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# Structure-based functional mechanisms and biotechnology applications of anti-CRISPR proteins

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Abstract | CRISPR loci and Cas proteins provide adaptive immunity in prokaryotes against invading bacteriophages and plasmids. In response, bacteriophages have evolved a broad spectrum of anti-CRISPR proteins (anti-CRISPRs) to counteract and overcome this immunity pathway. Numerous anti-CRISPRs have been identified to date, which suppress single-subunit Cas effectors (in CRISPR class 2, type II, V and VI systems) and multisubunit Cascade effectors (in CRISPR class 1, type I and III systems). Crystallography and cryo-electron microscopy structural studies of anti-CRISPRs bound to effector complexes, complemented by functional experiments in vitro and in vivo, have identified four major CRISPR–Cas suppression mechanisms: inhibition of CRISPR–Cas complex assembly, blocking of target binding, prevention of target cleavage, and degradation of cyclic oligonucleotide signalling molecules. In this Review, we discuss novel mechanistic insights into anti-CRISPR function that have emerged from X-ray crystallography and cryo-electron microscopy studies, and how these structures in combination with function studies provide valuable tools for the ever-growing CRISPR–Cas biotechnology toolbox, to be used for precise and robust genome editing and other applications.

Restriction–modification
A system in prokaryotes that typically contains a restriction endonuclease and a methylase that target pathogenic DNA.

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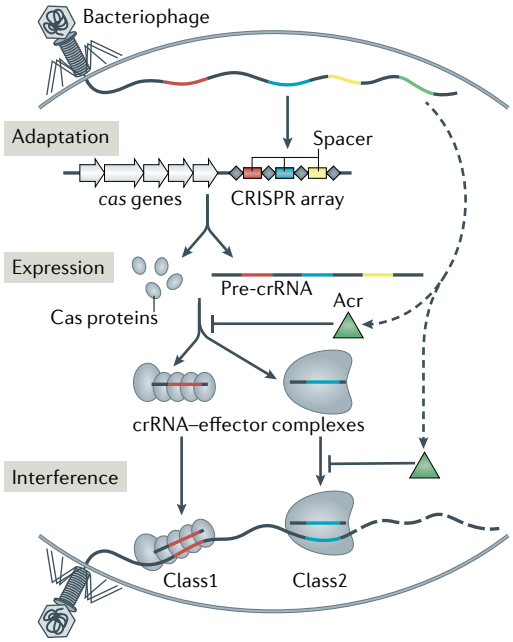
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Sensing foreign nucleic acids is one of most common cellular immunity strategies in all living organisms. In eukaryotes, the cGAS–STING signalling pathway can be activated by the presence of DNA in the cytosol, leading to innate-immunity activation through the production of type I interferons[1]. Unsurprisingly, pathogens have evolved various ways to overcome such defences[2,3]. In prokaryotes, bacteria and archaea have evolved multiple immune systems, both innate and adaptive, to protect against bacteriophage (phage) infections by targeting such 'invading' nucleic acids, including cleavage of invading phage genomes by innate restriction–modification systems[4], and CRISPR–Cas gene systems, which provide prokaryotes with adaptive immunity by memorization of past invading nucleic acids from phages[5] and plasmids[6]. In response, phages have evolved diverse counter-strategies to evade these antiviral systems, including modification of restriction sites or degradation of restriction–modification cofactors[7]. Evasion of CRISPR–Cas-based immunity has also been achieved through selection for point mutations in the CRISPR–Cas targeted sequence[8] or the formation of nucleus-like structures[9,10], which prevents target recognition or cleavage, or by generation of anti-CRISPR proteins (anti-CRISPRs) that directly inactivate the CRISPR–Cas effector complex[11–13].

CRISPR–Cas systems are widely distributed, having been found in approximately 85% of archaeal and approximately 40% of bacterial completely sequenced genomes[14]. Notably, CRISPR–Cas systems are highly diverse, and are currently classified into two broad classes, which together include six types that are categorized on the basis of phylogeny, *cas* gene composition and mechanisms of action[14,15]. Class 1 systems (which include types I, III and IV) are composed of multisubunit effector complexes, whereas class 2 systems (which include types II, V and VI) comprise single-subunit effector proteins. Despite these differences, all systems rely on the same basic principle of action, spanning three main stages: adaptation, expression and interference[16,17] (BOX 1). During the first, adaptation stage, the small DNA fragments derived from the invader, known as spacers, are integrated into the host CRISPR locus to immunize the host cell; this incorporation of spacers generates a genetic memory of the infection. In the second stage of defence, the CRISPR loci are transcribed into precursor CRISPR-derived RNAs (crRNAs) and further processed into short mature crRNAs. Finally, at the target interference stage, individual crRNAs assemble with Cas proteins to form crRNA–effector complexes, which are responsible for sequence-specific recognition and degradation of the invading nucleic acids. The requirement

Box 1 | **CRISPR–Cas adaptive immunity in prokaryotes**




CRISPR–Cas systems provide prokaryotes with adaptive immunity against invading bacteriophages[5] and plasmids[6]. This immunity involves three main stages: adaptation, expression and interference[16,17]. In the adaptation stage, short DNA fragments from the invading genomes are extracted and integrated into the host CRISPR array (see the figure). The CRISPR array consists of a series of direct repeats (grey diamonds) separated by the invading genetic elements (coloured boxes), known as spacers. The spacers function as a memory of the invader. During the expression stage, the CRISPR array is transcribed into long precursor CRISPR-derived RNAs (pre-crRNAs) that are processed into short mature crRNAs. Each crRNA consists of a single spacer that is typically flanked by parts of the repeat sequences. These crRNAs are assembled with one or more Cas proteins into crRNA-guided CRISPR–Cas effector complexes. On the basis of the composition of the effector complexes, CRISPR–Cas systems can be divided into two broad classes: class 1 systems, which use multiple-unit Cas proteins as effectors, and class 2 systems, which possess a single Cas protein[14,15]. In the interference stage, the crRNA guides the Cas effector (complex) to the target DNA or RNA site by recognizing a specific sequence through complementary base pairing, leading to the cleavage of the nucleic acids of the invading virus. As a result of the arms race between bacteriophages and their bacterial hosts, the bacteriophages have evolved various strategies to evade the host's CRISPR–Cas immunity. Anti-CRISPR (Acr) proteins encoded by bacteriophages are such counter-defence weapons that can directly inactivate CRISPR–Cas systems by interfering at different immunity stages, including by inhibiting CRISPR–Cas complex assembly, blocking of target DNA binding and preventing target cleavage[13,23–25,29,34,35].

of complementarity between crRNAs and phage nucleic acid targets allows phages to evade CRISPR–Cas immunity by undergoing rapid mutagenesis[8,18]. However, in contrast to other defence systems, the adaptive nature of CRISPR–Cas systems allows them to also acquire new spacers to counter these phage 'escapees'[18–20]; thus, mutagenesis alone is not sufficient for long-term phage survival.

To combat the adaptive nature of CRISPR–Cas surveillance complexes, phages have evolved protein inhibitors of CRISPR–Cas systems, known as anti-CRISPRs (Acr proteins), which were first discovered in 2013 (REF.[13]). Subsequently, knowledge of anti-CRISPRs has expanded rapidly, to date including 88 anti-CRISPR proteins identified in natural mobile genetic elements[21], and one synthetic small-molecule inhibitor that is not derived from a phage[22]. These reported anti-CRISPRs span five CRISPR–Cas subtypes, including class 2 types II, V and VI and class 1 types I and III, with none reported to date for type IV (TABLE 1). Distinct anti-CRISPRs sharing no sequence features between them have been discovered by diverse approaches, which are outlined in Supplementary Box 1. Biochemical and structural studies have uncovered specific mechanisms of function for 29 of these anti-CRISPRs, which revealed four principal strategies to counter CRISPR–Cas systems: inhibition of CRISPR–Cas complex assembly, blocking of target binding, prevention of target cleavage and degradation of cyclic oligonucleotide signalling molecules. Additional emerging strategies used by anti-CRISPRs include Cas inactivation through dimer formation and post-translational modification of Cas effectors (reviewed in REFS[23–35]).

Owing to their RNA-guided, sequence-specific recognition capacity, CRISPR–Cas systems have been repurposed as powerful genetic manipulation tools for gene and genome editing[36–38], gene regulation[39], imaging and molecular diagnosis[40–42]. The simplicity and robustness of this technology has transformed gene manipulation from being a fundamental-science technology to a tool used in a range of medical applications. Nevertheless, outstanding challenges remain related to the safety aspects of the gene manipulation process, which can lead to both off-target effects[43] and cellular toxicity[44], especially in the context of clinical development. Anti-CRISPRs are capable of interfering with CRISPR–Cas activities and potentially provide temporal, spatial and conditional control of CRISPR–Cas-derived genome editing tools. Applications of anti-CRISPRs as gene editing tools have been reported in prokaryotes and eukaryotes, especially in mammalian cells[33], and mirrored efforts to achieve a molecular understanding of the eukaryotic cGAS–STING pathway and its regulation, thereby facilitating the development of therapeutics for microbial infections and human diseases[45].

In this Review, we provide an up-to-date characterization and discussion of recent discoveries, mechanisms and applications of anti-CRISPRs. In contrast to previous reviews, we highlight novel mechanistic insights gained from recent X-ray crystallography and cryo-electron microscopy (cryo-EM) studies into anti-CRISPR suppression through competitive binding, allosteric control and enzymatic inhibition of Cas proteins and multisubunit Cascade complexes. Such a molecular understanding of CRISPR–Cas regulation should facilitate the development of precise and better-controlled CRISPR–Cas-based genome manipulation tools.

## Inhibiting class 1 CRISPR–Cas systems

Class 1 CRISPR–Cas systems, which include types I, III and IV, have effector complexes called 'Cascade', comprising multiple Cas proteins. Forty-six anti-CRISPRs have been identified in the type I system and two anti-CRISPRs have been identified in the type III system, but no anti-CRISPRs have been found yet in the comparatively rare type IV system (TABLE 1). These anti-CRISPRs inhibit CRISPR–Cas immunity by targeting various Cas proteins or through degradation of the second messenger.

### *Mechanisms of type I anti-CRISPRs*

Type I CRISPR–Cas immunity is achieved by the crRNA-guided Cascade complex and the helicase–nuclease Cas3 (REFS[17,46]) (FIG. 1a). A precursor crRNA is transcribed from the CRISPR array and processed into

Second messenger
An intracellular signalling molecule that transmits and amplifies the signal generated by extracellular signalling molecules (the first messengers).

R-loop
An RNA–DNA hybrid structure in which RNA pairs with a complementary DNA strand, causing the non-complementary DNA strand to loop out.

crRNAs by Cas6 (REFS[46,47]). The crRNA is then assembled with Cas proteins into a multisubunit Cascade complex, and its spacer hybridizes to a complementary sequence in the target DNA, known as the protospacer[46,48,49]. A short protospacer-adjacent motif (PAM) located immediately upstream of the DNA target is recognized by Cas8, a subunit of the Cascade complex (FIG. 1b). PAM recognition is required for subsequent unwinding of the target DNA, which is followed by its hybridization with the crRNA[50,51]. The displacement of the non-target DNA strand generates an R-loop and triggers the recruitment of Cas3, which degrades the non-target and target DNA strands[52–56]. Anti-CRISPR type I, subtype F1 (AcrIF1) and AcrIF2–AcrIF5 were the first anti-CRISPRs to be discovered, and the first CRISPR–Cas inhibition mechanisms were reported for AcrIF1–3: AcrIF1 and AcrIF2 (also known as AcrIC2)[57] sterically block binding of target DNA, and AcrIF3 prevents the recruitment of Cas3 (REF.[58]) (FIG. 1a,b). To date, nine anti-CRISPRs have been shown to inhibit CRISPR–Cas immunity by direct inhibition of DNA binding, either by blocking hybridization of target DNA with the crRNA guide (AcrF1, AcrIF8, AcrIF9 and AcrIF14) or by blocking the PAM-binding site on double-stranded DNA (dsDNA; AcrIF2, AcrIF6, AcrIF7, AcrIF10 and AcrIF11). Blocking of DNA binding is the most common function of type I anti-CRISPRs. Two other anti-CRISPRs (AcrIE1 and AcrIF3) directly bind to Cas3 to prevent it from cleaving DNA.

***Inhibition of DNA binding by blocking crRNA–DNA hybridization.*** In vitro biochemical studies have shown that AcrIF1 blocks target DNA recognition by directly binding to the Cas7 subunits of Cascade, with an estimated stoichiometry of 2.6 ± 0.3 AcrIF1 molecules per Csy (*Pseudomonas aeruginosa* Cascade)–crRNA complex[58]. Cryo-EM structures of the AcrIF1–Csy–crRNA complex consistently show that AcrIF1 directly interacts with the Cas7 backbone between two and three distinct sites, thereby sterically occluding target DNA and preventing its hybridization to the crRNA guide[59–61] (FIG. 1c). Nuclear magnetic resonance-based structural studies and site-directed mutagenesis of AcrIF1 identified three residues (Tyr6, Tyr20 and Glu31) that are crucial for AcrIF1-mediated anti-CRISPR activity[62]. A subsequent cryo-EM structure of Csy–crRNA–AcrIF1 revealed that these three AcrF1 residues interact with a conserved lysine (Lys85) on Cas7 (REFS[59,60]). Mutation of Lys85 to Ala disrupts not only Cas7 binding of AcrIF1 but also its binding of target DNA, indicating

Table 1 | **Main features of the six types of CRISPR–Cas systems**

| Class | Type | Subtype | Effector complex | Signature protein | Target | PAM requirement | Nuclease | Anti-CRISPRs (Acr proteins) |
|---|---|---|---|---|---|---|---|---|
| 1 | I | I-B | Multisubunit complex | Cas3 | dsDNA | Yes | Cas3 | AcrIB |
| | | I-C | | | | | | AcrIC1, AcrIF2 (also known as AcrIC2), AcrIC3, AcrIC4, AcrIC5, AcrIC6, AcrIC7, AcrIC8, AcrIC9, AcrIC10, AcrVA3 |
| | | I-D | | | | | Cas3 and Cas10d | AcrID1 |
| | | I-E | | | | | Cas3 | AcrIE1, AcrIE2, AcrIE3, AcrIE4, AcrIE5, AcrIE6, AcrIE7, AcrIE8, AcrIE9, AcrIE4-IF7 |
| | | I-F | | | | | | AcrIF1, AcrIF2, AcrIF3, AcrIF4, AcrIF5, AcrIF6, AcrIF7, AcrIF8, AcrIF9, AcrIF10, AcrIF11, AcrIF12, AcrIF13, AcrIF14, AcrIF15, AcrIF16, AcrIF17, AcrIF18, AcrIF19, AcrIF20, AcrIF21, AcrIF22, AcrIF23, AcrIF24, AcrIE4-IF7 |
| | III | III-A | | Csm1 (also known as Cas10) | ssRNA, ssDNA | No | Csm3, Csm1 (also known as Cas10) | AcrIII-1 |
| | | III-B | | | | | Cmr4, Cmr2 (also known as Cas10) | AcrIIIB1, AcrIII-1 |
| | IV | | | Unknown | Unknown | Unknown | Unknown | Unknown |
| 2 | II | II-A | Single protein | Cas9 | dsDNA | Yes | Cas9 RuvC and HNH domains | AcrIIA1, AcrIIA2, AcrIIA3, AcrIIA4, AcrIIA5, AcrIIA6, AcrIIA7, AcrIIA8, AcrIIA9, AcrIIA10, AcrIIA11, AcrIIA12, AcrIIA13, AcrIIA14, AcrIIA15, AcrIIA16, AcrIIA17, AcrIIA18, AcrIIA19, AcrIIA20, AcrIIA21, AcrIIA22, AcrIIA23, BRD0539 |
| | | II-C | | | | | | AcrIIC1, AcrIIC2, AcrIIC3, AcrIIC4, AcrIIC5 |
| | V | V-A | | Cas12 | dsDNA, ssRNA | Yes | Cas12 RuvC domain | AcrVA1, AcrVA2, AcrVA3, AcrVA4, AcrVA5 |
| | VI | VI-A | | Cas13 | ssRNA | No | Cas13 HEPN domain | AcrVIA1 (Lwa), AcrVIA2, AcrVIA3, AcrVIA4, AcrVIA5, AcrVIA6, AcrVIA7, AcrVIA1 (Lse) |

dsDNA, double-stranded DNA; HEPN domain, higher eukaryote and prokaryote nucleotide-binding domain; Lse, *Listeria seeligeri*; Lwa, *Leptotrichia wadeii*; ssDNA, single-stranded DNA; ssRNA, single-stranded RNA.

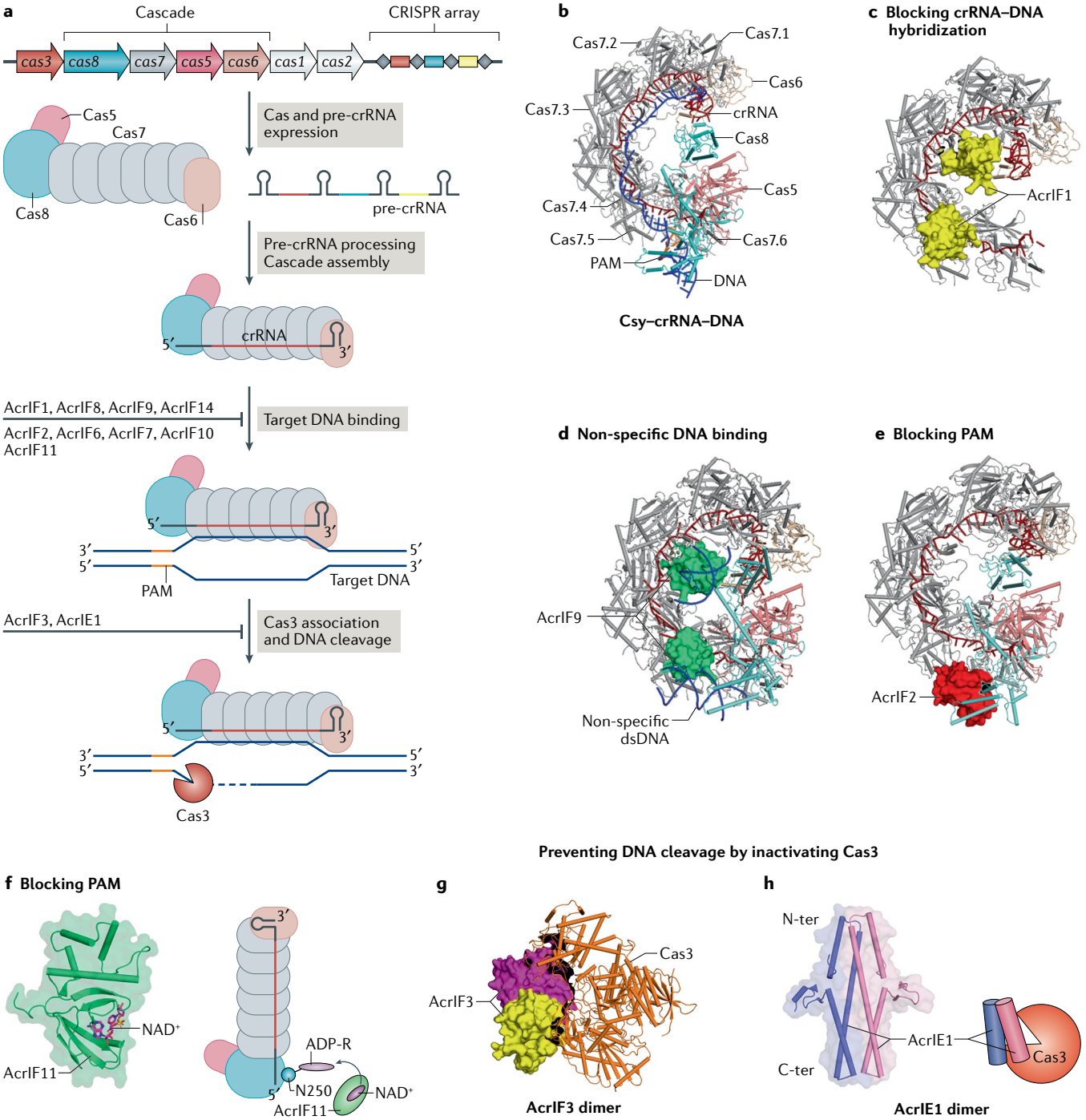



Fig. 1 | **Functional mechanisms of type I anti-CRISPRs. a** | Cas proteins are assembled with a CRISPR-derived RNA (crRNA) that is transcribed and processed from a CRISPR array to form the multisubunit Cascade complex. The crRNA guides the Cascade complex to recognize the protospacer-adjacent motif (PAM) and binds to the target DNA. This is followed by recruitment of the helicase–nuclease Cas3, which degrades both strands of the target DNA. To date, structure-based mechanistic insights are available for 11 anti-CRISPR (Acr) proteins in type I systems, which block target DNA binding and cleavage. **b** | Structure of Csy (*Pseudomonas aeruginosa* Cascade)–crRNA–DNA complex (Protein Data Bank (PDB) code 6B44). The PAM sequence is shown in orange. **c** | Two AcrIF1 molecules bound to the Csy–crRNA complex (PDB code 6B46) prevent hybridization of the crRNA guide to target DNA. **d** | Structure of the Csy–crRNA–AcrIF9 complex bound to non-specific double-stranded DNA (dsDNA) (PDB code 6WHI). AcrIF9 inhibits CRISPR–Cas activity by triggering non-specific dsDNA binding by the Csy–crRNA complex. **e** | Structure of AcrIF2 bound to a Csy–crRNA complex (PDB code 6B47). AcrIF2 sterically inhibits target DNA binding by blocking the PAM recognition site. **f** | Structure of AcrIF11 binding nicotinamide adenine dinucleotide ($NAD^+$) (PDB code 6KYF). The schematic representation shows that the key PAM recognition residue Asn250 (N250) in the Csy–crRNA complex undergoes ADP-ribosylation (ADP-ribose (ADP-R) is derived from $NAD^+$) by AcrIF11, resulting in the complete loss of DNA-binding ability of the Csy–crRNA complex. **g** | Structure of an AcrIF3 dimer bound to the *trans*-acting helicase–nuclease Cas3 (PDB code 5B7I). The direct interaction between AcrIF3 and Cas3 prevents the recruitment of Cas3 to the Csy–crRNA–DNA complex, thus inhibiting target DNA cleavage by Cas3. **h** | Structure of an AcrIE1 dimer (PDB code 6ARZ) (left panel) and a postulated schematic representation of dimeric AcrIE1 binding to and inhibiting Cas3 (right panel). C-ter, carboxy terminal; N-ter, amino terminal; pre-crRNA, precursor CRISPR-derived RNA.

that this conserved Cas7 residue targeted by AcrIF1 is also essential for crRNA guide–target DNA binding[59]. Furthermore, the observed structures of the web domain (composed of loops that interact with crRNA) of Cas7 between AcrIF1-free and AcrIF1-bound states suggest that AcrIF1 binding may induce a local conformational change in Cas7 that further restricts target DNA access[59]. Similarly to AcrIF1, two molecules of AcrIF14 (REF.[63]) and one molecule of AcrIF8 (REF.[64]) competitively bind to the target DNA-binding site on different Cas7 subunits of the Csy–crRNA complex, and sterically block crRNA–DNA hybridization.

***Inhibition of DNA binding by triggering non-specific dsDNA binding.*** The cryo-EM structure of Csy–crRNA–AcrIF9 shows two molecules of AcrIF9 (FIG. 1d) bound to a site on the Csy–crRNA complex similar to the one observed for AcrIF1 (FIG. 1c), probably sterically blocking crRNA–dsDNA hybridization[64,65]. Additionally, binding of AcrIF9 to Csy–crRNA triggers sequence-non-specific dsDNA binding by the complex, that is, binding of non-target DNA (FIG. 1d), potentially sequestering the Cascade complex away from the target DNA and further enhancing the anti-CRISPR activity[65]. Interestingly, a recent report showed that specific dsDNA and non-specific dsDNA can rapidly exchange binding to an overlapping site on the Csy–crRNA–AcrIF9 complex, suggesting that complementarity between dsDNA and crRNA does not contribute to the complex–dsDNA interaction[66]. By contrast, single-stranded DNA (ssDNA) complementary to crRNA shows stronger binding to the Csy–crRNA–AcrIF9 complex than non-specific ssDNA, suggesting that Csy–crRNA–AcrIF9 still maintains sequence-specific binding capacity for ssDNA. Mutation of the conserved positively charged DNA-binding surface of AcrIF9 abolished the non-specific dsDNA-binding ability of Csy–crRNA and decreased its activity in vivo, but still retained its specific dsDNA-binding ability, indicating that the non-specific DNA-binding ability plays a key role in the AcrIF9-mediated inhibition mechanism[66], which is distinct from the AcrIF1-mediated inhibition mechanism of sterically blocking specific crRNA–dsDNA hybridization[59].

***Inhibition of DNA binding by targeting PAM recognition sites.*** PAM recognition is the essential initial step for binding of target DNA in type I CRISPR–Cas systems. Therefore, binding PAM recognition sites is another strategy used by anti-CRISPRs to escape CRISPR–Cas immunity (FIG. 1e,f). A conserved Lys-containing loop (residues 246–250), designated 'K-wedge' in Cas8, is crucial for PAM recognition[60]. Mutation of Lys247 (K247E) led to severe defects in binding DNA targets[59]. The Cas8-K247E mutant also showed decreased binding ability for AcrIF2 (REF.[59]) and AcrIF7 (REF.[67]), and correspondingly mutation of Cas8 Lys247-binding residues in AcrIF6 breaks the binding interface mediated by Lys247 and impairs the inhibition of AcrIF6 for target DNA cleavage[64], suggesting that AcrIF2, AcrIF6 and AcrIF7 target the conserved PAM recognition site. In vitro biochemical assays also show that AcrIF2 directly interacts with the Cas8 and Cas5 subunits of the Cascade complex[58]. In addition to these biochemical assays, the conserved PAM recognition site is also shown to be engaged by AcrIF2 in the cryo-EM structure of the Csy–crRNA–AcrIF2 complex[59,60] (FIG. 1e), and similarly by AcrIF6 (REF.[64]), AcrIF7 (REF.[63]) and AcrIF10 (REF.[60]).

Interestingly, distinct from previously reported strategies of blocking PAM recognition by forming stable complexes between anti-CRISPRs and the Csy–crRNA Cascade complex, AcrIF11 is a mono-ADP-ribosyltransferase that specifically ADP-ribosylates the Asn250 residue of Cas8, which is required for PAM recognition, leading to complete loss of DNA binding by Csy–crRNA[68] (FIG. 1f). This post-translational protein modification-based mechanism could make AcrIF11 a more potent inhibitor than AcrIF2, AcrIF6, AcrIF7 or AcrIF10. Targeting the conserved PAM recognition site is a potent general strategy used by anti-CRISPRs to evade CRISPR–Cas immunity.

***Preventing DNA cleavage by inactivating Cas3.*** Preventing target DNA cleavage is another strategy used by anti-CRISPRs to inhibit CRISPR–Cas immunity. AcrIF3 inhibits target DNA cleavage by preventing the recruitment of the *trans*-acting helicase–nuclease Cas3 to the Csy–DNA complex[58]. Structures of AcrIF3–Cas3 complexes determined by X-ray crystallography[69] and cryo-EM[70] reveal that AcrIF3 forms a homodimer that directly binds to Cas3 (FIG. 1g), locks it in an ADP-bound form and thus blocks the DNA entrance tunnel of Cas3 to prevent it from accessing the non-complementary target DNA. Furthermore, structural similarity between AcrIF3 and the carboxy-terminal (C-terminal) domain of Cas8 suggests that AcrIF3 probably mimics the C-terminal domain of Cas8, which is involved in Cas3 recruitment, thus blocking the recruitment of Cas3 to the Csy–crRNA–DNA complex[59]. In addition, inhibition of Cas3-mediated DNA degradation by AcrIF3 also inhibits primed spacer acquisition[70,71]. Similarly to AcrIF3, AcrIE1 inactivates Cas3 in subtype I-E CRISPR–Cas systems by directly binding to Cas3 as a dimer, with the C-terminal region of AcrIE1 being crucial for Cas3 inhibition[72] (FIG. 1h).

Cas3 is composed of a superfamily 2 helicase domain, which unwinds dsDNA, and a His-Asp (HD) nuclease domain, which cleaves DNA[52,56]. An HD nuclease domain encompasses also the amino terminus (N terminus) of Cas10d[52,56,73], which is responsible for dsDNA cleavage in the subtype I-D effector complex and directly interacts with AcrID1, which is an anti-CRISPR from an archaeal virus[73–75]. The crystal structure of AcrID1 reveals that it is a head-to-tail dimer that has a tendency to form fibres[74], and that two AcrID1 dimers bind to two Cas10d proteins, forming a circular 4:2 complex. This complex sequesters Cas10d into a non-functional, higher-order state unable to form a cleavage-competent effector complex, thereby preventing DNA cleavage[75].

***Unclear mechanisms of type I anti-CRISPR function.*** AcrIF4 was also shown to block CRISPR interference (CRISPRi) activity in its native host *P. aeruginosa*, suggesting that it blocks binding of target DNA in vivo[58]. However, a recent in vitro biochemical study showed

Primed spacer acquisition
The preferential acquisition of new spacers from a target recognized by a CRISPR-derived RNA-guided effector complex.

CRISPR interference
(CRISPRi). A technique that typically exploits a catalytically dead CRISPR–Cas effector complex to block transcription and thereby silence genes.

Single guide RNA (sgRNA). A synthetic fusion of the *trans*-activating CRISPR RNA (tracrRNA) and the CRISPR-derived RNA (crRNA), which functions similarly to the native crRNA–tracrRNA duplex in guiding Cas9 to a DNA target site.

that AcrIF4 directly binds to the Csy–crRNA complex and inhibits subtype I-F CRISPR–Cas systems without preventing binding of target DNA in vitro[63]. The cryo-EM structure of the Csy–crRNA–AcrIF4 complex showed that AcrIF4 interacts with Cas8f without blocking sites for PAM recognition or crRNA–DNA hybridization, and it was proposed that AcrIF4 might lock Cas8f and prevent it from undergoing conformational changes required for Csy–crRNA complex activation[63]. This observation is reminiscent of AcrIIA16, which was shown to block CRISPRi activity in vivo but did not block target DNA binding in vitro[76]. Further studies have revealed that AcrIIA16 impairs Cas9 cleavage activity only when incubated with Cas9 and single guide RNA (sgRNA) separately before sgRNA–Cas9 complex formation, suggesting that it interferes with sgRNA loading onto Cas9 (REF.[76]). Thus, more studies are required to fully elucidate the mechanism of AcrIF4-mediated CRISPR–Cas inhibition.

A recent report showed that AcrIF2 also inhibits the subtype I-C system, but this dual inhibition has a cost, because the AcrIF2-mediated type I-C inhibition ability is weakened in the presence of the type I-F system. Mutation of eight AcrIF2 acidic residues into Ala resulted in loss of anti-I-C function and only partial weakening of anti-I-F function in the presence of both the type I-C system and the type I-F system, suggesting that AcrIF2 makes more contacts with the type I-F effector complex than with the type I-C effector complex[57]. AcrIF2, AcrIC4, AcrIC5, AcrIC7$_{Pst}$ and AcrIC8 can block CRISPRi, suggesting that they might inhibit type I-C systems by blocking target DNA binding and/or steps upstream of it[57].

A recent study showed that AcrIE2 directly binds to the subtype I-E Cascade complex, but does not prevent Cascade from binding to DNA[77]. Intriguingly, binding of the AcrIE2–Cascade complex to several gene promoters led to transcription activation from these promoters. AcrIE2 was proposed to inhibit DNA cleavage by blocking the subsequent recruitment of Cas3 to the Cascade complex[77]. Finally, AcrIC1 and AcrIC3 were shown to inhibit CRISPR–Cas activity but not to block CRISPRi, suggesting that they may prevent Cas3 recruitment or activity[57]. Further biochemical and structural studies are required to elucidate the principles underlying these anti-CRISPR-mediated inhibition mechanisms.

### *Mechanisms of type III anti-CRISPRs*

Type III CRISPR–Cas immunity is achieved by the crRNA-loaded multiprotein effector complexes Csm in subtype III-A or subtype III-D and Cmr in subtype III-B or subtype III-C[78–82] (FIG. 2a). crRNA maturation involves cleavage by Cas6, which is not part of the effector complex[83]. The effector complex uses crRNA to recognize complementary RNA targets[84], which triggers cleavage of non-specific ssDNA by the HD domain of the signature protein Csm1 or Cmr2 (also known as Cas10)[85,86]

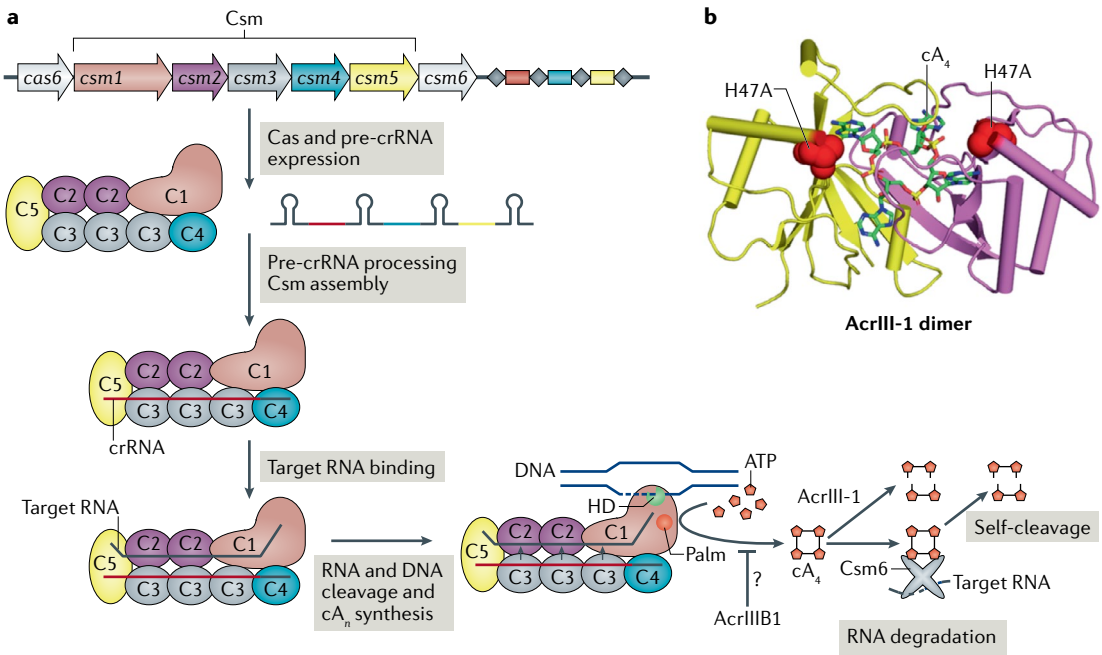



Fig. 2 | **Functional mechanisms of type III anti-CRISPRs. a** | Precursor CRISPR-derived RNA (pre-crRNA) is transcribed from the CRISPR array, processed into mature crRNAs and assembled with Csm proteins into effector complexes. The crRNA binds and guides the Csm–crRNA complex to target RNA, which is cleaved by Csm3 (C3) at six-nucleotide intervals (upwards arrows). Binding of target RNA triggers cleavage of non-specific single-stranded DNA by the His-Asp (HD) nuclease domain of Csm1 and synthesis of cyclic oligoadenylates (in this case, tetra-adenylate (cA$_4$)) from ATP by its pair of Palm domains. The produced cA$_4$ binds to Csm6 and allosterically simulates its single-strand RNase activity. Subsequently, cleavage of cA$_4$ to linear diadenylates shuts down its own RNase activity. The anti-CRISPR AcrIII-1 is a *trans*-acting ring nuclease that degrades cA$_4$. AcrIIIB1 probably interferes with cyclic oligoadenylate synthesis via the Cmr complex. **b** | Structure of dimeric AcrIII-1 bound to cA$_4$ (Protein Data Bank code 6SCF). The His47Ala (H47A) mutation is shown as a red sphere.

and synthesis of cyclic oligoadenylate (cOA) from ATP by the 'Palm' domains of Csm1 (REFS[87,88]). Subsequently, the Csm3 subunit or the Cmr4 subunit specifically cuts the RNA target at six-nucleotide intervals, thereby deactivating the HD and Palm domains of Csm1 or Cmr2, respectively[84,89–91]. The produced second messenger cOA is released from the Csm or Cmr complex[92], binds to the CRISPR-associated Rossmann fold (CARF) domain of non-specific dimeric RNases (Csm6 in subtype III-A or Csx1 in subtype III-B) and allosterically stimulates the single-strand RNase activity of its own higher eukaryotes and prokaryotes nucleotide-binding domains (HEPN domains), which are crucial for type III immunity in vivo[93–96]. Interestingly, *Thermococcus onnurineus* Csm6 (REF.[96]) and *Sulfolobus islandicus* Csx1 (REF.[97]) are not only RNases but are also ring nucleases that degrade tetra-adenylate rings ($cA_4$) into linear biadenylates containing terminal 2′,3′-cyclic phosphate through its CARF domain, thus terminating its RNase activity (FIG. 2a). Similarly, the second messenger $cA_6$ is degraded by *Enterococcus italicus* (REF.[98]) and *Streptococcus thermophilus* (REFS[98,99]) Csm6. This mechanism provides a time-restricted self-regulatory mechanism for controlling the RNase activity of Csm6 to limit self-toxicity.

The type III CRISPR–Cas cOA-based signalling pathway against invading phages is reminiscent of the bacterial cyclic oligonucleotide-based antiphage signalling system (CBASS)[100,101], which shares ancient common ancestry with the eukaryotic cGAS–STING pathway. Upon DNA binding, cGAS catalyses GTP and ATP into the second messenger 2′,3′-cGAMP, which subsequently binds to and activates STING, thereby leading to the production of type I interferons that mediate innate immune responses[45]. In turn, pathogens have evolved various strategies to evade host immunity in both prokaryotes and eukaryotes.

***Inhibition of second-messenger production.*** Targeting the second messenger cOA is an effective strategy used by viruses against both prokaryotic CRISPR–Cas immunity and eukaryotic cGAS–STING innate immunity. AcrIIIB1 inhibits an RNase-related process during RNA targeting, in which the RNase activity of Csx1 is required[102]. However, AcrIIIB1 does not directly interact with Csx1, but with the subtype III-B Cmr complex responsible for cOA production, suggesting that AcrIIIB1 inhibits Csx1-mediated RNase activity probably by interfering with cOA synthesis by the Cmr complex[102]. Similarly, some viral proteins inhibit cGAS enzymatic activity by directly binding cGAS[103] or through deamination of a crucial Asn residue in cGAS[104] to disrupt 2′,3′-cGAMP production.

***Degradation of the second messenger.*** AcrIII-1 is a protein of viral origin that directly binds to cOA, and degrades the second messenger $cA_4$ without direct interaction with the Cmr complex, thereby allowing viruses to overcome type III CRISPR–Cas immunity[105]. The cyclic tetra-adenylate is cleaved into two linear diadenylates (ApA>p), thus deactivating the downstream Csm6-mediated target RNA degradation. The crystal structure of AcrIII-1 in complex with $cA_4$ revealed that $cA_4$ bound to dimeric AcrIII-1 and is covered by a mobile loop (FIG. 2b). The conserved residue His47 of AcrIII-1 is crucial for its catalytic activity, as it decreased by 2,500-fold in the AcrIII-1-H47A mutant. Similarly, in eukaryotes, poxvirus immune nucleases (poxins) directly cleave the second messenger 2′,3′-cGAMP to inhibit STING-dependent innate immunity[106], indicating that degradation of second messengers is a widespread strategy used by viruses for immune evasion in both prokaryotes and eukaryotes.

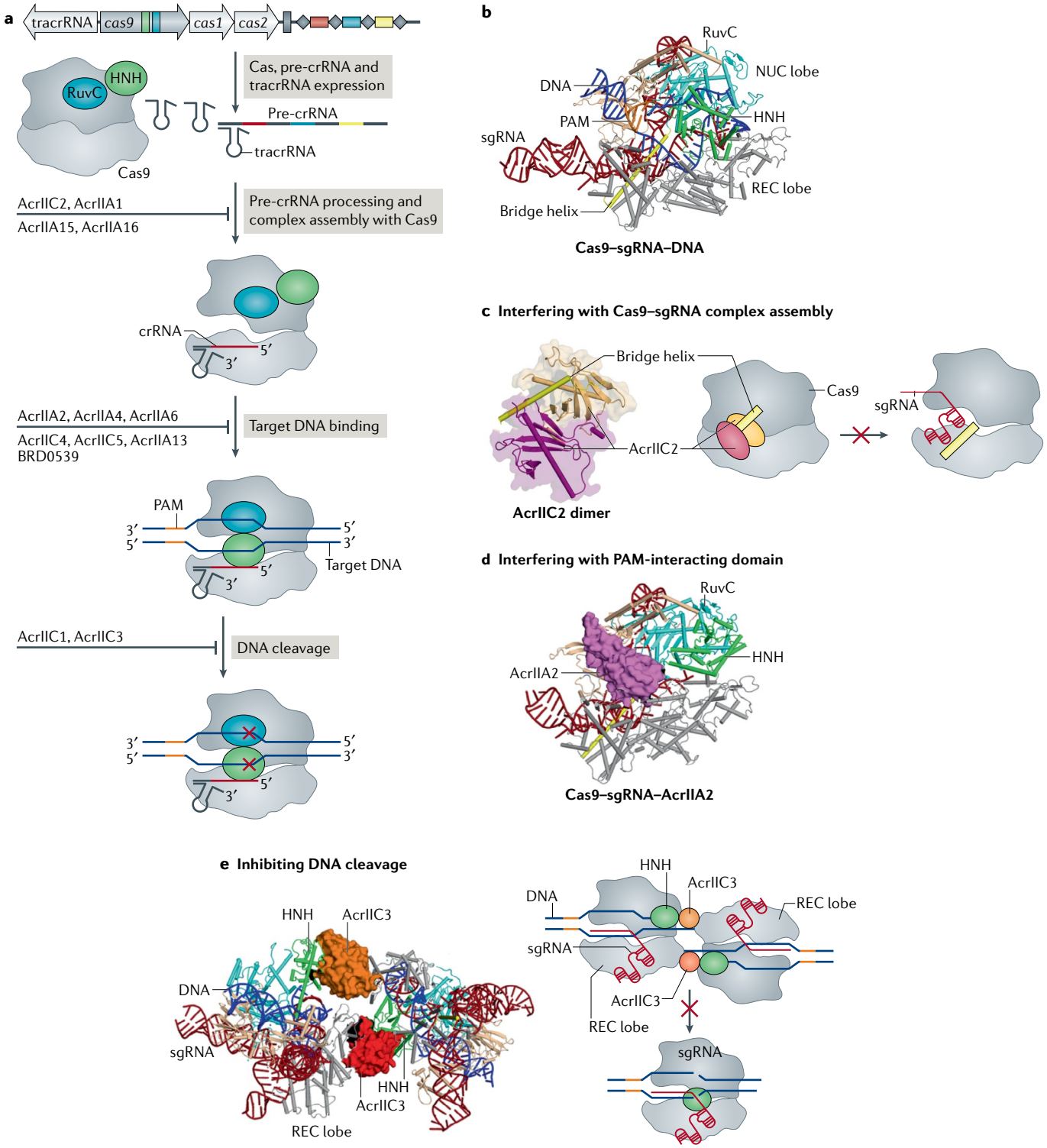
***Implications from eukaryotic cGAS–STING immunity.*** Pathogens have evolved other strategies to evade eukaryotic cGAS–STING immunity, such as degrading microbial nucleic acids, or directly degrading cGAS and STING. For example, the exonuclease TREX1 is hijacked by human immunodeficiency virus (HIV) to degrade excess HIV DNA and thus evade recognition by cGAS[107]. The dengue virus NS2B[108] and NS2B3 (REF.[109]) protease complexes inhibit cGAS–STING immunity by degrading cGAS and STING. Thus, it is likely that more strategies used by phages to counter the cOA-based signalling pathway of type III CRISPR–Cas systems remain to be discovered.

## Inhibiting class 2 CRISPR–Cas systems

Class 2 CRISPR–Cas systems, which include types II, V and VI, are defined by the presence of a single Cas effector molecule. Anti-CRISPRs have been discovered in all these types: 28 anti-CRISPRs have been identified in the type II system, 5 anti-CRISPRs has been identified in the type V system and 8 anti-CRISPRs have been identified in the type VI system (TABLE 1). Class 2 anti-CRISPRs inhibit CRISPR–Cas immunity mainly by interfering with CRISPR–Cas complex assembly, inhibiting target DNA (or RNA) binding or preventing target cleavage.

### *Mechanisms of type II anti-CRISPRs*

Type II CRISPR–Cas immunity is executed by a single Cas9 protein and two small RNAs: a crRNA and a *trans*-activating CRISPR RNA (tracrRNA)[110,111] (FIG. 3a). Portions of tracrRNA are complementary to the repeat region of the crRNA, and formation of such dsRNA is crucial for crRNA processing[111]. The mature crRNA and tracrRNA can be artificially covalently linked into a sgRNA, which is used for Cas9-mediated genome editing applications. Cas9 consists of two distinct lobes: the recognition lobe (REC lobe) and the nuclease lobe (NUC lobe)[112,113]. The NUC lobe contains two nuclease domains: HNH and RuvC, which are responsible for cleavage of target and non-target DNA strands, respectively. An arginine-rich bridge helix connecting the REC and NUC lobes is crucial for sgRNA–DNA recognition (FIG. 3b). Similarly to type I systems, PAM recognition, which is done by a PAM-interacting domain in the NUC lobe[113], is also required for type II CRISPR–Cas immunity[110]. The Cas9–sgRNA complex first recognizes the PAM sequence within the target DNA and then initiates melting of the target dsDNA, triggering R-loop formation and subsequently target cleavage.




***Interference with Cas9–sgRNA complex assembly.*** Dimeric AcrIIC2 forms an acidic groove that binds to the positively charged *Neisseria meningitides* Cas9 (NmeCas9) bridge helix motif, thereby preventing the formation of an active Cas9–sgRNA complex[114,115] (FIG. 3c). In addition, AcrIIC2 binding retains Cas9 in the apo (ligand-free) form, which is sensitive to cellular proteases, thus enhancing the anti-CRISPR activity of AcrIIC2. Further structural and biochemical studies revealed that AcrIIC2 can target a broad spectrum of Cas9 orthologues in a similar manner, which may benefit biotechnology applications of the most widely used Cas9 (REF.[114]).

AcrIIA1 can bind directly to the HNH domain of apo-Cas9 or sgRNA-loaded Cas9, triggering degradation of Cas9 during the lysogenic stage[116]. Mutagenesis analysis revealed that His840 in the HNH domain is the key residue responsible for AcrIIA1–Cas9 interaction

◀ Fig. 3 | **Functional mechanisms of type II anti-CRISPRs. a** | Cas9 proteins are assembled with a mature CRISPR-derived RNA (crRNA) and *trans*-activating CRISPR RNA (tracrRNA), which are both transcribed from the CRISPR locus. The mature crRNA and tracrRNA can be artificially covalently linked into a single guide RNA (sgRNA). The crRNA guides Cas9 to recognize the protospacer-adjacent motif (PAM) and subsequently to bind to and degrade both strands of the target DNA by the Cas9 RuvC and HNH nuclease domains. Thirteen type II anti-CRISPRs are shown to target Cas9–crRNA–tracrRNA complex assembly, target DNA binding and DNA cleavage. **b** | Structure of the Cas9–sgRNA–DNA complex (Protein Data Bank (PDB) code 5F9R). **c** | Structure and schematic representation of dimeric AcrIIC2 (in surface view) bound to the bridge helix of Cas9 (PDB code 6J9M). Dimeric AcrIIC2 binds to the positively charged Cas9 bridge helix motif, which is important for sgRNA recognition, thereby preventing sgRNA loading and the formation of an active Cas9–sgRNA complex. **d** | Structure of AcrIIA2 bound to the Cas9–sgRNA complex (PDB code 6IFO). AcrIIA2 occupies the PAM-interacting site in Cas9, thereby sterically blocking DNA recognition and binding by the Cas9–sgRNA complex. **e** | Structure of dimeric AcrIIC3 bound to a pair of Cas9–sgRNA–DNA complexes (PDB code 6JE4). The schematic representation shows how two AcrIIC3 molecules bind to Cas9–sgRNA–DNA and prevent DNA cleavage by directly locking the HNH domain of Cas9. NUC lobe, nuclease lobe; pre-crRNA, precursor CRISPR-derived RNA; REC lobe, recognition lobe.

and that Phe115 in an unstructured C-terminal loop of AcrIIA1 is also crucial for its interaction with Cas9 and anti-CRISPR activity. Interestingly, AcrIIA1 can induce degradation of Cas9 in vivo, but not in vitro, suggesting that an additional stimulus provided by the *Listeria monocytogenes* cellular environment is necessary for Cas9 degradation. AcrIIA1 triggers degradation of Cas9 in vivo, suggesting that AcrIIA1 may either destabilize apo-Cas9 or prevent sgRNA loading to apo-Cas9, similarly to what was previously reported for AcrIIC2 (REFS[114,115]). Furthermore, AcrIIA1, which functions as a dimer, is a bifunctional Cas9 inhibitor: its C-terminal domain binds and inhibits Cas9, and the N-terminal domain, which contains an HTH motif, acts as a transcriptional repressor of its own gene, thereby functioning as a Cas9 sensor that tunes its own expression to match Cas9 levels[117]. HTH-containing proteins have been reported to function as repressors of anti-CRISPR transcription[118,119], indicating that transcription autoregulation is conserved in HTH-containing proteins. In addition, AcrIIA1 broadly inhibits diverse Cas9 orthologues, probably owing to its recognition of the highly conserved catalytic residue His840 in the Cas9 HNH domain[116].

Similarly to AcrIIA1, the HTH-containing proteins AcrIIA13, AcrIIA14 and AcrIIA15 are also bifunctional inhibitors, which, in addition to inhibiting Cas9 immunity, also bind to their own promoters and negatively regulate their own transcription. AcrIIA15 inhibits Cas9 by blocking Cas9–sgRNA complex assembly, whereas AcrIIA13 inhibits Cas9-mediated DNA cleavage by preventing target DNA binding, resulting in robust inhibition of Cas9-mediated genome editing in human cells[120]. AcrIIA14 did not prevent Cas9 from binding to its target, but experimental evidence indicated that AcrIIA14 induces Cas9 to form an inactive conformation by triggering its dimerization[120], similarly to AcrIIC3 (REFS[114,121]). In addition, the recently identified AcrIIA16–AcrIIA19 were shown to block CRISPRi in vivo, indicating that they block target DNA binding or steps upstream of it[76]. Co-expressing AcrIIA16–AcrIIA19, *Streptococcus pyogenes* Cas9 (SpCas9) and sgRNA led to different levels of sgRNA degradation, suggesting that they might act through modulation of sgRNA levels. Specifically, AcrIIA16 inhibited SpCas9-mediated DNA cleavage when incubated with sgRNA and apo-SpCas9, but not when incubated with the sgRNA-loaded Cas9 complex, indicating it might interfere with sgRNA–Cas9 complex assembly[76].

***Inhibition of target DNA binding.*** The PAM-binding site of Cas9 is shown in FIG. 3b. Structural and biochemical analysis revealed that AcrIIA2 (REFS[122,123]) occupies the PAM-interacting site and locks the HNH domain, thereby sterically blocking dsDNA recognition and binding by the Cas9–sgRNA complex (FIG. 3d). Notably, AcrIIA4 binds to Cas9 in a similar position to AcrIIA2, occupying the PAM-interacting site, but also interacting with the RuvC domain, thereby blocking entry of the non-target DNA strand into the active site of the RuvC domain (REFS[124–127]). AcrIIA2 and AcrIIA4 show ~15-fold higher binding affinity for Cas9–sgRNA compared with the Cas9–sgRNA–DNA complex[124]. The affinity of AcrIIA4 for apo-Cas9 is ~8,000-fold lower than its affinity for sgRNA-loaded Cas9 (REF.[127]), but structural analysis shows that AcrIIA4 does not directly interact with sgRNA, indicating that the conformational changes of Cas9 induced by sgRNA[112] are required for AcrIIA4 binding. Both AcrIIA2 and AcrIIA4 can inhibit the activity of SpCas9 in bacterial and human cells, making them powerful tools for Cas9 regulation in gene editing applications[128].

In contrast to AcrIIA2 and AcrIIA4, AcrIIA6 reduces DNA-binding affinity by altering the conformational dynamics needed for PAM binding, but not by preventing direct PAM recognition[129]. The cryo-EM structure of the AcrIIA6–Cas9–sgRNA complex revealed a dimeric AcrIIA6 that binds to an allosteric site in what is the back face of Cas9 relative to the target-binding surface, inducing the dimerization of Cas9 and thereby constraining the dynamics needed for PAM binding[129]. AcrIIA6 can block Cas9 activity in bacteria and mammalian cells, implying that it has the potential to regulate gene editing[130].

AcrIIC4 and AcrIIC5 inhibit DNA cleavage by NmeCas9–sgRNA in vitro and NmeCas9-mediated genome editing in bacterial and mammalian cells[131]. Further biochemical studies revealed that AcrIIC4 and AcrIIC5 prevent stable DNA binding and reduce DNA affinity for Cas9–sgRNA by approximately ninefold and approximately sixfold, respectively[131].

***Inhibition of DNA cleavage by direct interaction.*** The structure of AcrIIC1 bound to the Cas9 HNH domain revealed that AcrIIC1 specifically interacts with the active sites in the HNH domain[121], possibly excluding the divalent cation required for target DNA cleavage[36]. Interestingly, AcrIIC1 traps Cas9 in a catalytically inactive but DNA-bound state by restraining the conformational changes required for DNA cleavage, converting Cas9 into catalytically dead Cas9 (dCas9). The interaction of AcrIIC1 with the HNH domain, which is the most conserved segment of Cas9, makes it a broad-spectrum inhibitor.

AcrIIC3 induces Cas9 dimerization and reduces the binding affinity of target DNA[121]. Recent biochemical and structural studies revealed that two AcrIIC3 molecules tether two Cas9 molecules together, so that each AcrIIC3 interacts with the HNH domain of one Cas9 molecule and the REC lobe of another Cas9 molecule, thereby sequestering the HNH domain away from the target strand cleavage site[114,132,133] (FIG. 3e). Furthermore, the inactive HNH conformation fails to activate the RuvC domain, thereby also inhibiting the cleavage of the non-target strand. Notably, AcrIIC3 can bind to apo-Cas9, the Cas9–sgRNA complex and even the DNA-bound Cas9–sgRNA complex. It has been suggested that AcrIIC3 can inhibit Cas9-mediated immunity also by preventing DNA cleavage through directly locking the HNH domain of Cas9, even when target DNA binding occurs[132].

***Unclear mechanisms.*** AcrIIA11 interacts with dsDNA, apo-SpCas9, sgRNA-loaded SpCas9 and SpCas9–sgRNA–DNA without preventing target recognition, probably representing a novel inhibition mechanism[134]. A recent preprint showed that AcrIIA23 enabled replication of the phage ϕAP1.1 in *S. pyogenes* during early stages of infection and the integration of the phage DNA into the direct repeats of the CRISPR locus; AcrIIA23 expression was immediately turned off following prophage DNA integration[135]. In addition to anti-CRISPRs, the small molecule BRD0539 was shown to block the formation of the SpCas9–crRNA–DNA complex in a screening assay probing the SpCas9–PAM interaction, indicating that BRD0539 inhibits SpCas9 binding of DNA either by competing with the PAM sequence or by binding to an allosteric site in Cas9 (REF.[22]).

AcrIIA5, a broad-spectrum inhibitor of type II CRISPR–Cas9 systems, inhibits Cas9 activity by preventing target DNA cleavage without blocking DNA binding both in vivo and in vitro, and it inhibits Cas9-mediated DNA cleavage activity only of the RuvC domain, but not of the HNH domain[136]. By contrast, another in vivo study indicated that AcrIIA5 inhibits Cas9 activity by preventing DNA binding, and leads to sgRNA cleavage[137]. However, sgRNA degradation by AcrIIA5 in vitro was not observed in a subsequent study[138], suggesting that other nucleases might be responsible for sgRNA cleavage in vivo[137]. In vitro studies in combination with elucidation of the solution structure of AcrIIA5 identified an intrinsically disordered N-terminal region that is essential for Cas9 inhibition by directly interacting with the Cas9–sgRNA complex[138]. Further studies will be necessary to clarify the mechanism of AcrIIA5-mediated Cas9 inhibition.

In addition to direct inactivation of the CRISPR–Cas effector complex by anti-CRISPRs, a recent preprint reported that AcrIIA22 modifies the torsion of target plasmid DNA[139]. AcrIIA22 is a DNA nickase capable of nicking the supercoiled plasmids and thus relieving their torsional stress, making the plasmids less susceptible to cleavage by SpCas9 both in vitro and in vivo. Thus, modification of DNA topology might be another effective strategy to counter CRISPR–Cas immunity.

### *Mechanisms of type V anti-CRISPRs*

Type V CRISPR–Cas immunity is mediated by the crRNA guide-bound Cas12 complex, leading to cleavage of dsDNA (FIG. 4a) or single-stranded RNA by a single RuvC domain[140]. Cas12a is the best-studied Cas12 subtype, consisting of REC and NUC lobes, which are connected by the wedge domain[141,142]. The NUC lobe contains the RuvC nuclease domain (FIG. 4b). Cas12a recognizes target DNA in a PAM-dependent manner[143–146]. Unlike Cas9, in the absence of an HNH domain, Cas12a creates a staggered DNA double-strand break through sequential cleavage of the non-target strand and the target strand solely by the conserved RuvC domain (FIG. 4a). The cleaved target strand remains bound to the Cas12a–crRNA complex, thereby enabling cleavage in *trans* of the non-target strand[142].

***Inhibition of target DNA binding by blocking the Cas12–PAM interaction.*** AcrVA1 broadly inhibits Cas12a orthologues in vitro and in human cells[147,148] by cleaving the spacer in Cas12a-bound crRNAs to permanently inactivate the Cas12a complex[149]. The cryo-EM structure of AcrVA1 bound to Cas12a–crRNA revealed two AcrVA1 molecules in the complex[150] (FIG. 4c). One AcrVA1 molecule (in violet in FIG. 4c) occupies the PAM-interacting site and interrupts the recognition of the PAM sequence by Cas12a, while also triggering cleavage of the Cas12a-bound crRNA, thereby inactivating the Cas12a–crRNA complex. The interaction of AcrVA1 with the Cas12–crRNA complex is essential for AcrVA1-mediated crRNA cleavage, as neither AcrVA1 nor Cas12a alone degrades crRNA. Mutation of three resides (Arg41, His42 and His45) in AcrVA1, which are positioned close to the crRNA, almost completely abolished crRNA cleavage, suggesting they have a catalytic role in crRNA cleavage. The second AcrVA1 molecule (in hot pink in FIG. 4c) is bound to the region of Cas12a that normally binds the downstream segment of the crRNA–ssDNA duplex, thereby sterically preventing crRNA–DNA duplex formation. Thus, AcrVA1 inhibits Cas12a-mediated immunity by two mechanisms: blocking of both PAM recognition and subsequently the formation of the crRNA–DNA duplex, and cleavage of the crRNA guide.

By contrast, AcrVA5 inhibits target DNA binding by acetylating Lys635 of Cas12a, which is crucial for PAM recognition[151] (FIG. 4d). The crystal structure of AcrVA5 with bound acetyl-CoA revealed that it adopts a typical acetyltransferase fold, with subsequent biochemical data showing that mutation of the AcrVA5 residues involved in acetyl-CoA recognition impaired Cas12a inhibition, further supporting AcrVA5 functioning as an acetyltransferase. The cryo-EM structure of Cas12a shows that Lys635, which is crucial for PAM recognition, is acetylated (FIG. 4d), thereby preventing DNA binding, and Cas12 bearing a naturally occurring mutation of Lys635 to Arg was insensitive to inhibition by AcrVA5 (REF.[151]). Thus, AcrVA5 acetylates Lys635 of Cas12a to sterically hinder PAM recognition by Cas12a.

***Inhibition of target DNA binding by locking Cas12a.*** AcrVA4 exists in solution as a dimer and induces dimerization of Cas12a–crRNA, thereby blocking dsDNA binding by Cas12a–crRNA[149]. However, the

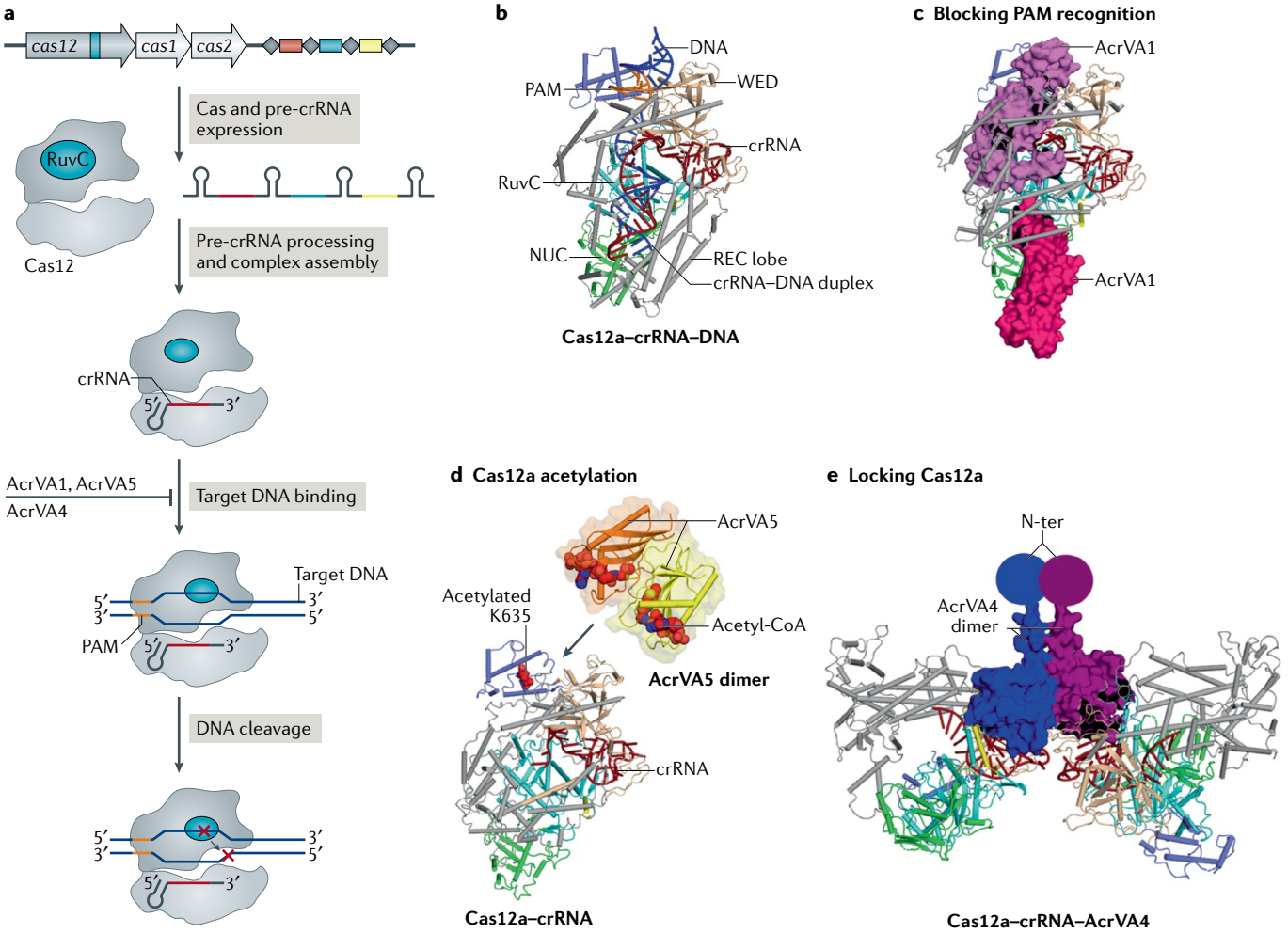



Fig. 4 | **Functional mechanisms of type V anti-CRISPRs. a** | Precursor CRISPR-derived RNA (pre-crRNA) is transcribed from the CRISPR array and processed into mature crRNAs, which assemble with Cas12 proteins to form the effector complex. The crRNA guides Cas12 (in this case, Cas12a) to the protospacer-adjacent motif (PAM) of the target DNA, which sequentially degrades the non-target and target strands using its sole RuvC nuclease domain. **b** | Structure of the Cas12a–crRNA–DNA complex (Protein Data Bank (PDB) code 5XUS). **c** | Structure of the Cas12a–crRNA complex bound by two AcrVA1 molecules (PDB code 6NMC). One AcrVA1 molecule (in violet) hinders PAM recognition and triggers the cleavage of the Cas12a-bound crRNA, while the second AcrVA1 molecule (in hot pink) sterically clashes with crRNA–single-stranded DNA duplex formation. **d** | The upper panel shows the dimeric AcrVA5 in complex with acetyl-CoA (PDB code 6IUF). AcrVA5 acetylates Lys635 of Cas12a. Acetylated Lys635 in the Cas12a–crRNA complex is shown in the lower panel (PDB code 6IV6). Acetylation of Lys635 in Cas12a by AcrVA5 sterically prevents Cas12a from recognizing the PAM. **e** | Structure of the Cas12a–crRNA complex bound to dimeric AcrVA4 (PDB code 6NM9). The disordered amino-terminal (N-ter) domain of AcrVA4 is shown as a sphere. AcrVA4 binds to Cas12a–crRNA and prevents the conformational changes required for crRNA–DNA duplex formation. NUC, nuclease lobe; REC lobe, recognition lobe; WED, wedge domain.

dimerization of AcrVA4 is not necessary for Cas12a inhibition, as the C-terminal binding domain of AcrVA4 alone, which is monomeric in solution, was sufficient for inhibition of Cas12a[152]. The cryo-EM structure of the Cas12a–crRNA–AcrVA4 complex reveals that AcrVA4 binds to either one or two molecules of Cas12a–crRNA, anchors the crRNA pseudoknot and prevents the conformational changes required for crRNA–DNA duplex formation[150,152,153] (FIG. 4e). AcrVA4 interacts with Cas12a–crRNA but not with apo-Cas12a, indicating that it binds to Cas12a in a crRNA-dependent manner[150].

AcrVA4 directly interacts with key Cas12a residues that are crucial for pre-crRNA processing by structurally mimicking the pre-crRNA substrate, as reflected in the contribution of the AcrVA4 acidic residues Glu184 and Glu204 (REF.[152]). Interestingly, AcrVA4 can also bind to the Cas12a–crRNA–DNA complex and induce release of the DNA before its cleavage[153]. In addition, AcrVA4 can also bind to a post-cleavage form of the Cas12a–crRNA–DNA complex with a cleaved R-loop, probably blocking the recycling of Cas12a–crRNA[153]. The multiple inhibition mechanisms used by AcrVA4 could ensure high efficiency of inhibiting CRISPR–Cas immunity.

### *Barring RNA binding in type VI systems*

Type VI CRISPR–Cas immunity is mediated by crRNA-guided Cas13, which triggers non-specific degradation of host and virus 'substrate' RNA upon

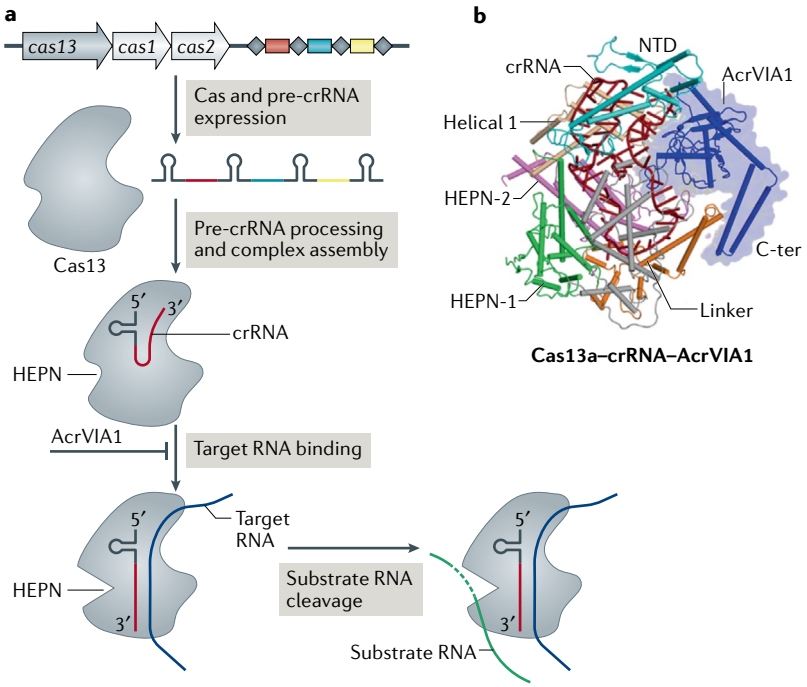



Fig. 5 | **Functional mechanisms of type VI anti-CRISPRs. a** | Precursor CRISPR-derived RNA (pre-crRNA) is transcribed from the CRISPR array and processed into mature crRNAs, which assemble with Cas13 proteins to form the effector complex. The crRNA guides Cas13 to target RNA, thereby triggering degradation of non-specific substrate RNA by the pair of Cas13 higher eukaryotes and prokaryotes nucleotide-binding domain 1 (HEPN-1) and HEPN-2. AcrVIA1 directly binds to Cas13a–crRNA and prevents target RNA binding and Cas13a conformational changes required for nuclease activation. **b** | Structure of the Cas13a–crRNA–AcrVIA1 complex (Protein Data Bank code 6VRB). C-ter, carboxy terminus; NTD, amino-terminal domain.

recognition of a crRNA-complementary phage RNA target[154] (FIG. 5a). Binding of target RNA triggers a considerable conformational change in Cas13a, which activates its composite catalytic site by bringing its two HEPN domains in close proximity and correct alignment[155]. The degradation of host transcripts leads to dormancy and prevents virus proliferation[156].

AcrVIA1, which is encoded by the genome of the ϕLS46 listeriophage, is capable of complete evasion of CRISPR–Cas13 immunity[157]. A cryo-EM structure of the Cas13a–crRNA–AcrVIA1 complex complemented by biochemical assays revealed that AcrVIA1 binds directly to the guide-exposed face of Cas13a and directly interacts with crRNA, as well as with residues in the helical 1, N-terminal domain, HEPN-2 and linker domains of Cas13a[157] (FIG. 5b). The interaction of AcrVIA1 with both crRNA and Cas13a prevented both access of the target RNA to the crRNA and the conformational changes required for HEPN-1 and HEPN-2 nuclease activation. Mutation of the key AcrVIA1 residues Tyr39A, Ser40, Asn43, Ser93 and Gln96 and truncation of two AcrVIA1 C-terminal helices (ΔN173–N232) led to complete loss of AcrVIA1 function. Anti-CRISPRs identified from *Leptotrichia wadei* F0279 prophages in type VI systems were able to control Cas13a activity in human cells[158]. Thus, anti-CRISPRs in type VI systems can be potentially used for control of CRISPR–Cas13a-based biotechnology tools.

## Anti-CRISPR biotechnology applications

CRISPR–Cas systems can be reliably repurposed for gene manipulation and are widely used for gene editing[36–38], regulation[39] and imaging in eukaryotic cells[40–42]. Thus, anti-CRISPRs have the potential to serve as useful tools for modulating CRISPR–Cas activities in prokaryotes and eukaryotes[33]. Elucidating the molecular mechanisms of anti-CRISPR functions could benefit the application of CRISPR–Cas tools for safe genetic manipulation, especially for clinical purposes. Such efforts at regulating prokaryotic CRISPR–Cas surveillance complexes undertaken in parallel with ongoing efforts of regulating the eukaryotic cGAS–STING pathway will collectively contribute to antimicrobial drug design and human disease therapy[45].

### *Reducing off-target activity*

Timed delivery of AcrIIA4 can reduce off-target editing in human cells without compromising on-target editing[126] (FIG. 6a). AcrVIA2 and AcrVIA5 also significantly reduced off-targets of *L. wadeii* Cas13a[158]. Of practical significance, fusion of anti-CRISPR variants to Cas9 also reduced off-targeting effects[159]. Base editing by a fusion of dCas9 with a cytidine deaminase can be used to efficiently correct point mutations relevant to genetic diseases without introducing potentially deleterious DNA double-strand breaks[160], but its use was shown to generate off-target single-nucleotide variants in rice and in mouse embryos[161,162]. AcrIIA2, AcrIIA4 and AcrIIA5 can efficiently inhibit dCas9-mediated base editing in human cells, and AcrIIA5 significantly reduced off-target effects of base editing systems in mammalian cells[163]. Furthermore, AcrVIA5 prevented dCas13-mediated RNA A-to-I editing (by an RNA adenosine deaminase) in mammalian cells[158]. Thus, anti-CRISPRs are an effective strategy to reduce off-target effects of CRISPR–Cas tools.

### *Reducing off-cell activity*

To restrict CRISPR–Cas function to specific tissues, an anti-CRISPR-based approach was developed to restrict Cas9 activity in all ancillary tissues. The approach is based on the use of endogenous cell type-specific microRNAs to repress anti-CRISPR expression in specific cell types. A microRNA inserted into either the 3′ untranslated region or the 5′ untranslated region of anti-CRISPR genes was shown to repress the expression of AcrIIA4, AcrIIC1 and AcrIIC3 (REFS[164–166]). The miR-122-repressed AcrIIC3 was transferred with Cas9–sgRNA into adult mice to allow gene editing in the liver (the only tissue in which miR-122 is expressed) and repress editing in unintended tissues, such as the heart[166] (FIG. 6b). This strategy provided the basis for application of anti-CRISPRs for cell-specific control of CRISPR–Cas9 gene editing.

### *Reducing cell cytotoxicity*

The persistent activation of Cas enzymes can be toxic to cells, so restriction of Cas nuclease activity is highly important for its successful application. Following CRISPR–Cas9 application in human haematopoietic stem cells, cytotoxicity and poor engraftment rates have been observed probably owing to the extended time

Gene drive
A genetic engineering technique for rapidly spreading a particular suite of genes through a population.

of Cas9 expression[44]. To restrict the activity of the Cas enzyme in the target cell, helper-dependent adenoviruses were developed that express a self-cleaving Cas9 (REF.[167]). The helper-dependent adenoviruses express CRISPR–Cas9, which directs the cleavage of the adenovirus's own genome following its transduction into target cells, thus allowing transient expression of Cas9 (FIG. 6c). To prevent self-cleavage during vector production, AcrIIA2 and AcrIIA4 were used to inhibit Cas9 activity in the producer cells and the helper virus. It was also reported that timed delivery of AcrIIA2 and AcrIIA4 controlled the duration of CRISPR–Cas9 activity, reduced human haematopoietic stem cell cytotoxicity and increased engraftment rates, without affecting on-target genome editing rates[44].

### *Controlling gene drives*

CRISPR–Cas-based gene drive is a genetic engineering technology that uses Cas9 to cut a chromosome at a specific site that does not contain a particular suite of drive genes, inducing the cell to repair the break by

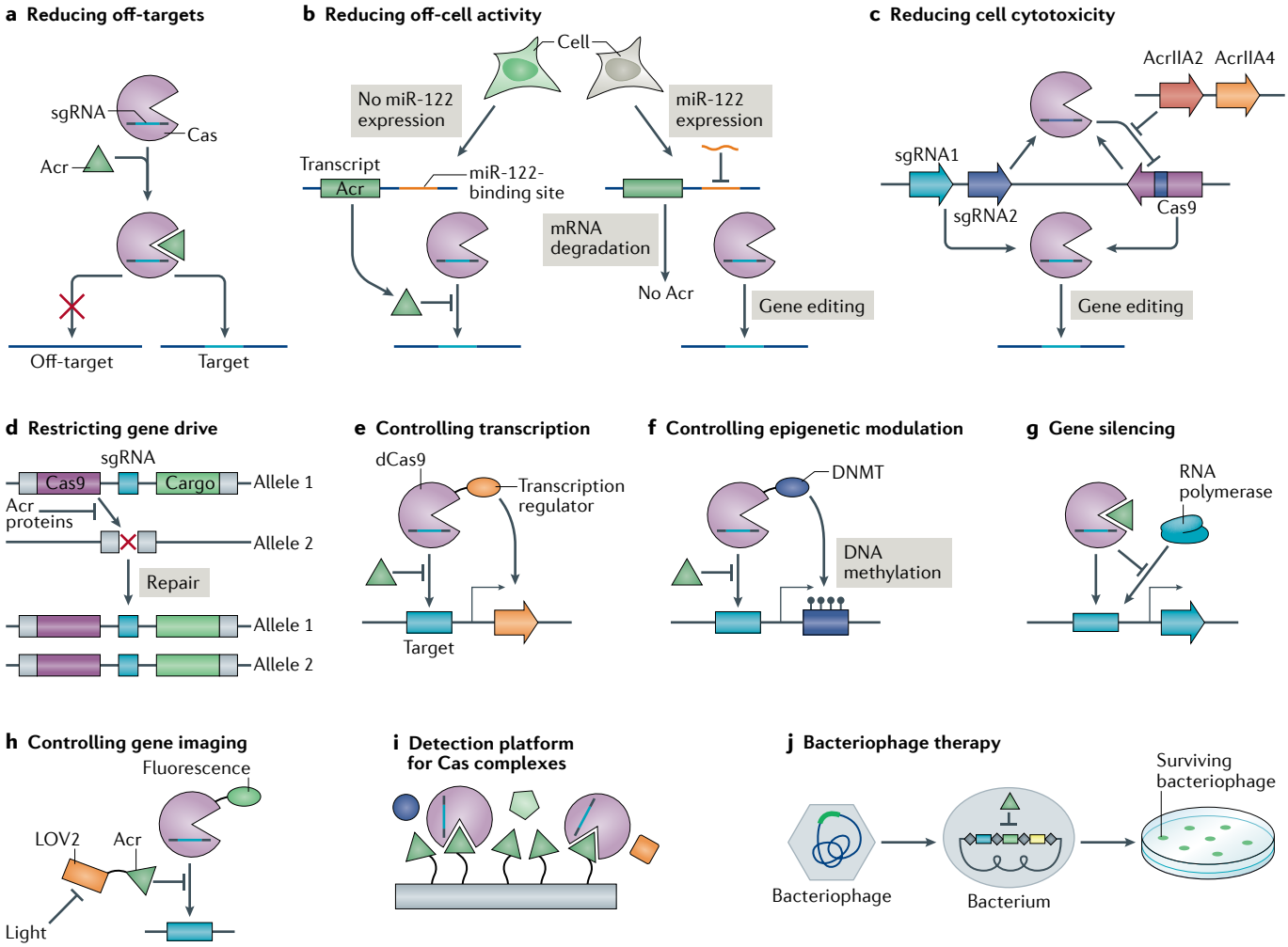



Fig. 6 | **Anti-CRISPR biotechnology applications. a** | Reducing off-target activity. The expression of an anti-CRISPR (Acr) decreases the cleavage of DNA or RNA off-targets by the CRISPR–Cas complex. **b** | Reducing off-cell activity by a microRNA (miR)-repressed anti-CRISPR to achieve cell type-specific gene editing. In cells expressing miR-122, the miRNA will bind to anti-CRISPR transcripts engineered to include miR-122-binding sites, leading to their degradation. Cells not expressing miR-122 will express the anti-CRISPRs, resulting in shutdown of gene editing. **c** | Reducing cell cytotoxicity by restricting the expression of Cas9. Schematic of a Cas9 self-cleavage system, which includes Cas9, single guide RNA 2 (sgRNA2) targeting the Cas9-encoding DNA and sgRNA1 targeting a site of interest in the genome. To prevent self-cleavage during the production of the viral vector, Cas9 activity is inhibited by AcrIIA2 and AcrIIA4 expressed from the producer cell and helper virus. **d** | Gene drive restriction by anti-CRISPRs. Gene drive uses Cas9 to cut a chromosome to induce the cell to repair the break and in doing so to copy the drive gene to the damaged chromosome and spread it through the population. Anti-CRISPRs block Cas9 activity and thus restrict gene drives. **e** | Controlling transcription using a fusion of catalytically dead Cas9 (dCas9) and a transcription regulator. Anti-CRISPRs that block target DNA binding can prevent the targeted function of the fusion protein at the desired genes or genes. **f** | Controlling epigenetic modulation. Anti-CRISPRs that block target DNA binding can prevent the targeted function of a fusion of dCas9 with an epigenetic regulator such as a DNA methyltransferase (DNMT). **g** | Gene silencing. Binding of anti-CRISPRs can turn Cas9 catalytically inactive without impairing its DNA-binding ability. The Cas9–anti-CRISPR complex blocks the binding of RNA polymerase, thus interfering with transcription. **h** | Dynamic control of dCas9-based imaging. Fusion of an anti-CRISPR with a photosensor protein such as LOV2, which is degraded by light, restricts dCas9 function to the presence of light. **i** | An anti-CRISPR-based platform for the detection of CRISPR–Cas complexes. **j** | Bacteriophage therapy. In bacteria, anti-CRISPRs inactivate CRISPR–Cas systems that include spacers (in green) targeting bacteriophages to increase the efficacy of phage therapy against bacteria. sgRNA, single guide RNA.

homologous recombination, thereby copying the drive genes onto the damaged chromosome. Gene drive systems have the potential to combat human diseases, including insect-borne diseases such as malaria[168,169]. Given the potential of genetically altering entire species by gene drive technology, safeguards are mandatory for the successful application of gene drives[170]. AcrIIA2 and AcrIIA4 were shown to be potent inhibitors of gene drive in *Saccharomyces cerevisiae* by inactivating Cas9 (REF.[171]) (FIG. 6d). The application of anti-CRISPRs in gene drive systems provides an effective option for controlling this promising gene editing tool.

### *Controlling dCas9-based tools*

The use of dCas9 has extended the application of CRISPR–Cas tools into transcription regulation, epigenetic modulation and live-cell chromatin imaging[41]. Many anti-CRISPRs interfere with CRISPR–Cas activities by preventing Cas enzymes from binding target sequences. The catalytically inactive Cas9 (dCas9) has been repurposed to interfere with gene transcription in bacterial and mammalian cells[39], and anti-CRISPRs have been used to control CRISPR–Cas-based gene regulatory circuits[172,173] (FIG. 6e). For example, dSpCas9 fused to the transcription repressor domain of MXI1 binds in the proximity of the promoter of a fluorescent reporter to repress its expression. Inducibly expressed AcrIIA2 and AcrIIA4 bind to dSpCas9–MXI1 and release the repression of the fluorescent reporter, resulting in enhancement of fluorescence[172]. Such anti-CRISPR-based gene circuits can be used to detect small molecules on the basis of the readout of fluorescence.

In addition to transcription regulation, anti-CRISPRs have been applied to control epigenetic modifications (FIG. 6f). For example, hypermethylation of the CGG repeat-expansion mutation at the 5′ untranslated region of the gene *FMR1* can silence the expression of the gene and cause fragile X syndrome. A fusion of dCas9 and the methylcytosine dioxygenase TET1 was used to demethylate the CGG repeats and restore the expression of *FMR1* (REF.[174]). In the presence of AcrIIA4, *FMR1* demethylation and expression were maintained for at least 2 weeks[174]. Thus, AcrIIA4 can be used to evaluate the persistence of epigenetic modification by dCas9 fused with a genetic modulator through the disruption of the DNA-binding ability of dCas9.

In *Escherichia coli*, endogenous type I CRISPR–Cas systems have been repurposed into programmable transcription repressors by deletion of the *cas3* nuclease gene[175]. In line with this concept, AcrIIC1 (REF.[121]), AcrIIC3 (REFS[114,132,133]), AcrIF3 (REFS[58,69,70]) and AcrIE1 (REF.[72]) are able to turn the CRISPR–Cas effector complex into a catalytically inactive state similar to dCas9, without impairing its DNA-binding ability, thereby repurposing these CRISPR–Cas systems into CRISPRi systems for gene silencing (FIG. 6g).

CASANOVA (CRISPR–Cas9 activity switching via a novel optogenetic variant of AcrIIA4) is a light-dependent anti-CRISPR tool based on a fusion of an optogenetic variant of AcrIIA4 and the LOV2 photosensor from *Avena sativa*. The system was used to monitor the kinetics of DNA targeting by dCas9 in living cells[176]. A dCas9–RFP fusion protein, guide RNA targeting telomeres and CASANOVA were expressed in the cells. AcrIIA4–LOV2 can bind and inhibit dCas9 in the dark; however, photoexcitation leads to the unfolding of LOV2, which induces the unfolding of AcrIIA4 and impairment of the binding affinity of AcrIIA4 for dCas9 (FIG. 6h). The comparison of telomere labelling under light and dark conditions can provide a direct confirmation of dCas9-based imaging in a dynamic manner.

### *Detecting CRISPR–Cas complexes*

Detection of the CRISPR–Cas effector complex within biological samples is important for evaluating the delivery efficiency, retention and distribution of CRISPR–Cas-based gene editing in target cells. Because of the high binding affinity of anti-CRISPRs for effector complexes, anti-CRISPR-based biosensing platforms provide an alternative to antibodies for effector complex detection, identification and quantification (FIG. 6i). AcrIIA4 can be immobilized as an affinity reagent for detection of the effector complex by electrochemical, fluorescent or colorimetric methods. The AcrIIA4-based biosensing platform can detect 280 pM CRISPR–Cas complex in reaction buffer and 8 nM CRISPR–Cas complex in biologically representative conditions[177]. Given that AcrIIC1 binds to the conserved HNH domain of diverse Cas9 proteins, it can be used as an affinity reagent to broadly detect CRISPR–Cas9 in different species[178]. These studies demonstrated that anti-CRISPRs have the potential to enable efficient and specific detection of CRISPR–Cas complexes.

### *CRISPR–Cas resistance in phage therapy*

Phage therapy is one of the most promising alternative methods to combat antibiotic-resistant bacteria[179]. However, many bacteria possess active CRISPR–Cas systems that may limit the range of application of phage therapy. Anti-CRISPRs that can naturally inactivate CRISPR–Cas systems in bacteria are a promising tool for achieving CRISPR–Cas resistance in phage therapy (FIG. 6j).

## Conclusions and future perspective

Since the discovery of anti-CRISPRs in 2013 (REF.[13]), their identification increased rapidly owing to the development of diverse approaches for their discovery. To date, 88 anti-CRISPRs and one synthetic small-molecule inhibitor have been identified, which span five CRISPR types (I, II, III, V and VI), with the exception of type IV (TABLE 1). The latter possibly reflects the relatively rare identification of type IV systems and consequently minimal structure–function characterization of their activity. The identification and characterization of anti-CRISPRs provides new insights into the arms race between prokaryotes and phages and a valuable addition to the CRISPR–Cas toolbox. However, only 29 of the 89 anti-CRISPRs have known mechanisms of activity, and research efforts have concentrated on the interference step, which is very relevant for biotechnology, and less on the adaptation and expression steps of the CRISPR–Cas surveillance pathway. The continuing study of anti-CRISPR mechanisms should increase our understanding of CRISPR–Cas functions and help in the development of the genome editing approaches.

Besides the phage-derived anti-CRISPRs, proteins associated with the CRISPR–Cas13b locus have been reported to regulate Cas13b-mediated RNA interference activity — which is repressed by Csx27 and enhanced by Csx28 (REF.[180]) — indicating that additional strategies for regulation of CRISPR–Cas systems exist and remain to be discovered. Furthermore, the ongoing evolutionary arms race between prokaryotes and phages enables prokaryotes to evolve additional strategies to counter phages, such as anti-anti-CRISPR strategies. For example, bacterial homologues of the AcrIIA1 N-terminal domain repress the expression of phage anti-CRISPRs[117]. Another anti-anti-CRISPR strategy is the fusion of Cas3 with Cas8 in subtype I-C systems, which can bypass inhibition by AcrIC3 (REF.[57]).

Studies on phage–prokaryote interactions led to the discovery of several important biotechnology tools, such as restriction enzymes[181], phage therapy[179] and CRISPR–Cas gene editing tools[36]. Notably, studies of the interplay between pathogens and eukaryotes provide important insights into the regulation of the cGAS–STING pathway, and facilitate the development of treatment for infections and human diseases, including cancer and inflammatory diseases. Continued study of the mechanisms of pathogen–host interactions in both prokaryotes and eukaryotes will enhance not only the development of potent and effective biotechnology tools but also of novel methods with potential medical and therapeutic applications.

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