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# How bacteria control the CRISPR-Cas arsenal

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CRISPR-Cas systems are adaptive immune systems that protect their hosts from predation by bacteriophages (phages) and parasitism by other mobile genetic elements (MGEs). Given the potent nuclease activity of CRISPR effectors, these enzymes must be carefully regulated to minimize toxicity and maximize anti-phage immunity. While attention has been given to the transcriptional regulation of these systems (reviewed in [1]), less consideration has been given to the crucial post-translational processes that govern enzyme activation and inactivation. Here, we review recent findings that describe how Cas nucleases are controlled in diverse systems to provide a robust anti-viral response while limiting auto-immunity. We also draw comparisons to a distinct bacterial immune system, restriction-modification.

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## Introduction

Bacteria face immense predation from bacteriophages (phages), generating a strong need for a diverse and effective panel of immune processes [2]. It has been estimated that approximately $10^{23}$ bacteriophage infections per second occur in the ocean [3,4], Pathways such as abortive infection [5,6], restriction modification [7,8], and CRISPR-Cas [9,10] can provide robust immunity against these pathogens. A common thread that weaves through these and other immune pathways is the need to precisely regulate the activity of the enzymes that enable function to prevent auto-immunity.

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (*cas*) genes constitute the only discovered 'adaptive' immune system in prokaryotes. These are categorized into two broad classes, six types (I–VI) and further subtypes [11]. Despite their diversity, these defense mechanisms show remarkable overlap. Immunological memory resides in the CRISPR array, a series of alternating repeats and spacers, with spacer sequences derived from foreign genetic elements in a process known as adaptation. The array is transcribed and processed to generate unit sized CRISPR RNAs (crRNA), which form a complex with one (Class 2, Types II, V, VI) or multiple (Class 1, Types I, III, IV) CRISPR-associated (Cas) proteins [12–16]. During interference, invading nucleic acids are recognized and destroyed via complementary base pairing between the crRNA and the foreign nucleic acid. The simplicity and programmability of DNA-encoded, RNA-guided nucleases has generated revolutionary prokaryotic [17] and eukaryotic [18] gene editing technologies utilizing the Class 2 Cas enzymes.

CRISPR-Cas immunity requires numerous RNA and DNA cleaving enzymes at all stages of function, to support this nucleic acid-based immune system. Nearly every core Cas protein superfamily has members that have been shown to possess DNA or RNA-targeting nuclease activity (Table 1). Many bacterial strains possess multiple Cas nucleases, which generates a potentially cytotoxic consequence to maintaining CRISPR-Cas. Therefore, the need for control and fine-tuned specificity is significant. The requirements for *in situ* control, regulation, localization, activation, and ultimately function are only starting to be understood with transcriptional-level regulation reviewed in [1]. Here, we summarize how microbes use post-translational regulation to activate, inactivate, and fine-tune Cas nucleases.

## Spacer acquisition

The ability to generate heritable immunity by acquiring fragments of invading DNA as new CRISPR spacers is a hallmark of CRISPR-Cas immunity. This activity requires the two most highly conserved *cas* genes, *cas1* and *cas2* [38,39]. This process must be highly selective, as acquiring new CRISPR spacers from the bacterial genome will lead to toxic 'self-targeting', as there is no intrinsic mechanism to tell self from non-self. A mechanism for avoiding CRISPR array cleavage is in place, however, as Types I and II systems require a 2–5 bp protospacer adjacent motif (PAM) that is recognized by Cas proteins and is not present in the chromosomal

**Table 1**

**CRISPR-Cas nucleases and associated substrates**

| Protein | Substrate | Process | Reference |
|---|---|---|---|
| Cas1 | dsDNA | Acquisition | [19] |
| Cas2 | ssRNA, ssDNA | Acquisition | [20,21] |
| Cas3 | ssDNA | Interference/acquisition | [22–24] |
| Cas4 | ssDNA | Acquisition | [25] |
| Cas5 | ssRNA | Biogenesis | [26] |
| Cas6 | ssRNA | Biogenesis | [16] |
| Csm6 | ssRNA | Interference | [27] |
| Cas7[a] | ssRNA | Interference | [28••,29,30] |
| Cas9 | dsDNA | Interference/acquisition | [31,32] |
| Cas10 | ssDNA | Interference | [28••,33•] |
| Cas12 | dsDNA, ssRNA | Processing/interference | [34,35•] |
| Cas13 | ssRNA | Processing/interference | [36,37•] |

[a] Cas7 family members from Type III systems include Csm5, Csm3, Cmr3, Cmr6 [38].

CRISPR array. Remarkably, Cas1 and Cas2 have been shown to preferentially incorporate foreign DNA into the CRISPR array [39,40••]. This trait appears to be explained by the enhanced frequency with which DNA fragments are generated from foreign DNA during replication initiation, stalls, double stranded breaks, and attack by host nucleases such as RecBCD [40••]. Additionally, DNA ends are exposed during phage injection, which was recently shown to bias spacer acquisition toward phage genome ends [41•]. Another hallmark of CRISPR immunity is the chronological order with which spacers are acquired, with new spacers appearing adjacent to the promoter-containing leader sequence. While the process of acquisition *per se* appears to be regulated only by the availability of free DNA and an 'acceptor' repeat sequence, the *Escherichia coli* integration host factor (IHF) was identified as being an instrumental regulator of the accurate insertion of a new spacer proximal to the leader [42•,43,44•].

Cas1 and Cas2 are the only Cas proteins required for naïve spacer acquisition *in vivo* [39,40••] and *in vitro* [45]. The most potent positive regulator of the acquisition machinery, however, is the CRISPR surveillance complex itself (Figure 1). Through a mechanism known as priming spacer acquisition [46], the recognition of a mismatched target triggers a positive feedback loop that stimulates new spacer acquisition from sequences flanking the initial target [46,47]. Moreover, when Cas3-mediated targeting takes place, the generated fragments are suitable substrates for spacer acquisition, again acting as a regulatory layer to reinforce the future targeting of *bona fide* foreign DNA [48,49•]. In Type II systems, spacer acquisition does not occur in the absence of Cas9, a process that ensures that DNA sequences acquired contain the correct PAM [50,51]. In sum, spacer acquisition is controlled post-translationally at multiple levels; by exposed DNA ends, repeat and leader sequences, IHF (in *E. coli*), and the effector machinery itself. These factors must operate in concert to both avoid a high rate of self-spacer acquisition, incorporate phage spacers in the appropriate location for downstream deployment, and to respond to mutant phages.

## crRNA biogenesis

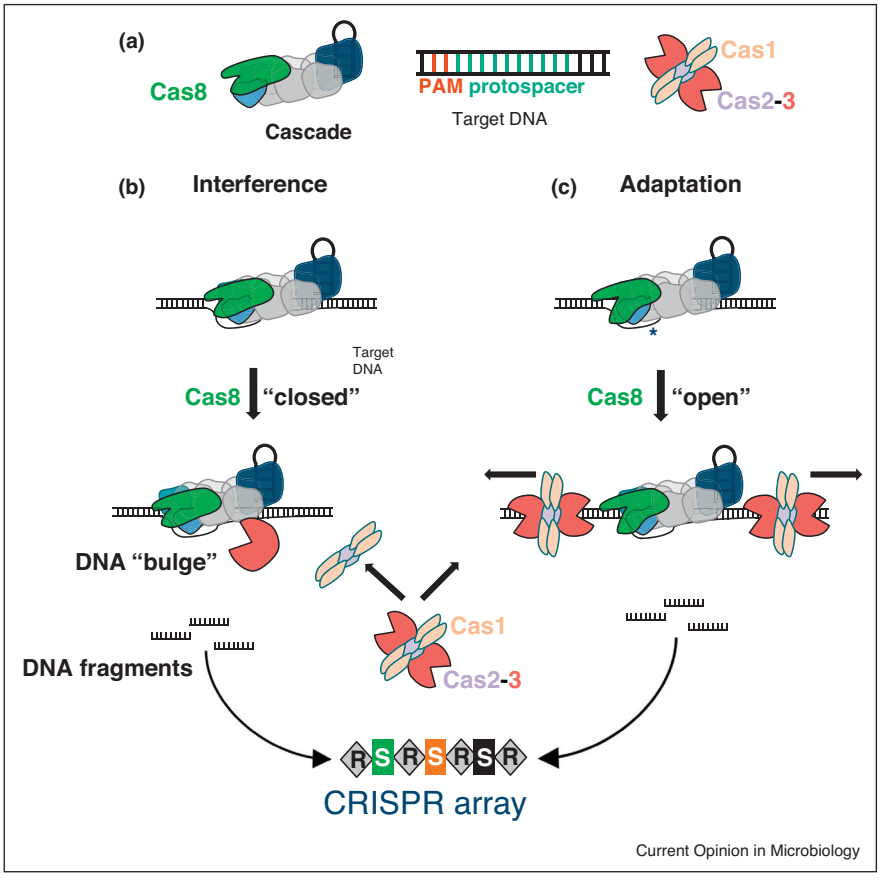
To generate surveillance complexes, the pre-crRNA transcript is cleaved to generate single crRNA units, a process that has now been attributed to multiple nucleases in their respective class and subtype, including Cas6 (Types I, III) [13,16,52], Cas5 (Type I-C) [26,53], RNAse III (Type II) [15], Cas 12 (Type V) [35•], and Cas13 (Type VI) [54]. Endoribonuclease specificity is dictated by the sequence and secondary structure of the CRISPR repeat. This structure is generated *in cis* for Types I, III, V, VI, due to the palindromic nature of the repeats, and *in trans* by the tracrRNA in Type II systems. While no further regulatory layers have been described for these ribonucleases, it remains to be seen whether other cellular RNAs with sequences that meet the rules for binding and cleavage are substrates for processing by these enzymes. Future work should consider whether unidentified proteins or factors play a role in directing Cas6 to the CRISPR repeat sequences, the same way that IHF directs Cas1-2 to the first repeat in the array. Exerting control over non-crRNA processing could be a particularly important consideration for heterologous expression of CRISPR-Cas systems.

## Interference (Type I)

Type I systems encode multi-protein surveillance complexes that, upon target recognition, recruit the *trans*-acting nuclease-helicase, Cas3, to degrade the target. To avoid general Cas3 toxicity, an envisioned strategy would necessitate the detection of a *bona fide* target for nuclease activation. Such targets have two traits, a PAM that is recognized by the large subunit in the surveillance complex, and near perfect identity between the DNA target and the crRNA sequence. Recent work has put forward models in two different Type I systems (I-E and I-F) that are consistent with localized activation of the Cas3 nuclease during target recognition, described below (Figure 1).

Type I-E: The large subunit of Cascade (CRISPR-associated complex for antiviral defense), Cas8, recruits Cas3 through the conformational changes it adopts when faced with a candidate target [55,56••]. As the surveillance complex samples sequences, Cas8's domains assume various conformations allowing it to exist in dynamic equilibrium between 'open' and 'closed' states, driving distinct Cas3 targeting outcomes (i.e. interference or priming). In the 'closed' conformation, Cas3 is recruited for DNA cleavage whereas in the 'open' conformation Cas3 is preferentially recruited in complex with Cas1 and Cas2, where together they mediate new spacer acquisition. In this model, a target bearing mismatches is a good substrate for spacer acquisition but will not necessarily trigger degradation. Similarly, positioning of the target

**Figure 1**




Type I activation and regulation. **(a)** The key players in Type I interference: Cascade (with Cas8 in green), target DNA with PAM highlighted in red and protospacer in teal, and the Cas1-2-3 protein complex. **(b)** During interference, Cascade initiates DNA binding by recognizing the PAM and target sequence. Cas3 (orange enzyme) recruitment is dependent on a 'closed' Cas8 (green protein) conformation and a non-target DNA strand 'bulge' which together signal a *bona fide* target. Upon recruitment, Cas3 likely sheds Cas1 and begins DNA cleavage and translocation in the 3′→5′ direction. **(c)** In the event of a mismatch (*) between the crRNA and target, Cas8 is in an 'open' conformation. Cas3 is recruited but does not dissociate from Cas1. The holoenzyme scans DNA bi-directionally for new spacers. In both panels, DNA fragments can be incorporated into the CRISPR array (repeats in gray diamonds) as new spacers (multi-colored rectangles).

DNA on the CRISPR complex, as well as DNA topology itself, may also influence Cas3 activity. A recent structure of a DNA-bound complex illustrates that the non-target strand has 'slack' in a displaced bulge that is necessary for Cas3 cleavage activity [57••]. While the distended DNA is not required for Cas3 to interact with the surveillance complex, it does enhance nucleolytic processivity, indicating its role in regulating Cas3 enzymatic activity.

Type I-F: Acquisition machinery can serve as a repressor of Cas3 activity. In Type I-F systems, a single gene encodes a fused Cas2-Cas3 protein [38]. Recent work has shown that the *Pseudomonas aeruginosa* Cas2-3 protein can form a complex with Cas1, which maintains Cas3 in a nuclease-inactive state [58••]. Conversely, a CRISPR-Cas complex bound to target DNA displaces Cas1 and activates the Cas3 nuclease. Interestingly, a similar Cas1-2-3 interaction was found in the *Pectobacterium atrosepticum* Type I-F system [44•], where the Cas1-2-3 complex maintains its role in new spacer acquisition. It is yet to be determined if Cas1 acts as a negative regulator in systems where Cas2 and Cas3 are encoded as separate polypeptides. Such a strategy would allow the cell to maintain the Cas3 enzyme ready for deployment in the event of an infection while limiting potentially toxic nuclease activity. Additionally, the protein and DNA conformational changes that occur when DNA is bound by the surveillance complex likely represent a unique molecular 'coincidence' to facilitate specific Cas3 activation.

## Interference (Type II)

The two nuclease domains (HNH and RuvC) in the Cas9 enzyme experience many checks and balances *en route* to a cleavage event. While ApoCas9 can associate non-specifically and weakly with DNA [59], it is not

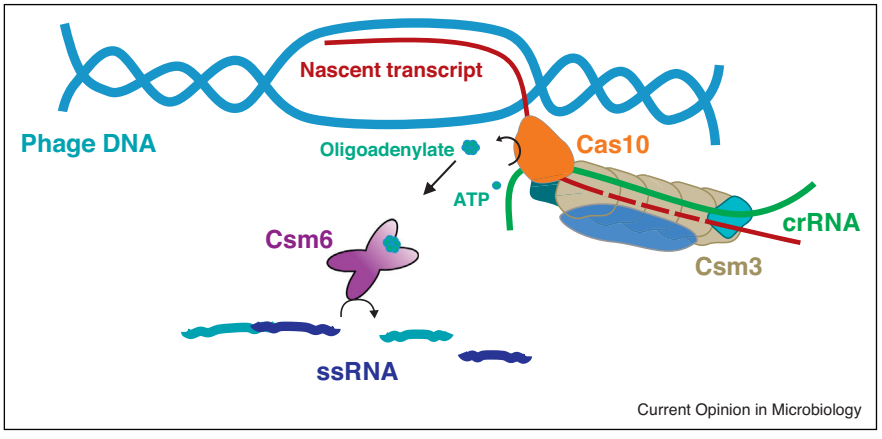
enzymatically active, and loading of the crRNA and the tracrRNA (or sgRNA in engineered forms) induces dramatic conformational changes that convert Cas9 into a sequence-specific, high-affinity DNA binding complex [60•,61,62]. This conformational rearrangement likely ensures that Cas9 is not constitutively enzymatically active within the cell. Emphasizing the role of the RNA-bound Cas9 as the active version of the protein, recently discovered bacteriophage encoded 'anti-CRISPR' proteins, AcrIIA2 and AcrIIA4, interact directly with RNA-loaded Cas9 but not ApoCas9 [63,64].

Upon recognition of the appropriate PAM and 'seed' region of the protospacer, Cas9 docks and unwinds the target DNA locally, base pairing with the complementary sequence [59,65,66]. Beyond correct PAM and seed sequences, further quality control measures are in place for Cas9. First, the HNH catalytic domain sits ~30 Å from the position where cleavage ultimately occurs. Fluorescence experiments revealed that the HNH domain only comes into the correct position when perfect or near perfect complementarity is present between the crRNA and the target [67•]. Remarkably, this movement also mediates an allosteric activation of the RuvC domain, the other catalytic domain in the protein, thereby ensuring that both nuclease domains 'fire' in a concerted manner to yield a double-strand break [67•,68]. Single molecule FRET studies have also shown that the HNH domain can sample an intermediate position that is between active and inactive conformations, and that more time is spent in the intermediate position for mismatched targets, including those with PAM-distal mutations [69,70•]. Again, emphasizing the importance of this process, bacteriophages have developed antagonists. Anti-CRISPR protein AcrIIC1 interacts with the HNH domain and prevents its movement, without interfering with DNA binding [71]. In sum, multiple conformational changes to this protein ensure not only a lack of basal enzymatic activity in the apo form, but also explain the general robustness of Cas9 to cleaving mostly on-target sequences, limiting genome damage.

## Interference (Type III)

The Type III system targeting machinery resemble the Type I surveillance complexes and also utilize a *trans*-acting nuclease, but they exhibit drastic mechanistic differences (Figure 2). Type III CRISPR complexes specifically recognize and base pair with RNA instead of DNA, but and are able to catalyze cleavage of both the bound RNA molecule as well as neighboring DNA [28••,29,72,73]. To combat phage infection, Type III systems deploy *three* distinct and interdependent nucleases, Cas10 (DNase), Csm3 or Cmr4 (RNases, Cas7 family members), and Csm6 (RNase), which are inactive in the absence of a threat. These are controlled by specific mechanisms that allow activation after a few requirements have been met: (i) Complementarity between the crRNA and target RNA [61], (ii) lack of complementarity between the crRNA 5′ handle and the target [74] to avoid CRISPR locus targeting, and (iii) active transcription across the target sequence [28••]. A nascent RNA transcript fitting these requirements is stably bound by a crRNA-guided surveillance complex and is cleaved via the pentameric backbone of Csm3 RNase subunits [28••]. This interaction triggers the second nuclease, Cas10, also a component of the surveillance complex, to cleave proximal DNA substrates [28••]. The third line of defense is a *trans*-acting RNase, Csm6, which is activated to non-specifically cleave RNA. The nature of Csm6 activation remained elusive until recent reports showed that Cas10's Palm domain, which contains a motif conserved among polymerases and cyclases, synthesizes a

**Figure 2**




Type III activation and regulation. Active phage transcription signals the surveillance complex via complementarity between the nascent RNA transcript and the crRNA. Csm3, an RNase, cleaves the complementary sequence. Cas10, a DNase, cleaves the proximal DNA template and synthesizes an oligoadenylate second-messenger which is sensed by the CARF domain on Csm6, activating its non-specific RNase activity.

cyclic oligoadenylate that controls Csm6 [75••,76••]. This molecule is sensed by the CARF (CRISPR-associated Rossman Fold [77]) domain on Csm6, leading to allosteric activation of the RNase. The Csm6 'clean-up' nuclease has been shown to be important when crRNAs recognize regions of phage genomes that are expressed late during infection [27] and may be a mechanism to limit the burst size of the phage.

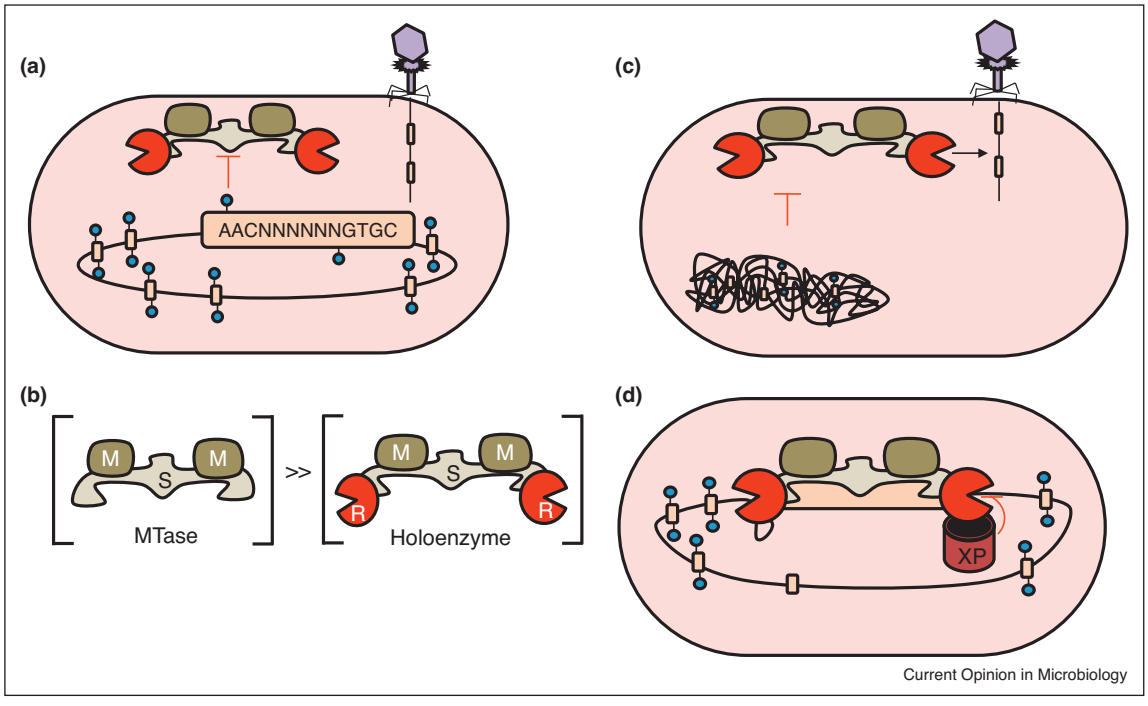
## Regulation of restriction modification

Some of the earliest discovered nucleolytic bacterial immune systems are restriction-modification (R-M) systems [78,79]. R-M systems encode restriction endonucleases (REN) that introduce double-stranded breaks in DNA substrates containing specific unmodified sequences [80,81,82]. While the bacterial genome is protected by DNA methylation (Figure 3a), R-M faces the same challenges as CRISPR-Cas: the need to balance potent, potentially bacteriocidal nucleases with a rapid response to phage infection. We illustrate this point with examples of self-avoidance from the *E. coli* K12 (EcoKI) Type I R-M system.

Type I R-M utilizes three host specificity determination genes: *hsdS* (sequence specificity), *hsdM* (modification activity), and *hsdR*, which translocates along and cleaves DNA [8,83,84]. The encoded proteins assemble into methyltransferases (MT, $S_1M_2$) and restriction-competent holoenzymes ($S_1M_2R_2$). Presumably to maintain protective chromosomal modifications, cellular concentrations of MT exceed that of holoenzymes [85] (Figure 3b). As DNA replication proceeds, hemi-methylated sequences are rapidly modified at specific adenines to $N_6$-methyladenine by Type I MT and holoenzymes [8]. The holoenzyme's MT activity is favored over its endonucleolytic activity in hemi-methylated substrates [86], which is critical for preventing nucleolytic degradation of the chromosome.

During time of extensive DNA recombination or repair, many unmodified sites will be generated which pose a great risk to the host chromosome. In these cases, a protective strategy known as restriction alleviation (RA) safeguards unmodified sites from cleavage. General RA is mediated by cationic polyamine-based genome condensation which physically occludes host DNA from nucleases [87] (Figure 3c). Further, it is proposed that DNA-binding proteins block holoenzyme translocation [88]. Importantly, incoming foreign DNA lacks these features and remains susceptible to restriction. Furthermore, Type I families IA and IB selectively degrade HsdR subunits during periods of stress. Treatment of

**Figure 3**




**(a)** HsdS and HsdM form a methyltransferase (MT) complex ($S_1M_2$) or a holoenzyme restriction endonuclease together with HsdR ($S_1M_2R_2$). The MT is found in higher cellular concentrations than the holoenzyme. **(b)** Modification of host substrate sequences protect the host chromosome from restriction, while foreign genetic elements that lack the protective modification are degraded. **(c)** Chromosomal condensation during cell stress will physically occlude host sites from restriction enzymes. **(d)** DNA damaging agents and incoming Type I restriction enzymes trigger family specific restriction alleviation. ClpXP specifically degrades HsdR subunits that translocate along the host chromosome.

bacteria with genotoxic agents leads to induction of a family-specific RA phenotype where specific degradation of HsdR is mediated by the disaggregase and protease complex, ClpXP [89,90] (Figure 3d). Although the degron on HsdR has not been identified, ClpXP binds chromosome-bound, translocating, holoenzyme-associated HsdR [91•].

Immunity-associated endonucleases in CRISPR-Cas and R-M systems deliver protection with the risk of aberrant, toxic activity against the host if improperly regulated. The described strategies by which CRISPR-Cas systems balance autoimmunity are distinct from those utilized by Type I REN, but it is interesting to consider the potential impacts of host DNA topology and chemistry on controlling CRISPR autoimmunity, and the possibility for active destruction of CRISPR complexes during times of cellular stress.

## Future considerations

Bacteria possess robust immunity pathways, which are fascinating in their basic biology and have revolutionized molecular biology. We envision that further advances in this field will reveal many new and exciting paradigms in microbiology.

Beyond post-translational control, we predict future mechanisms that operate post-transcriptionally (i.e. untranslated mRNAs that are translated during phage infection) could also be part of a robust CRISPR-Cas response. Furthermore, we imagine the possibility for novel proteins that interact with the CRISPR-Cas machinery and modulate or control its function. Indeed, recently discovered families of prophage-encoded anti-CRISPR proteins are interacting proteins that could also provide regulatory switches for turning CRISPR-Cas on and off, as well as enabling new modalities, such a transcriptional repression (CRISPRi) by inactivating nuclease function [63,64,71,92–94,95•,96]. Lastly, we envision that identifying key co-factors, and regulatory switches will be instrumental in continuing to expand the CRISPR-Cas toolbox to enable new functions and applications in various cell types. Continued studies on CRISPR-Cas in their natural settings will undoubtedly enable advancements in this direction [97].

## Conflict of interest

Nothing declared.

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