# Title Page

## Approach for *In vivo* Delivery of CRISPR/Cas System: A Recent Update and Future Prospect

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## Abstract

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system provides a groundbreaking genetic technology that allows scientists to modify genes by targeting specific genomic sites. Due to the relative simplicity and versatility of the CRISPR/Cas system, it has been extensively applied in human genetic research as well as in agricultural applications, such as improving crops. Since the gene editing activity of the CRISPR/Cas system largely depends on the efficiency of introducing the system into cells or tissues, an efficient and specific delivery system is critical for applying CRISPR/Cas technology. However, there are still some hurdles remaining for the translatability of CRISPR/Cas system. In this review, we summarized the approaches used for the delivery of the CRISPR/Cas system in mammals, plants and aquaculture. We further discussed the aspects of delivery that can be improved to elevate the potential for CRISPR/Cas translatability.



Keywords: CRISPR/Cas, Gene Editing, Gene Delivery, Gene therapy

## 1. Introduction

The innovation of gene editing has enabled the precise modification of specific genomic regions in a wide variety of organisms. Gene editing is mainly accomplished by using programmable nucleases that are highly specific. These nucleases create double-strand breaks (DSBs) in regions of interest of the genome. These DSBs are then repaired by nonhomologous end-joining (NHEJ), which is error-prone, or homology-directed repair (HDR), which is error-free; specific changes, such as insertions or deletions (indels), are thus introduced into desired regions of the genome [1-3]. By introducing HDR repair template, the defects in genes may be corrected, thus providing hope for correcting inherent errors in DNA.

Gene expression can be regulated by blocking messenger RNA through RNA interference (RNAi), including small interfering RNAs (siRNAs) and microRNAs (miRNAs), or by homologous recombination [4,5]. A recent new programmable nuclease technology, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas)-type RNA-guided nucleases system [6], has revolutionized the scientific field of gene editing. Although the versatility and ease of construction and target design make CRISPR/Cas extremely attractive for breakthrough gene therapy achievements and crop improvement, there are still important limitations to consider [7,8]. One of the obstacles is the immune response in animal systems; since the components of the CRISPR/Cas system are bacterially derived, this system is expected to trigger host immune responses. Another obstacle is the size of the components in the system, which are all macromolecules; thus, they are unable to spontaneously enter the cytosol and then the nucleus [8], which are essential for successful gene modification [9]. In addition, the large size of the CRISPR/Cas system may also make it difficult to package into delivery vehicles such as viral vectors. Another aspect of difficulty for delivery of the CRISPR/Cas system in mammals is its stability. The CRISPR/Cas system requires to be highly stable and functional; otherwise, it will be degraded or eliminated during circulation in the targeted organs or tissues. Efficient delivery is one of the last major hurdles to overcome in CRISPR/Cas-mediated gene editing. As such, developing stable and effective delivery approaches is critical for its application. In this review, we summarized the approaches used for the delivery of the CRISPR/Cas system in different biological systems, including mammals, aquacultures and plants (**Figure 1**). We also discussed the aspects of delivery that can be improved to elevate the potential translatability the CRISPR/Cas system.

## 2. CRISPR/Cas gene editing system

The CRISPR/Cas system was first identified as a prokaryotic adaptive immune system. It was the first programmable nuclease system that was found to function as ribonucleoprotein particles that utilized base pairing to recognize its targets [10]. This system for gene editing has been widely adopted since it is relatively easy to redesign to produce target specificity. Scientists have engineered and modified this system to allow CRISPR/Cas to act as a successful gene editing tool [6].

There are three key components in the CRISPR/Cas9 system: the tracrRNA, Cas9 protein, and pre-crRNA. The tracrRNA forms a complex with pre-crRNA after transcription. The Cas9 protein stabilizes the complex, and the pre-crRNA is then processed by RNase III to generate crRNA [11]. The Cas9/gRNA (made up of crRNA and tracrRNA) complex recognizes the protospacer adjacent motif (PAM), which is a short motif that is located adjacent to the target DNA sequence [12]. Then, the complex unwinds the target DNA beginning at the seed region (10-12 nucleotides) [13]. When the DNA sequence corresponds to the gRNA, two nuclease domains of Cas9 cleave target strands [14-16]. The Cas9/gRNA complex can tolerate single or sometimes multiple mismatches, with mismatches downstream of the seed region typically being more frequently tolerated [15,17].

There are six types of CRISPR/Cas systems (type I-VI) that are further classified into two classes: the class 1 CRISPR/Cas system and the class 2 CRISPR/Cas system [18,19]. The main feature of the class 1 CRISPR/Cas system, which is subclassified into types I, III, and IV, is that they have multisubunits of effector nuclease complexes. The class 2 CRISPR/Cas system differs from class 1 because it requires only a single effector nuclease; class 2 is subclassified into types II, V, and VI. Their programmable single effector nucleases enable nucleic acid detection and genome engineering [20,21,3,22,23]. Types II, V, and VI are based on Cas9, Cas12, and Cas13 effectors [24-26]. Among them, the CRISPR/Cas9 system is the most commonly used system to date.

## 3. Application of the CRISPR/Cas system for gene editing

### 3.1 Strategies based on Cas nuclease activity

Based on the nuclease activity of CRISPR/Cas, there are various gene editing strategies that have been developed for DNA (gene disruption, precise repair, targeted insertion, large-scale DNA editing) and RNA modification (***Supplementary Table S1***; **Figure 2a and 2b**).

#### 3.1.1 DNA editing

For gene disruption, NHEJ ligates DSBs introduced by Cas endonuclease, and this break and repair pattern takes place repeatedly until the target sequence is altered and an indel occurs [27]. An indel can cause frameshifting or exon skipping and subsequent gene disruption. Gene disruption can also

silence dominant negative mutations by disrupting the mutant allele while preserving the normal allele. In addition, HDR inserts donor template, which has homology arms to match target locus, to the genome and cause a deletion [28].

For precise repair, HDR uses a donor template that has the desired insertion or modification. This donor template has homology arms that enable it to match the target locus and insert the desired genetic material or modify the genome with high precision [27].

For targeted insertion, HDR allows precise insertion of exogenous DNA sequences into the genomes of dividing cells, while homology-independent targeted integration (HITI) allows insertion of exogenous DNA sequences into the genomes of nondividing cells using an NHEJ-based homology-independent strategy [29].

For large-scale DNA editing (editing a size of up to several megabase pairs [Mbp]), DNA fragments can be deleted by introducing the CRISPR/Cas system with two guide RNAs that target different sites. In addition, allelic exchange can correct recessive compound heterozygous mutations. This is achieved by generating homologous DNA breaks in both chromosomes, and the allelic exchange between mutated alleles can rescue the disease phenotype [30].

### 3.1.2 RNA editing

The CRISPR/Cas system acts not only on DNA but also RNA. Previous studies have identified an RNA-targeting CRISPR/Cas effector complex, termed the psiRNA-Cmr protein complex, which comprise prokaryotic silencing (psi)RNAs and Cmr Cas proteins. This complex cleaves target RNAs at a predetermined site, indicating that prokaryotes have their own unique RNA silencing system [31]. Cas endonuclease has also been shown to bind to and cleave ssRNA targets [32]. Strutt *et al*. showed that type II-A and II-C Cas9 endonucleases are capable of recognizing and cleaving ssRNA without a PAM. [33]. Recently, scientists have discovered the RNA-editing Cas13 family. The Cas13 family has been shown to be a programmable RNA-editing CRISPR/Cas system. Compared to other RNA targeting approaches, this system is more specific and efficient [34-36]. Recently, Konermann *et al.* discovered a Cas13d in *Ruminococcus flavefaciens* XPD3002 (CasRx), and it possesses high activity in human cells. CasRx is small, consisting of 930 amino acids, and it can be flexibly packaged into an Adeno-associated virus (AAV), making it suitable for delivery by AAV vectors. In addition to the knockdown activity, catalytically inactivated CasRx can be utilized to regulate pre-mRNA splicing by acting as a splice effector [37].

## 3.2 Strategies based on Cas-effector fusion protein activity

Since CRISPR/Cas possess DNA-binding properties, it may play a crucial role in important applications other than site-specific gene editing. A catalytically dead Cas9 enzyme (dCas9) has been developed to control gene expression [38]. dCas9, like Cas9, is capable of recognizing and binding to a target DNA sequence. However, instead of cleaving the target DNA sequence, dCas9 has been used for transcriptional inactivation, transcriptional activation, introducing epigenetic modifications or base editing. These functions are achieved by fusing dCas9 to gene activators, repressors, acetyltransferases or adenosine deaminases. Since dCas9 is in a catalytic inactive form, it is used here for precise targeting instead of its catalytic activity (***Supplementary Table S2*; Figure 2c and 2d**).

### 3.2.1 Transcriptional regulation

As dCas9 can be fused with transcriptional repressors or activators to target the promoter region of gene interest and result in transcriptional repression (CRISPR interference) or activation (CRISPR activation) without changing the genome; this activity has been demonstrated in *Escherichia coli* as well as in plant and mammalian cells [39,38,40]. In addition, studies have shown that a modifying sgRNA can also enhance the specificity of transcriptional regulation. For example, using the Cas9-VP64 transcriptional activator together with an sgRNA that has two MS2 RNA aptamer hairpin sequences added to it can successfully induce sequence-specific transcriptional activation [41].

The CRISPR/Cas system can also be utilized for epigenetic modification. Hilton *et al.* fused dCas9 with acetyltransferase that catalyze the acetylation of histone H3 at lysine 27. This modulation has been shown to strongly activate specific gene expression. Not only acetylation but also methylation may be accomplished using this approach [42].

### 3.2.2 Base and prime editing

CRISPR/dCas has been utilized for precise DNA and RNA editing. The CRISPR/nickase Cas9 (nCas9)-based base editor was first developed by Komor *et al.* and was used to convert a targeted C-G base pair to T-A by a DNA cytosine deaminase [43]. Gaudelli *et al.* subsequently developed a transfer RNA adenosine deaminase that, when fused to nCas9, can convert A-T base pairs to G-C base pairs [44]. This kind of CRISPR/Cas-mediated editing is powerful since single point mutations are accounted for a large category of genetic diseases.

Recently, a more powerful and versatile gene-editing method, prime editing, was discovered as a way to introduce indels and enable base conversions in both transitions and transversions [45]. The editor used in prime editing is termed prime editor. The prime editor is composed of nCas9 fused with reverse transcriptase. The prime editor is guided by a prime editing gRNA (pegRNA). After

nCas9 nicks the target site, the pegRNA binds to a single strand DNA (ssDNA) and initiates reverse transcription. The reverse transcribed pegRNA is then incorporated into the target site.

## 4. Current approaches for delivering the CRISPR/Cas system in mammals

CRISPR/Cas can be delivered using different modalities, including DNA, mRNA and protein. When it is delivered in a DNA mode, Cas and gRNA are delivered as a single plasmid. For the mRNA mode, Cas mRNA is delivered with a separate gRNA. For the protein mode, Cas protein is delivered with gRNA as a ribonucleoprotein complex (RNP). Each mode exhibits overall effectiveness but also includes some limitations. Packaging Cas9 and gRNA in the same plasmid makes the delivered cargo more stable than that of other methods; however, the large size of the plasmid increases the difficulty of delivery, and the integration of plasmids into the host genome and prolonged expression are potential limitations of this delivery method. Delivery of Cas mRNA enables faster gene editing; however, RNA is fragile, and the degradation of gRNA may initiate before Cas9 mRNA is successfully translated. The RNP is the most direct and fastest mode for gene editing. However, compared to plasmids or mRNAs, it is much more challenging to obtain a pure protein. In addition, the sudden introduction of bacterial proteins may induce an immune response in the host.

The delivery vehicles can be separated into two groups: viral and nonviral vectors. For *in vivo* delivery of CRISPR/Cas, viral vectors are the preferred vehicle. To date, nonviral vector delivery has not been as commonly used as viral-based delivery. However, nonviral vectors are comparable to viral vectors and are a topic of intense research. The delivery vehicles for the *in vivo* CRISPR/Cas system discussed below are summarized in **Figure 3 and Table 1**.

### 4.1 Viral-based CRISPR/Cas gene editing and delivery

Viral vectors are commonly used vehicles for introducing gene editing materials such as DNA. Lentivirus, adenovirus, and adeno-associated virus (AAV) are three major types of viral vectors widely used for the gene delivery of CRISPR/Cas system. Though viral delivery has high efficiency *in vivo*, there are some disadvantages, including safety issues. These viruses work by releasing the viral genome into host cells after infection. This means that the interactions between the virus and host cells must be strong; thus, viral delivery methods are more complicated than most of the nonviral methods under *in vivo* conditions.

#### 4.1.1 Lentivirus

Lentiviruses are RNA viruses with the capability to integrating into dividing and nondividing cells. Lentiviruses are an excellent delivery vehicle for cells that are hard to transfect by chemical methods. Furthermore, it has a large packaging capacity of ~10.7 kb [46]. This property allows it to carry multiple sgRNA sequences that can induce multiple gene editing at once [47]. Due to these advantages, lentiviruses have been used in many initial gene editing studies. Mouse models of myeloid malignancy [48] and lung cancer [49] have been generated using lentivirus delivery. However, there are some disadvantages of using lentivirus, including the integration of the viral genome, which may cause cancer via insertional activation of proto-oncogene [50].

To overcome these issues, lentiviral vectors have been further developed into integration-deficient lentiviral vectors (IDLVs) to reduce the undesired integration of the viral genome into the host cell genome [51,52]. IDLVs retain the property of being able to edit genes in hard-to-transfect cells [53,54]. Although IDLVs have been found to cause unwanted gene modifications, the study also showed that IDLVs have effective site-specific gene repair activity due to their active recruitment of host HDR proteins [55]. Therefore, pairing IDLVs with safer endonucleases such as SpCas9-HF or eSpCas9 may improve its application [56,57].

#### 4.1.2 Adenovirus

Adenoviruses are double-stranded DNA (dsDNA) viruses. Similar to a lentivirus, an adenovirus can infect both dividing and nondividing cells. However, since they do not generally induce genome integration in the host DNA, adenoviruses do not cause a potential off-target effects the way a lentivirus does. It has been shown that adenovirus-based delivery of the CRISPR/Cas system can result in the efficient editing of the *proprotein convertase subtilisin/kexin type 9* (*Pcsk9*) and *Pten* genes in adult mouse liver [58,59]. Moreover, adenovirus-based delivery also has been successfully used to induce specific chromosomal rearrangements to generate echinoderm microtubule-associated protein like 4-anaplastic lymphoma kinase (EML4-ALK)-driven lung cancer *in vivo* [60]. However, adenoviruses can elicit a significant immune response. Adenoviruses are also costly and difficult to produce in high volumes. These shortcomings set a limit for the applications of adenovirus-mediated delivery in clinical gene therapy [58].

#### 4.1.3 Adeno-Associated Virus

AAVs are small ssDNA viruses. Compared to lentivirus- and adenovirus-based delivery, AAV-based delivery is safe and efficient since it results in only minor cytotoxicity and immune responses [61,62]. Since AAVs have a wide range of serotypes, which helps to achieve a broad range of tissue tropisms

and used for efficient gene editing [63]. For example, Swiech *et al.* reported a first successful AAV-based CRISPR/Cas9 gene editing in the mouse brain [64]. A similar approach was used by Hung *et al.* for retinal gene editing and achieved high editing effects in the adult mouse retina [65]. Studies have also demonstrated successful AAV-based CRISPR/Cas9 gene editing in the retina of retinal degeneration mouse model [66,67]. AAV-based delivery of CRISPR/Cas components has also been used to knockdown IGF in the central nervous system [68]. In addition, studies have also demonstrated that muscle tissue-specific delivery of CRISPR/Cas components using AAV vectors can correct the mutated dystrophin gene in Duchenne muscular dystrophy (DMD), and functional recovery was observed *in vivo* [69,70,63,71]. Zhang et al. recently demonstrated improved CRISPR-Cas9–mediated gene editing efficiency in DMD mouse model using self-complementary AAV (scAAV) system [72]. AAV-based delivery of CRISPR/Cas9 has also been used to achieve effective gene correction in metabolic liver disease in newborn mice [73,74] . Moreover, delivery of sgRNAs using AAVs in a tissue-specific SpCas9 transgenic mouse can be employed to generate the disease animal model such as cardiomyopathy [75] and lung adenocarcinoma [76]. Also, Murlidharan et al. uses AAV chimeric (AAV2g9) to deliver gRNAs targeting the schizophrenia risk gene MIR137 into the brain of CRISPR/Cas9 knock-in mouse model to achieve brain-specific gene deletion [77]. Furthermore, delivery of sgRNAs using AAVs into CRISPR/Cas9 knock-in mice can be used to perform high-throughput mutagenesis to generate autochthonous mouse model of cancer [78,79]. Despite progress in using AAVs for CRISPR/Cas-based gene editing, the small cargo capacity (<4.7 kb) of AAVs can limit its application. Thus, when combining conventional SpCas9, which has a size of 4.2 kb, with the addition of sgRNA, another vector system is usually required. Later on, several smaller Cas9 orthologs (such as *Staphylococcus aureus* (SaCas9) [80], *Campylobacter jejuni* (CjCas9) [81], *Streptococcus thermophilus* (StCas9) [82] and *Neisseria meningitidis* (NmCas9) [81]) were developed by scientists to enable the *in vivo* gene editing by a single AAV vector.

### 4.2 Nonviral-based CRISPR/Cas gene editing and delivery

#### 4.2.1 DNA-based delivery

DNA-based delivery is commonly used for introducing the CRISPR/Cas system into cells because it is more stable than mRNA. CRISPR/Cas-encoding DNA facilitates greater gene editing efficiency than other methods [83-85]. For example, the CRISPR/Cas9 components were delivered in the form of DNA by tail-vein hydrodynamic injection to a mouse model of tyrosinemia and achieved >6% gene correction in the liver cells after a single application [86]. Furthermore, Zhen et al also reported that hydrodynamic injection of CRISPR/Cas9-encoding DNA can effectively disable the hepatitis B virus replication by creating mutations in virus DNA [87]. Apart from systemic administration, subretinal

injection of CRISPR/Cas components in a plasmid form in combination with electroporation has also been reported to enable an allele-specific gene editing in the retina of a rat model of retinitis pigmentosa [88]. A similar effect also found by Latella *et al.* in a mouse model of retinitis pigmentosa, which significantly reduced mutated protein levels and prevented major visual dysfunction [89]. In addition, Li et al. demonstrated an allele-specific gene editing in the retinas of Rho-P23H knock-in mice which selectively targeting the P23H allele that has a single-nucleotide mutation [90]. Moreover, Shinmyo *et al.* introduced a plasmid containing CRISPR/Cas components into the mouse brain using *in utero* electroporation for effective brain-specific gene editing *in vivo* [91]. These works demonstrated the applicability of DNA-based delivery of CRISPR/Cas9 *in vivo*.

**4.2.2 mRNA-based delivery**

mRNA-based delivery methods largely decrease the risk of host genome integration. However, the effective time of mRNA-based delivery methods is relatively fast, and there are some additional shortcomings of such delivery methods. For example, the stability of mRNA, and the need to deliver the components (Cas mRNA and sgRNA) separately are the two main concerns of this method. Yin *et al.* demonstrated a delivery method that utilized different vehicles for introduction of the CRISPR/Cas9 components, lipid nanoparticles delivered the Cas9 mRNA, and an AAV delivered the sgRNA/HDR template. By utilized this strategy, they showed an efficient correction of the *Fah* (*fumarylacetoacetate hydrolase*) gene in a mouse model of hereditary tyrosinemia [92]. However, it is important to note that this combination approach still requires viral codelivery to achieve certain efficacy, and compared to DNA and protein, RNA is unstable. Moreover, the degradation of sgRNA may significantly affect editing efficiency. Future research into increasing sgRNA stability is required to improve the efficiency of these methods. Studies have showed that modifying sgRNA has beneficial effects on the stability of sgRNA. Yin *et al.* modified sgRNA by switching the 2'OH group of RNA to 2'OMe and 2'F and added phosphorothioate bonds [93]. This study reported that a single injection induced more than 80% efficiency in editing *Pcsk9* in the livers of mice, demonstrating a potential modified method for improving the stability of mRNA in order to overcome the obstacles of mRNA-based delivery. In addition, other researchers reported a similar study in which modified sgRNA and Cas9-encoding mRNA were packaged into a lipid nanoparticle vehicle. With a single administration, a more than 97% reduction in the mouse *transthyretin* (*Ttr*) gene was shown in the serum protein levels of the liver. This study demonstrated efficient gene editing that could persist for at least 1 year [94]. Another study has also demonstrated a high editing efficacy (~80%) by unitizing a lipid nanoparticle with disulfide bonds (BAMEA-O16B) to deliver Cas9 mRNA and sgRNA *in vivo* [95].

### 4.2.3 Protein-based delivery

Delivering Cas protein with gRNA as a Cas9 RNP is the fastest and most direct pathway for gene editing, and it is suitable for *in vivo* therapeutic applications. To facilitate the delivery of Cas9 RNPs into target cells, a fusion protein of Cas9 and negatively supercharged proteins was created to enable the delivery by cationic lipid formulated transfection reagents such as RNAiMAX [96]. Delivery of the Cas9 RNP/RNAiMAX complex via injection into the cochlea of transgenic *Atoh1* (*Atonal BHLH Transcription Factor 1*)-*GFP* mice caused a 13% reduction in GFP in the ears of the transgenic mice. Mangeot *et al.* designed a vector based on murine leukemia virus (MLV), termed nanoblades, to deliver Cas9 RNPs for *in vivo* gene editing [97]. Moreover, an amphiphilic nanocomplex has also been developed to deliver Cas9 RNPs *in vivo* and showed effective gene editing in the brain of the mouse model of Alzheimer's disease [98]. Furthermore, to enhance endosomal escape, PEI polymers or combined PEI polymers with liposomes was used for Cas9 RNP delivery *in vivo*. Sun *et al.* coated a DNA nanoclew with PEI polymers to deliver Cas9 RNPs into the nuclei of human cells. By using this vehicle, target gene disruption can be achieved with negatively impacting cell viability [99]. The study also noted that the modification of DNA nanoclew to partially complementary with the sgRNA can further enhance the editing efficacy. In addition, the modification of Cas9 protein can also improve the efficacy of direct cytoplasmic/nuclear delivery of Cas9 RNP. Mout *et al*. developed the Cas9En protein, in which the N-terminus of Cas9 protein has an attached oligo glutamic acid tag that is negatively charged [100]. Cas9En RNPs were delivered using arginine-functionalized gold nanoparticles (Arg-AuNPs), which are positively charged. With the NLS attached, Cas9 RNPs were delivered directly to the cytosol, accumulated in the nucleus and provided ~30% editing efficiency. Recently, this nano-assembled platform has been used for Cas9 RNP delivery *in vivo* and achieved >8% gene editing efficiency [101].

AuNPs have also been used to deliver Cas9 RNPs *in vivo* for gene editing and correction in the disease models. AuNPs can be conjugated with donor DNA, Cas9 RNPs and the endosomal disruptive polymer PAsp (diethylenetriamine, DET) to form a vehicle termed CRISPR-Gold. Lee *et al*. reported that CRISPR-Gold-based Cas9 RNPs delivery can achieve 5.4% correction of the dystrophin gene in the muscle tissue of DMD mice [102]. Another study also showed that intracranial injection of CRISPR-Gold in the brain rescued mice from abnormal behaviors caused by fragile X syndrome [103]. CRISPR-Gold may offer the opportunity in the development of therapeutic approaches targeting the muscle and brain diseases, while effective endosomal escape is still required for higher delivery efficiency.

Overall, protein-based delivery offers reduced off-target effects and a low immune response compared to DNA and mRNA-based delivery [104]. Cas9 RNPs increase efficacy by avoiding the

degradation of sgRNA. However, transport of Cas9 RNPs into the cytosol or the nucleus is critical for therapeutic effects. Thus, endosomal entrapment is still a crucial obstacle to overcome [105].

## 5. Current approach of delivering the CRISPR/Cas system in aquaculture

Genomes of several aquaculture species, including Salmonidae, Nile tilapia, gilthead seabream, Siluridae, Cyprinidae, Northern Chinese Lamprey and Pacific oyster, have been successfully modified with the CRISPR/Cas system (**Table 2**). CRISPR/Cas protocols developed in model species such as zebrafish have been followed for gene editing in aquaculture species [106]. The standard gene transfer method used in aquaculture species is microinjection. Microinjection is performed using special equipment to inject the CRISPR/Cas complex into newly fertilized eggs; this method has high gene editing efficiency [107]. In most cases, NHEJ was used to induce mutations, while HDR has been successfully used in Rohu carp [108]. However, if gene editing continues at different stages of embryonic development, mosaicism could occur. These concerns are the focus of current research, which aim to enable more widespread adoption of CRISPR/Cas techniques in aquaculture. CRISPR/Cas techniques have been used to address characteristics such as sterility, growth and disease resistance of aquaculture species. The reason for inducing sterility in fish is to preserve the domesticated strains by preventing gene flow. For example, CRISPR/Cas techniques have been used to induce sterility in Atlantic salmon [109]. Several papers have demonstrated gene editing of the myostatin gene using the CRISPR/Cas approach to enhance the growth of fish, including channel catfish and common carp [110,111]. The CRISPR/Cas approach has also been used to investigate immunity and disease resistance in channel catfish, Rohu carp and grass carp [112,108,113]. Disruption of the *TLR22* gene in Rohu carp resulted in a model for studying immunology, demonstrating the capability of CRISPR/Cas to aid in the development of effective treatments for aquaculture. By understanding the underlying pathways of transcription and translation through CRISPR/Cas-based mechanisms, it is possible to strengthen disease resistance, decrease disease incidence, and improve species resilience in aquaculture. Aquaculture is highly suited for the application of CRISPR/Cas gene editing for numerous reasons. Sample sizes can be large without generating cumbersome costs; thousands of externally fertilized embryos enable microinjection by hand. The large sample size is impartial and useful for comparisons of successfully edited samples with controls and for the assessment of pathogen resistance. Furthermore, a large sample size enables the development of well-developed disease challenge models since extensive phenotypes are practical. With the technology becoming mature in aquaculture species, it is

becoming easier to study gene function, improve disease resistance, and generate new strains with selected characteristics that can improve economic value.

## 6. Current approach of delivering the CRISPR/Cas system in plants

As shown above, the CRISPR/Cas system is highly adept at modifying animal genomes. Studies have also demonstrated its ability to modify plant genomes. Conventionally, mixed dual promoter system is used to express CRISPR/Cas system in plants. In mixed dual promoter system, RNA polymerase II promoters are used to express Cas protein and RNA polymerase III promoters specifically expressed in plants, such as AtU6 for Arabidopsis or tomato, TaU6 for wheat, and OsU6 or OsU3 for rice, are used to express gRNA [114-117]. However, to utilize CRISPR/Cas9 technology in creating new traits in plants, efficient delivery of the CRISPR/Cas system into cells is essential. The two delivery methods utilized in plants are indirect and direct methods. Indirect methods (such as agroinfiltration, agroinfection, and viral infection/agroinfection) use plant bacteria or viruses to mediate the introduction of DNA constructs into target plant cells. By contrast, no biological organisms are used as mediators for direct delivery. Protoplast transfection and biolistic particle delivery are the most commonly used direct methods. Agroinfiltration is usually used as a transient assay and has been widely used for its versatility and simplicity [118-122]. Agroinfection, biolistic particle delivery and viral infection are usually used for stable editing. Protoplast transfection can be used for both transient and stable editing. The delivery methods used in plant gene editing (**Figure 4** and **Table 3**) will be summarized in the following sections.

### 6.1 Transient events

#### 6.1.1 Indirect method

***Agroinfiltration.*** Agrobacterium spp. are plant pathogens. When infecting plants, *Agrobacterium tumefaciens* causes tumor-like growth on aerial parts of the plant (crown gall), while *Agrobacterium rhizogenes* induces root tumors. Agrobacteria contain a large plasmid (exceeding 200 kb), which is named Ti in the case of *A. tumefaciens* or Ri in the case of *A. rhizogenes*, and it can transfer a specific DNA segment (transfer DNA or T-DNA) into the infected plant cells, enabling the T-DNA to integrate into the host genome. These two strains of agrobacterium have been modified to contain a disarmed Ti/Ri plasmid where tumor-inducing genes have been deleted. The essential parts of the T-DNA, border repeats (25 bp), are needed for plant transformation and are used to generate transgenic plants.

Agroinfiltration is a transient assay in which an *A. tumefaciens* culture containing modified T-DNA is directly injected into plant leaves (**Figure 4a**) [123-125]. For root hair transformation, *A. rhizogenes* is specifically used to evaluate editing efficiency in plant root hairs, and this method has mainly been used in legume species such as Medicago and soybean [126-128].

### 6.1.2 Direct method

***Protoplast transfection.*** A method for transfection and transient assays is protoplast transfection. This method enzymatically digests the cell walls of plant tissues and uses PEG for transfection or electroporation for delivery (**Figure 4b**). The same protoplasts can deliver several DNA constructs. Protoplast transfection has been proven to successfully deliver the CRISPR/Cas system and result in gene editing in *Arabidopsis thaliana, Nicotiana benthamiana*, rice, wheat, and maize, among others [129-131,115,132-134].

## 6.2 Stable events

### 6.2.1 Indirect method

***Agroinfection.*** Agrobacterium-mediated DNA delivery is the most commonly used method for almost all model plant species, main crop species, vegetable and fruit crops and forest crops. Similar to agroinfiltration, Agrobacterium can also create transgenic plants by genome integration in the plant nuclear DNA [135] (**Figure 4c**).

***Viral infection.*** The first viral vector used in plants was tobacco mosaic virus (TMV). Researchers used TMV to silence a gene in *N. benthamiana* [136]. The majority of plant viruses are RNA viruses whose genomes are ssRNAs, they can be synthesized *in vitro* and used to inoculate plants, or they can be synthesized *in vivo* as DNA viruses from a plasmid introduced directly to plants by mechanical means for gene delivery [137]. To accelerate the delivery process, the viral genome can be inserted as a cDNA fragment into a binary vector and then can be used for agroinfection-mediated delivery into a plant cell (**Figure 4d**).

Tobacco rattle virus (TRV) is an ssRNA virus that has two genome components, TRV1 (or RNA1) and TRV2 (or RNA2). Both genome components are required for inoculation. Plants edited using RNA viruses do not exhibit germline transmission of edits. For instance, Ali *et al.* used agroinfection to deliver the RNA1 genomic component of TRV and a vector derived from TRV RNA2 containing targeting gRNA into the leaves of *N. benthamiana* overexpressing Cas9 for gene editing in plant cells [138].

Geminiviruses, unlike TRV, do not require *in vitro* transcription prior to inoculation. Geminiviruses have a circular ssDNA genome [139]. Geminiviruses do not have a gene encoding DNA polymerase; therefore, their ssDNA genomes are converted into dsDNA genomes by host DNA polymerases in the nucleus. The dsDNA genome is then used as a template for virus transcription and rolling circle replication. Replication initiator protein (Rep) is essential for the initiation of rolling-circle replication. Rolling circle replication can either convert ssDNA genomes into dsDNA genomes or package ssDNA genomes into virions. Plant plasmodesmata pathways facilitate the transport of virions to adjacent cells [140,141]. Bean yellow dwarf virus (BeYDV), which is a geminivirus, has been used to deliver the CRISPR/Cas system [140]. Studies have demonstrated gene editing using BeYDV in tomato (*anthocyanin mutant 1* gene, *ANT1*), and a modified cabbage leaf curl virus (CaLCuV) has been used in tobacco [129,142]. Such approaches have also been applied in wheat, and researchers have enhanced the efficiency of this method by developing an optimized wheat dwarf virus (WDV) system [143].

### 6.2.2 Direct method

***Protoplast transfection.*** Unlike the transient method of protoplast transfection, the stable transformation method generated targeted genome modifications in whole plants that were regenerated from genome-edited protoplasts [132,133]. Two advantages of protoplast transfection are the ability to deliver multiple components and to do so at a high quantity. This method is highly suitable for gene editing using donor template repair. A high quantity of transfected cells can promote the recovery of gene editing via donor template repair. However, a disadvantage of protoplast transfection is the rate of plant regeneration in monocot plants. Protoplast transfection has been used for gene editing in potato [144], tobacco, and lettuce [133].

***Biolistic particle delivery.*** Biolistic particle delivery is accomplished by transfecting cells via bombardment. Gene guns can penetrate the cell wall of plant cells with physical force to deliver DNA (**Figure 4e**). This method is common in transforming plants due to its efficiency and its ability to deliver multiple DNA constructs simultaneously [145]. Most importantly, there is no plant species restriction to biolistic particle-based delivery. The main disadvantage of this method is that by introducing multiple copies of the DNA in the target plants, undesired effects such as gene suppression might occur in the recovered transgenic plants. Biolistic particle delivery has been used for gene editing in rice and wheat, soybean and maize using the CRISPR/Cas system [146-148]. In addition, this method is also used for efficient gene editing using CRISPR/Cas9 RNPs in crops, such as hexaploid wheat and maize [149,150].

## 7. Future prospects in CRISPR/Cas delivery

The CRISPR/Cas system is simple but versatile. The CRISPR/Cas system has great potential for gene editing, but the delivery of CRISPR/Cas into cells dramatically impacts editing efficiency. There are still some aspects of delivery that can be improved to elevate the potential for translatability.

### 7.1 Immunity to the CRISPR/Cas system and its delivery vehicle

It is known that the Cas gene must be delivered into cells to express the Cas protein, and the long-term and robust expression of bacterially derived protein is expected to activate the host immune system. One solution to this problem is to use a protein-based delivery of the CRISPR/Cas system, which may have less immunogenicity, as the Cas protein would only be present in the target cell for a short period of time [100]. When combined with immunogenic effects caused by certain delivery vehicles, the level of immunogenicity might make negligible the efficiency of the CRISPR/Cas system. It has been reported that exogenous RNA delivered by lipid nanoparticles might activate Toll-like receptors and subsequent immune responses [151]. Therefore, the type of delivery vector should be carefully chosen. Moreover, it is especially important to consider the side effects of viral vectors. When compared to lentiviruses, AAVs and adenoviruses can avoid the risk of undesired DNA integration into the host genome. Producing viral DNA or protein within the cells of host can generate a risk of for clinical applications [152,153].

### 7.2 Engineered biomaterials in improving the delivery efficiency

Among the delivery vectors, the most suitable vectors for *in vivo* delivery may be nonviral vectors rather than viral vectors. Nonviral delivery, compared with viral delivery, exhibits potential advantages. It reduces the risk of off-target effects by decreasing the expression period of nuclease and enables better control of dosing duration [92]. The emergence and development of nanotechnology and material sciences have produced versatile applications in gene editing. It has been shown that gold-based nanoparticles enable effective delivery of RNP both *in vitro and in vivo* [102]. In addition, polymeric-based and lipid-based nanoparticles exhibit low immunogenicity, especially in their ability to encapsulate large cargos [154]. Additionally, it has been demonstrated recently that PEI-magnetic nanoparticles can improve the delivery of CRISPR/Cas9 constructs *in vitro* with low cell toxicity and have been shown to be a promising delivery system that can improve the safety and utility of gene editing [155,156]. Moreover, researchers have demonstrated the delivery of the Cas9 RNP complex directly into cells using the nanoneedle array system and showed approximately 32% and 16% gene disruption efficiencies in HeLa cells and mouse breast cancer cells, respectively. Although the

efficiency needs to be improved, researchers were able to successfully demonstrate gene editing by the direct delivery of Cas9/sgRNA using a nanoneedle array, and this method of delivery may be applied to gene knock-in via HDR [157]. Recently, Chen *et al*. demonstrated a platform comprised of vertically aligned silicon nanotube (VA-SiNT) arrays for gene editing. They successfully delivered Cas9 RNP to the target gene and demonstrated more than 80% efficiency of SiNT-facilitated biocargo internalization. This indicated that the NT-facilitated molecular delivery platform has great potential to propel gene editing technologies [158]. However, nanoparticle-mediated protein delivery still has challenges, including the difficult process of packaging into small particles and the prevention of RNP degradation before it enters the nucleus. Therefore, biocompatible, well-tolerated, high capability and nonimmunogenic delivery vehicles are required to deliver cargos to the nucleus for effective gene editing, and these characteristics are essential when designing any nonviral delivery material.

### 7.3 Spatial and temporal regulation of Cas9 activity

As previously discussed, the unintended off-target effect of the CRISPR/Cas system is a major concern. Regulating delivery of the components of the CRISPR/Cas system to specific target sites before Cas9 is turned on and delivery of certain factors that switch on this machinery at a specific time point is critical. A number of teams have identified Cas9 endonuclease inhibitors. These anti-CRISPR (Acr) proteins, such as AcrIIA4, can shut off Cas9 activity [159,160]. Moreover, anti-CRISPRs could be used to limit editing activity to particular cells and tissues in the body. Researchers designed miRNA-responsive Acr switches, and delivery of this machinery with Cas9 or dCas9 enabled tissue-specific editing [161]. In a recent study, researchers generated Cas9 variants called ProCas9s that enabled the CRISPR/Cas9 system to be turned on only in target cells [162]. ProCas9 senses the type of cell it is in based on proteases. This machinery enables the safer translational application of CRISPR/Cas9 gene editing, and this technology could be used to help plants defend against viral pathogens.

Several strategies to control the activity or expression of Cas9 have also been demonstrated (**Table 4**). It has been reported that Cas9 can be expressed in a split [163-166] or inactive form [167,168]. In addition, an inducible system enabled Cas9 to be activated only when stimulated by a chemical inducer [169-173] or by exposure to certain types of light [166]. Studies have engineered a split-Cas9 system in which the activity of Cas9 is induced only when the two domains, recognition domain and nuclease domain, are assembled [174]. This split-Cas9 system is also utilized for gene editing using inteins. Inteins are protein introns that excise themselves out of host polypeptides to generate a functional protein [175]. The intein-based split-Cas9 system is composed of the split Cas9 domains, each of which is fused to intein sequences. Upon dimerization, these intein sequences will be spliced out, and fully active Cas9 can be generated [163]. Truong *et al*. demonstrated that Cas9

543 domains can be delivered by AAV vectors separately and retain comparable editing efficiencies as
544 full-length Cas9 [163]. Cas9 can also be chemically inducible by exposure to rapamycin, which
545 induces FK506-binding protein (FKBP)-FKBP Rapamycin binding (FRB) dimerization [176].
546 Rapamycin-inducible split-Cas9 is composed of split Cas9 fragments each fused with FRB and FKBP
547 fragments. In the presence of rapamycin, a fully active Cas9 is formed. Researchers have also
548 demonstrated a photoactivatable Cas9 (paCas9) system that utilized photoinducible dimerizing protein
549 domains termed Magnets [166]. This optically controlled split-Cas9 system was generated by fusing
550 each Cas9 fragment with Magnet fragments (pMagnet and nMagnet) and triggering Magnet
551 dimerization upon blue light treatment [177]. Several other optically controlled systems have also been
552 reported to enable CRISPR/Cas-based transcriptional activation and gene editing [178,177,179,180].
553 Nihongaki *et al*. developed a light-inducible system. They fused integrin binding protein 1 (CIB1) with
554 dCas9 and fused cryptochrome 2 (CRY2) with a transcriptional activator domain, and then they used
555 blue light to trigger dimerization of CIB1 and CRY2, resulting in subsequent expression of
556 downstream targets [177]. Shao *et al*. developed a optogenetic far-red light (FRL)-activated
557 CRISPR/dCas9 effector (FACE) system based on dCas9 [181-183] and the bacterial phytochrome
558 BphS [184] that induced transcription of target genes in the presence of FRL [180].
559 Other strategies can also enable tunable regulation of CRISPR/Cas9 systems. Wandless and
560 colleagues used small cell-permeable molecules to regulate protein stability. This chemical-genetic
561 approach allowed rapid and tunable expression of a specific protein by fusing the molecules to a
562 destabilizing domain [185]. The destabilizing domain acts as a degron that directs the fusion protein
563 to proteasome-dependent degradation without the presence of a small molecule ligand, which allows
564 tunable control of protein function. Ligand binding to the destabilizing domain protects the fusion
565 protein from degradation and allows the protein of interest to function normally. Thus far, several
566 ligand-destabilizing domain pairs have been discovered, including Shield-1 with mutant K506-binding
567 protein (FKBP) 12 (FKBP[DD]), trimethoprim with mutant DHFR (DHFR[DD]), and CMP8 with the
568 4-OHT-estrogen receptor destabilized domain (ER50[DD]) [185-187]. This concept can be utilized
569 for switchable gene editing and activation [188-190]. FKBP[DD], DHFR[DD] and ER50[DD] were
570 fused to Cas9 for drug inducible gene editing [189,190]. DHFR[DD] or ER50[DD] were fused to PP7-
571 activation domain [181], and DHFR[DD] can be fused directly to dCas9 activator [188] for drug
572 inducible gene activation. Multidimensional control can be achieved by pairing different ligand-
573 destabilizing domain pairs with different aptamers [189]. Another platform utilizes the hepatitis C
574 virus (HCV) nonstructural protein 3 (NS3) protease domain and its various inhibitors and has also
575 been used to regulate CRISPR/Cas activity [191]. Tague *et al*. integrated the NS3 protease domain and
576 its inhibitor into dCas9-VPR to form a ligand-inducible platform [191,192]. The NS3 protease domain

was inserted between the DNA binding scaffold and the C-terminal region, which is where NLS and VPR are located, to form a dCas9-NS3-NLS-VPR complex. NS3 protease, a self-cleaving proteinase, can separate VPR from dCas9 and subsequently inhibit transcriptional activation, while in the presence of protease inhibitor, transcriptional activation is achieved. Recently, Cas9 has been fused with small molecule-assisted shut-off tag (SMASh), which consists of the HCV NS3 and nonstructural protein 4a (NS4A, acting as a degron). Cas9 stability can be controlled by SMASh via asunaprevir, an HCV protease inhibitor. Cas9 protein is degraded when NS3-NS4A is inhibited in the presence of asunaprevir, while in the absence of asunaprevir, the gene editing activity of Cas9 was restored [193].

Unfortunately, there are still some obstacles to progressing with the application of the regulatory approach to the CRISPR/Cas system. Chemical inducers may elicit cytotoxicity, which would make application of this approach *in vivo* more difficult. Additionally, light-induced systems may be limited to *in vitro* studies since activating such a system with light *in vivo* would be invasive, and penetration of light into tissue may cause other problems. Further investigation, optimization and development are needed to overcome these challenges to advance the clinical translation of the CRISPR/Cas system.

## 8. Conclusion

The discovery and application of the CRISPR/Cas system offers great hope for the human disease treatment as well as revolutionize plant breeding. Although research on the CRISPR/Cas system in the life sciences community is well underway, there are still substantial barriers to efficient delivery that need to be overcome to achieve effective gene editing. Factors related to specificity, efficacy and regulatable expression are important to consider when selecting an approach. The development of new delivery methods has overcome many disadvantages that severely impede the translatability of the CRISPR/Cas system. With the rapid development of delivery methods, the successful translation of CRISPR/Cas technology into medical and agricultural applications is imperative and major improvements can be anticipated.

# 9. Declarations

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## 9.2 Conflicts of interest/Competing interests

The authors report no conflict of interest.

## 9.3 Ethics Approval

Not applicable

## 9.4 Consent to participate

Not applicable

## 9.5 Consent for publication

Not applicable

## 9.6 Availability of data and material

All data relevant to the study are available upon reasonable request from the corresponding author.

## 9.7 Code availability

Not applicable

## 9.8 Author contributions

Conceptualization- Y.F.C. and G.S.L. Writing (Original Draft)- Y.F.C. and G.S.L. Writing (Review & Editing)- F.L.L., A.J.P., V.H. A.W.H. and P.Y.W. Visualization, Y.F.C., A.J.P. and G.S.L. Funding Acquisition- P.Y.W. and G.S.L.

# References


1. Gaj T, Gersbach CA, Barbas CF, 3rd (2013) ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol 31 (7):397-405. doi:10.1016/j.tibtech.2013.04.004
2. Sander JD, Joung JK (2014) CRISPR-Cas systems for editing, regulating and targeting genomes. Nat Biotechnol 32 (4):347-355. doi:10.1038/nbt.2842
3. Cox DB, Platt RJ, Zhang F (2015) Therapeutic genome editing: prospects and challenges. Nat Med 21 (2):121-131. doi:10.1038/nm.3793
4. Davidson BL, McCray PB, Jr. (2011) Current prospects for RNA interference-based therapies. Nat Rev Genet 12 (5):329-340. doi:10.1038/nrg2968
5. Im W, Moon J, Kim M (2016) Applications of CRISPR/Cas9 for Gene Editing in Hereditary Movement Disorders. J Mov Disord 9 (3):136-143. doi:10.14802/jmd.16029
6. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337 (6096):816-821. doi:10.1126/science.1225829
7. Uddin F, Rudin CM, Sen T (2020) CRISPR Gene Therapy: Applications, Limitations, and Implications for the Future. Front Oncol 10:1387. doi:10.3389/fonc.2020.01387
8. Belting M, Sandgren S, Wittrup A (2005) Nuclear delivery of macromolecules: barriers and carriers. Adv Drug Deliv Rev 57 (4):505-527. doi:10.1016/j.addr.2004.10.004
9. Knight SC, Xie L, Deng W, Guglielmi B, Witkowsky LB, Bosanac L, Zhang ET, El Beheiry M, Masson JB, Dahan M, Liu Z, Doudna JA, Tjian R (2015) Dynamics of CRISPR-Cas9 genome interrogation in living cells. Science 350 (6262):823-826. doi:10.1126/science.aac6572
10. Jore MM, Lundgren M, van Duijn E, Bultema JB, Westra ER, Waghmare SP, Wiedenheft B, Pul U, Wurm R, Wagner R, Beijer MR, Barendregt A, Zhou K, Snijders AP, Dickman MJ, Doudna JA, Boekema EJ, Heck AJ, van der Oost J, Brouns SJ (2011) Structural basis for CRISPR RNA-guided DNA recognition by Cascade. Nat Struct Mol Biol 18 (5):529-536. doi:10.1038/nsmb.2019
11. Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, Eckert MR, Vogel J, Charpentier E (2011) CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature 471 (7340):602-607. doi:10.1038/nature09886
12. Chylinski K, Le Rhun A, Charpentier E (2013) The tracrRNA and Cas9 families of type II CRISPR-Cas immunity systems. RNA Biol 10 (5):726-737. doi:10.4161/rna.24321
13. Szczelkun MD, Tikhomirova MS, Sinkunas T, Gasiunas G, Karvelis T, Pschera P, Siksnys V, Seidel R (2014) Direct observation of R-loop formation by single RNA-guided Cas9 and Cascade effector complexes. Proc Natl Acad Sci U S A 111 (27):9798-9803. doi:10.1073/pnas.1402597111
14. Anders C, Niewoehner O, Duerst A, Jinek M (2014) Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. Nature 513 (7519):569-573. doi:10.1038/nature13579
15. Jinek M, Jiang F, Taylor DW, Sternberg SH, Kaya E, Ma E, Anders C, Hauer M, Zhou K, Lin S, Kaplan M, Iavarone AT, Charpentier E, Nogales E, Doudna JA (2014) Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. Science 343 (6176):1247997. doi:10.1126/science.1247997
16. Nishimasu H, Cong L, Yan WX, Ran FA, Zetsche B, Li Y, Kurabayashi A, Ishitani R, Zhang F, Nureki O (2015) Crystal Structure of Staphylococcus aureus Cas9. Cell 162 (5):1113-1126. doi:10.1016/j.cell.2015.08.007
17. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339 (6121):819-823. doi:10.1126/science.1231143
18. Wiedenheft B, Lander GC, Zhou K, Jore MM, Brouns SJJ, van der Oost J, Doudna JA, Nogales E (2011) Structures of the RNA-guided surveillance complex from a bacterial immune system. Nature 477 (7365):486-489. doi:10.1038/nature10402

19. Wright AV, Nunez JK, Doudna JA (2016) Biology and Applications of CRISPR Systems: Harnessing Nature's Toolbox for Genome Engineering. Cell 164 (1-2):29-44. doi:10.1016/j.cell.2015.12.035
20. Barrangou R, Horvath P (2017) A decade of discovery: CRISPR functions and applications. Nat Microbiol 2:17092. doi:10.1038/nmicrobiol.2017.92
21. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, Horvath P (2007) CRISPR provides acquired resistance against viruses in prokaryotes. Science 315 (5819):1709-1712. doi:10.1126/science.1138140
22. Klompe SE, Sternberg SH (2018) Harnessing "A Billion Years of Experimentation": The Ongoing Exploration and Exploitation of CRISPR-Cas Immune Systems. CRISPR J 1 (2):141-158. doi:10.1089/crispr.2018.0012
23. Knott GJ, Doudna JA (2018) CRISPR-Cas guides the future of genetic engineering. Science 361 (6405):866-869. doi:10.1126/science.aat5011
24. Makarova KS, Wolf YI, Alkhnbashi OS, Costa F, Shah SA, Saunders SJ, Barrangou R, Brouns SJ, Charpentier E, Haft DH, Horvath P, Moineau S, Mojica FJ, Terns RM, Terns MP, White MF, Yakunin AF, Garrett RA, van der Oost J, Backofen R, Koonin EV (2015) An updated evolutionary classification of CRISPR-Cas systems. Nat Rev Microbiol 13 (11):722-736. doi:10.1038/nrmicro3569
25. Shmakov S, Abudayyeh OO, Makarova KS, Wolf YI, Gootenberg JS, Semenova E, Minakhin L, Joung J, Konermann S, Severinov K, Zhang F, Koonin EV (2015) Discovery and Functional Characterization of Diverse Class 2 CRISPR-Cas Systems. Mol Cell 60 (3):385-397. doi:10.1016/j.molcel.2015.10.008
26. Shmakov S, Smargon A, Scott D, Cox D, Pyzocha N, Yan W, Abudayyeh OO, Gootenberg JS, Makarova KS, Wolf YI, Severinov K, Zhang F, Koonin EV (2017) Diversity and evolution of class 2 CRISPR-Cas systems. Nat Rev Microbiol 15 (3):169-182. doi:10.1038/nrmicro.2016.184
27. Chapman JR, Taylor MR, Boulton SJ (2012) Playing the end game: DNA double-strand break repair pathway choice. Mol Cell 47 (4):497-510. doi:10.1016/j.molcel.2012.07.029
28. Norris AD, Kim HM, Colaiacovo MP, Calarco JA (2015) Efficient Genome Editing in Caenorhabditis elegans with a Toolkit of Dual-Marker Selection Cassettes. Genetics 201 (2):449-458. doi:10.1534/genetics.115.180679
29. Suzuki K, Tsunekawa Y, Hernandez-Benitez R, Wu J, Zhu J, Kim EJ, Hatanaka F, Yamamoto M, Araoka T, Li Z, Kurita M, Hishida T, Li M, Aizawa E, Guo S, Chen S, Goebl A, Soligalla RD, Qu J, Jiang T, Fu X, Jafari M, Esteban CR, Berggren WT, Lajara J, Nunez-Delicado E, Guillen P, Campistol JM, Matsuzaki F, Liu GH, Magistretti P, Zhang K, Callaway EM, Zhang K, Belmonte JC (2016) In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. Nature 540 (7631):144-149. doi:10.1038/nature20565
30. Wang D, Li J, Song CQ, Tran K, Mou H, Wu PH, Tai PWL, Mendonca CA, Ren L, Wang BY, Su Q, Gessler DJ, Zamore PD, Xue W, Gao G (2018) Cas9-mediated allelic exchange repairs compound heterozygous recessive mutations in mice. Nat Biotechnol 36 (9):839-842. doi:10.1038/nbt.4219
31. Hale CR, Zhao P, Olson S, Duff MO, Graveley BR, Wells L, Terns RM, Terns MP (2009) RNA-guided RNA cleavage by a CRISPR RNA-Cas protein complex. Cell 139 (5):945-956. doi:10.1016/j.cell.2009.07.040
32. O'Connell MR, Oakes BL, Sternberg SH, East-Seletsky A, Kaplan M, Doudna JA (2014) Programmable RNA recognition and cleavage by CRISPR/Cas9. Nature 516 (7530):263-266. doi:10.1038/nature13769
33. Strutt SC, Torrez RM, Kaya E, Negrete OA, Doudna JA (2018) RNA-dependent RNA targeting by CRISPR-Cas9. Elife 7. doi:10.7554/eLife.32724

34. Abudayyeh OO, Gootenberg JS, Essletzbichler P, Han S, Joung J, Belanto JJ, Verdine V, Cox DBT, Kellner MJ, Regev A, Lander ES, Voytas DF, Ting AY, Zhang F (2017) RNA targeting with CRISPR-Cas13. Nature 550 (7675):280-284. doi:10.1038/nature24049
35. Cox DBT, Gootenberg JS, Abudayyeh OO, Franklin B, Kellner MJ, Joung J, Zhang F (2017) RNA editing with CRISPR-Cas13. Science 358 (6366):1019-1027. doi:10.1126/science.aaq0180
36. Liu L, Li X, Ma J, Li Z, You L, Wang J, Wang M, Zhang X, Wang Y (2017) The Molecular Architecture for RNA-Guided RNA Cleavage by Cas13a. Cell 170 (4):714-726 e710. doi:10.1016/j.cell.2017.06.050
37. Konermann S, Lotfy P, Brideau NJ, Oki J, Shokhirev MN, Hsu PD (2018) Transcriptome Engineering with RNA-Targeting Type VI-D CRISPR Effectors. Cell 173 (3):665-676 e614. doi:10.1016/j.cell.2018.02.033
38. Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 152 (5):1173-1183. doi:10.1016/j.cell.2013.02.022
39. Lowder LG, Paul JW, 3rd, Qi Y (2017) Multiplexed Transcriptional Activation or Repression in Plants Using CRISPR-dCas9-Based Systems. Methods Mol Biol 1629:167-184. doi:10.1007/978-1-4939-7125-1_12
40. Tanenbaum ME, Gilbert LA, Qi LS, Weissman JS, Vale RD (2014) A protein-tagging system for signal amplification in gene expression and fluorescence imaging. Cell 159 (3):635-646. doi:10.1016/j.cell.2014.09.039
41. Mali P, Aach J, Stranges PB, Esvelt KM, Moosburner M, Kosuri S, Yang L, Church GM (2013) CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. Nat Biotechnol 31 (9):833-838. doi:10.1038/nbt.2675
42. Hilton IB, D'Ippolito AM, Vockley CM, Thakore PI, Crawford GE, Reddy TE, Gersbach CA (2015) Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. Nat Biotechnol 33 (5):510-517. doi:10.1038/nbt.3199
43. Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR (2016) Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature 533 (7603):420-424. doi:10.1038/nature17946
44. Gaudelli NM, Komor AC, Rees HA, Packer MS, Badran AH, Bryson DI, Liu DR (2017) Programmable base editing of A*T to G*C in genomic DNA without DNA cleavage. Nature 551 (7681):464-471. doi:10.1038/nature24644
45. Anzalone AV, Randolph PB, Davis JR, Sousa AA, Koblan LW, Levy JM, Chen PJ, Wilson C, Newby GA, Raguram A, Liu DR (2019) Search-and-replace genome editing without double-strand breaks or donor DNA. Nature 576 (7785):149-157. doi:10.1038/s41586-019-1711-4
46. Rittiner JE, Moncalvo M, Chiba-Falek O, Kantor B (2020) Gene-Editing Technologies Paired With Viral Vectors for Translational Research Into Neurodegenerative Diseases. Front Mol Neurosci 13:148. doi:10.3389/fnmol.2020.00148
47. Kabadi AM, Ousterout DG, Hilton IB, Gersbach CA (2014) Multiplex CRISPR/Cas9-based genome engineering from a single lentiviral vector. Nucleic Acids Res 42 (19):e147. doi:10.1093/nar/gku749
48. Heckl D, Kowalczyk MS, Yudovich D, Belizaire R, Puram RV, McConkey ME, Thielke A, Aster JC, Regev A, Ebert BL (2014) Generation of mouse models of myeloid malignancy with combinatorial genetic lesions using CRISPR-Cas9 genome editing. Nat Biotechnol 32 (9):941-946. doi:10.1038/nbt.2951
49. Sanchez-Rivera FJ, Papagiannakopoulos T, Romero R, Tammela T, Bauer MR, Bhutkar A, Joshi NS, Subbaraj L, Bronson RT, Xue W, Jacks T (2014) Rapid modelling of cooperating genetic events in cancer through somatic genome editing. Nature 516 (7531):428-431. doi:10.1038/nature13906

50. Zhou S, Fatima S, Ma Z, Wang YD, Lu T, Janke LJ, Du Y, Sorrentino BP (2016) Evaluating the Safety of Retroviral Vectors Based on Insertional Oncogene Activation and Blocked Differentiation in Cultured Thymocytes. Mol Ther 24 (6):1090-1099. doi:10.1038/mt.2016.55
51. Joglekar AV, Hollis RP, Kuftinec G, Senadheera S, Chan R, Kohn DB (2013) Integrase-defective lentiviral vectors as a delivery platform for targeted modification of adenosine deaminase locus. Mol Ther 21 (9):1705-1717. doi:10.1038/mt.2013.106
52. Wanisch K, Yanez-Munoz RJ (2009) Integration-deficient lentiviral vectors: a slow coming of age. Mol Ther 17 (8):1316-1332. doi:10.1038/mt.2009.122
53. Lombardo A, Genovese P, Beausejour CM, Colleoni S, Lee YL, Kim KA, Ando D, Urnov FD, Galli C, Gregory PD, Holmes MC, Naldini L (2007) Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. Nat Biotechnol 25 (11):1298-1306. doi:10.1038/nbt1353
54. Sessa M, Lorioli L, Fumagalli F, Acquati S, Redaelli D, Baldoli C, Canale S, Lopez ID, Morena F, Calabria A, Fiori R, Silvani P, Rancoita PM, Gabaldo M, Benedicenti F, Antonioli G, Assanelli A, Cicalese MP, Del Carro U, Sora MG, Martino S, Quattrini A, Montini E, Di Serio C, Ciceri F, Roncarolo MG, Aiuti A, Naldini L, Biffi A (2016) Lentiviral haemopoietic stem-cell gene therapy in early-onset metachromatic leukodystrophy: an ad-hoc analysis of a non-randomised, open-label, phase 1/2 trial. Lancet 388 (10043):476-487. doi:10.1016/S0140-6736(16)30374-9
55. Wang Y, Wang Y, Chang T, Huang H, Yee JK (2017) Integration-defective lentiviral vector mediates efficient gene editing through homology-directed repair in human embryonic stem cells. Nucleic Acids Res 45 (5):e29. doi:10.1093/nar/gkw1057
56. Slaymaker IM, Gao L, Zetsche B, Scott DA, Yan WX, Zhang F (2016) Rationally engineered Cas9 nucleases with improved specificity. Science 351 (6268):84-88. doi:10.1126/science.aad5227
57. Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z, Joung JK (2016) High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. Nature 529 (7587):490-495. doi:10.1038/nature16526
58. Wang D, Mou H, Li S, Li Y, Hough S, Tran K, Li J, Yin H, Anderson DG, Sontheimer EJ, Weng Z, Gao G, Xue W (2015) Adenovirus-Mediated Somatic Genome Editing of Pten by CRISPR/Cas9 in Mouse Liver in Spite of Cas9-Specific Immune Responses. Hum Gene Ther 26 (7):432-442. doi:10.1089/hum.2015.087
59. Ding Q, Strong A, Patel KM, Ng SL, Gosis BS, Regan SN, Cowan CA, Rader DJ, Musunuru K (2014) Permanent alteration of PCSK9 with in vivo CRISPR-Cas9 genome editing. Circ Res 115 (5):488-492. doi:10.1161/CIRCRESAHA.115.304351
60. Maddalo D, Manchado E, Concepcion CP, Bonetti C, Vidigal JA, Han YC, Ogrodowski P, Crippa A, Rekhtman N, de Stanchina E, Lowe SW, Ventura A (2014) In vivo engineering of oncogenic chromosomal rearrangements with the CRISPR/Cas9 system. Nature 516 (7531):423-427. doi:10.1038/nature13902
61. Kotterman MA, Schaffer DV (2014) Engineering adeno-associated viruses for clinical gene therapy. Nat Rev Genet 15 (7):445-451. doi:10.1038/nrg3742
62. Nathwani AC, Reiss UM, Tuddenham EG, Rosales C, Chowdary P, McIntosh J, Della Peruta M, Lheriteau E, Patel N, Raj D, Riddell A, Pie J, Rangarajan S, Bevan D, Recht M, Shen YM, Halka KG, Basner-Tschakarjan E, Mingozzi F, High KA, Allay J, Kay MA, Ng CY, Zhou J, Cancio M, Morton CL, Gray JT, Srivastava D, Nienhuis AW, Davidoff AM (2014) Long-term safety and efficacy of factor IX gene therapy in hemophilia B. N Engl J Med 371 (21):1994-2004. doi:10.1056/NEJMoa1407309
63. Nelson CE, Gersbach CA (2016) Engineering Delivery Vehicles for Genome Editing. Annu Rev Chem Biomol Eng 7:637-662. doi:10.1146/annurev-chembioeng-080615-034711
64. Swiech L, Heidenreich M, Banerjee A, Habib N, Li Y, Trombetta J, Sur M, Zhang F (2015) In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9. Nat Biotechnol 33 (1):102-106. doi:10.1038/nbt.3055

65. Hung SS, Chrysostomou V, Li F, Lim JK, Wang JH, Powell JE, Tu L, Daniszewski M, Lo C, Wong RC, Crowston JG, Pebay A, King AE, Bui BV, Liu GS, Hewitt AW (2016) AAV-Mediated CRISPR/Cas Gene Editing of Retinal Cells In Vivo. Invest Ophthalmol Vis Sci 57 (7):3470-3476. doi:10.1167/iovs.16-19316
66. Yu W, Mookherjee S, Chaitankar V, Hiriyanna S, Kim JW, Brooks M, Ataeijannati Y, Sun X, Dong L, Li T, Swaroop A, Wu Z (2017) Nrl knockdown by AAV-delivered CRISPR/Cas9 prevents retinal degeneration in mice. Nat Commun 8:14716. doi:10.1038/ncomms14716
67. Hu S, Du J, Chen N, Jia R, Zhang J, Liu X, Yang L (2020) In Vivo CRISPR/Cas9-Mediated Genome Editing Mitigates Photoreceptor Degeneration in a Mouse Model of X-Linked Retinitis Pigmentosa. Invest Ophthalmol Vis Sci 61 (4):31. doi:10.1167/iovs.61.4.31
68. Lin H, Hu H, Duan W, Liu Y, Tan G, Li Z, Liu Y, Deng B, Song X, Wang W, Wen D, Wang Y, Li C (2018) Intramuscular Delivery of scAAV9-hIGF1 Prolongs Survival in the hSOD1(G93A) ALS Mouse Model via Upregulation of D-Amino Acid Oxidase. Mol Neurobiol 55 (1):682-695. doi:10.1007/s12035-016-0335-z
69. Bengtsson NE, Hall JK, Odom GL, Phelps MP, Andrus CR, Hawkins RD, Hauschka SD, Chamberlain JR, Chamberlain JS (2017) Muscle-specific CRISPR/Cas9 dystrophin gene editing ameliorates pathophysiology in a mouse model for Duchenne muscular dystrophy. Nat Commun 8:14454. doi:10.1038/ncomms14454
70. Long C, Amoasii L, Mireault AA, McAnally JR, Li H, Sanchez-Ortiz E, Bhattacharyya S, Shelton JM, Bassel-Duby R, Olson EN (2016) Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy. Science 351 (6271):400-403. doi:10.1126/science.aad5725
71. Tabebordbar M, Zhu K, Cheng JKW, Chew WL, Widrick JJ, Yan WX, Maesner C, Wu EY, Xiao R, Ran FA, Cong L, Zhang F, Vandenberghe LH, Church GM, Wagers AJ (2016) In vivo gene editing in dystrophic mouse muscle and muscle stem cells. Science 351 (6271):407-411. doi:10.1126/science.aad5177
72. Zhang Y, Li H, Min YL, Sanchez-Ortiz E, Huang J, Mireault AA, Shelton JM, Kim J, Mammen PPA, Bassel-Duby R, Olson EN (2020) Enhanced CRISPR-Cas9 correction of Duchenne muscular dystrophy in mice by a self-complementary AAV delivery system. Sci Adv 6 (8):eaay6812. doi:10.1126/sciadv.aay6812
73. Wang L, Yang Y, Breton C, Bell P, Li M, Zhang J, Che Y, Saveliev A, He Z, White J, Latshaw C, Xu C, McMenamin D, Yu H, Morizono H, Batshaw ML, Wilson JM (2020) A mutation-independent CRISPR-Cas9-mediated gene targeting approach to treat a murine model of ornithine transcarbamylase deficiency. Sci Adv 6 (7):eaax5701. doi:10.1126/sciadv.aax5701
74. Yang Y, Wang L, Bell P, McMenamin D, He Z, White J, Yu H, Xu C, Morizono H, Musunuru K, Batshaw ML, Wilson JM (2016) A dual AAV system enables the Cas9-mediated correction of a metabolic liver disease in newborn mice. Nat Biotechnol 34 (3):334-338. doi:10.1038/nbt.3469
75. Carroll KJ, Makarewich CA, McAnally J, Anderson DM, Zentilin L, Liu N, Giacca M, Bassel-Duby R, Olson EN (2016) A mouse model for adult cardiac-specific gene deletion with CRISPR/Cas9. Proc Natl Acad Sci U S A 113 (2):338-343. doi:10.1073/pnas.1523918113
76. Platt RJ, Chen S, Zhou Y, Yim MJ, Swiech L, Kempton HR, Dahlman JE, Parnas O, Eisenhaure TM, Jovanovic M, Graham DB, Jhunjhunwala S, Heidenreich M, Xavier RJ, Langer R, Anderson DG, Hacohen N, Regev A, Feng G, Sharp PA, Zhang F (2014) CRISPR-Cas9 knockin mice for genome editing and cancer modeling. Cell 159 (2):440-455. doi:10.1016/j.cell.2014.09.014
77. Murlidharan G, Sakamoto K, Rao L, Corriher T, Wang D, Gao G, Sullivan P, Asokan A (2016) CNS-restricted Transduction and CRISPR/Cas9-mediated Gene Deletion with an Engineered AAV Vector. Mol Ther Nucleic Acids 5 (7):e338. doi:10.1038/mtna.2016.49
78. Chow RD, Guzman CD, Wang G, Schmidt F, Youngblood MW, Ye L, Errami Y, Dong MB, Martinez MA, Zhang S, Renauer P, Bilguvar K, Gunel M, Sharp PA, Zhang F, Platt RJ, Chen S

(2017) AAV-mediated direct in vivo CRISPR screen identifies functional suppressors in glioblastoma. Nat Neurosci 20 (10):1329-1341. doi:10.1038/nn.4620
79. Wang G, Chow RD, Ye L, Guzman CD, Dai X, Dong MB, Zhang F, Sharp PA, Platt RJ, Chen S (2018) Mapping a functional cancer genome atlas of tumor suppressors in mouse liver using AAV-CRISPR-mediated direct in vivo screening. Sci Adv 4 (2):eaao5508. doi:10.1126/sciadv.aao5508
80. Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, Zetsche B, Shalem O, Wu X, Makarova KS, Koonin EV, Sharp PA, Zhang F (2015) In vivo genome editing using Staphylococcus aureus Cas9. Nature 520 (7546):186-191. doi:10.1038/nature14299
81. Kim E, Koo T, Park SW, Kim D, Kim K, Cho HY, Song DW, Lee KJ, Jung MH, Kim S, Kim JH, Kim JH, Kim JS (2017) In vivo genome editing with a small Cas9 orthologue derived from Campylobacter jejuni. Nat Commun 8:14500. doi:10.1038/ncomms14500
82. Muller M, Lee CM, Gasiunas G, Davis TH, Cradick TJ, Siksnys V, Bao G, Cathomen T, Mussolino C (2016) Streptococcus thermophilus CRISPR-Cas9 Systems Enable Specific Editing of the Human Genome. Mol Ther 24 (3):636-644. doi:10.1038/mt.2015.218
83. Patsali P, Kleanthous M, Lederer CW (2019) Disruptive Technology: CRISPR/Cas-Based Tools and Approaches. Mol Diagn Ther 23 (2):187-200. doi:10.1007/s40291-019-00391-4
84. Manghwar H, Li B, Ding X, Hussain A, Lindsey K, Zhang X, Jin S (2020) CRISPR/Cas Systems in Genome Editing: Methodologies and Tools for sgRNA Design, Off-Target Evaluation, and Strategies to Mitigate Off-Target Effects. Adv Sci (Weinh) 7 (6):1902312. doi:10.1002/advs.201902312
85. Mali P, Esvelt KM, Church GM (2013) Cas9 as a versatile tool for engineering biology. Nat Methods 10 (10):957-963. doi:10.1038/nmeth.2649
86. Yin H, Xue W, Chen S, Bogorad RL, Benedetti E, Grompe M, Koteliansky V, Sharp PA, Jacks T, Anderson DG (2014) Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. Nat Biotechnol 32 (6):551-553. doi:10.1038/nbt.2884
87. Zhen S, Hua L, Liu YH, Gao LC, Fu J, Wan DY, Dong LH, Song HF, Gao X (2015) Harnessing the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated Cas9 system to disrupt the hepatitis B virus. Gene Ther 22 (5):404-412. doi:10.1038/gt.2015.2
88. Bakondi B, Lv W, Lu B, Jones MK, Tsai Y, Kim KJ, Levy R, Akhtar AA, Breunig JJ, Svendsen CN, Wang S (2016) In Vivo CRISPR/Cas9 Gene Editing Corrects Retinal Dystrophy in the S334ter-3 Rat Model of Autosomal Dominant Retinitis Pigmentosa. Mol Ther 24 (3):556-563. doi:10.1038/mt.2015.220
89. Latella MC, Di Salvo MT, Cocchiarella F, Benati D, Grisendi G, Comitato A, Marigo V, Recchia A (2016) In vivo Editing of the Human Mutant Rhodopsin Gene by Electroporation of Plasmid-based CRISPR/Cas9 in the Mouse Retina. Mol Ther Nucleic Acids 5 (11):e389. doi:10.1038/mtna.2016.92
90. Li P, Kleinstiver BP, Leon MY, Prew MS, Navarro-Gomez D, Greenwald SH, Pierce EA, Joung JK, Liu Q (2018) Allele-Specific CRISPR-Cas9 Genome Editing of the Single-Base P23H Mutation for Rhodopsin-Associated Dominant Retinitis Pigmentosa. CRISPR J 1:55-64. doi:10.1089/crispr.2017.0009
91. Shinmyo Y, Tanaka S, Tsunoda S, Hosomichi K, Tajima A, Kawasaki H (2016) CRISPR/Cas9-mediated gene knockout in the mouse brain using in utero electroporation. Sci Rep 6:20611. doi:10.1038/srep20611
92. Yin H, Song CQ, Dorkin JR, Zhu LJ, Li Y, Wu Q, Park A, Yang J, Suresh S, Bizhanova A, Gupta A, Bolukbasi MF, Walsh S, Bogorad RL, Gao G, Weng Z, Dong Y, Koteliansky V, Wolfe SA, Langer R, Xue W, Anderson DG (2016) Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components in vivo. Nat Biotechnol 34 (3):328-333. doi:10.1038/nbt.3471
93. Yin H, Song CQ, Suresh S, Wu Q, Walsh S, Rhym LH, Mintzer E, Bolukbasi MF, Zhu LJ, Kauffman K, Mou H, Oberholzer A, Ding J, Kwan SY, Bogorad RL, Zatsepin T, Koteliansky V,

Wolfe SA, Xue W, Langer R, Anderson DG (2017) Structure-guided chemical modification of guide RNA enables potent non-viral in vivo genome editing. Nat Biotechnol 35 (12):1179-1187. doi:10.1038/nbt.4005
94. Finn JD, Smith AR, Patel MC, Shaw L, Youniss MR, van Heteren J, Dirstine T, Ciullo C, Lescarbeau R, Seitzer J, Shah RR, Shah A, Ling D, Growe J, Pink M, Rohde E, Wood KM, Salomon WE, Harrington WF, Dombrowski C, Strapps WR, Chang Y, Morrissey DV (2018) A Single Administration of CRISPR/Cas9 Lipid Nanoparticles Achieves Robust and Persistent In Vivo Genome Editing. Cell Rep 22 (9):2227-2235. doi:10.1016/j.celrep.2018.02.014
95. Liu J, Chang J, Jiang Y, Meng X, Sun T, Mao L, Xu Q, Wang M (2019) Fast and Efficient CRISPR/Cas9 Genome Editing In Vivo Enabled by Bioreducible Lipid and Messenger RNA Nanoparticles. Adv Mater 31 (33):e1902575. doi:10.1002/adma.201902575
96. Zuris JA, Thompson DB, Shu Y, Guilinger JP, Bessen JL, Hu JH, Maeder ML, Joung JK, Chen ZY, Liu DR (2015) Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. Nat Biotechnol 33 (1):73-80. doi:10.1038/nbt.3081
97. Mangeot PE, Risson V, Fusil F, Marnef A, Laurent E, Blin J, Mournetas V, Massourides E, Sohier TJM, Corbin A, Aube F, Teixeira M, Pinset C, Schaeffer L, Legube G, Cosset FL, Verhoeyen E, Ohlmann T, Ricci EP (2019) Genome editing in primary cells and in vivo using viral-derived Nanoblades loaded with Cas9-sgRNA ribonucleoproteins. Nat Commun 10 (1):45. doi:10.1038/s41467-018-07845-z
98. Park H, Oh J, Shim G, Cho B, Chang Y, Kim S, Baek S, Kim H, Shin J, Choi H, Yoo J, Kim J, Jun W, Lee M, Lengner CJ, Oh YK, Kim J (2019) In vivo neuronal gene editing via CRISPR-Cas9 amphiphilic nanocomplexes alleviates deficits in mouse models of Alzheimer's disease. Nat Neurosci 22 (4):524-528. doi:10.1038/s41593-019-0352-0
99. Sun W, Ji W, Hall JM, Hu Q, Wang C, Beisel CL, Gu Z (2015) Self-assembled DNA nanoclews for the efficient delivery of CRISPR-Cas9 for genome editing. Angew Chem Int Ed Engl 54 (41):12029-12033. doi:10.1002/anie.201506030
100. Mout R, Ray M, Yesilbag Tonga G, Lee YW, Tay T, Sasaki K, Rotello VM (2017) Direct Cytosolic Delivery of CRISPR/Cas9-Ribonucleoprotein for Efficient Gene Editing. ACS Nano 11 (3):2452-2458. doi:10.1021/acsnano.6b07600
101. Lee YW, Mout R, Luther DC, Liu YC, Castellanos-García L, Burnside AS, Ray M, Tonga GY, Hardie J, Nagaraj H, Das R, Phillips EL, Tay T, Vachet RW, Rotello VM (2019) In Vivo Editing of Macrophages through Systemic Delivery of CRISPR-Cas9-Ribonucleoprotein-Nanoparticle Nanoassemblies. Advanced Therapeutics 2 (10):7. doi:10.1002/adtp.201900041
102. Lee K, Conboy M, Park HM, Jiang F, Kim HJ, Dewitt MA, Mackley VA, Chang K, Rao A, Skinner C, Shobha T, Mehdipour M, Liu H, Huang WC, Lan F, Bray NL, Li S, Corn JE, Kataoka K, Doudna JA, Conboy I, Murthy N (2017) Nanoparticle delivery of Cas9 ribonucleoprotein and donor DNA in vivo induces homology-directed DNA repair. Nat Biomed Eng 1:889-901. doi:10.1038/s41551-017-0137-2
103. Lee B, Lee K, Panda S, Gonzales-Rojas R, Chong A, Bugay V, Park HM, Brenner R, Murthy N, Lee HY (2018) Nanoparticle delivery of CRISPR into the brain rescues a mouse model of fragile X syndrome from exaggerated repetitive behaviours. Nat Biomed Eng 2 (7):497-507. doi:10.1038/s41551-018-0252-8
104. Mout R, Ray M, Lee YW, Scaletti F, Rotello VM (2017) In Vivo Delivery of CRISPR/Cas9 for Therapeutic Gene Editing: Progress and Challenges. Bioconjug Chem 28 (4):880-884. doi:10.1021/acs.bioconjchem.7b00057
105. Bareford LM, Swaan PW (2007) Endocytic mechanisms for targeted drug delivery. Adv Drug Deliv Rev 59 (8):748-758. doi:10.1016/j.addr.2007.06.008
106. Jao LE, Wente SR, Chen W (2013) Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. Proc Natl Acad Sci U S A 110 (34):13904-13909. doi:10.1073/pnas.1308335110

969 107. Goto R, Saito T, Matsubara T, Yamaha E (2019) Microinjection of Marine Fish Eggs. Methods
970 Mol Biol 1874:475-487. doi:10.1007/978-1-4939-8831-0_27
971 108. Chakrapani V, Patra SK, Panda RP, Rasal KD, Jayasankar P, Barman HK (2016) Establishing
972 targeted carp TLR22 gene disruption via homologous recombination using CRISPR/Cas9. Dev
973 Comp Immunol 61:242-247. doi:10.1016/j.dci.2016.04.009
974 109. Wargelius A, Leininger S, Skaftnesmo KO, Kleppe L, Andersson E, Taranger GL, Schulz RW,
975 Edvardsen RB (2016) Dnd knockout ablates germ cells and demonstrates germ cell independent sex
976 differentiation in Atlantic salmon. Sci Rep 6:21284. doi:10.1038/srep21284
977 110. Khalil K, Elayat M, Khalifa E, Daghash S, Elaswad A, Miller M, Abdelrahman H, Ye Z, Odin
978 R, Drescher D, Vo K, Gosh K, Bugg W, Robinson D, Dunham R (2017) Generation of Myostatin
979 Gene-Edited Channel Catfish (Ictalurus punctatus) via Zygote Injection of CRISPR/Cas9 System.
980 Sci Rep 7 (1):7301. doi:10.1038/s41598-017-07223-7
981 111. Zhong Z, Niu P, Wang M, Huang G, Xu S, Sun Y, Xu X, Hou Y, Sun X, Yan Y, Wang H
982 (2016) Targeted disruption of sp7 and myostatin with CRISPR-Cas9 results in severe bone defects
983 and more muscular cells in common carp. Sci Rep 6:22953. doi:10.1038/srep22953
984 112. Elaswad A, Khalil K, Ye Z, Liu Z, Liu S, Peatman E, Odin R, Vo K, Drescher D, Gosh K, Qin
985 G, Bugg W, Backenstose N, Dunham R (2018) Effects of CRISPR/Cas9 dosage on TICAM1 and
986 RBL gene mutation rate, embryonic development, hatchability and fry survival in channel catfish.
987 Sci Rep 8 (1):16499. doi:10.1038/s41598-018-34738-4
988 113. Ma J, Fan Y, Zhou Y, Liu W, Jiang N, Zhang J, Zeng L (2018) Efficient resistance to grass carp
989 reovirus infection in JAM-A knockout cells using CRISPR/Cas9. Fish Shellfish Immunol 76:206-
990 215. doi:10.1016/j.fsi.2018.02.039
991 114. Kim JS (2018) Precision genome engineering through adenine and cytosine base editing. Nat
992 Plants 4 (3):148-151. doi:10.1038/s41477-018-0115-z
993 115. Shan Q, Wang Y, Li J, Gao C (2014) Genome editing in rice and wheat using the CRISPR/Cas
994 system. Nat Protoc 9 (10):2395-2410. doi:10.1038/nprot.2014.157
995 116. Shimatani Z, Kashojiya S, Takayama M, Terada R, Arazoe T, Ishii H, Teramura H, Yamamoto
996 T, Komatsu H, Miura K, Ezura H, Nishida K, Ariizumi T, Kondo A (2017) Targeted base editing in
997 rice and tomato using a CRISPR-Cas9 cytidine deaminase fusion. Nat Biotechnol 35 (5):441-443.
998 doi:10.1038/nbt.3833
999 117. Xie K, Yang Y (2013) RNA-guided genome editing in plants using a CRISPR-Cas system. Mol
1000 Plant 6 (6):1975-1983. doi:10.1093/mp/sst119
1001 118. Bhaskar PB, Venkateshwaran M, Wu L, Ane JM, Jiang J (2009) Agrobacterium-mediated
1002 transient gene expression and silencing: a rapid tool for functional gene assay in potato. PLoS One 4
1003 (6):e5812. doi:10.1371/journal.pone.0005812
1004 119. Circelli P, Donini M, Villani ME, Benvenuto E, Marusic C (2010) Efficient Agrobacterium-
1005 based transient expression system for the production of biopharmaceuticals in plants. Bioeng Bugs 1
1006 (3):221-224. doi:10.4161/bbug.1.3.11722
1007 120. Figueiredo JF, Romer P, Lahaye T, Graham JH, White FF, Jones JB (2011) Agrobacterium-
1008 mediated transient expression in citrus leaves: a rapid tool for gene expression and functional gene
1009 assay. Plant Cell Rep 30 (7):1339-1345. doi:10.1007/s00299-011-1045-7
1010 121. Kim MJ, Baek K, Park CM (2009) Optimization of conditions for transient Agrobacterium-
1011 mediated gene expression assays in Arabidopsis. Plant Cell Rep 28 (8):1159-1167.
1012 doi:10.1007/s00299-009-0717-z
1013 122. Zheng L, Liu G, Meng X, Li Y, Wang Y (2012) A versatile Agrobacterium-mediated transient
1014 gene expression system for herbaceous plants and trees. Biochem Genet 50 (9-10):761-769.
1015 doi:10.1007/s10528-012-9518-0
1016 123. Belhaj K, Chaparro-Garcia A, Kamoun S, Nekrasov V (2013) Plant genome editing made easy:
1017 targeted mutagenesis in model and crop plants using the CRISPR/Cas system. Plant Methods 9
1018 (1):39. doi:10.1186/1746-4811-9-39

124. Feng Z, Zhang B, Ding W, Liu X, Yang DL, Wei P, Cao F, Zhu S, Zhang F, Mao Y, Zhu JK (2013) Efficient genome editing in plants using a CRISPR/Cas system. Cell Res 23 (10):1229-1232. doi:10.1038/cr.2013.114
125. Piatek A, Ali Z, Baazim H, Li L, Abulfaraj A, Al-Shareef S, Aouida M, Mahfouz MM (2015) RNA-guided transcriptional regulation in planta via synthetic dCas9-based transcription factors. Plant Biotechnol J 13 (4):578-589. doi:10.1111/pbi.12284
126. Zhang H, Cao Y, Zhang H, Xu Y, Zhou C, Liu W, Zhu R, Shang C, Li J, Shen Z, Guo S, Hu Z, Fu C, Sun D (2020) Efficient Generation of CRISPR/Cas9-Mediated Homozygous/Biallelic Medicago truncatula Mutants Using a Hairy Root System. Front Plant Sci 11:294. doi:10.3389/fpls.2020.00294
127. Michno JM, Wang X, Liu J, Curtin SJ, Kono TJ, Stupar RM (2015) CRISPR/Cas mutagenesis of soybean and Medicago truncatula using a new web-tool and a modified Cas9 enzyme. GM Crops Food 6 (4):243-252. doi:10.1080/21645698.2015.1106063
128. Zhang S, Kondorosi E, Kereszt A (2019) An anthocyanin marker for direct visualization of plant transformation and its use to study nitrogen-fixing nodule development. J Plant Res 132 (5):695-703. doi:10.1007/s10265-019-01126-6
129. Cermak T, Baltes NJ, Cegan R, Zhang Y, Voytas DF (2015) High-frequency, precise modification of the tomato genome. Genome Biol 16:232. doi:10.1186/s13059-015-0796-9
130. Liang Z, Zhang K, Chen K, Gao C (2014) Targeted mutagenesis in Zea mays using TALENs and the CRISPR/Cas system. J Genet Genomics 41 (2):63-68. doi:10.1016/j.jgg.2013.12.001
131. Lin Q, Zong Y, Xue C, Wang S, Jin S, Zhu Z, Wang Y, Anzalone AV, Raguram A, Doman JL, Liu DR, Gao C (2020) Prime genome editing in rice and wheat. Nat Biotechnol 38 (5):582-585. doi:10.1038/s41587-020-0455-x
132. Park J, Choe S (2019) DNA-free genome editing with preassembled CRISPR/Cas9 ribonucleoproteins in plants. Transgenic Res 28 (Suppl 2):61-64. doi:10.1007/s11248-019-00136-3
133. Woo JW, Kim J, Kwon SI, Corvalan C, Cho SW, Kim H, Kim SG, Kim ST, Choe S, Kim JS (2015) DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. Nat Biotechnol 33 (11):1162-1164. doi:10.1038/nbt.3389
134. Lin CS, Hsu CT, Yang LH, Lee LY, Fu JY, Cheng QW, Wu FH, Hsiao HC, Zhang Y, Zhang R, Chang WJ, Yu CT, Wang W, Liao LJ, Gelvin SB, Shih MC (2018) Application of protoplast technology to CRISPR/Cas9 mutagenesis: from single-cell mutation detection to mutant plant regeneration. Plant Biotechnol J 16 (7):1295-1310. doi:10.1111/pbi.12870
135. Hwang HH, Yu M, Lai EM (2017) Agrobacterium-mediated plant transformation: biology and applications. Arabidopsis Book 15:e0186. doi:10.1199/tab.0186
136. Kumagai MH, Donson J, della-Cioppa G, Harvey D, Hanley K, Grill LK (1995) Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. Proc Natl Acad Sci U S A 92 (5):1679-1683. doi:10.1073/pnas.92.5.1679
137. Nagyova A, Subr Z (2007) Infectious full-length clones of plant viruses and their use for construction of viral vectors. Acta Virol 51 (4):223-237
138. Ali Z, Abul-faraj A, Li L, Ghosh N, Piatek M, Mahjoub A, Aouida M, Piatek A, Baltes NJ, Voytas DF, Dinesh-Kumar S, Mahfouz MM (2015) Efficient Virus-Mediated Genome Editing in Plants Using the CRISPR/Cas9 System. Mol Plant 8 (8):1288-1291. doi:10.1016/j.molp.2015.02.011
139. Hanley-Bowdoin L, Settlage SB, Orozco BM, Nagar S, Robertson D (2000) Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation. Crit Rev Biochem Mol Biol 35 (2):105-140
140. Baltes NJ, Gil-Humanes J, Cermak T, Atkins PA, Voytas DF (2014) DNA replicons for plant genome engineering. Plant Cell 26 (1):151-163. doi:10.1105/tpc.113.119792
141. Wawrzyniak P, Plucienniczak G, Bartosik D (2017) The Different Faces of Rolling-Circle Replication and Its Multifunctional Initiator Proteins. Front Microbiol 8:2353. doi:10.3389/fmicb.2017.02353

142. Yin K, Han T, Liu G, Chen T, Wang Y, Yu AY, Liu Y (2015) A geminivirus-based guide RNA delivery system for CRISPR/Cas9 mediated plant genome editing. Sci Rep 5:14926. doi:10.1038/srep14926
143. Gil-Humanes J, Wang Y, Liang Z, Shan Q, Ozuna CV, Sanchez-Leon S, Baltes NJ, Starker C, Barro F, Gao C, Voytas DF (2017) High-efficiency gene targeting in hexaploid wheat using DNA replicons and CRISPR/Cas9. Plant J 89 (6):1251-1262. doi:10.1111/tpj.13446
144. Andersson M, Turesson H, Nicolia A, Falt AS, Samuelsson M, Hofvander P (2017) Efficient targeted multiallelic mutagenesis in tetraploid potato (Solanum tuberosum) by transient CRISPR-Cas9 expression in protoplasts. Plant Cell Rep 36 (1):117-128. doi:10.1007/s00299-016-2062-3
145. Kikkert JR, Vidal JR, Reisch BI (2005) Stable transformation of plant cells by particle bombardment/biolistics. Methods Mol Biol 286:61-78. doi:10.1385/1-59259-827-7:061
146. Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z, Zhang K, Liu J, Xi JJ, Qiu JL, Gao C (2013) Targeted genome modification of crop plants using a CRISPR-Cas system. Nat Biotechnol 31 (8):686-688. doi:10.1038/nbt.2650
147. Ma X, Zhang Q, Zhu Q, Liu W, Chen Y, Qiu R, Wang B, Yang Z, Li H, Lin Y, Xie Y, Shen R, Chen S, Wang Z, Chen Y, Guo J, Chen L, Zhao X, Dong Z, Liu YG (2015) A Robust CRISPR/Cas9 System for Convenient, High-Efficiency Multiplex Genome Editing in Monocot and Dicot Plants. Mol Plant 8 (8):1274-1284. doi:10.1016/j.molp.2015.04.007
148. Svitashev S, Young JK, Schwartz C, Gao H, Falco SC, Cigan AM (2015) Targeted Mutagenesis, Precise Gene Editing, and Site-Specific Gene Insertion in Maize Using Cas9 and Guide RNA. Plant Physiol 169 (2):931-945. doi:10.1104/pp.15.00793
149. Liang Z, Chen K, Li T, Zhang Y, Wang Y, Zhao Q, Liu J, Zhang H, Liu C, Ran Y, Gao C (2017) Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. Nat Commun 8:14261. doi:10.1038/ncomms14261
150. Svitashev S, Schwartz C, Lenderts B, Young JK, Mark Cigan A (2016) Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes. Nat Commun 7:13274. doi:10.1038/ncomms13274
151. Kedmi R, Ben-Arie N, Peer D (2010) The systemic toxicity of positively charged lipid nanoparticles and the role of Toll-like receptor 4 in immune activation. Biomaterials 31 (26):6867-6875. doi:10.1016/j.biomaterials.2010.05.027
152. Nayak S, Herzog RW (2010) Progress and prospects: immune responses to viral vectors. Gene Ther 17 (3):295-304. doi:10.1038/gt.2009.148
153. Shirley JL, de Jong YP, Terhorst C, Herzog RW (2020) Immune Responses to Viral Gene Therapy Vectors. Mol Ther 28 (3):709-722. doi:10.1016/j.ymthe.2020.01.001
154. Yin H, Kanasty RL, Eltoukhy AA, Vegas AJ, Dorkin JR, Anderson DG (2014) Non-viral vectors for gene-based therapy. Nat Rev Genet 15 (8):541-555. doi:10.1038/nrg3763
155. Hryhorowicz M, Grzeskowiak B, Mazurkiewicz N, Sledzinski P, Lipinski D, Slomski R (2019) Improved Delivery of CRISPR/Cas9 System Using Magnetic Nanoparticles into Porcine Fibroblast. Mol Biotechnol 61 (3):173-180. doi:10.1007/s12033-018-0145-9
156. Rohiwal SS, Dvorakova N, Klima J, Vaskovicova M, Senigl F, Slouf M, Pavlova E, Stepanek P, Babuka D, Benes H, Ellederova Z, Stieger K (2020) Polyethylenimine based magnetic nanoparticles mediated non-viral CRISPR/Cas9 system for genome editing. Sci Rep 10 (1):4619. doi:10.1038/s41598-020-61465-6
157. Ayana Yamagishi, Daisuke Matsumoto, Yoshio Kato, Yuki Honda, Mone Morikawa, Futoshi Iwata, Takeshi Kobayashi, Nakamura C (2019) Direct Delivery of Cas9-sgRNA Ribonucleoproteins into Cells Using a Nanoneedle Array. Applied Sciences 9:965
158. Chen Y, Aslanoglou S, Murayama T, Gervinskas G, Fitzgerald LI, Sriram S, Tian J, Johnston APR, Morikawa Y, Suu K, Elnathan R, Voelcker NH (2020) Silicon-Nanotube-Mediated Intracellular Delivery Enables Ex Vivo Gene Editing. Adv Mater:e2000036. doi:10.1002/adma.202000036

159. Rauch BJ, Silvis MR, Hultquist JF, Waters CS, McGregor MJ, Krogan NJ, Bondy-Denomy J (2017) Inhibition of CRISPR-Cas9 with Bacteriophage Proteins. Cell 168 (1-2):150-158 e110. doi:10.1016/j.cell.2016.12.009
160. Dong, Guo M, Wang S, Zhu Y, Wang S, Xiong Z, Yang J, Xu Z, Huang Z (2017) Structural basis of CRISPR-SpyCas9 inhibition by an anti-CRISPR protein. Nature 546 (7658):436-439. doi:10.1038/nature22377
161. Hoffmann MD, Aschenbrenner S, Grosse S, Rapti K, Domenger C, Fakhiri J, Mastel M, Borner K, Eils R, Grimm D, Niopek D (2019) Cell-specific CRISPR-Cas9 activation by microRNA-dependent expression of anti-CRISPR proteins. Nucleic Acids Res 47 (13):e75. doi:10.1093/nar/gkz271
162. Oakes BL, Fellmann C, Rishi H, Taylor KL, Ren SM, Nadler DC, Yokoo R, Arkin AP, Doudna JA, Savage DF (2019) CRISPR-Cas9 Circular Permutants as Programmable Scaffolds for Genome Modification. Cell 176 (1-2):254-267 e216. doi:10.1016/j.cell.2018.11.052
163. Truong DJ, Kuhner K, Kuhn R, Werfel S, Engelhardt S, Wurst W, Ortiz O (2015) Development of an intein-mediated split-Cas9 system for gene therapy. Nucleic Acids Res 43 (13):6450-6458. doi:10.1093/nar/gkv601
164. Davis KM, Pattanayak V, Thompson DB, Zuris JA, Liu DR (2015) Small molecule-triggered Cas9 protein with improved genome-editing specificity. Nat Chem Biol 11 (5):316-318. doi:10.1038/nchembio.1793
165. Chew WL, Tabebordbar M, Cheng JK, Mali P, Wu EY, Ng AH, Zhu K, Wagers AJ, Church GM (2016) A multifunctional AAV-CRISPR-Cas9 and its host response. Nat Methods 13 (10):868-874. doi:10.1038/nmeth.3993
166. Nihongaki Y, Kawano F, Nakajima T, Sato M (2015) Photoactivatable CRISPR-Cas9 for optogenetic genome editing. Nat Biotechnol 33 (7):755-760. doi:10.1038/nbt.3245
167. Tsai SQ, Wyvekens N, Khayter C, Foden JA, Thapar V, Reyon D, Goodwin MJ, Aryee MJ, Joung JK (2014) Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing. Nat Biotechnol 32 (6):569-576. doi:10.1038/nbt.2908
168. Guilinger JP, Thompson DB, Liu DR (2014) Fusion of catalytically inactive Cas9 to FokI nuclease improves the specificity of genome modification. Nat Biotechnol 32 (6):577-582. doi:10.1038/nbt.2909
169. Cao J, Wu L, Zhang SM, Lu M, Cheung WK, Cai W, Gale M, Xu Q, Yan Q (2016) An easy and efficient inducible CRISPR/Cas9 platform with improved specificity for multiple gene targeting. Nucleic Acids Res 44 (19):e149. doi:10.1093/nar/gkw660
170. Dow LE, Fisher J, O'Rourke KP, Muley A, Kastenhuber ER, Livshits G, Tschaharganeh DF, Socci ND, Lowe SW (2015) Inducible in vivo genome editing with CRISPR-Cas9. Nat Biotechnol 33 (4):390-394. doi:10.1038/nbt.3155
171. Zetsche B, Volz SE, Zhang F (2015) A split-Cas9 architecture for inducible genome editing and transcription modulation. Nat Biotechnol 33 (2):139-142. doi:10.1038/nbt.3149
172. Liu KI, Ramli MN, Woo CW, Wang Y, Zhao T, Zhang X, Yim GR, Chong BY, Gowher A, Chua MZ, Jung J, Lee JH, Tan MH (2016) A chemical-inducible CRISPR-Cas9 system for rapid control of genome editing. Nat Chem Biol 12 (11):980-987. doi:10.1038/nchembio.2179
173. Lu J, Zhao C, Zhao Y, Zhang J, Zhang Y, Chen L, Han Q, Ying Y, Peng S, Ai R, Wang Y (2018) Multimode drug inducible CRISPR/Cas9 devices for transcriptional activation and genome editing. Nucleic Acids Res 46 (5):e25. doi:10.1093/nar/gkx1222
174. Wright AV, Sternberg SH, Taylor DW, Staahl BT, Bardales JA, Kornfeld JE, Doudna JA (2015) Rational design of a split-Cas9 enzyme complex. Proc Natl Acad Sci U S A 112 (10):2984-2989. doi:10.1073/pnas.1501698112
175. Shah NH, Muir TW (2014) Inteins: Nature's Gift to Protein Chemists. Chem Sci 5 (1):446-461. doi:10.1039/C3SC52951G

176. Banaszynski LA, Liu CW, Wandless TJ (2005) Characterization of the FKBP.rapamycin.FRB ternary complex. J Am Chem Soc 127 (13):4715-4721. doi:10.1021/ja043277y
177. Nihongaki Y, Yamamoto S, Kawano F, Suzuki H, Sato M (2015) CRISPR-Cas9-based photoactivatable transcription system. Chem Biol 22 (2):169-174. doi:10.1016/j.chembiol.2014.12.011
178. Hemphill J, Borchardt EK, Brown K, Asokan A, Deiters A (2015) Optical Control of CRISPR/Cas9 Gene Editing. J Am Chem Soc 137 (17):5642-5645. doi:10.1021/ja512664v
179. Polstein LR, Gersbach CA (2015) A light-inducible CRISPR-Cas9 system for control of endogenous gene activation. Nat Chem Biol 11 (3):198-200. doi:10.1038/nchembio.1753
180. Shao J, Wang M, Yu G, Zhu S, Yu Y, Heng BC, Wu J, Ye H (2018) Synthetic far-red light-mediated CRISPR-dCas9 device for inducing functional neuronal differentiation. Proc Natl Acad Sci U S A 115 (29):E6722-E6730. doi:10.1073/pnas.1802448115
181. Zalatan JG, Lee ME, Almeida R, Gilbert LA, Whitehead EH, La Russa M, Tsai JC, Weissman JS, Dueber JE, Qi LS, Lim WA (2015) Engineering complex synthetic transcriptional programs with CRISPR RNA scaffolds. Cell 160 (1-2):339-350. doi:10.1016/j.cell.2014.11.052
182. Du D, Roguev A, Gordon DE, Chen M, Chen SH, Shales M, Shen JP, Ideker T, Mali P, Qi LS, Krogan NJ (2017) Genetic interaction mapping in mammalian cells using CRISPR interference. Nat Methods 14 (6):577-580. doi:10.1038/nmeth.4286
183. Gao Y, Xiong X, Wong S, Charles EJ, Lim WA, Qi LS (2016) Complex transcriptional modulation with orthogonal and inducible dCas9 regulators. Nat Methods 13 (12):1043-1049. doi:10.1038/nmeth.4042
184. Ryu MH, Gomelsky M (2014) Near-infrared light responsive synthetic c-di-GMP module for optogenetic applications. ACS Synth Biol 3 (11):802-810. doi:10.1021/sb400182x
185. Banaszynski LA, Chen LC, Maynard-Smith LA, Ooi AG, Wandless TJ (2006) A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. Cell 126 (5):995-1004. doi:10.1016/j.cell.2006.07.025
186. Iwamoto M, Bjorklund T, Lundberg C, Kirik D, Wandless TJ (2010) A general chemical method to regulate protein stability in the mammalian central nervous system. Chem Biol 17 (9):981-988. doi:10.1016/j.chembiol.2010.07.009
187. Miyazaki Y, Imoto H, Chen LC, Wandless TJ (2012) Destabilizing domains derived from the human estrogen receptor. J Am Chem Soc 134 (9):3942-3945. doi:10.1021/ja209933r
188. Balboa D, Weltner J, Eurola S, Trokovic R, Wartiovaara K, Otonkoski T (2015) Conditionally Stabilized dCas9 Activator for Controlling Gene Expression in Human Cell Reprogramming and Differentiation. Stem Cell Reports 5 (3):448-459. doi:10.1016/j.stemcr.2015.08.001
189. Maji B, Moore CL, Zetsche B, Volz SE, Zhang F, Shoulders MD, Choudhary A (2017) Multidimensional chemical control of CRISPR-Cas9. Nat Chem Biol 13 (1):9-11. doi:10.1038/nchembio.2224
190. Senturk S, Shirole NH, Nowak DG, Corbo V, Pal D, Vaughan A, Tuveson DA, Trotman LC, Kinney JB, Sordella R (2017) Rapid and tunable method to temporally control gene editing based on conditional Cas9 stabilization. Nat Commun 8:14370. doi:10.1038/ncomms14370
191. Tague EP, Dotson HL, Tunney SN, Sloas DC, Ngo JT (2018) Chemogenetic control of gene expression and cell signaling with antiviral drugs. Nat Methods 15 (7):519-522. doi:10.1038/s41592-018-0042-y
192. Chavez A, Scheiman J, Vora S, Pruitt BW, Tuttle M, E PRI, Lin S, Kiani S, Guzman CD, Wiegand DJ, Ter-Ovanesyan D, Braff JL, Davidsohn N, Housden BE, Perrimon N, Weiss R, Aach J, Collins JJ, Church GM (2015) Highly efficient Cas9-mediated transcriptional programming. Nat Methods 12 (4):326-328. doi:10.1038/nmeth.3312
193. Wu Y, Yang L, Chang T, Kandeel F, Yee JK (2020) A Small Molecule-Controlled Cas9 Repressible System. Mol Ther Nucleic Acids 19:922-932. doi:10.1016/j.omtn.2019.12.026

194. Monteys AM, Ebanks SA, Keiser MS, Davidson BL (2017) CRISPR/Cas9 Editing of the Mutant Huntingtin Allele In Vitro and In Vivo. Mol Ther 25 (1):12-23. doi:10.1016/j.ymthe.2016.11.010
195. Nishiyama J, Mikuni T, Yasuda R (2017) Virus-Mediated Genome Editing via Homology-Directed Repair in Mitotic and Postmitotic Cells in Mammalian Brain. Neuron 96 (4):755-768 e755. doi:10.1016/j.neuron.2017.10.004
196. Kemaladewi DU, Maino E, Hyatt E, Hou H, Ding M, Place KM, Zhu X, Bassi P, Baghestani Z, Deshwar AG, Merico D, Xiong HY, Frey BJ, Wilson MD, Ivakine EA, Cohn RD (2017) Correction of a splicing defect in a mouse model of congenital muscular dystrophy type 1A using a homology-directed-repair-independent mechanism. Nat Med 23 (8):984-989. doi:10.1038/nm.4367
197. Yin C, Zhang T, Qu X, Zhang Y, Putatunda R, Xiao X, Li F, Xiao W, Zhao H, Dai S, Qin X, Mo X, Young WB, Khalili K, Hu W (2017) In Vivo Excision of HIV-1 Provirus by saCas9 and Multiplex Single-Guide RNAs in Animal Models. Mol Ther 25 (5):1168-1186. doi:10.1016/j.ymthe.2017.03.012
198. Singh K, Evens H, Nair N, Rincon MY, Sarcar S, Samara-Kuko E, Chuah MK, VandenDriessche T (2018) Efficient In Vivo Liver-Directed Gene Editing Using CRISPR/Cas9. Mol Ther 26 (5):1241-1254. doi:10.1016/j.ymthe.2018.02.023
199. Kayesh MEH, Amako Y, Hashem MA, Murakami S, Ogawa S, Yamamoto N, Hifumi T, Miyoshi N, Sugiyama M, Tanaka Y, Mizokami M, Kohara M, Tsukiyama-Kohara K (2020) Development of an in vivo delivery system for CRISPR/Cas9-mediated targeting of hepatitis B virus cccDNA. Virus Res 290:198191. doi:10.1016/j.virusres.2020.198191
200. Krooss SA, Dai Z, Schmidt F, Rovai A, Fakhiri J, Dhingra A, Yuan Q, Yang T, Balakrishnan A, Steinbruck L, Srivaratharajan S, Manns MP, Schambach A, Grimm D, Bohne J, Sharma AD, Buning H, Ott M (2020) Ex Vivo/In vivo Gene Editing in Hepatocytes Using "All-in-One" CRISPR-Adeno-Associated Virus Vectors with a Self-Linearizing Repair Template. iScience 23 (1):100764. doi:10.1016/j.isci.2019.100764
201. Zuckermann M, Hovestadt V, Knobbe-Thomsen CB, Zapatka M, Northcott PA, Schramm K, Belic J, Jones DT, Tschida B, Moriarity B, Largaespada D, Roussel MF, Korshunov A, Reifenberger G, Pfister SM, Lichter P, Kawauchi D, Gronych J (2015) Somatic CRISPR/Cas9-mediated tumour suppressor disruption enables versatile brain tumour modelling. Nat Commun 6:7391. doi:10.1038/ncomms8391
202. Miller JB, Zhang S, Kos P, Xiong H, Zhou K, Perelman SS, Zhu H, Siegwart DJ (2017) Non-Viral CRISPR/Cas Gene Editing In Vitro and In Vivo Enabled by Synthetic Nanoparticle Co-Delivery of Cas9 mRNA and sgRNA. Angew Chem Int Ed Engl 56 (4):1059-1063. doi:10.1002/anie.201610209
203. Edvardsen RB, Leininger S, Kleppe L, Skaftnesmo KO, Wargelius A (2014) Targeted mutagenesis in Atlantic salmon (Salmo salar L.) using the CRISPR/Cas9 system induces complete knockout individuals in the F0 generation. PLoS One 9 (9):e108622. doi:10.1371/journal.pone.0108622
204. Datsomor AK, Zic N, Li K, Olsen RE, Jin Y, Vik JO, Edvardsen RB, Grammes F, Wargelius A, Winge P (2019) CRISPR/Cas9-mediated ablation of elovl2 in Atlantic salmon (Salmo salar L.) inhibits elongation of polyunsaturated fatty acids and induces Srebp-1 and target genes. Sci Rep 9 (1):7533. doi:10.1038/s41598-019-43862-8
205. Li M, Yang H, Zhao J, Fang L, Shi H, Li M, Sun Y, Zhang X, Jiang D, Zhou L, Wang D (2014) Efficient and heritable gene targeting in tilapia by CRISPR/Cas9. Genetics 197 (2):591-599. doi:10.1534/genetics.114.163667
206. Jiang DN, Yang HH, Li MH, Shi HJ, Zhang XB, Wang DS (2016) gsdf is a downstream gene of dmrt1 that functions in the male sex determination pathway of the Nile tilapia. Mol Reprod Dev 83 (6):497-508. doi:10.1002/mrd.22642

207. Feng R, Fang L, Cheng Y, He X, Jiang W, Dong R, Shi H, Jiang D, Sun L, Wang D (2015) Retinoic acid homeostasis through aldh1a2 and cyp26a1 mediates meiotic entry in Nile tilapia (Oreochromis niloticus). Sci Rep 5:10131. doi:10.1038/srep10131
208. Xie QP, He X, Sui YN, Chen LL, Sun LN, Wang DS (2016) Haploinsufficiency of SF-1 Causes Female to Male Sex Reversal in Nile Tilapia, Oreochromis niloticus. Endocrinology 157 (6):2500-2514. doi:10.1210/en.2015-2049
209. Zhang X, Wang H, Li M, Cheng Y, Jiang D, Sun L, Tao W, Zhou L, Wang Z, Wang D (2014) Isolation of doublesex- and mab-3-related transcription factor 6 and its involvement in spermatogenesis in tilapia. Biol Reprod 91 (6):136. doi:10.1095/biolreprod.114.121418
210. Li M, Sun Y, Zhao J, Shi H, Zeng S, Ye K, Jiang D, Zhou L, Sun L, Tao W, Nagahama Y, Kocher TD, Wang D (2015) A Tandem Duplicate of Anti-Mullerian Hormone with a Missense SNP on the Y Chromosome Is Essential for Male Sex Determination in Nile Tilapia, Oreochromis niloticus. PLoS Genet 11 (11):e1005678. doi:10.1371/journal.pgen.1005678
211. Jiang D, Chen J, Fan Z, Tan D, Zhao J, Shi H, Liu Z, Tao W, Li M, Wang D (2017) CRISPR/Cas9-induced disruption of wt1a and wt1b reveals their different roles in kidney and gonad development in Nile tilapia. Dev Biol 428 (1):63-73. doi:10.1016/j.ydbio.2017.05.017
212. Li M, Feng R, Ma H, Dong R, Liu Z, Jiang W, Tao W, Wang D (2016) Retinoic acid triggers meiosis initiation via stra8-dependent pathway in Southern catfish, Silurus meridionalis. Gen Comp Endocrinol 232:191-198. doi:10.1016/j.ygcen.2016.01.003
213. Kishimoto K WY, Yoshiura Y, Toyoda A, Ueno T, Fukuyama H, Kato K, Kinoshita M (2018) Production of a breed of red sea bream Pagrus major with an increase of skeletal muscle mass and reduced body length by genome editing with CRISPR/Cas9. Aquaculture 495:415-427
214. Cleveland BM, Yamaguchi G, Radler LM, Shimizu M (2018) Editing the duplicated insulin-like growth factor binding protein-2b gene in rainbow trout (Oncorhynchus mykiss). Sci Rep 8 (1):16054. doi:10.1038/s41598-018-34326-6
215. Yu H, Li H, Li Q, Xu R, Yue C, Du S (2019) Targeted Gene Disruption in Pacific Oyster Based on CRISPR/Cas9 Ribonucleoprotein Complexes. Mar Biotechnol (NY) 21 (3):301-309. doi:10.1007/s10126-019-09885-y
216. Zu Y, Zhang X, Ren J, Dong X, Zhu Z, Jia L, Zhang Q, Li W (2016) Biallelic editing of a lamprey genome using the CRISPR/Cas9 system. Sci Rep 6:23496. doi:10.1038/srep23496
217. Jiang W, Zhou H, Bi H, Fromm M, Yang B, Weeks DP (2013) Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. Nucleic Acids Res 41 (20):e188. doi:10.1093/nar/gkt780
218. Jiang W, Yang B, Weeks DP (2014) Efficient CRISPR/Cas9-mediated gene editing in Arabidopsis thaliana and inheritance of modified genes in the T2 and T3 generations. PLoS One 9 (6):e99225. doi:10.1371/journal.pone.0099225
219. Feng Z, Mao Y, Xu N, Zhang B, Wei P, Yang DL, Wang Z, Zhang Z, Zheng R, Yang L, Zeng L, Liu X, Zhu JK (2014) Multigeneration analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in Arabidopsis. Proc Natl Acad Sci U S A 111 (12):4632-4637. doi:10.1073/pnas.1400822111
220. Mao Y, Zhang H, Xu N, Zhang B, Gou F, Zhu JK (2013) Application of the CRISPR-Cas system for efficient genome engineering in plants. Mol Plant 6 (6):2008-2011. doi:10.1093/mp/sst121
221. Schiml S, Fauser F, Puchta H (2014) The CRISPR/Cas system can be used as nuclease for in planta gene targeting and as paired nickases for directed mutagenesis in Arabidopsis resulting in heritable progeny. Plant J 80 (6):1139-1150. doi:10.1111/tpj.12704
222. Steinert J, Schiml S, Fauser F, Puchta H (2015) Highly efficient heritable plant genome engineering using Cas9 orthologues from Streptococcus thermophilus and Staphylococcus aureus. Plant J 84 (6):1295-1305. doi:10.1111/tpj.13078

223. Xing HL, Dong L, Wang ZP, Zhang HY, Han CY, Liu B, Wang XC, Chen QJ (2014) A CRISPR/Cas9 toolkit for multiplex genome editing in plants. BMC Plant Biol 14:327. doi:10.1186/s12870-014-0327-y
224. Fauser F, Schiml S, Puchta H (2014) Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in Arabidopsis thaliana. Plant J 79 (2):348-359. doi:10.1111/tpj.12554
225. Wang S, Zhang S, Wang W, Xiong X, Meng F, Cui X (2015) Efficient targeted mutagenesis in potato by the CRISPR/Cas9 system. Plant Cell Rep 34 (9):1473-1476. doi:10.1007/s00299-015-1816-7
226. Yan L, Wei S, Wu Y, Hu R, Li H, Yang W, Xie Q (2015) High-Efficiency Genome Editing in Arabidopsis Using YAO Promoter-Driven CRISPR/Cas9 System. Mol Plant 8 (12):1820-1823. doi:10.1016/j.molp.2015.10.004
227. Wolter F, Klemm J, Puchta H (2018) Efficient in planta gene targeting in Arabidopsis using egg cell-specific expression of the Cas9 nuclease of Staphylococcus aureus. Plant J 94 (4):735-746. doi:10.1111/tpj.13893
228. Wang ZP, Xing HL, Dong L, Zhang HY, Han CY, Wang XC, Chen QJ (2015) Egg cell-specific promoter-controlled CRISPR/Cas9 efficiently generates homozygous mutants for multiple target genes in Arabidopsis in a single generation. Genome Biol 16:144. doi:10.1186/s13059-015-0715-0
229. Hyun Y, Kim J, Cho SW, Choi Y, Kim JS, Coupland G (2015) Site-directed mutagenesis in Arabidopsis thaliana using dividing tissue-targeted RGEN of the CRISPR/Cas system to generate heritable null alleles. Planta 241 (1):271-284. doi:10.1007/s00425-014-2180-5
230. Mao Y, Zhang Z, Feng Z, Wei P, Zhang H, Botella JR, Zhu JK (2016) Development of germ-line-specific CRISPR-Cas9 systems to improve the production of heritable gene modifications in Arabidopsis. Plant Biotechnol J 14 (2):519-532. doi:10.1111/pbi.12468
231. Tsutsui H, Higashiyama T (2017) pKAMA-ITACHI Vectors for Highly Efficient CRISPR/Cas9-Mediated Gene Knockout in Arabidopsis thaliana. Plant Cell Physiol 58 (1):46-56. doi:10.1093/pcp/pcw191
232. Wu R, Lucke M, Jang YT, Zhu W, Symeonidi E, Wang C, Fitz J, Xi W, Schwab R, Weigel D (2018) An efficient CRISPR vector toolbox for engineering large deletions in Arabidopsis thaliana. Plant Methods 14:65. doi:10.1186/s13007-018-0330-7
233. Upadhyay SK, Kumar J, Alok A, Tuli R (2013) RNA-guided genome editing for target gene mutations in wheat. G3 (Bethesda) 3 (12):2233-2238. doi:10.1534/g3.113.008847
234. Gao J, Wang G, Ma S, Xie X, Wu X, Zhang X, Wu Y, Zhao P, Xia Q (2015) CRISPR/Cas9-mediated targeted mutagenesis in Nicotiana tabacum. Plant Mol Biol 87 (1-2):99-110. doi:10.1007/s11103-014-0263-0
235. Endo A, Masafumi M, Kaya H, Toki S (2016) Efficient targeted mutagenesis of rice and tobacco genomes using Cpf1 from Francisella novicida. Sci Rep 6:38169. doi:10.1038/srep38169
236. Kim H, Kim ST, Ryu J, Kang BC, Kim JS, Kim SG (2017) CRISPR/Cpf1-mediated DNA-free plant genome editing. Nat Commun 8:14406. doi:10.1038/ncomms14406
237. Fan D, Liu T, Li C, Jiao B, Li S, Hou Y, Luo K (2015) Efficient CRISPR/Cas9-mediated Targeted Mutagenesis in Populus in the First Generation. Sci Rep 5:12217. doi:10.1038/srep12217
238. Xie K, Minkenberg B, Yang Y (2015) Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. Proc Natl Acad Sci U S A 112 (11):3570-3575. doi:10.1073/pnas.1420294112
239. Miao J, Guo D, Zhang J, Huang Q, Qin G, Zhang X, Wan J, Gu H, Qu LJ (2013) Targeted mutagenesis in rice using CRISPR-Cas system. Cell Res 23 (10):1233-1236. doi:10.1038/cr.2013.123
240. Xu R, Li H, Qin R, Wang L, Li L, Wei P, Yang J (2014) Gene targeting using the Agrobacterium tumefaciens-mediated CRISPR-Cas system in rice. Rice (N Y) 7 (1):5. doi:10.1186/s12284-014-0005-6

241. Zhou H, Liu B, Weeks DP, Spalding MH, Yang B (2014) Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. Nucleic Acids Res 42 (17):10903-10914. doi:10.1093/nar/gku806
242. Zhang H, Zhang J, Wei P, Zhang B, Gou F, Feng Z, Mao Y, Yang L, Zhang H, Xu N, Zhu JK (2014) The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. Plant Biotechnol J 12 (6):797-807. doi:10.1111/pbi.12200
243. Mikami M, Toki S, Endo M (2016) Precision Targeted Mutagenesis via Cas9 Paired Nickases in Rice. Plant Cell Physiol 57 (5):1058-1068. doi:10.1093/pcp/pcw049
244. Tang X, Lowder LG, Zhang T, Malzahn AA, Zheng X, Voytas DF, Zhong Z, Chen Y, Ren Q, Li Q, Kirkland ER, Zhang Y, Qi Y (2017) A CRISPR-Cpf1 system for efficient genome editing and transcriptional repression in plants. Nat Plants 3:17103. doi:10.1038/nplants.2017.103
245. Shi J, Gao H, Wang H, Lafitte HR, Archibald RL, Yang M, Hakimi SM, Mo H, Habben JE (2017) ARGOS8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions. Plant Biotechnol J 15 (2):207-216. doi:10.1111/pbi.12603
246. Jacobs TB, LaFayette PR, Schmitz RJ, Parrott WA (2015) Targeted genome modifications in soybean with CRISPR/Cas9. BMC Biotechnol 15:16. doi:10.1186/s12896-015-0131-2
247. Cai Y, Chen L, Liu X, Sun S, Wu C, Jiang B, Han T, Hou W (2015) CRISPR/Cas9-Mediated Genome Editing in Soybean Hairy Roots. PLoS One 10 (8):e0136064. doi:10.1371/journal.pone.0136064
248. Sun X, Hu Z, Chen R, Jiang Q, Song G, Zhang H, Xi Y (2015) Targeted mutagenesis in soybean using the CRISPR-Cas9 system. Sci Rep 5:10342. doi:10.1038/srep10342
249. Du H, Zeng X, Zhao M, Cui X, Wang Q, Yang H, Cheng H, Yu D (2016) Efficient targeted mutagenesis in soybean by TALENs and CRISPR/Cas9. J Biotechnol 217:90-97. doi:10.1016/j.jbiotec.2015.11.005
250. Butler NM, Atkins PA, Voytas DF, Douches DS (2015) Generation and Inheritance of Targeted Mutations in Potato (Solanum tuberosum L.) Using the CRISPR/Cas System. PLoS One 10 (12):e0144591. doi:10.1371/journal.pone.0144591
251. Lawrenson T, Shorinola O, Stacey N, Li C, Ostergaard L, Patron N, Uauy C, Harwood W (2015) Induction of targeted, heritable mutations in barley and Brassica oleracea using RNA-guided Cas9 nuclease. Genome Biol 16:258. doi:10.1186/s13059-015-0826-7
252. Sugano SS, Shirakawa M, Takagi J, Matsuda Y, Shimada T, Hara-Nishimura I, Kohchi T (2014) CRISPR/Cas9-mediated targeted mutagenesis in the liverwort Marchantia polymorpha L. Plant Cell Physiol 55 (3):475-481. doi:10.1093/pcp/pcu014
253. Brooks C, Nekrasov V, Lippman ZB, Van Eck J (2014) Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated9 system. Plant Physiol 166 (3):1292-1297. doi:10.1104/pp.114.247577
254. Ito Y, Nishizawa-Yokoi A, Endo M, Mikami M, Toki S (2015) CRISPR/Cas9-mediated mutagenesis of the RIN locus that regulates tomato fruit ripening. Biochem Biophys Res Commun 467 (1):76-82. doi:10.1016/j.bbrc.2015.09.117
255. Alagoz Y, Gurkok T, Zhang B, Unver T (2016) Manipulating the Biosynthesis of Bioactive Compound Alkaloids for Next-Generation Metabolic Engineering in Opium Poppy Using CRISPR-Cas 9 Genome Editing Technology. Sci Rep 6:30910. doi:10.1038/srep30910
256. Chandrasekaran J, Brumin M, Wolf D, Leibman D, Klap C, Pearlsman M, Sherman A, Arazi T, Gal-On A (2016) Development of broad virus resistance in non-transgenic cucumber using CRISPR/Cas9 technology. Mol Plant Pathol 17 (7):1140-1153. doi:10.1111/mpp.12375
257. Jia H, Wang N (2014) Xcc-facilitated agroinfiltration of citrus leaves: a tool for rapid functional analysis of transgenes in citrus leaves. Plant Cell Rep 33 (12):1993-2001. doi:10.1007/s00299-014-1673-9
258. Jia H, Orbovic V, Jones JB, Wang N (2016) Modification of the PthA4 effector binding elements in Type I CsLOB1 promoter using Cas9/sgRNA to produce transgenic Duncan grapefruit

alleviating XccDeltapthA4:dCsLOB1.3 infection. Plant Biotechnol J 14 (5):1291-1301. doi:10.1111/pbi.12495
259. Ren C, Liu X, Zhang Z, Wang Y, Duan W, Li S, Liang Z (2016) CRISPR/Cas9-mediated efficient targeted mutagenesis in Chardonnay (Vitis vinifera L.). Sci Rep 6:32289. doi:10.1038/srep32289
260. Malnoy M, Viola R, Jung MH, Koo OJ, Kim S, Kim JS, Velasco R, Nagamangala Kanchiswamy C (2016) DNA-Free Genetically Edited Grapevine and Apple Protoplast Using CRISPR/Cas9 Ribonucleoproteins. Front Plant Sci 7:1904. doi:10.3389/fpls.2016.01904
261. Levy JM, Yeh WH, Pendse N, Davis JR, Hennessey E, Butcher R, Koblan LW, Comander J, Liu Q, Liu DR (2020) Cytosine and adenine base editing of the brain, liver, retina, heart and skeletal muscle of mice via adeno-associated viruses. Nat Biomed Eng 4 (1):97-110. doi:10.1038/s41551-019-0501-5
262. Bao J, Liu W, Xie J, Xu L, Guan M, Lei F, Zhao Y, Huang Y, Xia J, Li H (2019) Nix Co3-x O4 Nanoneedle Arrays Grown on Ni Foam as an Efficient Bifunctional Electrocatalyst for Full Water Splitting. Chem Asian J 14 (3):480-485. doi:10.1002/asia.201801710

## Figure captions

**Fig. 1** Schematic Diagrams of *in vivo* CRISPR/Cas delivery modes and vehicles in the different biological system**.** Systems used for delivery of CRISPR/Cas components (a) can be separated into two major categories, CRISPR/Cas delivery mode (b) and delivery vehicle (c). Three CRISPR/Cas delivery models including DNA (plasmid encoding both the Cas protein and the gRNA), mRNA (RNA for Cas protein translation and a separate gRNA) and protein (Cas protein with gRNA as a ribonucleoprotein complex, RNP) can be delivered in to mammalians, aquacultures or plants via bacterial or viral vectors, non-viral carriers and physically direct delivery (d)

**Fig. 2** CRISPR/Cas-mediated gene editing strategies. The versatile CRISPR/Cas system is a powerful tool for DNA, RNA editing, gene modulation and base, prime editing by leveraging different approaches (a) to achieve numerous gene editing outcomes (b)

**Fig. 3** Representation of different delivery methods of the CRISPR/Cas system to target organs in the rodent. Delivery methods including virus-based (lentivirus, adenovirus and adeno-associated virus) and non-virus-based (Plasmid-, RNA- or Protein-based) delivery have been used to deliver CRISPR/Cas system to different organs in the rodent

**Fig. 4** Schematic representation of main methods used to modify plant genome by CRISPR/Cas system. The schematic diagram showing major steps involved in the generation of gene edited plants using direct and indirect methods including agroinfiltration (a), protoplast transfection (b), agroinfection (c), and virus infection (d) and biolistic particle delivery (e)

# Tables

**Table 1** Delivery methods for CRISPR/Cas system in mammals

| Model- Target tissue (disease) | Delivery methods | | Editing gene | Reference |
|---|---|---|---|---|
| | Viral delivery system | No viral delivery system | | |
| Mouse – Blood (Myeloid malignancy) | Lentivirus | | *Tet2, Runx1, Dnmt3a, Ezh2, Nf1, Smc3, p53 and Asxl1* | [48] |
| Mouse – Lung (Lung Cancer) | Lentivirus | | *Pten, Nkx2-1* | [49] |
| Mouse – Liver (NASH) | Adenovirus | | *Pten* | [58] |
| Mouse – Liver (Cardiovascular disease) | Adenovirus | | *Pcsk9* | [59] |
| Mouse – Lung (Lung Cancer) | Adenovirus | | *Eml4, Alk* | [60] |
| Mouse – Brain | Adeno-associated virus | | *Mecp2, Dnmt1, Dnmt3a and Dnmt3b* | [64] |
| Mouse – Eye (LCA) | Adeno-associated virus | | *Yfp* | [65] |
| Mouse – Muscle (DMD) | Adeno-associated virus | | *Dmd* | [70] [71] |
| Mouse – Lung (Lung Cancer) | Adeno-associated virus | | *Kras, p53, and Lkb1* | [76] |
| Mouse – Liver (OTC deficiency) | Adeno-associated virus | | *Otc* | [74] |
| Mouse – Liver | Adeno-associated virus | | *Pcsk9* | [80] |

| | | | |
|---|---|---|---|
| (Cardiovascular disease) | | | |
| Mouse – Brain<br>(Huntington disease) | Adeno-associated virus | *Htt* | [194] |
| Mouse – Brain<br>(GBM) | Adeno-associated virus | *Trp53, Nf1, or Rb1* | [78] |
| Mouse – Brain | Adeno-associated virus | *Camk2a, Erk2, Actb* | [195] |
| Mouse – Muscle<br>(DMD) | Adeno-associated virus | *Dmd* | [69] |
| Mouse – Muscle<br>(MDC1A) | Adeno-associated virus | *Lama2* | [196] |
| Mouse – Eye<br>(Retinal degeneration) | Adeno-associated virus | *Nrl* | [66] |
| Mouse – Spleen, lungs, heart, colon, and brain<br>(HIV/AIDS) | Adeno-associated virus | *HIV-1 DNA* | [197] |
| Mouse – Liver<br>(Hemophilia B) | Adeno-associated virus | *F9* | [198] |
| Mouse – Liver<br>(Cancer) | Adeno-associated virus | *Tsgs* | [79] |
| Mouse – Liver | Adeno-associated virus | *HBV cccDNA* | [199] |
| Mouse – Eye<br>(X-Linked Retinitis Pigmentosa) | Adeno-associated virus | *Rpgr* | [67] |
| Mouse – Liver<br>(Tyrosinemia) | Adeno-associated virus | *Fah* | [200] |

| | | | | |
|---|---|---|---|---|
| Mouse – Liver (OTC deficiency) | Adeno-associated virus | | *Otc* | [73] |
| Mouse – Muscle (DMD) | Adeno-associated virus | | *Dmd* | [72] |
| Mouse – Brain (Schizophrenia) | Adeno-associated virus | | *Mir137* | [77] |
| Mouse – Muscle (ALS) | Adeno-associated virus | | *Igf1* | [68] |
| Mouse – Liver (Tyrosinemia) | | Plasmid – based | *Fah* | [86] |
| Mouse – Liver | | Plasmid – based | *HBsAg* | [87] |
| Rat – Eye Retinitis pigmentosa) | | Plasmid – based | *(Rho(S334))* | [88] |
| Mouse – Eye (Retinitis pigmentosa) | | Plasmid – based | *Rho* | [89] |
| Mouse – Brain (MB and GBM) | | Plasmid – based | *Trp53, Pten, Nf1* | [201] |
| Mouse – Brain | | Plasmid – based | *Satbs* | [91] |
| Mouse – Eye (IRDs) | | Plasmid- based | *(Rho)-P23H* | [90] |
| Mouse – Liver (Tyrosinemia) | | mRNA-based | *Fah* | [92] |
| Mouse – Liver (Cardiovascular disease) | | mRNA-based | *Pcsk9* | [93] [95] |
| Mouse – Liver | | mRNA-based | *Ttr* | [94] |

| | | | |
|---|---|---|---|
| (Tyrosinemia) | | | |
| Mouse – Liver, Kidney, Lung | mRNA-based | *floxed tdTomato* | [202] |
| Mouse – Ear | Protein – based | *Egfp* | [96] |
| Mouse – Muscle (DMD) | Protein – based | *Dmd* | [102] |
| Mouse – Brain (FXS) | Protein – based | *Grm5* | [103] |
| Mouse – Brain (Alzheimer's disease) | Protein – based | *Th, Bace1* | [98] |
| Mouse – Liver and spleen | Protein – based | *Pten* | [101] |
| Mouse – Liver and spleen (HT1) | Protein – based | *Hpd* | [97] |

ALS, Amyotrophic lateral sclerosis; DMD, Duchenne muscular dystrophy; FXS, Fragile X syndrome; GBM, Glioblastoma; HIV/AIDS, Human immunodeficiency virus/acquired immunodeficiency syndrome; HT1, Hereditary tyrosinemia type I; IRDs, Inherited retinal degenerations; LCA, Leber congenital amaurosis; MB, Medulloblastoma; MDC1A, congenital muscular dystrophy type 1A; NASH, Non-alcoholic steatohepatitis; OTC, Ornithine transcarbamylase

1462 **Table 2** Delivery methods for CRISPR/Cas system in aquacultures
1463

| Species | Applications | References |
|---|---|---|
| Zebrafish | Gene editing of multiple genes | [106] |
| Rohu carp | Gene editing of Tlr22 gene | [108] |
| Atlantic salmon | Gene editing of Dnd gene | [109] |
| | Gene editing of Tyr and Slc45a2 genes | [203] |
| | Gene editing of Elovl2 gene | [204] |
| Channel catfish | Gene editing of Mstn gene | [110] |
| | Gene editing of Ticam and Rbl gene | [112] |
| Common carp | Gene editing of Sp7 and Mstn genes | [111] |
| Grass carp | Gene editing of *Gcjam-a* gene | [37] |
| Tilapia | Gene editing of *Nanos2, Nanos3, Dmrt1* and *Foxl2* genes | [205] |
| | Gene editing of *Gsdf* gene | [206] |
| | Gene editing of *Aldh1a2* and *Cyp26a1* genes | [207] |
| | Gene editing of *Sf-1* gene | [208] |
| | Gene editing of *Dmrt6* gene | [209] |
| | Gene editing of *Amhy* gene | [210] |
| | Gene editing of *Wt1a* and *Wt1b* genes | [211] |
| Southern catfish | Gene editing of *Aldh1a2* gene | [212] |
| Sea bream | Gene editing of Mstn gene | [213] |

| | | |
|---|---|---|
| Rainbow trout | Gene editing of Igfbp2b1 and Igfbp2b2 genes | [214] |
| Pacific Oyster | Gene editing of Mstn and Twist genes | [215] |
| Northern Chinese lamprey | Gene editing of multiple gene | [216] |

1466 **Table 3** Delivery methods for CRISPR/Cas system in plants
1467

| Species | Delivery methods | | Edited gene | References |
|---|---|---|---|---|
| | **Stable** | **Transient** | | |
| Arabidopsis thaliana | | Protoplast, Agroinfiltration | *Pds3, Fls2, Rack1b* and *Rack1c* | [124] |
| Arabidopsis thaliana | | Agroinfiltration | *Gfp* | [217] |
| Arabidopsis thaliana | Agrobacterium | | *Gfp* | [218] |
| Arabidopsis thaliana | Agrobacterium | Protoplast | *Bri1, Jaz1, Gai* and *Yfp* | [124] |
| Arabidopsis thaliana | Agrobacterium | | *Bri1, Jaz1, Gai, Chli, Ap1, Tt4* and *Guus* | [219] |
| Arabidopsis thaliana | Agrobacterium | Protoplast | *Chl1, Chl2* and *Tt4i* | [220] |
| Arabidopsis thaliana | Agrobacterium | | *Adh1* | [221,222] |
| Arabidopsis thaliana | Agrobacterium | | *Try, Cpc* and *Etc2* | [223] |
| Arabidopsis thaliana | Agrobacterium | | *5g55580* | [147] |
| Arabidopsis thaliana | Agrobacterium | | *Adh1, Tt4, Rtel1* and *Guus* | [224] |
| Arabidopsis thaliana | Agrobacterium | | *Etc2, Try* and *Cpc* | [225] |
| Arabidopsis thaliana | Agrobacterium | | *Bri1* | [226] |
| Arabidopsis thaliana | Agrobacterium | | *Als* | [227] |
| Arabidopsis thaliana | Agrobacterium | | *Etc2, Try, Cpc* and *Chli1/2* | [228] |
| Arabidopsis thaliana | Agrobacterium | | *Ft* and *SplA4* | [229] |
| Arabidopsis thaliana | Agrobacterium | | *Ap1, Tt4* and *Gl2* | [230] |
| Arabidopsis thaliana | Agrobacterium | | *Pds3, Ag, Duo1* and *Adh1* | [231] |

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|---|---|---|---|---|
| Arabidopsis thaliana | Agrobacterium | | *At3g04220* | [232] |
| Nicotiana benthamiana | | Protoplast, Agroinfiltration | *Pds3* | [124] |
| Nicotiana benthamiana | | Agroinfiltration | *Pds* | [123] |
| Nicotiana benthamiana | Agrobacterium | Agroinfiltration | *Pds* | [123] |
| Nicotiana benthamiana | | Agroinfiltration | of *Pds* | [233] |
| Nicotiana benthamiana | | Agroinfiltration | *Edll* and *Srdx* | [125] |
| Nicotiana benthamiana | Virus | | *Pcna* and *Pds* | [138] |
| Nicotiana benthamiana | | Agroinfiltration | *Gfp* | [217] |
| Nicotiana benthamiana | Virus | | *Pds, Isph* and *Fsgus* | [142] |
| Nicotiana tabacum | | Protoplast | *Pds* and *Pdr6* | [234] |
| Nicotiana tabacum | Virus | | *SurA* and *SurB* | [140] |
| Nicotiana tabacum | | Protoplast | *Pds* | [134] |
| Nicotiana tabacum | Agrobacterium | | *Pds* and *Stf1* | [235] |
| Nicotiana tabacum | | Protoplast | *Aoc* genes | [236] |
| Populus | Agrobacterium | | *Pds* | [237] |
| Oryza sativa | Agrobacterium | | *Roc5, Spp* and *Ysa* | [124] |
| Oryza sativa | | Protoplast | *Sweet11, Sweet14* and *Dsred* | [217] |
| Oryza sativa | | Agroinfiltration | *Pds* | [123] |
| Oryza sativa | Biolistic | Protoplast | *Pds-sp1, Badh2, 02g23823* and *Mpk2* | [146] |
| Oryza sativa | Agrobacterium | Protoplast | *Myb*1 | [220] |

| | | | | |
|---|---|---|---|---|
| Oryza sativa | | Protoplast | *Mpk5* gene | [238] |
| Oryza sativa | Agrobacterium | | *Cao* and *Lazy1* | [239] |
| Oryza sativa | Agrobacterium | | *Ptg1, Ptg2, Ptg3, Ptg4, Ptg5, Ptg6, Ptg7, Ptg8* and *Ptg9* | [238] |
| Oryza sativa | Agrobacterium | | *Bel* | [240] |
| Oryza sativa | Agrobacterium | | *Ftl, Gstu, Mrp15* and *Anp waxy* | [147] |
| Oryza sativa | Agrobacterium | Protoplast | *Sweet1a, Sweet1b, Sweet 11, Sweet13* and *P450* | [241] |
| Oryza sativa | Agrobacterium | | *Pds, Pms3, Epsps, Derf1, Msh1, Myb5, Myb1, Roc5, Spp* and *Ysa* | [242] |
| Oryza sativa | Agrobacterium | | *Dmc1a* | [243] |
| Oryza sativa | | Protoplast | *Pds, Dep1, Roc5* and *miR159b* | [244] |
| Oryza sativa | Agrobacterium | | *Dl* and *Als* | [235] |
| Oryza sativa | | Protoplast | *Epsps, Hct* and *Pds* | [34] |
| Triticum aestivum | | Protoplast | *Mlo* | [146] |
| Triticum aestivum | Biolistic | Protoplast | *Gw2* | [149] |
| Triticum aestivum | | Agroinfiltration | *Inox* and *Pds* | [233] |
| Zea mays | | Protoplast | *Ipk* | [130] |
| Zea mays | Agrobacterium | Protoplast | *Hkt1* | [223] |
| Zea mays | Biolistic | | *Lig1, Ms26, Ms45, Als1* and *Als2* | [148] |
| Zea mays | Biolistic | | *Lig, Ms26, Ms45* and *Als2* | [150] |
| Zea mays | Biolistic | | *Argos8* | [245] |

| | | | | |
|---|---|---|---|---|
| Glycine max | Agrobacterium | | *Gfp, 07g14530, 01gDDM1, 11gDDM1, Met1-04g, Met1-06g, miR1514* and *miR1509* | [246] |
| Glycine max | Biolistic | | *Dd20, Dd43* and *Als1* | [247] |
| Glycine max | Agrobacterium | | *06g14180, 08g02290* and *Glyma12g37050* | [248] |
| Glycine max | Agrobacterium | | *Bar, Fei, Fei2* and *Shr* | [247] |
| Glycine max | Agrobacterium | | *Pds11* and *GlymaPds18* | [249] |
| Glycine max | | Protoplast | *Fad2-1a* and *Fad2-1b* | [236] |
| Solanum tuberosum | Agrobacterium | | *Iaa2* | [225] |
| Solanum tuberosum | Agrobacterium | | *Als1* | [250] |
| Solanum tuberosum | | Protoplast | *Gbss* | [144] |
| Hordeum vulgare | Agrobacterium | | *Pm19* | [251] |
| Liverwort | Agrobacterium | | *Arf1* | [252] |
| Solanum lycopersicum | Agrobacterium | | *Ago7* | [253] |
| Solanum lycopersicum | Virus | | *Ant1* | [129] |
| Solanum lycopersicum | Agrobacterium | | *Rin* | [254] |
| Brassica oleracea | Agrobacterium | | *C.ga4.a* | [251] |
| Opium poppy | | Agroinfiltration | *4'Omt2* | [255] |
| Cucumis sativus | Agrobacterium | | *EIf4e* | [256] |
| Citrus x sinensis | | Agroinfiltration | *Pds* | [257] |
| Citrus x paradisi | Agrobacterium | Agroinfiltration | *Cspds* | [257] |

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|---|---|---|---|---|
| Citrus x paradisi | Agrobacterium | Agroinfiltration | *Cslob1* | [258] |
| Vitis vinifera | Agrobacterium | | *Idndh* | [259] |
| Vitis vinifera | | Protoplast | *Mlo7* | [260] |
| Malus malus | | Protoplast | *Dipm-1, Dipm-2* and *Dipm-4* | [260] |

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**Table 4** Summary of regulatory CRISPR/Cas systems

| **Type of system** | Split-Cas9 | | | Light-inducible | Destabilizing domain | NS3 domain |
|---|---|---|---|---|---|---|
| | Intein-inducible | Rapamycin-inducible | Photoactivatable | | | |
| ***In vivo* studies** | [261] | n/a | n/a | [180] | [262] | n/a |
| **Delivery vehicle** | Viral-based delivery: AAV | n/a | n/a | DNA-based delivery: Electroporation | DNA-based delivery: tail vein hydrodynamic injection | n/a |