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The next generation of CRISPR–Cas technologies and applications

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Abstract

The prokaryote-derived CRISPR–Cas genome editing systems have transformed our ability to manipulate, detect, image and annotate specific DNA and RNA sequences in living cells of diverse species. The ease of use and robustness of this technology have revolutionized genome editing for research spanning from fundamental science to translational medicine. Initial successes have inspired efforts to discover new systems for targeting and manipulating nucleic acids, including those from Cas9, Cas12, Cascade and Cas13 orthologs. Genome editing by CRISPR–Cas can utilize non-homologous end joining (NHEJ) and homologous-directed repair (HDR) for DNA repair, as well as single-base editing enzymes. In addition to targeting DNA, CRISPR–Cas-based RNA-targeting tools are being developed for research, medicine and diagnostics. Nuclease-inactive and RNA-targeting Cas proteins have been fused to a plethora of effector proteins to regulate gene expression, epigenetic modifications and chromatin interactions. Collectively, these advances are considerably advancing our understanding of biology and propelling CRISPR–Cas-based tools towards clinical use in gene and cell therapies.

INTRODUCTION

The ability to modulate and edit genetic information is crucial for studying gene function and uncovering biological mechanisms. Since the first demonstration of producing specific DNA fragments with restriction enzymes in 1971, scientists have been harnessing prokaryotic molecules for gene editing¹. In addition to restriction enzymes², classes of DNA-modifying tools include recombinases³ and programmable nucleases such as

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meganucleases, zinc finger nucleases, transcription activator-like effector nucleases and clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated endonuclease (Cas) systems⁴. DNA-binding proteins that modify specific loci have tremendously advanced science, biotechnology and medicine. However, the complexity of developing modular DNA-binding proteins to bind at custom targets often requires protein engineering expertise. In the past decade, the CRISPR–Cas9 technology has transformed genome engineering by removing the need for any expertise in engineering custom targeted DNA-binding proteins, because the target specificity of CRISPR–Cas9 relies on base pairing of nucleic acids rather than protein–DNA recognition.

In nature, the CRISPR-Cas system is a prokaryotic adaptive immunity mechanism used to cleave invading nucleic acids⁵. An assortment of CRISPR-Cas systems exists across diverse species of bacteria and archaea, which differ in their components and mechanisms of action. For example, class 1 CRISPR–Cas systems comprise multi-protein effector complexes whereas class 2 systems have a single effector protein; overall there are 6 CRISPR–Cas types and at least 29 subtypes^{6–8}, and this list of types and subtypes is undergoing rapid expansion. All CRISPR-Cas systems rely on CRISPR RNA (crRNA) or, in experimental CRISPR–Cas9 systems, on the guide RNA (gRNA) for guidance and targeting specificity (Figure 1). Following hybridization of the spacer [G] part of the crRNA to a target sequence that is positioned next to a protospacer adjacent motif (PAM) (or a protospacer flanking sequence (PFS) in type VI systems), the Cas nuclease cleaves the target nucleic acid. Thus, site-specific cleavage at any locus containing a PAM or PFS can be achieved by retargeting CRISPR-Cas systems with designed crRNAs containing appropriate spacer sequences. The discovery and development of type II CRISPR-Cas9 systems and the ease of their use have led to rapid adoption and development of a great range of applications, spanning from fundamental to translational science and medicine⁹. In turn, the early successes have inspired efforts to discover new CRISPR–Cas systems and develop novel genome engineering applications.

In this review, we discuss recent advances in CRISPR–Cas tools for gene editing and epigenetic modulation, before describing a diverse range of new CRISPR–Cas functions. We discuss next-generation applications such as perturbation of the transcriptome and non-coding genome, single-base editing, genome-wide pooled screens, chromatin reorganization and therapeutic potential moving towards clinical studies.

Advances in genome editing

CRISPR-Cas systems are modular DNA-binding or RNA-binding proteins that can be engineered to bind specific sequences by designing crRNAs or gRNAs containing spacers that complement the target sequence. In addition to binding specific nucleic acid sequences, these proteins also function as nucleases and thus can be used for programmable genome editing.

Broader targeting capacity

By harnessing the unique attributes of various CRISPR-Cas systems, such as PAM specificity, protein size and nuclease activity, a range of CRISPR–Cas-based DNA-targeting

tools have been developed for genome editing applications. Additionally, the development of methods for the detection of on-target and off-target interactions has advanced the targeting specificity of CRISPR-Cas tools (Supplementary information Box 1).

CRISPR-Cas9 tools—Cas9 belongs to the class 2 type II CRISPR systems and is the most widely used genome editing tool. Specifically, *Streptococcus pyogenes* Cas9 (SpCas9) was the first to be used outside of prokaryotic cells¹⁰ and reprogrammed for genome editing in mammalian cells^{11,12}; it remains the most commonly used Cas9. Following DNA target recognition, SpCas9 typically generates a blunt double-strand break (DSB) (Figure 1a)¹³. DNA targeting by SpCas9 relies on the 20-nucleotides long spacer and on the PAM 5'-NGG^{10,14}. Cas9 systems are dual-RNA-guided: a crRNA is responsible for DNA targeting and also hybridizes with the trans-activating crRNA (tracrRNA), which is responsible for forming the complex with Cas9^{15,16}. The crRNA and tracrRNA functions can be recapitulated with an engineered single guide RNA (gRNA)¹⁰ (Figure 1a).

Recognition of the PAM 5'-NGG (N represents any nucleotide) limits the availability of SpCas9 target sites in the human genome to an average of one target site for every eight base pairs⁹. To increase the availability of target sites, directed evolution [G] approaches have generated variants with altered PAM specificities (Table 1)^{17,18}. For example, an expanded-PAM SpCas9 variant, xCas9, recognizes 5'-NG, 5'-GAA and 5'-GAT PAM sequences¹⁸. Another motivation for engineering Cas9 variants is to increase targeting specificity. In fact, several studies have described mutated Cas9 variants with reduced off-target cleavage following expression of Cas9 and gRNAs from plasmids¹⁹⁻²² or their delivery as ribonucleoprotein (RNP) complexes²³. Alternatively, on-target CRISPR-Cas specificity has been increased by engineering secondary structures in the form of RNA hairpins on the spacer region of gRNAs, which increase the thermodynamic barrier to crRNA or gRNA strand invasion at off-target sites while generally maintaining on-target activity²⁴.

The discovery and development of additional Cas9 orthologs that recognize different PAM sequences has provided a greater choice of target sites. For example, *Streptococcus thermophilus* Cas9 recognizes the PAM 5'-NNAGAAW (W represents A or T)^{11,25} and *Neisseria meningitidis* Cas9 recognizes 5'-NNNNGATT²⁶⁻²⁸. These Cas9 orthologs have been repurposed for DNA targeting in bacteria and mammalian cells. Furthermore, the PAM recognized by *Staphylococcus aureus* Cas9 (SaCas9) is 5'-NNGRRT (R represents A or G)²⁹. Notably, SaCas9 gene editing efficiencies are comparable to SpCas9 and the smaller size of SaCas9 (1,053 amino acids compared to 1,368 amino acids of SpCas9) has enabled its use in size-restricted delivery vectors such as adeno-associated virus (AAV)²⁹. More recently, an even smaller Cas9 ortholog, from *Campylobacter jejuni* (984 amino acids), was reported to recognize the PAM 5'-NNNRYM (V represents A, C or G; Y represents C or T)³⁰ and used for targeted genome editing *in vivo*³¹. Additional efforts to identify Cas9 orthologs has resulted in the discovery of CasX (980 amino acids), the smallest Cas9 to date³².

CRISPR-Cas12a—Another class II RNA-guided endonuclease that has been reprogrammed for gene editing in human cells is Cas12a (formerly Cpf1)³³. As a type V system, Cas12a generates a staggered cut with a 5' overhang at DNA target sites and does

not use a tracrRNA (Figure 1b). In contrast to the generation of blunt ends by Cas9, production of staggered ends by Cas12a may be advantageous for applications such as integrating DNA sequences in a precise orientation. Additionally, Cas12a can cleave crRNA arrays [G] to generate its own crRNAs. This crRNA processing ability facilitates the use of a single customized crRNA array for simplified multiplexed genome editing with multiple crRNAs³⁴.

Cas12a from *Acidaminococcus* spp. (AsCas12a) and *Lachnospiraceae* spp. (LbCas12a), the first Cas12a orthologs that were shown to have activity in mammalian cells, recognize the PAM sequence 5'-TTTV upstream of the target sequence. To improve their genome editing activity, an enhanced AsCas12a variant (enAsCas12a) has been engineered³⁵. To increase the targeting range of Cas12a, AsCas12a variants have recently been engineered to recognize the PAMs 5'-TYCV and 5'-TATV³⁶, or PAMs 5'-VTTV, 5'-TTTT, 5'-TTCN and 5'-TATV³⁵. The unique features and cutting mechanism of Cas12a provides a genome editing tool that expands the CRISPR toolbox.

Cascade and Cas3—Type I systems of the class 1 category are the most common type of CRISPR-Cas systems in nature, comprising a multimeric DNA-targeting complex termed Cascade and the endonuclease Cas3 (Figure 1c). Before recruiting Cas3 to a target DNA sequence, Cascade must first bind to DNA through PAM and spacer recognition³⁷⁻⁴². Cascade offers greater target site flexibility owing to its promiscuous recognition of PAM sequences⁴³. Recruitment of Cas3 generates a single-stranded nick followed by target DNA degradation through 3' to 5' exonuclease activity^{37,39,44,45}. Both the nickase and helicase activities of Cas3 are essential for the degradation of foreign DNA in prokaryotes³⁸. The unique cutting mechanism of Cas3 is being harnessed as an antimicrobial tool by directing native or exogenous type I systems to bacterial genomes for degradation and subsequent cell death⁴⁶. Exploration to repurpose the nickase, helicase and exonuclease activities of Cas3 may lead to new applications in mammalian cells.

In type I systems, crRNA arrays are processed by the Cascade subunit Csy4⁴⁷. Like Cas12a, this endonuclease activity has been repurposed for directed RNA processing. For example, the *Pseudomonas aeruginosa* type I-F Csy4 has been used for generating multiple Cas9 gRNAs in human cells^{48,49}.

Mechanisms and uses of gene editing

Gene editing nucleases, including Cas9, function by generating targeted DNA breaks that induce the DNA damage response and stimulate repair by various endogenous mechanisms⁵⁰. Use of the unique characteristics of the different DNA repair mechanisms has enabled the development of specific genome editing strategies.

NHEJ versus HDR—Eukaryotes predominantly repair DSBs through the error-prone non-homologous end joining (NHEJ) pathway, which leads to accumulation of small insertions or deletions (indels [G]) following repeated cycles of break and repair (Figure 2a). Alternatively, a repair template with homology to the target site can be delivered with Cas9 to stimulate the error-free homology-directed repair (HDR), but typically at a lower efficiency than NHEJ-mediated repair (Figure 2b). NHEJ can be used to produce gene

knockouts (deletions) whereas HDR can be used to introduce a specific change in the targeted genomic site, such as a point mutation or insertion of a longer segment of DNA. Increasing the efficiency of HDR following nuclease-mediated DNA breakage is widely pursued to fully harness the power of genome editing to introduce precise genomic alterations⁵¹⁻⁵⁵.

Gene deletions—Following Cas9 cleavage, NHEJ-mediated DNA repair can be harnessed to create gene knockouts. When targeting a coding exon, indel-mediated frameshift mutations, which also typically introduce premature stop codons downstream of the target site, will disrupt gene expression. Alternatively, by simultaneously targeting two sites in a gene, a deletion can be generated between the DSBs^{11,56-58}, including megabase-size deletions⁵⁹ (Figure 2a). A systematic exploration of Cas9-mediated deletion efficiencies showed an inverse correlation between deletion size and its frequency⁶⁰. In addition to studies in cells, strategies have been developed for facilitating heritable genomic deletions in organisms such as zebrafish⁶¹ and mice⁶²⁻⁶⁴. The wide spectrum of possible Cas9-mediated genomic deletions is accelerating the investigation of genes and genetic elements.

Gene insertions—Inserting a DNA sequence encoding an epitope tag or a fluorescent protein into protein-coding genes to monitor endogenously-expressed proteins is a valuable strategy for studying protein function in native cellular settings. Cas9-mediated and NHEJ-mediated gene tagging strategies have been developed based on the integration of linear DNA fragments at nuclease cleavage sites. In homology-independent targeted integration (HITI), a tag is flanked with gRNA target sites, so that Cas9 can simultaneously release it from a plasmid and cleave a recipient genomic target adjacent to the gene of interest⁶⁵ (Figure 2a). Generic plasmid-based systems to create endogenous amino-terminal⁶⁶ or carboxy-terminal^{66,67} gene–tag fusions using non-target-specific universal donor sequences have also been developed. Large-scale gene tagging is now possible due to the simplicity of these modular Cas9-mediated systems. HITI utilizes NHEJ for DSB repair, creating two problems: generation of indels and donor integration in random orientation. To overcome these obstacles, donor sequences can be flanked with homology arms. To circumvent the need for molecular cloning of target-specific donor sequences, single-strand DNA (ssDNA) were used to tag endogenous human genes with GFP-coding sequences⁶⁸ (Figure 2b). Mice with multiple precise single point mutations were generated using multiplexed HDR in mouse embryonic stem cells⁶⁹. Cancer modeling in mice can also be achieved by HDR-mediated insertion of missense gain-of-function mutations⁷⁰. To generate conditional knockout mice at high efficiency by inserting large regulatable cassettes, RNPs were co-delivered with long ssDNA donors containing short homology arms⁷¹. Recently, an HDR-dependent strategy termed CORRECT (consecutive re-guide or re-Cas steps to erase CRISPR–Cas-blocked targets) was developed for producing scarless targeted knock-in of disease-relevant mutations⁷². By making variations in the donor template, edited cell lines, including human pluripotent stem cells, can be generated with pathogenic mutations and with additional, silent mutations that block subsequent target-site recognition by the nucleases and formation of NHEJ-mediated indels⁷³ (Figure 2b).

Translocations—During cancer development, oncogenic fusion genes are frequently created through chromosomal translocations. Translocations can be mediated by illegitimate NHEJ of DSBs located at two non-homologous chromosomes. To generate models for studying the oncogenic properties of fusion proteins, simultaneous Cas9-mediated cleavage at two genomic loci has been used to engineer cancer-relevant translocations in human cells^{74,75}. Cas9-induced chromosomal rearrangements leading to oncogenic gene fusions have been recapitulated also in mice⁷⁶. These genetically engineered models are important for understanding tumorigenesis and for developing therapeutic strategies against oncogenic fusion proteins.

Single-base editing—The most common genetic variants associated with human disease are point mutations. An ability to edit single nucleotide bases is important for creating genetic disease models and developing corrective therapeutics. Targeted HDR-mediated single-base editing can be achieved by co-delivering Cas9 with a homologous donor sequence that contains the edited nucleotide of choice⁷². However, such strategies remain inefficient, particularly in post-mitotic cells with decreased HDR activity. Additionally, the need to create DSBs to induce efficient HDR carries the possibility of off-target mutagenesis, and even on-target activation of DNA repair pathways can have adverse consequences on cell viability^{77,78}.

For improved single-base editing, tools have been developed that utilize Cas9 nickase (nCas9) or catalytically deficient Cas9 (dCas9) for site-specific targeting without generating DSBs. For direct conversion of single nucleotides, dCas9 or nCas9 have been fused to cytidine deaminases. Fusion with deaminases such as rat APOBEC1 and lamprey cytidine deaminase 1 can achieve targeted C→T (or G→A) nucleotide conversions within a 5-bp activity window located within the spacer sequence^{79,80} (Figure 2c). Cellular DNA repair responses can antagonize this process and restore edited bases, therefore a uracil glycosylase inhibitor was also used to prevent base excision repair and increase the efficiency of base editing⁷⁹⁻⁸¹. A third generation editor (BE3) containing APOBEC1 fused to a 16-residue XTEN linker, nCas9 and a uracil glycosylase inhibitor (APOBEC1–XTEN–dCas9(A840H)-UGI) can achieve permanent conversion of 15–75% of a target nucleotide in mammalian cells⁷⁹. Furthermore, BE3 has accomplished base editing *in vivo* through RNP-mediated protein delivery to mouse and zebrafish embryos^{82,83}, AAV-mediated delivery *in utero* to mice⁸⁴, and injection of mRNA and gRNA to human embryos^{85,86}. In adult mice, BE3 was used to introduce site-specific nonsense mutations into the *Pcsk9* gene, which resulted in lowered cholesterol levels⁸⁷.

Continued development of BE3 has resulted in improved single-base editing. For example, improved Cas9-mediated targeting specificity has been achieved by combining BE3 with a high-fidelity Cas9⁸³. For optimization of base editing, lengthening of the linker between the fused proteins and adding a second copy of the uracil glycosylase inhibitor has led to fourth generation base editors engineered from SpCas9 (BE4) and SaCas9 (SaBE4)⁸⁸. The base-targeting range has continued to expand following fusion of APOBEC1 to catalytically inactive dLbCas12a, which recognizes a T-rich PAM and has a 6-bp activity window⁸⁹. Recently, enAsCas12a was used for enhanced base-editing activity³⁵. To narrow the editing window at targets with potential C→T bystander alterations, base editors have been

developed with human APOBEC3A for use in human cells^{90,91} and plants⁹². Specifically, eA3A-BE3, an engineered APOBEC3A domain (eA3A) fused to BE3, preferentially deaminates cytidines according to a TCR>TCY>VCN hierarchy⁹⁰. Additionally, APOBEC3A-mediated base editing can be achieved in regions with high DNA methylation levels and CpG dinucleotide content⁹¹.

Recently, the base editor toolbox has been expanded to adenine base editors (ABEs), which can perform targeted A→G (or T→C) nucleotide conversions⁹³. A seventh-generation ABE with the highest reported editing efficiencies and on-target activities was developed using directed evolution and protein engineering of a tRNA adenosine deaminase⁹³. Optimized and enhanced cytidine and adenine base editors include BE4max, AncBE4max and ABEmax⁹⁴.

Unbiased analyses of base-editing specificity is particularly difficult given the prevalence of single-base substitutions in the human genome and the frequency of sequencing errors. An early analysis of base editor specificity revealed off-target sites that are different from what was detected in cells treated with Cas9 alone⁹⁵. More recently, widespread gRNA-independent off-target activity was reported for cytosine base editors in both plants and mice,^{96,97} indicating the existence of base-editing activity that is independent of Cas9–DNA interactions. Therefore, future efforts will likely focus on strategies to restrict base editing activity to intended targeted sites.

With the abundance of known point mutations associated with genetic disease, single base editors can be used to make animal models with nonsense mutations or single amino acid substitutions. Moreover, the therapeutic potential of base editors for correction or knockout of clinically relevant human diseases is being explored.⁹⁸ Base editing may prove particularly useful for multiple-gene targeting, where avoiding the formation of multiple DSBs on different chromosomes that could generate translocations would be particularly desirable.

High-throughput loss-of-function screens—RNA interference (RNAi) has been the primary system for large-scale gene perturbation in mammalian cells, but the limitations of RNAi include incomplete suppression of target genes and frequent off-target effects. These limitations can largely be overcome by Cas9-based methods for gene knockout screening (Box 1). Indeed, Cas9-based high-throughput screens achieve high rates of target validation.⁹⁹⁻¹⁰² The ease of producing large gRNA libraries coupled with efficient lentiviral delivery platforms — for example, a genomic CRISPR–Cas9 pooled lentivirus library, which can knockout over 18,000 human genes using 3–4 gRNAs per gene⁹⁹ — has made genomic knockout screens possible in mouse^{100,101,103} and human cells^{99,104,105}. Such a mouse-genome targeting library was delivered to a mouse model of tumor growth and metastasis, and loss-of-function mutations in known tumor suppressor and novel genes were identified *in vivo*¹⁰². For higher content readout of pooled screens, the modularity of Cas9 has been coupled with single-cell RNA sequencing¹⁰⁶⁻¹⁰⁸. By pairing genomic perturbation and transcriptomic analysis within the same cell, higher order interactions can be elucidated including the function of combinatorial interactions. Considerable advances in the

optimization gRNA-library design are also improving the quality and throughput of these screens¹⁰⁹.

Beyond single gene perturbation for therapeutic targeting strategies, combinatorial studies can be used to dissect genetic interactions. For cancer therapy, simultaneous knockout of a synthetic-lethal gene pair can achieve cell killing via multiplexed targeting. Therefore CRISPR–Cas-based double knockout screening has been developed for dissecting genetic interactions and identifying synthetic-lethal drug target pairs of cancer genes^{110,111}.

Excessive DNA damage and cell death resulting from Cas9-induced DSBs may muddle conclusions drawn from knockout screens. Another point raised from recent Cas9 loss-of-function screens is that not all indels result in gene knockout. To address these issues, a DSB-independent knockout method, termed CRISPR-STOP⁷⁹, was developed using CRISPR base editors to create stop codons by single nucleotide conversion. To expand this induction of stop codons (iSTOP) method, a database of over 3.4 million gRNAs targeting 97-99% of genes in eight eukaryotic species was compiled¹¹². These Cas9-based knockout screens have confirmed known essential genes and mediators of resistance to drugs and toxins and provided novel genetic insights.

Although many initial applications of CRISPR–Cas-based gene editing were directed at studying gene function, a particularly important use of this technology lies in annotating the non-coding genome in ways that have not been previously possible. For example, the *BCL11A* gene encodes a transcription factor that controls the levels of fetal hemoglobin¹¹³; modulation of *BCL11A* expression by perturbing cell type-specific enhancers could be used as a therapeutic approach for β -haemoglobin disorders. By tiling the 10 kb of the *BCL11A* enhancer region with a gRNA library, divergence in enhancer–gene interactions was revealed between mice and humans and crucial minimal genetic elements were revealed and validated as targets for fetal hemoglobin reinduction¹¹³. This work involved the introduction of indel mutations at noncoding sequences to identify functional gene regulatory elements, which later led to the development of therapeutic strategies to target these sites in preclinical models of sickle cell disease and beta thalassemia¹¹⁴. Alternatively, HDR was used to introduce all possible nucleotide substitutions into a putative gene regulatory element to decipher its function¹¹⁵. Numerous other high-throughput tiling approaches are being used to identify functional elements in regulatory regions¹¹⁶⁻¹²³. Finally, a genomic screening method that targets splice sites was used to identify long non-coding RNAs (lncRNAs) that are essential for cellular growth¹²⁴. By using the same library to screen multiple cell lines, cell-type-specific differences in lncRNA essentially were identified.

Molecular recording—To better understand cellular dynamics in response to external (and internal) stimuli, CRISPR–Cas-based tools have been developed to function as molecular recorders by tracking cellular responses in the form of nucleotide alterations. Self-targeting gRNAs (stgRNAs) can be generated so that expression of Cas9 and the stgRNA will result in cleavage and indel mutation accumulation at the stgRNA loci^{125,126}. Thus, a cellular response can be ‘recorded’ by linking cellular responses with the expression of the stgRNA or Cas9. By sequencing the stgRNA locus and determining the level of accumulated mutations, the duration or intensity of the stimulus can be measured. Alternatively, cellular

activity can be recorded as individual nucleotide alterations using single-base editors targeted to designated positions on plasmid or genomic DNA¹²⁷. These CRISPR–Cas-based molecular recording systems have been used to track cellular behavior in response to the presence of small molecules, virus infection, light exposure and multiplexed stimuli in bacteria and human cells^{125,127}.

Cas9-mediated nucleotide alterations are inherited from the founder cell to its descendants, and therefore indels can be used for cell-lineage tracing. To perform whole-organism lineage tracing, accumulation of indel scars over multiple rounds of cell division was recorded following Cas9 and gRNA injection into 1-cell zebrafish embryos containing a compact DNA barcode with multiple Cas9 target sites¹²⁸. By tracking these scars in hundreds of thousands of cells from individual zebrafish, it was found that most organs derive from relatively few embryonic progenitors¹²⁸. To increase the number of traceable scars, Cas9 was targeted to its own gRNA spacer sequences. DNA repair mechanisms that form indels within the spacer sequences results in increased scarring complexity, which provides more information for improved phylogenetic annotation¹²⁶. Another synthetic recording system, termed memory by engineered mutagenesis with optical in situ readout (MEMOIR), was developed to record and subsequently read lineage information out of single cells in situ¹²⁹. This system combines Cas9-based targeted mutagenesis with multiplexed single-molecule RNA fluorescence hybridization (smFISH) to visualize recorded editing events for studying lineage tracing while maintaining the relative spatial positioning of cells¹²⁹.

An alternative recording strategy is based on integrating nucleotides into bacterial genomic crRNA arrays as trackable molecular events. This mechanism utilizes the natural adaptation process of prokaryotic CRISPR-Cas systems, in which Cas1 and Cas2 proteins capture short fragments of invading plasmid or phage genetic material and integrate the exogenous sequences as spacers into a crRNA array^{5,130}. Since new spacers are preferentially inserted at the 5' end of crRNA arrays⁵, this mechanism can be harnessed for tracking sequential spacer acquisition as a means of recording the temporal order of molecular events. In a population of bacterial cells overexpressing Cas1 and Cas2, synthetic oligonucleotides can be serially electroporated to generate stable genomic recordings of multiple molecular events¹³¹. Recently, this technique has been scaled to store synthetic sequences encoding pixel values of black and white images and a short movie into the genomes of living bacteria¹³². These studies demonstrate the capacity of DNA to encode and store analog data.

CRISPR–Cas TARGETING RNA

Although CRISPR–Cas systems have been valuable for targeting DNA, manipulating RNA is limited by lack of precise and efficient RNA-targeting molecular tools. RNAi and antisense oligonucleotides can inhibit gene expression, but additional tools are needed to expand RNA-targeting applications. Recently, development of CRISPR–Cas technology for binding or cleaving specific RNAs has advanced RNA manipulation in living cells (Figure 3).

RCas9

Cas9 can be made to cleave ssDNA targets by providing a PAM-presenting oligonucleotide (PAMmer) that anneals to ssDNA¹³³. Similarly, a PAMmer can be provided to direct Cas9 to ssRNA targets¹³⁴ (Figure 3a). To specifically target RNA while avoiding DNA, PAMmers can be designed for RNA sequences that lack PAMs at the corresponding genomic DNA sites. This RNA-targeting Cas9 system, termed RCas9, only requires the design and synthesis of a matching gRNA and complementary PAMmer¹³⁴. By targeting dCas9 to RNA, RCas9 can be utilized as a programmable RNA-binding protein for RNA recognition (Figure 3b). This modular tool permits detection of endogenous RNA without the need to genetically encode affinity tags on transcripts. RCas9 binding to specific mRNAs has been utilized for their visualization and tracking into stress granules [G] in living cells¹³⁵. Further development of this technology may provide a useful tool for RNA visualization of mRNAs of low abundance or concentration.

Catalytically active RCas9 can stimulate site-specific cleavage of ssRNA¹³⁴. Thus, RCas9 can be used to control cellular processes at the transcript level. Therapeutic strategies to block the expression of toxic RNA can utilize genome editing through DNA targeting, however this involves a risk of causing permanent off-target DNA edits. By contrast, the diagnostic and therapeutic potential for RCas9 has been demonstrated by visualizing and eliminating toxic RNA species associated with microsatellite-repeat expansion [G] diseases¹³⁶ (Figure 3c). Specific RNA targeting and elimination were observed in patient cells *ex vivo*, but *in vivo* efficacy remains to be demonstrated. Although the development of RNA-targeting therapies is hindered by the need for continuously targeting newly synthesized transcripts, AAV delivery is known to support long-term transgene expression¹³⁷, and truncated versions of RCas9 have been generated that are compatible with the limited AAV packaging capacity¹³⁶.

Cas9 orthologs

Although in nature Cas9 is thought to preferentially target phage and DNA in bacteria, Cas9 orthologs have the capacity to also target RNA. SaCas9 and *Campylobacter jejuni* Cas9 (CjCas9) can directly cleave ssRNA in a PAM-independent manner¹³⁸ (Figure 3a). When targeted to RNA, SaCas9 repressed gene expression in *Escherichia coli*¹³⁸ and for CjCas9, crRNA-dependent but PAM-independent binding and cleavage of endogenous RNAs was shown¹³⁹.

Francisella novicida Cas9 (FnCas9) was originally shown to target bacterial mRNA and alter gene expression¹⁴⁰, and has been repurposed to target the RNA genome of hepatitis C virus (HCV) in eukaryotic cells¹⁴¹. This positive-sense ssRNA virus has a cytosolic life cycle and its RNA does not undergo reverse transcription and genomic integration. By targeting the 5' or 3' untranslated regions of the HCV genome, FnCas9 inhibited both viral protein production and replication (Figure 3c). Unlike RCas9, RNA targeting by FnCas9 is PAM-independent and thus does not require PAMmers¹⁴¹. In future, FnCas9 could also potentially be used to target negative-sense ssRNA viruses such as those belonging to the filoviridae, paramyxoviridae, or orthomyxoviridae families. Additional studies are needed to clarify the potential physiological consequences of RNA targeting by Cas9 in eukaryotic cells.

Cas13

CRISPR–Cas systems containing naturally RNA-targeting endonucleases have been recently discovered. In bacteria, Cas13a (formerly known as C2c2) is an RNA-guided RNA-targeting nuclease. This class 2 type VI CRISPR protein is activated upon recognition of ssRNA targets¹⁴² (Figure 3a). Similar to a PAM sequence, some type VI CRISPR proteins require recognition of a PFS¹⁴², however Cas13a from *Leptotrichia wadei* (LwaCas13a)¹⁴³ and Cas13b from *Prevotella sp. P5-125* (PspCas13b)¹⁴⁴ do not. Following target binding, Cas13a cuts at uracil bases anywhere in its vicinity, and this ‘collateral’ cleavage extends also to nearby, untargeted RNAs. Cas13a has been programmed to cleave specific mRNAs in both bacteria and eukaryotic cells^{142,143}. Unexpectedly, collateral cleavage by activated Cas13a was not observed in eukaryotic cells, but the mechanism for this difference remains unknown¹⁴³. A catalytically inactive Cas13a variant, dCas13a, maintains the ability to bind targeted RNA and was used for live cell imaging of RNA¹⁴³. Similar to RCas9, dCas13a has been targeted to mRNA to visualize the formation of stress granules^{135,143}.

The collateral cleavage observed following programmed mRNA targeting in bacteria cells has also been demonstrated *in vitro* with purified Cas13a protein^{143,145}. This promiscuous RNase activity, which is induced upon target recognition has been utilized as a molecular detection platform termed SHERLOCK (specific high sensitivity enzymatic reporter unlocking)¹⁴⁶. Following detection of target RNA, Cas13a is activated for collateral-RNA cleavage-mediated release of a reporter signal. On the basis of this method, a diagnostic test was developed to detect viral RNA of specific strains of Zika and Dengue viruses¹⁴⁶. Additionally, amplified DNA can be converted to RNA for subsequent Cas13-mediated detection¹⁴⁶. Following conversion to RNA, SHERLOCK can be used to detect species-specific bacterial pathogens, discriminate between single-nucleotide polymorphisms in the human genome, and identify cell-free, mutated tumor DNA. Further development has resulted in the improved SHERLOCKV2 molecular detection platform, which can perform quantitative detection, has increased sensitivity, and can be used to detect simultaneously up to four targets¹⁴⁷. Recently, Cas12a has also been repurposed as a detection tool. Following targeted activation by dsDNA, Cas12a nonspecifically cleaves ssDNA¹⁴⁸. By providing a quenched ssDNA reporter, the collateral cleavage of Cas12a can be used to detect viral DNA in patient samples^{146,148}.

A single-base RNA editing application has been developed by fusing dCas13 to adenosine deaminase acting on RNA (ADAR) enzymes (Figure 3b). This system, termed REPAIR (RNA editing for programmable A to I replacement), can make directed adenosine-to-inosine edits in eukaryotic cells¹⁴⁴. In translation and splicing, inosine is functionally equivalent to guanine^{149,150}. To broaden the base conversions achievable by REPAIR, dCas13 could be fused with other RNA editing domains such as that of APOBEC for potential cytidine-to-uridine editing. Additional applications for site-specific binding of dCas13a include studying RNA–protein interactions, visualizing RNA trafficking and localization with fluorescently tagged dCas13a or modulating the function or translation of transcripts with dCas13a fused to different effectors. The application of this RNA editing tool for treating genetic diseases remains to be explored.

Scanning of bacterial genome sequences has led to the identification of a class 2 type VI-D CRISPR effector, termed Cas13d. Similarly to Cas13a, Cas13d-mediated cleavage promotes collateral RNA cleavage in bacteria¹⁵¹ but not when expressed in mammalian cells¹⁵². RNA recognition by Cas13d is PFS-independent. dCas13d lacks target-RNA cleavage activity but retains

rRNA array processing activity, and notably, its smaller size makes packaging into vectors like AAV possible for *in vivo* applications^{151,152}. These characteristics have been utilized to deliver dCas13d and a crRNA array targeting *cis* elements in pre-mRNAs to manipulate alternative splicing in a neuronal model of frontotemporal dementia¹⁵² (Figure 3b).

GENE REGULATION by CRISPR–Cas

Beyond gene editing through the formation of DNA breaks, site-specific gene regulation is possible by engineering Cas9 as a DNA recognition complex rather than a targeted nuclease¹⁵³. Mutations in the RuvC (D10A) and HNH (H840A) nuclease domains destroy the catalytic activity of Cas9 while maintaining its RNA-guided DNA targeting capacity^{10,154}. The CRISPR–Cas toolbox has been expanded by fusing this dCas9 with diverse effectors such as transcription repressors or activators, epigenetic modifiers, and fluorophores (Figure 4).

Transcription regulators

The modularity of dCas9 is exemplified by the ability to tether protein effectors to dCas9 or to the gRNA and still maintain dCas9-mediated DNA targeting. Thus, a versatile DNA-targeting platform can be combined with various protein effectors for a broad range of applications.

CRISPRi—Binding of dCas9 to DNA elements may repress transcription by sterically hindering the RNA polymerase machinery¹⁵⁴. dCas9-mediated steric interference, termed CRISPR interference (CRISPRi), works efficiently in prokaryotic cells but is less effective in eukaryotic cells¹⁵⁴⁻¹⁵⁶. To enhance the repressive capacity of CRISPR in eukaryotic cells, dCas9 has been tethered to transcription repressor domains such as that of Krüppel-associated box (KRAB)¹⁵⁶, which is found in many natural zinc-finger transcription factors¹⁵⁷. KRAB is known to induce heterochromatin formation, and changes in chromatin structure often accompany dCas9–KRAB-targeted transcription repression¹⁵⁸. dCas9–KRAB is a robust tool in mammalian cells that can effectively silence single genes and noncoding RNAs by targeting promoter regions, 5' untranslated regions and proximal and distal enhancer elements^{156,159-161} (Figure 4a). For improved repressive capabilities, dCas9 was fused to a bipartite repressor consisting of the transcription repression domains of KRAB and of methyl-CpG-binding protein 2¹⁶². The versatility of dCas9–KRAB is highlighted by its capacity to repress transcription by targeting both genes and gene-regulatory regions¹⁶¹.

CRISPRa—dCas9 can also be fused to activator effectors for programmed transcription activation, termed CRISPR activation (CRISPRa). In eukaryotes, both reporter genes and endogenous genes can be activated by dCas9 fused to the transcription activation domains of

the NF- κ B transactivating subunit (p65) or to VP64 (four repeats of the herpes simplex VP16 activation domain)^{156,163-165}. Synergistic gene activation has frequently been observed with these synthetic transcription factors by targeting multiple gRNAs to a promoter region^{163,164}. In addition, synergy can be achieved by combining different activator domains¹⁶⁶⁻¹⁷⁰. Multiplexed activation of endogenous genes can also be used for cellular reprogramming¹⁷¹. For example, direct conversion of primary mouse embryonic fibroblasts to induced neuronal cells was achieved following activation of lineage-specific transcription factors by targeting VP64–dCas9–VP64 (dCas9 fused to VP64 at each of its termini) to the endogenous *Brn2*, *Ascl1* and *Myt1l* genes¹⁷² (Figure 4a), and similar approaches have been applied to reprogramming cells into pluripotency¹⁷³ or to myogenic cells¹⁶⁷.

Epigenome editing—Targeted epigenetic modifications, such as acetylation and methylation of histones and methylation of DNA, can be achieved using dCas9-based tools¹⁵³. For example, the fusion of dCas9 to the catalytic core of the human histone acetyltransferase p300 was targeted to promoters and enhancers for catalyzing the acetylation of histone H3 Lys 27, leading to robust gene activation¹⁷⁴. DNA demethylation was achieved using dCas9 fusions with the catalytic domain of methylcytosine dioxygenase TET1. Targeting dCas9–TET1 to the *BRCA1* promoter resulted in transcription up-regulation¹⁷⁵. As a potential therapy, dCas9–TET1 was used to de-methylate the CGG-expansion mutation in the 5' untranslated region of the gene *FMR1* and reverse its silencing, which is associated with Fragile X syndrome¹⁷⁶ (Figure 4a). Importantly, *FMR1* expression was maintained following engraftment of edited cells into mouse brains.

For heritable transcriptional silencing, dCas9–KRAB can be used in combination with DNA methyltransferases (DNMTs). Stable silencing of the β 2-microglobulin promoter–enhancer was achieved in up to 78% of K562 cells by the transient expression of dCas9 fused to the KRAB domain and to the catalytic domains of DNMT3A and DNMT3L, along with seven gRNAs¹⁷⁷. Combined with the robustness of dCas9-mediated targeting, the plethora of potential epigenetic effectors provides many applications for epigenetic studies.

Dynamic control of Cas9 function

Inducible systems function by requiring particular stimuli for gene activation. Based on the type of stimulus, various strategies have been developed for generating inducible Cas9-based systems that permit temporal control of Cas9-mediated gene targeting (Figure 4b).

Chemical induction—Chemical compounds can activate Cas9 expression through inducible promoters. This may be desirable to precisely time gene knockout in certain cell types, rather than use constitutive knockout cell lines. Doxycycline-inducible expression of Cas9 has been used in human pluripotent stem cells^{178,179} and in adult mice¹⁸⁰. However, doxycycline-independent mutagenesis has been observed in the transfected cells, suggesting the expression of Cas9 is leaky in some of these systems¹⁸⁰.

Inducible dCas9-based systems also offer versatility in epigenome engineering. A doxycycline-inducible CRISPRi system enabled efficient, tunable and reversible disease modeling in induced pluripotent stem cell (iPSC)-derived cardiomyocytes¹⁸¹. Chemically

inducible CRISPRa systems have been developed using conditionally stabilized dCas9–activators¹⁸², or split dCas9–activators that dimerize following chemical induction^{183,184} (Figure 4b). Beneficial uses of inducible split dCas9–activators include minimizing leaky dCas9 expression and targeting multiple genes for multiplexed temporal regulation¹⁸⁴.

Optogenetics—Light-inducible dCas9 systems enable precise dynamic regulation of endogenous genes and the possibility of spatial control. For example, light-inducible dimerization of the plant-derived cytochrome proteins CRY2 and CIB1 has been used to create photoactivatable dCas9–p65¹⁸⁵ and dCas9–VP64¹⁸⁶ (Figure 4b). An anti-CRISPR protein [G] was engineered for light-mediated spatiotemporal control of genome and epigenome editing in human cells by pairing a photosensor from *Avena sativa* with a SpCas9 inhibitor¹⁸⁷. A second-generation optogenetic split-protein system was developed and targeted to upregulate the expression of the gene neurogenic differentiation 1 to induce neuronal differentiation in iPSCs¹⁸⁸. For more complex regulation, multiple chemical- and light-inducible systems have been used to dynamically manipulate the activation or repression of multiple genes¹⁸⁹ (Figure 4b). These light-inducible systems hold promise for modeling development and disease with reversible and temporal control of gene expression.

Other genomic dCas9 applications

CRISPR–dCas9 gene regulation systems are proving immensely valuable for elucidating the function of transcribed genes. Another important application of these tools lies in understanding the function of the noncoding genome. Cas9 and dCas9-engineered effectors provide an opportunity to explore these genomic regions, for which there are no other tools for direct perturbation.

Annotating the non-coding genome

With the inception of dCas9–effector tools, CRISPRi and CRISPRa methods are also being developed for high-throughput screening to annotate the non-coding genome^{170,190–194} (Box 1). Epigenome editing with these methods permits efficient perturbation of regulatory elements without mutating the DNA, and CRISPRa-based methods enable gain-of-function studies. CRISPRi and CRISPRa have been combined in parallel screens to target DNaseI hypersensitive sites [G] that surround genes of interest¹⁹³. This unique approach identified regulatory elements that may be dependent on the direction of dCas9-based transcription perturbation. Collectively, these dCas9-based methods enable the elucidation of the roles of regulatory sequences in their native genomic contexts and allow the screening of lncRNAs whose function might not be altered by introduction of indels with Cas9 nucleases^{195,196}.

Chromatin interactions

Chromatin structure modulates genome function, however elucidating the molecular basis of this modulation has been limited by an inadequate availability of methods to study chromatin–protein interactions. To identify proteins that interact with specific genome loci, the chromatin can be immunoprecipitated using an antibody against a dCas9–tag fusion protein, which is co-expressed with a gRNA that targets the desired DNA sequence. This method, named engineered DNA-binding molecule-mediated chromatin

immunoprecipitation (enChIP) is then followed by mass spectrometry to identify the locus-associated proteins¹⁹⁷. enChIP was used for biochemical analysis of transcription and epigenetic regulation at specific genomic loci in living cells¹⁹⁸. Alternatively, dCas9 has been tethered to APEX2, which is an engineered peroxidase that promiscuously labels nearby proteins with biotin^{199,200}. dCas9–APEX2 can be used to biotinylate proteins in the vicinity of a targeted genomic locus; these proteins can then be identified following affinity purification and mass spectrometry (Figure 4c)²⁰⁰.

Regulation of gene expression is also influenced by the formation of long-range chromatin interactions, often referred to as chromatin looping. To better understand the role of chromatin interactions, dCas9-based methods have been developed for precisely modifying chromatin looping. Biotinylated dCas9 has been used to identify chromatin-associated proteins and study long-range chromatin interactions²⁰¹. Chromatin loop reorganization with CRISPR–dCas9 (CLOuD9) can selectively and reversibly establish chromatin loops and modulate the expression of associated genes²⁰² (Figure 4c). As an alternative to the chemically induced CLOuD9 system, a light-inducible dCas9 system was developed for directing rearrangement of chromatin looping on faster time scales²⁰³. A chemically inducible and reversible system termed CRISPR-GO can control spatial genome organization within the cell²⁰⁴. CRISPR-GO enables studying chromatin interactions within nuclear compartments to help elucidate their function. CRISPRi tools such as dCas9–KRAB have also been used to disrupt anchored looping interactions that coordinate changes in gene expression²⁰⁵. These studies have helped to confirm the roles of interactions between loci in the maintenance of gene expression. Chromatin restructuring facilitated by these technologies will be greatly beneficial for studying the dynamic roles of genome architecture in gene regulation.

Imaging loci

Methods to image specific DNA sequences are useful for studying the spatial organization of the genome. Fluorescence in situ hybridization techniques have been valuable for this purpose, however they require cell fixation. For live cell imaging, EGFP-tagged dCas9 and structurally optimized gRNAs have been targeted to repetitive elements and to coding genes²⁰⁶. By targeting a large number of loci, labeling of an entire chromosome was made possible for live cell imaging²⁰⁷. Depending on chromosome length, painting entire human chromosomes could require about 100–800 gRNAs.

To expand these tools to multicolor genome imaging, orthogonal dCas9s have been tagged with different fluorescent proteins^{208,209}. Other dCas9-based multicolor, live cell imaging methods have focused on engineering gRNA scaffolds [G]. By adapting gRNA scaffolds to bind sets of fluorescent proteins, up to 6 targeted chromosomal loci were visualized simultaneously²¹⁰. Additionally, gRNA aptamer [G] insertions have been engineered that concurrently bind two different fluorescent protein tags²¹¹. This dual-color approach is tolerant to photobleaching [G], which makes it useful for long-term imaging of genomic loci.

BIOMEDICAL APPLICATIONS OF CRISPR TOOLS

CRISPR–Cas-based gene editing and epigenome engineering tools have revolutionized our ability to manipulate the genomic functions. Importantly, these tools are now being applied in gene therapy and in enhancing cell therapy.

Pre-clinical gene therapy

Genome editing technologies have transformed the gene therapy paradigm from delivery of an exogenous transgene to editing human genome sequences. The therapeutic potential of making precise, targeted genome modifications includes a wide variety of diseases and disorders, but potential limitations must be overcome as CRISPR–Cas-based technologies advance to the clinic (Box 2). Although the most obvious therapeutic applications of genome editing are correcting mutations that cause genetic diseases, a variety of editing strategies exist that manipulate genes involved in more common, complex disease. For example, by targeting SpCas9 to the mouse cholesterol homeostasis gene *Pcsk9* through adenovirus delivery, a reduction in low-density lipoprotein cholesterol was demonstrated following gene disruption and silencing *in vivo*²¹². For preclinical assessment of somatic genome editing applications, this work has been expanded to successfully target the human *PCSK9* gene in mice engrafted with human hepatocytes²¹³. Similar approaches have been explored using epigenetic silencing of *Pcsk9* by viral delivery of dCas9–KRAB.²¹⁴

Therapeutic genome editing strategies are currently being explored for ocular diseases such as retinitis pigmentosa, which can result in blindness. Cas9-mediated disruption of the gene *Nrl* by indel formation preserved the function of cone photoreceptors in three different mouse models of retinal degradation²¹⁵. A HITI-mediated Cas9 insertion repaired the 1.9kb deletion in the kinase gene *Mertk* in a retinitis pigmentosa rat model and restored MERTK function⁶⁵. The gene therapeutics were delivered to the eye using AAV vectors. Importantly, AAV is the most frequently used gene-therapy delivery vehicle due to its effective and safe track record and wide range of tissue targeting. AAV delivery to skeletal and cardiac muscle can be used for treatment of neuromuscular disorders such as Duchenne muscular dystrophy (DMD). In most individuals with DMD, a hotspot of various deletions exists that disturbs the open reading frame of the *DMD* gene, which encodes dystrophin²¹⁶. Restoration of the reading frame *in vivo* has been achieved in several studies following AAV-mediated delivery of CRISPR–Cas9 to excise additional exons through NHEJ around the inherited deletion²¹⁷⁻²¹⁹, including a mutation correction that has been sustained for at least one year after CRISPR–Cas9 administration^{220,221}. These deletion-based editing approaches resulted in the expression of a truncated but partially functional dystrophin. Importantly, progress has been made in advancing these approaches to testing in large-animal models of DMD²²². In order to avoid the generation of DSBs, Cas9-mediated single-base editing of splice site donors and acceptors has also been explored in these models^{223,224}.

In addition to viral delivery methods, lipid nanoparticles (LNPs) can be utilized to deliver Cas9 *in vivo*. Recently, LNPs containing SpCas9 mRNA and a chemically modified gRNA targeting the mouse *Ttr* gene were delivered to mice²²⁵. Following a single administration, a reduction of TTR serum protein levels was observed and levels of *in vivo* genome editing required for therapeutic benefit were achieved. The clinical significance of CRISPR–Cas-

based therapeutics relies on coupling genome editing developments with continued advancements in delivery methods²²⁶. In particular, transient, non-viral-mediated delivery strategies may be useful in addressing concerns about long-term expression of immunogenic Cas proteins and integration of DNA vectors into the genome^{221,227}.

Translation to the clinic

The most clinically advanced gene editing strategies rely on *ex vivo* cell manipulation that provides therapeutic effects following the administration of the cells back to the donor. In particular, engineered autologous T cells have been successful in adoptive T-cell immunotherapy²²⁸. Gene editing approaches have been used for enhancing the properties of these engineered cells. For example, the insertion of transgenes encoding programmable chimeric antigen receptors [G] (CARs) into the endogenous T cell receptor alpha constant gene, rather than overexpression of CARs from viral vectors, prevents the exhaustion of T cells from overstimulation^{229,230}. Another important therapeutic application of genome editing is in knocking out components of the human leukocyte antigen system to generate universal cell donors [G],²³¹ which would address the practical and economic challenges of patient-specific autologous cell therapies. Researchers have also targeted programmed cell death protein 1 (*PD-1*) to block inhibitory signals that prevent T-cell recognition of tumor cells^{230,232,233}. In fact, autologous T cells that were treated *ex vivo* with Cas9 to knock out PD-1 were infused back into individuals with cancer, in the first use of CRISPR–Cas gene editing in a human clinical trial in the United States or Europe (NCT03399448)^{234,235}. Also currently underway are the first human trial of CRISPR–Cas to treat a genetic disease, β -thalassemia (NCT03655678), and the first trial of *in vivo* genome editing by CRISPR–Cas in retina to treat a rare form of blindness (NCT03872479).²²⁷ Importantly, these CRISPR–Cas-based clinical trials build on a foundation of several genome editing clinical trials using zinc finger nucleases²³⁶. Collectively, these clinical trials will establish the therapeutic potential of recently developed genome engineering tools.

CONCLUSIONS AND FUTURE DIRECTIONS

Repurposing CRISPR–Cas systems for use in eukaryotic cells has revolutionized the genome engineering field. Even with the extensive use of type II CRISPR–Cas systems, continued discovery and development of CRISPR systems from prokaryotic species has resulted in new, beneficial technologies, such as Cas13a-based RNA targeting tools. Fusing dCas9 to the plethora of effectors will continue to expand possibilities for targeted epigenetic modulation.

The ease of gRNA-library generation for large-scale Cas9 targeting coupled with advancements in next-generation sequencing has made genome-wide genetic and epigenetic screens readily available. Perturbation at this magnitude will advance our understanding of biological mechanisms and aid the discovery of new therapeutic targets. Additionally, the multiplexed targeting potential of CRISPR–Cas systems will enable more complex and sophisticated manipulation of cellular processes.

As CRISPR–Cas-based therapeutics enter clinical testing, they hold great potential for correcting genetic diseases and enhancing cell therapies. Preclinical results are promising

but safety and efficacy need to be monitored closely during these studies. A potential risk of using gene editing methods is the introduction of off-target changes to genome sequence, and thus enhancing methods for detecting rare mutations and quantifying their potential risks will be important for future clinical advancement.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

Spacer

The interchangeable portion of the guide RNA that is complementary to the targeted sequence

Directed evolution

Method to generate and select for nucleic-acid or protein variants with desirable properties

crRNA arrays

In bacterial genomes, series of spacers flanked by repeats, which are transcribed as a single pre-crRNA array and subsequently processed into individual crRNAs

Indels

Small insertions or deletions of nucleotides at repair sites of DNA double-strand breaks

Stress granules

Denote a type of cytosolic membraneless bodies with high concentrations of RNA and/or proteins, which form in different cell stress conditions

Microsatellite-repeat expansion

Repetitive DNA sequences that can expand between generations and encode RNAs that are toxic to cells and cause neurological disorders

Anti-CRISPR protein

A protein that interacts with and inhibits CRISPR–Cas activity

DNaseI hypersensitive sites

Chromatin regions accessible to the enzyme DNase I; generally denote gene-activity-permissive chromatin

gRNA scaffolds

The backbone (invariable) portions of gRNAs, which are recognized by Cas proteins

gRNA aptamer

RNA structures added to the gRNA scaffold, which can bind specific effector molecules

Photobleaching

Reduction in the intensity of fluorescence emission owing to the imaging of a sample over time

Seed region

PAM-proximal nucleotides in the target sequence, where spacer mismatches are less tolerated for on-target gRNA binding

Chimeric antigen receptors

T cell receptors engineered to recognize a specific antigen

Universal cell donors

Cells engineered to avoid recognition by a recipient immune system

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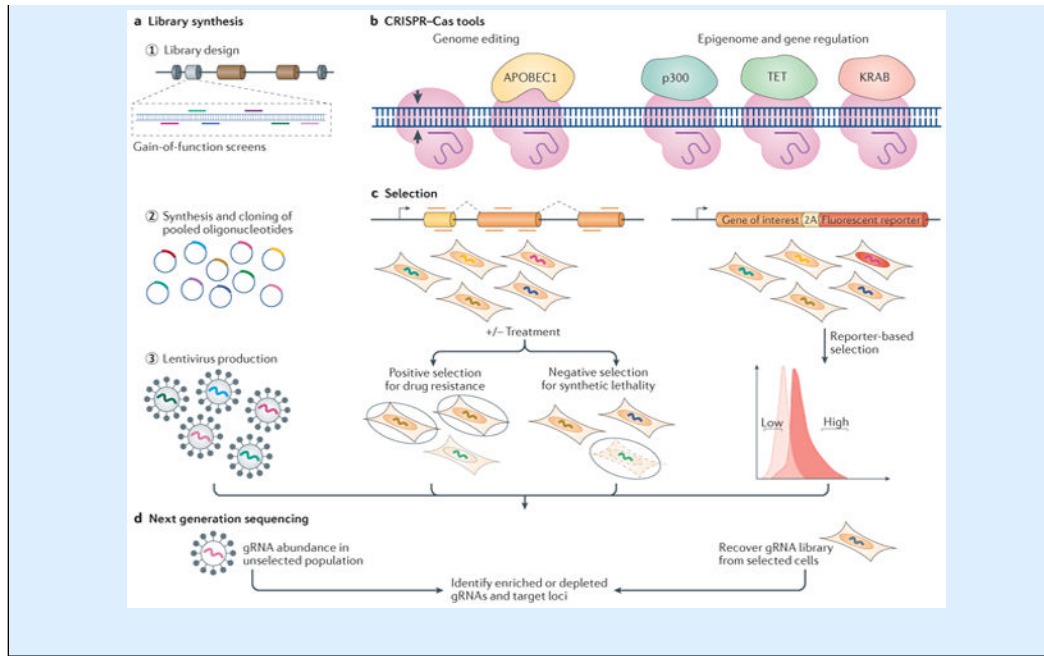
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Box 1 |**Genome-wide pooled screens using CRISPR–Cas-based tools.**

The simplicity of targeting CRISPR–Cas tools to the genome has facilitated high-throughput genetic screening. Genome-scale targeting of Cas9 is possible with synthesis of a guide RNA (gRNA) library. The breadth of the gRNA library can be customized, for example, loss-of-function screens may use saturation mutagenesis and target only exons of human genes^{99,104,105}, and screens to annotate the noncoding genome may target sites of accessible chromatin¹⁹³ or transcription factor motifs¹²³. The gRNA libraries are generated by synthesizing pools of oligonucleotides, cloning them into plasmids and producing a lentivirus library that encodes the gRNAs (see the figure, part **a**). A Cas9-expressing cell line can be generated prior to gRNA delivery, or cells can be co-transduced with Cas9 and the gRNA library. Fusion of Cas9 nickase (nCas9) or catalytically deficient Cas9 (dCas9) to different effector proteins can enable genome editing (for example, by the cytidine deaminase APOBEC1) or epigenome and gene regulation (for example, histone acetylation by p300, DNA demethylation by TET dioxygenases or transcription repression by KRAB domains; see the figure, part **b**). To screen for functional elements, gRNAs that elicit the phenotype of interest must be enriched or depleted. For example, positive selection can identify elements that function in drug resistance²³⁷ and negative selection can identify elements involved in synthetic lethality²³⁸ (see the figure, part **c**). Alternatively, gene regulatory elements can be identified by selecting cells with altered gene expression either through direct immunofluorescence staining or through tagging an endogenous gene with a reporter¹⁹³. By selecting cells with low or high reporter expression, factors that affect gene expression can be identified (see the figure, part **c**). Following selection, next-generation sequencing and bioinformatics are used to compare the unselected gRNA library with the selected gRNA library and identify enriched and depleted gRNAs and thus specific genomic loci (see the figure, **d**). A wide range of applications are possible with CRISPR-based screens. Interrogation of gene function can identify genes involved in cell survival and proliferation or cancer genes^{100,105,238,239}; drug targets can be identified based on resistance or sensitivity to drugs, toxins or pathogens^{238,240}. Targeted screens are also mapping the function of the noncoding genome by perturbing enhancer sequences¹¹³ or modulating particular sets of genes – for example, targeted activation of all transcription factor genes to identify factors involved in stem cell differentiation²⁴¹. Although pooled CRISPR-based screens have so far used Cas9-based tools, in the future other Cas proteins could be used for other functions or for orthogonal screening.



Box 2 |**Potential limitations of CRISPR–Cas medical applications.**

Despite the advances in CRISPR–Cas-based genome engineering technologies, some challenges remain for translating these tools to the clinic:

- Adeno-associated virus (AAV), which is the most frequently used gene-therapy delivery vehicle, provides limited packaging capacity of genetic information. This restriction has led to continued development and *in vivo* testing of smaller Cas9 orthologs such as *Staphylococcus aureus* Cas9 (SaCas9)²⁹ and *Campylobacter jejuni* Cas9³¹. Nevertheless, prolonged expression of Cas9 from AAV vectors and integration of AAV vectors into DSBs remain undesirable consequences of AAV delivery.²²¹
- Off-target effects, which remain a major concern, can be reduced with preliminary guide RNA selection and optimization. For example, VIVO (verification of *in vivo* off-targets)²⁴² can be used with CIRCLE-seq (cleavage effects by sequencing)²⁴³ to screen off-targets using the genomic DNA from the specific patient or organism. More-sensitive methods are necessary to detect possible off-target editing and to understand the possible implications of any unintended genome changes.
- Immunogenicity of Cas proteins is another potential obstacle to their clinical application. Immune responses to Cas9 following its delivery into mouse models is well-documented^{221,244}, but the implications of this for therapeutic approaches are still unclear. Recently, pre-existing adaptive immunity to *Streptococcus pyogenes* Cas9 and SaCas9 has been detected in human blood samples²⁴⁵⁻²⁴⁷. In the case of intracellular expression of virus-delivered Cas9, T cell responses may be worrisome if they are reactive to Cas9 peptides displayed by treated cells. More studies are needed to decipher the implications for clinical use of pre-existing immunity. The high prevalence of exposure of the human population to *S. pyogenes* and *S. aureus* is an additional motivation for ongoing testing of novel Cas9 orthologs. Other approaches to limit immunogenicity include the reengineering of immunogenic epitopes of Cas proteins, the use of transient immunosuppressive drugs during treatment, or *ex vivo* cell modification.
- A potential limitation is the observation that CRISPR–Cas-mediated gene editing is more efficient in cells that have lost the function of the tumor suppressor *p53*^{77,78}.

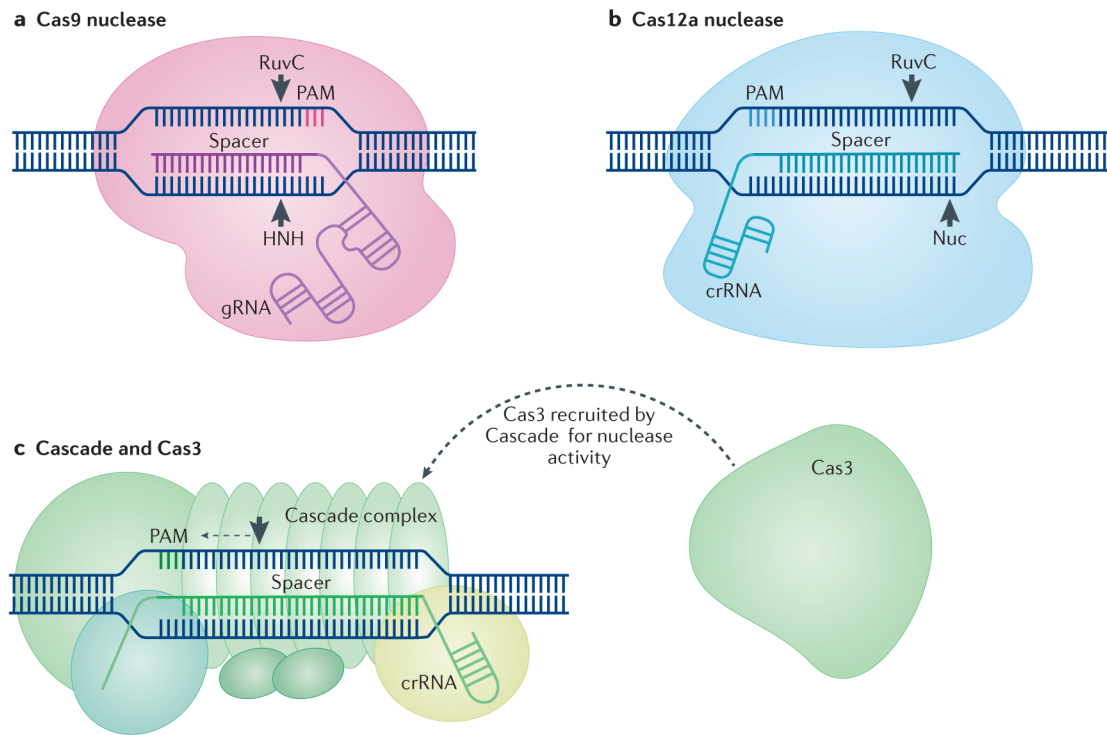


Figure 1. Overview of the main CRISPR–Cas gene editing tools.

a | CRISPR-associated endonuclease 9 (Cas9) proteins rely on RNA guidance for targeting specificity. In engineered CRISPR–Cas9 systems, Cas9 interacts with the backbone of the guide RNA (gRNA). Complementary pairing of the spacer portion of the gRNA to a DNA target sequence positioned next to a 5' protospacer adjacent motif (PAM) results in generation of a blunt DNA double-strand break by the two Cas9 nuclease domains, RuvC and HNH¹¹⁻¹³. **b** | Cas12a nucleases recognize DNA target sequences with complementarity to the crRNA spacer positioned next to a 3' PAM. Target recognition results in generation of a staggered DNA double-strand break by a RuvC domain and a putative nuclease (Nuc) domain³³. **c** | Cascade is a multimeric complex that targets DNA that has complementarity to the spacer portion of a crRNA and that is positioned next to a 3' PAM³⁷⁻⁴². Following target recognition, Cascade recruits Cas3 to generate a single-strand nick, which is followed by 3' to 5' degradation of the targeted DNA^{37,39,44,45}.

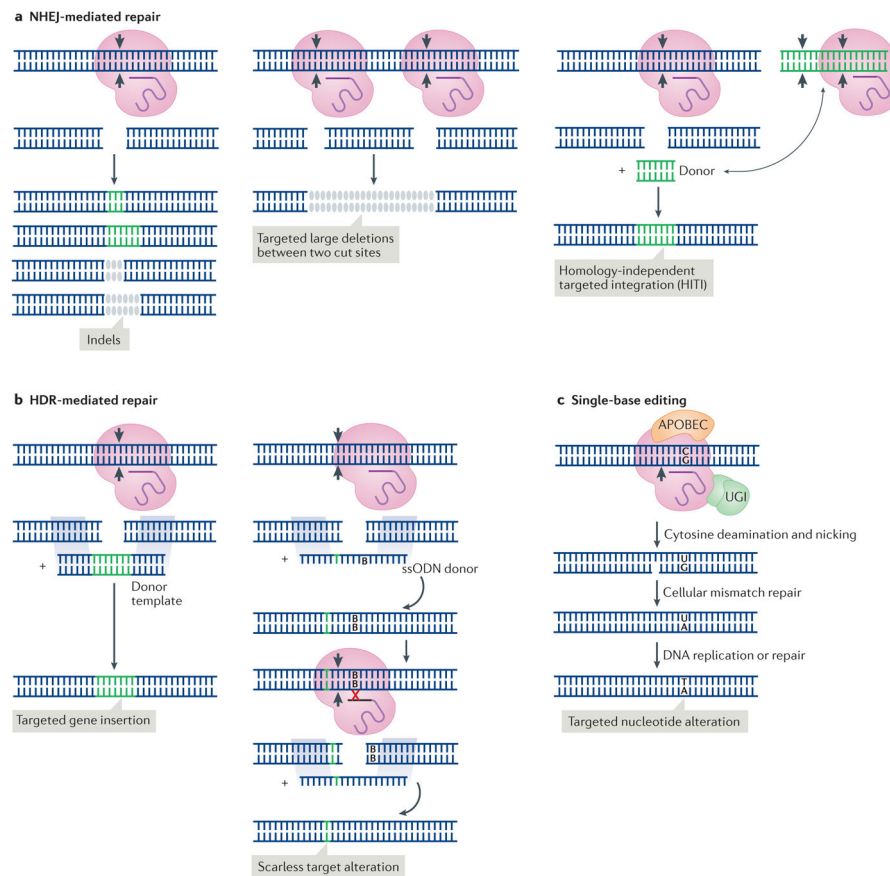


Figure 2. Genome editing strategies.

Nucleases generate targeted DNA double-strand breaks (DSBs), which can be repaired by different repair pathways. **a** | Non-homologous end joining (NHEJ)-mediated repair is error-prone and induces small insertion or deletion mutations (indels). Large, targeted deletions can be produced through repair between two DSBs produced by simultaneously targeting nucleases to two genomic sites. Alternatively, homology-independent targeted integrations (HITI) can be directed to a single cut site by providing donor DNA that is independently targeted for cutting⁶⁵. **b** | The homology-directed repair (HDR) pathway can be utilized for genome editing by providing either double-strand or single-strand oligodeoxynucleotide (ssODN) donor templates that contain homology arms to the cut target site. Single nucleotide alterations or insertion of larger sequences can be mediated by introducing variations into the donor template, which may also consist of plasmid DNA, viral DNA²⁴⁸ or long single-stranded DNA⁷¹. Following HDR, silent mutations — also referred to as blocking mutations (B) — that prevent subsequent target site recognition by the nucleases and formation of NHEJ-mediated indels, can be incorporated into the donor template along with the intended alterations⁷³. **c** | For single nucleotide C→T (or G→A) conversion, Cas9 nickase has been fused to cytidine deaminases such as APOBEC1⁷⁹. For increased base editing efficiency, two uracil glycosylase inhibitors (UGI) have been fused to a base editor for preventing cellular base excision repair⁸⁸.

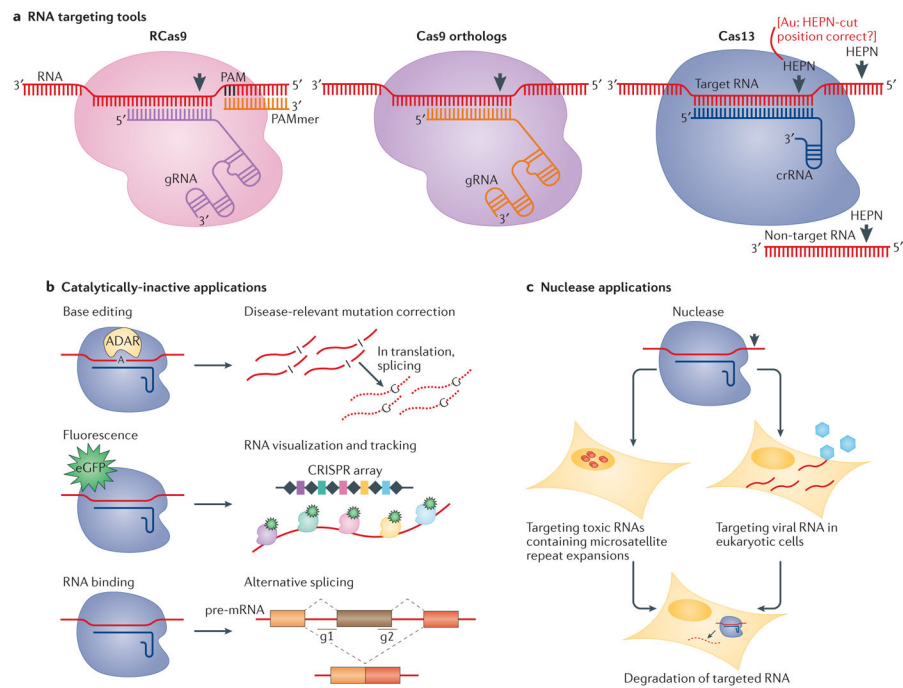


Figure 3. RNA targeting tools and their applications.

a | *Streptococcus pyogenes* Cas9 was repurposed to target RNA (RCas9) by providing it a matching guide RNA (gRNA) and a complementary PAM-presenting oligonucleotide (PAMmer)¹³⁴. Cas9 orthologs such as *Staphylococcus aureus* Cas9 and *Campylobacter jejuni* Cas9, can target RNA in the absence of a PAMmer, thereby demonstrating PAM-independent RNA cleavage¹³⁸. Cas13 proteins are RNA-guided RNA-targeting nucleases, some requiring recognition of a protospacer flanking sequence (PFS), that generate cuts along target and non-target RNA molecules using two HEPN domains, which are nucleotide-binding domains with RNA cutting activity¹⁴². **b** | Similar to catalytically deficient Cas9 (dCas9), dCas13 maintains the capacity to bind the targeted RNA. For RNA visualization and tracking purposes, a fluorescent protein can be fused to the dCas protein and co-localize with an array of crRNAs or gRNAs^{135,143}. Adenosine deaminase RNA specific (ADAR) enzymes can be fused to dCas for RNA A→I base editing to correct disease-relevant mutations. To promote alternative splicing, dCas13 can be targeted to bind splicing regulating *cis* elements¹⁵². **c** | Cas13 can be used for targeted RNA degradation in eukaryotic cells for applications such as targeting viral RNA or toxic RNAs that contain microsatellite repeat expansions¹³⁶. *Francisella novicida* Cas9 has been repurposed in eukaryotic cells to target the RNA genome of hepatitis C virus¹⁴¹.

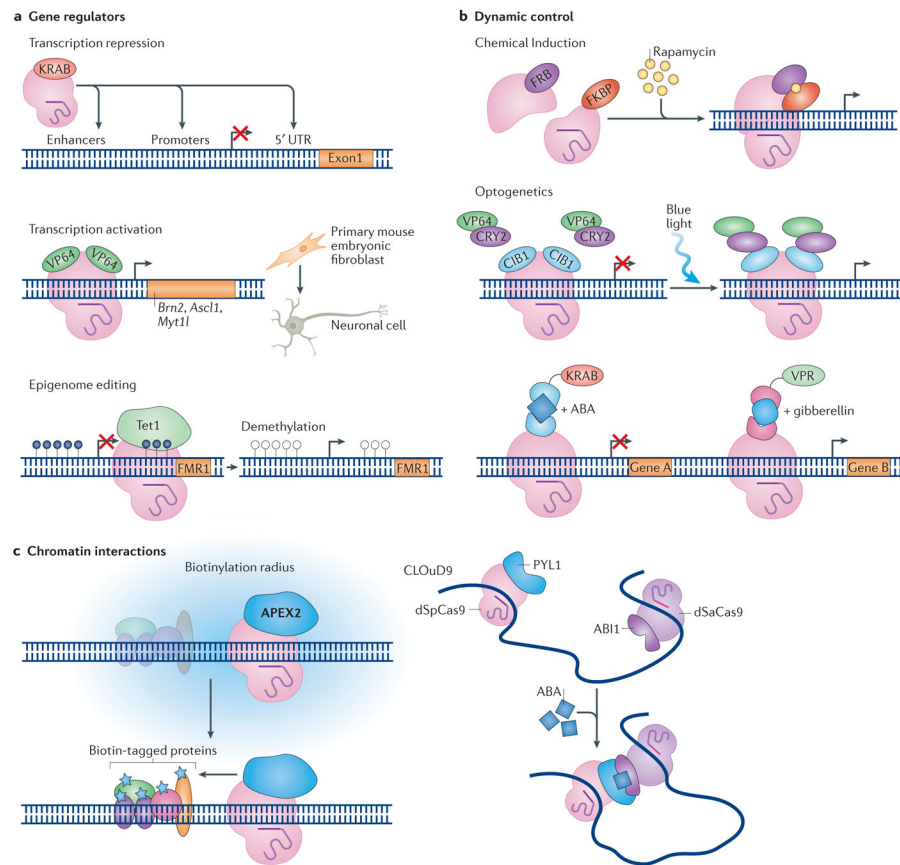


Figure 4. Targeted gene regulation and other applications.

a | For transcription repression, catalytically-deficient Cas9 (dCas9) alone or dCas9 fused to effectors such as the transcription repression domain of Krüppel-associated box domain (KRAB)¹⁵⁷ can be targeted to promoters, 5' untranslated region (5' UTR) enhancers^{156,159-161}. Transcription activation can be targeted by fusing dCas9 to transcription activation domains such as VP64: VP64–dCas9–VP64 activated the expression of the neuronal transcription-factor genes *Brn2*, *Ascl1* and *Myt1l* and thus directed the conversion of primary mouse embryonic fibroblasts into neuronal cells¹⁷². Similarly, dCas9 was fused to the catalytic domain of methylcytosine dioxygenase TET1 and targeted to the *FMR1* gene, to reverse the hypermethylation and silencing of the gene, which is associated with Fragile X syndrome¹⁷⁶. **b** | Inducible Cas9-based systems allow dynamic control of gene targeting. For example, chemical induction by rapamycin of the dimerization of split dCas9 fused to the rapamycin-binding domains of FKBP and FRB activates target-gene expression. Alternatively, light-inducible dimerization of the cytochrome proteins CRY2 and CIBN can be used in photoactivatable systems. Combinations of inducible dCas9-ortholog-based systems can be used for dynamic manipulation of multiple targets simultaneously. For example, dimerization of *Streptococcus pyogenes* dCas9 (dSpCas9)–KRAB by the addition of abscisic acid (ABA) can repress one gene while dimerization of *Staphylococcus aureus* dCas9 (dSaCas9)–VP64–p65–Rta (VPR) by the addition of gibberellin can lead to activation of another gene¹⁸⁹. **c** | CRISPR–dCas9 tools can monitor or manipulate chromatin interactions that regulate gene expression. The fusion of dCas9 to the peroxidase APEX2

can be used to biotinylate proteins in the vicinity of a targeted genomic locus; the proteins are then identified using mass spectrometry²⁰⁰. Distal loci can be brought into proximity using chromatin loop reorganization with CRISPR–dCas9 (CLOuD9). In the CLOuD9 system, dSpCas9 and dSaCas9 are fused to the dimerizing, ABA-binding proteins PYL1 and ABI1²⁰². ABA induces targeted protein dimerization and chromatin looping, which can be reversed following its removal to restore the endogenous chromatin conformation.

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Table 1.

Cas9 variants with altered PAM and targeting specificities

Name	Included mutations	PAM (5' to 3')	Notes
SpCas9	Native <i>Streptococcus pyogenes</i> Cas9	NGG ²⁴⁹	1368 amino acids
VRER SpCas9	D1135V, G1218R, R1335E, T1337R	NGCG ¹⁷	Altered PAM variant; Bacterial-selection-based screening
VQR SpCas9	D1135V, R1335Q, T1337R	NGAN or NGNG ¹⁷	Altered PAM variant; Bacterial-selection-based screening
EQR SpCas9	D1135E, R1335Q, T1337R	NGAG ¹⁷	Altered PAM variant; Bacterial-selection-based screening
xCas9-3.7	A262T, R324L, S409I, E480K, E543D, M694I, E1219V	NG, GAA, GAT ¹⁸	Altered PAM variant; Phage-assisted continuous evolution
eSpCas9 (1.0)	K810A, K1003A, R1060A	NGG	Enhanced specificity; Structure-guided protein engineering ¹⁹
eSpCas9 (1.1)	K810A, K1003A, R1060A	NGG	Enhanced specificity; Structure-guided protein engineering ¹⁹
Cas9-HF1	N497A, R661A, Q695A, Q926A	NGG	Enhanced specificity ²⁰
HypaCas9	N692A, M694A, Q695A, H698A	NGG	Enhanced specificity ²¹
evoCas9	M495V, Y515N, K526E, R661Q	NGG	Enhanced specificity; yeast-based screening ²²
HiFi Cas9	R691A	NGG	Enhanced specificity for ribonucleoprotein delivery ²³
ScCas9	Native <i>Streptococcus canis</i> Cas9	NNG ²⁵⁰	1375 amino acids
StCas9	Native <i>Streptococcus thermophilus</i> Cas9	NNAGAAW ^{11,25}	1121 amino acids
NmCas9	Native <i>Neisseria meningitidis</i> Cas9	NNNGATT ²⁶⁻²⁸	1082 amino acids
SaCas9	Native <i>Staphylococcus aureus</i> Cas9	NNGRRT ²⁹	1053 amino acids
CjCas9	Native <i>Campylobacter jejuni</i> Cas9	NNNVRYM ³⁰	984 amino acids
CasX	Deltaproteobacteria and Planctomycetes phyla	TTCN ³²	980 amino acids

AAV, adeno-associated virus

ABA, abscisic acid

ABE, adenine base editor

ABII, dimerizing ABA-binding protein

ADAR, adenosine deaminase acting on RNA

APEX2, engineered apurinic/aprimidinic endodeoxyribonuclease 2 peroxidase

APOBEC1, apolipoprotein B mRNA editing enzyme complex-1

BE3, third generation base editor

BLESS, breaks labeling, enrichment on streptavidin and next-generation sequencing

Cas9-HF1, high fidelity Cas9 variant

CIB1, plant-derived cytochrome protein

CIRCLE-seq, circularization for in vitro reporting of cleavage effects by sequencing

CLOuD9, chromatin loop reorganization with CRISPR-dCas9

CORRECT, consecutive re-guide or re-Cas steps to erase CRISPR/Cas-blocked targets

CRISPR, clustered regularly interspaced short palindromic repeats

CRISPRa, CRISPR activation
CRISPRi, CRISPR interference
crRNA, CRISPR RNA
CRY2, plant-derived cytochrome protein
dCas9, catalytically deficient Cas9
Digenome-seq, *in vitro* Cas9-digested whole-genome sequencing
DNA, deoxyribonucleic acid
DNMT, DNA methyltransferase
DSB, double-strand break
dsDNA, double-stranded DNA
enAsCas12a, enhanced AsCas12a variant
enCHIP, engineered DNA-binding molecule-mediated chromatin immunoprecipitation
EQR, Cas9 variant with triple mutations
eSpCas9, enhanced specificity SpCas9 variant
evoCas9, evolved high fidelity Cas9 variant
FKBP, rapamycin-binding domain
FRB, rapamycin-binding domain
GFP, green fluorescent protein
gRNA, guide RNA
GUIDE-seq, genome-wide, unbiased identification of DSBs enabled by sequencing
HCV, hepatitis C virus
HDR, homologous-directed repair
HEPN, higher eukaryotes and prokaryotes nucleotide binding domain
HiFi Cas9, high fidelity Cas9 variant
HITI, homology-independent targeted integration
HNH, Cas9 nuclease domain
HTGTS, high-throughput, genome-wide, translocation sequencing
HypaCas9, hyper-accurate Cas9 variant
Indels, insertions or deletions
iPSC, induced pluripotent stem cell
lncRNA, long non-coding RNA
KRAB, Krüppel-associated box
LNP, lipid Nanoparticle
MEMOIR, mutagenesis with optical in situ readout
mRNA, messenger RNA
nCas9, Cas9 nickase
NHEJ, non-homologous end joining
p65, NF- κ B transactivating subunit

PAM, protospacer adjacent motif
PAMmer, PAM-presenting oligonucleotide
PFS, protospacer flanking sequence
PYL1, dimerizing ABA-binding protein
REPAIR, RNA editing for programmable A to I replacement
RNA, ribonucleic acid
RNAi, RNA interference
RNP, ribonucleoprotein
RuvC, Cas9 nuclease domain
SHERLOCK, specific high sensitivity enzymatic reporter unlocking
smFISH, single-molecule RNA fluorescence hybridization
ssDNA, single-stranded DNA
ssODN, single-stranded oligodeoxynucleotide
stgRNA, self-targeting gRNA
TET1, ten-eleven translocation methylcytosine dioxygenase 1
tracrRNA, trans-activating crRNA
tRNA, transfer RNA
UGI, uracil glycosylase inhibitor
VIVO, verification of in vivo off-targets
VP64, four repeats of the herpes simplex VP16 activation domain
VQR, Cas9 variant with triple mutations
VRER, Cas9 variant with quadruple mutations
xCas9, expanded PAM SpCas9 variant
AsCas12a – *Acidaminococcus* spp. Cas12a
CjCas9 – *Campylobacter jejuni* Cas9
FnCas9 – *Francisella novicida* Cas9
LbCas12a – *Lachnospiraceae* spp. Cas12a
LwaCas13a – *Leptotrichia wadei* Cas13a
NmCas9 – *Neisseria meningitidis* Cas9
PaCsy4 – *Pseudomonas aeruginosa* Csy4
PspCas13b – *Prevotella sp. P5-125* Cas13b
SaCas9 – *Staphylococcus aureus* Cas9
ScCas9 – *Streptococcus canis* Cas9
SpCas9 – *Streptococcus pyogenes* Cas9
StCas9 – *Streptococcus thermophilus* Cas9