Topic Introduction

Overview of CRISPR–Cas9 Biology

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Prokaryotes use diverse strategies to improve fitness in the face of different environmental threats and stresses, including those posed by mobile genetic elements (e.g., bacteriophages and plasmids). To defend against these elements, many bacteria and archaea use elegant, RNA-directed, nucleic acid–targeting adaptive restriction machineries called CRISPR–Cas (CRISPR-associated) systems. While providing an effective defense against foreign genetic elements, these systems have also been observed to play critical roles in regulating bacterial physiology during environmental stress. Increasingly, CRISPR–Cas systems, in particular the Type II systems containing the Cas9 endonuclease, have been exploited for their ability to bind desired nucleic acid sequences, as well as direct sequence-specific cleavage of their targets. Cas9-mediated genome engineering is transcending biological research as a versatile and portable platform for manipulating genetic content in myriad systems. Here, we present a systematic overview of CRISPR–Cas history and biology, highlighting the revolutionary tools derived from these systems, which greatly expand the molecular biologists' toolkit.

INTRODUCTION AND HISTORY

For decades, the function and purpose of CRISPR (clustered, regularly interspaced, short, palindromic repeats)–Cas (CRISPR-associated) systems remained an enigma, until a series of astute observations paved the way for an exploding field of research on the biology of these prokaryotic adaptive immune systems and the exploration of how they can be exploited for directed genome modification. The rapid evolution of this field has been dubbed the "CRISPR craze" and is widely recognized throughout the scientific community as having already revolutionized genetic engineering (Pennisi 2013; Barrangou 2014; Doudna and Charpentier 2014). Only 3 years after the first proof-of-principle experiments demonstrating that these systems could be reprogrammed and exploited as genome engineering tools, Cas9 technologies have not only been used to generate genetic knockout mutants in diverse organisms and model systems, but for a variety of other applications including, but not limited to, transcriptional repression and activation and live-cell imaging of DNA localization (Jinek et al. 2012; Chen et al. 2013; Cong et al. 2013; DiCarlo et al. 2013; Jiang et al. 2013; Mali et al. 2013; Perez-Pinera et al. 2013; Qi et al. 2013; Doudna and Charpentier 2014; Sampson and Weiss 2014).

It is a little-known fact that the study of CRISPR–Cas systems unknowingly began more than 25 years ago when an array of short, repetitive DNA sequences (~20–40 bp in length, termed "repeats") inter-

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spaced with nonrepetitive sequences (termed "spacers") was identified following the sequencing of the gene encoding alkaline phosphatase isozyme conversion enzyme (*iap*) in the *Escherichia coli* genome (Ishino et al. 1987). At the time, the function and purpose of these sequences were unknown. However, two decades later, computational analyses led to the discovery that these repetitive arrays were present in numerous bacteria and archaea and, notably, that the spacers were identical to many sequences present in exogenous mobile genetic elements such as plasmids, transposons, and bacteriophages (Bolotin et al. 2005; Mojica et al. 2005). Further bioinformatic studies revealed that these arrays, termed CRISPR arrays, were often associated with a core set of Cas genes (Jansen et al. 2002; Haft et al. 2005). Many of the Cas genes had sequence similarity to endonuclease and helicase families or genes encoding other nucleic acid binding proteins (Jansen et al. 2002; Haft et al. 2005; Makarova et al. 2006). In conjunction with the fact that many spacers were identical to mobile genetic elements, these findings gave rise to the postulation that CRISPR–Cas systems may act as a form of RNA-directed interference against foreign genetic elements (Makarova et al. 2006). This hypothesis was solidified in 2007 by a set of foundational experiments that provided the first direct evidence that CRISPR sequences and the associated Cas proteins directed interference against bacteriophage infection (Barrangou et al. 2007). Perhaps even more interestingly, new spacer sequences were naturally acquired into the CRISPR array following bacteriophage infection, subsequently facilitating sequence-specific resistance to the offending phage, and revealing a mechanism of adaptive immunity in prokaryotes (Barrangou et al. 2007; Brouns et al. 2008; Gasiunas et al. 2012; Westra et al. 2012; Staals et al. 2013).

Over the last 8 years, the mechanism of RNA-directed interference by CRISPR–Cas systems has been largely uncovered (Barrangou and Marraffini 2014; Plagens et al. 2015; Rath et al. 2015). Briefly, CRISPR-mediated interference occurs in three primary stages: (1) spacer acquisition, (2) crRNA transcription and maturation, and (3) target identification and cleavage (Fig. 1). During spacer acquisition, foreign nucleic acids are identified and processed into short, spacer-sized sequences that are inserted into the CRISPR array, to be flanked by a pair of repeat sequences (Fig. 1A–D; Heler et al. 2014). The CRISPR array is then transcribed and processed into mature small RNAs, called crRNAs, that each contain portions of the repeat sequences and a single spacer that facilitates identification of a target nucleic acid with significant sequence complementarity to the spacer sequence (Fig. 1E,F). The crRNAs complex with Cas protein(s) and, in some cases, additional RNAs to bind the target, resulting in target cleavage (Fig. 1G,H; Barrangou and Marraffini 2014; Plagens et al. 2015; Rath et al. 2015).

The field of CRISPR–Cas biology continues to rapidly expand. Numerous groups have elegantly revealed not only the molecular function of CRISPR–Cas systems in defense against foreign nucleic acids (Barrangou et al. 2007; Brouns et al. 2008; Marraffini and Sontheimer 2008; Hale et al. 2009; Garneau et al. 2010; Bikard et al. 2012; Gasiunas et al. 2012) but also uncovered clues about the evolution of these systems (Makarova et al. 2011; Chylinski et al. 2014; Krupovic et al. 2014; Koonin and Krupovic 2015) and their functions in other physiological processes (Bikard and Marraffini 2013; Westra et al. 2014; Barrangou 2015; Ratner et al. 2015). Most recently, and as is the topic of this collection, this foundational work has led to the discovery of how these systems, and specifically the CRISPR-associated endonuclease Cas9, can be engineered for myriad biotechnological applications.

TYPES OF CRISPR–Cas SYSTEMS

CRISPR–Cas systems can be subdivided into three main types (Type I, II, and III) that are each distinguished by the presence of unique Cas proteins, encoded adjacent to the CRISPR array (Makarova et al. 2011). Despite their conserved function in prokaryotic adaptive immunity, CRISPR–Cas systems are structurally and mechanistically diverse (Makarova et al. 2011, 2013; Vestergaard et al. 2014). The adaptation stage of immunity is the most conserved between the three CRISPR–Cas subtypes, with all known systems encoding the Cas proteins involved in this process, Cas1 and Cas2 (Fig. 1A–C; Heler et al. 2014). These two metal-dependent nucleases are both necessary and sufficient for spacer acquisition, but dispensable for target interference (Datsenko et al. 2012; Yosef et al. 2012; Nunez et al. 2014, 2015; Heler et al. 2015). Recently solved crystal structures of Cas1 and

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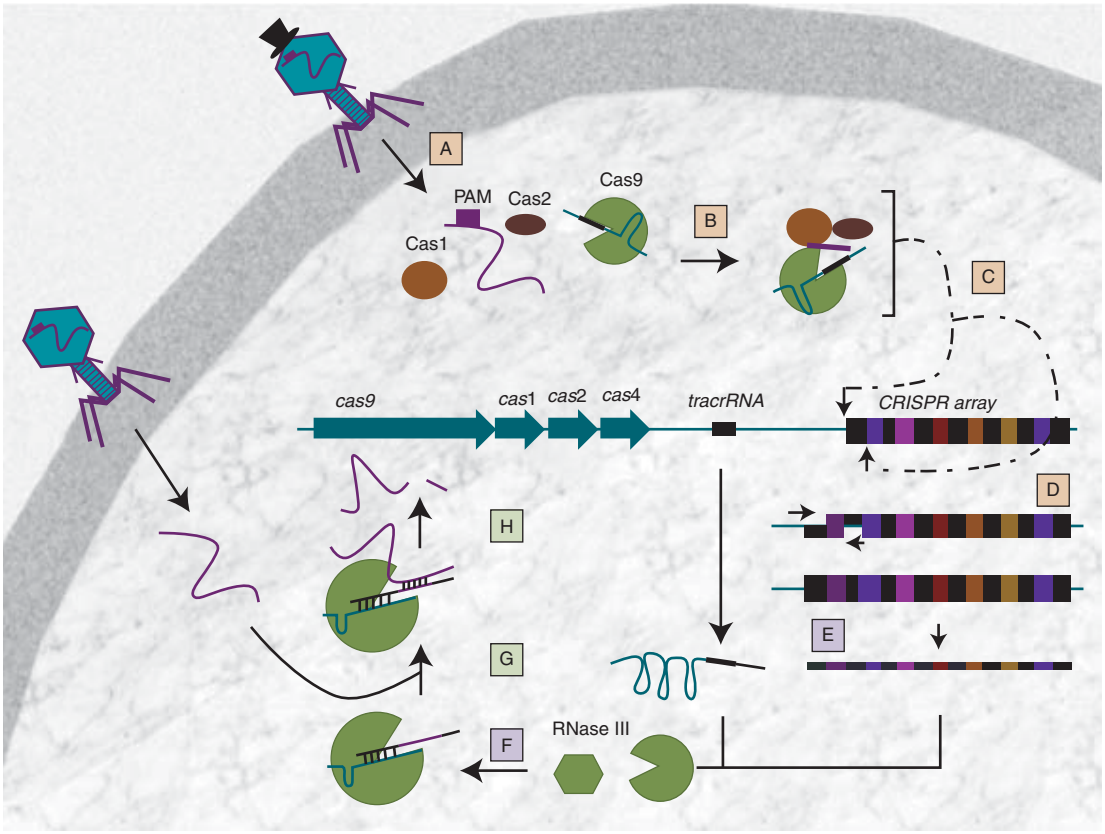

FIGURE 1. The three stages of adaptive immunity by Type II-C CRISPR–Cas systems. (*A–D*) Spacer acquisition: (*A*) foreign DNA (dark purple) enters the cell, and (*B*) *Cas1*, *Cas2*, and *Cas9* in complex with tracrRNA (blue) select a spacer sequence on the target through Cas9-mediated identification of a protospacer adjacent motif (PAM; dark purple rectangle on the foreign DNA). The PAM adjacent sequence is processed into a spacer-sized fragment. (*C*) The Cas protein complex attached to the spacer identifies the CRISPR array and creates staggered single-stranded breaks on each side on a repeat. (*D*) The new spacer sequence is inserted into the array and the single-stranded repeats on either side of the new spacer are repaired by DNA polymerase I. (*E,F*) crRNA transcription and maturation: (*E*) the CRISPR array and tracrRNA are transcribed. (*F*) Cas9 binds tracrRNA and the CRISPR transcript, which is then cleaved into mature, spacer-specific crRNAs by RNase III. The mature dual crRNA:tracrRNA remains bound to Cas9 as a heteroduplex. (*G,H*) Target identification and cleavage: (*G*) Upon re-infection with foreign DNA, the spacer on the crRNA of the Cas9:RNA heteroduplex binds to its complementary sequence on the foreign nucleic acid. (*H*) Cas9 adopts a conformationally active state and cleaves both DNA strands in the target, protecting the cell.

Cas2 indicate that these proteins form stable, heterodimeric complexes in vitro, and that in vivo, the interaction between Cas1 and Cas2 is necessary for recognizing the DNA secondary structure of the CRISPR repeat sequence during integration of new spacers (Nunez et al. 2014). The catalytic activity of Cas1 is essential for spacer acquisition, whereas the predicted nuclease active site of Cas2 is not (Nunez et al. 2014, 2015). Evidence from multiple types of CRISPR–Cas systems indicates that Cas1 and Cas2 may form complexes with Cas proteins involved in target identification and cleavage (Datsenko et al. 2012; Plagens et al. 2012; Swarts et al. 2012; van der Oost et al. 2014; Heler et al. 2015; Wei et al. 2015). Spacer acquisition may require these other Cas proteins to accurately select sequences in a way that prevents the CRISPR–Cas system from targeting its own chromosomal spacer sequences with the crRNAs transcribed from it; the details of this are described in sections below for the Type II systems (Barrangou et al. 2007; Datsenko et al. 2012; Heler et al. 2015).

The differences between the distinct types of CRISPR–Cas systems become increasingly clear at the crRNA maturation, target identification, and interference stages of immunity. Notably, Type I and III systems (described in this section) use large, multimeric protein complexes for these activities, whereas the Type II systems (described in detail in subsequent sections) require a single protein for these diverse

functions (van der Oost et al. 2014). Type I systems use the endonucleases Cas6 or Cas5d to cleave the CRISPR array transcript within the repeat sequences flanking each spacer, resulting in a short 5′ repeat-derived sequence and a 3′ hairpin, including a repeat-derived sequence (Carte et al. 2010; Gesner et al. 2011; Jore et al. 2011; Sashital et al. 2011; Garside et al. 2012; Nam et al. 2012; Koo et al. 2013; Reeks et al. 2013). The Cas6 protein then transports the mature crRNA to a complex of Cas proteins called Cascade (CRISPR-associated complex for antiviral defense), which functions in interference, in some cases remaining attached to the crRNA and becoming a part of the interference complex (Brouns et al. 2008; Carte et al. 2008; Haurwitz et al. 2010, 2012; Hatoum-Aslan et al. 2011; Jore et al. 2011; Wang et al. 2011; Sternberg et al. 2012; Niewoehner et al. 2014). Type I systems form an interference complex of four to five distinct Cas proteins, each with different stoichiometry (Brouns et al. 2008; Jore et al. 2011; Sashital et al. 2011; Nam et al. 2012; van Duijn et al. 2012). Cryoelectron microscopy (CryoEM) structures of this complex indicate that six copies of Cas7, a protein with a ferredoxin fold that resembles an RNA Recognition Motif, form an RNA-binding ridge (Wiedenheft et al. 2011a, b). This ridge binds the crRNA, which is anchored by other Cas proteins on either end of the Cas7 multimer (Lintner et al. 2011). When the crRNA binds the target DNA, conformational changes result in the recruitment of the Cas3 endonuclease, which mediates target degradation and is the defining Cas protein of Type I systems (Jore et al. 2011; Wiedenheft et al. 2011b; Westra et al. 2012).

Like the Type I systems, Type III systems also use Cas6 for crRNA processing and form multiprotein complexes for target interference (Reeks et al. 2013). However, the Cas proteins in the Type III complexes are different (Spilman et al. 2013; Staals et al. 2013). Cas10 is a component of Type III interference complexes and is the defining Cas protein of these systems, although its function has not been fully elucidated (Makarova et al. 2011). CryoEM structures of Type III systems show that the crRNA is positioned along a backbone of a Cas protein complex consisting of repeat units of Csm3 (III-A) or Cmr4 (III-B), much like the Cas7 repeats in Type I systems (Zhang et al. 2012; Hrle et al. 2013; Rouillon et al. 2013; Spilman et al. 2013; Staals et al. 2013, 2014). Interestingly, both Type III-A and III-B systems are capable of targeting DNA and RNA (Hale et al. 2009; Peng et al. 2015; Samai et al. 2015). In Type III-A systems, degradation of DNA requires Cas10 and cleavage occurs directly adjacent to the 3′ end of the bound crRNA (Samai et al. 2015). Degradation of RNA targets by Type III-A systems occurs in even, 6-nucleotide intervals via the Csm3 active site, with each identical subunit in the backbone individually cleaving the target to collectively fragment the invading nucleic acid into consistent and precisely sized sequences (Staals et al. 2013; Samai et al. 2015). It is likely that the backbone repeat of Cmr4 in Type III-B systems has a similar mechanism of target cleavage (Staals et al. 2013; van der Oost et al. 2014).

Specificity of the crRNA for the target is enhanced through distinct mechanisms in different systems to avoid off-target effects that could occur because of binding of fully or partially complementary sequences, as mistargeting of the host chromosome is likely lethal to the bacteria. Types I and II systems improve specificity through recognition of a specific nucleotide sequence adjacent to the target but on the complementary strand of DNA, called the PAM (protospacer adjacent motif) (Bolotin et al. 2005; Deveau et al. 2008; Mojica et al. 2009; Marraffini and Sontheimer 2010). PAM recognition facilitates Cas interference complex binding, DNA melting, and RNA:DNA heteroduplex formation (described in detail below for Type II systems) and prevents self-targeting of similar or identical sequences lacking a PAM (Marraffini and Sontheimer 2010). Interestingly, some Type III-A systems may avoid cleavage of sequences incorporated into the host genome through a unique transcription-dependent DNA targeting mechanism that enables tolerance of lysogenic phages while preventing lytic phage production (Goldberg et al. 2014).

Cas9-MEDIATED crRNA MATURATION

In contrast to Type I and III systems, Type II systems require a single Cas protein, the Cas9 endonuclease, to mediate crRNA maturation (Deltcheva et al. 2011). The CRISPR array is first transcribed

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as a single, long transcript. Subsequently, this pre-crRNA transcript is processed into individual crRNAs, each specific for a different target (Fig. 1E,F). A single, matured, spacer-specific crRNA is then complexed with Cas9 as well as the *trans*-activating crRNA (tracrRNA), a small RNA encoded within the CRISPR–Cas locus, and unique to Type II systems. The tracrRNA contains multiple stem-loop structures and a sequence with partial complementarity to the CRISPR repeat sequence, allowing binding to the crRNA to facilitate maturation and complex formation with Cas9 (Deltcheva et al. 2011; Jinek et al. 2012; Chylinski et al. 2013, 2014; Fonfara et al. 2014). The dsRNA endonuclease, RNase III, which is typically encoded distal from the CRISPR locus, is also required for crRNA maturation (Deltcheva et al. 2011). RNase III recognizes the dsRNA structure created by the tracrRNA:crRNA duplex and cleaves both strands of RNA within the double-stranded repeat region (Deltcheva et al. 2011). The tracrRNA:crRNA duplex binds tightly to Cas9 and undergoes additional processing through an unknown mechanism that likely involves additional bacterial RNases (Deltcheva et al. 2011). The dual RNA:Cas9 complex is then able to identify and cleave targets with sequence complementarity to the crRNA spacer (Fig. 1G,H; Deltcheva et al. 2011; Gasiunas et al. 2012; Jinek et al. 2012; Chylinski et al. 2013; Fonfara et al. 2014). In some Type II systems, notably that encoded by the pathogen *Neisseria meningitidis*, maturation of the crRNAs is independent of RNase III and tracrRNA (Zhang et al. 2013). In this case, internal promoter sequences within each repeat sequence allow for transcription of individual crRNAs. These crRNAs still require tracrRNA to associate with Cas9, highlighting the importance of the RNA duplex for interactions with this protein (Zhang et al. 2013).

TARGET INTERFERENCE BY Cas9

The mechanism of target interference by Type II CRISPR–Cas systems has been well established and sophisticatedly elucidated, greatly informed by the solving of the crystal structures of Cas9 alone and bound to DNA and RNA (Deltcheva et al. 2011; Gasiunas et al. 2012; Jinek et al. 2012, 2014; Fonfara et al. 2014; Nishimasu et al. 2014). Similar to its role in crRNA maturation, Cas9 is the sole Type II Cas protein involved in target surveillance and interference (Deltcheva et al. 2011; Jinek et al. 2012).

Cas9 has a two-lobed morphology, with a larger α-helical lobe and smaller nuclease lobe that together form a clam-like shape with a central channel to position the target (Fig. 2A,B; Jinek et al. 2014; Nishimasu et al. 2014). Cas9 first binds the crRNA:tracrRNA duplex via a positively charged arginine-rich motif located on the inner surface of the α-helical lobe, where the two lobes come together at the end of the central cavity (Jinek et al. 2014; Nishimasu et al. 2014). Upon RNA binding, Cas9 undergoes a first conformational change to create the channel that positions the nucleic acids along the length of the protein, by rotating the nuclease lobe around the nucleic acid binding pocket of the α-helical lobe (Jinek et al. 2014; Nishimasu et al. 2014). This reorients the endonuclease domains to either side of the channel, into a favorable conformation for subsequent target cleavage (Figs. 1G,H and 2B,C) (Jinek et al. 2014; Nishimasu et al. 2014).

Cas9 must then scan DNA to identify target sequences with a high degree of accuracy so as not to target its own chromosome. This is partially accomplished by the requirement for the PAM motif (typically ~3 bp) adjacent to the targeted region on the target DNA (Figs. 1 and 2B,C) (Gasiunas et al. 2012; Jinek et al. 2012; Fonfara et al. 2014). Cas9 associates and dissociates randomly along a DNA strand until encountering a PAM sequence (Sternberg et al. 2014). Subsequently, the PAM-interacting domain of Cas9 (located in the carboxyl terminus) binds tightly to the target DNA through two binding loops that interact with the major and minor grooves of the PAM (Jinek et al. 2014; Nishimasu et al. 2014). Cas9 then undergoes a second conformational change, locking the DNA target into place along the length of the central cavity between the two lobes (Jinek et al. 2014; Nishimasu et al. 2014). Interaction with the PAM leads to destabilization of adjacent double-stranded DNA and orients the target sequence to facilitate binding to the seed region of the crRNA (Jinek et al. 2014; Nishimasu et al. 2014). If the target sequence has near-perfect complementarity in the PAM-proximal

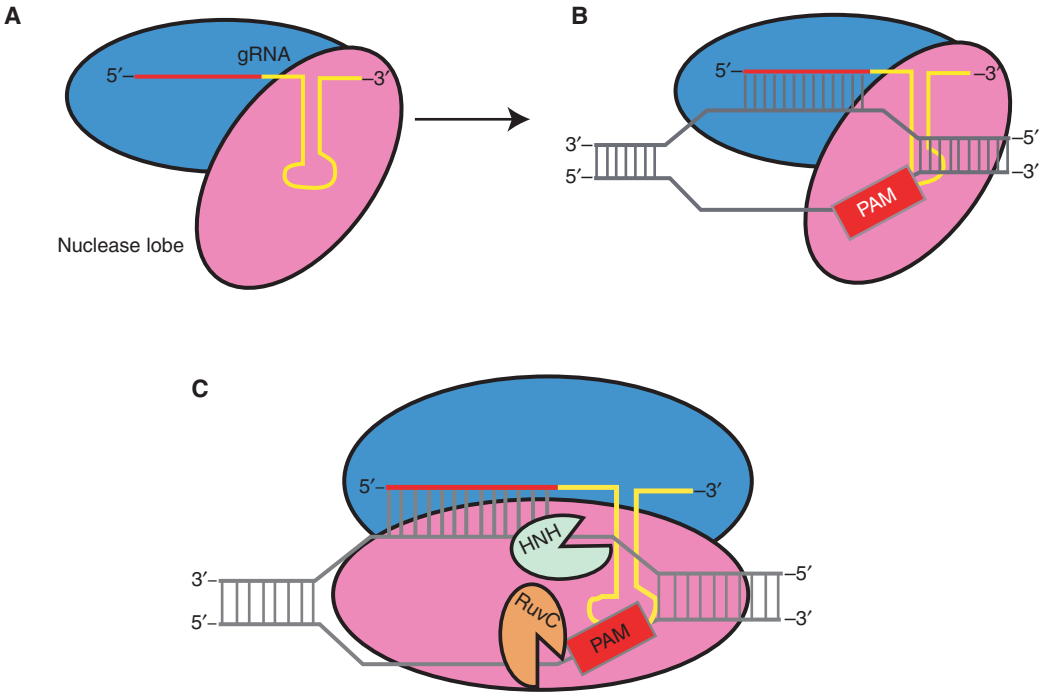

FIGURE 2. Schematic of Cas9:gRNA interactions. (*A*) Upon association with a chimeric gRNA, consisting of an ssRNA targeting region similar to the crRNA (red) and a dsRNA structure similar to that created by the crRNA:tracrRNA complex (yellow), the α-helical lobe (blue) and the nuclease lobe (pink) of Cas9 are opened into a conformation that reveals a channel for DNA targets to bind. (*B*) When DNA containing a PAM sequence is identified by Cas9, and the targeting sequence of the gRNA (red) has significant sequence complementarity to the immediately adjacent DNA sequence, the DNA is melted and unwound, generating a DNA:RNA hybrid. (*C*) Cas9 then undergoes a conformational change, clamping its nuclease lobe across the targeted DNA and positioning each strand into the HNH and RuvC active sites of the nuclease lobe. The HNH and RuvC endonuclease domains then cleave the complementary and noncomplementary strands, respectively, resulting in a double-strand break in the target immediately adjacent to the PAM.

region of the spacer, melting along the DNA will occur as one strand of the target base pairs along the remainder of the complementary spacer, forming an RNA:DNA heteroduplex (Anders et al. 2014; Jinek et al. 2014; Nishimasu et al. 2014). This results in separation of the two DNA strands into distinct, metal ion-dependent endonuclease active sites (Jinek et al. 2014; Nishimasu et al. 2014).

The HNH endonuclease domain cleaves the DNA strand bound to the RNA three nucleotides upstream of the PAM, whereas the noncomplementary strand is also bound by the nuclease lobe of Cas9 but cleaved by a separate RuvC domain (Jinek et al. 2012, 2014; Nishimasu et al. 2014). These active sites preferentially use magnesium as a divalent ion but can tolerate manganese (although with a lower cleavage efficiency), whereas calcium inhibits activity (Jinek et al. 2012; Anders et al. 2014). Interestingly, recent in vitro kinetic studies suggest that Cas9 is a single turnover enzyme that remains bound to the DNA target following cleavage, and the fate of Cas9 that has completed cleavage is currently unknown (Sternberg et al. 2014).

SPACER ACQUISITION IN Cas9-DEPENDENT CRISPR–Cas SYSTEMS

Adaptation, through the acquisition of new spacers into the CRISPR array, is the least understood stage of canonical CRISPR–Cas function. In Type II-A systems, all components of the CRISPR–Cas system form a complex that is required for adaptation (Cas1, Cas2, Cas9, Csn2, and tracrRNA) (Heler et al. 2015; Wei et al. 2015). A similar mechanism is likely used by other Type II subtypes that contain these components, excluding Csn2, which is absent from Type II-C and is replaced by Cas4 in Type II-

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B subtypes (Chylinski et al. 2013, 2014). Both Csn2 and Cas4 resemble RecB-like nucleases and may therefore play a similar role in adaptation, although their precise functions are not known (van der Oost et al. 2014). Csn2 and Cas4, as well as Cas1 and Cas2, are all dispensable for crRNA processing and target interference in Type II CRISPR–Cas systems (Deltcheva et al. 2011; Jinek et al. 2012). Interestingly, the Cas1 proteins present in Type II CRISPR–Cas systems cluster phylogenetically with those of Type I systems (Chylinski et al. 2014). This may indicate that the distinct functions of Type II systems arose via recombination events with Cas9 and other types of CRISPR–Cas systems, such as the Type I system (Chylinski et al. 2014).

Upon invasion by a foreign nucleic acid, CRISPR–Cas systems must select spacer sequences in a manner that prevents autoimmunity (Stern et al. 2010; Heler et al. 2015). Type II systems accomplish this by requiring a specific PAM sequence adjacent to the one that will ultimately be integrated as the spacer (i.e., the protospacer) (Díez-Villaseñor et al. 2013; Nunez et al. 2014; Heler et al. 2015). In Type II-A systems, Cas9, in complex with Cas1, Cas2, and Csn1 and bound to tracrRNA, identifies PAMs on the invading DNA to facilitate spacer selection using the PAM-interacting domain (Jinek et al. 2014; Nishimasu et al. 2014; Heler et al. 2015; Wei et al. 2015). There may be additional requirements for the selection of the spacer sequence, as there is an enrichment for certain spacer sequences that cannot be accounted for by the sequence of the PAM alone; however, these requirements have yet to be identified (Heler et al. 2014).

Mutations in the PAM-interacting domain of Cas9 do not prevent spacer acquisition but instead result in incorporation of spacers that are not adjacent to a PAM in the target (Heler et al. 2015). The endonuclease activity of Cas9 is dispensable for acquisition, suggesting that the role for Cas9 is to select spacers by binding to the PAM and protospacer sequence, whereas Cas1 (whose nonspecific nuclease activity is required for adaptation) of the associated Cas1–Cas2–Csn1 complex cleaves the adjacent sequence, yielding a precisely selected spacer sequence (Heler et al. 2015). There are many unknowns in the mechanism of adaptation, but a general model has been developed (Fig. 1A–D; Heler et al. 2014, 2015; Nunez et al. 2014, 2015). Cas1–Cas2 together interact with the secondary structures of the CRISPR repeat sequences within the array, preferentially near the leader sequence, which also acts as a promoter (Nunez et al. 2014, 2015). A repeat sequence within the chromosomal array is then nicked at the 3′ end, allowing for ligation of the free hydroxyl to the spacer fragment (Nunez et al. 2015). The spacer is inserted into the array, flanked by the single complementary strands of the first CRISPR repeat (Nunez et al. 2015). These are repaired into double-stranded repeats by DNA polymerase, resulting in a new repeat-flanked spacer in the chromosome, to be transcribed and processed into a crRNA that can protect against future invasion by complementary, PAM-flanked sequences (Nunez et al. 2015).

ALTERNATIVE FUNCTIONS OF Cas9 IN BACTERIAL PHYSIOLOGY

Although CRISPR–Cas systems have been very well established to promote prokaryotic defense against foreign nucleic acids, there is increasing evidence that these systems, and Cas9 in particular, play important roles in bacterial physiology (Bikard and Marraffini 2013; Westra et al. 2014; Barrangou 2015; Ratner et al. 2015). These additional Cas9-mediated functions include endogenous gene regulation and facilitate the strengthening of envelope structure, resistance to antibiotics and ultimately allow certain bacterial pathogens to dampen host immune activation (Bikard and Marraffini 2013; Westra et al. 2014; Barrangou 2015; Ratner et al. 2015).

Some alternative Cas9 functions have been revealed through the study of the intracellular pathogen *Francisella novicida* (Sampson and Weiss 2013). Using a regulatory axis comprised of Cas9, tracrRNA, and a unique small RNA encoded adjacent to the CRISPR array, the scaRNA (small, CRISPR–Cas associated RNA), *F. novicida* represses the production of a specific endogenous bacterial lipoprotein (BLP) (Sampson et al. 2013; Chylinski et al. 2014). Repression of this BLP by the *F. novicida* Cas9 regulatory axis allows the bacterial cell to strengthen the integrity of its envelope, decreasing envelope permeability and promoting resistance to certain antibiotics (Sampson et al.

2014). Furthermore, because BLPs are recognized by the host innate immune receptor, Toll-like receptor 2 (TLR2), repression of BLP allows *F. novicida* to dampen the activation of TLR2 and prevent inflammatory immune signaling, ultimately promoting survival and replication in the host (Jones et al. 2012; Sampson et al. 2013, 2014). The precise mechanism of Cas9-mediated gene repression in this system is unknown. In the absence of Cas9, tracrRNA, or the scaRNA, levels of the BLP transcript are drastically increased, as these components together act to decrease the stability of the mRNA (Sampson et al. 2013). Interestingly, the catalytic residues within Cas9 that are involved in DNA cleavage are not essential to maintain low levels of BLP transcript, most likely suggesting that stability is altered by currently unidentified accessory RNases (Heidrich and Vogel 2013; Sampson et al. 2013).

F. novicida is not the only bacterium known to use Cas9 in a fashion distinct from defense against invading nucleic acid. Cas9 encoded by *Neisseria meningitidis* is necessary for attachment, entry, and intracellular survival of the bacteria in human epithelial cells (Sampson et al. 2013). *Campylobacter jejuni* also uses Cas9 for attachment and invasion of epithelial cells (Louwen et al. 2013). In the absence of Cas9, *C. jejuni* displays increased envelope permeability, antibiotic susceptibility, and surface antibody binding, which may suggest that Cas9 acts to regulate components of the *C. jejuni* envelope (Louwen et al. 2013; Sampson et al. 2014). Numerous other examples of alternative CRISPR–Cas activities that do not use Cas9 have been observed and are growing in number. Some notable examples include biofilm formation in *Pseudomonas aeruginosa* (Zegans et al. 2009; Cady and O'Toole 2011), fruiting body formation in *Myxococcus xanthus* (Viswanathan et al. 2007; Wallace et al. 2014), intra-amoeba survival in *Legionella pneumophila* (Gunderson and Cianciotto 2013; Gunderson et al. 2015), colonization of nematodes by *Xenorhabdus nematophila* (Veesenmeyer et al. 2014), and many others. However, as these systems do not use Cas9, they are beyond the scope of this discussion and have been reviewed extensively elsewhere (Bikard and Marraffini 2013; Westra et al. 2014; Barrangou 2015; Ratner et al. 2015). Nonetheless, such examples of moonlighting functions of CRISPR–Cas systems in bacterial physiology may provide the framework to understand the evolution of these systems as well as how they may be further used and exploited for biotechnological purposes.

USE OF Cas9 FOR GENOME ENGINEERING

The insights into the mechanism of Cas9 function led to the hypothesis that the spacer sequence of the crRNA targeting region could be reprogrammed such that this machinery would mediate target cleavage at sites of interest. This activity was subsequently shown, in vitro, only 5 years after the first functional description of these systems (Barrangou et al. 2007; Jinek et al. 2012). It was clearly shown that synthetic crRNAs could be produced that were capable of hybridizing to DNA sequences of interest, allowing *Streptococcus pyogenes* Cas9 to catalyze a double-stranded DNA break at that site (Jinek et al. 2012). Although still exceedingly less complex than synthetically engineering other site-specific nucleases, such as zinc finger nucleases or TALENs (transcription-like effector nucleases), the expression of two separate small RNAs nonetheless represented added difficulty. The requirement for tracrRNA was relieved and the system simplified even further with the engineering of a synthetic, double-stranded targeting RNA (a guide RNA, or gRNA) (Jinek et al. 2012). (See Introduction: **Guide RNAs: A Glimpse at the Sequences that Drive CRISPR–Cas Systems** [Briner and Barrangou 2016].) The gRNA retains the double-stranded sequence and structural elements of the tracrRNA:crRNA duplex that are necessary for interaction with Cas9 but is transcribed as a single RNA (Jinek et al. 2012; Chylinski et al. 2013). This chimeric RNA therefore does not require RNase III processing. The spacer sequence, which directs Cas9 targeting, can easily be modified, facilitating reprogramming against diverse targets (Deltcheva et al. 2011; Jinek et al. 2012). The generation of the gRNA significantly increased the ease of engineering new targeting sequences and, together with the elucidation of Cas9 activity, helped pave the way for a revolutionary, highly cost-effective, and efficient method of genome engineering.

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These developments have now sprung the so-called CRISPR craze of Cas9-mediated genome engineering in many systems, both prokaryotic and eukaryotic. Cas9 from multiple bacterial species (including *S. pyogenes*, *S. thermophilus*, *Staphylococcus aureus*, *N. meningitidis*, and *Treponema denticola*) have been successfully used to edit the genomes of cells from diverse organisms including the human (discussed in Protocol: **Protocol for Genome Editing in Human Pluripotent Stem Cells** [Smith et al. 2016]), bacteria, yeast (discussed in Protocol: **CRISPR–Cas9 Genome Engineering in *Saccharomyces cerevisiae* Cells** [Ryan et al. 2016]), nematode, plants, fruitfly (discussed in Introduction: **Cas9-Mediated Genome Engineering in *Drosophila melanogaster*** [Housden and Perrimon 2016]), zebrafish (discussed in Protocol: **Optimized CRISPR–Cas9 System for Genome Editing in Zebrafish** [Vejnar et al. 2016]), salamander, frog, and rodent (discussed in Protocol: **Protocol for the Generation of Genetically Modified Mice Using the CRISPR–Cas9 Genome-Editing System** [Henao-Mejia et al. 2016]), with target modification efficiencies reported up to 80% (Jinek et al. 2012, 2013; Belhaj et al. 2013; Cho et al. 2013; Cong et al. 2013; DiCarlo et al. 2013; Gratz et al. 2013; Hou et al. 2013; Hwang et al. 2013; Jiang et al. 2013; Lo et al. 2013; Nakayama et al. 2013; Nekrasov et al. 2013; Ren et al. 2013; Wang et al. 2013; Yu et al. 2013; Flowers et al. 2014; Ryan and Cate 2014; Ran et al. 2015). Such rapid utilization across these varied systems serves to highlight the ease of use and portability of Cas9-based technologies.

In the simplest use of Cas9 genome editing, random mutations are introduced at the site of cleavage. Because Cas9 catalyzes a double-strand break at its cleavage site adjacent to the PAM, cells can undergo nonhomologous end joining (NHEJ) to repair the cleaved DNA (Cong et al. 2013; DiCarlo et al. 2013; Jinek et al. 2013). With varying efficacies based on the cellular repair machineries, NHEJ can restore the cleaved sequence to the original, but it can also result in the loss or addition of nucleotides (Cong et al. 2013; DiCarlo et al. 2013; Jinek et al. 2013). The majority of mutations that are generated following Cas9-mediated cleavage are either single-base insertions or deletions or nine-base deletions (Cradick et al. 2013). Such NHEJ-mediated repair can therefore result in early stop codons or other frameshift mutations that can cause loss of function of the targeted gene. Ultimately, this can provide a quick and simple method to generate null mutations in genes of interest.

An alternative repair pathway to NHEJ can also occur within the cell, termed homology-directed repair (HDR). HDR transpires when DNA containing sequence homology with the region surrounding the cleavage site is used as a template for homologous recombination. By introducing linear or circular DNA containing a sequence of interest (such as a selectable or nonselectable marker) flanked by regions homologous to those adjacent to the Cas9 cleavage site, integration of this donor construct can occur by HDR. This allows Cas9 to effectively generate desired insertions of DNA into sequence-specific sites of interest (Cong et al. 2013; DiCarlo et al. 2013). (The detection of HDR events is discussed in Introduction: **Detecting Single-Nucleotide Substitutions Induced by Genome Editing** [Miyaoka et al. 2016].) Furthermore, to increase the likelihood of HDR and limit the chances of NHEJ, a partially mutated Cas9 protein can be used. Engineered point mutations in either one of the two Cas9 endonuclease domains (RuvC or HNH) results in a protein that is capable of only cleaving a single strand of its DNA target (Jinek et al. 2012; Cong et al. 2013; DiCarlo et al. 2013). This decreases the frequency of NHEJ repair, and in the presence of a donor construct, these single-strand nicks are preferentially repaired by HDR. To further increase the rate of HDR, NHEJ can be inhibited (Chu et al. 2015; Maruyama et al. 2015). This has successfully been accomplished by either transcriptionally silencing the NHEJ machinery or through a small molecule inhibitor of the NHEJ polymerase (DNA Pol IV). By blocking NHEJ, HDR repair rates have been increased by four- to 19-fold, facilitating much more efficient integration of desired sequences into targeted sites (Chu et al. 2015; Maruyama et al. 2015).

The ability to easily target Cas9 to diverse sequences within the same cell allows large-scale screens of genetic knockouts to be performed (a process described in Introduction: **Large-Scale Single Guide RNA Library Construction and Use for Genetic Screens** [Wang et al. 2016]), a method previously relegated to the world of prokaryotic genetics. Recent studies have used pools of more than 70,000 gRNAs in both positive and negative screens (Bell et al. 2014; Shalem et al. 2014; Wang et al. 2014; Yin et al. 2014; Zhou et al. 2014). Cas9-based screens allow genes to be fully inactivated, not only repressed

as occurs during canonical RNA interference-based screens. This loss-of-function method may allow the identification of genes that maintain functional roles even when repressed to very low expression levels through RNAi methods. Cas9 deletion screens, therefore, will potentially uncover previously masked functions of critical genes.

Although the ability of Cas9 to catalyze sequence-specific DNA breaks has revolutionized the introduction of insertions and deletions into DNA, a number of other technologies have been invented that exploit Cas9's ability to bind and strongly associate with desired DNA sequences. Cas9 can be engineered to be completely catalytically inactive through alanine substitutions in both the RuvC and HNH domains, resulting in a variant termed nuclease-deficient Cas9, or dCas9 (Jinek et al. 2012; Jiang et al. 2013; Qi et al. 2013). dCas9 binds targeted DNA sequences as specified by the gRNA, but rather than cleaving the target, instead prevents transcription by blocking the binding or elongation of RNA polymerase (Jiang et al. 2013; Qi et al. 2013); see Protocol: **CRISPR Technology for Genome Activation and Repression in Mammalian Cells** (Du and Qi 2016). The level of transcriptional inhibition, or CRISPR interference (CRISPRi), can be tuned with different strategies to titrate the expression level of a transcript. Simultaneously targeting dCas9 to multiple sites in the same gene increases repression, as does increasing the proximity of dCas9 binding to the promoter (Jiang et al. 2013; Qi et al. 2013). Whereas repression can occur via dCas9 alone, this protein can be tethered to other proteins and molecules to facilitate increased efficacy or perform other actions at discrete sites in a genome. Fusion of dCas9 to the KRAB or SID4X repressors in eukaryotic systems can increase targeted repression (Jiang et al. 2013; Konermann et al. 2013; Perez-Pinera et al. 2013; Qi et al. 2013). Similar to transcriptional repression, dCas9 can also be fused to a transcriptional activator, such as VP64 in eukaryotic systems or the omega subunit of RNA polymerase in prokaryotic systems (Cheng et al. 2013; Jiang et al. 2013; Mali et al. 2013; Perez-Pinera et al. 2013; Qi et al. 2013). When guided to a promoter, these dCas9-activator fusions can efficiently recruit RNA polymerase and activate transcription of genes of interest.

The programmable DNA binding activity of dCas9 has been exploited even further. For instance, a fluorescently tagged dCas9 can be guided to specific genetic loci in live cells, allowing the spatiotemporal dynamics of specific sequences within the chromatin to be observed (Chen et al. 2013). Additionally, dCas9 has also been used to purify specific DNA sequences from live cells, in an enhanced form of chromatin immunoprecipitation (enCHiP) (Fujita and Fujii 2015). Cas9-mediated enCHiP has allowed the identification of previously unknown proteins that associate with specific DNA sequences in mammalian chromosomes (Fujita and Fujii 2013, 2014). Furthermore, fusion of dCas9 to the human acetyltransferase p300 allows the site-specific acetylation of histone H3 on lysine 27 (Hilton et al. 2015). This facilitates the activation of genes at enhancer sites distal to the targeted gene and also allows heritable epigenetic changes to be passed into a population (Hilton et al. 2015). Future Cas9 technologies may use other effector proteins to drive sequence-specific epigenetic modifications, such as DNA and/or histone methylation.

One of the most powerful attributes of the Cas9 system is the ability to be multiplexed to distinct targets within the same cell (Cheng et al. 2013; Cong et al. 2013; Ryan and Cate 2014); see Introduction: **Characterization of Cas9–Guide RNA Orthologs** (Braff et al. 2016). In fact, the simultaneous utilization of Cas9 orthologs from distinct species has allowed the generation of mutations, as well as transcriptional activation and repression to occur within the same cell (Esvelt et al. 2013). Such methods lay the foundation for the engineering of incredibly detailed genetic circuits or to intricately probe genetic networks. In theory, the multiplexing capacity of Cas9 could be used to generate double- and triple-mutant libraries, facilitating the study of redundant systems and more easily exploring complex genetic circuits.

Despite the unprecedented utility and efficiency of the Cas9-dependent tools that have been created, one nontrivial challenge facing these technologies is off-target effects. Outside of a seed sequence located up to 12 bases proximal to the PAM, Cas9 can tolerate a range of mismatches, allowing it to bind and cleave sequences that are not the exact target (Jinek et al. 2012; Cradick et al. 2013; Pattanayak et al. 2013; Lin et al. 2014b). To prevent nontarget interactions, a number of databases have been developed (such as E-Crisp, Off-Spotter, and CRISPRdirect) that allow researchers to design gRNAs with op-

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timized targeting and few to no off-target possibilities (Heigwer et al. 2014; Naito et al. 2015; Pliatsika and Rigoutsos 2015). However, such optimized Cas9 targeting can still be somewhat imperfect.

One method to drastically reduce off-target effects involves guiding Cas9 nickases to offset sites on the opposite strands, flanking the target, and creating a pair of ssDNA nicks (Mali et al. 2013; Ran et al. 2013). In conjunction with a donor construct containing homology with the sequences adjacent to those that have been cleaved, this method allows very high specificity of gene replacement at the site flanked by the offset nicks. Off-target effects are significantly limited, as the likelihood of nicked pairs at sites other than the desired sequence is extremely low (Mali et al. 2013; Ran et al. 2013). Furthermore, ssDNA nicks are easily repaired by the cell with almost undetectable levels of mutation. Thus, even if a single Cas9 nickase cleaves an off target site, the likelihood of a detrimental effect is limited. Cas9 has also been recently engineered to contain a photocaged lysine, rendering the protein catalytically inactive until stimulated with UV light, allowing it to become active and capable of cleaving DNA targets (Hemphill et al. 2015). Although still in infancy, such approaches will allow a fine-tuning of the regulation of Cas9 catalytic activity. These methods to overcome the potential off-target and other undesired effects of Cas9 will greatly increase the utility and acceptance of this technology, not only in a research setting, but also in therapeutic and clinical applications.

Cas9 technologies hold promise for use in mediating gene therapy, although numerous significant hurdles and questions remain. Although delivery (described in Protocol: **Adeno-Associated Virus–Mediated Delivery of CRISPR–Cas Systems for Genome Engineering in Mammalian Cells** [Gaj and Schaffer 2016]) is a major roadblock, supplying Cas9, specific gRNAs, and repair constructs may allow the treatment of defined genetic disorders, by introducing or removing genetic information. Although large in size, Cas9 may be packaged into adeno- and lentiviral vectors (Shalem et al. 2014; Ran et al. 2015), but recent studies have also showed that Cas9 in complex with gRNAs can enter cells directly using lipid-based transfection techniques, fusion to cell-penetrating peptides, and nanoparticle delivery (Ramakrishna et al. 2014; McNeer et al. 2015; Zuris et al. 2015). Furthermore, the study of various Cas9 variants from different species may reveal a minimally sized Cas9 enzyme that retains programmable DNA binding and cleavage function (Jinek et al. 2014; Ran et al. 2015). An additional layer of security in delivery has also been successfully used whereby Cas9 is controlled by cell-specific promoters, allowing its activity to be limited to very specific cell types, such as neurons (Swiech et al. 2015). Further approaches using optogenetics have allowed the regulation of dCas9-mediated gene activation only in response to light stimulation (Konermann et al. 2013; Hemphill et al. 2015; Nihongaki et al. 2015).

The pathway toward translational uses of Cas9-directed repair has been exemplified recently in a number of systems. For instance, a common mutation in the CFTR locus that contributes to cystic fibrosis was repaired by Cas9 in primary human intestinal cells (Schwank et al. 2013). Similarly, in human induced pluripotent stem cells (iPSCs) derived from a myeloproliferative neoplasm, Cas9 was used to repair the oncogenic mutation (Smith et al. 2015), and mutations in the *crygc* gene that is responsible for cataracts were repaired in mouse zygotes and spermatogonial stem cells (Ren et al. 2013; Wu et al. 2015). Additionally, HIV proviruses have been removed from infected cells using Cas9-directed cleavage, and hepatitis B and hepatitis C viruses have been targeted, perhaps providing a framework for future antiviral therapeutics (Hu et al. 2014; Lin et al. 2014a; Kennedy et al. 2015; Liao et al. 2015; Price et al. 2015). Such repair has not been limited to tissue culture studies ex vivo. In mice (Ren et al. 2013), the *Fah* mutation, which induces tyrosinemia, and recently a *cftr* mutation in a mouse model of cystic fibrosis were both successfully corrected through Cas9-mediated repair (Yin et al. 2014; McNeer et al. 2015). Although proofs of concept, these groundbreaking studies highlight the therapeutic potential of emerging Cas9 technologies in treating genetic disorders.

FUTURE DIRECTIONS

From their first identification as unique genetic elements to the elucidation of their function as a prokaryotic adaptive immune system, CRISPR–Cas systems have been one of the most exciting fields

in biology. Being able to exploit these systems for biotechnological purposes serves to emphasize the power that the study of seemingly "basic" biological mechanisms can have on extremely far reaching biotechnological and clinical applications. Already, Cas9-mediated engineering has been used throughout multiple fields and is rapidly changing the face of eukaryotic genetics.

Continued study of natural CRISPR–Cas systems, both in their canonical function as restriction systems against nucleic acids and in their alternative roles in bacterial physiology, will provide further insights into how these systems can be exploited for bioengineering applications. As more Cas9 orthologs are analyzed, these variants will allow researchers to further understand the structural and sequence requirements that determine PAM specificity, crRNA sequence requirements, and DNA binding stringency, allowing Cas9 proteins to be engineered for increased specificity and efficacy. Likewise, there remain large, unanswered questions in the field of CRISPR–Cas biology that will certainly lead to the development of even more tools for molecular biology. Already, other Cas proteins have been predicted to have diverse and conserved functions. For example, Cas1 and Cas2 have been proposed to act as a toxin–antitoxin system, becoming autotoxic in the presence of bacteriophage infections that are not successfully controlled by the canonical nucleic acid targeting activity of CRISPR–Cas systems, perhaps by cleaving endogenous mRNA (Makarova et al. 2012). This second line of defense would prevent bacteriophages from replicating and subsequently infecting other cells but, if true, could also form the platform for a Cas2-based RNA interference technology. At the same time, continued study of how Cas1 and Cas2 act to integrate new sequences into the bacterial chromosome may further allow the generation of new technologies that are more efficient at mediating site-directed DNA integration. Thus, as we learn more about the functions of diverse Cas proteins, we will greatly expand our ability to develop novel molecular tools for interrogation of pressing biological mysteries.

The power of proteins that can be programmed to recognize specific sequences of DNA is enormous. Given the ease and accessibility of the Cas9 system, incredible progress has been made in developing this system for a plethora of purposes that have already left their mark on numerous disciplines from molecular biology to translational medicine. Cas9 has shaped and will continue to shape modern biology now and for the foreseeable future. The technological possibilities of Cas9 are seemingly endless and limited only by our creativity and imagination.

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