Narrative Review Article

From Double-Strand Breaks to Precision Edits: A Comparative Review of Modern Genome Editing Tools

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ABSTRACT

Over the past decade, genome editing has been transformed by RNA-guided CRISPR-Cas systems, which have enabled increasingly precise and programmable DNA modification. Since CRISPR-Cas9 was repurposed for genome editing in 2012, base editing (2016) and prime editing (2019) have expanded the field beyond double-strand break–dependent repair. Two classes of DNA base-editors have been developed, cytosine base-editors (CBEs) and adenine base-editors (ABEs). Recently, prime editing has further expanded the CRISPR editing toolkit to all twelve possible transition and transversion mutations, as well as small insertion or deletion mutations. Base editors enable precise transition mutations without double-strand breaks (DSBs). In HEK293T cells, CBE3 has demonstrated a 2- to 6-fold improvement over CBE2 while maintaining low indel formation (~1.1%). Advances in ABE development include seventh-generation variants achieving ~50% efficiency with ≥99.9% product purity and minimal indels in human cells. In HEK293T cells, third-generation prime editors (PE3) achieved editing efficiencies of up to ~55%, while the subsequent PE3b strategy reduced indel formation by up to 13-fold without compromising editing efficiency. CRISPR-Cas9 has achieved clinical success in the treatment of sickle cell disease and β-thalassemia, while base editing and prime editing have shown promising preclinical potential for disorders such as cystic fibrosis and Tay-Sachs disease. Together, these findings highlight the rapid progress of genome editing while underscoring remaining challenges in delivery, specificity, and clinical translation. This review compares Cas9-mediated homology-directed repair (HDR) with generations of cytosine base editors (CBE1–CBE3), adenine base editors (ABE1-ABE7), and prime editors (PE1–PE3b), focusing on their mechanistic distinctions, efficiencies, delivery challenges, and therapeutic applications.

Keywords: CRISPR-Cas9; Base editing; Prime editing; Adenine base editors; Cytosine base editors; viral delivery

INTRODUCTION

CRISPR-Cas9 revolutionized genome editing by providing a programmable and efficient method for modifying DNA, building on earlier platforms such as zinc finger nucleases (ZFNs) and transcription activator–like effector nucleases (TALENs) (1, 2). However, its reliance on double-strand breaks (DSBs) and endogenous repair pathways introduces limitations in precision, efficiency, and the risk of unintended mutations (3, 4).

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To address these challenges, next-generation genome editing tools have been developed that enable precise DNA modification without inducing DSBs (5, 6). Base editors allow targeted single-nucleotide changes (5), while prime editors expand this capability to include small insertions, deletions, and all types of base substitutions (7). Successive generations of cytosine and adenine base editors have improved editing efficiency and product purity (5, 8), while prime editing systems have enhanced versatility and precision through optimized reverse transcriptase activity and strand-biasing strategies (7).

Despite these advances, efficient delivery remains a central challenge for clinical translation of CRISPR-Cas9 technology (9). Adeno-associated virus (AAV) vectors have emerged as a leading in vivo delivery platform due to their favorable safety profile (9), although their limited packaging capacity has driven the development of compact editors and dual-AAV strategies (9, 10). This review examines the mechanistic foundations, generational improvements, delivery challenges, and therapeutic applications of CRISPR-Cas9, base editing, and prime editing technologies, highlighting the field's progression toward safer and more precise genome editing approaches.

CRISPR-CAS9 AND ITS LIMITATIONS

Prior to the development of CRISPR, genome editing depended on engineered nucleases such as ZFNs and TALENs. These platforms relied on complex protein–DNA interactions, requiring laborious protein engineering, high production costs, and limited flexibility in target selection. ZFNs operated through triplet-based zinc finger motifs, while TALENs required large repetitive DNA-binding arrays, making customization for each new locus technically demanding and time-consuming. The introduction of CRISPR-Cas9 in 2012 fundamentally transformed the field by replacing protein-based targeting with a programmable RNA-guided mechanism. Simply altering the guide RNA enables rapid retargeting of Cas9, providing far greater efficiency, lower cost, and the capacity for multiplex editing, which collectively established CRISPR as a revolutionary genome engineering tool (1, 2).

Origin of CRISPR

The CRISPR-Cas9 system is a naturally occurring defense mechanism found in bacteria and archaea. Clustered regularly interspaced short palindromic repeats (CRISPR) refers to a region in the bacterial or archaeal genome that stores fragments of viral DNA, enabling the organism to recognize and respond to future infections—a form of adaptive immunity. This phenomenon was first observed in E. coli DNA sequences in 1987 by Ishino *et al*. (11). In these microorganisms, CRISPR functions as an RNA-guided immune system: the Cas complex uses CRISPR RNAs (crRNAs) to identify foreign DNA and directs Cas proteins to induce DSBs within the foreign DNA. When bacteria encounter unfamiliar genetic material, they capture short DNA fragments of the invading DNA and integrate them into their genome as part of a "repeat-spacer" array. If the same invader returns, the system uses the stored sequences to recognize matching targets and guides Cas proteins to eliminate the intruding DNA, preventing reinfection (12). Scientists have since adapted this system to utilise its precise targeting mechanism for genome editing purposes, especially for research applications and the treatment of human diseases (13).

CRISPR-Cas9 Mechanism

In 2012, Jennifer Doudna and Emmanuelle Charpentier released their groundbreaking paper on repurposing the bacterial immune system, CRISPR-Cas9, to create a programmable genetic tool now known as the CRISPR-Cas9 system (14). As of today, there are multiple CRISPR-Cas systems and Cas nucleases, but this review will focus exclusively on the CRISPR-SpCas9 system. SpCas9 refers to a Cas9 homolog from Streptococcus pyogenes. Its high efficiency and ease of use have made SpCas9 the most widely used Cas9 ortholog (15, 16). The natural CRISPR system is composed of two separate RNA molecular entities: crRNA and trans-activating CRISPR RNA (tracrRNA). The crRNA is a nucleotide sequence complementary to the target sequence, acting as a guide, while the tracrRNA acts as a scaffold that binds the crRNA to the Cas9 protein. For genome editing, these two components were fused into a single chimeric RNA molecule called the single guide RNA (sgRNA), which makes the system much more affordable and easier to use. The sgRNA and the Cas9 protein make up the effector complex (Figure 1). The sgRNA contains a sequence complementary to the target DNA. Cas9 interacts with the protospacer-adjacent motif (PAM) of its DNA target through its PAM-interacting (PI) domain at its C terminus. The PAM sequence is a short motif located next to the target DNA sequence. In prokaryotic systems, PAMs help distinguish between self and non-self-DNA: the invader DNA contains PAMs, while the host's CRISPR array does not. SpCas9

typically recognizes an "NGG" PAM sequence, where 'N' can be any base followed by two guanine bases. The relatively simple NGG PAM requirement contributes to the versatility and widespread adoption of SpCas9 in genome editing applications. The PAM essentially acts as a recognition signal for the Cas9 enzyme to cleave at a particular location. PAM sequences are naturally present in the genomes of humans and other eukaryotic organisms, which enables CRISPR-Cas9 to be used as an effective genome editing tool in these systems. Upon locating a PAM, the sgRNA binds to the complementary sequence on the opposite strand of the target DNA sequence. Once both these criteria have been fulfilled, Cas9 uses its two nuclease domains (HNH and RuvC) to cleave the double-strand target DNA, creating a DSB three base pairs upstream of the PAM. The requirement for a PAM restricts Cas9's access to certain sequences, as not all DNA regions contain the appropriate PAM in the correct location, which has driven scientists in search of PAM-free nucleases (17, 18).

Homologous vs Non-homologous DNA repair

After cleavage, there are two basic repair pathways that eukaryotic cells can use for DNA repair: Non-Homologous End Joining (NHEJ) and Homology-Directed Repair (HDR) (Figure 2). NHEJ is an error-prone mechanism that occurs when no homologous DNA template is available. In this case, random insertions or deletions (indels) may occur through blunt-end ligation. If the indel causes a frameshift mutation, it may introduce a premature stop codon and disrupt the gene function, creating what's known as a knockout. NHEJ is the most common pathway for repairing DSBs. HDR, on the other hand, is a slower and more energy-consuming pathway, but it is more accurate at fixing the DSB with fewer mutations. HDR uses a homologous DNA template to guide the repair of the DSB. Once a break occurs, enzymes perform long end resection, removing nucleotides from the 5' ends to generate 3' single-stranded DNA (ssDNA) overhangs. These overhangs invade the homologous DNA template, align with the

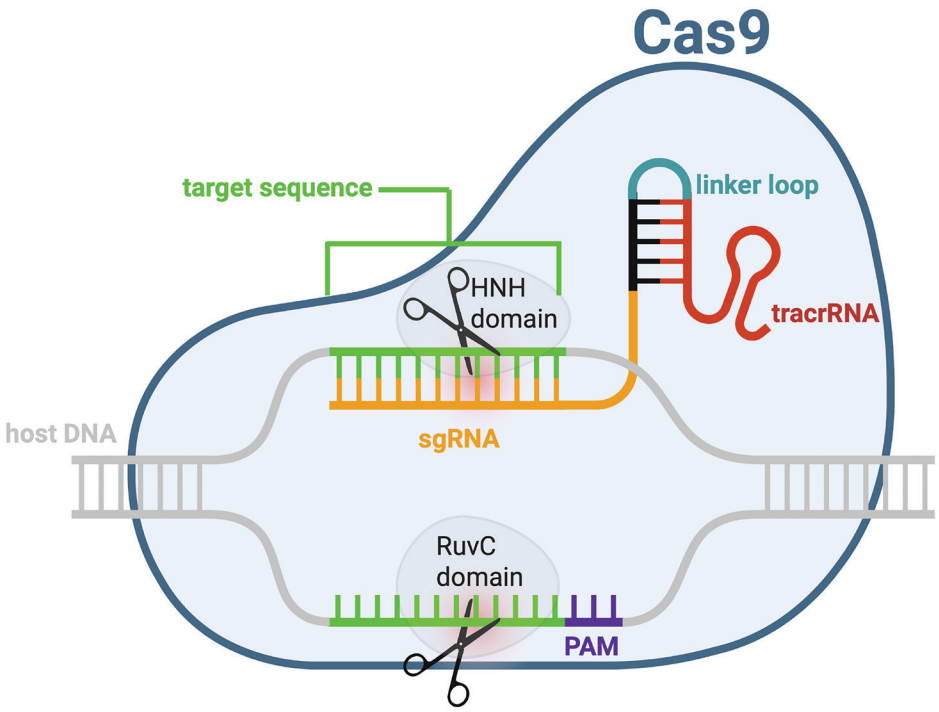

Figure 1. ***Schematic of the CRISPR-Cas9 gene editing mechanism.*** *The Cas9 nuclease is guided by an sgRNA, which directs it to the complementary DNA target sequence adjacent to a PAM. The HNH domain cleaves the target strand, while the RuvC domain cleaves the non-target strand, producing a DSB. The tracrRNA and linker loop stabilize sgRNA binding within the Cas9 protein. Created in BioRender. Sinha, A. (2025) https://BioRender.com/rpp5p9x.*

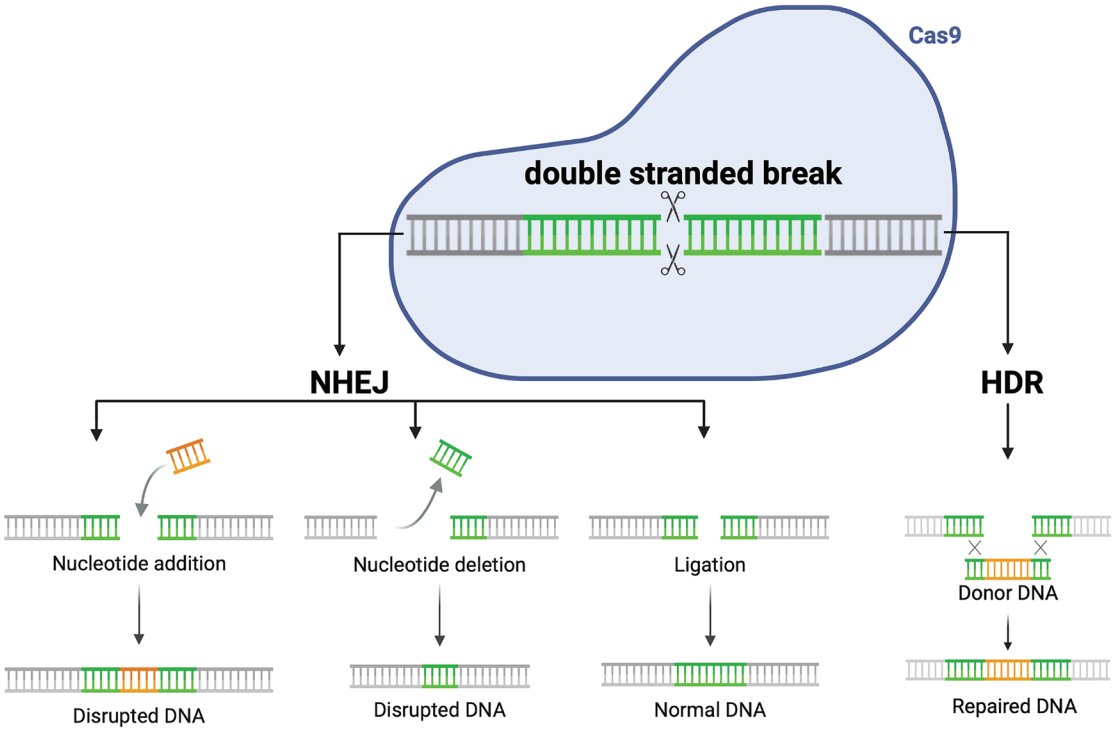

Figure 2. DNA repair pathways in response to double-strand breaks. *Cas9 introduces a DSB that can be repaired by either NHEJ or HDR. NHEJ repairs often result in nucleotide additions, deletions, or ligation, producing either disrupted or normal DNA. In contrast, HDR uses a donor DNA template to precisely repair the break, enabling accurate gene modification. Created in BioRender. Sinha, A. (2025) https://BioRender.com/gmgethw.*

complementary strand, and guide DNA synthesis to restore the missing information. The second end of the DSB is captured in a step known as second-end capture, ensuring accurate pairing of both ends with the template. Finally, the crossover structures are resolved, and the DNA backbone is ligated to complete the repair. In the context of CRISPR, HDR can be harnessed to introduce specific DNA sequences, allowing for targeted insertions or "knock-ins." By providing an engineered DNA template with the desired sequence flanked by homology arms, researchers can insert new genes, correct mutations, or add functional tags at precise genomic locations (3).

Problems with efficiency and accuracy:

Despite its high performance, the CRISPR-Cas9 system still faces limitations in both efficiency and accuracy. After a DSB is introduced, cells overwhelmingly favor NHEJ over HDR, largely because NHEJ is faster and more active in most cell types. However, NHEJ is error-prone and frequently leads to indels, translocations, or other unintended DNA rearrangements. DSBs, in general, are among the most dangerous types of DNA damage and can trigger genomic instability. In therapeutic genome editing applications, unintended DSBs may increase the risk of adverse outcomes, including mutations associated with cancer development (4).

These risks make Cas9-induced breaks problematic in contexts where precise edits are needed. For clinical treatment of genetic diseases, the goal is not gene disruption but the accurate correction of a point mutation in the target locus. However, existing strategies to correct point mutations through HDR remain highly inefficient under therapeutically relevant conditions — particularly in unmodified, non-dividing cells — with correction rates typically ranging from ~0.1–5% as reported across prior CRISPR-Cas9 studies (5). As a result, scientists began seeking alternatives that could improve precision without relying on the cell's natural repair mechanisms. This pursuit led to the development of CRISPR-based base editing by Komor *et al.*

BASE EDITING

Base editing enables precise single-nucleotide changes without introducing DSBs, making it more efficient and less error-prone than traditional CRISPR-Cas9 editing. Since many human genetic diseases are caused by single-nucleotide polymorphisms (SNPs), this approach has significant therapeutic potential. Notably, approximately 62% of pathogenic SNPs located within genes can be corrected through base editing (19).

A base editor consists of a Cas protein that is directed to a specific DNA sequence by an sgRNA and a DNA-modifying enzyme that enables the nucleotide conversion. Two main classes exist: cytosine base editors (CBEs), which convert C→T, and adenine base editors (ABEs), which convert A→G. Together, these systems enable all four transition mutations (A↔G, C↔T). To perform these edits without generating DSBs, base editors use modified forms of Cas9. Wild-type Cas9 contains two nuclease domains, RuvC and HNH, which together produce a DSB. Mutation of the RuvC domain (D10A) prevents cleavage of the non-target strand, while mutation of the HNH domain (H840A) prevents cleavage of the target strand. When one domain is inactivated, the resulting Cas9 nickase (nCas9) introduces a single-strand nick; when both domains are inactivated, catalytically dead Cas9 (dCas9) binds DNA without cleavage (20).

Cytosine Base Editors

CBEs mediate C:G to T:A transitions using a catalytically impaired Cas nuclease (dCas9 or nCas9) fused to a cytidine deaminase, typically Apolipoprotein B mRNA Editing Enzyme Catalytic Subunit 1 (APOBEC1). The enzyme deaminates cytosine to uracil within a defined editing window on the non-target strand. During DNA replication or repair, the resulting U:G mismatch is resolved into a stable T:A base pair. This review focuses on the first three generations of CBEs, which differ in design and efficiency (5).

The first-generation CBEs (CBE1) consists of APOBEC1 fused to dCas9, enabling targeted C→T conversion without generating a DSB. While efficient in vitro, CBE1 shows reduced activity in human cells because uracil intermediates are recognized and removed by uracil-DNA glycosylase (UDG), a key enzyme in the base excision repair (BER) pathway, preventing stable incorporation of the edit (5, 22–25).

To address this limitation, second-generation CBEs

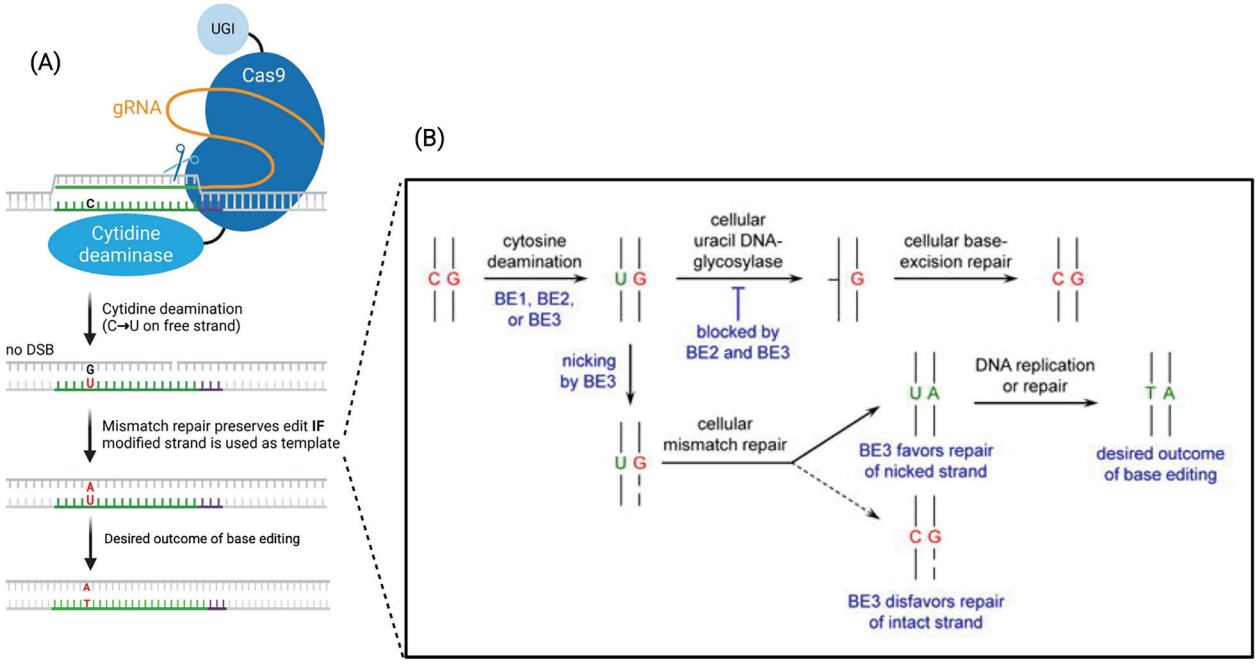

Figure 3. Mechanism of cytosine base editing. *(A) A cytidine deaminase fused to nCas9 mediates targeted C→U conversion on the non-complementary DNA strand. Following DNA repair, the modification can yield a stable C:G to T:A substitution. Adapted from Addgene Blog (21). Created with BioRender.com. (B) Cytosine deamination pathways, illustrating the mechanisms preferred by successive generations of CBEs. Adapted from Komor et al. (5).*

(CBE2) incorporate a uracil-DNA glycosylase inhibitor (UGI) to block BER activity and improve retention of the edited intermediate. UGI binds tightly to UNG, preventing it from excising uracil from DNA. This modification increases editing efficiency in HEK293T cells via plasmid transfection, while maintaining low indel formation (≤0.1%) (5, 26).

Third-generation CBEs (CBE3) further improve editing efficiency by exploiting the mismatch repair (MMR) pathway. Following cytosine deamination, a U:G mismatch is formed, which can be resolved in two ways: either the G on the unedited strand is replaced with an A to produce the desired T:A base pair, or the U on the edited strand is replaced with a C, restoring the original C:G pair. The outcome depends on which DNA strand is selected as the repair template. To bias this process toward the desired edit, CBE3 uses an nCas9 to introduce a nick in the non-edited strand, directing the MMR machinery to preferentially correct that strand. This results in a 2- to 6-fold increase in editing efficiency compared to earlier generations in HEK293T cells via plasmid transfection, while maintaining low indel formation (~1.1%) (5). These differences in design and function across CBEs are summarized in Figure 4.

Adenine Base Editors

ABEs convert A:T base pairs to G:C base pairs. As established earlier, transition mutations account for a large proportion of pathogenic SNPs, making ABEs particularly valuable for correcting disease-causing mutations. ABEs operate under a similar principle as CBEs—both rely on deamination of a nucleobase. ABEs operate through deamination of adenine to inosine, which is interpreted as guanine by the cell's replication and repair machinery. However, a major challenge in developing ABEs was that no known enzyme naturally deaminates adenine in DNA.

To initiate this development, Liu and coworkers evolved a deoxyadenosine deaminase capable of acting on ssDNA. This engineered enzyme was derived from Escherichia coli's tRNA adenosine deaminase, TadA (8). In their experiment, E. coli cells were given TadA mutants along with defective antibiotic resistance genes. To survive in the presence of antibiotics, the TadA-dCas9 fusion protein (TadA*) had to convert a deoxyadenosine to deoxyinosine in the defective gene, effectively restoring its function. In this experiment, dCas9 was used instead of nCas9—despite nCas9 yielding higher editing efficiencies in eukaryotic systems like CBE3—because bacteria lack nick-directed mismatch repair machinery. As a result, nicking would not enhance base editing in E. coli, making dCas9 sufficient for the selection process (28). Ultimately, TadA*-dCas9 fusions achieved efficient adenine-to-inosine conversion in E. coli.

The first-generation ABE (ABE1.2) was constructed by fusing the evolved TadA* to the N-terminus of nCas9, along with a flexible linker sequence (XTEN) and a nuclear localization signal (NLS), forming the TadA*-XTEN-nCas9-NLS construct (8). Although ABE1.2 demonstrated a low editing efficiency of 3.2% in HEK293T cells via plasmid transfection, it proved the concept was viable. Further generations of ABEs improved on this design to increase editing efficiency and reduce unwanted indels. One notable improvement was ABE5.3. TadA normally functions as a homodimer, with one monomer catalyzing deamination and the other aiding tRNA binding. In E. coli, wild-type TadA could dimerize with the engineered TadA*-dCas9 complex. However, mammalian cells lack endogenous TadA, preventing this heterodimerization.

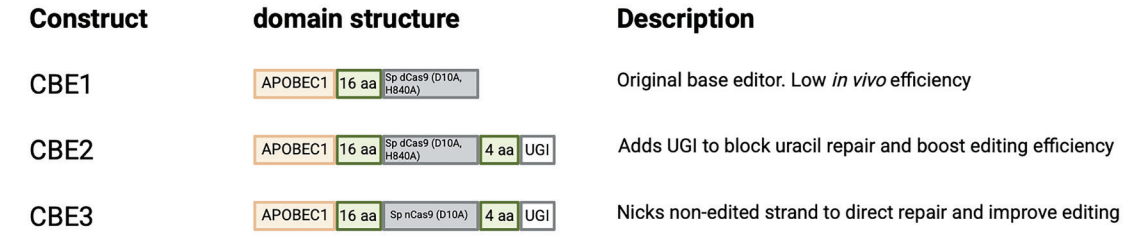

Figure 4. ***Cytosine base editor variants (CBE1–CBE3) and domain structure.*** *Protein schematics highlighting domains and functions of the first three generations of CBEs. BE1: APOBEC1 cytidine deaminase fused to dCas9, D10A/H840A, showing low in vivo efficiency due to uracil excision. BE2: BE1 plus a UGI to block UDG-initiated base-excision repair and improve product retention. BE3: APOBEC1 fused to nCas9, D10A and UGI; nicking of the non-edited strand biases repair toward installing the desired C•G→T•A conversion. Adapted from Rees & Liu (27). Created in BioRender. Sinha, A. (2025) https://BioRender.com/0xoori8.*

To overcome this, Liu and coworkers developed a heterodimeric protein in which a wild-type TadA monomer, an evolved TadA* monomer, and an nCas9 were combined into a single polypeptide (wtTadA–TadA*–nCas9). This design, termed ABE5.3, boosted editing efficiency to 39% in HEK293T cells via plasmid transfection (8). Subsequent engineering efforts led to seventh-generation ABEs, which achieved up to 50% efficiency in HEK293T cells via plasmid transfection, with extremely high product purity (≥99.9%) and minimal indel formation (≤0.1%), resulting in even cleaner edits than those produced by CBEs (8, 29, 30).

Advantages and Limitations of Base Editing

One of the major advantages of base editing is that it represents the first type of CRISPR-based genome editing that does not rely on creating DSBs in the DNA. Traditional CRISPR-Cas systems, such as those using Cas9 nucleases, introduce DSBs at the target site, which can lead to unintended consequences such as large deletions, insertions, chromosomal rearrangements, or activation of DNA damage responses. In contrast, base editing employs a modified, catalytically impaired Cas protein fused to a deaminase enzyme, allowing for the direct conversion of one DNA base into another without cutting both DNA strands.

This mechanism of action makes base editing a significantly safer alternative to conventional Cas nucleases, particularly in therapeutic contexts. By avoiding DSBs, base editing greatly reduces the risk of off-target effects and genomic instability. This is especially beneficial when correcting SNPs, which are the most common type of genetic variation linked to a wide range of diseases. The precision and efficiency of base editors enable the correction of these point mutations with high specificity and minimal disruption to the surrounding genome, making them a powerful tool for both research and clinical applications.

There are several limitations to base editing, with some of the most relevant ones being those regarding bystander editing, targeting limitations, and the constraint to 4 transition mutations. Bystander editing can occur when multiple editable C's or A's are present within the activity window, resulting in unintended modification of nearby bases at the target site. Based on computational modeling, the probability of a non-silent bystander mutation is 47% for third-generation CBEs and 38% for ABEs (6). Some of the most effective ways to reduce bystander mutations are to limit how long the enzyme remains bound to its substrate—ensuring it dissociates after only a few reactions instead of persisting for longer—and to design a narrower activity window. Based on the same computational analysis, when the activity window is restricted to roughly two nucleotides in width, the probability of a non-silent bystander mutation drops to about 12% (26).

In addition to bystander editing, base editors are limited by targeting constraints. Successful DNA target binding requires a PAM, and the SpCas9 is the most widely used ortholog, as it offers the least restrictive PAM. However, based on computational analysis of the ClinVar database, only 26% of known pathogenic SNPs that are of the four types of transitions can be targeted by SpCas9-derived base editors, which has highlighted the need to develop base editors with additional PAM compatibilities (31).

Additionally, base editing is limited in that it can only perform four types of transition mutations. This constraint has driven the development of prime editing, which expands the range of possible edits beyond what base editing can achieve.

PRIME EDITING

In October 2019, David R. Liu's group introduced a groundbreaking genome editing technique called Prime Editing. Like base editing, prime editing enables precise genetic modifications without introducing DSBs. It offers greater versatility, capable of performing all twelve possible single–base-pair substitutions—including the four transition mutations (A↔G, C↔T) and the eight transversion mutations (A↔C, A↔T, G↔C, G↔T)—as well as small insertions and deletions. In principle, prime editing can correct up to 89% of known pathogenic human genetic variants (7).

Mechanism of Prime Editing

The prime editing system consists of three key components: an nCas9, an engineered reverse transcriptase (RT) fused to the C-terminus of the nickase, and a specially designed prime editing guide RNA (pegRNA) (Figure 5). The pegRNA is more complex than traditional guide RNAs used in CRISPR-Cas9 and base editing. It includes a spacer (guide sequence), a reverse transcriptase template (RTT), a primer binding site (PBS), and a scaffold region that binds both the Cas9 and Reverse Transcriptase (6, 32). Notably, the pegRNA carries the intended genetic edit in the form of the RTT, serving as an integrated donor template.

The editing process begins when the complex locates

and binds to the DNA at a PAM site. The modified nCas9 induces a single-stranded break on the target strand three base pairs upstream of the PAM, generating a 3' hydroxyl group. This exposed 3' end acts as a primer that is needed for the reverse transcriptase. The PBS portion of the pegRNA hybridizes to this region, allowing the reverse transcriptase to extend the DNA strand using the RTT as a template.

This results in the formation of two redundant DNA flaps: a 5' flap containing the original (unedited) sequence and a 3' flap carrying the edited version. Since both flaps cannot be incorporated simultaneously, one must be removed. Thermodynamically, the cell prefers the perfectly complementary 5' flap, which would eliminate the edit. However, in practice, the 5' flap is often preferentially cleaved by endogenous endonucleases such as FEN1 and EXO1 (7, 33). Both FEN1 and EXO1 display 5′–3′ exonuclease and 5′ flap endonuclease activity, enabling them to remove the unedited flap and improve editing outcomes (34). Generations of Prime editors (PE1-PE3b) discussed below.

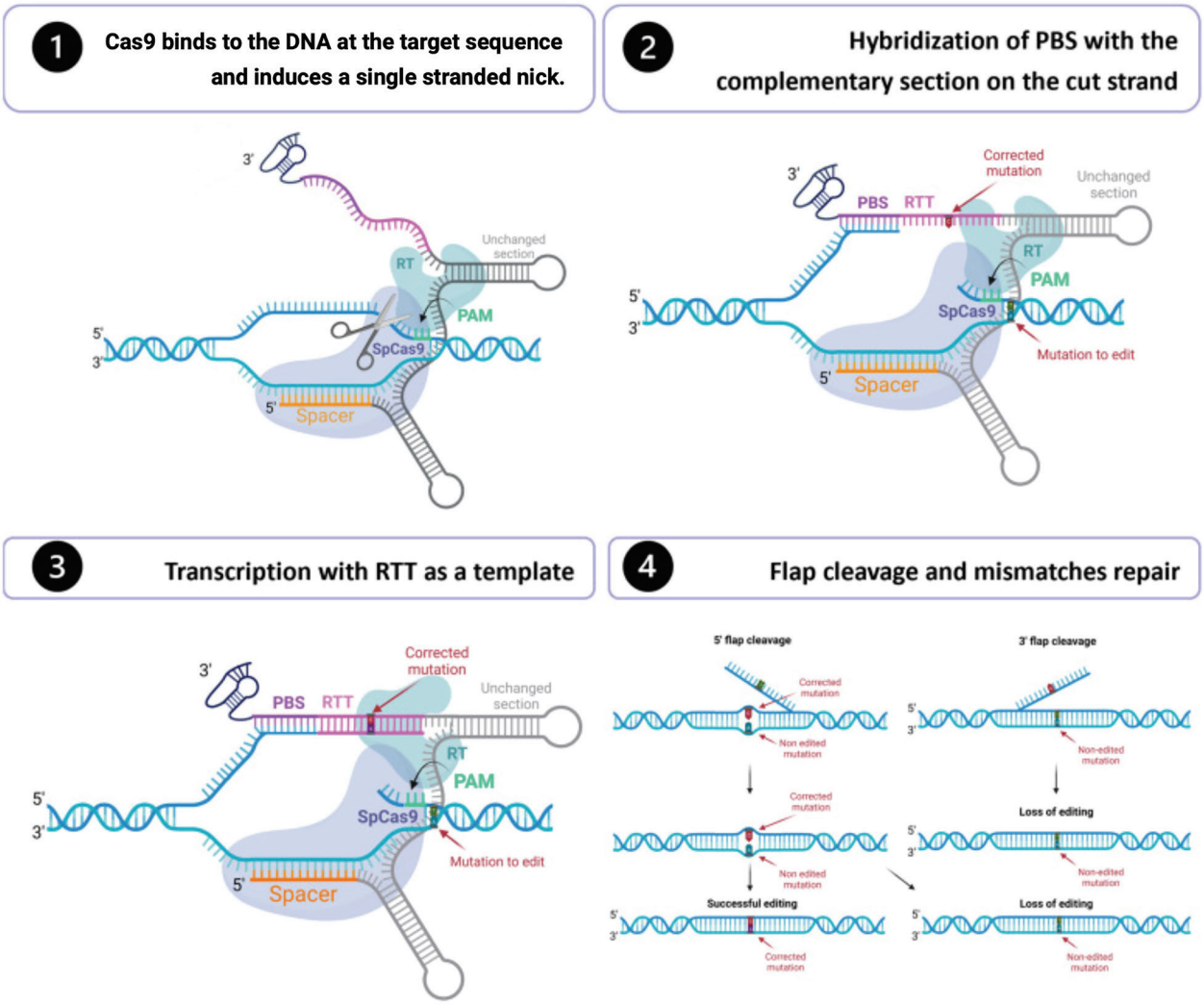

Figure 5. ***Mechanism of prime editing.*** *Step 1: Cas9 fused with a reverse transcriptase (RT) binds to the DNA at the target sequence and induces a single stranded nick. Step 2: The Primer Binding Site hybridizes to its complementary sequence on the cut strand. Step 3: The RT will use the RTT as a template to transcribe the cut strand. Step 4: Mismatches will be repaired by a 5′ flap or a 3′ flap. Adapted from Goodbout & Tremblay (35). Created in BioRender. Sinha, A. (2025) https://BioRender.com/hgbcvy2.*

Generations of Prime Editors

The first generation of prime editors (PE1) consisted of a wild-type Moloney Murine Leukemia Virus reverse transcriptase fused to nCas9 (H840A) and guided by a pegRNA. Editing efficiency was influenced by the length of the PBS, reaching between 0.7% and 5.5%, while indels remained minimal at an average of 0.2% in HEK293T cells via plasmid transfection (7). PE1 established that prime editing could install targeted transversions, insertions, and deletions without the need for DSBs or donor DNA templates.

To improve efficiency and accuracy, second generation prime editors (PE2) incorporated five specific mutations into the Moloney Murine Leukemia Virus reverse transcriptase, producing a pentamutant enzyme. These mutations were selected to enhance thermostability, strengthen binding to the DNA:RNA substrate, and allow the reverse transcriptase to remain engaged long enough to synthesize DNA more efficiently during prime editing.

This modified Reverse Transcriptase replaced the wild-type version in PE1. As a result, PE2 achieved a 1.6- to 5.1-fold improvement in the efficiency of prime editing point mutations over PE1 in HEK293T cells via plasmid transfection (7). It also demonstrated improved performance in generating targeted insertions and deletions.

Building on strategies previously used in base editing, Liu and colleagues developed third generation prime editors (PE3) by adding sgRNA to PE2 to nick the non-edited DNA strand. In HEK293T cells via plasmid transfection, this strategy boosted overall editing efficiency by 1.5- to 4.2-fold compared to PE2, with efficiencies as high as 55% (7).

However, introducing a second nick also increased the risk of indel formation. To address this, the researchers developed PE3b, which uses a modified sgRNA that only matches the edited allele. This design creates a mismatch with the unedited allele, preventing nicking until after the edit has occurred. In HEK293T cells via plasmid transfection, PE3b minimizes concurrent nicks and significantly reduces indel rates by up to 13-fold (to 0.74%) --- without compromising editing efficiency (7). For ease of reference, the comparative characteristics of the PE systems discussed above are summarized in Figure 6.

Advantages and Limitations of Prime Editing

Prime editing offers notable improvements over Cas nuclease-mediated HDR and base editing. It can perform all twelve types of single-base substitutions, along with small insertions (up to dozens of nucleotides) and deletions (up to hundreds of nucleotides), with relatively high precision.

Prime editing is more specific than CRISPR Cas9 and base editing. Its specificity stems from three key checkpoints of base pairing: (1) binding and nicking of the target DNA by the prime editor guided by the pegRNA spacer, (2) hybridization of the PBS to the 3' end of the nicked DNA to start reverse transcription, and (3) successful integration of the new sequence via

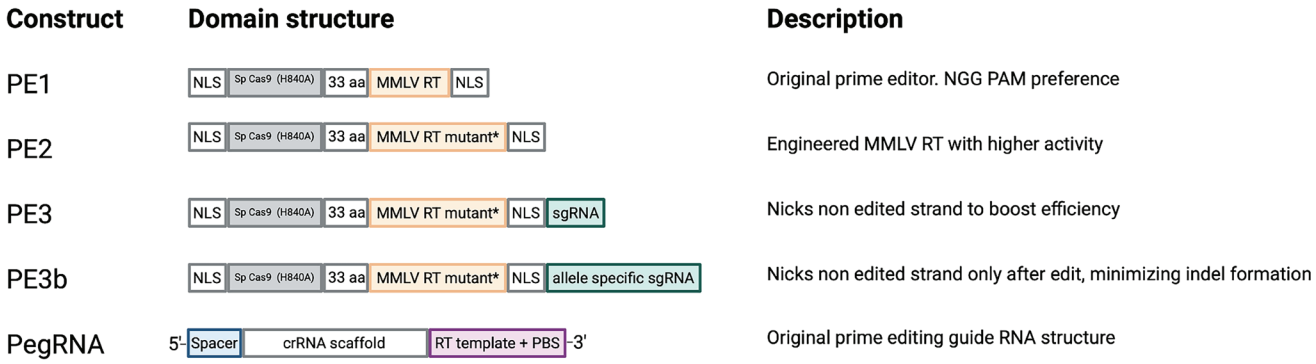

Figure 6. ***Prime editor (PE1–PE3b) architectures and pegRNA design.*** *Domain structures and functional distinctions of prime editors and their guide RNAs. PE1: SpnCas9 (H840A) fused to Moloney murine leukemia virus reverse transcriptase (MMLV RT), guided by an NGG PAM. PE2: PE1 containing an engineered MMLV RT mutant with enhanced activity. PE3: PE2 combined with an additional sgRNA that nicks the non-edited strand to increase efficiency. PE3b: A modified PE3 using an allele-specific sgRNA that nicks the non-edited strand only after the desired edit, thereby reducing indel formation. The pegRNA consists of a spacer sequence, a crRNA scaffold, and an extended 3' region encoding both the PBS and the RTT for introducing edits. Adapted from Chen et al. (36). Created in BioRender. Sinha, A. (2025) https://BioRender.com/vgcckrk.*

hybridization of the 3' flap to the downstream DNA.

In PE3 systems, a second sgRNA directs the prime editor to nick the opposite strand, improving editing efficiency. Despite this, prime editing still produces fewer indels than Cas nucleases. It also shows lower off-target activity and can edit regions farther from the PAM site, offering greater flexibility than other methods (36).

Prime editing still faces limitations despite all of its advantages. Most notably, prime editing has relatively low editing efficiency due to the requirement of three sequential base-pairing checkpoints. Each checkpoint presents a potential barrier to success. Efforts to improve each step have led to the development of enhanced pegRNAs with greater efficiency. Additionally, due to the size of pegRNAs, prime editing faces size constraints when considering delivery mechanisms, which can hinder the success rate of larger edits. Current research explores strategies like dual pegRNAs to overcome this barrier (37). Related to this limitation, prime editing also requires more components than base editing or Cas9-mediated HDR, which complicates delivery. The next part of this review will address the delivery system methods.

Two classes of DNA base-editors have been described thus far, CBEs and ABEs. Recently, prime editing has further expanded the CRISPR editing toolkit to all twelve possible transition and transversion mutations, as well as small insertion or deletion mutations, as tabulated below in Table 1.

Table 1. *Comparison of CRISPR-Cas9, base editing, and prime editing technologies.*

Category	CRISPR-Cas9	Base editing	Prime editing	References
Off-target effects	Present; off-target indel frequencies commonly range from ~1–20% at mismatched sites and large deletions (>100 bp) have been reported due to DSB repair	Low; indel rates typically ≤0.1–1%, but bystander editing occurs in ~38–47% of editable windows depending on editor generation	Low; indel frequencies generally ≤0.1–0.8%, with significantly reduced off-target activity compared to Cas9-mediated HDR	(4, 5, 6, 26, 36)
Possible Edits (including Transitions and Transversions)	Insertions, deletions, and all base substitutions via HDR or NHEJ	Transitions only: A↔G, C↔T; bystander edits may restrict targeting	All 12 base substitutions (A↔C, A↔T, G↔C, G↔T), small insertions, and deletions; less stringent PAM requirements	(5, 6, 7, 19)
Programability	Requires donor DNA template for precise edits	Yes; programmable via sgRNA and editing window	Yes; programmable via pegRNA encoding desired edit	(5, 8, 29)
Efficient delivery	Currently possible; most mature delivery pipelines	Possible but more challenging due to editor size	Needs improvement due to large editor and pegRNA size	(9, 10)
Therapeutic Applications	Gene knockouts, exon disruption, large deletions; clinically approved for sickle cell disease and β-thalassemia, with additional applications (LCA10) under clinical investigation.	Correction of pathogenic point mutations; Preclinical correction demonstrated in cell-based and animal models of HGPS and DMD.	Precise correction of insertions, deletions, and complex mutations; demonstrated in cell-based models of CF and used to generate animal models of TSD.	(38-45)
Enzymes involved	Cas9 nuclease	nCas9 or dCas9 fused to cytidine or adenosine deaminases (e.g., APOBEC1, TadA*)	nCas9 (H840A) fused to engineered reverse transcriptase	(5, 6, 8)

DELIVERY CHALLENGES IN GENOME EDITING

The most crucial and challenging factor of therapeutic success is the safe and efficient delivery of editors to target tissues or cells. Gene delivery systems (GDS) can be classified into two groups based on the origin of the gene carrier: non-viral delivery and viral delivery. Non-viral vectors face many issues such as low delivery efficiency, cytotoxicity, and mutagenicity of the DNA and as such, viral vectors are more often used.

Viral vectors are created by modifying a certain virus in the laboratory for use in gene therapy applications. A range of viruses is used, each of which has its unique strengths and weaknesses. Some examples include retrovirus, lentivirus, herpes simplex virus, adenovirus, and AAV. Among these, AAV vectors have unique therapeutically beneficial features. AAV has not been linked with any human diseases, causes a mild immune response, rarely integrates into the host genome, can be preserved for long periods of time in episomal form, can be specifically targeted to several different cell types, and has a well-characterized genome, so the consequences are more predictable. For these reasons, AAV vectors are the leading platform for in vivo delivery of gene therapies (9).

AAV belongs to the parvovirus family and is dependent on co-infection with other viruses in order to replicate. It is composed of an icosahedral protein capsid and a ssDNA genome about 4.8 kb long, which contains three genes: Rep (Replication), Cap (Capsid), and Aap (Assembly). The genome is flanked by two T-shaped inverted terminal repeats (ITRs) at the ends that are needed for replication and packaging (10). Recombinant AAVs (rAAVs) have the rep and cap genes replaced by the CRISPR-Cas9 genetic material. The ITRs are needed to guide genome replication and packaging during vector production. Since these genes are required for delivery, they reduce the workable size of the construct from 4.8 kb to 4.7 kb. The low immunogenicity and cytotoxicity of AAVs are largely due to the removal of viral coding sequences.

One of the most significant limitations of AAV vectors when it comes to base and prime editing is the low carrying capacity of 4.7 kb. A base editor consisting of a CBE or an ABE and a guide RNA is approximately 6 kb, while prime editors are more than 7 kb. Therefore, delivering gene therapy has become a problem for some of these more sophisticated forms of gene editing, which have many components and therefore are of larger sizes.

There are strategies that are trying to mitigate these problems, such as using Cas9 proteins with smaller sequences and dual AAV systems. The commonly used SpCas9 has a size of 4.1 kb, leading engineers to use Cas9 homologs with smaller sequences. For example, SaCas9, which is from Staphylococcus aureus, has a size of 3.7 kb. A smaller size is easier to deliver; however, it has a smaller targeting range, which can affect editing efficiency. Selecting alternative Cas9 homologs requires careful evaluation of their specific advantages and limitations.

A dual AAV system refers to splitting the genetic material among two AAV vectors to overcome problems with carrying capacity. To do CRISPR-Cas9 editing, it is generally done by engineering one of the vectors (AAV1) to express the Cas9 enzyme, and engineering the other vector (AAV2) to express the gRNA and the donor template. Although the dual AAV system can be a way to expand space for genetic material, it relies on delivering two AAV vectors simultaneously through a cell, which reduces editing efficiency. So, even though these two solutions could expand packaging space, the length of the gene remains constrained by the size of the AAV.

Delivery strategies for genome editing systems vary across platforms and remain a major limitation in therapeutic applications. CRISPR-Cas9 benefits from relatively mature delivery methods, including viral vectors such as AAV and non-viral approaches such as electroporation, reflecting its broader clinical development and higher study frequency. In contrast, base editors and prime editors offer improved precision but face greater delivery challenges due to their larger construct sizes, which can exceed the packaging capacity of standard AAV vectors and often require dual-vector systems or alternative delivery strategies. While viral delivery provides high efficiency, it is limited by payload constraints and potential immunogenicity, whereas non-viral approaches such as lipid nanoparticles offer greater flexibility but may have lower efficiency and limited tissue specificity. Overall, delivery remains a key bottleneck across all genome editing platforms, with ongoing research focused on improving efficiency, targeting, and clinical scalability. Despite these limitations, each platform retains strong therapeutic potential, as explored in the next section.

THERAPEUTIC APPLICATIONS

Therapeutic applications of genome editing highlight the transformative potential of CRISPR-Cas9, base

editing, and prime editing in addressing genetic diseases. Each of these technologies offers distinct advantages and limitations, making them particularly suited for different classes of mutations and disorders. This section will examine representative examples where each editing strategy has been applied or explored, underscoring their promise in advancing precision medicine.

CRISPR-Cas9

CRISPR-Cas9 introduces DSBs in DNA to induce targeted insertions or deletions through NHEJ or precise changes via HDR. CRISPR–Cas9 is generally preferred over base or prime editing when simple gene disruption can restore normal function, such as in cases like reactivating fetal hemoglobin in β-thalassemia and sickle cell disease by knocking out BCL11A or removing aberrant splice sites in Leber congenital amaurosis 10 (LCA10). In these cases, a gene knockout is more efficient and clinically applicable than precise nucleotide correction. Notably, CRISPR-Cas9 is currently the only genome-editing platform to achieve regulatory approval, with Casgevy approved for the treatment of sickle cell disease and transfusion-dependent β-thalassemia, while other therapeutic applications, including LCA10, are currently being evaluated in clinical trials. Overall, CRISPR-Cas9 is best suited for gene knockouts, correction of larger deletions or duplications, and treatment strategies where inducing a loss of function can mitigate disease.

β-thalassemia and sickle cell disease

β-thalassemia and sickle cell disease are both inherited disorders caused by mutations in the beta-globin gene (HBB). β-thalassemia is characterized by reduced or absent production of beta-globin chains, resulting in impaired hemoglobin synthesis and chronic anemia. In contrast, sickle cell disease is caused by a mutation that produces abnormal hemoglobin (HbS), leading red blood cells to become rigid and sickle-shaped.

CRISPR-Cas9 has demonstrated success as a treatment strategy for both disorders through reactivation of fetal hemoglobin (HbF). BCL11A is a transcription factor that represses HbF production in adults, and CRISPR-Cas9 can be used to disrupt the BCL11A erythroid enhancer, thereby restoring HbF expression (38). Increased HbF levels can compensate for the reduced beta-globin production seen in β-thalassemia and can inhibit HbS polymerization in sickle cell disease, reducing disease severity. This strategy culminated in the approval of Casgevy, the first CRISPR-based therapy for sickle cell disease and transfusion-dependent β-thalassemia (39).

Leber congenital amaurosis

LCA is a group of inherited retinal dystrophies that cause severe vision loss, often leading to blindness, beginning in infancy. LCA10, the most common subtype of LCA, is caused by mutations in the CEP290 gene, which is responsible for making a protein that is crucial in the development and function of cilia. Cilia on photoreceptor cells are essential for detecting light and color.

The most frequent mutation is a deep intronic mutation in CEP290 that generates a cryptic splice donor site, leading to abnormal intron splicing resulting in a non-functional protein. The large size of the CEP290 gene prevents its use in AAV-mediated gene augmentation therapy. Ongoing clinical trials are using CRISPR Cas9 to remove the intronic splice mutation to restore expression of wild-type CEP290 by using two guide RNAs to cleave the cryptic splice donor site (40).

Base Editing

Base editing enables precise single-nucleotide conversions without introducing DSBs, significantly reducing the likelihood of indels or chromosomal rearrangements. CBEs and ABEs allow for correction of the four most common transition mutations (C→T, T→C, A→G, G→A), which account for the majority of disease-causing SNPs. Progeria and muscular dystrophy are two diseases for which base editing has shown promising preclinical potential, as both are often caused by specific types of single-base mutations. To date, these therapeutic applications remain primarily at the preclinical stage, with successful demonstrations in cell-based and animal models rather than approved clinical therapies.

Progeria

Hutchinson-Gilford Progeria Syndrome (HGPS) is a rare and fatal disease characterized by accelerated aging. Most cases are caused by a C→T point mutation of the LMNA gene that results in the protein progerin. A characteristic feature of HGPS arteries is loss of smooth muscle, which contributes to heart attacks, the primary cause of death in HGPS. In a cell-based model with induced pluripotent stem cells (iPSCs) ABEs demonstrated promising therapeutic potential by improving the phenotype of tissue-engineered blood vessels derived from patients with HGPS. Edited cells did not express progerin and exhibited phenotypes comparable to healthy control cells (41).

Muscular dystrophy

Duchenne muscular dystrophy (DMD) is a fatal muscle disease caused by mutations in the dystrophin gene that disrupt the reading frame, leading to a non-functional protein. Dystrophin maintains muscle membrane integrity, and a lack of it causes progressive muscle degeneration. Base editing has shown promising preclinical potential for treating DMD. Base editing can be used to change a single nucleotide in the splice donor site, a recognition sequence for cellular machinery during splicing. The modified splice donor site is unrecognizable, and as a result, the exon gets skipped during splicing. This exon skipping restores the reading frame, allowing the gene to produce a truncated but functional dystrophin protein. These findings were demonstrated in preclinical studies using mouse models, large animal models and human iPSC-derived cardiomyocytes (42). Prime editing is an alternative approach that is more focused but also less efficient than base editing.

Prime Editing

Prime editing is the most flexible CRISPR-derived tool to date, capable of introducing all 12 types of base substitutions, as well as small insertions and deletions, without relying on DSBs or donor templates. While its versatility is unmatched, prime editing currently suffers from relatively lower editing efficiencies and more complex delivery due to the size and number of components involved. To date, prime editing has shown promising preclinical potential for disorders requiring precise sequence correction. Cystic fibrosis (CF) and Tay-Sachs disease (TSD) illustrate the preclinical potential of prime editing because of its ability to precisely correct insertions and deletions. Overall, prime editing is best suited for therapeutic interventions where transition editing is insufficient and exact sequence rewriting is required.

Cystic fibrosis

CF is caused by mutations in the CFTR gene, which is responsible for producing a protein that regulates the movement of chloride ions and water across cell membranes, resulting in the buildup of thick, sticky mucus. The predominant cause of CF is a 3-nucleotide deletion referred to as F508del. Current studies reveal the ability of prime editing to correct F508del in immortalized bronchial epithelial cells with a 58% editing efficiency and minimal off-target effects. A study by Sousa *et al*. demonstrates the feasibility of prime editing as a potential one-time therapeutic approach for CF in a preclinical cell-based model (43). Other studies have further demonstrated the potential of prime editing to correct additional CF-causing mutations, including L227R and N1303K (44).

Tay-Sachs

TSD is a neurodegenerative disorder caused by deficiency of β-hexosaminidase A (HexA), an enzyme essential for lysosomal waste degradation. The most common pathogenic variant is a 4-bp insertion in the HEXA gene. Although prime editing has the theoretical capacity to correct this mutation, the study by Qian *et al*. used PE3 to generate a rabbit model carrying the disease-associated insertion rather than to treat the disorder. The resulting rabbit model, produced with editing efficiencies of up to 68%, provides a valuable platform for studying TSD pathogenesis and evaluating future therapeutic strategies (45).

Representative studies discussed throughout this review are summarized in Table 2, highlighting the editing platform, mutation type, experimental model, delivery method, editing efficiency, and therapeutic application or research objective.

CONCLUSION

As gene editing technologies continue to advance, the ability to correct genetic disease at the molecular level is becoming increasingly realistic. Cas9, base editing, and prime editing now each offer powerful and complementary approaches: Cas9 provides efficient gene disruption and has already reached clinical approval through therapies like Casgevy for sickle cell disease and β-thalassemia; base editors have recently achieved high correction efficiencies in blood stem cells using next-generation ABE8e variants; and newer compact editors, such as mini-IscB systems, are helping overcome delivery limitations (47-49). Prime editing continues to expand in precision and versatility, most notably through strategies like PERT, which can rescue a wide range of nonsense mutations without affecting normal stop codons (50).

Despite these promising developments, delivery remains the primary challenge across all editing platforms, particularly for large systems such as prime editors. While CRISPR-Cas9 is supported by a robust body of evidence and has reached clinical application, much of the data for base editing and prime editing remains derived from in vitro and preclinical models, highlighting the need for further clinical validation.

Table 2. *Representative studies of genome editing technologies and key characteristics.*

Study	Editing Platform	Mutation Type	Cell Type/Model	Delivery Method	Editing Efficiency	Therapeutic Application
Cong et al. (46), 2013	CRISPR-Cas9 (HDR)	Insertions, deletions, substitutions	HEK293T cells	Plasmid transfection	Up to ~5%	Proof-of-concept genome editing
Komor et al. (5), 2016	CBE3	C→T transition	HEK293T cells; mouse astrocytes	Plasmid transfection; nucleofection	Up to 37% (HEK293T); 58–75% (mouse astrocytes)	Proof-of-concept CBE
Gaudelli et al. (8), 2017	ABE7	A→G transition	HEK293T cells	Plasmid transfection	Up to ~50%	Proof-of-concept ABE
Anzalone et al. (7), 2019	PE3/PE3b	All 12 base substitutions, insertions, deletions	HEK293T cells	Plasmid transfection	Up to 55% (PE3); 13-fold indel reduction (PE3b)	Proof-of-concept prime editing platform
Frangoul et al. (38), 2020	CRISPR-Cas9 (CTX001)	Gene disruption (BCL11A erythroid enhancer)	CD34+ hematopoietic stem and progenitor cells	Electroporation	Up to ~80% allelic editing	Clinical treatment of sickle cell disease and β-thalassemia
Chemello et al. (42), 2021	ABEmax-SpCas9-NG (split-intein)	A→G transition (splice donor site disruption)	Mouse N2a cells; ΔEx51 mice; human iPSC-derived cardiomyocytes	Dual AAV9, intramuscular injection	51.0 ± 2.3%	Preclinical correction of DMD
Qian et al. (45), 2021	PE3	4-bp insertion (HEXA ins TATC)	Rabbit embryos; HEK293FT cells	Microinjection	Up to 68% in rabbit pups; up to 37.5% in rabbit embryos	Generation of TSD rabbit model
Sousa et al. (43), 2024	PE3	3-bp deletion (F508del)	Immortalized bronchial epithelial cells	Plasmid transfection	Up to 58%	Preclinical correction of CF
Abutaleb et al. (41), 2025	ABE7.10max	C→T correction (LMNA c.1824 C>T)	HGPS iPSC-derived vascular smooth muscle cells and endothelial cells	Lentiviral transduction	Not explicitly reported; functional rescue observed with ≥50% edited cells	Preclinical rescue of HGPS vascular phenotype

Continued improvements in editor compactness, targeting flexibility, and specificity—such as near-PAM-less Cas enzymes and high-fidelity variants—will further broaden therapeutic possibilities (51-53). Future research should focus on improving delivery systems, expanding targetable regions, and evaluating long-term safety and clinical outcomes. Together, these advances signal a rapidly progressing field that is steadily moving toward safe, efficient, and accessible treatments for a wide range of genetic diseases.

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CONFLICT OF INTEREST

The author declares that there are no conflicts of interest related to this work.

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