CellPress

Trends in Biotechnology

Review

Next-Generation CRISPR Technologies and Their Applications in Gene and Cell Therapy

M. Alejandra Zeballos C.[1] and Thomas Gaj[1,2,*]

The emergence of clustered regularly interspaced short palindromic repeat (CRISPR) nucleases has transformed biotechnology by providing an easy, efficient, and versatile platform for editing DNA. However, traditional CRISPR-based technologies initiate editing by activating DNA double-strand break (DSB) repair pathways, which can cause adverse effects in cells and restrict certain therapeutic applications of the technology. To this end, several new CRISPR-based modalities have been developed that are capable of catalyzing editing without the requirement for a DSB. Here, we review three of these technologies: base editors, prime editors, and RNA-targeting CRISPR-associated protein (Cas)13 effectors. We discuss their strengths compared to traditional gene-modifying systems, we highlight their emerging therapeutic applications, and we examine challenges facing their safe and effective clinical implementation.

Highlights

Next-generation CRISPR technologies hold the potential to expand the capabilities of therapeutic editing.

Advanced CRISPR modalities harness the versatility and programmability of traditional CRISPR systems but can catalyze highly precise editing outcomes without a DNA double-strand break.

Next-generation CRISPR technologies include base editors, prime editors, and RNA-targeting Cas13 effectors.

The continued refinement of these technologies could enable their safe and effective implementation to treat a range of disorders.

Next-Generation CRISPR Technologies: the Need to Move Beyond the DNA DSB for Therapeutic Gene Editing

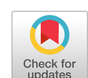
The rise of programmable and sequence-specific technologies capable of correcting, replacing, and deleting specific DNA sequences in cells has transformed modern biology, making the creation of designer cell lines and whole organisms a largely routine endeavor, and providing a highly efficient means for implementing therapeutic gene correction. The core technology behind these advances are targeted nucleases [1], which stimulate the process of gene-editing by introducing a targeted DNA **double-strand break** (**DSB**, see Glossary) that then activates cellular DNA repair pathways [2–4] which, in turn, can be harnessed to modify the targeted genomic site. Although several platforms have been developed to facilitate this process, including zinc-finger nucleases [5] and transcription activator-like effector nucleases [6], technologies derived from naturally occurring CRISPR systems have emerged as the method of choice for most genome engineers [7–11], as CRISPR systems are versatile, efficient and easy to use.

Traditional CRISPR technologies typically consist of two components: a Cas protein that cleaves nucleic acids and a **single guide RNA** (sgRNA) that binds to the Cas protein and directs it to a specific nucleic acid sequence – always adjacent to a conserved and compatible **protospacer adjacent motif** (PAM) or **protospacer flanking site** (PFS) – for its cleavage [9]. Because of the general efficiency and the overall specificity with which they carry out this process, CRISPR technologies have emerged as promising therapeutic modalities. In fact, within only 6 years of the first reports demonstrating their ability to edit DNA in mammalian cells [8,10,11], CRISPR systems were used to successfully edit immune cells which were then safely transplanted to cancer patients [12,13]. Clinical trials aimed at evaluating the ability of CRISPR nucleases to safely correct sickle cell disease (ClinicalTrials.gov Identifier: NCT03745287) and β-thalassemia (NCT03655678), two inherited blood disorders, as well as Leber congenital amaurosis type 10 (NCT03872479), an inherited retinal disorder, have also been initiated, which further demonstrates the therapeutic potential of the technology. However, despite the immense promise that CRISPR nucleases hold for treating a number of diseases, their reliance on DNA breaks to

[1]Department of Bioengineering, University of Illinois, Urbana, IL 61801, USA
[2]Carl R. Woese Institute for Genomic Biology, University of Illinois, Urbana, IL 61801, USA

*Correspondence: gaj@illinois.edu (T. Gaj).

 https://doi.org/10.1016/j.tibtech.2020.10.010
© 2020 Elsevier Ltd. All rights reserved.



stimulate the gene-editing process could undermine their safety and, in some cases, their efficacy (Box 1). For example, it is appreciated that DSBs induced by CRISPR nucleases have the potential to induce adverse effects in cells. These outcomes can include chromosomal translocations [14–16], genomic deletions [15,17], reduced fitness [18], cell cycle dysfunction [19], and the activation of the tumor suppressor protein TP53 [20,21]. Additionally, homology-directed repair (HDR), the DNA repair pathway most often used by CRISPR nucleases to faithfully introduce a specific change to a DNA sequence, is often limited by its efficiency in certain cell types [22–24]. For instance, HDR, which functions by incorporating genetic material from a co-delivered donor template into the target site, is typically only capable of achieving therapeutic rates of modification in dividing cells, which can prevent its implementation for therapeutic gene correction in postmitotic cells [25]. Importantly, HDR can be further limited by its competition with nonhomologous end joining (NHEJ), an error-prone DNA repair pathway that can create mutagenic base insertions and deletions (indels) at the target site [26,27]. Recently however, several new technologies have emerged that hold the potential to overcome many of these fundamental limitations. These tools in particular possess the programmability and flexibility characteristic of CRISPR nucleases but carry expanded functional capabilities that enable editing without the traditional requirement of a DSB. Here, we review three of these modalities: **base editors**, **prime editors**, and Cas13 effectors. We discuss the individual strengths of these technologies compared to traditional gene-editing systems, and we examine their emerging applications for gene and cell therapy.

Base Editors as a Therapeutic Gene-Editing Modality: Early Preclinical Successes and Future Challenges

Mechanisms for DNA Editing

CRISPR base editors are a technology that can facilitate the introduction of targeted point mutations in DNA, but without the requirement for a DSB. Most broadly, base editors consist of fusions of a **Cas9 nickase** (nCas9), a variant of the Cas9 nuclease that creates a single-strand rather than a DSB, with a nucleobase deaminase enzyme that initiates base editing by catalyzing a targeted deamination reaction. To date, several different base editors have been developed for use in mammalian cells (Figure 1A,B), including cytosine base editors (CBEs), which can catalyze the deamination of a target cytosine to facilitate its conversion to a thymine [28,29], adenosine base editors (ABEs), which can deaminate a target adenosine to facilitate its transition to a guanosine [30] and, most recently, guanosine base editors (GBEs), which can create C > G transversions in certain settings [31].

To catalyze a single-base modification, base editors, as directed by the sgRNA, first bind to a specific genomic target. Following the formation of a nCas9–sgRNA–DNA ternary complex that denatures the local target DNA sequence, the deaminase enzyme binds to its cognate bases, typically within a narrow stretch or **window** of nucleotides in the exposed DNA strand, and then catalyzes a deamination reaction that, in the case of a CBE, results in the transition of cytosine to uracil, a nucleobase which is recognized by cells as thymidine. The resulting U–G base mismatch is then resolved into the target T–A pair via cellular DNA replication or repair mechanisms that are activated in part by nCas9-induced nicking of the nonedited strand. Importantly, the enzymatic machinery and the mechanisms underlying this process vary by the base editor.

Box 1. Editing DNA with nickases

Similar to DSBs, single-stranded breaks in DNA, known as nicks, can stimulate HDR. To this end, nickase enzymes, including Cas9 nickases, can be used to create targeted DNA nicks that can facilitate the integration of DNA but without a DSB [110–112]. However, this approach relies on HDR to mediate editing and thus remains restricted to dividing cells.

Glossary

Base editors: a class of gene-editing proteins that can create certain single-base substitutions without creating a DSB. Base editors typically consist of a nCas9 variant fused to either a cytosine deaminase for C > T editing or an engineered adenosine deaminase for A > G editing.

Cas9 nickase (nCas9): a Cas9 variant that carries a mutation within one of its two catalytic domains that inactivates its ability to cleave both strands of DNA, thereby resulting in the cutting of only one strand. The D10A mutation inactivates the RuvC domain whereas the H840A mutation inactivates the HNH domain. Base editors typically consist of the nCas9-D10A variant, while prime editors have thus far relied on the nCas9-H840A variant.

Double-strand break (DSB): a type of DNA damage that results when both strands of a DNA duplex are cut. Traditional Cas nucleases stimulate DNA editing by inducing a targeted DSB, which in turn activates cellular repair pathways that can modify the targeted sequence.

Editing window: a stretch of sequence, typically 5–8 nucleotides in length, in the sgRNA binding site that is particularly favorable for deamination by a base editor. The size and location of this window can vary depending on the nCas9 variant, the linker composition, and deaminase domain.

Indel byproducts: a typically undesirable outcome usually resulting from the inadvertent activation of the nonhomologous end-joining repair pathway and its subsequent generation of small insertion and deletion mutations at the target site.

On-target editing: the desired editing outcome at the targeted base. An optimal editing platform will generate high rates of on-target editing and avoid forming nontarget bystander and indel byproducts.

Prime editing guide RNA (pegRNA): a modified sgRNA that directs the prime editor to a specific genomic site to initiate editing but also carries the edit-containing template for the reverse transcriptase domain that is part of the prime editor.

Prime editor: a gene-editing protein consisting of a nCas9 fused to a reverse transcriptase domain that creates edits within a target sequence using a pegRNA. Prime editors can in theory be

For example, the most efficient ABEs rely on an engineered tRNA adenosine deaminase (TadA) enzyme that deaminates a target adenosine in an exposed DNA strand to yield an inosine intermediate, which is then recognized as guanosine during repair. However, unlike with HDR, the repair mechanisms underlying these processes appear to be sufficiently active in both dividing and nondividing cells [32], which suggests that base editors could serve as a broadly applicable therapeutic modality.

used to correct all types of point mutations and introduce a number of small insertions and deletions in a target sequence.
Protospacer adjacent motif (PAM): a short but essential DNA motif adjacent to the sgRNA binding site that is required by Cas proteins to cleave DNA.
Protospacer flanking site (PFS): a single nucleotide motif that is analogous to the PAM and required by many Cas13 proteins to engage with a target RNA.
Single-guide RNA (sgRNA): an engineered RNA molecule that, via base complementarity, directs Cas proteins to bind to and cleave a specific DNA sequence.

Therapeutic Applications of Base Editing

Given their considerable strengths and their potential to enable new opportunities for therapeutic editing, base editors have been rapidly deployed *in vivo* to treat a number of disorders (Table 1). Among the first successful examples demonstrating their potential was the finding that ABEs could be delivered to skeletal muscle to restore dystrophin expression in a mouse model of Duchenne muscular dystrophy (DMD) [33]; a genetic disorder characterized by progressive muscle degeneration and weakness due to mutations in the dystrophin protein. Specifically, an ABE was used to revert a premature stop codon in a mutant dystrophin gene via A > G editing, which resulted in restored dystrophin expression. However, since most base editor proteins exceed the carrying capacity of a single adeno-associated virus (AAV) vector (Table 2), a particularly promising *in vivo* gene delivery vehicle [34], two AAV particles were needed to deliver the ABE. In this particular case, the base editor transgene was split in two halves and delivered across two AAV vectors that were subsequently joined together by a recombination reaction between the inverted terminal repeat (ITR) sequences present in each vector [35,36] (Figure 2A).

As an alternative to vector-mediated recombination, intein-mediated protein trans-splicing, an approach that harnesses self-splicing intein domains to catalyze the ligation of two separately expressed polypeptides into a single protein chain, has also been used to reassemble two base editor halves into the full-length protein [37–39] (Figure 2B). The potential of intein-mediated trans-splicing for *in vivo* gene correction was in fact first demonstrated in a mouse model of phenylketonuria, a recessive liver disease that is caused by mutations in the phenylalanine hydroxylase (PAH) gene. In this particular case, dual vector delivery to the liver of an intein-containing split CBE led to the correction of up to 25% of mutant PAH alleles [40]. Similar intein-mediated trans-splicing approaches have been used to deliver base editors to the brain to correct loss-of-function point mutations in the *Npc1* gene, which gives rise to the lysosomal storage disorder Niemann–Pick disease type C [41] and to the inner ear to correct a mutation in the *TMC1* gene in a neonatal mouse model of hereditary deafness, which resulted in partially restored auditory function [42]. Additionally, intein-mediated trans-splicing has been utilized to create an in-frame start codon in the fumarylacetoacetate hydrolase (*Fah*) gene to restore its expression and mitigate a pathogenic phenotype in a murine model of hereditary tyrosinemia type I (HTI) [43], further solidifying the potential of dual vector delivery for enabling therapeutic base editing. Notably, nonviral approaches (Table 2) involving lipid nanoparticles (LNPs) have also been used to deliver base-editor-encoding mRNA and have enabled correction in a mouse model of HTI [44,45]. Importantly, LNP-delivered mRNA is only transiently maintained, which can reduce the risk for accumulating off-target effects from persistently expressing a base editor protein in cells from a viral vector.

In addition to correcting loss-of-function mutations, base editors can also be used to silence gene expression in a manner that overcomes a key limitation underlying the use of CRISPR-Cas9 nucleases for the same application, namely their reliance on NHEJ. Nuclease-induced NHEJ, in particular, carries a risk for creating in-frame mutations in the target gene that could lead to the production of unique mutant protein isoforms, which can not only diminish the therapeutic

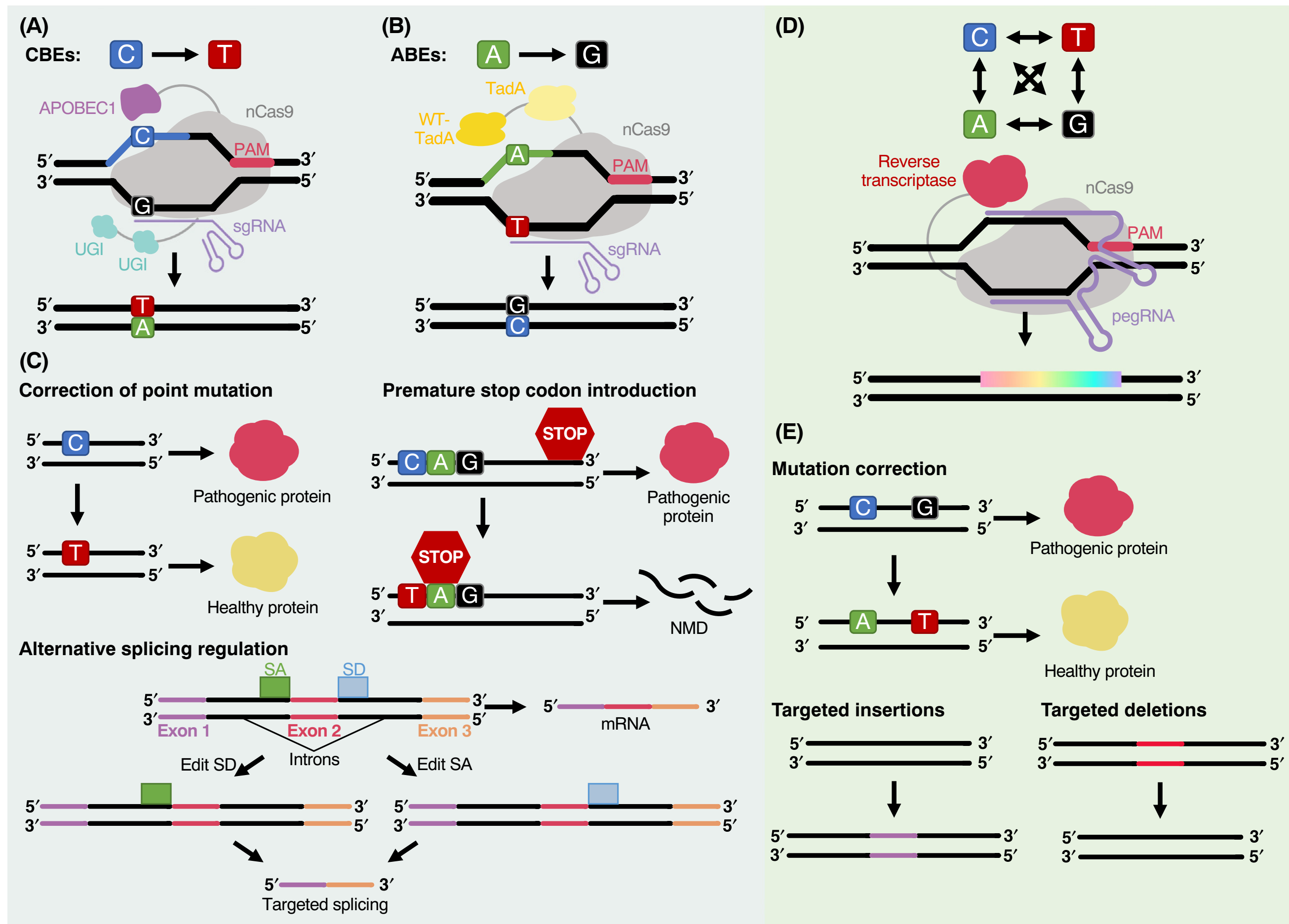


Figure 1. Overview of Next-Generation DNA-Editing Technologies. (A) Fourth-generation CBEs (BE4) consist of fusions of nCas9 with a cytosine deaminase domain (APOBEC1) that catalyzes the deamination of cytosine and two UGI domains, which can prevent the unwanted excision of uracil by endogenous uracil N-glycosylase enzymes. To catalyze base editing, CBEs are directed to a specific DNA sequence via an sgRNA. Then, following nCas9 binding and denaturation of the target DNA sequence, the APOBEC1 enzyme binds to cytosines within a stretch of sequence in the exposed DNA strand and catalyzes a deamination reaction that results in the conversion of cytosine to uracil, which is recognized by cells as thymine during replication and/or repair. (B) ABE7.10 catalyzes A > G transitions and consists of a tandem fusion of a wild-type TadA and an evolved TadA domain, engineered to accept DNA as a substrate, fused with nCas9. Deamination of adenosine yields inosine, which is recognized by cells as guanosine. (C) Therapeutic outcomes of base editing include: (i) the correction of single point mutations that encode for pathogenic proteins; (ii) the introduction of premature stop codons to eliminate pathogenic transcripts via the NMD surveillance pathway; and (iii) regulation of mRNA splicing by editing splicing motifs – SA and SD – to induce exon skipping. (D) Prime editors consist of a pegRNA, which both specifies the target sequence and encodes the desired edits, and nCas9 fused to a reverse transcriptase (RT) domain, which promotes the incorporation of the edit encoded in the pegRNA template into the target DNA sequence. The RT fusion provides high levels of design versatility for achieving a range of editing outcomes. (E) Examples of therapeutic prime editing outcomes include: (i) the correction of single or multiple mutations that result in pathogenic proteins; and (ii) the insertion or deletion of pathogenic sequences. Abbreviations: APOBEC1, cytosine deaminase domain; CBE, cytosine base editor; nCas9, CRISPR-associated protein 9 nickase; NMD, nonsense-mediated decay; pegRNA, prime editing guide RNA; SA, splice acceptor site; SD, splice donor site; sgRNA, single-guide RNA; TadA, tRNA adenosine deaminase; UGI, uracil DNA glycosylase inhibitor.

efficacy of this approach but, in some cases, also potentially cause adverse effects [46–48]. In contrast, by catalyzing targeted C > T transitions at CGA, CAG, CAA and TGG triplets, CBEs can create premature stop codons that can result in the more uniform degradation of the target mRNA by nonsense-mediated decay (NMD) [49,50], a surveillance mechanism used by cells to prevent the formation of truncated proteins (Figure 1C). Along similar lines, ABEs can be used to prevent gene expression by altering the ATG start codon [51].

Table 1. Preclinical Gene and Cell Therapy Applications of Next-Generation CRISPR Technologies[a]

Disorder	Strategy	Delivery	Therapeutic outcomes	Refs
DMD	Base-editor-mediated correction of nonsense mutation	*In vivo* via dual AAV vectors	Restored dystrophin expression in myofibers in an adult DMD mouse model	[33]
PKU	Base-editor-mediated correction of nonsense mutation	*In vivo* via dual AAV vectors	Restored physiological blood L-Phe levels, enhanced PAH enzyme activity, and reversed a light fur phenotype in PAH^{enu2} adult mice	[40]
Niemann–Pick disease type C	Base-editor-mediated correction of loss of function mutation	*In vivo* via dual AAV vectors	Slowed neurodegeneration and increased lifespan in Npc1^{I1061T} homozygous mice	[41]
Deafness	Base-editor-mediated correction of loss of function mutation	*In vivo* via dual AAV vectors	Restored inner hair cell sensory transduction and hair cell morphology and transiently rescued low-frequency hearing in neonatal mice carrying a loss-of-function point mutation in *TMC1*	[42]
Coronary heart disease	Base-editor-mediated gene silencing	*In vivo* via an adenoviral vector	Reduced plasma PCSK9 protein levels and reduced plasma cholesterol levels in adult mice	[53]
	Base-editor-mediated gene silencing	*In utero* via an adenoviral vector	Reduced plasma PCSK9 protein levels and reduced plasma cholesterol levels in mouse fetuses	[52]
	Base-editor-mediated gene silencing	*In vivo* via functionalized lipid-like nanoparticles	Reduced postnatal serum PCSK9 protein levels in mice	[113]
ALS	Base-editor-mediated gene silencing	*In vivo* via dual AAV vectors	Reduced the rate of muscle atrophy and muscle denervation, improved neuromuscular function, and slowed disease progression in the G93A-SOD1 mouse model of ALS	[54]
HTI	Base-editor-mediated gene silencing	*In utero* via an adenoviral vector	Rescue of the lethal phenotype of HTI, as indicated by weight gain and prolonged survival in *Fah*$^{-/-}$ mice, a model of HTI	[52]
	Base-editor-mediated splice site correction	*In vivo* via hydrodynamic injection of plasmid DNA	Partially restored splicing, rapid expansion of Fah^{+} hepatocytes in the liver and rescued weight loss in adult *Fah*$^{mut/mut}$ mice	[45]
		In vivo via lipid nanoparticle delivery	Edited hepatocytes in adult *Fah*$^{mut/mut}$ mice	
	Base-editor-mediated correction of mutation	*In vivo* via lipid nanoparticle delivery	Rescued weight loss and restored Fah expression in liver tissue of mice carrying a mutant version of Fah	[44]
	Base-editor-mediated generation of *de novo* in frame start codon	*In vivo* via dual AAV vectors	Restored Fah expression and halted weight loss in an HTI mouse model	[43]
β-Thalassemia and SCD	Base editor-mediated modification of transcription factor binding sites	*Ex vivo* via electroporation of RNP	Engraftment of edited HSCs in NBSGW mice resulted in HbF induction	[56]
Pancreatic cancer	Cas13-mediated transcript knockdown	*In vivo* via an AAV vector	Slowed tumor growth and reduced tumor cell proliferation in mice bearing subcutaneous AsPC-1 xenografts	[98]
Retinal injury	Cas13-mediated transcript knockdown	*In vivo* via an AAV vector	Induced the conversion of glia cells into retinal ganglion cells, which resulted in partially restored visual responses in a drug-induced retinal injury mouse model	[99]
PD	Cas13-mediated transcript knockdown	*In vivo* via an AAV vector	Induced the conversion of glia cells into dopaminergic neurons, which resulted in reduced motor dysfunction in a PD mouse model created by inducing lesions with 6-OHDA	[99]

[a]Abbreviations: 6-OHDA, 6-hydroxydopamine; ALS, Amyotrophic lateral sclerosis; DMD, Duchenne muscular dystrophy; Fah, fumarylacetoacetate hydrolase; HbF, fetal hemoglobin; HSCs, hematopoietic stem cells; HTI, hereditary tyrosinemia type I; PAH, phenylalanine hydroxylase; PCSK9, proprotein convertase subtilisin/kexin type 9; PD, Parkinson's disease; PKU, phenylketonuria; RNP, ribonucleoprotein; SCD, sickle cell disease; SOD1, superoxide dismutase 1; TMC1, transmembrane channel-like 1.

The therapeutic potential of this approach has now been demonstrated in multiple studies. Specifically, adenoviral vectors, which, unlike AAV, can accommodate the delivery of a full-length base editor protein within a single viral particle (Table 2), have been used to deliver base editors to the liver to silence PCSK9, a protein that can regulate LDL receptor degradation and has emerged as a potential target for hypercholesterolemia, in both adult [52] and prenatal [53]

Table 2. Delivery Strategies for CRISPR Technologies[a]

Viral methods								
Vector	Integrating/ nonintegrating	Pathogenicity	Packaging capacity	Duration of expression	Cargo	Diameter	Primary setting for genome editing	Refs
Lentivirus	Integrating[b]	Low to moderate	~8 kb	Long term	RNA	~130 nm	*Ex vivo*	[114]
Adenovirus	Nonintegrating	Moderate to high	~8 kb	Long term	DNA	~100 nm	*In vivo*	[114]
AAV	Can integrate at low frequencies (0.1–1%)	Low	~4.7 kb	Long term in quiescent cells	DNA	~25 nm	*In vivo*	[114]

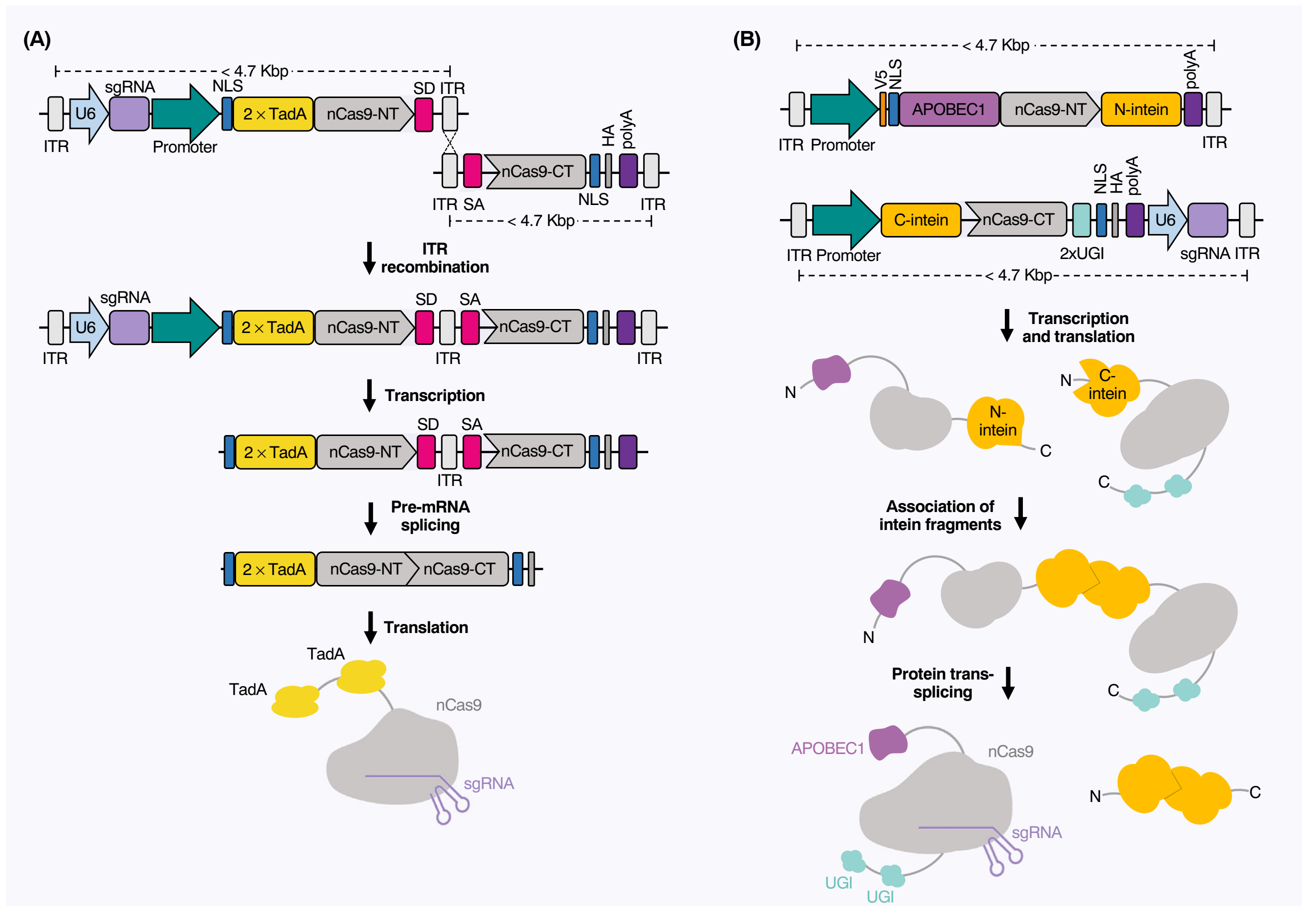
Nonviral methods								
System	Composition	Pathogenicity	Distribution	Duration of expression	Cargo	Size	Primary setting for genome-editing	Refs
Lipid nanoparticles	Ionizable lipids, cholesterol, PEG, DOPE, supplemental cationic components	Low	- Systemic administration (liver, lung)[c] - Local administration	Short term	Protein, DNA, RNA	150–200 nm	*In vivo*	[115]
Lipid-like nanoparticles	TT derivatives, PEG, DOPE	Low	- Systemic administration (liver and blood)	Short term	RNA	~100 nm	*In vivo*	[113,116,117]
Gold nanoparticles	Gold nanoparticles conjugated to thiol-modified DNA	Low	Local administration	Short term	Protein, DNA, RNA	~500 nm	*In vivo*	[118]
Electroporation	Wave pulse	Low	Local administration (skin, retina)	Short term	Protein, DNA, RNA	NA	*In vivo, ex vivo*	[119,120]

[a]Abbreviations: PEG, polyethylene glycol; DOPE, 1,2-dioleyl-sn-glycero-3-phosphoethanolamine; NA, not applicable; TT, *N*[1],*N*[3],*N*[5]-tris(2-aminoethyl)benzene-1,3,5-tricarboxamide.
[b]Integration-deficient lentiviruses also exist.
[c]Cationic component level dependent.

mice. Similarly, a prenatal strategy was undertaken in mice to silence the HPD gene to rescue HTI [53], a metabolic disorder characterized by elevated blood levels of the amino acid tyrosine. These studies altogether demonstrated the potential of base editors to not only silence gene expression *in vivo* by introducing nonsense mutations, but also the potential of *in utero* gene therapy for correcting inherited genetic disorders.

In addition to reprogramming metabolic flux *in vivo*, base editors can be used to silence the expression of genes with toxic gain-of-function mutations. Specifically, dual AAV particle delivery of a split-intein CBE engineered to disable a mutant gene causative for a form of amyotrophic lateral sclerosis, a currently incurable disorder that involves the selective loss of motor neurons in the spinal cord and brain, prolonged survival and slowed the deterioration of motor function in a mouse model of the disease [54], further reinforcing the potential of dual vector delivery approaches for achieving meaningful therapeutic effects in preclinical rodent models.

Similar to CRISPR nucleases, base editors also have the potential to advance cell therapy. For instance, base editors have been used to simultaneously disable the expression of multiple targets known to boost the potency of allogeneic CAR-T cells, including the *TRAC* locus, the



Trends in Biotechnology

Figure 2. Methods of Reconstituting Large Gene-Editing Proteins Delivered by Dual Adeno-associated Virus Vectors. (A) ITR recombination can mediate the joining of two ITR-flanked viral genomes that each encode a portion of a base editor sequence. In the case of an adenosine base editor (ABE), the first vector encodes: (i) an sgRNA driven by the U6 promoter; and (ii) a promoter to drive expression of the NLS-tagged tandem TadA domains fused to the NT half of a split nCas9 transgene and an SD sequence, while the second vector carries: (i) an SA signal sequence; (ii) the CT half of the split nCas9 fused to an NLS tag and an HA epitope tag; and (iii) a bGH polyA signal sequence. Once in the target cell, the two viral vectors are joined by a recombination reaction between the two ITRs and the full-length ABE pre-mRNA is transcribed. pre-mRNA processing removes the intron and the intervening ITR, resulting in the translation of a full-length ABE protein. (B) Intein-mediated protein trans-splicing of two separately expressed base editor halves can be used to assemble a full-length base editor protein. In the case of a CBE, the first vector encodes: (i) a promoter to drive expression of the NT of the base editor; (ii) a V5 epitope and NLS tag followed by the APOBEC1 domain fused to the NT fragment of the split nCas9 half and a connected N-terminal intein domain; and (iii) a bGH poly A signal sequence, while the second vector carries: (i) a promoter to drive the expression of the CT intein domain fused to the CT half of nCas9 linked to two UGI domains, an NLS and an HA epitope tag; and (iii) a polyA signal sequence. This vector also encodes an sgRNA expression cassette. Both vectors are packaged and co-delivered *in vivo*, where, once internalized by the cells, the separate intein-split base editor protein halves are expressed. Following their translation, the N and C intein fragments associate and catalyze a trans-splicing reaction that removes the intein moieties and results in the formation of the full-length CBE. Abbreviations: ABE, adenosine base editor; APOBEC1, cytosine deaminase domain; bGH, bovine growth hormone; CBE, cytosine base editor; CT, C-terminal; ITR, inverted terminal repeat; nCas9, CRISPR-associated protein 9 nickase; NLS, nuclear localization signal; NT, N-terminal; polyA, polyadenylation; SA, splice acceptor; SD, splice donor; sgRNA, single-guide RNA; TadA, tRNA adenosine deaminase; UGI, uracil DNA glycosylase inhibitor.

β-2 microglobulin gene, and the programmed cell death 1 gene, which altogether resulted in not only a more efficient CAR-T cell therapy but also higher quality cells, as measured by a reduction in DSB-induced translocations compared to Cas9-edited cells [55]. Along similar lines, base editors have been used to correct inherited blood disorders by *ex vivo* gene therapy. This was achieved by electroporating purified ribonucleoproteins (RNPs) to cells, which, unlike base editors expressed from viral vectors, exist in cells only transiently. In this case, C > T editing in blood progenitor cells was used to activate fetal hemoglobin by mutating a key transcription factor binding site within the

CellPress

BCL11A enhancer, a repressor of fetal hemoglobin [56]. Thus, base editing technologies can be used to tune gene expression by modifying functional elements within noncoding sequences.

Moving forward, the finding that base editors can modulate alternative splicing by modifying conserved intronic motifs in genes [57,58], which can include both donor and acceptor signals, should extend their use to therapeutic exon skipping, as demonstrated against the mutant dystrophin gene in patient-derived induced pluripotent stem cells (iPSCs) [58] (Figure 1C).

Challenges for Therapeutic Base Editing

While the therapeutic potential for base editing has been demonstrated now in multiple studies, the clinical implementation of the technology, like CRISPR-Cas9 nucleases and other gene-editing modalities before them, must overcome a number of hurdles, particularly with respect to safety and *in vivo* efficacy. In particular, it is now well appreciated that, in addition to both Cas9:sgRNA-dependent [28,59–62] and -independent [63] off-target effects in DNA, base editors can nonspecifically modify RNA [64]. Therefore, a number of protein engineering strategies have been implemented to create base editor scaffolds with improved specificity, including variants with not only decreased off-target DNA editing activity [62,65–69] but also reduced RNA targeting capabilities [64,69–71]. Additionally, base editors can sometimes create an incorrect edit at the target base or produce bystander mutations via their editing of nontarget bases within the targeted sequence. As a result, base editor variants with improved **on-target editing** capabilities have been developed. These proteins in fact have been reported to possess not only improved on-target editing frequencies [72–77] but also lower rates of unwanted bystander mutations [66,68,77,78] and fewer **indel byproducts** [73,75].

Additionally, as AAV vectors have emerged as a highly promising therapeutic gene delivery vehicle [34], it will be important to evaluate the effectiveness and safety of dual vector strategies for delivering split-intein-containing base editors *in vivo*, particularly within large animal models, which can better mimic many aspects of human physiology relevant to vector delivery than rodent models. Such models will also be important for studying the potential immunogenicity and long-term safety of base editor proteins. Finally, considering recent observations indicating their integration into the genome when delivering Cas9 [79,80], it will be important to determine at what frequency base editor-encoding AAV vectors are also integrated into a host cells' genome.

Prime Editors: A Newly Emerged Gene-Editing Technology with Broad Therapeutic Potential

Though base editors have expanded the range of therapeutic applications possible for gene editing, the technology remains limited in part by the functional capabilities of current deaminase domains, which, while enabling transition mutations and C > G transversions, cannot currently insert or delete DNA sequences. However, a recently reported alternative gene-editing technology named 'prime editing' [81] holds the potential to overcome current functional limitations.

Prime editors consist of nCas9 tethered to a reverse transcriptase (RT) domain and a modified sgRNA, referred to as a **prime editing guide RNA** (pegRNA), that not only specifies the target site for nCas9 but also serves as a template for the RT domain during DNA repair (Figure 1D). To catalyze editing, prime editors, as directed by the pegRNA, first bind to a specific target DNA sequence, which then facilitates nCas9-induced nicking of the target strand and hybridization of the nicked 3′ DNA strand with a complementary primer binding site encoded in the pegRNA. Following heteroduplexation, the RT domain then catalyzes the synthesis of a new DNA strand, which encodes the edit, from the template specified by the pegRNA. This new strand is then introduced into the target site following DNA repair.

Currently the most efficient version of this system, termed PE3, consists of the prime editor platform described above and a second sgRNA that facilitates nicking of the nonedited strand, which can enhance the rate of editing.

Compared to base editors, prime editors offer increased targeting flexibility, since they do not have as restricted an editing window, and the potential to generate all possible base transitions and transversions, thereby offering a means to correct a wide range of possible gene mutations (Figure 1D), as evidenced by proof-of-principle studies demonstrating their ability to correct mutations known to cause sickle cell disease and Tay–Sachs disease in cell culture [81]. However, compared to similarly active CBEs and ABEs, PE3, the prime editing system currently found to be the most efficient for editing DNA, has been reported to generate increased rates of indel byproducts [81]. Because high product purity and a relative lack of indel mutations are both defining features of many single-base editing technologies, refining prime editors to generate fewer collateral indels will be a high priority moving forward. Additionally, streamlining the design of an optimal pegRNA configuration to minimize laboratory screening efforts is also likely a high priority, as recently demonstrated by two studies [82,83]. Along these lines, machine-learning strategies, which have proven effective for not only predicting Cas9-specific indel patterns [84,85] but also base-editor-mediated editing outcomes [86–88], could be used to improve pegRNA design and enable more efficient prime editing. Nonetheless, given their additional capacity for inserting and deleting new DNA sequences, prime editing technologies hold potential therapeutic editing applications beyond base correction.

However, while the versatility and the reported efficiency of prime editors makes them an attractive platform for correcting potential disease-causing mutations, additional studies are required to comprehensively interrogate their genome-wide specificities, although it is likely that the combination of pegRNA-mediated targeting specificity and RT-based templating requirements will ensure similar, if not greater, specificity than observed using base editors. Nonetheless, the RT domain itself could induce off-target effects. Finally, similar to base editor proteins, efficiently delivering prime editors via AAVs could prove challenging given their large size. However, established dual vector delivery strategies that rely on ITR recombination [33] and intein-mediated protein trans-splicing [32,40,41,54,89] to reassemble the full-length prime editor protein should be compatible with the technology and enable its *in vivo* delivery for further preclinical study.

Targeting, Skipping, and Editing RNA Using CRISPR-Cas13 Effectors: Expanding the CRISPR Toolbox Beyond DNA Editing

Although CRISPR technology is most commonly associated with DNA editing, the discovery of Cas13 enzymes [90–95], which cleave a target RNA via an intrinsic RNase activity that is activated by the binding of a CRISPR RNA (crRNA) guide molecule, has facilitated the creation of a flexible and programmable toolbox capable of targeting RNA (Figure 3A).

From a therapeutic perspective, similar to antisense oligonucleotides (ASOs) and RNAi, CRISPR-Cas13 effectors offer a means to suppress gene expression without the risk for inducing DNA damage to cells, as Cas13 proteins have been demonstrated to generally lack DNase activity [96]. Cas13 targeting is also, in theory, reversible. For example, because Cas13 engages with RNA, its expression could potentially be terminated by an engineered kill-switch, which, if implemented, could return the cell's transcriptome back to its original state. Cas13 proteins may also offer distinct targeting advantages to RNAi, a clinically promising platform capable of efficiently knocking down target gene expression. For example, side-by-side comparisons

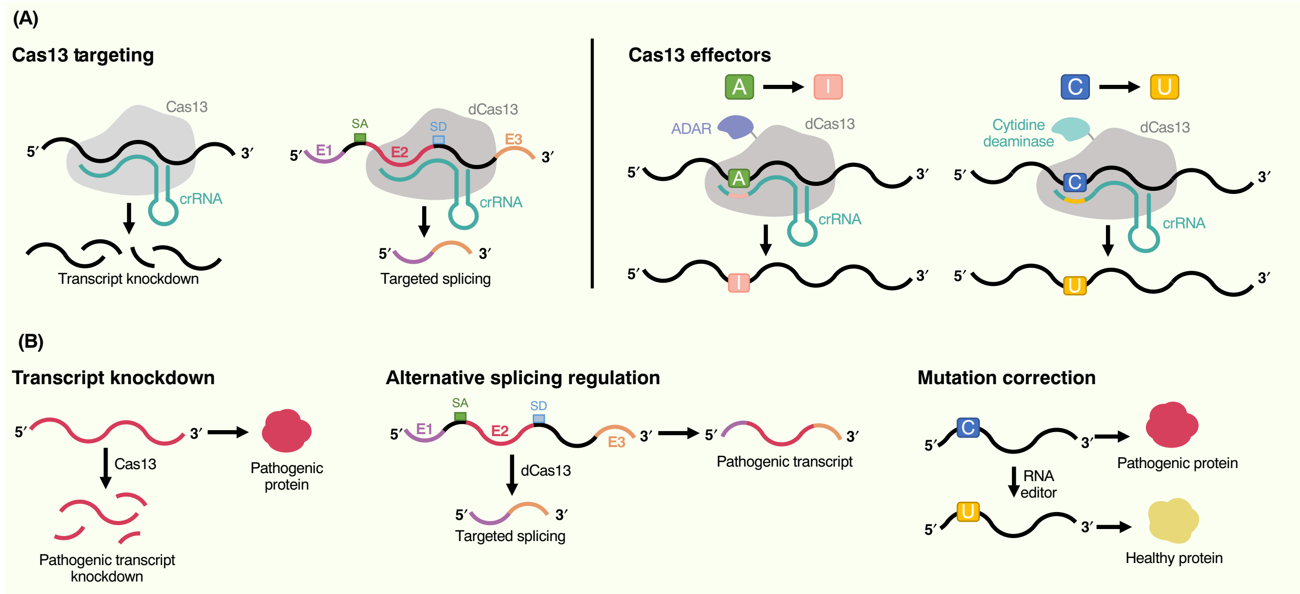


Figure 3. Overview of RNA-Targeting Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) Systems. (A, left) To catalyze RNA cleavage, a crRNA molecule binds to Cas13 and directs it to a target RNA site, which stimulates the RNase catalytic activity of the HEPN domains of Cas13, triggering its cleavage. Cas13 systems can also mediate targeted splicing utilizing dCas13 and a single or multiple crRNA molecules that bind to splice site motifs – SA and SD – and likely block them from recognition by the splicing machinery. (A, right) Additionally, Cas13 effectors can serve as scaffolds to enable targeted RNA editing. RNA editors consist of fusions of dCas13 to ADAR to stimulate A to I edits and cytidine deaminase domains to mediate C to U conversions. (B) Examples of therapeutic RNA targeting outcomes include the use of Cas13 protein to cleave pathogenic transcripts to prevent the translation of mutated proteins and the use of dCas13 to bind splice donor and acceptor sites to block spliceosome protein complexes from triggering exon inclusion that may encode for pathogenic isoforms. Additionally, RNA editing can be used to correct single-point mutations in mRNA that encode for pathogenic proteins. Abbreviations: ADAR, adenosine deaminase acting on RNA; Cas13, CRISPR-associated protein 13; crRNA, CRISPR RNA; E1, exon 1; E2, exon 2; E3, exon 3; SA, splice acceptor site; SD, splice donor site.

with spacer-matching short-hairpin RNAs (shRNAs) have revealed that Cas13d proteins induce fewer off-target effects in cultured cells, indicating that they may have superior targeting abilities [90,96,97], though additional studies comparing Cas13 to preclinically optimized gene-silencing technologies will be needed to establish this.

Given these advantages, Cas13 proteins have been deployed *in vivo* to facilitate therapeutic outcomes (Table 1). For instance, repeated injections of a Cas13 RNP designed to target a mutant *KRAS* transcript that can trigger cancer development was demonstrated to shrink the mass of a tumor xenograft [98]. Additionally, because of their compact size, certain Cas13 effectors, such as the Cas13 protein from *Ruminococcus flavefaciens* (RfxCas13), can be packaged into a single AAV vector particle to enable continuous *in vivo* targeting. In fact, RfxCas13 has been delivered *in vivo* by AAV to knockdown PTBP1, a suppressor of neuronal differentiation, to mediate the conversion of retinal glial cells into ganglion cells, a type of neuron located in the retina, to restore visual responses in a retinal injury mouse model [99]. A similar strategy was undertaken to induce the expression of dopaminergic neurons in the brain to slow motor dysfunction in a mouse model of Parkinson's disease [99]. In both cases, Cas13 targeting was found to induce few off-target effects.

In addition to knocking down a target mRNA, Cas13 proteins can effectively modulate alternative splicing (Figure 3B). In particular, catalytically inactivated Cas13 protein variants (dubbed dCas13) can be directed by a crRNA to bind to – and thus interfere with – positive and negative splicing factors to mediate the inclusion or exclusion of specific exons [96,100].

The therapeutic potential of this approach was demonstrated by multiplexed targeting of a splice acceptor site and two putative exonic splice enhancer sites in MAPT pre-mRNA, which facilitated correction of a mis-splicing event in a patient-derived neuronal model of dementia [96], and the restoration of exon 7 splicing within the survival motor neuron 2 gene in spinal muscular atrophy (SMA) patient fibroblasts [100].

Additionally, akin to current base editing technologies, Cas13 can also serve as a scaffold to direct RNA editing in cells (Figure 3A). Specifically, dCas13 proteins have been fused to adenosine deaminases acting on RNA (ADAR) domains to mediate A > I conversions in a target RNA [97] and an evolved ADAR domain that instead acts as a cytidine deaminase to create C > U edits [101]. Though the full therapeutic potential of Cas13-based RNA editing has yet to be harnessed, RNA editing systems have been used to correct a point mutation in MECP2 mRNA, which enabled rescue of certain phenotypes associated with Rett syndrome in a mouse model of the disease [102].

Moving forward however, the clinical implementation of Cas13 effectors could face several obstacles. In particular, similar to ASOs and RNAi, Cas13 proteins must continuously engage with a target RNA to sustain their therapeutic effect. Given the finding that many individuals may have pre-existing antibodies and/or reactive T cells to CRISPR proteins [103,104], the persistent expression of Cas13 proteins could pose a risk for stimulating a specific immune response (although the surface of Cas13 proteins could be re-engineered to potentially evade common neutralizing responses [105]). Cas13 proteins may carry additional risks owing to the fact that, in certain situations, they have been reported to cleave nontarget RNAs after activation [91,106]. Thus, long-term study of the immunogenicity and targeting specificity of Cas13 proteins will be important for determining their potential as a therapeutic.

Concluding Remarks and Future Perspectives

The past several years has seen a rapid rise in the number of technologies capable of editing DNA and RNA with both high precision and with high frequency. Owing to their potential to correct disease-causing mutations via repair mechanisms that are generally available in most cell types, these technologies hold immense potential as therapeutics for a range of disorders. It should be noted though that many of these platforms have overlapping capabilities. For instance, both base editors and prime editors are capable of creating C > T and A > G transitions. Thus, when identifying a target editor, multiple factors, including ontarget editing efficiency and nontarget editing byproducts should all be considered and weighed appropriately. Additionally, despite the rapid emergence of base editor scaffolds with improved targeting capabilities, it likely will be necessary to thoroughly optimize individual base editor proteins for their specific therapeutic targets in order to ensure maximally efficient formation of the ontarget product and to further minimize potentially counterproductive nontarget editing outcomes.

In addition to developing methods to further refine the capabilities of these increasingly sophisticated gene-editing machines, special attention must be paid to their delivery [107]. In particular, large animal studies will be needed to determine the overall effectiveness for dual AAV-based strategies for delivering base editors and potentially prime editors. Additionally, any optimization of a gene-editing protein should go hand in hand with optimization of: (i) the viral capsid to ensure efficient delivery to the most relevant cell populations; and (ii) the promoter sequence to enable efficient and also preferably cell type specific editing. Effectively implementing next-generation CRISPR technologies will also require extensively assessing their immunogenicity. Specifically, while DNA-editing technologies have, in theory, the capacity to self-inactivate their own expression, RNA-targeting technologies must be continuously expressed in order to sustain a

Outstanding Questions

Can the expression of next-generation CRISPR technologies be effectively controlled *in vivo* to prevent adverse effects?

Can base editing technologies be engineered to possess the specificity and efficiency needed to safely edit DNA *in vivo*?

Will prime editing technology emerge as an effective gene therapy agent?

Which delivery methods will prove most effective for enabling *in vivo* base editing and prime editing?

Will the long-term expression of Cas13 proteins cause adverse effects?

therapeutic effect, which could increase their risk for eliciting an immune response. Studies in large animal models will therefore be important for helping to establish whether continuously expressing Cas13 or any other next-generation CRISPR technology causes long-term adverse effects (see Outstanding Questions). Finally, in addition to the single-base DNA and RNA editing technologies described here, targetable technologies for inserting large transgenes into DNA through RNA-guided transposition mechanisms [108,109] are actively being developed and could soon enable a new means of accomplishing therapeutic gene integration into safe-harbor sites, among other possible applications.

In summary, next-generation DNA and RNA editing technologies have enormous potential to advance gene and cell therapies.

Acknowledgments

M.A.Z.C. was supported by the National Institute of Biomedical Imaging and Bioengineering of the National Institutes of Health (T32EB019944), the Mavis Future Faculty Fellows Program, and by a University of Illinois Aspire Fellowship. This work was also supported by the Muscular Dystrophy Association (MDA602798).

References

1. Kim, H. and Kim, J.S. (2014) A guide to genome engineering with programmable nucleases. *Nat. Rev. Genet.* 15, 321–334
2. Lukacsovich, T. *et al.* (1994) Repair of a specific double-strand break generated within a mammalian chromosome by yeast endonuclease I-SceI. *Nucleic Acids Res.* 22, 5649–5657
3. Rouet, P. *et al.* (1994) Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. *Proc. Natl. Acad. Sci. U. S. A.* 91, 6064–6068
4. Wyman, C. and Kanaar, R. (2006) DNA double-strand break repair: all's well that ends well. *Annu. Rev. Genet.* 40, 363–383
5. Urnov, F.D. *et al.* (2010) Genome editing with engineered zinc finger nucleases. *Nat. Rev. Genet.* 11, 636–646
6. Joung, J.K. and Sander, J.D. (2013) TALENs: a widely applicable technology for targeted genome editing. *Nat. Rev. Mol. Cell Biol.* 14, 49–55
7. Cho, S.W. *et al.* (2013) Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nat. Biotechnol.* 31, 230–232
8. Cong, L. *et al.* (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819–823
9. Jinek, M. *et al.* (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816–821
10. Jinek, M. *et al.* (2013) RNA-programmed genome editing in human cells. *eLife* 2, e00471
11. Mali, P. *et al.* (2013) RNA-guided human genome engineering via Cas9. *Science* 339, 823–826
12. Stadtmauer, E.A. *et al.* (2020) CRISPR-engineered T cells in patients with refractory cancer. *Science* 367, eaba7365
13. Lu, Y. *et al.* (2020) Safety and feasibility of CRISPR-edited T cells in patients with refractory non-small-cell lung cancer. *Nat. Med.* 26, 732–740
14. Brunet, E. and Jasin, M. (2018) Induction of chromosomal translocations with CRISPR-Cas9 and other nucleases: understanding the repair mechanisms that give rise to translocations. *Adv. Exp. Med. Biol.* 1044, 15–25
15. Kosicki, M. *et al.* (2018) Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. *Nat. Biotechnol.* 36, 765–771
16. Rayner, E. *et al.* (2019) CRISPR-Cas9 causes chromosomal instability and rearrangements in cancer cell lines, detectable by cytogenetic methods. *CRISPR J.* 2, 406–416
17. Cullot, G. *et al.* (2019) CRISPR-Cas9 genome editing induces megabase-scale chromosomal truncations. *Nat. Commun.* 10, 1136
18. Chen, X. *et al.* (2020) Expanding the editable genome and CRISPR-Cas9 versatility using DNA cutting-free gene targeting based on in trans paired nicking. *Nucleic Acids Res.* 48, 974–995
19. van den Berg, J. *et al.* (2018) A limited number of double-strand DNA breaks is sufficient to delay cell cycle progression. *Nucleic Acids Res.* 46, 10132–10144
20. Haapaniemi, E. *et al.* (2018) CRISPR-Cas9 genome editing induces a p53-mediated DNA damage response. *Nat. Med.* 24, 927–930
21. Ihry, R.J. *et al.* (2018) p53 inhibits CRISPR-Cas9 engineering in human pluripotent stem cells. *Nat. Med.* 24, 939–946
22. Cox, D.B. *et al.* (2015) Therapeutic genome editing: prospects and challenges. *Nat. Med.* 21, 121–131
23. Nami, F. *et al.* (2018) Strategies for *in vivo* genome editing in nondividing cells. *Trends Biotechnol.* 36, 770–786
24. Rothkamm, K. *et al.* (2003) Pathways of DNA double-strand break repair during the mammalian cell cycle. *Mol. Cell. Biol.* 23, 5706–5715
25. Lin, S. *et al.* (2014) Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. *eLife* 3, e04766
26. Howden, S.E. *et al.* (2015) Simultaneous reprogramming and gene correction of patient fibroblasts. *Stem Cell Rep.* 5, 1109–1118
27. Merkle, F.T. *et al.* (2015) Efficient CRISPR-Cas9-mediated generation of knockin human pluripotent stem cells lacking undesired mutations at the targeted locus. *Cell Rep.* 11, 875–883
28. Komor, A.C. *et al.* (2016) Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* 533, 420–424
29. Nishida, K. *et al.* (2016) Targeted nucleotide editing using hybrid prokaryotic and vertebrate adaptive immune systems. *Science* 353, aaf8729
30. Gaudelli, N.M. *et al.* (2017) Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage. *Nature* 551, 464
31. Kurt, I.C. *et al.* (2021) CRISPR C-to-G base editors for inducing targeted DNA transversions in human cells. *Nat. Biotechnol.* 39, 41–46
32. Yeh, W.H. *et al.* (2018) *In vivo* base editing of post-mitotic sensory cells. *Nat. Commun.* 9, 2184
33. Ryu, S.M. *et al.* (2018) Adenine base editing in mouse embryos and an adult mouse model of Duchenne muscular dystrophy. *Nat. Biotechnol.* 36, 536–539
34. Wang, D. *et al.* (2019) Adeno-associated virus vector as a platform for gene therapy delivery. *Nat. Rev. Drug Discov.* 18, 358–378
35. Lai, Y. *et al.* (2005) Efficient *in vivo* gene expression by trans-splicing adeno-associated viral vectors. *Nat. Biotechnol.* 23, 1435–1439

36. Sun, L. *et al.* (2000) Overcoming adeno-associated virus vector size limitation through viral DNA heterodimerization. *Nat. Med.* 6, 599–602
37. Fine, E.J. *et al.* (2015) Trans-spliced Cas9 allows cleavage of HBB and CCR5 genes in human cells using compact expression cassettes. *Sci. Rep.* 5, 10777
38. Li, J. *et al.* (2008) Protein trans-splicing as a means for viral vector-mediated *in vivo* gene therapy. *Hum. Gene Ther.* 19, 958–964
39. Truong, D.J. *et al.* (2015) Development of an intein-mediated split-Cas9 system for gene therapy. *Nucleic Acids Res.* 43, 6450–6458
40. Villiger, L. *et al.* (2018) Treatment of a metabolic liver disease by *in vivo* genome base editing in adult mice. *Nat. Med.* 24, 1519–1525
41. Levy, J.M. *et al.* (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, 97–110
42. Yeh, W.H. *et al.* (2020) *In vivo* base editing restores sensory transduction and transiently improves auditory function in a mouse model of recessive deafness. *Sci. Transl. Med.* 12, eaay9101
43. Yang, L. *et al.* (2020) Amelioration of an inherited metabolic liver disease through creation of a de novo start codon by cytidine base editing. *Mol. Ther.* 28, 1673–1683
44. Jiang, T. *et al.* (2020) Chemical modifications of adenine base editor mRNA and guide RNA expand its application scope. *Nat. Commun.* 11, 1979
45. Song, C.Q. *et al.* (2020) Adenine base editing in an adult mouse model of tyrosinaemia. *Nat. Biomed. Eng.* 4, 125–130
46. Lalonde, S. *et al.* (2017) Frameshift indels introduced by genome editing can lead to in-frame exon skipping. *PLoS One* 12, e0178700
47. Mou, H. *et al.* (2017) CRISPR/Cas9-mediated genome editing induces exon skipping by alternative splicing or exon deletion. *Genome Biol.* 18, 108
48. Tuladhar, R. *et al.* (2019) CRISPR-Cas9-based mutagenesis frequently provokes on-target mRNA misregulation. *Nat. Commun.* 10, 4056
49. Billon, P. *et al.* (2017) CRISPR-mediated base editing enables efficient disruption of eukaryotic genes through induction of STOP codons. *Mol. Cell* 67, 1068–1079 e4
50. Kuscu, C. *et al.* (2017) CRISPR-STOP: gene silencing through base-editing-induced nonsense mutations. *Nat. Methods* 14, 710–712
51. Wang, X. *et al.* (2020) Efficient gene silencing by adenine base editor-mediated start codon mutation. *Mol. Ther.* 28, 431–440
52. Rossidis, A.C. *et al.* (2018) *In utero* CRISPR-mediated therapeutic editing of metabolic genes. *Nat. Med.* 24, 1513–1518
53. Chadwick, A.C. *et al.* (2017) *In vivo* base editing of PCSK9 (Proprotein Convertase Subtilisin/Kexin Type 9) as a therapeutic alternative to genome editing. *Arterioscler. Thromb. Vasc. Biol.* 37, 1741–1747
54. Lim, C.K.W. *et al.* (2020) Treatment of a mouse model of ALS by *in vivo* base editing. *Mol. Ther.* 28, 1177–1189
55. Webber, B.R. *et al.* (2019) Highly efficient multiplex human T cell engineering without double-strand breaks using Cas9 base. *Nat. Commun.* 10, 5222
56. Zeng, J. *et al.* (2020) Therapeutic base editing of human hematopoietic stem cells. *Nat. Med.* 26, 535–541
57. Gapinske, M. *et al.* (2018) CRISPR-SKIP: programmable gene splicing with single base editors. *Genome Biol.* 19, 107
58. Yuan, J. *et al.* (2018) Genetic modulation of RNA splicing with a CRISPR-guided cytidine deaminase. *Mol. Cell* 72, 380–394 e7
59. Gaudelli, N.M. *et al.* (2017) Programmable base editing of A*T to G*C in genomic DNA without DNA cleavage. *Nature* 551, 464–471
60. Kim, D. *et al.* (2017) Genome-wide target specificities of CRISPR RNA-guided programmable deaminases. *Nat. Biotechnol.* 35, 475–480
61. Liang, P. *et al.* (2019) Genome-wide profiling of adenine base editor specificity by EndoV-seq. *Nat. Commun.* 10, 67
62. Rees, H.A. *et al.* (2017) Improving the DNA specificity and applicability of base editing through protein engineering and protein delivery. *Nat. Commun.* 8, 15790
63. Zuo, E. *et al.* (2019) Cytosine base editor generates substantial off-target single-nucleotide variants in mouse embryos. *Science* 364, 289–292
64. Grunewald, J. *et al.* (2019) Transcriptome-wide off-target RNA editing induced by CRISPR-guided DNA base editors. *Nature* 569, 433–437
65. Doman, J.L. *et al.* (2020) Evaluation and minimization of Cas9-independent off-target DNA editing by cytosine base editors. *Nat. Biotechnol.* 38, 620–628
66. Gehrke, J.M. *et al.* (2018) An APOBEC3A-Cas9 base editor with minimized bystander and off-target activities. *Nat. Biotechnol.* 36, 977–982
67. Hu, J.H. *et al.* (2018) Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. *Nature* 556, 57–63
68. Kim, Y.B. *et al.* (2017) Increasing the genome-targeting scope and precision of base editing with engineered Cas9-cytidine deaminase fusions. *Nat. Biotechnol.* 35, 371–376
69. Yu, Y. *et al.* (2020) Cytosine base editors with minimized unguided DNA and RNA off-target events and high on-target activity. *Nat. Commun.* 11, 2052
70. Grunewald, J. *et al.* (2019) CRISPR DNA base editors with reduced RNA off-target and self-editing activities. *Nat. Biotechnol.* 37, 1041–1048
71. Rees, H.A. *et al.* (2019) Analysis and minimization of cellular RNA editing by DNA adenine base editors. *Sci. Adv.* 5, eaax5717
72. Jiang, W. *et al.* (2018) BE-PLUS: a new base editing tool with broadened editing window and enhanced fidelity. *Cell Res.* 28, 855–861
73. Komor, A.C. *et al.* (2017) Improved base excision repair inhibition and bacteriophage Mu Gam protein yields C:G-to-T:A base editors with higher efficiency and product purity. *Sci. Adv.* 3, eaao4774
74. Wang, L. *et al.* (2017) Enhanced base editing by co-expression of free uracil DNA glycosylase inhibitor. *Cell Res.* 27, 1289–1292
75. Gaudelli, N.M. *et al.* (2020) Directed evolution of adenine base editors with increased activity and therapeutic application. *Nat. Biotechnol.* 38, 892–900
76. Richter, M.F. *et al.* (2020) Phage-assisted evolution of an adenine base editor with improved Cas domain compatibility and activity. *Nat. Biotechnol.* 38, 883–891
77. Zhang, X. *et al.* (2020) Increasing the efficiency and targeting range of cytidine base editors through fusion of a single-stranded DNA-binding protein domain. *Nat. Cell Biol.* 22, 740–750
78. Liu, Z. *et al.* (2020) Efficient base editing with high precision in rabbits using YFE-BE4max. *Cell Death Dis.* 11, 36
79. Hanlon, K.S. *et al.* (2019) High levels of AAV vector integration into CRISPR-induced DNA breaks. *Nat. Commun.* 10, 4439
80. Nelson, C.E. *et al.* (2019) Long-term evaluation of AAV-CRISPR genome editing for Duchenne muscular dystrophy. *Nat. Med.* 25, 427–432
81. Anzalone, A.V. *et al.* (2019) Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* 576, 149–157
82. Chow, R.D. *et al.* (2021) A web tool for the design of prime-editing guide RNAs. *Nat. Biomed. Eng.* 5, 190–194
83. Kim, H.K. *et al.* (2021) Predicting the efficiency of prime editing guide RNAs in human cells. *Nat. Biotechnol.* 39, 198–206
84. Chakrabarti, A.M. *et al.* (2019) Target-specific precision of CRISPR-mediated genome editing. *Mol. Cell* 73, 699–713 e6
85. Shen, M.W. *et al.* (2018) Predictable and precise template-free CRISPR editing of pathogenic variants. *Nature* 563, 646–651
86. Arbab, M. *et al.* (2020) Determinants of base editing outcomes from target library analysis and machine learning. *Cell* 182, 463–480.e30
87. Dandage, R. *et al.* (2019) beditor: A computational workflow for designing libraries of guide RNAs for CRISPR-mediated base editing. *Genetics* 212, 377–385
88. Hwang, G.H. *et al.* (2018) Web-based design and analysis tools for CRISPR base editing. *BMC Bioinformatics* 19, 542
89. Winter, J. *et al.* (2019) Targeted exon skipping with AAV-mediated split adenine base editors. *Cell Discov.* 5, 41
90. Abudayyeh, O.O. *et al.* (2017) RNA targeting with CRISPR-Cas13. *Nature* 550, 280–284

91. Abudayyeh, O.O. *et al.* (2016) C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. *Science* 353, aaf5573
92. East-Seletsky, A. *et al.* (2016) Two distinct RNase activities of CRISPR-C2c2 enable guide-RNA processing and RNA detection. *Nature* 538, 270–273
93. Meeske, A.J. *et al.* (2019) Cas13-induced cellular dormancy prevents the rise of CRISPR-resistant bacteriophage. *Nature* 570, 241–245
94. Smargon, A.A. *et al.* (2017) Cas13b is a type VI-B CRISPR-associated RNA-guided RNase differentially regulated by accessory proteins Csx27 and Csx28. *Mol. Cell* 65, 618–630 e7
95. Yan, W.X. *et al.* (2018) Cas13d is a compact RNA-targeting type VI CRISPR effector positively modulated by a WYL-domain-containing accessory protein. *Mol. Cell* 70, 327–339 e5
96. Konermann, S. *et al.* (2018) Transcriptome engineering with RNA-targeting type VI-D CRISPR effectors. *Cell* 173, 665–676 e14
97. Cox, D.B.T. *et al.* (2017) RNA editing with CRISPR-Cas13. *Science* 358, 1019–1027
98. Zhao, X. *et al.* (2018) A CRISPR-Cas13a system for efficient and specific therapeutic targeting of mutant KRAS for pancreatic cancer treatment. *Cancer Lett.* 431, 171–181
99. Zhou, H. *et al.* (2020) Glia-to-neuron conversion by CRISPR-CasRx alleviates symptoms of neurological disease in mice. *Cell* 181, 590–603 e16
100. Du, M. *et al.* (2020) CRISPR artificial splicing factors. *Nat. Commun.* 11, 2973
101. Abudayyeh, O.O. *et al.* (2019) A cytosine deaminase for programmable single-base RNA editing. *Science* 365, 382–386
102. Sinnamon, J.R. *et al.* (2017) Site-directed RNA repair of endogenous Mecp2 RNA in neurons. *Proc. Natl. Acad. Sci. U. S. A.* 114, E9395–E9402
103. Charlesworth, C.T. *et al.* (2019) Identification of preexisting adaptive immunity to Cas9 proteins in humans. *Nat. Med.* 25, 249–254
104. Simhadri, V.L. *et al.* (2018) Prevalence of pre-existing antibodies to CRISPR-associated nuclease Cas9 in the USA population. *Mol. Ther. Methods Clin. Dev.* 10, 105–112
105. Ferdosi, S.R. *et al.* (2019) Multifunctional CRISPR-Cas9 with engineered immunosilenced human T cell epitopes. *Nat. Commun.* 10, 1842
106. Varble, A. and Marraffini, L.A. (2019) Three new Cs for CRISPR: collateral, communicate, cooperate. *Trends Genet.* 35, 446–456
107. van Haasteren, J. *et al.* (2020) The delivery challenge: fulfilling the promise of therapeutic genome editing. *Nat. Biotechnol.* 38, 845–855
108. Klompe, S.E. *et al.* (2019) Transposon-encoded CRISPR-Cas systems direct RNA-guided DNA integration. *Nature* 571, 219–225
109. Strecker, J. *et al.* (2019) RNA-guided DNA insertion with CRISPR-associated transposases. *Science* 365, 48–53
110. Chen, X. *et al.* (2017) In trans paired nicking triggers seamless genome editing without double-stranded DNA cutting. *Nat. Commun.* 8, 657
111. Hyodo, T. *et al.* (2020) Tandem paired nicking promotes precise genome editing with scarce interference by p53. *Cell Rep.* 30, 1195–1207 e7
112. Nakajima, K. *et al.* (2018) Precise and efficient nucleotide substitution near genomic nick via noncanonical homology-directed repair. *Genome Res.* 28, 223–230
113. Zhang, X. *et al.* (2020) Functionalized lipid-like nanoparticles for *in vivo* mRNA delivery and base editing. *Sci. Adv.* 6, eabc2315
114. Chen, X. and Goncalves, M.A. (2016) Engineered viruses as genome editing devices. *Mol. Ther.* 24, 447–457
115. Yin, H. *et al.* (2017) Structure-guided chemical modification of guide RNA enables potent non-viral *in vivo* genome editing. *Nat. Biotechnol.* 35, 1179–1187
116. Jiang, C. *et al.* (2017) A non-viral CRISPR/Cas9 delivery system for therapeutically targeting HBV DNA and pcsk9 *in vivo*. *Cell Res.* 27, 440–443
117. Wei, T. *et al.* (2020) Systemic nanoparticle delivery of CRISPR-Cas9 ribonucleoproteins for effective tissue specific genome editing. *Nat. Commun.* 11, 3232
118. Lee, K. *et al.* (2017) Nanoparticle delivery of Cas9 ribonucleoprotein and donor DNA in vivo induces homology-directed DNA repair. *Nat. Biomed. Eng.* 1, 889–901
119. Latella, M.C. *et al.* (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, e389
120. Wu, W. *et al.* (2017) Efficient *in vivo* gene editing using ribonucleoproteins in skin stem cells of recessive dystrophic epidermolysis bullosa mouse model. *Proc. Natl. Acad. Sci. U. S. A.* 114, 1660–1665