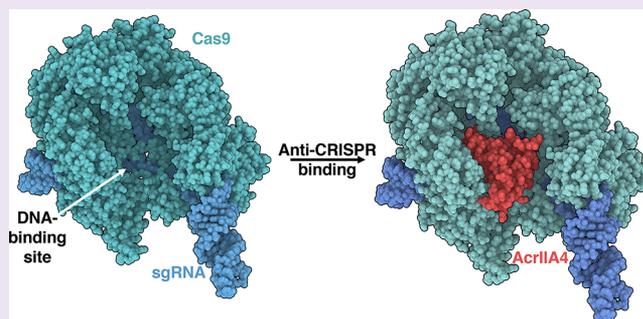


Protein Inhibitors of CRISPR-Cas9

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ABSTRACT: Bacteria are under constant predation from viruses, called bacteriophages (phages). This threat has driven the evolution of multiple defense systems, including the CRISPR-Cas (clustered regularly interspaced short palindromic repeats and CRISPR associated genes) immune pathway. Phages are not passive bystanders in their CRISPR-mediated demise, however, as many have developed potent protein inhibitors of the bacterial adaptive immune system. Here, I review the work that led to the discovery of many distinct “anti-CRISPR” proteins. Furthermore, I outline how understanding their mechanisms of action has provided a suite of specific and high-affinity reagents to modulate and study CRISPR-Cas applications.



Many powerful biotechnologies have been derived from the molecular arms race between bacteria and their viruses. Bacteriophages (phages) or “bacteria eaters” were discovered >100 years ago¹ and are still shaping our understanding of molecular biology and providing new tools.² There are an estimated 10^{23} phage infections per second on the planet, a number driven by the massive numbers of phages and bacteria in the ocean.^{3,4} Advances in high throughput sequencing technologies, microscopy, and extensive sampling have led to the realization that phages are a prominent member of nearly all ecological niches, including the human microbiome.⁵ This appreciation of their abundance, but a poor understanding of their roles, in combination with a dire need for new mechanisms to combat antimicrobial resistance, has led phage biology into a renaissance in recent years. Historically, elucidating the mechanisms by which phages infect their host bacteria led to the identification of ligases, polymerases, recombinases, and restriction enzymes, among many other reagents.⁶ More recently, efforts to identify new ways that bacteria protect themselves from phages led to the discovery of a novel and powerful new immune system, known as CRISPR-Cas.⁷

Clustered regularly interspaced short palindromic repeats (CRISPR) are arrays of repetitive DNA found in the genomes of bacteria and archaea. The spacing sequences between the direct repeats can possess sequence identity to phage genomes, representing a vaccination card or memory component of the first adaptive immune system identified in prokaryotes. Together with CRISPR-associated (cas) genes, this system harvests small sequences (~30 bp) from a phage genome, incorporates it into the CRISPR array, and subsequently transcribes, processes and packages these CRISPR RNAs (crRNAs) into Cas protein complexes that surveil the microbial cell for invasion. Detection of a foreign invader *via*

complementarity between the crRNA sequence and the phage RNA or DNA mediates recognition of the target, which is subsequently cleaved with remarkable specificity.

Six distinct types of CRISPR-Cas systems (types I–VI) have been discovered to date,⁸ divided broadly into two classes, those that utilize a multiprotein surveillance complex (class 1: types I, III, IV) and those that utilize a single protein effector nuclease (class 2: types II, V, VI). The discovery that microbes program sequence-specific nucleases with RNA guides has been harnessed since 2012 to design and unleash precision double stranded breaks on genomes from many organisms, including humans, leading to the “CRISPR-Cas revolution” in genome editing.^{9–12} While this technology initially focused on the Cas9 nuclease, other class 2 effectors such as Cas12 (Cpf1) and Cas13 (C2c2) have recently been utilized due to the simplicity of single protein effectors guided by a single RNA guide.^{13–15}

As is the case with any formidable immune system, nature has developed powerful antagonists of CRISPR-Cas systems. Here, I describe the latest iteration in our understanding of CRISPR-Cas evolution, and yet another reagent borne out of the phage–bacteria arms race, anti-CRISPR proteins (see Table 1).

THE PHAGE COUNTERATTACK

A recurrent theme in studying the molecular battle between phages and their hosts has been the emergence of defense and counter-defense strategies. The ability of viruses to shut down immune pathways has also been well documented in

Special Issue: Chemical Biology of CRISPR

Received: September 25, 2017

Accepted: December 18, 2017

Published: December 18, 2017

Table 1. Anti-CRISPR Proteins^a

name	type	source	target (organism) ^b	accession
AcrIF1	I-F	<i>Pseudomonas aeruginosa</i> phage	Cascade (Pae, Pec)	YP_007392342.1
AcrIF2	I-F	<i>P. aeruginosa</i> phage	Cascade (Pae, Pec)	YP_002332454.1
AcrIF3	I-F	<i>P. aeruginosa</i> phage	Cas3 (Pae)	YP_007392440.1
AcrIF4	I-F	<i>P. aeruginosa</i> phage	Cascade (Pae)	WP_016068584.1
AcrIF5	I-F	<i>P. aeruginosa</i> phage	target unknown (Pae)	YP_007392740.1
AcrIF6	I-E/I-F	<i>P. aeruginosa</i> prophage	target unknown (Pae, Pec)	WP_043884810.1
AcrIF7	I-F	<i>P. aeruginosa</i> phage	target unknown (Pae, Pec)	YP_009146150.1
AcrIF8	I-F	<i>Pectobacterium atrosepticum</i> phage	target unknown (Pae, Pec)	YP_007006940.1
AcrIF9	I-F	<i>Vibrio parahemolyticus</i> prophage	target unknown (Pae, Pec)	WP_031500045
AcrIF10	I-F	<i>Shewanella xiamenensis</i> prophage	target unknown (Pae, Pec)	WP_037415910.1
AcrIE1	I-E	<i>P. aeruginosa</i> phage	target unknown (Pae)	YP_007392738.1
AcrIE2	I-E	<i>P. aeruginosa</i> phage	target unknown (Pae)	YP_007392439.1
AcrIE3	I-E	<i>P. aeruginosa</i> phage	target unknown (Pae)	YP_950454.1
AcrIE4	I-E	<i>P. aeruginosa</i> phage	target unknown (Pae)	NP_938238.1
AcrIIA1	II-A	<i>L. monocytogenes</i> prophage	target unknown (Lmo)	WP_003722518.1
AcrIIA2	II-A	<i>L. monocytogenes</i> prophage	Cas9 (Lmo, Spy)	WP_003722517.1
AcrIIA3	II-A	<i>L. monocytogenes</i> prophage	target unknown (Lmo)	WP_014930691.1
AcrIIA4	II-A	<i>L. monocytogenes</i> prophage	Cas9 (Lmo, Spy)	WP_003723290.1
AcrIIA5	II-A	<i>Streptococcus thermophilus</i> phage	target unknown (Sth, Spy)	ASD50988.1
AcrIIC1	II-C	<i>Neisseria meningitidis</i> mobile element	Cas9 (Nme, Cje, Geo)	WP_049360089.1
AcrIIC2	II-C	<i>N. meningitidis</i> prophage	Cas9 (Nme)	WP_042743678.1
AcrIIC3	II-C	<i>N. meningitidis</i> prophage	Cas9 (Nme)	WP_042743676.1

^aNote: Anti-CRISPR nomenclature has been updated to reflect the type and the subtype. For example, what was formerly AcrF1 is now AcrIF1.

^bPae, *Pseudomonas aeruginosa*; Pec, *Pectobacterium atrosepticum*; Lmo, *Listeria monocytogenes*; Spy, *Streptococcus pyogenes*; Sth, *Streptococcus thermophilus*; Nme, *Neisseria meningitidis*; Cje, *Campylobacter jejuni*; Geo, *Geobacillus stearothermophilus*.

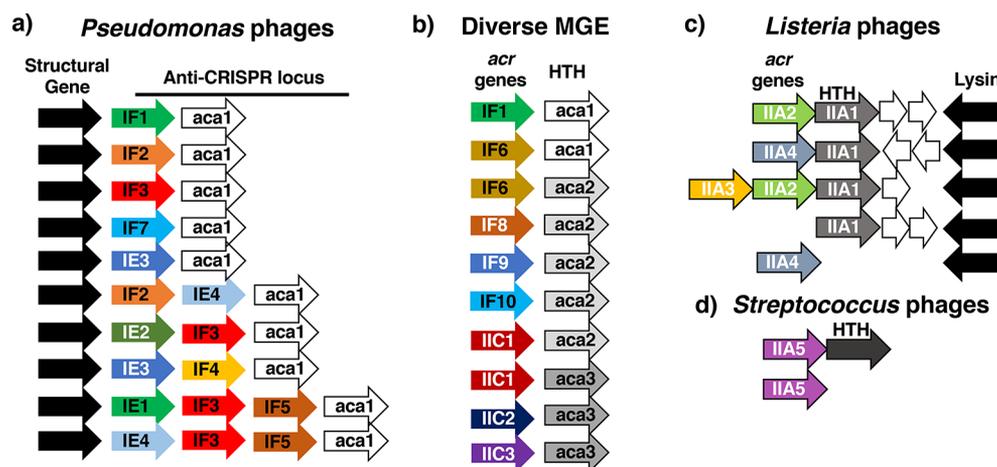


Figure 1. (a) The *Pseudomonas aeruginosa* phage anti-CRISPR locus. A syntenic region from 10 different phage genomes is shown to highlight the varied assortment of type I-F (IF1–IF5, IF7) and I-E (IE1–IE4) anti-CRISPR genes that are found in related phage genomes, anchored adjacent to a conserved structural gene (black). The presence of anti-CRISPR associated gene 1 (*aca1*) is highly conserved in these anti-CRISPR loci. (b) The helix–turn–helix (HTH) encoding *aca1* gene enabled identification of new anti-CRISPR genes (IF6, IF8–IF10), found next to novel HTH proteins (*aca2*, *aca3*), leading to the identification of type II-C anti-CRISPRs (IIC1–3). (c) Type II-A anti-CRISPR genes (IIA1–4) are shown with their associated HTH gene, which is also an anti-CRISPR (IIA1). (d) AcrIIA5, found in *Streptococcus* phages, associated with an HTH in certain phages. The gene size and spacing are not to scale. MGE, mobile genetic elements. HTH, helix–turn–helix. acr, anti-CRISPR.

eukaryotes.^{16,17} Decades of work on a bacterial “innate” immune system, restriction–modification (R–M), has generated a wealth of literature to inform searches for similar mechanisms of CRISPR–Cas evasion. Phage-encoded inhibitors of R–M systems take many shapes and forms, largely following three themes: (i) modifying the target of the immune system, (ii) mimicking the target of the immune system, or (iii) disabling the immune system.¹⁸ These strategies have been paralleled by anti-CRISPR proteins, which function by either

mimicking or occluding the target DNA, or directly disabling CRISPR nucleases, as described below.

The first report of proteins inhibiting CRISPR–Cas function emerged in 2013, encoded by phages that infect the opportunistic human pathogen *Pseudomonas aeruginosa*.¹⁹ This discovery was enabled by the observation that integrated phage genomes were inactivating the endogenous CRISPR–Cas system, suggesting that they expressed inhibitor proteins. These anti-CRISPR proteins inactivate the Class 1, type I CRISPR–Cas system, which is comprised of a multisubunit ribonucleo-

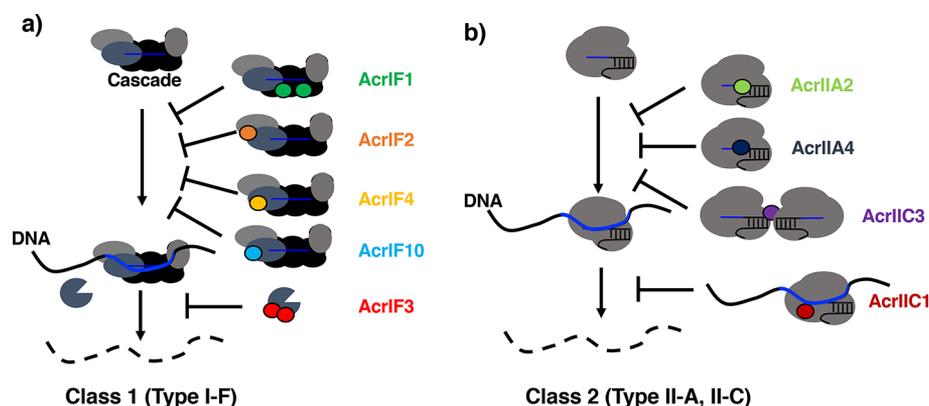


Figure 2. Summary of known mechanisms of CRISPR-Cas inhibition. (a) In the multiprotein subunit class 1 CRISPR-Cas system, type I-F inhibitors function by binding to the RNA-guided Cascade complex and preventing DNA binding (AcrIF1, AcrIF2, AcrIF4), or by interacting with the trans-acting nuclease Cas3 and preventing recruitment to the DNA target (AcrIF3). (b) In the single protein effector class 2 CRISPR-Cas system, type II-A and II-C inhibitors bind directly to Cas9 and block DNA binding (AcrIIA2, AcrIIA4, AcrIIC3) or block DNA cutting by limiting the movement of the HNH nuclease domain upon DNA binding (AcrIIC1). In the absence of these inhibitor proteins, the DNA is degraded (dashed lines). Note: the exact binding locations of AcrIF4 and AcrIIA2 are unknown and only approximated in this figure.

protein effector complex and a *trans*-acting nuclease/helicase, Cas3.^{20–22} The most remarkable aspect of the first set of five “anti-CRISPR” proteins discovered was their diversity. One anti-CRISPR was initially identified in a phage that was inactivating the CRISPR-Cas system, and others were found by testing a set of syntenic genes with no obvious identity to the first. The only commonality between these genes was that they were small (~150–450 base pairs) and encoded by closely related phages at the same genomic position, or “anti-CRISPR locus” (Figure 1a). By expressing these proteins in *P. aeruginosa*, it was revealed that they each possessed anti-CRISPR activity against either the type I-F or type I-E CRISPR-Cas system,^{19,23} both of which are found in *P. aeruginosa* isolates.^{24,25} Anti-CRISPR genes are named in the order with which they were discovered and describe the system they inhibit, for example *acrIF1* (anti-CRISPR for type I-F). Note that this has been updated from earlier nomenclature, *acrF1*.

While the anti-CRISPR genes themselves did not possess informative homologues or domain structures, biochemical experiments revealed that type I-F anti-CRISPR proteins interact directly with Cas proteins (Figure 2a). For example, two distinct anti-CRISPR proteins, AcrIF1 and AcrIF2, interact with the Cascade surveillance complex members Cas7 and Cas8, respectively, which blocks DNA binding.²⁶ A third anti-CRISPR protein, AcrIF3, prevents the recruitment of the Cas3 nuclease *via* a direct interaction.²⁶ Subsequent structures of anti-CRISPRs both bound and unbound to their targets confirmed the truly distinct nature of these proteins, as they possessed no common fold.^{27,28} However, thorough analysis of structures of the I-F Cascade complex bound to various anti-CRISPRs (i.e., AcrIF1, AcrIF2, AcrIF10) has revealed common strategies including steric occlusion of DNA binding and DNA mimicry.^{29,30} Anti-CRISPR diversity across even closely related phages underscores the strong selective pressures placed on an immune/anti-immune effector battle and emphasizes how this conflict is a “hot spot” for the generation of novel genes.³¹ With 14 type I anti-CRISPR proteins having been identified,^{19,23,32,33} the only commonality that unites them is often a shared genomic locus within a mobile element such as a prophage and an association with a putative regulatory helix–turn–helix (HTH) protein.

■ CRISPR-CAS9 INHIBITORS

The predictive power and importance of anti-CRISPR locus organization was clearly demonstrated by the discovery of the first inhibitors of the type II CRISPR-Cas9 system. The loci where type I anti-CRISPRs (*acrIF1–IF5*, *acrIF7*, *acrIE1–IE4*) were found always possessed a conserved, predicted helix–turn–helix protein (HTH, anti-CRISPR associated protein 1, *aca1*) adjacent to anti-CRISPR genes. While the function of this gene is still unknown, it served as a powerful indicator gene for discovery of new anti-CRISPRs *acrIF6* and *acrIF8–IF10* (Figure 1b). While identifying new type I anti-CRISPRs, Pawluk and colleagues identified a new HTH anti-CRISPR associated gene, *aca2*.³² Homologues of this gene were identified next to new anti-CRISPRs, which in turn, led to a new gene encoding a predicted HTH protein, *aca3*. Remarkably, this bioinformatic “guilt-by-association” based search led to anti-CRISPR loci in *Neisseria meningitidis* mobile elements, an organism with a well characterized type II-C CRISPR-Cas9 system.^{34,35} Three candidates identified in this way possessed inhibitory activity against *NmCas9* in natural *Neisseria* hosts, *in vitro*, and in human cells.³⁶ Subsequent structural and biochemical work has revealed that AcrIIC1 is a broad spectrum type II-C Cas9 inhibitor, which prevents movement of the HNH nuclease domain toward its DNA substrate, while AcrIIC3 inhibits Cas9 through a mechanism involving a forced dimerization of Cas9³⁷ (Figure 2b).

Cas9 has been the most widely utilized CRISPR-Cas enzyme for biotechnological applications. Specifically, the type II-A orthologue from *Streptococcus pyogenes* (*SpyCas9*) has been deployed for gene editing in numerous organisms, as well as many DNA-binding applications derived from catalytically dead Cas9 (dCas9).¹² To identify anti-CRISPRs that function against the type II-A system, a bioinformatics approach was developed, which surveyed genomes for “self-targeting” (Figure 3). This describes a situation where one or more endogenous CRISPR spacers encode for the production of a crRNA complex that would mediate the cleavage of a target in the same genome. Cleavage of a bacterial genome in this manner would be lethal, and therefore, broadly speaking, the self-targeting CRISPR system must be disabled in some manner for the continued survival of the cell. There are many possible ways to inactivate CRISPR systems, including mutation, transcriptional repres-

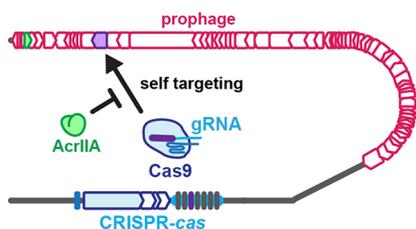


Figure 3. Spacers in a CRISPR array dictate the identity of the guide RNAs (gRNAs) that will be generated by that strain. In this example, a “purple” gRNA possesses perfect complementarity to a sequence in the bacterial genome, specifically in a prophage. This will result in the destruction of the genome and cellular death, if not for an “AcrIIA” protein that neutralizes the threat.

sion, or anti-CRISPR inhibition. In the bacterium *Listeria monocytogenes*, however, self-targeting was revealed to be directly stabilized by one of four anti-CRISPR proteins (AcrIIA1–4), present in numerous related prophages (Figure 1c). Again, a characteristic anti-CRISPR locus was observed where conserved phage genes flanked a diverse locus with genes possessing anti-CRISPR function.³⁸

The recent discovery of a fifth type II-A Cas9 inhibitor (AcrIIA5) was made by immunizing strains of *Streptococcus thermophilus* with spacers that target various phages and identifying a phage that resists targeting³⁹ (Figure 1d). Comprehensive cloning of phage open reading frames led to the identification of *acrIIA5*, which possessed Cas9-inhibitory activity when expressed in isolation. Notably, *acrIIA5* is encoded both by lytic and temperate phages, a property which is also shared by *acrIIA4*. AcrIIA5 was also functional against *SpyCas9*, much like AcrIIA4, suggesting broad spectrum inhibition.

Proteins AcrIIA2 and A4 were shown to block Cas9-DNA binding using CRISPRi assays in *E. coli*. This mechanism was later supported by direct biochemical evidence that these proteins interact tightly with Cas9 after sgRNA loading ($K_d = 4$ nM) and prevent DNA binding *in vitro*⁴⁰ (Figure 2b). Structural studies revealed that AcrIIA4 mimics DNA and binds to the Cas9 residues that are required for binding to the PAM nucleotides in the DNA template.^{40–42} No structural or biochemical mechanisms have been demonstrated for the other type II-A Cas9 inhibitors, but it can be expected that diverse routes to Cas9 inhibition will be advantageous for downstream applications.

■ APPLICATIONS FOR PHAGE INHIBITOR PROTEINS

Phage enzymes such as T4 DNA ligase, T7 polymerase, and Cre recombinase have been applied broadly as *in vitro* and *in vivo* biotechnologies for decades. Phages and phage proteins are also being widely studied and utilized in the fight against antimicrobial resistant pathogens. Some well-studied phages such as M13 and T7 have even been co-opted for numerous phage display-based technologies, which enable screens for protein–protein interactions. It is less common for non-enzymatic phage-derived proteins (such as anti-CRISPRs) to have been co-opted for biotechnology. One powerful example, however, is the use of the uracil glycosylase inhibitor (UGI) from *Bacillus subtilis* phage PBS1, which displays remarkably strong inhibition of the human enzyme. This has recently enabled precise base editing technologies with fused domains to dCas9.^{43,44} Given the strong binding affinity that many phage-

encoded inhibitors display for their target (e.g., UGI $IC_{50} = 12$ pM), uses in eukaryotic systems may be plentiful.

All anti-CRISPR proteins are defined by their ability to block CRISPR-Cas-mediated DNA cleavage *in bacteria*. Moreover, all characterized anti-CRISPRs function through direct interactions with a target Cas protein.^{26,37,40} Therefore, Cas9 inhibitors have utility as a specific and direct “off-switch” for Cas9 gene editing in heterologous hosts such as human cells. One of the largest hurdles currently faced by gene editing applications is off-target mutations that can occur during editing.^{45,46} While on-target events occur with faster kinetics than off-targeting, the long-term stability of Cas9 in the host nucleus also leads to off-target mutations. The extent of this problem can be mitigated by thoughtful sgRNA design⁴⁷ and varies depending on many factors including cell type, Cas9 concentration, and Cas9 delivery modality.^{48,49} For therapeutic applications, a limitation arises with the need to target a particular site during allele-specific targeting, where investigators are limited to a single target site and sgRNA sequence. In these cases, anti-CRISPRs may represent a particularly useful reagent to limit off-target editing. As a proof-of-principle, the specific anti-Cas9 activity of AcrIIA4 (*SpyCas9* inhibitor) has been shown to reduce off-target editing in cell culture⁴¹ when deployed *via* protein or plasmid delivery. Many other strategies are also being used to limit off-target editing such as mutagenesis of Cas9 to enhance accuracy, leading to the generation of e*SpyCas9*(1.1), *SpyCas9*-HF1, and most recently, *HypaCas9*.^{50–52} Other strategies to enable enhanced control in cells include the addition of ligand binding domains to generate an allosteric switch⁵³ and small molecule-mediated degradation.⁵⁴ While not yet experimentally tested, inhibitor proteins are likely compatible with these engineering efforts and could be applied in concert to enable enhanced precision and safety for future applications where preventing off-target editing is absolutely critical.

Many anti-CRISPRs function by inhibiting DNA-binding by CRISPR-Cas complexes (AcrIF1, IF2, IF4; AcrIIA2, IIA4; AcrIIC1, IIC3).^{26,36,38} This inhibits various CRISPR-Cas applications such as gene editing itself,^{36,38,41} CRISPRi,³⁸ and CRISPR-based imaging.³⁶ For applications that do not result in a change to the DNA sequence (i.e., CRISPRi, imaging), inhibitors are also likely of use due to their ability to impede a dCas9 based application such as transcriptional repression. Although AcrIIA4 is unable to displace Cas9 from bound DNA, cellular factors that likely contribute to Cas9 displacement could provide free Cas9 for AcrIIA4 to bind, ultimately titrating dCas9 away from its DNA target. At a fundamental level, stoichiometric inhibitor proteins are also useful to understand the dynamics of dCas9 based activities, prevent leaky CRISPRi activity, and build circuits that cycle dCas9 based modalities between tightly controlled on and off states.

Not all anti-CRISPR proteins are complete off switches for CRISPR-Cas function. Remarkably, some specifically disable DNA cleavage, while still permitting DNA binding. In both type I systems (AcrIF3 inhibits Cas3 recruitment) and in type II systems (AcrIIC1 inhibits Cas9 HNH domain activation), cleavage inhibition has been reported, which serves to enable CRISPRi (Figure 2).^{26,28,37} While the physiological importance of this partial inactivation of CRISPR-Cas in bacteria is not yet clear, these proteins clearly enable CRISPRi transcriptional repression with CRISPR-Cas systems that are otherwise genetically wild-type.²⁶ AcrIIC1 performing this function was only recently reported,³⁷ and thus applications have not yet

been fully explored. However, the ability to toggle Cas9 between catalytically active and dead states in this manner could enable new applications.

FUTURE CONSIDERATIONS

Future work to explore applications will focus, like CRISPR-Cas9 technology itself, on delivery. Inhibitors have been successfully provided on plasmids or as protein⁴¹ and will need to be continually evaluated for their impact on the ratio of on- to off-target editing in any particular setting. Of particular note is the advantage of a genetically encoded Cas9 off-switch as CRISPR-Cas9 technology is applied to living organisms. For example, provision of an inhibitor gene in the same viral vector as a CRISPR effector nuclease would ensure that every cell in a given organism receives both the nuclease and the off-switch. Additionally, there are valid concerns that gene-drive technology (which forces non-Mendelian inheritance of a mutant allele using CRISPR-Cas9^{55,56}) could become uncontrolled or used for nefarious purposes. In theory, Cas9 inhibitor expression would be able to halt a gene drive in its tracks.

As demonstrated by Figure 1, the identification of an anti-CRISPR locus for any CRISPR-Cas subtype is a remarkable finding, as it can function as a key to discovering numerous independent proteins that inhibit CRISPR-Cas function through diverse mechanisms. Once a locus is identified, there is massive potential for novel gene discovery, limited only by the extent to which the relevant elements have been sequenced. As researchers continue to discover and mine these mysterious loci, which are seemingly assembled as a “grab bag” of anti-CRISPRs, these proteins provide novel reagents for mechanistic and structural studies as well as applications described here. For more consideration of the natural biology and evolutionary trajectory of anti-CRISPRs, their loci, and how bacteria might fight back, I direct the reader to recently published reviews.^{33,57} Although only a small number of such anti-CRISPR loci have been identified thus far, discovering more will continue to bring large sums of phage genomic “dark matter” constituents into the light.

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Notes

The author declares no competing financial interest.

ACKNOWLEDGMENTS

I thank A. Borges for critical reading of this manuscript and A. Borges, B. Rauch, and F. Jiang (J. Doudna lab) for contributing to the figures. CRISPR-Cas work in the Bondy-Denomy lab is supported by the University of California San Francisco Program for Breakthrough in Biomedical Research, funded in part by the Sandler Foundation, and a National Institutes of Health Office of the Director Early Independence Award (DP5-OD021344).

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