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Harnessing CRISPR-Cas9 immunity for genetic engineering

Emmanuelle Charpentier^{1,2,3} and Luciano A. Marraffini⁴

Emmanuelle Charpentier: emmanuelle.charpentier@helmholtz-hzi.de; Luciano A. Marraffini: marraffini@rockefeller.edu

¹Helmholtz Centre for Infection Research, Department of Regulation in Infection Biology, Braunschweig 38124, Germany

²The Laboratory for Molecular Infection Medicine Sweden (MIMS), Umeå Centre for Microbial Research (UCMR), Department of Molecular Biology, Umeå University, Umeå 90187, Sweden

³Hannover Medical School, Hannover 30625, Germany

⁴Laboratory of Bacteriology, The Rockefeller University, 1230 York Ave., New York, NY 10065, USA

Summary

CRISPR-Cas encodes an adaptive immune system that defends prokaryotes against infectious viruses and plasmids. Immunity is mediated by Cas nucleases, which use small RNA guides (the crRNAs) to specify a cleavage site within the genome of invading nucleic acids. In type II CRISPR-Cas systems, the DNA-cleaving activity is performed by a single enzyme Cas9 guided by an RNA duplex. Using synthetic single RNA guides, Cas9 can be reprogrammed to create specific double-stranded DNA breaks in the genomes of a variety of organisms, ranging from human cells to bacteria, and thus constitutes a powerful tool for genetic engineering. Here we describe recent advancements in our understanding of type II CRISPR-Cas immunity and how these studies led to revolutionary genome editing applications.

Introduction

While seminal studies at the end of the XIX century established that human immunity is adaptive [1], the recognition that prokaryotic organisms also harbor an adaptive immune system had to wait until the new millennium. The prokaryotic adaptive immune system is encoded by Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and their associated proteins (Cas). CRISPR loci constitute an array of short repetitive sequences (30–40 bp-long) separated by equally short unique intervening sequences called “spacers” [2–4]. Spacer sequences with hits on Genebank match short regions of the genome of viruses and plasmids of bacteria and archaea. Early work established the existence of these repetitive

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Correspondence to: Emmanuelle Charpentier, emmanuelle.charpentier@helmholtz-hzi.de; Luciano A. Marraffini, marraffini@rockefeller.edu.

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loci in many prokaryotic genomes [5], the genetic linkage of CRISPR repeats with conserved *cas* genes [6, 7], the homology of spacers to sequences of plasmid and viral origin [8–10], and the transcription of CRISPR small RNAs [11]. These early observations led to the proposal that CRISPR-Cas systems work as a genetic interference mechanism to control the spread of viruses and plasmids [12], a prediction that was later corroborated experimentally [13, 14]. A great body of work that followed these initial studies allows us today to formulate the following model for CRISPR-Cas immunity (reviewed in [2–4]). Immunity is divided into three stages. In the first stage, known as the adaptation phase, upon infection by viruses or plasmids Cas proteins promote the incorporation of a short region of the invader's genome into the CRISPR array as a new spacer. The second stage involves the biogenesis of CRISPR RNAs (crRNAs), in which the repeat-spacer array is transcribed as a long precursor that is cleaved at the repeat sequences by Cas endoribonucleases. Finally, the small crRNAs produced are used as guides to direct a Cas ribonucleoprotein complex to their cognate target in the viral or plasmid genome. This is known as the interference stage and results in the destruction of the invader's genome, with the concomitant protection of the infected cell.

Similarly to restriction-modification systems [15], CRISPR-Cas systems provide a tool for sequence-specific cleavage of nucleic acids that can be equally exploited for biotechnological purposes. Cleavage specificity is determined by the crRNA, enabling precise control and easy re-programming of cleavage. CRISPR-Cas systems, however, are extremely diverse. Based on the *cas* gene content, these systems have been classified into three distinct groups [16] that differ in the molecular mechanisms of the three stages. For example, while most CRISPR-Cas systems target DNA molecules [14, 17–19], type III-B systems target RNA [20, 21]. Also, whereas targeting is mediated by large Cas ribonucleoprotein complexes in type I and type III CRISPR-Cas systems [20, 22], type II systems require a single Cas protein (Cas9) [23] and two small RNAs (the crRNA guide and the tracrRNA, see below) [24, 25]. Among this variety of targeting mechanisms, the type II CRISPR-Cas9 systems arose as the optimal system to develop for biotechnological applications for two reasons. First, Cas9 provides very efficient dsDNA cleavage [25, 26] as opposed to the RNA cleavage of type III-B systems [20], the relatively under-characterized DNA cleavage activity of the other type III systems [27], or the ssDNA cleavage of type I systems [18, 19]. Second, Cas9 cleavage requires a minimal set of components [24, 25], facilitating the optimization of the system in heterologous organisms [23, 26]. In this short review we detail the mechanisms of crRNA biogenesis and dsDNA cleavage of type II CRISPR-Cas systems and how they can be engineered to introduce genetic modifications and control gene expression in different organisms.

The bacterial type II CRISPR-Cas system

Back early 2000's, Cas9 (formerly COG3513, Csx12, Cas5 or Csn1) was predicted as a large multi-functional protein [28] containing two putative conserved domains, HNH [7, 8, 12] and RuvC-like [12] that would confer the nucleic acid-cleaving activity in the interference step of type II CRISPR-Cas systems (formerly Nmeni/CASS4). Some years later, a series of studies in streptococcal species provided genetic evidence for the Cas9 function. Cas9 of *S. thermophilus* is necessary and sufficient for interference and the HNH

and RuvC domains are critical in this step [23]. The protein acts by introducing double stranded DNA breaks (DSBs) site-specifically into target phages and plasmids [17]. Targeting by Cas9 is also strictly dependent on the presence of a protospacer adjacent motif (PAM) [29–31] juxtaposed to the crRNA-targeted sequence on the invading DNA [17]. A study in *Streptococcus pyogenes* later identified tracrRNA as a novel small RNA encoded in the vicinity of the type II CRISPR-Cas locus [24]. tracrRNA in this bacterial species is expressed as two primary transcripts that contain an anti-repeat sequence enabling tracrRNA to form duplexes with the repeats of the precursor CRISPR transcript (pre-crRNA) [24]. The tracrRNA:pre-crRNA duplexes are then used as templates of the bacterial endoribonuclease III that cleaves the RNAs at the level of the anti-repeat:repeat regions in a process requiring RNA stabilization by Cas9 [24]. The resulting intermediate forms of crRNAs bound to mature tracrRNAs undergo a second maturation event of still unknown nature to generate the mature tracrRNA:crRNAs in which crRNAs consist of a DNA targeting spacer sequence in 5' and repeat sequence in 3' [24]. tracrRNA and RNase III in addition to pre-crRNA and Cas9 are essential components in temperate phage defense in the Group A *Streptococcus* [24]. Biochemical followed by structural characterization of the targeting system demonstrated a unique mechanism for Cas9 [25, 32–34]. In brief, the dual-tracrRNA:crRNAs guide the enzyme Cas9 to the target DNA [25]. Target recognition is initiated by scanning the invading DNA molecule for both the PAM and the homology to the spacer sequence of the dual-RNA [25, 32]). An R-loop is formed and Cas9 subsequently introduces DSBs in target DNA using the HNH motif to cleave the strand complementary to the crRNA spacer sequence and the RuvC-like domain to cleave the non-complementary strand [25, 26].

The type II CRISPR-Cas system has recently been divided into three sub-types [35–37]. Analyses of Cas9 phylogeny and variability of sequences among tracrRNA anti-repeats, CRISPR repeats and Cas9 orthologs resulted in the proposal that the dual-tracrRNA:crRNAs have functionally co-evolved with Cas9 [24, 25, 35–37]. DNA cleavage by dual-tracrRNA:crRNA-guided Cas9 orthologs was reported in various bacterial species, closely or distantly related to *S. pyogenes* Cas9 [25, 35, 38]. Functional exchangeability of dual-RNA and Cas9 orthologs was established and the secondary structure of the tracrRNA anti-repeat:crRNA repeat duplex dictating the specific RNA recognition by Cas9 was revealed to be critical in the resulting orthogonality [25, 35].

Development of the CRISPR-Cas9 technology

The CRISPR-Cas9 technology is based on the engineering of the dual-tracrRNA:crRNA into a single-guide RNA (sgRNA) [25]. A sgRNA consists of the targeting sequence located in 5' that can form Watson-Crick base-pairing with the target DNA and the tracrRNA:crRNA mimicking double-stranded region located in 3' that binds to Cas9 [25]. Thus, Cas9 can be programmed with sgRNAs to target any specific DNA sequences of interest owing to the presence of the PAM flanking the targeted sequence on the DNA by simply exchanging the guide sequence of the sgRNAs [25]. Given the simplicity of design, efficiency and versatility of the system, CRISPR-Cas9 was proposed as a promising alternative technology to zinc-finger [39, 40] and TALE nucleases [41, 42] for genome-targeting and genome-editing applications [25] (Fig. 1). Within a short time, the CRISPR-Cas9 technology was

demonstrated to function efficiently as a genome editing tool in human cells [43–45]. The system originating from *S. pyogenes* has since been broadly used by the scientific community to edit or modify genomes in a vast range of cells and organisms (For reviews: [46–48]). Biochemical studies have revealed that Cas9 can be repurposed into either a nickase variant by mutating the HNH or RuvC-like domain or a catalytically inactive DNA binding variant (dCas9 for dead Cas9) by mutating both domains simultaneously [25, 26]. These CRISPR-Cas9 variants have enabled the development of the technology into DNA targeting functions other than dsDNA cleavage such as modulation of transcription or modification of DNA (For reviews: [46–48]) (see below). Furthermore, the diversity of naturally evolving dual-RNA-Cas9 enzymes constitutes a large source of CRISPR-Cas9 systems that can provide multiple alternatives for gene targeting ([25, 35–37]). A few of these orthologous CRISPR-Cas9 systems have been tested in human cells [43, 49, 50].

CRISPR-Cas9 genome editing in bacteria

In most eukaryotic organisms the generation of dsDNA breaks by Cas9 is repaired by either the homologous recombination or non-homologous end joining (NHEJ) mechanisms [43–45, 51] (Fig. 1). In the bacterium *Streptococcus pneumoniae* however, early work showed that Cas9 cleavage of chromosomal sequences is lethal [52]. While the molecular mechanism behind this phenomenon is yet to be elucidated, it can be used as a powerful counter-selection strategy for the introduction of mutations in bacteria. Cas9-mediated mutagenesis has been tested in both *S. pneumoniae* and *E. coli* and requires the co-transformation of a Cas9 construct guided by an RNA designed to target the desired sequence along with a recombination template harboring mutations in the target seed or PAM sequences that abrogate Cas9 cleavage [53]. During co-transformation, the mutated template is recombined into the Cas9 target site and this prevents Cas9 chromosomal cleavage and cell death. Wild-type cells, where recombination did not take place or that did not receive the Cas9 construct or the editing template, are selected against and only mutated cells survive. Particularly in *E. coli* this approach complements the recombineering of mutagenic oligonucleotides to increase the frequency of the recovery of mutant cells by several orders of magnitude [53].

Regulation of bacterial gene expression using dCas9

As mentioned above, mutation of the active site residues of Cas9 (D10A and H840A in *S. pyogenes* Cas9) converts the enzyme into a programmable, RNA-guided dsDNA binding protein, dCas9 [25, 26]. This has been exploited to develop a tool that interferes with gene expression by directing dCas9 either to promoter or open reading frame sequences to prevent transcription initiation or elongation, respectively [54, 55] (Fig. 1). In the latter case, dCas9 abruptly stops transcription at the binding site and the repression is considerably stronger when the coding strand is targeted (i.e., it anneals with the spacer sequence of the guide RNA). In both cases repression is highly efficient, leading to a reduction in gene expression of several orders of magnitude.

In addition to direct repression of transcription, it is possible to create dCas9 protein fusions with activator domains to activate gene expression. This was demonstrated by fusing dCas9

to the ω subunit of RNA polymerase (RNAP) and expressing the fusion in *E. coli rpoZ* cells (lacking the gene for this subunit). The ω polypeptide recruits RNAP through interactions with the β' subunit [56]; thus the dCas9- ω fusion can activate the transcription of poorly expressed promoters by recruiting RNAP [54]. Activation, however, is much less efficient than repression by dCas9 alone. In eukaryotes, dCas9 alone [55] or fused to transcription repression domains such as KRAB or SID effectors [57] or to VP16/VP64 or p65 activator domains [58–60] have also been used to modulate gene expression. As it is the case for prokaryotes, approaches that rely on the generation of active dCas9 fusions are less efficient.

Although the development of successful dCas9-effector fusions requires additional fine-tuning work, these fusions can greatly expand the repertoire of genetic tools based on dCas9. In a recent study a GFP-dCas9 fusion was used to label specific DNA loci, providing a powerful live cell-imaging alternative technique [61]. In theory, the possibilities are endless: dCas9 could be fused to epigenetic modifiers, chromatin remodeling domains or recombinases. Future work will undoubtedly explore these exciting variations of the Cas9-based technologies.

Conclusions

RNA-programmable CRISPR-Cas9 originating from the type II CRISPR-Cas bacterial immune system has recently emerged as a next generation powerful and flexible targeted genome engineering technology for applications in a large variety of cells and organisms. In bacteria, CRISPR-Cas9 provides a counter-selection methodology for the introduction of mutations. Moreover, catalytically inactive CRISPR-Cas9 (dCas9) can either be targeted directly to prevent transcription initiation or elongation or be engineered with activator domains to enhance gene expression, a strategy also known as CRISPRi (for CRISPR interference). The silencing methodology could thus enable the generation of knockdown libraries for basic research or other more biotechnology-oriented purposes. Given the speed of the recent development of CRISPR-Cas9-based technologies, it is expected that innovative variations of the system for the manipulation of genes and modulation of gene expression in bacteria should soon emerge. Future studies aiming at a better understanding of the biochemical and structural properties of the large source of orthologous CRISPR-Cas9 systems should also provide new insights to exploit and improve further the technology. Last but not least, efforts of researchers to decipher the still not fully characterized nucleic acid targeting mechanisms of other CRISPR-Cas systems may result in the development of additional tools and applications in biology.

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Highlights

- CRISPR-Cas is an RNA-mediated adaptive immune system against mobile genomes
- RNA-programmable CRISPR-Cas9 enables targeted genome editing
- Catalytically inactive CRISPR-Cas9 allows targeted modulation of transcription
- Cas9 can be fused to diverse functional domains to manipulate and modify DNA

Figure 1. Cas9-based genetic applications

(A) Wild-type Cas9 loaded with a single guide RNA (sgRNA) generates dsDNA breaks that can be used to introduce target mutations. Chromosomal breaks can be repaired by non-homologous end joining (NHEJ), creating indels that introduce knock-out frameshift mutations. If a sequence homologous to the Cas9 target is provided (the editing template; either linear dsDNA or a short oligonucleotide), the break can be repaired by homologous recombination. In this case, site-specific mutations in the editing template can also be incorporated in the genome. (B) A catalytically dead Cas9 (dCas9, containing mutations in both the RuvC and HNH active sites) can be used as an RNA-guided DNA binding protein that can repress both transcription initiation when bound to promoter sequences or

transcription elongation when bound to the template strand within an open reading frame. Arrows indicate transcription start sites.