Sculpting the genome and beyond: novel tools for DNA and RNA targeting

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Chapter 3

Prime editing: advances and therapeutic applications

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Abstract

CRISPR—Cas-mediated genome editing has revolutionized biomedical research and will likely change the therapeutic and diagnostic landscape. However, CRISPR—Cas9, which edits DNA by activating DNA double-strand break (DSB) repair pathways, is not always sufficient for gene therapy applications where precise mutation repair is required. Prime editing, the latest revolution in genome editing technologies, can achieve any possible base substitution, insertions and deletions without the requirement for DSBs. However, prime editing is still in its infancy, further development is needed to improve editing efficiency and delivery strategies for therapeutic application. Here, we summarize the latest developments in the optimization of prime editor (PE) variants with improved editing efficiency and precision. Moreover, we highlight some potential therapeutic applications.

CRISPR—Cas-mediated precise genome editing

The simplicity and versatility of CRISPR—Cas systems has led to their rapid adoption as the most widely used genome editing technology for site-specific DNA manipulations [1-3]. CRISPR—Cas-based tools typically consist of a nuclease that induces DNA double-strand breaks (DSBs) (see Glossary) at a specific genomic locus targeted by a programmable guide RNA [4, 5]. The DSBs are mostly resolved through one of the two major DSB repair pathways: non-homologous end joining (NHEJ) and homology-directed repair (HDR) [6-8]. Although NHEJ can efficiently re-ligate two DSB ends, it is error-prone and generates insertions or deletions (indels) [9, 10]. On the other hand, HDR precisely introduces desired alterations including insertions, deletions or substitutions, based on a DNA repair template [11, 12] (Figure 3.1A). However, HDR is restricted to the S/G2 phase of the cell cycle and is inefficient in most therapeutically relevant cell types [11, 13]. Indeed, in most somatic cells, NHEJ outcompetes HDR in repairing the DSBs, resulting in a complex range of editing outcomes [4, 5, 14]. In addition, the generation of DSBs is associated with undesired editing outcomes, such as large deletions [15], inversions [16], translocations [15] and initiation of cellular stress response mechanisms to preserve genome stability, such as p53 activation [17-19]. To overcome this, HDR strategies that rely on single-strand breaks (SSBs) generated by CRISPR—Cas9 nickase (nCas9, D10A or H840A) [20] have been developed, albeit with low editing efficiency [21]. These challenges exemplify the need for the development of alternative precise genome editing tools.

CRISPR—Cas-based Base editors (BEs) can incorporate a desired modification without the requirement for a DSB, HDR and donor DNA templates and can be applied in both dividing and non-dividing cells (Figure 3.1B) [22, 23]. BEs are engineered fusion proteins consisting of a D10A nCas9 and a DNA deaminase domain that catalyzes the deamination of either cytidine (C) or adenosine (A) resulting in base conversions to thymine (T) or guanine (G), respectively (Figure 3.1B) [24, 25]. As such, BEs are not universally applicable for “fixing” disease-causing alleles that arise from specific insertions, deletions, or some base substitutions. Moreover, the editing efficiency and outcomes of BEs can be affected by the sequence context of the target base. For example, CBE was more efficient when the target C was in the context of TC and ABE was was more efficient when the target A was in the

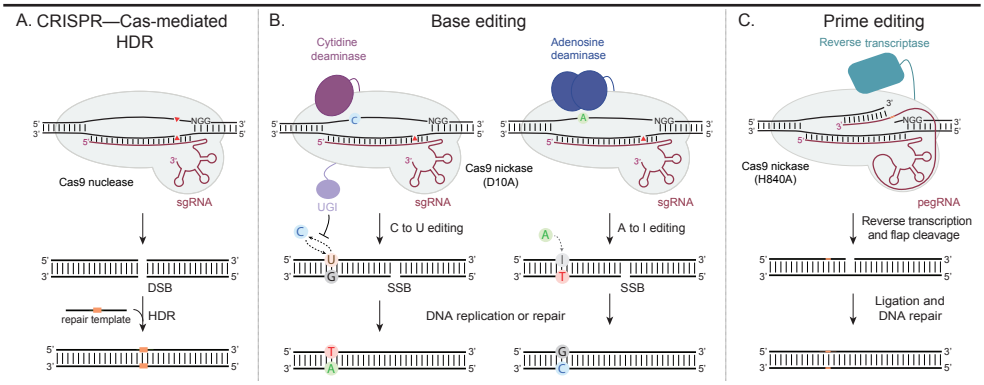

Figure 3.1. CRISPR—Cas-mediated precise genome editing systems. (A) Cas9 nuclease or Cas9 nickase is guided by a sgRNA to generate a targeted DNA double-strand break (DSB) or single-strand break (SSB). Upon the presence of a repair template carrying desired edits (orange), the cellular HDR machinery fixes the DSB or SSB using the repair template allowing for precise incorporation of the template-carried edits in the genome. (B) Base editors (BEs) are engineered fusion proteins composed of nCas9 (D10A) and deaminase domain(s). Cytosine base editor (CBE) can convert C to U in a single strand. Then the resulting U:G heteroduplex can be converted to a T:A base pair following DNA replication or repair. Another functional domain fused to the CBE, the uracil glycosylase inhibitor (UGI) domain, prevents U from reverting to C, thereby favoring the C to T conversion. Adenine base editor (ABE) can deaminate A to form Inosine (I), which has the same base pairing preferences as G. Thus, the I:T heteroduplex can be converted to G:C following DNA replication and repair. For both ABE and CBE, nCas9 (D10A) nicks the target strand and cause a single strand break (SSB) which favors mismatch repair (MMR) machinery to pair the deaminase-converted base with a properly matched base. Collectively, CBE and ABE can install all four transition mutations (C to T, T to C, A to G, and G to A). (C) Prime editor is an engineered fusion protein composed of nickase Cas9 (nCas9) and a reverse transcriptase (RT). The nCas9 creates an SSB on the non-target strand. The released 3'-end then hybridizes to the 3' end of the prime editing guide RNA (pegRNA) and is reverse transcribed by the RT domain. The reverse transcription incorporates the edits encoded in the pegRNA (orange) to the newly synthesized DNA strand. Equilibration between the edited 3′ flap and the unedited 5′ flap, endogenous 5′ flap cleavage and ligation, and DNA repair results in the stable incorporation of the desired edit in the genome.

context of YAC (Y is T or C) [24, 25]. Besides, the target base must be in the activity window, where the positions are susceptible to deamination [24]. However, the presence of multiple editable bases within the BE activity window can result in undesired 'bystander' mutations [24-26].

Prime editing is the first precise genome editing technology that allows for all 12 possible base-to-base conversions as well as insertions, and deletions that does not require DSBs or donor DNA [27]. Its broad editing spectrum potentially allows the correction of up to 89% of human genetic diseases [27]. Prime editors (PE) consist of a nCas9 (H840A) conjugated with an engineered reverse transcriptase (RT) paired with a prime editing gRNA (pegRNA) that both specifies the target site and encodes the desired edit (an overview in Figure 3.1C, more details in Figure 3.2). In this review, we will describe the mechanism of prime editing, highlight various efforts to enhance its efficiency, and summarize some recent potential applications of prime editing in therapeutics.

Prime editing: basic mechanism and specificity

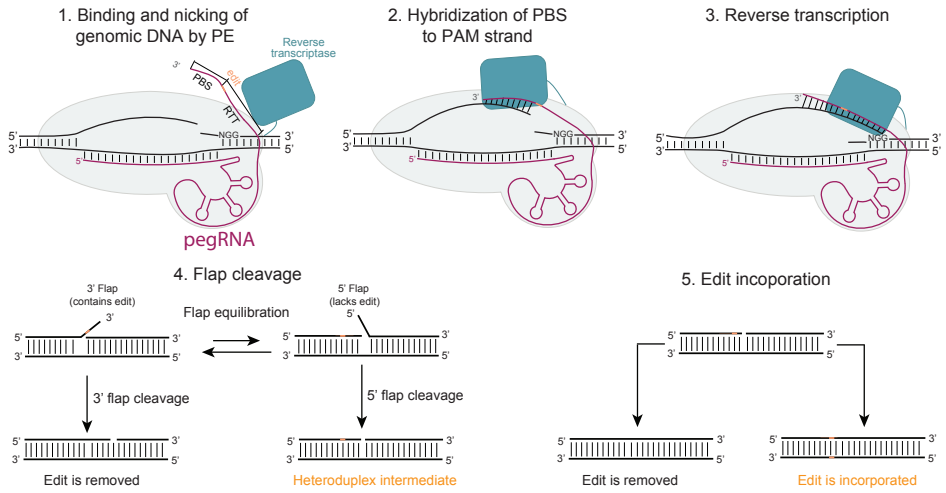

Figure 3.2. Mechanism of prime editing. Illustration detailing the putative steps of the prime editing system. (1) Cas9 nickase is guided to the target by the pegRNA and generates a nick on the non-target strand exposing a 3′-hydroxyl group. (2) The resulting 3’ end hybridizes to the PBS and (3) primes the reverse transcriptase to synthesize DNA using the sequence containing the desired edit encoded in the RTT of the pegRNA. (4) The newly synthesized strand leads to an equilibration between the intermediates with 3’ flap containing the desired edit and 5’ flap that does not contain the desired edit. The 5′ flap is degraded by cellular enzymes. The edited 3’ flap is ligated into the genome. (5) Finally, repair of the complementary genomic DNA strand, using the edited strand as the template by DNA repair or replication results in stable installation of the edit.

The PE protein is a fusion between a nCas9 (H840A) and an engineered Moloney murine leukemia reverse transcriptase (MMLV-RT). This nCas9-RT fusion protein combines with a prime-editing guide RNA (pegRNA), which comprises a spacer sequence that hybridizes to the target strand, the Cas9-binding scaffold part of the guide RNA and an RT template encoding the desired modification and a primer binding site (PBS) (Figure 3.2, Step 1) [27]. The nCas9 of PE nicks the non-target strand exposing a 3′-single-stranded DNA (ssDNA) (Figure 3.2, Step 1) that hybridizes to the PBS (Figure 3.2, Step 2) allowing the associated RT to extend the nicked 3’-ssDNA using the RT template (Figure 3.2, Step 3). The action of RT results in two redundant ssDNA flaps: a 5′ flap that contains the unedited sequence and a 3′ flap that contains the edited sequence (Figure 3.2, Step 4). The fully complementary 5′ flap is thermodynamically favored to hybridize with the unedited strand. Nonetheless, the inherent susceptibility of 5’ flaps to excision by endogenous structure-specific endonucleases likely leads to the hybridization of the edited 3’ flap, resulting in a heteroduplex. Finally, ligation and DNA mismatch repair resolve heteroduplexes by copying information from the edited strand to the unedited strand, resulting in the permanent incorporation of the desired modification (Figure 3.2, Step 5) [27].

In the first PE report [27], the authors made note of the remarkably low frequency of off-target prime editing and demonstrated that in HEK293T cells, the average off-target prime editing frequency of pegRNAs targeting *HEK3*, *HEK4*, *EMX1*, and *FANCF* at the top four known Cas9 off-target sites was <0.1%, <2.2 ± 5.2%, <0.1%,

and <0.13 ± 0.11%, respectively. In comparison, off-target editing of traditional Cas9-sgRNA 16 ± 16%, 60 ± 26%, 48 ± 28%, and 4.3 ± 5.6%, respectively. The low off-targeting frequency of prime editing was confirmed in subsequent independent studies in a variety of model organisms, such as mouse embryos (<0.1%) [28-31], organoid lines (no observable off-target mutations) [32, 33], and plants (0.00-0.23%) [34]. The high specificity of the prime editing system is likely due to the additional two-step DNA hybridization of the target DNA–PBS and target DNA–RT template required by PE ensuing low hybridization and editing at off-target loci [27]. Nonetheless, reverse transcription of 3'-extended pegRNAs can proceed into the guide scaffold, resulting in scaffold sequence insertion at the target locus. Indeed, while the frequency of such events was low, unwanted pegRNA-mediated scaffold insertions were demonstrated in both HEK293T cells and zebrafish [27, 35]. The promiscuous activity of deaminase domains in BEs was shown to sometimes lead to Cas9-independent off-target editing in both DNA and RNA [36-38]. Similarly, broad overexpression of RT in the nucleus may also lead to PE-independent off-target reverse transcription and genome editing. However, no significant genome-wide or transcriptome-wide PE-independent off-target mutations or perturbations have been observed [27, 39, 40].

Overcoming current challenges in using PE

PE has been demonstrated to allow specific gene modification in various cell types [27, 28, 35, 41-44], organoids [32, 33], zebrafish [35], drosophila [39], mice [29, 45-48] and plants [49-53]. However, the technology is still in its infancy and needs to overcome several limitations to realize its full potential. In particular, the low editing efficiency remains a critical challenge. The results of an experimental analysis of PE editing efficiency on thousands of tested sites using a lentiviral second-generation PE (PE2) showed that the editing efficiency of PE2 is typically below 20% in immortalized cell lines and drops even further in primary cells [54]. Moreover, the editing efficiency greatly varies across target loci and cell types, hampering its broad application [27]. In addition to the efficiency of the prime editor itself, the effective delivery of prime editing systems into the target cells remains a challenge as well (Box 3.1). The size of full-length PE precludes its incorporation into a single adeno-associated viral (AAV) vector system, conferring a considerable challenge for its safe *in vivo* delivery. Thus, approaches to enhance the efficiency and fidelity of PE in different cell types, and to survey potential undesired consequences of PE in different cell lines needs to be developed. Moreover, novel delivery technologies need to be developed for the successful deployment of PE in therapeutics. Below, we discuss some of the emerging strategies to address these limitations.

Improving the PE protein

The first-generation PE (PE1) uses the wild-type M-MLV RT fused to the C-terminus of the nCas9 (H840A) (Figure 3.3A) [27]. This resulted in 0.7-5.5% base substitution efficiency. Engineering of five specific mutations (D200N, L603W, and T330P to improve thermostability; T306K and W313F to enhance binding of RT to the pegRNA) [55] in the M-MLV RT domain yielded PE2 (Figure 3.3A) with a 5.1-fold increase in base substitution efficiency over the PE1 [27]. Addition of an N-terminal c-Myc nuclear localization signal (NLS) and inclusion of both a variant bipartite

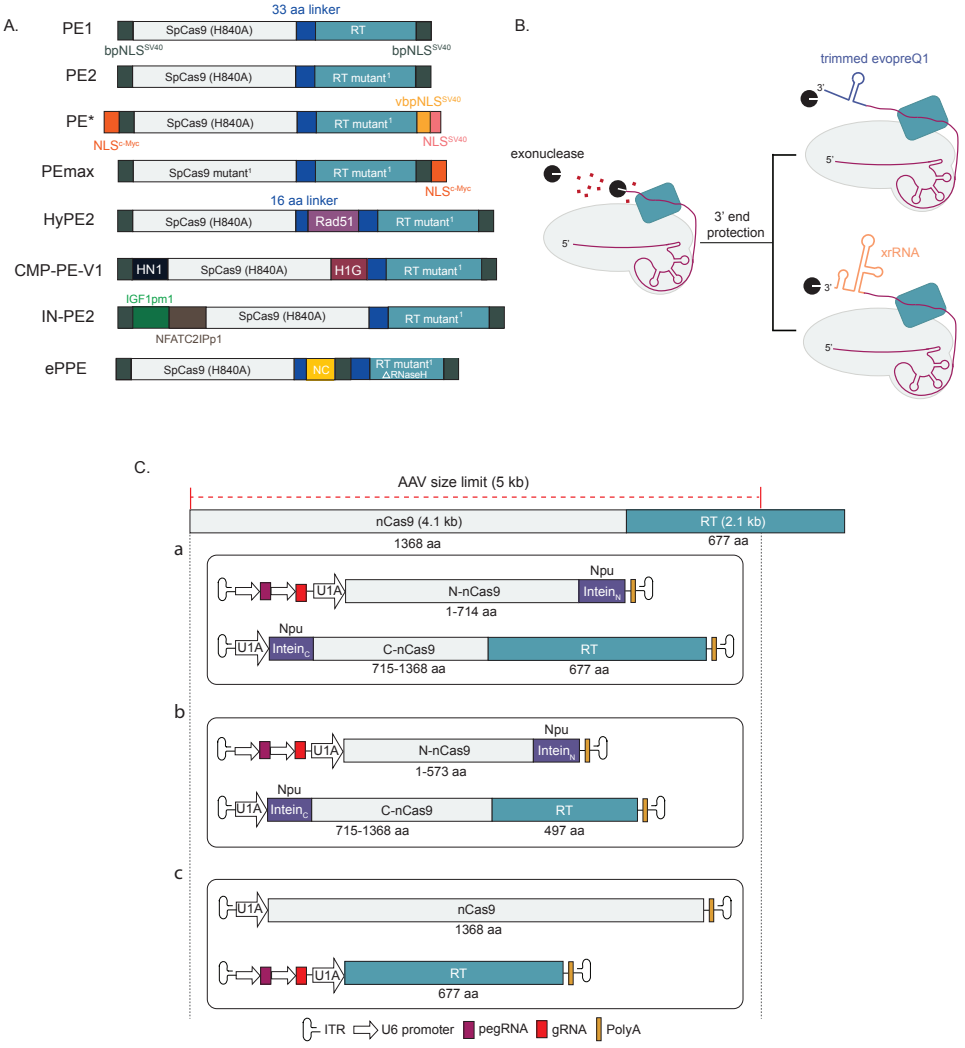

Figure 3.3. Strategies to improve the efficiency of prime editing. (A) Optimizing the PE protein. Schematic architectures of PE1, PE2, PE*, PEmax, HyPE2, CMP-PE-V1, IN-PE2 editors and ePPE. RT mutant1: M-MLV RT with five mutations (D200N, T306K, W313F, T330P and L603W), SpCas9 mutant 1: SpCas9 with three mutations (R221K, N394K and H840A), Rad51: a single stranded DNA binding protein domain, HN1: high mobility group nucleosome binding domain 1, H1G: histone H1 central globular domain, IGF1pm1 (I) and NFATC2IPp1 (N): two peptides derived from DNA repair proteins, NC: a viral nucleocapsid protein. (B) Stabilization of the pegRNA. Addition of the structured RNA motifs (trimmed evopre Q1 and xrRNA) to the 3' terminus of pegRNA protects the 3'extension from degradation by exonucleases. evopreQ1 is a modified prequeosine1-1 riboswitch aptamer and xrRNA is an exoribonuclease-resistant RNA motif. (C) Current strategies for in vivo delivery. Schematic of a split-intein dual AAV prime editor. Full-length PE2 (a) or PE2 with truncated RT (b) was reconstituted from two PE2 fragments (N-nCas9 and C-nCas9-RT) employing the Npu DnaE split intein. (c) nCas9 and M-MLV RT were subcloned into two separate AAV8 vectors without inteins.
Abbreviations: Npu: Nostoc punctiforme; DnaE: α subunit of the DNA polymerase III; ITR: inverted terminal repeat; C, carboxy-terminal; N, amino-terminal.

SV40 NLS (vBP-SV40) and a SV40 NLS at the C-terminus of PE (PE*) (Figure 3.3A) results in its nearly complete nuclear localization, improving overall base substitution efficiency by 1.9-fold over PE2 [48]. Combining a human codon-optimized RT, an additional C-terminal c-Myc NLS and activity-improving mutations in nCas9 results in a more efficient PE variant (PE max) (Figure 3.3A). PE max exhibits 2.5-fold and 1.2-fold increase in base substitution efficiency as compared to PE2 in HeLa and HEK293T cells, respectively [56].

The fusion of additional functional domains to prime editor has also been employed to augment the editing efficiency of PE. Addition of a of Rad51 DNA-binding domain between the nCas9 and RT domains, named hyPE2 (Figure 3.3A), increases PE2 efficiency by a median 1.4- and 1.5-fold in HEK293T and HCT116 cells [44]. The improvement was notably high when using a PBS with low melting temperature (T_m), suggesting that the Rad51 presumably facilitates the binding of the PBS to the 3'-ssDNA generated by the nCas9 nick, thereby enhancing reverse transcription [44]. Moreover, fusion of chromatin-modulating peptides to PE3 (where an additional gRNA is added to the PE2 system to nick the unedited strand, details below), named CMP-PE3-V1, improves chromatin accessibility, resulting in 2.55- and 3.92-fold higher targeting efficiency than PE3 at *Igf2* and *Adamts20* loci in the mouse cell lines (Figure 3.3A) [28]. Likewise, fusion of a dual DNA repair-related peptide, IGFpm1-NFATC2IPp1 to a PE (IN-PE2) leads to a median of 1.63-, 1.31- and 1.23-fold increase in prime editing across dozens of target sites in mESC, HEK293T and U2OS cells, respectively (Figure 3.3A) [57]. Interestingly, this peptide fusion is unlikely to function through interactions with mismatch repair machinery, rather increases cellular protein expression of the PE. Consequently, the resulting high levels of PE in the cell increases the overall prime editing efficiency. More recently, based on a previously reported plant prime editor (PPE) [50], authors from the same lab constructed an enhanced PPE – ePPE, in which a viral nucleocapsid protein that has nucleic acid chaperone activity is inserted between the nCas9 and the RNase H domain-deleted M-MLV-RT (Figure 3.3A). In plant cells, this ePPE achieves a 1.8-3.4-fold improvement in prime editing efficiency compared with PPE [58].

Optimizing the pegRNA

While the programmability of prime editing allows for straightforward editing, defining a suitable pegRNA design necessitates thorough optimization. The lengths of the PBS and RT template significantly affect PE efficiency [27]. Therefore, different pegRNAs with varying PBS and RT template lengths must be tested for any given target site. Typically, the PBS and RT template are in the range of 8-15 nt and 10-20 nt, respectively [27]. Several computational tools to aide in optimal pegRNA design have been developed, such as pegFinder [59], PrimeDesign [60], PE-Designer [61], PnB Designer [62], and pegIT [63].

Given that the PBS is complementary to part of the spacer at the 5' end of pegRNA, their annealing could cause pegRNA circularization potentially hampering editing. To inhibit circularization, a 20-nt hairpin forming Csy4 recognition site was fused to the 3′ end of canonical pegRNA [64]. Moreover, combining co-expression

of the sgRNA and pegRNA in a single transcript to optimize their stoichiometry for the PE3 system and mutating the fourth uracil of consecutive uracils in the scaffold of pegRNA into a cytosine to eliminate a putative transcription termination signal led to higher pegRNA expression. This system, called enhanced PE (ePE), results in a 1.9-fold increase in base substitution efficiency over PE3 without increasing indels [64]. Furthermore, to stabilize the secondary structure of pegRNA, non-C/G pair was changed to C/G pair in the small hairpin (apegRNA), resulting in higher frequencies of targeted insertions and deletions when compared to the regular pegRNA [65]. The incorporation of structured RNA motifs, such as an exoribonuclease-resistant RNA motif (xrRNA) or a trimmed prequeosine$_1$-1 riboswitch aptamer (tevopre Q1) to the 3' terminus of pegRNAs can enhance their stability and prevent degradation by cellular exonucleases, resulting in 2.5-4.5 fold improved prime editing outcomes compared to a regular pegRNA in a variety of cell types (Figure 3.3B) [66, 67]. Hence, the use of engineered pegRNAs will likely reduce the need for exhaustive screening and can substantially advance the application scope of prime editing.

Manipulating cellular DNA repair pathways

The overall prime editing efficiency depends on the ability to strongly favor the incorporation of a desired edit. In PE3, this can be achieved by using an extra single guide RNA (sgRNA) that introduces a nick in the unedited strand distal to the edit site, which likely triggers the intrinsic DNA repair response that favors the editing outcome [27]. PE3 results in 1.5-4.2-fold increase in base substitution efficiency over PE2 in HEK293T cells. Further improvement of this system called PE3b also uses a nicking sgRNA, but only targets the edited sequence, resulting in 13-fold decreased levels of indel products by preventing nicking of the non-edited DNA strand until the other strand is converted to the edited sequence [27]. Akin to PE3, nuclease-based PE (PEn) outperforms nickase-based PE at hard-to-edit targets by introducing a nick in the unedited strand, directing the DNA repair response towards the edited outcome. PEn exhibits a 2-3-fold higher insertion and base substitution over PE3 [42]. Besides, introduction of silent mutations at proper positions in the RTT (spegRNA) enhances the intended base substitution efficiency with an average of 353-fold compared to regular pegRNA in PE3 [65]. Moreover, the apegRNA and the mutations underlying spegRNA can be combined (aspegRNA) to further enhance PE editing efficiency in human cells [65]. Using paired pegRNAs to encode the same edits in both DNA strands can improve editing efficiency in human cells and rice protoplasts [53]. Yet, this paired-pegRNA strategy seems to perturb genomic integrity by promoting large DNA fragment deletions (1 kb to 10 kb), integration (> 5kb) and inversion (40 kb) [68-72].

The cellular factors and pathways involved in the 3' flap cleavage have not been well established, but it's shown that mismatch repair (MMR) (Box 3.2) activity strongly impedes efficiency and homogeny of prime editing outcomes [56, 73]. Inhibiting key mismatch repair (MMR) factors such as MLH1 and MSH2 using an engineered MMR-inhibiting protein (MLH1dn) indeed increased PE efficiency [56, 73]. The PE4 (PE2+MLH1dn) and PE5 (PE3+MLH1dn) systems include transient expression of MLH1dn, which enhances the efficiency of different prime edits by

an average 7.7-fold and 2.0-fold compared to PE2 and PE3, respectively. Moreover, strategic installation of synonymous mutations near the intended edit site also enhances prime editing outcomes by evading MMR recognition, even without MLH1dn [56].

Increasing targeting scope

The conventional PE are limited in their target scope due to the restraints imposed by the protospacer adjacent motif (PAM) sequence of the *Streptococcus pyogenes* Cas9 (SpCas9). Previously developed SpCas9 PAM variants have been successfully integrated into PEs to relax the canonical PAM preferences [74-76]. Three engineered PEs extend the target scope without sacrificing editing efficiency: PE-SpG (PAM:5'-NG, N is any nucleotide base), PE-NG (PAM:5'-NG) and PE-SpRY (PAM independent) [77]. Notably, the PE-SpRY variant extends the coverage of human genetic diseases to 94.4% and increases the number of PE3b applicable sites for improved editing efficiency without introducing more indels [77]. The PE3b-SpRY variant has been used to introduce the clinically significant BRAF V600E mutation with an editing efficiency of 11.8%, which was not possible to use conventional PEs [77].

Developing PE delivery strategies

Now most *in vivo* studies are based on adeno-associated virus (AAV) delivery due to its efficient cellular uptake, low immunogenicity, and range of serotype specificities [78]. However, PE exceeds the cargo capacity of a single AAV vector, making safe and efficient delivery challenging. To counteract this, split inteins, which can reassemble two split PE (sPE) parts into the full-length protein have been applied for delivery *in vivo* in mouse liver and retina. One such variant consists of the PE split into two parts at Ser 714. Dual AAV8s were used to deliver the split-inteins (sPE714) into mouse liver to correct the E342K mutations in SERPINA1. A 3.1% ± 0.6% correction rate after 10 weeks was observed (Figure 3.3C) [48]. Another study tested several split sites (Thr994, Ser1005, Ser1024 and Thr1032 in nCas9) and revealed that sPE1024 performed best to edit *Dnmt1* in mouse retina, paving the way for *in vivo* gene editing therapy using PE [79]. In addition, a compact PE (cPE) was created by removing the RNase H domain of RT (Figure 3.3C). cPE can efficiently induce precise editing *in vivo*, with an efficiency similar to the full-length PE. After testing several split sites, split-cPE2-573 was delivered via dual-AAV8 into mouse liver to edit *Pcsk9* with 13.5% editing efficiency after 4 weeks [45]. Interestingly, a sPE, in which the nCas9 is not tethered to the RT, was delivered using two AAV8 vectors (the first AAV8 expressing nCas9, and the second expressing the RT, the pegRNA and a nicking sgRNA) (Figure 3.3C) [80]. This sPE variant corrected 1.3% of A to G mutation in a mouse model of type I tyrosinemia. In addition to viral delivery methods described above, electroporation of PE2 ribonucleoprotein complex (RNP) into zebrafish zygotes was shown to yield 30% editing efficiency in somatic cells and even resulted in germline transmission [35]. As such, mRNA- or RNP- based delivery systems may help to overcome the size restrictions imposed by viral vectors and provide attractive and safe means for the application of PE systems in target cells.

Overall, prime editing represents a promising strategy for precise genome editing, with remarkable flexibility and reliability. Recent efforts to improve efficiency, editing scope and delivery of prime editing have resulted in more rapid uptake of the technology, especially in the context of biotechnological and pharmacological applications, as we will discuss in the next section.

Prime editing in therapeutic applications

In vivo gene editing to treat patients with genetic diseases is a long-standing goal of modern medicine. Given that prime editing has some considerable strengths over other CRISPR—Cas9-mediated genome editing methods, it has been rapidly deployed *in vivo*, and its potential to treat several genetic disorders has been examined [81]. While PEs have not yet reached clinical trials, a few proof-of-principle studies using mammalian animal models provide a foundation for future clinical trials. Here, we highlight several recent examples in which use of PE may eventually lead to new clinical solutions (Table 3.1).

First, PE3 has been used to insert 2 nucleotides in exon 52 of the ΔEx51 iPSC model, the most common single-exon deletion mutations in Duchenne Muscular Dystrophy (DMD) patients, with an efficiency of 54% [82]. Isolation and differentiation of these prime-edited iPSCs into cardiomyocytes resolved the contractile abnormalities that were observed in unedited cells. In addition, subretinal injection of split AAV-PE2 in rd12 mice, a mouse model of human Leber Congenital Amaurosis (LCA), with a nonsense mutation caused by a C to T change in exon 3 of *RPE65*, showed an average editing efficiency of 28% without any unintended edits. Moreover, the AAV-PE2 treated rd12 mice revealed more light-induced electrical responses compared to the control mice [83]. In contrast, in the same mouse model, the correction efficiency of Cas9-mediated HDR was only 1.2% ± 0.3% with the indel frequency of 17% ± 8%; and for base editor, the correct efficiency was 11% ± 7% with substantial 7.7% ± 5% frequencies of bystander edits, demonstrating the advantage of the PE2 system over other precise genome editing methods in both editing efficiency and editing-outcome homogeneity [83].

PEs have also been applied in three mouse models of liver disease. The *Fah*$^{mut/mut}$ mouse model of tyrosinemia type I is caused by inactivating the *fumarylacetoacetate hydrolase* (*Fah*) gene with G to A mutation. Hydrodynamic injection of PE3 related plasmids in *Fah*$^{mut/mut}$ mice yielded an average editing efficiency of 11.5% [83]. Moreover, all PE3-edited mice survived until the end of the experiment (40 days), while mice injected with phosphate-buffered saline showed substantial weight loss and died before 30 days [83]. PE was also applied in a PiZ transgenic mouse model of alpha-1 antitrypsin deficiency (AATD), caused by an inactivating G to A mutation in the *Serpin Peptidase Inhibitor Family A member 1* (*SERPINA1*) gene. Hydrodynamic injection of plasmid encoding NLS-optimized PE2 (PE2*) (Figure 3.3A) in the mouse liver increased A-to-G correction in PiZ *SERPINA1* (6.7% on average) by 3.1-fold compared to PE2 (2.1%) [48]. Finally, PE was used in a *Pah*^{enu2} mouse model of Phenylketonuria (PKU) is caused by inactivating the phenylalanine hydroxylase (*Pah*) gene with T to C mutation, resulting in abnormally high blood L-Phe concentrations [84]. Human adenoviral vector 5 (AdV) was used to deliver PE3$^{\Delta RnH}$

Table 3.1. Key examples of preclinical gene and cell therapy applications of prime editing

Disease model	Key organ (cell type)	Prime editor variant	Prime editing strategy	Delivery vehicle/ method	Editing outcomes	Refs
Duchenne Muscular Dystrophy in human ΔEx51 iPSC model	iPSC	PE3	GT insertion at exon 52 of DMD	Electroporation	- Successful restoration of dystrophin protein expression was demonstrated in prime-edited iPSC-derived cardiomyocytes - The arrhythmic defect was alleviated in prime-edited iPSC-derived cardiomyocytes	[82]
Leber Congenital Amaurosis in rd12 mice	Retina	PE2	T to C transition at exon 3 of Rpe65	Triple AAV2 vectors	- A distinct membranous and cytoplasmic expression of the RPE65 protein in RPE tissue was observed in the edited mice. - The edited mice revealed better dark-adapted light-induced electrical responses	[83]
Tyrosinemia type I in Fah $^{mut/mut}$ mice	Liver	PE3	A to G transition at exon 8 of Fah	Hydrodynaic mdiated non-viral vector delivery	- The edited mice showed an average of 61% FAH+ cells in the liver at day 40 - The edited mice exhibited lower weight loss and survived at day 40	[83]
Alpha-1 antitrypsin deficiency in mice	Liver	PE2*	A to G transition at exon 5 of SERPINA1	Hydrodynaicic mediated non-viral vector delivery	- 6.7 % gene correction with 2.7% indel at day 45	[48]
		PE3		Dual AAV8 vectors	- 3.1% gene correction with 0.4% indel at day 70	

Phenylketonuria in Pah^{enu2} mice	Liver	PE3 lacking RNase H domain	C to T transition at exon 7 of Pah	Dual AAV8 vectors	- < 2% gene correction	[47]
				Human adenoviral vector 5	- 11.1% gene correction with < 0.2% indel - L-Phe concentrations reduced to therapeutically satisfactory levels of 100 ± 34 μM	

(RNase H domain of RT in PE protein was deleted) to the *Pah^{enu2}* mouse model. Average correction efficiencies of 11.1% (up to 17.4%) in neonates led to therapeutic reduction of blood phenylalanine, without inducing detectable off-target mutations or prolonged liver inflammation, demonstrating the potential role of PE in the clinical treatment of PKU [85].

Taken together, these examples highlight that PE systems have the potential to correct monogenic diseases caused by a variety of mutations. In addition, PE allows the introduction of multiple simultaneous edits using tandem arrays of pegRNAs [86-88]. This has rendered it a valuable tool for treating polygenic disease, such as for example coronary artery disease. Moreover, using paired pegRNAs allows for the insertion of large DNA fragments (>100bp), making the correction of multiple gene variants located in a hotspot possible [69]. Yet, the efficiency of PE systems remains relatively low, even using more recent augmented versions of the system. Furthermore, the large size of PE effectors make the therapeutic delivery of these systems a challenge. Finally, an additional work is needed to assess its long-term safety. Combined, these concerns may restrain PE being applied directly in human bodies in the near future. Further optimization of PE editing efficiency, taking the advantage of advanced whole genome NGS technologies and advancing new delivery strategies, the *ex vivo* applications of prime editing may first bring its benefits to cancer or blood disease therapies.

Box 3.1. CRISPR delivery systems

Gene editing agents can be delivered into cells either as DNA, mRNA, or directly as protein and ribonucleoprotein complexes (RNPs) [89]. In general, delivery techniques can be divided into viral and non-viral delivery [90]. Viral vectors, especially adeno-associated virus (AAV), can deliver nucleic acid cargos to many cells and have shown great promise in clinical trials due to their efficient cellular uptake, high biocompatibility and multiple serotype specificities [78]. However, its packaging capacity is limited and the prolonged expression leads to increased off-targeting frequency [91, 92]. Non-viral delivery vectors include electroporation [93], lipid–mediated transfection [94], nanoparticles [95], cell-penetrating peptide [96], hydrodynamic delivery [97], microinjection [98], induced transduction by osmocytosis and propanebetaine (iTOP) [99] and virus-like particles (VLPs) [100] can transiently deliver RNP-based gene editing agents, thereby reducing off-target editing. From a safety perspective, RNP delivery vehicles are the most attractive, and the development of improved RNP delivery vehicles will be highly impactful for future therapeutic applications [89].

Box 3.2. DNA Mismatch repair (MMR)

DNA MMR is a highly conserved pathway from prokaryotes to eukaryotes that plays a key role in maintaining genomic integrity [101]. It repairs base mismatches and small insertions or deletions caused by misincorporation errors during DNA replication [102]. In eukaryotes, the nicked strand will be replaced by MMR through resolving DNA heteroduplexes containing mismatches [102, 103]. During this process, MutS, MutL, exonuclease, polymerase and ligase are involved [56, 104]. First the MutSα (MSH2-MSH6)

or MutS (MSH2-MSH3) will bind to the heteroduplex to recognize mismatches to initiate MMR [105, 106]. Afterwards, MutLα, recruited by MSH2 cuts the strand that contains the nick [107, 108]. Finally, exonuclease 1, a 5' to 3' exonuclease will excise the heteroduplex from these cuts, the polymerase will then synthesize the excised DNA strand and the ligase will seal the nascent stand [109, 110]. Thus, in prime editing, the strand containing the desired edits will preferentially be removed. Identifying ways to inhibit this process can significantly improve editing outcomes [56].

Concluding remarks and future perspectives

The progression from initial discovery and characterization of the prime editing system to their potential applications in biomedical sciences has occurred at a breath-taking pace. The improved flexibility and specificity of prime editing over other CRISPR—Cas9-mediated genome editing strategies has made the use of PE highly attractive. However, there are several issues that need to be addressed, most importantly the issue of low efficiency. To further enhance the efficiency of prime editing, the structure of the PE-pegRNA-DNA complex need to be elucidated (see Outstanding questions). Further optimization of PE effector could be achieved by exploiting more active nCas9 or RT variants by rational modification or *in vitro* evolution approaches and fusion of additional or combinatorial functional protein domains. In the absence of further improvements of PE efficiency, therapeutic application of PE systems would be limited to disorders that require minimal functional restoration of the corrected genes, or situations where there are more than the typical two autosomal copies of a gene present, increasing the likelihood of functional gene correction. This is for example the case in $p47^{phox}$-deficient chronic granulomatous disease, where the NCF1 gene is inactivated by the transfer of a ΔGT mutation from one of two processed pseudogenes, resulting in a non-sense mutation that prevents translation into functional protein. In the context of this defect, correction of either the *NCF1* gene itself, or any of the processed pseudogenes will correct the disorder, increasing the likelihood of functional therapeutic benefit [111]. Another example is genetic skeletal muscle disorders and some metabolic disorders. Since skeletal muscle fibers are syncytia, containing thousands of nuclei per cell this elevates the chance of gene correction per cell. Question remains of course what level of gene correction is needed on a per-cell basis to have functional therapeutic benefit. In addition, *in vivo* delivery of the large PE systems in combination with its pegRNA remains a substantial hurdle. To this end, development of new viral vectors [112], nano-particle vectors [113, 114] and RNP- or mRNA- based delivery approaches [28-30, 115, 116] becomes urgent (see Outstanding questions). In addition, we need to better understand how cell-state and/or cell-type influence prime editing efficiency, and how different DNA repair mechanisms result in productive or unproductive prime editing (see Outstanding questions). Collectively, these developments have the potential to improve the performance of PE, and we expect that the number and diversity of PE applications, including *in vivo*, will continue to grow.

Declaration of interests

The authors have no interests to declare.

References

1. Adli, M., *The CRISPR tool kit for genome editing and beyond.* Nature Communications, 2018. **9**(1): p. 1-13.
2. Moon, S.B., et al., *Recent advances in the CRISPR genome editing tool set.* Experimental & Molecular Medicine, 2019. **51**(11): p. 1-11.
3. Hsu, P.D., E.S. Lander, and F. Zhang, *Development and applications of CRISPR-Cas9 for genome engineering.* Cell, 2014. **157**(6): p. 1262-1278.
4. Jinek, M., et al., *A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity.* Science, 2012. **337**(6096): p. 816-821.
5. Cong, L., et al., *Multiplex genome engineering using CRISPR/Cas systems.* Science, 2013. **339**(6121): p. 819-823.
6. Liang, F., et al., *Homology-directed repair is a major double-strand break repair pathway in mammalian cells.* Proceedings of the National Academy of Sciences, 1998. **95**(9): p. 5172-5177.
7. Jeggo, P., *5 DNA Breakage and Repair.* Advances in genetics, 1998. **38**: p. 185-218.
8. Ceccaldi, R., B. Rondinelli, and A.D. D'Andrea, *Repair pathway choices and consequences at the double-strand break.* Trends in Cell Biology, 2016. **26**(1): p. 52-64.
9. Lieber, M.R., *The mechanism of double-strand DNA break repair by the nonhomologous DNA end joining pathway.* Annual Review of Biochemistry, 2010. **79**: p. 181.
10. Chiruvella, K.K., Z. Liang, and T.E. Wilson, *Repair of double-strand breaks by end joining.* Cold Spring Harbor Perspectives in Biology, 2013. **5**(5): p. a012757.
11. Heyer, W.-D., K.T. Ehmsen, and J. Liu, *Regulation of homologous recombination in eukaryotes.* Annual Review of Genetics, 2010. **44**: p. 113.
12. Yeh, C.D., C.D. Richardson, and J.E. Corn, *Advances in genome editing through control of DNA repair pathways.* Nature Cell Biology, 2019. **21**(12): p. 1468-1478.
13. Lin, S., et al., *Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery.* Elife, 2014. **3**: p. e04766.
14. Mali, P., et al., *RNA-guided human genome engineering via Cas9.* Science, 2013. **339**(6121): p. 823-826.
15. Kosicki, M., K. Tomberg, and A. Bradley, *Repair of double-strand breaks induced by CRISPR–Cas9 leads to large deletions and complex rearrangements.* Nature Biotechnology, 2018. **36**(8): p. 765-771.
16. Mani, R.S. and A.M. Chinnaiyan, *Triggers for genomic rearrangements: insights into genomic, cellular and environmental influences.* Nat Review Genetics, 2010. **11**(12): p. 819-29.
17. Ihry, R.J., et al., *p53 inhibits CRISPR–Cas9 engineering in human pluripotent stem cells.* Nature Medicine, 2018. **24**(7): p. 939-946.
18. Enache, O.M., et al., *Cas9 activates the p53 pathway and selects for p53-inactivating mutations.* Nat Genetics, 2020. **52**(7): p. 662-668.
19. Haapaniemi, E., et al., *CRISPR–Cas9 genome editing induces a p53-mediated DNA damage response.* Nature Medicine, 2018. **24**(7): p. 927-930.
20. Nishimasu, H., et al., *Crystal structure of Cas9 in complex with guide RNA and target DNA.* Cell, 2014. **156**(5): p. 935-949.
21. Davis, L. and N. Maizels, *Homology-directed repair of DNA nicks via pathways distinct from canonical double-strand break repair.* Proceedings of the National Academy of Sciences, 2014. **111**(10): p. E924-E932.
22. Porto, E.M., et al., *Base editing: advances and therapeutic opportunities.* Nature Review Drug Discovery, 2020. **19**(12): p. 839-859.
23. Anzalone, A.V., L.W. Koblan, and D.R. Liu, *Genome editing with CRISPR-Cas nucleases, base editors, transposases and prime editors.* Nat Biotechnology, 2020. **38**(7): p. 824-844.
24. Komor, A.C., et al., *Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage.* Nature, 2016. **533**(7603): p. 420-4.
25. Gaudelli, N.M., et al., *Programmable base editing of A*T to G*C in genomic DNA without DNA cleavage.* Nature, 2017. **551**(7681): p. 464-471.
26. Gehrke, J.M., et al., *An APOBEC3A-Cas9 base editor with minimized bystander and off-target activities.* Nature Biotechnology, 2018. **36**(10): p. 977-982.
27. Anzalone, A.V., et al., *Search-and-replace genome editing without double-strand breaks or donor DNA.* Nature, 2019. **576**(7785): p. 149-157.

28. Park, S.J., et al., *Targeted mutagenesis in mouse cells and embryos using an enhanced prime editor.* Genome Biology, 2021. **22**(1): p. 170.
29. Liu, Y., et al., *Efficient generation of mouse models with the prime editing system.* Cell Discovery, 2020. **6**: p. 27.
30. Lin, J., et al., *Modeling a cataract disorder in mice with prime editing.* Molecular Therapy Nucleic Acids, 2021. **25**: p. 494-501.
31. Gao, P., et al., *Prime editing in mice reveals the essentiality of a single base in driving tissue-specific gene expression.* Genome Biology, 2021. **22**(1): p. 83.
32. Schene, I.F., et al., *Prime editing for functional repair in patient-derived disease models.* Nature Communications, 2020. **11**(1): p. 5352.
33. Geurts, M.H., et al., *Evaluating CRISPR-based prime editing for cancer modeling and CFTR repair in organoids.* Life Science Alliance, 2021. **4**(10).
34. Jin, S., et al., *Genome-wide specificity of prime editors in plants.* Nature Biotechnology, 2021. **39**(10): p. 1292-1299.
35. Petri, K., et al., *CRISPR prime editing with ribonucleoprotein complexes in zebrafish and primary human cells.* Nature Biotechnology, 2022. **40**(2): p. 189-193.
36. Doman, J.L., et al., *Evaluation and minimization of Cas9-independent off-target DNA editing by cytosine base editors.* Nature Biotechnology, 2020. **38**(5): p. 620-628.
37. Grünewald, J., et al., *Transcriptome-wide off-target RNA editing induced by CRISPR-guided DNA base editors.* Nature, 2019. **569**(7756): p. 433-437.
38. Fan, J., et al., *Cytosine and adenine deaminase base-editors induce broad and nonspecific changes in gene expression and splicing.* Communications Biology, 2021. **4**(1): p. 1-12.
39. Bosch, J.A., G. Birchak, and N. Perrimon, *Precise genome engineering in Drosophila using prime editing.* Proceedings of the National Academy of Sciences, 2021. **118**(1).
40. Kim, D.Y., et al., *Unbiased investigation of specificities of prime editing systems in human cells.* Nucleic Acids Research, 2020. **48**(18): p. 10576-10589.
41. Habib, O., et al., *Comprehensive analysis of prime editing outcomes in human embryonic stem cells.* Nucleic Acids Resarch, 2022. **50**(2): p. 1187-1197.
42. Adikusuma, F., et al., *Optimized nickase- and nuclease-based prime editing in human and mouse cells.* Nucleic Acids Resarch, 2021. **49**(18): p. 10785-10795.
43. Surun, D., et al., *Efficient Generation and Correction of Mutations in Human iPS Cells Utilizing mRNAs of CRISPR Base Editors and Prime Editors.* Genes (Basel), 2020. **11**(5).
44. Song, M., et al., *Generation of a more efficient prime editor 2 by addition of the Rad51 DNA-binding domain.* Nature Communications, 2021. **12**(1): p. 5617.
45. Zheng, C., et al., *A flexible split prime editor using truncated reverse transcriptase improves dual-AAV delivery in mouse liver.* Molecular Therapy, 2022. **30**(3): p. 1343-1351.
46. Aida, T., et al., *Prime editing primarily induces undesired outcomes in mice.* bioRxiv, 2020, doi:10.1101/2020.08.06.239723.
47. Böck, D., et al., *In vivo prime editing of a metabolic liver disease in mice.* Science Translational Medicine, 2022. **14**(636): p. eabl9238.
48. Liu, P., et al., *Improved prime editors enable pathogenic allele correction and cancer modelling in adult mice.* Nature Communications, 2021. **12**(1): p. 2121.
49. Wang, L., et al., *Spelling Changes and Fluorescent Tagging With Prime Editing Vectors for Plants.* Frontiers in Genome Editing, 2021. **3**: p. 617553.
50. Lin, Q., et al., *Prime genome editing in rice and wheat.* Nat Biotechnol, 2020. **38**(5): p. 582-585.
51. Jiang, Y.Y., et al., *Prime editing efficiently generates W542L and S621I double mutations in two ALS genes in maize.* Genome Biology, 2020. **21**(1): p. 257.
52. Lu, Y., et al., *Precise genome modification in tomato using an improved prime editing system.* Plant Biotechnol Journal, 2021. **19**(3): p. 415-417.
53. Lin, Q., et al., *High-efficiency prime editing with optimized, paired pegRNAs in plants.* Nature Biotechnology, 2021. **39**(8): p. 923-927.
54. Kim, H.K., et al., *Predicting the efficiency of prime editing guide RNAs in human cells.* Nature Biotechnology, 2021. **39**(2): p. 198-206.
55. Arezi, B. and H. Hogrefe, *Novel mutations in Moloney Murine Leukemia Virus reverse transcriptase increase thermostability through tighter binding to template-primer.* Nucleic Acids Research, 2009. **37**(2): p. 473-481.

56. Chen, P.J., et al., *Enhanced prime editing systems by manipulating cellular determinants of editing outcomes.* Cell, 2021. **184**(22): p. 5635-5652 e29.
57. Velimirovic, M., et al., *Peptide fusion improves prime editing efficiency.* Nature Communications, 2022. **13**(1): p. 3512.
58. Zong, Y., et al., *An engineered prime editor with enhanced editing efficiency in plants.* Nature Biotechnology, 2022. **40**(9): p. 1394-1402.
59. Chow, R.D., et al., *A web tool for the design of prime-editing guide RNAs.* Nature Biomedical Engineering, 2021. **5**(2): p. 190-194.
60. Hsu, J.Y., et al., *PrimeDesign software for rapid and simplified design of prime editing guide RNAs.* Nature Communications, 2021. **12**(1): p. 1034.
61. Hwang, G.H., et al., *PE-Designer and PE-Analyzer: web-based design and analysis tools for CRISPR prime editing.* Nucleic Acids Research, 2021. **49**(W1): p. W499-w504.
62. Siegner, S.M., et al., *PnB Designer: a web application to design prime and base editor guide RNAs for animals and plants.* BMC Bioinformatics, 2021. **22**(1): p. 101.
63. Anderson, M.V., et al., *pegIT - a web-based design tool for prime editing.* Nucleic Acids Research, 2021. **49**(W1): p. W505-w509.
64. Liu, Y., et al., *Enhancing prime editing by Csy4-mediated processing of pegRNA.* Cell Research, 2021. **31**(10): p. 1134-1136.
65. Li, X., et al., *Highly efficient prime editing by introducing same-sense mutations in pegRNA or stabilizing its structure.* Nature Communications, 2022. **13**(1): p. 1669.
66. Zhang, G., et al., *Enhancement of prime editing via xrRNA motif-joined pegRNA.* Nature Communications, 2022. **13**(1): p. 1856.
67. Nelson, J.W., et al., *Engineered pegRNAs improve prime editing efficiency.* Nature Biotechnology, 2022. **40**(3): p. 402-410.
68. Choi, J., et al., *Precise genomic deletions using paired prime editing.* Nature Biotechnology, 2022. **40**(2): p. 218-226.
69. Anzalone, A.V., et al., *Programmable deletion, replacement, integration and inversion of large DNA sequences with twin prime editing.* Nature Biotechnology, 2022. **40**(5): p. 731-740.
70. Jiang, T., et al., *Deletion and replacement of long genomic sequences using prime editing.* Nature Biotechnology, 2022. **40**(2): p. 227-234.
71. Tao, R., et al., *Bi-PE: bi-directional priming improves CRISPR/Cas9 prime editing in mammalian cells.* Nucleic Acids Research, 2022. **50**(11): p. 6423-6434.
72. Wang, J., et al., *Efficient targeted insertion of large DNA fragments without DNA donors.* Nature Methods, 2022. **19**(3): p. 331-340.
73. Ferreira da Silva, J., et al., *Prime editing efficiency and fidelity are enhanced in the absence of mismatch repair.* Nature Communications, 2022. **13**(1): p. 760.
74. Kleinstiver, B.P., et al., *Engineered CRISPR-Cas9 nucleases with altered PAM specificities.* Nature, 2015. **523**(7561): p. 481-485.
75. Hu, J.H., et al., *Evolved Cas9 variants with broad PAM compatibility and high DNA specificity.* Nature, 2018. **556**(7699): p. 57-63.
76. Nishimasu, H., et al., *Engineered CRISPR-Cas9 nuclease with expanded targeting space.* Science, 2018. **361**(6408): p. 1259-1262.
77. Kweon, J., et al., *Engineered prime editors with PAM flexibility.* Molecular Therapy, 2021. **29**(6): p. 2001-2007.
78. Wu, Z., A. Asokan, and R.J. Samulski, *Adeno-associated virus serotypes: vector toolkit for human gene therapy.* Molecular Therapy, 2006. **14**(3): p. 316-327.
79. Zhi, S., et al., *Dual-AAV delivering split prime editor system for in vivo genome editing.* Molecular Therapy, 2022. **30**(1): p. 283-294.
80. Liu, B., et al., *A split prime editor with untethered reverse transcriptase and circular RNA template.* Nat ure Biotechnology, 2022. **40**(9): p. 1388-1393.
81. Doudna, J.A., *The promise and challenge of therapeutic genome editing.* Nature, 2020. **578**(7794): p. 229-236.
82. Chemello, F., et al., *Precise correction of Duchenne muscular dystrophy exon deletion mutations by base and prime editing.* Science Advance, 2021. **7**(18).
83. Jang, H., et al., *Application of prime editing to the correction of mutations and phenotypes in adult mice with liver and eye diseases.* Nature Biomedical Engineering, 2022. **6**(2): p. 181-194.
84. Shedlovsky, A., et al., *Mouse models of human phenylketonuria.* Genetics, 1993. **134**(4): p. 1205-1210.

85. Böck, D., et al., *In vivo prime editing of a metabolic liver disease in mice.* Science Translational Medicine, 2022. **14**(636): p. eabl9238.
86. Aulicino, F., et al., *Highly efficient CRISPR-mediated large DNA docking and multiplexed prime editing using a single baculovirus.* Nucleic Acids Research, 2022. **50**(13): p. 7783-7799.
87. Li, H., et al., *Multiplex precision gene editing by a surrogate prime editor in rice.* Molecular Plant, 2022. **15**(7): p. 1077-1080.
88. Torkamani, A., N.E. Wineinger, and E.J. Topol, *The personal and clinical utility of polygenic risk scores.* Nature Review Genetics, 2018. **19**(9): p. 581-590.
89. Raguram, A., S. Banskota, and D.R. Liu, *Therapeutic in vivo delivery of gene editing agents.* Cell, 2022. **185**(15): p. 2806-2827.
90. Lino, C.A., et al., *Delivering CRISPR: a review of the challenges and approaches.* Drug Delivery, 2018. **25**(1): p. 1234-1257.
91. Verdera, H.C., K. Kuranda, and F. Mingozzi, *AAV vector immunogenicity in humans: a long journey to successful gene transfer.* Molecular Therapy, 2020. **28**(3): p. 723-746.
92. Asmamaw, M. and B. Zawdie, *Mechanism and applications of CRISPR/Cas-9-mediated genome editing.* Biologics: Targets & Therapy, 2021. **15**: p. 353.
93. Chen, S., et al., *Highly efficient mouse genome editing by CRISPR ribonucleoprotein electroporation of zygotes.* Journal of Biological Chemistry, 2016. **291**(28): p. 14457-14467.
94. Yin, H., et al., *Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components in vivo.* Nature Biotechnology, 2016. **34**(3): p. 328-333.
95. Zuris, J.A., et al., *Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo.* Nature Biotechnology, 2015. **33**(1): p. 73-80.
96. Ramakrishna, S., et al., *Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA.* Genome Research, 2014. **24**(6): p. 1020-1027.
97. Gori, J.L., et al., *Delivery and specificity of CRISPR/Cas9 genome editing technologies for human gene therapy.* Human Gene Therapy, 2015. **26**(7): p. 443-451.
98. Yang, H., et al., *One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering.* Cell, 2013. **154**(6): p. 1370-1379.
99. D'Astolfo, D.S., et al., *Efficient intracellular delivery of native proteins.* Cell, 2015. **161**(3): p. 674-690.
100. Lyu, P., L. Wang, and B. Lu, *Virus-like particle mediated CRISPR/Cas9 delivery for efficient and safe genome editing.* Life, 2020. **10**(12): p. 366.
101. Larrea, A.A., S.A. Lujan, and T.A. Kunkel, *SnapShot: DNA mismatch repair.* Cell, 2010. **141**(4): p. 730. e1-730. e1.
102. Iyer, R.R., et al., *DNA mismatch repair: functions and mechanisms.* Chemical Reviews, 2006. **106**(2): p. 302-323.
103. Kunkel, T.A. and D.A. Erie, *DNA mismatch repair.* Annual Review Biochemistry, 2005. **74**: p. 681-710.
104. Li, G.-M., *Mechanisms and functions of DNA mismatch repair.* Cell Research, 2008. **18**(1): p. 85-98.
105. Gupta, S., M. Gellert, and W. Yang, *Mechanism of mismatch recognition revealed by human MutSβ bound to unpaired DNA loops.* Nature Structural & Molecular biology, 2012. **19**(1): p. 72-78.
106. Warren, J.J., et al., *Structure of the human MutSα DNA lesion recognition complex.* Molecular Cell, 2007. **26**(4): p. 579-592.
107. Kadyrov, F.A., et al., *Endonucleolytic function of MutLα in human mismatch repair.* Cell, 2006. **126**(2): p. 297-308.
108. Pluciennik, A., et al., *PCNA function in the activation and strand direction of MutLα endonuclease in mismatch repair.* Proceedings of the National Academy of Sciences, 2010. **107**(37): p. 16066-16071.
109. Genschel, J., L.R. Bazemore, and P. Modrich, *Human Exonuclease I Is Required for 5' and 3' Mismatch Repair.* Journal of Biological Chemistry, 2002. **277**(15): p. 13302-13311.
110. Zhang, Y., et al., *Reconstitution of 5'-directed human mismatch repair in a purified system.* Cell, 2005. **122**(5): p. 693-705.
111. Wrona, D., et al., *CRISPR-Directed Therapeutic Correction at the NCF1 Locus Is Challenged by Frequent Incidence of Chromosomal Deletions.* Molecular Therapy Methods Clinical Development, 2020. **17**: p. 936-943.

112. Asmamaw Mengstie, M., *Viral Vectors for the in Vivo Delivery of CRISPR Components: Advances and Challenges.* Frontiers in Bioengineering and Biotechnology, 2022. **10**: p. 895713.
113. Taha, E.A., J. Lee, and A. Hotta, *Delivery of CRISPR-Cas tools for in vivo genome editing therapy: Trends and challenges.* Journal of Controlled Release, 2022. **342**: p. 345-361.
114. Kazemian, P., et al., *Lipid-Nanoparticle-Based Delivery of CRISPR/Cas9 Genome-Editing Components.* Molecular Pharmaceutics, 2022. **19**(6): p. 1669-1686.
115. D'Astolfo, D.S., et al., *Efficient intracellular delivery of native proteins.* Cell, 2015. **161**(3): p. 674-690.
116. Staahl, B.T., et al., *Efficient genome editing in the mouse brain by local delivery of engineered Cas9 ribonucleoprotein complexes.* Nature Biotechnology, 2017. **35**(5): p. 431-434.