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# CRISPR Editing in Biological and Biomedical Investigation

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## ABSTRACT

The revolutionary technology for genome editing known as the clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR-associated protein 9 (Cas9) system has sparked advancements in biological and biomedical research. The scientific breakthrough of the development of CRISPR-Cas9 technology has allowed us to recapitulate human diseases by generating animal models of interest ranging from zebrafish to non-human primates. The CRISPR-Cas9 system can also be used to delineate the mechanisms underlying the development of human disorders and to precisely correct disease-causing mutations. Repurposing this technology enables wider applications in transcriptome and epigenome manipulation and holds promise to reach the clinic. In this review, we highlight the latest advances of the CRISPR-Cas9 system in different platforms and discuss the hurdles and challenges this technology is facing. J. Cell. Biochem. 118: 4152–4162, 2017. 

**KEY WORDS:** CRISPR-Cas9; DISEASE MODELS; GENOME EDITING; GENE CORRECTION; CRISPRi; CRISPRa; IMAGING

Earlier genome-editing technologies, such as zinc-finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN), have enabled the manipulation of genes by targeting DNA double-stranded breaks (DSBs) via non-homologous end-joining (NHEJ) or homology-directed repair (HDR) pathways [Rudin et al., 1989; Rouet et al., 1994; Choulika et al., 1995; Bibikova et al., 2002; Moscou and Bogdanove, 2009]. NHEJ-mediated error-prone DNA repair generates insertion/deletions (indels) at the site of the break that disrupts the translational reading frame, leading to frameshift mutations. Alternatively, DNA damage can be repaired through the HDR pathway in a DNA template-dependent manner that results in precise gene insertions or corrections (Fig. 1a). Despite these advances, wider adoption of this technology has been limited by low specificity and complex procedures [Sanchez-Rivera and Jacks, 2015]. In the past several years, the most revolutionary technology for genome editing, the clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR-associated protein 9 (Cas9) system, has achieved great accomplishments worldwide. The development of the CRISPR-Cas9 system has opened a new era of precise genome manipulation that has almost reached the clinic [Go and Stottmann, 2016].

The CRISPR story began in 1987 when a Japanese group reported an odd 29-nucleotide repeat sequence that had 32-nucleotide spacing [Ishino et al., 1987]. Thereafter, scientists spent nearly 20 years identifying the CRISPR as being part of an adaptive immune system that defends against invading infections [Lander, 2016]. Of the three types of CRISPR systems, the type II CRISPR nuclease system is the simplest for genome editing technology, which requires only a Cas9 protein and two RNAs: CRISPR RNAs (crRNA) and trans-activating crRNAs (tracrRNA) [Brouns et al., 2008; Wiedenheft et al., 2011; Jinek et al., 2012]. In the native system, the Cas9 nuclease is guided by a duplex formed by a crRNA that contains a 20-nucleotide region for DNA binding and a tracrRNA that activates Cas9 to cleave the DNA [Barrangou et al., 2007]. Target recognition and DNA cleavage require the presence of the protospacer-adjacent motif (PAM), a consensus NGG or NAG sequence adjacent to the 3′ end of the target DNA [Gasiunas et al., 2012; Jinek et al., 2012]. The crRNA: tracrRNA duplex was further simplified with a programmed chimeric single-guide RNA (sgRNA), which directs Cas9 nuclease to create DSBs in DNA at the desired positions of interest [Jinek et al., 2012; Mali et al., 2013] (Fig. 1b). This development has facilitated the targeting of DNA by simply designing a sgRNA and introducing it in the presence of a Cas9 protein. The experimental protocol for sgRNA design and construction has been well-documented [Sanjana et al., 2014; Shalem et al., 2014].

## GENERATION OF ANIMAL MODELS

Inspired by the successful release of many solid studies of the CRISPR-Cas9 system in bacteria [Gasiunas et al., 2012; Jinek et al., 2012] and mammalian cells [Cong et al., 2013; Mali et al., 2013],

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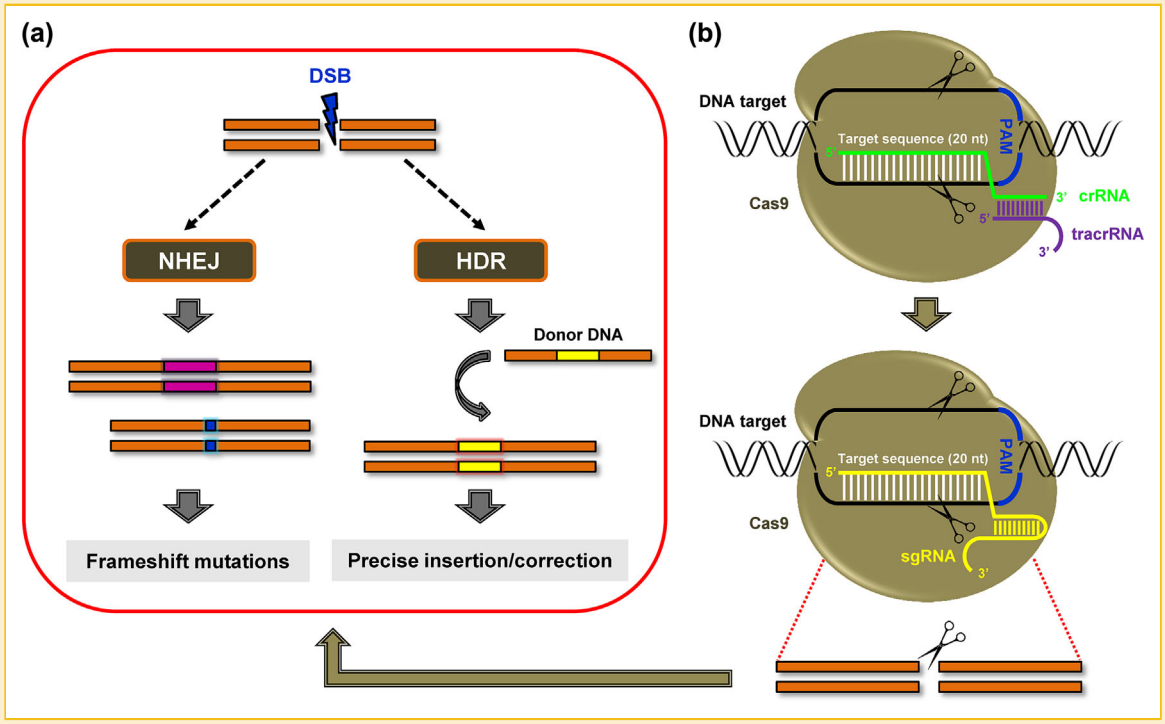


Fig. 1. Genome editing with the CRISPR-Cas9 system. (a) DNA double-stranded breaks (DSB) can be repaired by non-homologous end-joining (NHEJ) or homology-directed repair (HDR). The NHEJ repair pathway results in random insertion/deletions (indels) at the site of the break, leading to frameshift mutations. The HDR-mediated pathway achieves precise gene insertion/correction in the presence of a donor DNA template. (b) Cas9 endonuclease (in dark tan) can be guided by a crRNA: tracrRNA duplex to the specific target DNA sequence that is adjacent to the protospace-adjacent motif (PAM, in dark blue). The crRNA: tracrRNA duplex can be simplified with a chimeric single-guide RNA (sgRNA, in yellow), which directs the Cas9 protein to create a DSB at the desired position of the DNA target, thereby stimulating intracellular DNA repair pathways.

scientists have attempted to apply a CRISPR-Cas9 system in multiple organisms ranging from zebrafish [Hwang et al., 2013], drosophilae [Akiyama and Gibson, 2015; Gantz and Bier, 2015], mosquitos [Gantz et al., 2015; Hammond et al., 2016], rodents [Jackson et al., 2005; Soda et al., 2007; Li et al., 2013; Maddalo et al., 2014; Xue et al., 2014], dogs [Zou et al., 2015], pigs [Redel and Prather, 2015; Park et al., 2017a], to non-human primates [Niu et al., 2014; Chen et al., 2015].

Compared with conventional embryonic stem cell (ESC) manipulation, CRISPR-Cas9-mediated gene editing in ESCs has dramatically increased the gene editing efficiency by reducing the time required from years to months [Fellmann et al., 2017]. In addition to targeting only a single gene, the CRISPR-Cas9 technology has been extended to simultaneously disrupt five genes in mouse ESCs with high efficiency in only one step [Wang et al., 2013]. This finding, as well as the later report of a CRISPR-Cas9-mediated inducible mouse modeling platform [Yang et al., 2013], has facilitated the rapid generation of large repositories of ESCs with unprecedented speed and precision [Sanchez-Rivera and Jacks, 2015]. Alternatively, Cas9 and the appropriately designed sgRNA can be directly microinjected or simply electroporated into fertilized zygotes to achieve heritable gene modification. Co-injection of Cas9 mRNA and sgRNAs into mouse zygotes resulted in up to 80% of the total mice carrying biallelic mutations in the targeted genes; importantly, these models can be generated in only 1 month, a much shorter time than it takes using the conventional method of gene targeting in ESCs [Wang et al., 2013]. Last year, one research team reported the generation of a robust mouse model for Middle East respiratory syndrome coronavirus (MERS-CoV) [Cockrell et al., 2016]. Because small animal models are naturally resistant to MERS-CoV, the authors introduced two human mutations (at positions 288 and 330) into the mouse *Dpp4* gene via pronuclear microinjection into fertilized zygotes, making the mice highly susceptible to MERS-CoV infection and replication. This discovery holds great promise in the field of therapeutic design and offers new strategies to combat emerging viruses in the near future.

In addition to mice, CRISPR-Cas9 editing has been performed to target malaria mosquito embryos, leading to malaria eradication by either impairing the fertility of female mosquitos [Hammond et al., 2016] or inserting an antimalarial gene into malaria mosquitos [Gantz et al., 2015]. Recently, researchers revealed the first CRISPR-Cas9-targeted porcine model by directly injecting the Cas9 ribonucleoprotein complex and sgRNA sequences into porcine zygotes [Park et al., 2017a]. Zoology experts from China further extended the application of the CRISPR-Cas9 system to monkeys by targeting one-cell-stage embryos [Niu et al., 2014] and have generated primate models for Duchenne muscular dystrophy (DMD) [Chen et al., 2015]. These promising CRISPR-Cas9 models, which faithfully recapitulate human disease, pave the way for better understanding diseases and identifying vaccines or drugs that have high efficacy in humans.

Nonetheless, genome editing in zygotes always causes genetic mosaicism, or even embryonic lethality, making zygotes difficult to assess and manipulate [Fellmann et al., 2017]; on the other hand, germline editing is infeasible in human embryos due to bioethical concerns [Olson, 2016]. Such limitations allow researchers to directly perform somatic genome editing ex vivo or in vivo within certain tissues in adult animals.

Malina et al. [2013] first reported a CRISPR-Cas9-mediated ex vivo disruption of *Tp53* in the Eμ-*myc* mouse model. Subsequently, several leading groups have reported CRISPR-Cas9-mediated ex vivo somatic genome editing in mice for modeling hematological malignancies [Chen et al., 2014; Heckl et al., 2014]. These findings highlight the feasibility of using CRISPR-Cas9-based ex vivo genome editing for modeling human cancer. In 2014, the Jacks group successively delivered plasmids encoding Cas9 and sgRNAs directly into murine liver cells in vivo [Xue et al., 2014] and delivered all-in-one lentiviruses expressing the CRISPR components into murine lung cells [Sanchez-Rivera et al., 2014]. Later that year, another group mimicked oncogenic chromosomal translocation (*Eml4-Alk*) in vivo using an adenoviral-mediated CRISPR-Cas9 delivery system in wild-type mice [Maddalo et al., 2014]. Meanwhile, researchers noted that both viral and non-viral nanoparticle-sgRNA delivery methods are efficient in facilitating genome editing in multiple murine tissues in vivo, including neurons, immune cells and lung endothelial cells [Platt et al., 2014]. In 2016, *Science* simultaneously published three exciting in vivo somatic gene-editing studies using a mouse model of DMD, a fatal X-linked recessive inherited disease caused by mutations in the human *dystrophin* (*DMD*) gene [Long et al., 2016; Nelson et al., 2016; Tabebordbar et al., 2016]. The lack of functional DMD protein leads to progressive muscle deterioration, and no effective treatments have been developed. Using a DMD mouse model harboring the nonsense mutation in exon 23 of the *Dmd* gene, researchers developed adeno-associated virus (AAV)-mediated CRISPR-Cas9 strategies and restored expression of a truncated DMD variant by excision of exon 23. Amazingly, the amount of DMD expression that could be restored could be enough to provide a potential therapeutic benefit in humans [Koch, 2016], although the safety and efficacy of systemic administration of AAV vectors as well as the risk of off-target integrations still need to be assessed [VandenDriessche and Chuah, 2016]. Overall, these studies empower a broader range of disease modeling applications via CRISPR-Cas9-mediated genome engineering, allowing researchers to uncover fundamental mechanisms in disease initiation, maintenance and procession, and explore the therapeutic potential of the CRISPR-Cas9 system to correct disease-causing mutations. The application of CRISPR-Cas9 strategies in the generation of animal models is summarized in Figure 2.

## PRECISE MODELING OF GENE FUNCTIONS IN VITRO

Despite the exciting advances in generating in vivo models, most CRISPR-Cas9 applications to date have been limited to bench studies due to their streamlined and straightforward process.

### NHEJ-MEDIATED GENE DELETION

The introduction of a targeted knockout is the simplest way to delineate the roles of certain genes. Because it is a precise and flexible editing tool for complete silencing, CRISPR-Cas9-based mutagenesis has been overwhelmingly applied in biological and biomedical fields throughout the world. Unlike RNA interference (RNAi)-based silencing, which is limited by partial depletion of target gene levels, CRISPR-Cas9 enables a complete loss of gene function by NHEJ-mediated error prone repair. Moreover, this system works in all cell culture systems in a similar manner, with the major difference in the delivery methods of the CRISPR-Cas9 system [Fellmann et al., 2017]. For example, the transient transfection of plasmids encoding Cas9 and sgRNAs in cells has been reported in many studies [Cho et al., 2013; Cong et al., 2013; Mali et al., 2013]; in contrast, virus-based stable delivery is a more efficient way to modify cells [Shalem et al., 2014], especially in the hard-to-transfect blood cells [Tagde et al., 2016; Zhang et al., 2016a,b].

Human cancers are the most targeted diseases using the CRISPR-Cas9 technology because isogenic knockout cells allow for the rapid identification of the causative roles of oncogenes or tumor suppressors, excluding other interferences. In addition, the faster and more economical reprogramming abilities of CRISPR-Cas9 provide exciting opportunities to unravel the mechanisms of drug resistance and to identify potential therapeutic targets. Last year, one research group unveiled the oncogenic features of transglutaminase 2 (TGM2) in mantle cell lymphoma (MCL) using a lentiviral-based CRISPR-Cas9-mediated knockout system. They uncovered a positive feedback loop involving TGM2-NFKB signaling, IL6, and autophagy [Zhang et al., 2016a]. Disruption of this network may be a promising therapeutic target and introduce novel strategies to overcome chemoresistance in MCL [Zhang and McCarty, 2017]. Recently, a multicenter team identified a novel mechanistic contribution of the glucose transport inhibitors *NR3C1*, *TXNIP* and *CNR2* to pre-B-cell acute lymphoblastic leukemia (ALL) [Chan et al., 2017]. These genes are downstream targets highly induced by PAX5; CRISPR-Cas9-mediated deletion of these genes significantly enhanced glucose uptake and increased ATP levels, revealing an important function of PAX5 as a metabolic gatekeeper and providing promising therapeutic targets. Another illustrative example is the application of genome editing tools to modify chimeric antigen receptor (CAR) T cells, which has emerged as a powerful therapy in treating various hematological malignancies [Maus et al., 2014]. To prevent unwanted response from CAR T cell immunotherapy, such as graft-versus-host disease (GVHD) or graft rejection, researchers have attempted to knock out endogenous T cell receptor (TCR) genes using ZFN and TALEN genome editing [Torikai et al., 2012; Qasim et al., 2017]. Last year, one Chinese group was the first to inject CRISPR-Cas9-edited T cells back into a patient that had metastatic lung cancer. The researchers disabled the gene encoding programmed cell death protein 1 (PD-1), which prevents T cells from attacking other cells. Without PD-1, the edited T cells will defeat and attack the cancer; however, the efficacy of CRISPR-enabled attack has not been fully determined until 6 months of observation [Cyranoski, 2016].

A major limitation for CRISPR-Cas9-edited cell lines is adaptive changes caused by secondary mutations. Two recent publications

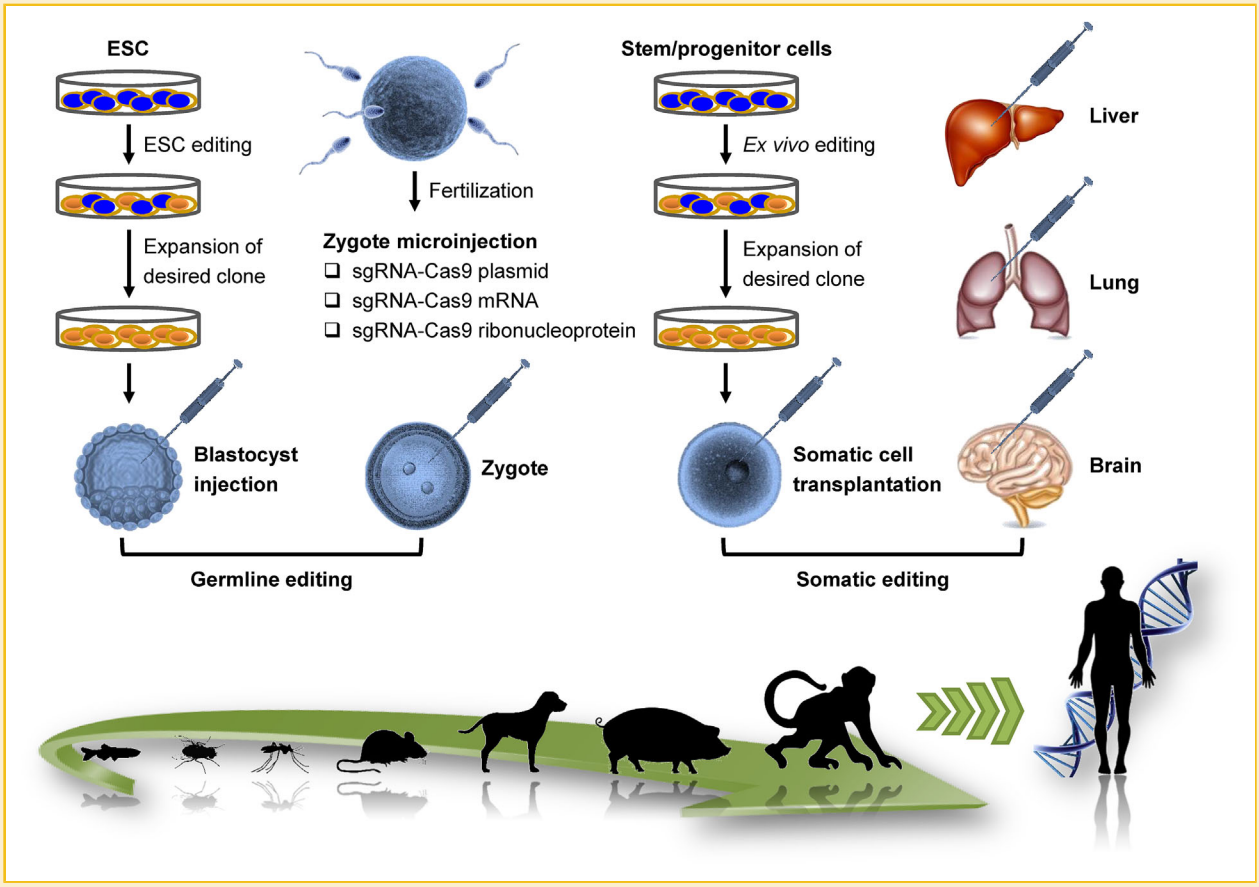


**Fig. 2.** Applications of the CRISPR-Cas9 system in disease modeling. CRISPR-Cas9-mediated germline editing can be achieved by the electroporation of embryonic stem cells (ESC), which are further validated and expanded before being injected into host blastocysts. Alternatively, CRISPR components can be introduced by direct microinjection into fertilized zygotes. In addition to germline editing, primary stem/progenitor cells can be ex vivo manipulated and expanded prior to transplantation into somatic cells. Editing reagents can also be delivered to certain host tissues (e.g., liver, lung or brain) via local injection. These CRISPR strategies allow for a direct and accurate recapitulation of human diseases and offer a potential therapeutic benefit to humans.

reported unexpected results: researchers showed human immunodeficiency virus (HIV) evolution and escape from CRISPR gene-programmed attack due to Cas9/sgRNA-derived mutations [Wang et al., 2016a,b]. Further deep sequencing analysis showed that such mutations are generated by cellular NHEJ mutagenesis at the cleavage site. These indels result in a change in the target DNA sequence, thereby preventing sgRNA from binding and ultimately leading to resistance to Cas9/sgRNA [Wang et al., 2016b]. These findings highlight the importance of further strategies and solutions to overcome viral resistance to the CRISPR-Cas9 system [Liang et al., 2016].

Owing to its ability to completely disrupt target genes and the simplicity of designing potent sgRNAs, the CRISPR-Cas9 system has been extended to large-scale loss-of-function (LOF) genome screens in human cells [Koike-Yusa et al., 2014; Shalem et al., 2014; Wang et al., 2014; Zhou et al., 2014]. One pioneer study established a lentivirus-mediated library containing 73,000 sgRNAs to generate knockout collections for genome-wide screening in myeloid leukemia cells [Wang et al., 2014]. The following year, the same group further identified essential genes required for cell proliferation and survival in four leukemia and lymphoma cell lines [Wang et al., 2015]. Compared with RNAi-based screens, CRISPR-Cas9 screens achieve robust hits and exhibit significantly lower false-negative rates [Munoz et al., 2016]. Nonetheless, one study highlighted the importance of combining data from both CRISPR-Cas9 and RNAi screens [Morgens et al., 2016]. In this study, the authors performed parallel CRISPR-Cas9 and RNAi screens to identify essential genes in the human chronic myelogenous leukemia (CML) cell line K562. Although CRISPR-Cas9 and RNAi screens achieved similar levels of precision, numerous identified genes were not overlapped in these two screens. This observation suggests that CRISPR-Cas9 and RNAi screens can detect distinct aspects of biology; one plausible reason is that certain genes that are only partially knocked down lead to totally different phenotypes compared to those resulting from complete loss via knockout. More recently, one team from Stanford University developed a CRISPR-based double knockout system that can disable two genes at one time in cells. Using this modified system, they knocked out 21,321 pairs of drug targets in K562 leukemia cells and identified synthetic lethal drug target pairs. This work demonstrates a promising high-throughput CRISPR-based

screening tool to determine functional pairwise genetic interactions [Han et al., 2017].

One should keep in mind that genotoxic stress can be induced by large numbers of DNA breaks when performing CRISPR-based LOF screening. Two independent experiments noted that genome targeting by CRISPR-Cas9 in highly amplified regions leads to increased DNA damage and a profound anti-proliferative response, revealing an unanticipated class of false-positive hits [Aguirre et al., 2016; Munoz et al., 2016]. These findings are alarming for CRISPR-based screening in highly amplified loci. In this regard, appropriate sgRNA design and careful control to not make excessive genomic cuts are urgently needed. Another important challenge for the CRISPR-Cas9-mediated knockout system is cell lethality at an early stage caused by the loss of essential genes. One solution is to use temperature-sensitive mutant alleles, which have recently been well-described [Housden et al., 2017]. The inducible CRISPR-Cas9 system provides an alternative option to identify the biological functions of critical genes [Zhang and McCarty, 2016].

### HDR-MEDIATED GENE CORRECTION

Compared to NHEJ-mediated gene disruption, HDR-induced gene correction has a broader application spectrum, although the HDR pathway is less efficient in mammalian cells. Human inherited disorders such as β-hemoglobinopathies (i.e., β-thalassemia and sickle cell disease) [Dever et al., 2016; Traxler et al., 2016; Ye et al., 2016; Park et al., 2017b], anemias [Bluteau et al., 2016; Osborn et al., 2016], and coagulation disorders [Guan et al., 2016; Park et al., 2016] represent ideal targets for CRISPR-Cas9-based gene therapy because the partial restoration by gene correction is sufficient to reverse the symptoms. In addition to blood disorders, which have been extensively discussed in detail [Zhang and McCarty, 2016], other disease-causing mutations can be precisely targeted and corrected using CRISPR-Cas9. In 2013, one group repaired a mutation in the cystic fibrosis transmembrane conductor receptor (CFTR) locus via HDR in primary intestinal stem cells derived from cystic fibrosis patients that had a single-gene hereditary defect [Schwank et al., 2013]. Later, HDR-induced gene correction was successfully applied to efficiently correct the mutations and phenotypes in mouse models of hereditary tyrosinemia [Yin et al., 2014, 2016], hearing loss [Mianne et al., 2016] or eye diseases [Kim et al., 2017]. Recently, one research team fully replaced, instead of excised, a defective mutation by introducing AAV-mediated CRISPR components and a DMD homology region in a mouse model of DMD [Bengtsson et al., 2017]. Indeed, this approach allows for the replacement of a mutation with a correct sequence. Regardless, moving CRISPR-Cas9-based gene correction from the bench to the clinic has a long way to go. Reasons for this are that (i) the human immune system may recognize the corrected proteins as foreign and thus eliminate the proteins by cytotoxic T lymphocytes; (ii) the phenotype of the edited cells may not fully reflect the actual response in humans since epigenetic and microenvironment alterations are not considered through simple ex vivo or in vitro experiments; and (iii) despite successful modeling in vivo, many uncertain safety issues and delivery efficiency remain unaddressed in humans [Fellmann et al., 2017; Housden et al., 2017].

## TRANSCRIPTIONAL MODULATION

To expand the potential of the CRISPR-Cas9 system, Weissman's group introduced two mutations at the RuvC and HNH sites of the Cas9 endonuclease and created a catalytically inactive Cas9 protein (dCas9) that had no endonuclease activity. This repurposed CRISPR-dCas9 system is called CRISPR interference (CRISPRi) and this system efficiently represses the expression of targeted genes by directly blocking transcription initiation or elongation with no off-target effects [Qi et al., 2013]. Unlike the permanent genetic modifications induced by Cas9 endonuclease, CRISPRi-mediated gene repression is inducible and reversible, offering an adjustable platform for RNA-guided transcription regulation [Qi et al., 2013]. To achieve enhanced transcription regulation, the researchers fused dCas9 to either repressor domains such as KRAB (Krüppel associated box) or activator domains such as VP64 (four copies of the VP16 activators) and p65 (p65AD), resulting in a system capable of repressing (CRISPRi) or activating (CRISPRa) the transcription of target genes [Gilbert et al., 2013] (Fig. 3a). This CRISPRi/a approach was further optimized to control the transcription levels of endogenous genes, including non-coding transcripts, across a high dynamic range; more importantly, CRISPRi/a can be used to rapidly screen for both LOF and gain-of-function (GOF) phenotypes in a pooled setting, providing complementary information for mapping complex pathways that has not yet been achieved [Gilbert et al., 2014]. In contrast, Konermann et al. [2015] developed an independent CRISPRa system mediated by a synergistic activation mediator (SAM) complex consisting of multiple distinct transcriptional activators, thus achieving robust gene upregulation mediated by a single sgRNA. These advances facilitate whole-transcriptome control in the human genome and help discover therapeutic targets through genome-scale CRISPRi/a screening [Heckl and Charpentier, 2015]. Amazingly, repression and activation of genes can be simultaneously achieved using programmed scaffold RNAs (scRNAs) that encode both target DNA and regulatory effectors [Zalatan et al., 2015]. This strategy enables the simultaneous control of multiple genes in the same cell by turning on one set of genes while turning off another set, thus offering a wide range of biomedical and biotechnological uses [Koch, 2015].

Given its uncomplicated design and versatile features, the CRISPRi/a method has been applied to multiple organisms. For instance, CRISPRi has been extensively used to repress multiple genes in *Mycobacterium* [Choudhary et al., 2015], *Cyanobacterium* [Yao et al., 2016], *Corynebacterium glutamincum* [Cleto et al., 2016], and *Escherichia coli* [Kim et al., 2016b] and is thus capable of regulating molecules of interest in bacterial microbes. Meanwhile, multiple animal models, such as worms and zebrafish [Long et al., 2015; Rossi et al., 2015], have been successfully generated using CRISPRi. Soon after these studies in simple organisms, this approach was further exploited to precisely modulate endogenous gene expression in human breast cancer cells [Shen et al., 2016] and in human induced pluripotent stem cells (iPSC)-derived neurons [Heman-Ackah et al., 2016]. Recently, one group from Netherlands compared the performance of CRISPR, CRISPRi, and a traditional short hairpin RNA (shRNA) system in their abilities to identify essential genes in human bladder cancer cells [Evers et al., 2016].

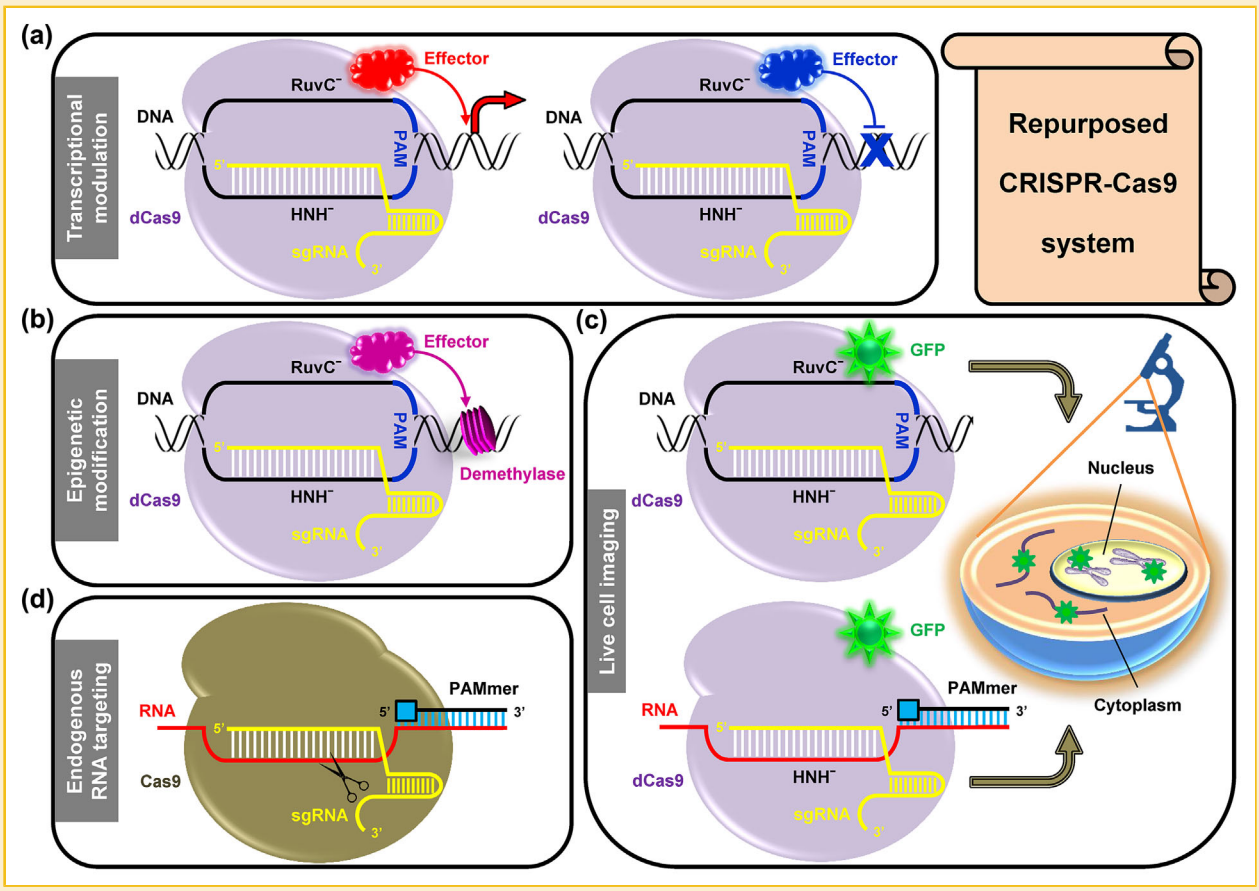


Fig. 3. Repurposed CRISPR–Cas9 system. (a) Transcriptional modulation. A catalytically inactive Cas9 protein (dCas9, in light purple) with no endonuclease activity can be fused to activating effectors (in red) or repressive effectors (in dark blue), leading to the activation (CRISPRa) or the repression (CRISPRi) of transcription of the target genes. (b) Epigenetic modification. The dCas9 protein can be extended to fuse with epigenetic effectors (e.g., DNA demethylase) for epigenomic engineering. (c) Live cell imaging. The CRISPR–Cas9 system has been repurposed as a genome imaging tool by fusing with fluorescent reporters (e.g., GFP, in light green) to track DNA or RNA in live human cells. (d) Endogenous RNA targeting. The Cas9 protein (in dark tan) can be directed to bind or cleave target RNA at specific sites using specially designed PAMmers, enabling transcriptome editing.

Interestingly, they found that the CRISPR-based knockout system outperformed the CRISPRi- and shRNA-based technologies in discriminating hits from non-hits in functional genetic screens because of its low noise, absence of off-target effects and better consistency across cell lines.

Likewise, the CRISPRa method has been utilized to create in vivo models in drosophilae [Lin et al., 2015], worms and zebrafish [Long et al., 2015]. In addition to the typical dCas9-VP64 activator, other "second-generation" activators have been tested across multiple cell types and species [Chavez et al., 2016]. In particular, three activators, SunTag [Tanenbaum et al., 2014], VPR (VP64-p65-Rta) [Chavez et al., 2015], and SAM [Konermann et al., 2015], appear to be the most potent systems compared to dCas9-VP64. This study provides an extensive set of systems to generate more reliable tools for improved activation, especially for the highly repressed genes, although most uses of the CRISPRa method have thus far been limited to preliminary function studies [Hu et al., 2016; Guo et al., 2017; Koirala et al., 2017]. Very recently, a group from the University of Oxford reported on the novel design of a spacer-blocking hairpin (SBH) structure at the 5′ end of sgRNA [Ferry et al., 2017]. This strategy can abrogate the function of CRISPR transcriptional activators. By replacing the SBH loop with conditional RNA-cleaving unit enables an inducible SBH (iSBH) platform, which can modulate the CRISPRa effect in the presence of specific inducers. This iSBH-based CRISPR system presents a versatile and simple "plug and play" tool for the accurate, conditional activation of the CRIPSR-based system in different disease states, and enriches the design of more complex networks for research and therapeutic purposes.

## EPIGENETIC MODIFICATION

Apart from its use in transcriptional modulation, CRISPR-dCas9 has been utilized to alter epigenetic marks for remodeling the aberrant epigenetic landscape. In 2015, two groups fused dCas9 with the histone demethylase [Kearns et al., 2015] or acetyltransferase p300 [Hilton et al., 2015] to target enhancers or promoters, thus leading to

robust changes in gene expression. Another group targeted dCas9-KRAB to the distal enhancer of DNase I-hypersensitive sites (DHS), resulting in highly specific histone methylation at the enhancers and decreased chromatin accessibility at both the enhancer and its associated promoters [Thakore et al., 2015]. Recently, one team developed a CRISPR-based tool to specifically increase CpG methylation by fusing dCas9 with the catalytic domain of the DNA methyltransferase DNMT3A, enabling methylation of a larger part of promoters in the genomes of mammalian cells [Vojta et al., 2016]. Furthermore, CRISPR-based epigenomic editing has recently been used in high-throughput LOF/GOF screens of regulatory elements in the human genome, serving as a highly scalable platform for epigenomic engineering [Klann et al., 2017]. These studies strengthen the potential of the CRISPR-Cas9 system to target regulatory regions rather than the target gene itself and provide a powerful tool for dissecting the regulatory networks that coordinate gene expression (Fig. 3b).

## LIVE-CELL IMAGING OF GENOMIC LOCI

Beyond gene editing and regulation, another urgent challenge in the field of cell biology is efficiently labeling and tracing specific DNA sequences within live cells, although conventional fluorescence in situ hybridization (FISH) has been developed during the last few decades. To overcome these hurdles, scientists have further repurposed the CRISPR-Cas9 system as a genome imaging tool for imaging live human cells [Chen et al., 2013]. This CRISPR imaging system, consisting of an enhanced green fluorescent protein (EGFP)-tagged dCas9 protein and an optimized sgRNA, has enabled the robust imaging of repetitive elements in both telomeres and protein-coding genes and allowed for the visualization of arbitrary non-repetitive genomic sequences in the human genome (Fig. 3c). Undoubtedly, this new CRISPR-based imaging technology serves as a complementary approach to FISH, allowing simpler and more direct tracking of telomere dynamics, subnuclear localization and chromatin organization throughout the cell cycle. The same system was later reported in live mouse embryonic stem cells [Anton et al., 2014]. Unlike the previous study that mainly focused on detection sensitivity [Chen et al., 2013], Anton et al. [2014] performed a thorough comparison of the CRISPR-based imaging method with the 3D-FISH and immune-labeling methods and explored the potential applications of using the CRISPR-based imaging technology in high resolution microscopy studies. Later that year, Tanenbaum et al. [2014] developed a synthetic SunTag system to recruit up to 24 copies of GFP to a target polypeptide chain. Using a modified dCas9 tagged with the SunTag, the authors created nearly 20-fold brighter fluorescent signals than those resulting from the dCas9 directly fused to GFP, thus providing a magnified fluorescence imaging platform in live cells. Subsequently, dCas9 variants fused with different fluorescent proteins were introduced into human U2OS cells, allowing for the simultaneous detection of multiple colors in genomic loci with high spatial resolution; the distance between two loci on the same chromosome was also mapped using this same strategy [Ma et al., 2015]. In addition, one group achieved imaging of low- and non-repetitive chromosome loci by integrating a sgRNA with up to 16 MS2 motifs to enable robust fluorescent signal amplification [Qin et al., 2017]. Recently, researchers even painted an entire chromosome by introducing hundreds of specific and non-repetitive sgRNAs in a live cell for fluorescence imaging; amazingly, the arrangements of the homologous chromosomes as well as the movement of a particular chromosome in dividing cells could be clearly visualized [Zhou et al., 2017]. Overall, a CRISPR-based imaging tool facilitates modeling spatiotemporal dynamics in different cell stages and enables a panorama of interactions between genomic loci within live human cells.

## TARGETING ENDOGENOUS RNAs

In addition to targeting double-stranded DNA (dsDNA), the CRISPR-Cas9 system has been revolutionarily developed to cleave single-stranded RNA (ssRNA) at specific target sites by providing the PAM as part of an oligonucleotide (PAMmer) that hybridizes to the target RNA (Fig. 3d). This system enables Cas9 to recognize and cleave RNA targets in a programmable fashion and facilitates direct transcript detection [O'Connell et al., 2014]. On the other hand, this system may be a helpful alternative when RNAi cannot be used for RNA degradation [Ghodsizadeh, 2014]. Last year, the same group further developed RNA-targeting Cas9 (RCas9) as a fluorescence-based probe to track RNA in live cells [Nelles et al., 2016] (Fig. 3c). This innovative strategy allows RCas9 to recognize RNA while avoiding the encoding DNA; importantly, the subcellular distribution of RCas9 was highly correlated with RNA-FISH, depicting the ability of RCas9 to track the trafficking and localization of RNA in living cells. This RCas9-based method lays a solid foundation for transcriptome editing by CRISPR-Cas9 and enables scientists to measure diverse transcript types in multiple cell systems and manipulate target RNA, not only by detecting RNA but also by altering RNA modifications [Burgess, 2016]. In addition to the type II CRISPR-Cas9 system, scientists have uncovered the features of type I CRISPR-Cas systems, which comprise approximately 90% of all CRISPR-Cas loci identified in bacteria and archaea, and can target both DNA and RNA [Makarova et al., 2017]. Beyond tracking RNA in live cells, future developments of the RCas9 system, such as the modulation of multiple RNA-processing steps, the generation of disease modeling, and the promotion of clinical translation, deserve further exploration.

## FINAL REMARKS

Although CRISPR-Cas9-based tools have been adopted across numerous fields worldwide, the challenges regarding ethical concerns and clinical applications of CRISPR-Cas9 have been underscored [Olson, 2016]. Successful bench-to-bed translations rely on efficient delivery systems and refined administrative procedures to target human diseases with high specificity. In this respect, applying CRISPR-Cas9 editing to large animal species, such as dogs, pigs, or non-human primates, will help gain better understanding of human diseases and provide a more comprehensive safety report and therapeutic strategies. One should note that adaptive evolutionary changes in pathogens, such as viruses, may develop anti-CRISPR strategies to escape from CRISPR-mediated

attack, resulting in treatment failure. Despite these unaddressed barriers, CRISPR-Cas9-enabled functional research has strengthened our grasp of potential underpinnings of the initiation and development of human diseases. More inspiringly, novel editing tools such as Cas9-fused deaminases [Komor et al., 2016] or Cpf1 nucleases [Zetsche et al., 2015; Kim et al., 2016a] have demonstrated the unlimited possibilities of using CRISPR technology for genome editing, defining an unprecedented path to the next generation of transformative therapies and treatment in the foreseeable future.

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