 2015; 16(1):87. [PubMed: 25924609]
25. Inui M, et al. Rapid generation of mouse models with defined point mutations by the CRISPR/Cas9 system. *Sci Rep*. 2014; 4:5396. [PubMed: 24953798]
26. Yang H, et al. One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. *Cell*. 2013; 154(6):1370–9. [PubMed: 23992847]
27. Malina A, et al. Repurposing CRISPR/Cas9 for in situ functional assays. *Genes & Development*. 2013; 27(23):2602–14. [PubMed: 24298059]
28. Heckl D, et al. Generation of mouse models of myeloid malignancy with combinatorial genetic lesions using CRISPR-Cas9 genome editing. *Nat Biotechnol*. 2014; 32(9):941–946. [PubMed: 24952903]
29. Chen S, et al. Genome-wide CRISPR screen in a mouse model of tumor growth and metastasis. *Cell*. 2015; 160(6):1246–1260. [PubMed: 25748654]
30. Shalem O, et al. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science*. 2014; 343(6166):84–7. [PubMed: 24336571]
31. Wang T, et al. Genetic screens in human cells using the CRISPR-Cas9 system. *Science*. 2014; 343(6166):80–4. [PubMed: 24336569]
32. Shi J, et al. Discovery of cancer drug targets by CRISPR-Cas9 screening of protein domains. *Nat Biotechnol*. 2015; 33(6):661–7. [PubMed: 25961408]
33. Swiech L, et al. In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9. *Nat Biotechnol*. 2015; 33(1):102–106. [PubMed: 25326897]
34. Sanchez-Rivera FJ, et al. Rapid modelling of cooperating genetic events in cancer through somatic genome editing. *Nature*. 2014; 516(7531):428–31. [PubMed: 25337879]
35. Cheng R, et al. Efficient gene editing in adult mouse livers via adenoviral delivery of CRISPR/Cas9. *FEBS Lett*. 2014; 588(21):3954–8. [PubMed: 25241167]
36. Ding Q, et al. Permanent alteration of PCSK9 with in vivo CRISPR-Cas9 genome editing. *Circ Res*. 2014; 115(5):488–92. [PubMed: 24916110]
37. Ran FA, et al. In vivo genome editing using *Staphylococcus aureus* Cas9. *Nature*. 2015; 520(7546):186–191. [PubMed: 25830891]
38. Xue W, et al. CRISPR-mediated direct mutation of cancer genes in the mouse liver. *Nature*. 2014; 514(7522):380–4. [PubMed: 25119044]
39. Yin H, et al. Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. *Nat Biotechnol*. 2014; 32(6):551–3. [PubMed: 24681508]
40. Hendrickson EA. Cell-cycle regulation of mammalian DNA double-strand-break repair. *Am J Hum Genet*. 1997; 61(4):795–800. [PubMed: 9382087]

41. Shrivastav M, De Haro LP, Nickoloff JA. Regulation of DNA double-strand break repair pathway choice. *Cell Res.* 2008; 18(1):134–47. [PubMed: 18157161]
42. Zuris JA, et al. Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. *Nat Biotechnol.* 2015; 33(1):73–80. [PubMed: 25357182]
43. Dow LE, et al. Inducible in vivo genome editing with CRISPR-Cas9. *Nat Biotechnol.* 2015; 33(4):390–4. [PubMed: 25690852]
44. Platt RJ, et al. CRISPR-Cas9 Knockin Mice for Genome Editing and Cancer Modeling. *Cell.* 2014; 159(2):440–55. [PubMed: 25263330]
45. Wu X, et al. Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells. *Nature biotechnology.* 2014; 32(7):670–6.
46. Davis KM, et al. Small molecule-triggered Cas9 protein with improved genome-editing specificity. *Nat Chem Biol.* 2015; 11(5):316–8. [PubMed: 25848930]
47. Zetsche B, Volz SE, Zhang F. A split-Cas9 architecture for inducible genome editing and transcription modulation. *Nat Biotechnol.* 2015; 33(2):139–42. [PubMed: 25643054]
48. Blasco RB, et al. Simple and rapid in vivo generation of chromosomal rearrangements using CRISPR/Cas9 technology. *Cell reports.* 2014; 9(4):1219–1227. [PubMed: 25456124]
49. Choi PS, Meyerson M. Targeted genomic rearrangements using CRISPR/Cas technology. *Nat Commun.* 2014; 5:3728. [PubMed: 24759083]
50. Maddalo D, et al. In vivo engineering of oncogenic chromosomal rearrangements with the CRISPR/Cas9 system. *Nature.* 2014; 516(7531):423–427. [PubMed: 25337876]
51. Torres R, et al. Engineering human tumour-associated chromosomal translocations with the RNA-guided CRISPR-Cas9 system. *Nat Commun.* 2014; 5:3964. [PubMed: 24888982]
52. Lupianez DG, et al. Disruptions of topological chromatin domains cause pathogenic rewiring of gene-enhancer interactions. *Cell.* 2015; 161(5):1012–25. [PubMed: 25959774]
53. Lagutina IV, et al. Modeling of the human alveolar rhabdomyosarcoma Pax3-Foxo1 chromosome translocation in mouse myoblasts using CRISPR-Cas9 nuclease. *PLoS Genet.* 2015; 11(2):e1004951. [PubMed: 25659124]
54. Frock RL, et al. Genome-wide detection of DNA double-stranded breaks induced by engineered nucleases. *Nat Biotechnol.* 2015; 33(2):179–86. [PubMed: 25503383]
55. Gonzales APW, Yeh J-RJ. Cas9-based genome editing in zebrafish. *Methods in enzymology.* 2014; 546:377–413. [PubMed: 25398350]
56. Housden BE, Lin S, Perrimon N. Cas9-based genome editing in *Drosophila*. *Methods in enzymology.* 2014; 546:415–439. [PubMed: 25398351]
57. Waaijers S, Boxem M. Engineering the *Caenorhabditis elegans* genome with CRISPR/Cas9. *Methods (San Diego, Calif.)*. 2014; 68(3):381–388.
58. Gratz SJ, et al. Highly specific and efficient CRISPR/Cas9-catalyzed homology-directed repair in *Drosophila*. *Genetics.* 2014; 196(4):961–971. [PubMed: 24478335]
59. Bassett AR, et al. Highly efficient targeted mutagenesis of *Drosophila* with the CRISPR/Cas9 system. *Cell reports.* 2013; 4(1):220–228. [PubMed: 23827738]
60. Gratz SJ, et al. Genome engineering of *Drosophila* with the CRISPR RNA-guided Cas9 nuclease. *Genetics.* 2013; 194(4):1029–1035. [PubMed: 23709638]
61. Ren X, et al. Optimized gene editing technology for *Drosophila melanogaster* using germ line-specific Cas9. *Proc Natl Acad Sci U S A.* 2013; 110(47):19012–19017. [PubMed: 24191015]
62. Yu Z, et al. Highly efficient genome modifications mediated by CRISPR/Cas9 in *Drosophila*. *Genetics.* 2013; 195(1):289–91. [PubMed: 23833182]
63. Ablain J, et al. A CRISPR/Cas9 vector system for tissue-specific gene disruption in zebrafish. *Dev Cell.* 2015; 32(6):756–64. [PubMed: 25752963]
64. Hruscha A, et al. Efficient CRISPR/Cas9 genome editing with low off-target effects in zebrafish. *Development (Cambridge, England).* 2013; 140(24):4982–4987.
65. Hwang WY, et al. Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol.* 2013; 31(3):227–229. [PubMed: 23360964]

66. Jao L-E, Wente SR, Chen W. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proc Natl Acad Sci U S A*. 2013; 110(34):13904–13909. [PubMed: 23918387]
67. Xiao A, et al. Chromosomal deletions and inversions mediated by TALENs and CRISPR/Cas in zebrafish. *Nucleic acids research*. 2013; 41(14):e141. [PubMed: 23748566]
68. Chiu H, et al. Transgene-free genome editing in *Caenorhabditis elegans* using CRISPR-Cas. *Genetics*. 2013; 195(3):1167–1171. [PubMed: 23979577]
69. Cho SW, et al. Heritable gene knockout in *Caenorhabditis elegans* by direct injection of Cas9-sgRNA ribonucleoproteins. *Genetics*. 2013; 195(3):1177–1180. [PubMed: 23979576]
70. Dickinson DJ, et al. Engineering the *Caenorhabditis elegans* genome using Cas9-triggered homologous recombination. *Nat Methods*. 2013; 10(10):1028–1034. [PubMed: 23995389]
71. Friedland AE, et al. Heritable genome editing in *C. elegans* via a CRISPR-Cas9 system. *Nat Methods*. 2013; 10(8):741–743. [PubMed: 23817069]
72. Katic I, Großhans H. Targeted heritable mutation and gene conversion by Cas9-CRISPR in *Caenorhabditis elegans*. *Genetics*. 2013; 195(3):1173–1176. [PubMed: 23979578]
73. Tzur YB, et al. Heritable custom genomic modifications in *Caenorhabditis elegans* via a CRISPR-Cas9 system. *Genetics*. 2013; 195(3):1181–1185. [PubMed: 23979579]
74. Waaijers S, et al. CRISPR/Cas9-targeted mutagenesis in *Caenorhabditis elegans*. *Genetics*. 2013; 195(3):1187–1191. [PubMed: 23979586]
75. Chapman KM, et al. Targeted Germline Modifications in Rats Using CRISPR/Cas9 and Spermatogonial Stem Cells. *Cell Rep*. 2015; 10(11):1828–35. [PubMed: 25772367]
76. Hu X, et al. Heritable gene-targeting with gRNA/Cas9 in rats. *Cell Res*. 2013; 23(11):1322–5. [PubMed: 24145754]
77. Li D, et al. Heritable gene targeting in the mouse and rat using a CRISPR-Cas system. *Nat Biotechnol*. 2013; 31(8):681–683. [PubMed: 23929336]
78. Ma Y, et al. Heritable multiplex genetic engineering in rats using CRISPR/Cas9. *PLoS One*. 2014; 9(3):e89413. [PubMed: 24598943]
79. Yoshimi K, et al. Allele-specific genome editing and correction of disease-associated phenotypes in rats using the CRISPR-Cas platform. *Nat Commun*. 2014; 5:4240. [PubMed: 24967838]
80. Ma Y, et al. Generation of eGFP and Cre knockin rats by CRISPR/Cas9. *FEBS J*. 2014; 281(17):3779–90. [PubMed: 25039742]
81. Nakamura K, et al. Generation of muscular dystrophy model rats with a CRISPR/Cas system. *Sci Rep*. 2014; 4:5635. [PubMed: 25005781]
82. Flisikowska T, et al. A porcine model of familial adenomatous polyposis. *Gastroenterology*. 2012; 143(5):1173–5. e1–7. [PubMed: 22864254]
83. Rogers CS, et al. Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. *Science*. 2008; 321(5897):1837–1841. [PubMed: 18818360]
84. Hai T, et al. One-step generation of knockout pigs by zygote injection of CRISPR/Cas system. *Cell research*. 2014; 24(3):372–375. [PubMed: 24481528]
85. Niu Y, et al. Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. *Cell*. 2014; 156(4):836–43. [PubMed: 24486104]
86. Liu H, et al. TALEN-mediated gene mutagenesis in rhesus and cynomolgus monkeys. *Cell Stem Cell*. 2014; 14(3):323–8. [PubMed: 24529597]
87. Konermann S, et al. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature*. 2015; 517(7536):583–8. [PubMed: 25494202]
88. Mali P, et al. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nature biotechnology*. 2013; 31(9):833–8.
89. Cheng AW, et al. Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell research*. 2013; 23(10):1163–1171. [PubMed: 23979020]
90. Gilbert LA, et al. Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. *Cell*. 2014; 159(3):647–61. [PubMed: 25307932]
91. Gilbert LA, et al. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell*. 2013; 154(2):442–51. [PubMed: 23849981]

92. Maeder ML, et al. CRISPR RNA-guided activation of endogenous human genes. *Nat Methods*. 2013; 10(10):977–979. [PubMed: 23892898]
93. Guil S, Esteller M. Cis-acting noncoding RNAs: friends and foes. *Nat Struct Mol Biol*. 2012; 19(11):1068–75. [PubMed: 23132386]
94. Hilton IB, et al. Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nat Biotechnol*. 2015; 33(5):510–7. [PubMed: 25849900]
95. Fu Y, et al. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat Biotechnol*. 2014; 32(3):279–284. [PubMed: 24463574]
96. Fu Y, et al. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nature biotechnology*. 2013; 31(9):822–6.
97. Ran FA, et al. Double Nicking by RNA-Guided CRISPR Cas9 for Enhanced Genome Editing Specificity. *Cell*. 2013
98. Iyer V, et al. Off-target mutations are rare in Cas9-modified mice. *Nat Methods*. 2015; 12(6):479. [PubMed: 26020497]
99. Smith C, et al. Whole-Genome Sequencing Analysis Reveals High Specificity of CRISPR/Cas9 and TALEN-Based Genome Editing in Human iPSCs. *Cell Stem Cell*. 2014; 15(1):12–3. [PubMed: 24996165]
100. Suzuki K, et al. Targeted gene correction minimally impacts whole-genome mutational load in human-disease-specific induced pluripotent stem cell clones. *Cell Stem Cell*. 2014; 15(1):31–6. [PubMed: 24996168]
101. Veres A, et al. Low incidence of off-target mutations in individual CRISPR-Cas9 and TALEN targeted human stem cell clones detected by whole-genome sequencing. *Cell Stem Cell*. 2014; 15(1):27–30. [PubMed: 24996167]
102. Tsai SQ, et al. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat Biotechnol*. 2015; 33(2):187–197. [PubMed: 25513782]
103. Wang X, et al. Unbiased detection of off-target cleavage by CRISPR-Cas9 and TALENs using integrase-defective lentiviral vectors. *Nat Biotechnol*. 2015; 33(2):175–8. [PubMed: 25599175]
104. Cencic R, et al. Protospacer adjacent motif (PAM)-distal sequences engage CRISPR Cas9 DNA target cleavage. *PLoS ONE*. 2014; 9(10):e109213. [PubMed: 25275497]
105. Drost J, et al. Sequential cancer mutations in cultured human intestinal stem cells. *Nature*. 2015; 521(7550):43–47. [PubMed: 25924068]
106. Matano M, et al. Modeling colorectal cancer using CRISPR-Cas9-mediated engineering of human intestinal organoids. *Nature medicine*. 2015; 21(3):256–262.
107. Li HL, et al. Precise correction of the dystrophin gene in duchenne muscular dystrophy patient induced pluripotent stem cells by TALEN and CRISPR-Cas9. *Stem Cell Reports*. 2015; 4(1):143–54. [PubMed: 25434822]
108. Osborn MJ, et al. Fanconi anemia gene editing by the CRISPR/Cas9 system. *Hum Gene Ther*. 2015; 26(2):114–26. [PubMed: 25545896]
109. Schwank G, et al. Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell*. 2013; 13(6):653–8. [PubMed: 24315439]
110. Xie F, et al. Seamless gene correction of beta-thalassemia mutations in patient-specific iPSCs using CRISPR/Cas9 and piggyBac. *Genome Res*. 2014; 24(9):1526–33. [PubMed: 25096406]
111. Baltimore D, et al. Biotechnology. A prudent path forward for genomic engineering and germline gene modification. *Science*. 2015; 348(6230):36–38. [PubMed: 25791083]
112. Lanphier E, et al. Don't edit the human germ line. *Nature*. 2015; 519(7544):410–411. [PubMed: 25810189]
113. Liang P, et al. CRISPR/Cas9-mediated gene editing in human trippronuclear zygotes. *Protein Cell*. 2015; 6(5):363–72. [PubMed: 25894090]

Outstanding Questions Box

- Targeted gene editing/replacement, rather easily achieved in cell culture and zygotes, remains a significant challenge *in vivo* in adult mammalian tissues. For accurate disease modeling and clinical application, this is a key hurdle to overcome.
- Injecting fertilized eggs is the fastest path to CRISPR-modified animals, such as mice, rats, pigs and monkeys, but can lead to significant genetic mosaicism. Can improved delivery or expression systems eliminate this problem?
- Will CRISPR/Cas9-based approaches provide a suitable platform to comprehensively explore the impact of chromosome structural variations and disruptions in the non-coding genome in a way that other scalable genomic technologies (i.e. shRNAs) have not?
- Wide variation in reported off-target effects of different sgRNAs suggests there are features that predict high-fidelity genomic interactions. Further understanding and exploiting such features will significantly enhance every application of CRISPR/Cas9-driven genome modification.

Trends Box

- CRISPR-based genome editing is a technology derived from bacteria that is revolutionizing how we study gene function in almost all model organisms, from flies to primates.
- CRISPR creates new possibilities for genome manipulation, offering a flexible and user-friendly platform to explore the genetic causes of disease.
- First described as a means to create simple genetic mutants only 3 years ago, CRISPR tools are now being adapted to replace, rearrange, silence, activate, and remodel genomic elements.
- CRISPR changes the way we study biology *in vivo*.

Figure 1. DNA repair mechanisms dictate genome editing outcomes

Following CRISPR/Cas9-induced DNA double-strand breaks, DNA is usually repaired by either homology directed repair (HDR) or non-homologous end joining (NHEJ). HDR enables integration of exogenous 'repair templates' such as single-stranded donor oligonucleotides (ssODN) or double-stranded DNA (dsDNA). NHEJ results in the generation of random insertions and deletions (indels) that may disrupt coding regions, or catalyze genomic rearrangements. The cellular preference for HDR or NHEJ following DNA damage can be augmented by small molecules that interfere with each mechanism, and thus bias toward the other. *Abbreviations:* ATM = Ataxia telangiectasia mutated; ATR = ataxia telangiectasia and Rad3-related; RAD51 = Rad51 recombinase; KU70 = XRCC6, X-ray repair complementing defective repair in Chinese hamster cells 6; KU80 = XRCC5, X-ray repair complementing defective repair in Chinese hamster cells 5; AZT = Azidothymidine; TFT = Trifluridine.

Figure 2. Genomic targeting of Cas9/sgrRNA complexes

Genomic targeting of Cas9/sgrRNA complexes is mediated by complementarity between the sgRNA (orange) and a genomic DNA (gDNA) target site (green) that lies immediately upstream of a protospacer adjacent motif (PAM, purple). For Cas9 derived from *Streptococcus pyogenes*, the PAM sequence is NGG, or less commonly, NAG. Cas9 variants from different bacterial species have alternative PAM requirements. Effective recognition and unwinding of the gDNA from the PAM results in double-strand cleavage by two independent nuclease domains in Cas9 (blue arrows).

Figure 3. CRISPR dominates papers published with genome editing technologies

Dotplot representing the average number of articles indexed on PubMed containing the search terms “CRISPR”, “TALEN”, or “ZFN” in the title or abstract, published over the last 10 years. Although it is the most recent addition to the genome editing toolbox, papers reporting CRISPR far outnumber both TALEN and ZFN based studies.

Table 1

Applications of CRISPR tools for genome modification^{a,b}

	Advantages	Disadvantages	Examples	Summary
Cut	<ul style="list-style-type: none"> • Fast and simple • Efficient NHEJ-mediated mutagenesis 	<ul style="list-style-type: none"> • Random mutagenesis • Off-target cutting 	<p>[21]</p> <p>[77]</p> <p>[44]</p> <p>[34]</p> <p>[38]</p> <p>[43]</p> <p>[24]</p>	<p>Production of single and multiplex mutant mice via direct zygote injections of Cas9/sgRNAs</p> <p>Production of mutant mice and rats via direct zygote injections of Cas9/sgRNAs</p> <p>Cre-dependent (LSL) Cas9 transgene, enabling CRISPR editing following delivery of an exogenous sgRNA</p> <p>Somatic gene editing in the lung via lentiviral delivery of Cas9 and sgRNAs</p> <p>Somatic gene editing in the liver using hydrodynamic transfection of plasmid-based Cas9 and sgRNAs</p> <p>Inducible genome editing in mice carrying a dox-responsive Cas9 and single or multiple sgRNAs in one transgene</p> <p>Efficient knock-in alleles using Cas9-sgRNA (protein-RNA) complexes, delivered by zygote injection</p>
Nick	<ul style="list-style-type: none"> • Fast and simple • Efficient NHEJ-mediated mutagenesis • Limited off-target effects 	<ul style="list-style-type: none"> • Random mutagenesis • Requires 2 sgRNAs/target 	<p>[35]</p> <p>[25]</p> <p>[43]</p>	<p>Adenoviral-mediated delivery of Cas9 and sgRNAs to the mouse liver</p> <p>Generation of knock-in and mutant mice by direct zygote injection of Cas9n/sgRNA/donor template</p> <p>Inducible genome editing using a dox-responsive Cas9n transgene and multiple sgRNAs in one transgene</p>

	Advantages	Disadvantages	Examples	Summary
Knock-in		<ul style="list-style-type: none"> • Specific mutations • Endogenous protein tags & fluorescent reporters 	<ul style="list-style-type: none"> • Low efficiency • Frequent mutations in non-targeted allele • Requires HDR machinery 	<p>[26]</p> <p>Production of LoxP, epitope and fluorescent reporter mice via zygote injection of Cas9/sgRNAs/donor template</p> <p>[25]</p> <p>Generation of knock-in and mutant mice by direct zygote injection of Cas9/sgRNA/donor template</p> <p>[38]</p> <p>Somatic Ctnnb1 gene substitution using hydrodynamic transfection of plasmid-based Cas9/sgRNA/donor</p> <p>[39]</p> <p>Somatic Fah gene substitution using hydrodynamic transfection of plasmid-based Cas9/sgRNA/donor</p>
Rearrange		<ul style="list-style-type: none"> • Deletions, inversions & translocations • Endogenous rearrangements (regulation maintained) 	<ul style="list-style-type: none"> • Low efficiency • No way to specify type of rearrangement desired 	<p>[50]</p> <p>Somatic chromosomal rearrangement of EML4-Alk locus in the lung via adenoviral delivery of Cas9 and sgRNAs</p> <p>[48]</p> <p>Somatic chromosomal rearrangement of EML4-Alk locus in the lung via lentiviral delivery of Cas9 and sgRNAs</p> <p>[52]</p> <p>Disruption of chromatin topology domains by inversion and deletion of chromosome regions in ESCs</p>
Activate		<ul style="list-style-type: none"> • Reversible enforced expression • Potential for multiplexed activation • Endogenous transcript regulation maintained 	<ul style="list-style-type: none"> • May require multiple sgRNAs or accessory proteins • Thorough testing required to identify appropriate sgRNA combinations 	<p>N/D</p>
Repress		<ul style="list-style-type: none"> • Inhibition of RNA production • Reversible gene silencing • Multiplexed gene suppression 	<ul style="list-style-type: none"> • Potential for irreversible repression 	<p>N/D</p>

	Advantages	Disadvantages	Examples	Summary
Remodel		<ul style="list-style-type: none">• Epigenome remodeling• Activation or suppression of promoters & enhancers	<ul style="list-style-type: none">• Potential for irreversible repression• Off-target effects not well characterized	N/D

^aThis list does not include studies that used manipulation of cells *ex vivo*. To date, no studies have reported the use of CRISPRi, CRISPRa, or epigenome modifying Cas9 variants in *in vivo* models.

^b_{ssODN} = single-stranded donor oligonucleotide; VP64 = Tetrameric VP16 transcription activator domain; KRAB = Kruppel associated box