TECHNOLOGIES AND TECHNIQUES

The next generation of CRISPR–Cas technologies and applications

Adrian Pickar-Oliver[1,2] *and Charles A. Gersbach*[1,2,3]*

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 ranging 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 orthologues. Genome editing by CRISPR–Cas can utilize non-homologous end joining and homology-directed repair 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, the new advances are considerably improving our understanding of biological processes and are propelling CRISPR–Cas-based tools towards clinical use in gene and cell therapies.

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

The ability to modulate and edit genetic information is crucial for studying gene function and uncovering biological mechanisms. Since the first demonstration of the production of specific DNA fragments with restriction enzymes in 1971, scientists have been harnessing prokaryotic molecules for gene editing[1]. In addition to restriction enzymes[2], classes of DNA-modifying tools include recombinases[3] and programmable nucleases such as meganucleases, zinc finger nucleases, transcription activator-like effector nucleases and CRISPR–Cas systems[4]. 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 acids[5]. An assortment of CRISPR–Cas systems exist across diverse species of bacteria and archaea, which differ in their components and mechanisms of action. For example, class 1 CRISPR–Cas systems comprise multiprotein effector complexes, whereas class 2 systems have a single effector protein; overall there are six 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 (FIG. 1). Following hybridization of the spacer 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 cleaves the target nucleic acid. Thus, site-specific cleavage at any locus containing a PAM or a 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, from fundamental science to translational science and medicine[9]. 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 the therapeutic potential moving towards clinical studies.

[1]*Department of Biomedical Engineering, Duke University, Durham, NC, USA.*
[2]*Center for Genomic and Computational Biology, Duke University, Durham, NC, USA.*
[3]*Department of Surgery, Duke University Medical Center, Durham, NC, USA.*
**e-mail: charles.gersbach@duke.edu*

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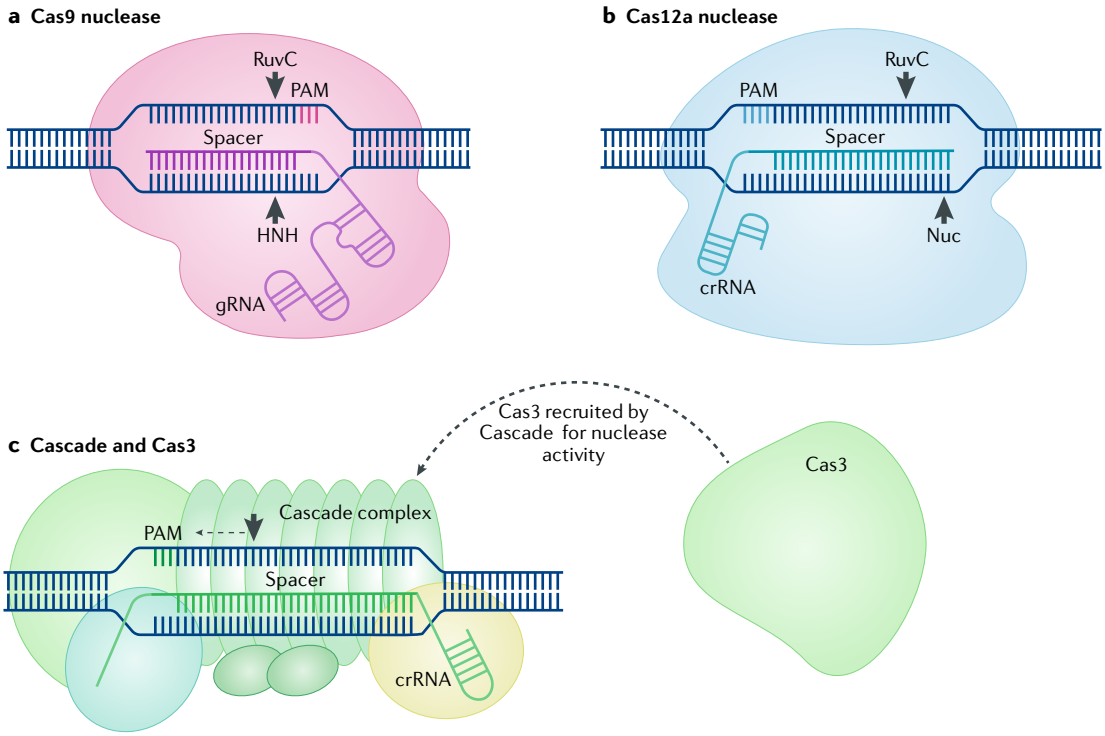


Fig. 1 | **Overview of the main CRISPR–Cas gene editing tools. a** | Cas9 proteins rely on RNA guidance for targeting specificity. In engineered CRISPR–Cas9 systems, Cas9 interacts with the backbone of the guide RNA (gRNA). Complementary pairing of the spacer portion of the gRNA to a DNA target sequence 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 HNH[11–13]. **b** | Cas12a nucleases recognize DNA target sequences with complementarity to the CRISPR RNA (crRNA) spacer positioned next to a 3′ PAM. Target recognition results in the generation of a staggered DNA double-strand break by a RuvC domain and a putative nuclease (Nuc) domain[33]. **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′ PAM[37–42]. 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].

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 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 prokaryotic cells[10] 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)[13] (FIG. 1a). DNA targeting by SpCas9 relies on the 20-nucleotide-long spacer and on the PAM 5′-NGG (N represents any nucleotide)[10,14]. Cas9 systems are dual-RNA guided: a crRNA is responsible for DNA targeting and also hybridizes with the transactivating crRNA, which is responsible for forming the complex with Cas9 (REFS[15,16]). The crRNA and transactivating crRNA functions can be recapitulated with an engineered single gRNA[10] (FIG. 1a).

Recognition of the PAM 5′-NGG limits the availability of SpCas9 target sites in the human genome to an average of one target site for every eight base pairs[9]. To increase the availability of target sites, directed evolution approaches have generated variants with altered PAM specificities[17,18] (TABLE 1). For example, an expanded-PAM SpCas9 variant, xCas9, recognizes 5′-NG, 5′-GAA and 5′-GAT PAM sequences[18]. Another motivation for engineering Cas9 variants is to increase targeting specificity. Several studies have described mutated Cas9 variants with reduced off-target cleavage following expression of Cas9 and gRNAs from plasmids[19–22] or their delivery as ribonucleoprotein complexes[23]. Alternatively, on-target CRISPR–Cas specificity has been increased by

Directed evolution
Method of generating and selecting 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-CRISPR RNA array and subsequently processed into individual CRISPR RNAs.

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 activity[24].

The discovery and development of additional Cas9 orthologues 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′-NNNNGATT[26–28]. These Cas9 orthologues 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 those of SpCas9, and the smaller size of SaCas9 (1,053 amino acids compared with 1,368 amino acids of SpCas9) has allowed its use in size-restricted delivery vectors such as adeno-associated virus (AAV)[29]. More recently, an even smaller Cas9 orthologue, from *Campylobacter jejuni* (984 amino acids), was reported to recognize the PAM 5′-NNNVRYM (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 orthologues resulted in the discovery of CasX (980 amino acids), the smallest Cas9 to date[32].

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 transactivating crRNA (FIG. 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 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 crRNAs[34].

Cas12a from *Acidaminococcus* spp. (AsCas12a) and Cas12a from Lachnospiraceae spp. (LbCas12a), the first Cas12a orthologues that were shown to have activity in mammalian cells, recognize the PAM sequence 5′-TTTV upstream of the target sequence. To increase their genome editing activity, an enhanced AsCas12a variant (enAsCas12a) has been engineered[35]. To increase the targeting range of Cas12a, AsCas12a variants were recently engineered to recognize the PAMs 5′-TYCV and 5′-TATV[36] or the PAMs 5′-VTTV, 5′-TTTT, 5′-TTCN and 5′-TATV[35]. The unique features and cutting mechanism of Cas12a provide 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 (FIG. 1c). Before recruiting Cas3 to a target DNA sequence, Cascade must first bind to DNA through PAM and spacer recognition[37–42]. Cascade offers greater target site flexibility owing to its promiscuous recognition

Table 1 | **Cas9 variants with altered protospacer adjacent motif and targeting specificities**

Name	Description of protein variant or mutations	PAM (5′ to 3′)	Notes
SpCas9	Native *Streptococcus pyogenes* Cas9	NGG[24,48]	1,368 amino acids
VRER SpCas9[a]	D1135V, G1218R, R1335E, T1337R	NGCG[17]	Altered PAM variant; bacterial selection-based screening
VQR SpCas9[b]	D1135V, R1335Q, T1337R	NGAN or NGNG[17]	Altered PAM variant; bacterial selection-based screening
EQR SpCas9[b]	D1135E, R1335Q, T1337R	NGAG[17]	Altered PAM variant; bacterial selection-based screening
xCas9-3.7[c]	A262T, R324L, S409I, E480K, E543D, M694I, E1219V	NG, GAA, GAT[18]	Altered PAM variant; phage-assisted continuous evolution
eSpCas9 (1.0)[d]	K810A, K1003A, R1060A	NGG	Enhanced specificity; structure-guided protein engineering[19]
eSpCas9 (1.1)[d]	K810A, K1003A, R1060A	NGG	Enhanced specificity; structure-guided protein engineering[19]
Cas9-HF1[e]	N497A, R661A, Q695A, Q926A	NGG	Enhanced specificity[20]
HypaCas9[f]	N692A, M694A, Q695A, H698A	NGG	Enhanced specificity[21]
evoCas9[g]	M495V, Y515N, K526E, R661Q	NGG	Enhanced specificity; yeast-based screening[22]
HiFi Cas9[e]	R691A	NGG	Enhanced specificity for ribonucleoprotein delivery[23]
ScCas9	Native *Streptococcus canis* Cas9	NNG[249]	1,375 amino acids
StCas9	Native *Streptococcus thermophilus* Cas9	NNAGAAW[11,25]	1,121 amino acids
NmCas9	Native *Neisseria meningitidis* Cas9	NNNNGATT[26–28]	1,082 amino acids
SaCas9	Native *Staphylococcus aureus* Cas9	NNGRRT[29]	1,053 amino acids
CjCas9	Native *Campylobacter jejuni* Cas9	NNNVRYM[30]	984 amino acids
CasX	Phyla Deltaproteobacteria and Planctomycetes	TTCN[32]	980 amino acids

PAM, protospacer adjacent motif. [a]*S. pyogenes* Cas9 variant with quadruple mutations; [b]*S. pyogenes* Cas9 variant with triple mutations; [c]expanded PAM *S. pyogenes* Cas9 variant; [d]enhanced-specificity *S. pyogenes* Cas9 variant; [e]high-fidelity Cas9 variant; [f]hyperaccurate Cas9 variant; [g]evolved high-fidelity Cas9 variant.

of PAM sequences[43]. 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 activity and the helicase activity of Cas3 are essential for the degradation of foreign DNA in prokaryotes[38]. 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 death[46]. 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 Csy4 (REF.[47]). Like Cas12a, this endonuclease activity has been repurposed for directed RNA processing. For example, the *Pseudomonas aeruginosa* type IF Csy4 has been used to generate 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 mechanisms[50]. Use of the unique characteristics of the different DNA repair mechanisms has allowed the development of specific genome editing strategies.

Non-homologous end joining versus homology-directed repair. Eukaryotes predominantly repair DSBs through the error-prone non-homologous end joining (NHEJ) pathway, which leads to accumulation of small insertions or deletions (indels) following repeated cycles of break and repair (FIG. 2a). Alternatively, a repair template with homology to the target site can be delivered with Cas9 to stimulate error-free homology-directed repair (HDR), but typically at a lower efficiency than NHEJ-mediated repair (FIG. 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 alterations[51–55].

Gene deletions. Following Cas9 cleavage, NHEJ-mediated DNA repair can be harnessed to create gene knockouts. When a coding exon is targeted, 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 deletions[59] (FIG. 2a). A systematic exploration of Cas9-mediated deletion efficiencies showed an inverse correlation between deletion size and its frequency[60]. In addition to studies in cells, strategies have been developed to facilitate heritable genomic deletions in organisms such as zebrafish[61] and mice[62–64]. The wide spectrum of possible Cas9-mediated genomic deletions is accelerating the investigation of genes and genetic elements.

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

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 on the basis of 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 interest[65] (FIG. 2a). Generic plasmid-based systems to create endogenous amino-terminal[66] 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 owing to the simplicity of these modular Cas9-mediated systems. HITI uses NHEJ for DSB repair, creating two problems: generation of indels and donor integration in a 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-stranded DNA (ssDNA) was used to tag endogenous human genes with GFP-coding sequences[68] (FIG. 2b). Mice with multiple precise single-point mutations were generated by multiplexed HDR in mouse embryonic stem cells[69]. Cancer modelling in mice can also be achieved by HDR-mediated insertion of missense gain-of-function mutations[70]. To generate conditional knockout mice at high efficiency by insertion of large regulatable cassettes, ribonucleoproteins were co-delivered with long ssDNA donors containing short homology arms[71]. Recently, an HDR-dependent strategy termed 'CORRECT' (consecutive reguide or re-Cas steps to erase CRISPR–Cas-blocked targets) was developed to produce scarless targeted knock-in of disease-relevant mutations[72]. If variations in the donor template are made, 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 indels[73] (FIG. 2b).

Translocations. During cancer development, oncogenic fusion genes are frequently created through chromosomal translocations. Translocations can be mediated by illegitimate NHEJ of DSBs located in 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 mice[76]. 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 the creation of genetic disease models and the development

of corrective therapeutics. Targeted HDR-mediated single-base editing can be achieved by co-delivery of Cas9 and a homologous donor sequence that contains the edited nucleotide of choice[72]. However, such strategies remain inefficient, particularly in postmitotic 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

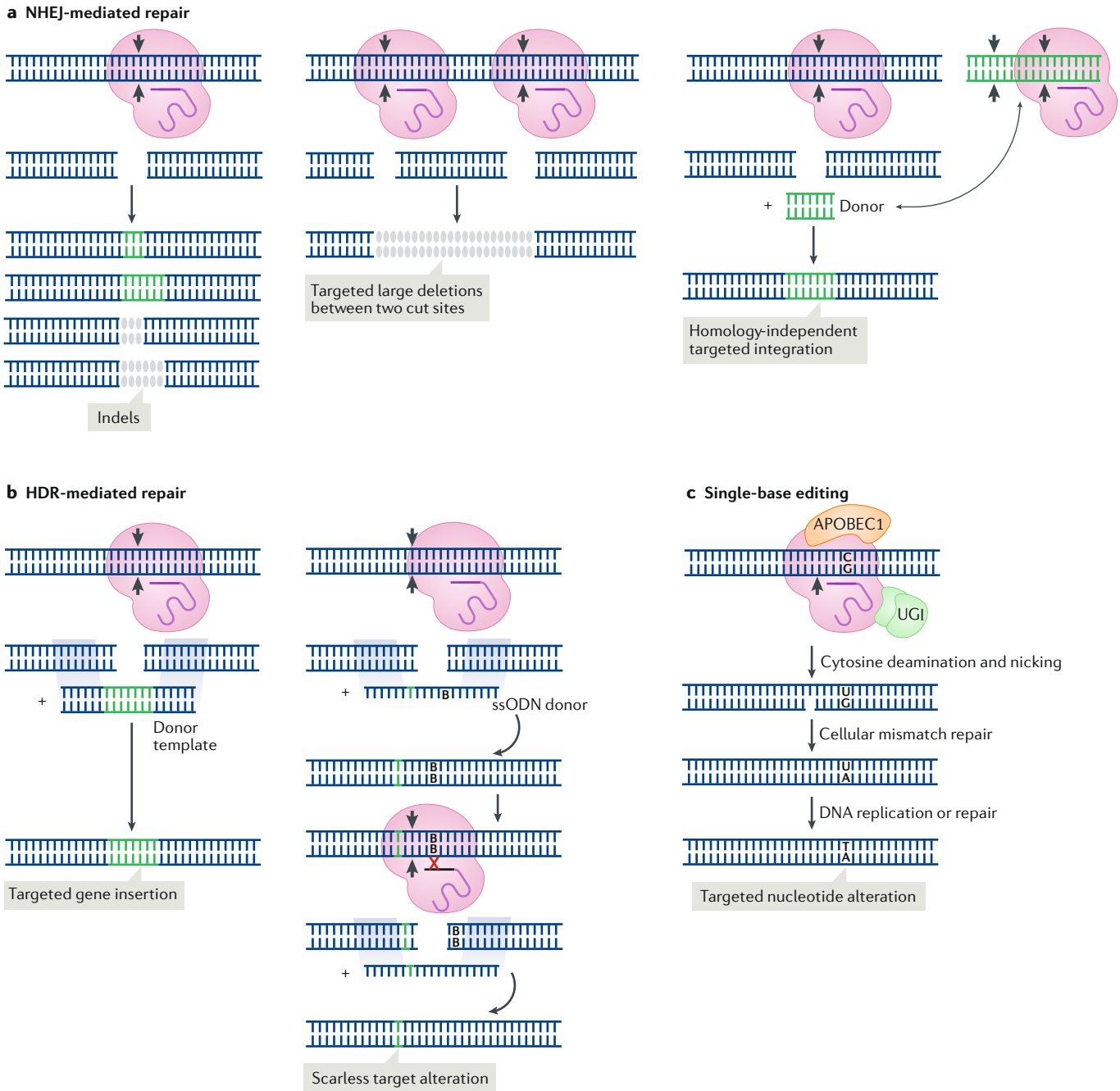


Fig. 2 | **Genome editing strategies.** Nucleases generate targeted DNA double-strand breaks, which can be repaired by different repair pathways. **a** | Non-homologous end joining (NHEJ)-mediated repair is error-prone and induces small insertion or deletion mutations (indels). Large, targeted deletions can be produced through repair between two double-strand breaks produced by simultaneous targeting of nucleases to two genomic sites. Alternatively, homology-independent targeted integrations can be directed to a single cut site by providing donor DNA that is independently targeted for cutting[65]. **b** | The homology-directed repair (HDR) pathway can be used for genome editing by providing either double-stranded or single-stranded oligodeoxynucleotide (ssODN) donor templates that contain homology arms (grey rectangles) 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 DNA[247] or long single-stranded DNA[71]. 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 alterations[73]. **c** | For single-nucleotide C→T (or G→A) conversion, Cas9 nickase has been fused to cytidine deaminases such as APOBEC1 (REF. [79]). For increased base-editing efficiency, two uracil glycosylase inhibitors (UGIs) have been fused to a base editor for prevention of cellular base excision repair[88].

activation of DNA repair pathways can have adverse effects on cell viability[77,78].

For improved single-base editing, tools have been developed that utilize Cas9 nickase or catalytically deficient Cas9 (dCas9) for site-specific targeting without generating DSBs. For direct conversion of single nucleotides, dCas9 or Cas9 nickase has 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] (FIG. 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 editing[79–81]. A third generation editor (BE3) containing APOBEC1 fused to a 16-residue XTEN linker, Cas9 nickase and a uracil glycosylase inhibitor (APOBEC1–XTEN–dCas9(A840H)–UGI) can achieve permanent conversion of 15–75% of a target nucleotide in mammalian cells[79]. Furthermore, BE3 has accomplished base editing in vivo through ribonucleoprotein-mediated protein delivery to mouse and zebrafish embryos[82,83], AAV-mediated delivery in utero to mice[84] and injection of mRNA and gRNA into 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 levels[87].

Continued development of BE3 has resulted in improved single-base editing. For example, increased Cas9-mediated targeting specificity has been achieved by combining BE3 with a high-fidelity Cas9 (REF.[83]). For optimization of base editing, lengthening of the linker between the fused proteins and addition of 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 LbCas12a, which recognizes a T-rich PAM and has a 6-bp activity window[89]. Recently, enAsCas12a was used for enhanced base-editing activity[35]. 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 plants[92]. Specifically, eA3A–BE3, an engineered APOBEC3A domain (eA3A) fused to BE3, preferentially deaminates cytidines according to a TCR>TCY>VCN hierarchy[90]. Additionally, APOBEC3A-mediated base editing can be achieved in regions with high DNA methylation levels and CpG dinucleotide content[91].

Recently, the base editor toolbox has been expanded to adenine base editors (ABEs), which can perform targeted A→G (or T→C) nucleotide conversions[93]. A seventh-generation ABE with the highest reported editing efficiencies and on-target activities was developed by directed evolution and protein engineering of a tRNA adenosine deaminase[93]. Optimized and enhanced cytidine and ABEs include BE4max, AncBE4max and ABEmax[94].

Unbiased analysis 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 alone[95]. 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 in different chromosomes that could generate translocations would be particularly desirable.

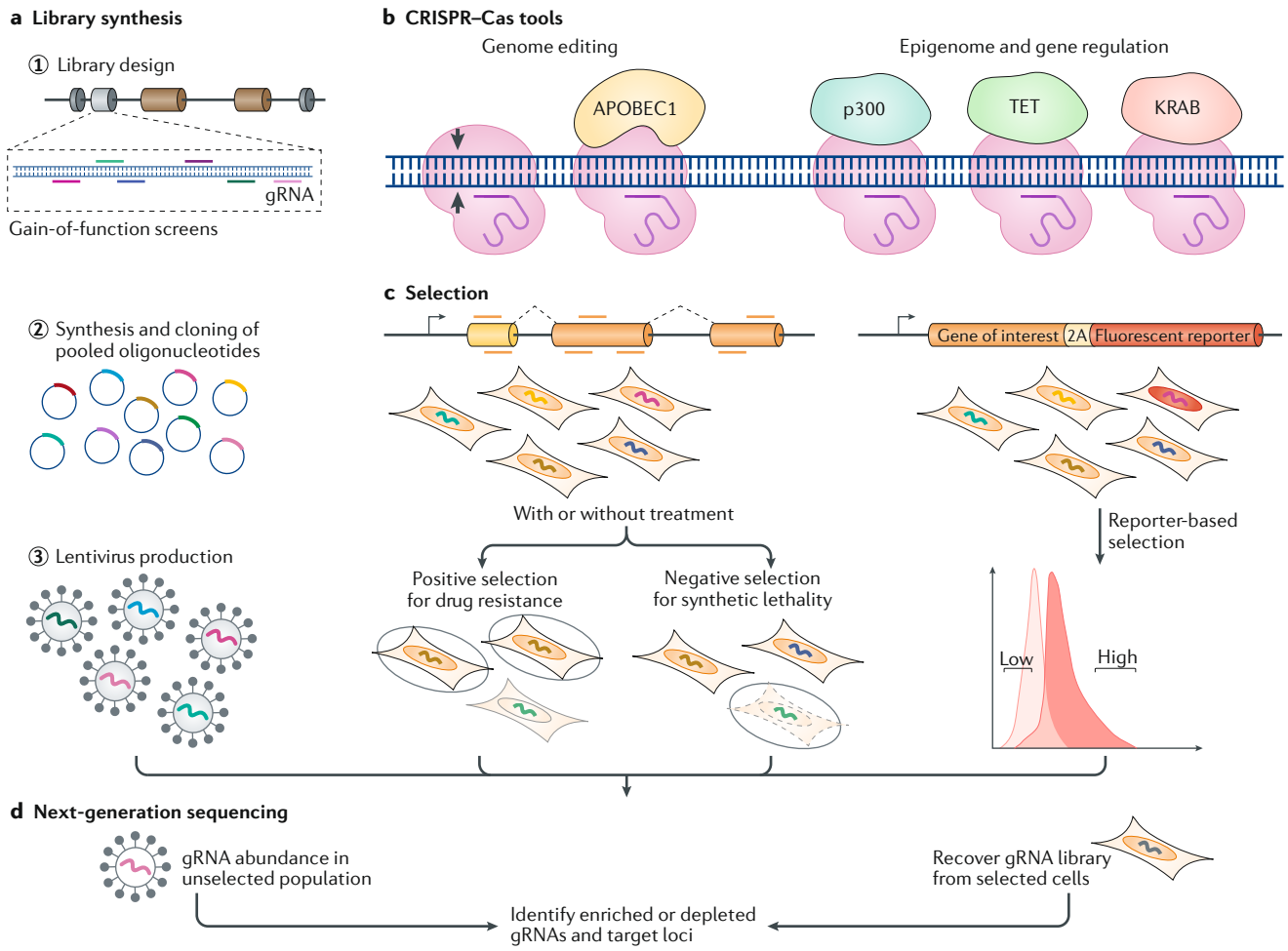
High-throughput loss-of-function screens. RNA interference has been the primary system for large-scale gene perturbation in mammalian cells, but its limitations include incomplete suppression of target genes and frequent off-target effects. These limitations can largely be overcome by Cas9-based methods 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 knock out more than 18,000 human genes using three to four gRNAs per gene[99] — has made genomic knockout screens possible in mouse cells[100,101,103] and human cells[99,104,105]. Such a mouse-genome-targeting library was delivered to a mouse model of tumour growth and metastasis, and loss-of-function mutations in known tumour 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 sequencing[106–108]. 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 of gRNA-library design are also improving the quality and increasing the throughput of these screens[109].

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 dissection of genetic interactions and identification of 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

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 non-coding genome may target sites of accessible chromatin[193] or transcription factor motifs[123]. 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 before gRNA delivery, or cells can be co-transduced with Cas9 and the gRNA library. Fusion of Cas9 nickase or catalytically deficient Cas9 to different effector proteins can allow 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 Krüppel-associated box (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 resistance[236] and negative selection can identify elements involved in synthetic lethality[237] (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 reporter[193]. 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, part **d**). A wide range of applications is possible with CRISPR-based screens. Interrogation of gene function can identify genes involved in cell survival and proliferation or cancer genes[100,105,237,238]; drug targets can be identified on the basis of resistance or sensitivity to drugs, toxins or pathogens[237,239]. Targeted screens are also mapping the function of the non-coding genome by perturbing enhancer sequences[113] or modulating particular sets of genes; for example, targeted activation of all transcription factor genes to identify factors involved in stem cell differentiation[240]. 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.



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-STOP'[79], was developed using CRISPR base editors to create stop codons by single nucleotide conversion. To expand this induction of stop codons method, a database of more than 3.4 million gRNAs targeting 97–99% of genes in eight eukaryotic species was compiled[112]. 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 were not previously possible. For example, the *BCL11A* gene encodes a transcription factor that controls the levels of fetal haemoglobin[113]; modulation of *BCL11A* expression by perturbing cell type-specific enhancers could be used as a therapeutic approach for β-haemoglobin disorders. By tiling of 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 haemoglobin reinduction[113]. This work involved the introduction of indel mutations at non-coding 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 β-thalassaemia[114]. Alternatively, HDR was used to introduce all possible nucleotide substitutions into a putative gene regulatory element to decipher its function[115]. Numerous other high-throughput tiling approaches are being used to identify functional elements in regulatory regions[116–123]. Finally, a genomic screening method that targets splice sites was used to identify long non-coding RNAs that are essential for cellular growth[124]. By use of the same library to screen multiple cell lines, cell type-specific differences in long non-coding RNAs 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 in plasmid or genomic DNA[127]. These CRISPR–Cas-based molecular recording systems have been used to track cellular behaviour 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 by 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 one-cell zebrafish embryos containing a compact DNA barcode with multiple Cas9 target sites[128]. By tracking these scars in hundreds of thousands of cells from individual zebrafish, it was found that most organs derive from relatively few embryonic progenitors[128]. 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 result in increased scarring complexity, which provides more information for improved phylogenetic annotation[126]. Another synthetic recording system, termed MEMOIR (memory by engineered mutagenesis with optical in situ readout), was developed to record and subsequently read lineage information out of single cells in situ[129]. This system combines Cas9-based targeted mutagenesis with multiplexed single-molecule RNA fluorescence hybridization to visualize recorded editing events for studying lineage tracing while maintaining the relative spatial positioning of cells[129].

An alternative recording strategy is based on integrating nucleotides into bacterial genomic crRNA arrays as trackable molecular events. This mechanism uses the natural adaptation process of prokaryotic CRISPR–Cas systems, in which Cas1 and Cas2 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 arrays[5], this mechanism can be harnessed to track 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 events[131]. Recently, this technique was scaled to store synthetic sequences encoding pixel values of black-and-white images and a short movie in the genomes of living bacteria[132]. These studies demonstrate the capacity of DNA to encode and store analogue 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, the development of CRISPR–Cas technology for binding or cleaving specific RNAs has advanced RNA manipulation in living cells (FIG. 3).

RCas9

Cas9 can be made to cleave ssDNA targets by providing a PAM-presenting oligonucleotide (PAMmer) that anneals to ssDNA[133]. Similarly, a PAMmer can be provided to direct Cas9 to single-stranded RNA (ssRNA) targets[134] (FIG. 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', requires only the design and synthesis of a matching gRNA and complementary PAMmer[134]. By targeting dCas9 to RNA, RCas9 can be used as a programmable RNA-binding protein for RNA recognition (FIG. 3b). This modular tool permits detection of endogenous RNA without the need to genetically encode affinity tags on transcripts. RCas9

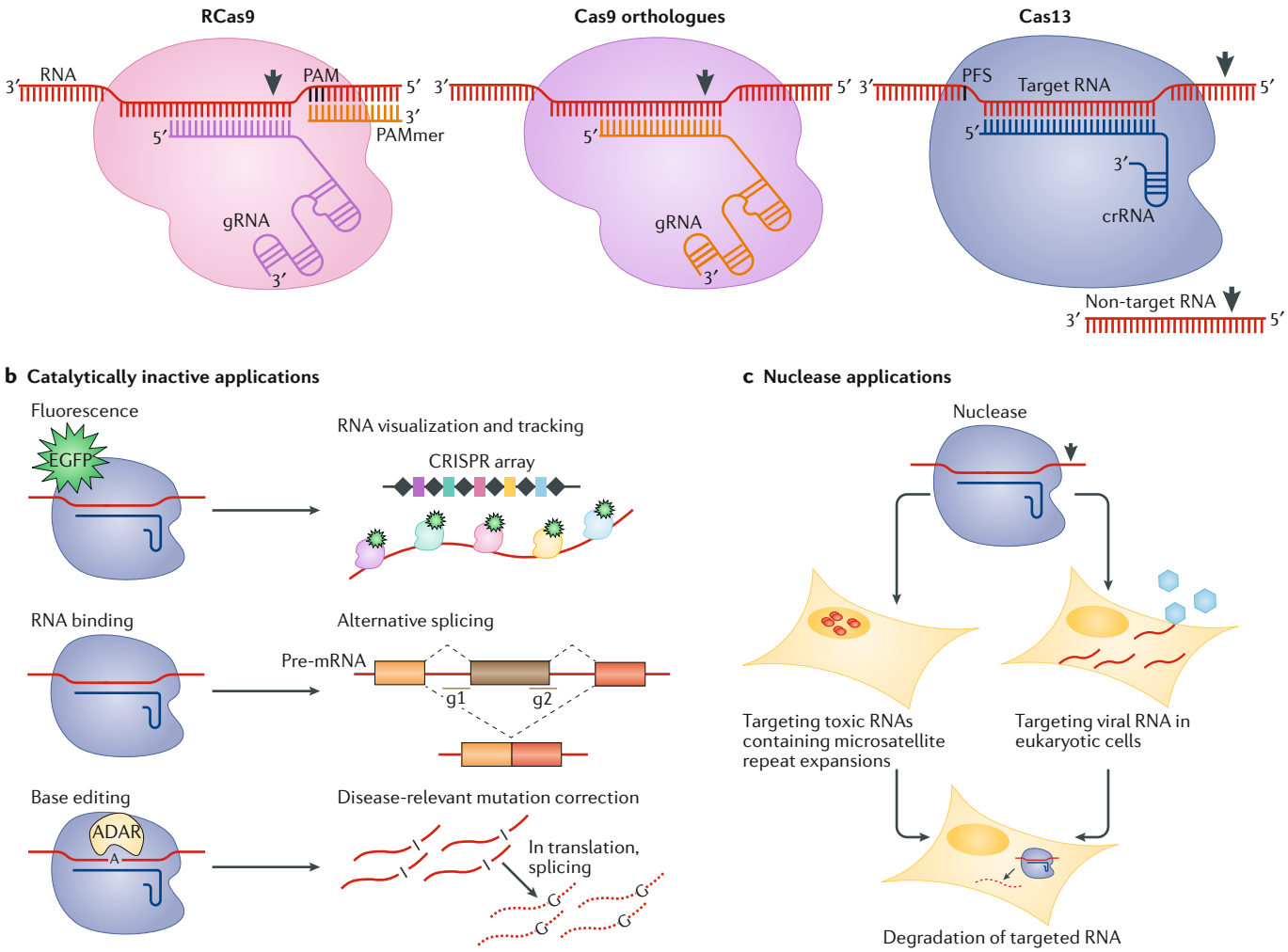


Fig. 3 | **RNA-targeting tools and their applications. a** | *Streptococcus pyogenes* Cas9 was repurposed to target RNA (RCas9) by providing it with a matching guide RNA (gRNA) and a complementary protospacer adjacent motif (PAM)-presenting oligonucleotide (PAMmer)[134]. Cas9 orthologues such as *Staphylococcus aureus* Cas9 and *Campylobacter jejuni* Cas9 can target RNA in the absence of a PAMmer, thereby demonstrating PAM-independent RNA cleavage[138]. Cas13 proteins are RNA-guided RNA-targeting nucleases, some requiring recognition of a protospacer flanking sequence (PFS). Cas13 generates cuts along target and non-target RNA molecules using two HEPN domains, which are nucleotide-binding domains with RNA-cutting activity[142]. **b** | Similarly to catalytically deficient Cas9, catalytically deficient Cas13 maintains the capacity to bind to the targeted RNA. For RNA visualization and tracking purposes, a fluorescent protein can be fused to the catalytically deficient Cas and colocalize with an array of CRISPR RNAs (crRNAs) or gRNAs[135,143]. Adenosine deaminase acting on RNA (ADAR) can be fused to catalytically deficient Cas for RNA A→I base editing to correct disease-relevant mutations. To promote alternative splicing, catalytically deficient Cas13 can be targeted with gRNAs (g1 and g2) to bind splicing regulating *cis* elements[152]. **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 expansions[136]. *Francisella novicida* Cas9 has been repurposed in eukaryotic cells to target the RNA genome of hepatitis C virus[141]. EGFP, enhanced GFP.

Stress granules
Cytosolic membraneless bodies with high concentrations of RNA and/or proteins that 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.

binding to specific mRNAs has been used for their visualization and tracking into stress granules in living cells[135]. Further development of this technology may provide a useful tool for RNA visualization of mRNAs of low abundance or low concentration.

Catalytically active RCas9 can stimulate site-specific cleavage of ssRNA[134]. Thus, RCas9 can be used to control cellular processes at the transcript level. Therapeutic strategies to block the expression of toxic RNA can use 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 visualization and elimination of toxic RNA species associated with microsatellite-repeat expansion diseases[136] (FIG. 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 expression[137], and truncated versions of RCas9 have been generated that are compatible with the limited AAV packaging capacity[136].

Cas9 orthologues

Although in nature Cas9 is thought to preferentially target phages and DNA in bacteria, Cas9 orthologues have the capacity to also target RNA. SaCas9 and *Campylobacter jejuni* Cas9 can directly cleave ssRNA in a PAM-independent manner[138] (FIG. 3a). When targeted to RNA, SaCas9 repressed gene expression in *Escherichia coli*[138], and for *C. jejuni* Cas9, crRNA-dependent but PAM-independent binding and cleavage of endogenous RNAs was shown[139].

Francisella novicida Cas9 (FnCas9) was originally shown to target bacterial mRNA and alter gene expression[140], and has been repurposed to target the RNA genome of hepatitis C virus in eukaryotic cells[141]. 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 hepatitis C virus genome, FnCas9 inhibited both viral protein production and viral replication (FIG. 3c). Unlike RCas9, RNA targeting by FnCas9 is PAM independent and thus does not require PAMmers[141]. FnCas9 could also potentially be used to target negative-sense ssRNA viruses such as those belonging to the families *Filoviridae*, *Paramyxoviridae* and *Orthomyxoviridae*. 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 were 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 on recognition of ssRNA targets[142] (FIG. 3a). Similarly to a PAM sequence, some type VI CRISPR proteins require recognition of a PFS[142]; however, Cas13a from *Leptotrichia wadei*[143] and Cas13b from *Prevotella* sp. P5-125 (REF.[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 unknown[143]. A catalytically inactive Cas13a variant, dCas13a, maintains the ability to bind targeted RNA and was used for live-cell imaging of RNA[143]. Similarly 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 bacterial cells has also been demonstrated in vitro with purified Cas13a[143,145]. This promiscuous RNase activity, which is induced on target recognition, has been used 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 virus and dengue virus[146]. Additionally, amplified DNA can be converted into RNA for subsequent Cas13-mediated detection[146]. Following this conversion into 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 tumour 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 simultaneously detect up to four targets[147]. Recently, Cas12a was repurposed as a detection tool. Following targeted activation by double-stranded DNA, Cas12a non-specifically cleaves ssDNA[148]. By providing a quenched ssDNA reporter, the collateral cleavage by Cas12a can be used to detect viral DNA in patient samples[146,148].

A single-base RNA editing application was developed by fusion of dCas13 to adenosine deaminase acting on RNA (FIG. 3b). This system, termed 'REPAIR' (RNA editing for programmable A to I replacement), can make directed adenosine-to-inosine edits in eukaryotic cells[144]. 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 APOBEC1 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 and 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-mediated cleavage, Cas13d-mediated cleavage promotes collateral RNA cleavage in bacteria[151] but not when expressed in mammalian cells[152]. RNA recognition by Cas13d is PFS independent. Furthermore, dCas13d lacks target-RNA cleavage activity but retains ribosomal RNA array processing activity, and, notably its smaller size makes packaging into vectors such as AAV possible for in vivo applications[151,152]. These characteristics have been used to deliver dCas13d and a crRNA array targeting *cis* elements in pre-mRNAs to manipulate alternative splicing in a neuronal model of frontotemporal dementia[152] (FIG. 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 nuclease[153]. 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 fusion of this dCas9 with diverse effectors such as transcription repressors or activators, epigenetic modifiers and fluorophores (FIG. 4).

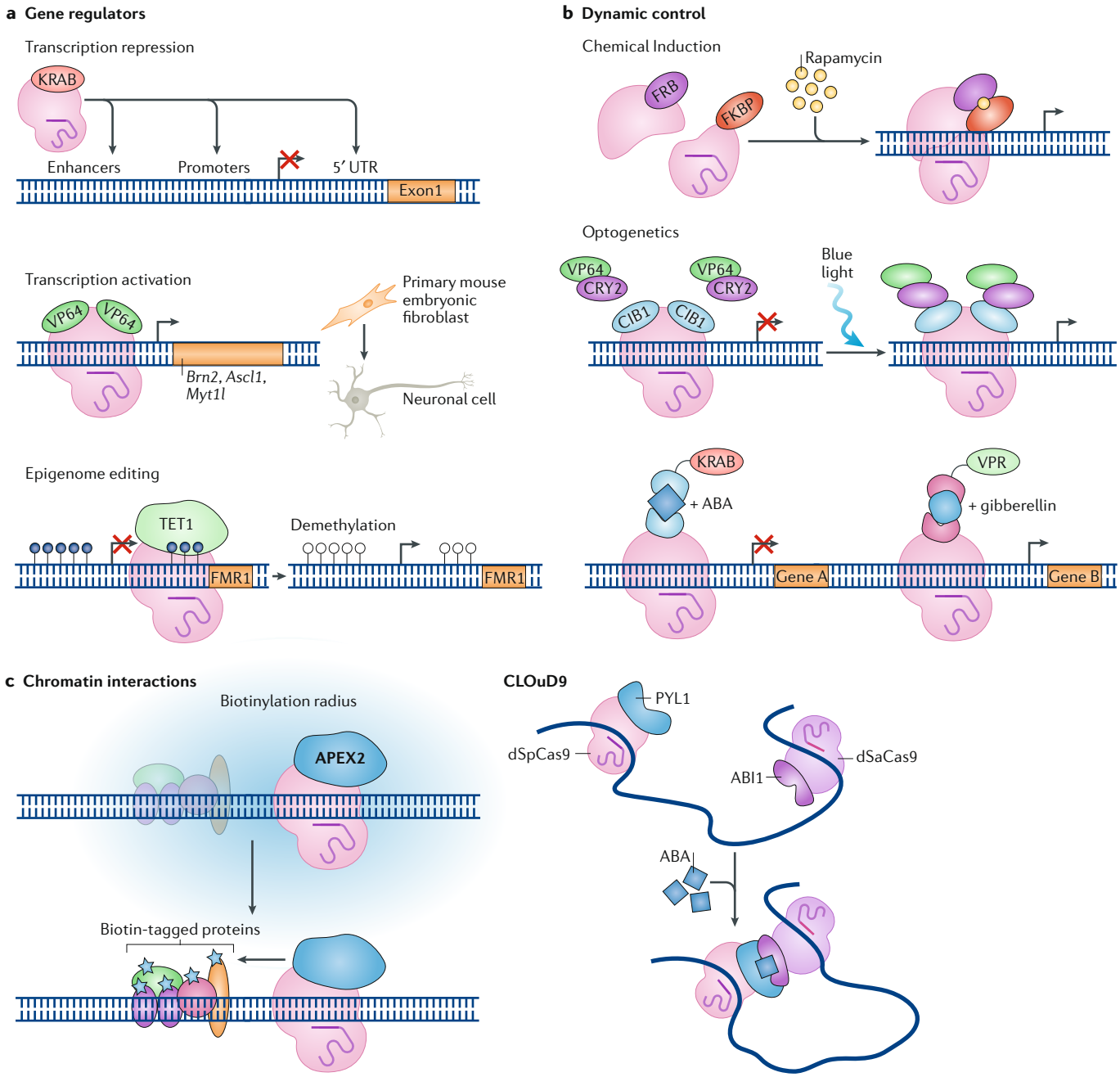


Fig. 4 | **Targeted gene regulation and other applications. a** | For transcription repression, catalytically deficient Cas9 (dCas9) alone or dCas9 fused to effectors such as the transcription repression domain of Krüppel-associated box (KRAB)[157] can be targeted to promoters, 5′ untranslated regions (UTRs) or enhancers[156,159–161]. Transcription activation can be targeted by fusion of 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 cells[172]. 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 syndrome[176]. **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 FKBP and FRB activates target-gene expression. Alternatively, light-inducible dimerization of the cytochrome CRY2 with its binding partner, CIB1, can be used in photoactivatable systems. Combinations of inducible dCas9 orthologue-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 gene[189]. **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 by mass spectrometry[200]. Distal loci can be brought into proximity using 'chromatin loop reorganization with CRISPR–dCas9' (CLOuD9). In the CLOuD9 system, dSpCas9 and dSaCas9 targeted to distal loci are fused to the dimerizing ABA-binding proteins PYL1 and ABI1 (REF.[202]). ABA induces targeted protein dimerization and chromatin looping, which can be reversed following its removal to restore the endogenous chromatin conformation.

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.

CRISPR interference. Binding of dCas9 to DNA elements may repress transcription by sterically hindering the RNA polymerase machinery[154]. dCas9-mediated steric interference, termed 'CRISPR interference' (CRISPRi), works efficiently in prokaryotic cells but is less effective in eukaryotic cells[154–156]. To enhance the repressive capacity of CRISPR in eukaryotic cells, dCas9 was tethered to transcription repressor domains such as that of Krüppel-associated box (KRAB)[156], which is found in many natural zinc finger transcription factors[157]. KRAB is known to induce heterochromatin formation, and changes in chromatin structure often accompany dCas9–KRAB-targeted transcription repression[158]. dCas9–KRAB is a robust tool in mammalian cells that can effectively silence single genes and non-coding RNAs by targeting promoter regions, 5′ untranslated regions and proximal and distal enhancer elements[156,159–161] (FIG. 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 2 (REF.[162]). The versatility of dCas9–KRAB is highlighted by its capacity to repress transcription by targeting both genes and gene-regulatory regions[161].

CRISPR activation. 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 nuclear factor-κB 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 domains[166–170]. Multiplexed activation of endogenous genes can also be used for cellular reprogramming[171]. For example, direct conversion of primary mouse embryonic fibroblasts into 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* (also known as *Pou3f2*), *Ascl1* and *Myt1l* genes[172] (FIG. 4a), and similar approaches have been applied to reprogramming cells into pluripotency[173] or to myogenic cells[167].

Epigenome editing. Targeted epigenetic modifications, such as acetylation and methylation of histones and methylation of DNA, can be achieved using dCas9-based tools[153]. For example, the fusion of dCas9 to the catalytic core of the human histone acetyltransferase p300 was targeted to promoters and enhancers for catalysis of the acetylation of histone H3 Lys27, leading to robust gene activation[174]. DNA demethylation was achieved using dCas9 fusions with the catalytic domain of methylcytosine dioxygenase TET1. Targeting of dCas9–TET1 to the *BRCA1* promoter resulted in transcription upregulation[175]. As a potential therapy, dCas9–TET1 was used to demethylate the CGG-expansion mutation in the 5′ untranslated region of the *FMR1* gene and reverse its silencing, which is associated with fragile X syndrome[176] (FIG. 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 gRNAs[177]. 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. On the basis of the type of stimulus, various strategies have been developed to generate inducible Cas9-based systems that permit temporal control of Cas9-mediated gene targeting (FIG. 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 mice[180]. However, doxycycline-independent mutagenesis was observed in the transfected cells, suggesting that the expression of Cas9 is leaky in some of these systems[180].

Inducible dCas9-based systems also offer versatility in epigenome engineering. A doxycycline-inducible CRISPRi system allowed efficient, tunable and reversible disease modelling in induced pluripotent stem cell-derived cardiomyocytes[181]. Chemically inducible CRISPRa systems have been developed with use of conditionally stabilized dCas9 activators[182] and with use of split dCas9 activators that dimerize following chemical induction[183,184] (FIG. 4b). The beneficial uses of inducible split dCas9 activators include minimizing leaky dCas9 expression and targeting of multiple genes for multiplexed temporal regulation[184].

Optogenetics. Light-inducible dCas9 systems allow precise dynamic regulation of endogenous genes and the possibility of spatial control. For example, light-inducible dimerization of the plant-derived cytochrome CRY2 with its binding partner, CIB1, has been used to create photoactivatable dCas9–p65 (REF.[185]) and dCas9–VP64 (REF.[186]) (FIG. 4b). An anti-CRISPR protein was engineered for light-mediated spatiotemporal control of genome and epigenome editing in human cells by pairing a photosensor from *Avena sativa* with an SpCas9 inhibitor[187]. A second-generation optogenetic split-protein system was developed and targeted to upregulate the expression of the *NEUROD1* gene to induce neuronal

Anti-CRISPR protein
A protein that interacts with and inhibits CRISPR–Cas activity

differentiation in induced pluripotent stem cells[188]. For more complex regulation, multiple chemical-inducible and light-inducible systems have been used to dynamically manipulate the activation or repression of multiple genes[189] (FIG. 4b). These light-inducible systems hold promise for modelling 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 non-coding genome. Cas9-engineered 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, the 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 allow gain-of-function studies. CRISPRi and CRISPRa have been combined in parallel screens to target DNase I hypersensitive sites that surround genes of interest[193]. 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 long non-coding RNAs whose function might not be altered by introduction of indels with Cas9 nucleases[195,196].

Chromatin interactions

Chromatin structure modulates genome function; however, elucidation of 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 with 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 proteins[197]. Specifically, enChIP was used for biochemical analysis of transcription and epigenetic regulation at specific genomic loci in living cells[198]. 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 after affinity purification and mass spectrometry[200] (FIG. 4c).

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 interactions[201]. Chromatin loop reorganization with CRISPR–dCas9 (CLOuD9) can selectively and reversibly establish chromatin loops and modulate the expression of associated genes[202] (FIG. 4c). As an alternative to the chemically induced CLOuD9 system, a light-inducible dCas9 system was developed to direct rearrangement of chromatin looping on faster timescales[203]. A chemically inducible and reversible system termed 'CRISPR-GO' can control spatial genome organization within the cell[204]. CRISPR-GO allows for the study of 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 expression[205]. 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, enhanced GFP-tagged dCas9 and structurally optimized gRNAs have been targeted to repetitive elements and to coding genes[206]. By the targeting of a large number of loci, labelling of an entire chromosome was made possible for live-cell imaging[207]. Depending on chromosome length, painting entire human chromosomes could require about 100–800 gRNAs.

To expand these tools to multicolour genome imaging, orthogonal dCas9 regulators have been tagged with different fluorescent proteins[208,209]. Other dCas9-based multicolour, live-cell imaging methods have focused on engineering gRNA scaffolds. By adaptation of gRNA scaffolds to bind sets of fluorescent proteins, up to six targeted chromosomal loci were visualized simultaneously[210]. Additionally, gRNA aptamer insertions have been engineered that concurrently bind two different fluorescent protein tags[211]. This dual-colour approach is tolerant of photobleaching, 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.

Preclinical 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

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

gRNA scaffolds
The backbone (invariable) portions of guide RNAs, which are recognized by Cas proteins.

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

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

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

Despite the advances in CRISPR–Cas-based genome engineering technologies, some challenges remain to translate 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 orthologues such as *Staphylococcus aureus* Cas9 (REF.[29]) and *Campylobacter jejuni* Cas9 (REF.[31]). Nevertheless, prolonged expression of Cas9 from AAV vectors and integration of AAV vectors into double-strand breaks 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)[241] can be used with CIRCLE-seq (cleavage effects by sequencing)[242] 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[221,243] but the implications of this for therapeutic approaches are still unclear. Recently, pre-existing adaptive immunity to *Streptococcus pyogenes* Cas9 and *S. aureus* Cas9 was detected in human blood samples[244–246]. 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 orthologues. Other approaches to limit immunogenicity include the re-engineering of immunogenic epitopes of Cas proteins, the use of transient immunosuppressive drugs during treatment and 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 tumour suppressor p53 (REFS[77,78]).

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, there are a variety of editing strategies that manipulate genes involved in more common, complex diseases. For example, by targeting SpCas9 to the mouse cholesterol homeostasis gene *Pcsk9* through adenovirus delivery, a reduction in the level of low-density lipoprotein cholesterol was demonstrated following gene disruption and silencing in vivo[212]. For preclinical assessment of somatic genome editing applications, this work was expanded to successfully target the human *PCSK9* gene in mice in which human hepatocytes had been engrafted[213]. 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 degradation[215]. An HITI-mediated Cas9 insertion repaired the 1.9-kb deletion in the kinase gene *Mertk* in a retinitis pigmentosa rat model and restored MERTK function[65]. The gene therapeutics were delivered to the eye by 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 dystrophin[216]. Restoration of the reading frame in vivo was achieved in several studies following AAV-mediated delivery of CRISPR–Cas9 to excise additional exons through NHEJ around the inherited deletion[217–219], including a mutation correction that was sustained for at least 1 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 DMD[222]. 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 can be used to deliver Cas9 in vivo. Recently, lipid nanoparticles containing SpCas9 mRNA and a chemically modified gRNA targeting the mouse *Ttr* gene were delivered to mice[225]. Following a single administration, a reduction in serum transthyretin 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 methods[226]. In particular, transient, non-virally 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 immunotherapy[228]. Gene editing approaches have been used to enhance the properties of these engineered cells. For example, the insertion of transgenes encoding programmable chimeric antigen receptors into the endogenous T cell receptor-α constant gene, rather than overexpression of chimeric antigen receptors from viral vectors, prevents the exhaustion of T cells from overstimulation while also eliminating endogenous T cell receptor expression that could direct graft-versus-host disease[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[231], which would address the practical and economic challenges of patient-specific autologous cell therapies. Researchers have also targeted programmed cell death protein 1 to block inhibitory signals that prevent T cell recognition of tumour cells[230,232,233]. Autologous T cells that were treated ex vivo with Cas9 to knock out the genes encoding PD1 and the α and β chains of the T cell receptor were infused back into individuals with cancer in the first use of CRISPR–Cas gene

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.

editing in a human clinical trial in the United States or Europe (NCT03399448)[231,234]. Also currently under way are the first human trial of CRISPR–Cas to treat a genetic disease, β-thalassaemia (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 nucleases[235]. 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 the 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 allow 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 the genome sequence, and thus improving methods for detection of rare mutations and quantifying their potential risks will be important for future clinical advancement.

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C.A.G. and A.P.-O. are inventors on patent applications related to CRISPR technologies. C.A.G. is a scientific adviser to Element Genomics, Locus Biosciences and Sarepta Therapeutics.

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