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Review

Past, present, and future of CRISPR genome editing technologies

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SUMMARY

Genome editing has been a transformative force in the life sciences and human medicine, offering unprecedented opportunities to dissect complex biological processes and treat the underlying causes of many genetic diseases. CRISPR-based technologies, with their remarkable efficiency and easy programmability, stand at the forefront of this revolution. In this Review, we discuss the current state of CRISPR gene editing technologies in both research and therapy, highlighting limitations that constrain them and the technological innovations that have been developed in recent years to address them. Additionally, we examine and summarize the current landscape of gene editing applications in the context of human health and therapeutics. Finally, we outline potential future developments that could shape gene editing technologies and their applications in the coming years.

INTRODUCTION

Genome editing—the precise and targeted modification of the genetic material of living organisms—represents one of the most significant advancements in molecular biology. It has far-reaching applications, from unraveling fundamental biological processes to driving advancements in medicine, agriculture, and biotechnology. With the approval of the first CRISPR-based human therapy in late 2023,[1] CRISPR genome editing is entering a new era. In this review, we aim to provide a panoramic view of the CRISPR genome editing landscape, emphasizing its current state, potential future developments, and the hurdles that must be overcome to fully realize its promise for human medicine.

THE PAST: DEVELOPMENT AND LIMITATIONS OF CRISPR GENOME EDITING

Historical background

Genome editing originally arose from advancements in the field of eukaryotic DNA repair. Pioneering studies in the early 1990s using homing endonucleases such as I-SceI, which recognizes 18-bp DNA sequences, showed that induction of a targeted double-strand break (DSB) in mammalian cells stimulated homologous recombination at the target site.[2] This established the concept of genome editing using DSB-generating nucleases. The need for diverse nucleases with long recognition sites, which would be capable of targeting single sites in eukaryotic genomes, spurred the subsequent development of engineered nuclease enzymes based on complex fusions of non-specific DNA endonucleases with tandem arrays of sequence-specific DNA binding modules. These were initially zinc-finger nucleases (ZFNs), introduced in the early 2000s, and later transcription activator-like effector nucleases (TALENs) in 2010–2011.[3,4] These developments set the stage for genome editing, but the laborious design and generation of ZFNs and TALENs limited their use. The discovery and development of RNA-guided, CRISPR-associated (Cas) nucleases marked a revolutionary shift due to their simplicity of programming, specificity, and versatility.[5]

In 2007, prokaryotic CRISPR-Cas systems were shown to function as adaptive genome defense mechanisms that recognize and target foreign nucleic acids associated with viruses (phages) and other mobile genetic elements.[6] In these systems, fragments of invader DNA are acquired and stored in repetitive arrays, whose transcription and subsequent processing yields CRISPR RNAs (crRNAs). crRNAs function as molecular guides and program molecular machineries composed of Cas proteins to recognize invading nucleic acids and target them for destruction.[7] Subsequent research revealed that so-called type II CRISPR-Cas systems site-specifically cleaved phage DNA,[8] that the activity was dependent on the protein Cas9,[9] and that an additional RNA component, *trans*-activating crRNA (tracrRNA), was essential for crRNA maturation.[10] Finally, biochemical studies published in 2012 demonstrated that Cas9 functioned as a DNA cleaving endonuclease[11,12] whose specificity was determined by a dual-RNA guide structure composed of crRNA and tracrRNA.[12] The CRISPR-Cas9 system was further streamlined by integrating tracrRNA and crRNA into a single guide RNA (sgRNA), thus providing a fully programmable one-nuclease-one-guide-RNA design.[12] This became the basis for

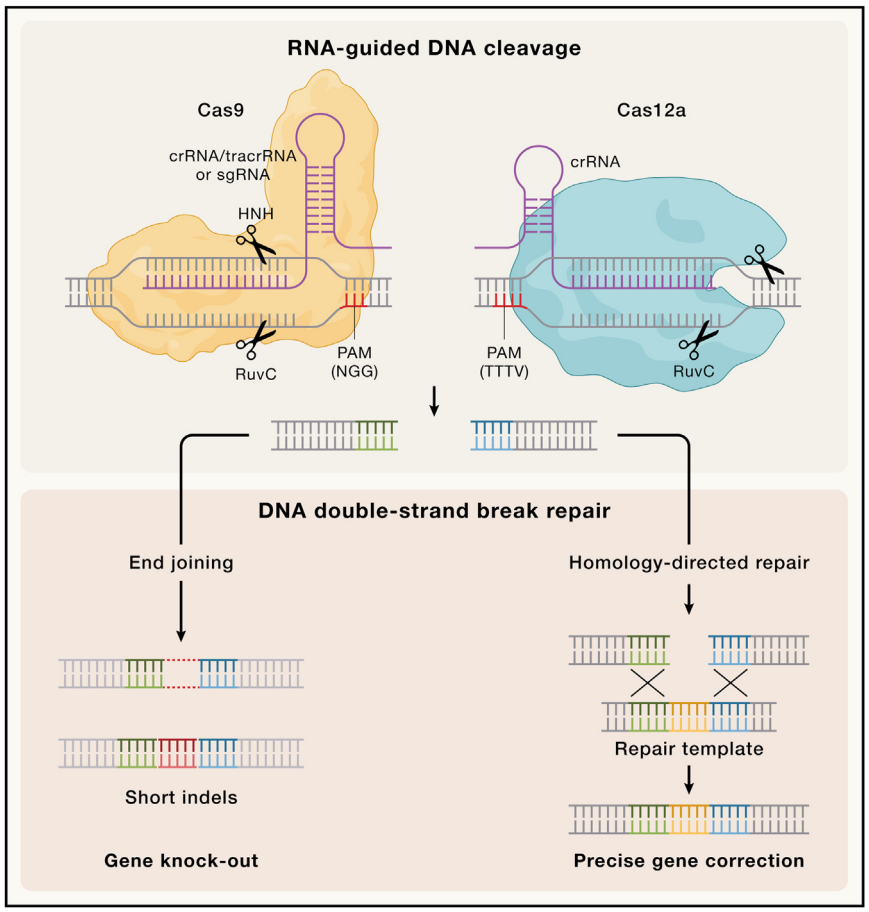

Figure 1. Molecular principles of CRISPR genome editing

CRISPR genome editing relies on RNA-guided nucleases such as Cas9 and Cas12a for site-specific target DNA recognition and cleavage. Cas9 utilizes a dual-guide RNA composed of a CRISPR RNA (crRNA)-*trans*-activating CRISPR RNA (tracrRNA) pair or a single-guide RNA (sgRNA), whereas Cas12a is programmed with a crRNA only. Target DNA recognition is dependent on complementarity with the spacer sequence of the guide RNA as well as the presence of a protospacer adjacent motif (PAM). Cas9 recognizes an NGG PAM, whereas Cas12a requires a TTTV PAM (V = G, C, or A). Upon target binding, the nucleases catalyze DNA cleavage, generating a DNA double-strand break (DSB). DSB repair by cellular DNA repair pathways leads to the introduction of genetic modifications (edits). The end-joining pathways result in short insertions or deletions (indels), whereas homology-directed repair (HDR) using an exogenous DNA repair template can be used to engineer precise modifications.

the development of CRISPR genome editing technologies, whereby targeting Cas9 to a specific genomic site could thus be achieved by designing an sgRNA with a matching sequence.[12] The discovery of the RNA-guided DNA cutting activity of Cas9 set off a race to repurpose it for genome editing, which culminated in several studies published in early 2013 reporting that expression of Cas9 and specific sgRNAs in eukaryotic cells led to the introduction of genetic modification at target genomic loci.[13–17]

CRISPR nuclease-based genome editing

The programmability of CRISPR-Cas nucleases to generate site-specific double-strand DNA breaks has enabled their rapid adaptation for genome editing technologies (Figure 1). The archetypical Cas9 protein originating from *Streptococcus pyogenes* (SpCas9), the first Cas nuclease to be repurposed for genome editing, remains the most widely used gene editor due to its intrinsically high activity and specificity.[12,18] Cas9 forms an active nuclease in association with either crRNA-tracrRNA complexes or sgRNA guides.[11,12] To direct the Cas9 nuclease to the genomic locus of interest, the 20-nt guide sequence on the 5′ end of the crRNA can be altered to enable canonical base pairing with the DNA target. Target binding is additionally dependent on the presence of a short protospacer adjacent motif (PAM) located on the non-target strand (NTS) of the DNA, immediately downstream of the target site. Initial recognition of the PAM results in local unwinding of the target DNA, whereas the guide RNA base pairs with the target strand (TS) of the DNA in a 5′-3′ directional manner starting at the PAM-proximal end of the target site, triggering conformational changes in Cas9 that lead to nuclease domain activation.[19–21] Cas9 subsequently cleaves the double-stranded DNA (dsDNA) substrate three nucleotides upstream of the PAM sequence, generating DSBs with either blunt ends or single-nucleotide 5′ overhangs.[12] DSB formation is catalyzed by the Cas9 HNH and RuvC domains, which cleave the TS and NTS, respectively.[11,12] Selective inactivation of either nuclease domain converts Cas9 into RNA-guided nickases, whereas inactivation of both domains results in an RNA-guided DNA binding protein that can serve as a platform for delivery of fused proteins to specific genomic loci.[5,12]

Cas12a, a Cas nuclease originating from type V CRISPR-Cas systems, was discovered a few years after Cas9 and likewise repurposed for genome editing.[22] In contrast to Cas9, Cas12a does not require a tracrRNA for activation and instead catalyzes nucleolytic processing of its own guides by recognizing a conserved pseudoknot structure in the repeat-derived segment of the crRNA,[23,24] a feature that has been exploited for multiplexed editing *in vivo*.[25,26] Cas12a targets DNAs containing a 5′-terminal TTTV PAM and cleaves both strands within the PAM-distal part of the target site in a sequential manner using its single RuvC domain catalytic site, which results in the generation of 5-nt 5′ overhangs.[22,27] The PAM-distal DSB product then dissociates from the protein, whereas Cas12a remains in a catalytically active state, able to cleave additional single-stranded DNA (ssDNA) substrates in *trans*.[27,28] Cas12a has proved to be a highly efficient nuclease capable of precise gene editing, with complementary properties and functionality to Cas9.[25,29,30] The *trans*-nuclease activity of Cas12a has

additionally been utilized for sequence-specific nucleic acid detection.[28,31]

Conventional genome editing approaches rely on the introduction of site-specific double-strand DNA breaks in the genome and their subsequent resolution by endogenous cellular DNA repair pathways (Figure 1). DSBs generated by Cas9 or Cas12a enzymes are generally repaired by end-joining pathways, which are typically error-prone, or by precise homology-directed repair (HDR) mechanisms.[32,33] End-joining is the predominant mode of DNA repair in mammalian cells and relies on the direct religation of broken DNA ends by the non-homologous end joining (NHEJ) or microhomology-mediated end joining (MMEJ) pathways.[32,33] Processing of the exposed DNA ends before religation leads to the addition or removal of nucleotides, resulting in short insertions or deletions (collectively termed indels) at the site of the DSB, an outcome thought to be facilitated by repeated cleavage of precisely repaired DSBs until accumulated indels preclude further cleavage.[34] This is most commonly used to selectively disrupt protein-coding gene sequences to achieve gene knockouts, or gene deletions, by the simultaneous introduction of two DSBs in close proximity.[13–16] Editing outcomes resulting from end-joining repair of Cas9-induced DSBs are reproducible and depend on the local sequence context, comprising single-nucleotide insertions or small deletions due to NHEJ, as well as MMEJ-mediated deletions.[35–37]

Conversely, HDR is a precise DSB repair pathway that relies on the presence of a homologous DNA molecule to guide the outcome of the repair.[32] By exogenously providing an artificial homology repair template, HDR can be exploited to introduce desired mutations, insertions, or deletions precisely within the targeted genomic locus. The repair templates, delivered either as double-stranded DNAs (typically via plasmids or viral vectors) or synthetic single-strand DNA oligonucleotides (ssODNs), carry the desired mutation flanked by sequences homologous to regions on either side of the DSB. Although this approach in principle enables editing with nucleotide precision, HDR is mostly active only in actively dividing cells, as it requires repair factors that are commonly expressed only in the S and G2 phases of the cell cycle.[32] The efficiency of the HDR outcome thus depends on the type of repair template, the delivery method, cell type, local chromatin context, and other factors that can affect DNA repair pathway choice to preferentially enhance HDR and suppress end-joining repair.[33,38]

Limitations of CRISPR genome editing

The repurposing of CRISPR-Cas systems as simple and effective programmable gene editing tools has greatly advanced many areas of basic and applied research, setting the stage for the development of targeted gene therapies and various biotechnological applications.[39,40] However, the functional features of a highly evolved biological defense system differ from the functionalities expected from a precise genome editing tool. Consequently, the application potential of first-generation CRISPR-based gene editing tools is limited by several key factors, the principal ones being specificity, targeting scope, and the need to rely on endogenous DSB repair mechanisms to achieve genomic edits (Figure 2). Finally, the delivery of CRISPR components is limited by specific constraints of the delivery vectors and target cells or organisms.

Off-target activity

Natural CRISPR-Cas systems tolerate, to a certain degree, mismatches between the guide RNA and the target, a likely evolutionary consequence of the need to counter the high mutational rate of phages. This property is, however, undesired for genome engineering applications, as it may result in the targeting and editing of partially complementary off-target sites elsewhere in the genome in addition to the intended, on-target locus. The off-target activity of Cas9 has been documented by numerous studies showing that the enzyme tolerates a considerable number and variety of nucleotide mismatches within the guide-target heteroduplex in a guide-dependent manner.[41–50] Off-targets can range from sites harboring a single base mismatch to targets containing multiple consecutive mismatches, or even nucleotide insertions or deletions.[45–51] Despite the mismatch tolerance of Cas9, the majority of potential off-target sites are merely bound and do not result in dsDNA cleavage and editing due to intrinsic checkpoints in the DNA binding and cleavage mechanism of Cas9.[20,42–44,48,50,52] Moreover, off-target profiling studies have shown that the frequency of off-target cleavage events is consistently lower *in vivo* as compared with isolated genomic DNA, suggesting that additional factors, including genome structure, might govern the editing activity of Cas9 in cells.[47,53] Nevertheless, simultaneous off-target cleavage at multiple sites within the genome can ultimately result in genomic rearrangements such as deletions, inversions, or chromosomal translocations and trigger DNA damage and stress response pathways.[54–56] Off-target editing remains a major concern for therapeutic applications and has prompted major efforts to develop robust and sensitive methods for the prediction and detection of off-target edits and to improve the specificity of CRISPR genome editors by molecular engineering.[45,47,57,58]

Targeting scope: PAM requirements

The DNA-binding mechanism of CRISPR nucleases restricts their targeting scope to genomic target sites flanked by a PAM sequence. Although the NGG PAM of SpCas9, the most widely used genome editor nuclease, theoretically permits finding a suitable target site every 8 nucleotides on average, some genomic regions are not easily targetable by SpCas9 due to a high A/T content. A number of naturally occurring Cas9 orthologs with alternative PAM specificities have been identified and adopted for genome editing; however, many of these have even more restrictive PAM requirements.[59–61] Although this provides greater specificity of targeting[19,62] and reduces off-target activity,[60,63] it often results in suboptimal DNA cleavage and editing efficiencies. Cas12a nucleases recognize T-rich PAM sequences,[22,27] typically TTTV, but their targeting scope is likewise restricted. To overcome the limitation of PAM requirements, a number of artificial Cas9 variants with modified or relaxed PAM specificities have been developed in recent years, as outlined in the next section. Although these greatly expand the range of targetable sites, relaxed PAM targeting is associated with a strong decrease in targeting specificity,[64] increasing the likelihood of off-target effects and reduced on-target editing efficiency due to sequestration at off-target sites.

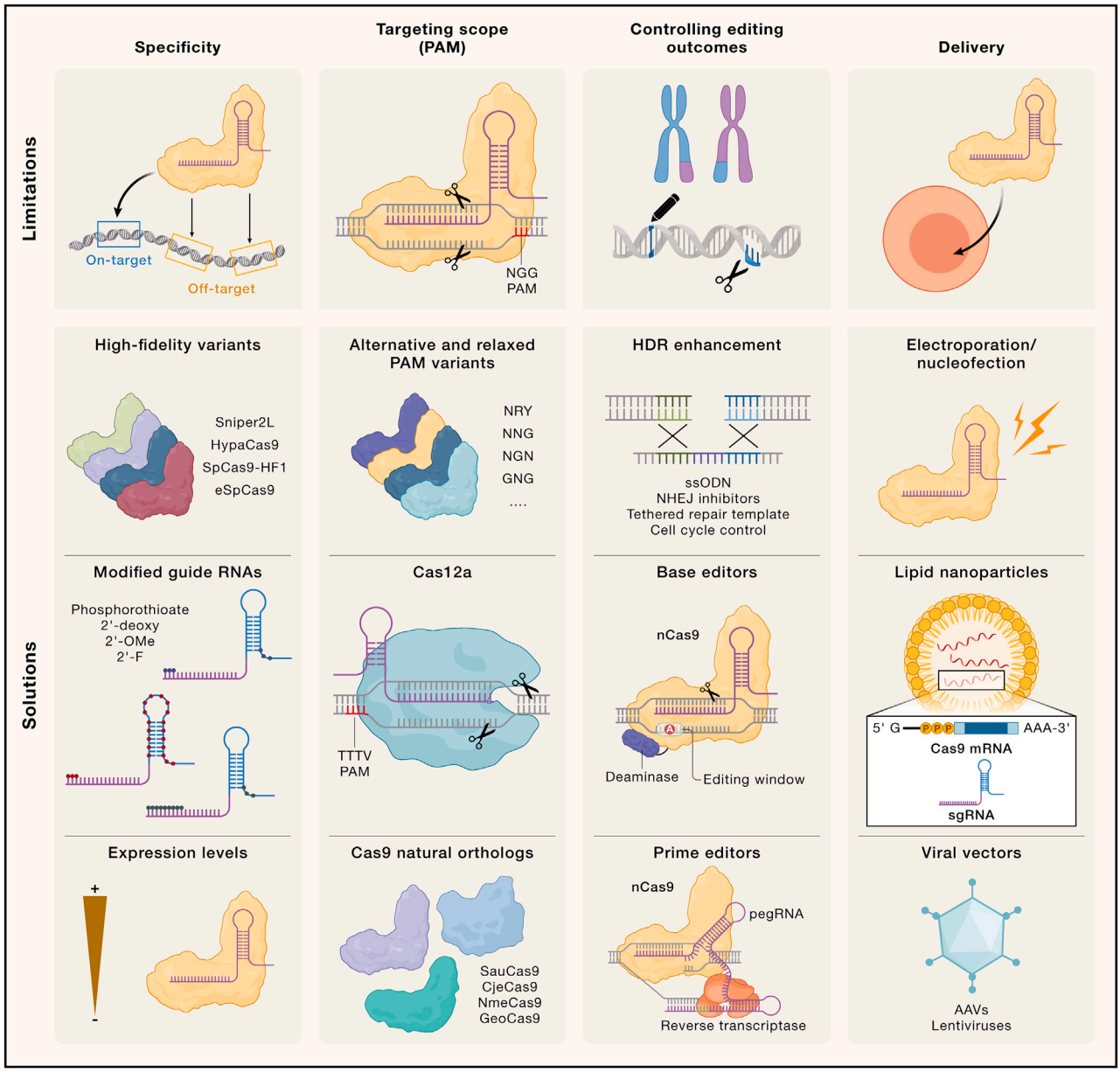

Figure 2. Limitations of CRISPR genome editing

CRISPR genome editing faces four principal limitations, each addressed by specific technological solutions. Specificity: off-target activities of genome editors have been addressed by the development of high-fidelity nuclease variants, chemically modified guide RNAs, and controlled expression of genome editor nucleases. Targeting scope: the NGG PAM sequence requirement of SpCas9 restricts the scope of targetable genomic sites. This is addressed using engineered variants of Cas9 with alternative or relaxed PAM requirements, other naturally derived Cas9 orthologs with alternative PAM requirements, and Cas12a enzymes. Control of editing outcomes: various approaches, including asymmetric or tethered HDR repair templates, cell cycle synchronization, and NHEJ inhibitors, have been developed to enhance the efficiency of HDR and suppress the formation of indels by end-joining pathways. Second-generation technologies such as base or prime editing enable the introduction of precise modifications independently of HDR. Delivery: cellular delivery of genome editor components is facilitated by electroporation/nucleofection, lipid nanoparticles, and viral vectors.

Controlling editing outcomes

The generation of DSBs within the genome using targeted nucleases significantly enhances the rate of HDR in mammalian cells.[65] Despite this, the use of HDR is restricted to dividing cells and often results in heteroallelic editing outcomes due to simultaneous editing resulting from end joining pathways.[66,67] Moreover, the precision of editing at the intended genomic target site is limited by other adverse outcomes, including large deletions and chromosomal rearrangements and even chromosome loss.[54,68,69] As a result, much research has been dedicated to developing approaches to control DNA repair outcomes, particularly to stimulate the rate of HDR repair and thus increase the

efficiency of knockin (insertion) mutations.[70] Most importantly, it has been demonstrated that the choice of repair template and its delivery greatly affects the efficiency of HDR.[38,70] Methods to enhance HDR include the use of asymmetric ssODN templates,[71] introduction of silent mutations to block recurrent cleavage at the target site,[72] or the tethering of the repair donor template to the break site.[73,74] Furthermore, manipulation of the cell cycle combined with the delivery of pre-assembled Cas9-ribonucleoparticles also results in enhanced HDR.[75] Cell-cycle synchronization can be achieved by using small molecule inhibitors for temporarily slowing down the S phase[76] or by increasing the proportion of cells in the G2/M phase.[77] Other methods focus on shifting the balance between NHEJ and HDR pathway choice toward HDR by suppressing key NHEJ factors.[78,79] Analogously, direct association of Cas9 with repair factors involved in HDR, such as Rad51, Rad52, or Mre11, can substantially improve the rates of knockin mutations.[80–82] In primary human T cells, HDR editing efficiencies exceeding 80%–90% could be achieved with engineered ssDNA repair templates designed to form dsDNA ends recognizable by Cas9-RNPs, used in conjunction with small-molecule DNA repair modulators.[83] Finally, recent studies have shown that the efficiency of HDR can be improved by retargeting of NHEJ editing byproducts using secondary guide RNAs.[84,85] Despite these advances, the process of introducing knockin mutations, particularly long insertions, using homology templates remains challenging. Post-mitotic, terminally differentiated cells such as neurons, in which HDR does not occur at appreciable levels,[33,86] thus remain largely recalcitrant to precise editing using canonical DSB-based strategies. These constraints, together with the potential genotoxic effects of DSBs, have motivated the development of genome editing technologies that do not rely on DSB and obviate the need for HDR, including base editing and prime editing, and most recently CRISPR-based recombinases and transposases.

Delivery

The targeted delivery of gene editors remains the limiting factor for most *in vivo* and *ex vivo* gene editing applications. In particular, safe, specific, and efficient delivery of CRISPR components to targeted cells is a prerequisite for successful therapeutic genome editing. Furthermore, the immunogenicity of CRISPR components and their delivery vectors presents a concern for *in vivo* therapeutic applications.[87] Pre-existing anti-Cas9 antibodies and reactive T cells have been identified in humans,[88,89] and Cas9 immunity has been associated with compromised therapeutic outcomes in canine and non-human primate disease models.[90,91] Several strategies for overcoming pre-existing immunity have been proposed, for example, engineering Cas9 to eliminate immunogenic epitopes, modulating the immune responses, as well as limiting the duration of Cas9 expression.[39,88,92]

Cas9/Cas12a enzymes and their guide RNAs can be delivered in multiple formats, depending on the target cell type or organism. For most *in vitro* (i.e., *ex vivo*) applications in cultured cells, electroporation (nucleofection) or liposome-mediated transfection remain the most widely used delivery modality due to their high efficiencies.[93] Cas9 and guide RNA components can be delivered as RNA, plasmid DNA, or *in vitro* reconstituted ribonucleoprotein (RNP) complexes. Transient RNP-based delivery has become the preferred choice of gene editing for *ex vivo* therapeutic applications, as long-term expression of the Cas9 complex from a plasmid can result in high rates of off-target editing and random plasmid integration.[94] For germline genome editing in many model organisms, Cas9 RNPs or mRNA are typically delivered via microinjection or electroporation.[95–97]

In vivo delivery of CRISPR-Cas9 into mammalian cells is commonly achieved using viral vectors. Adenoviruses, lentiviruses, and adeno-associated viruses (AAVs) can all be engineered to replace the viral genes within the vector with gene editing modules.[98] AAVs remain the preferred vectors for *in vivo* delivery due to their low immunogenicity, high transduction efficiency, and diverse cell tropism.[98] However, AAVs are relatively small viruses with limited (~4.7 kb) cargo packaging capacity.[98] As a result, it is difficult to package the genes encoding SpCas9 (4.2 kb) and its sgRNA (~100 nt) into a single AAV vector unless ultracompact promoters are used.[98,99] To overcome this limitation, there has been much focus on adapting smaller Cas9 orthologs[59–61,63,100] and compact Cas12-family enzymes.[101–105]

Lastly, Cas9 mRNA in combination with synthetic guide RNAs, or *in vitro* reconstituted RNP particles, can be delivered into cells *in vivo* using non-viral approaches such as lipid-based nanoparticles (LNPs).[106,107] The advantage of these methods is that they are generally safer and exhibit lower immunogenicity than viral-based vectors. LNPs are internalized by endocytosis upon which the LNP contents either escape the endosome and are transferred into the nucleus, or they are degraded by the lysosome, limiting their efficiency.[108] New LNP formulations have recently emerged that result in improved efficiency of tissue-specific and organ targeting.[106,109] Altogether, the delivery of Cas9 and other genome editors using lipid nanoparticles has been successfully applied in a variety of cell types and organisms.[106,107]

THE PRESENT: CURRENT TECHNOLOGIES AND THEIR APPLICATIONS

Since the first demonstration of CRISPR-based gene editing, the field has evolved at an unprecedented pace. The capabilities of first-generation DSB-dependent genome editors based on Cas9 and Cas12a nucleases have been enhanced by continuous innovations that have not only increased the versatility of these tools but have also refined their precision and minimized unintended editing consequences. However, concerns about their safety persist, both due to off-target editing activity and potential genotoxic effects of on-target DSBs,[43,54] including the induction of p53.[56] To reduce the occurrence of unintended edits, a number of approaches have been explored for precise spatiotemporal control of CRISPR genome editors,[110,111] for example, the use of naturally occurring anti-CRISPR protein inhibitors for tissue-restricted editing.[112,113] Concerns about the genotoxicity of DSBs and the need to address the low efficiency of HDR have furthermore prompted the development of "second-generation" CRISPR technologies that mediate genome editing without relying on DBS formation and HDR—notably base editors (BEs) and prime editors (PEs). The current, expanded landscape of available technologies (Figure 3) thus offers a much more tailored approach to genome editing, with specific technologies particularly suited for certain types of edits or delivery modalities.

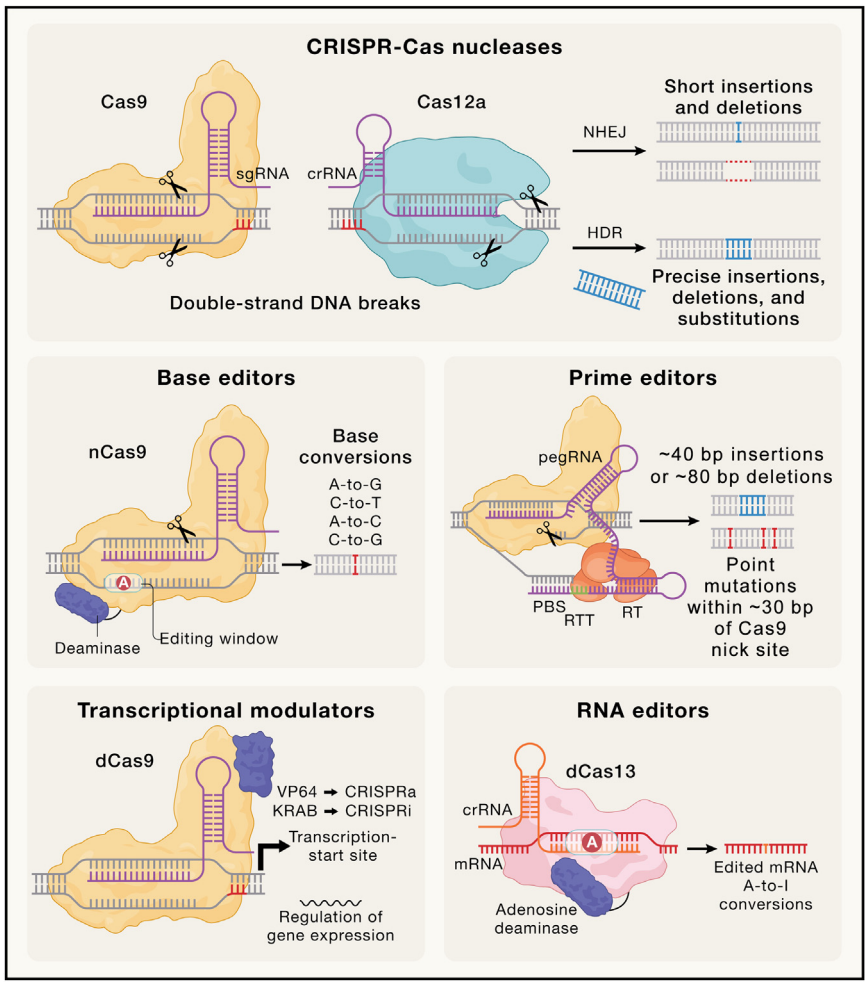

Figure 3. Current CRISPR editing technologies

An overview of currently utilized genome editor technologies based on CRISPR-associated nucleases and their derivatives. DSB-based genome editing: Cas9 and Cas12a nucleases enable efficient gene knockouts and provide limited capability to generate HDR-mediated knockin edits. Base editing: this approach employs a fusion of a Cas9 nickase (nCas9) with nucleobase modifying enzymes. Base editors enable the direct conversion of a single-nucleotide base into another without the need for double-strand breaks. This approach is particularly effective for introducing specific point mutations (A-to-G or C-to-T, and also A-to-C or C-to-G), enabling precise gene correction or the introduction of stop codons for precise gene knockouts. Prime editing: this technology combines a Cas9 nickase with a reverse transcriptase (RT) and uses a prime editing guide RNA (pegRNA) consisting of a Cas9 sgRNA fused to an RT template (RTT) and a primer-binding site (PBS). Nicking the non-target DNA strand enables its extension by RT upon hybridization to the PBS in the pegRNA, thereby copying the RTT sequence into the target locus. Prime editing enables the insertion, deletion, and replacement of short DNA sequences up to several tens of nucleotides. Transcriptional modulators: these tools enable RNA-guided control of gene transcription by targeting a deactivated Cas9 (dCas9) fused with transcriptional modulation domains (such as VP64 or KRAB) to gene promoters. RNA editors: diverging from genome editing, RNA editors utilize RNA-targeting Cas13 nucleases, either for targeted transcript degradation (when catalytically active) or for transcript editing (when rendered catalytically inactive and fused to adenosine deaminases).

Although these additions to the genome editing toolkit have significantly contributed to addressing the many constraints associated with canonical CRISPR genome editing, they nevertheless still have limits on their activities, specificity, and delivery. This section outlines how current technologies have emerged, which limitations they have helped to mitigate, and what remaining constraints they still face.

Nucleases beyond Cas9

The initial development of Cas9 as a genome editor nuclease motivated follow-up studies aiming to identify new naturally occurring Cas enzymes with potential applications in genome engineering. Thanks to these efforts, more than a dozen of new evolutionarily diverse RNA-guided nucleases have been discovered and adopted for genome editing in recent years.[22,101,114,115] The discovery of Cas12a in 2015 marked a pivotal expansion beyond Cas9, offering an alternative nuclease with a distinct PAM requirement, guide RNA format, and DNA cleavage pattern.[22,27] Cas12a enzymes have been reported to exhibit higher specificity and lower off-target activity *in vivo*, in part due to their slower rates of DNA cleavage.[116] Subsequent bioinformatic explorations have unearthed a wide array of type V Cas effectors with distinct PAM and guide RNA requirements.[101,105,114,117,118] Of particular interest are "minimal," compact type V nucleases that hold promise for applications in which packaging the gene editor components into size-limited viral vectors (such as AAV) is necessary.[104,105,115,119,120] Finally, RNA-targeting RNA-guided nucleases such as Cas13,[114,121] Cas12a2,[122,123] and Cas12g[101] have introduced a new modality to the molecular editing toolbox, allowing target degradation or editing of mRNAs,[124–126] and nucleic acid detection.[31,127,128] Although these discoveries have brought about new capabilities, each Cas nuclease comes with its own set of limitations, including restricted PAM targeting, variable *in vivo* activities and off-target profiles, or potential immunogenicity. Nevertheless, the availability of multiple enzymes now permits taking a more tailored approach to genome editing, enabling flexibility for various applications. The orthogonality of many of these enzymes with respect to their guide RNAs is particularly useful for multiplexing applications.

High-fidelity Cas9 variants

To address the issue of off-target activity, engineered variants of SpCas9 with improved specificity have been developed using two complementary approaches. The first involves

structure-based rational design of fidelity-enhancing mutations, based on the idea that eliminating specific contacts between the Cas9 protein and bound DNA target makes the Cas9-guide RNA complex more sensitive to mismatches in the substrate DNA and thus reduces the probability of off-target binding and cleavage.[129–131] Biophysical and biochemical studies of these variants have revealed that the mutations substantially slow down the DNA cleavage rate, thereby promoting off-target release.[131,132] The second approach utilizes directed evolution methods to select for mutations that reduce off-target editing.[133–135] Similar efforts have been made to engineer high-fidelity variants of other Cas9 and Cas12a enzymes. Although the high-fidelity variants developed to date offer considerably increased specificities over wild-type enzymes, their efficiencies can vary across different DNA targets and applications.[136,137] Moreover, as each target is associated with a unique set of off-targets with variable editing frequencies,[48,49,137] none of the currently available high-fidelity nuclease variants is likely to be universally applicable.

Guide RNA modifications

Engineering the guide RNAs of genome editors to increase specificity offers a compelling alternative to employing high-fidelity nuclease mutants. The first of such efforts focused on the use of truncated guide RNAs for Cas9, in which the guide segment is truncated from 20 to 17–18 nucleotides.[138] These have been shown to significantly reduce the off-target activity of SpCas9 at a variety of sites[45,138] but also exhibit decreased efficiency or trigger editing at new off-target sites.[45,130] 5′ end modifications of the guide segment, either through the introduction of secondary structures[139] or unpaired nucleotides,[140] have also been shown to mitigate off-target recognition. Several studies have demonstrated the functionality of "hybrid" RNA-DNA guides and found that 2′-deoxynucleotide substitutions within the guide segment substantially increase the specificity of gene editing in cells.[141–144] The introduction of other chemical modifications such as 2′-O-methyl or 2′-fluoro nucleotides and phosphorothioate linkages within the guide RNA has also emerged as a powerful strategy to increase Cas9 specificity and enhance guide stability.[145–147] Some of these modifications have shown robust gene editing activity *in vivo*, but the effects of the modified nucleotides are highly position-dependent, similarly to DNA substitutions.[143,145–147]

Alternative PAM genome editors

To overcome the PAM-dependent target site constraints of Cas nucleases, several studies have sought to expand the PAM targeting scope of SpCas9 either by structure-based rational engineering or directed evolution to introduce specificity-altering amino acid substitutions in the PAM-interacting domain. This initially led to the development of VQR, EQR, and VRER SpCas9 variants capable of targeting NGAN, NGNG, and NGCG PAMs, respectively.[148–150] The PAM selectivity of SpCas9 has been further relaxed in additional engineered variants targeting non-G PAMs.[151] To make an even a wider range of genomic sites accessible to CRISPR-Cas9, recent efforts succeeded in engineering SpCas9 variants capable of NRN PAM targeting,[152–154] including nearly "PAM-less" Cas9 designs.[154,155] Although the *in vivo* editing activity of these variants is highly variable, they have significantly expanded the targeting potential of Cas9 and simplified target site selection, complementing wild-type SpCas9 and other naturally derived Cas9 orthologs. Similarly, Cas12a variants with relaxed PAM specificities have also been developed for editing applications.[156,157]

Base editing

CRISPR-derived base editors (BEs) have been developed as a versatile technology to generate targeted point mutations without the need for generating DSBs and providing homology repair templates, thereby enabling editing in HDR-deficient cells.[158,159] BEs are modular fusions of a RuvC-inactivated nickase version of Cas9 with a nucleotide deaminase enzyme.[160] Initially, two classes of BEs were developed. Cytosine BEs (CBEs), which contain catalytic domains derived from cytidine deaminases such as APOBEC1, as well as an uracil glycosylase inhibitor (UGI) domain, mediate C-to-T conversion.[159] In turn, adenine BEs (ABEs) generate A-to-G conversions using an adenosine deaminase domain from the tRNA-specific deaminase TadA that has been engineered by directed evolution to act on ssDNA.[158] Upon binding of the Cas9 module, BEs deaminate a cytosine or adenine within an "editing window" in the PAM-distal segment of the displaced non-target DNA strand to uracil or inosine, respectively. These are read out during DNA replication as thymine and guanine, respectively, inducing transition point mutations. Since their invention, the original CBE and ABE editors have gone through several design iterations to improve activity and reduce the amount of deaminase-induced off-target edits,[161–164] and Cas12a BEs have also been developed.[165] The base editing repertoire has also been expanded to also cover A-to-C,[166] A-to-Y,[167] and C-to-G[168] transversions. Due to their largely predictable editing outcomes, BEs have been applied for genome-wide knockout and mutational screens.[169] The precision of BEs makes them suitable for therapeutic corrections of diseases caused by single-point mutations.[170–172] Although BEs allow for more precise control over editing outcomes, they do suffer from several limitations. These include limited efficiency, bystander editing, broad editing windows, and substantial off-target activity.[173–175] Latest-generation ABE variants exhibit higher editing efficiencies and lower frequencies of Cas9-independent off-target editing than CBEs,[161,175–177] which has prompted the engineering of new CBE variants by directed evolution of ABEs.[163,164,178] Finally, both ABEs and CBEs can have undesired genotoxic effects by generating DSBs, deletions, and translocations at the on-target locus, albeit at lower frequencies than canonical nuclease-based Cas9 editing.[173] These can be partially mitigated by modulating delivery timing and editor expression levels.[173]

Prime editing

Prime editing is a Cas9-based approach developed to generate targeted point mutations, as well as insertions or deletions in an HDR-independent manner.[179] The prime editor consists of a prime editing guide RNA (pegRNA) and a fusion protein construct composed of Cas9 nickase with an inactivated HNH domain and an engineered reverse transcriptase (RT) domain.[179] The pegRNA contains a 3′-terminal sequence extension that is

complementary to the NTS of the intended genomic target and contains the desired mutation(s). Cas9 generates a nick in the NTS, which then base pairs with the complementary pegRNA extension.[179] The mutation is then introduced by RT-catalyzed extension of the 3′ end of the NTS using the pegRNA as a template.[179] This is followed by reannealing of the DNA strands to produce a 5′ flap intermediate that undergoes excision and ligation, fixing the edit in the genomic DNA. Such targeted strand synthesis allows the introduction of insertions of up to ~40 bp or deletions of up to ~80 bp in length, as well as point mutations as far as ~30 bp from the Cas9 nicking site.[179] Starting from the first-generation prime editor design, in which a nickase Cas9 was fused with a wild-type RT from the Moloney murine leukemia virus (MMLV), subsequent PE design generations brought improvements in prime editing efficiency by including engineered MMLV RT domains with enhanced thermostability and introducing a second sgRNA that to generate a nick on the non-edited strand to promote retention of the edit in the genomic DNA.[179] Additional improvements have been achieved by inhibiting the DNA mismatch repair pathway, as well as by improving nuclear localization, expression, and DNA nicking.[180,181] Furthermore, the addition of stabilizing secondary structures to the 3′ end of the pegRNA, which counteracts its degradation, has been demonstrated to enhance prime editing efficiency.[182] To date, prime editing has been successfully applied in a variety of organisms and cell types.[183–186] However, depending on the intended edit, target site sequence, and cell type, prime editing efficiencies are highly variable and often low.[180] Additionally, prime editing does not entirely avoid the generation of on-target DSBs, resulting in unintended and potentially genotoxic effects.[173] To address these limitations, novel PE variants with improved performance continue to be developed,[187–190] including systems that enable the generation of more extensive edits using pegRNA pairs.[191–195],

Transcriptional modulation: CRISPRi and CRISPRa

CRISPR technologies are not only applicable for genome editing but have also enabled transient manipulation of gene expression. Catalytically inactive mutants of Cas9 were initially used in bacteria to target gene promoters and sterically block RNA polymerases, thereby inhibiting RNA transcription.[196] To suppress gene expression in eukaryotic cells, nuclease-inactive Cas9 can be fused to various transcriptional and epigenetic modulators, for example, the KRAB transcriptional repressor domain,[197,198] and targeted to promoter regions of actively transcribed genes. The resulting approach, known as CRISPR interference (CRISPRi), enables efficient knockdowns of gene expression as an alternative to small interfering RNA (siRNA)-based RNA interference. Analogously, nuclease-dead Cas9 protein fusions can be used to activate the expression of specific genes, either by directly recruiting transcriptional activation factors or by modulating the chromatin state. CRISPR activation (CRISPRa) has been achieved by fusing dCas9 to transactivation domains such as VP64 and its derivatives.[199–201] Alternatively, fusions with histone-modifying enzymes, such as demethylases or acetylases, can site-specifically alter the epigenetic markers at a particular site and induce active chromatin states to drive gene expression.[202,203] Such approaches have been readily adapted for genome-wide loss-of-function (CRISPRi) or gain-of-function (CRISPRa) screening.[204] However, as DNA binding by Cas9 is generally more promiscuous than cleavage,[42,48,50] CRISPR-directed transcriptional modulation suffers from considerable off-target activity.

Targeted RNA silencing and modifications

The discovery of RNA-guided, ssRNA-targeting Cas13 enzymes from type VI CRISPR-Cas systems[114] has led to the development of molecular tools for targeted RNA silencing, as well as editing and modification.[124,126] As RNA edits are transient, transcriptome editing potentially offers an attractive alternative to genome editing in certain contexts. For instance, in acute diseases like pain, inflammation, or viral infections, transient RNA editing could provide temporary therapeutic effects without introducing permanent genetic changes.[205] Current RNA editing approaches are based on fusing nuclease-inactive dCas13 proteins with adenosine deaminase acting on RNA (ADAR) enzymes to catalyze adenosine deamination within target RNA molecules, generating an inosine base.[206] The hybridization of the Cas13 guide RNA with the target RNA produces a dsRNA substrate for the ADAR enzyme. The resulting inosine can effectively base pair with a cytosine and is therefore read out as a guanosine during translation, resulting in the introduction of codon changes into mRNA equivalent to an A-to-G conversion.[207] Subsequent protein engineering efforts have resulted in the development of an ADAR2 variant capable of Cas13-guided C-to-U conversion in RNA.[125] The concept of targeted RNA modification using dCas13 protein fusions has been further extended to develop "epitranscriptomic editors" capable of site-specifically installing the N6-methyladenosine (m6A) modification in target mRNAs.[208]

Current applications in basic research and human medicine

The technological advancements in CRISPR genome editing have led to a host of applications with significant potential to enhance human health, spanning from advancements in fundamental research to the development of new therapeutic treatments (Figure 4). Firstly, CRISPR has transformed genetic research by allowing scientists to mimic disease-causing mutations in various experimental models, create large-scale genome-wide screening methods, and to develop synthetic genetic recording devices for studying normal development and disease progression. Although not reviewed here in detail, CRISPR systems have been repurposed to develop molecular diagnostics enabling specific, rapid, and sensitive detection of viral DNA or RNA.[209] CRISPR technologies have also been used to establish strategies for the elimination of viral or bacterial human pathogens, the latter through the development of engineered bacteriophages.[210,211] A specific example of limiting pathogen spread is CRISPR-based gene drives, in which particular suppressive traits, such as female sterility, are introduced to collapse pathogen-harboring populations of insects, primarily malaria-transmitting mosquitoes.[212] Finally, the past decade of CRISPR genome editing has culminated in the development of a multitude of therapeutic approaches for genetic diseases, several of which have moved from preclinical studies in cell-based and animal models into human clinical trials. This includes both *in vivo* and *ex vivo* therapeutic correction

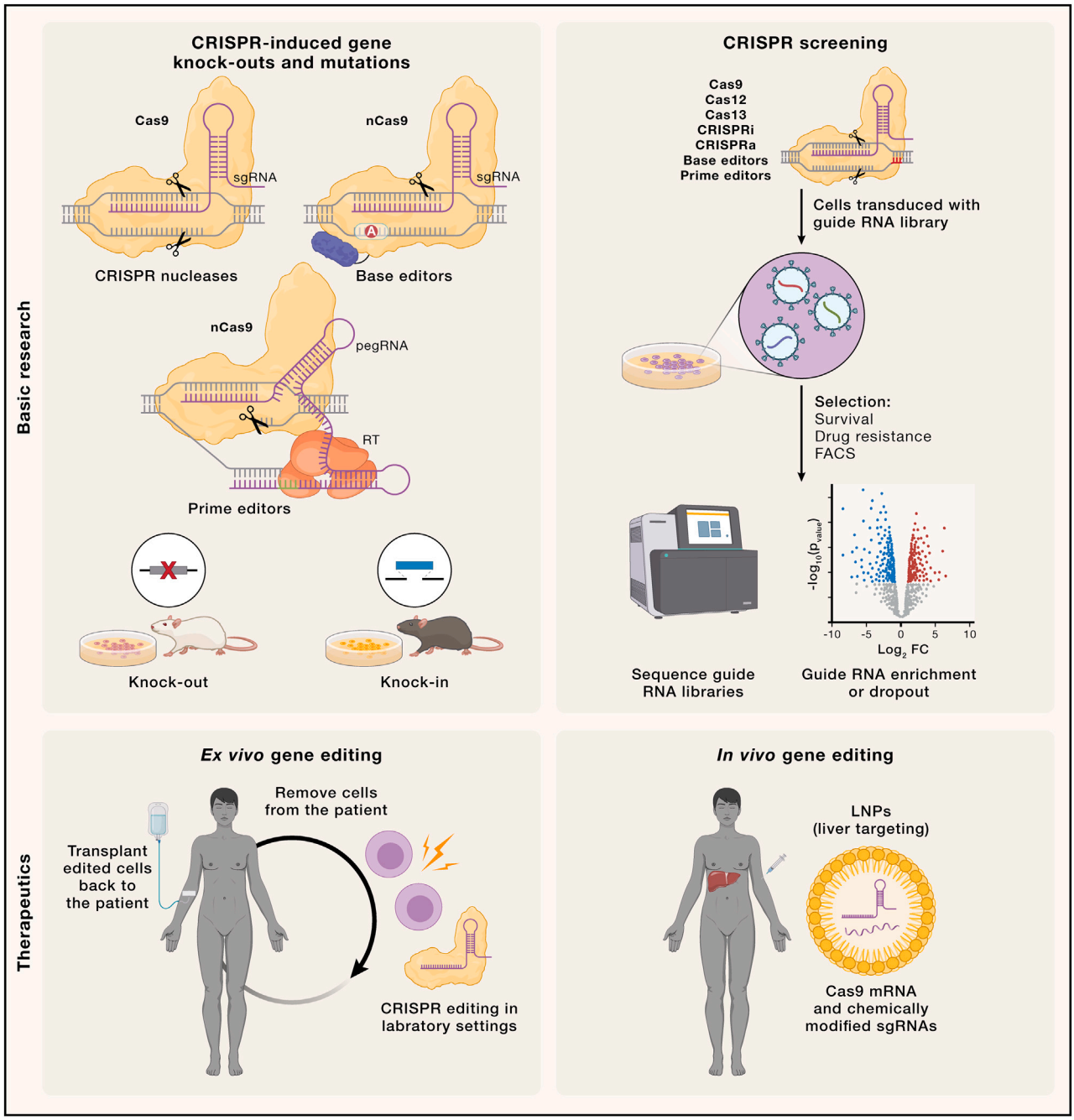

Figure 4. Applications of CRISPR genome editors relevant to human health

An overview of CRISPR-based genome editing applications in basic research and gene therapies. CRISPR-induced gene knockouts or mutations: CRISPR nucleases, base editors, and prime editors facilitate the generation of specific genetic alterations, including gene knockouts, knockins, and targeted mutations in cultured cell lines and animals, enabling the modeling of human genetic disease variants. CRISPR screens: these applications involve the use of guide RNA libraries for high-throughput gene function analysis. Cells transduced with these libraries are subjected to selection-based assays, and next-generation sequencing is used to analyze guide RNA enrichment or depletion in the cell population, thus identifying genes involved in a specific biological process. CRISPR screens can be conducted using CRISPR nucleases, CRISPRi/a transcriptional modulators, as well as base and prime editors. *Ex vivo* therapeutic gene editing: these therapeutic approaches involve CRISPR editing of cells derived from patients or healthy donors in a controlled laboratory environment. The modified cells are then (re)introduced into patients. *In vivo* therapeutic genome editing: this approach involves direct delivery of CRISPR genome editors into patients, targeting specific organs or tissues, typically using methods based on lipid nanoparticles or viral vectors.

strategies. *In vivo* therapeutic correction approaches involve delivery of gene editing components to the affected tissues inside the human body.[213] By contrast, *ex vivo* approaches involve collecting cells from a patient, editing them in a laboratory, followed by transplantation of edited cells back into the patient. *Ex vivo* CRISPR editing has furthermore enabled the generation of autologous and allogeneic genome-modified cell therapeutics, primarily intended for cancer immunotherapy.[214]

Reverse genetics: Disease modeling in cells, organoids, and animals

Identifying the specific alleles that cause human disorders is a key focus in genetic research. Next-generation sequencing technologies have advanced our ability to pinpoint mutations that may be responsible for these conditions. However, to conclusively determine that a specific variant is causative for a genetic disease, experimental validation through reverse genetic approaches is essential.[215] In particular, CRISPR genome editing has been instrumental for the generation of isogenic cell-based disease models, enabling researchers to introduce specific mutations into wild-type cells or, conversely, revert mutations in patient-derived cells to wild type to generate control cell lines. Shortly after demonstrating the genome editing activity of Cas9 in human cells,[13–15] CRISPR genome editing gained widespread use in research focused on modeling disease phenotypes in patient-derived cells,[216] organoids,[217] or model organisms.[218,219] Human induced pluripotent stem cells (iPSCs) offer a powerful method for modeling disease phenotypes. By editing these cells and guiding them to differentiate into the specific cell types affected by a disease, researchers can investigate disorders in relevant cellular contexts.[216] CRISPR-based iPSC models are especially valuable for diseases that impact otherwise inaccessible tissues or organs, such as the brain.[220,221] CRISPR-engineered organoids, which can mimic the complexity of disease-affected organs,[217] have been used for modeling cancers by engineering mutations in tumor suppressor genes or oncogenes.[222] CRISPR-engineered model organisms, including *C. elegans* and zebrafish, are also useful for establishing links between specific mutations and human genetic diseases.[219] These models have proven particularly successful in replicating rare human developmental disorders.[223–225] Rodents, primarily mice, are the most commonly used animal disease models in which CRISPR editing has facilitated the generation of multiplexed or conditional gene knockouts or knockins.[226,227]

Larger animal models, including non-human primates, pigs, and dogs, exhibit greater physiological and genetic resemblance to humans. Their extended lifespans make them particularly valuable for studying aging and the progression of chronic disorders, offering a closer approximation to human pathologies over time.[218,228] CRISPR-engineered non-human primates have provided deeper insights into developmental processes.[229] Similarly, human cardiovascular diseases have been successfully modeled in CRISPR-edited pigs[230] or dogs.[231] Moreover, the ability to generate rodent and large animal models is crucial beyond the validation of disease-relevant variants and understanding disease mechanisms, as these models provide a vital platform for the preclinical evaluation of therapeutic interventions aimed at curing such conditions.[218] CRISPR genome editing enables generation of large animal models without the need for maintaining large breeding populations, with substantial economic and animal welfare benefits. A major advantage of CRISPR genome editing is the possibility to engineer multiplexed edits by targeting several genomic loci simultaneously using distinct guide RNAs.[232] This approach has enabled the generation of animal models harboring more than one gene mutation[227,233] and multiplexed inactivation of endogenous retroviruses by targeting up to 62 unique genetic sites in a single cell using distinct sgRNA sequences.[234] Beyond modeling mutations that cause diseases, multiplexed genome editing using CRISPR has also transformed other areas of biotechnology, notably plant engineering for agricultural applications and metabolic pathway engineering in microorganisms for industrial bioproduction.[232,235,236]

Forward genetics: CRISPR screens, lineage tracing, and molecular recorders

Forward genetic approaches such as genome-wide knockout screens are powerful tools for investigating biological functions in health and disease. Leveraging ongoing advancements in genome editing, CRISPR screens enable systematic perturbation of thousands of individual genes and non-coding genomic elements within cells, facilitating the identification of genes associated with specific biological pathways and their interactions. In these high-throughput screens, guide RNA libraries along with Cas enzymes are delivered into cells, typically by transduction with a pool of lentiviral vectors, ensuring that each cell receives a distinct sgRNA.[237] Upon applying cell survival- or readout-based selection, next-generation sequencing is used to identify which guide RNAs are enriched or conversely dropped from the cell population, thus identifying the target genes linked to the specific phenotype. This general approach has been adapted to use diverse types of CRISPR technologies, including CRISPR nucleases for knockout screens,[46,238,239] CRISPRi,[204,240] CRISPRa,[204,241] BEs,[242] PEs,[243] as well as Cas13,[244] allowing for a wide range of genetic modifications to be studied. Early pooled CRISPR screens were designed to identify genes that contribute to easily selectable phenotypes like cell growth or drug resistance.[245,246] Multiplexed CRISPR screens have also been utilized to discover pairwise genetic interactions that suppress the growth of cancer cells.[247–249] Furthermore, *in vivo* pooled CRISPR screens have also been performed in zebrafish embryos, inactivating individual genes and isolating embryos with targeted phenotypes to identify the responsible genetic alterations.[250] BEs, which enable efficient introduction of point mutations, have become particularly powerful tools for both loss- and gain-of-function genetic variant screening.[169,251]

The integration of CRISPR screens with single-cell omics methodologies has greatly enhanced our ability to study gene function.[237] Methods like PERTURB-seq,[252] CRISP-seq,[239] CROP-seq,[238] and Mosaic-seq[253] use single-cell RNA sequencing to track sgRNAs in individual cells and simultaneously monitor the full spectrum of gene expression changes after CRISPR-induced perturbations. Further refinements in these methodologies have enabled direct investigation of gene function *in vivo*, such as analyzing genes associated with neurodevelopmental disorders in the mouse brain.[240] Additionally, these methods have been combined with spatially resolved single-cell RNA sequencing to dissect the intricate interactions between cancer and immune cells in tumors,[254] or linked with single-cell mass spectrometry for detailed protein expression profiling.[255]

Beyond genome-wide screens, CRISPR genome editing tools have also facilitated the development of lineage-tracing approaches to enable monitoring of cell proliferation and the reconstruction of cell lineage trees.[256] Lineage tracing is achieved by introducing artificial DNA barcodes into cells and their continuous editing by Cas9 over time, which is detected using single-cell RNA sequencing to map cell phylogenies and to integrate them with single-cell transcriptomic outputs.[257,258] For example, these approaches have enabled successful reconstitution of cell lineages during zebrafish development[259,260] and mouse embryogenesis,[261] and tracked the evolution and dynamics of metastatic cancer cells.[262,263] CRISPR genome editing has enabled the development of synthetic memory devices to record information of transient molecular events into DNA barcodes.[264] This includes information on past transcriptional history,[265–267] the activity of specific cis-regulatory elements,[268] or exposure to certain environmental factors.[269,270] Such molecular recorders function by linking a particular molecular event to the generation of a unique, detectable genome-encoded edit, facilitating the tracking of the presence, intensity, duration, and relative timing of these events.[264,271] In bacteria, transcriptional recording based on a CRISPR spacer acquisition machinery containing a RT enables direct, chronological capture of sequences from transcribed RNA in the genome.[266] The application of this technology has been extended to *in vivo* studies of the gut microbiome in mice.[267]

Ex vivo *therapeutic applications*

CRISPR-based genome editing of patient-derived cells *ex vivo*, followed by their expansion and retransplantation, is a powerful strategy for treating genetic disorders manifested in blood cells. This is exemplified by successful efforts to develop treatments for genetic hemoglobinopathies, the first of which has now been approved in Europe, the UK, and the USA. Sickle cell disease (SCD) and transfusion-dependent β-thalassemia (TDT) result from a range of mutations in the hemoglobin subunit β (HBB).[272,273] Although these mutations could in principle be address by corrective genome editing, reactivating fetal γ-globin (HBG) expression, which is normally silenced after birth by the transcriptional repressor BCL11A, offers a more tractable solution.[274] This has been achieved by targeted disruption of an erythroid enhancer in the BCL11A gene in CD34+ hematopoietic stem cells by Cas9 RNP electroporation.[275]

Chimeric antigen receptor (CAR) T cell therapies involve *ex vivo* engineering of T cells to target cancer cell markers like CD19 or B cell maturation antigen (BCMA) in B cell malignancies and multiple myeloma, respectively, thus destroying the cancer cells following infusion into patients.[214] In currently approved autologous CAR-T cell therapies, the CAR-encoding gene is randomly inserted in the genome by transduction with a lentiviral vector. Moreover, the cells require a logistically complex, failure-prone manufacturing process. To address these limitations, allogeneic CAR-T cells generated by CRISPR editing of donor-derived T cells could offer enhanced functionality, streamlined production, and enhanced quality control.[276] CRISPR-directed insertion of the CAR construct at the T cell receptor α constant (TRAC) locus using HDR avoids graft-versus-host disease,[277] whereas knockouts of PDCD1, Regnase-1, and TGFBR2 genes are designed to reduce CAR-T cell exhaustion and increase persistence.[276,278,279] Several clinical trials are currently underway for CRISPR-edited CAR-T cell therapies that target a panel of blood cancers. In a notable example, multiplexed base-edited CAR-T cells have been used to treat relapsed T cell leukemia, a previously incurable condition, in childhood patients.[280]

A potentially powerful strategy for future genome therapies combines *ex vivo* CRISPR genome editing with iPSC technologies. A recently developed approach designed for managing type 1 diabetes involves genome engineering iPSCs, differentiating them into pancreatic endoderm cells, and encapsulating within biocompatible devices for implantation in patients to enable insulin production, which is vital for managing type 1 diabetes.[281] Within these complex protocols, CRISPR editing could be used to delete a set of genes to promote graft acceptance, to protect engineered cells from stress, and to optimize insulin production.[213] CRISPR gene editing is also being used in animal xenotransplantation studies. For example, targeted genetic modifications that prevent immune rejection in pig lung xenografts have improved graft acceptance in non-human primate recipients.[282]

In vivo *therapeutic applications*

Contrasting with *ex vivo* strategies, *in vivo* therapeutic gene editing involves direct administration of CRISPR genome editors to affected tissues in the body via localized or systemic delivery.[213] A number of proof-of-principle preclinical studies have already successfully demonstrated therapeutic genome editing *in vivo*, notably exemplified by efforts to restore dystrophin protein expression in animal models of Duchenne muscular dystrophy by muscle-directed delivery of Cas9 and guide RNAs using AAV9 vectors,[283–285] and rescue spinal muscular atrophy in a mouse model of the disease by AAV9-mediated delivery of CBE into the brain.[171] Another early approach involves direct injection of CRISPR components into the eye to mitigate Leber congenital amaurosis type 10, a retinal dystrophy caused by pathogenic mutations in the CEP290 gene. In this case, Cas9, driven by tissue-specific promoters for precision, was packaged alongside guide RNAs within AAV5 vectors and delivered to the affected area via sub-retinal injection to restore vision in mice and non-human primates.[286]

The liver is an excellent target for *in vivo* gene editing due to its discontinuous capillary structures that facilitate uptake of systemically administered lipid nanoparticles through the low-density lipoprotein (LDL) receptor pathway. Transthyretin (TTR) amyloidosis is a disease caused by harmful buildup of misfolded TTR in the heart or the nervous system. As TTR is predominantly produced in the liver, an effective therapeutic strategy for TTR amyloidosis has used LNP-mediated delivery of Cas9 mRNA and synthetic sgRNAs to knock out this gene in the liver. In a clinical trial, this approach demonstrated significant reduction of serum TTR levels,[287] the first successful case of a systemically delivered *in vivo* CRISPR gene therapy.

Clinical trials are also exploring treatments for familial hypercholesterolemia, a condition characterized by high blood LDL cholesterol levels. One of the innovative approaches involves LNP-based systemic delivery of CRISPR ABEs to target the proprotein convertase subtilisin/kexin type 9 (PCSK9) gene in the liver, aiming to lower blood LDL cholesterol by knocking out this gene.[288] In non-human primates, this approach has significantly decreased PCSK9 levels and serum LDL cholesterol

without affecting germline tissues,[90,170,288] setting the stage for phase I clinical trials. An enhanced therapeutic strategy uses encapsulation of ABE mRNA together with PCSK9-specific sgRNAs within LNPs that are uniquely formulated with multi-valent N-acetylgalactosamine (GalNAc) ligands, improving uptake by liver cells in patients with deficient LDL receptors.[289]

THE FUTURE: EMERGING TECHNOLOGIES

As the limitations of current CRISPR technologies have become increasingly clear over the past decade, novel approaches and methodologies continue to be developed and fine-tuned to address these constraints and improve the efficacy and versatility of CRISPR-based genome editing. These emergent, third-generation tools and technologies (Figure 5) include recently discovered classes of compact RNA-guided nucleases that have been adapted for DSB-based editing and could also serve as RNA-guided DNA binding platforms for other genome editor modalities such as BEs and PEs. The insertion of long, gene-sized DNA sequences, particularly in post-mitotic cells lacking HDR, remains a major unmet need in the genome editing field. In this context, the development of CRISPR-guided recombinases and transposons presents a promising and potentially powerful avenue to fill this technology gap. New approaches have also emerged for genome editing technologies based on retrotransposons and for editing RNA transcripts. Finally, the creation of new genome editor tools continues to go hand in hand with advances in the development of delivery methods, which represent a major challenge for therapeutic applications. Overall, these developments reflect the dynamic of the rapidly advancing genome editing field, where each new method offers complementary strengths to address the diverse needs and challenges of genetic manipulations.

Minimal ancestral RNA-guided nucleases

The ongoing search for novel RNA-guided nucleases is largely motivated by the need for alternative enzymes with orthogonal guide RNA scaffolds and targeting capabilities, ideally with a minimal size that would facilitate the construction of fusion proteins (such as BEs and PEs) and their efficient cellular delivery using viral vectors. This is exemplified by recent development and engineering of the ultracompact Cas12f nuclease (422 amino acids) to enhance its *in vivo* editing activity by deep mutational scanning and structure-guided design.[290] The molecular genome editing toolkit has also recently expanded thanks to ongoing studies aiming to discover new naturally occurring RNA-guided nucleases.[117,291] These efforts have focused on the evolutionary ancestors of Cas9 and Cas12 enzymes, namely IscB/IsrB[117] and TnpB,[291] respectively. These proteins, also collectively referred to as HEARO (HNH endonuclease-associated RNA and ORF) or OMEGA (obligate mobile element guided activity) nucleases, are encoded within prokaryotic transposable elements in which they contribute to the transposition mechanism and promote transposon retention.[292] Both IscB and TnpB nucleases have been demonstrated to mediate programmable genome editing in human cells.[117,291] The discovery of eukaryotic TnpB-like proteins termed Fanzors[293–295] (~400–700 amino acids), which have also been shown to catalyze RNA-guided DNA cleavage and support programmable genome editing, has highlighted the universality of RNA-guided nucleases in all domains of life. The compact size of these nucleases should facilitate their delivery; however, further molecular engineering efforts will be needed to increase their currently low efficiency and limited targeting scope.

DNA polymerase editors

Unlike PEs, in which a RT is fused to a Cas9 nickase to introduce modifications at the target site RNA template using part of the guide RNA as a template, novel approaches are being explored that utilize DNA polymerases to introduce targeted mutations into the genome. In an early attempt, continuous diversification of nucleotides within a tunable window of up to 350 nucleotides from a target site was achieved using an engineered error-prone DNA polymerase fused to a Cas9 nickase variant.[296] A more recent study demonstrated that a phage-derived DNA polymerase (provided in *trans*) can introduce edits at a Cas9-nicked site using a tethered linear DNA template.[297] In contrast to RT-based prime editing, this approach avoids autoinhibitory intramolecular base pairing within the guide RNA and enables longer insertions of over 100 nucleotides. Another novel approach, termed click editing, combines Cas9 with DNA-dependent DNA polymerases (DDPs) and HUH endonucleases to enable the introduction of diverse genome edits, including all single-nucleotide substitutions, as well as short insertions and deletions.[298] The process exploits the bioconjugation activity of HUH endonucleases to covalently attach "click DNA" templates to a HUH-nCas9-DDP protein fusion. This approach not only facilitates precise genome editing with minimal indels but also avoids unintended insertions. DNA-polymerase-based editing technologies stand out for their potential to induce a wide spectrum of genetic alterations, offering a high degree of control and diversity of outcomes.

CRISPR-guided recombinases and transposons

Genome editing relying on the template-directed repair of dsDNA breaks is largely restricted to proliferating cells in which HDR is active and does not function efficiently in post-mitotic cells.[299] Moreover, the efficiency of HDR-mediated insertions inversely scales with insert size, severely limiting the ability to inserting long DNA fragments or entire new genes into the genome. Although prime editing facilitates the introduction of insertions without relying on HDR, the inserts are currently restricted to a few tens of nucleotides.[179] The capabilities of PEs have recently been extended by methods that combine them with serine recombinases/integrases, whereby prime editor-mediated installation of a recombinase recognition site enables subsequent insertion of multi-kilobase DNA sequences by the recombinase.[188,191,300] The systematic discovery and characterization of novel recombinases will facilitate further optimization of these methods to improve their efficiency and specificity.[301]

Transposons are capable of autonomously catalyzing the insertion of large DNA insertion independent of DSB generation and repair. Several studies have attempted to fuse nuclease-inactive Cas9 to various transposases, including Sleeping Beauty, mariner, and piggyBac, for RNA-mediated transposition in cells.[302–305] Although these approaches enhance the frequency of transposition events in the vicinity of the target site,

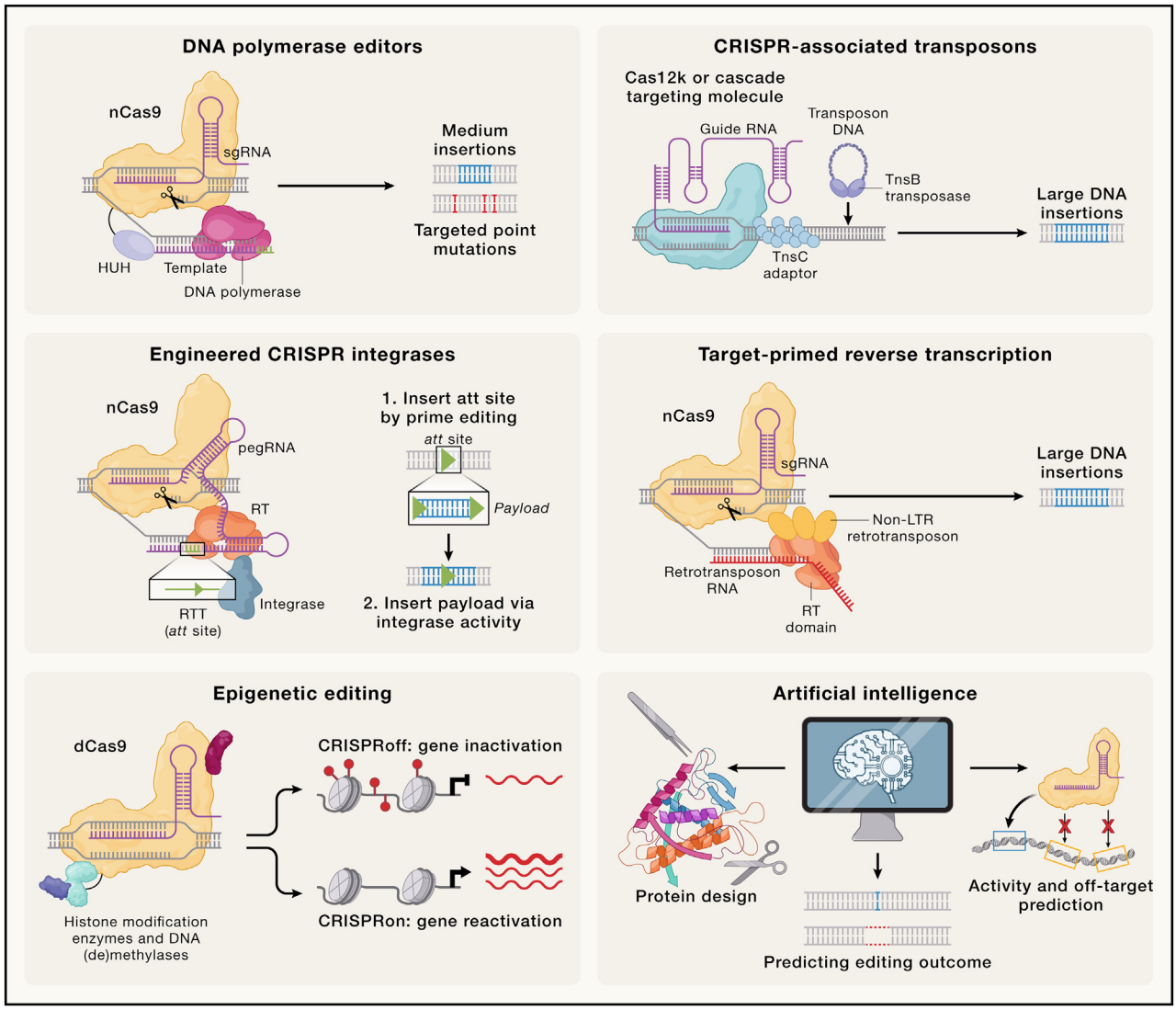

Figure 5. Emerging technologies in genome editing

A summary of emerging technologies in the genome editing field. DNA polymerase editors: this technology combines Cas9 nickases with DNA polymerases and tethering of a single-stranded DNA template, for example, using an HUH endonuclease. A key difference from prime editing lies in its use of DNA polymerase rather than reverse transcriptase and the delivery of the DNA template in *trans*. CRISPR-associated transposons: these naturally occurring mobile genetic elements utilize CRISPR effector complexes in conjunction with transposase proteins for RNA-guided transposition to insert long DNA sequences into specific genomic sites. Engineered CRISPR integrases: these technologies are based on combining prime editors with site-specific serine recombinases. The prime editing initially introduces a recombinase *att* site at the target DNA location, subsequently enabling recombinase-catalyzed insertion of large DNA payloads. Target-primed reverse transcription: this process involves fusing nickase Cas9 with non-long terminal repeat (non-LTR) retrotransposon-derived reverse transcriptases and RNAs. It operates by nicking the target DNA to generate a free 3′ end to prime reverse transcription of the retrotransposon-associated RNA, resulting in targeted DNA insertion. Epigenetic editors: fusions of deactivated dCas9 with DNA methylases and histone-modification enzymes enable targeted chromatin modifications at specific genomic locations, leading to the heritable repression of gene expression (CRISPRoff) without altering the underlying DNA sequence. Gene reactivation (CRISPRon) involves targeting repressed genes using Cas9 fusions with DNA demethylases and transcriptional activator domains. Artificial intelligence in gene editing: AI is making significant inroads in *de novo* protein and guide design, as well as in computational prediction of off-target sites and editing outcomes.

the overall low efficiency and high frequency of off-target transposition events currently precludes their widespread use as targeted gene insertion technologies. In contrast to engineered Cas9-transposase fusions, CRISPR-associated transposon (CAST) systems are naturally occurring Tn7-like transposable elements that have co-opted type I or V CRISPR-Cas systems as targeting modules to mediate RNA-guided DNA transposition. The systems are capable of highly efficient, site-specific insertion in bacteria.[306–308] Extensive structural and functional studies have shed light on the mechanisms of CASTs,[309–313] providing a framework for molecular engineering of these systems to enable targeted DNA transposition in mammalian cells.[314,315] Although the efficiency of type V CASTs remains rather low, type I CASTs show promising signs of activity in human cells,

highlighting the potential of these systems as technologies for site-specific insertion of large genetic payloads. Nevertheless, further systematic functional profiling of naturally occurring CASTs, along with mechanistic investigations and engineering efforts, will be needed to achieve the levels of efficacy necessary for robust genome editing and therapeutic gene delivery.

Retroelement-based editing

Distinct from CASTs, retroelements, particularly non-long terminal repeat (non-LTR) retrotransposons,[316] represent an emerging approach to enable programmable insertion of long DNA sequences with potentially higher efficiency and specificity. Like PEs, retroelements catalyze DNA insertion by target-primed reverse transcription (TPRT), a mechanism that involves nicking the target DNA and using the exposed 3′ end of the nick to prime reverse transcription of the retrotransposon RNA.[316,317] Recent structural studies of the silkworm R2 element have revealed its mechanisms of target DNA recognition and TPRT initiation.[318,319] Using a Cas9 nickase, the retroelement could be retargeted to non-native DNA sequences, outlining a possible strategy for adapting retroelements for targeted DNA insertion.[318] Compared with other methods, retroelements might offer unique advantages for the delivery of larger gene-sized DNA payloads. However, the precision of insertion and the control of off-target effects will require further validation. Moreover, the efficiency of retroelement-mediated integration might vary depending on the target cell type and genomic context, presenting a limitation in its universal applicability.

Epigenome editing

The introduction of permanent genetic modifications poses significant risks, such as unintended mutations, and raises ethical concerns in the context of germline genome editing. CRISPR-based epigenome editing—introducing targeted epigenetic modifications that do not affect germline DNA yet can persist through numerous cellular generations offers a promising alternative.[320] By fusing DNA- and histone-modifying enzymes to nuclease-dead Cas9, chromatin can be restructured at a specific genomic locus to induce or repress target gene expression. A recently established epigenome editor approach termed CRISPRoff exploits DNA methyltransferases such as Dnmt3A or Dnmt3L in conjunction with KRAB transcriptional repressor domains to efficiently and persistently silence gene expression in proliferating cells over multiple generations.[321] The silencing can be reversed by CRISPRon, a multi-partite editor combining Cas9 with the DNA demethylase TET1 and transcriptional activation domains, to reactivate expression.[321] Acetylation is another form of epigenetic histone modification in which histone acetyltransferases (HATs) and histone deacetylases (HDACs) can be used to modify the chromatin and thus activate or repress gene expression, respectively.[202,322] However, certain limitations persist. As Cas9 can transiently bind to off-target sites based on the seed sequence of the guide RNA alone,[42–44] histone and chromatin modifiers can inadvertently affect the transcription status of off-target genes. Additionally, the long-term effects and stability of these epigenetic changes remain to be researched in more detail to fully understand their consequences, especially in the context of complex diseases.

RNA editing

As with epigenome editing, introducing targeted mutations directly into the mRNA offers a potentially safer alternative to editing DNA due to the limited lifetime of mRNAs within the cell.[323] RNA editing approaches have evolved to offer more precise and versatile control over the editing outcomes. One such recent advancement arose from the discovery of the Cas7-11 system, a novel single-protein CRISPR-Cas effector that uses guide RNAs to cleave target RNAs.[324,325] The system has been re-engineered to knockdown transcripts with minimal off-target activity and apparently no collateral cleavage activity in mammalian cells, as compared with Cas13a-mediated knockdown approaches.[324,325] Additionally, a novel approach for CRISPR-mediated *trans*-splicing of long RNA transcripts has recently been reported.[326] By utilizing RNA-targeting Cas13 enzymes in combination with a *trans*-splicing RNA, the technology enables replacement or insertion of large segments in mammalian mRNA transcripts. These novel technologies have significant implications for therapeutic development, particularly for genetic diseases that could be addressed by transient gene correction. RNA editing thus represents an opportunity for more sophisticated and targeted approaches in genomic medicine and expanding the potential for treatments that require transient or reversible genetic modulation.

Novel delivery methods

Efficient and precise delivery of the gene editor complexes into target cells or organs remains the most significant limiting factor for genome editing. A number of strategies are currently under development to enhance delivery efficiency and minimize potential immunogenicity of CRISPR-based genome editors. Firstly, new LNP formulations have recently been developed to enable tissue-specific targeting, thus offering a versatile approach for packaging and delivering CRISPR components with enhanced cellular uptake and reduced off-target editing.[109] Cell-penetrating peptides have also shown considerable potential as a delivery method for CRISPR enzymes, particularly in the editing of primary human lymphocytes,[327] neuronal cells,[328] and airway epithelia.[329] Bacterial contractile injection systems have been re-engineered to permit transient and cell-specific delivery of protein cargo, including genome editor nucleases, with high efficiencies.[330] Engineered virus-like particles that mimic viruses but are devoid of viral genetic material have emerged as a powerful alternative to viral vectors for *in vivo* applications.[331] Another promising technology based on the engineering of endogenous viral particles is based on ancient viruses encoded in eukaryotic genomes, which could potentially permit delivery with reduced immunogenic response.[332] Finally, with the advent of accurate computational protein design approaches, *de novo* designed protein cages are being developed that could provide modular strategies for delivering gene editor complexes to biological environments that are not accessible to natural systems.[333,334] Despite the great promise that these advancements hold, the cytotoxicity, off-target, and immunogenicity profiles of these technologies will need to be rigorously assessed across various cell types and tissues. Furthermore, the scalability of some of these methods for clinical applications poses a significant challenge, as does the cost of developing and manufacturing such complex delivery systems. Overall, there is

a critical need for further research to refine these techniques to ensure their safety and efficacy for therapeutic use.

Artificial intelligence

The recent and rapid adoption of artificial intelligence (AI) and deep learning computational algorithms in biological sciences has also had far-reaching implications for the genome editing field. It has already made considerable progress in improving its precision and efficiency by predictive modeling of the outcomes and efficiency of edits, including off-target activity, which are crucial for minimizing unintended genetic alterations, particularly for therapeutic applications where safety and efficacy are of paramount interest.[335–338] Potentially, AI methods might be used in the future to further personalize gene editing approaches in therapeutic applications. The power of AI is also being harnessed for the engineering of novel, more efficient, and specific nucleases through novel computational protein design methods.[339–342] However, AI methods also come with their own set of challenges. Predictive models are heavily dependent on the availability and quality of the training data, which can be limited and highly variable depending on the experimental approach. Moreover, the "black-box" nature of AI models precludes understanding of how these models make decisions, making predictions difficult to interpret and trust. Nevertheless, the potential applications of AI in gene editing are immense but will require careful consideration moving forward.

OUTLOOK

The past decade has seen the field of genome editing transitioned from a nascent scientific pursuit to a transformative biotechnological force. First-generation CRISPR technologies, primarily based on endogenous repair or site-specific DSBs, have been complemented by second-generation technologies such as base editing and prime editing that enable direct target DNA modification without DSB generation and are generally considered safer and yield more predictable editing outcomes. Emerging (third-generation) CRISPR technologies are being developed to address two major unmet needs in the field: achieving precise insertion of large (gene-sized) payloads, and gene regulation without any genome editing through epigenome engineering. The ongoing advancement of these and other technologies is enabled by two approaches: on the one hand, by mining the metagenome to discover novel molecular systems, and on the other, by synthetic biology and (AI-supported) molecular engineering.

The present state of genome editing is thus best exemplified by an ever-expanding repertoire of novel technologies and methods, primarily based on RNA-guided molecular tools derived from CRISPR-Cas systems, that have revolutionized our ability to manipulate the genetic material with precision and ease. The current trajectory suggests that these technologies will continue to be refined, with gradual improvements in specificity, efficiency, and delivery mechanisms. Areas warranting further investigation will include the development of more specific and less immunogenic delivery vectors, reduction of off-target effects, and enhanced control over editing outcomes, improving both the accuracy and precision of genome editing. As we look to the future, it is likely that nucleic acid-guided systems will remain central to genome editing due to their simple programmability and adaptability, as compared with purely protein-based technologies. However, the mode of their application may evolve, with potential shifts toward more transient editing approaches to minimize the risk of unintended long-term genetic changes or transient delivery to minimize genomic disruption and immune response.

As first-generation technologies have already been approved for clinical use in treating conditions like SCD and β-thalassemia through *ex vivo* editing, with *in vivo* approaches not far behind, the bottleneck constraining the development of future genome-directed therapeutics for a wide range of diseases will no longer necessarily be the lack of safe, efficient, or precise genome editors. The primary challenges will lie in delivery methods, especially for organs other than the blood and liver and for certain cell types *ex vivo*. Advancements in *in vivo* delivery methods, such as those seen in mRNA vaccine development, are likely to significantly catalyze the application of CRISPR technologies. Additionally, even with the tissues and cells that are currently editable using available methods, more basic research will be needed to identify safe editing targets. The range of diseases that could be treated through CRISPR will thus expand with the development of new delivery vectors and the characterization of disease-causing variants and their corrective strategies. It is expected that advances outside the CRISPR field will help drive the technologies in new directions, enhancing their capabilities. In this context, the rise of AI-based methods will allow us to accurately model complex genomic editing landscapes, predicting on- and off-target editing outcomes, and design more capable genome editors, thereby accelerating the pace of implementing safe therapeutic approaches.

Ethical and societal implications, particularly concerning human germline genome modifications, will remain at the forefront of genome editing discussions. With human somatic cell editing becoming reality, the prospect of therapeutic and non-therapeutic germline editing, with the potential to make heritable changes to the human genome, raises profound ethical questions that the global community must address. As research studies in human embryos have shown, CRISPR genome editing technologies are not sufficiently safe or efficacious to use for germline editing for reproductive purposes.[343,344] Moreover, the therapeutic utility of germline editing is limited, likely to benefit only a small number of individuals. Nevertheless, the urgency for international consensus on the governance and responsible stewardship of genome editing technologies cannot be overstated, especially in light of their rapid development, continuous improvement, and widespread adoption.

In conclusion, despite the existing challenges, the future of CRISPR genome editing is bright. It has the potential not only to drive research breakthroughs and revolutionize human medicine but also to enhance agriculture and address ecological challenges, thereby laying the foundation for a healthier and more sustainable future for generations to come.

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DECLARATION OF INTERESTS

M.J. is a co-founder, equity holder, and member of the Scientific Advisory Board of Caribou Biosciences, Inc. M.J. is a named inventor on patents and patent applications related to CRISPR genome editing technologies.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES USED IN THE WRITING PROCESS

During the preparation of this work, the authors used ChatGPT4 in order to improve text readability. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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