Leading Edge
Review

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The Biology of CRISPR-Cas: Backward and Forward

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SUMMARY

In bacteria and archaea, clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins constitute an adaptive immune system against phages and other foreign genetic elements. Here, we review the biology of the diverse CRISPR-Cas systems and the major progress achieved in recent years in understanding the underlying mechanisms of the three stages of CRISPR-Cas immunity: adaptation, crRNA biogenesis, and interference. The ecology and regulation of CRISPR-Cas in the context of phage infection, the roles of these systems beyond immunity, and the open questions that propel the field forward are also discussed.

Introduction

More than a billion years of coevolution have produced a multitude of sophisticated evasion and defense strategies in prokaryotes and their viral predators. Suppression of bacteriophage adsorption, restriction modification of the invading phage genome, and abortive infection are among the well-characterized innate defense mechanisms against phage predation (Labrie et al., 2010). One of the most exciting discoveries in microbiology of the last decades was the revelation that prokaryotes also display adaptive, heritable immunity. Prokaryotic CRISPR-Cas adaptive immune systems store memory of past infections, and upon reinfection, deploy RNA-guided nucleases for sequence-specific silencing of phages and other mobile genetic elements (MGEs), such as plasmids and transposons.

The defining feature of these systems, which are found in 50% and nearly 90% of complete bacterial and archaeal genomes, respectively (Grissa et al., 2007; Makarova et al., 2015), is the CRISPR array. This genomic locus is composed of alternating identical repeats and unique spacers (Ishino et al., 1987; Jansen et al., 2002). A milestone in CRISPR research was the realization that the spacer sequences match plasmids and phage genomes, as it was the first hint that CRISPR-Cas might function as a prokaryotic defense mechanism (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005). The function of CRISPR-Cas as an adaptive immune system in which the CRISPR array serves as an archive of previous infections was ultimately demonstrated by the observation that phage challenge of *Streptococcus thermophilus* stimulates expansion of the CRISPR array by acquisition of phage-derived spacers that immunize against subsequent infection (Barrangou et al., 2007). The ability of the immune system to prevent infection by other MGEs was demonstrated shortly afterward by showing CRISPR-driven inhibition of plasmid conjugation and transformation in *Staphylococcus epidermidis* (Marraffini and Sontheimer, 2008).

Adjacent to the CRISPR array is a series of genes encoding the Cas proteins that drive the three phases of immunity: adaptation, CRISPR RNA (crRNA) biogenesis, and interference. During adaptation, foreign nucleic acids are selected, processed, and integrated into the CRISPR array to provide a memory of infection. Memory is retrieved when the CRISPR array is transcribed to produce a long precursor crRNA (pre-crRNA) that is processed within the repeat sequences to yield mature crRNAs. Upon subsequent infection, the interference machinery is guided by crRNAs to cleave complementary sequences, termed protospacers, in the foreign nucleic acids (Figure 1). By compromising the selfish, often hostile programs encoded by MGEs, CRISPR-Cas systems protect prokaryotes from succumbing to infection. According to the assortment of *cas* genes and the nature of the interference complex, CRISPR-Cas systems have been assigned to two classes, which are further subdivided into six types and several subtypes that each possess signature *cas* genes. Class 1 CRISPR-Cas systems (types I, III, and IV) employ multi-Cas protein complexes for interference, whereas in class 2 systems (types II, V, and VI), interference is accomplished by a single effector protein (Makarova et al., 2011, 2013, 2015; Shmakov et al., 2015).

CRISPR-Cas has garnered much attention in recent years as a genome-engineering tool that has revolutionized the life sciences and is recognized for its potentially transformative applications in biotechnology, agriculture, and medicine. The focus of this Review, however, is the biology of CRISPR-Cas systems and the recent progress in understanding the underlying mechanisms. In addition to outlining the common themes of CRISPR-Cas-mediated immunity across the various types and sub-types, we also highlight the diversity of proteins and mechanisms in these systems. Interference is presented first, followed by the preceding processes that license this final stage of immunity. The role of these immune systems in the ecology of phage-prokaryote interactions, and the strategies that phages have

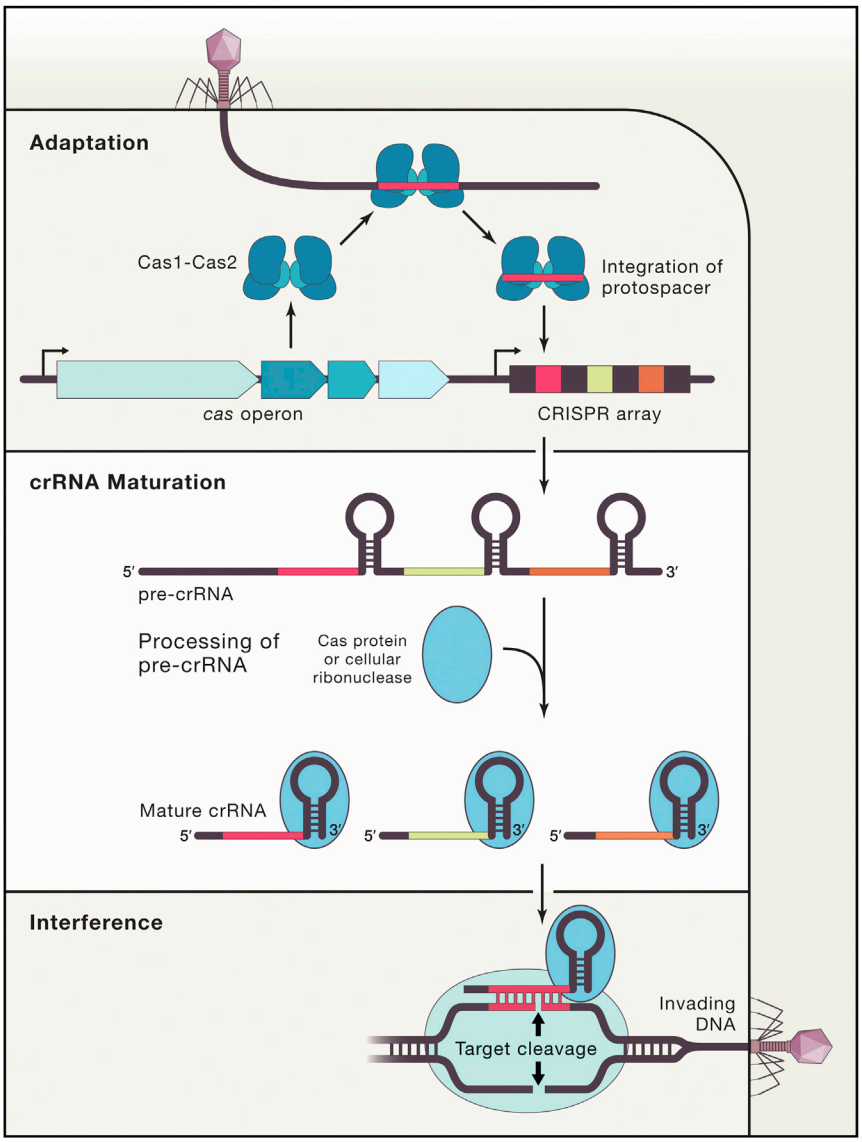


Figure 1. The Three Stages of CRISPR Immunity

During adaptation, the Cas1-Cas2 complex selects a part of the foreign DNA and integrates it into the host's CRISPR array. In the next stage (crRNA maturation), the CRISPR array is transcribed into a long pre-crRNA that is further processed by Cas proteins or, in some cases, by cellular RNases. In the interference stage, the mature crRNAs guide Cas nucleases to the cognate foreign DNA. The Cas proteins cleave the foreign nucleic acid upon binding of the crRNA to the target sequence. In class 1 systems, the interference machinery is a multi-Cas-protein complex, whereas class 2 systems utilize a single Cas protein for target cleavage.

evolved to counter CRISPR-Cas are addressed. We also discuss the regulation of CRISPR-Cas, the roles of these systems beyond immunity, and other emerging topics in the field.

Interference: Cleaving DNA and RNA Invaders

Sequence-specific destruction of invading MGEs is the basis for CRISPR-Cas defense. In the final stage of CRISPR-Cas-mediated immunity, mature crRNAs guide the interference machinery to cleave invading nucleic acids. In order to store the genetic information of a parasitic MGE, a part of the foreign DNA must be integrated in the genomic CRISPR locus of the host. This, however, raises an inherent problem for the interference machinery: the sole reliance on sequence complementarity between the crRNA and the target sequence would result in cleavage of the CRISPR array. Hence, nearly all characterized CRISPR-Cas systems (except type III) have evolved an authentication and discrimination mechanism that involves coordinated recognition of a short sequence, called the protospacer adjacent motif (PAM), by both the adaptation and interference machinery. The presence of a PAM proximal to the acquired spacer and targeted protospacer and its absence in the CRISPR array facilitates robust immunity while averting auto-immune targeting of the CRISPR array.

Interference in Class 1 CRISPR-Cas Systems

Type I

Type I systems are the most widespread CRISPR-Cas systems (Koonin et al., 2017; Makarova et al., 2015) and employ a crRNA-bound multiprotein complex termed CRISPR-associated complex for antiviral defense (Cascade) for target recognition, as well as the nuclease Cas3 for target cleavage (Figure 2) (Brouns et al., 2008). Cas3 is the hallmark protein of type I systems and is recruited upon target binding by Cascade to cleave the foreign DNA. Although the overall architecture of Cascade is conserved, its composition can vary between different subtypes and homology of the subunits has often been established on the basis of functional similarities rather than sequence similarities (for details, see Koonin et al., 2017; Makarova et al., 2015). Among the seven subtypes that have been identified to date (I-A to I-F and I-U) (Makarova et al., 2015), the I-E system of *Escherichia coli* is most thoroughly characterized and has the full complement of subunits that are found in type I systems, thus serving as a model for understanding type I interference.

Cascade of the type I-E CRISPR-Cas system has a molecular weight of 405 kDa and displays the following composition: $(Cas5e)_1$-$(Cas6e)_1$-$(Cas7e)_6$-$(Cas8e)_1$- $(Cas11e)_2$ (Brouns et al., 2008; Jore et al., 2011). According to the former nomenclature, Cas8e and Cas11 were known as Cse1 and Cse2, respectively. In almost all type I systems, pre-crRNA is processed by an RNase of the Cas6 family (or Cas5d in subtype I-C). In *E. coli*,

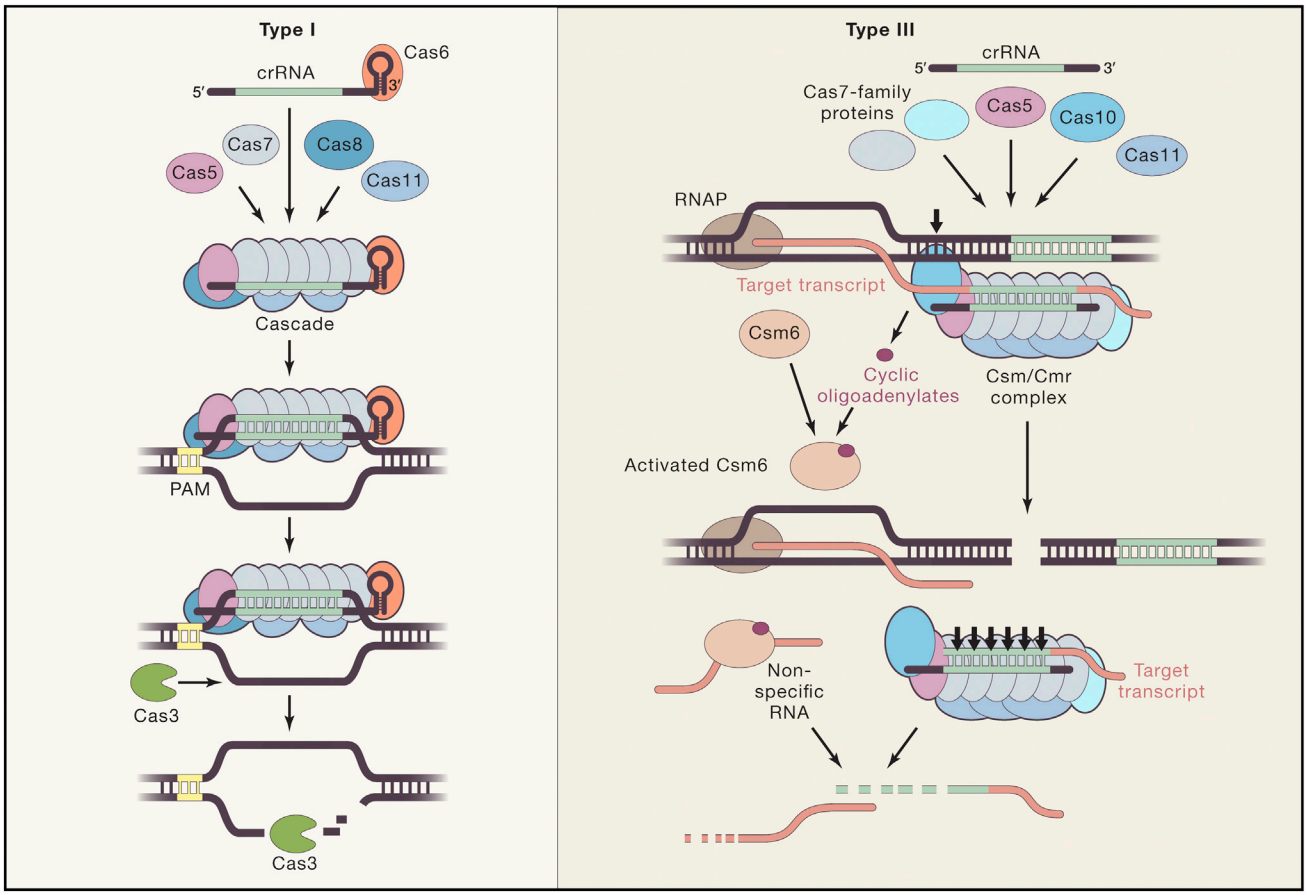


Figure 2. The Interference Pathways of Class 1 CRISPR-Cas Systems

Two general pathways for class 1 interference exist. In the first pathway, exemplified by the type I-E system of *E. coli*, crRNA bound by Cas6 serves as a scaffold for Cascade assembly. Cascade first recognizes the PAM (yellow) on the invader DNA. R-loop formation is induced when crRNA base pairs with the target strand of the DNA. The presence of the R-loop triggers the recruitment of the endonuclease Cas3, which initiates degradation of the non-target strand. Similar to type I systems, type III systems form multi-Cas-protein complexes for interference (Csm and Cmr for type III-A and type III-B, respectively) using the crRNA as a scaffold. Type III-A is shown here as an example. Unlike in type I, Cas6 of type III is not an integral part of the interference complex. The crRNA within the type III complexes binds to complementary regions in target RNA transcripts. Binding triggers a Cas10-mediated double-strand break within the corresponding template DNA, after which Cas7 (Csm3) cleaves the transcript RNA. Upon target binding, Cas10 also generates cyclic oligoadenylates, which activate the RNase Csm6 to degrade non-specific RNAs.

pre-crRNA processing by Cas6e yields 61-nt-long mature crRNAs that encompass the full spacer sequence, flanked on both sides by repeat portions (see crRNA Biogenesis: Generating Guides for Cas Proteins). Cas6e remains bound to the 3′ repeat portion of the crRNA after processing (Gesner et al., 2011; Sashital et al., 2011). Subsequently, Cascade assembles into a seahorse-like shape. The crRNA is an integral part of Cascade and is bound along the backbone of the complex and capped by Cas5e at the 5′ end (Jackson et al., 2014; Jore et al., 2011; Mulepati et al., 2014; Wiedenheft et al., 2011a; Zhao et al., 2014). The helical backbone is composed of six tightly connected Cas7e proteins that adopt hand-like shapes with the thumb domains responsible for tight connection of the subunits. Starting from the last nucleotide of the 5′ repeat handle, the thumbs of the subunits kink the crRNA at every sixth nucleotide. The five nucleotides in between are aligned along the palm domain to enable efficient base pairing of the crRNA with the target DNA (Jackson et al., 2014; Mulepati et al., 2014; Zhao et al., 2014). Cas11e and Cas8e are defined as the small and large subunits of Cascade, respectively. Two Cas11e subunits interact directly with the Cas7e backbone and form the "belly" of the complex; Cas8e interacts with Cas5e, Cas7e, and Cas11e and forms the tail (Jackson et al., 2014; Mulepati et al., 2014).

Recognition of the PAM in the double-stranded target DNA is mediated by the large subunit, which also initiates the local unwinding of DNA and the subsequent binding of crRNA to the cDNA strand of the protospacer (Hayes et al., 2016; Xiao et al., 2017a). Crucial for protospacer binding of the Cascade complex are the first eight PAM-proximal nucleotides of the crRNA (termed seed sequence), with the exception of the sixth nucleotide, which does not bind to the target. Mutations in the seed sequence greatly impair binding of Cascade to the target in *E. coli* (Semenova et al., 2011; Xiao et al., 2017a). The non-target strand is bound by two Cas11e subunits, leading to the formation and stabilization of the so-called R-loop structure, which is

accompanied by substantial conformational changes of the small and large subunits and thus allows recruitment of the nuclease Cas3 for target cleavage (Hayes et al., 2016; Jackson et al., 2014; Jore et al., 2011; Mulepati et al., 2014; Xiao et al., 2017a). The HD domain of Cas3 nicks the displaced non-target DNA strand, inducing structural changes in the protein that activate its ATP-dependent helicase activity. As a result, Cas3 translocates and successively degrades the non-target DNA strand in the 3′ to 5′ direction, leaving a single-stranded DNA (ssDNA) gap of roughly 200–300 nt in the target genome (Huo et al., 2014; Redding et al., 2015; Westra et al., 2012; Zhao et al., 2014). This, however, might be an intermediate degradation product, as partially ssDNA might not lead to full destruction of the invader. It is thought that the complete degradation of the target DNA is mediated either by other host nucleases or by the potent, Cascade-independent ssDNA nuclease activity of Cas3 that has been observed *in vitro* (Mulepati and Bailey, 2013; Redding et al., 2015; Sinkunas et al., 2013; Wright et al., 2016).

Although the overall structure of Cascade and involvement of Cas3 are conserved, there are several notable subtype-specific differences in the type I interference machinery. Several subtypes lack certain Cascade subunits found in type I-E. In fact, the subtypes I-A and I-E are the only systems that harbor a separate gene for the small subunit. In the other subtypes, the small subunit is either fused to or functionally replaced by Cas8 (Koonin et al., 2017; Richter et al., 2017). An even more minimal Cascade architecture is seen in type I-C, which lacks a Cas6 homolog (Hochstrasser et al., 2016; Nam et al., 2012), and type I-Fv (a variant of type I-F), where the large and small subunits are absent and functionally replaced by Cas5fv and Cas7fv (Gleditzsch et al., 2016; Pausch et al., 2017). An interesting variation in the overall shape of Cascade was found in type I-F, in which the backbone of the surveillance complex (known as Csy in this subtype) has a short helical pitch and almost forms a closed ring (Chowdhury et al., 2017; Peng et al., 2017; Wiedenheft et al., 2011b) but subsequently "unwinds" upon target DNA recognition (Guo et al., 2017). Although Cas3 is the signature protein of type I systems, fusion or fission of the *cas3* gene is seen in several subtypes (Koonin et al., 2017). Collectively, these studies suggest that there is significant genetic and functional plasticity in the components of the type I interference machinery but that the overall architecture and modules for crRNA binding and processing (Cas6 and/or Cas5), the backbone (Cas7), PAM-recognition and R-loop stabilization, and target cleavage (Cas3) are conserved. The seed sequence is crucial for type I-E and I-F interference (Semenova et al., 2011; Wiedenheft et al., 2011b; Xiao et al., 2017a) and is therefore likely another common feature among type I systems. High-resolution structures and detailed insight into target recognition and cleavage are still awaited for subtypes I-A, I-D and I-U. However, given the presence of genes encoding the core functional units of the interference machinery (Koonin et al., 2017; Makarova et al., 2015), it is likely that they follow the same principles that have been established by investigation of interference in the other type I systems.

Type III

Type III CRISPR-Cas systems employ Cascade-like complexes (termed Csm for III-A and Cmr for III-B) that display high similarity to type I effector complexes in their overall composition and structure (Figure 2) (Hochstrasser et al., 2014; Jackson et al., 2014; Makarova et al., 2011; Mulepati et al., 2014; Osawa et al., 2015; Staals et al., 2014; Taylor et al., 2015; Zhao et al., 2014). However, in contrast to other described interference mechanisms, type III systems target both RNA and DNA substrates. Here, DNA cleavage strictly depends on the transcription of the target sequence (Deng et al., 2013; Elmore et al., 2016; Estrella et al., 2016; Goldberg et al., 2014; Kazlauskiene et al., 2016; Samai et al., 2015). In the following, we will describe the type III-A and III-B complexes according to the new nomenclature presented by Koonin et al., (2017). The protein names according to the former nomenclature will be given in parentheses.

Similar to Cascade, the Csm and Cmr complexes assemble along the mature crRNA, which is bound by Cas5 (Csm4/Cmr3) at the 5′ repeat handle. The backbone is composed of Cas7-family proteins (Csm3 and Csm5 for type III-A/Cmr4, Cmr6, and Cmr1 for type III-B), while Cas11 (Csm2/Cmr5) and Cas10 are the small and large subunits, respectively (Mulepati et al., 2014; Osawa et al., 2015; Staals et al., 2014; Taylor et al., 2015). Target cleavage is initiated by binding of the type III effector complex to the nascent target transcript in a crRNA-dependent manner. The Cas7 subunits (Csm3/Cmr4) cleave the ssRNA at every sixth nucleotide. DNA cleavage is carried out by the palm domain of the Cas10 subunit and strictly requires transcription of the target in both type III systems (Osawa et al., 2015; Samai et al., 2015; Staals et al., 2014; Tamulaitis et al., 2014; Taylor et al., 2015). RNases belonging to the Csm6 or related Csx1 families are frequently associated with type III CRISPR-Cas systems (Koonin et al., 2017; Makarova et al., 2011). Both Csm6 and Csx1 non-specifically degrade foreign transcripts and have auxiliary or sometimes essential functions during type III interference even though they are not part of the effector complex (Deng et al., 2013; Hatoum-Aslan et al., 2013, 2014; Jiang et al., 2016b; Niewoehner and Jinek, 2016; Sheppard et al., 2016). Recently, it has been revealed that the Cas10 subunit of the Csm complex not only mediates target DNA cleavage, but also converts ATP into cyclic adenylates that act as second messengers to activate the Csm6 RNase. The production of the messenger by Cas10 depends on binding of the Csm complex to the target RNA and thus constitutes a regulatory mechanism that triggers robust interference in the presence of an invader (Kazlauskiene et al., 2017; Niewoehner et al., 2017).

In most type III systems, binding of the 5′ repeat portion of the crRNA to the target inhibits DNA cleavage and serves as a PAM-independent mechanism of self- versus non-self-discrimination (Marraffini and Sontheimer, 2010). However, the necessity of a PAM sequence (rather than the 5′ repeat tag incompatibility) was revealed for the type III-B system of *Pyrococcus furiosus*. Here, the so-called RNA-PAM (rPAM) is located 3′ of the crRNA-complementary sequence on the target RNA and was shown to be crucial for DNA cleavage by the Cmr complex (Elmore et al., 2016). In contrast, a recent study on the type III-A system of *S. epidermidis* found no evidence for the necessity of a PAM or rPAM in this system (Pyenson et al., 2017), indicating subtype or species-related differences in self- versus non-self-discrimination in type III systems.

The subtypes III-C and III-D were only recently identified (Makarova et al., 2015), and little is known about their interference

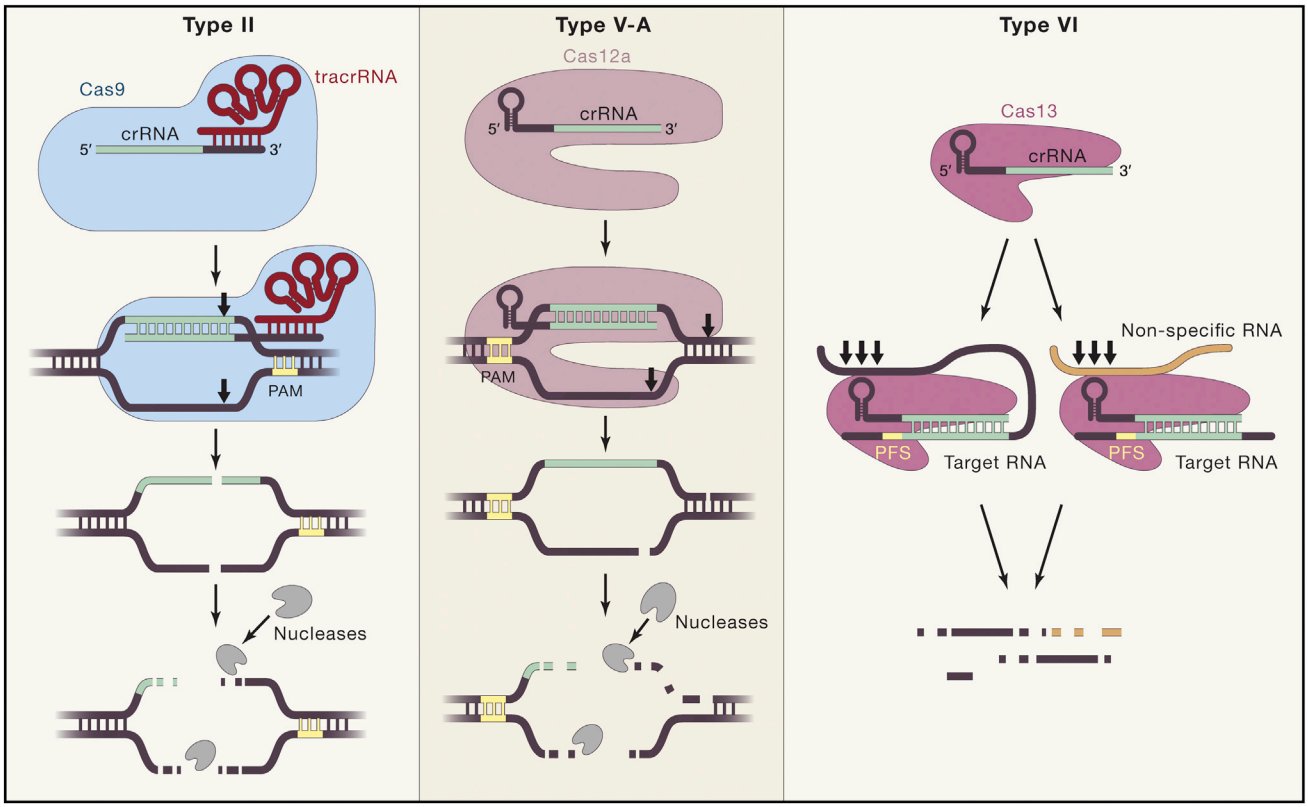


Figure 3. The Interference Pathways of Class 2 CRISPR-Cas Systems

In class 2 systems, interference is accomplished by a single effector protein. In the three well-characterized examples of class 2 interference (type II, type V-A, and type VI), the effector proteins (Cas9, Cas12a, and Cas13, respectively) participate in crRNA maturation and are therefore already bound to the guide RNA prior to target selection and cleavage. During type II interference, Cas9 in complex with tracrRNA (red) and crRNA interrogates the target DNA for the correct PAM (yellow) sequences before probing for complementarity to the crRNA. Base pairing of crRNA to the target strand induces an R-loop structure that finally triggers cleavage of the target and non-target strands by the HNH and RuvC domains, respectively, to yield a blunt double-strand break in the DNA. Type V systems utilize Cas12 proteins for interference. Cas12a of the type V-A system does not require tracrRNA. Following PAM recognition, the RuvC domain of Cas12a cleaves the target sequence at the PAM-distal end, yielding a staggered cut. The tracrRNA requirement and mechanisms may differ for other Cas12 proteins. In type VI, Cas13 is guided by the crRNA to target complementary ssRNAs. Target binding requires a protospacer flanking site (PFS, yellow) and induces conformational changes of Cas13, resulting in an activated catalytic site within the two HEPN domains of the protein. In the activated state, Cas13 acts as a RNase, indiscriminately cutting any exposed RNA, including target RNA, resulting in the global degradation of RNA.

mechanisms. Despite the absence of the conserved adaptation module (composed of the genes *cas1* and *cas2*) and sequence divergence in their *cas10* genes, their overall genetic composition is comparable to the well-characterized types III-A and III-B (Makarova et al., 2015), suggesting similar interference mechanisms. Type IV systems are also categorized as class 1 CRISPR-Cas systems and harbor genes resembling *cas5*, *cas7*, and *cas8* (Koonin et al., 2017; Makarova et al., 2015). However, comprehensive data on type IV-mediated CRISPR-Cas immunity is still missing.

Interference in Class 2 CRISPR-Cas Systems

Type II

Cas9 is a dual RNA-guided DNA endonuclease that is required for interference and immunity in type II systems (Figure 3) (Barrangou et al., 2007; Gasiunas et al., 2012; Jinek et al., 2012; Makarova et al., 2011; Sapranauskas et al., 2011). The type II-A, II-B, and II-C systems are differentiated on the basis of the size of the *cas9* gene and the presence of subtype-specific genes (Chylinski et al., 2013; Fonfara et al., 2014). In addition to crRNA, Cas9 requires *trans*-activating crRNA (tracrRNA), a small RNA that bears complementarity to the repeat regions of crRNA (Deltcheva et al., 2011). Once bound to mature dual RNA (tracrRNA:crRNA) or the engineered single-guide RNA (sgRNA) chimera that has been developed for genome engineering applications, Cas9 identifies target DNA through PAM recognition and subsequent base pairing of the guide RNA with the DNA. If the target displays sufficient complementarity to the RNA guide, Cas9 generates a blunt, double-strand break 3 bp upstream of the PAM (Garneau et al., 2010; Jinek et al., 2012).

Cas9 has a bilobed structure with a central cleft that accommodates the crRNA:DNA duplex. The α-helical recognition (REC) lobe and the nuclease (NUC) lobe are joined by a disordered linker and by the highly conserved arginine-rich bridge helix that forms multiple contacts to the sgRNA. The NUC lobe contains the conserved HNH and RuvC nuclease domains and a variable C-terminal domain that interacts with the PAM (Anders

et al., 2014; Hirano et al., 2016; Jiang et al., 2015, 2016a; Jinek et al., 2014; Nishimasu et al., 2014, 2015; Yamada et al., 2017).

Detailed structural analysis of Cas9 in inactive and active nucleic-acid-bound states, together with numerous biochemical studies, have substantially contributed to our understanding of the interference mechanism in type II systems and are the subject of several in-depth reviews (Jiang and Doudna, 2017; van der Oost et al., 2014). Structural studies have confirmed that guide RNA binding regulates Cas9 activity by inducing a large conformational rearrangement in the protein. This results in ordering of the PAM-interacting residues and the seed sequence of the guide RNA to render the protein competent for DNA binding and PAM recognition (Jiang et al., 2015; Jinek et al., 2014). The guide RNA-bound surveillance complex scans the DNA, and upon recognition of its cognate PAM in the non-target strand, it induces local DNA duplex unwinding to allow the guide RNA to probe for complementarity of the 10- to 12-nt seed sequence in the PAM-proximal region of the target strand (Anders et al., 2014; Jinek et al., 2012; Mekler et al., 2017; Sternberg et al., 2014).

Base pairing between the guide RNA and target DNA and additional conformational changes in Cas9 promote further invasion of the guide RNA beyond the seed sequence, thus stabilizing the R-loop structure (Jiang et al., 2016a). PAM-distal complementarity and divalent cations are necessary for conformational activation of the HNH domain into the cleavage-competent state (Dagdas et al., 2017). Conformational activation of the HNH domain is coupled to rearrangements of the linker loops between the HNH and RuvC domains. This allosteric communication between the nuclease domains results in concerted cleavage of the target strand by the HNH domain and the non-target strand by the RuvC domain (Jinek et al., 2012; Sternberg et al., 2015).

Type V

Type V CRISPR-Cas systems are divided into subtypes V-A, V-B, and V-C that are characterized by effector proteins Cas12a (formerly called Cpf1), Cas12b (C2c1), and Cas12c (C2c3), respectively (Shmakov et al., 2015). Phylogenetic analysis and the low amino acid similarity of these proteins to one another and to Cas9 suggest that they all evolved independently from distinct transposon-associated nucleases of the TnpB family (Shmakov et al., 2015). Whereas Cas12c awaits detailed characterization, the structure and activity of Cas12a and Cas12b have been recently investigated and are described below. Furthermore, several loci tentatively annotated as type V-U lack the *cas1-cas2* adaptation module but encode small putative effector proteins containing RuvC-like and zinc finger motifs whose potential regulatory or defense functions will require further investigation (Shmakov et al., 2017).

Unlike Cas9 and Cas12b, Cas12a of type V-A does not require tracrRNA for activity (Figure 3) (Fonfara et al., 2016; Shmakov et al., 2015; Zetsche et al., 2015). After PAM recognition and sufficient base pairing between the crRNA and target DNA, Cas12a and Cas12b cleave both DNA strands, resulting in staggered double-stranded breaks with 5- and 7-nt overhangs distal to the PAM, respectively (Fonfara et al., 2016; Shmakov et al., 2015; Yang et al., 2016; Zetsche et al., 2015). In contrast to type II systems, which utilize diverse PAMs (Fonfara et al., 2014) that are located on the non-target strand, Cas12 proteins recognize the PAM on both DNA strands, with the non-target PAM sequence being T-rich (Fonfara et al., 2016; Yamano et al., 2016; Yang et al., 2016; Zetsche et al., 2015). Interestingly, Cas12b does not possess a PAM recognition domain like Cas9 or Cas12a (Liu et al., 2017a; Yang et al., 2016). Moreover, Cas12a and Cas12b require a seed sequence of approximately 18 nt, implying that they could be highly specific alternatives to Cas9 for genome editing applications (Kim et al., 2016a, 2017; Kleinstiver et al., 2016; Liu et al., 2017a). Cas12a and Cas12b share the bilobed structure of Cas9, composed of the REC and NUC lobes (Dong et al., 2016; Gao et al., 2016; Liu et al., 2017a; Stella et al., 2017; Swarts et al., 2017; Yamano et al., 2016; Yang et al., 2016). In Cas12a, cleavage of both DNA strands occurs in a single catalytic site of the RuvC domain (Swarts et al., 2017). How both strands are positioned in the catalytic site and whether they are cleaved successively will require further investigation. Structures of Cas12b ternary complex with extended target and non-target DNA strands suggest that both strands can be positioned in the same RuvC catalytic pocket and that at least the target DNA strand is cleaved by the RuvC domain (Yang et al., 2016). Details of the catalytic reaction remain unknown, but it is plausible that Cas12a and Cas12b utilize a similar mechanism.

Type VI

Recent computational searches led to the identification of type VI systems, which are defined by the presence of proteins containing two RxxxxH motifs. These motifs are characteristic of higher eukaryotes and prokaryotes nucleotide (HEPN)-binding domains, which are commonly found in RNases (Anantharaman et al., 2013; Shmakov et al., 2015, 2017). Type VI systems encode the HEPN-containing effector protein Cas13, which, unlike other class 2 effectors, cleaves ssRNA (Figure 3) (Abudayyeh et al., 2016; East-Seletsky et al., 2016; Shmakov et al., 2015; Smargon et al., 2017). Cas13 is activated by "target" ssRNAs complementary to the crRNA to degrade not only the target ssRNA, but also collateral ssRNAs, similar to Csm6 and Csx1 enzymes in type III systems (Abudayyeh et al., 2016; East-Seletsky et al., 2016, 2017; Liu et al., 2017b; Smargon et al., 2017). Though Cas13 enzymes can, in principle, cleave any ssRNA by employing the conserved arginine and histidine residues within the two HEPN domains of the NUC lobe, Cas13a subfamilies display divergent preference for either uridine or adenine 5′ to the scissile bond (Abudayyeh et al., 2016; East-Seletsky et al., 2016; Liu et al., 2017b; Smargon et al., 2017).

Cas13 enzymes are programmed by crRNA and activated by target ssRNA but do not require tracrRNA (Abudayyeh et al., 2016; Liu et al., 2017b; Shmakov et al., 2015). Cas13a tolerates peripheral mismatches in the crRNA:target ssRNA duplex but requires a central seed sequence for RNase activity. In addition, a non-G protospacer flanking site (PFS) 3′ of the target ssRNA is important for activation of *Leptotrichia shahii* Cas13a (Abudayyeh et al., 2016; Liu et al., 2017b) but might differ in other species (East-Seletsky et al., 2017). In contrast, Cas13b activity requires a PFS at each side of the protospacer; the 5′ PFS is non-C, whereas NAN or NNA are preferred at the 3′ PFS (Smargon et al., 2017).

Cas13a and Cas13b also process the repeat regions of pre-crRNA, but biochemical and structural studies suggest distinct

active sites for RNA-activated RNA degradation and pre-crRNA processing by Cas13a (East-Seletsky et al., 2016; Liu et al., 2017b). Furthermore, crRNA maturation does not appear to be an absolute requirement for interference in type VI systems (East-Seletsky et al., 2017). Upon binding to (pre-)crRNA, Cas13a undergoes conformational changes that stabilize the crRNA and facilitate target binding (Liu et al., 2017b). Binding of target ssRNA activates the RNase activity of Cas13a by inducing further conformational changes that bring the catalytic sites of the HEPN domains into close proximity (Liu et al., 2017c). In contrast to the internal active sites of other class 2 effectors (Dong et al., 2016; Jinek et al., 2014; Yang et al., 2016), the two HEPN domains of Cas13a form a composite active site at the external surface of the enzyme that is proposed to account for non-specific RNA degradation (Liu et al., 2017c, 2017b).

When expressed heterologously in *E. coli* with phage-specific crRNAs, Cas13a and Cas13b can confer protection against ssRNA phages, potentially by targeted degradation of the phage genome and/or mRNAs (Abudayyeh et al., 2016; Smargon et al., 2017). However, the indiscriminate RNase activity of Cas13 enzymes leads to restriction of bacterial growth, implying that type VI might degrade host RNAs to induce death or dormancy of infected cells (Abudayyeh et al., 2016; East-Seletsky et al., 2016). The balance between death versus dormancy and between self- versus non-self-targeting in type VI interference might be determined by the intrinsic activity of Cas13 (East-Seletsky et al., 2017), the relative phage load, or by inhibitors or activators of Cas13, such as the recently identified Csx27 and Csx28 proteins (Smargon et al., 2017).

crRNA Biogenesis: Generating Guides for Cas Proteins

The hallmark of CRISPR-Cas defense is the utilization of crRNAs for sequence-specific targeting of invading genetic elements. The transcription start point of the precursor crRNA (pre-crRNAs) usually lies within the leader sequence preceding the CRISPR array. The transcript is subsequently processed within the repeats to generate mature crRNAs, which are usually composed of a repeat segment that is recognized by Cas proteins in a structure- and/or sequence-dependent manner and a spacer portion that is important for target binding (Figure 4).

Class 1 crRNA Maturation

The process of crRNA maturation shows great similarity between type I and type III systems. Both typically employ Cas6 enzymes (Carte et al., 2008; Gesner et al., 2011; Haurwitz et al., 2010; Richter et al., 2012b; Sashital et al., 2011) to specifically process the repeat within the pre-crRNA. A notable exception is the type I-C system, which does not code for a Cas6 homolog. Here, Cas5d functionally replaces Cas6 (Garside et al., 2012; Nam et al., 2012). The majority of type I pre-crRNAs harbor palindromic sequences within their repeats and are thus able to form stable stem-loop structures that are recognized by Cas6 or Cas5d (the affix "d" in Cas5d refers to "Dvulg," the former name of this protein in the type I-C system [Haft et al., 2005]). The nucleases cleave the RNA directly downstream of the hairpin, yielding mature crRNAs that are composed of a full spacer flanked by a short repeat-derived 5′ handle and the 3′ stem loop (Gesner et al., 2011; Haurwitz et al., 2010; Nam et al., 2012; Sashital et al., 2011). Most Cas6 enzymes remain bound to the crRNA after repeat cleavage and therefore act as scaffolds for the formation of Cascade (Jore et al., 2011; Sashital et al., 2011). In contrast, all so-far described homologs of Cas6a (type I-A) and some homologs of Cas6b (type I-B) release the crRNA after the processing event (Charpentier et al., 2015). In contrast to the other subtypes, type I-A and I-B repeats are non-palindromic (Kunin et al., 2007), and it was believed that in these cases, Cas6 solely recognizes the repeat sequence. However, recent studies revealed the significance of a stem-loop structure for repeat cleavage and suggest that Cas6 remodels the repeats to form the requisite stem-loop structure and to reposition the cleavage site (Sefcikova et al., 2017; Shao and Li, 2013; Shao et al., 2016). In contrast to most other monomeric Cas6 proteins, Cas6 proteins in systems with non-palindromic crRNA repeats (mainly I-A and I-B) form dimers (Reeks et al., 2013; Richter et al., 2013; Shao and Li, 2013), thus raising the idea that dimerization might be related to the remodeling function of Cas6.

As some repeat sequences in type III can either be unstructured or only form weak stem loops, they might also rely on Cas6 to remodel crRNA (Kunin et al., 2007). Supporting this assumption, type III Cas6 proteins show high sequence similarity to Cas6 homologs of type I-A and I-B. In addition, the overall processing mechanism in type III is highly similar to type I-A and I-B: in these systems, Cas6 cleaves the pre-crRNA within the repeat region and the processed crRNA undergoes further trimming at the 3′ end, thus removing the hairpin. Moreover, like the Cas6 homologs of type I-A and I-B, type III Cas6 proteins are not part of the interference complex (Carte et al., 2008, 2010; Hatoum-Aslan et al., 2011; Plagens et al., 2014; Richter et al., 2012b). Homologs of Cas6 are not present in the subtypes III-C and III-D. Here, Cas5 proteins might be involved in pre-crRNA processing comparable to type I-C. The mechanism of crRNA processing in the recently classified subtype IV awaits characterization. However, the presence of *cas5* orthologs and *cas6*-like genes in this type suggests a processing mechanism similar to other class 1 types.

Class 2 crRNA Maturation

Class 2 systems co-opt the interference machinery and, in some cases non-Cas proteins, for crRNA maturation. Type II systems and type V-B systems require tracrRNA for CRISPR-mediated immunity (Deltcheva et al., 2011; Shmakov et al., 2015; Zhang et al., 2013). The effector protein of the specific type—for example, Cas9 of type II-A—binds and stabilizes the tracrRNA:crRNA duplex and further recruits the host protein RNase III for processing within the duplexed repeat (Deltcheva et al., 2011). After a second cleavage by an unknown RNase—which removes the 5′ repeat-derived tag—the effector complex composed of Cas9 and the tracrRNA:crRNA duplex is ready for interference (Deltcheva et al., 2011; Gasiunas et al., 2012; Jinek et al., 2012). Type II-C systems of *Neisseria meningitidis* and *Campylobacter jejuni* also utilize a tracrRNA:crRNA duplex for target interference (Dugar et al., 2013; Zhang et al., 2013). Here, however, it was described that the repeats of the type II-C arrays contain promoter elements that lead to the transcription of individual crRNAs. These crRNAs can be processed by RNase III, but this is not a prerequisite for a functional interference complex (Zhang et al., 2013).

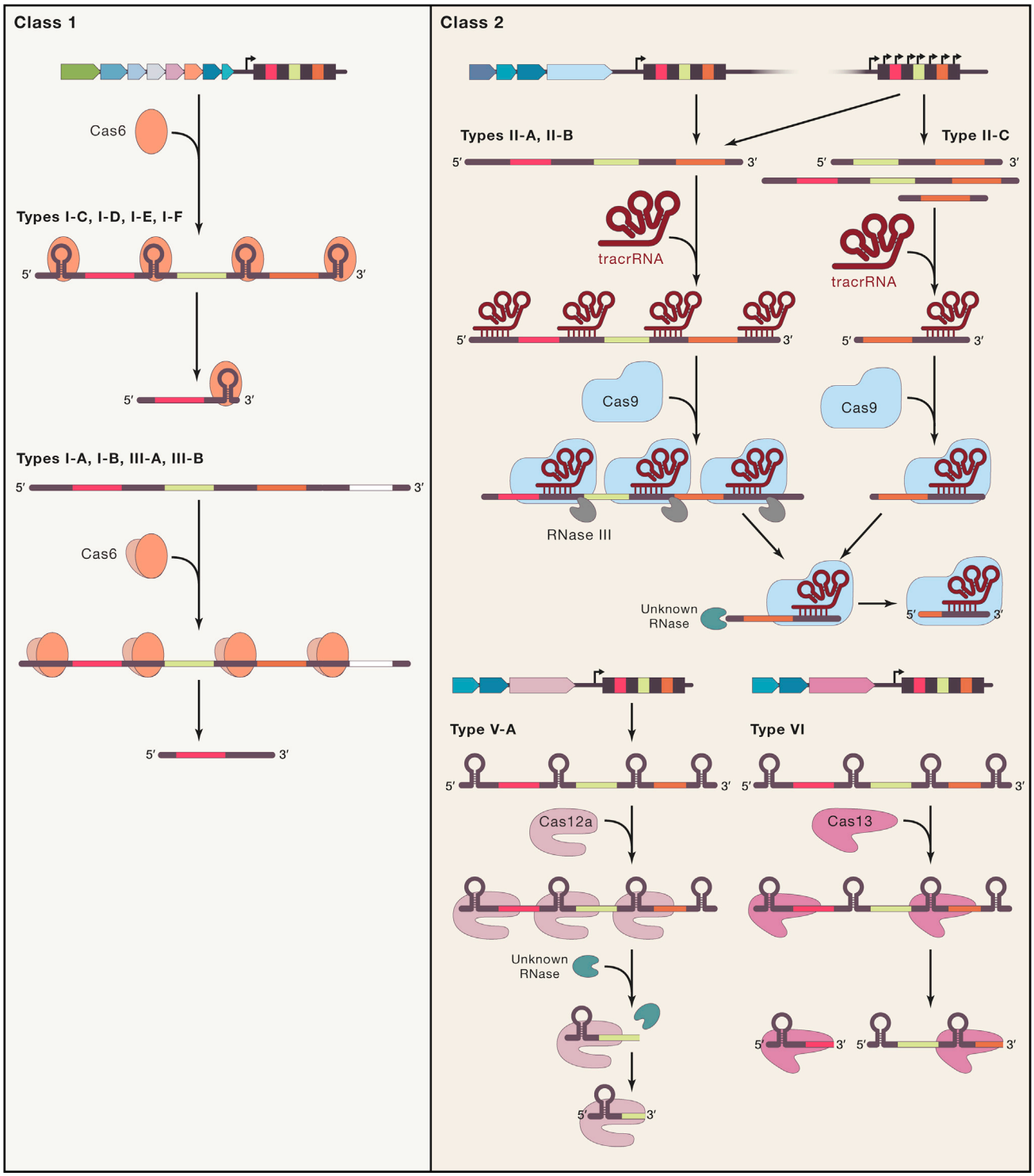


Figure 4. The crRNA Maturation Pathways of Class 1 and Class 2 CRISPR-Cas Systems

In class 1 systems, the CRISPR array is transcribed yielding a long pre-crRNA. Cas6-family enzymes recognize the repeat structure and/or sequence and process the RNA into intermediate or mature crRNAs. In some cases (e.g., type I-A and type I-B), Cas6 acts as a dimer to process the unstructured pre-crRNA. crRNA maturation in class 2 systems differs significantly. In type II, tracrRNA (red) and pre-crRNA form duplexes, which are bound and stabilized by Cas9. This enables the processing by the host protein RNase III. The intermediate crRNA is further matured by an unknown RNase. Type II-C systems were described to employ an RNase-III-independent pathway. Here, promoter sequences within the repeats enable internal transcription and formation of

(legend continued on next page)

In type V and type VI systems, the effector proteins Cas12 and Cas13, respectively, possess dual nuclease activity for crRNA processing and target interference. In type V-A systems, Cas12a recognizes the repeat hairpin structure and cleaves within the repeat to generate crRNAs with 5′ repeat-derived tags (Fonfara et al., 2016). For type V-B and type V-C systems, comprehensive data are still missing, but it seems that their effector proteins Cas12b and Cas12c process precursor crRNA and that the former also requires tracrRNA (Shmakov et al., 2015). Similar to Cas12a, the Cas13 effector protein of type VI systems does not require tracrRNA for crRNA processing (Abudayyeh et al., 2016; Shmakov et al., 2015). Cas13a recognizes the sequence and structure of the repeat within the pre-crRNA and processes upstream of the hairpin (East-Seletsky et al., 2016). Interestingly, crRNA maturation is not a strict requirement for target cleavage in type VI-A, as pre-crRNAs can also serve as functional guides (East-Seletsky et al., 2017). In type VI-B systems, the repeats can vary in length within one CRISPR array, while spacer size remains the same. Therefore, Cas13b-mediated processing results in the generation of mature crRNAs that harbor a 30-nt spacer portion combined with either a 36-nt or an 88-nt repeat sequence. Both repeat architectures were shown to promote target cleavage by the effector (Smargon et al., 2017).

Adaptation: Memorizing Invading Nucleic Acids

Adaptation, also known as spacer acquisition, is the step in which memory of previous infections is formed and is the reason why CRISPR-Cas immunity is adaptive and heritable. The CRISPR array serves as a genetic memory bank, and spacer acquisition into the array is accomplished in several steps: the detection of an MGE, protospacer selection, protospacer processing, and spacer integration into the CRISPR array. The key players of spacer acquisition are Cas1 and Cas2, which are present in nearly all CRISPR-Cas systems (Koonin et al., 2017). In the type I-E CRISPR-Cas system of *E. coli*, the best-studied model for adaptation, a stable complex composed of two Cas1 dimers bridged by one Cas2 dimer (abbreviated as Cas1-Cas2) acts as an integrase in which Cas1 is catalytic and Cas2 has a structural function (Nuñez et al., 2014). Cas1 and Cas2 are the only Cas proteins required for naive spacer acquisition in the type I-E system (Figure 5A); however, additional Cas proteins are required in other systems (Heler et al., 2015; Li et al., 2014; Vorontsova et al., 2015; Wei et al., 2015). In the type II-A CRISPR-Cas system of *Streptococcus pyogenes* and *S. thermophilus*, all Cas proteins (Cas9, Cas1, Cas2, and Csn2) and tracrRNA are essential for spacer integration (Heler et al., 2015; Wei et al., 2015). The adaptation mechanisms of type I and type II are the most thoroughly characterized. They provide a model for our current understanding of spacer acquisition and are discussed below. We also address recent progress on other acquisition mechanisms, as well as notable subtype-specific differences.

The Origin of Protospacer

Spacer acquisition begins with the detection of foreign genetic elements that are subsequently processed and integrated into the CRISPR array. In order to avoid auto-immunity, it is important that the adaptation machinery display a preference for foreign versus self DNA and/or that the activity of the adaptation machinery is enhanced by signals of (imminent) infection (see The Ecology and Regulation of CRISPR-Cas). A study in *E. coli* revealed that the degraded DNA fragments generated during the repair of double-stranded DNA (dsDNA) breaks (DSBs) are an important source of protospacers (Levy et al., 2015). The RecBCD repair complex is recruited to DSB sites, which are often found at replication forks (Dillingham and Kowalczykowski, 2008; Smith, 2012; Wigley, 2013). RecBCD unwinds and degrades the DNA until it reaches a crossover hotspot instigator (Chi) site. Sequences proximal to the Chi sites, as well as sites of replication fork stalling, were shown to be the protospacer-sampling hotspots, suggesting that the RecBCD degradation fragments are captured by the adaptation machinery (Levy et al., 2015). The underrepresentation of Chi sites on foreign DNA compared to genomic DNA of *E. coli* (Dillingham and Kowalczykowski, 2008) allows RecBCD to degrade larger portions of the foreign genome and serves as a basis of preferential acquisition of non-self DNA (Levy et al., 2015).

A similar mechanism was recently described in the type II-A system of *S. pyogenes*, where regions between exposed DNA ends and Chi sites were highly favored for spacer sampling (Modell et al., 2017). In phage DNA, sequences between the injected linear DNA ends and the closest Chi site are spacer-sampling hotspots. It has been demonstrated that the AddAB machinery (the Gram-positive paralogs of RecBCD) was necessary for efficient spacer acquisition and thus suggests a similar self- versus non-self-discrimination strategy as observed in *E. coli* (Modell et al., 2017). The reliance on other host proteins suggest that the adaptation machinery lacks an intrinsic ability to distinguish between self- and non-self-DNA. Indeed, overexpression of catalytically inactive Cas9, which abolishes interference and thus prevents auto-immunity, resulted in a surplus of genome-derived spacers over plasmid-derived spacers in the type II-A system of *S. thermophilus* (Wei et al., 2015). Considering that spacer integration is a rare event (Wei et al., 2015), a low acquisition rate might be a strategy to compensate for inefficient self- versus non-self-discrimination in order to reduce the chance of auto-immunity and/or allow beneficial horizontal gene transfer.

Protospacer Selection and Processing

In addition to preferential fragmentation of foreign DNA by the RecBCD/AddAB machinery, selection of specific protospacers by the adaptation machinery is often non-random. In type I and type II systems, the adaptation machinery selects protospacers with a PAM that is compatible with the interference machinery (Datsenko et al., 2012; Deveau et al., 2008; Mojica et al., 2009; Swarts et al., 2012). Studies in *E. coli* showed that the

mature crRNAs, which form crRNA:tracrRNA duplexes that bind Cas9. In type V-A and type VI, Cas12a and Cas13, respectively, recognize the structure and sequence of their repeats in order to cleave the pre-crRNA upstream of the stem structure. In type V-A, an additional uncharacterized processing event occurs. Processing by Cas13 of type VI yields mature crRNA. However, crRNA maturation is not an absolute requirement for interference in this system.

Cas1-Cas2 complex is sufficient for PAM recognition (Datsenko et al., 2012; Swarts et al., 2012); the Cas1 subunits preferably bind the PAM-complementary sequence (Wang et al., 2015). Moreover, type I-E Cas1-Cas2 prefers protospacers with 3′-single-stranded overhangs of at least 7 nt at both ends, showing that both PAM and structure affect protospacer selection (Wang et al., 2015). These dual-forked DNA substrates are likely derived from the partial re-annealing of the ssDNA fragments generated by RecBCD (Levy et al., 2015) or by the interference machinery during interference-driven adaptation (described below) (Künne et al., 2016; Staals et al., 2016). Two Cas1 tyrosine wedges splay the dual-fork DNA and stabilize the 23-bp duplex. This positions the 3′ overhangs near the active sites of the Cas1 dimers (Wang et al., 2015). Cas1 cleaves the 3′ overhangs to generate a 33-nt product with a 3′OH on each overhang. Two nucleotides of the PAM-complementary sequence are removed in this process, thus preventing acquisition of spacers that would result in cleavage of the CRISPR array. The structure of the Cas1-Cas2 complex seems to serve as a molecular ruler that determines the protospacer size and thus prepares the protospacer for integration into the CRISPR array (Nuñez et al., 2015; Wang et al., 2015).

Unlike type I-E, Cas1 and Cas2 alone are not sufficient for naive spacer acquisition in type II-A systems. Here, Cas9, Csn2 and tracrRNA are additional requirements (Heler et al., 2015). Cas9 selects protospacers that are adjacent to a PAM while random protospacers are selected when the PAM recognition domain of Cas9 is mutated. Cas9 catalytic activity is dispensable for protospacer acquisition, indicating that Cas9 is not involved in protospacer processing (Heler et al., 2015; Wei et al., 2015).

Spacer Integration

The CRISPR array is preceded by an AT-rich leader sequence. Spacer integration preferentially occurs at the leader end of the CRISPR array and thus keeps a chronological record of previous infections (Barrangou et al., 2007; Pourcel et al., 2005). The mechanism of protospacer integration has been studied in detail in the type I-E system of *E. coli*. *In vitro* studies showed that the mechanism by which Cas1-Cas2 integrates new spacers is similar to that of viral integrases and transposases (Nuñez et al., 2015). First, the 3′OH of the protospacer performs a nucleophilic attack at the target site and thus attaches to the 5′ phosphate of the leader-proximal repeat. This process depends on the recognition of the leader-repeat boundary, which is specified through binding of the leader sequence by a CRISPR-independent protein called integration host factor (IHF) (Nuñez et al., 2015, 2016; Yoganand et al., 2017). IHF sharply bends the DNA, which results in a U-shaped leader structure and favors recognition of the leader-repeat boundary by Cas1-Cas2 (Wright et al., 2017) (Figure 5A; inset). In the second step, the 3′OH of the other protospacer strand is ligated to the opposite end of the first repeat (Nuñez et al., 2015, 2016). Important during this step are two inverted repeat motifs in the CRISPR repeat, which serve as anchors for the Cas1-Cas2 complex and determine the position of the second integration site (Goren et al., 2016). Upon complex binding, the repeat becomes distorted, which is crucial for making the second integration site accessible to Cas1 (Wright et al., 2017). The incorporation of the new spacer in the correct orientation is ensured by the presence of the partial PAM on the protospacer. Though some PAM nucleotides are removed prior to integration, this likely occurs after binding of the acquisition complex to the leader-repeat junction, so directionality is preserved (Shipman et al., 2016; Shmakov et al., 2014; Wang et al., 2015) (Figure 5A; inset).

Unlike type I-E, recognition of the leader-repeat end in type II-A is IHF independent and requires a short motif termed leader-anchoring site (LAS), which consists of 5 bp of the repeat-proximal leader end and is directly recognized by Cas1-Cas2. Interestingly, mutations in the LAS can lead to ectopic spacer integration within the CRISPR array. Although spacer acquisition is less effective in this case, the recognition of alternative anchoring sites gives the system flexibility to overcome alterations of the canonical LAS by integrating new spacer at an alternative anchoring site (McGinn and Marraffini, 2016). However, spacer sequences within the CRISPR array provide less resistance against phages than leader-proximal spacers, likely due to the lower abundance of distally encoded crRNAs (McGinn and Marraffini, 2016).

After recognition of the LAS, the type II-A Cas1-Cas2 complex can conduct the first integration reaction at either end of the first repeat, although integration at the leader boundary is usually preferred (Wright and Doudna, 2016). Structural data supporting this model were recently presented for the type II-A integration complex of *Enterococcus faecalis* (Xiao et al., 2017b). Here, terminal sequences on both sides of the repeat were shown to be sufficient but suboptimal for target recognition. Additional interactions of Cas1 with the first four repeat-proximal nucleotides of the leader, however, allow a more efficient interaction with the target and thus explaining the preference for first integration event at the leader side of the first repeat (Xiao et al., 2017b). The first reaction is characterized by generation of a half-site integration intermediate where only one strand of the protospacer is ligated to one end of the repeat. The second integration event depends on proper protospacer size, the recognition of the opposite repeat end (Wright and Doudna, 2016), and bending of the repeat by the Cas1-Cas2 complex (Xiao et al., 2017b). In case these requirements are not fulfilled, full-site integration cannot occur and the acquisition complex presumably reverses the first integration reaction, or the half-site integration intermediate is removed by DNA repair proteins (Wright and Doudna, 2016).

Primed Spacer Acquisition

While naive spacer acquisition generates memory against previously unconfronted MGEs (Yosef et al., 2012) (Figure 5A), primed acquisition refers to intensified spacer uptake that is stimulated by pre-existing spacers that bear complete or partial complementarity to previously encountered MGEs. This serves as a counter-strategy against phage escape mutants (Datsenko et al., 2012; Fineran et al., 2014; Li et al., 2014; Richter et al., 2014; Swarts et al., 2012). So far, priming has only been observed in type I systems, but further research will be required to determine whether priming also exists in other CRISPR-Cas systems. In addition to Cas1 and Cas2, primed acquisition requires the interference machinery (Datsenko et al., 2012; Fineran et al., 2014; Savitskaya et al., 2013; Swarts et al., 2012).

In the type I-E system, CRISPR interference is aborted by mutations in the PAM sequence. Although Cascade still binds the

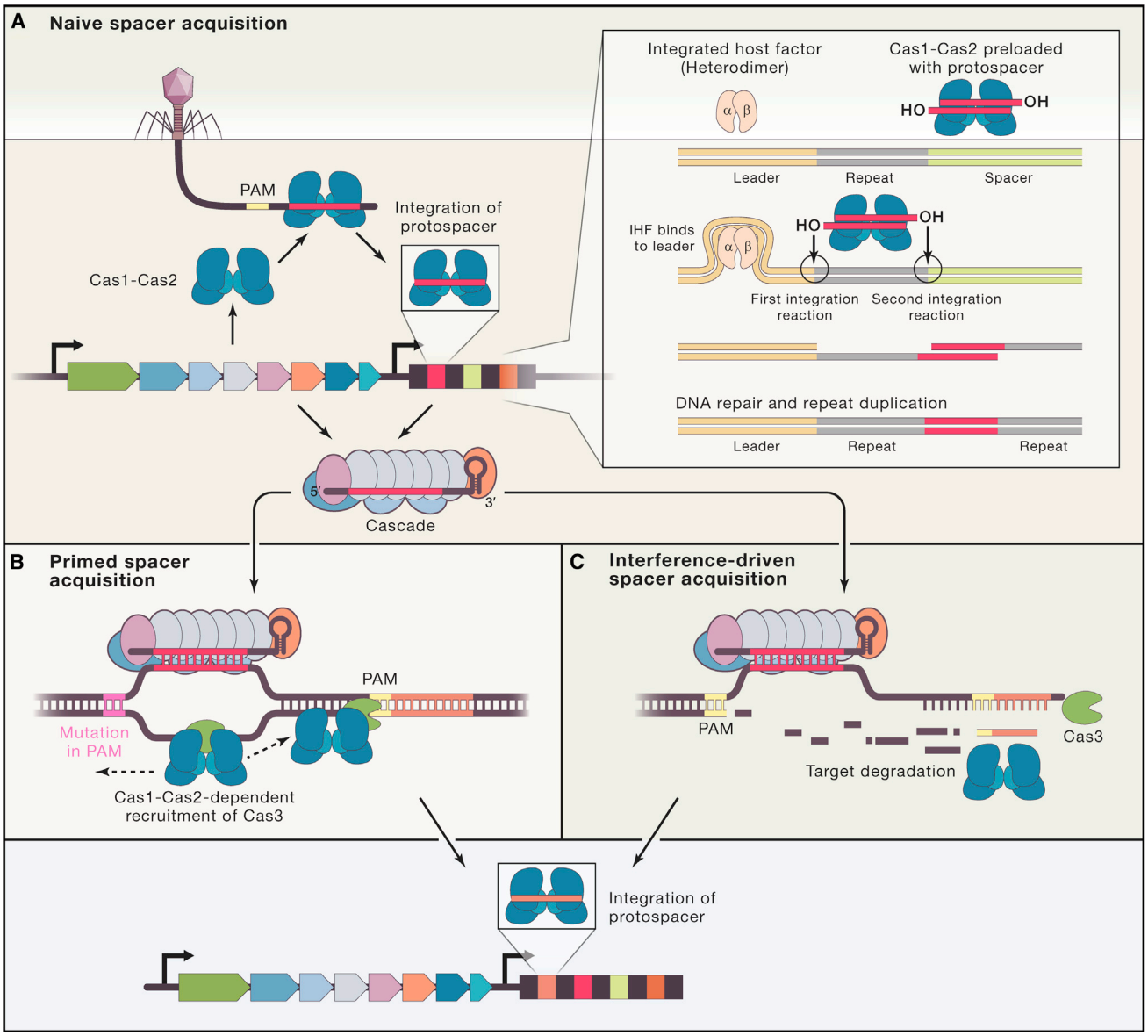


Figure 5. Spacer Acquisition in Type I Systems

(A) During naive spacer acquisition in type I-E systems, the Cas1-Cas2 complex is sufficient for the recognition of a canonical PAM. After initial fragmentation of the invading DNA by RecBCD (not shown), suitable protospacers are integrated at the leader-proximal end of the CRISPR array (inset). The CRISPR-unrelated integrated host factor (IHF) is essential for this process, as it binds a specific sequence of the leader, yielding a sharply bent DNA structure. DNA bending allows the Cas1-Cas2 complex to recognize and bind the leader-proximal repeat. The 3′OH ends of the protospacer perform nucleophilic attacks on the leader side and spacer side of the repeat backbone. During the first integration reaction, the leader-repeat boundary is nicked and ligated to one strand of the protospacer. During the second integration reaction, the other protospacer strand is ligated to the opposite end of the repeat, leading to the duplication of the first repeat. DNA polymerase and ligase subsequently fill the single-strand gaps.

(B) Primed spacer acquisition requires an existing spacer matching the target. Mutations in the seed sequence or the PAM, however, abolish interference. In some cases, crRNA guides Cascade to bind the imperfect target sequence, but the complex fails to recruit Cas3 for DNA degradation. Here, Cas1-Cas2 recruits Cas3, and the complex translocates bidirectionally (dashed arrows) away from the target site without degrading the DNA. Cas1-Cas2 selects proper protospacer with canonical PAM for spacer integration.

(C) Interference-driven spacer acquisition also requires the presence of an existing spacer against the invader, which results in target cleavage by the interference machinery. Following the degradation of target DNA by Cas3, Cas1-Cas2 captures DNA fragments and subsequently integrates them into the CRISPR array.

target site in a PAM-independent manner, it fails to recruit Cas3 (Blosser et al., 2015; Redding et al., 2015; Xue et al., 2016). Intriguingly, the presence of Cas1-Cas2 restores Cas3 recruitment and results in bi-directional translocation of Cas3 without target degradation. The underlying mechanism is not yet fully understood. Presumably, Cas3 moves along the DNA together with Cas1-Cas2, selecting new protospacers for integration (Redding et al., 2015) (Figure 5B). This model is supported by presence of a

large Cas1-Cas2-3 complex (in which Cas3 is naturally fused to the C terminus of Cas2) in the related type I-F system of *Pectobacterium atrosepticum* (Fagerlund et al., 2017; Richter et al., 2012a) and *Pseudomonas aeruginosa* (Rollins et al., 2017), and the interaction between Cas1 and the subunits of Cascade in *E. coli* (Babu et al., 2011).

Analysis of newly acquired spacers revealed subtype-dependent sampling patterns during priming. In type I-E systems, protospacers that lie on the same strand as the priming spacer are preferentially sampled (Shmakov et al., 2014; Swarts et al., 2012). In contrast, the type I-B system of *Haloarcula hispanica* (Li et al., 2014) and the type I-F of *P. atrosepticum* sample protospacers from both strands (Richter et al., 2014) but display a preference for the sequences proximal to the priming protospacers. Altogether, this indicates that there are yet unknown mechanistic variations during priming between different subtypes.

Interference-driven acquisition displays a variation of the priming process. In this case, mutations do not abolish interference, so the target DNA is degraded and subsequently captured by Cas1-Cas2 (Figure 5C). This creates a positive feedback loop that diversifies the spacer repertoire against a mutating target and thus severely limits the chance of phage escape (Künne et al., 2016; Staals et al., 2016).

Further Acquisition Mechanisms

Although the mechanism behind type I-E and type II-A spacer acquisition is well understood, it is unclear to what degree this is conserved in other CRISPR systems. The presence of Cas1 and Cas2 in nearly all CRISPR systems likely reflects a conserved function of this complex during adaptation. In several systems, additional Cas proteins have been implicated in spacer acquisition. Cas4 is encoded by type II-B CRISPR-Cas systems and several type I and type V systems (Koonin et al., 2017; Makarova et al., 2015; Shmakov et al., 2015). The fusion of Cas4 to Cas1 in types I-U and V-B (Koonin et al., 2017; Mohanraju et al., 2016) and the formation of a complex containing Cas4, Cas1, Cas2, and Csa1 in *Thermoproteus tenax* (Plagens et al., 2012) is also suggestive of a role of Cas4 in adaptation. It is believed that the 5′–3′ exonuclease activity of Cas4 participates in the processing of protospacers by generating single-stranded 3′ overhangs that are required for integration by Cas1-Cas2, as described above (Zhang et al., 2012). In the type II-A system, Csn2 is also essential for spacer acquisition *in vivo* (Barrangou et al., 2007; Heler et al., 2015; Wei et al., 2015). Csn2 interacts with other type II-A proteins (Heler et al., 2015; Ka et al., 2016) and can bind free DNA ends and slide along dsDNA in an energy-independent manner, which is suggestive of an accessory function of Csn2 during spacer integration (Arslan et al., 2013). However, the precise nature of its involvement in the integration process remains enigmatic, particularly because it is not required for the integration reaction *in vitro* (Wright and Doudna, 2016).

An interesting variation of the thus far described spacer acquisition modes is seen in the type III-B CRISPR-Cas system of *Marinomonas mediterranea*, which harbors a Cas1 homolog that is fused to a reverse transcriptase (RT). In this system, RNA and DNA oligonucleotides can be inserted into the CRISPR array. Integration of RNA is followed by reverse transcription of the inserted fragment into cDNA by the RT domain of Cas1 (Silas et al., 2016). Despite the substantial progress of recent years, spacer acquisition is still the least understood step in CRISPR immunity. Most of our knowledge derives only from type I and II systems. It will be interesting to see to what extent the underlying mechanisms are conserved in other types. Intriguingly, the *cas1-cas2* adaptation module is missing in types IV, V-U, and some type III and type VI systems, implying that they either have regulatory functions or that adaptation is accomplished by novel mechanisms or by co-opting the CRISPR-Cas machinery of other systems in *trans* (Koonin et al., 2017).

The Ecology and Regulation of CRISPR-Cas

Although the mechanisms underlying CRISPR-mediated immunity are becoming clear, we are only beginning to understand how CRISPR-Cas and other prokaryotic defenses are regulated and how they shape the ecology of phage-host interactions (reviewed by Westra et al., 2016). The term ''arms race'' has be used to describe the relationship between prokaryotes and phages and implies they are engaged in incessant adaptation and counter-adaptation of defense and offense strategies. However, in many circumstances, it seems that phage and bacterial populations coexist and exhibit stable fluctuations without extensive antagonistic coevolution (Hall et al., 2011; Koskella and Brockhurst, 2014; Lenski, 1984; Lenski and Levin, 1985). In this section, we discuss factors influencing the reciprocal regulation of CRISPR-Cas and the ecology of phage-host interactions.

Phages Counter CRISPR-Cas

An important facet of the role of CRISPR-Cas in the ecology of phage-host interactions are the strategies that phages utilize to counter CRISPR-Cas. Sequence specificity is the key feature of these immune systems and can be overcome by random mutations in the phage genome. For both CRISPR classes, escape mutations are typically located in the PAM sequence or the seed region of the protospacer and lead to inefficient cleavage by the Cas protein machinery (Deveau et al., 2008; Semenova et al., 2011). Type III CRISPR-Cas systems seem to be less affected by this evasion strategy, as they lack a PAM sequence and show a higher tolerance toward mismatches (Gudbergsdottir et al., 2011; Manica et al., 2011). In fact, the type III-A system of *S. epidermidis* was recently shown to be highly flexible toward mutations in the protospacer and adjacent sites, resulting in a drastic decrease of viral escape rates, which could not be accomplished by type II-A CRISPR-Cas immunity (Pyenson et al., 2017). Some type I systems have developed primed adaptation as a counter strategy for phage escape mutants. Here, mismatches between the targeting crRNA and the protospacer can trigger enhanced uptake of new spacers. However, the effectiveness of mutation as an evasion strategy may become obsolete when the predator is exposed to extensive evolutionary pressure (for instance, in bacterial populations with a highly diverse spacer content). Here, phages undergo rapid extinction, as they cannot effectively adapt to the multiple spacer genotypes within a population, even though infection of individual cells may be accomplished (van Houte et al., 2016). Long-term coevolutionary experiments showed that the addition of a second phage at a later time point leads to genomic rearrangement

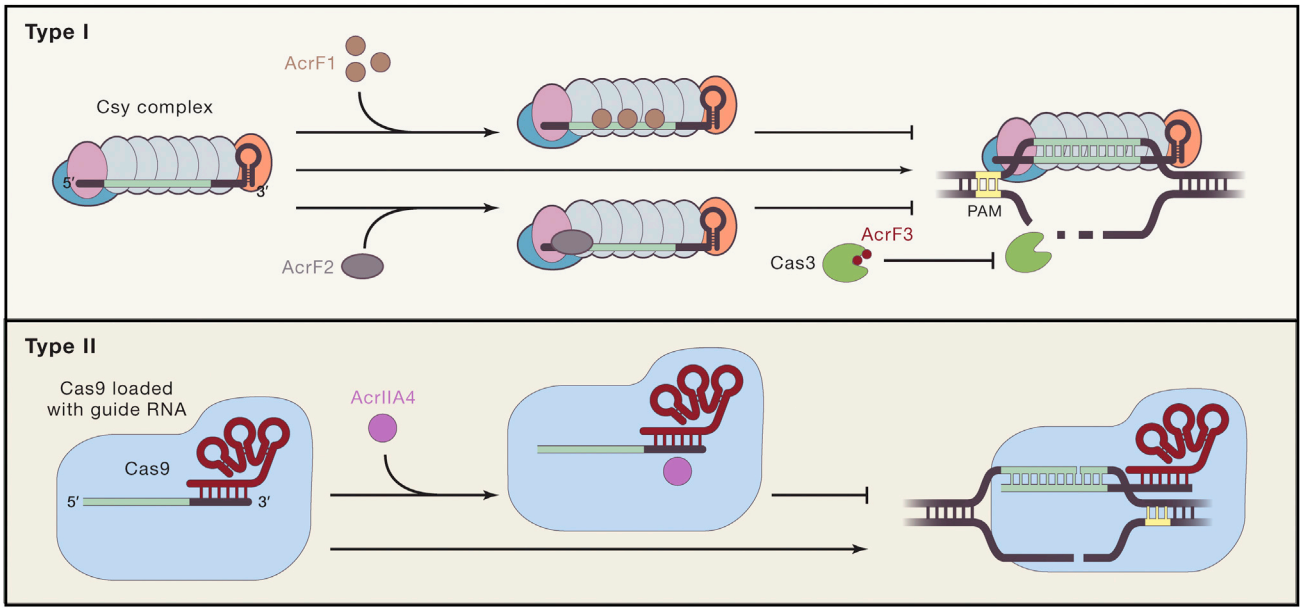


Figure 6. Inhibition of Interference by Acr Proteins

In type I-F, the Csy-crRNA complex (subtype-specific term for Cascade) binds foreign DNA and subsequently recruits Cas3 for target cleavage. The phage anti-CRISPR protein AcrF1 (brown) interacts with the Csy3 backbone of the complex (gray), whereas AcrF2 (dark purple) binds to the Csy1-Csy2 heterodimer (blue and pink). Both proteins thereby block the access to the foreign DNA. AcrF3 (red) prevents target degradation by directly inhibiting the Cas3 nuclease. Type II interference can be inhibited by the AcrIIA4 protein (pink), which binds the PAM-interaction domain of Cas9 and thereby impairs target binding and cleavage.

and recombination events between the two species and thus increases the genetic variability to give rise to *de novo* CRISPR-Cas evasion. However, phage loss eventually occurred, raising the idea that constant influx of predatory populations may be crucial for phage survival (Paez-Espino et al., 2015).

Some phages have evolved more sophisticated strategies to circumvent CRISPR-Cas immunity, one of which employs small proteins that directly inhibit the interference machinery. Several phages that infect *P. aeruginosa* express anti-CRISPR (Acr) proteins that directly inhibit the activity of the bacterial type I-F interference complex to impair anti-phage immunity (Bondy-Denomy et al., 2013). AcrF1 and AcrF2 interact with different subunits of the Cascade and prevent proper binding of the complex to the target DNA (Bondy-Denomy et al., 2015; Chowdhury et al., 2017; Peng et al., 2017). AcrF3 sterically blocks the recruitment of the endonuclease Cas3 and thus inhibits DNA cleavage (Bondy-Denomy et al., 2015) (Figure 6). Several Acr genes targeting the type I-E system of *P. aeruginosa* have been identified. Interestingly, these did not prevent type I-F CRISPR immunity in the same bacterial species, nor were they able to block the activity of the related type I-E system of *E. coli*, suggesting that certain Acr proteins are highly specific for a given CRISPR-Cas subtype (Bondy-Denomy et al., 2013; Pawluk et al., 2014). Recently, however, new *acr* gene families have been discovered, one of them encoding the protein AcrF6, which inhibits both the type I-F and I-E systems of *P. aeruginosa*, thus displaying very broad specificity (Pawluk et al., 2016a). Type II anti-CRISPR (AcrII) proteins employ a strategy similar to AcrF1 and AcrF2 by inhibiting the interference mechanism (Pawluk et al., 2016b; Rauch et al., 2017). For example, AcrIIA4 of a *Listeria monocytogenes* prophage blocks the PAM-interacting region of Cas9 (Dong et al., 2017; Rauch et al., 2017; Shin et al., 2017; Yang and Patel, 2017) (Figure 6). Moreover, AcrIIA4 blocks target binding of Cas9 in other bacterial species (Rauch et al., 2017), indicating its ability for cross-species inhibition of type II-A interference. Interestingly, the inhibitory effect of AcrIIA4 and several anti-type II-C Acr proteins has also been confirmed in human cells (Pawluk et al., 2016b; Rauch et al., 2017), thus demonstrating their potential as a tool to regulate for Cas9-mediated genome engineering (Sontheimer and Davidson, 2017). All Acr proteins that have been reported thus far inhibit the interference machinery. Interestingly, adaptation requires the Csy complex in the subtype I-F system of *P. aeruginosa*. As a consequence, spacer acquisition is also inhibited in the presence of several AcrF proteins targeting the interference complex (Vorontsova et al., 2015). Further research and mining of the phage metagenome may uncover phage inhibitors of crRNA biogenesis or the adaptation complex or novel protein- or RNA-based strategies for phages to evade CRISPR-mediated immunity.

Instead of preventing CRISPR-Cas immunity, some phages turn the tides and use CRISPR-Cas defense against their host. *Vibrio cholerae* phages encode a fully functional type I-F system that targets a host genomic island to compromise a CRISPR-unrelated anti-phage defense system (Seed et al., 2013). *C. jejuni* harbors a minimal type II-C CRISPR-Cas system composed of Cas9, Cas1, Cas2, and tracrRNA and is susceptible to some phages that encode their own Cas4 protein. During infection with these phages, the CRISPR array acquires new spacers that exclusively target the genome of *C. jejuni*, indicating that the phage is able to utilize the bacterial CRISPR-Cas system

against its host. It is worth mentioning that *C. jejuni* and the associated phage coexist in stable equilibrium (Siringan et al., 2014). The acquisition of host-derived spacers may be important for retaining this balance (Hooton and Connerton, 2015).

Balancing the Benefits and Costs of CRISPR-Cas

The robust, heritable immunity conferred by CRISPR-Cas systems can constitute a major fitness advantage that is consistent with the presence of these systems in diverse environments and species (Grissa et al., 2007). The evolution of primed adaptation and the aforementioned phage strategies to counteract CRISPR-Cas, as well as observations that CRISPR array spacer content in natural bacterial ecosystems can evolve rapidly and matches current and historical phage threats (reviewed by Westra et al., 2016), strongly suggest that CRISPR-Cas can play an important role in the antagonistic coevolution of prokaryotes and their viral predators.

Their benefits notwithstanding, CRISPR-Cas systems are notably absent from half of bacterial species (Grissa et al., 2007). The reasons for this are not entirely understood, but there are several mutually non-exclusive explanations. In addition to the adaptive immunity provided by CRISPR-Cas, prokaryotes can defend themselves from phages by blocking phage adsorption through receptor or cell-wall modifications, preventing injection of the phage genome, or destroying the phage genome by restriction-modification systems (Labrie et al., 2010). The recent identification of additional anti-phage mechanisms, such as bacteriophage exclusion (BREX) systems, prokaryotic Argonaute proteins, and serine/threonine kinases suggest that yet-unknown strategies may exist (Depardieu et al., 2016; Goldfarb et al., 2015; Olovnikov et al., 2013; Swarts et al., 2014). In some situations, these constitutive innate defenses can provide sufficient protection against phages.

Under certain circumstances, CRISPR-Cas can compromise bacterial adaptation and fitness. These systems not only target potentially lethal phages, but also limit the acquisition of plasmid- or prophage-encoded traits that might otherwise promote fitness by enhancing resistance or pathogenicity (Bikard et al., 2012; Jiang et al., 2013; Marraffini and Sontheimer, 2008). Although most spacers correspond to foreign nucleic acids, there is a non-negligible risk of acquiring self-targeting spacers that can result in auto-immunity (Bikard et al., 2012; Jiang et al., 2013; Stern et al., 2010; Vercoe et al., 2013). Furthermore, the costs of maintaining these complex systems can outweigh their benefits, as has been observed for the type II-A systems of *S. thermophilus* (Vale et al., 2015). Loss of CRISPR-Cas might therefore provide autonomous benefit to a bacterium and its progeny. In addition, recent reports suggest that loss of CRISPR-mediated immunity in a small subset of the host population sustains the phage population to preserve mild selective pressure and thus keep immunity prevalent among the bacteria (Levin et al., 2013; Weissman et al., 2017). Moreover, exceeding costs of antagonistic coevolution is another crucial factor explaining why bacteria and phage populations transit into a balanced state of coexistence with mild coevolution rather than a continuous arms race (Koskella and Brockhurst, 2014). Due to the fitness trade-off, it has been proposed that CRISPR-Cas systems are more likely to provide an overall benefit when phage diversity and load are low (Iranzo et al., 2013; Weinberger et al., 2012). These parameters are lower in thermophilic environments, potentially explaining the enrichment of CRISPR-Cas in thermophiles (Makarova et al., 2006).

Several recent studies suggest that context-dependent induction of CRISPR-Cas immunity represents an alternative strategy for mitigating the costs and risks of CRISPR-Cas activity (reviewed by Patterson et al., 2017). In dense microbial populations, such as biofilms, vulnerability to phage predation is high (Abedon, 2012; Kasman et al., 2002). Quorum sensing pathways that govern coordinated, density-dependent behaviors of bacterial populations have been shown to upregulate Cas protein expression to enhance CRISPR-mediated immunity (Høyland-Kroghsbo et al., 2017; Patterson et al., 2016). Likewise, a number of studies have found that phage infection induces expression of the CRISPR array and *cas* genes (Agari et al., 2010; Fusco et al., 2015; Quax et al., 2013; Young et al., 2012). Phage adsorption induces envelope stress, and this has been implicated in the upregulation of CRISPR-Cas activity (Perez-Rodriguez et al., 2011). Auto-regulation has been shown for the type I-A CRISPR system in the archaeon *Sulfolobus solfataricus*, where Cascade and the transcriptional regulator Csa3b occupy the promoter region and suppress expression of the *cas* gene cassette. Upon encountering a matching protospacer during phage infection, Cascade binds the protospacer and therefore dissociates from the promoter region, leading to de-repression of *cas* gene expression (He et al., 2016). Type III CRISPR-Cas systems intrinsically assess risk by targeting prophages only when they are actively transcribed (Goldberg et al., 2014; Samai et al., 2015). Future studies are likely to reveal additional mechanisms by which bacteria couple the threat of predation to induction of CRISPR-Cas and how this allows them to balance the costs and benefits of these defense systems.

Functions of CRISPR-Cas beyond Immunity

Adaptive immunity is the canonical function of CRISPR-Cas. However, the high conservation but slow evolution of some CRISPR-Cas systems suggests they may have important functions beyond immunity (Touchon et al., 2011). Indeed, several additional functions of CRISPR have been reported in diverse bacterial species (reviewed by Westra et al., 2014). These functions range from genetic regulation of bacterial physiology and virulence, as discussed below, to promoting genome evolution (Vercoe et al., 2013) or DNA repair (Babu et al., 2011). Some of these functions appear to be novel and selected, whereas others are probably by-products of the role of CRISPR-Cas in immunity.

In *Myxococcus xanthus* and *P. aeruginosa*, CRISPR-Cas systems modulate expression of endogenous genes to regulate group behavior. During starvation, coordinated movement of the *M. xanthus* population leads to aggregation and the formation of a mound that further develops into a fruiting body containing spores that can endure environmental stress (Kaiser et al., 2010). Sporulation in *M. xanthus* involves the *dev* locus, which encodes a type I-C CRISPR-Cas system. Mutations in *cas7* (*devR*), *cas5* (*devS*), and *cas8c* (*devT*) of this CRISPR-Cas system markedly impair sporulation, possibly by reducing the expression of FruA response regulator (Boysen et al., 2002; Thöny-Meyer and Kaiser, 1993; Viswanathan et al., 2007). The type III-B system of *M. xanthus* has also be implicated in fruiting body formation (Wallace et al., 2014). It is currently unclear

whether *M. xanthus* Cas proteins regulate sporulation independent of CRISPR or whether partial crRNA complementarity might guide these proteins to modulate the expression of sporulation genes. In contrast, it is thought that suppression of *P. aeruginosa* biofilm formation and swarming ability by its type I-F CRISPR-Cas system involves crRNA (Cady and O'Toole, 2011; Heussler et al., 2015; Zegans et al., 2009). Here, a model has been proposed in which partial complementarity between a crRNA and the lysogen induces nicking by Cas3 to produce ssDNA overhangs that recruit RecA. This leads to activation of the SOS response and de-repression of lysis genes. The detailed pathway has yet to be elucidated, but it is believed that in order to be disruptive, produced lysis proteins require an external signal, which is triggered by attachment of the cell to a surface. Hence, only biofilm-associated cells, but not planktonic cells, become lysed (Heussler et al., 2015).

Although CRISPR-Cas can attenuate virulence by preventing horizontal transfer of virulence and antibiotic resistance genes (Bikard et al., 2012; Jiang et al., 2013; Marraffini and Sontheimer, 2008), type II CRISPR-Cas systems are highly represented among pathogens (Fonfara et al., 2014) and have been shown to enhance virulence in several bacterial species (Louwen et al., 2013; Sampson et al., 2013). In *Francisella novicida*, a small CRISPR-associated RNA (scaRNA) forms a complex with tracrRNA and Cas9 that is described to trigger degradation of the mRNA of a bacterial lipoprotein (*blp*). Reduced levels of the lipoprotein in the bacterial membrane lead to decreased detection by the host immune system, thus increasing virulence of *F. novicida* (Sampson et al., 2013). In *C. jejuni*, deletion of Cas9 enhances antibody adsorption (Louwen et al., 2013). Furthermore, Cas9 promotes the invasiveness of *N. meningitidis* (Sampson et al., 2013). Although the further investigation of underlying mechanisms will be necessary, it is tempting to speculate that Cas9 might have a conserved role controlling the composition of the cell envelope in Gram-negative species. Modulation of virulence has also been reported for other CRISPR-Cas elements. For instance, the nuclease activity of Cas2 of the type II-B system of *Legionella pneumophila* is crucial for infection of amoebae (Gunderson and Cianciotto, 2013; Gunderson et al., 2015). Intriguingly, recent research uncovered similar regulatory functions displayed by further CRISPR types. The type I-F system of *P. aeruginosa* is able to digest the transcript of the quorum-sensing regulator LasR, resulting in an impaired immune response by the host (Li et al., 2016). Furthermore, a *L. monocytogenes* CRISPR transcript associated to type I-A (Mandin et al., 2007) enhances virulence by promoting expression of a ferrous iron transporter (Mandin et al., 2007; Toledo-Arana et al., 2009).

Although only a few examples have been reported so far, the involvement of CRISPR in processes beyond immunity is becoming increasingly apparent. With the discovery of novel CRISPR-Cas interference mechanisms (e.g., sole RNA targeting by type VI), new regulatory functions will likely be uncovered.

Conclusions and Future Directions

The CRISPR-Cas prokaryotic immune system is undoubtedly one of the most significant discoveries in modern microbiology. The efficiency and simplicity of CRISPR-Cas9 and other select interference mechanisms has been leveraged in powerful genome editing tools (Komor et al., 2017). However, research on the biology of CRISPR-Cas has revealed the true complexity and diversity of these prokaryotic immune systems. Although extensive research in the past decade has built a framework for our understanding of CRISPR-Cas, several questions regarding the very basics of these defense systems still await a comprehensive scientific explanation. While insight into the adaptation phase has rapidly accrued, our knowledge is limited to few CRISPR-Cas types. It will be interesting to see to what extent the mechanisms involved in protospacer selection and integration are conserved among different types. In particular, proteins like Cas4 and Csn2 have been implicated in adaptation, but the nature of their involvement remains elusive. The notable absence of Cas1 and Cas2 or the CRISPR array in some systems further suggests inter-system cooperation in species with multiple CRISPR-Cas loci (Koonin et al., 2017). Reminiscent of vertebrate immunity are reports of synergy between innate and adaptive prokaryotic defenses (Dupuis et al., 2013; Price et al., 2016; Swarts et al., 2015), as well as activation by small cyclic nucleotides (Kazlauskiene et al., 2017; Niewoehner et al., 2017). Moreover, the cooperation of CRISPR-Cas and toxin-antitoxin (TA) systems has been proposed (Koonin and Makarova, 2013). The dormant state induced by TA systems has the potential to decelerate viral reproduction and thus allow time for induction of a CRISPR-mediated immune response during a phage infection. In fact, Cas1 and Cas2 resemble TA modules and might have functioned as such prior to their role as a CRISPR integrase (Koonin and Makarova, 2013). It will be interesting to see whether these ancient roles might be preserved in some CRISPR-Cas systems. An expanding niche in CRISPR research that has highlighted the antagonistic coevolution of prokaryotes and phages and revealed strategies for the control of CRISPR-based technologies is the investigation of anti-CRISPR proteins (Rauch et al., 2017; Sontheimer and Davidson, 2017). The increasing availability of metagenome data is expected to reveal novel phage strategies to control CRISPR-Cas, as well as new CRISPR loci and Cas proteins, some of which might show unique modes of interference and regulation (e.g., the Csx27- and Csx28-mediated control of Cas13b RNase activity [Smargon et al., 2017]). Considering the momentum with which we enter the second decade of CRISPR, we anticipate that discoveries in the near future will bring us closer to holistic understanding of the intricacy and diversity of CRISPR-Cas biology both within and beyond its role in immunity.

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