HHS Public Access

Author manuscript

*Trends Genet*. Author manuscript; available in PMC 2020 June 01.

Published in final edited form as:

*Trends Genet*. 2019 June ; 35(6): 446–456. doi:10.1016/j.tig.2019.03.009.

# Three new C's for CRISPR: Collateral, Communicate, Cooperate

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## Abstract

Clustered regularly interspaced short palindromic repeats (CRISPR) loci and their associated (*cas*) genes provide protection against invading phages and plasmids in prokaryotes. Typically, short sequences are captured from the invader's genome, integrated into the CRISPR locus, and transcribed into short RNAs that direct RNA-guided Cas nucleases to the invader's nucleic acids for their degradation. Recent work in the field has revealed unexpected features of the CRISPR-Cas mechanism: (1) collateral, non-specific, cleavage of host nucleic acids, (2) secondary messengers that amplify the immune response, and (3) immune-suppression of CRISPR targeting by phage-encoded inhibitors. Here we review these new and exciting findings.

## Introduction

Born out of necessity for survival, the interplay between pathogens and their hosts drives the evolution of immune and counter-immune responses. Prokaryotic biology has provided us with some of the most interesting and useful exponents of this co-evolution. CRISPR-Cas systems provide acquired immunity towards viruses [1] and plasmids [2], and constitute a fascinating example of the bacteria vs phage arms race. Upon infection, a small fraction of the bacterial cells integrate a short sequence (30-40 base pairs) from the invader's genome into the CRISPR array [1]. These DNA fragments, known as spacers, are transcribed and processed into short CRISPR RNAs (crRNAs) [3-5] that associate with effector CRISPR-associated (Cas) proteins and guide them to the target phage or plasmid [6-9] using base-pair complementarity. Cleavage of the invader's nucleic acids by the crRNA-guided Cas nuclease abrogates infection. There is a great diversity of CRISPR-Cas systems, which are classified in different types (I-VI) and contain distinct effector Cas nucleases that target different nucleic acids (Box. 1) [10]. While all CRISPR types have in common the use of RNA-

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**Declaration of interests.** L.A.M. is a cofounder and Scientific Advisory Board member of Intellia Therapeutics, and a co-founder of Eligo Biosciences.

guided ribonucleoprotein complexes that recognize foreign invaders to trigger host defense, recent work has uncovered additional features for some of these types (Table 1).

## Collateral: Non-specific nucleic acid degradation by Cas nucleases

One of the fundamental features of CRISPR-Cas immunity is that it relies on programmable, sequence specific nucleases [8, 9, 11]. This property is the basis of the revolutionary genome editing techniques based on the specific DNA cleavage by Cas nucleases [12, 13]. However, recent work has demonstrated that nucleic acid cleavage is often not limited to the target specified by the crRNA, and non-specific cleavage may be providing or enhancing immunity within the bacterial cell.

Type VI CRISPR-Cas systems employ the single effector protein Cas13 (previously called C2c2), harboring two higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domains that were predicted to target RNA instead of DNA [14]. This was experimentally confirmed *in vivo*, both in *Escherichia coli* cells expressing Cas13a from *Leptotrichia shahii* [15], as well as in native *Listeria seeligeri* cells [16]. Surprisingly, when Cas13a targeted plasmid transcripts, a significant decrease in cell growth was observed [15, 16]. This phenotype suggested that Cas13a possesses a non-specific RNase activity that not only degrades target RNA, but also cellular transcripts (Figure 1A). In vitro studies showed that RNA targets complementary to the crRNA guide are cleaved by Cas13, but also trigger the degradation of non-specific RNA, with both activities dependent on the presence of the two HPEN domains of Cas13 [15, 17]. Structural studies have given further insight into this mechanism: the two HEPN nuclease domains are located on the surface of complex, removed from the crRNA:target RNA duplex, where they have access to RNA molecules not complementary to the crRNA [18]. Upon binding of the target RNA, the HEPN domains undergo a conformational shift that brings them together to form a catalytic site that can degrade non-target RNA in *trans* [19, 20]. The precise mechanism of *cis*-cleavage of the target RNA still remains unclear.

Cas9, the crRNA guided nuclease of type II CRISPR-Cas systems that is widely used for gene editing [12, 13], creates double-stranded DNA (dsDNA) breaks at the target site using two DNA-cutting domains: RuvC and HNH [8, 9]. Cas12 (previously known as Cpf1), the effector nuclease of type V systems, is a close relative of Cas9 that uses a single RuvC domain to introduce staggered dsDNA breaks [21]. Recently, it was found that Cas12a also degrades non-specific single-stranded DNA (ssDNA) upon crRNA-mediated, specific, binding of either ssDNA or dsDNA (Figure 1B) [22, 23]. Furthermore, collateral ssDNA degradation seems to be broadly conserved across type V effectors, such as Cas12i and Cas12g, the latter being able to recognize and cleave RNA in a sequence specific manner, an event that also triggers collateral ssDNA and RNA degradation [24]. Cas14, a more distant relative of Cas12, recognizes only ssDNA targets *in vitro* to engage in non-specific ssDNA destruction [25]. Structural studies of different type V effectors have shown that both the target and non-target strand of a specific dsDNA substrate can occupy the RuvC domain, thus implicating the RuvC domain in the cleavage of both strands [26-28]. More recently, FRET and cryo-EM experiments demonstrated that Cas12a undergoes a series of checkpoints during target binding that culminates in exposure of the RuvC domain, which

initially cleaves the unwound dsDNA target by first cutting the non-target strand, then the target strand, and subsequently remains activated, allowing for indiscriminate ssDNA cleavage [29, 30].

Type III CRIPSR-Cas systems are notable for their complex mechanism, which requires transcription of the target DNA for immunity [31, 32] and includes non-specific degradation of both ssDNA and RNA molecules (Figure 1C). The Csm (type III-A) or Cmr (type III-B) multi-subunit effector complexes use the crRNA guide to bind a target transcript [33-35]. Target recognition triggers the activities of two different domains of Cas10, the signature subunit of type III CRISPR systems. First, activation of the HD domain results in non-specific ssDNA degradation [33-35]. However, the ssDNA degraded is confined to the vicinity of the target sequence [7], presumably because it is part of the transcription bubble formed after RNA polymerase synthesizes the target RNA. Second, activation of the Cas10 Palm domain converts ATP into cyclic oligoadenylates of 4 or 6 units (cOA) [36-39]. This second messenger is bound by Csm6 (for type III-A systems, Csx1 in other type III subtypes) and activates its non-specific RNase activity (see below). Finally, target recognition by the type III effector complexes triggers the cleavage of the RNA complementary to the crRNA by the Csm3 (type III-A) or Cmr4 (type III-B) subunits of the complex [40-42], causing the inactivation of both the HD and Palm domains of Cas10 [35], an event that limits the possible toxic effects of the collateral nucleic acid degradation carried on by type III systems.

Similarly to the specific target cleavage of Cas nucleases, the non-specific degradation of nucleic acids is important for both the CRISPR-Cas immune response and for the development of biotechnological tools. Regarding bacterial immunity, collateral destruction of RNA can provide a secondary layer of defense. This seems to be the function of Csm6, which enhances the type III-A CRISPR-Cas immune response against “difficult” targets. It has been shown that during type III-A immunity against plasmids, Csm6-mediated degradation of both host and plasmid transcripts leads to a growth arrest that is essential for the clearance of plasmids harboring poorly transcribed targets, which are not efficiently cleaved by the ssDNA activity of Cas10 [43]. In the case of the type III-A anti-phage response, Csm6 activation is required for efficient immunity against late-expressed or mutated targets, both of which prevent efficient clearance of the phage [44]. Presumably, by the time late-expressed genes are transcribed, the phage genome has switched to rolling circle replication for the packaging of the concatemeric genomes [45], a process that interferes with transcription and thus with the targeting by the Csm complex. In the case of mutated targets, it is hypothesized that mismatches between the crRNA and the target RNA affect the activation of Cas10’s HD, but not Palm, domain.

Although not clear yet, the indiscriminate transcript degradation promoted by Csm6 could affect the availability of both host- and plasmid/phage-derived factors that are important for the replication of these mobile genetic elements. The growth arrest generated during plasmid targeting is temporary and the eventual disappearance of the target DNA, for which the Cas10 ssDNA degradation activity is required, allows the cells to recover [43]. It would be interesting to determine if and how type VI systems use the collateral RNA degradation produced by Cas13 to provide immunity (see Outstanding Questions). It is conceivable that

in the absence of specific target DNA degradation, the constant synthesis of the target RNA could lead to cell death instead of the growth arrest observed for type III-A CRISPR-Cas immunity [46]. In such cases, and perhaps also in other CRISPR types where the collateral host nucleic acid degradation is irreversible, the CRISPR-Cas immune response could operate in a manner similar to other immune mechanisms involving programmed cell death, such as abortive infection and toxin-antitoxin systems [47], where the elimination of the infected cells stops the spread of the invader to protect the uninfected population. Future studies of the environmental and selection forces that favor CRISPR-mediated collateral degradation of nucleic acids (and its associated cell death) over specific targeting CRISPR functions will allow us to understand the diversification of these immune systems.

Little is known about the role of non-specific ssDNA degradation in the CRISPR-Cas immune response. In the case of type III-A immunity, the non-specific ssDNA degradation performed by Cas10 seems to be the core, rather than an accessory activity of this system. Several observations support this idea: (i) an intact HD domain is absolutely required for efficient type III-A CRISPR-Cas immunity, even when Csm6 is activated [43]; (ii) as already noted, the degradation is restricted to the transcription bubble [7] and (iii) Cas10 activation does not trigger a growth arrest [43]. The role of the non-specific ssDNA degradation produced by type V systems is not known. Although this activity has yet to be studied in the context of bacterial defense, different functions can be hypothesized. One possibility is for protection against ssDNA phage infection [48]. Another function could be the degradation of the ssDNA intermediates generated during DNA repair or rolling circle replication of phages and plasmids [49]. It is not known if Cas12 and Cas14 can affect the host's ssDNA and cause cellular toxicity, and if this is the case, whether their activities can be regulated to prevent cell death (see Outstanding Questions). Experiments designed to map the extent of ssDNA degradation *in vivo* will be essential in assessing its relevance during the CRISPR-Cas immune response and in determining whether the Cas nucleases can compete with endogenous proteins that typically bind these ssDNA intermediates, such as RecA and Ssb [50].

Regarding biotechnological applications, indiscriminate nucleic acid destruction allows the amplification of a signal generated from the detection of low levels of specific nucleic acid targets. The Cas nucleases use the crRNA to query a given sample for the presence of a specific RNA or DNA sequence (from an infecting virus or bacteria, or a particular allele in human DNA, for example). If found, the target nucleic acid will activate the non-specific activity of the Cas nuclease, which then will degrade a reporter nucleic acid. The technology is simple, rapid, and very sensitive, allowing the detection of specific sequence variants present at attomolar concentrations. For Cas13a and Csm6, a method called SHERLOCK (Specific High-Sensitivity Enzymatic Reporter UnLOCKing) has been developed to detect low levels of DNA (after it is transcribed) and RNA viruses [51, 52]. For Cas12a and Cas14, DETECTR (DNA Endonuclease-TargetEd CRISPR Trans Reporter) can identify single-nucleotide polymorphisms in human and viral DNA [22, 25]. In addition, the *cis* RNA cleavage of Cas13a has been repurposed for the knockdown of specific transcripts in plants and mammals [53, 54]. Notably, the collateral cleavage generated by Cas13a's *trans* activity has not been detected in these eukaryotic cells. It is unknown if the non-specific RNA substrates are not accessible or the trans RNA degradation of Cas13a is regulated in these

hosts; either of these scenarios would have interesting biological and technological implications.

## Communicate: secondary messenger signaling in CRISPR immunity

Secondary messenger signaling plays important roles in eukaryotic immune signaling, best demonstrated in the cGAS-STING DNA sensing pathway [55] and the 2'-5' oligoadenylate synthetase/RNaseL pathway [56]. The generation of a secondary messenger is particularly well suited for the response to an invading pathogen, as it allows for rapid production and amplification of a danger signal in the cell. A series of bioinformatic studies coupled with early genetic and structural work proposed the existence of such a signaling pathway during type III-A CRISPR-Cas immunity. As previously mentioned, these systems harbor Csm6, an accessory RNase with an HEPN domain [57]. Early experimental work demonstrated that this nuclease does not directly associate with the Csm complex, but it is essential for anti-plasmid immunity [58]. Bioinformatic and structural analysis of Csm6 revealed the presence of a second domain, CARF (CRISPR-Associated Rossman Fold) [59, 60], with the predicted ability to bind dinucleotides that could function as second messengers during the type III CRISPR-Cas immune response [61]. Two studies [37, 38] found that upon binding to a target RNA, the Palm domain of Cas10 (typically found in polymerases and nucleotide cyclases [62]) within the Csm complex generates cyclic oligoadenylates (cOA) of 4-6 subunits (Figure 1C). In turn the cOAs are bound by the CARF-domains of Csm6 (a dimer) to license its HEPN-dependent RNase activity. In vivo, it was found that individual HEPN or palm domain mutants phenocopy the double mutant in anti-phage type III-A immunity [38], demonstrating that both domains are part of the same genetic pathway. More recently a similar pathway has been shown to be in place for the activation of the Csx1 RNase by the Cmr complex in other type III systems [36, 39], proving that second messenger signaling is a property of different type III CRISPR-Cas systems.

Due to the toxicity of the global RNA degradation triggered by Csm6/Csx1 activation, it would be desirable to regulate the cOA second messenger pathway. As mentioned above, the cleavage of the target transcript deactivates both the HD and Palm domains of Cas10 [35]. While this stops the production of cOA, it would not deactivate the Csm6/Csx1 molecules that remain bound to the second messenger. A solution for this problem has been found in *S. solfataricus*, where ring nucleases that contain catalytic CARF domains are able to cleave cOA substrates *in vitro* [63]. Notably, these are not associated with type III CRISPR-*cas* loci and are not universally present in other prokaryotes harboring these immune systems, suggesting that different modes of Csm6/Csx1 deactivation likely remain undiscovered (see Outstanding Questions).

Finally, it is possible that second messenger pathways play diverse roles during CRISPR immunity. Bioinformatic searches have found a wide variety of CARF-domain encoding proteins associated with type III loci. These domains are typically fused to a multitude of effector domains, including various nucleases and proteases [59, 64, 65]. For example, some CARF domains are fused to transmembrane domains with homology to Lon proteases, raising the possibility of intracellular localization of CRISPR function or, more intriguingly, an extracellular effect of type III CRISPR activation [65]. Also, transcription factors

containing CARF proteins have been found to transcriptionally control different host genes, among them type I loci [66, 67], suggesting that type III immunity may regulate other cellular pathways, including the CRISPR defense by other systems (see Outstanding Questions). It is also likely that secondary messenger signaling is not limited to the cOA pathway. Particularly interesting are the WYL domain proteins that contain a ligand binding groove related to Sm-like SH3 β-barrels [59]. These proteins have already been shown to be important in the transcriptional control of both type I and type VI CRISPR systems [68, 69], but it remains to be determined if they are under control of a ligand and if so, what the nature of that ligand is.

## Cooperate: the mechanism of CRISPR inhibitors

The arms race between host and virus results in constant evolutionary pressure to maintain a balance between the immune response and viral infectivity. With the exception of type III systems, which provide robust immunity even in the presence of multiple target mutations [70], single-nucleotide changes of the target sequence can abrogate the CRISPR immunity provided by a particular spacer [71]. To neutralize these “escapers”, the CRISPR-Cas immune response mediates the acquisition of multiple different spacer sequences that collectively protect the population, rather than a single host cell [72]. To counteract this, phages have evolved inhibitors of CRISPR targeting that can overcome the immunity of not one, but all spacer sequences. Since the initial discovery of anti-CRISPRs (Acrs) [73], many inhibitors have been identified that antagonize type I [74-77], type II [78-83] and most recently type V [84, 85] CRISPR-Cas systems.

Although Acrs are extremely diverse in protein sequence, they fall into two different functional groups that block either target binding or target cleavage. The first class includes inhibitors AcrIF1, AcrIF2, and AcrIF10 that inhibit the type I-F CRISPR-Cas system in *Pseudomonas aeruginosa* (Figure 2A). Type I loci encode a complex, known as Cascade, that holds the crRNA guide to locate and bind a complementary target [3]. Target recognition recruits the Cas3 nuclease to cleave the DNA [86]. Biochemical and structural studies have shown that AcrIF1 binds the backbone subunit of the type I-F Cascade complex, Cas7f, locking the complex into a confirmation that occludes the binding of dsDNA by the crRNA [87-89]. AcrIF2 acts a dsDNA mimic and binds the foot of the type I-F Cascade complex between the Cas7F and Cas8f subunits, blocking the entrance of dsDNA into the CRISPR complex [87-89]. A third inhibitor of type I-F CRISPR immunity, AcrIF10, binds the Cas5f and Cas8F Cascade subunits causing a conformational change that blocks dsDNA recognition [88]. Type II-C inhibitor AcrIIC3 [78], causes Cas9 dimerization that prevents the binding of dsDNA (Figure 2B) [90, 91]. Type II-A inhibitors AcrIIA2 and AcrIIA4 [79], while binding in different arrangements, prevent the recognition of the protospacer adjacent motif (PAM), a sequence located downstream of the target required for Cas9 DNA cleavage [92, 93]. Lastly, AcrIIC2 forms a dimer and binds the bridge helix of Cas9 and inhibits the interaction of Cas9 with the crRNA, thereby preventing the binding of dsDNA targets [91]. The second class of CRISPR inhibitors allows binding of the target DNA but prevents its cleavage. The type I-F inhibitor AcrIF3 binds to Cas3 to inhibit its recruitment to the target DNA by Cascade [89]. Structural studies indicated that an AcrIF3 dimer obstructs the DNA channel for non-target strand DNA entrance into the Cas3

nuclease, while also blocking a linker important for the interaction of Cas3 with the type I-F Cascade complex [94]. The type II-C inhibitor AcrIIC1 [78], binds the HNH domain of Cas9 to prevent the cleavage of the DNA strand complementary to the crRNA, while also blocking the conformational shift needed for RuvC-mediated cleavage of the non-complementary strand [90].

Although *in vitro* work allowed for the elucidations of the molecular mechanism of inhibition of most Acrs, their mode of inhibition *in vivo* has only recently been addressed. Since none of the inhibitors described so far are injected along with the phage genome, *acr* genes need to be transcribed and translated before they can inhibit CRISPR targeting. Therefore, the timing of their inhibition has been a major question: how can they prevent cleavage within a host that has an already established CRISPR machinery ready to attack the phage genome immediately following injection? Two studies [95, 96] solved this conundrum by showing that the Acrs actually do not prevent complete destruction of the virus, as previously hypothesized. Instead, Acrs partially suppress CRISPR immunity, leaving the surviving hosts much less ready to attack the next wave of infecting phages (Figure 2C). The cooperation of multiple phages that sequentially infect the host is critical for inhibition, with each round of infection leading to additional immunosuppression of the CRISPR-Cas response. As a consequence of this mode of action, the success of the Acr-harboring phage depends on the multiplicity of infection, the strength of the Acrs (a combination of their level of expression and affinity for the Cas protein they inhibit) and the strength of the initial CRISPR response (also a combination of their level of expression and targeting efficiency).

Finally, although only inhibitors of specific CRISPR targeting mechanisms have been found so far, a more general obstruction of the CRISPR-Cas immune response could be equally and even more beneficial for the phage. For example, recently AcrVA3.1 was identified as a broad-spectrum inhibitor with the ability to block both type I-C and type VA targeting [84], and therefore its mechanism of action is intriguing. It is interesting to speculate that phages may contain inhibitors that act either upstream (preventing spacer acquisition) or downstream (repairing the cleaved nucleic acids) of the targeting event (see Outstanding Questions). Most likely, the strong forces of evolution in place during the arms race between prokaryotes and their parasites not only forged the extreme diversity of CRISPR-Cas systems, but also an immense assortment of CRISPR inhibitors that are yet to be discovered.

## Concluding remarks

The original, groundbreaking, work in the CRISPR field identified a straightforward relationship between invaders and the CRISPR immune system, where CRISPR spacers are acquired, foreign nucleic acids are cleaved, and the infected cells are protected. Recent advances have added nuance to this paradigm by increasing the complexity of the interaction between the CRISPR immune system and both the phage and the host cell (Table 1). As understanding of these interactions expands, a more complete and dynamic view of the CRISPR immune response is emerging.

## Acknowledgements.

AV is supported by the Arnold O. Beckman Postdoctoral Fellowship. LAM is supported by the Rita Allen Scholars Program and an NIH Director's Pioneer Award (DP1GM128184-01). LAM is an investigator of the Howard Hughes Medical Institute.

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**Box. 1**

### CRISPR-Cas systems

CRISPR-Cas systems are divided into 2 main classes, determined by the composition of their effector subunits [10, 97]. Class 1 effectors are multi-subunit complexes, and include types I, III, and IV. Although these types share little sequence homology, they have an overall conserved architecture. Repeating subunits of Cas proteins create a protein backbone, with distinct Cas proteins located at the head and foot of the crRNA complex to form the intact effector unit. Type I effector complexes recognize double-stranded DNA (dsDNA), and mediate cleavage of DNA via recruitment of nuclease/helicase protein, Cas3. The type III complex recognizes RNA and carries out a multitude of effector functions once activated. The Cas10 subunit cleaves ssDNA that is being transcribed and activates the non-specific-RNase activity of Csm6, while the Csm3 cleaves the RNA target. Lastly, type IV systems lack the components for spacer acquisition and their function remains undetermined. Class 2 effectors are composed of a single Cas unit that associates with the crRNA to carry out targeting. Due to their relative simplicity, they have been widely exploited for biotechnological applications.

This class includes CRISPR types II, V, and VI. Type II uses Cas9 to recognize and cleave dsDNA. The majority of type V CRISPR systems can also recognize dsDNA targets and create double-stranded breaks, although through a different mechanism than by Cas9. Lastly, Type IV is unique in that it recognizes RNA targets, which subsequently activates a non-specific RNase activity.

## Outstanding Questions

- Is the non-specific degradation of nucleic acids a new mode of CRISPR defense based on the death of the host? Can spacers be acquired in such scenario?
- Are there additional mechanisms to turn off secondary messenger signaling during type III CRISPR-Cas immunity?
- Do the type III CRISPR secondary messengers (cOAs) activate other pathways within the host?
- Do phages harbor global inhibitors of the CRISPR-Cas response; i.e., that inhibit spacer acquisition or enhance phage DNA repair?
- Is the immense diversity of CRISPR-Cas systems a result of their need to overcome anti-CRISPR inhibitors?

## Highlights

- The core feature of CRISPR-Cas systems is the use of RNA-guided Cas nucleases that use a short RNA to locate and cleave complementary nucleic acids.
- Recent studies uncovered features that add substantial complexity to this central mechanism.
- Upon crRNA-guided recognition of complementary nucleic acids, many CRISPR-Cas systems also degrade non-target RNA or ssDNA.
- The RNA-guided recognition of target RNA molecules triggers the production of cyclic oligoadenylate, a secondary messenger that activates the non-specific degradation of RNA during type III CRISPR-Cas immunity.
- Many inhibitors of Cas nucleases have been found in phage genomes, which require the cooperative infection of viruses to gradually suppress the CRISPR-Cas immune response.

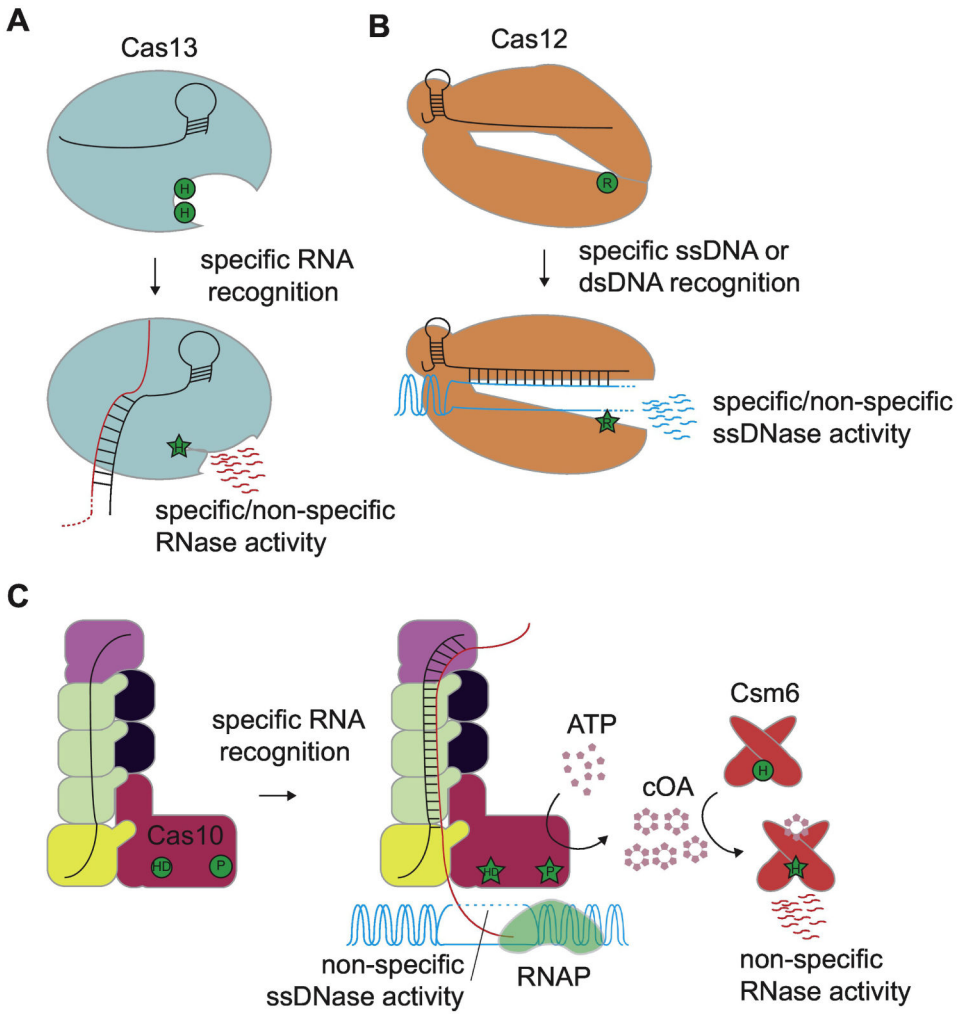


**Figure 1. Collateral nucleic acid degradation by Cas nucleases and CRISPR secondary messenger signaling.**

**(A)** RNA targeting by the type VI effector Cas13a. Cas13a possesses two HEPN domains (green circle, H). Binding of target RNA (red line) to the crRNA (black line) induces a conformational shift that brings the domains in close proximity to form an active HEPN site (green star). The active site then cleaves RNA in a sequence-independent manner. **(B)** Nucleic acid targeting by the prototypical type V effector protein, Cas12a. The RuvC domain (green circle, R) is responsible for the nucleolytic activity of Cas12a. Before target recognition, this domain is obscured by the Rec lobe. Upon binding of either double-stranded DNA (dsDNA) or single-stranded DNA (ssDNA) targets (blue lines) to the crRNA (black line), the effector undergoes a conformational shift that exposes and activates the RuvC domain (green star) to license cleavage of the target DNA and non-specific cleavage of ssDNA. **(C)** Nucleic acid targeting and secondary messenger signaling by the type III-A CRISPR-Cas system. Upon recognition of a target transcript (red line) to the crRNA (black line), the type III complex activates multiple effector functions. The HD domain of the Cas10 subunit (green circle, H) is able to non-specifically cleave ssDNA (blue line) from the transcription bubble. The Palm domain of Cas10 (green circle, P) is also activated and

converts ATP into 4 or 6 member rings of cyclic oligoadenylates (cOA). cOA then acts as a secondary messenger and activates Csm6 by binding to its CARF domain causing activation of the HEPN domain (green circle, inactive, or green star, active; H), unleashing non-specific RNA cleavage. Additionally, not depicted, a subunit of the type III complex, Csm3, is a specific RNase that cleaves the bound transcript.

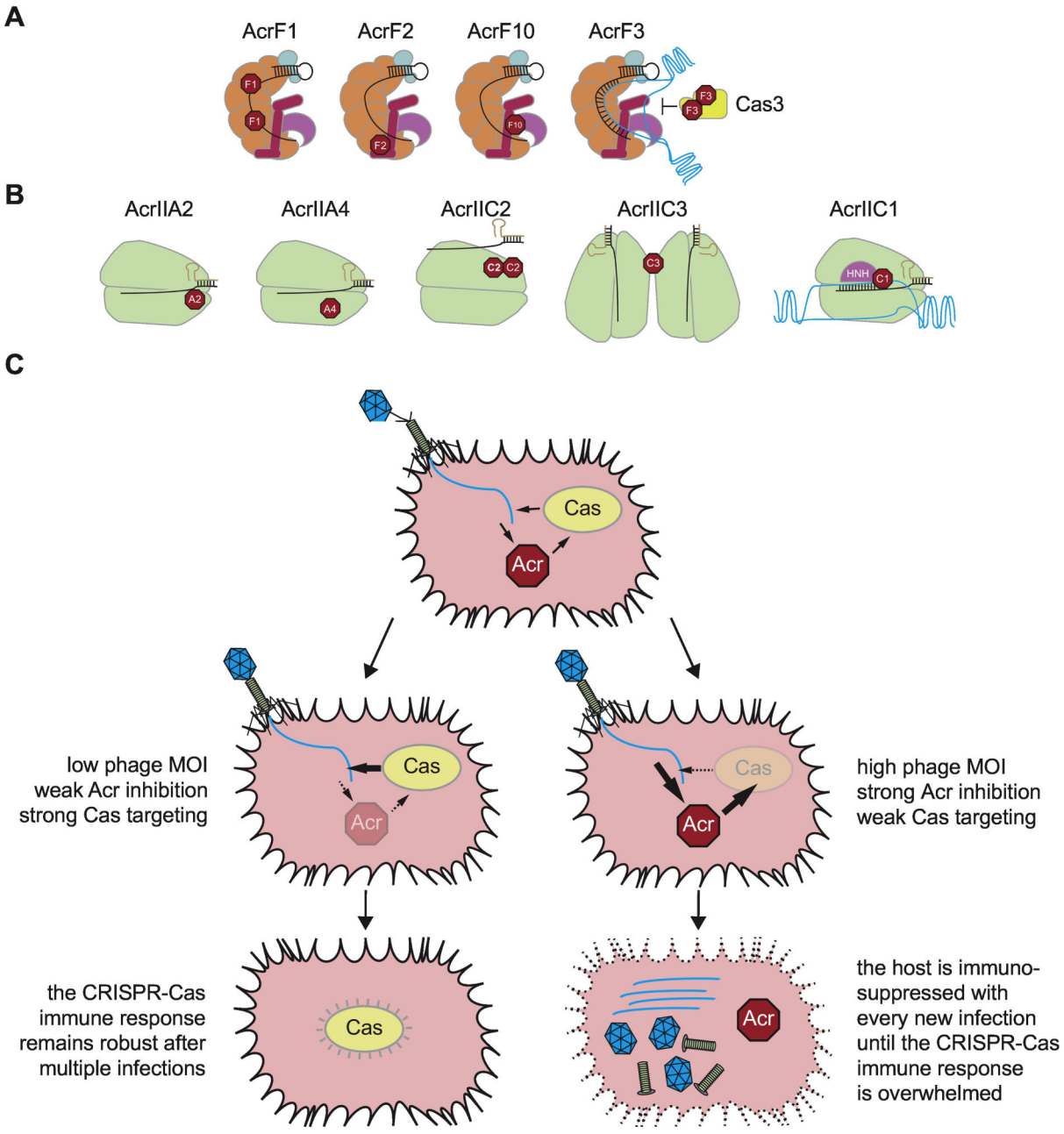


**Figure. 2. Mechanisms of phage CRISPR inhibitors.**

**(A)** CRISPR inhibitors of type I-F systems. The first class of inhibitors bind to the type I-F Cascade complex and prevent the binding of dsDNA. AcrIF1 binds the between Cas7f subunits at regular intervals, locking the complex in a formation that cannot bind dsDNA. AcrIF2 binds between the Cas7f and Cas8f subunits and AcrIF10 binds between Cas8f and Cas5f. Both inhibitors prevent the entry of DNA into the targeting complex. After the crRNA within Cascade (black line) has bound to its DNA target (blue line), the complex recruits Cas3, a DNase that degrades the target DNA. AcrIF3 forms a dimer that prevents recruitment of Cas3 to the targeting complex and blocks DNA entry into the Cas3 nucleolytic site. **(B)** CRISPR inhibitors of type II systems. AcrIIA2 and AcrIIA4 are

inhibitors of the type II-A Cas9 nuclease. During target search Cas9 scans DNA for protospacer adjacent motif (PAM), which recognition is the first step in licensing DNA cleavage. AcrIIA2 and AcrIIA4 both bind the portion of Cas9 that recognizes the PAM, thus blocking DNA loading into the nuclease. AcrIIC2 binds the bridge helix of Cas9 to interfere with crRNA/tracrRNA binding. AcrIIC3 is a type II-C Cas9 inhibitor that causes Cas9 dimerization and also prevents DNA loading. AcrIIC1 binds the HNH domain, preventing cleavage of the target strand of DNA, along with blocking the conformational shift required to license RuvC-mediated cleavage of the non-target strand. **(C)** Phage cooperation is required for effective CRISPR inhibition by Acrs. Of the currently identified Acrs, none are injected during phage infection, indicating they must be transcribed and translated post-injection. Therefore, the host is immuno-suppressed with every infection wave. The success of inhibition depends on three factors: the multiplicity of infection (MOI), the strength of the Acr and the strength of CRISPR targeting.

**Table 1.**

Features of CRISPR-Cas systems

| CRISPR type | Specific cleavage | Collateral cleavage | Secondary messenger | CRISPR inhibitors |
|---|---|---|---|---|
| I | DNA | no | no | yes |
| II | DNA | no | no | yes |
| III | RNA | RNA, ssDNA | cOA | no |
| IV | - | - | - | - |
| V | DNA, RNA | ssDNA | no | yes |
| VI | ? | RNA | no | no |

"?": not clear yet

"-": not studied yet

"no": not identified to date

"cOA": cyclic oligo-adenosine