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Structures and Strategies of Anti-CRISPR-Mediated Immune Suppression

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Abstract

More than 50 protein families have been identified that inhibit CRISPR (clustered regularly interspaced short palindromic repeats)-Cas-mediated adaptive immune systems. Here, we analyze the available anti-CRISPR (Acr) structures and describe common themes and unique mechanisms of stoichiometric and enzymatic suppressors of CRISPR-Cas. Stoichiometric inhibitors often function as molecular decoys of protein-binding partners or nucleic acid targets, while enzymatic suppressors covalently modify Cas ribonucleoprotein complexes or degrade immune signaling molecules. We review mechanistic insights that have been revealed by structures of Acrs, discuss some of the trade-offs associated with each of these strategies, and highlight how Acrs are regulated and deployed in the race to overcome adaptive immunity.

Contents

INTRODUCTION

CRISPRs (clustered regularly interspaced short palindromic repeats) and associated *cas* genes are essential components of diverse adaptive immune systems that defend bacteria and archaea from infection by foreign genetic elements. These immune systems are partitioned into two classes that have evolved independently but have been exchanged horizontally across taxa (47, 50). Class 1 systems are divided into three types (I, III, IV) and 18 subtypes (A, B, C, etc.), and all class 1 systems consist of a multi-subunit RNA-guided surveillance complex (50, 51). Similarly, class 2 systems are divided into three types (II, V, VI) and 26 subtypes, and all class 2 systems consist of a single-protein effector that is guided by a CRISPR RNA (crRNA) (50, 51). Despite the phylogenetic and functional diversity of these systems, they all seem to participate in defense.

Considerable effort has been dedicated to understanding how Cas proteins integrate fragments of foreign DNA at one end of the CRISPR locus and how CRISPR DNA is transcribed and processed into short crRNAs that guide Cas nucleases to the DNA or RNA of invading parasites (**Figure 1**). Progress in this field has been frenetic, and numerous reviews dedicated to mechanisms of CRISPR adaptation (1, 38, 53, 80), crRNA biogenesis (16, 17), and crRNA-guided target degradation (32, 43, 64) are available. As sophisticated and diverse as these immune systems are, phages and other genetic parasites have evolved mechanisms to neutralize these immune systems. Originally discovered in 2013, anti-CRISPRs (Acrs) appear to mirror the diversity of the CRISPR systems themselves (9, 10, 59). Much like CRISPR systems, Acrs have attracted considerable attention, and several reviews have recently been published that address Acr function, evolution, and methods of discovery (11, 35, 60, 77). Here we complement existing reviews by first introducing recent work on the expression and regulation of Acrs. The implications of *acr* regulation are discussed in the context of CRISPR-Cas expression, before we move on to a discussion focused on the structures of Acr proteins and what they have taught us about the vulnerabilities of crRNA-guided defense systems.

TIMING IS EVERYTHING

How do crRNA-guided surveillance complexes find complementary targets in a crowded intracellular environment, and on a timescale that affords protection from an invading virus that will (in some cases) program the cell for lysis (i.e., death) in the first few minutes after infection? The mechanisms that explain effective surveillance are complicated, but we know that detection of a complementary DNA target does not initially rely on unwinding the double-stranded DNA (dsDNA) duplex, which would be slow and energetically expensive (71, 79). Instead, detection of invading dsDNA (crRNA-guided detection of RNA relies on alternative mechanisms) starts with

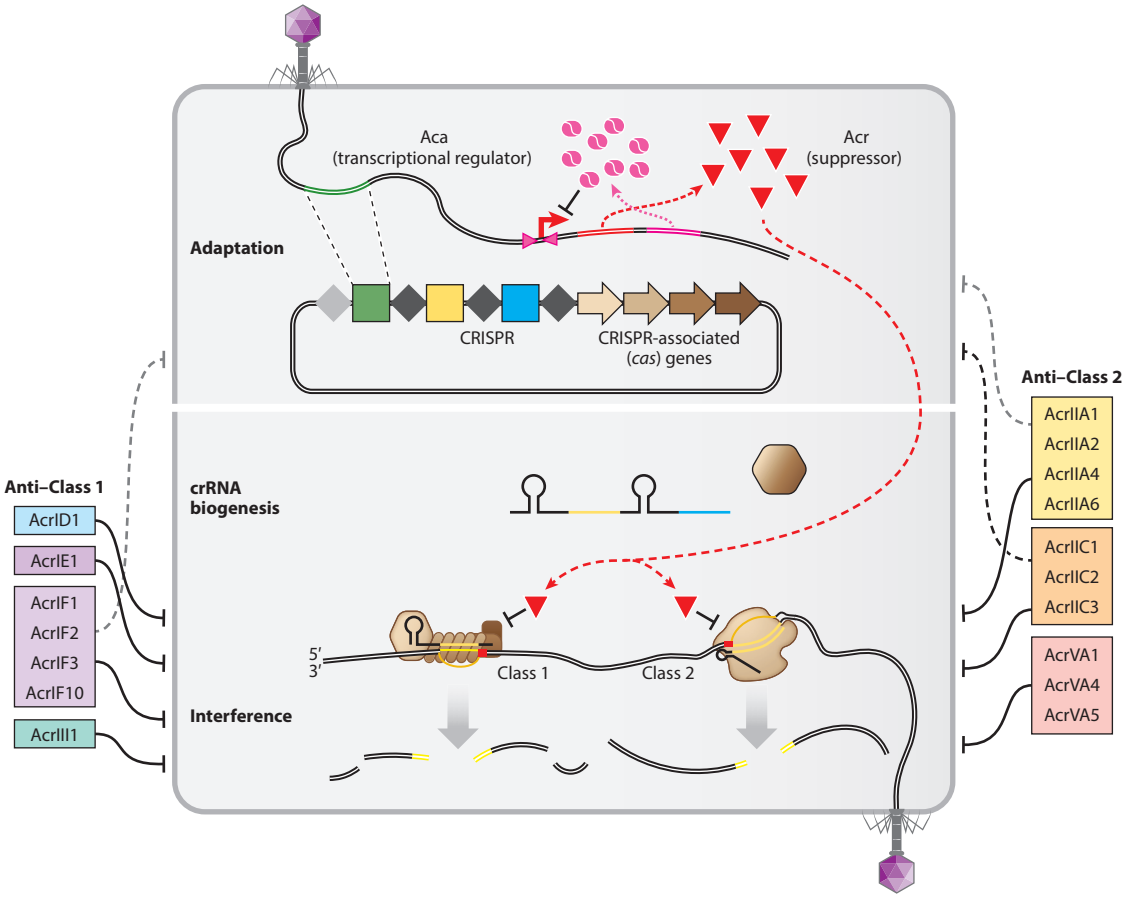


Figure 1

CRISPR-Cas adaptive immunity and structurally determined anti-CRISPRs (Acrs). Homodimeric Acr-associated (Aca) proteins (*pink*) repress Acr (*red triangles*) expression by binding to an upstream inverted repeat. Most suppressors of class 1 (*left*) and 2 (*right*) immune systems target the CRISPR RNA (crRNA)-guided surveillance complex and block DNA binding or nuclease activation.

the identification of a short-duplexed sequence motif called a PAM (protospacer-adjacent motif) (54, 73, 79). PAM binding is thought to destabilize the duplex and thereby facilitate crRNA-guided strand invasion (2, 69, 79). If the adjacent sequence is not complementary, then the interaction is ephemeral, and the search continues (75, 85). In contrast, a PAM with an adjacent complementary sequence triggers a conformational change that activates the nuclease and prompts target destruction (37, 57, 67, 82, 84, 95, 97). Collectively, this is an efficient process, and some crRNA-guided surveillance complexes (e.g., Cas9 and Cascade) are predicted to find their targets in less than a minute (19, 40, 85).

But if crRNA-guided immune surveillance is rapid and efficient, then how do phages escape detection and elimination? DNA mutations and modifications play an important role in phage escape (13, 20, 72, 86), but here we focus on the delivery of immune suppressors. To effectively suppress the immune system, phages must quickly produce or deliver Acrs before the genome is identified and destroyed by crRNA-guided immune complexes (12, 48). It is conceivable (maybe even probable) that some phages package and inject Acr proteins along with their genomes. In

fact, Stone et al. (81) recently determined the structure of a phage decoration protein that is a structural homolog of AcrIIC1. Although this structural similarity raises the intriguing possibility that proteins associated with the virion could serve as readymade CRISPR-Cas antidotes, recent evidence now indicates that *acr* genes are among the first genes to be transcribed and translated during an infection (**Figure 1**) (7, 76).

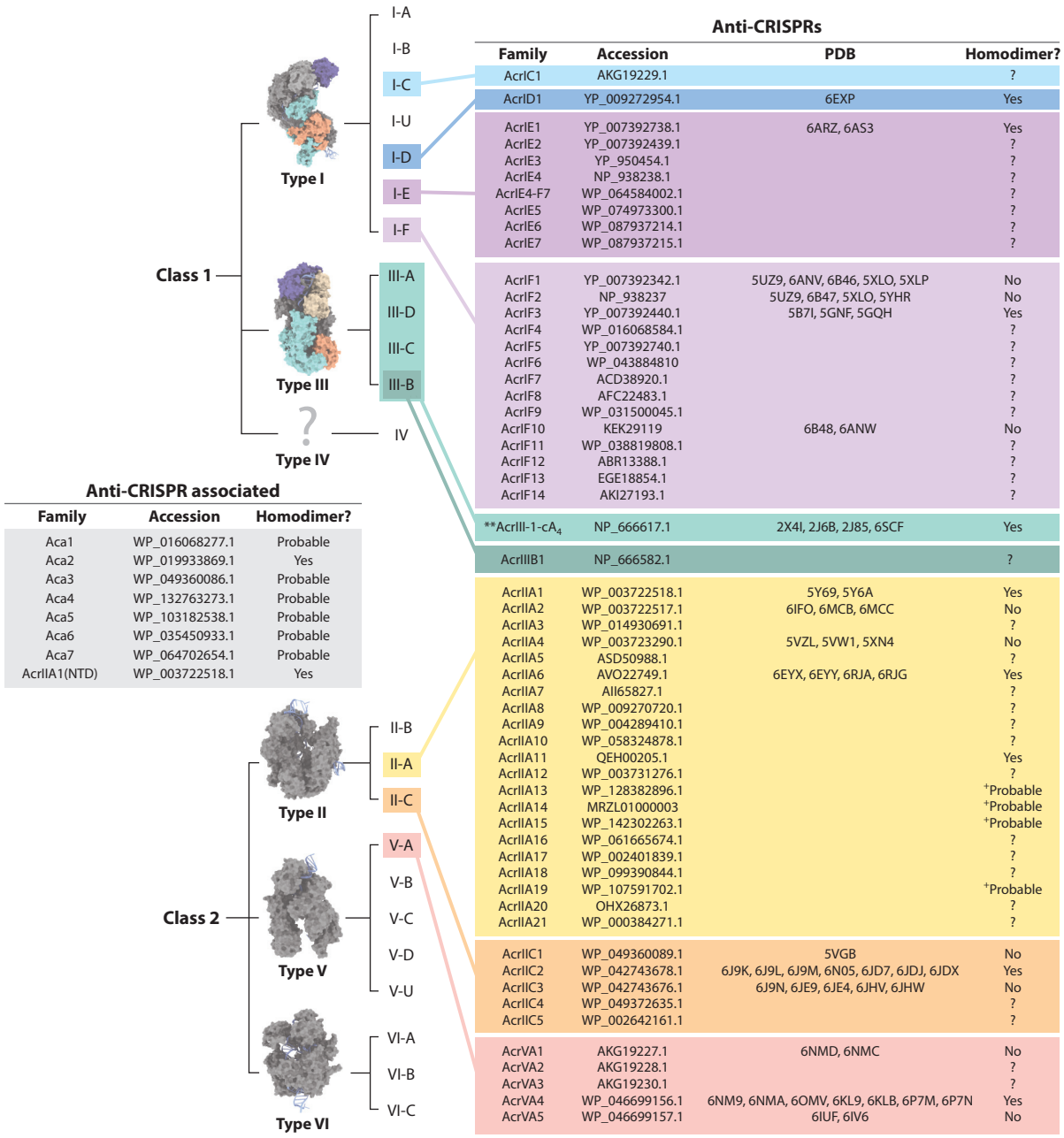
To date, more than 50 families of unique Acrs (some containing just a single known homolog) have been experimentally demonstrated to suppress type I, II, III, or V CRISPR immunity (8) (**Figures 1**, **2**). These proteins are small (52–333 amino acids) and diverse, sharing little to no sequence similarity with other proteins (10, 59). The small size and diverse sequences make it difficult to identify new Acrs using standard homology-based search methods. However, Pawluk et al. (61) first identified a conserved gene with a helix-turn-helix motif that is encoded downstream of known anti-CRISPR genes but absent in related phages lacking anti-CRISPRs. These anti-CRISPR-associated (*aca*) genes have become effective new tools that serve as genetic landmarks for locating new Acrs, but until recently their functions have gone unreported. Stanley et al. (76) recently demonstrated that *acr* and *aca* genes are immediately transcribed as a single RNA from the upstream promoter (i.e., polycistronic). Collectively, this work and a paper from Birkholz et al. (7) now show that Aca proteins are homodimers that repress expression of the operon by binding to inverted repeats in the promoter (76) (**Figure 1**). This results in a temporally controlled negative-feedback loop that helps explain how phages deliver an early dose of Acrs, without the detrimental effects of runaway gene expression that occur in the absence of the repressor (7, 76). However, since Acr delivery appears to require transcription and translation (as opposed to delivery of the proteins directly), there is an intrinsic delay, and this delay provides an initial advantage to a previously "vaccinated" cell containing bespoke crRNA-guided complexes targeting that phage (12, 48). Borges et al. (12) and Landsberger et al. (48) recently demonstrated that infections of cells containing a CRISPR system targeting that phage are typically cleared, but each infection delivers small doses of Acr proteins that temporarily immunocompromise the cell. Thus, at high viral titers, Acrs accumulate to a critical intracellular threshold that eventually overwhelms the immune system (12, 48). Remarkably, the intracellular threshold necessary for immunosuppression differs between Acrs and correlates with the Acr binding affinity (i.e., weak binders require higher Acr concentrations and vice versa) (12, 48).

While the role of Acrs in blocking crRNA-guided target interference in previously vaccinated cells has been well established, very little work has been done to understand how Acrs impact adaptation or crRNA biogenesis (**Figure 1**). With the exception of a single paper indicating that Acrs inhibit new sequence acquisition in type I-F systems (87), little work has been published on the role of Acrs in processes upstream of interference. This is especially surprising given that Cas9 plays a critical role in both interference and new sequence adaptation (30, 91). We anticipate that Acr proteins play an underappreciated role in limiting the efficiency of new sequence integration and that efforts to interrogate this aspect of the biology will reveal new insights into the CRISPR-anti-CRISPR dynamic.

Stoichiometric Inhibitors of CRISPR Defense

Stochiometric inhibitors are often decoys that bind to the crRNA-guided surveillance complexes and prevent target binding or nuclease activation.

Anti-CRISPR proteins that masquerade as dsDNA. Parasites routinely use molecular mimicry to evade host immune responses (34, 65). Since many of the CRISPR-Cas systems target dsDNA (types I, II, and V), it is not surprising that phages have evolved a diverse repertoire of Acr proteins



Anti-CRISPR associated

Family	Accession	Homodimer?
Aca1	WP_016068277.1	Probable
Aca2	WP_019933869.1	Yes
Aca3	WP_049360086.1	Probable
Aca4	WP_132763273.1	Probable
Aca5	WP_103182538.1	Probable
Aca6	WP_035450933.1	Probable
Aca7	WP_064702654.1	Probable
AcrIIA1(NTD)	WP_003722518.1	Yes

Anti-CRISPRs

Family	Accession	PDB	Homodimer?
AcrIC1	AKG19229.1		?
AcrID1	YP_009272954.1	6EXP	Yes
AcrIE1	YP_007392738.1	6ARZ, 6AS3	Yes
AcrIE2	YP_007392439.1		?
AcrIE3	YP_950454.1		?
AcrIE4	NP_938238.1		?
AcrIE4-F7	WP_064584002.1		?
AcrIE5	WP_074973300.1		?
AcrIE6	WP_087937214.1		?
AcrIE7	WP_087937215.1		?
AcrIF1	YP_007392342.1	5UZ9, 6ANV, 6B46, 5XLO, 5XLP	No
AcrIF2	NP_938237	5UZ9, 6B47, 5XLO, 5YHR	No
AcrIF3	YP_007392440.1	5B7I, 5GNF, 5GQH	Yes
AcrIF4	WP_016068584.1		?
AcrIF5	YP_007392740.1		?
AcrIF6	WP_043884810		?
AcrIF7	ACD38920.1		?
AcrIF8	AFC22483.1		?
AcrIF9	WP_031500045.1		?
AcrIF10	KEK29119	6B48, 6ANW	No
AcrIF11	WP_038819808.1		?
AcrIF12	ABR13388.1		?
AcrIF13	EGE18854.1		?
AcrIF14	AKI27193.1		?
**AcrIII-1-cA$_4$	NP_666617.1	2X4I, 2J6B, 2J85, 6SCF	Yes
AcrIIIB1	NP_666582.1		?
AcrIIA1	WP_003722518.1	5Y69, 5Y6A	Yes
AcrIIA2	WP_003722517.1	6IFO, 6MCB, 6MCC	No
AcrIIA3	WP_014930691.1		?
AcrIIA4	WP_003723290.1	5VZL, 5VW1, 5XN4	No
AcrIIA5	ASD50988.1		?
AcrIIA6	AVO22749.1	6EYX, 6EYY, 6RJA, 6RJG	Yes
AcrIIA7	AII65827.1		?
AcrIIA8	WP_009270720.1		?
AcrIIA9	WP_004289410.1		?
AcrIIA10	WP_058324878.1		?
AcrIIA11	QEH00205.1		Yes
AcrIIA12	WP_003731276.1		?
AcrIIA13	WP_128382896.1		+Probable
AcrIIA14	MRZL01000003		+Probable
AcrIIA15	WP_142302263.1		+Probable
AcrIIA16	WP_061665674.1		?
AcrIIA17	WP_002401839.1		?
AcrIIA18	WP_099390844.1		?
AcrIIA19	WP_107591702.1		+Probable
AcrIIA20	OHX26873.1		?
AcrIIA21	WP_000384271.1		?
AcrIIC1	WP_049360089.1	5VGB	No
AcrIIC2	WP_042743678.1	6J9K, 6J9L, 6J9M, 6N05, 6JD7, 6JDJ, 6JDX	Yes
AcrIIC3	WP_042743676.1	6J9N, 6JE9, 6JE4, 6JHV, 6JHW	No
AcrIIC4	WP_049372635.1		?
AcrIIC5	WP_002642161.1		?
AcrVA1	AKG19227.1	6NMD, 6NMC	No
AcrVA2	AKG19228.1		?
AcrVA3	AKG19230.1		?
AcrVA4	WP_046699156.1	6NM9, 6NMA, 6OMV, 6KL9, 6KLB, 6P7M, 6P7N	Yes
AcrVA5	WP_046699157.1	6IUF, 6IV6	No

Figure 2

Anti-CRISPR (Acr) and anti-CRISPR-associated (Aca) proteins. Acr proteins that have been experimentally demonstrated to prevent CRISPR interference, with representative protein accession numbers, Protein Data Bank (PDB) identifiers, and propensity to form homodimers. Proteins with helix-turn-helix domains, but not confirmed to form homodimers are listed as probable. Double asterisks (**) indicate Acrs with a suggested naming convention that includes the secondary messenger.

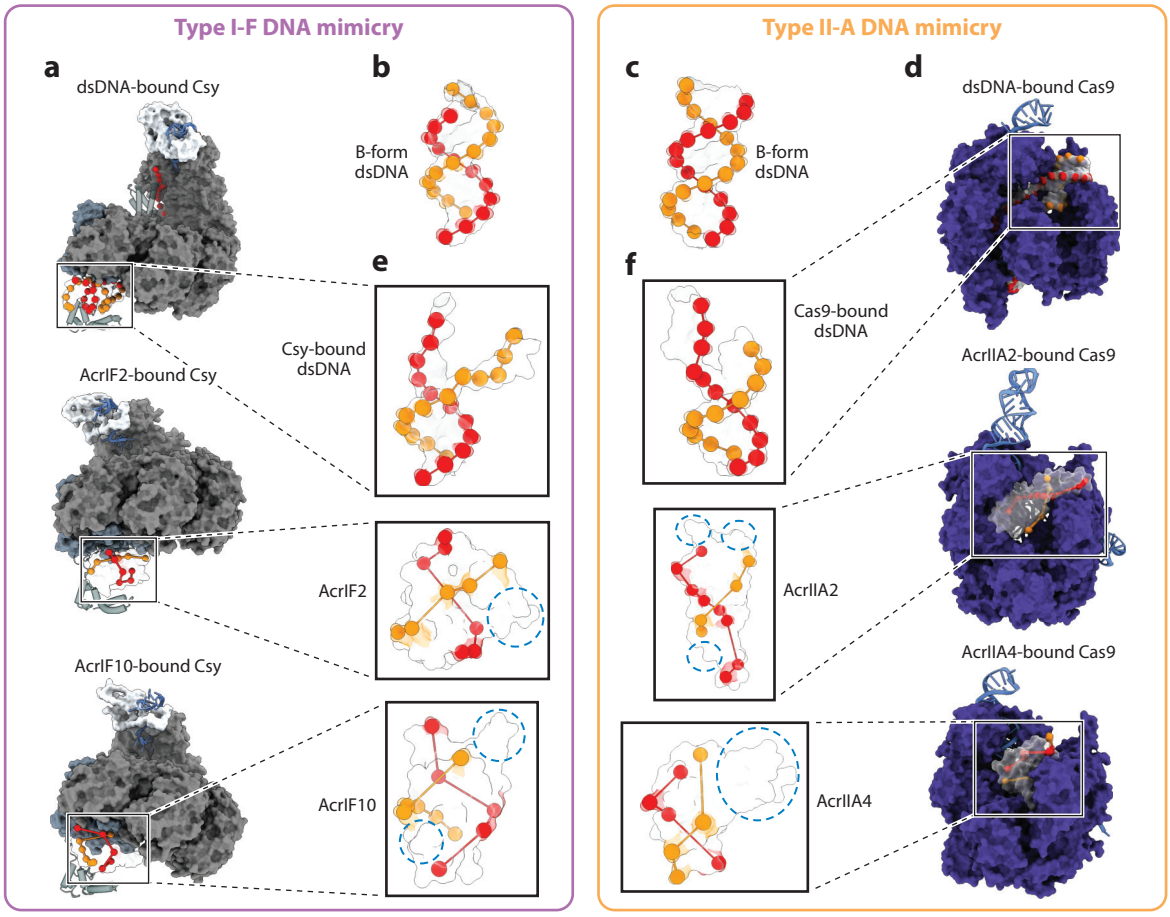


Figure 3

Anti-CRISPRs more closely mimic bound double-stranded DNA (dsDNA) than idealized B-form DNA. (*a*) Surface representation of Csy bound to dsDNA, AcrIF2, and AcrIF10 [Protein Data Bank (PDB): 6NE0, 5UZ9, 6B48]. (*b*,*c*) Surface representation of modeled B-form DNA with orbs highlighting phosphate groups of the DNA backbone. (*d*) Surface representation of Cas9 bound to dsDNA, AcrIIA2, and AcrIIA4 (PDB: 4UN3, 6MCB, 5VW1). (*e*,*f*) Surface representation of DNA bound by surveillance complex with orbs highlighting phosphate groups of the DNA backbone (*top*) and surface representations of bound anti-CRISPRs with orbs highlighting pseudohelical arrangement of acidic residues and auxiliary structural features (*dashed blue circles* in *bottom two insets*).

that serve as dsDNA decoys that intercept the immune systems and prevent detection of invading DNA (18, 22, 28, 39, 49, 63, 74, 96). However, if dsDNA mimicry is a shared mechanism for diverse Acrs, then why does each Acr only interact with a specific surveillance complex? To address this question, we compared Acr structures to idealized B-form DNA and to the distorted DNA bound by each of the corresponding surveillance complexes (**Figure 3*a*,*d***). These comparisons reveal a negative charge distribution on Acrs that more closely resembles the pattern of phosphates on DNA bound by the surveillance complex than idealized B-form (**Figure 3*b*,*c*,*e*,*f***). In addition to the pseudohelical presentation of negatively charged residues, we found that Acrs disguised as (bound) dsDNA are also often adorned with additional structural features (**Figure 3*e*,*f***). These ancillary structural decorations often obscure the helical charge distribution characteristic of dsDNA, but they are expected to play important roles in affinity and specificity of Acr target

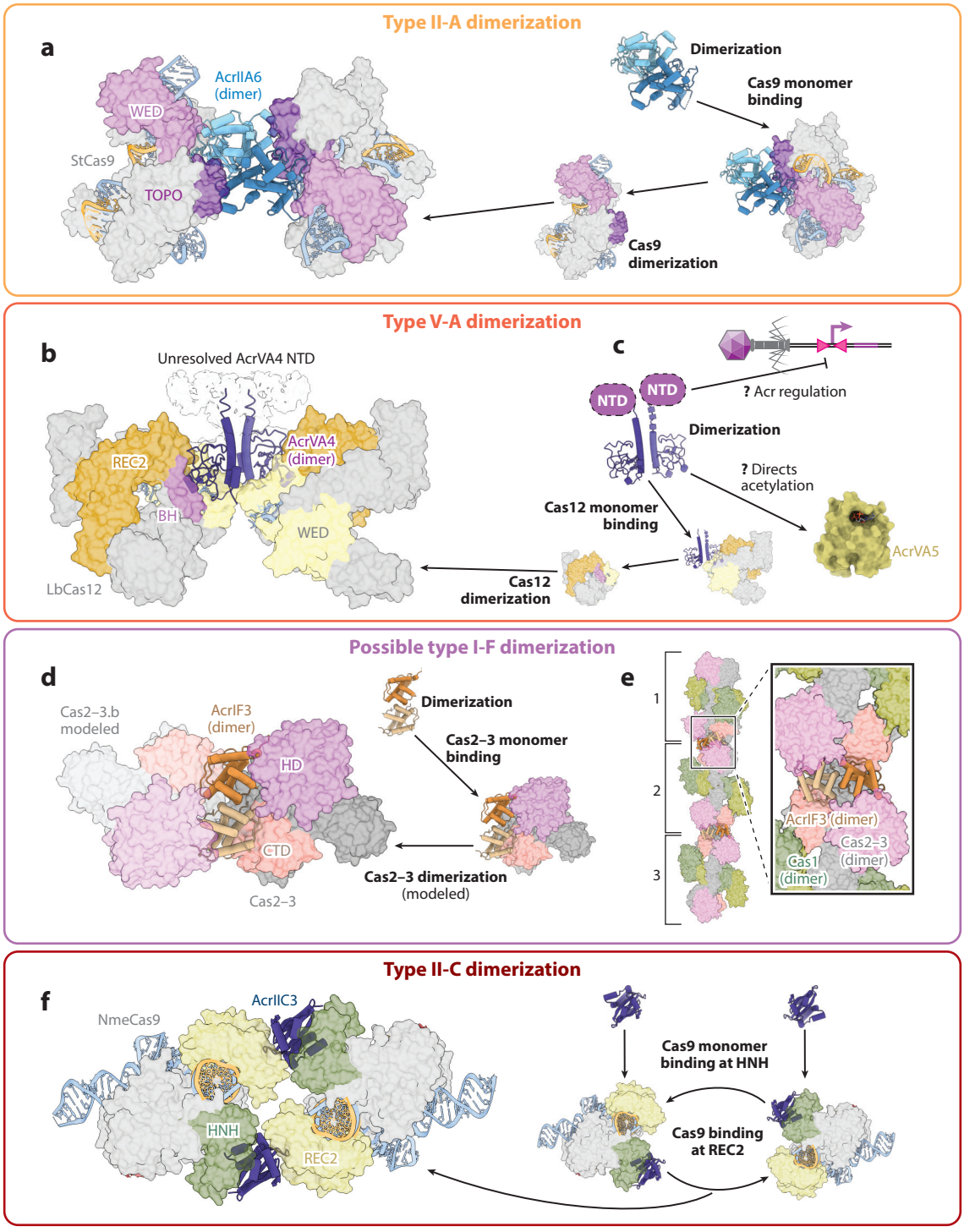
selection. Collectively, the shape and charge distribution of a specific Acr, as well as the additional structural adornments that are unique to each Acr, may help explain why each DNA mimic exclusively targets the surveillance complex of a specific subtype.

Acr-MEDIATED DIMERIZATION

Anti-CRISPRs that target diverse immune subtypes often form homodimers (27, 36, 41, 45, 46, 62, 83, 88, 89, 98, 99). This strategy may increase the effective size of an otherwise small Acr, which may in turn increase the affinity and specificity of target interactions. Moreover, homodimeric-Acrs often dimerize their corresponding immune system targets (e.g., AcrIIA6 and AcrVA4), which may balance the costs of making more Acrs upon infection (i.e., two Acrs neutralize two Cas). AcrIIA6, for example, forms a stable homodimer that recognizes one molecule of Cas9 through a series of high-affinity interactions contributed by each Acr subunit (27, 83, 99) (**Figure 4*a***). Residues that produce interactions on one face of the homodimer are symmetrically presented on the opposing face, and these residues are free to engage with an additional molecule of Cas9. Thus, one homodimer neutralizes two molecules of Cas9, and formation of the homodimeric Acr simultaneously increases the affinity for both molecules of the Cas9 target.

The benefits of homodimer formation are less clear in the case of AcrVA4. In contrast to the coordinated binding interactions by AcrIIA6, one subunit of an AcrVA4 dimer forms the majority of contacts with a single molecule of Cas12 (45, 62, 98) (**Figure 4*b***). Theoretically, a monomer of AcrVA4 would be just as effective as the dimer. Consistent with this idea, the flexible N-terminal domain (NTD) of AcrVA4 (unresolved in cryo–electron microscopy structures) was shown to be required for Acr dimerization but dispensable for Cas12 inhibition (45). However, additional selective pressures may be at play. Dimerization may be critical for regulating expression of *acrVA4*. Since the anti-CRISPR is not flanked by a known *aca* gene but does occur immediately downstream of two inverted repeats (90), AcrVA4 may be regulated through an *aca*-like mechanism. While the role of the AcrVA4 NTD remains unknown, recent work from Osuna et al. (58) shows that the NTD regions of some Acrs (e.g., AcrIIA1) are not required for immune system inhibition but rather function as Aca-type regulators of *acr* expression (58). In fact, the NTD of AcrIIA1 is also required for dimerization (41), again suggesting that while an Acr may form a dimer, this is not always necessary for Acr function. The NTD of AcrVA4 may play a similar role in regulating expression of the Acr, and this function may be dependent on dimerization of the suppressor. An alternate possibility for the biological role of AcrVA4 dimerization is that two molecules of the anti-CRISPR may be needed for interactions with AcrVA5. These Acrs co-occur in phage genomes and have been reported to form a complex (90); however, this assembly dissociates upon AcrVA4-LbCas12 binding (98). Since AcrVA5 is an enzymatic inhibitor with little substrate specificity (see below), its association with AcrVA4 may impart target specificity on an otherwise nonspecific enzyme.

Unlike Acrs that dimerize the crRNA-guided surveillance complex, some anti-CRISPRs require two molecules to neutralize a single Cas protein. AcrIIC2 forms a homodimer but fails to dimerize Cas9, suggesting two molecules of the suppressor are needed for each Cas target (83, 99). The AcrIIC2 dimer binds to apo-Cas9 (i.e., sans RNA) and prevents the RNA guide, which may increase proteolytic degradation of newly expressed Cas9 proteins (83, 99). This makes AcrIIC2 one of the more confounding and potentially interesting Acrs, since this mechanism implies that it is unable to prevent interference by Cas9 that is already loaded with a guide. This Acr would have limited utility in a cell with preformed Cas9s carrying crRNAs targeting that phage. However, since Cas9 must associate with the crRNA to fulfill its role in new sequence acquisition (30), it is



(Caption appears on following page)

Figure 4 (*Figure appears on preceding page*)

Acrs that oligomerize Cas protein targets. (*a*,*b*) AcrIIA6 and AcrVA4 dimerize StCas9 and LbCas12, respectively (PDB: 6RJA, 6JE4; EMDB: 9398). (*c*) AcrVA4 homodimer formation may facilitate *acr* regulation and/or guide AcrVA5 to the appropriate target (PDB: 6NMA, 6IUF). (*d*) Model for AcrIF3-mediated oligomerization of the Cas1–2/3 integration complex of the type I-F immune system. The AcrIF3 dimer interacts with the HD nuclease domain and CTD of Cas2/3 (PDB: 5B7I). (*e*) Proposed model for AcrIF3-mediated dimerization would enable bidirectional oligomerization of Cas1–2/3 heterohexamers, three of which are illustrated. Modeled Cas1–2/3 supercomplex joined by AcrIF3 (PDB: 5B7I, 3GOD; EMDB: 8558). (*f*) Two AcrIIC3 molecules join two molecules of NmeCas9. Domains of contact colored (PDB: 6JE4). Abbreviations: ACR, anti-CRISPR; BH, bridge-helix domain; CTD, carboxy terminal domain; EMDB, Electron Microscopy Data Bank; HD, histidine-aspartate nuclease domain; HNH, HNH nuclease domain; NTD, N terminal domain; PDB, Protein Data Bank; REC2, recognition domain; TOPO, topoisomerase-homology domain; WED, wedge domain.

possible that AcrIIC2 serves as a suppressor that inhibits both interference and Cas9-dependent integration of foreign DNA into the CRISPR (83).

Based on the emerging theme of homodimeric Acrs that trigger dimerization of their targets, we wondered if other Acr dimers might be capable of achieving a similar function. AcrIF3 is a molecular mimic of a helical bundle on the type I-F surveillance complex and forms a homodimer that binds the type I-F transacting nuclease/helicase (Cas2/3) (67, 68, 88, 89). Structures of the AcrIF3 homodimer bound to Cas2/3 reveal a series of contacts made by each AcrIF3 molecule that are arranged along one face of the homodimer (88, 89). To determine if AcrIF3 might be capable of dimerizing Cas2/3, we modeled an additional molecule of Cas2/3 onto the crystal structure of dimeric AcrIF3 bound to Cas2/3 (**Figure 4*c***). In this model, there are no major structural impediments that preclude AcrIF3-mediated dimerization of Cas2/3, and the majority of residues on the solvent-exposed binding face of AcrIF3 are available to interact with a second molecule of Cas2/3. This suggests that AcrIF3 may tether two molecules of the helicase-nuclease to prevent interference. AcrIF3 has also been shown to block adaptation (87). The mechanism for blocking adaption has not been well established, but Rollins et al. (68) previously showed that AcrIF3 binds to Cas2/3 alone and to Cas2/3 in complex with Cas1. In most type I systems, Cas2 and Cas3 are separate proteins involved in adaptation and interference, respectively. However, in I-F systems, these proteins are fused into a single polypeptide that forms a large (∼375 kDa) heterohexameric complex with Cas1 ($Cas1_4$-$Cas2/3_2$) (26, 66, 68) (**Figure 4*d***). This suggests that AcrIF3 might be a more efficient immune suppressor than has previously been appreciated, and its ability to block adaptation and interference might be complemented by the ability to trigger oligomerization of the Cas1–2/3 complex.

WHY TWO Acrs?

Unlike the homodimeric Acrs that benefit from larger binding surfaces, and in many cases dimerize their targets, a few Acrs function as monomers that bind a single surveillance complex at multiple sites (e.g., AcrIF1 and AcrIIC3). The advantage of this strength-in-numbers strategy is unclear, since multiple Acrs binding a single Cas protein will increase the anti-CRISPR concentration needed to saturate targets and slow down the process of immune suppression. For example, AcrIF1 blocks access to the crRNA guide by binding to two different sites on the Csy complex (18, 28, 63), but it is unknown whether binding at both sites is necessary to prevent crRNA-guided target engagement. After PAM binding, the crRNA is expected to directionally unwind the DNA duplex, starting at the PAM proximal end of the guide. Mismatches nearest the PAM (referred to as the seed region of the crRNA) prevent target binding, indicating that hybridization within the seed region of crRNA is critical for target recognition (15, 92). One of the two AcrIF1 molecules that bind the Csy complex blocks access to the crRNA seed. However, it is unclear whether this binding

site is sufficient to impede target binding or whether a second molecule of AcrIF1 must also bind further up the crRNA backbone to prevent target hybridization.

The advantages of multiple binding sites are more obvious for AcrIIC3, which binds to both the recognition lobe (REC2) and the HNH nuclease domain of Cas9 (42, 82, 99). In fact, AcrIIC3-mediated dimerization of Cas9 is necessary for suppression, as a single molecule of AcrIIC3 is unable to prevent Cas9 target cleavage (99). Recently published structures reveal that two molecules of AcrIIC3 bridge oppositely oriented molecules of Cas9, creating a ring-shaped structure (82) (**Figure 4*e***). AcrIIC3 prevents activation of the Cas9 HNH nuclease (82), but it is unclear why one Acr protein is unable to block this conformational rearrangement. To understand the mechanism of AcrIIC3, we superimposed a molecule of the Acr onto structures of Cas9 in multiple conformational states. This analysis reveals that Cas9 conformational changes induced by target binding and nuclease activation disrupt contacts with AcrIIC3, and these conformational changes must dislodge the anti-CRISPR in the absence of additional stabilizing contacts with a second molecule of Cas9. This indicates that assembly of the ring-shaped heterotetramer provides additional contacts to both molecules of AcrIIC3, allowing each suppressor to pin the HNH domains of the two Cas9s in inactive conformations. Collectively, this suggests a model where two AcrIIC3-Cas9 heterodimers bind one another to neutralize each surveillance complex (**Figure 4*e***), and that a monomer of AcrIIC3 is insufficient to inhibit one molecule of Cas9 but two Acrs collectively inhibit two surveillance complexes.

Enzymatic Suppressors of CRISPR Immunity

Enzymatic suppressors are generally larger than stoichiometric inhibitors, which may be a slight disadvantage with respect to expression and delivery to the cell, but enzymes are multiple-turnover, which suggests that fewer Acr molecules are necessary to do the job.

Covalent modification of the surveillance complex. In contrast to Acrs with steric and allosteric mechanisms of immunosuppression, AcrVA1 and AcrVA5 are enzymes that covalently modify the Cas12-crRNA complex, making them potent suppressors even at substoichiometric concentrations (23, 46, 98). This may be especially beneficial for type V suppressors, since Cas12 is capable of multiple-turnover targeting (78). AcrVA1 is an endoribonuclease that cleaves between the fifth and sixth positions of the crRNA (**Figure 5*a*,*b***), while AcrVA5 is a GCN5-related *N*-acetyltransferase (GNAT) that acetylates lysines (**Figure 5*c*,*d***) (23, 46, 98). Initially, AcrVA5 was thought to target acetylation to a specific PAM-sensing residue (i.e., K635), but Dong et al. (23) detected widespread acetylation of Cas12, which may indicate that AcrVA5 is more promiscuous than previously appreciated (**Figure 5*c***). The biochemical promiscuity of AcrVA5 is consistent with the lack of an N-terminal specificity domain that is found in other GNAT homologs (70) (**Figure 5*d***). However, indiscriminate acetylation seems like a dangerous and ineffective strategy for a bacteriophage dependent on host resources for replication. The previously reported formation of a complex between AcrVA5 and AcrVA4 (98) suggests that AcrVA4 may direct the acetyltransferase activity of AcrVA5 to the appropriate substrate (i.e., Cas12).

Degradation of Second Messenger Molecules

Cyclic oligonucleotides are important regulators of immune systems in all domains of life (33, 56). Recently it has been demonstrated that detection of invading nucleic acids by the type III CRISPR-Cas immune systems triggers synthesis of cyclic-oligoadenylate (cA_4), which is a signaling molecule that activates additional nucleases that are essential for immune clearance (55). The

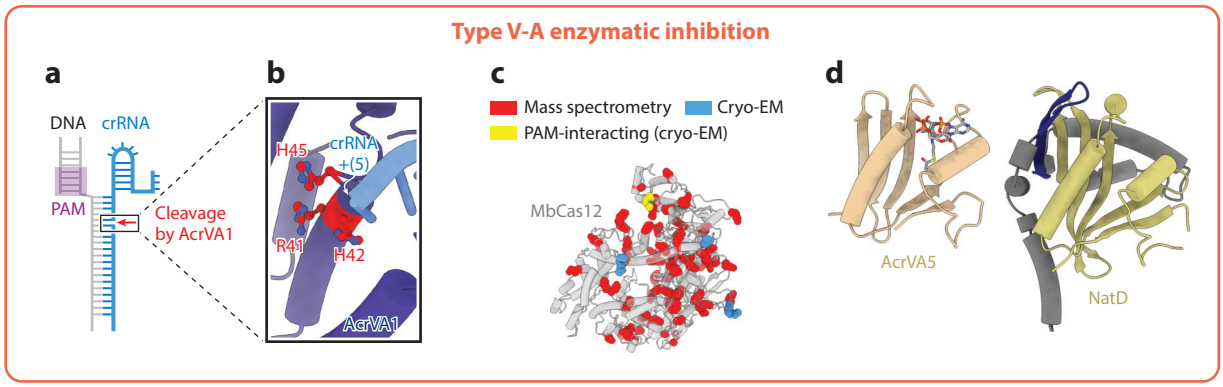


Figure 5

Enzymatic Acrs that inhibit type V-A CRISPR-Cas systems. (*a*) AcrVA1 is an endoribonuclease that cleaves between the fifth and sixth positions of the crRNA guide. (*b*) Active-site residues of AcrVA1 (*red*) responsible for crRNA cleavage (PDB: 6NMD). (*c*) Residues of MbCas12 acetylated by AcrVA5 according to mass spectrometry (*red*) or cryo-EM and mass spectrometry (*blue* and *yellow*) (PDB: 6IV6). (*d*) Comparison of AcrVA5 (*tan*) and closest structural homolog (NatD from *Homo sapiens*) (RMSD = 0.82 Å for 36 equivalently positioned C-α atoms). Structural features of NatD shared with AcrVA5 colored in olive. Two NatD β strands that determine acceptor substrate specificity (*dark blue*) and additional N-terminal structural features (*gray*) (PDB: 6IUF, 4U9W). Abbreviations: Acr, anti-CRISPR; crRNA, CRISPR RNA; EM, electron microscopy; PAM, protospacer-adjacent motif; PDB, Protein Data Bank; RMSD, root-mean-square deviation.

importance of cA_4 in immune signaling is highlighted by the recent discovery of a widespread anti-CRISPR (i.e., AcrIII-1) that enzymatically degrades cA_4, thereby neutralizing the immune response (4). AcrIII-1, which has a novel fold, assembles into a homodimer that specifically recognizes the symmetry of cA_4. Though it may be a coincidence, we find the importance of symmetry imposed by homodimers to be remarkable. This includes homodimeric Acrs that dimerize the Cas proteins they target, homodimeric Acas that recognize the symmetry of inverted DNA repeats in the promoter, and now homodimeric Acrs that recognize the symmetry of second messengers.

In our opinion, AcrIII-1 represents an emerging class of anti-CRISPRs that transcend traditional subtype boundaries because of their unique mechanisms of action. Given the lack of reliance on a specific protein-protein interaction, this anti-CRISPR is presumed to inhibit any system that relies on cA4, regardless of subtype (4). This suggests that, like Cas proteins, second messengers are also in conflict, and we expect that Acrs will mirror the diversity of the messengers themselves. At a practical level, Acrs that target second messengers, rather than a subtype-specific protein, may defy the existing Acr naming scheme, which typically includes a letter to designate a specific subtype rather than a second messenger that might be present in many different subtypes. For this new generation of Acrs, we suggest a naming scheme that incorporates the specific messenger (e.g., AcrIII-1-cA4).

THE FUTURE OF ANTI-CRISPR

Gazing into the anti-CRISPR crystal ball, it seems that only one thing is certain. The discovery of new anti-CRISPRs will continue to provide new insights about Acr mechanisms, while simultaneously revealing mechanistic vulnerabilities of the immune systems that they inhibit. To date, we are aware of 14 stoichiometric inhibitors that either block DNA binding (PAM recognition or hybridization to the guide) or allow binding but prevent nuclease activation, and DNA mimics appear to be a common theme in the type I, II, and V systems. We anticipate the discovery of Acrs

that adopt similar strategies for RNA-targeting systems (type III and type VI). Protein mimics, like AcrIF3 (which mimics the helical bundle of Cas8f), also appear to be effective suppressors, and we expect that protein mimics will be identified with increasing frequency. Similarly, we anticipate that enzymatic inhibitors are currently underrepresented, and we expect that multiple-turnover enzymes that posttranslationally modify Cas proteins or posttranscriptionally modify crRNAs will continue to reveal the versatility of these inhibitors.

As the repertoire of crRNA-guided nuclease continues to expand, the unique features of each system (i.e., PAM requirements, protein sizes, and cleavage mechanisms) are seamlessly incorporated into the growing quiver of genome-editing tools that each have specialized chemical properties that make them more appropriate for particular applications (55, 94). Much like the CRISPRs, anti-CRISPRs are now being repurposed for applications in genome engineering. The most frequent use of Acr proteins has been as rheostats for tunable and tissue-specific control of gene editing or gene repression/activation (52). Anti-CRISPRs have now been deployed using optogenetic and ligand-based posttranslational switches for control of Cas9 or dCas9 activity (14, 52). Additional proof-of-principle work has demonstrated the ability of anti-CRISPRs to reverse or limit Cas9-based gene drives (5) and reduce off-target gene editing by limiting excessive Cas protein expression/activity through delayed Acr delivery (74) or direct fusion of Acr mutants to the Cas9 nuclease (3). We predict similar applications will emerge for Acrs that target Cas12- and Cas13-based systems, as well as Class 1 systems that have recently been domesticated for gene editing (21, 31) or RNA-guided delivery of large DNA cargos to specific genetic addresses (44, 93).

Given the prevalence of CRISPR systems in archaeal genomes (~85%) (48), we expect that archaeal viruses will deploy the most diverse, abundant, and innovative Acr-based solutions for subverting CRISPR-based immunity and that these Acrs will in turn drive immunological innovation. Aside from AcrIII-1, only two other archaeal anti-CRISPR proteins have been described to date (i.e., AcrID1 and AcrIIIB1) (6, 29). Remarkably, SIRV2 contains up to 12 copies of AcrID1 (29). This observation suggests that duplicated Acrs may be involved in dosage compensations, in a way that is conceptually similar to the expansions of K3L suppressors in poxviruses (DNA accordions) (25). Alternatively, or perhaps in addition, it is possible that each of these paralogs have nuanced, nonredundant functions.

The work on AcrIII-1 highlights the biological innovation that occurs in response to the selective pressures imposed by viruses and encourages us to think beyond the stoichiometric or enzymatic proteins that bind directly to Cas proteins or crRNAs. We anticipate that, like eukaryotic viruses, phages and viruses that infect archaea will also use noncoding RNAs or peptide-based inhibitors that serve as decoys or molecular jamming devices that block or redirect the immune systems. We are inspired by the recent work on Acr proteins that degrade cyclic nucleotides, and we are reminded of recent work demonstrating that poxviruses also degrade cyclic nucleotides that are essential for immune signaling in eukaryotic immune systems (24). We anticipate that eukaryotic and prokaryotic immunologists will continue to identify common themes in immune defense and viral counterdefense, and based on historical precedence, we anticipate that many of these new discoveries will lead to disruptive new technologies that will, once again, transform industry and medicine.

DISCLOSURE STATEMENT

B.W. is the founder of SurGene, LLC, and is an inventor on patent applications related to CRISPR-Cas systems and applications thereof. J.B.-D. is a scientific advisory board member of SNIPR Biome and Excision Biotherapeutics and a scientific advisory board member and cofounder of Acrigen Biosciences.

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