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CRISPR-Based Technologies: Prokaryotic Defense Weapons Repurposed

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Summary

To combat potentially deadly viral infections, prokaryotic microbes enlist small RNA-based adaptive immune systems (CRISPR-Cas systems) that protect through sequence-specific recognition and targeted destruction of viral nucleic acids (either DNA or RNA depending on the system). Here, we summarize rapid progress made in redirecting the nuclease activities of these microbial immune systems to bind and cleave DNA or RNA targets of choice, by reprogramming the small guide RNAs of the various CRISPR-Cas complexes. These studies have demonstrated the potential of Type II CRISPR-Cas systems both as efficient and versatile genome editing tools and as potent and specific regulators of gene expression in a very broad range of cell types (including human) and organisms. Progress is also being made in developing a Type III RNA-targeting CRISPR-Cas system as a novel gene knockdown platform to investigate gene function and modulate gene expression for metabolic engineering in microbes.

Keywords

CRISPR; Cas; genome editing; RNA silencing; biotechnology; metabolic engineering

CRISPR-Cas: Prokaryotic small RNA-based defense systems

Prokaryotic microbes (bacteria and archaea) are under constant attack by potentially deadly viruses and other harmful invading mobile genetic elements. The viruses evolve rapidly and are estimated to outnumber the prokaryotes 10 to 1 [1]. Not surprisingly, prokaryotes have evolved a variety of innate defense mechanisms to ward off these predators (reviewed in [2–5]). The CRISPR-Cas systems are the most recently discovered armament in the prokaryotic arsenal (recently reviewed in [4, 6–9]). The unique features of these RNA-guided adaptive immune systems have captivated the scientific community, and the molecular mechanisms that underlie the pathways (Figure 1) are the subject of intense research. In turn, the newly acquired knowledge is being rapidly deployed in the development of exciting new CRISPR-based technologies with important biotechnological, industrial, and biomedical applications.

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Components of the multiple types of CRISPR-Cas systems: Arsenal inventory

At least eleven distinct CRISPR-Cas systems have been identified [4, 6–11]. The systems can be grouped into three broad classes, Types I, II and III, which include eleven distinct subtypes: Types I A–F, Types II A–C and Types III A and B [6, 11–13] (Table 1). Each of the systems and its specific Cas protein components are referred to by a name derived from a model organism for the system (e.g., Cse or Cas subtype *ME. coli* for Type I–E) [10, 14]. Most of the CRISPR-Cas systems that have been characterized target invader DNA [4, 15–21]; however, the Type III-B Cmr system cleaves target RNAs [22–26].

The guide RNAs of the CRISPR-Cas systems arise from the CRISPRs (clustered regularly interspaced short palindromic repeats): loci present in prokaryotic genomes that contain tandem arrays of a short direct repeat sequence (~30–40 basepairs) separated by a series of similarly-sized “spacer” sequences acquired from viruses and other invaders (where the same sequence is known as a “protospacer”) [16, 27–31] (Figure 1). Transcription of CRISPR loci and subsequent processing of CRISPR transcripts produces mature CRISPR (cr)RNAs that each contain an invader-derived (guide) sequence flanked by the signature CRISPR repeat (tag or handle) sequence at the 5′ and/or 3′ end, depending on the system [24, 32–38].

crRNAs interact with CRISPR-associated (Cas) proteins to form ribonucleoprotein (crRNP) effector complexes that recognize (via crRNA basepairing) and cleave the DNA or RNA of the invader [17, 18, 23, 32, 39–43] (Figure 1). Each CRISPR-Cas system includes a characteristic module of Cas proteins typically encoded near the CRISPR locus (Table 1). Some of the Cas proteins are members of broad superfamilies found in multiple CRISPR-Cas systems [10, 11, 14]. Cas3 and Cas9 superfamily proteins are known effector nucleases (i.e., DNases that destroy the invader DNA) for Type I and Type II systems, respectively [17, 18, 27, 32, 44–46]. The identity of the effector nuclease component of Type III CRISPR-Cas systems is not yet known.

Exploiting the CRISPR-Cas systems: strain surveillance and protection

The discovery and understanding of the CRISPR-Cas systems used by prokaryotes to defend themselves against invaders encountered in their environments is being leveraged in a number of ways [47–49]. CRISPR locus sequences can evolve rapidly in response to local predators and thus vary among isolates of related strains. This feature is being exploited for fast and precise strain identification in epidemiological investigations (e.g., to track pathogenic strains involved in disease outbreaks) and to probe microbial diversity and host-virus interactions in natural environments including human microbiomes [50–55].

A variety of bacteria and archaea are employed to make important products, including pharmaceuticals, biofuels, commodity chemicals, and food. Unique CRISPR locus sequences can be used to tag and identify valuable industrial strains [47]. Moreover, priming or manipulation of CRISPR-Cas systems can generate phage-resistant commercial strains, improving product quality and reducing economic losses due to culture infections [51]. Resistant strains can be isolated by deliberate exposure to phages and selection of strains with arrays of phage-targeting sequences in their CRISPR loci [51]. Alternatively, resistant strains can be deliberately engineered by introduction of invader sequences into existing CRISPR loci or by introduction of entire CRISPR-Cas modules into strains lacking defense [56, 57].

As described below, individual CRISPR-Cas pathway enzymes are also being repurposed as powerful weapons for the research and medical fronts.

Repurposing Type II CRISPR-Cas effector complexes: powerful new RNA-guided genome editing tools

The ability to eliminate, replace, or modify sequences within a genome in a highly targeted manner (genome editing) has a broad range of important basic and applied applications including fundamental gene discovery, plant and microbe bioengineering and correction of human gene disorders (gene therapy). Targeted gene editing *in vivo* can be accomplished through ectopic expression of artificial protein nucleases (typically, zinc finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs)) individually designed to recognize a specific target DNA site and introduce a double-stranded (ds)DNA break [8, 58–60]. Different types of mutations can be obtained depending upon which endogenous DNA repair pathway is engaged to repair the dsDNA break. If the dsDNA break occurs in the presence of a co-introduced, editing template (donor DNA fragment that shares homology with target DNA), this can trigger DNA repair by the high fidelity, homologous recombination (HR) pathway to replace, remove, or alter/correct the target gene in a manner specified by the editing template. In the absence of an editing template, cells typically repair the dsDNA break by the relatively error-prone mechanism of non-homologous end joining (NHEJ), which typically leads to short gene insertions or deletions (indels) and thus can yield gene inactivation mutations.

The “Cas9” Type II CRISPR-Cas immune system effector complex has recently emerged as a powerfully adaptable, next-generation genome editing tool: an attractive alternative to the development of individual protein enzymes to induce individual genome modifications (Figure 2) [48, 61–63]. In a short period of time, a hail of studies have reported successful RNA-guided genome editing with Cas9 crRNP complexes in diverse cell types and model organisms, including several cultured human cell lines [63–75], rodents [69, 76–80], zebra fish [81–84], frog [85, 86], plants [86–93], fruit fly [94–99], worm [100–108], yeast [109] and bacteria [110]. Cas9-based genome editing systems have been used for large scale (including genome-wide) loss-of-function genetic screens in cultured human cells [74, 75] as well as for correction of disease-associated genetic mutations in adult human stem cell-derived organoids and mouse zygotes [73, 80].

Type II CRISPR-Cas system effector complexes are comprised of a single protein: Cas9, an RNA-guided endonuclease that catalyzes blunt-ended dsDNA breaks [16–18, 35] (Figure 2). The system utilizes two RNAs: a crRNA that guides Cas9 to DNA target sequences via base-pairing and a tracrRNA that pairs with the crRNA and plays an unspecified but essential role in target DNA cleavage [18, 35]. The two essential RNAs (crRNA and tracrRNA) can be experimentally joined (by a connecting RNA loop) into a single guide RNA (sgRNA) [18] to simplify the system to just one protein (Cas9) and one RNA (sgRNA).

The only known constraint on target selection for Cas9 crRNPs is the requirement for a very short sequence motif known as the PAM (protospacer adjacent motif) immediately downstream of the desired target site in the genome (protospacer). The most widely used Cas9 enzyme is derived from the bacterium *Streptococcus pyogenes*, and its activity simply requires a 5'-GG-3' (or, with reduced activity, 5'-AG-3') PAM sequence located one base-pair downstream of the target site [13, 18, 63, 65, 110, 111]. Cas9 from *Streptococcus thermophilus* [13, 17, 46, 65, 112, 113], *Neisseria meningitidis* [13, 67, 113] and *Treponema denticola* [113] require different PAMs (5'-NNAGAAW-3', 5'-NNNGA/CTT-3', 5'-NAAAAN-3' respectively), providing additional options for targeting a specific

genome site. Codon-optimized versions of Cas9 are used to facilitate expression of the protein in heterologous cells or organisms, and nuclear localization signals are required for function when eukaryotic species are used.

RNA-directed Cas9 genome editing enzymes have been found to perform with comparable and typically improved efficiencies relative to ZFNs or TALENs in side-by-side comparisons in multiple systems [63–66, 81]. Moreover, Cas9 RNA-based genome editing offers several advantages over protein-guided ZFN and TALEN systems. Targeting new DNA sites with Cas9 simply entails designing an sgRNA that is complementary to the target DNA making the approach much faster, cheaper, and easier to implement (as compared to developing a new ZNF and TALEN protein for each target site). In addition, the Cas9 platform allows simultaneous editing of more than one site (multiplexing) through expression of more than one guide crRNA [65, 66, 110]. The multiplex approach has been used to create mice carrying five different mutant genes in a single step [79], for example, and to introduce large deletions of genomic regions by directing Cas9 cleavages at the two sites flanking the desired deletion [65]. The independent mechanisms by which Cas9 cuts the two strands of a DNA target also provide a distinct advantage. Cas9 employs different nuclease active sites (RuvC and HNH nuclease domains) to cut each DNA strand (Figure 2), which can be independently inactivated (by mutation) to generate a Cas9 enzyme that cleaves one strand [11, 17, 18, 46 Jinek, 2013 #216]. Single-strand nicking variants of Cas9 used for genome editing have been found to lower NHEJ (error prone) DNA repair and favor more predictable changes made as the results of HR DNA repair in human cells [65, 66]. Thus, wildtype Cas9 can be used for double-stranded DNA cleavage, while active site mutants (RuvC or HNH) cleave a single strand, offering a way of reliably introducing predictable mutations into genomes (Table 2).

Weapons modification: nuclease-defective Cas9 enzymes developed to control transcription

Inactivation of both nuclease domains of Cas9 (by double mutation) produces a protein capable of RNA-guided transcription repression in *E. coli*, and less reliably, in a human cell line [114]. This novel platform, dubbed “CRISPRi” (for CRISPR interference), appears to function via the Cas9 complex binding (but not cleaving) the DNA target site and blocking transcription initiation or elongation (Table 2). Targeting (of either DNA strand) at key transcriptional promoter elements results in significant impairment of gene expression (presumably blocking initiation) [114]. By contrast, targeting only the non-template strand of intragenic sites results in gene expression repression (presumably blocking elongation). An innovative inducible Cas9-based CRISPRi system permitted controlled and reversible target gene inhibition in *E. coli*. Targeting expression of regulatory and structural genes of the well-characterized lactose utilization pathway produced expected phenotypes, demonstrating the effectiveness and utility of the CRISPRi knockdown technology [114]. The nuclease-defective Cas9 is also capable of activating (rather than repressing) gene expression when directed (by the sgRNA) to interact with transcriptional repressor binding sites [114].

The Cas9 complex has been further modified by the fusion of specific transcription activators or repressors to the nuclease-defective protein to precisely activate or silence expression of a target gene (Table 2) [115–118]. These “CRISPR transcription factors” (Cas9-transcriptional effector domain fusion proteins) can be programmed with sgRNAs that recognize specific transcription control elements to obtain predictable effects on endogenous gene expression at targeted loci. The effects can be modulated by multiplex targeting (employing multiple sgRNAs) at different positions along the promoter region of

the gene [115, 116]. CRISPR transcription factors have been successfully employed in human [115–117], mouse [115], yeast [116, 117], and bacterial [118] cells.

The Cas9 complex provides a versatile platform for RNA-guided delivery of various agents and Cas9 fusion proteins with other functionalities will certainly be developed. For example, epigenetic effector domain fusions could be constructed, such as a methyltransferase fusion for RNA-guided DNA methylation or a histone modifying enzyme fusion to effect gene-specific changes in chromatin organization. In addition, multiple Cas9-based tools can be independently deployed in a single cell (e.g., to activate transcription of one gene and inhibit transcription of another) using Cas9 proteins from different organisms and corresponding sgRNAs (which differ in the repeat and tracrRNA sequences) [13, 113].

Assessment of target specificity of Cas9 systems: Avoiding collateral damage

Clearly, the various RNA-programmable Cas9 systems hold tremendous promise in site-specific genome editing and gene expression control applications. A critical issue that remains to be thoroughly addressed is the degree to which off-target effects (cleavage or binding of unintended DNA sequences) occur in the various cellular contexts where these Cas9-based tools are being used and how these can be mitigated.

Both the homology between the ~20 nucleotide guide region of the sgRNA and the requirement for the immediately adjacent PAM motif in the target DNA contribute to the specificity of the Cas9 reagents, however some off-target mutations have been documented. DNA cleavage has been observed at a few unintended sites with various extents of sgRNA/target sequence mismatch in mammalian cells [70, 72]. Studies aimed at deciphering Cas9 targeting rules indicate that extensive - but not necessarily 100% - homology with the sgRNA guide element is required for targeting [17, 18, 65, 69–71, 110, 112, 119]. The most recent in-depth studies reveal that Cas9-sgRNA targeting is quite complex, with mismatch tolerance depending upon the exact number, type, and position of RNA/DNA mismatches and varying for different sgRNA/target combinations [65, 69–71, 110, 119]. The mismatch tolerance of the Cas9 complex may be advantageous for silencing rapidly evolving viral target sequences.

Evidence indicates that off-target effects in Cas9 applications can be reduced by judicious choice of target sites (guided by recently established targeting rules) and by optimizing the expression levels of the Cas9-sgRNA complexes [71]. In addition, a “double-nicking” approach has been developed to reduce the possibility of off-target effects. Single active site mutants of Cas9 are used with two distinct sgRNAs to simultaneously generate offset single-strand nicks (yielding a 5' overhang) on each of the opposing two strands of the target sequence [69, 111]. This co-nicking strategy appears to generate double-stranded breaks with efficiency comparable to wildtype Cas9 (with a single sgRNA), but with greatly improved target site specificity [69].

Another weapon in the arsenal: deploying the Type III effector complex for gene knockdown

Another CRISPR-Cas effector complex is in development for applied RNA-guided gene knockdown. The Type III-B Cmr complex is the only CRISPR-Cas complex so far found to target RNAs [22, 23] (Figure 3). Functional Cmr complexes can be reconstituted with the six recombinant Cmr proteins (Cmr1-6) and synthetic crRNAs [23]. The crRNAs have an essential 8-nucleotide 5' tag sequence (derived from the CRISPR repeat) and can have guide regions of various lengths [23, 24]. crRNAs found in native Cmr complexes in *P. furiosus*

contain 31- and 37-nucleotide guide elements [23, 24]. The Cmr complexes specifically cleave RNA targets in the region base-paired by the crRNA guide sequence [22–25]. Identifying the nuclease component may allow further refinement of this complex as a tool. The overall architecture and subunit organization of functional Cmr complexes was recently revealed by a combination of cryo electron microscopy and biochemical analyses [22, 25, 26].

The Cmr complex can be programmed to cleave selected target RNAs using customized crRNAs *in vitro* [24] suggesting that Cmr complexes can be engineered to destroy selected mRNAs and manipulate gene expression similar to eukaryotic RNAi technologies (Figure 3). As a basic research tool, the Cmr technology has the potential to rapidly advance gene discovery and pathway analysis (especially in prokaryotes which lack the RNAi machinery naturally present in many eukaryotic systems). crRNA-mediated knockdowns would allow rapid screening of multiple gene targets in parallel. In industry, the potential of the Cmr system to simultaneously modulate the expression of several genes in a pathway could be of tremendous value in metabolic engineering of microbes to optimize production of biofuels, pharmaceuticals, foods, etc. Relative to DNA-targeting technologies, the Cmr system offers more straightforward capabilities for partial and temporary gene knockdown. Furthermore, the Cmr complex appears not to have target site limitations imposed by the requirement for a PAM sequence flanking the target sequence (unlike Cas9-based reagents and other DNA targeting CRISPR-Cas systems) [24]. Although the potential for off-target effects has not been experimentally tested, the Cmr complexes employ longer guide sequences for target recognition than the Cas9-based reagents.

Enlistment of crRNA biogenesis nucleases: novel endoribonucleases

The site-specific RNA cleavage capabilities of Cas6 superfamily crRNA processing endoribonucleases are also being exploited as valuable research tools. These protein enzymes recognize and cleave at specific sites within the CRISPR repeat sequences in CRISPR locus transcripts to release individual crRNAs [32, 34, 38]. There are multiple potential applications for the Cas6 site-specific endoribonucleases. The Cas6y (or Csy4) nuclease has been used to optimize protein expression in *E. coli* expression systems [120]. Cleavages at Cas6y sites inserted upstream of the ribosomal binding sites in monocistronic or polycistronic mRNAs leads to significantly improved predictable expression [120]. An inventive, cleavable RNA affinity tagging procedure to isolate and purify RNA-associated proteins has also been developed using Cas6y [121]. Using the related Cas6e (or Cse3) enzyme, this general approach has been adapted to generate T7 *in vitro* transcripts with homogenous 5' ends [122].

Concluding remarks

The effector complexes of the CRISPR-Cas immune systems utilize guide RNAs to effectively recognize and target foreign DNAs or RNAs for destruction. RNA-guided recognition makes these immune systems highly and rapidly adaptable to diverse targets – recognizing a new target simply requires a new guide RNA sequence (that can be obtained directly from the invader). This same attribute makes the CRISPR-Cas effector complexes exceptionally flexible and accessible tools for the multiple applications to which they can be adapted, which currently include incredibly valuable uses in genome editing and modulation of gene expression. We are currently witnessing the power of a naturally evolved prokaryotic defense system being repurposed to obtain effective new weapons for industry, research and medicine. Notably, our understanding of the multiple CRISPR-Cas systems is still far from complete and additional tools and applications are yet to come from these fertile research grounds.

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Glossary

Cas	<u>CRISPR-associated</u> . Refers to a conserved gene or corresponding protein, that functions with (and is often encoded adjacent to) the CRISPR locus as part of a CRISPR-Cas immune system that protects prokaryotes from viruses and other molecular invaders.
CRISPR	<u>Clustered regularly interspaced short palindromic repeat</u> . A CRISPR locus consists of a regular array of copies of a short direct repeat interspaced with similarly-sized sequences that typically originate from viruses or plasmids termed spacers.
crRNA	CRISPR RNA. Small non-coding RNAs that are produced by transcription and processing of CRISPR transcripts. crRNAs contain guide sequence (complementary to an invasive nucleic acid) and a CRISPR-repeat-derived sequence or tag at the 5' and/or 3' end.
crRNA tag	CRISPR RNA repeat-derived sequence present at the 5' and/or 3' end of a mature crRNA. The tag element contributes to specific assembly of crRNPs and is required for invader silencing. Also referred to as handles in the literature.
crRNP	A complex between a crRNA and cognate Cas proteins responsible for recognition and defense against invading nucleic acids.
Protospacer	The sequence in the foreign target DNA that corresponds with the (spacer) sequence integrated into the CRISPR locus and is recognized by the guide region of the encoded crRNA in the DNA targeting systems.
PAM	<u>Protospacer adjacent motif</u> . A short (typically 2–5 nucleotide) conserved sequence element that is located immediately adjacent to the protospacer in the foreign target DNA. This motif (or a close sequence variant) is important for target DNA identification and cleavage in Type I and II CRISPR-Cas systems.
sgRNA	An engineered chimeric RNA in which the crRNA and tracrRNA of Type II CRISPR-Cas systems are linked in a single molecule. Capable of directing Cas9 to cleave a corresponding target DNA sequence.
Spacer	Historical term for a DNA sequence found between the repeat elements of the CRISPR locus. Spacer sequences in the CRISPR locus match protospacer sequences in invading DNAs and encode the guide elements of the crRNAs.
tracrRNA	<u>Trans-activating crRNA</u> . A small non-coding RNA of Type II CRISPR-Cas systems that base-pairs with the 3' tag of the crRNA and is required for Cas9 DNA targeting activity.

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Highlights

CRISPR-Cas systems use RNA-guided effector complexes to destroy invading nucleic acid

Powerful technologies are rapidly arising from these prokaryotic defense systems

Uses range from pathogen tracking to genetic defect repair to metabolic engineering

The RNA-guided effector complexes offer particularly adaptable application platforms

Figure 1.

Overview of the CRISPR-Cas pathways. To obtain the ability to recognize and silence a specific virus or other foreign genetic element, a short fragment of invader DNA is acquired and integrated into the host CRISPR locus near the leader (Adaptation). The CRISPR locus contains copies of a short direct repeat sequence (black) that separate the invader-derived sequences (multiple colors). Cas genes encoding the protein components of the system are typically encoded adjacent to CRISPR loci (not shown). Protospacer adjacent motifs (PAMs) are found immediately adjacent to viral sequences selected for CRISPR integration (protospacers). CRISPR locus transcripts are processed to generate multiple individual crRNAs (crRNA Biogenesis). Mature crRNAs retain repeat sequence at the 5' end (shown)

and/or 3' end of the invader-derived guide sequence (colored region). crRNAs associate with 1 to 6 Cas proteins (depending on the system) to form crRNA-Cas protein (crRNP) effector complexes. The crRNP specifically recognizes foreign DNA or RNA (depending on the system) via base-pairing of the crRNA and cleaves (Invader Silencing). PAMs provide important auxiliary signals for the recognition and silencing of invaders for some Type I and II DNA-targeting systems.

Figure 2.

RNA-programmable genome editing by Type II Cas9 CRISPR-Cas systems. The Cas9 endonuclease (yellow) has two nuclease active sites (RuvC and HNH) that cleave opposing strands of a DNA molecule to generate a blunt-ended, double-strand DNA break (DSB). The cleavage is directed by the associated crRNA that contains a guide region that recognizes one strand of the target sequence by base-pairing. The enzyme requires a short protospacer adjacent motif (PAM) immediately adjacent to the region of homology as an auxiliary target identification signal. Cas9 activity also requires a second RNA (tracrRNA) that base-pairs with the repeat-derived tag of the crRNA. The crRNA and tracrRNA can be combined through covalent linkage into a single-guide RNA (sgRNA). In human and many other cell types, the Cas9-induced, double-strand DNA breaks undergo repair by either non-homologous end joining (NHEJ) or homologous recombination (HR). NHEJ repair results in indels (short insertions or deletions) and provides a means to selectively disrupt or inactivate target genes. When provided with a homologous donor DNA, HR repair can affect sequence replacement to insert, modify or delete sequence at the site.

Figure 3. RNA-programmable gene knockdown by Type III-B Cmr CRISPR-Cas systems. The Cmr effector complex (blue) consists of six Cmr proteins and a crRNA. The guide region of the crRNA base-pairs with the homologous sequence in the mRNA target and the target RNA is cleaved by the complex. Applications of the Cmr system include RNA-directed gene knockdown to investigate gene function or to facilitate metabolic engineering.

Multiple CRISPR-Cas systems with distinct crRNAs, Cas proteins, and mechanism of action against target nucleic acids.

Table 1

CRISPR/CAS system	crRNA	Known or predicted Cas proteins of effector crRNP	Target	Effector Nuclease
I		Cas8a, Cas5, Cas7, Cas5, Cas3 ^{+/#}	DNA	Cas3
		Cas8b, Cas7, Cas5, Cas3		
		Cas8c, Cas7, Cas5, Cas3		
		Cas10d, Csc1, Csc2,
	Cse1-2, Cas7, Cas5, Cas6e, Cas3		
		Csy1-3, Cas6y, Cas2/3		
II		Cas9 (+ tracrRNA)	DNA	Cas9
III		Csm1(Cas10a)-Csm5	DNA	?
		Cmr1, Cmr2 (Cas10b), Cmr3-6	RNA	?

Table 2

Cas9 variants for genome editing or gene expression control applications.

Cas9	Activity	Application
Wild-type (Cas9 RuvC ⁺ , HNH ⁺)	Double-stranded DNA break (cleavage)	Genome editing
Single active site mutants (Cas9 RuvC ⁻ , HNH ⁺ or Cas9 RuvC ⁺ , HNH ⁻)	Single-stranded DNA break (nickase)	Genome editing
Double active site mutant (Cas9 RuvC ⁻ , HNH ⁻)	DNA binding (no cutting)	Gene knockdown/interference (CRISPRi)
Double active site mutants with fusion proteins (Cas9, RuvC ⁻ , HNH ⁻ & transcription activator or repressor domain)	DNA binding (no cutting) & transcriptional control	Modulation of gene expression (activation or repression)