

Review Article

Harnessing CRISPR for the Future: Innovations, Applications, and Challenges in Genome Editing

ABSTRACT:

The advent of genome editing has opened up hitherto unimaginable avenues for research into agriculture, treatment of hereditary disorders and other intricate biological processes, revolutionising the fields which are at core of human civilization. CRISPR-based technologies are leading the charge in this transformation due to their facile programmability and exceptional efficiency. Here in this comprehensive review, we take a look at where CRISPR techniques for gene editing are at the moment, both in terms of research and in general, and we point out the constraints they face as well as the technical advances that have come up to overcome them. In addition, we take a look at the present state of gene editing approaches and summarise them as they pertain to human food security and health aspect. Lastly, we discuss some upcoming changes that may affect gene editing technology and its uses in the years to come.

Keywords: genome editing, CRISPR, cas, gRNA, off-target

1. INTRODUCTION

Genome editing, the process of precisely and selectively altering an organism's DNA, is a major step forward in molecular biology. From advancing our understanding of basic biological processes to facilitating breakthroughs in biotechnology, agriculture, and medicine, its potential uses are many and varied. Genome editing with CRISPR is entering a new age with the regulatory approval of the very first CRISPR-based human treatment in late 2023. By highlighting its present status, prospective future advancements, and the obstacles that need to be addressed to completely realize its potential for agriculture and medicine, this study seeks to offer a comprehensive overview of the genome-editing CRISPR landscape.

2. GLIMPSE INTO HISTORY

Gene editing originally sprang from a need for better methods of fixing DNA damage in eukaryotic creatures. To induce homologous recombination at particular locations, researchers in the 1990s demonstrated that homing endonucleases, such as I-SceI, could recognize 18-bp DNA sequences and generate an intended DNA double-strand break (DSB) in a cell. Originating from this was the concept of genome editing using nucleases that produce double-strand breaks. In order to build unique nucleases via long recognition sites that might target specific sites in eukaryotic genomes, scientists fused vague DNA endonucleases with tandem arrangements of sequence-specific DNA linking modules. These engineered nuclease enzymes were then developed. The zinc-finger nucleases (ZFNs) were the first to emerge in the early 2000s, but TALENs took their position in 2010 and 2011 (Andrew et al.,2011). These developments cleared the path for genome editing, but the stringent design and generation procedures of ZFNs and TALENs made them challenging to use. Because of their adaptability, specificity, and ease of programming, RNA-guided, CRISPR-associated nucleases (Cas) were a game-changer upon their introduction (Joung et al.,2013).

Table 1. Differences between ZFN, TALEN and CRISPR: Genome editing tools.

Features	ZFN (Zinc Finger Nuclease)	TALEN (Transcription Activator-Like Domain)	CRISPR (Clustured Regularly Interspaced Short Palindromic Repeats Spaced)
Discovery	1990s	2010	2012
Target Mechanism	DNA binding zinc finger domain	TALE domains	Guide RNA
Nuclease	Fok1	Fok1	Cas9

Complexity	High	High	Low
Specificity	High	High	High
Scalability	Limited	Limited	High
Cost	High	High	Low
Target size limitations	Limited to 3 bases	Not specific	Not specific
Delivery methods	Virus, electroporation, plasmid	Virus, electroporation, plasmid	Virus, electroporation, plasmid, ribonucleoprotein
Off target	Moderate	Moderate	Moderate to low

Evidence from 2007 suggests that bacterial CRISPR-Cas systems function as adaptive genome defense mechanisms, able to detect and target foreign nucleic acids associated with phages and other mobile genetic components (Rodolphe et al.,2007). The DNA fragments acquired from intruders and stored in repeating matrices in these systems are processed and converted into CRISPR RNAs (crRNAs) through transcription. By acting as molecular beacons and instructing molecular machines composed of Cas proteins, crRNAs destroy invading nucleic acids. Newer research has linked crRNA maturation to an additional RNA component known as trans-activating crRNA (tracrRNA), and it has been demonstrated that type II CRISPR-Cas systems can break phage DNA at specific locations (Carte et al.,2008). This process is facilitated by the enzyme Cas. Biochemical studies in 2012 finally established that Cas9 is a DNA-cleaving endonuclease with selectivity determined by a dual-RNA guide architecture consisting of crRNA and tracrRNA (Hatoum et al.,2011). Merging tracrRNA and crRNA into an individual guide RNA (sgRNA) simplified the CRISPR-Cas9 system and allowed for a completely configurable "one-nuclease-one-guide RNA" approach. This breakthrough laid the groundwork for CRISPR genome editing methods, which allow for the targeted delivery of Cas9 to a particular region of the genome through the use of complementary sgRNAs. The following table 1 shows the difference between different techniques for genome editing.

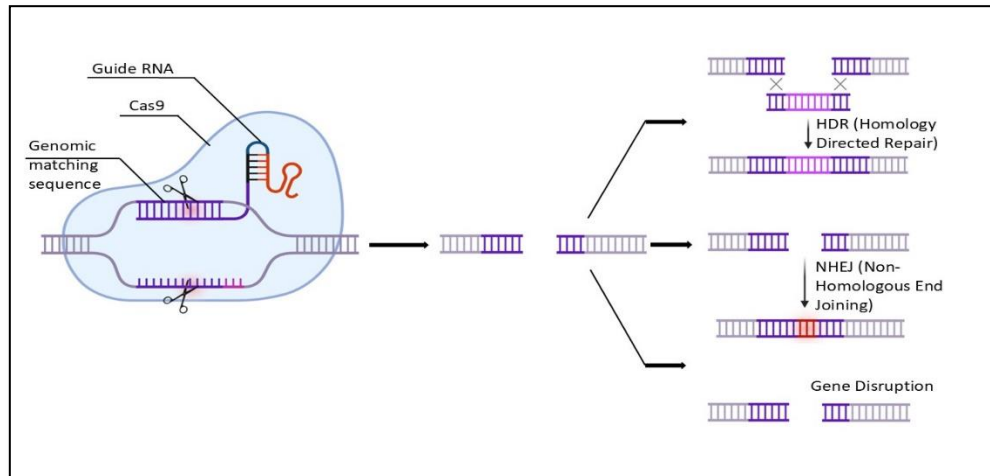
3. GENOME EDITING WITH CRISPR NUCLEASES

The ability to precisely target specific locations on DNA to cause double-strand breaks makes CRISPR-Cas nucleases highly versatile for use in genome editing technologies. *Streptococcus pyogenes* prototypical Cas9 protein (SpCas9) was one of the first Cas nucleases to be successfully used for genome editing (Urnow et al.,2018). It remains the most popular gene editor due to its naturally high activity and specificity. Cas9 may trigger its nuclease activity when it detects crRNA-tracrRNA clusters or sgRNA guidance. To direct the Cas9 nuclease to the desired genomic region, one can alter the 20-nt guide sequence on the 5-prime end of the crRNA. This will allow for canonical base pairing with the DNA target. The presence of a short protospacer adjacent motif (PAM) on the DNA's non-target strand (NTS), approximately downstream of the target site, provides further proof of target binding (Pacesa et al.,2022). The initial recognition of the PAM causes local unwinding of the target DNA. Subsequently, starting at the terminus nearest to the PAM, the guide RNA sequence bases-pairs with the TS of the DNA in a 50/30 directed fashion. Because of this, Cas9's nuclease domain becomes active through conformational changes (LeCong et al.,2013). Three nucleotides upstream of the PAM sequence, Cas9 cleaves the double-stranded DNA substrate, resulting in double-strand breaks (DSBs) with blunt ends or single-nucleotide overhangs.

In order to create double-strand breaks (DSBs), two domains-the RuvC domain and the Cas9 HNH domain cleave the TS and NTS, respectively (Hovarth et al.,2010). When one domain of Cas9 is inactivated, it becomes an RNA-guided nickase; when both domains are inactivated simultaneously, it becomes an RNA-guided DNA binding protein that can transport fused proteins to specific genomic sites (Chuwang et al.,2021). A few years after Cas9, another Cas nuclease originating from type V CRISPR-Cas systems, Cas12a, was discovered and repurposed for genome editing (Zhang et al.,2021). While Cas9 needs a tracrRNA to activate, Cas12a may be able to catalyze nucleolytic breakage of its own guides by recognizing a conserved pseudoknot structure in the repeat-derived section of the crRNA. Multiplexed manipulation in vivo has made use of this feature. By using the single RuvC domain enzymatic site to progressively cleave both strands inside the PAM-distal portion of the target site, Cas12a produces 50 nucleotide overhangs (Jiao et al.,2024). Any DNA with a 5-prime terminal TTTV PAM is its intended target. The PAM-distal DSB byproducts then dissociate from the protein, leaving Cas12a in a functioning catalytic state that can cleave more ssDNA substrates trans. When it comes to precise gene editing, Cas12a is just as effective as Cas9, another nuclease with many practical uses (Hilary et al.,2023). Traditional approaches to genome editing rely on stimulating the body's own DNA repair machinery to repair damaged DNA at precise locations. DSBs

generated by Cas9 or Cas12a enzymes are often repaired using end-joining pathways or accurate homology-directed repair mechanisms (Bai et al.,2023). Nevertheless, mistakes can occur along these channels. End-joining repair of DNA in mammals mostly involves re-ligation of broken DNA terminals through mechanisms mediated by microhomology or non-homologous end joining. Prior to re-ligation, which introduces or deletes nucleotides, the exposed ends of the DNA are processed. At the location of the double-strand break, this results in tiny insertions or deletions known as indels (Min et al.,2023).

Figure 1: Mechanism of gene editing by CRISPR Cas9



In theory, this may be accelerated by repeatedly cleaving repaired DSBs until the number of indels becomes too high to proceed. The main use of this technique is to achieve knockouts or deletions in protein-coding sequences of genes by precisely introducing two double-strand breaks (DSBs) close together (Liao et al.,2024). The ends-joining repair of Cas9-induced double-strand breaks (DSBs) leads to reproducible editing, which includes deletions mediated by mismatch repair (MMEJ), small deletions produced by non-homologous end joining (NHEJ) (Chaoyo et al.,2015), and single nucleotide insertions (SNIs).

On the other hand, homologous DNA repair (HDR) is an exact DSB repair route that uses a homologous DNA molecule to dictate the repair's outcome. The targeted genomic locus can be precisely modified, inserted, or deleted via HDR by externally offering a synthetic homology repair template (Tretbar et al.,2024). The repair templates, which can be artificial single-strand DNA oligonucleotides or double-stranded DNAs (usually delivered by plasmids or viral vectors), include the target mutation surrounded by sequences that are homologous to the sections on either side of the double-strand break (DSB). Since HDR relies on repair proteins that are typically expressed only during the S and G2 stages of the cell cycle, it is primarily active in actively dividing cells (Lu et al.,2024). Nevertheless, this method does allow for altering with nucleotide precision in theory. Consequently, the effectiveness of the HDR result is conditional on a number of variables that influence the selection of DNA repair pathways to favorably boost HDR while suppressing end-joining repair, such as the repair template type, delivery mechanism, cell type, and local chromatin context (Shakirova et al.,2023). The figure 1 diagrammatically shows the mechanism of CRISPR Cas9 based genome editing.

4. CRISPR LIMITATIONS

Many fields of basic and applied research have been significantly enhanced by repurposing CRISPR-Cas systems as effective and simple programmable gene editing tools. This has paved the way for the creation of targeted gene therapies and different biotechnological applications (Doduna et al.,2020). In contrast to the expected functionalities of a precise genome editing tool, the functional aspects of an extremely established biological defense system are different. Therefore, there are a number of important issues that restrict the potential applications of first-generation CRISPR-based gene editing tools (Pacesa et al.,2024). These include specificity, the scope of targeting, and the necessity to depend on endogenous DSB repair processes to accomplish genomic alterations. Lastly, there are certain limitations associated with the transfer of vectors and the cells or creatures that are intended to receive CRISPR components (Li et al.,2023).

4.1 OFF-TARGET ACTIVITY

Natural CRISPR-Cas systems likely tolerate some degree of guide RNA discrepancies with targets as an evolutionary reaction to the phages' enormous mutation rate (Pattanayak et al.,2020). The potential for editing partially complementary off-target regions elsewhere in the genome in addition to the planned on-target locus makes this feature less than desirable for genome engineering. Multiple studies have demonstrated Cas9's off-target activity by showing that it can guide-dependently tolerate a wide variety of nucleotide mismatches within the guide-target heteroduplex (Guo et al.,2023). However, when multiple locations throughout the genome are cut simultaneously, off-target, it can trigger DNA damage and response to stress pathways. Genomic inversions, deletions, and chromosomal translocations are all possible outcomes of this (Redelings et al.,2024).

4.2 CONTROLLING THE OUTCOMES OF EDITS

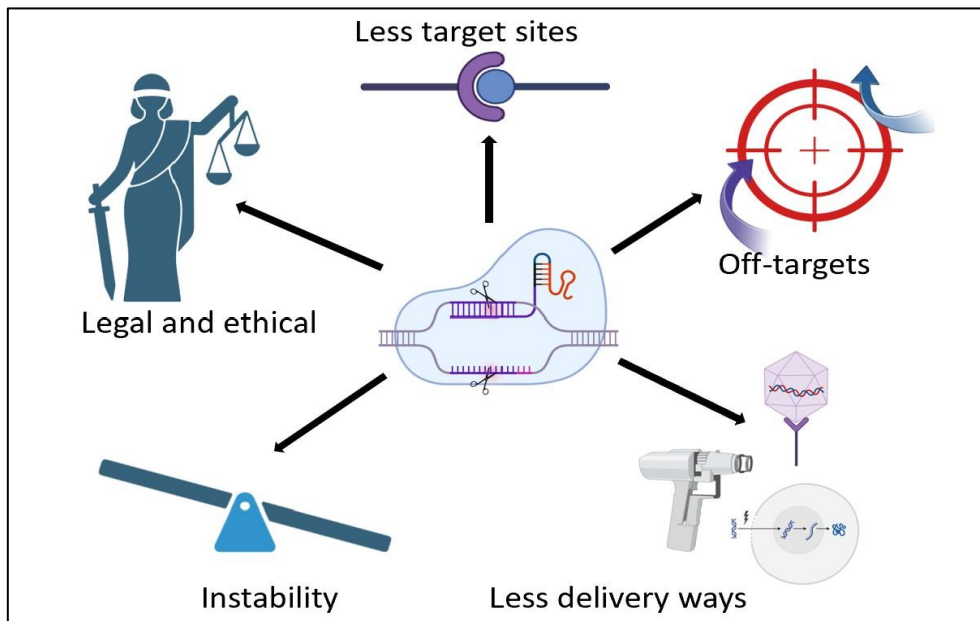
By inserting targeted nucleases into the genome, the frequency of HDR in the cells of mammals is substantially enhanced (Rouet et al.,1994). Regardless, HDR is only applicable to cells that are dividing, and it often yields heteroallelic editing results due to the simultaneous editing caused by end joining pathways (Moynahan et al.,2010). Chromosome rearrangements, large deletions, or even chromosome loss are some of the other unforeseen outcomes that make it difficult to edit precisely at the desired genomic site. As a result, researchers have focused on methods to control the outcomes of DNA repair, such as improving the efficiency of knockin (insertion) mutations and accelerating HDR repair (Lacombe et al.,2024). Recent research has shown that HDR can be made more efficient by retargeting NHEJ edit outputs using secondary guide RNAs. Despite recent advances, utilizing homology templates to deliver knockin mutations-particularly lengthy insertions-remains a challenging strategy (Hermantara et al.,2024).

4.3 DELIVERY

A key limitation of gene editing applications both in-vivo and ex-vivo is the difficulty in achieving tailored gene editor dispersion. Getting CRISPR elements to specific cells quickly, safely, and precisely is crucial for therapeutic genome editing to work. Another issue with CRISPR elements and delivery vectors for in-vivo therapeutic purposes is their possible immunogenicity (Wagner et al.,2019). In disease models, Cas9 immunity has been associated with reduced treatment efficacy. The target cell type or organism dictates the delivery format of the Cas9/Cas12a enzymes and their guide RNAs. Electroporation (nucleofection) and liposome-mediated transfection continue to be the most popular delivery modalities for the majority of in vitro (i.e., ex vivo) uses in cultivated cells because of their efficient operations (Berdeckaa et al.,2023). A variety of delivery vehicles are available for Cas9 and guide RNA components, including plasmid DNA, RNA, and in vitro reassembled ribonucleoprotein (RNP) complexes. The use of intermittent RNP-based delivery has largely replaced long-term production of the Cas9 complex using a plasmid in gene editing for ex vivo medicinal applications due to the substantial amount of off-target editing and randomized plasmid integration that can occur with this method (Eftekhari et al.,2023). Microinjection or electroporation are the usual methods for delivering Cas9 RNPs or mRNA to modify germline genomes in numerous model species. Viral vectors are frequently used to deliver CRISPR-Cas9 inside cells in vivo (Wang et al.,2023).

Gene editing modules can be built into adenoviruses, lentiviruses, and adenoassociated viruses (AAVs) to substitute the viral genes within the vector. AAVs continue to be the go-to vectors for in vivo delivery because of their wide range of high transduction, cell tropism, efficiency, and minimal immunogenicity. On the other hand, AAVs are little viruses that can only carry a cargo of about 4.7 kilobytes. Because of this, using ultracompact promoters for packing the genes expressing SpCas9 (4.2 kb) and its sgRNA (100 nucleotides) within a single AAV vector is no easy feat (Wang et al.,202). Finally, lipid-based nanoparticles (LNPs) or synthetic guide RNAs can be used to deliver Cas9 mRNA into cells in vivo, rather than relying on viral methods. These approaches have the benefit of lessen the likelihood of immune responses and are safer than vectors derived from viruses (Chen et al.,2020). Many different kinds of cells and creatures have benefited from the use of lipid nanoparticles for the transmission of Cas9 and other genome editors (Rosenblum et al.,2020). The figure below shows the limitations pertaining to CRISPR based editing of genome.

Figure 2: Limitations of CRISPR: legal, ethical, off target effects, instability, delivery constraints.



5. THE PRESENT TECHNOLOGIES AND ITS APPLICATIONS

Rapid advancements have been made in the field of CRISPR-based modification of genes since its initial demonstration. The variety, precision, and reduction of unwanted editing consequences of and Cas12a nucleases are all being improved by ongoing advancements (Asmamaw et al.,2024). Nevertheless, worries over their safety continue, owing to off-target editing behavior and the possibility of genotoxic effects from on-target DSBs, such as p53 activation (Shumega et al.,2024). The use of naturally existing anti-CRISPR protein antagonists for tissue limited editing is one of several methods that have been investigated for accurate spatiotemporal regulation of CRISPR genome editors, with the goal of reducing the occurrence of unintentional edits (Weigand et al.,2020). Additional "second-generation" CRISPR technologies, such as base editors (BEs) along with prime editors (PEs), mediate genome editing independently of DSB formation and HDR, in response to worries regarding the genotoxicity of DSB and the necessity to address the poor efficiency of HDR (Averina et al.,2024). Even though these genome editing tools have helped a lot, regarding canonical CRISPR genome editing's many limitations, they still have some work to do in terms of specificity, transport, and activities. In this section, we will go over the history of contemporary technologies, the limits many have helped to alleviate, and the limitations they currently encounter.

5.1 BEYOND NUCLEASE CAS9

Following Cas9's initial development as a genome editing nuclease, researchers set out to discover more naturally occurring Cas enzymes that may be used for genome engineering. An alternate nuclease with a different PAM requirement, guide RNA design, and DNA cleavage mechanism was discovered in 2015 with the discovery of Cas12a, marking a key expansion beyond Cas9 (Strohkendl et al.,2018). Due in part towards their lower of DNA cleavage, Cas12a enzymes have been found to display higher selectivity and reduced off target activity in vivo. Bioinformatic investigations that followed identified several type V Cas effectors that needed different amounts of PAM and guide RNA (Mendoza et al.,2018). "Minimal," small type V nucleases are of special interest because they show potential for uses that call for enclosing gene editing components within size-limited viral vectors (like AAV) (Karvelis et al.,2020). Cas13 and Cas12g are RNA-targeting RNA-guided nucleases that have added a new tool to the molecular editing toolkit (Yang et al.,2024). These nucleases may detect nucleic acids, edit or degrade mRNAs, or both. While these advancements have opened up new possibilities, there are still some limits to each Cas nuclease. These include, but are not limited to, limited PAM targeting, varied in vivo activities and off-target profiles, and potential immunogenicity (Amiri et al.,2024). However, with the availability of several enzymes, genome editing may now be approached more precisely, opening up a range of possibilities

for different applications. The majority of these enzymes are ideal for multiplexing because they are orthogonal to their guide RNAs (Cown et al.,2024).

5.2 HIGH-FIDELITY VARIANTS OF CAS9

To tackle the problem of off-target activity, two supplementary methods were used to produce tailored SpCas9 variants with enhanced specificity (Acharya et al.,2024). The first one uses structure-based rational design to create mutations that improve fidelity (Chen et al.,2017). This approach is based on the theory that when specific contacts between the Cas9 protein and bound DNA target are eliminated, the Cas9-guide RNA complex becomes more sensitive to substrate DNA mismatches (Baranova et al.,2024). As a result, the likelihood of off target binding and cleavage is reduced. The mutations significantly reduce the DNA cleavage rate, which promotes off-target release, according to biophysical and biochemical investigations of these variants. The second strategy involves selecting mutations that decrease off-target editing using directed evolution methods (Vakulskas et al.,2018). Additional Cas9 and Cas12a enzymes have also been the target of similar attempts to develop high-fidelity versions. The current crop of high-fidelity variations includes enzymes with far higher specificities than wild-type enzymes, although their efficiency varies depending on the DNA target and the task at hand (Schmid et al.,2022).

5.3 MODIFICATIONS OF GUIDE RNA

An attractive substitute for using high-fidelity nuclease mutants is to enhance specificity by altering the guide RNAs of genome editors (Fu et al.,2014). An early attempt at this sort of thing centred on Cas9 truncated guide RNAs, where the guide segment is cut short at 22 nucleotides in the middle (Reautschnig et al.,2024). These have been demonstrated to drastically lower SpCas9 off-target activity at several locations, but they also display reduced efficiency or initiate editing at novel off-target sites. Additional evidence that 5 prime end alterations of the guide segment, such as the addition of unpaired nucleotides and secondary structures can reduce off-target recognition is available (Alipanahi et al.,2024). Addition of 2-methyl or 2-fluoro nucleotides and phosphorothioate links to the guide RNA is another effective way to improve guide stability and increase Cas9 specificity (Chatterjee et al.,2024). The effects of the changed nucleotides are position-dependent, similar to DNA substitutions, although some of these alterations have demonstrated substantial gene editing activity in vivo (Kliuchnikov et al.,2024).

5.4 PAM GENOME ALTERNATIVE

Various studies have attempted to introduce specificity-altering substitutions for amino acids in the PAM-interacting domain or use structure-based rational engineering to broaden the PAM targeting scope of SpCas9 (Kleinstiver et al.,2016). This is in an effort to circumvent the PAM-dependent target location constraints of Cas nucleases. Additional designed variants targeting non-G PAMs have further lowered the PAM selectivity of SpCas9. Engineered SpCas9 variations capable of NRN PAM targeting, including practically "PAM-less" Cas9 designs, have recently allowed CRISPR-Cas9 to reach an even broader spectrum of genomic locations (Sun et al.,2024). They have greatly increased Cas9's targeting capability and made target site selection much simpler.

5.5 BASE EDITING

Editing in cells lacking homology repair (HDR) is now possible with the use of CRISPR-derived base editors (BEs), a flexible method that may produce specific point mutations without creating double-strand breaks (DSBs) and supplying homology repair templates (Hwang et al.,2024). BEs are constructed by fusing a nucleotide deaminase enzyme with a Cas9 variant that is RuvC-inactivated, in a modular fashion. It all started with two types of BEs. A cytosine BE (CBE) mediates the conversion of C to T by incorporating catalytic domains from cytidine deaminases (APOBEC1, for example) and a uracil glycosylase inhibitor (UGI) domain (Tong et al.,2024). On the other hand, adenine BEs (ABEs) convert A to G by utilizing an adenosine deaminase region from the tRNA-specific deaminase TadA, which has been modified to function on ssDNA by directed evolution (Zhao et al.,2024). After the Cas9 module binds, BEs convert a cytosine or adenine to uracil or inosine, respectively, inside a "editing window" in the PAM-distal section of the relocated non-target DNA strand. During DNA replication, these are read out as thymine and guanine, respectively, which causes mutations at transition points. Several design revisions have been undertaken by the preliminary CBE and ABE editors since their conception to enhance activity and decrease the number of off-target edits caused by deaminase (Liu et al.,2024).

Additionally, Cas12a BEs have been produced. Additional C-to-G, A-to-C and A-to-Y transversions are now part of the standard editing toolkit (Chen et al.,2024). For mutational screens and genome-wide knockouts, BEs have been used since their editing results are often predictable. Because of their pinpoint accuracy, BEs can be employed to treat disorders brought on by single-point mutations. Even though BEs provide more control over editing results, they have a few drawbacks. Some of these issues include low efficiency, editing by uninvolved parties, wide editing windows, and significant off-target behaviour (Alharbi et al.,2024). New CBE variants have been engineered through directed evolution of ABEs because, compared to CBEs, latest-generation ABE variants show better editing efficiency and lower rates of Cas9-independent off-target editing (Fiumara et al.,2022). In conclusion, while canonical nuclease-based Cas9 editing is more commonly associated with undesirable genotoxic effects, ABEs and CBEs can also cause deletions, DSBs and translocations at the on-target locus, but to a lesser extent.

5.6 PRIME EDITING

Prime editing is an HDR-independent method for producing targeted point mutations, insertions, or deletions using a Cas9-based technique. The prime editor is made up of a fusion protein construct that includes a Cas9 nickase with an inactivated HNH domain and an altered reverse transcriptase (RT) domain, as well as a prime editing guide RNA (pegRNA) (Mikhaylova et al.,2024). A 3 prime terminal sequence extension of the pegRNA carries the targeted mutation(s) and is complementary to the NTS at the target genomic region. Following Cas9's nick generation in the NTS, the complementary pegRNA extension is base paired with. Next, the three primary ends of the NTS are extended utilizing the pegRNA as a template through RT-catalyzed synthesis, introducing the mutation. After that, the DNA strands are reannealed to create a 5-flap intermediate. Then, the intermediate is subjected to excision and ligation, which fixes the alteration in the genomic DNA. This type of targeted strand synthesis enables the introduction of point mutations up to 30 bp at the Cas9 nicking site, as well as insertions or deletions up to 80 bp in length (Vu et al.,2024). Improving prime editing efficiency was a goal of successive generations of PE designs, which began with the first-generation design that fused a nickase Cas9 with a the wild-type strain RT derived from Moloney murine leukemia virus (MMLV). These designs included engineered MMLV RT domains with improved thermostability and introduced a second sgRNA to generate an insertion on the nonedited strand, which helped to promote edit retention in the genomic DNA, preventing the DNA mismatch repair pathway, enhancing nuclear localization and expression, and repairing DNA nicks have all contributed to further advances (Ferreira et al.,2024).

6. MODULATION AT TRANSCRIPTIONAL LEVEL: CRISPRi, CRISPRa

In addition to genome editing, CRISPR technologies have made it possible to temporarily alter gene expression. The first application of catalytically inactive Cas9 mutants was in bacterial gene promoter targeting and sterically blocking RNA polymerases, which inhibited RNA transcription (Allemailem et al.,2024). The KRAB transcriptional repressor region is one example of an epigenetic modulator that can be linked to nuclease-inactive Cas9 and then directed to the promoter regions of genes that are transcribed in order to decrease expression of genes in eukaryotic cells (Monteferrario et al.,2024). The subsequent method, called CRISPR interference (CRISPRi), allows for effective reductions of gene expression and serves as a substitute for RNA interference based on small interfering RNA (siRNA) (Schlosshauer et al.,2024). Similarly, chromatin state modulation or direct recruitment of transcriptional activation factors are two ways in which nuclease-dead Cas9 protein fusions can be effectively utilized to trigger the expression of targeted genes (Chen et al.,2024). Integrating dCas9 with transactivation domains like VP64 or its derivatives has allowed for CRISPR activation, or CRISPRa. Another option is to form combinations with histone-modifying enzymes like acetylases or demethylases (Huang et al.,2024). These enzymes can then modify epigenetic markers at specific sites and produce active chromatin states, which in turn promote gene expression. The CRISPR-directed transcriptional modulation method has a lot of off-target action since Cas9 DNA binding is often more promiscuous than cleavage.

7. THE EMERGING FUTURE

The limitations of existing CRISPR technologies have been more apparent over the last decade, prompting the development and refinement of new techniques and methodologies to enhance the effectiveness and adaptability of CRISPR-based editing of genomes. These new, third-generation tools and technologies encompass a wide range of possibilities, including DSB-based

editing using newly found classes of compacted RNA-guided nucleases and potential RNA-guided DNA binding frameworks for additional genome editing modalities like BEs and Pes (Villiger et al.,2024). A significant unfulfilled requirement in the realm of genome editing is the incorporation of lengthy, gene-sized DNA sequences, especially in post-mitotic cells devoid of HDR. One possible and encouraging way to close this technological gap is the creation of CRISPR-guided recombinases and transposons (Hseih et al.,2024). Additionally, novel strategies for altering RNA transcripts and genomes using retrotransposons-based technologies have surfaced (Chen et al.,2024). The development of new delivery techniques is closely tied to the production of novel genome editor tools, which poses a significant barrier for therapeutic applications. All things considered, these advancements mirror the rapid pace of the fast-developing area of genome editing, where new methods provide complementing strengths to tackle the many needs and obstacles of genetic modifications.

7.1 MINIMAL RNA-GUIDED ACTIVITY OF NUCLEASES

A minimally sized enzyme with targeting capabilities and orthogonal guide RNA scaffolds would greatly aid in the development of fusion proteins (like PEs and BEs) and their effective cellular delivery through viral vectors, which is a major driving force behind the continuing quest for new RNA-guided nucleases. An example of this is the recent advancements in engineering and development of the ultracompact Cas12f nuclease, which have been designed to improve its *in vivo* editing activity by structure-guided design and extensive mutational scanning (Tang et al.,2024). New molecular genome editing tools have been added to the toolbox as a result of continuing research to find new RNA-guided nucleases that occur naturally.

7.2 EDITORS AS DNA POLYMERASE

By fusing a Cas9 nickase variation with a designed error-prone DNA polymerase, an early effort was made to continuously diversify nucleotides in a configurable window of as many as 300 nucleotides from a target site (Halperin et al.,2018). A recent work proved that a trans-provided phage-derived DNA polymerase may make modifications at a Cas9-nicked spot with the help of a linear DNA template that is attached (Chang et al.,2024). Bypassing autoinhibitory intramolecular base pairing in the guide RNA, this method allows for longer insertions of more than 100 nucleotides, in contrast to RT-based prime editing. Click editing is an innovative method that uses Cas9 in conjunction with DNA-dependent DNA polymerases (DDPs) and HUH endonucleases to generate a wide variety of genome edits, such as small insertions and deletions, all single-nucleotide substitutions, and more (da Silva et al.,2023). Covalently attaching "click DNA" templates to a fusion of HUH-nCas9 and DDP protein is achieved through the technique, which takes use of the biological conjugation action of HUH endonucleases. This method prevents accidental inserts while simultaneously allowing for accurate genome editing with few indels.

7.3 CRISPR-GUIDED TRANSPOSONS AND RECOMBINASES

Independent of DSB production and repair, transposons can catalyze the insertion of long DNA strands on their own. A number of investigations have sought to achieve RNA-guided transposition in cells by fusing nuclease inactive Cas9 to other transposases, such as piggyBac, mariner, and Sleeping Beauty (Ochmann et al.,2024). These methods improve transposition rates close to the target location, but they are not yet practical for targeted gene insertion due to their poor efficiency and large off-target transposition rates. Natural Tn7-like transposable elements called CRISPR-associated transposon (CAST) systems facilitate RNA-guided DNA transposition by utilizing either type I or V CRISPR-Cas systems in targeting modules, as opposed to synthetic Cas9-transposase fusions (Hsieh et al.,2024). These technologies can implant themselves into bacteria precisely where they are needed. To reach the levels of effectiveness needed for comprehensive genome editing and clinical gene delivery, additional comprehensive functional characterization of naturally existing CASTs, mechanistic research, and engineering efforts are required (Durrant et al.,2023).

7.4 EDITING BASED ON RETROELEMENT

Retroelements, in particular non-long terminal repeat (non-LTR) retrotransposons (Lee et al.,2024), are a new kind of retroelement that differ from CASTs and offer a way to programmatically insert long DNA sequences that may be more efficient and specific than CASTs (Tang et al.,2023). In the same way as PEs facilitate DNA insertion, retroelements do the same by nicking the target DNA and priming the retrotransposon RNA for reverse transcription with the exposed three prime ends of the nick. This process is called target-primed reverse transcription (TPRT).

7.5 RNA EDITING

Due to the short half-life of messenger RNAs (mRNAs) inside the cell, adding targeted changes straight into the mRNA provides a different approach to editing DNA that may be safer, similar to epigenome editing (Dolken et al.,2008). More nuanced and flexible control over the edited results is now possible with RNA editing techniques. The Cas7-11 system is an innovative single-protein CRISPR-Cas effector which utilizes guide RNAs to cleave target RNAs (Hong et al.,2024). Its discovery led to one such improvement recently. When compared to Cas13a-mediated knockdown techniques, the re-engineered system appears to have no additional cleavage activity and limited off-target activity when knocking down transcripts in mammalian cells (Borrajó et al.,2023). Furthermore, a new method for trans splicing long RNA transcripts using CRISPR has just been published. This approach allows for the replacement or insertion of substantial portions in mammalian mRNA transcripts by combining RNA-targeting Cas13 enzymes with a trans-splicing RNA.

7.6 NOVEL METHODS FOR DELIVERY

To improve the efficiency of delivery and reduce the possibility of immunogenicity in CRISPR-based genome editors, several techniques are presently being developed. Firstly, there have been advancements in LNP formulations that allow for tissue-specific targeting (Gao et al.,2024). This opens up new possibilities for the packaging and delivery of CRISPR components, since they have been shown to have improved cellular absorption and decreased off-target editing. The use of cell-penetrating peptides to deliver CRISPR enzymes has also demonstrated promising results, especially in the editing of human primary neuronal cells, lymphocytes, and airway epithelium (de Moraes et al.,2024). It is now possible to efficiently deliver temporary and cell-specific protein cargo, such as genome editing nucleases, by re-engineering the contractile injection mechanisms of bacteria (Kreitz et al.,2024). A potent substitute for viral transmission vectors for in vivo applications has arisen engineered virus-like particles. These particles resemble viruses but do not contain any viral genetic material (Sharma et al.,2024). One further exciting development in the field of viral particle engineering is the possibility of delivering therapeutics with a diminished immunogenic response. These particles are derived from long-extinct viruses that are encoded in the genomes of eukaryotic organisms (Segel et al.,2021). Lastly, new computational protein design methods are enabling the development of purpose-built protein cages, which may offer modular solutions for transporting gene editing complexes to inaccessible biological settings (Wang et al.,2023). The immunogenicity profiles, cytotoxicity and off-target of these technologies must be thoroughly evaluated across different cell types and tissues, notwithstanding the immense promise that these breakthroughs hold.

7.7 THE BLACK BOX- ARTIFICIAL INTELLIGENCE

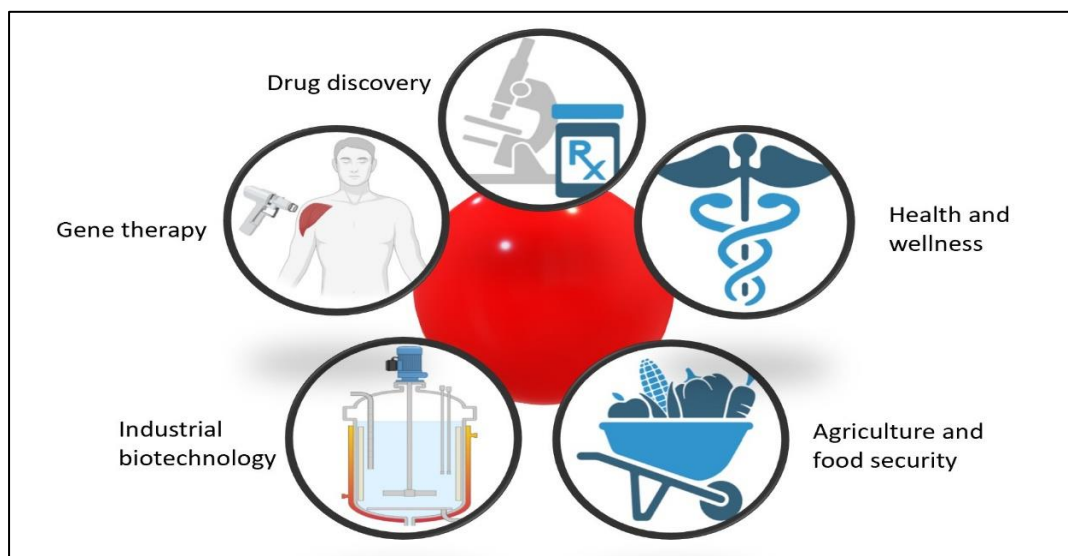
There have been far-reaching consequences for genome editing from the present and rapid adoption of deep learning computing algorithms and artificial intelligence (AI) in the biological sciences. There is vital need for reducing unintended genetic alterations. Further customization of gene editing technologies for biological purposes may be possible in the future with the help of AI methods. New computational protein design methods are also being used to develop nucleases that are more selective, efficient, and powered by AI. On the other hand, there are certain difficulties associated with AI approaches. The training data used to train predictive models can be scarce and of varying quality, depending on the method used to conduct the experiments. It is also difficult to understand and trust predictions made by AI models due to their "black-box" nature, which makes it impossible to know how these algorithms make decisions (Hassija et al.,2024).

8. LOOK INTO THE OUTLOOK

Genome editing has gone from being an emerging scientific discipline to a powerful biotech industry in the last decade. Base editing and prime editing are examples of second-generation CRISPR technologies that build upon first-generation methods. These methods allow for direct target DNA modification without DSB generation, which is seen as safer and more reliable than first-

generation methods, which relied on intrinsic repair or site-specific double-strand breaks. Achieving accurate insertion of big (gene-sized) payloads and regulating genes without genome editing via epigenome engineering are two significant unfulfilled requirements in the field that are being addressed by emerging (third generation) CRISPR technologies.

Fig.3. Sectors and application of genome editing tools



Two methods are facilitating the continuous improvement of these as well as other technologies: first, synthetic biology and molecular engineering assisted by artificial intelligence; and second, metagenome mining for the discovery of new molecular systems.

Thus, the most striking feature of modern genome editing is the ever-growing toolbox of innovative technologies and approaches, most of which are based upon RNA-guided molecular techniques inherited from CRISPR-Cas systems, which have completely transformed our capacity to manipulate genetic material with ease and precision.

9. CONCLUSION

Genome editing discussions will always center on the ethical and societal ramifications, especially when it comes to human germline genome changes. As the technology to edit cells becomes widely available, the ethical concerns surrounding the editing which could alter the genome in a way that is passed down from generation to generation beg to be addressed. It is crucial that we get an international agreement on how to regulate and responsibly handle genome editing technologies as soon as possible, given their fast advancement, ongoing improvement, and extensive use. CRISPR genome editing has a promising future, despite certain obstacles. This has the ability to improve agriculture, solve ecological problems, and drive medical discoveries, all of which could lead to a more prosperous and sustainable future for future generations. The figure below diagrammatically emphasises on the wide application of genome editing tools.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declares that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during the writing or editing of manuscripts.

REFERENCES

1. Andrew J. Wood *et al.*, Targeted Genome Editing Across Species Using ZFNs and TALENs. *Science* **333**,307-307(2011). DOI:10.1126/science.1207773
2. Joung, J., Sander, J. TALENs: a widely applicable technology for targeted genome editing. *Nat Rev Mol Cell Biol* **14**, 49–55 (2013). <https://doi.org/10.1038/nrm3486>

3. Rodolphe Barrangou *et al.*, CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes. *Science* **315**,1709-1712(2007). DOI:10.1126/science.1138140
4. Carte J, Wang R, Li H, Terns RM, Terns MP. Cas6 is an endoribonuclease that generates guide RNAs for invader defense in prokaryotes. *Genes Dev.* 2008 Dec 15;22(24):3489-96. doi: 10.1101/gad.1742908. PMID: 19141480; PMCID: PMC2607076.
5. Hatoum-Aslan A, Maniv I, Marraffini LA. Mature clustered, regularly interspaced, short palindromic repeats RNA (crRNA) length is measured by a ruler mechanism anchored at the precursor processing site. *Proc Natl Acad Sci U S A.* 2011 Dec 27;108(52):21218-22. doi: 10.1073/pnas.1112832108. Epub 2011 Dec 12. PMID: 22160698; PMCID: PMC3248500.
6. Urnov FD. Genome Editing B.C. (Before CRISPR): Lasting Lessons from the "Old Testament". *CRISPR J.* 2018 Feb;1(1):34-46. doi: 10.1089/crispr.2018.29007.fyu. PMID: 31021186.
7. Pacesa, M., Loeff, L., Querques, I. *et al.* R-loop formation and conformational activation mechanisms of Cas9. *Nature* **609**, 191–196 (2022). <https://doi.org/10.1038/s41586-022-05114-0>
8. Le Cong *et al.*, Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science* **339**,819-823(2013). DOI:10.1126/science.1231143
9. Horvath P, Barrangou R. CRISPR/Cas, the immune system of bacteria and archaea. *Science.* 2010 Jan 8;327(5962):167-70. doi: 10.1126/science.1179555. PMID: 20056882.
10. Chuang, C. K., & Lin, W. M. (2021). Points of view on the tools for genome/gene editing. *International Journal of Molecular Sciences*, 22(18), 9872.
11. Zhang, Y., Ren, Q., Tang, X., Liu, S., Malzahn, A. A., Zhou, J., and Qi, Y. (2021). Expanding the scope of plant genome engineering with Cas12a orthologs and highly multiplexable editing systems. *Nature communications*, 12(1), 1944.
12. Jiao, C., Peeck, N. L., Yu, J., Ghaem Maghami, M., Kono, S., Collias, D., and Beisel, C. L. (2024). TracrRNA reprogramming enables direct PAM-independent detection of RNA with diverse DNA-targeting Cas12 nucleases. *Nature Communications*, 15(1), 5909.
13. Hillary, V. E., & Ceasar, S. A. (2023). A review on the mechanism and applications of CRISPR/Cas9/Cas12/Cas13/Cas14 proteins utilized for genome engineering. *Molecular Biotechnology*, 65(3), 311-325.
14. Bai, W., Huang, M., Li, C., & Li, J. (2023). The biological principles and advanced applications of DSB repair in CRISPR-mediated yeast genome editing. *Synthetic and Systems Biotechnology*, 8(4), 584-596.
15. Min, J., Zhao, J., Zigelbaum, J., Lee, J., Takahashi, S., Cummings, P., and Gautier, J. (2023). Mechanisms of insertions at a DNA double-strand break. *Molecular Cell*, 83(14), 2434-2448.
16. Liao, H., Wu, J., VanDusen, N. J., Li, Y., & Zheng, Y. (2024). CRISPR-Cas9-mediated homology-directed repair for precise gene editing. *Molecular Therapy Nucleic Acids*, 35(4).
17. Tretbar, U. S., Rurik, J. G., Rustad, E. H., Sürün, D., Köhl, U., Olweus, J., and Blache, U. (2024). Non-viral vectors for chimeric antigen receptor immunotherapy. *Nature Reviews Methods Primers*, 4(1), 74.
18. Lu, M., & Billerbeck, S. (2024). Improving homology-directed repair by small molecule agents for genetic engineering in unconventional yeast? —Learning from the engineering of mammalian systems. *Microbial Biotechnology*, 17(2), e14398.
19. Shakirova, A., Karpov, T., Komarova, Y., & Lepik, K. (2023). In search of an ideal template for therapeutic genome editing: A review of current developments for structure optimization. *Frontiers in Genome Editing*, 5, 1068637.
20. Doudna, J.A. The promise and challenge of therapeutic genome editing. *Nature* **578**, 229–236 (2020). <https://doi.org/10.1038/s41586-020-1978-5>
21. Pacesa, M., Loeff, L., Querques, I. *et al.* R-loop formation and conformational activation mechanisms of Cas9. *Nature* **609**, 191–196 (2022). <https://doi.org/10.1038/s41586-022-05114-0>
22. Li, T., Yang, Y., Qi, H., Cui, W., Zhang, L., Fu, X., and Yu, T. (2023). CRISPR/Cas9 therapeutics: progress and prospects. *Signal Transduction and Targeted Therapy*, 8(1), 36.
23. Pattanayak, V., Lin, S., Guilinger, J. *et al.* High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nat Biotechnol* **31**, 839–843 (2013). <https://doi.org/10.1038/nbt.2673>
24. Guo, C., Ma, X., Gao, F., & Guo, Y. (2023). Off-target effects in CRISPR/Cas9 gene editing. *Frontiers in bioengineering and biotechnology*, 11, 1143157.

25. Boyle EA, Becker WR, Bai HB, Chen JS, Doudna JA, Greenleaf WJ. Quantification of Cas9 binding and cleavage across diverse guide sequences maps landscapes of target engagement. *Sci Adv.* 2021 Feb 19;7(8): eabe5496. doi: 10.1126/sciadv. abe5496. PMID: 33608277; PMCID: PMC7895440.
26. Ding, S., Liu, J., Han, X., & Tang, M. (2023). CRISPR/Cas9-Mediated Genome Editing in Cancer Therapy. *International Journal of Molecular Sciences*, 24(22), 16325.
27. Redelings, B. D., Holmes, I., Lunter, G., Pupko, T., & Anisimova, M. (2024). Insertions and deletions: Computational methods, evolutionary dynamics, and biological applications. *Molecular biology and evolution*, 41(9), msae177.
28. P. Rouet, F. Smih, M. Jasin, Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells., *Proc. Natl. Acad. Sci. U.S.A.* 91 (13) 6064-6068, <https://doi.org/10.1073/pnas.91.13.6064> (1994).
29. Moynahan, M., Jasin, M. Mitotic homologous recombination maintains genomic stability and suppresses tumorigenesis. *Nat Rev Mol Cell Biol* 11, 196–207 (2010). <https://doi.org/10.1038/nrm2851>
30. Lacombe, L. (2024). *CRISPR-Cas9-based strategies for enhanced targeted integration* (Doctoral dissertation, Université Paris-Saclay).
31. Hermantara, R., Richmond, L., Taqi, A. F., Chilaka, S., Jeantet, V., Guerrini, I., and West, A. (2024). Improving CRISPR–Cas9 directed faithful transgene integration outcomes by reducing unwanted random DNA integration. *Journal of Biomedical Science*, 31(1), 32.
32. Wagner, D.L., Amini, L., Wendering, D.J., Burkhardt, L.M., Akyuz, L., Reinke, P., Volk, H.D., and Schmueck-Henneresse, M. (2019). High prevalence of *Streptococcus pyogenes* Cas9-reactive T cells within the adult human population. *Nat. Med.* 25, 242–248.
33. Berdeckaa, D., De Smedta, S. C., De Vosb, W. H., & Braeckmansa, K. (2023). Non-viral delivery of RNA for therapeutic T cell engineering ex vivo. *Towards minimally invasive delivery of large molecules into human T cells by photoporation with polydopamine nanosensitizers and photothermal nanofibers*, 9.
34. Eftekhari, Z., Zohrabi, H., Oghalaie, A., Ebrahimi, T., Shariati, F. S., Behdani, M., & Kazemi-Lomedasht, F. (2024). Advancements and challenges in mRNA and ribonucleoprotein-based therapies: From delivery systems to clinical applications. *Molecular Therapy-Nucleic Acids*.
35. Wang, J., Lin, J., Chen, Y., Liu, J., Zheng, Q., Deng, M., Wang, R., Zhang, Y., Feng, S., Xu, Z., et al. (2023). An ultra-compact promoter drives wide spread neuronal expression in mouse and monkey brains. *Cell Rep.* 42, 113348.
36. Wang, D., Zhang, F., and Gao, G. (2020). CRISPR-Based Therapeutic Genome Editing: Strategies and In Vivo Delivery by AAV Vectors. *Cell* 181, 136–150.
37. Chen, F., Alphonse, M., and Liu, Q. (2020). Strategies for nonviral nano particle-based delivery of CRISPR/Cas9 therapeutics. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* 12, e1609.
38. Rosenblum, D., Gutkin, A., Kedmi, R., Ramishetti, S., Veiga, N., Jacobi, A.M., Schubert, M.S., Friedmann-Morvinski, D., Cohen, Z.R., Behlke, M.A., et al. (2020). CRISPR-Cas9 genome editing using targeted lipid nanoparticles for cancer therapy. *Sci. Adv.* 6, eabc9450.
39. Asmamaw Mengstie, M., Teshome Azezew, M., Asmamaw Dejenie, T., Teshome, A. A., Tadele Admasu, F., Behaile Teklemariam, A., and Abebe, E. C. (2024). Recent Advancements in Reducing the Off-Target Effect of CRISPR-Cas9 Genome Editing. *Biologics: Targets and Therapy*, 21-28.
40. Shumega, A. R., Pavlov, Y. I., Chirinskaite, A. V., Rubel, A. A., Inge-Vechtomov, S. G., & Stepchenkova, E. I. (2024). CRISPR/Cas9 as a Mutagenic Factor. *International journal of molecular sciences*, 25(2), 823.
41. Wiegand, T., Karambelkar, S., Bondy-Denomy, J., and Wiedenheft, B. (2020). Structures and Strategies of Anti-CRISPR-Mediated Immune Suppression. *Annu. Rev. Microbiol.* 74, 21–37.
42. Averina, O. A., Kuznetsova, S. A., Permyakov, O. A., & Sergiev, P. V. (2024). Current knowledge of base editing and prime editing. *Molecular Biology*, 58(4), 571-587.
43. Strohkendl, I., Saifuddin, F. A., Rybarski, J. R., Finkelstein, I. J., & Russell, R. (2018). Kinetic basis for DNA target specificity of CRISPR-Cas12a. *Molecular cell*, 71(5), 816-824.
44. Mendoza, B. J., & Trinh, C. T. (2018). In silico processing of the complete CRISPR-Cas spacer space for identification of PAM sequences. *Biotechnology Journal*, 13(9), 1700595.
45. Karvelis, T., Bigelyte, G., Young, J.K., Hou, Z., Zedaveinyte, R., Budre, K., Paulraj, S., Djukanovic, V., Gasiar, S., Silanskas, A., et al. (2020). PAM recognition by miniature CRISPR–Cas12f nucleases triggers programmable double-stranded DNA target cleavage. *Nucleic Acids Res.* 48, 5016–5023.

46. Amiri, M., Moaveni, A. K., Majidi Zolbin, M., Shademan, B., & Nourazarian, A. (2024). Optimizing cancer treatment: the synergistic potential of CAR-T cell therapy and CRISPR/Cas9. *Frontiers in Immunology*, *15*, 1462697.
47. Cowan, Q. T., Gu, S., Gu, W., Ranzau, B. L., Simonson, T. S., & Komor, A. C. (2024). Development of multiplexed orthogonal base editor (MOBE) systems. *Nature Biotechnology*, 1-15.
48. Acharya, S., Ansari, A. H., Kumar Das, P., Hirano, S., Aich, M., Rauthan, R., and Chakraborty, D. (2024). PAM-flexible Engineered FnCas9 variants for robust and ultra-precise genome editing and diagnostics. *Nature Communications*, *15*(1), 5471.
49. Chen, J.S., Dagdas, Y.S., Kleinstiver, B.P., Welch, M.M., Sousa, A.A., Harrington, L.B., Sternberg, S.H., Joung, J.K., Yildiz, A., and Doudna, J.A. (2017). Enhanced proofreading governs CRISPR-Cas9 targeting accuracy. *Nature* *550*, 407–410.
50. Baranova, S. V., Zhdanova, P. V., Koveshnikova, A. D., Pestryakov, P. E., Vokhtantsev, I. P., Chernonosov, A. A., & Koval, V. V. (2024). Cleavage of DNA Substrate Containing Nucleotide Mismatch in the Complementary Region to sgRNA by Cas9 Endonuclease: Thermodynamic and Structural Features. *International Journal of Molecular Sciences*, *25*(19), 10862.
51. Vakulskas, C.A., Dever, D.P., Rettig, G.R., Turk, R., Jacobi, A.M., Collingwood, M.A., Bode, N.M., McNeill, M.S., Yan, S., Camarena, J., et al. (2018). A high-fidelity Cas9 mutant delivered as a ribonucleoprotein complex enables efficient gene editing in human hematopoietic stem and progenitor cells. *Nat. Med.* *24*, 1216–1224.
52. Schmid-Burgk, J.L., Gao, L., Li, D., Gardner, Z., Strecker, J., Lash, B., and Zhang, F. (2020). Highly parallel profiling of Cas9 variant specificity. *Mol. Cell* *78*, 794–800.e8.
53. Fu, Y., Sander, J.D., Reyon, D., Cascio, V.M., and Joung, J.K. (2014). Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat. Biotechnol.* *32*, 279–284.
54. Reautschnig, P., Fruhner, C., Wahn, N., Wiegand, C. P., Kragness, S., Yung, J. F., and Stafforst, T. (2024). Precise in vivo RNA base editing with a wobble-enhanced circular CLUSTER guide RNA. *Nature biotechnology*, 1-13.
55. Alipanahi, R., Safari, L., & Khanteymooori, A. (2024). DTMP-prime: A deep transformer-based model for predicting prime editing efficiency and PegRNA activity. *Molecular Therapy Nucleic Acids*, *35*(4).
56. Chatterjee, S., Bhattacharya, M., Agoramoorthy, G., & Chakraborty, C. (2024). Different cellular barriers to RNA therapeutics and strategies to overcome. *International Journal of Surgery*, *110*(1), 571-573.
57. Kliuchnikov, E., Maksudov, F., Zuber, J., Hyde, S., Castoreno, A., Waldron, S., and Barsegov, V. (2024). Improving the Potency Prediction for Chemically Modified siRNAs through Insights from Molecular Modeling Focused on Individual Sequence Positions. *Molecular Therapy Nucleic Acids*.
58. Kleinstiver, B.P., Prew, M.S., Tsai, S.Q., Topkar, V.V., Nguyen, N.T., Zheng, Z., Gonzales, A.P.W., Li, Z., Peterson, R.T., Yeh, J.R., et al. (2015). Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature* *523*, 481–485.
59. Sun, L., Zhang, T., Lan, X., Zhang, N., Wang, R., Ma, S., and Xia, Q. (2024). High-Throughput Screening of PAM-Flexible Cas9 Variants for Expanded Genome Editing in the Silkworm (*Bombyx mori*). *Insects*, *15*(4), 241.
60. Hwang, G. H., Kim, S. H., Oh, M., Kim, S., Habib, O., Jang, H. K., and Bae, S. (2024). Detailed mechanisms for unintended large DNA deletions with CRISPR, base editors, and prime editors. *bioRxiv*, 2024-01.
61. Tong, H., Wang, H., Wang, X., Liu, N., Li, G., Wu, D., and Yang, H. (2024). Development of deaminase-free T-to-S base editor and C-to-G base editor by engineered human uracil DNA glycosylase. *Nature Communications*, *15*(1), 4897.
62. Zhao, G., Ma, Q., Yang, H., Jiang, H., Xu, Q., Luo, S., and Lu, Z. (2024). Base editing of the mutated TERT promoter inhibits liver tumor growth. *Hepatology*, *79*(6), 1310-1323.
63. Liu, Z., Chen, S., Davis, A. E., Lo, C. H., Wang, Q., Li, T., and Sun, Y. (2024). Efficient Rescue of Retinal Degeneration in Pde6a Mice by Engineered Base Editing and Prime Editing. *Advanced Science*, *11*(42), 2405628.
64. Chen, L., Hong, M., Luan, C., Gao, H., Ru, G., Guo, X., and Li, D. (2024). Adenine transversion editors enable precise, efficient A• T-to-C• G base editing in mammalian cells and embryos. *Nature biotechnology*, *42*(4), 638-650.
65. Alharbi, S., Al-Dakhil, M., & Alotaibi, F. S. (2024). Precise Genome Editing of Plants Through Base and Prime Editor. *OMICs-based Techniques for Global Food Security*.

66. Fiumara, M., Ferrari, S., Omer-Javed, A., Beretta, S., Albano, L., Canarutto, D., Varesi, A., Gaddoni, C., Brombin, C., Cugnata, F., et al. (2023). Genotoxic effects of base and prime editing in human hematopoietic stem cells. Published online September 7, 2023. *Nat. Biotechnol.*
67. Mikhaylova, E. V., Kuluev, B. R., Gerashchenkov, G. A., Chemeris, D. A., Garafutdinov, R. R., Kuluev, A. R., and Chemeris, A. V. (2024). Prime-Editing Methods and pegRNA Design Programs. *Molecular Biology*, 58(1), 17-32.
68. Vu, T. V., Nguyen, N. T., Kim, J., Hong, J. C., & Kim, J. Y. (2024). Prime editing: Mechanism insight and recent applications in plants. *Plant Biotechnology Journal*, 22(1), 19-36.
69. Ferreira da Silva, J., Tou, C. J., King, E. M., Eller, M. L., Rufino-Ramos, D., Ma, L., and Kleinstiver, B. P. (2024). Click editing enables programmable genome writing using DNA polymerases and HUH endonucleases. *Nature Biotechnology*, 1-13.
70. Allemailem, K. S., Almatroudi, A., Alrumaihi, F., Alradhi, A. E., Theyab, A., Algahtani, M., and Khan, A. A. (2024). Current updates of CRISPR/Cas system and anti-CRISPR proteins: innovative applications to improve the genome editing strategies. *International Journal of Nanomedicine*, 10185-10212.
71. Schlosshauer, J. J., Shamloo, S., Schamrin, K., & Imig, J. (2024). Dual CRISPR-Interference Strategy for Targeting Synthetic Lethal Interactions between Non-Coding RNAs in Cancer Cells. *bioRxiv*, 2024-11.
72. Chen, R., Shi, X., Yao, X., Gao, T., Huang, G., Ning, D., and Chen, W. (2024). Specific multivalent molecules boost CRISPR-mediated transcriptional activation. *Nature Communications*, 15(1), 7222.
73. Huang, M., Wang, K., Li, A., Zhu, X., Zhou, Z., Yang, C., and Zhang, X. (2024). Mini and enhanced CRISPR activators for cancer therapies. *Journal of Advanced Research*.
74. Villiger, L., Joung, J., Koblan, L., Weissman, J., Abudayyeh, O. O., & Gootenberg, J. S. (2024). CRISPR technologies for genome, epigenome and transcriptome editing. *Nature Reviews Molecular Cell Biology*, 1-24.
75. Hsieh, S. C., & Peters, J. E. (2024). Natural and Engineered Guide RNA-directed Transposition with CRISPR-Associated Tn7-like Transposons. *Annual Review of Biochemistry*, 93.
76. Chen, Y., Luo, S., Hu, Y., Mao, B., Wang, X., Lu, Z., and Li, W. (2024). All-RNA-mediated targeted gene integration in mammalian cells with rationally engineered R2 retrotransposons. *Cell*, 187(17), 4674-4689.
77. Tang, N., & Ji, Q. (2024). Miniature CRISPR-Cas12 systems: Mechanisms, engineering, and genome editing applications. *ACS Chemical Biology*, 19(7), 1399-1408.
78. Halperin, S.O., Tou, C.J., Wong, E.B., Modavi, C., Schaffer, D.V., and Dueber, J.E. (2018). CRISPR-guided DNA polymerases enable diversification of all nucleotides in a tunable window. *Nature* 560, 248–252.
79. Chang, C. W., Truong, V. A., Pham, N. N., & Hu, Y. C. (2024). RNA-guided genome engineering: paradigm shift towards transposons. *Trends in Biotechnology*.
80. daSilva, J.F., Tou, C.J., King, E.M., Eller, M.L., Ma, L., Rufino-Ramos, D., and Kleinstiver, B.P. (2023). Click editing enables programmable genome writing using DNA polymerases and HUH endonucleases. Preprint at bioRxiv.
81. Ochmann, M. T., Miskey, C., Botezatu, L., Sandoval-Villegas, N., Diem, T., & Ivics, Z. (2024). A novel hyperactive variant of the Sleeping Beauty transposase facilitates non-viral genome engineering. *Molecular Therapy Nucleic Acids*, 35(4).
82. Hsieh, S. C., & Peters, J. E. (2024). Natural and Engineered Guide RNA-directed Transposition with CRISPR-Associated Tn7-like Transposons. *Annual Review of Biochemistry*, 93.
83. Durrant, M.G., Fanton, A., Tycko, J., Hinks, M., Chandrasekaran, S.S., Perry, N.T., Schaepe, J., Du, P.P., Lotfy, P., Bassik, M.C., et al. (2023). Systematic discovery of recombinases for efficient integration of large DNA sequences into the human genome. *Nat. Biotechnol.* 41, 488–499.
84. Lee, R. J., Horton, C. A., Van Treeck, B., McIntyre, J. J., & Collins, K. (2024). Conserved and divergent DNA recognition specificities and functions of R2 retrotransposon N-terminal domains. *Cell Reports*, 43(5).
85. Tang, S., and Sternberg, S.H. (2023). Genome editing with retroelements. *Science* 382, 370–371.
86. Dolken, L., Ruzsics, Z., Radde, B., Friedel, C.C., Zimmer, R., Mages, J., Hoffmann, R., Dickinson, P., Forster, T., Ghazal, P., et al. (2008). High resolution gene expression profiling

- for simultaneous kinetic parameter analysis of RNA synthesis and decay. *RNA* 14, 1959–1972.
87. Hong, T., Luo, Q., Ma, H., Wang, X., Li, X., Shen, C., and Tang, X. (2024). Structural basis of negative regulation of CRISPR-Cas7-11 by TPR-CHAT. *Signal Transduction and Targeted Therapy*, 9(1), 111.
 88. Borrajo, J., Javanmardi, K., Griffin, J., St Martin, S.J.St., Yao, D., Hill, K., Blainey, P.C., and Al-Shayeb, B. (2023). Programmable multi-kilobase RNA editing using CRISPR-mediated trans-splicing. Preprint at bioRxiv.
 89. Gao, P. (2024). Advancements in Lipid Nanoparticle Technologies for Precision Targeting in Therapeutic Delivery. *Precision Nanomedicine*, 7(3), 1318-1330.
 90. de Moraes, C. C. P. D. L., Correia, E. M., Bonamino, M. H., & Vasconcelos, Z. F. M. D. (2024). Cell-Penetrating Peptides and CRISPR-Cas9: A Combined Strategy for Human Genetic Disease Therapy. *Human Gene Therapy*, 35(19-20), 781-797.
 91. Kreitz, J., Friedrich, M.J., Guru, A., Lash, B., Saito, M., Macrae, R.K., and Zhang, F. (2023). Programmable protein delivery with a bacterial contractile injection system. *Nature* 616, 357–364.
 92. Sharma, R., & Malviya, R. (2024). Virus-like particles for disease diagnosis and drug delivery applications. *Current Nanoscience*, 20(5), 613-629.
 93. Segel, M., Lash, B., Song, J., Ladha, A., Liu, C.C., Jin, X., Mekhedov, S.L., Macrae, R.K., Koonin, E.V., and Zhang, F. (2021). Mammalian retro virus-like protein PEG10 packages its own mRNA and can be pseudo typed for mRNA delivery. *Science* 373, 882–889.
 94. Wang, J.Y.J., Khmelinskaia, A., Sheffler, W., Miranda, M.C., Antanasi jevic, A., Borst, A.J., Torres, S.V., Shu, C., Hsia, Y., Nattermann, U., et al. (2023). Improving the secretion of designed protein assemblies through negative design of cryptic transmembrane domains. *Proc. Natl. Acad. Sci. USA* 120. e2214556120.
 95. Hassija, V., Chamola, V., Mahapatra, A., Singal, A., Goel, D., Huang, K. and Hussain, A. (2024). Interpreting black-box models: a review on explainable artificial intelligence. *Cognitive Computation*, 16(1), 45-74.