

Precision gene editing: The power of CRISPR-Cas in modern genetics

Jeong H. Joo,^{1,2} Soogene Lee,^{1,2} and Keun P. Kim¹

¹Department of Life Science, Chung-Ang University, Seoul 06974, Korea

Gene editing has transformed molecular biology by enabling precise modifications to genomic DNA across a wide variety of organisms. Gene editing technologies make it possible to add, remove, or modify specific DNA sequences, with a range of applications including gene knockouts, therapeutic gene correction, and the design of targeted genetic traits. These techniques depend on two main DNA repair mechanisms: homology-directed repair (HDR), which facilitates precise changes to the genome, and non-homologous end joining (NHEJ), which often results in mutations such as deletions or frameshift errors. Among the diverse gene-editing platforms, the CRISPR-Cas system has emerged as the most extensively employed, owing to its simplicity, low cost, and efficiency. This review presents the evolution of gene-editing technologies, with a particular emphasis on the CRISPR-Cas system and its expanding applications in genetics, biotechnology, agriculture, and medicine. Furthermore, advanced gene editing approaches are discussed, offering an overview of emerging trends.

INTRODUCTION

Gene editing technologies have become a powerful tool in molecular biology, allowing for precise changes to DNA sequences.^{1–5} These techniques allow for targeted alterations at specific genomic loci, including gene insertion, deletion, or replacement. This versatile approach supports precise gene regulation by utilizing endogenous cellular repair mechanisms activated in response to DNA double-strand breaks (DSBs).^{1–3} After DSBs occur, cells can repair themselves through two main repair pathways: homology-directed repair or non-homologous end joining.⁶ The homology-directed repair pathway facilitates high-fidelity genetic modifications through the use of a donor template, allowing for the accurate introduction of new genetic material. In contrast, the non-homologous end joining pathway is typically an error-prone repair mechanism that can lead to insertions or deletions that may introduce frameshift mutations or premature stop codons, thereby disrupting gene function and enabling gene knockout (Figure 1C).^{7–9} Gene editing enables researchers to manipulate genetic sequences with high precision, expanding its applications across genetics, biotechnology, and medicine. In recent years, gene editing technologies have rapidly transformed in the life sciences and positioned these approaches as pivotal tools for gene therapy. The principal genome-editing platforms include meganucleases, zinc finger nucleases (ZFNs), transcription-activator-like effector nucleases (TALENs), and clustered

regularly interspaced short palindromic repeats (CRISPRs) (Figures 1A and 1B; Table 1).^{2,3,9–11,29–33} Based on these nuclease-based platforms, next-generation technologies such as base editing, prime editing, RNA editing, and epigenome editing have rapidly emerged, allowing for precise modifications without inducing DSBs. These advances significantly improve the precision, safety, and therapeutic potential of genome engineering. Accordingly, this review provides an overview of the basic nuclease-based approaches and advanced CRISPR-derived platforms shaping the future of gene editing, along with gene delivery techniques, therapeutic applications of CRISPR technology, and emerging trends in gene editing.

HISTORICAL BACKGROUND OF GENOME EDITING TECHNIQUES

Meganucleases

Meganucleases, also known as homing endonucleases, are one of the earliest classes of programmable nucleases used in genome editing (Figure 1A).^{10–12} These naturally occurring endonucleases typically recognize large DNA target sequences (14–40 base pairs [bp]) (Figure 1B). They recognize DNA cleavage sites and induce site-specific DSBs.¹¹ Meganucleases exhibit inherently high specificity that minimizes off-target activity; their relatively small size facilitates delivery and enables efficient homologous recombination, making them advantageous for therapeutic applications.^{13–15} These advantages are accompanied by lower potential cytotoxicity. However, meganucleases were historically limited due to the difficulty of reprogramming target specificity. Recently, iECURE and Precision BioSciences have developed technologies that enable the efficient redesign of meganuclease DNA recognition domains. These engineered platforms are now being applied in clinical trials, leveraging the advantages of meganucleases.

ZFNs

An ZFN is a chimeric protein composed of a zinc finger DNA-binding domain and a FokI restriction endonuclease domain

<https://doi.org/10.1016/j.omtn.2025.102733>.

²These authors contributed equally

Correspondence: Jeong H. Joo, Department of Life Science, Chung-Ang University, Seoul 06974, Korea.

E-mail: jkiiklovehot@naver.com

Correspondence: Keun P. Kim, Department of Life Science, Chung-Ang University, Seoul 06974, Korea.

E-mail: kpkim@cau.ac.kr



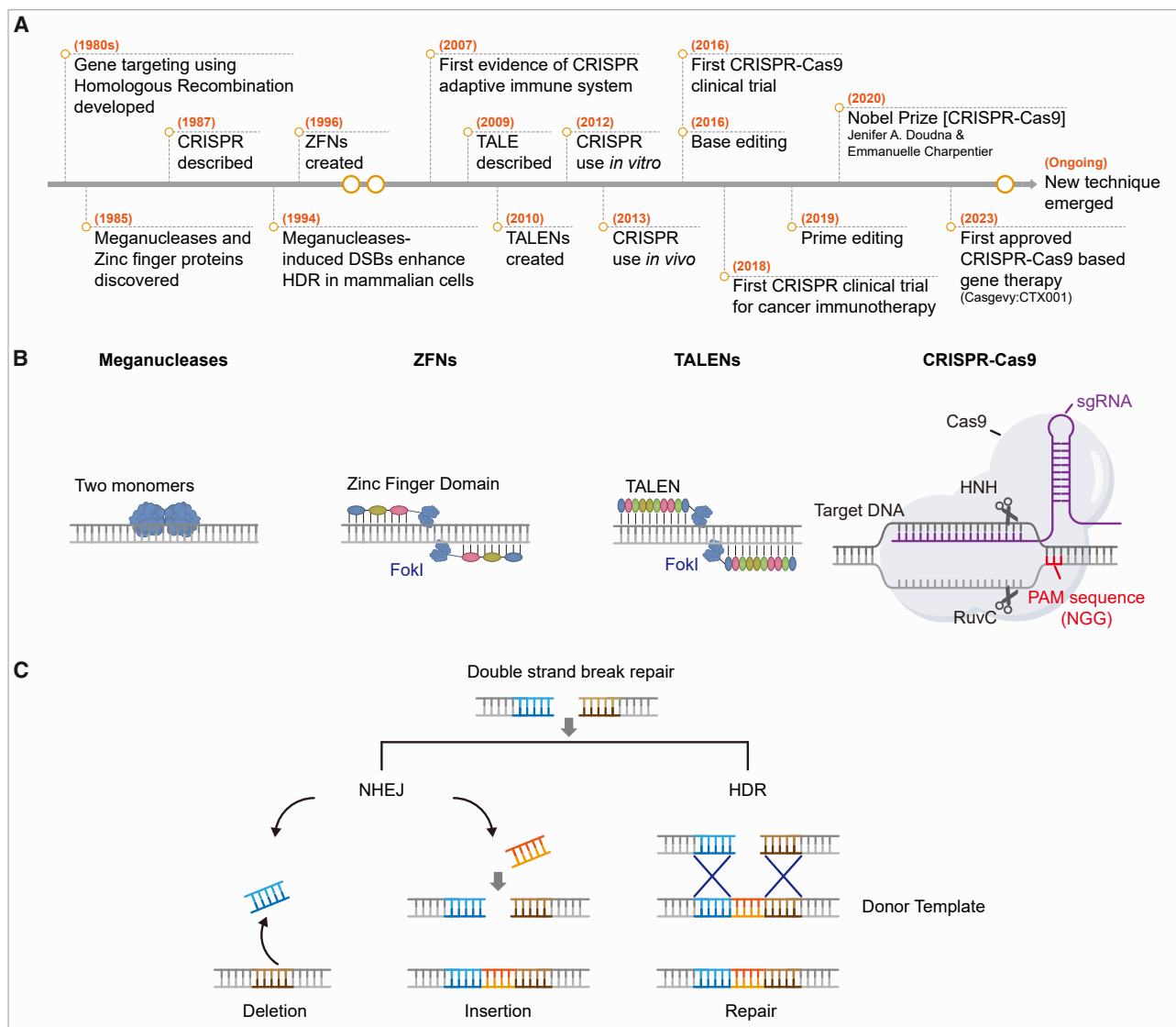


Figure 1. Genome editing platforms and DSB repair mechanisms

(A) Schematic timeline of the development of gene editing. (B) Overview of four currently used genome editing technologies: Meganucleases; ZFN, zinc finger nuclease; TALEN, transcription activator-like effector nuclease; CRISPR-Cas9, clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (Cas9). (C) DSB repair mechanisms. DSBs formed by genome editing are repaired through non-homologous end joining (NHEJ) or homology-directed repair (HDR) using a donor template. NHEJ can lead to insertions or deletions of various lengths, whereas HDR can induce gene replacement when a donor DNA template is present. NHEJ, non-homologous end joining; HDR, homology-directed repair.

(Figure 1B).^{16,17} The first zinc-finger protein, transcription factor IIIA (TFIIIA)—a cysteine-rich regulatory protein—was identified in 1985 in *Xenopus* oocytes (Figure 1A).^{34,35} Proteins belonging to the TFIIIA superfamily possess Cys₂His₂ zinc-finger domains, which facilitate nucleic acid binding. The Cys₂His₂ zinc-finger domain, consisting of approximately 30 amino acids, exhibits a conserved structural motif of two antiparallel β -strands followed by an α -helix.^{34–39} This domain is among the most prevalent DNA-binding structures found in eukaryotic transcription factors. Each zinc-finger motif recognizes a three-bp DNA sequence through specific interac-

tions between the α -helix of the domain and the major groove of DNA (Figure 1B).¹⁸ This recognition mechanism permits selective binding to nucleotide triplets, thereby enabling customization of target specificity.^{17,35,40–42} The zinc-finger domain directs sequence-specific DNA binding, while the FokI domain mediates DNA cleavage to generate DSBs at predefined loci, facilitating DNA recombination. Typically, three to six zinc-finger motifs are linked together to target DNA sequences ranging from 9 to 18 bp.^{17,35,40–42} In functional terms, ZFN monomers bind to opposite DNA strands with a spacer sequence of approximately 5–6 bp

Table 1. Comparison of meganuclease, ZFN, TALEN, and CRISPR-Cas9

	Meganuclease	ZFN	TALEN	CRISPR-Cas9
DNA recognition	protein-based	zinc finger protein	TALE protein	guide RNA
Nuclease	endonuclease	FokI	FokI	Cas9
Repair system	DSBs repaired by HDR or NHEJ			
Off-target effect	low	lower than CRISPR-Cas9	lower than CRISPR-Cas9	high
Design	complex (1–6 months)	complex (~1 month)	complex (~1 month)	very simple (within a week)
Price	high	high	medium	low
References	Arnould et al. ^{10–15}	Li ^{16–22}	Gaj, ¹⁸ ; Shao, ¹⁹ ; Cui et al. ^{22–25}	Gaj, ¹⁸ ; Cui, ²² ; Jinek, ^{26–28}

ZFN, zinc-finger nuclease; TALEN, transcription-activator-like effector nuclease; CRISPR, clustered regularly interspaced short palindromic repeats; DSB, double-strand break; HDR, homology-directed repair; NHEJ, non-homologous end joining.

separating them. Dimerization of the FokI nuclease across this spacer region is required for enzymatic activation and subsequent DNA cleavage.^{43,44}

TALENs

Subsequently, a second-generation gene editing platform was developed: TALENs.^{9,18,30–32} Similar to ZFNs, TALENs consist of a DNA-binding domain fused to a FokI nuclease domain (Figure 1B). The DNA-binding domain of TALENs is composed of transcription-activator-like effector (TALE) proteins derived from the plant pathogen *Xanthomonas*.^{23,24} The TALE domain recognizes DNA sequences in a modular fashion akin to the zinc-finger domain, with each TALE repeat—comprising 33–35 amino acids—recognizing a single nucleotide. TALEs contain repeat-variable di-residues, where the 12th and 13th amino acid residues determine nucleotide specificity.^{45,46} Specifically, repeat-variable di-residues recognize “T” with NG; “A” with NI; “C” with HD; and “G” with NN, HN, or NK.^{45–47} As with ZFNs, TALENs require dimerization for DNA cleavage, and each monomer can recognize up to 20 bp.^{18,48} The target site must begin with a “T” and include a spacer of 12–19 bp.¹⁸ Compared to ZFNs, TALENs utilize a simpler recognition code, which facilitates more straightforward design.

Although ZFN and TALEN systems have several advantages, both systems require custom DNA-binding proteins for each genomic target. While the construction of synthetic genes encoding these proteins was historically a challenge, advanced modern DNA synthesis and assembly technologies have significantly simplified this process. Nevertheless, there are still some limitations. First, achieving high target specificity can be challenging. The modular DNA-binding domains of both systems may exhibit context-dependent off-target activity, affecting target specificity. Second, efficient intracellular delivery can be difficult. This is a particular obstacle for the large TALEN proteins, which are challenging to package into size-limited viral vectors. In contrast, the more compact ZFNs offer greater flexibility in this regard. Considering these remaining limitations, the emergence of CRISPR and CRISPR-associated (Cas) proteins provided a more flexible and efficient alternative for genome editing.

CRISPR-Cas

The CRISPR-Cas system is currently the most widely utilized genome editing platform in molecular biology laboratories, owing to its straightforward design, low cost, high efficiency, reproducibility, and short experimental cycle. In 2012, the function of the CRISPR-Cas system was precisely elucidated, and its potential as a genome editing tool was demonstrated.^{26,49} This system provides protection against bacteriophages and exogenous DNA through the integration of invader-derived spacers into the CRISPR array and subsequent cleavage of invading genetic material.^{50,51} CRISPR systems are broadly categorized into two classes: class I systems, which are characterized by multi-protein effector complexes (types I, III, and IV), and class II systems, which rely on single-protein effectors (types II, V, and VI).^{52,53} An overview of the classification and principal features of each CRISPR-Cas subtype is presented in Table 2. Among them, CRISPR-Cas9 (class II, type II) is one of the most thoroughly investigated and widely applied genome editing tools.^{52,58} In addition to the widely used type II-A subtype *Streptococcus pyogenes* Cas9 (SpCas9), other Cas9 orthologs such as SaCas9 (type II-A), FnCas9 (type II-B), NmeCas9 (type II-C), and CjCas9 (type II-C) are increasingly being utilized for their unique properties, including smaller size and alternative protospacer-adjacent motif (PAM) compatibility (Table 2).^{26,59–62} Moreover, several non-Cas9 CRISPR systems, such as Cas12, Cas13, and Cas14, as well as next-generation editing technologies including base editors and prime editors, are expanding the genome editing toolbox with enhanced versatility and precision (Tables 2 and 3). CRISPR-Cas9 remains the most technologically advanced and widely implemented genome editing tool, warranting a more in-depth discussion of its mechanisms and applications in the following section.

Structure and mechanism of the CRISPR-Cas9 system

The CRISPR-Cas9 system comprises two essential components: a guide RNA (gRNA) and the CRISPR-associated protein 9 (Cas9). Referred to as “genetic scissors,” CRISPR-Cas9 cleaves double-stranded DNA at targeted sites via its endonuclease activity.⁶³ The gRNA consists of two subunits: the crRNA and the tracrRNA (Figure 1B).^{26,27} The CRISPR RNA (crRNA), approximately 18–20

Table 2. Classification of CRISPR-Cas systems

Class	Type	Cas endonuclease	Target	Subtypes	Special features	Reference
Class I ^a	I	Cas3	DNA	subtypes I-[A-F, U]	• uses the Cascade complex for target recognition • requires a PAM sequence during the interference phase • Cas3 works with protein complexes for DNA degradation • Cas3 has been explored for selective bacterial targeting	Jinek et al. ²⁶ ; Makarova et al. ⁵² ; Jore et al. ⁵⁴
Class I ^a	III	Cas10	RNA/DNA	Subtypes III-[A-F]	• type III-A mediates DNA interference • type III-B mediates RNA targeting and interference	Makarova et al. ⁵²
Class I ^a	IV	Cas6-like, Cas7, Cas11	RNA/DNA	Subtypes IV-[A-C]	• type IV-A contains a CRISPR array and utilizes Cas6-like and Cas7 proteins to target foreign nucleic acids • type IV-A performs RNA-guided interference and plays a key role in plasmid competition • type IV-B contains Cas11 and is involved in plasmid propagation and cellular interaction	Makarova et al. ⁵² ; Pinilla-Redondo et al. ⁵⁵ ; Taylor et al. ⁵⁶ ; Čepaitė et al. ⁵⁷
Class II ^b	II	Cas9	DNA	Subtypes II-[A-C]	• requires tracrRNA and crRNA • requires a PAM sequence during the interference phase • type II-A: SpCas9 (<i>Streptococcus pyogenes</i>); SaCas9 (<i>Staphylococcus aureus</i>). • type II-B: FnCas9 (<i>Francisella novicida</i>) • type II-C: NmeCas9 (<i>Neisseria meningitidis</i>); CjCas9 (<i>Campylobacter jejuni</i>)	Jinek et al. ²⁶ ; Makarova et al. ⁵² ; Cong et al. ⁵⁸ ; Friedland et al. ⁵⁹ ; Hirano et al. ⁶⁰ ; Amrani et al. ⁶¹ ; Kim et al. ⁶² ; Mei et al. ⁶³
Class II ^b	V	Cas12 (Cpf1)	RNA/DNA	Subtypes V-[A-E, V]	• Cas12a produces 5' overhangs • Processes precursor crRNA without tracrRNA or RNase III • Cas12a displays enhanced efficiency in AT-rich genomic regions • Exhibits lower off-target activity • Used for epigenome editing	Makarova et al. ⁵² ; Zetsche et al. ⁶⁴ ; Yamano et al. ⁶⁵ ; Swarts and Jinek ⁶⁶
Class II ^b	VI	Cas13	RNA	Subtypes IV-[A-C]	• a nonspecific RNase that digests nearby RNA • two major subtypes: Cas13a (<i>Leptotrichia wadei</i>) and Cas13b (<i>Prevotella</i> spp.)	Makarova et al. ⁵² ; Cox et al. ⁶⁷ ; Abudayyeh et al. ⁶⁸

^aMultiprotein effector modules.^bSingle-protein effector modules.PAM, protospacer adjacent motif; crRNA, CRISPR RNA; tracrRNA, *trans*-activating crRNA

nucleotides in length, hybridizes with the target DNA sequence, whereas the *trans*-activating crRNA (tracrRNA) serves as a scaffold for Cas9 binding. The tracrRNA interacts complementarily with specific regions of the crRNA, thereby facilitating proper assembly and activation of the gRNA-Cas9 complex. Both the crRNA and tracrRNA are required to functionally activate the CRISPR-Cas9 system. The Cas9 protein consists of two major structural lobes: the recognition (REC) lobe and the nuclease (NUC) lobe.^{26,27,80} The REC lobe includes REC1 and REC2 domains, which mediate gRNA binding. The NUC lobe contains three functional domains: RuvC and HNH, which cleave opposite strands of the DNA, and the PAM-interacting domain, which identifies the PAM required for target DNA recognition.^{26,27,80}

The mechanism of CRISPR-Cas9-mediated genome editing follows three primary steps: (1) recognition of the target sequence, (2) induction of double-stranded DNA cleavage, and (3) DNA repair.¹⁹ In the initial step, a designed single-guide RNA (sgRNA) directs Cas9 to the specific DNA target by base-pairing to the target via its spacer sequence. Concurrently, Cas9 engages with a PAM sequence located immediately downstream of the target site. The PAM is a short, conserved DNA motif, generally 2–5 nucleotides in length, with sequence variation depending on the originating bacterial species.^{58,81,82} SpCas9 typically recognizes PAM sequences with the consensus 5'-NGG-3', where N represents any nucleotide.^{58,81,82} Upon PAM recognition, an RNA-DNA hybrid is formed, and Cas9 induces a DSB by cleaving the complementary DNA strand

Table 3. CRISPR editing technologies

	CRISPR-Cas9	Base editor	Prime editor	RNA editor	Transcriptional modulator
Target	DNA	DNA	DNA	RNA	DNA
Applications	insertion, deletion	substitution	substitution, insertion, deletion	substitution	gene activation or repression
Editing capability	–	• editing window of 5–6 bases (single or multiple adjacent base substitutions possible)	• substitution up to ~30 bp • insertion up to ~40 bp • deletion up to ~80 bp	–	• regulation of transcription without altering the DNA sequence
Accuracy	low	high	high	high	high
Apply	mainly used for insertions and deletions	mainly used for substitution	broad variety of edits	RNA	programmable control of gene expression
Advantages	• easy, fast	• low off-target effect • high accuracy base pair substitution • no DSBs or donor DNA required	• low off-target effects • high accuracy • all base-to-base conversions are possible • no DSBs or donor DNA required	• enables temporary gene therapy • limited-duration gene therapy	• no DSBs; reversible
Limitations	• high on- and off-target mutations	• nickases can lead to DSBs, including off-target effects • limited targetable sites • bystander mutations • constrained editing window	• nickases can lead to DSBs, including off-target effects • low editing efficiency • large size	• risks of bystander and off-target editing	• limited to target genes with accessible promoters • effect transient
References	Jinek et al. ²⁶ ; Makarova et al. ⁵² ; Cong ⁵⁸ ; Mei et al. ⁶³	Gaudelli et al. ⁶⁹ ; Komor et al. ⁷⁰	Anzalone et al. ⁷¹ ; Ponnienelvan et al. ⁷²	Cox et al. ⁶⁷ ; Booth et al. ⁷³	Qi et al. ^{74–79}

via the HNH domain and the non-complementary strand via the RuvC domain.^{19,63,83} The resulting DSB is subsequently repaired by the cell's endogenous DNA repair pathways.

Limitations of the CRISPR-Cas system

A significant limitation of the CRISPR-Cas9 platform is the occurrence of off-target effects, which occur when the CRISPR-Cas9 complex binds and cleaves unintended genomic sites.⁸⁴ Such off-target activity can disrupt genes critical for normal cellular function or regulation of proliferation, potentially resulting in deleterious mutations and disease development.⁸⁵ To address this issue, the development of advanced genome editing tools with improved specificity is essential to enhance the precision and safety of CRISPR-based therapeutics. There are several genome-wide profiling methods to detect off-target activity, for example, (1) identifying Cas9-induced DSBs: GUIDE-seq and CIRCLE-seq, (2) genome-wide mapping of Cas9 cleavage sites: Digenome-seq and SITE-seq, and (3) estimating potential off-target sites: *in silico* prediction tools.^{86–91} Another limitation of the CRISPR-Cas9 platform is the recognition of PAM sequences, which restricts the genomic locations that CRISPR can target. Scientists are developing Cas9 variants with altered PAM specificities. For example, SpCas9-NG can recognize NG instead of NGG, and xCas9 can recognize a variant PAM sequence.⁹² These variants expand targetable sites and mitigate PAM constraints.

CURRENT CRISPR EDITING TECHNOLOGIES

Beyond the conventional CRISPR-Cas9 and other DSB-dependent genome editing approaches, a range of novel technologies has emerged. These platforms are derived from CRISPR systems and are engineered to edit DNA, RNA, or gene expression with varying characteristics. They are categorized based on their specificity, efficiency, and practical application. A comparative summary of current CRISPR editing tools—including CRISPR-Cas9, base editing, prime editing, and RNA editing—is provided in Table 3.

Base editing

Base editing was first reported in 2016 by David Liu's laboratory. It is a refined CRISPR-Cas9-based technique that enables precise nucleotide modifications without generating DSBs. This approach involves the fusion of a RuvC-inactivated Cas9 nickase [nCAs9 (D10A)] with a nucleotide deaminase enzyme, which facilitates direct base conversion at targeted genomic loci (Figure 2).^{69,70} To increase editing efficiency, nCas9 is designed to introduce a nick on the non-edited strand, encouraging the cell's DNA repair machinery to preferentially resolve mismatches by using the edited strand as a template.^{69,70} By enabling single-nucleotide changes, base editing allows for highly specific and efficient genomic alterations.

Two principal classes of base editors have been developed: cytosine base editors (CBEs) and adenine base editors (ABEs) (Figure 2).

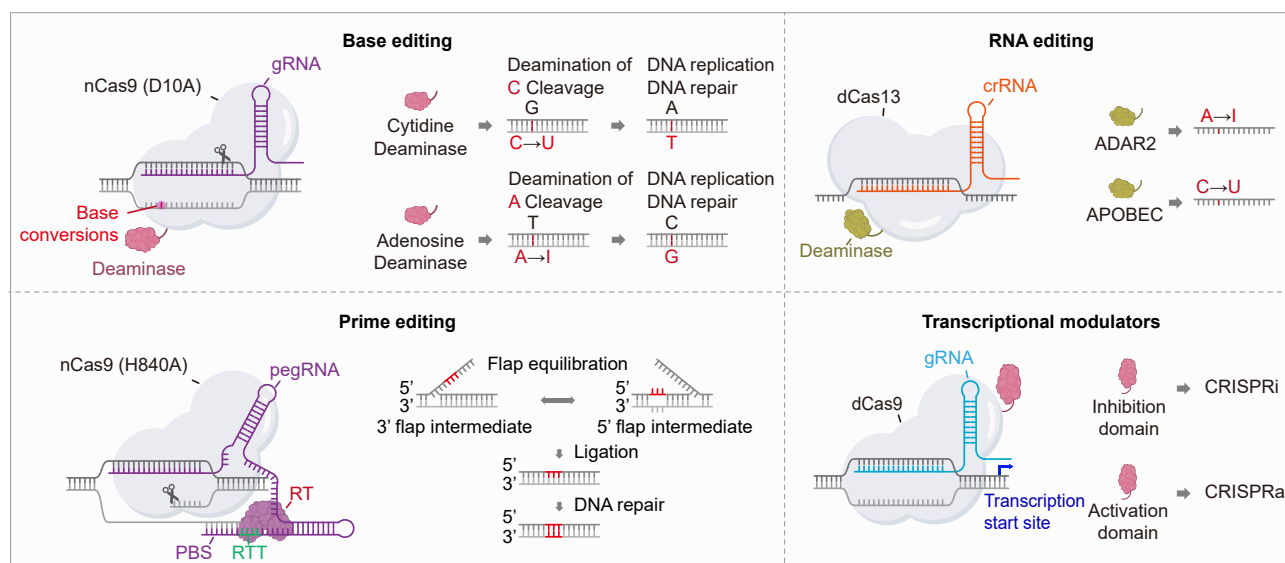


Figure 2. Overview of base editing and prime editing systems

Base editing involves the fusion of Cas9 nickase [nCas9(D10A)] with a nucleobase-modifying enzyme to convert one nucleotide into another without generating double-strand breaks. Cytidine deaminase converts C to U, which is subsequently repaired into T, while adenosine deaminase converts A to I, which is then repaired into G. This technique is particularly useful for introducing specific point mutations, precise gene corrections, or stop codons for gene knockout. Prime editing integrates nCas9(H840A) with reverse transcriptase (RT) and employs prime editing guide RNA (pegRNA), which contains a single-guide RNA (sgRNA), a reverse transcription template (RTT), and a primer binding site (PBS). Following nicking of the non-target DNA strand, the PBS of the pegRNA binds, and, through RT extension, the RTT sequence is copied to the target site. Prime editing enables the insertion, deletion, or replacement of short DNA sequences. RNA editing utilizes Cas13 nuclease fused to a deaminase enzyme to alter RNA sequences, thereby modifying protein production. Transcriptional modulators regulate gene transcription using catalytically inactive Cas9 (dCas9) fused to transcriptional modulation domains.

Both platforms enable efficient correction of point mutations in living cells, offering a permanent and accurate means of genetic modification.^{69,70} CBEs utilize a cytidine deaminase to catalyze the conversion of cytosine to uracil, which subsequently pairs with adenine, resulting in a C-G to T-A substitution upon DNA repair (Figure 2).⁷⁰ Following the success of CBEs, ABEs were engineered to broaden the spectrum of targetable mutations. ABEs incorporate an adenosine deaminase that converts adenine to inosine, which is interpreted as guanine by the cellular replication and transcription machinery, leading to an A-T to G-C substitution (Figure 2).⁶⁹ Beyond these foundational systems, the base editing toolbox has expanded to include editors capable of other conversions, such as C-to-G base editors (CGBEs) (Table S2).⁹³ Furthermore, continuous protein engineering has produced state-of-the-art variants with dramatically improved efficiency, such as CBE6b and ABE8e, and dual base editors such as SPACE and A&C-BE-max.^{69,93–98} Advanced base editing tools are summarized in Table S2.

A major advantage of base editing is its exceptional precision. Because the system alters only a single nucleotide without introducing DSBs, it minimizes the risk of indels and off-target effects often associated with conventional CRISPR-Cas9 editing. This specificity renders base editing a highly promising platform for therapeutic interventions targeting monogenic disorders.

Prime editing

Prime editing is a versatile and high-precision genome editing strategy that integrates an nCas9(H840A) variant, reverse transcriptase (RT), and a specially engineered prime editing guide RNA (pegRNA).⁷¹ The pegRNA comprises three components: an sgRNA, a primer binding site (PBS), and a reverse transcriptase template (RTT) (Figure 2).⁷¹ The PBS hybridizes with the non-target strand of the DNA at the target locus, while the RTT contains the desired sequence modification, facilitating accurate sequence replacement during DNA synthesis.

The prime editing process is initiated when the pegRNA directs the nCas9-RT complex to the genomic site of interest. The nCas9 introduces a single-strand break in the non-target strand, permitting the RT to copy the RTT sequence into the genome. This enables the incorporation of desired edits, including substitutions, short insertions, and short deletions.⁷¹ Moreover, recently evolved prime editing enables large-scale gene deletions. Compared with CRISPR-Cas9, prime editing enhances the precision of genome editing primarily by minimizing the generation of unwanted indels at the target site.^{71,72} Similar to base editing, prime editing avoids the creation of DSBs, which significantly reduces the frequency of off-target mutations compared to standard CRISPR-Cas9, making it a safer option for therapeutic applications. Its ability to introduce diverse and accurate edits without requiring donor DNA templates or DSBs makes prime editing an attractive platform for correcting pathogenic mutations

implicated in genetic diseases.⁷² Recently, several versions of the prime editor, PE1–PE7, PEmax, and epegRNA, have been developed with different efficiencies and fidelities, summarized in Table S3.^{99–103}

RNA editing

RNA editing is a post-transcriptional approach that enables targeted modification of RNA sequences, thereby modulating protein expression without inducing permanent changes to the genome (Figure 2).^{67,73} In contrast to base editing, which alters the DNA sequence, RNA editing introduces transient modifications at the RNA level. Cas13 and Cas7-11 have emerged as leading candidate systems for RNA editing. Cas13 and Cas7-11 are protein complexes that represent major RNA editing technologies, showing different mechanisms and functionality from conventional Cas systems. These systems allow RNA degradation, editing, and modulation.^{67,68,104} By leveraging fusion proteins, such as ADAR2, which catalyzes the conversion of adenosine to inosine, or APOBEC, which deaminates cytosine to uridine, RNA editing can effectively reprogram transcripts to modulate protein function.⁶⁷ This technology offers considerable therapeutic potential due to its reversibility and reduced risk of off-target genomic alterations.⁷³

Transcriptional modulators

The first programmable transcriptional modulators were developed using engineered ZFPs and TALEs, before the emergence of CRISPR-based systems. Advances in CRISPR technologies have led to the development of transcriptional modulators that regulate gene expression without altering the DNA sequence. This is achieved using a catalytically inactive Cas9 (dCas9), which binds to target genomic loci without inducing DSBs, thereby facilitating transcriptional repression or activation (Figure 2).⁷⁴ CRISPR interference (CRISPRi) suppresses gene expression by guiding dCas9 to promoter regions, where it can repress transcription initiation or recruit transcriptional repressor domains. For example, dCas9 fused to the KRAB repressor domain can recruit chromatin-modifying complexes that silence gene expression.^{75,76} Conversely, CRISPR activation (CRISPRa) enhances transcription through the fusion of dCas9 to transcriptional activators, such as VP64.^{77–79} Furthermore, more advanced systems like the CRISPRa synergistic activation mediator (SAM) system achieve stronger activation by using a modified sgRNA containing RNA aptamers to recruit additional activation domains such as p65 and HSF1.⁷⁸ This enables targeted upregulation of endogenous genes, providing a robust method for dissecting gene function and engineering gene regulatory networks. Because these systems modulate gene expression without inducing genomic modifications, they offer a reversible and tunable platform for functional genomics and potential therapeutic applications. Beyond transient transcriptional modulation, related approaches have been developed to achieve heritable changes in gene expression via targeted epigenetic modification, as discussed in the epigenetic editing section.

PRECISION GENE DELIVERY TECHNIQUES

The CRISPR delivery system plays a critical role in the efficient transport of CRISPR-Cas components into target cells. The success of

gene editing is highly dependent on precise and effective delivery of elements such as the Cas protein and sgRNA. The CRISPR-Cas system may be introduced in one of three formats: (1) DNA encoding the Cas protein and sgRNA, (2) RNA comprising Cas mRNA and sgRNA, or (3) a ribonucleoprotein (RNP) complex consisting of the Cas protein bound to sgRNA.¹⁰⁵ The delivery efficiency of these formats varies depending on how nucleic acid materials are delivered to therapeutic interventions. CRISPR-Cas systems are generally administered using *in vitro*, *in vivo*, or *ex vivo* approaches to facilitate therapeutic interventions.¹⁰⁵ Delivery modalities can be categorized into viral-vector-based, non-viral-vector-based, and physical techniques. Multiple strategies have been developed to enhance these delivery processes and optimize gene-editing efficacy.

Viral vector delivery

Viral vectors are among the most widely utilized platforms for gene delivery. As early as the 1980s, researchers recognized the potential of viruses as carriers for therapeutic gene transfer.¹⁰⁶ They engineered viruses by eliminating their pathogenic elements and incorporating therapeutic genes.¹⁰⁶ These vectors serve as delivery vehicles that encapsulate genetic material, protect it from premature degradation, and facilitate efficient transduction into target cells. To ensure safety, viral genomes are modified to remove sequences that prevent replication and pathogenicity. A typical delivery method involves producing viral particles containing Cas9 and sgRNA in HEK293T cells, which are subsequently used to transduce target cells either *in vivo* or *in vitro*.¹⁰⁵ By leveraging the inherent transduction mechanisms of viruses, this approach enables effective gene transfer (Figure 3). Commonly used viral vectors include adenoviruses, adeno-associated viruses (AAVs), and retroviruses.¹⁰⁵

Retroviruses

The first gene therapy clinical trial in 1990 used a retroviral vector to transduce autologous T cells *ex vivo* with a cDNA encoding human adenosine deaminase.¹⁰⁷ This landmark study highlighted the promise of gene therapy and the utility of modified retroviruses as nucleic acid delivery tools. Retroviruses can accommodate up to 8 kb of nucleic acid and stably integrate the transgene into the host genome, enabling sustained gene expression.¹⁰⁸ However, the integration mechanism also introduces risks of insertional mutagenesis, which can result in genotoxic effects, including activation of proto-oncogenes. Furthermore, retroviruses are limited to infecting actively dividing cells, which restricts their applicability and raises concerns about safety.¹⁰⁸

Lentiviruses

Lentiviruses, a subclass of retroviruses, can infect both dividing and non-dividing cells, making them more versatile as delivery vectors. A key advantage is their ability to be pseudotyped with envelope proteins from other viruses, which enhances their flexibility for various applications.¹⁰⁸ However, lentiviral vectors integrate into the host genome, raising concerns about unintended gene disruption when applied in CRISPR-Cas9 systems.¹⁰⁹ Such integration may compromise genomic integrity; thus, careful assessment is needed when using lentiviruses for therapeutic gene editing.

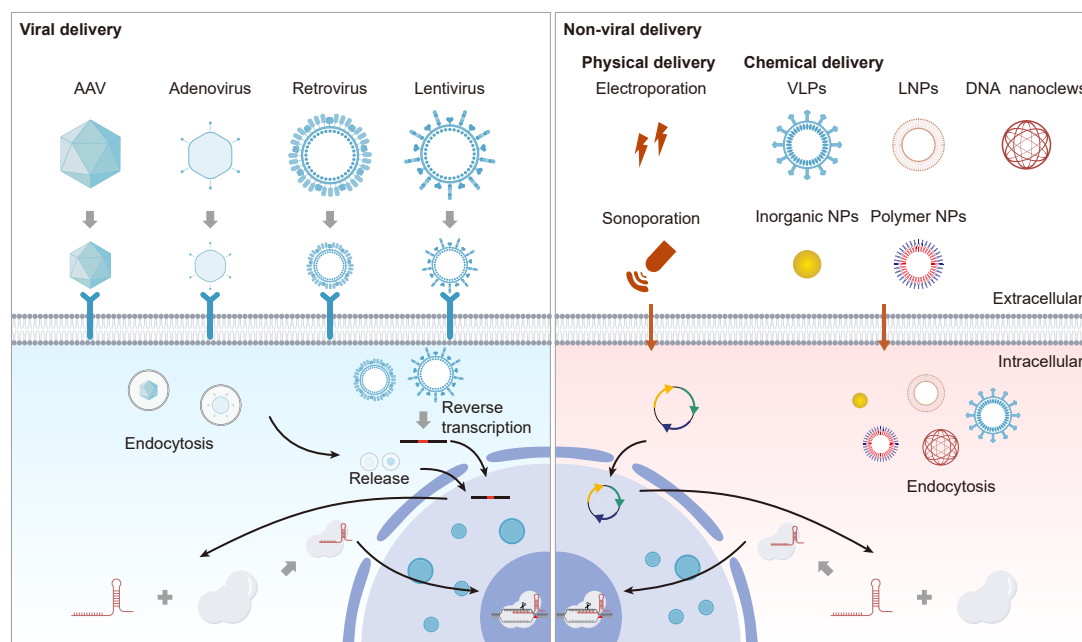


Figure 3. Gene delivery systems for genome editing technology

A schematic diagram illustrating gene delivery systems based on both viral and non-viral approaches. Viral vectors include AAV, retrovirus, lentivirus, and adenovirus. Non-viral delivery methods encompass physical approaches such as electroporation and sonoporation, as well as chemical approaches utilizing virus-like particles (VLPs), lipid nanoparticles (LNPs), polymer nanoparticles, DNA nanoclews, and inorganic nanoparticles.

Adenoviruses

Adenoviruses are non-enveloped, double-stranded DNA viruses with an icosahedral nucleocapsid. Adenoviruses can infect both dividing and non-dividing cells. Although they are versatile, concerns about pathogenicity and host immune responses have limited their broader clinical application. Therefore, the therapeutic potential of adenoviruses should be approached with caution in clinical applications.¹¹⁰

AAVs

AAVs are frequently employed in CRISPR-mediated gene editing owing to their high stability, replication-defective nature, and low immunogenicity. Their capsid-receptor interactions govern host range across species and enable efficient delivery of genetic constructs to diverse cell types.^{111,112} Furthermore, different AAV serotypes exhibit natural tropism for specific tissues, and several serotypes show high affinity for muscle or neuronal cells, which can be leveraged for targeted therapies.^{110,113} Nevertheless, their limited cargo capacity restricts them to delivering only small genetic payloads. This limitation presents a significant barrier for applications requiring the transfer of larger constructs.

Non-viral delivery

Non-viral vectors enable the delivery of CRISPR components into cells through the physical and chemical properties of synthetic or naturally derived carriers, thereby eliminating the need for viral vectors.¹⁰⁵ Non-viral delivery vectors commonly used for virus-like par-

ticles (VLPs) and nanomaterials employed for CRISPR delivery include lipid nanoparticles (LNPs), polymer nanoparticles, DNA nanoclews, and inorganic nanoparticles.^{105,114–120} Because they lack viral elements, these vectors can be used both *in vitro* and *in vivo*, offering improved safety profiles and reduced immunogenicity compared with viral-vector-based systems (Figure 3).¹⁰⁵

VLPs

VLPs are nanoscale, self-assembling protein complexes that mimic the structural features of native viruses but lack genetic material. The absence of a viral genome eliminates the risk of replication or integration into the host genome, addressing major safety concerns associated with traditional viral vectors such as lentiviral or adenoviral vectors.^{114–117} VLPs are classified by structural characteristics into enveloped and non-enveloped types.^{114–117} Enveloped VLPs contain a lipid bilayer derived from the host cell membrane and embedded with viral envelope proteins, whereas non-enveloped VLPs are composed of capsid proteins.^{114–117} The production of VLPs generally involves the recombinant expression of viral capsid proteins in host cells. These proteins self-assemble into highly ordered particles that structurally resemble authentic virions but are devoid of nucleic acids.^{114–117} VLPs can be engineered for tissue-specific targeting by displaying ligand peptides or antibodies on their surface, enhancing delivery efficiency to specific cell types or tissues. Chemical or genetic modifications can also be employed to improve the stability, cargo capacity, and endosomal escape of VLPs, optimizing them for various therapeutic applications.^{114–117} The

enveloped delivery vehicle (EDV), developed by Jennifer Doudna's laboratory, is designed to deliver CRISPR-Cas9 RNPs for *in vivo* genome editing.¹¹⁸ A key feature is the development of smaller mini-EDVs that improve delivery efficiency.¹¹⁸ In parallel, engineered VLPs (eVLPs), developed by David Liu's laboratory, offer a versatile approach. eVLPs can carry diverse cargo, such as proteins and RNA, and are distinguished by enabling transient cargo activity.¹¹⁹ This transient activity is crucial for minimizing side effects while preserving therapeutic efficacy.

LNPs

LNPs rely on electrostatic interactions between negatively charged nucleic acids and ionizable cationic lipids, resulting in nanoparticles capable of crossing the cell membrane to deliver genetic cargo to target cells.¹²⁰ This delivery strategy underpins multiple products approved by the US Food and Drug Administration (FDA) and demonstrates high transfection efficiency. LNPs also protect RNA from premature degradation and enable streamlined manufacturing processes, facilitating their widespread application in RNA vaccine development and CRISPR-based delivery systems.¹²¹ However, LNPs passively accumulate in the liver due to physiological clearance mechanisms, which makes liver-directed therapies feasible but poses a major challenge for targeting other tissues. LNPs require cold-chain storage to maintain stability, which complicates distribution logistics, and concerns persist regarding their cytotoxicity and immunogenic potential. Despite these challenges, LNPs remain a promising delivery vehicle for liver-targeted clinical applications.¹²⁰

Polymer nanoparticles

Polymer nanoparticles, derived either from natural sources or through chemical synthesis, are extensively utilized for delivering CRISPR components. Their surfaces can be easily functionalized with targeting elements to facilitate tissue-specific delivery and controlled release of therapeutic cargo. For example, attaching ligands or antibodies that bind to cancer cell receptors can guide the nanoparticles to tumors, enhancing therapeutic precision. Commonly investigated polymers include polyethyleneimine, polyamidoamine, and chitosan. However, many polymeric materials exhibit substantial cytotoxicity, requiring strategies to reduce safety risks concerns, reduce production costs, and improve delivery efficacy. As a result, current efforts are focused on developing next-generation polymers and optimizing existing polymer-based formulations.¹²²

DNA nanoclews

DNA nanoclews are self-assembled DNA nanostructures designed to mediate the intracellular delivery of CRISPR components. These constructs exhibit high compatibility with biological systems and are degradable by DNases, enabling controlled release under specific intracellular conditions, similar to anticancer drug delivery systems. Recent studies have demonstrated the utility of DNA nanoclews in delivering CRISPR-Cas9 RNPs, providing targeted delivery and responsive release capabilities.¹²³

Inorganic nanoparticles

Inorganic nanoparticles—including gold, iron oxide, and silica-based particles—serve as platforms for constructing nanostructured materials used in both therapeutic delivery and biomedical imaging. Among these, gold nanoparticles are the most extensively studied for CRISPR-Cas9 RNP delivery due to their controllable size distribution, low immunogenicity, and potent therapeutic efficacy in various disease models. Their application enables efficient *in vivo* delivery with high targeting specificity at pathological sites.¹²⁴

Other non-viral vectors

Additional non-viral vectors include traditional drug delivery systems such as cell-penetrating peptides, which possess a positive charge and facilitate membrane translocation via electrostatic interactions. These peptides are commonly used for the direct intracellular delivery of CRISPR-Cas9 systems, and ongoing research continues to explore their potential in enhancing gene-editing efficiency.¹²⁵

Physical delivery

Physical delivery strategies involve transient disruption of the cell membrane to enable the intracellular transfer of CRISPR components, including Cas proteins, sgRNA, and other molecular cargo (Figure 3).

Electroporation

Electroporation is a commonly used physical delivery approach in which an electric field transiently permeabilizes the lipid bilayer of the plasma membrane, thereby facilitating the intracellular delivery of RNA or DNA.^{126,127} This method applies to both *in vitro* and *in vivo* delivery of CRISPR-Cas9 components, offering considerable versatility. Compared with research methods such as microinjection, electroporation generally achieves higher embryo survival rates, reducing the number of animals required for the generation of transgenic mouse models.^{126,127} However, electroporation is associated with certain drawbacks, including the potential for cell death and a reduction in cellular stemness, which may adversely affect gene-editing efficiency in specific cell types.

Membrane-deformation-based delivery

Membrane deformation involves the mechanical perturbation of the cell membrane to transiently enhance its permeability, thereby allowing the entry of CRISPR components. Examples include microfluidic cell squeezing and lance array nanoinjection, which uses microfabricated arrays of nanometer-scale needles to puncture the cell membrane and directly deliver CRISPR components into cells.^{128–130} While both approaches offer precise physical access to the intracellular space, they are still under active investigation and have not yet been widely adopted in clinical gene or cell therapy.

Sonoporation

Sonoporation leverages acoustic cavitation generated by ultrasound waves to create transient pores in the cell membrane, enabling the uptake of molecular cargo. This targeted method allows site-specific

delivery with high precision and efficiency. The improved delivery efficiency permits the use of lower therapeutic doses, thereby minimizing systemic side effects. Notably, sonoporation is minimally invasive and, when appropriately controlled, can minimize tissue damage. It also holds therapeutic promise for neurological disorders, as ultrasound can transiently enhance permeability across the blood-brain barrier.¹³¹

CHALLENGES WITH DELIVERY SYSTEMS

Transfection efficiency and immune responses

Viral vector delivery systems demonstrate high transduction efficiency due to their innate ability to protect cargo from degradation and facilitate cellular entry. Nonetheless, their application is associated with several limitations inherent to viral biology. A primary challenge is the host immune response, which may compromise therapeutic efficacy, particularly in individuals with prior exposure to the virus. Moreover, the immunogenicity of viral vectors generally restricts their use to a single administration.¹³² Another limitation involves their constrained payload capacity, which becomes particularly problematic for large genetic elements such as the Cas9 gene, approximately 4.2 kb in length. Safety concerns also persist, including the risk of pathogenic reversion and the potential for insertional mutagenesis. In contrast, non-viral vectors offer greater cargo capacity, facilitating the delivery of larger therapeutic constructs. These vectors also exhibit reduced immunogenicity and lower cytotoxicity, thereby supporting repeated high-dose administrations.¹³² However, systemic delivery remains a major challenge, particularly when targeting extrahepatic tissues such as the brain, muscle, or lung. Many non-viral platforms show preferential accumulation in the liver, limiting delivery efficiency to other organs. Furthermore, toxicity can arise from mechanisms such as lipid accumulation, inflammation, and cellular stress, especially at high doses or with repeated exposure.

Physical delivery: Surgical intervention

Physical delivery techniques operate via straightforward mechanisms of action, allowing for precise control over the transfection site and yielding more predictable outcomes. However, access to deep or internal organs frequently necessitates surgical intervention. Many of these methods are also associated with pain and damage to adjacent tissues.

THERAPEUTIC APPLICATIONS OF CRISPR TECHNOLOGY

CRISPR-based gene editing technology enables precise genetic modifications and offers promising therapeutic avenues for diseases previously deemed untreatable. The therapeutic applications of CRISPR are generally categorized into two primary strategies: *ex vivo* gene editing and *in vivo* gene editing. Each modality possesses distinct advantages and limitations, serving complementary roles within the broader landscape of gene therapy (Figure 4).^{132–134} A summary of ongoing clinical trials utilizing genome editing is presented in Table S1.

Ex vivo

Ex vivo gene editing involves the modification of autologous cells outside the patient's body, followed by their reintroduction. This process includes three critical steps: cell isolation, gene editing, and reinfusion. Initially, somatic cells are harvested from the patient. Subsequently, CRISPR and other gene editing tools are applied to modify the extracted cells. Finally, the edited cells are reinfused into the patient (Figure 4).^{133,134} A key advantage of the *ex vivo* approach is that the patient is not directly exposed to gene editing components, thereby reducing systemic exposure and potentially lowering the risk of off-target effects. However, off-target edits can still occur during the *in vitro* modification phase, necessitating thorough screening and quality control before reinfusion to avoid systemic toxicity.^{132–134} Furthermore, the ability to selectively reintroduce only modified cells permits precise control over cell dosage. Casgevy (exa-cel) is a successful representative case of *ex vivo* CRISPR therapy (Figures 1A; Table S1; NCT03745287 and NCT03655678). It was developed by Vertex Pharmaceuticals and CRISPR Therapeutics. In December 2023, it became the first CRISPR-based treatment approved by both the US FDA and the UK's Medicines and Healthcare products Regulatory Agency (MHRA). Casgevy treats sickle cell disease (SCD) and transfusion-dependent β -thalassemia (TDT) by editing a patient's hematopoietic stem cells to produce fetal hemoglobin, easing symptoms of these severe blood disorders. Its approval marks a milestone in genetic medicine, paving the way for other *ex vivo* gene editing therapies in development.

Another *ex vivo* approach is CAR T cell therapy, which involves modifying autologous T cells outside the body to recognize tumor markers, such as CD19 or BCMA, and reinfusing them to eliminate cancer cells.¹³⁵ However, the autologous process is time-consuming and costly due to the need to manufacture a personalized product for each patient. To overcome these limitations, allogeneic ("off-the-shelf") CAR T cell therapies using donor-derived cells are under development. Through CRISPR editing, immune rejection can be minimized and the production process streamlined.¹³⁶ Additionally, a promising strategy involves the combination of iPSC technology with CRISPR genome editing. Induced pluripotent stem cells (iPSCs) can be genetically edited with CRISPR and subsequently differentiated into specific cell types for transplantation, offering potential for personalized cell therapy and tissue replacement. Notably, this approach has been applied to the treatment of type 1 diabetes, where engineered iPSCs are differentiated into pancreatic endoderm cells and transplanted to restore insulin production (Table S1; NCT05210530).¹³⁷ However, this method is restricted to cells that can be reliably cultured and manipulated *ex vivo*, presenting challenges for targeting certain pathological cell types, such as malignant cells that necessitate direct *in vivo* intervention.

In vivo

In vivo gene editing involves the direct modification of genetic material within the patient's body. Although technically more complex, this strategy offers considerable therapeutic potential, particularly in

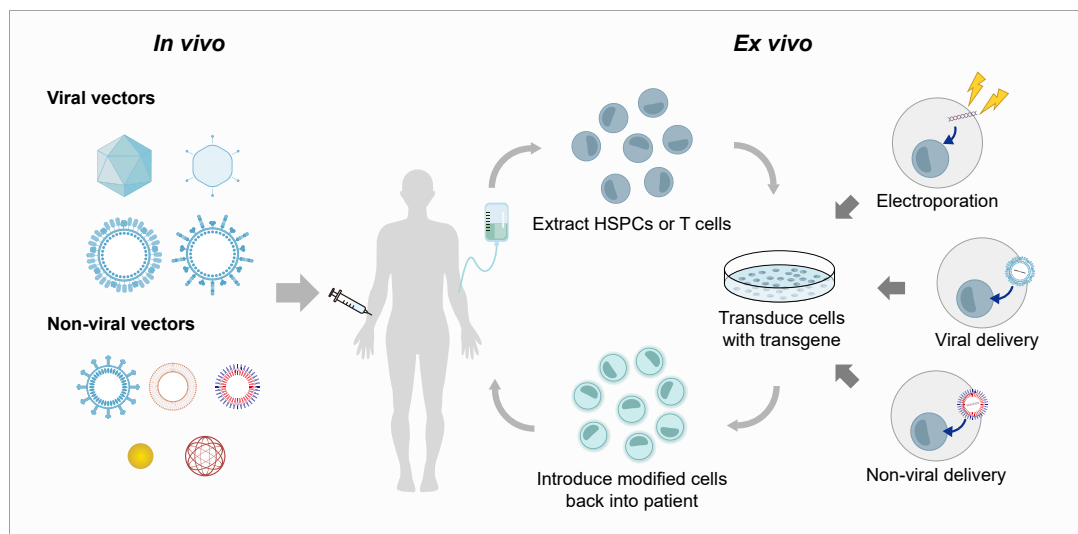


Figure 4. Applications of CRISPR genome editing for human gene therapy

Ex vivo and *in vivo* strategies leveraging CRISPR genome editors. In the *in vivo* approach, viral or non-viral vectors are directly injected into the patient. In the *ex vivo* strategy, hematopoietic stem and progenitor cells (HSPCs) or T cells are extracted from the patient, and gene therapy agents are introduced into the cells using electroporation, viral vectors, or non-viral vectors. The modified cells are subsequently reintroduced into the patient. The decision between *in vivo* and *ex vivo* strategies depends on the specific CRISPR genome editing technology employed.

scenarios where cell transplantation is not feasible.^{133,134} The success of *in vivo* gene editing depends heavily on the development of efficient and specific delivery systems that enhance target cell uptake while minimizing off-target activity and genotoxic effects.^{132–134} Additionally, achieving precise targeting of specific tissues or organs remains a critical requirement. By circumventing the limitations inherent to *ex vivo* culture, *in vivo* gene editing expands therapeutic applicability across a broader spectrum of diseases. Nevertheless, this approach still faces challenges, including delivery specificity, immune responses, and potential cellular toxicity.

EMERGING TRENDS IN GENE EDITING

DNA polymerase editors

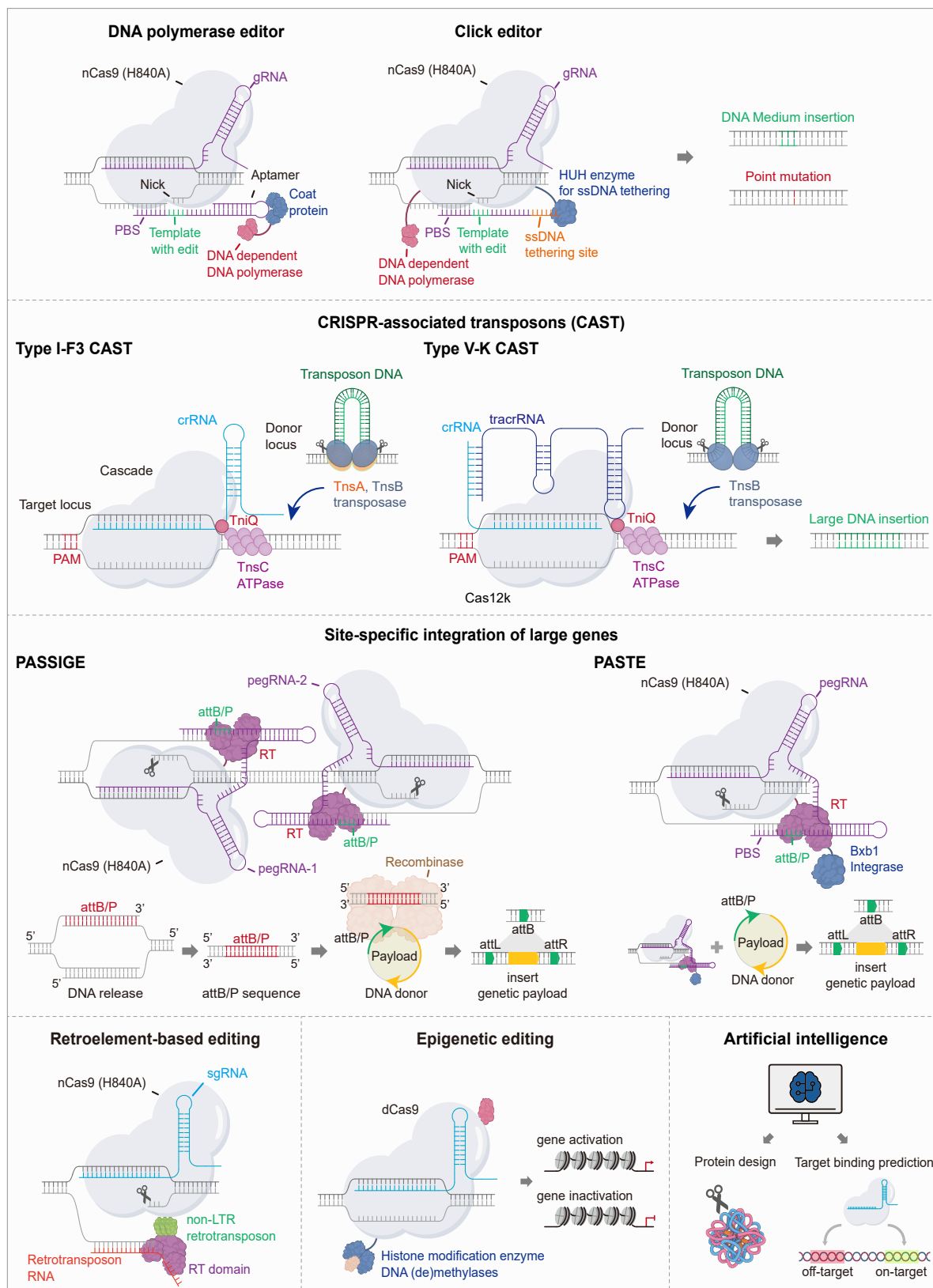
The DNA polymerase editor represents an advancement in CRISPR-Cas9-based technologies, in which nCas9(H840A) and a DNA polymerase are tethered to single-stranded DNA template through an HUH endonuclease to enable gene editing (Figure 5).^{138,139} Unlike prime editing, which employs reverse transcriptase and RNA templates, this system utilizes DNA polymerases and DNA templates for the modification or replication of DNA.¹⁴⁰ During the editing process, nCas9 targets a specific genomic locus and introduces a single-strand break.¹⁴⁰ This break serves as the priming site for DNA-polymerase-mediated synthesis of a new DNA sequence. A single-stranded DNA template, supplied exogenously, is conjugated to the target site via enzymes such as the HUH endonuclease.^{138,139} The DNA polymerase then uses this template to replicate and insert the new sequence at the cleavage site. A key advantage of the DNA polymerase editor is its ability to avoid self-inhibitory base pairing within the gRNA, thereby enhancing editing precision and enabling the insertion of sequences exceeding 100 nucleotides.¹⁴¹ This capacity

substantially broadens the scope and fidelity of gene editing, particularly for applications requiring the insertion of long DNA fragments, which are challenging to achieve using conventional CRISPR systems. However, using exogenous DNA templates in DNA-polymerase-mediated editing presents significant challenges. Effectively delivering DNA templates into target cells or tissues remains a major obstacle, especially for *in vivo* applications. Furthermore, exogenous DNA can trigger innate immune responses, potentially leading to cytotoxicity and decreased editing efficiency. Overcoming these challenges is essential for expanding the therapeutic potential of this technology.

A refined variant of this platform, termed the click editor, incorporates click chemistry to enhance the efficiency and accuracy of DNA-polymerase-mediated editing (Figure 5).¹³⁹ Click chemistry facilitates the formation of stable and specific bonds between molecular components, improving the alignment of the target DNA with the DNA template. This optimized interaction ensures precise template attachment and supports high-fidelity sequence modification by DNA polymerase. By accelerating and stabilizing the template-binding step, this method enhances the overall efficiency and accuracy of gene editing.¹³⁹ More recently, the implementation of T4 DNA polymerase has further advanced this approach by mitigating harmful end modifications at nCas9-induced DNA termini and promoting the efficient filling of 5' overhangs, thereby improving editing precision.¹⁴²

CRISPR-associated transposons

CRISPR-associated transposons (CASTs) are mobile genetic elements that integrate crRNA-guided DNA transposition functionalities (Figure 5).^{143–146} These systems combine crRNA with associated



(legend on next page)

Cas proteins to mediate site-specific transposition, analogous to the mechanism employed by the Tn7 transposon.^{147–149} CAST offers several advantages over traditional genome editing approaches, including enhanced programmability, flexibility in PAM recognition, and the ability to insert sequences into the genome without generating DSBs or requiring endogenous DNA repair pathways. Although CAST systems adopt a Tn7-like mechanism of action, their integration of CRISPR proteins distinguishes them from conventional transposons.^{147–149} Core elements of CAST include the transposition machinery (TnsA and TnsB), regulatory protein TnsC, and CRISPR-associated components. The TniQ protein acts as a molecular bridge between the CRISPR system and the transposition enzymes.^{147–149} CAST can be adapted to various CRISPR subtypes, with type I-F (employing the Cascade complex) and type V-K (utilizing Cas12K) being the most extensively characterized.^{149,150} The type I-F3 system, originally identified in *Vibrio cholerae*, functions through a multi-protein Cascade complex comprising Cas6, Cas7, and a Cas5-Cas8 fusion protein, in association with TniQ. During the initial phase of transposition, the TniQ-Cascade complex binds to the target genomic locus, whereupon TnsA and TnsB cleave the DNA. TnsC subsequently associates with TniQ to complete the integration process.^{149,150} Conversely, the type V-K system, discovered in *Scytonema hofmanni*, relies on a single Cas protein, Cas12K, which operates in conjunction with tracrRNA.¹⁴⁸ Similar to the Tn7 transposition pathway, this system mediates DNA insertion but lacks the TnsA component, relying instead on TnsB to perform DNA cleavage. The Cas12K-tracrRNA complex recognizes and binds the target site, while TnsC and TniQ orchestrate the subsequent steps of transposition. CAST technologies are currently being employed in a wide range of *in vitro* and *in vivo* genome editing applications, targeting diverse loci, organisms, and genetic payloads.¹⁴⁸ However, their low efficiency in mammalian cells has been a major challenge. To overcome this limitation, the laboratories of David Liu and Samuel Sternberg developed an evolved CRISPR-associated transposase.¹⁵¹ This engineered transposase enables the highly efficient and programmable insertion of multi-kilobase gene sequences into specific sites within the human genome. This innovation represents a significant advancement, greatly expanding the potential of CAST-mediated therapeutic gene insertion in human cells.¹⁵¹

Site-specific integration of large genes

Prime-editing-assisted site-specific integrase gene editing (PASSIGE) and a related approach, programmable addition via site-specific tar-

geting elements (PASTE), are techniques that integrate prime editing with site-specific recombinases—specifically, large serine recombinases—to facilitate efficient insertion of multi-kilobase DNA sequences at defined genomic loci (Figure 5).^{71,152–154} PASSIGE utilizes single-flap or dual-flap prime editing to install a recombinase recognition site at the target locus, thereby enabling site-specific insertion of large DNA fragments by the corresponding recombinase. This strategy can be implemented via a single transfection, delivering all components concurrently, or through two sequential transfections involving initial prime editing followed by recombinase-mediated integration.^{71,152–154} PASTE employs CRISPR-nCas9(H840A), a reverse transcriptase, and a serine integrase (Bxb1), in combination with two gRNAs, to install an integrase recognition sequence (attB) and stimulate prime editing (PE3), thereby enhancing integration efficiency. This method relies on serine recombinases, such as Bxb1, to mediate accurate and efficient DNA insertion.¹⁵³ Both PASSIGE and PASTE depend on prime editing to introduce a recombinase att site, followed by targeted integration of large DNA payloads by the recombinase.^{153,154} Historically, PASTE showed higher integration efficiency than the original PASSIGE; however, the continuously evolved eePASSIGE, developed by David Liu's group using an evolved Bxb1 integrase, now outperforms PASTE in multiple contexts, with each approach employing distinct optimization strategies.¹⁵⁴ For instance, PASTE incorporates pegRNA scaffold mutants, linker modifications between Cas9 and reverse transcriptase, and engineered reverse transcriptase mutations to enhance performance, although some modifications have paradoxically led to reduced integration efficiency.¹⁵⁴ Furthermore, fusion of recombinases to prime editors has been associated with diminished integration rates, and both approaches exhibit decreased efficiency with increasing donor DNA size.

Retroelement-based editing

Target-primed reverse transcription (TPRT) is an emerging gene editing modality within the CRISPR framework that is integral to the mechanism of prime editing (Figure 5).¹⁵⁵ TPRT is modeled on retroelements (retrotransposons), naturally occurring genetic elements that integrate via a nick-primed reverse transcription mechanism rather than relying on double-strand DNA cleavage.^{156,157} These retrotransposons, classified as non-long terminal repeat elements, include LINE-1 and R2—both of which have gained prominence in gene editing research.^{156,158,159} In TPRT, a site-specific nick exposes a 3'-OH that primes reverse transcription of an RNA template; in prime editing, nCas9(H840A) supplies this nick, and the reverse

Figure 5. Advanced CRISPR editing technologies

A summary of emerging technologies in genome editing. DNA polymerase editing (click editor) integrates nCas9(H840A) with DNA polymerase and uses an endonuclease with single-stranded DNA templates. CRISPR-associated transposons involve fusing CRISPR effector complexes with transposase proteins to facilitate RNA-guided transposition of long DNA sequences into specific loci, with commonly used systems including Type I-F3 CAST and Type V-K CAST. Site-specific integration of large genes combines prime editors with site-specific serine recombinases to guide recombinase-mediated insertion at att sites within target DNA loci, enabling efficient integration of large DNA sequences. PASSIGE and PASTE methodologies support large-gene integration. Retroelement-based editing employs nCas9(H840A) with a non-long terminal repeat (non-LTR) reverse transcriptase and RNA, generating a free 3' end that enables reverse transcription of the RNA and its insertion into the target DNA. Epigenetic editing fuses dCas9 with DNA methyltransferases and histone-modifying enzymes to modify chromatin at specific gene loci, leading to gene silencing (CRISPR-off) without altering the DNA sequence. Alternatively, dCas9 can be fused with DNA demethylases and transcriptional activation domains to restore gene expression (CRISPR-on). AI-based gene editing employs AI to design novel proteins and guide RNAs, predict off-target effects, and forecast editing outcomes.

transcriptase copies the pegRNA-encoded template sequence. The newly synthesized DNA is subsequently incorporated into the genome through flap resolution and ligation, without generating a DSB at the target site.¹⁵⁷ This mechanism facilitates the incorporation of RNA-encoded sequences into genomic DNA. Importantly, by avoiding DSBs, TPRT-based editors reduce DSB-associated by-products, enabling precise and reliable gene insertions or modifications.^{156,157} Through this mechanism, prime editing technologies overcome many of the limitations inherent to traditional CRISPR-Cas9 platforms, offering a pathway for high-fidelity genomic correction.

Epigenetic editing

Epigenetic editing based on the principles of transcriptional modulation enables stable and potentially heritable gene expression changes. This technique modulates transcriptional activity and regulates gene expression without altering the underlying DNA sequence, typically achieved by fusing dCas9 to DNA methyltransferases or histone-modifying enzymes (Figure 5).^{160,161} Earlier non-CRISPR platforms, such as ZFPs and TALEs, have also been utilized for targeted epigenetic modification, offering delivery advantages in size-limited systems.^{162,163} This facilitates DNA methylation at defined gene regions, leading to heritable transcriptional repression (CRISPRoff) without permanent genomic alterations. Conversely, gene activation (CRISPRon) can be achieved by fusing dCas9 to DNA demethylases or transcriptional activators, thereby restoring gene expression at previously silenced loci.^{160,161} As a highly precise strategy for modulating gene expression, epigenetic editing is increasingly being investigated as a potential therapeutic modality for various diseases.

CRISPR-enabled autonomous transposable element

The CRISPR-enabled autonomous transposable element (CREATE) system is a novel gene-editing platform that integrates CRISPR-Cas9 with RNA-mediated transposable elements, such as the LINE-1 transposon.¹⁶⁴ Unlike conventional approaches that rely on DNA donor templates, this system enables targeted gene insertion through RNA-based transposons. This allows for the precise integration of large genetic payloads (approximately 1 kb) into specific genomic loci in human cell lines and primary T cells, demonstrating high specificity with minimal off-target effects.¹⁶⁴ Moreover, this approach eliminates the requirement for viral vectors to deliver DNA donor constructs, thereby mitigating associated safety concerns and addressing manufacturing limitations.¹⁶⁴

Artificial intelligence in CRISPR design and optimization

Artificial intelligence (AI) is increasingly integrated into CRISPR-based gene editing workflows and is employed across multiple domains (Figure 5). AI is instrumental in the design of CRISPR components, prediction of off-target events, optimization of guide RNAs (gRNAs), and simulation of editing outcomes. Given the critical importance of minimizing off-target effects in gene editing, AI facilitates the development of highly specific gRNAs with reduced unintended activity.^{165–168} By applying deep learning algo-

rithms, AI can identify sequence patterns associated with effective gRNAs in previous CRISPR experiments and predict gRNA sequences with enhanced target specificity.^{169–173} Furthermore, AI simulations enable the prediction of likely outcomes following gene editing—such as insertions, deletions, or point mutations—thereby reducing experimental time and cost. In addition, AI is employed to predict optimal CRISPR delivery strategies, improving transfection efficiency while minimizing cytotoxicity.^{165–168,173} AI also plays a significant role in synthetic biology by supporting the design of microbial systems engineered to produce biofuels, pharmaceuticals, and other biologically derived compounds. It contributes to the identification of novel therapeutic gene targets and the refinement of CRISPR-based gene therapy approaches.¹⁷⁴ Moreover, AI facilitates the comparative analysis of gene editing tools, thereby guiding the selection of the most suitable CRISPR system for specific experimental or clinical applications.¹⁷³ Notably, Marcus Noyes and colleagues have developed AI-guided high-throughput screening strategies for the efficient engineering of highly specific ZFPs.¹⁷² These approaches enable scalable, precise targeting of DNA sequences for both research and therapeutic purposes, underscoring the growing role of AI across diverse gene editing platforms.¹⁷³

CONCLUSIONS AND FUTURE PERSPECTIVES

Ongoing research efforts continue to focus on enhancing the efficiency and versatility of CRISPR systems, as well as improving gene delivery platforms. In particular, substantial progress is being made in optimizing *in vivo* delivery approaches to maximize editing efficiency while minimizing off-target effects. These advancements have significantly expanded the therapeutic potential of CRISPR, supporting the development of both *ex vivo* and *in vivo* strategies for the treatment of genetic disorders, malignancies, and other complex conditions. The future success of CRISPR-based therapies will depend on further improvements in editing precision and delivery technologies. Persistent technical challenges—such as enhancing delivery efficiency, minimizing immune responses, and reducing unintended genetic modifications—must be addressed through continued investigation.

ACKNOWLEDGMENTS

This work was supported by grants from the National Research Foundation of Korea (NRF), funded by the Korean government (MSIT) (RS-2023-00208191 and 2022M3A9J4079468).

AUTHOR CONTRIBUTIONS

J.H.J., S.L., and K.P.K. wrote the manuscript. J.H.J. and K.P.K. created the artwork.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omtn.2025.102733>.

REFERENCES

- Li, H., Yang, Y., Hong, W., Huang, M., Wu, M., and Zhao, X. (2020). Applications of genome editing technology in the targeted therapy of human diseases: mechanisms, advances and prospects. *Signal Transduct. Target. Ther.* 5, 1. <https://doi.org/10.1038/s41392-019-0089-y>.
- Urnov, F.D., Rebar, E.J., Holmes, M.C., Zhang, H.S., and Gregory, P.D. (2010). Genome editing with engineered zinc finger nucleases. *Nat. Rev. Genet.* 11, 636–646. <https://doi.org/10.1038/nrg2842>.
- Carroll, D. (2011). Genome engineering with zinc-finger nucleases. *Genetics* 188, 773–782. <https://doi.org/10.1534/genetics.111.131433>.
- Jiang, C., Meng, L., Yang, B., and Luo, X. (2020). Application of CRISPR/Cas9 gene editing technique in the study of cancer treatment. *Clin. Genet.* 97, 73–88. <https://doi.org/10.1111/cge.13589>.
- Manghwar, H., Lindsey, K., Zhang, X., and Jin, S. (2019). CRISPR/Cas system: recent advances and future prospects for genome editing. *Trends Plant Sci.* 24, 1102–1125. <https://doi.org/10.1016/j.tplants.2019.09.006>.
- Wyman, C., and Kanaar, R. (2006). DNA double-strand break repair: all's well that ends well. *Annu. Rev. Genet.* 40, 363–383. <https://doi.org/10.1146/annurev.genet.40.110405.090451>.
- Sung, P., and Klein, H. (2006). Mechanism of homologous recombination: mediators and helicases take on regulatory functions. *Nat. Rev. Mol. Cell Biol.* 7, 739–750. <https://doi.org/10.1038/nrm2008>.
- San Filippo, J., Sung, P., and Klein, H. (2008). Mechanism of eukaryotic homologous recombination. *Annu. Rev. Biochem.* 77, 229–257. <https://doi.org/10.1146/annurev.biochem.77.061306.125255>.
- Zheng, Y., Li, Y., Zhou, K., Li, T., VanDusen, N.J., and Hua, Y. (2024). Precise genome-editing in human diseases: mechanisms, strategies and applications. *Signal Transduct. Target. Ther.* 9, 47. <https://doi.org/10.1038/s41392-024-01750-2>.
- Arnould, S., Delenda, C., Grizot, S., Desseaux, C., Pâques, F., Silva, G.H., and Smith, J. (2011). The I-CreI meganuclease and its engineered derivatives: applications from cell modification to gene therapy. *Protein Eng. Des. Sel.* 24, 27–31. <https://doi.org/10.1093/protein/gzq083>.
- Stoddard, B.L. (2005). Homing endonuclease structure and function. *Q. Rev. Biophys.* 38, 49–95. <https://doi.org/10.1017/S0033583505004063>.
- Jacquier, A., and Dujon, B. (1985). An intron-encoded protein is active in a gene conversion process that spreads an intron into a mitochondrial gene. *Cell* 41, 383–394. [https://doi.org/10.1016/s0092-8674\(85\)80011-8](https://doi.org/10.1016/s0092-8674(85)80011-8).
- Pâques, F., and Duchateau, P. (2007). Meganucleases and DNA double-strand break-induced recombination: perspectives for gene therapy. *Curr. Gene Ther.* 7, 49–66. <https://doi.org/10.2174/156652307779940216>.
- Mandip, K.C., and Steer, C.J. (2019). A new era of gene editing for the treatment of human diseases. *Swiss Med. Wkly.* 149, w20021. <https://doi.org/10.4414/smww.2019.20021>.
- Khalil, A.M. (2020). The genome editing revolution: review. *J. Genet. Eng. Biotechnol.* 18, 68. <https://doi.org/10.1186/s43141-020-00078-y>.
- Li, L., Wu, L.P., and Chandrasegaran, S. (1992). Functional domains in Fok I restriction endonuclease. *Proc. Natl. Acad. Sci. USA* 89, 4275–4279. <https://doi.org/10.1073/pnas.89.10.4275>.
- Kim, C.A., and Berg, J.M. (1996). A 2.2 Å resolution crystal structure of a designed zinc finger protein bound to DNA. *Nat. Struct. Biol.* 3, 940–945. <https://doi.org/10.1038/nsb1196-940>.
- Gaj, T., Gersbach, C.A., and Barbas, C.F., 3rd (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol.* 31, 397–405. <https://doi.org/10.1016/j.tibtech.2013.04.004>.
- Shao, M., Xu, T.R., and Chen, C.S. (2016). The big bang of genome editing technology: development and application of the CRISPR/Cas9 system in disease animal models. *Dongwuxue Yanjiu* 37, 191–204. <https://doi.org/10.13918/j.issn.2095-8137.2016.4.191>.
- Kim, H.J., Lee, H.J., Kim, H., Cho, S.W., and Kim, J.S. (2009). Targeted genome editing in human cells with zinc finger nucleases constructed via modular assembly. *Genome Res.* 19, 1279–1288. <https://doi.org/10.1101/gr.089417.108>.
- Kim, J.S., Lee, H.J., and Carroll, D. (2010). Genome editing with modularly assembled zinc-finger nucleases. *Nat. Methods* 7, 91. <https://doi.org/10.1038/nmeth0210-91a>.
- Cui, Z., Liu, H., Zhang, H., Huang, Z., Tian, R., Li, L., Fan, W., Chen, Y., Chen, L., Zhang, S., et al. (2021). The comparison of ZFNs, TALENs, and SpCas9 by GUIDE-seq in HPV-targeted gene therapy. *Mol. Ther. Nucleic Acids* 26, 1466–1478. <https://doi.org/10.1016/j.omtn.2021.08.008>.
- Boch, J., Scholze, H., Schornack, S., Landgraf, A., Hahn, S., Kay, S., Lahaye, T., Nickstadt, A., and Bonas, U. (2009). Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* 326, 1509–1512. <https://doi.org/10.1126/science.1178811>.
- Moscou, M.J., and Bogdanove, A.J. (2009). A simple cipher governs DNA recognition by TAL effectors. *Science* 326, 1501. <https://doi.org/10.1126/science.1178817>.
- Ul Ain, Q., Chung, J.Y., and Kim, Y.H. (2015). Current and future delivery systems for engineered nucleases: ZFN, TALEN and RGEN. *J. Control. Release* 205, 120–127. <https://doi.org/10.1016/j.jconrel.2014.12.036>.
- Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., and Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816–821. <https://doi.org/10.1126/science.1225829>.
- Gasiunas, G., Barrangou, R., Horvath, P., and Siksnys, V. (2012). Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc. Natl. Acad. Sci. USA* 109, E2579–E2586. <https://doi.org/10.1073/pnas.1208507109>.
- Gu, S., Zhang, J., Li, L., and Zhong, J. (2022). Repurposing the endogenous CRISPR-Cas9 system for high-efficiency genome editing in *Lactocaseibacillus paracasei*. *ACS Synth. Biol.* 11, 4031–4042. <https://doi.org/10.1021/acssynbio.2c00374>.
- Rahman, S.H., Maeder, M.L., Joung, J.K., and Cathomen, T. (2011). Zinc-finger nucleases for somatic gene therapy: the next frontier. *Hum. Gene Ther.* 22, 925–933. <https://doi.org/10.1089/hum.2011.087>.
- 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. <https://doi.org/10.1038/nrm3486>.
- Chen, K., and Gao, C. (2013). TALENs: customizable molecular DNA scissors for genome engineering of plants. *J. Genet. Genomics* 40, 271–279. <https://doi.org/10.1016/j.jgg.2013.03.009>.
- Sun, N., and Zhao, H. (2013). Transcription activator-like effector nucleases (TALENs): a highly efficient and versatile tool for genome editing. *Biotechnol. Bioeng.* 110, 1811–1821. <https://doi.org/10.1002/bit.24890>.
- Daboussi, F., Zaslavskiy, M., Poirot, L., Loperfido, M., Gouble, A., Guyot, V., Leduc, S., Galetto, R., Grizot, S., Oficjalska, D., et al. (2012). Chromosomal context and epigenetic mechanisms control the efficacy of genome editing by rare-cutting designer endonucleases. *Nucleic Acids Res.* 40, 6367–6379. <https://doi.org/10.1093/nar/gks268>.
- Miller, J., McLachlan, A.D., and Klug, A. (1985). Repetitive zinc-binding domains in the protein transcription factor IIIA from *Xenopus* oocytes. *EMBO J.* 4, 1609–1614. <https://doi.org/10.1002/j.1460-2075.1985.tb03825.x>.
- Pavletich, N.P., and Pabo, C.O. (1991). Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 Å. *Science* 252, 809–817. <https://doi.org/10.1126/science.2028256>.
- Brown, R.S., Sander, C., and Argos, P. (1985). The primary structure of transcription factor TFIID has 12 consecutive repeats. *FEBS Lett.* 186, 271–274. [https://doi.org/10.1016/0014-5793\(85\)80723-7](https://doi.org/10.1016/0014-5793(85)80723-7).
- Engelke, D.R., Ng, S.Y., Shastry, B.S., and Roeder, R.G. (1980). Specific interaction of a purified transcription factor with an internal control region of 5S RNA genes. *Cell* 19, 717–728. [https://doi.org/10.1016/s0092-8674\(80\)80048-1](https://doi.org/10.1016/s0092-8674(80)80048-1).
- Hanas, J.S., Hazuda, D.J., Bogenhagen, D.F., Wu, F.Y., and Wu, C.W. (1983). *Xenopus* transcription factor A requires zinc for binding to the 5 S RNA gene. *J. Biol. Chem.* 258, 14120–14125. [https://doi.org/10.1016/S0021-9258\(17\)43831-2](https://doi.org/10.1016/S0021-9258(17)43831-2).
- Beerli, R.R., and Barbas, C.F., 3rd (2002). Engineering polydactyl zinc-finger transcription factors. *Nat. Biotechnol.* 20, 135–141. <https://doi.org/10.1038/nbt0202-135>.

40. Liu, Q., Segal, D.J., Ghiara, J.B., and Barbas, C.F. (1997). Design of polydactyl zinc-finger proteins for unique addressing within complex genomes. *Proc. Natl. Acad. Sci. USA* 94, 5525–5530. <https://doi.org/10.1073/pnas.94.11.5525>.
41. Elrod-Erickson, M., Rould, M.A., Nekudova, L., and Pabo, C.O. (1996). Zif268 protein-DNA complex refined at 1.6 Å: a model system for understanding zinc finger-DNA interactions. *Structure* 4, 1171–1180. [https://doi.org/10.1016/s0969-2126\(96\)00125-6](https://doi.org/10.1016/s0969-2126(96)00125-6).
42. Elrod-Erickson, M., Benson, T.E., and Pabo, C.O. (1998). High-resolution structures of variant Zif268-DNA complexes: implications for understanding zinc finger-DNA recognition. *Structure* 6, 451–464. [https://doi.org/10.1016/s0969-2126\(98\)00047-1](https://doi.org/10.1016/s0969-2126(98)00047-1).
43. Shimizu, Y., Bhakta, M.S., and Segal, D.J. (2009). Restricted spacer tolerance of a zinc finger nuclease with a six amino acid linker. *Bioorg. Med. Chem. Lett.* 19, 3970–3972. <https://doi.org/10.1016/j.bmcl.2009.02.109>.
44. Händel, E.M., Alwin, S., and Cathomen, T. (2009). Expanding or restricting the target site repertoire of zinc-finger nucleases: the inter-domain linker as a major determinant of target site selectivity. *Mol. Ther.* 17, 104–111. <https://doi.org/10.1038/mt.2008.233>.
45. Mak, A.N.S., Bradley, P., Cernadas, R.A., Bogdanove, A.J., and Stoddard, B.L. (2012). The crystal structure of TAL effector PthXo1 bound to its DNA target. *Science* 335, 716–719. <https://doi.org/10.1126/science.1216211>.
46. Deng, D., Yan, C., Pan, X., Mahfouz, M., Wang, J., Zhu, J.K., Shi, Y., and Yan, N. (2012). Structural basis for sequence-specific recognition of DNA by TAL effectors. *Science* 335, 720–723. <https://doi.org/10.1126/science.1215670>.
47. Yang, J., Zhang, Y., Yuan, P., Zhou, Y., Cai, C., Ren, Q., Wen, D., Chu, C., Qi, H., and Wei, W. (2014). Complete decoding of TAL effectors for DNA recognition. *Cell Res.* 24, 628–631. <https://doi.org/10.1038/cr.2014.19>.
48. Smith, J., Bibikova, M., Whitby, F.G., Reddy, A.R., Chandrasegaran, S., and Carroll, D. (2000). Requirements for double-strand cleavage by chimeric restriction enzymes with zinc finger DNA-recognition domains. *Nucleic Acids Res.* 28, 3361–3369. <https://doi.org/10.1093/nar/28.17.3361>.
49. Knott, G.J., and Doudna, J.A. (2018). CRISPR-Cas guides the future of genetic engineering. *Science* 361, 866–869. <https://doi.org/10.1126/science.aat5011>.
50. Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D.A., and Horvath, P. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 315, 1709–1712. <https://doi.org/10.1126/science.1138140>.
51. Marraffini, L.A., and Sontheimer, E.J. (2008). CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science* 322, 1843–1845. <https://doi.org/10.1126/science.1165771>.
52. Makarova, K.S., Wolf, Y.I., Alkhnbashi, O.S., Costa, F., Shah, S.A., Saunders, S.J., Barrangou, R., Brouns, S.J.J., Charpentier, E., Haft, D.H., et al. (2015). An updated evolutionary classification of CRISPR-Cas systems. *Nat. Rev. Microbiol.* 13, 722–736. <https://doi.org/10.1038/nrmicro3569>.
53. Shmakov, S., Abudayyeh, O.O., Makarova, K.S., Wolf, Y.I., Gootenberg, J.S., Semenova, E., Minakhin, L., Joung, J., Konermann, S., Severinov, K., et al. (2015). Discovery and functional characterization of diverse class 2 CRISPR-Cas systems. *Mol. Cell* 60, 385–397. <https://doi.org/10.1016/j.molcel.2015.10.008>.
54. Jore, M.M., Lundgren, M., van Duijn, E., Bultema, J.B., Westra, E.R., Waghmare, S. P., Wiedenheft, B., Pul, U., Wurm, R., Wagner, R., et al. (2011). Structural basis for CRISPR RNA-guided DNA recognition by Cascade. *Nat. Struct. Mol. Biol.* 18, 529–536. <https://doi.org/10.1038/nsmb.2019>.
55. Pinilla-Redondo, R., Mayo-Muñoz, D., Russel, J., Garrett, R.A., Randau, L., Sørensen, S.J., and Shah, S.A. (2020). Type IV CRISPR-Cas systems are highly diverse and involved in competition between plasmids. *Nucleic Acids Res.* 48, 2000–2012. <https://doi.org/10.1093/nar/gkz1197>.
56. Taylor, H.N., Laderman, E., Armbrust, M., Hallmark, T., Keiser, D., Bondy-Denomy, J., and Jackson, R.N. (2021). Positioning diverse type IV structures and functions within class 1 CRISPR-Cas systems. *Front. Microbiol.* 12, 671522. <https://doi.org/10.3389/fmicb.2021.671522>.
57. Čepaitė, R., Klein, N., Mikšys, A., Camara-Wilpert, S., Ragozius, V., Benz, F., Skorupskaitė, A., Becker, H., Žvejiūtė, G., Steube, N., et al. (2024). Structural variation of types IV-A1- and IV-A3-mediated CRISPR interference. *Nat. Commun.* 15, 9306. <https://doi.org/10.1038/s41467-024-53778-1>.
58. Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., and Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819–823. <https://doi.org/10.1126/science.1231143>.
59. Friedland, A.E., Baral, R., Singhal, P., Loveluck, K., Shen, S., Sanchez, M., Marco, E., Gotta, G.M., Maeder, M.L., Kennedy, E.M., et al. (2015). Characterization of *Staphylococcus aureus* Cas9: a smaller Cas9 for all-in-one adeno-associated virus delivery and paired nickase applications. *Genome Biol.* 16, 257. <https://doi.org/10.1186/s13059-015-0817-8>.
60. Hirano, H., Gootenberg, J.S., Horii, T., Abudayyeh, O.O., Kimura, M., Hsu, P.D., Nakane, T., Ishitani, R., Hatada, I., Zhang, F., et al. (2016). Structure and engineering of *Francisella novicida* Cas9. *Cell* 164, 950–961. <https://doi.org/10.1016/j.cell.2016.01.039>.
61. Amrani, N., Gao, X.D., Liu, P., Edraki, A., Mir, A., Ibraheem, R., Gupta, A., Sasaki, K.E., Wu, T., Donohoue, P.D., et al. (2018). NmeCas9 is an intrinsically high-fidelity genome-editing platform. *Genome Biol.* 19, 214. <https://doi.org/10.1186/s13059-018-1591-1>.
62. Kim, E., Koo, T., Park, S.W., Kim, D., Kim, K., Cho, H.Y., Song, D.W., Lee, K.J., Jung, M.H., Kim, S., et al. (2017). In vivo genome editing with a small Cas9 orthologue derived from *Campylobacter jejuni*. *Nat. Commun.* 8, 14500. <https://doi.org/10.1038/ncomms14500>.
63. Mei, Y., Wang, Y., Chen, H., Sun, Z.S., and Ju, X.D. (2016). Recent progress in CRISPR/Cas9 technology. *J. Genet. Genomics* 43, 63–75. <https://doi.org/10.1016/j.jgg.2016.01.001>.
64. Zetsche, B., Gootenberg, J.S., Abudayyeh, O.O., Slaymaker, I.M., Makarova, K.S., Essletzbichler, P., Volz, S.E., Joung, J., van der Oost, J., Regev, A., et al. (2015). Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* 163, 759–771. <https://doi.org/10.1016/j.cell.2015.09.038>.
65. Yamano, T., Nishimasu, H., Zetsche, B., Hirano, H., Slaymaker, I.M., Li, Y., Fedorova, I., Nakane, T., Makarova, K.S., Koonin, E.V., et al. (2016). Crystal structure of Cpf1 in complex with guide RNA and target DNA. *Cell* 165, 949–962. <https://doi.org/10.1016/j.cell.2016.04.003>.
66. Swarts, D.C., and Jinek, M. (2018). Cas9 versus Cas12a/Cpf1: Structure-function comparisons and implications for genome editing. *Wiley Interdiscip. Rev. RNA* 9, e1481. <https://doi.org/10.1002/wrna.1481>.
67. Cox, D.B.T., Gootenberg, J.S., Abudayyeh, O.O., Franklin, B., Kellner, M.J., Joung, J., and Zhang, F. (2017). RNA editing with CRISPR-Cas13. *Science* 358, 1019–1027. <https://doi.org/10.1126/science.aag0180>.
68. Abudayyeh, O.O., Gootenberg, J.S., Essletzbichler, P., Han, S., Joung, J., Belanto, J. J., Verdine, V., Cox, D.B.T., Kellner, M.J., Regev, A., et al. (2017). RNA targeting with CRISPR-Cas13. *Nature* 550, 280–284. <https://doi.org/10.1038/nature24049>.
69. Gaudelli, N.M., Komor, A.C., Rees, H.A., Packer, M.S., Badran, A.H., Bryson, D.I., and Liu, D.R. (2017). Programmable base editing of A-T to G-C in genomic DNA without DNA cleavage. *Nature* 551, 464–471. <https://doi.org/10.1038/nature24644>.
70. Komor, A.C., Kim, Y.B., Packer, M.S., Zuris, J.A., and Liu, D.R. (2016). Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* 533, 420–424. <https://doi.org/10.1038/nature17946>.
71. Anzalone, A.V., Randolph, P.B., Davis, J.R., Sousa, A.A., Koblan, L.W., Levy, J.M., Chen, P.J., Wilson, C., Newby, G.A., Raguram, A., and Liu, D.R. (2019). Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* 576, 149–157. <https://doi.org/10.1038/s41586-019-1711-4>.
72. Ponnienselman, K., Liu, P., Nyalile, T., Oikemus, S., Maitland, S.A., Lawson, N.D., Luban, J., and Wolfe, S.A. (2023). Reducing the inherent auto-inhibitory interaction within the pegRNA enhances prime editing efficiency. *Nucleic Acids Res.* 51, 6966–6980. <https://doi.org/10.1093/nar/gkad456>.
73. Booth, B.J., Nourreddine, S., Katrekara, D., Savva, Y., Bose, D., Long, T.J., Huss, D.J., and Mali, P. (2023). RNA editing: expanding the potential of RNA therapeutics. *Mol. Ther.* 31, 1533–1549. <https://doi.org/10.1016/j.ymthe.2023.01.005>.
74. Qi, L.S., Larson, M.H., Gilbert, L.A., Doudna, J.A., Weissman, J.S., Arkin, A.P., and Lim, W.A. (2013). Repurposing CRISPR as an RNA-guided platform for

- sequence-specific control of gene expression. *Cell* 152, 1173–1183. <https://doi.org/10.1016/j.cell.2013.02.022>.
75. Gilbert, L.A., Larson, M.H., Morsut, L., Liu, Z., Brar, G.A., Torres, S.E., Stern-Ginossar, N., Brandman, O., Whitehead, E.H., Doudna, J.A., et al. (2013). CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* 154, 442–451. <https://doi.org/10.1016/j.cell.2013.06.044>.
 76. Thakore, P.I., D'Ippolito, A.M., Song, L., Safi, A., Shivakumar, N.K., Kabadi, A.M., Reddy, T.E., Crawford, G.E., and Gersbach, C.A. (2015). Highly specific epigenome editing by CRISPR-Cas9 repressors for silencing of distal regulatory elements. *Nat. Methods* 12, 1143–1149. <https://doi.org/10.1038/nmeth.3630>.
 77. Maeder, M.L., Linder, S.J., Cascio, V.M., Fu, Y., Ho, Q.H., and Joung, J.K. (2013). CRISPR RNA-guided activation of endogenous human genes. *Nat. Methods* 10, 977–979. <https://doi.org/10.1038/nmeth.2598>.
 78. Konermann, S., Brigham, M.D., Trevino, A.E., Joung, J., Abudayyeh, O.O., Barcena, C., Hsu, P.D., Habib, N., Gootenberg, J.S., Nishimasu, H., et al. (2015). Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature* 517, 583–588. <https://doi.org/10.1038/nature14136>.
 79. Weltner, J., Balboa, D., Katayama, S., Bessalov, M., Krut'skov, K., Jouhilahti, E.M., Trokovic, R., Kere, J., and Otonkoski, T. (2018). Human pluripotent reprogramming with CRISPR activators. *Nat. Commun.* 9, 2643. <https://doi.org/10.1038/s41467-018-05067-x>.
 80. Nishimasu, H., Ran, F.A., Hsu, P.D., Konermann, S., Shehata, S.I., Dohmae, N., Ishitani, R., Zhang, F., and Nureki, O. (2014). Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell* 156, 935–949. <https://doi.org/10.1016/j.cell.2014.02.001>.
 81. Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., and Church, G.M. (2013). RNA-guided human genome engineering via Cas9. *Science* 339, 823–826. <https://doi.org/10.1126/science.1232033>.
 82. Tang, L., Yang, F., He, X., Xie, H., Liu, X., Fu, J., Xi, H., Lu, X., Liu, C., Song, Z., et al. (2019). Efficient cleavage resolves PAM preferences of CRISPR-Cas in human cells. *Cell Regen.* 8, 44–50. <https://doi.org/10.1016/j.cr.2019.08.002>.
 83. Jiang, F., and Doudna, J.A. (2017). CRISPR-Cas9 structures and mechanisms. *Annu. Rev. Biophys.* 46, 505–529. <https://doi.org/10.1146/annurev-biophys-062215-010822>.
 84. Fu, Y., Foden, J.A., Khayter, C., Maeder, M.L., Reyon, D., Joung, J.K., and Sander, J.D. (2013). High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat. Biotechnol.* 31, 822–826. <https://doi.org/10.1038/nbt.2623>.
 85. Guo, C., Ma, X., Gao, F., and Guo, Y. (2023). Off-target effects in CRISPR/Cas9 gene editing. *Front. Bioeng. Biotechnol.* 11, 1143157. <https://doi.org/10.3389/fbioe.2023.1143157>.
 86. Hsu, P.D., Lander, E.S., and Zhang, F. (2014). Development and applications of CRISPR-Cas9 for genome engineering. *Cell* 157, 1262–1278. <https://doi.org/10.1016/j.cell.2014.05.010>.
 87. Tsai, S.Q., Zheng, Z., Nguyen, N.T., Liebers, M., Topkar, V.V., Thapar, V., Wyvekens, N., Khayter, C., Iafrate, A.J., Le, L.P., et al. (2015). GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat. Biotechnol.* 33, 187–197. <https://doi.org/10.1038/nbt.3117>.
 88. Kim, D., and Kim, J.S. (2018). DIG-seq: a genome-wide CRISPR off-target profiling method using chromatin DNA. *Genome Res.* 28, 1894–1900. <https://doi.org/10.1101/gr.236620.118>.
 89. Cameron, P., Fuller, C.K., Donohoue, P.D., Jones, B.N., Thompson, M.S., Carter, M.M., Gradia, S., Vidal, B., Garner, E., Slorach, E.M., et al. (2017). Mapping the genomic landscape of CRISPR-Cas9 cleavage. *Nat. Methods* 14, 600–606. <https://doi.org/10.1038/nmeth.4284>.
 90. Yan, J., Xue, D., Chuai, G., Gao, Y., Zhang, G., and Liu, Q. (2020). Benchmarking and integrating genome-wide CRISPR off-target detection and prediction. *Nucleic Acids Res.* 48, 11370–11379. <https://doi.org/10.1093/nar/gkaa930>.
 91. Sherkatghanad, Z., Abdar, M., Charlier, J., and Makarenkov, V. (2023). Using traditional machine learning and deep learning methods for on- and off-target prediction in CRISPR/Cas9: a review. *Brief. Bioinform.* 24, bbad131.
 92. Zeng, D., Li, X., Huang, J., Li, Y., Cai, S., Yu, W., Li, Y., Huang, Y., Xie, X., Gong, Q., et al. (2020). Engineered Cas9 variant tools expand targeting scope of genome and base editing in rice. *Plant Biotechnol. J.* 18, 1348–1350. <https://doi.org/10.1111/pbi.13293>.
 93. Kurt, I.C., Zhou, R., Iyer, S., Garcia, S.P., Miller, B.R., Langner, L.M., Grünwald, J., and Joung, J.K. (2021). CRISPR C-to-G base editors for inducing targeted DNA transversions in human cells. *Nat. Biotechnol.* 39, 41–46. <https://doi.org/10.1038/s41587-020-0609-x>.
 94. Zhang, E., Neugebauer, M.E., Krasnow, N.A., and Liu, D.R. (2024). Phage-assisted evolution of highly active cytosine base editors with enhanced selectivity and minimal sequence context preference. *Nat. Commun.* 15, 1697. <https://doi.org/10.1038/s41467-024-45969-7>.
 95. Liang, F., Zhang, Y., Li, L., Yang, Y., Fei, J.F., Liu, Y., and Qin, W. (2022). SpG and SpRY variants expand the CRISPR toolbox for genome editing in zebrafish. *Nat. Commun.* 13, 3421. <https://doi.org/10.1038/s41467-022-31034-8>.
 96. Zheng, S., Liu, Y., Xia, X., Xiao, J., Ma, H., Yuan, X., Zhang, Y., Chen, Z., Peng, G., Li, W., et al. (2025). Sequence context-agnostic TadA-derived cytosine base editors for genome-wide editing in zebrafish. *Adv. Sci.* 12, e2411478. <https://doi.org/10.1002/adv.202411478>.
 97. Grünwald, J., Zhou, R., Lareau, C.A., Garcia, S.P., Iyer, S., Miller, B.R., Langner, L.M., Hsu, J.Y., Aryee, M.J., and Joung, J.K. (2020). A dual-deaminase CRISPR base editor enables concurrent adenine and cytosine editing. *Nat. Biotechnol.* 38, 861–864. <https://doi.org/10.1038/s41587-020-0535-y>.
 98. Xue, N., Liu, X., Zhang, D., Wu, Y., Zhong, Y., Wang, J., Fan, W., Jiang, H., Zhu, B., Ge, X., et al. (2023). Improving adenine and dual base editors through introduction of TadA-8e and Rad51DBD. *Nat. Commun.* 14, 1224. <https://doi.org/10.1038/s41467-023-36887-1>.
 99. Anzalone, A.V., Koblan, L.W., and Liu, D.R. (2020). Genome editing with CRISPR-Cas nucleases, base editors, transposases and prime editors. *Nat. Biotechnol.* 38, 824–844. <https://doi.org/10.1038/s41587-020-0561-9>.
 100. Chen, P.J., Hussmann, J.A., Yan, J., Knipping, F., Ravisankar, P., Chen, P.F., Chen, C., Nelson, J.W., Newby, G.A., Sahin, M., et al. (2021). Enhanced prime editing systems by manipulating cellular determinants of editing outcomes. *Cell* 184, 5635–5652.e29. <https://doi.org/10.1016/j.cell.2021.09.018>.
 101. Doman, J.L., Pandey, S., Neugebauer, M.E., An, M., Davis, J.R., Randolph, P.B., McElroy, A., Gao, X.D., Raguram, A., Richter, M.F., et al. (2023). Phage-assisted evolution and protein engineering yield compact, efficient prime editors. *Cell* 186, 3983–4002.e26. <https://doi.org/10.1016/j.cell.2023.07.039>.
 102. Nelson, J.W., Randolph, P.B., Shen, S.P., Everette, K.A., Chen, P.J., Anzalone, A.V., An, M., Newby, G.A., Chen, J.C., Hsu, A., and Liu, D.R. (2022). Engineered pegRNAs improve prime editing efficiency. *Nat. Biotechnol.* 40, 402–410. <https://doi.org/10.1038/s41587-021-01039-7>.
 103. Yan, J., Oyler-Castrillo, P., Ravisankar, P., Ward, C.C., Levesque, S., Jing, Y., Simpson, D., Zhao, A., Li, H., Yan, W., et al. (2024). Improving prime editing with an endogenous small RNA-binding protein. *Nature* 628, 639–647. <https://doi.org/10.1038/s41586-024-07259-6>.
 104. Catchpole, R.J., and Terns, M.P. (2021). New type III CRISPR variant and programmable RNA targeting tool: oh, thank heaven for Cas7-11. *Mol. Cell* 81, 4354–4356. <https://doi.org/10.1016/j.molcel.2021.10.014>.
 105. Huang, J., Zhou, Y., Li, J., Lu, A., and Liang, C. (2022). CRISPR/Cas systems: delivery and application in gene therapy. *Front. Bioeng. Biotechnol.* 10, 942325. <https://doi.org/10.3389/fbioe.2022.942325>.
 106. Cline, M.J. (1985). Perspectives for gene therapy: inserting new genetic information into mammalian cells by physical techniques and viral vectors. *Pharmacol. Ther.* 29, 69–92. [https://doi.org/10.1016/0163-7258\(85\)90017-8](https://doi.org/10.1016/0163-7258(85)90017-8).
 107. Muul, L.M., Tuschoff, L.M., Soenen, S.L., Jagadeesh, G.J., Ramsey, W.J., Long, Z., Carter, C.S., Garabedian, E.K., Alleyne, M., Brown, M., et al. (2003). Persistence and expression of the adenosine deaminase gene for 12 years and immune reaction to gene transfer components: long-term results of the first clinical gene therapy trial. *Blood* 101, 2563–2569. <https://doi.org/10.1182/blood-2002-09-2800>.
 108. Howarth, J.L., Lee, Y.B., and Uney, J.B. (2010). Using viral vectors as gene transfer tools (Cell Biology and Toxicology Special Issue: ECTS-UK 1 day meeting on

- genetic manipulation of cells). *Cell Biol. Toxicol.* 26, 1–20. <https://doi.org/10.1007/s10565-009-9139-5>.
109. Kim, H.S., Hwang, G.H., Lee, H.K., Bae, T., Park, S.H., Kim, Y.J., Lee, S., Park, J.H., Bae, S., and Hur, J.K. (2021). CREVIS-Seq: a highly accurate and multiplexable method for genome-wide mapping of lentiviral integration sites. *Mol. Ther. Methods Clin. Dev.* 20, 792–800. <https://doi.org/10.1016/j.omtm.2020.10.012>.
 110. Wang, J.H., Gessler, D.J., Zhan, W., Gallagher, T.L., and Gao, G. (2024). Adeno-associated virus as a delivery vector for gene therapy of human diseases. *Signal Transduct. Target. Ther.* 9, 78. <https://doi.org/10.1038/s41392-024-01780-w>.
 111. Hanlon, K.S., Kleinstiver, B.P., Garcia, S.P., Zaborowski, M.P., Volak, A., Spirig, S.E., Muller, A., Sousa, A.A., Tsai, S.Q., Bengtsson, N.E., et al. (2019). High levels of AAV vector integration into CRISPR-induced DNA breaks. *Nat. Commun.* 10, 4439. <https://doi.org/10.1038/s41467-019-12449-2>.
 112. Naso, M.F., Tomkowicz, B., Perry, W.L., 3rd, and Strohl, W.R. (2017). Adeno-associated virus (AAV) as a vector for gene therapy. *BioDrugs* 31, 317–334. <https://doi.org/10.1007/s40259-017-0234-5>.
 113. Srivastava, A. (2016). In vivo tissue-tropism of adeno-associated viral vectors. *Curr. Opin. Virol.* 21, 75–80. <https://doi.org/10.1016/j.coviro.2016.08.003>.
 114. Kushnir, N., Streatfield, S.J., and Yusibov, V. (2012). Virus-like particles as a highly efficient vaccine platform: diversity of targets and production systems and advances in clinical development. *Vaccine* 31, 58–83. <https://doi.org/10.1016/j.vaccine.2012.10.083>.
 115. Roldão, A., Mellado, M.C.M., Castilho, L.R., Carrondo, M.J.T., and Alves, P.M. (2010). Virus-like particles in vaccine development. *Expert Rev. Vaccines* 9, 1149–1176. <https://doi.org/10.1586/erv.10.115>.
 116. Mohsen, M.O., and Bachmann, M.F. (2022). Virus-like particle vaccinology, from bench to bedside. *Cell. Mol. Immunol.* 19, 993–1011. <https://doi.org/10.1038/s41423-022-00897-8>.
 117. Nooraei, S., Bahrulolum, H., Hoseini, Z.S., Katalani, C., Hajizade, A., Easton, A.J., and Ahmadian, G. (2021). Virus-like particles: preparation, immunogenicity and their roles as nanovaccines and drug nanocarriers. *J. Nanobiotechnol.* 19, 59. <https://doi.org/10.1186/s12951-021-00806-7>.
 118. Ngo, W., Peukes, J., Baldwin, A., Xue, Z.W., Hwang, S., Stickels, R.R., Lin, Z., Satpathy, A.T., Wells, J.A., Schekman, R., et al. (2025). Mechanism-guided engineering of a minimal biological particle for genome editing. *Proc. Natl. Acad. Sci. USA* 122, e2413519121. <https://doi.org/10.1073/pnas.2413519121>.
 119. Raguram, A., An, M., Chen, P.Z., and Liu, D.R. (2025). Directed evolution of engineered virus-like particles with improved production and transduction efficiencies. *Nat. Biotechnol.* 43, 1635–1647. <https://doi.org/10.1038/s41587-024-02467-x>.
 120. Wang, M., Zuris, J.A., Meng, F., Rees, H., Sun, S., Deng, P., Han, Y., Gao, X., Pouli, D., Wu, Q., et al. (2016). Efficient delivery of genome-editing proteins using bio-reducible lipid nanoparticles. *Proc. Natl. Acad. Sci. USA* 113, 2868–2873. <https://doi.org/10.1073/pnas.1520244113>.
 121. Kulkarni, J.A., Witzigmann, D., Chen, S., Cullis, P.R., and van der Meel, R. (2019). Lipid nanoparticle technology for clinical translation of siRNA therapeutics. *Acc. Chem. Res.* 52, 2435–2444. <https://doi.org/10.1021/acs.accounts.9b00368>.
 122. Yin, H., Kauffman, K.J., and Anderson, D.G. (2017). Delivery technologies for genome editing. *Nat. Rev. Drug Discov.* 16, 387–399. <https://doi.org/10.1038/nrd.2016.280>.
 123. Sun, W., Ji, W., Hall, J.M., Hu, Q., Wang, C., Beisel, C.L., and Gu, Z. (2015). Self-assembled DNA nanoclews for the efficient delivery of CRISPR-Cas9 for genome editing. *Angew. Chem. Int. Ed. Engl.* 54, 12029–12033. <https://doi.org/10.1002/anie.201506030>.
 124. Lee, K., Conboy, M., Park, H.M., Jiang, F., Kim, H.J., Dewitt, M.A., Mackley, V.A., Chang, K., Rao, A., Skinner, C., et al. (2017). Nanoparticle delivery of Cas9 ribonucleoprotein and donor DNA in vivo induces homology-directed DNA repair. *Nat. Biomed. Eng.* 1, 889–901. <https://doi.org/10.1038/s41551-017-0137-2>.
 125. Derakhshankhah, H., and Jafari, S. (2018). Cell penetrating peptides: a concise review with emphasis on biomedical applications. *Biomed. Pharmacother.* 108, 1090–1096. <https://doi.org/10.1016/j.biopha.2018.09.097>.
 126. Kaneko, T., Sakuma, T., Yamamoto, T., and Mashimo, T. (2014). Simple knockout by electroporation of engineered endonucleases into intact rat embryos. *Sci. Rep.* 4, 6382. <https://doi.org/10.1038/srep06382>.
 127. Qin, W., Dion, S.L., Kutny, P.M., Zhang, Y., Cheng, A.W., Jillette, N.L., Malhotra, A., Geurts, A.M., Chen, Y.G., and Wang, H. (2015). Efficient CRISPR/Cas9-mediated genome editing in mice by zygote electroporation of nuclease. *Genetics* 200, 423–430. <https://doi.org/10.1534/genetics.115.176594>.
 128. Chakrabarty, P., Gupta, P., Illath, K., Kar, S., Nagai, M., Tseng, F.G., and Santra, T. S. (2022). Microfluidic mechanoporation for cellular delivery and analysis. *Mater. Today Bio* 13, 100193. <https://doi.org/10.1016/j.mtbio.2021.100193>.
 129. Sessions, J.W., Lewis, T.E., Skousen, C.S., Hope, S., and Jensen, B.D. (2016). The effect of injection speed and serial injection on propidium iodide entry into cultured HeLa and primary neonatal fibroblast cells using lance array nanoinjection. *SpringerPlus* 5, 1093. <https://doi.org/10.1186/s40064-016-2757-5>.
 130. Sharei, A., Cho, N., Mao, S., Jackson, E., Pocevicute, R., Adamo, A., Zoldan, J., Langer, R., and Jensen, K.F. (2013). Cell squeezing as a robust, microfluidic intracellular delivery platform. *J. Vis. Exp.* 7, e50980. <https://doi.org/10.3791/50980>.
 131. Tu, J., and Yu, A.C.H. (2022). Ultrasound-mediated drug delivery: sonoporation mechanisms, biophysics, and critical factors. *BME Front.* 2022, 9807347. <https://doi.org/10.34133/2022/9807347>.
 132. Li, L., Hu, S., and Chen, X. (2018). Non-viral delivery systems for CRISPR/Cas9-based genome editing: challenges and opportunities. *Biomaterials* 171, 207–218. <https://doi.org/10.1016/j.biomaterials.2018.04.031>.
 133. Dunbar, C.E., High, K.A., Joung, J.K., Kohn, D.B., Ozawa, K., and Sadelain, M. (2018). Gene therapy comes of age. *Science* 359, eaan4672. <https://doi.org/10.1126/science.aan4672>.
 134. High, K.A., and Roncarolo, M.G. (2019). Gene therapy. *N. Engl. J. Med.* 381, 455–464. <https://doi.org/10.1056/NEJMr1706910>.
 135. Cappell, K.M., and Kochenderfer, J.N. (2023). Long-term outcomes following CAR T cell therapy: what we know so far. *Nat. Rev. Clin. Oncol.* 20, 359–371. <https://doi.org/10.1038/s41571-023-00754-1>.
 136. Chen, S., and van den Brink, M.R.M. (2024). Allogeneic “off-the-shelf” CAR T cells: challenges and advances. *Best Pract. Res. Clin. Haematol.* 37, 101566. <https://doi.org/10.1016/j.beha.2024.101566>.
 137. Shapiro, A.M.J., Thompson, D., Donner, T.W., Bellin, M.D., Hsueh, W., Pettus, J., Wilensky, J., Daniels, M., Wang, R.M., Brandon, E.P., et al. (2021). Insulin expression and C-peptide in type 1 diabetes subjects implanted with stem cell-derived pancreatic endoderm cells in an encapsulation device. *Cell Rep. Med.* 2, 100466. <https://doi.org/10.1016/j.xcrm.2021.100466>.
 138. Aird, E.J., Lovendahl, K.N., St Martin, A., Harris, R.S., and Gordon, W.R. (2018). Increasing Cas9-mediated homology-directed repair efficiency through covalent tethering of DNA repair template. *Commun. Biol.* 1, 54. <https://doi.org/10.1038/s42003-018-0054-2>.
 139. Ferreira da Silva, J., Tou, C.J., King, E.M., Eller, M.L., Rufino-Ramos, D., Ma, L., Cromwell, C.R., Metovic, J., Benning, F.M.C., Chao, L.H., et al. (2025). Click editing enables programmable genome writing using DNA polymerases and HUH endonucleases. *Nat. Biotechnol.* 43, 923–935. <https://doi.org/10.1038/s41587-024-02324-x>.
 140. Halperin, S.O., Tou, C.J., Wong, E.B., Modavi, C., Schaffer, D.V., and Dueber, J.E. (2018). CRISPR-guided DNA polymerases enable diversification of all nucleotides in a tunable window. *Nature* 560, 248–252. <https://doi.org/10.1038/s41586-018-0384-8>.
 141. Liu, B., Dong, X., Zheng, C., Keener, D., Chen, Z., Cheng, H., Watts, J.K., Xue, W., and Sontheimer, E.J. (2024). Targeted genome editing with a DNA-dependent DNA polymerase and exogenous DNA-containing templates. *Nat. Biotechnol.* 42, 1039–1045. <https://doi.org/10.1038/s41587-023-01947-w>.
 142. Yang, Q., Abebe, J.S., Mai, M., Rudy, G., Kim, S.Y., Devinsky, O., and Long, C. (2024). T4 DNA polymerase prevents deleterious on-target DNA damage and enhances precise CRISPR editing. *EMBO J.* 43, 3733–3751. <https://doi.org/10.1038/s44318-024-00158-6>.

143. Bhatt, S., and Chalmers, R. (2019). Targeted DNA transposition in vitro using a dCas9-transposase fusion protein. *Nucleic Acids Res.* 47, 8126–8135. <https://doi.org/10.1093/nar/gkz552>.
144. Chen, S.P., and Wang, H.H. (2019). An engineered Cas-transposon system for programmable and site-directed DNA transpositions. *CRISPR J.* 2, 376–394. <https://doi.org/10.1089/crispr.2019.0030>.
145. Hew, B.E., Sato, R., Mauro, D., Stoytchev, I., and Owens, J.B. (2019). RNA-guided piggyBac transposition in human cells. *Synth. Biol. (Oxf.)* 4, ysz018. <https://doi.org/10.1093/synbio/ysz018>.
146. Kovač, A., Miskey, C., Menzel, M., Grueso, E., Gogol-Döring, A., and Ivics, Z. (2020). RNA-guided retargeting of Sleeping Beauty transposition in human cells. *eLife* 9, e53868. <https://doi.org/10.7554/eLife.53868>.
147. Peters, J.E., Makarova, K.S., Shmakov, S., and Koonin, E.V. (2017). Recruitment of CRISPR-Cas systems by Tn7-like transposons. *Proc. Natl. Acad. Sci. USA* 114, E7358–E7366. <https://doi.org/10.1073/pnas.1709035114>.
148. Strecker, J., Ladha, A., Gardner, Z., Schmid-Burgk, J.L., Makarova, K.S., Koonin, E.V., and Zhang, F. (2019). RNA-guided DNA insertion with CRISPR-associated transposases. *Science* 365, 48–53. <https://doi.org/10.1126/science.aax9181>.
149. Klompe, S.E., Vo, P.L.H., Halpin-Healy, T.S., and Sternberg, S.H. (2019). Transposon-encoded CRISPR-Cas systems direct RNA-guided DNA integration. *Nature* 571, 219–225. <https://doi.org/10.1038/s41586-019-1323-z>.
150. Petassi, M.T., Hsieh, S.C., and Peters, J.E. (2020). Guide RNA categorization enables target site choice in Tn7-CRISPR-Cas transposons. *Cell* 183, 1757–1771. <https://doi.org/10.1016/j.cell.2020.11.005>.
151. Witte, I.P., Lampe, G.D., Eitzinger, S., Miller, S.M., Berrios, K.N., McElroy, A.N., King, R.T., Stringham, O.G., Gelsinger, D.R., Vo, P.L.H., et al. (2025). Programmable gene insertion in human cells with a laboratory-evolved CRISPR-associated transposase. *Science* 388, eadt5199. <https://doi.org/10.1126/science.adt5199>.
152. Anzalone, A.V., Gao, X.D., Podracky, C.J., Nelson, A.T., Koblan, L.W., Raguram, A., Levy, J.M., Mercer, J.A.M., and Liu, D.R. (2022). Programmable deletion, replacement, integration and inversion of large DNA sequences with twin prime editing. *Nat. Biotechnol.* 40, 731–740. <https://doi.org/10.1038/s41587-021-01133-w>.
153. Yarnall, M.T.N., Ioannidi, E.I., Schmitt-Ulms, C., Krajewski, R.N., Lim, J., Villiger, L., Zhou, W., Jiang, K., Garushyants, S.K., Roberts, N., et al. (2023). Drag-and-drop genome insertion of large sequences without double-strand DNA cleavage using CRISPR-directed integrases. *Nat. Biotechnol.* 41, 500–512. <https://doi.org/10.1038/s41587-022-01527-4>.
154. Pandey, S., Gao, X.D., Krasnow, N.A., McElroy, A., Tao, Y.A., Duby, J.E., Steinbeck, B.J., McCreary, J., Pierce, S.E., Tolar, J., et al. (2025). Efficient site-specific integration of large genes in mammalian cells via continuously evolved recombinases and prime editing. *Nat. Biomed. Eng.* 9, 22–39. <https://doi.org/10.1038/s41551-024-01227-1>.
155. Zheng, C., Liu, B., Dong, X., Gaston, N., Sontheimer, E.J., and Xue, W. (2023). Template-jumping prime editing enables large insertion and exon rewriting in vivo. *Nat. Commun.* 14, 3369. <https://doi.org/10.1038/s41467-023-39137-6>.
156. Han, J.S. (2010). Non-long terminal repeat (non-LTR) retrotransposons: mechanisms, recent developments, and unanswered questions. *Mob. DNA* 1, 15. <https://doi.org/10.1186/1759-8753-1-15>.
157. Tang, S., and Sternberg, S.H. (2023). Genome editing with retroelements. *Science* 382, 370–371. <https://doi.org/10.1126/science.ad3183>.
158. Wilkinson, M.E., Frangieh, C.J., Macrae, R.K., and Zhang, F. (2023). Structure of the R2 non-LTR retrotransposon initiating target-primed reverse transcription. *Science* 380, 301–308. <https://doi.org/10.1126/science.adg7883>.
159. Deng, P., Tan, S.Q., Yang, Q.Y., Fu, L., Wu, Y., Zhu, H.Z., Sun, L., Bao, Z., Lin, Y., Zhang, Q.C., et al. (2023). Structural RNA components supervise the sequential DNA cleavage in R2 retrotransposon. *Cell* 186, 2865–2879.e20. <https://doi.org/10.1016/j.cell.2023.05.032>.
160. Saunderson, E.A., Stepper, P., Gomm, J.J., Hoa, L., Morgan, A., Allen, M.D., Jones, J.L., Gribben, J.G., Jurkowski, T.P., and Ficiz, G. (2017). Hit-and-run epigenetic editing prevents senescence entry in primary breast cells from healthy donors. *Nat. Commun.* 8, 1450. <https://doi.org/10.1038/s41467-017-01078-2>.
161. Nuñez, J.K., Chen, J., Pommier, G.C., Cogan, J.Z., Replogle, J.M., Adriaens, C., Ramadoss, G.N., Shi, Q., Hung, K.L., Samelson, A.J., et al. (2021). Genome-wide programmable transcriptional memory by CRISPR-based epigenome editing. *Cell* 184, 2503–2519.e17. <https://doi.org/10.1016/j.cell.2021.03.025>.
162. Neumann, E.N., Bertozzi, T.M., Wu, E., Serack, F., Harvey, J.W., Brauer, P.P., Pirtle, C.P., Coffey, A., Howard, M., Kamath, N., et al. (2024). Brainwide silencing of prion protein by AAV-mediated delivery of an engineered compact epigenetic editor. *Science* 384, ado7082. <https://doi.org/10.1126/science.ado7082>.
163. Cappelluti, M.A., Mollica Poeta, V., Valsoni, S., Quarato, P., Merlin, S., Merelli, I., and Lombardo, A. (2024). Durable and efficient gene silencing in vivo by hit-and-run epigenome editing. *Nature* 627, 416–423. <https://doi.org/10.1038/s41586-024-07087-8>.
164. Wang, Y., Lin, R.Z., Harris, M., Lavayen, B., Diwanji, N., McCreedy, B., Hofmeister, R., and Getts, D. (2025). CRISPR-enabled autonomous transposable element (CREATE) for RNA-based gene editing and delivery. *EMBO Rep.* 26, 1062–1083. <https://doi.org/10.1038/s44319-024-00364-7>.
165. Xiang, X., Corsi, G.I., Anthon, C., Qu, K., Pan, X., Liang, X., Han, P., Dong, Z., Liu, L., Zhong, J., et al. (2021). Enhancing CRISPR-Cas9 gRNA efficiency prediction by data integration and deep learning. *Nat. Commun.* 12, 3238. <https://doi.org/10.1038/s41467-021-23576-0>.
166. Marquart, K.F., Allam, A., Janjuha, S., Sintsova, A., Villiger, L., Frey, N., Krauthammer, M., and Schwank, G. (2021). Predicting base editing outcomes with an attention-based deep learning algorithm trained on high-throughput target library screens. *Nat. Commun.* 12, 5114. <https://doi.org/10.1038/s41467-021-25375-z>.
167. Wessels, H.H., Stirn, A., Méndez-Mancilla, A., Kim, E.J., Hart, S.K., Knowles, D.A., and Sanjana, N.E. (2024). Prediction of on-target and off-target activity of CRISPR-Cas13d guide RNAs using deep learning. *Nat. Biotechnol.* 42, 628–637. <https://doi.org/10.1038/s41587-023-01830-8>.
168. Mathis, N., Allam, A., Kissling, L., Marquart, K.F., Schmidheini, L., Solari, C., Balázs, Z., Krauthammer, M., and Schwank, G. (2023). Predicting prime editing efficiency and product purity by deep learning. *Nat. Biotechnol.* 41, 1151–1159. <https://doi.org/10.1038/s41587-022-01613-7>.
169. Khakzad, H., Igashov, I., Schneuing, A., Goverde, C., Bronstein, M., and Correia, B. (2023). A new age in protein design empowered by deep learning. *Cell Syst.* 14, 925–939. <https://doi.org/10.1016/j.cels.2023.10.006>.
170. Malbrancke, C., Rostain, W., Depardieu, F., Cocco, S., Monasson, R., and Bikard, D. (2023). Computational design of novel Cas9 PAM-interacting domains using evolution-based modelling and structural quality assessment. *PLoS Comput. Biol.* 19, e1011621. <https://doi.org/10.1371/journal.pcbi.1011621>.
171. Huang, J., Lin, Q., Fei, H., He, Z., Xu, H., Li, Y., Qu, K., Han, P., Gao, Q., Li, B., et al. (2023). Discovery of deaminase functions by structure-based protein clustering. *Cell* 186, 3182–3195.e14. <https://doi.org/10.1016/j.cell.2023.05.041>.
172. Ichikawa, D.M., Abidin, O., Alerasool, N., Kogenaru, M., Mueller, A.L., Wen, H., Giganti, D.O., Goldberg, G.W., Adams, S., Spencer, J.M., et al. (2023). A universal deep-learning model for zinc finger design enables transcription factor reprogramming. *Nat. Biotechnol.* 41, 1117–1129. <https://doi.org/10.1038/s41587-022-01624-4>.
173. Dixit, S., Kumar, A., Srinivasan, K., Vincent, P.M.D.R., and Ramu Krishnan, N. (2023). Advancing genome editing with artificial intelligence: opportunities, challenges, and future directions. *Front. Bioeng. Biotechnol.* 11, 1335901. <https://doi.org/10.3389/fbioe.2023.1335901>.
174. Marino, N.D., Pinilla-Redondo, R., Csörgő, B., and Bondy-Denomy, J. (2020). Anti-CRISPR protein applications: natural brakes for CRISPR-Cas technologies. *Nat. Methods* 17, 471–479. <https://doi.org/10.1038/s41592-020-0771-6>.