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Review article

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Chemical engineering of CRISPR–Cas systems for therapeutic application

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Abstract

Clustered regularly interspaced short palindromic repeats (CRISPR) technology has transformed molecular biology and the future of gene-targeted therapeutics. CRISPR systems comprise a CRISPR-associated (Cas) endonuclease and a guide RNA (gRNA) that can be programmed to guide sequence-specific binding, cleavage, or modification of complementary DNA or RNA. However, the application of CRISPR-based therapeutics is challenged by factors such as molecular size, prokaryotic or phage origins, and an essential gRNA cofactor requirement, which impact efficacy, delivery and safety. This Review focuses on chemical modification and engineering approaches for gRNAs to enhance or enable CRISPR-based therapeutics, emphasizing Cas9 and Cas12a as therapeutic paradigms. Issues that chemically modified gRNAs seek to address, including drug delivery, physiological stability, editing efficiency and off-target effects, as well as challenges that remain, are discussed.

Sections

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Introduction

The ability to change a gene at will profoundly impacts our ability to understand biology and treat genetic diseases[1–3]. Clustered regularly interspaced short palindromic repeats (CRISPR) and their CRISPR-associated (Cas) endonucleases are at the forefront of gene editing technologies due to their straightforward programmability[4–6]. CRISPR–Cas effector enzymes, such as Cas9 from *Streptococcus pyogenes* (*Sp*Cas9), were initially discovered as prokaryotic defence mechanisms against viral infection[6–9]. However, their broad utility for gene editing quickly became apparent, culminating in Nobel Prize recognition in 2020 and CRISPR-based therapeutics that are now approved for clinical use[6,8,10–12]. More traditional technologies, like zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), require the careful design of a new protein for each new gene. By contrast, CRISPR–Cas systems can be rapidly programmed for conceivably any gene by changing the guide RNA (gRNA) sequence following highly predictable nucleic acid base pairing rules[1,3,13].

When double-stranded DNA-targeting Cas enzymes are guided to their target, they unwind the DNA duplex and cleave the phosphodiester bonds of both strands[14,15]. Gene editing occurs when double-strand breaks (DSBs) in DNA are subsequently repaired by cellular mechanisms, such as non-homologous end joining (NHEJ) and homology-directed repair (HDR)[16]. The NHEJ pathway is imprecise, leading to insertion-deletion (indel) mutations, but efficient for creating 'knockouts' of genes, given that indels usually introduce premature stop codons or missense mutations that translate into nonfunctional proteins[1,17–20]. The HDR pathway requires a 'donor' DNA template, typically having termini with homology to sequences flanking the DSB[1,21]. This recombination mechanism is much more precise due to target homology, but it is also less efficient[17]. In principle, the HDR pathway can be commandeered to insert any sequence of interest introduced via the donor DNA[19,20,22]. Although Cas9-based systems are the most widely utilized, CRISPR discovery spans two classes, six types and nearly forty subtypes. Class 2 systems, which possess single multi-domain effector proteins, namely Cas9 and Cas12a enzymes, are the most heavily studied for biotechnology and therapeutics[19,23–25].

Developing CRISPR-based drugs, especially for systemic treatments, will require conventional pharmacologic optimizations, including delivery, half-life, efficacy, specificity and effect duration. In addition, special considerations for CRISPR–Cas systems must also be made, such as reduction of off-target editing (that is, unintended editing of the wrong gene) given that it is difficult to reverse or correct it, unlike traditional drugs. As CRISPR–Cas systems are protein-based and RNA-based, optimizing both components is necessary for therapeutic development. Lessons learned from the successes and failures of nucleic acid therapeutics have helped guide the chemical exploration of gRNAs. Oligonucleotide therapeutics, such as antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs), are at the forefront of modern medicine, with approximately two dozen now approved for the clinic[26–28]. To achieve this, two pivotal chemical innovations occurred. One was the development of chemical modifications of the phosphate backbone, ribose or deoxyribose sugar, and nucleobases to enhance delivery and nuclease stability as well as to reduce immune stimulation[29,30]. Equally important has been the more recent identification of methods for efficient delivery[31,32]. This Review focuses on the role of chemical gRNA engineering in CRISPR–Cas systems and discusses recent developments, potential applications and critical issues that remain for the therapeutic translation of this technology.

Overview of CRISPR–Cas systems

Drug design and development pose many challenges, including delivery, safety and metabolic stability. For biologics and gene-targeted medicines, this is further complicated by complex structure–activity relationships, including folding and molecular interactions of biological polymers like proteins and nucleic acids. To appreciate the challenges and solutions to CRISPR–Cas gRNA chemical engineering and development of CRISPR therapeutics broadly, we discuss the structure and mechanism of CRISPR–Cas9 and CRISPR–Cas12a enzymes, which represent prototypical systems being currently developed for therapeutic use.

Cas protein, guide RNA and ribonucleoprotein architecture

Cas9 is a class 2 type II CRISPR effector enzyme with a bilobed architecture enveloping the gRNA:target DNA heteroduplex[13]. The recognition (REC) lobe is responsible for interaction with the heteroduplex. The nuclease (NUC) lobe includes the protospacer adjacent motif (PAM)-interacting (PI) domain and HNH and RuvC catalytic domains. The PI domain interacts with the target DNA's PAM sequence. The HNH and RuvC domains contain active sites responsible for Mg^{2+}-dependent endonucleolytic cleavage of the target and non-target DNA phosphodiester bonds, respectively[13]. Finally, the bridge helix connects the two lobes[33,34].

Natural CRISPR–Cas9 is a dual RNA-guided system requiring both a CRISPR RNA (crRNA) and a trans-activating CRISPR RNA (tracrRNA) for target acquisition and cleavage[13,35,36] (Fig. 1a). The crRNA has two distinct regions: a guide region derived naturally from CRISPR array spacers, which is complementary to the target sequence, and a tracrRNA-pairing region derived from CRISPR array repeats, which is complementary to the 5′ end of the tracrRNA[6,37,38]. The tracrRNA anchors the gRNA to Cas9 through folded stem loop structures. In bacteria harbouring Cas9 effectors, such as *S. pyogenes*, processing of the precursor crRNA within the CRISPR array is mediated by RNase III endonucleases upon hybridization with the tracrRNA, yielding mature crRNA:tracrRNA complexes. This dual-guide system is often simplified into a single-guide RNA (sgRNA), approximately 100 nucleotides in length, by linking the crRNA and tracrRNA together via a GAAA tetraloop[6,39,40].

Cas12a, previously known as CRISPR from *Prevotella* and *Francisella* 1 (Cpf1), is a class 2 type V effector enzyme[23,37]. Although multiple subtype V systems exist, Cas12a is the best-studied subtype, with orthologues from various species that differ in length and gene-editing efficiency[23,41]. Cas12a from *Acidaminococcus* species (*As*Cas12a), *Francisella novicida* (*Fn*Cas12a) and *Lachnospiraceae* bacterium (*Lb*Cas12a) are often used in human gene editing applications[42,43]. Cas12a domains are named based on functional similarity to those identified in Cas9[40], although they do not have structural similarities[33,44]. Cas12a also has a wedge domain responsible for interacting with its crRNA[45]. Additionally, Cas12a contains a single RuvC active site responsible for cleavage of both the target strand and the non-target strand[23]. Its auxiliary Nuc domain was previously thought to be analogous to the HNH domain in Cas9[33]; however, it does not contain an active site and is instead involved in orienting the target strand for cleavage within the RuvC domain[43].

Cas12a has a crRNA self-processing mechanism that separates the crRNA sequences within CRISPR arrays via an additional independent active site located in the wedge domain[40], yielding mature crRNAs approximately 39–43 nucleotides in length[23,46] (Fig. 1b). This unique endoribonuclease activity has been utilized for multiplexing of Cas12a-based editing systems[47]. Additionally, Cas12a does not require a tracrRNA like Cas9, making it a naturally compact single RNA-guided system[39]. This feature may be valuable for clinical applications, given

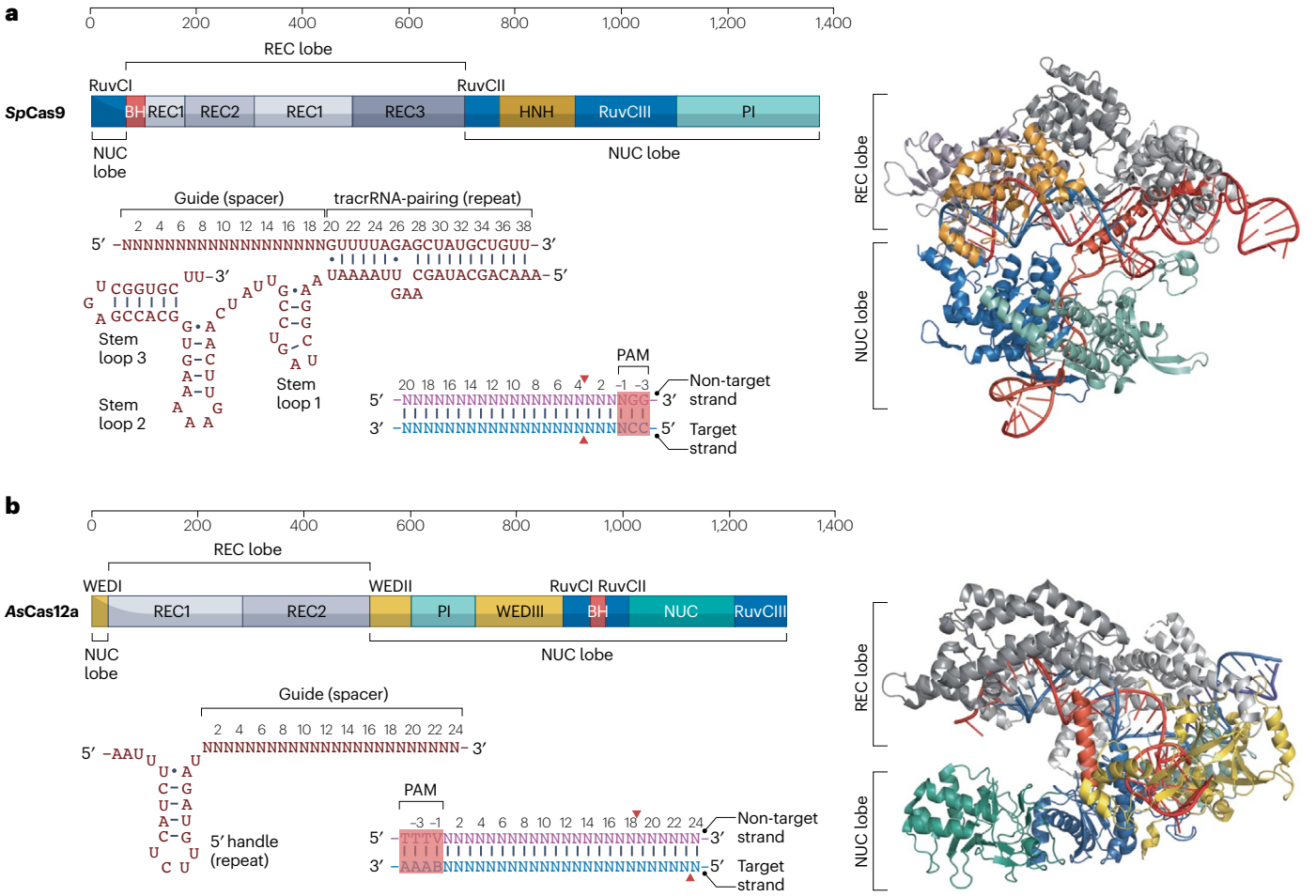


Fig. 1 | Structural organization of the natural CRISPR–Cas9 and CRISPR–Cas12a complexes. a, Schematic representation of *Streptococcus pyogenes* Cas9 (*Sp*Cas9) structural domains and their arrangement within the nuclease (NUC) and recognition (REC) lobes. The crystal structure of *Sp*Cas9 in complex with guide RNA and target DNA (PDB ID: 4OO8) is on the right[34]. The locations of the NUC and REC lobes are labelled. The domains are coloured according to the schematic on the left. Diagram of the *Sp*Cas9 natural dual-guide RNA depicting the CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA). The target DNA duplex is shown, with the non-target strand in purple and the target strand in blue. The protospacer adjacent motif (PAM) sequence (NGG; N = A, C, G, T) on the non-target strand is highlighted in an orange box. Location of the cleavage sites are shown (red arrowheads). **b**, Schematic representation of *Acidaminococcus sp.* Cas12a (*As*Cas12a) structural domains and their arrangement within the NUC and REC lobes. The crystal structure of *As*Cas12a in complex with guide RNA and target DNA (PDB ID: 5B43) is on the right[33]. The locations of the NUC and REC lobes are labelled. The domains are coloured according to the schematic on the left. Diagram of the *As*Cas12a crRNA depicting the guide (spacer) and 5′ handle (repeat) regions. The target DNA duplex is shown, with the non-target strand in purple and the target strand in blue. The PAM sequence (TTTV; V = A, C, G) on the non-target strand is highlighted in an orange box. Location of the cleavage sites are shown (red arrowheads). BH, bridge helix; PDB, Protein Data Bank; PI, PAM-interacting domain; WED, wedge domain. Part **a** adapted with permission from ref. 34, Elsevier. Part **b** adapted with permission from ref. 33, Elsevier.

that a single, shorter crRNA can be readily prepared using standard solid-phase oligonucleotide synthesis[40,48]. The Cas12a crRNA comprises a repeat-derived region, often called the 5′ handle, that folds into a pseudoknot structure and a spacer-derived guide region[42]. Due to the precise folding requirements of the pseudoknot structure and its role in binding to Cas12a, its sequence is highly conserved amongst Cas12a orthologues[23,33,43,48].

The gRNAs bind to Cas proteins through multiple weak interactions. These include charge–charge interactions between the negatively charged phosphate backbone and positively charged amino acids, hydrophobic packing of bases and aliphatic side chains, and hydrogen bonding networks[40]. Polar contacts between ribose 2′-hydroxyl (2′-OH) groups and the Cas protein, usually as hydrogen bonds with the peptide backbone (Fig. 2), appear particularly relevant for gRNA chemical modification. Cas protein function has proven to be quite sensitive to loss of the 2′-OH group at these residue positions[40]. The binding of the gRNA to the Cas protein results in conformational changes within the protein structure, which assemble an active ribonucleoprotein (RNP) complex[46,49,50].

Mechanism of target DNA binding and cleavage

The mechanism for target DNA cleavage by CRISPR–Cas systems can be summarized in three key steps: (1) PAM recognition, (2) heteroduplex 'R-loop' formation and (3) DNA cleavage (Fig. 3a). PAM recognition by

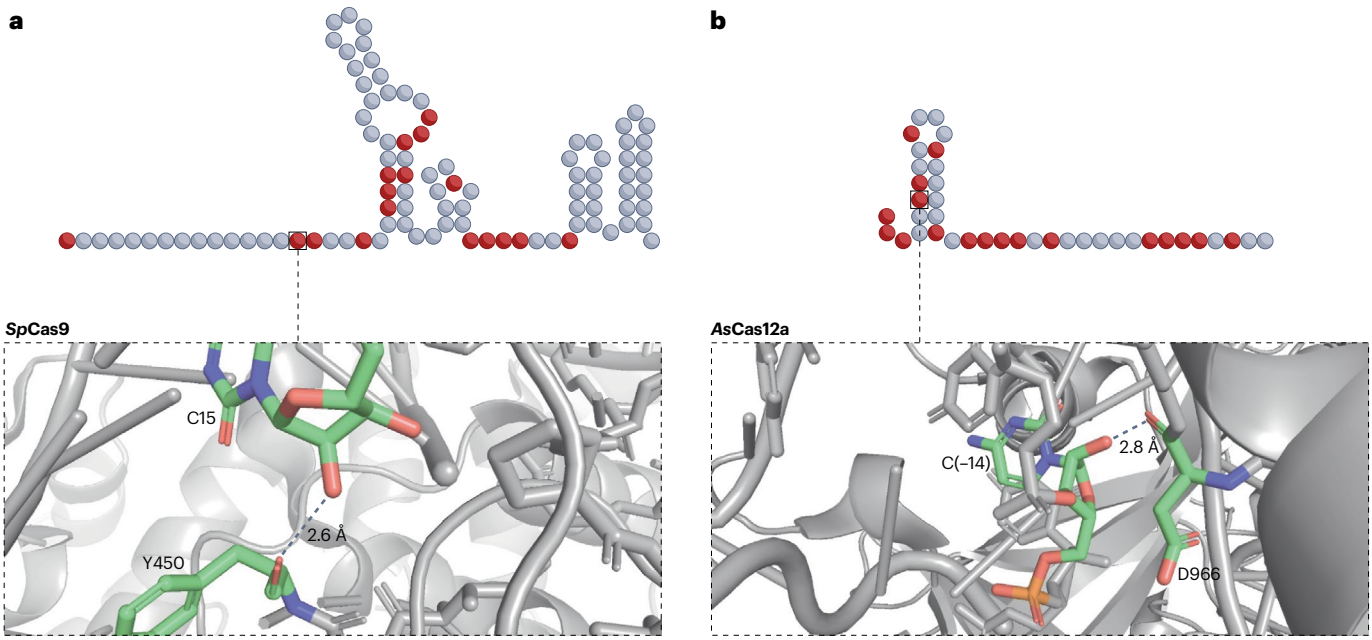


Fig. 2 | Representative 2′-OH contacts between guide RNA and Cas proteins. Nucleotides that form 2′-hydroxyl (2′-OH) contacts with the Cas protein are coloured in red. **a**, The 2′-OH group in the guide region of the Cas9 single-guide RNA forms a hydrogen bond with the carbonyl oxygen of Y450 in the Cas9 protein from *Streptococcus pyogenes* (*Sp*Cas9) (PDB ID: 4OO8)[34]. **b**, The 2′-OH group in the 5′ handle of the Cas12a CRISPR RNA forms a hydrogen bond with the carbonyl oxygen of D966 in the *Acidaminococcus sp*. *As*Cas12a protein (PDB ID: 5B43)[33]. PDB, Protein Data Bank.

the Cas RNP appears to be accomplished through rapid collisions with DNA molecules rather than sliding along the DNA, which could explain why the binding of non-PAM regions of DNA is short-lived[5]. When the target DNA interacts with the RNP complex, it is positioned so that the negatively charged DNA backbone is held in a positively charged central channel through electrostatic interactions with the REC lobe, while the minor groove of the PAM sequence is in the PI domain[33,43]. The amino acids in this domain sample base interactions through a base-and-shape readout mechanism to determine whether they fit correctly[33,43]. Cas9 recognizes G-rich PAMs, specifically NGG (N = A, C, G, T) for *Sp*Cas9, on the non-target strand downstream of the target binding region[13]. Conversely, Cas12a recognizes T-rich PAMs, specifically TTTV (V = A, C, G) for *As*Cas12a, on the non-target strand upstream of the target-binding region[42]. If the PAM sequence is a satisfactory fit, an amino acid residue will be inserted into the double-stranded DNA, disrupting duplex hybridization and causing the +1 phosphate group on the target strand to rotate away from the non-target strand. This promotes local unwinding of the double-stranded DNA and allows the crRNA guide region to begin sampling base pairing potential with the target strand. The incorrect base sequence in the DNA results in steric exclusion, preventing favourable interactions with the PI domain and rotation of the +1 phosphate group. However, this domain of the Cas12a protein has greater flexibility than its Cas9 counterpart, allowing it to recognize cytosine (C) residue-containing non-canonical PAM sequences, albeit with weaker interactions[46].

The second step involves the formation of a heteroduplex between the crRNA guide region and the complementary DNA target strand; this heteroduplex is also known as an ‘R-loop’. Upon satisfying the PAM requirement, base pairing is initiated with the target strand. The non-target strand becomes aligned within the RuvC active site, and the separated DNA duplex is stabilized by hydrogen bonding and van der Waals contacts throughout the NUC lobe[40]. The first 5–10 PAM-proximal nucleotides within the crRNA spacer region, known as the seed region[46], are structurally pre-ordered through various interactions with Cas9 or Cas12a; such pre-ordering facilitates nucleation of heteroduplex base pairing[40]. Consequently, these crRNA nucleotides are highly sensitive to mismatched bases, and Cas amino acids are highly conserved[40]. Improper or poorly formed R-loops are unstable and induce dissociation of Cas RNPs from the DNA. Mismatches or bulges farther from the seed sequence result in more tolerable structural perturbations that often allow reduced levels of DNA cleavage activity[51]. After a 20-base-pair R-loop is formed in both systems, hydrophobic amino acids form disruptive interactions with the heteroduplex, preventing additional base pairing[33].

The final step is DNA cleavage. Conformational changes within the Cas protein are induced to unlock the active site, when a satisfactory R-loop structure is formed. In the case of Cas9, proper R-loop formation is sensed by REC domains and linker amino acids flanking the HNH domain[34,52]. This gatekeeping step is referred to as the REC domain conformational checkpoint[53]. The scissile phosphates of the DNA strands are then drawn into the active sites in the correct orientation and proximity to catalytic residues, facilitating hydrolysis[46]. Activation of the HNH domain triggers additional conformational changes to activate the RuvC domain for non-target strand cleavage[54]. For Cas9, this sequence of events most often creates a ‘blunt’ DSB between nucleotides 3 and 4 upstream of the PAM[55] (Fig. 3b).

For Cas12a, cleavage of the non-target strand in the RuvC domain between nucleotides 18 and 19 downstream of the PAM sequence leads to the DNA duplex fraying at the PAM-distal end of the R-loop[43]. The Nuc

domain coordinates with the target strand to fold it back into the RuvC active site with the same polarity as the non-target strand for cleavage approximately between nucleotides 23 and 24, yielding a 'staggered' DSB[33,43,46] (Fig. 3b). The non-target strand can be further processed through trimming in the active site to yield longer overhangs[56] that may be advantageous in promoting HDR. Given that the target strand is cleaved downstream of the nucleotides involved in base pairing in the PAM-distal region, this strand can undergo multiple rounds of cleavage before the heteroduplex is impacted. This results in substantial deletion of the target sequence, potentially increasing the chance that an indel mutation will lead to gene knockout[40].

Chemical engineering of CRISPR–Cas systems

Chemical engineering of CRISPR–Cas gRNA achieves several goals. They include increased stability against nucleases and tuning of Cas protein activity and specificity, as well as ease of synthesis, control and therapeutic delivery. An array of studies has tackled these challenges with various strategies. This section emphasizes chemical modifications to the gRNA that enable therapeutic applications and compatibility with Cas enzyme activity or the desired therapeutic outcome. With regard to the chemical modifications discussed, we refer the reader to Boxes 1 and 2 for a more in-depth description of the properties of chemical modifications that have been incorporated into gRNAs. We do not speculate on the optimal chemical modification patterns for gRNAs in this Review, as it may be premature to draw broad design rules. Many of the studies conducted to date are diverse and cannot be easily or systematically compared with each other to tease out the best modification patterns. This variability between studies includes in vitro biochemical assays versus cell-based assays as well as different cell lines, delivery methods, target sequences and chemistries unique to each study. Furthermore, many modifications have not been tested at each position individually and are very often placed in the context of multiple modifications.

Fusing the natural Cas9 dual-guide RNA into a single-guide RNA

One of the first published alterations to gRNA was the conversion of the natural dual-guide system of *Sp*Cas9 into a sgRNA by truncating the crRNA and tracrRNA and fusing them via a GAAA tetraloop. The sgRNA simplifies expression from vectors and has been shown to enhance editing activity in mouse liver relative to a dual RNA-guided system[57]. Chemical engineering has shown that the tetraloop can be replaced with triazole linkages or other chemical groups introduced via 'click chemistry' and related reactions (Fig. 4).

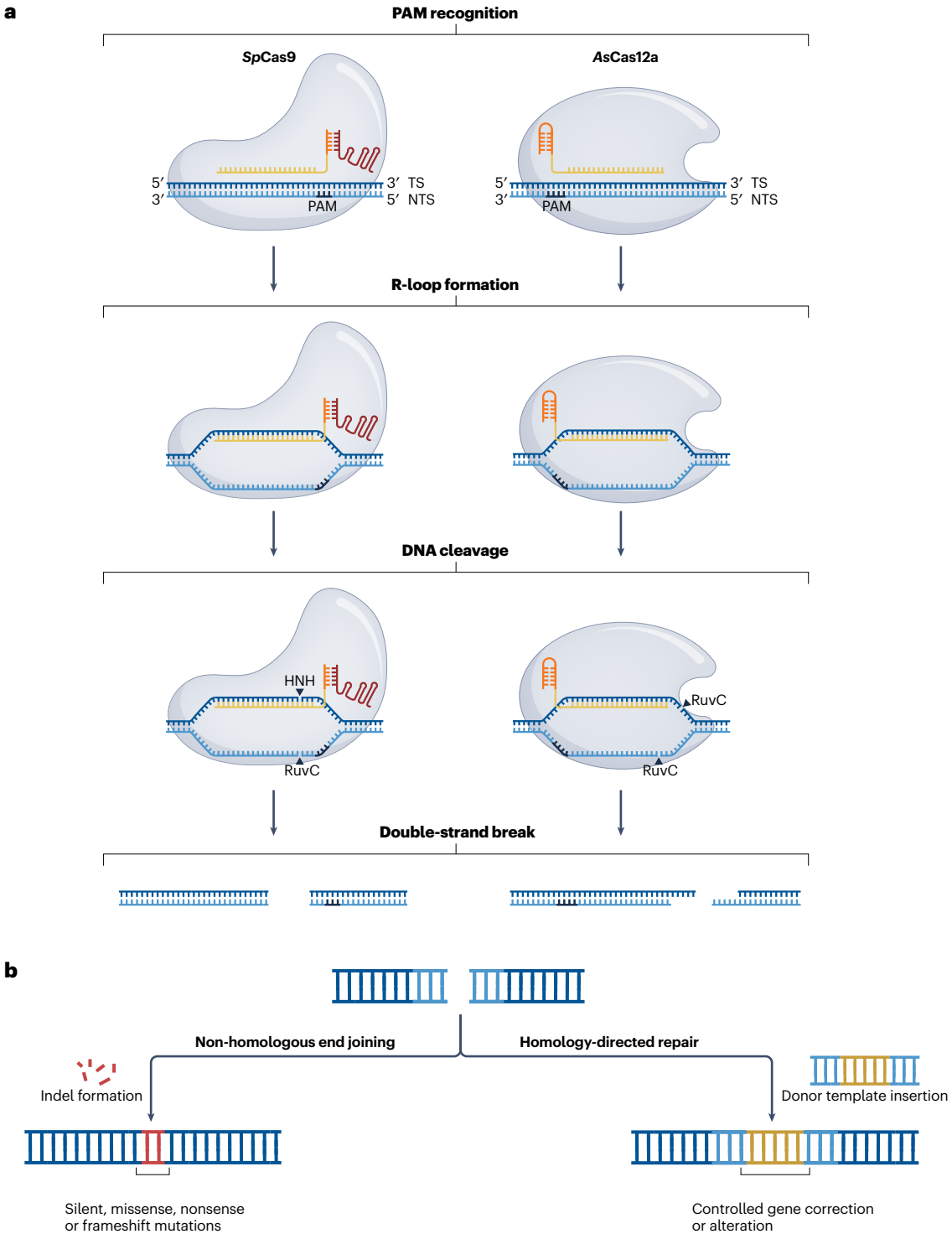
Click reactions have been used successfully in various applications to link two molecules together. They are bio-orthogonal and generally quick, high-yielding reactions[36,58]. The two most common types of click reactions that produce triazole linkages are the copper(I)-catalysed alkyne–azide cycloaddition and the strain-promoted alkyne–azide cycloaddition[36]. Copper(I)-catalysed alkyne–azide cycloaddition can use any unstrained alkyne group, but it relies on a copper catalyst, which is toxic to cells and difficult to remove during nucleic acid purification. Therefore, it is unlikely to be readily used in therapeutic applications[59] (Fig. 4a). In the strain-promoted alkyne–azide cycloaddition, a strained alkyne, such as cyclooctyne and dibenzocyclooctyne, increases reactivity towards the azide without a metal catalyst (Fig. 4b). Triazole linkages showed high compatibility when used to replace the whole tetraloop or its phosphate groups, even showing a modest increase in editing compared with unmodified sgRNA[60,61]. Given that the Cas9 protein does not interact with the sgRNA tetraloop, it should tolerate a range of modifications and chemistry. Another method for linking the crRNA and tracrRNA is to utilize the tetrazine-based inverse electron-demand Diels–Alder reaction, which has favourable conditions similar to those of alkyne–azide click reactions[62,63] (Fig. 4c,d).

Extending half-life, boosting editing and evading immune responses

RNA's poor nuclease stability and immune activation make it an unsuitable therapeutic drug candidate[31]. Chemical modifications to the gRNA have therefore sought to address these shortcomings (Table 1). One of the simplest approaches to address these challenges is minimal modification of the 5′ and 3′ termini of gRNAs to inhibit exonuclease degradation and extend cell half-life (Fig. 5). The first study reporting chemical modification of gRNA replaced the three terminal nucleotides with 2′-*O*-methyl (2′-OMe), 2′-OMe with phosphorothioate (PS) linkages or 2′-OMe with thiophosphonoacetate linkages[64] (Boxes 1 and 2), with the latter two modifications significantly increasing editing activity when introduced into primary human cells, which are typically challenging to transfect and edit. Other groups subsequently demonstrated the utility of terminal modifications to improve editing efficiencies by Cas9 and Cas12a, including 2′-deoxy (DNA) and 2′-fluoro RNA (2′F-RNA) with and without PS linkages[47,65,66] (Box 1). Capping the ends of synthetic gRNAs with modified nucleotides and backbone linkages is now standard practice in biomedical CRISPR applications. Other linkages used include amide linkages, tolerated in the PAM-distal region[67,68], and stimuli-responsive phosphorothioates, which activate editing in response to stimuli such as light or hydrogen peroxide[69]. A stimuli-responsive approach has also been utilized on the ribose sugar[70].

Nucleobase modifications are fundamental to the regulation of biological systems. They modulate gene and protein expression at the DNA (epigenetics) or mRNA (epitranscriptomics) level[71]. Examples include 5-methylcytosine (m^5C), 5-hydroxymethylcytosine and N^1-methyladenosine[71,72]. Many natural nucleobase modifications are found in transfer RNA (tRNA) and play critical roles in tRNA folding, stability and interactions[73]. Modifying the nucleobases of other nucleic acid therapeutics reduces immune responses, enhances nuclease stability, and alters thermal stability and affinity to the target[32]. The 2023 Nobel Prize in Physiology or Medicine was based on the value of identifying and incorporating natural nucleobase modifications, like pseudouridine (Ψ), to convert mRNA into a viable vaccine. Ψ can reduce innate immune system detection and increase protein expression. Although substantial research has been conducted on nucleobase modifications, only three have been approved clinically (m^5C, Ψ and N^1-methylpseudouridine)[74–76].

Multiple studies have explored the potential of nucleobase modifications to gRNAs (Fig. 5). In vitro or cell-free Cas9 cleavage assays have reported that m^5C, Ψ and other base modifications are well-tolerated in both the tracrRNA and crRNA and can increase specificity[77]. In cells, these modified gRNAs reduce immune recognition and cytotoxicity[77], but they also decrease editing activity[78]. This research highlights the discrepancy between in vitro and cell-based assays for gRNA modifications, which is also observed for other base modifications like N^6-methyladenosine, 2-thiouridine, 4-thiouridine and N^1-methylpseudouridine[63,78]. Interestingly, utilizing uridine depletion to increase the incorporation of nucleobase modifications (m^5C and Ψ) into a Cas9 mRNA, enhanced editing and decreased immunogenicity[79]. For Cas12a, N^6-methyladenosine



reduces cleavage in vitro and in cells in a manner dependent on cellular demethylase activity[80]. Cas proteins engage in interactions with nucleobases, which can be disrupted when certain modifications are present. For instance, N^1-methylpseudouridine assumes a *syn* conformation of the base, causing destabilization of the RNA:DNA R-loop hybrid[81].

Probing structure–activity relationships and expanding therapeutic utility

Chemically altering the structure of nucleic acids has a profound impact on their biophysical properties, like hybridization affinity and helical structure, and their pharmacological properties, such as hydrophobicity, nuclease resistance, immunogenicity, pharmacokinetics and

Fig. 3 | General mechanism of double-stranded DNA cleavage and repair by natural CRISPR–Cas9 and CRISPR–Cas12a systems. a, (Left) The Cas9 protein (*Sp*Cas9 specifically) recognizes the target site through an NGG (N = A, C, G, T) protospacer adjacent motif (PAM) sequence on the non-target strand (NTS), the CRISPR RNA (crRNA) spacer sequence hybridizes to the target strand upstream of the PAM sequence and forms an R-loop structure, and the Cas9 protein induces a double-strand break in the PAM-proximal region of the target DNA, creating a blunt cleavage site. The crRNA spacer-derived guide region is coloured in yellow, the repeat-derived trans-activating crRNA (tracrRNA)-pairing region in orange, the tracrRNA in red, the target strand (TS) in dark blue, the NTS in light blue and the PAM sequence in black. (Right) The Cas12a protein (*As*Cas12a specifically) recognizes a TTTV (V = A, C, G) PAM sequence; its crRNA spacer sequence hybridizes and forms an R-loop structure downstream of this site, and the Cas12a protein induces a double-strand break in the PAM-distal region, creating a staggered cleavage site. **b**, (Left) The non-homologous end joining DNA repair mechanism uses random insertion of nucleotides and results in insertion-deletion (indel) formation, leading to various mutations. (Right) The homology-directed repair DNA repair mechanism uses a donor template with ends homologous to the cleavage site and results in insertion of the template for either controlled gene correction or alteration. *As*Cas12a, Cas12a from *Acidaminococcus sp.*; *Sp*Cas9, Cas9 from *Streptococcus pyogenes*.

pharmacodynamics, and accumulation or half-life in tissues[29]. These changes can become key determining factors in clinical translation. Chemical modification has been crucial for the success of nucleic acid therapeutics, with each of the therapies approved by the US Food and Drug Administration (FDA) bearing at least one essential chemical modification[26]. This includes PS modifications, which have been utilized in early antisense drugs like fomivirsen[82,83]. The 2′-OMe and 2′F-RNA modifications have contributed to the efficacy of siRNA therapeutics such as givosiran and lumasiran[84,85]. The 2′-*O*-methoxyethyl modification present in ASOs, such as mipomersen, inotersen and volanesorsen, improves target binding and stability[86–88]. Phosphorodiamidate morpholino oligomers result in a neutral backbone resistant to nucleases. They are used in splice-switching oligonucleotides such as eteplirsen and golodirsen for the treatment of Duchenne muscular dystrophy[89–91]. From a fundamental science perspective, chemical modification of nucleic acids can also serve as a probe to better understand the structure–activity relationships and mechanisms of action when interacting with cofactors (that is, Cas9) or molecular targets (that is, DNA or RNA).

Modifying the ribose moiety of RNA nucleotides can enhance therapeutic properties by reducing immune recognition, increasing half-life and stability, and modulating target hybridization affinity[29,31]. One of the most straightforward modifications is the replacement of the 2′-OH group with a hydrogen (H) to make 2′-deoxyribose, or DNA (Box 1). Multiple groups have explored DNA substitutions in Cas9 and Cas12a crRNAs, both as a probe to understand the structure–activity relationship and as a way to improve the potential therapeutic utility[38,48,92–94]. Complete conversion to DNA inactivates the crRNA, presumably due to the loss of sufficient A-form structure and hydrogen bonding networks[92,95]. However, partial DNA modification, particularly at positions that do not substantially interact with Cas proteins, like loop regions, at the termini and away from predicted 2′-OH contacts (Fig. 2), can be well-tolerated, or they can even enhance enzymatic activity and specificity[38,92–94].

DNA is a naturally occurring nucleotide and is still subject to enzymatic degradation and recognition by cellular factors involved in innate immune sensing[29,96]. Therefore, other ribose modifications with well-characterized properties and a track record of success in nucleic acid therapeutics have been more heavily investigated (Fig. 5). These include 2′-OMe, 2′F-RNA and 2′-4′ bridged nucleic acids (BNAs), which better mimic RNA nucleotide structure and either block RNase recognition or prohibit the usual intramolecular attack of the 2′ oxygen on the adjacent 3′ phosphate[29,30].

Methylation at the 2′ position, generating the naturally occurring 2′-OMe, has been used to substitute terminal 5′ and 3′ nucleotides at the ends of Cas9 crRNAs, tracrRNAs and sgRNAs, as well as Cas12a crRNAs (Box 1). This modification has been reported to increase stability, activity and specificity, although higher activity may result from longer half-lives in cells[97]. Placement of 2′-OMe internally can also be well-tolerated at certain positions in which the slightly bulky methyl group does not cause steric constraints or disrupt predicted polar contacts mediated by the 2′-OH group[38,48,98].

The 2′F-RNA modification has been incorporated into dual-guide systems (that is, crRNA and tracrRNA) and sgRNAs for Cas9 and the crRNA for Cas12a[98,99] (Box 1). When placed in the guide region of a heavily 2′-OMe-modified Cas9 sgRNA, it improved in vivo editing activity and reduced off-target editing events[99]. In the dual-guide context, a fully 2′F-RNA-modified crRNA maintains in vitro editing activity, but such activity is almost entirely lost in cells. An alternating 2′F-RNA/2′-OMe pattern in the guide region completely abolished in vitro editing activity[100]. Placing 2′F-RNA in the 3′ end of the seed region showed tolerability in vitro in the context of a primarily all-DNA-modified or unmodified crRNA. However, when these crRNAs were used in cells, gene editing was significantly decreased. One of the more effective positions for the 2′F-RNA is in the 5′ end of the gRNA spacer region, outside the seed region[100]. In the tracrRNA for Cas9, 2′F-RNA is effective at improving cell editing, especially when combined with other modifications such as 2′-OMe[98].

When 2′F-RNA is incorporated into the crRNA of Cas12a, internal 2′F-RNA substitutions are better tolerated than 2′OMe, allowing the incorporation of more modifications[65]. Additionally, substituting up to five consecutive 2′F-RNAs at the 3′ end increases activity relative to unmodified crRNA. This is likely due to fluorine's small atomic radius, which minimizes its impact on the overall structure and protects the RNA strand towards endonuclease stability, particularly at pyrimidine sites[30]. This modification pattern was broadly applicable across different target genes and can be used to enhance the editing activity of low-yielding orthologues such as *Lb*Cas12a[65]. However, more extensive modification of the crRNA with 2′F-RNA or other nucleotide analogues requires consideration of 2′-OH contacts sensitive to this substitution[48].

Placement of BNAs, like LNA, cEt, and BNA^{NC}, in the spacer seed region for Cas9 increases PAM specificity in cells but lowers cleavage kinetics and activity in vitro[97,101] (Box 1). This was attributed to the BNA modifications helping induce a strong A-form helical character, resulting in better binding to the target DNA. As a result of its steric bulk, the incorporation of BNA^{NC} at select sites in the gRNA spacer region leads to increased target specificity compared with locked nucleic acid. Although BNA analogues are conformationally 'locked,' modifications that enhance the flexibility of an oligonucleotide are useful when targeting complex secondary structures[102,103]. When utilized in the dual-guide system, Cas9 crRNA modified with unlocked

Box 1 | Properties of ribose and phosphodiester modifications commonly incorporated into therapeutic nucleic acids

Chemical modifications play a crucial role in modern therapeutic nucleic acids. Chemical properties like nuclease resistance, thermal affinity and helical structure profoundly influence their biological activity. The conformation of the sugar ring determines the helical structure of each modification. Modifications are broadly categorized into two types: those that form an A-form helical structure (similar to RNA) and those that form a B-form helical structure (similar to DNA).

2′-Deoxy (DNA)

DNA lacks a 2′-hydroxyl (2′-OH), which increases the flexibility of the ribose, allowing various sugar pucker conformations[214]. Therefore, DNA can assume an A-form or B-form helical geometry depending on its local environment[215]. For example, when DNA is placed in the context of neighbouring RNA nucleotides, it will readily acquire the preferred A-form-like helical structure of RNA[215]. DNA has enhanced nuclease stability with respect to RNA nucleotides, but it cannot form hydrogen bonding interactions.

2′-O-methyl (2′-OMe)

When it comes to oligonucleotide therapeutics, the addition of 2′-OMe not only boosts target binding affinity and nuclease stability, but it also helps lower the immune response[29]. This groundbreaking modification has achieved remarkable success in clinical applications and is a key component of the effectiveness of small interfering RNA drugs patisiran, givosiran and inclisiran[29]. Due to the methyl group replacing the hydrogen, the newly introduced group cannot act as a hydrogen-bond donor, but it can act as an acceptor. Interestingly, RNA can be naturally modified to 2′-OMe, such as by site-specific RNA-guided box C/D small nucleolar RNP complexes[216].

2′-Fluoro RNA (2′F-RNA)

This modification, in which the 2′-OH group is replaced by a fluorine atom, was one of the first studied synthetic RNA mimics, and it is utilized in several FDA-approved oligonucleotide therapeutics, including pegaptanib and patisiran[196,217]. 2′F-RNA increases the thermal affinity of complementary nucleic acids (ΔT_m = +2–3 °C per modification; T_m, melting temperature) as a result of the highly electronegative fluorine enhancing the strength of hydrogen bonding between nucleobases in the base pairs[218,219]. The fluorine atom can act as a hydrogen-bond acceptor but not as a donor. Curiously, although this modification enhances endonuclease stability, particularly for pyrimidine nucleotides, 2′F-RNA-modified oligonucleotides are still prone to degradation by exonucleases[30].

Bridged nucleic acids (BNAs)

BNAs have also been shown to enhance cellular uptake, binding affinity and nuclease stability[29]. The simplest of these, locked nucleic acid (LNA), remarkably increases the thermal affinity for RNA (ΔT_m = +5–9 °C per modification)[29,30]. The preorganization of the sugar can explain the enhancement in binding affinity into an RNA-like conformation as well as improved hydrophobic interactions by the 2′-O,4′-C methylene bridge. Other bicyclic analogues tested in CRISPR–Cas9 systems are constrained 2′-O-ethyl (cEt) and *N*-methyl substituted BNAs (BNA^{NC})[101].

Phosphorothioate (PS)

One of the most widely researched phosphate modifications is the PS linkage, in which a non-bridging oxygen atom is replaced by a sulfur atom. This subtle alteration slightly reduces binding affinity to complementary RNA (ΔT_m = −0.5 °C per modification) but enhances binding to serum proteins such as albumin, aiding in circulation[30,220,221]. The synthesis of a fully PS-modified oligonucleotide of *n* nucleotides in length creates 2^{n-1} possible diastereoisomers due to the chiral nature of the internucleotide PS linkage[222]. PS linkages with the R_p stereochemistry are more vulnerable to nuclease degradation, whereas the S_p stereochemistry is less detrimental to binding affinity[223,224]. As a result, the epimeric (R_p+S_p) mixtures strike a good balance between binding affinity and nuclease resistance.

nucleic acid (UNA) (Fig. 5) showed minimal gene-editing activity in vitro. However, UNA was only tested in a single position at the 3′ end of the spacer region in the context of other chemical modifications[38]. Another class that enhances flexibility are non-nucleotide linkers such as butanediol[102] (Fig. 5), which has also shown minimal editing activity when used in the same position and chemically modified context as UNA. Therefore, more research is warranted to determine if flexible modifications such as UNA and butanediol can enhance gRNA properties in other positions.

Modifications that do not fit the prototypical RNA mimic properties but have been investigated are 2′-arabinonucleic acid and 2′-fluoro-arabinonucleic acid (Fig. 5 and Box 2). Both have been utilized to examine the role of A-form helical structure, flexibility and 2′-OH requirements implicated in previous studies, given that they prefer a DNA-like sugar conformation[38,104,105]. These modifications generally decreased in vitro editing activity when used in Cas9 and Cas12a crRNAs[38,48]. However, when used in the tracrRNA-pairing region of Cas9 crRNA, in vitro cleavage activity was maintained[38,48].

The 2′-hydroxyl barrier

At present, all ribose modifications evaluated in the literature have failed to satisfactorily replace most residues in which the gRNA is predicted to make 2′-OH polar contacts with the Cas9 or Cas12a protein (Fig. 2). We have colloquially termed this bottleneck, which effectively prevents full chemical tuning of gRNAs, the '2′-hydroxyl barrier.' A puzzling phenomenon surrounding the 2′-hydroxyl barrier is the observation that some modifications are well-tolerated at these positions in vitro, providing robust enzymatic cleavage even with fully chemically modified gRNA[38], yet, when introduced into cells, these same gRNAs often do not support efficient gene editing[95,98,100]. These results suggest that unique features about the cellular environment or chromatin substrate confer critical 2′-OH contact sensitivity to CRISPR–Cas enzymes. Several factors inherent to the cellular environment may contribute to this discrepancy. One key consideration is the concentration of active RNP complexes. In vitro assays typically utilize higher concentrations of RNP and target DNA and are not limited by cellular uptake, compartmentalization, localization and molecular crowding. Furthermore,

chromatin accessibility of genomic DNA makes it more complex to bind and cleave than purified DNA[106]. Therefore, slight alterations in 2′-OH contacts and their role in Cas activity may be inconsequential in vitro yet impact gene editing in cellular contexts.

When trying to replace native RNA residues at critical 2′-OH positions, it has been observed that bulky modifications, such as 2′F-4′OMe and 2′4′-diOMe modifications in Cas9 crRNA[38] and the seven-membered ring oxepane nucleic acid in Cas12a crRNA, are poorly compatible[48] (Fig. 5 and Box 2). Conversely, more minor modifications, such as 2′F-RNA and DNA, that are less structurally perturbing can be better tolerated in certain positions. Unfortunately, DNA offers no hydrogen bonding capacity and 2′F-RNA can only provide some hydrogen-bond-acceptor properties but not hydrogen-bond-donor properties[107,108]. Substitution of 2′-arabinonucleic acid, which is a stereoisomer of RNA but points the 2′-OH in the opposite orientation, at a few critical 2′-OH residues was unsuccessful in supporting gene editing by Cas12a, despite exhibiting in vitro cleavage activity[48]. In an attempt to substitute for H-bond-donating and H-bond-accepting properties at the 2′ position, 2′-amino (2′-NH_2) was incorporated at individual residues in the 5′ handle of the Cas12a crRNA (Fig. 5). Walking both 2′-NH_2 (Box 2) and 2′F-RNA across critical residues revealed an inability to replace the 2′-OH at most contact positions, although some relative position-dependence and sequence-dependence was observed for each[48].

Although critical 2′-OH residues must be largely maintained as native RNA nucleotides, they can still be partially protected by modifications of neighbouring phosphodiester bonds (Fig. 5) like PS linkages, albeit with often reduced editing activity[48]. Given that PS linkages alter nuanced protein interactions and mildly decrease hybridization

Box 2 | Other diverse chemical modifications incorporated into CRISPR RNA

4′-O-methyl (4′-OMe) combined with 2′-F and 2′-OMe

2′F-4′OMe and 2′4′-diOMe modifications were shown to adopt an RNA-like conformation. Both of these modifications enhance nuclease stability, increase thermal affinity and are tolerated in small interfering RNA[225,226].

Unlocked nucleic acid (UNA) and non-nucleotide linkers

UNA, which takes its name from the lack of a C2′–C3′ bond in the ribose sugar[227,228], significantly decreases binding affinity in RNA duplexes (ΔT_m=−10 °C per modification; T_m, melting temperature) but increases the oligonucleotide's flexibility to target a secondary structure. Although it lowers binding affinity, it does enhance mismatch discrimination and nuclease resistance with respect to natural RNA[227,229].

Non-nucleotide structures such as the butanediol (C4) linker have been shown to destabilize a duplex structure but enhance its flexibility. This modification maintains the phosphate distances and has been shown to destabilize a duplex (ΔT_m=−5 to −8 °C per modification), but, when incorporated into the antisense strand of a DNA:RNA duplex, it increases the rate of RNase H cleavage 3.5-fold, which is relevant for antisense oligonucleotide applications. In comparison, the UNA modification does not show such an enhancement[102].

Arabinonucleic acid (ANA) and 2′-fluoro-arabinonucleic acid (2′F-ANA)

ANA is a stereoisomer of RNA, with a conformation similar to DNA due to the 2′-OH being on the opposite face of the ribose sugar. ANA can bind to RNA but not DNA complements, and it exhibits higher resistance to degradation by 3′ exonucleases than both DNA and RNA[104,105].

The 2′F-ANA modification is the epimer of 2′F-RNA, and it adopts a DNA-like sugar conformation[230,231]. A special feature of 2′F-ANA:RNA hybrids is the formation of internucleotide fluorine–hydrogen bonds (2′F–H8) at purine:pyrimidine steps (for example, TG and TA)[107,232]. Evidence shows that these interactions also occur intramolecularly in both pyrimidine and purine nucleosides[233]. There are many applications of 2′F-ANA oligonucleotides, including their use in antisense oligonucleotides[234,235], small interfering RNAs[236] and aptamers[237,238]. Similar to ANA, 2′F-ANA also confers enhanced 3′ exonuclease resistance.

2′,5′-RNA

The 2′,5′ internucleotide linkage is an alternative to the canonical 3′,5′ linkage most commonly observed in nucleic acids[111]. Although this linkage destabilizes RNA duplexes (ΔT_m=−2.5 to −4.3 °C per modification), the sugar pucker's ability to adapt its conformation, especially when single or minimal insertions are used in an otherwise 3′,5′-RNA strand, allows for the molecule's structural flexibility.

2′-Amino (2′-NH_2)

2′-NH_2 contains an amine group instead of the 2′-OH group, which can function as both a hydrogen acceptor and a hydrogen donor. At physiological pH, the major form of 2′-NH_2 is non-protonated, owing to the pK_a of the 2′-NH_2 group being approximately 6.2 (ref. 239). This increases the probability of forming hydrogen-bonding interactions. This modification adopts a conformation that is similar to DNA.

Oxepane nucleic acid

Oxepane nucleic acid (OxNA) has an expanded ring structure with a seven-membered ring. With the increased ring size, it can possess hydroxyl groups at several positions and is, in principle, able to form multiple hydrogen-bond interactions. The expanded ring structure of ONA makes it possible to place the internucleotide linkage at various positions on the sugar ring[240].

Phosphonoacetate and thiophosphonoacetate

The backbone modifications phosphonoacetate (PACE) and thiophosphonoacetate (thioPACE) have a phosphorus–carbon bond and either a non-bridging oxygen or sulfur atom, respectively. They have been shown to recruit RNase H when used in DNA and are resistant to nucleases[241].

Phosphoryl guanidine

The phosphoryl guanidine (PG) linkage is a backbone modification that substitutes one of the non-bridging oxygen atoms for a phosphoryl guanidine group, making the backbone charge-neutral[113]. This modification enhances both cellular uptake and nuclease resistance. Additionally, unlike other backbone modifications, this linkage enhances hybridization affinity to DNA and RNA targets and confers low toxicity in cells.

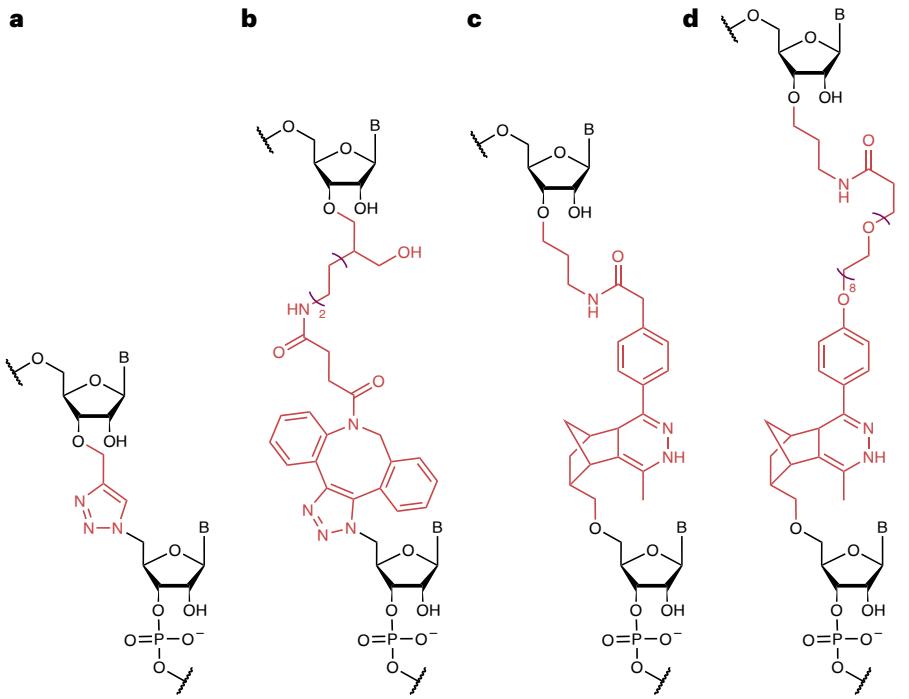


Fig. 4 | Chemical modifications that link the crRNA and tracrRNA together to form a sgRNA in the CRISPR–Cas9 system. Such chemical modifications include the unstrained (part **a**) and strained (part **b**) triazole-based linkers and the short (part **c**) and long (part **d**) tetrazine-based linkers. crRNA, CRISPR RNA; sgRNA, single-guide RNA; tracrRNA, trans-activating crRNA.

affinity, these observations suggest that the 2′-OH group plays additional roles, such as supporting enzyme dynamics, beyond simple hydrogen bonding. In addition to thiophosphonoacetate, phosphonoacetate (PACE) backbone modifications have also been explored in the spacer region of Cas9 crRNA (Box 2). PACE incorporation at certain positions significantly reduced off-target effects[109,110]. Although PACE incorporation was not performed in the context of specific 2′-OH contacts, these results suggest that critical 2′-OH contacts may help control specificity and activity and argue for more investigations into linkage modifications. For example, the use of 2′,5′ linkages throughout the Cas9 crRNA was explored (Box 2), as this linkage maintains a hydroxyl group on the sugar while providing enhanced nuclease resistance[111,112]. However, multiple consecutive insertions of this non-canonical linkage throughout the Cas9 crRNA led to either reduced or abolished editing activity[38]. Finally, phosphoryl guanidine (Box 2) is a charge-neutral backbone modification that, when strategically incorporated at specific positions in the PAM-distal spacer region of the Cas9 crRNA, away from 2′-OH contacts, is found to increase target specificity without substantially impacting editing activity[113].

To address the 2′-hydroxyl barrier, novel chemistries that maintain 2′-OH contacts but confer nuclease resistance, such as 4′ ribose modifications, should be explored further[38,63,114]. However, their effects on ribose ring conformation, base pairing efficiency and steric constraints may play a role in function and will need to be considered. Other engineering alternatives may provide solutions, such as the recently demonstrated use of a chemically modified oligonucleotide hybridized to sgRNA across the labile RNA nucleotides, which enabled 'naked' unformulated delivery of sgRNAs in vivo[115]. Likewise, covalent conjugates have been minimally explored, but they could mask gRNAs or direct them efficiently to specific tissues[116,117]. For example, cell-penetrating peptides fused to the *Sp*Cas9a protein have been shown to enhance delivery to the striatum. Protein engineering of individual Cas enzymes may also provide a workaround for certain therapeutic applications. Although substantial effort has been dedicated to Cas protein engineering, there remains a substantial gap in combining the chemical engineering of CRISPR gRNAs with Cas protein engineering.

The 2′-hydroxyl barrier has been observed for both Cas9 and Cas12a systems, suggesting the possibility of a broader theme shared by RNA-guided CRISPR enzymes. One hypothesis is that dependence on 2′-OH contacts emerged from the intimate co-evolution of RNA guides and their protein counterparts to help regulate enzyme activity, as many cofactors do, or to ensure that DNA could not be alternatively used to promote cleavage activity[48]. Notably, for both Cas9 and Cas12a, 2′-OH contacts primarily occur between gRNA and the peptide backbone, not amino acid side chains[33,51] (Fig. 2). These contacts could play roles in regulating enzyme activity through conformational dynamics. However, their status during the many conformational state transitions that Cas enzymes undergo for catalysis remains unclear. Although complete modification of the gRNA may not be necessary for many applications, a deeper understanding of the rules governing modification tolerability are essential for precisely tailoring CRISPR–Cas systems to meet clinical efficacy criteria and for unlocking broader therapeutic use. Importantly, a similar 2′-hydroxyl barrier might be anticipated for other RNA-guided enzymes. Therefore, understanding the 2′-hydroxyl barrier will likely not only accelerate CRISPR-based therapeutics but also impact other RNA-guided systems beyond CRISPR that might eventually find therapeutic applications, such as the recently discovered bridge RNA-guided IS110-based recombination system[118].

The delivery dilemma

Similar to many nucleic acid therapeutics, targeted and safe delivery presents a substantial challenge for the effective use of CRISPR–Cas systems in clinical applications[119] (Fig. 6). Difficulties arise from the large size of the system's components, negative charge and variable stability.

Ongoing clinical trials mostly utilize adeno-associated virus expression or lipid nanoparticle (LNP) formulations for delivery[120–122]. Viral vectors, especially adeno-associated virus, have been extensively explored for gene editing applications due to their small size, high delivery efficiencies and tissue-specific tropisms (Fig. 6b). However, their packaging capacity is limited to ~4.7 kb, which restricts the delivery of larger CRISPR systems. This barrier can be overcome by using different viral delivery vehicles such as lentivirus[123,124] and adenovirus particles[125–127], which vary in loading capacity, type of genetic material they encapsulate, immunogenicity and tissue tropism[121,128]. Alternatively, the CRISPR components can be split into two viral vectors, and smaller Cas enzymes, such as *Staphylococcus aureus* Cas9 (*Sa*Cas9) and Cas12a from *Erysipelotrichia* (*Eb*Cas12a)[122], or intein-split Cas9[129,130], can be used to reduce the overall cargo[124]. Virus-like particles have emerged as a promising method that offers the benefits of viral and non-viral delivery (Fig. 6b). Virus-like particles are derived from retroviruses like HIV-1 and Moloney murine leukaemia virus and have the potential to deliver mRNAs, proteins or RNPs directly, instead of an engineered viral genome[131]. DNA expression vectors can be assembled into nanostructures with cell-penetrating peptides as an alternative to viral vectors or virus-like particles[132]. These short peptides are cationic and associate with the vector DNA through electrostatic interactions. They can also be conjugated to nuclear localization sequences to facilitate nuclear translocation or to aptamers for cell-targeting capabilities.

Instead of delivering DNA to encode gene editing systems, CRISPR components can be delivered to cells as separate RNA molecules, specifically as mRNAs encoding Cas proteins and chemically stabilized gRNAs (Fig. 6a). The Cas protein must be translated from mRNA, which creates a temporal lag before RNP assembly, making chemical stabilization of the gRNA especially important. The most common method for RNA-based delivery is using LNPs, as previously demonstrated by clinically approved siRNA therapeutics[133] and mRNA vaccines[134,135]. Additionally, due to their transient and non-viral properties, as well as an inability to become permanently integrated into the genome of patient cells, RNA-based delivery of CRISPR–Cas systems using LNPs mitigates the dangers of expression from DNA[57,136]. LNPs are capable of targeting a variety of tissues following either systemic administration or direct administration to the desired tissue based on their size and lipid composition. They can also be co-formulated to encapsulate the Cas mRNA and gRNA within the same particle, ensuring delivery to the exact location[137]. LNPs can be further functionalized with targeting ligands for higher specificity. For example, *N*-acetylgalactosamine-functionalized LNPs preferentially target the liver[138] and are safe and robust in clinically approved siRNA[139] and ASO delivery[140]. LNPs are primarily endocytosed by hepatocytes following complexation with apolipoprotein E (ApoE) in serum. Endocytosis follows *N*-acetylgalactosamine binding to asialoglycoprotein receptors (ASGPRs), which are expressed at significantly higher concentrations in hepatocytes than in other cell types, leading to targeted uptake[141].

In RNP-based delivery methods, pre-assembled RNP complexes are directly delivered to cells, providing precise control over the Cas protein and gRNA composition and concentrations. This approach can help minimize chemical modification requirements for the gRNA and removes RNP assembly as a limiting factor, often resulting in high editing efficiency[142]. However, similar to plasmids and mRNA, the RNP complex must be protected and cannot readily cross cell membranes. Electroporation is beneficial for zygotic and stem cell ex vivo gene editing[12,142]. However, this method is neither safe nor practical for in vivo gene editing. There are several direct injection routes for the brain, such as intrathecal, intrastriatal and intracerebroventricular injections[143]. However, researchers primarily utilize intravenous or subcutaneous administration for the delivery of RNP complexes in extrahepatic tissues. LNP formulations and exosomes can encapsulate and deliver RNP complexes systemically to cells[144]. Exosomes are natural extracellular vesicles composed of a lipid bilayer. This bilayer

Table 1 | Summary of the effect of chemical modifications tested in vitro and in cell-based assays

Chemical modification	Cas9		Refs.	Cas12a		Refs.
	in vitro	cell-based		in vitro	cell-based	
m^1A	×		63			
m^6A	✓	×	63,77,78	✓	×	80
m^5C	✓	×	77,78			
Ψ	✓	×	77,78			
$m^1Ψ$	✓	×	78			
s^2U	✓	×	63			
s^4U	✓		63			
DNA	✓	✓	38,92,93, 95,97	✓	✓	48,94
2′-OMe	✓	✓	38,64,66, 97–100	✓	✓	47,48,65
2′F-RNA	✓	✓	38,97–100	✓	✓	48,65
2′F-ANA	✓	×	38			
2′-NH_2				✓	✓	48
2′,4′-diOMe	×		38			
2′F-4′OMe	✓	×	38			
LNA	✓	✓	38,97			
cEt		✓	100			
BNA^{NC}	✓	✓	101			
ANA				✓	×	48
UNA	×		38			
OxNA				×		48
C4	×		38			
PS	✓	✓	64,66, 69,99	✓	✓	47,48,65
PACE	✓	✓	109,110			
thioPACE	✓	✓	64			
2′,5′-RNA	×		38			
PG	✓		113			

✓ = improved or unaffected editing activity; × = reduced or abolished editing activity. This table aims to serve as a general guide for which chemical modifications are effective versus ineffective in each CRISPR–Cas system. However, it should be noted that effects are often position-dependent and context-dependent. Therefore, these findings are not conclusive, and many chemistries are worth exploring further. ANA, arabinonucleic acid; BNA^{NC} *N*-methyl substituted bridged nucleic acid; C4, butanediol linker; cEt, constrained 2′-*O*-ethyl; 2′F-ANA, 2′-fluoro-arabinonucleic acid; 2′F-RNA, 2′-fluoro RNA; LNA, locked nucleic acid; m^1A, N^1-methyladenosine; $m^1Ψ$, N^1-methylpseudouridine; m^5C, 5-methylcytosine; m^6A, N^6-methyladenosine; 2′-NH_2, 2′-amino; 2′-OMe, 2′-*O*-methyl; OxNA, oxepane nucleic acid; PACE, phosphonoacetate; PG, phosphoryl guanidine; PS, phosphorothioate; Ψ, pseudouridine; s^2U, 2-thiouridine; s^4U, 4-thiouridine; thioPACE, thiophosphonoacetate; UNA, unlocked nucleic acid.

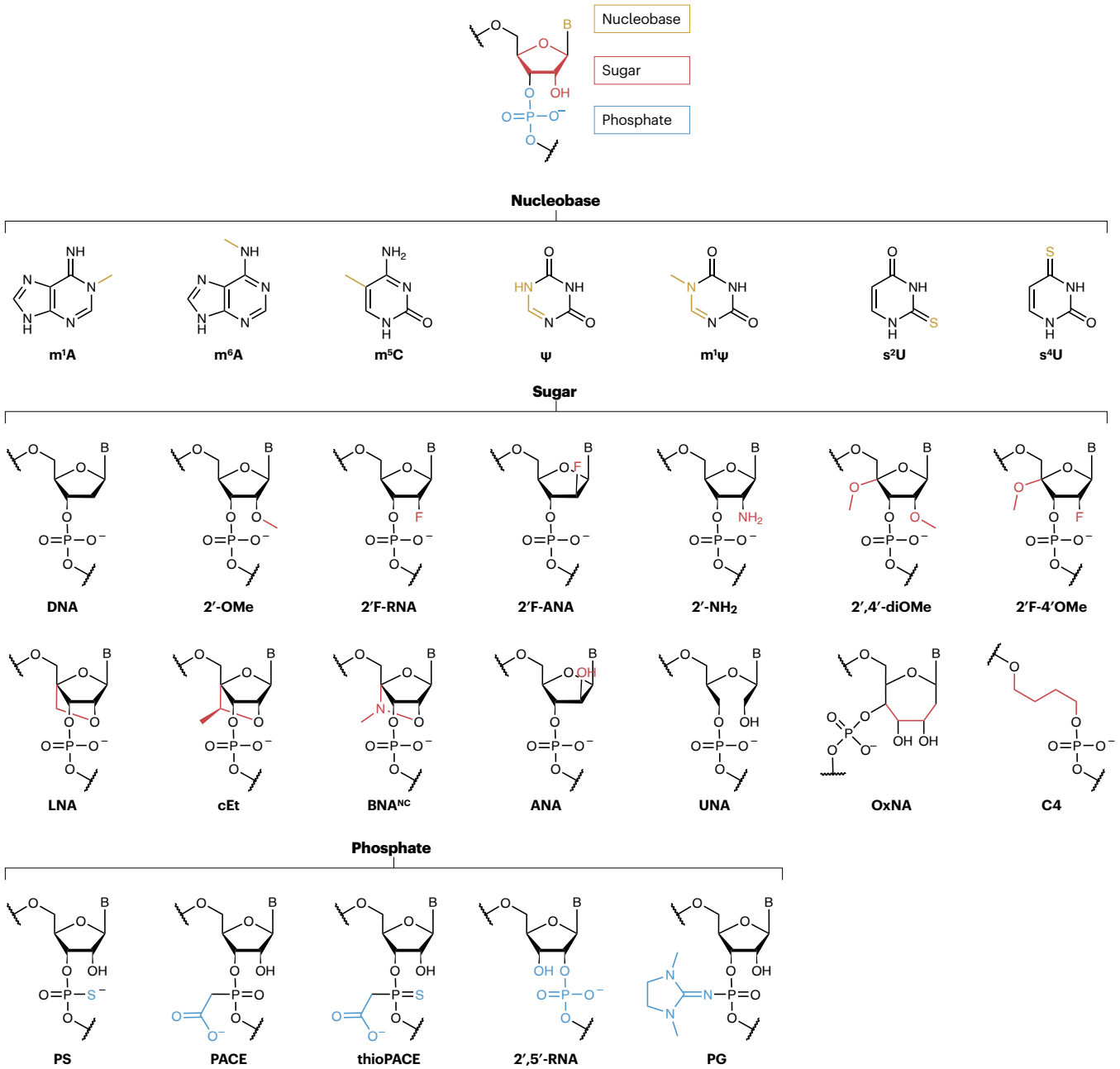


Fig. 5 | Chemical modifications used in CRISPR–Cas systems. These modifications fall into three categories: nucleobase (yellow), sugar (red) and phosphate (blue) modifications.

protects the RNP complex during circulation and prevents its recognition and destruction by immune cells[145]. Exosomes can be either derived from the target cell type or artificially functionalized with peptides for targeted delivery.

Next-generation CRISPR–Cas systems

CRISPR–Cas systems evolved as prokaryotic defence mechanisms against phages and selfish genetic elements, not for precise genetic manipulation in clinical applications[146]. Their introduction into mammalian cells to generate DSBs often causes undesirable side effects beyond the occasional off-target editing, to induce genotoxicity[147], large deletions[148], chromosomal translocations[149] and chromothrypsis[150]. The difficulty in controlling DNA repair outcomes has led to the development of alternative CRISPR–Cas effectors that do not generate DSBs. These systems utilize catalytically inactive 'dead' (that is, dCas9) or nickase (that is, nCas9) versions fused to other enzymes or factors that elicit a desired effect. Examples include DNA nucleobase conversion (base editing), introduction of new, short stretches of templated

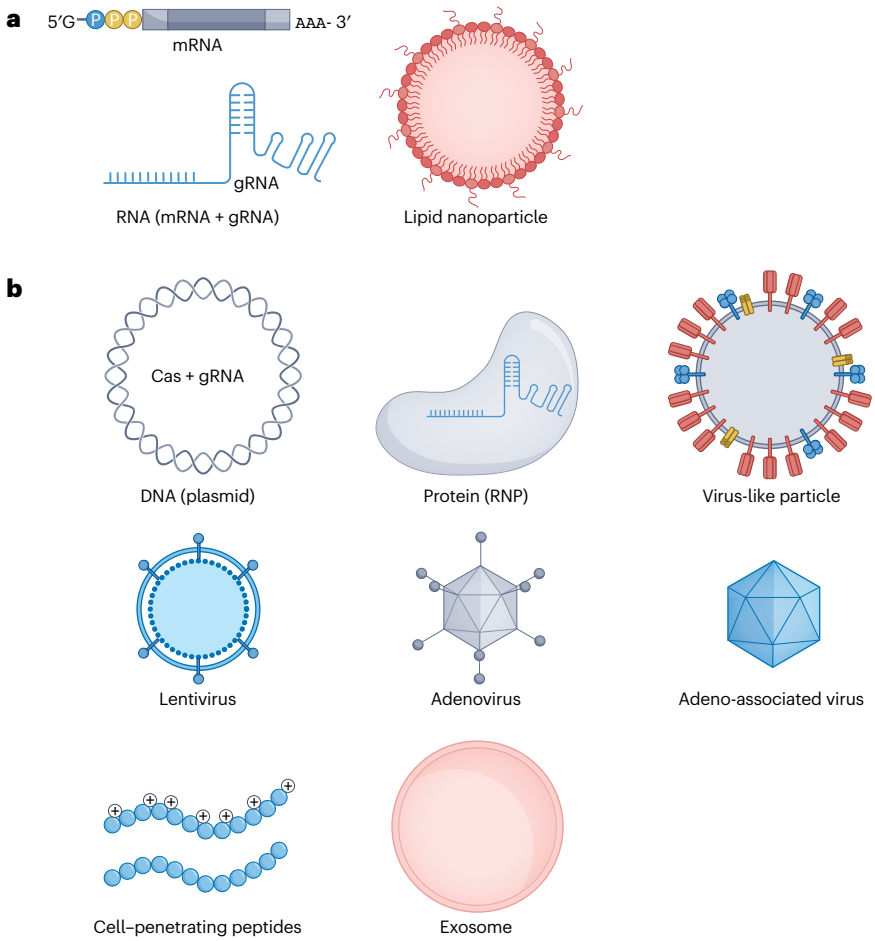


Fig. 6 | Delivery methods for CRISPR–Cas systems. a, CRISPR components (mRNA and guide RNA (gRNA)) and delivery system (lipid nanoparticles) that are being utilized in clinical trials. **b**, Components and delivery systems, including DNA plasmid (Cas protein and gRNA) and ribonucleoprotein (RNP), virus-like particles, lentivirus, adenovirus, adeno-associated virus, peptides and exosomes, that are in preclinical development.

sequence by reverse transcription (prime editing), and transient epigenome editing techniques such as transcriptional activation (CRISPRa) or transcriptional interference (CRISPRi)[3,151–157] (Fig. 7). In addition, targeting RNA with CRISPR–Cas systems like Cas13 has created new opportunities for gene-targeted therapies[158–161]. Importantly, many of the benefits and challenges of chemical crRNA engineering will extend to these next-generation systems, given that they still rely on RNA guides. For a more in-depth discussion of epigenome editing, we refer the reader to the review written by Villiger et al.[157].

Base editors and prime editors

Base editors (BEs) change nucleobase pairs from C·G to T·A or A·T to G·C, without creating DSBs or requiring donor templates[151,162] (Fig. 7a). They are composed of the gRNA and dCas9, or in some cases nCas9, fused to cytidine or adenosine deaminase enzymes. The RNA-guided system directs the deaminases to catalyse the chemical conversion of targeted bases, thereby changing the DNA sequence. Cytosine base editors convert C to U, which is then corrected to T during DNA replication or repair. Similarly, adenine base editors convert A to inosine (I), which is further corrected to G.

Chemical modifications of the mRNA encoding the BE protein and the sgRNA were shown to enhance base editing efficiency[109,163]. In non-human primates, LNP-based in vivo delivery utilized synthetic gRNAs with terminal 2′-OMe and PS modifications[138]. Substituting all uridine residues in mRNA with 5-methoxyuridine, alongside terminal 2′-OMe and PS modifications of the sgRNA, increased protein expression and significantly boosted A-to-G conversion rates[163]. Furthermore, using modified sgRNA (2′-OMe with PACE) in conjunction with BE4 mRNA led to a 40% enhancement in cytidine base editing efficiency compared with 2′-OMe-PS modifications, with a 250-fold increase in residual sgRNA levels[109].

Unlike BEs, which are limited to specific types of nucleobase conversions, prime editors allow for a wider range of high-precision editing, including small insertions, deletions and all 12 possible base-to-base conversions, without inducing DSBs[164] (Fig. 7b). These systems rely on nCas9 fused to a reverse transcriptase (RT) and an engineered gRNA referred to as prime editing guide RNA (pegRNA). The pegRNA consists of a spacer sequence that targets the genomic site, a primer binding site, which serves as the initiation sequence for RT-mediated synthesis, and the RT template sequence that encodes the intended edit.

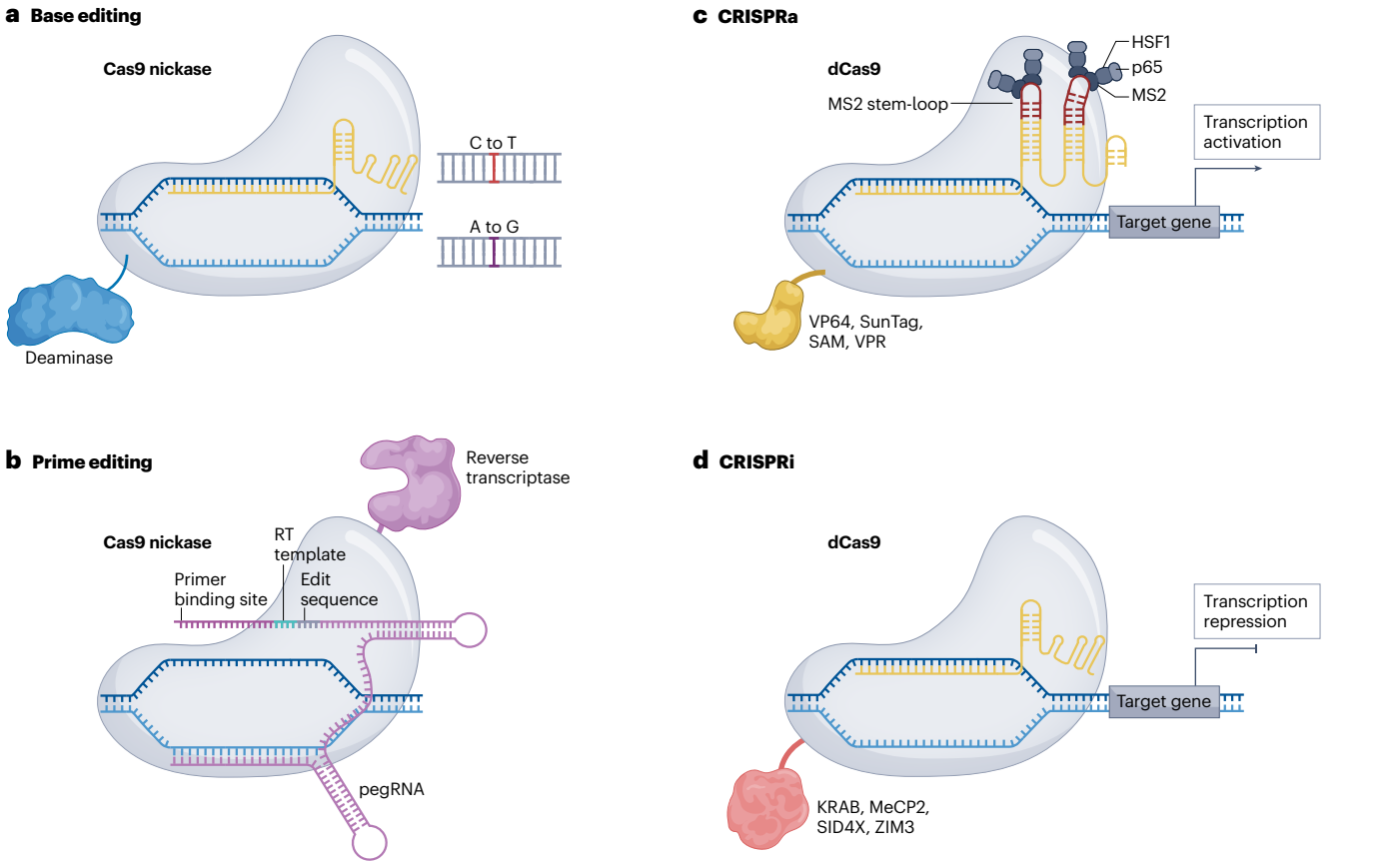


Fig. 7 | Engineered CRISPR–Cas-based editing without double-strand breaks. a, Base editing combines a Cas9 nickase (nCas9) with a deaminase enzyme, enabling single-base substitutions without causing double-strand DNA breaks. **b**, Prime editor comprises an nCas9 and reverse transcriptase (RT) that utilizes prime editing guide RNA (pegRNA) for precise DNA edits. The pegRNA comprises a single-guide RNA linked to an RT template and a primer binding site. **c**, Transcriptional activation (CRISPRa) consists of catalytically inactive (dead) Cas9 (dCas9) fused to transcriptional activators (such as VP64, VPR and SunTag). An engineered single-guide RNA incorporating MS2 aptamers recruits transcriptional activators P65 and HSF1 linked to an MS2 coat protein. The guide RNA targets a gene, the promoter, resulting in increased target gene expression. **d**, Transcriptional interference (CRISPRi) features dCas9 fused to a transcriptional repressor domain, like KRAB. The complex is directed to the gene target site to repress transcription.

The stability of the pegRNA greatly influences the efficacy of prime editing[165]. Exonucleolytic degradation results in truncated pegRNAs that compete with the full-length RNAs, resulting in decreased efficiency. Structured motifs at the 3′ end of pegRNA have been used to address the stability issue. For example, 3′ pseudoknots (evopreQ1 or mpknot) resulted in a three–four-fold enhancement in prime editing efficiency in various genomic contexts and cell types. Other motifs have included the Zikavirus exoribonuclease-resistant RNA motif (xr-pegRNA) and G-quadruplex structures, which provide 4.5-fold improvements in prime editing[166,167].

Chemical modifications of pegRNAs have also improved editing efficiency. Three terminal 2′-OMe and PS linkages were shown to enhance editing up to 3.1-fold in human cells[168]. In vivo studies have also demonstrated that an all-2′-OMe primer binding site with three terminal PS linkages in the pegRNA resulted in 2.8-fold enhancement[169]. Conversely, full 2′-OMe replacement of the RT template resulted in a significant decrease in activity. Full 2′-OMe modifications of the primer binding site were also well-tolerated in a split prime editor using DNA polymerase instead of a reverse transcriptase[170]. Unlike traditional prime editors with reverse transcriptase and pegRNAs, these systems utilize DNA polymerase and an unlinked sgRNA termed linear RNA prime editing template or a DNA polymerase template. Notably, introducing DNA modifications into the RT template enhanced the activity of this system.

Transcriptional activation (CRISPRa) or interference (CRISPRi)

Systems such as CRISPRa[156,171] and CRISPRi[156] utilize dCas9 for target DNA binding without cleavage. When guided to gene promoters, dCas9 can be fused to various transcription factors or epigenetic enzymes to upregulate or downregulate gene expression[3] (Fig. 7c,d). CRISPRa fuses dCas9 to multiple copies of the VP16 transactivator domain[172] or other activation domains, such as a tripartite activation module VP64-p65-Rta (VPR)[173]. These domains interact with the cell's transcriptional machinery, resulting in transcriptional upregulation. Alternatively, SunTag utilizes a repeating peptide array to fuse multiple VP64 activation domains per dCas9[174]. Synergistic activation mediator (SAM) utilizes the dCas9-VP64 with an engineered sgRNA scaffold that contains two MS2 aptamers that bind MS2 coat proteins fused to HS1

and p65 activation domains. This approach results in higher levels of gene activation than VPR and SunTag[175]. First-generation CRISPRi used a KRAB repressor domain fused to dCas9. This domain binds to DNA through its zinc finger motifs and recruits TRIM 28 to induce heterochromatin spreading that results in gene expression[176]. The addition of effectors like MeCP2 to KRAB has further increased transcriptional repression[177], and other domains, like SALL1-SDS3, have been reported to perform exceptionally well[178]. Few studies have explored chemically modified guides for CRISPRa or CRISPRi. Only CRISPRi has been reported to utilize chemically modified gRNAs by incorporating two relatively standard terminal 2′-OMe-PS linkages[178].

Targeting RNA

CRISPR–Cas systems that naturally target RNA instead of DNA, such as the Cas13 family of CRISPR effectors, are an emerging tool for therapeutic development[159,179]. The endonuclease activity of RNA-targeted Cas enzymes presents an alternative to RNAi or ASO-based gene-targeted therapeutics. However, nonspecific collateral cleavage of non-targeted RNA is an ongoing challenge that limits safety[180,181]. Whether chemically modified crRNAs can help control collateral cleavage or on/off-target editing for Cas13 systems is unknown. Only one study to date has specifically investigated the chemical modification of Cas13d crRNA, concluding that the most notable gains in activity and duration of RNA knockdown came from replacing three 3′ terminal nucleotides with 2′-OMe or PS, but not both[158]. Substituting larger sections or the entire crRNA with 2′-OMe led to a substantial loss in activity. However, a high-resolution molecular structure for RfxCas13d (formerly CasRx) has not been available, precluding any investigation of potential 2′-OH contacts between crRNA and Cas13d. RNA-targeted therapeutic applications will likely turn to catalytically inactive (dCas13) versions. These include recent attempts to improve *trans*-splicing technology, which can replace entire exons during pre-mRNA splicing[161,182], or dCas13 fusions to other effector enzymes, such as ADAR catalytic domains for site-specific and targeted A-to-I RNA editing[160].

CRISPR-based therapeutics

Exa-cel/CTX001 and reni-cel/EDIT-301 for blood disorders

The first CRISPR-based therapeutic to be granted clinical approval was exagamglogene autotemcel (exa-cel), formerly known as CTX001 (ref. 28) (Table 2). It was developed jointly by Vertex Pharmaceuticals and CRISPR Therapeutics to treat patients with either transfusion-dependent β-thalassemia (TDT) or sickle cell disease (SCD)[12]. Due to their monogenic nature and the Mendelian inheritance pattern of haemoglobin disorders, which simplify the targeting and contribute to their high prevalence in various populations, these diseases represent promising candidates for intervention by CRISPR-based medicines[183].

Both of these diseases are caused by mutations in the *HBB* gene, which encodes the β-globin subunit of adult haemoglobin (HbA). These mutations lead to defective or deformed erythrocytes[184–186]. Although TDT and SCD impact the correct formation of HbA, fetal haemoglobin (HbF) is unaffected[12]. HbF production is developmentally regulated by the transcription factor BCL11A, which represses γ-globin expression. Typically, HbF is replaced with HbA around birth; however, individuals with hereditary persistence of HbF have inactivated BCL11A variants that allow for γ-globin expression postnatally, with no clinical consequences[183]. Hereditary persistence of HbF imposes protection against clinical manifestations of the disease.

Exa-cel editing with CRISPR–Cas9 mimics naturally occurring BCL11A variants to restore erythrocyte function in patients with TDT and SCD[12]. The therapy involves CRISPR-edited autologous $CD34^+$ haematopoietic stem and progenitor cells (HPSCs) in which patient cells are collected, edited ex vivo, then returned via intravenous infusion[12,64]. Editing uses Cas9 RNPs assembled with sgRNAs bearing three terminal 2′-OMe-PS modifications. The sgRNA targets the *BCL11A* gene at the erythroid-specific enhancer region, thereby reducing its expression. This optimized strategy results in minimal cytotoxicity, high levels of gene editing and undetectable off-target editing[12].

An initial phase I clinical trial of exa-cel showed a favourable response in one patient with TDT and one with SCD. The approach resulted in 68.9–82.7% of allelic editing frequencies in both patients[12]. At month 15, the patient with TDT had 100% peripheral erythrocytes expressing HbF (F-cells), and the patient with SCD had 98.1% peripheral F-cells. A total of 97 patients were subsequently enrolled in the study, and a significant majority achieved the endpoints of transfusion-independence and freedom from vaso-occlusive crises[187,188]. In November 2023, exa-cel was granted conditional authorization by the Medicines and Healthcare products Regulatory Agency in the UK, making it the first CRISPR-based therapeutic approved by a regulatory agency. The FDA followed shortly after with their approval in December 2023 and January 2024 of the use of exa-cel to treat patients with TDT and SCD with vaso-occlusive crises, respectively.

Table 2 | Selected CRISPR-based medicines approved or in clinical development

Drug Name	Company	Disease	Gene Target	Platform	Current Clinical Development Stage	Ref.
Exagamglogene autotemcel or exa-cel (formerly CTX001)	Vertex Pharmaceuticals CRISPR Therapeutics	Transfusion-dependent β-thalassemia (TDT) Sickle cell disease (SCD)	*BCL11A*	Ex vivo	Clinically approved	12
Renizgamglogene autogedtemcel or reni-cel (formerly EDIT-301)	Editas Medicine	TDT SCD	*HBG1/2*	Ex vivo	TDT: phase I/II (NCT05444894) SCD: phase I/II (NCT04853576)	194
Nexiguran ziclumeran or nex-z (formerly NTLA-2001)	Intellia Therapeutics Regeneron Pharmaceuticals	Hereditary transthyretin amyloidosis	*TTR*	In vivo	Phase III (NCT06128629)	195
NTLA-2002	Intellia Therapeutics	Hereditary angioedema	*KLKB1*	In vivo	Phase III (NCT06634420)	200
VERVE-101, VERVE-102	Verve Therapeutics	Heterozygous familial hypercholesterolaemia	*PCSK9*	In vivo	VERVE-101: phase I (NCT05398029) VERVE-102: phase I (NCT06164730)	210

Similar to exa-cel, another ex vivo CRISPR-based therapeutic for TDT and SCD is renizgamglogene autogedtemcel (reni-cel, formerly known as EDIT-301), developed by Editas Medicine[189] (Table 2). Reni-cel also consists of CRISPR-edited autologous $CD34^+$ HPSCs; however, the CRISPR–Cas12a system is used and the *HBG1/2* gene is the target. The *HBG1/2* gene, which encodes the γ-globin subunit, is included in the β-globin locus along with the *HBB* gene[190]. The *HBG1/2* promoter region contains a proximal CCAAT-box motif near the activator NF-Y binding site and a distal CCAAT-box motif near the BCL11A repressor binding site. In adult-stage erythroid cells, BCL11A and its corepressors sterically block NF-Y binding, leading NF-Y and its coactivators to bind to the *HBB* promoter instead. Multiple single-nucleotide polymorphisms (SNPs) within the distal CCAAT-box motif that prevent BCL11A binding are often found in patients with hereditary persistence of HbF[191]. Reni-cel mimics disruptive SNPs in the BCL11A binding region by using CRISPR–Cas12a[189]. Use of the proprietary Cas12a system results in higher specificity and editing efficiency, larger deletions, and greater indel formation than the Cas9 system[189,192].

In preclinical studies, approximately 90% editing was observed in donor $CD34^+$ HPSCs, which confers 40–50% HbF expression in mice[193]. These levels were deemed therapeutically relevant, given that approximately 30% HbF compared with total haemoglobin is associated with the absence of symptoms for both diseases[189]. In a limited phase I/II clinical trial, two patients with severe TDT and seven patients with severe SCD were treated with reni-cel[194]. Treatment led to an increase in both HbF and total haemoglobin, with over 40% HbF observed after 4 months, and it was sustained throughout the follow-up period, thereby achieving the target levels[189]. Treatment also allowed patients with TDT to be transfusion-independent and patients with SCD to be vaso-occlusive crisis-free.

NTLA-2001 for hereditary transthyretin amyloidosis

Although blood disorders are readily targeted using the ex vivo approach, it is more challenging to target tissues. One CRISPR-based therapeutic that utilizes in vivo delivery is NTLA-2001, developed by Intellia Therapeutics and Regeneron Pharmaceuticals to treat patients with hereditary transthyretin (hATTR) amyloidosis[195] (Table 2). This progressive and fatal disease is caused by misfolding and aggregation of the TTR protein, leading to clinical manifestations such as polyneuropathy and cardiomyopathy. NTLA-2001 aims to target and knockout the *TTR* gene, which results in reduced TTR protein and improved patient outcomes. Given that the disease is monogenic and TTR is primarily expressed in the liver, hATTR amyloidosis is a common target for RNA therapeutics, including the first FDA-approved siRNA therapeutic, patisiran[196]. NTLA-2001 consists of a sgRNA targeting the *TTR* gene and an mRNA encoding the *Sp*Cas9 protein co-formulated in an LNP with lipid tropism. Given that the RNP complex is not assembled prior to entering cells, the sgRNA requires more extensive chemical modification. Based on an earlier publication, Intellia demonstrated the in vivo activity of a sgRNA modified by 2′-OMe-PS on the ends and 2′-OMe within internal stem loops[57,64].

Preclinical studies in transgenic mice showed that NTLA-2001 knocks down TTR expression after a single administration[195]. Additionally, no off-target editing events were observed at any of the seven top predicted loci in primary human hepatocytes, even at multiples of the intended therapeutic exposure. Editing in cynomolgus monkeys with a surrogate sgRNA revealed that the gene was repaired via the NHEJ mechanism to create a single-nucleotide insertion of 'A' in 98.8% of sequences, resulting in a frameshift mutation[195]. In a limited phase I clinical trial involving six patients with hATTR amyloidosis with polyneuropathy, a single intravenous infusion dose of 0.3 mg kg^{-1} showed an 87% mean reduction in baseline serum TTR protein levels at day 28 (ref. 195). The cohort treated with the higher dose of 1 mg kg^{-1} experienced a 93% mean reduction in baseline serum TTR at day 28 (ref. 137). Following these positive results, the phase I clinical trial expanded to include 72 patients, with a portion of these patients enrolled in the cardiomyopathy arm. NTLA-2001, now known as nexiguran ziclumeran or nex-z, has advanced to pivotal clinical trials for both polyneuropathy and cardiomyopathy[197].

NTLA-2002 for hereditary angioedema

Following the success of the NTLA-2001 studies, Intellia Therapeutics developed NTLA-2002 to treat patients with hereditary angioedema (Table 2). This disease is primarily caused by multiple mutations throughout the *SERPING1* gene, which cause either deficiency (type 1) or dysfunction (type 2) of the C1 esterase inhibitor (C1-INH)[198]. C1-INH regulates the contact activation pathway, including the cleavage and release of bradykinin high-molecular-weight kininogen by plasma kallikrein. Bradykinin plays a role in inflammation and swelling seen in angioedema attacks by increasing vascular permeability. In rare cases of individuals with Fletcher factor deficiency, reduction in plasma prekallikrein has no major clinical consequences, aside from prolonged activated partial thromboplastin time[199]. Therefore, it is suggested that knockout of the *KLKB1* gene, which encodes plasma kallikrein B1, should be an effective way to treat patients with hereditary angioedema by reducing total plasma kallikrein levels[200].

Given that plasma kallikrein is primarily expressed in the liver, NTLA-2002 uses the same design as NTLA-2001 with the sequence of the sgRNA guide region reprogrammed to target the *KLKB1* gene[200], demonstrating the modularity of this platform. In a phase I clinical trial involving ten patients with either type 1 or type 2 hereditary angioedema, a single administration of NTLA-2002 resulted in sustained reduction in total plasma kallikrein levels[200]. Relative to baseline, patients treated with 25 mg, 50 mg or 75 mg saw reductions of 67%, 84% and 95%, respectively. Based on previous studies, it was determined that a reduction of >60% of total plasma kallikrein is associated with near-complete control of attacks[201,202]. This is further supported by the data in this study, given that, although patients were allowed to remain on long-term prophylaxis drugs, all patients prescribed these drugs withdrew from using them following NTLA-2002 treatment[200]. Additionally, patients were allowed to continue to use on-demand therapies during attacks; however, the number of monthly attacks either decreased or stopped altogether for each patient, so that frequent use of on-demand treatment was no longer necessary. As seen with NTLA-2001, all adverse events were graded as mild to moderate. Based on these positive data, the study advanced to a phase II clinical trial, testing the two lowest doses and the requirement of prophylaxis withdrawal before NTLA-2002 administration.

VERVE-101 and VERVE-102 for heterozygous familial hypercholesterolaemia

Additional examples of LNP-delivered liver therapies are VERVE-101 and VERVE-102, developed by Verve Therapeutics[197] (Table 2). These therapies utilize ABE8.8-m base editing technology along with gRNA incorporating 2′-OMe and PS modifications, designed for the treatment of heterozygous familial hypercholesterolaemia (HeFH). HeFH is an autosomal co-dominant disorder characterized by high levels of low-density lipoprotein cholesterol (LDL-C), resulting in premature atherosclerotic

Glossary

2′-OH contacts
Hydrogen bonding interactions between the 2′-hydroxyl (2′-OH) groups of the gRNA nucleotides and the Cas amino acids, as well as between nucleotides.

Base editors
(BEs). Systems consisting of a dCas9 fused to either a cytidine deaminase, for cytosine base editors, or an adenosine deaminase, for adenine base editors, which allow for the precise conversion of specific DNA bases (such as, C to T or A to G) without double-strand breaks.

Chemical modification
Either a naturally occurring or synthetic modification to the phosphate, sugar or nucleobase of nucleotides.

Click chemistry
A biocompatible chemical reaction that allows for efficient and specific joining of two biomolecules.

CRISPR RNA
(crRNA). An RNA molecule composed of a spacer-derived guide region and a repeat-derived trans-activating CRISPR RNA-pairing or 5′ handle region.

dCas9
A Cas9 protein with mutations in both nuclease domains (RuvC and HNH), resulting in a catalytically inactive enzyme still capable of binding to DNA via guide RNA.

Double-strand breaks
(DSBs). DNA damage types in which both strands of the DNA double helix are cleaved, forming either a blunt or staggered break.

Ex vivo gene editing
A therapeutic approach in which patient cells are isolated, edited outside of the body and subsequently reintroduced back into the patient.

Guide RNA
(gRNA). A generalized term for an RNA molecule that directs the CRISPR–Cas effector enzyme to a specific DNA sequence through complementary base pairing.

Homology-directed repair
(HDR) A precise DNA repair pathway guided by a homologous DNA template to repair double-strand breaks.

In vivo gene editing
A therapeutic approach in which the gene editing components are delivered directly into a patient via local or systemic delivery.

Lipid nanoparticle
(LNP). A vesicle composed of lipid moieties used to encapsulate nucleic acids or proteins and deliver these therapeutic agents into cells.

nCas9
A Cas9 protein with either the RuvC or HNH catalytic domain mutated, resulting in a Cas9 that can introduce a single-strand break (that is, a nick) into DNA by cleaving only one DNA strand.

Non-homologous end joining
(NHEJ). A DNA repair pathway in which double-strand breaks are repaired by directly joining the ends, often resulting in insertions or deletions at the site.

Non-target strand
The DNA strand opposite the target strand that contains the protospacer adjacent motif and remains unbound during CRISPR–Cas-mediated cleavage.

Off-target effects
Unintended cleavages or modifications of DNA at sites other than the intended target sequence.

Prime editing guide RNA
(pegRNA). An RNA molecule used in prime editing that combines the properties of a gRNA, a reverse transcriptase template and a template sequence encoding the desired edit to direct the fusion protein to the target DNA site.

Prime editors
Systems consisting of an nCas9 fused to a reverse transcriptase enzyme and guided by a pegRNA to enable precise edits without double-strand breaks.

Protospacer adjacent motif
(PAM). A short, specific DNA sequence adjacent to the target site that is essential for the recognition and binding of the Cas enzyme.

Ribonucleoprotein (RNP) complex
A complex composed of both RNA and protein.

R-loop
A nucleic acid structure that forms when an RNA strand binds to one strand of a DNA double helix, creating an RNA:DNA hybrid duplex and displacing the other DNA strand.

Seed region
The first 5–10 nucleotides directly adjacent to the PAM that initiate base pairing between the gRNA and the target strand.

Single-guide RNA
(sgRNA). An RNA molecule created by fusing the Cas9 crRNA and trans-activating CRISPR RNA into a single construct.

Sugar pucker conformations
Conformations of the ribose ring in a nucleotide, with C2′-endo corresponding to DNA-like sugars with B-form helical structures and C3′-endo corresponding to RNA-like sugars with A-form helical structures.

Target strand
The DNA strand that is complementary to the gRNA.

Trans-activating CRISPR RNA
(tracrRNA). An RNA molecule that hybridizes to the crRNA and anchors the gRNA construct to Cas9 through its stem loop structures.

Transcriptional activation
(CRISPRa). A technique using dCas9 fused to transcriptional activation domains and directed by a gRNA to specific genomic loci to upregulate gene expression.

Transcriptional interference
(CRISPRi). A method using dCas9 fused to repressor domains to repress gene expression when guided to specific genomic loci by a gRNA.

Viral vectors
Modified viruses used to deliver nucleic acids into cells for therapeutic purposes.

cardiovascular hypercholesterolaemia[203]. HeFH is caused by mutations in several genes responsible for cholesterol metabolism, including *PCSK9*, *LDLR* and *APOB*, resulting in elevated LDL-C levels[204–207]. Proprotein convertase subtilisin/kexin type 9 (PCSK9) is produced in the liver and released into the bloodstream, where it binds to LDL receptors (LDLRs) on hepatocyte surfaces and targets them for degradation[208]. PCSK9 reduces the number of LDLR on the liver surface, thereby limiting the liver's ability to remove LDL-C from the bloodstream. Notably,

loss-of-function mutations in *PCSK9* have been shown to lower LDL-C levels in the blood[209]. Individuals with loss-of-function mutations in the *PCSK9* gene had an 88% decrease in the occurrence of coronary heart disease[209]. Therefore, reducing PCSK9 levels can serve as an effective strategy for lowering LDL-C in patients with HeFH and thereby reduce coronary heart disease incidents.

Both VERVE-101 and VERVE-102 utilize the same payloads; however, VERVE-102 uses a *N*-acetylgalactosamine-LNP delivery system to enhance hepatic delivery[138,210]. These systems target the *PCSK9* gene, which is predominantly expressed in hepatocytes. The introduction of an A-to-G base edit in the *PCSK9* gene effectively disrupts the production of PCSK9, leading to the reduction of LDL-C in the blood. Preclinical studies in non-human primates demonstrated significant reduction in both PCSK9 protein serum levels and LDL-C blood levels. Specifically, a single dose of 0.75 and 1.5 mg kg^{-1} of VERVE-101 resulted in a 45% and 69% reduction in LDL-C, respectively. A clinical trial in patients with HeFH showed that a single infusion of VERVE-101 at a dose of 0.45 mg kg^{-1} reduced LDL-C cholesterol by 39–48%[211,212]. Patients receiving a higher dose of 0.6 mg kg^{-1} experienced a 55% reduction in LDL-C levels, which was maintained for up to 180 days. However, Verve Therapeutics suspended enrolment for the clinical trial of VERVE-101 due to one patient experiencing a severe increase in serum alanine aminotransferase levels[213]. Fortunately, the adverse events were fully resolved within a few days. The company has redirected its focus towards the VERVE-102 trial.

Conclusions

CRISPR technology has revolutionized molecular biology, biomedical research and medicine. It allows researchers to knockout genes, add new genes and sequence elements, turn transcription on or off, and add epigenetic markers to the genomes of organisms. It has also more recently enabled editing at the RNA sequence level, potentially allowing similar gains in gene therapy with lower risks. Human gene editing using CRISPR has already demonstrated the ability to treat previously incurable diseases in the clinic, including TDT, SCD and hATTR amyloidosis, by intentionally changing the DNA inside cells of patients[35,159].

Chemical modification of gRNA has contributed to their success as therapeutics. Commonly used modifications include PS linkages, 2′F-RNA and 2′-OMe, which are well-tolerated at the termini and select internal positions of Cas9 and Cas12a gRNAs. However, these chemical modifications, and even those that have been utilized in CRISPR–Cas studies broadly, represent only a fraction of chemistries explored previously for nucleic acid therapeutics. Therefore, there remains a long list of others that might be applied to CRISPR–Cas systems. These include modifications to nucleobases, internal modifications to protect against endonucleases and ribose modifications to both understand and overcome the 2′-hydroxyl barrier. Although there is still progress to be made, the future of CRISPR-based therapeutics is incredibly bright, with clinical successes in hand and many exciting applications on the horizon.

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