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A CRISPR method for genome engineering

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Abstract

Clustered regularly interspaced short palindromic repeat (CRISPR) RNA-guided adaptive immune systems that protect bacteria and archaea from infection by viruses are now being routinely repurposed for genome engineering in a wide variety of cell types and multicellular organisms.

Introduction and content

Clustered regularly interspaced short palindromic repeats (CRISPRs) and their associated genes (*cas*) are essential components of nucleic acid-based adaptive immune systems that are widespread in bacteria and archaea [1-5]. Similar to RNA interference (RNAi) pathways in eukaryotes, CRISPR-mediated immune systems rely on small RNAs for the sequence-specific delivery of dedicated nucleases to invading nucleic acids, such as viruses [2,5]. However, CRISPR systems are phylogenetically and mechanistically distinct from RNAi. Here we provide a brief overview of CRISPR-mediated immunity and explain how the emerging new properties of this defense system are being repurposed for genome engineering in bacteria [6-9], yeast [10,11], human cells [10,12-28], insects [29-32], fish [33-37], worms [38-46], plants [47-54], frogs [55], pigs [56], and rodents [14, 57-61]. The advent of these new genome engineering techniques illustrates how basic research can lead to unexpected innovations with applications in environmental and medical sciences.

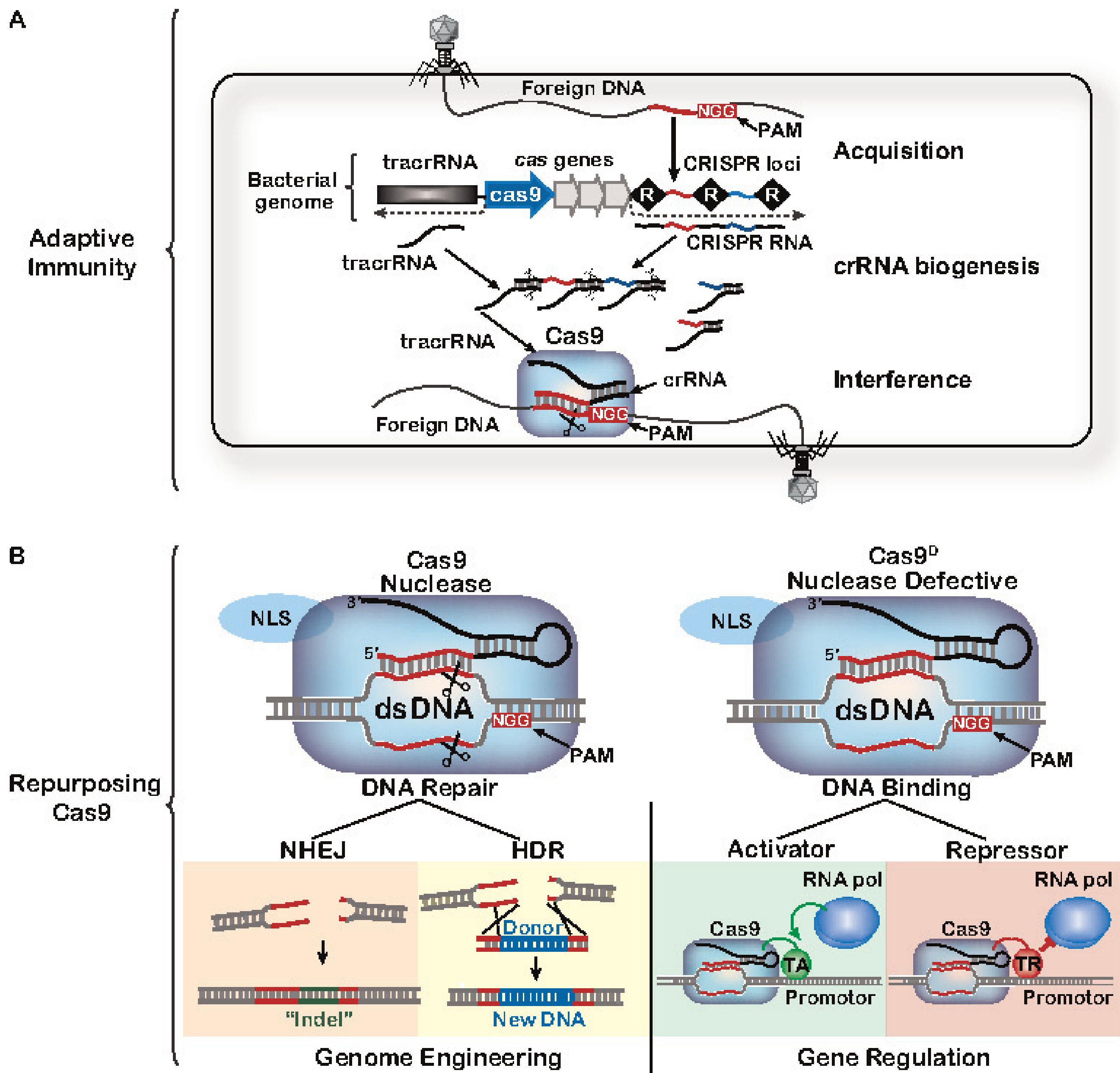
Discovering CRISPRs

Each CRISPR locus consists of a series of short repeats that are separated by non-repetitive spacer sequences derived from foreign genetic elements (Figure 1). This conserved repeat-spacer-repeat architecture was originally observed in the *Escherichia coli* genome in 1987 [62], but the function of these repeats remained enigmatic until 2005, when three groups reported that the spacer sequences in CRISPR loci are often identical to sequences in bacteriophage (phage) genomes and plasmids [63-65]. These observations suggested that CRISPRs might be part of a novel nucleic acid-based immune system designed to protect bacteria and archaea from infection by viruses and other genetic parasites. To test this hypothesis, Barrangou *et al.* challenged cultures of *Streptococcus thermophilus* with different phages and then screened for phage-resistant mutants [66]. DNA sequencing of the CRISPR loci from phage-resistant strains of *S. thermophilus* revealed that the CRISPR locus contained new "spacers" that were derived from the invading phage DNA, and the number of new phage-derived spacers correlated with the degree of phage resistance [66]. In addition, Barrangou and colleagues demonstrated that phage resistance could be genetically enhanced or reduced through insertion or deletion (respectively) of phage-targeting spacer sequences, suggesting that CRISPR-based vaccination programs might be used to protect industrial strains of bacteria from common phage infections.

Stages of CRISPR-mediated defense

Phylogenetic studies have identified distinct versions of the CRISPR system, but adaptive immunity in all of these systems proceeds in three distinct stages: acquisition of foreign DNA, CRISPR RNA (crRNA) biogenesis, and target interference [3,4,67] (Figure 1A). During new sequence acquisition, short fragments of foreign DNA are non-randomly selected and preferentially integrated at one end of the CRISPR locus [68-73]. The addition

Figure 1. Repurposing RNA-guided nucleases from the CRISPR-mediated adaptive immune system in bacteria



A) CRISPR-mediated adaptive immunity proceeds in three distinct stages: acquisition of foreign DNA, CRISPR RNA (crRNA) biogenesis, and target interference. Bacteria acquire resistance to viral and plasmid challengers by integrating short fragments of foreign nucleic acid (called protospacers) into CRISPR loci encoded in the bacterial genome. Protospacers are selected from regions of the genome that are flanked by a short sequence motif called a protospacer adjacent motif (PAM). CRISPR loci consist of a series of short repeats (R, black diamonds) and unique spacers (red and blue lines). CRISPR loci are transcribed and the RNA is processed into a library of small CRISPR-derived RNAs (crRNAs). In some CRISPR systems (i.e. Type II), a trans-activating CRISPR RNA (tracrRNA) is essential for RNA processing and for recognition by Cas9 (CRISPR-associated protein 9). Cas9 is an RNA-guided, dsDNA binding protein that uses two nuclease domains to cleave both strands of target DNA.
B) Cas9 targeting relies on PAM recognition and base pairing between the crRNA and the target DNA. The Cas9 nuclease can be easily programmed to target any DNA sequence with an adjacent PAM by designing a crRNA complementary to the target sequence. Genomic double-stranded DNA breaks are repaired by non-homologous end joining (NHEJ) or homology directed repair (HDR). NHEJ is error-prone, resulting in insertions or deletions (indels) that disrupt the target site. HDR relies on a donor template that can be used to deliver foreign DNA at a specific location. Alternatively, nuclease defective versions of Cas9 (Cas9^{D}) have been tethered to transcription activators (TA) that promote gene transcription, or transcriptional repressors (TR) that inhibit transcription.

of each new "spacer" sequence is accompanied by the duplication of the terminal repeat, thus maintaining the repeat-spacer-repeat architecture of the CRISPR locus [72]. CRISPR loci are transcribed into a long primary transcript that is processed into a library of small crRNAs, each containing a "guide" sequence complementary to a previously encountered invader [74-79]. Each crRNA is bound by one or more CRISPR-associated (Cas) protein(s) and the resulting ribonucleoprotein complex patrols the intracellular environment for targets that are complementary to the crRNA-guide sequence [80-88]. Identified target sequences are cleaved by dedicated nucleases [2,4,5,89]. Some CRISPR systems use crRNAs to target and cleave complementary RNAs, in a process that conceptually resembles eukaryotic RNAi [80,81,90]. However, most CRISPR systems use crRNAs to target invading DNA [91-95]. Many of these DNA targeting systems rely on sophisticated multi-subunit complexes, but one of these systems relies on a single protein called Cas9. Cas9 can be programmed with an RNA to target virtually any complementary DNA sequence [91,96]. The simplicity of the Cas9 system has recently been adopted by a rapidly expanding community of scientists for programmable genetic engineering in microorganisms, cell lines, plants, and animals (Table 1).

Table 1. Applications for RNA-guided Cas9 proteins

Origin	Gene	Purpose	Application	Addgene #	Reference
S. pyogenes	Cas9	DSB	in vitro	39312	[96]
	$Cas9^{CSN}$	SSN	in vitro	39316	
	$Cas9^{NSN}$	SSN	in vitro	39315	
	$Cas9^{D}$	dead	in vitro	39318	
L. innocua	Cas9	DSB	in vitro	39313	
S. thermophilus	Cas9	DSB	in vitro	39314	
N. meningitidis	Cas9	DSB	in vitro	39317	
S. pyogenes	hCas9	editing	hum/mus	42234	[22]
S. pyogenes	hCas9	editing	hum/mus	42230	[16]
	$hCas9^{NSN}$	editing	hum/mus	42335	
	hCas9	editing	hum/mus	42229	
	$hCas9^{NSN}$	editing	hum/mus	42333	
S. pyogenes	hCas9	editing	hum/mus	41815	[25]
	$hCas9^{NSN}$	editing	hum/mus	41816	
S. pyogenes	Cas9	editing	Bacteria	44250	[8]
	$Cas9^{D}$	CRISPRi	Bacteria	44249	
	$hCas9^{D}$	CRISPRi	hum/mus	44246	
	$hCas9^{D}$	CRISPRi	hum/mus	44247	
S. pyogenes	Cas9	editing	Bacteria	42876	[7]
S. pyogenes	Cas9	editing	Zebrafish	42251	[35]
	Cas9	editing	hum/mus	42252	
S. pyogenes	hCas9	editing	hum/mus	43945	[15]
S. pyogenes	hCas9	editing	hum/mus	44719	[18]
	$hCas9^{NSN}$	editing	hum/mus	44720	
S. pyogenes	hCas9	editing	Yeast	43802	[11]
	hCas9	editing	Yeast	43804	
S. pyogenes	hCas9	editing	hum/mus	44758	[59]
S. pyogenes	$hCas9^{D}$	tracking	hum/mus	46910	[10]
	$hCas9^{D}$	repression	hum/mus	46911	
	$hCas9^{D}$	activation	hum/mus	46912	
	$hCas9^{D}$	activation	hum/mus	46913	
	$hCas9^{D}$	repression	Yeast	46920	
	$hCas9^{D}$	repression	Yeast	46921	
S. pyogenes	hCas9	editing	*Drosophila*	45945	[30]
	hCas9	editing	*Drosophila*	46294	
S. pyogenes	wCas9	editing	*C. elegans*	46168	[41]
S. pyogenes	hCas9	editing	hum/mus	43861	Unpublished data (Joung Lab)
S. pyogenes	hCas9	editing	Plants	46965	[49]
S. pyogenes	zCas9	editing	Zebrafish	46757	[36]
	zCas9	editing	Zebrafish	47929	
N. meningitidis	Cas9	editing	hum/mus	47867	[13]
S. pyogenes	wCas9	editing	*C. elegans*	47549	[46]
S. pyogenes	wCas9	editing	*C. elegans*	47911	[39]
S. pyogenes	wCas9	editing	*C. elegans*	47933	[42]

$Cas9^{CSN}$, (H840A mutant) complementary strand nickase; $Cas9^{D}$, catalytically inactive/dead; $Cas9^{NSN}$, (D10A mutant) non-complementary strand nickase; CRISPRi, CRISPR-interference; DSB, double-strand DNA break; hCas9, mammalian codon optimized; hum/mus, human and mouse; SSN, single-strand nick; wCas9, worm codon optimized; zCas9, zebrafish codon optimized.

A CRISPR boom in biotechnology

Basic research on bacteriophages led to the discovery of DNA restriction endonucleases in the 1970s [97,98]. These enzymes transformed molecular biology by making it possible to cleave specific DNA sequences. Like restriction enzymes, CRISPR systems evolved as components of prokaryotic immune systems that efficiently target nucleic acids for sequence-specific cleavage. However, unlike DNA restriction enzymes, which typically bind to specific 4-8 bp regions of double-stranded DNA (dsDNA), CRISPR RNA-guided systems are extremely versatile and can be easily programmed to target virtually any RNA or DNA substrate. These new RNA-guided nucleases are now being exploited by genome engineers for programmed manipulation of nucleic acids in diverse model systems.

In 2007, Barrangou *et al.* demonstrated that the CRISPR-mediated immune system in *S. thermophilus* relied on the *cas9* gene for CRISPR-mediated protection from invading viruses [66]. To investigate the mechanism of protection and the fate of phage DNA during the infection, Garneau *et al.* sequenced viral DNA isolated from infected cells and showed that both stands of the target DNA were cleaved, resulting in a blunt-ended cleavage product [91]. However, at this time the mechanism of generating small CRISPR derived RNA's in this system was not understood. In 2011, Emmanuelle Charpentier's laboratory reported the identification of a trans-activating crRNA (tracrRNA) with sequences complementary to the repeat sequences of the CRISPR RNA [99]. They show that processing of the long primary CRISPR transcript was dependent on the tracrRNA and an endogenous RNAase III enzyme. Subsequently, Jinek *et al.* purified the Cas9 protein from *Streptococcus pyogenes* and showed that Cas9-mediated cleavage of dsDNA relied on both the crRNA-guide and the tracrRNA (Figure 1A) [96]. To simplify these two RNA systems, Jinek *et al.* made a single chimeric RNA by fusing the 3′ end of the crRNA to the 5′ end of the tracrRNA, and demonstrated that this RNA could target Cas9 to cleave virtually any DNA sequence by design. Similarly, Gasiunas *et al.* reported purification of the Cas9 protein from *Streptococcus thermophilus* and demonstrated programmable cleavage of dsDNA targets [100]. Together, mechanistic insights in these papers offered the exciting new possibility of using RNA-guided nucleases to generate dsDNA breaks for targeted genome "editing."

The principles of genome editing rely on cellular DNA repair systems. The dsDNA breaks introduced by designer nucleases are repaired by either non-homologous end-joining (NHEJ) [101] or homology-directed repair (HDR) [102] (Figure 1B). NHEJ is an error-prone process that is often accompanied by insertion or deletion of nucleotides (indels) at the targeted site, resulting in a genetic knockout of the targeted region of the genome due to frameshift mutations or the insertion of a premature stop codon. Alternatively, HDR relies on template DNA containing sequences homologous to the targeted site to repair the double stranded break. Prior to the discovery of CRISPR RNA-guided nucleases, the most advanced methods for genome editing involved sophisticated protein engineering of zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), or homing meganucleases [103,104]. However, protein engineering is expensive and the engineered enzymes sometimes cleave non-target sequences, resulting in off-target effects that are difficult to identify and sometimes toxic. In contrast to the previously existing technologies, CRISPR RNA-guided nucleases rely on simple Watson-Crick base pairing rules that abrogate the need for sophisticated protein engineering. The efficiency and accuracy of RNA-guided genome editing is currently the subject of intense investigation [7,14,17,20,21,24,26,61].

In 2013, less than six months after the reports by Jinek *et al.* and Gasiunas *et al.* on the programmable cleavage of dsDNA by Cas9, two Science papers by Cong *et al.* and Mali *et al.* demonstrated how RNA-guided Cas9 nucleases could be used to edit genes in mouse or human cell lines [16,25]. To repurpose the Cas9 nuclease for targeted genome editing, the authors fused nuclear localization signals (NLSs) to a codon-optimized version of the *cas9* gene and co-expressed this gene with plasmids expressing the tracrRNA and a crRNA-guide, or a single chimeric guide RNA (gRNA) [16,25]. Editing efficiencies by Cas9 were comparable to what has been achieved using ZFNs and TALENs, but using RNAs to program Cas9 for sequence-specific dsDNA breaks is simple, reliable, and cheap. In fact, Ding *et al.* recently compared the editing efficiency between TALENs and Cas9 at eight different loci in pluripotent stem cells and found that the Cas9-based system "consistently and substantially outperformed" TALENs across all loci [18]. In addition to relying on NHEJ to introduce genetic lesions at programmed cleavage sites, several papers have also now demonstrated that simultaneous delivery of either single-stranded or dsDNA donors can be used to promote HDR [9,11,13,14,18,24,25,28,30,34,38,42,44, 46,47,50,56]. A DNA donor identical to the wildtype sequence can be used to restore the original sequence, but DNA donors can also be used to introduce single nucleotide mutations or new genes (Figure 1B). Programmed delivery of foreign DNA to specific locations in the genome suggests that CRISPR RNA-guided nucleases could be used for gene therapy to repair or replace defective genes.

These initial studies have been followed by a rapid succession of papers demonstrating the versatility of RNA-guided Cas9 nucleases for genome engineering. In the last eight months, there have been over 60 independent publications demonstrating how different versions of the guide RNA can be used to target Cas9 to specific sequences for genome engineering in cells as well as multicellular organisms. Cas9-based systems have been used to efficiently generate allelic modifications in early stage embryos [14,29,30,33-37,55,57-61]. This method has been used to make biallelic transgenetic knockouts in animals using a single-step process that is profoundly accelerating *in vivo* genetic studies in live animal systems [14,33-37,55,57-61]. Furthermore, delivery of multiple guide RNAs can be used to edit several genes in a single genome simultaneously or excise large genomic segments located between two different cleavage sites [30,37,44,61]. This approach, called "multiplexing", has been used to knock out up to five genes in a single embryo [36,57,58,60]. Multiplexing may be particularly useful for knocking out redundant genes or parallel pathways.

The early founders of Cas9-based genome engineering established precedence for resource sharing by making their expression plasmids available to the scientific community at Addgene.org (Table 1). The accessibility of these plasmids, combined with the simplicity of programming Cas9, has contributed to the rapid implementation of this system for target genome engineering. However, the versatility of this platform permits the development of novel applications that go beyond site-specific double-stranded breaks for traditional genome editing. Recently, nuclease defective mutants of Cas9 ($Cas9^D$) have been used as a programmable DNA-binding protein with the potential to deliver diverse cargos to specific locations. To date, $Cas9^D$ has been used to regulate gene transcription in bacteria [6,8], yeast [10] and human cells [8,10,12,23,24,27] by fusing it to transcription factors and directing it to promoter regions of specific genes (Figure 1B). Together, the gene repression and activation capabilities of $Cas9^D$-based systems provide a simple and efficient method for controlling global gene expression that will help untangle complex gene networks and facilitate the development of synthetic organisms with controllable gene expression patterns.

Defining the rules of engagement

Understanding the molecular basis of RNA-guided DNA recognition by Cas9 is critical for implementing this system in a clinical setting. To understand the "rules of engagement", it is prudent to consider the context in which Cas9 evolved. In the first stage of adaptive immunity, foreign DNA (viral or plasmid) is inserted into the CRISPR locus of the host (Figure 1). Since the CRISPR locus is the template for generating crRNAs, each crRNA is complementary to at least two distinct targets: an invading phage or plasmid sequence (called a protospacer), and the "spacer" sequence in the CRISPR locus of the host. Cas9 avoids "self" (i.e. spacers in the CRISPR) and efficiently targets "non-self" (i.e. protospacers) through protein-mediated recognition of a short sequence motif called a protospacer adjacent motif (PAM). The PAM is an antigenic signature that may promote duplex destabilizations so that the crRNA can access the single-stranded regions of the adjacent DNA sequence for complementary base pairing [96,100].

To quantify how each of these recognition sequences contribute to target cleavage efficiencies by Cas9 from *S. pyogenes*, Jiang *et al.* generated a library of targets containing all possible nucleotide substitutions at each position in the protospacer and the PAM [7]. Their results clearly indicate that the NGG motif in the PAM region is the most potent antigen for Cas9 targeting, but a NAG or NNGGN PAM can also elicit Cas9 targeting. In addition to the PAM, they identified a 12-nucleotide "seed sequence" immediately upstream of the PAM that is critical for target recognition. However, the rules of engagement are complex and different mutations display significantly different targeting defects [17]. Furthermore, there are many different variations of the Cas9 protein and many of these proteins have different PAM recognition sequences [13]. Generally speaking, the 12 bases proximal to the PAM are crucial for target recognition, but there are position- and nucleotide-specific effects that alter targeting efficiencies. To determine how these rules apply in the context of human cells, several studies have recently evaluated Cas9-mediated off-target cleavage effects in cultured human cell lines [14,18,20,21,24,26]. These studies reveal that the length of the gRNA can alter efficiency of Cas9 targeting and higher concentrations of the gRNA and Cas9 result in higher frequencies of off-target cleavage effects. These observations are critical to consider during experimental design, and Hsu *et al.* have developed web-based software to help experimentalists select target sequences that will minimize potential off-target effects [21].

More than Cas9

In the midst of the Cas9 frenzy, other important applications for the CRISPR machinery have been developed. In 2008, Brouns *et al.* identified a protein in *Escherichia coli* that exclusively binds and selectively cleaves long CRISPR transcripts into small crRNAs [74]. This protein, called Cas6e (formerly CasE or Cse3) is a member of a large family of extremely diverse proteins that bind and cleave different RNA sequences. These

proteins represent a new class of RNA restriction enzymes with the potential to advance RNA biology in the same way that DNA restriction enzymes did 40 years ago. Recently, activatable CRISPR-associated RNA restriction endoribonucleases have been used for targeted gene regulation in *E. coli* [105] and for affinity purification of RNAs and ribonucleoprotein complexes [106-108].

A CRISPR future

The simplicity of programming RNA-guided Cas9 nucleases has contributed to their rapid implementation by the genome engineering community. However, the extent of off-target cleavage and the influence of chromatin structure and modification states on Cas9 cleavage efficiencies remain poorly understood. Early screens for off-target modifications focused on a subset of loci with sequences similar to the authentic target. These initial efforts failed to reveal off-target modifications, but more recent studies performed using high-throughput techniques indicated that high concentrations of Cas9-based nucleases promote off-target cleavage [7,18,24,26,61]. Nevertheless, using higher concentrations of Cas9 also leads to more efficient gene disruption. This suggests a delicate balance between efficiency and accuracy of these nucleases, and methods that enhance their specificity may have significant utility.

We anticipate that the pace of Cas9-based gene modifications will continue to accelerate and that this system will be implemented in increasingly diverse model systems. The simplicity of this system will permit a rapid generation of genome-scale knockout libraries for complex model systems, including human cells, with the potential for *ex vivo* gene therapy in humans. Furthermore, nuclease defective mutants of Cas9 will be tethered to an increasingly diverse array of accessory domains with functions that can be regulated with light (e.g. optogenetics) or chemical treatments. Collectively, Cas9-based technologies are revolutionizing contemporary molecular genetics.

We are still in the infancy of a rapidly evolving field that has focused almost exclusively on the utility of Cas9 proteins from a few organisms (Table 1). While the versatility of Cas9 proteins appears to be limitless, we anticipate that the biochemical, biophysical and target recognition properties of CRISPR RNA-guided complexes from other systems (i.e. Cascade, Cmr, and Csm) will have functional attributes that are desirable in certain contexts. Moreover, we anticipate that anti-CRISPR proteins encoded by some viruses will interact with the components of these systems in unanticipated ways that may lead to new applications for regulating or altering the function of these systems [67,109]. Basic research on the mechanisms of these systems will continue to be the fuel that drives innovation.

Abbreviations

Cas, CRISPR-associated; Cas9, CRISPR-associated protein 9; Cas9^{D}, nuclease defective mutant of Cas9; CRISPR, clustered regularly interspaced short palindromic repeat; crRNA, CRISPR RNA; dsDNA, double-stranded DNA; gRNA, guide RNA; HDR, homology directed repair; indel, insertion or deletion; NHEJ, non-homologous end-joining; NLS, nuclear localization signal; PAM, protospacer adjacent motif; TA, transcription activator; TALEN, transcription activator-like effector nuclease; TR, transcriptional receptor; tracrRNA, trans-activating crRNA; ZFN, zinc finger nuclease.

Disclosures

Blake Wiedenheft is a (co-)inventor on patents related to the application of CRISPRs and their associated genes.

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