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Harnessing Nature's Toolbox for Genome Engineering

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Bacteria and archaea possess a range of defense mechanisms to combat plasmids and viral infections. Unique among these are the CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR associated) systems, which provide adaptive immunity against foreign nucleic acids. CRISPR systems function by acquiring genetic records of invaders to facilitate robust interference upon reinfection. In this Review, we discuss recent advances in understanding the diverse mechanisms by which Cas proteins respond to foreign nucleic acids and how these systems have been harnessed for precision genome manipulation in a wide array of organisms.

CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR associated) adaptive immune systems are found in roughly 50% of bacteria and 90% of archaea (Makarova et al., 2015). These systems function alongside restriction-modification systems, abortive infections, and adsorption blocks to defend prokaryotic populations against phage infection (Labrie et al., 2010). Unlike other mechanisms of cellular defense, which provide generalized protection against any invaders not possessing countermeasures, CRISPR immunity functions analogously to vertebrate adaptive immunity by generating records of previous infections to elicit a rapid and robust response upon reinfection.

CRISPR-Cas systems are generally defined by a genomic locus called the CRISPR array, a series of ~20–50 base-pair (bp) direct repeats separated by unique “spacers” of similar length and preceded by an AT-rich “leader” sequence (Jansen et al., 2002; Kunin et al., 2007). Nearly two decades after CRISPR loci were first identified in Escherichia coli, spacers were found to derive from viral genomes and conjugative plasmids, serving as records of previous infection (Bolotin et al., 2005; Ishino et al., 1987; Mojica et al., 2005; Pourcel et al., 2005). Sequences in foreign DNA matching spacers are referred to as “protospacers.” In 2007, it was shown that a spacer matching a phage genome immunizes the host microbe against the corresponding phage and that infection by a novel phage leads to the expansion of the CRISPR array by addition of new spacers originating from the phage genome (Barrangou et al., 2007).

CRISPR immunity is divided into three stages: spacer acquisition, CRISPR RNA (crRNA) biogenesis, and interference (Figure 1A) (Makarova et al., 2011b; van der Oost et al., 2009). During spacer acquisition, also known as adaptation, foreign DNA is identified, processed, and integrated into the CRISPR locus as a new spacer. The crRNA biogenesis or expression

stage involves CRISPR locus transcription, often as a single pre-crRNA, and its subsequent processing into mature crRNAs that each contain a single spacer. In the interference stage, an effector complex uses the

crRNA to identify and destroy any phage or plasmid bearing sequence complementarity to the spacer sequence of the crRNA.

These steps are carried out primarily by Cas proteins, which are encoded by cas genes flanking the CRISPR arrays. The specific complement of cas genes varies widely. CRISPR-Cas systems can be classified based on the presence of “signature genes” into six types, which are additionally grouped into two classes (Figure 1B) (Makarova et al., 2011b; Makarova et al., 2015; Shmakov et al., 2015). Types I–III are the best studied, while Types IV–VI have only recently been identified (Makarova and Koonin, 2015; Makarova et al., 2015; Shmakov et al., 2015). The signature protein of Type I systems is Cas3, a protein with nuclease and helicas domains that functions in foreign DNA degradation to cleave DNA that is recognized by the multi-protein–crRNA complex Cascade (CRISPR-associated complex for antiviral defense). In Type II systems, the signature cas9 gene encodes the sole protein necessary for interference. Type III systems are signified by Cas10, which assemblies into a Cascade-like interference complex for target search and destruction. Type IV systems have Csf1, an uncharacterized protein proposed to form part of a Cascade-like complex, though these systems are often found as isolated cas genes without an associated CRISPR array (Makarova and Koonin, 2015). Type V systems also contain a Cas9-like single nuclease, either Cpf1, C2c1, or C2c3, depending on the subtype (Shmakov et al., 2015; Zetsche et al., 2015a). Type VI systems have C2c2, a large protein with two predicted HEPN (higher eukaryotes and prokaryotes nucleotide-binding) RNase domains (Shmakov et al., 2015). Type I, III, and IV systems are considered

Class 1 systems based on their multi-subunit effector complexes, while the single-subunit effector Type II, V, and VI systems are grouped into Class 2 (Makarova et al., 2015; Shmakov et al., 2015).

The study of CRISPR biology has revealed enzyme mechanisms that can be harnessed for precision genome engineering and other applications, leading to an explosion of interest in both native CRISPR pathways and the use of these systems for applications in animals, plants, microbes, and humans. In this Review, we discuss recent advancements in the field that reveal unexpected divergence, as well as unifying themes underlying the three stages of CRISPR immunity. In each case, we highlight the ways in which these systems are being harnessed for applications across many areas of biology.

Figure 1. Function and Organization of CRISPR Systems

(A) CRISPR immunity occurs in three stages. Upon introduction of foreign DNA, the adaptation machinery selects protospacers and inserts them into the leader end of the CRISPR locus. During crRNA biogenesis, the CRISPR locus is transcribed and sequence elements in the repeats direct processing of the pre-crRNA into crRNAs each with a single spacer. The crRNA then assemblies with Cas proteins to form the effector complex, which acts in the interference stage to recognize foreign nucleic acid upon subsequent infection and degrade it.

(B) CRISPR systems are extremely diverse but can largely be classified into six major types. Representative operons for each type are shown here. Genes only present in some subtypes are shown with dashed outlines. Genes involved in interference are colored red, those involved in crRNA biogenesis are colored yellow, and those involved in adaptation are colored blue. Type IV systems are notable for their frequent occurrence in the absence of CRISPR loci.

Acquisition: Creating Genetic Records of Past Infections

CRISPR immunity begins with the detection and integration of foreign DNA into the host cell's chromosome. In the Streptococcus thermophilus Type II-A system, where acquisition was first detected experimentally, new spacers from bacteriophageal DNA are inserted into the leader end of the CRISPR locus, causing duplication of the first repeat to maintain the repeat-spacear architecture (Figure 1A) (Barrangou et al., 2007). Subsequent studies using the E. coli Type I-E system verified that Cas1 and Cas2 mediate spacer acquisition (Datsenko et al., 2012; Swarts et al., 2012; Yosef et al., 2012). The selection of new protospaceer sequences is nonrandom and, in most systems, depends on the presence of a 2–5 nucleotide protospaceer adjacent motif (PAM) found next to the protospaceer

sequence (Deveau et al., 2008; Mojica et al., 2009). PAM-specific selection of protospacers is critical for immunity, as crRNA-guided interference in most systems depends on the PAM sequence for foreign DNA detection and destruction, which avoids self-targeting at the PAM-free CRISPR locus. Interestingly, spacers originating from the host genome are present in almost 20% of CRISPR-containing organisms, suggesting alternative roles of the CRISPR-Cas machinery in directing other processes such as endogenous gene regulation and genome evolution (Westra et al., 2014). Spacer acquisition has been observed experimentally in various systems across Types I – III. Here, we focus on recent mechanistic studies of acquisition in Type I – E and Type II – A systems, in which the most comprehensive studies have been done.

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Type I Acquisition

Acquisition in E. coli occurs via two mechanisms—naive and primed (Figure 2A). Naive acquisition initiates upon infection by previously unencounded DNA and relies on the Cas1-Cas2 integrate complex to recognize and acquire new spacers from foreign DNA. Overexpression of Cas1 and Cas2 in the absence of other Cas proteins leads to the acquisition of 33 bp spacers at the leader-proximal end of the CRISPR array (Datsenko et al., 2012; Yosef et al., 2012). The PAM of the E. coli CRISPR-Cas system was identified as 5'-AWG-3', with the G becoming the first nucleotide of the integrated spacer (Datsenko et al., 2012; Diez-Villasefr et al., 2013; Levy et al., 2015; Nuez et al., 2014; Savitskaya et al., 2013; Shmakov et al., 2014; Swarts et al., 2012; Yosef et al., 2012; Yosef et al., 2013). In addition to the PAM, a dinucleotide motif, AA, found at the 3' end of the protospacer was also shown to be present in a disproportionately large number of spacers (Yosef et al., 2013). A recent crystal structure of the Cas1-Cas2 complex bound to an unprocessed protospacer revealed sequence-specific contacts with the 5'-CTT-3' sequence on the PAM-comple-

Figure 2. Protocolscer Selection and Integration in Adaptation

(A) The selection of protospacers for acquisition is poorly understood, but studies suggest at least three distinct mechanisms for the selection of substrates for integration. In Type I systems, primed adaptation occurs when Cascade binds a partially mismatched target. The nuclease/helicase Cas3 is recruited to the target site and then likely translocates along the target DNA to a new site. The new location is then selected as a protospacer to be used by Cas1-Cas2 in the integration reaction. In E. coli, naive adaptation involves the nuclease/helicase RecBCD. The degradation products appear to serve as substrates for Cas1-Cas2, but how the variable-length single-stranded products of RecBCD activity are converted into double-stranded protospacers of appropriate size is unknown. In Type II systems, Cas9 recognizes PAM sites and likely circuits Cas1-Cas2 to acquire the flanking sequence.

(B) Cas1-Cas2 act as an integrase to insert protospacers into the CRISPR locus as new spacers. The complex with protospace bound recognizes the leader-adjacent repeat and catalyzes a pair of transesterification reactions. The 3' OH of each protospace strand makes a nucleophilic attack on the repeat backbone, one at the leader-side and one at the spacer side. The resulting gapped product is then repaired, causing duplication of the first repeat.

mentary strand, suggesting that Cas1 recognizes PAM sites on potential protospacers before they are processed for integration (Wang et al., 2015).

After a spacer is acquired from a new invader, the resulting crRNA assemblies with Cas proteins to form Cascade, the interference complex capable of targeting PAM-adjacent DNA sequences matching the spacer sequence of the crRNA (Brouns et al., 2008; Jore et al., 2011; Lint-

ner et al., 2011). Upon target binding, the helicase/nuclease Cas3 is recruited to the site and processively degrades the foreign DNA (Hochstrasser et al., 2014; Mulepati and Bailey, 2011; Sinkunas et al., 2011; Sinkunas et al., 2013; Westra et al., 2012). Strikingly, when Cascade encounters a mutant PAM or protospacer that prevents Cas3 degradation, hyperactive spacer acquisition from the targeted plasmid or genome is triggered in a process called “priming” (Figure 2A) (Datsenko et al., 2012; Li et al., 2014; Richter et al., 2014; Savitskaya et al., 2013; Swarts et al., 2012). Priming increases the host’s repertoire of functional spacers, allowing the host to adapt to invaders that evade the CRISPR-Cas system

by mutation. Cascade is capable of binding escape mutant target sites, and recent single-molecule studies showed that the presence of Cas1 and Cas2 allows for the recruitment of Cas3 to these sites (Blosser et al., 2015; Redding et al., 2015; Richter et al., 2014). The recruited Cas3 can then trans locate in either direction, in contrast to the unidirectional movement observed at perfect targets, without degrading the target DNA (Redding et al., 2015). Cas1 and Cas2 may accompany the translocating Cas3 and
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be activated for protospacer selection, allowing for robust acquis-sition on either side of the target site.

Primed acquisition has also been shown experimentally in the P. atrosepticum Type I-F system, in which Cas2 and Cas3 are naturally fused as a single polypeptide that associates with Cas1, as well as in the Haloarcula hispanica Type I-B system, where naive acquisition was not experimentally observed (Li et al., 2014; Richter et al., 2014; Richter et al., 2012). Acquisition in H. hispanica also requires Cas4, a 5' → 3' exonuclease found in most Type I subtypes as well as Type II-B and Type V systems, and which might be involved in generating 3' overhangs on pro-tospacers prior to integration (Lemak et al., 2013; Li et al., 2014; Makarova et al., 2015). Although Cas1 and Cas2 may be the minimal proteins required for spacer acquisition in some systems, the association of Cas1, Cas2, and the interference machinery allows the host to coordinate robust adaptive immunity in Type I systems.

Self-versus Non-Self-Recognition

The mechanism underlying the preference for foreign over self DNA during protospaces selection remained poorly understood until a recent study on spacer acquisition during naive acquisition. Spacer acquisition in E. coli was shown to be highly dependent on DNA replication, and foreign-derived spacers were preferred over self-derived spacers by about 100- to 1,000-fold (Levy et al., 2015). Analysis of the source of self-derived spacers demonstrated that protospaces were acquired largely from genomic loci predicted to frequently generate stalled replication forks and double-stranded DNA breaks (Levy et al., 2015). Such harmful dsDNA breaks are repaired by the helicase/nuclease RecBCD complex, which degrades the broken ends until reaching a Chi-site, after which only the 5' end is degraded (Dillingham and Kowalczykowski, 2008). Due to the lower frequency of Chi sites in foreign DNA, RecBCD is predicted to preferentially degrade plasmids and viral DNA, resulting in the generation of candidate protospaces substrates for Cas1 and Cas2 (Levy et al., 2015) (Figure 2A). RecBCD degrades DNA asymmetrically, yielding single-stranded fragments ranging from tens to hundreds of nucleotides long from one strand and kilobases long from the other (Dillingham and Kowalczykowski, 2008). It is unclear how Cas1-Cas2 substrates, which are 33 bp long and partially double stranded with 3' overhangs, are generated from RecBCD products (Nuez et al., 2015a; Nuez et al., 2015b; Wang et al., 2015). It is possible that ssDNA products re-anneal to produce partial duplexes, followed by processing to 33 bp by an unknown mechanism prior to integration into the CRISPR locus. Recent crystal structures of Cas1-Cas2 with bound protospaces reveal that the complex defines the length of the duplex region of the protospaces via a ruler mechanism and may cleave the 3' overhangs to their final length (Nuez et al., 2015b; Wang et al., 2015). The involvement of a helicase/nuclease in both Type I-E primed and naive acquisition (Cas3 and RecBCD, respectively), as well as in Cas4-containing subtypes, hints at a conserved mechanism for protospace generation. It is also worth noting that RecBCD is conserved primarily in Gram-negative bacteria, while Gram-positive bacteria and archaea rely on AddAB and HerA-NurA, respectively, to fill a similar role (Blackwood et al., 2013; Dillingham and Kowalczykowski, 2008). Whether CRISPR-Cas systems in these organ-
isms have evolved to cooperate with these evolutionarily distinct machineries remains to be tested.

Mechanism of Protocolsacer Integration

Cas1 and Cas2 play central roles in the acquisition of new spacers, where they function as a complex (Nuez et al., 2014). Crystal structures of Cas1 and Cas2, with or without bound protospacer, revealed two copies of a Cas1 dimer bridged by a central Cas2 dimer (Nuez et al., 2014; Nuez et al., 2015b; Wang et al., 2015). Cas1 functions catalytically, while Cas2 appears to serve a primarily structural role (Arslan et al., 2014; Datsenko et al., 2012; Nuez et al., 2014; Yosef et al., 2012).

The first insight into the mechanism of protospacer integration was gained by Southern blot analysis of the genomic CRISPR locus of E. coli cells overexpressing Cas1 and Cas2 (Arslan et al., 2014). This revealed integration intermediates consistent with two transesterification reactions, where each strand of the protospacer is integrated into opposite sides of the leader-proximal repeat (Figure 2B). This integrate-like model was further bolstered by the in vitro reconstitution of protospacer integration into a plasmid-encoded CRISPR locus using purified Cas1-Cas2 complex (Nuez et al., 2015a). The integration reaction required double-stranded DNA protospacers with 3'-OH ends that are integrated into plasmid DNA via a direct nucleophilic transesterification reaction, reminiscent of retroviral integrases and DNA transposases (Engelman et al., 1991; Mizuuchi and Adzuma, 1991).

Although deep sequencing of in vitro integration products revealed preferential protospac integration adjacent to the first repeat, confirming that Cas1-Cas2 directly recognize the CRISPR locus, integration also occurred at the borders of every repeat at varying levels (Nuez et al., 2015a). This contrasts with spacer acquisition only occurring at the first repeat in E. coli in vivo (Datsenko et al., 2012; Swarts et al., 2012; Yosef et al., 2012). To determine if the Cas1-Cas2 complex has sequence specificity for the leader-repeat sequence, a recent study took advantage of the Cas1-catalyzed disintegration reaction, a reversal of the integration reaction also observed with retroviral integrases and transposases (Chow et al., 1992; Rollie et al., 2015). Disintegration activity was stimulated when using the correct leader-repeat border sequences, highlighting intrinsic sequence-specific recognition by Cas1. Furthermore, disintegration was faster at the leader-repeat junction compared to the repeat distal end (Rollie et al., 2015). Taken together, protospac integration likely begins at the leader-repeat junction via sequence-specific recognition by Cas1, followed by a second nucleophilic attack at the repeat distal end. This ensures precise duplication of the first repeat, as observed in vivo, after DNA repair by host proteins. The integration mechanism is hypothesized to be highly specific, as almost all acquired spacers with a corresponding AAG PAM are oriented with the 5'-G at the leader-proximal end, leading to functional crRNA-dependent targeting by Cascade and Cas3 (Shmakov et al., 2014). A preference for integration in the proper orientation was observed in vitro when protospacers with a 5'-G were used (Nuez et al., 2015a); however, inclusion of part of the PAM in spacers has only been observed in E. coli, raising the question of how Cas1-Cas2 in other systems properly orient the integration reaction.

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Type II Acquisition

While most mechanistic work on acquisition has been performed in Type I systems, recent studies in Type II systems have also shed light on key aspects of spacer acquisition. One generalizable finding in Type II systems is the dependence of acquisition on infection by defective phage (Hynes et al., 2014). A significant problem with CRISPR immunity is the time required for foreign DNA to be identified, integrated into the CRISPR locus, transcribed, processed, and assembled into an interference complex that must then begin the search for appropriate targets. Since lytic phage can kill cells within 20 min, providing insufficient time for this multi-step process, Hynes and colleagues tested the hypothesis that initial communication takes place from infection by a defective phage. Supplementation of active phage with UV-irradiated phage or phage susceptible to a restriction-modification system stimulated spacer acquisition compared to that observed with active phage alone (Hynes et al., 2014). The authors speculate that acquisition from compromised phage might also represent the dominant mode of acquisition in wild populations, allowing for a small subset of the population to acquire resistance and escape without needing to outpace a rapidly reducing phage.

Type II Acquisition Machinery

Type II systems are subdivided into II-A, II-B, and II-C based on the presence or absence of an additional cas gene alongside the minimal complement of cas1, cas2, and cas9. Type II-A systems contain csn2, while Type II-B systems, which are least commonly found, contain cas4 (Chylinski et al., 2014; Makarova et al., 2011b). Type II-C systems comprise only the minimal three genes. Csn2 has been shown to be essential for acquisition in several Type II-A systems (Barrangou et al., 2007; Heler et al., 2015; Wei et al., 2015b).

It forms a tetramer with a toroidal architecture that binds and slides along free DNA ends, though its function in CRISPR systems is unclear (Arslan et al., 2013; Ellinger et al., 2012; Koo et al., 2012; Lee et al., 2012). Cas4, discussed above, is likely involved in acquisition in Type II-B systems. Type II-C systems, which constitute the majority of identified Type II systems (Chylinski et al., 2014; Makarova et al., 2015), are possibly functional for acquisition in the absence of auxiliary acquisition factors, though in the case of the Campylo-bacter jejuni system, acquisition was only observed following infection by phage encoding a Cas4 homolog (Hooton and Con-nerton, 2014).

Recently, two simultaneous studies demonstrated that, in addition to Cas1, Cas2, and Csn2, Cas9 plays a necessary role in the acquisition of new spacers in Type II systems (Heler et al., 2015; Wei et al., 2015b). Both groups, one working with the CRISPR1 Type II-A system of S. thermophilus, the other with the Type II-A system of Streptococcus pyogenes and the CRISPR3 system of S. thermophilus, also Type II-A, showed that wild-type or catalytically inactive Cas9 (dCas9) supported robust spacer acquisition, whereas deletion of Cas9 abolished spacer acquisition. It is proposed that Cas9 serves to recognize PAM sites in potential protospacers and mark them for recognition by Cas1 and Cas2 (Figure 2A). This hypothesis was confirmed by mutating the PAM-interacting residues of Cas9, resulting in complete loss in PAM-specificity in the newly acquired spacers (Heler et al., 2015). This presents a striking contrast to the E. coli Type I-E system, where Cas1-Cas2 recognize PAM sequences independently.

Intriguingly, expression of dCas9 results in the acquisition of primarily self-targeting spacers, suggesting that many acquisition events lead to self-targeting and suicide (Wei et al., 2015b). Microbial populations may rely on a few individuals to acquire phage resistance while the rest succumb to infection or CRISPR-mediated suicide. Some systems, such as that found in E. coli, may evolve to use host processes to bias acquisition away from self-targeting. Alternatively, S. thermophilus might have mechanisms of self-non-self-discrimination that were masked in the strain overexpressing CRISPR proteins. Phage challenge experiments with wild-type S. thermophilus revealed that some sequences were acquired as spacers disproportionately often across multiple experiments, suggesting that the Type II acquisition machinery has preferences in addition to Cas9-dependent PAM selection, though no clear pattern emerged with respect to the genomic location or sequence of protospacers that indicated a basis for the preferences (Paez-Espino et al., 2013).

Additionally, it was demonstrated that the four proteins of the S. pyogenes CRISPR system (Cas1, Cas2, Csn2, and Cas9) form a complex, suggesting that Cas9 directly circuits the acquisition proteins to potential targets (Heler et al., 2015). While drawing comparisons between the involvement of Cas9 in acquisition and primed acquisition in Type I systems is tempting, neither group saw evidence that acquisition was affected by the presence of existing spacers matching or closely matching the infecting phage or plasmid (Heler et al., 2015; Wei et al., 2015b). In addition, while the trans-activating crRNA (tracrRNA) that forms a complex with Cas9 and the crRNA is necessary for acquisition, it is unclear whether a corresponding crRNA is also required (Heler et al., 2015; Wei et al., 2015b). Future mechanistic work will be required to shed light on the similarities between Cas9-mediated spacer acquisition and the primed acquisition in Type I systems.

Type II Protocolsacer Integration

The sequence requirements for protospace integration in Type II-A systems were recently demonstrated in S. thermophilus (Wei et al., 2015a). Similar to E. coli, the leader and a single repeat were sufficient to direct integration. Furthermore, only the ten nucleotides of the leader proximal to the first repeat are required to license the integration of new spacers, in contrast to the 60 nt minimal requirement in E. coli (Wei et al., 2015a; Yosef et al., 2012). A limited mutational study of the repeat showed that the first two nucleotides are necessary for acquisition, while the final two nucleotides can be mutated without consequence (Wei et al., 2015a). Thus, Cas1-Cas2-catalyzed integration at the leader-repeat junction is sequence specific, while the attack at the repeat-space junction is determined by a ruler mechanism, in agreement with observations from experiments in the E. coli system (Diez-Villasehor et al., 2013). Together, these findings support the functional conservation of the Cas1-Cas2 integrase complex

despite divergent mechanisms of protospace selection between Types I and II CRISPR-Cas systems.

CRISPR Integrases as Genome-Modifying Tools

As with many other Cas proteins, the Cas1-Cas2 integrate complex shows promise for use in modifying genomes. While Cas1-Cas2 catalyze a reaction similar to that of many integrases

and transposases, they exhibit several fundamental differences that make them uniquely suited to certain applications. Cas1-Cas2 complexes lack sequence specificity for the DNA substrate to be integrated, a property that could make the system ideal for barcoding genomes. Genome barcoding allows for tracking lineages originating from individual cells, facilitating studies of population evolution, cancer, development, and infection (Blundell and Levy, 2014). Cas1-Cas2 complexes integrate short DNA sequences, in contrast with current techniques based on recombinases that integrate entire plasmids, resulting in potential fitness costs and unwanted negative selection (Blundell and Levy, 2014). Interestingly, in vitro integration of DNA substrates into plasmid targets revealed integration into non-CRISPR sites (Nuez et al., 2015a), suggesting that Cas1-Cas2 can be harnessed to integrate into a wide array of target sequences. A greater understanding of the minimal functional recognition motif for various Cas1-Cas2 integrases will facilitate the development of this technology.

crRNP Biogenesis: Generating Molecular Sentinels for the Cell

CRISPR immune systems use RNA-programmed proteins to patrol the cell in search of DNA molecules bearing sequences complementary to the crRNA. Assembly of these molecular sentinels begins with transcription of the CRISPR locus to generate long, precursor CRISPR RNAs (pre-crRNAs), followed by processing into short crRNA guides (Brouns et al., 2008; Carte et al., 2008). The promoter is embedded within the AT-rich leader sequence upstream of the repeat-spacer array, or sometimes within the repeat sequences (Zhang et al., 2013). Here, we briefly review the processing of pre-crRNAs catalyzed by the Cas6 endoribonuclease family in Type I and III systems and a distinct processing pathway in Type II systems that involves endogenous RNase III, Cas9, and a tracrRNA. The crRNA biogenesis pathway has been extensively reviewed elsewhere (Charpentier et al., 2015; Hochstrasser and Doudna, 2015).

Processing by Cas6 Endoribonucleases

Type I and Type III systems employ Cas6 endoribonucleases to cleave pre-crRNAs sequence specifically within each repeat (Brouns et al., 2008; Carte et al., 2008; Haurwitz et al., 2010). Although Cas6 homologs are variable in sequence, they share a conserved cleavage mechanism that results in crRNA guides comprising an entire spacer sequence flanked by portions of the repeat sequence on the 5′ and 3′ ends. Mature crRNA guides consist of an 8 nt 5′ handle derived from the repeat sequence and variable lengths of the repeat at the 3′ handle, which is further trimmed by as-yet-unidentified cellular nuclease(s) in Type III systems (Hale et al., 2008). A notable exception is in Type I-C systems, which utilize a Cas5 variant for crRNA processing, leaving an 11 nt 5′ handle and 21–26 nt at the 3′ end (Garside et al., 2012; Nam et al., 2012b). In other Type I systems, Cas5 subunits serve a non-catalytic role capping the 5′ end of the crRNA in Cascade complexes.

In Type I-C, I-D, I-E, and I-F systems, the repeats form stable hairpin structures that allow for structure- and sequence-specific cleavage by Cas6 at the base of the hairpin (Gesner et al., 2011; Haurwitz et al., 2010; Sashital et al., 2011). After cleavage, the hairpin constitutes the 3′ handle of the crRNA. The Cas6 pro-

teins in Haloferax volcanii (Cas6b), E. coli and T. thermophilus (Cas6e), and Pseudomonas aeruginosa (Cas6f) remain stably bound to the 3′ handle and eventually become part of the Cascade complex (Brendel et al., 2014; Brouns et al., 2008; Gesner et al., 2011; Haurwitz et al., 2010; Sashital et al., 2011).

Type I-A, I-B, III-A, and III-B repeat sequences are non-palindromic and predicted to be unstructured in solution (Kunin et al., 2007). Thus, the respective Cas6 is thought to rely on sequence for specificity rather than structure. Interestingly, a crystal structure of the Type I-A Cas6 bound to its cognate RNA structure reveals Cas6 inducing a 3 bp hairpin in the RNA that positions the scissile phosphate in the enzyme active site (Shao and Li, 2013). It remains unknown whether other Cas6s that recognize

non-palindromic repeats have a similar mechanism of RNA stabilization. Following or concurrent with the maturation of the crRNAs, the Cas proteins involved in interference assemble into the final effector complex that functions to recognize and destroy targets bearing sequence complementarity to the crRNA. In systems where Cas6 remains bound to the crRNA, it may serve to nucleate the assembly of the subunits that constitute the effector complex backbone along the crRNA. In type III systems, the number of backbone subunits defining the complex length is variable, and any unprotected crRNA remaining is degraded (Hale et al., 2008; Staals et al., 2014).

Processing in Type II Systems

Type II systems rely on a different mechanism to process pre-crRNAs. In Types II-A and II-B, pre-crRNA cleavage specificity is aided by a tracrRNA that has sequence complementarity to the CRISPR repeat sequence (Deltcheva et al., 2011). The gene encoding the tracrRNA is typically located either proximal to or within the CRISPR-cas locus (Chylinski et al., 2014). Upon crRNA:tracrRNA base pairing, which is stabilized by Cas9, endogenous RNase III cleaves the pre-crRNA at the repeat. The reliance on RNase III, which is not found in archaea, may explain why Type II systems are limited to bacteria (Garrett et al., 2015). An unknown nuclease trims the 5' end of the crRNA to remove the flanking repeat sequence and portions of the spacer. In S. pyogenes, the 30 nt spacer sequence is trimmed to the 20 nt that base-pairs with complementary foreign sequences during interference (Deltcheva et al., 2011; Jinek et al., 2012). In the Neisseria meningitidis and C. jejuni Type II-C systems, each repeat sequence encodes a promoter, resulting in varying lengths of pre-crRNAs depending on the transcription start site (Dugar et al., 2013; Zhang et al., 2013). Although RNase III-mediated pre-crRNA processing can still occur, RNase III is dispensable for interference in these systems (Zhang et al., 2013). Thus, Cas9 is able to complex with the pre-crRNA and unprocessed tracrRNA for functional target interference without further processing of the pre-crRNAs.

Cas6 as a Biotechnology Tool

The Cas6 homolog from Type I-F systems, Cas6f (also known as Csy4), was the first Cas protein to be repurposed as a tool. Following demonstration of the sequence specificity of Cas6f binding and cleavage, the protein has been used for the purification of tagged RNA transcripts from cells (Haurwitz et al., 2010; Lee et al., 2013; Salvail-Lacoste et al., 2013; Sternberg et al., 2012). Subsequent studies showed that Cas6f could be used to alter the translation and stability of tagged rRNAs, allowing

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Figure 3. Interference by Class 1 Systems (A) Interference in Type I systems is carried out by Cascade and Cas3. Cascade is a large complex composed of the crRNA, bound at either end by Cas5 and Cas6, multiple Cas7 subunits along the crRNA, a large subunit (Cse1, Csy1, Cas8, or Cas10), and sometimes small subunits (Cse2 and Csa5). The Type I-E complex is schematized here. The large subunit recognizes the PAM in foreign DNA and initiates unwinding of the target DNA and annealing to the crRNA. Cas3 is recruited to the resulting R-loop and makes a nick. It then translocates along the displaced strand and processively degrades it.

(B) Type III systems contain either Csm or Cmr complexes, which share a similar architecture. The Csm complex from Type III-A systems is shown here. The crRNA is bound at either end by Csm5/Cmr1 and Csm4/Cmr3, which have homology to Cas6 and Cas5, respectively. Csm3/Cmr4 form the backbone of the complex, Cas10 serves as the large subunit, and Csm2/Cmr5 are the small subunit. These complexes can target both RNA and actively transcribed DNA. Cas10 catalyzes cleavage of target DNA, while the backbone subunit catalyzes cleavage of the target RNA at every sixth base, which is unpaired with the crRNA. Rather than recognizing a PAM sequence, these complexes only leave if the 5' and 3' handles of the crRNA do not anneal to the target.

for post-transcriptional regulation of protein expression (Borchardt et al., 2015; Du et al., 2015; Nissim et al., 2014). Cas6f has also been used alongside Cas9 to process multiple guide RNAs from a single transcript, greatly facilitating multiplexed editing (Tsai et al., 2014).

Interference: Precise, Programmable DNA Binding and Cleavage

Implementation of CRISPR systems to provide immunity involves RNA-guided recognition and precision cutting of DNA molecules, a property that makes them useful for genome engineering and control of gene expression. The extreme diversity of the crRNP targeting complexes is largely responsible for the variability observed in different CRISPR types. Whereas Types I and III use multi-protein complexes, Types II and V rely on a single protein for interference. Extensive studies have elucidated the mechanisms and structures of several complexes from each of the three major types, revealing the commonality of target binding through crRNA base-pairing and high divergence in the machineries and modes of target cleavage. For more in-depth recent reviews focused exclusively on CRISPR interference, refer to Tsui and Li (2015) and Plagens et al. (2015).

Type I Interference

In Type I systems, the roles of target DNA recognition and degradation are segregated into two distinct components. The crRNA-guided Cascade complex binds and unwinds the DNA target sequence (Brouns et al., 2008) and then recruits Cas3 to degrade the target in a processive manner through the combined action of its HD nuclease and helicase domains (Figure 3A) (Makarova et al., 2011b; Mulepati and Bailey, 2013; Sinkunas et al., 2013; Westra et al., 2012). Each Type I subtype (I-A through I-F) has a distinct complement of Cascade components and, in some cases, significant variation of the cas3 gene (Makarova et al., 2011b).

The E. coli Cascade complex has served as the model system for understanding the mechanism of Type I interference. In addition to the central 61 nt crRNA bearing the 32 nt spacer, the complex comprises five proteins in different stoichiometries: (Cse1)1, (Cse2)2, (Cas5), (Cas7)6, and (Cas6)1. The Cas7 subunits form the "backbone" that polymerizes along the crRNA and determines the crescent-shaped, semi-helical architecture seen in all structurally characterized Cascade complexes (Hochstrasser et al., 2014; Jackson et al., 2014; Jore et al., 2011; Mulepati et al., 2014; Wiedenheft et al., 2011a; Zhao et al., 2014). Cas6 (Cas6e in Type I-E systems) remains bound to the 3' hairpin following CRISPR maturation, while Cas5 binds the 5' handle (Brouns et al., 2008; Jore et al., 2011). A "small subunit" (Cse2 in Type I-E) is often found in two copies forming the "belly" of the structure and helps stabilize the crRNA and target DNA (Jackson et al., 2014; Mulepati et al., 2014; Zhao et al., 2014). A "large subunit" (Cse1 in Type I-E, Cas8 in most other subtypes) binds at the 5' end of the crRNA and recognizes the PAM sequences and recruits Cas3 to an authenticated target (Figure 3A) (Hochstrasser et al., 2014; Sashital et al., 2012). While Cas6 does not always remain with the complex and the small subunit is often found as a fusion with the large subunit, the overall architecture of Cascade complexes is generally conserved (Makarova et al., 2011b; Plagens et al., 2012; Sokolowski et al., 2014).

Cascade pre-arranges the spacer segment of the crRNA in six five-base segments of pseudo A-form conformation, with the sixth base flipped out and bound by a Cas7 subunit (Jackson et al., 2014; Mulepati et al., 2014; Zhao et al., 2014). To initiate

Figure 4. Interference by Class 2 Systems

(A) In Type II systems, Cas9 forms the effector complex with a crRNA and a tracrRNA. Cas9 is composed of the nuclease lobe and the α-helical lobe. The nuclease lobe contains both the HNH and RuvC-like nuclease domains as well as the PAM-interacting domain. The 3' hairpins of the tracrRNA bind the nuclease lobe, while the stemloop and spacer line the channel between the two lobes. Binding to a matching, PAM-adjacent target causes the HNH domain to move into position to cleave the annealed strand, while the displaced strand is fed into the RuvC active site for cleavage.

(B) Cpf1 is the effector protein in Type V-A systems, the best characterized Type V subtype. It binds the crRNA alone. The structure of Cpf1 is unknown, but it contains an active RuvC-like nuclease domain for target cleavage. Cpf1 recognizes a PAM and makes two staggered cuts in a matching sequence. It has been proposed that Cpf1 acts as a dimer, with each monomer providing a RuvC active site, though there may be another unidentified nuclease domain.

interference, Cascade first recognizes trinucleotide PAM sites in the target strand of foreign DNA through

specific interactions with Cse1 (Sashital et al., 2012). Upon PAM binding, the DNA target is unwound starting at the PAM-proximal end of the proto-spacer to form an R loop structure (Hochstrasser et al., 2014; Rollins et al., 2015; Rutkauskas et al., 2015; Sashital et al., 2012; Szczelkun et al., 2014; van Erp et al., 2015). Each stretch of five exposed bases in the crRNA is free to bind the target DNA, leading to a stable but highly distorted and discontinuous crRNA:target strand duplex (Mulepati et al., 2014; Szczelkun et al., 2014). Cascade undergoes a conformational change upon target binding that enables recruitment of Cas3 to the
Cse1 subunit (Hochstrasser et al., 2014; Mulepati et al., 2014). Cas3 binds and nicks the displaced strand using its catalytic center of the HD nuclease domain (Gong et al., 2014; Huo et al., 2014; Mulepati and Bailey, 2013; Sinkunas et al., 2013; Westra et al., 2012). The ATP-dependent helicase activity of Cas3 is then activated, causing metal- and ATP-dependent 3' → 5' translocation and processive degradation of the nontarget strand (Gong et al., 2014; Huo et al., 2014; Westra et al., 2012). Cas3 initially degrades only 200–300 nt of the nontarget strand, though it continues translocating for many kilobases (Redding et al., 2015). Exposed ssDNA on the target strand may then become a substrate for other ssDNA nucleases or an additional Cas3 molecule to complete the degradation of foreign DNA (Mulepati and Bailey, 2013; Redding et al., 2015; Sinkunas et al., 2013). In addition to the PAM, target interference also relies on a seed region at the 3' end of the spacer segment of the crRNA (Semenova et al., 2011; Wiedenheft et al., 2011b). Single point mutations of the seed region of the E. coli Cascade complex, at the 1 to 5 and 7 to 8 position of the spacer, is enough to decrease target DNA binding and subsequent interference (Semenova et al., 2011).
Differences in the cas3 gene among Type I subtypes suggest some variability in interference mechanism. In some Type I-E species, Cas3 is fused to Cse1 by a linker that allows it to stably associate with the Cascade complex (Westra et al., 2012). In Type I-A systems, the Cas3 helicase and nuclease domains exist as separate polypeptides that both associate with the Cascade complex (Plagens et al., 2014). In Type I-F systems, Cas3 is fused to Cas2, lending further genetic support for the interaction between the interference and acquisition machinery during primed acquisition (Makarova et al., 2015; Richter and Fineran, 2013; Richter et al., 2012). How these fusions and domain separations affect the processive degradation observed in Type I-E systems requires further study.
Type II Interference
In contrast to the multi-subunit effector complexes seen in Type I and Type III systems (but similar to Cpf1 of Type V systems), the Type II signature protein Cas9 functions as an individual protein, along with a crRNA and tracrRNA, to interrogate DNA targets and destroy matching sequences by cleaving both strands of the target (Figure 4A) (Gasiunas et al., 2012; Jinek et al., 2012). Extensive studies on Cas9 have yielded a range of structures of S. pyogenes Cas9 in different substrate-bound states, as well as structures of several orthologs (Anders et al., 2014; Jiang et al., 2015; Jinek et al., 2014; Nishimasu et al., 2015; Nishimasu et al., 2014). Many of these structures, as well as the mechanism of Cas9 target search and recognition, are reviewed elsewhere (van der Oost et al., 2014); here, we focus on the most recent advances.
Structures of Cas9 have revealed two distinct lobes, the nuclease lobe and the α-helical or REC lobe (Anders et al., 2014; Jinek et al., 2014; Nishimasu et al., 2015; Nishimasu et al., 2014). The nuclease lobe is composed of the HNH nuclease domain, which cleaves the target strand, a RuvC-like nuclease domain, which cleaves the non-target strand and is separated into three distinct regions in the primary sequence by the intervening α-helical lobe and the HNH domain, and a C-terminal PAM-interacting domain (Anders et al., 2014; Jinek
Cell
et al., 2014; Nishimasu et al., 2015; Nishimasu et al., 2014). The α-heical lobe contains an arginine-rich “bridge helix,” which connects the two lobes and interacts with the guide RNA, and is the most variable region of Cas9, with insertions or deletions accounting for much of the wide variation in size seen in Cas9 orthologs (Chylinski et al., 2014; Jinek et al., 2014; Nishimasu et al., 2014).

Cas9 initiates its target search by probing duplexed DNA for an appropriate PAM before initiating target unwinding (Sternberg et al., 2014). The target unwinds from the seed region, the first 10-12 nucleotides following the PAM, toward the PAM-distal end (Szczelkun et al., 2014). A perfect or near-perfect match leads to cleavage of both DNA strands, with mismaches being more tolerated outside of the seed region (Cong et al., 2013; Jiang et al., 2013; Jinek et al., 2012; Sternberg et al., 2014). The mechanism by which mismates distant from the cleavage site prevent cleavage appears to rely on the structural flexibility of the HNH domain, which has yet to be crystallized in proximity to the scissile phosphate (Anders et al., 2014; Nishimasu et al., 2015; Nishimasu et al., 2014). FRET assays show that the HNH domain swings into a catalytically competent position only upon binding to a cognate double-stranded DNA substrate, underscoring the multiple steps of conformational control of Cas9-catalyzed DNA cleavage (Sternberg et al., 2015). The RuvC domain is in turn allosterically regulated by the HNH domain. Cleavage of the non-target strand requires movement of the HNH domain into an active position, even when the mismatched substrates allow full unwinding of the non-target strand (Sternberg et al., 2015).

Recent crystal structures of S. pyogenes Cas9-sgRNA surveillance complex and of the smaller Staphylococcus aureus Cas9 in a target-bound state provided new insights into Cas9 function (Jiang et al., 2015; Nishimasu et al., 2015). The sgRNA-bound structure revealed how binding of sgRNA shifts Cas9 from the auto-inhibited state observed in the apo form to a conformation competent for target search (Jiang et al., 2015; Jinek et al., 2014). As previously observed in low-resolution electron microscopy structures, a nucleic acid binding cleft is formed between the two lobes upon sgRNA binding (Jinek et al., 2014). Furthermore, two PAM-interacting arginine residues are pre-positioned to allow for scanning of potential target DNA, a finding that may explain the necessity of tracrRNA in directing PAM-dependent spacer acquisition. Surprisingly, while the 3' hairpins of the tracrRNA have been shown to provide nearly all of the binding energy and specificity for Cas9, the repeat-anti-repeat region of the sgRNA as well as the seed sequence were required to induce the conformational rearrangement (Briner et al., 2014; Jiang et al., 2015; Wright et al., 2015). The seed sequence of the sgRNA was also found to be pre-ordered in an A-form helix, analogous to the pre-ordered seed region of guide RNA observed in ukaryotic Argonaute structures and the Type I and Type III effector complexes, where the entire crRNA is pre-arranged in a target-binding-competent state (Jackson et al., 2014; Kuhn and Joshua-Tor, 2013; Mulepati et al., 2014; Osawa et al., 2015; Taylor et al., 2015; Zhao et al., 2014). The observed pre-ordering of the guide RNA provides an energetic compensation for the unwinding of the target duplex to facilitate binding.

Cas9 from the Type II-C CRISPR system of S. aureus was crystallized in complex with sgRNA and a single-stranded DNA target sequence, providing insight into the structural variation between more distantly related Cas9 (Nishimasu et al., 2015). S. aureus Cas9 is significantly smaller than the Cas9 of S. pyogenes (1,053 versus 1,368 amino acids) and recognizes a significantly different guide RNA and PAM site. The S. aureus Cas9 structure revealed a smaller α-helical lobe, with domains in the middle and PAM-proximal side notably absent, while the nuclease lobe is largely conserved (Nishimasu et al., 2015). The authors proposed a new domain designation, the wedge domain, which diverges significantly between the two proteins and appears integral to determining guide RNA orthogonality. Another small Cas9, that from Actinomyces naeslundi, was previously crystallized in the apo form, but the absence of bound substrate and significant disordered regions limited detailed exploration of the differences between the orthologs (Jinek et al., 2014). Other recent work with Type II-C Cas9 proteins from N. meningitidis and Corynebacterium diphtheriae, among other Type II-C orthologs, revealed that these enzymes have a reduced ability to unwind dsDNA compared to S. pyogenes Cas9 and exhibit efficient PAM-independent and in some cases tracrRNA-independent cleavage of ssDNA (Ma et al., 2015; Zhang et al., 2015). This activity may allow for more efficient interference with ssDNA plasmid or phage or represent a more ancestral activity that predates the expansion of the α-helical lobe to facilitate more robust DNA unwinding.

Type III Interference

Type III systems are classified into Type III-A and Type III-B based on their effector complexes (Type III-C and III-D have also been identified, but not yet characterized) (Makarova et al., 2015). The former

is constituted by the Csm complex, and the latter by the Cmr complex (Makarova et al., 2011b). Phylogenetic studies suggested that some csm and cmr genes are distant homologs of cas genes that compose the Cascade complex of Type I systems, and subsequent structural studies have revealed a striking structural conservation between Cascade and the Csm and Cmr complexes (Hochstrasser et al., 2014; Jackson et al., 2014; Makarova et al., 2013; Mulpati et al., 2014; Osawa et al., 2015; Staals et al., 2014; Taylor et al., 2015; Zhao et al., 2014). For a detailed discussion of the structural similarities between these complexes, refer to Jackson and Wiedenheft (2015). Briefly, Csm3 (in III-A systems) or Cmr4 (in III-B) polymerizes along the crRNA as a helical backbone, analogously to Cas7, while Csm2 or Cmr5 take the role of Cse2 as the small subunit (Figure 3B) of Jackson and Wiedenheft, 2015). Similar to Cascade, the crRNA is pre-arranged for binding with kinks every six nucleotides. The target nucleic acid (RNA in all solved Type III structures) binds in a distorted manner, forming five-nucleotide helical stretches with the sixth base flipped out to allow for the extreme deviation from helical nucleic acid observed in all structures (Osawa et al., 2015; Taylor et al., 2015). Cmr3 and Csm4 bind the 5' crRNA handle, while Cas10 (also referred to as Csm1 and Cmr2) serves as the large subunit (Makarova et al., 2011a; Osawa et al., 2015; Staals et al., 2014; Taylor et al., 2015). Csm5, Cmr6, and Cmr1 also share homology with Cas7 and cap the helical backbone at the 3' end of the crRNA. In Type III-B systems, two major crRNA species are

generally observed, differing by six nucleotides (Juranek et al., 2012; Staals et al., 2014). Cryo-electron microscopy captured two Cmr complexes of different sizes, with one complex having one fewer Cmr4 and Cmr5 subunit, suggesting that the different crRNA lengths are the result of different complex sizes, or vice versa (Taylor et al., 2015).

Despite the structural similarities, the Type III interference complexes function quite distinctly from Cascade. The substrate specificity of Csm and Cmr complexes has only recently been clarified. Early in vivo genetic experiments suggested Csm targeted DNA, while in vitro studies of Cmr showed binding and cleavage activity against RNA only (Hale et al., 2009; Marraffini and Sontheimer, 2008), leading to a model wherein the two subtypes had evolved distinct and complementary substrate preferences. This simple model was soon complicated by the observation that Csm complexes in vitro also bind and cleave RNA while exhibiting no activity against DNA (Staals et al., 2014; Tamulaitis et al., 2014). Meanwhile, the in vivo DNA-targeting activity of III-A (systems was shown to depend on transcription at the target site, in contrast to the transcription-independent targeting seen in Type I and Type II systems, and a similar activity was observed for a III-B system in vivo (Deng et al., 2013; Goldberg et al., 2014). These observations were reconciled by the discovery that the Csm complex from Staphylococcus epidermidis exhibits both RNA cleavage and DNA cleavage when directed against the non-template strand of actively transcribed DNA (Samai et al., 2015).

DNA and RNA interference are carried out by distinct subunits of the Type III complexes. RNA interference is mediated by the backbone subunit Csm3 (or Cmr4 in III-B systems), which cleaves the target every six nucleotides in the active site of a separate subunit by activating the ribose 2' OH for nucleophilic attack in a manner typical of metal-independent RNases (Osawa et al., 2015; Staals et al., 2014; Tamulaitis et al., 2014; Taylor et al., 2015). Cas10 cleaves DNA exposed by a transcription bubble using a single catalytic site in its palm polymerase domain (Samai et al., 2015). The details of DNA targeting by Cmr have not been independently confirmed, but the conservation of Cas10 and evidence for transcription-dependent plasmid clearing supports a similar mechanism (Deng et al., 2013; Makarova et al., 2011b).

The distinct behavior of Type III systems provides the host microbe with the ability to tolerance temperate phases (Goldberg et al., 2014). While Type I and Type II systems target and degrade any protospace-containing DNA, Type III systems ignore foreign DNA until transcription begins that poses a threat to the cell. This has the advantage of allowing cells to acquire advantageous genes contained in prophages, such as antibiotic resistance genes, and causing cell suicide in the event that a lysogenic phage becomes lytic and begins transcribing genes with matching spacers (Goldberg et al., 2014). However,

the strand-specific nature of both the RNA targeting and transcription-dependent DNA targeting imposes an additional restriction on the integration step of acquisition, as only one direction of integration will yield productive interference. The means by which this apparent limitation is overcome are unclear. Type III systems are also frequently found coexisting with Type I systems, in which case their distinct target specificity might allow
for interference with targets that somehow avoid recognition by Cascade (Makarova et al., 2011b).
Type III systems are also unique in their lack of a PAM. Rather than recognizing a distinct motif to avoid auto-immunity at the CRISPR locus, the Csm and Cmr complexes instead check for complementarity between the repeat-derived region of the crRNA with the target and do not cleave if a full match is detected (Marraffini and Sontheimer, 2010; Samai et al., 2015; Staats et al., 2014; Tamulaitis et al., 2014). The specificity of Type III effector complexes for single-stranded targets might provide a rationale for their distinct mode of target authentication. For Type I and Type II effector complexes, which target dsDNA, PAM recognition allows for an initial binding event to facilitate subsequent unwinding of the target to probe for complementarity to the crRNA (Hochstrasser et al., 2014; Rollins et al., 2015; Sternberg et al., 2014; Szczelkun et al., 2014; Westra et al., 2012). Type III complexes can immediately probe a potential single-stranded target for complementarity to their bound crRNA without a need to license initial unwinding, and the exposed nature of a single-stranded target facilitates the check for complementarity to the repeat-derived region of the guide.

Type V Interference

Type V systems have only recently been classified, but initial work demonstrated that these systems are functional for interference (Makarova et al., 2015; Zetsche et al., 2015a). The systems appear most similar to Type II systems, possessing only the acquisition machinery and a single additional protein (Makarova et al., 2015; Schunder et al., 2013; Vestergaard et al., 2014). Three subtypes of Class V systems have been identified with widely varying interference proteins (Shmakov et al., 2015). Type V-A, V-B, and V-C are characterized by the presence of Cpf1, C2c1, and C2c3, respectively (Shmakov et al., 2015). All three proteins are evolved from the same family of transposon-associated TpnB proteins as Cas9 and have a C-terminal RuvC domain and arginine-rich bridge helix (Shmakov et al., 2015). However, the proteins show little similarity to each other, and the phylogenetic grouping of the associated cas1 genes with various branches of Type I and Type III cas1 genes suggests that each of these subtypes originated from distinct recombination events between CRISPR systems and tpnB genes (Shmakov et al., 2015).
While some Type V-B systems have an identifiable tracrRNA necessary for activity, Type V-A and V-C systems lack both a tracrRNA and Cas6 or Cas5-like endonuclease, making it unclear how the crRNA is processed (Makarova et al., 2015; Shmakov et al., 2015). The crRNA of Type V-A systems has a conserved stem-loop and can be processed to a functional form when transcribed in E. coli in the presence of Cpf1 (Zetsche et al., 2015a). Whether Cpf1 is also required for processing and the potential involvement host factors remains unknown. The Cpf1 from Francisella novicida can successfully interfere with transformed plasmids and recognizes a 5'-TTN-3' PAM at the 5' end of the protospaceser sequence, similar to the PAM location of Type I systems and in contrast to the 3' PAM observed in Type II systems. The enzyme makes a double-strand break, resulting in five-nucleotide 5' overhangs distal to the PAM site (Figure 4B). Mutation of catalytic residues in the RuvC active site prevents cleavage of either strand (Zetsche et al., 2015a).

The authors propose that Cpf1 might act as a dimer, with each monomer providing a RuvC active site but only one recognizing the target. If this is the case, whether one or both monomers has a bound crRNA is unclear. Alternatively, an as-of-yet undiscovered active site might be present in the protein, in which case its activity must be tightly coupled to that of RuvC to explain the phenotype observed for the RuvC mutant. A C2c1, which also has only one identifiable nuclease domain, has also been shown to be active for cleavage in vivo and in vitro, where it recognizes a 5'-TTN-3' PAM and requires a tracrRNA (Shmakov et al., 2015). Many mechanisms in these newly discovered systems, both Type V and the essentially uncharacterized Type VI, remain unknown and open for future study.

Interference Complexes as Genome Editing Tools

Most tool development of Cas proteins has focused on exploiting the programmable sequence-specific DNA recognition of interference complexes. Cas9 from S. pyogenes in particular has proven enormously useful for genome engineering. The ability to render Cas9 a two-component system by fusing the crRNA and tracrRNA into a single guide RNA (sgRNA) has allowed for its easy use for genome editing, transcriptional control, RNA targeting, and imaging (for recent reviews, see Jiang and Marraffini, 2015; Sternberg and Doudna, 2015). Cas9 has been used in various cell types and organisms ranging from mice and monkeys to primary human T cells and stem cells, as well as plants, bacteria, and fungi (Jiang and Marraffini, 2015; Sternberg and Doudna, 2015). Recent work has focused on developing various chemical- and light-inducible Cas9 constructs to allow for greater spatiotemporal control and on employing Cas9 orthologs with different PAM sequences and smaller sizes, allowing for easier packaging in adeno-associated virus vectors (Davis et al., 2015; Nihongaki et al., 2015; Polstein and Gersbach, 2015; Ran et al., 2015; Zetsche et al., 2015b).

Other interference complexes have already been used or have the potential to be useful for genome manipulation as well. Although the multi-subunit composition of Cascade makes it less tractable for genome engineering compared to Cas9, its large size and stable binding has been used for transcriptional silencing in E. coli (Rath et al., 2015). No published work has shown the application of Csm or Cmr complexes, but either could likely be used for various RNA modulation applications in cells. Two Cpf1 homologs, out of 16 that were tested, have been shown to facilitate genome editing in human cells (Zetsche et al., 2015a). The alternate PAM specificity of Cpf1 may prove useful for targeting sites without an appropriate PAM for Cas9, and the staggered cuts might prove to favor distinct pathways of DNA repair. However, a thorough investigation of the efficiency and off-target editing of Cpf1 will be needed to determine if this protein will see significant use alongside Cas9.

While Cas9 has already seen extensive use in the research setting, challenges remain for its application in the clinic. While making programmed cuts has become largely trivial, biasing DNA repair toward homology-directed repair rather than non-homologous end joining remains a challenge (Chu et al., 2015; Maruyama et al., 2015). Delivery of Cas9, either as an RNP or on a plasmid or viral vector, to particular tissues in whole organisms

is another challenge that must be addressed to enable clinical applications (D'Astolfo et al., 2015; Gori et al., 2015; Howes and Schofield, 2015; Lin et al., 2014; Zuris et al., 2015). As the field continues to advance rapidly, clinical trials may occur within a few years, with therapies possibly following within a decade. Engineering of crop plants with Cas9 is already underway: regulatory rulings have so far considered knockout plants not to be genetically modified organisms, but the regulatory fate of other modifications is currently being considered (Servick, 2015).

Concluding remarks

Despite the rapid progress of the field since the first demonstration of CRISPR immunity in 2007, many mechanistic questions remain un answered. Fundamental aspects of acquisition, such as how substrates for Cas1-Cas2-mediated integration are generated and the mechanism and extent of self-versus non-self-discrimination in different CRISPR subtypes, are still a mystery. While crRNA biogenesis and interference are reasonably well understood for certain model subtypes (Type I-E, Type II-A), the sheer diversity of CRISPR systems means that many subtypes with potentially distinct mechanisms remain unexplored. Type V and VI systems have only begun to be analyzed, and Type IV systems, bearing some familiar cas genes but no identifiable CRISPR locus, have yet to be characterized experimentally and almost certainly rely on mechanisms distinct from those of traditional CRISPR systems (Makarova and Koonin, 2015).

Other aspects of CRISPR-Cas systems lie beyond the scope of this Review. We have not discussed the non-immune functions of CRISPR-Cas systems, some of which appear to have evolved to serve regulatory rather than defense roles (for reviews, see Westra et al., 2014, and Ratner et al., 2015). Phage evasion of CRISPR immunity is another active area of research, with identified mechanisms including DNA modification, specialized anti-CRISPR proteins, and mutational escape (Bondy-Denomy et al., 2013;

Bondy-Denomy et al., 2015; Bryson et al., 2015; Deveau et al., 2008; Paez-Espino et al., 2015; Pawluk et al., 2014). The context-dependent regulation of CRISPR-Cas systems in response to phage infection and stress signals has also been explored but requires further study (Bondy-Denomy and Davidson, 2014; Garrett et al., 2015; Kenchappa et al., 2013; Patterson et al., 2015; Pul et al., 2010). The rapid development of technology derived from CRISPR-Cas systems, most notably Cas9 but also Cas6f/Csy4, Cascade, and Cpf1, has fueled intense interest in the field. The arms race between bacteria and bacteriophage has generated powerful molecular biology tools, from restriction enzymes that enabled recombinant DNA technology to Cas9, which started the "CRISPR revolution" in modern genome engineering. CRISPR systems haven proven to be both fascinating and enormously useful. Further study of bacterial immune systems, both CRISPR systems and those yet undiscovered, promises to yield further unforeseen discoveries and exciting new technologies.

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