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4 **Diversity of molecular mechanisms used by anti-CRISPR proteins:**
5 **the tip of an iceberg?**
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Abstract

Bacteriophages (phages) and their preys are engaged in an evolutionary arms race driving the co-adaptation of their attack and defense mechanisms. In this context, phages have evolved diverse anti-CRISPR proteins to evade the bacterial CRISPR-Cas immune system, and propagate. Anti-CRISPR proteins do not share much resemblance with each other and with proteins of known function, which raises intriguing questions particularly relating to their modes of action. In recent years, there have been many structure-function studies shedding light on different CRISPR-Cas inhibition strategies. As the anti-CRISPR field of research is rapidly growing, it is opportune to review the current knowledge on these proteins, with particular emphasis on the molecular strategies deployed to inactivate distinct steps of CRISPR-Cas immunity. Anti-CRISPR proteins can be orthosteric or allosteric inhibitors of CRISPR-Cas machineries, as well as enzymes that irreversibly modify CRISPR-Cas components. This repertoire of CRISPR-Cas inhibition mechanisms will likely expand in the future, providing fundamental knowledge on phage-bacteria interactions and offering great perspectives for the development of biotechnological tools to fine-tune CRISPR-Cas-based gene edition.

Introduction

In the battle for survival, bacteria and archaea have evolved mechanistically diverse defense mechanisms to resist their predators [1]. The prokaryotic CRISPR-Cas immune system uniquely provides a memory of past infections in the form of small pieces of DNA, taken from foreign genetic material and integrated into a CRISPR locus as spacers flanked by short repeats. These spacers are transcribed into CRISPR RNAs (crRNAs) that associate with Cas proteins to form effector complexes that patrol the cells for potential invasion. Upon recognition and binding to nucleic acid sequences complementary to crRNAs (referred to as protospacers), the Cas nuclease function is activated and the invading genetic material is destroyed [2] (Figure 1). CRISPR-Cas systems are highly diverse and currently classified into two broad classes, based on the composition of Cas proteins, six types and twenty-five subtypes. Class 1 systems (types I, III and IV) use multi-protein effector complexes to bind and cleave target DNA, whereas Class 2 systems (types II, V, and VI) use a single, multi-domain Cas protein for nucleic acid binding and cleavage [3].

Without much surprise, bacteriophages (phages) - the most abundant biological entities on the planet [4] - have evolved different mechanisms to protect against CRISPR-Cas immunity. For instance, they can hide from effector complexes through modifications, deletions or mutations in regions of their genome that require perfect complementarity with crRNAs [2, 5-7]. Additionally, phages produce anti-CRISPR proteins (Acr) at the early infection stage that directly interact with, or modify, CRISPR-Cas components, and block their activity. Anti-CRISPR proteins were first identified in *Pseudomonas aeruginosa* prophages in 2013 [8]. However, the first 3D structure of an anti-CRISPR protein was actually determined in 2009 with the crystal structure of the ORF 99 from the archaeal virus *Acidianus* filamentous virus 1 (AFV1) [13]. Indeed, He and colleagues characterized the first anti-CRISPR protein encoded by the archaeal viruses *Sulfolobus islandicus* rod-shape viruses 2 and 3 (SIRV2 and SIRV3), AcrID1, which was none other than an homolog of AFV1 ORF 99 [15]. To date, nearly fifty anti-CRISPR proteins inhibiting Class 1 and Class 2 CRISPR-Cas systems have been identified in prophages, virulent viruses, and other mobile genetic elements [9-12]. Strikingly, these proteins are different from one another and do not share much sequence similarities with proteins of known function. The diversity and broad distribution of anti-CRISPR proteins raise many questions relating to their origins and

evolution, their roles in the diversification of CRISPR-Cas systems and emergence of anti-anti-CRISPR mechanisms, and their modes of action.

In recent years, the highly dynamic anti-CRISPR field of research has emerged and accumulated important knowledge on the molecular mechanisms deployed by these natural CRISPR-Cas inhibitors. Many reviews that thoroughly present the discovery of anti-CRISPR proteins and their molecular mechanisms have recently been published [14, 64, 65, 66]. Here, we intend to provide a complementary, global perspective on their mode of action. We summarize the latest findings focusing on the different routes, and their interplay, to interfere with CRISPR-Cas immunity, and on the variety of molecular tactics to inactivate CRISPR-Cas machineries, which can be differentiated into orthosteric, allosteric, and enzymatic inhibition mechanisms. In the current context where the characterization of anti-CRISPR proteins is rapidly growing, these few functional and mechanistic parameters can be used to simply sort out diverse CRISPR-Cas inhibitors.

Different routes to inactivate CRISPR-Cas immunity

CRISPR-Cas immunity divides into 1) the adaptation step, to build up the immunological memory through the acquisition of foreign DNA sequences, 2) the expression and assembly step, to produce functional effector complexes, and 3) the interference step, to detect and cleave foreign nucleic acids, provided they are flanked by a protospacer-adjacent motif (PAM), with the exception of type III and type VI CRISPR-Cas systems, that allows self and non-self discrimination by the host [5, 16] (Figure 1). In type I and type II CRISPR-Cas systems, the PAM is also important for appropriate spacers selection during adaptation [17, 18]. Anti-CRISPR proteins have therefore the possibility to inactivate CRISPR-Cas immunity by interfering with one, or several, of these steps. However, most of the anti-CRISPR proteins characterized so far are anti-interference molecules that directly interact with effector complexes. The anti-CRISPR field of research having emerged recently, this trend may change in the future.

Inhibition of CRISPR-Cas adaptation

Cas1 and Cas2 are essential proteins for spacer acquisition in all studied CRISPR-Cas systems [17], but they do not seem to have any role in the expression or interference steps. Therefore, any anti-CRISPR protein inhibiting the production or the

activity of these Cas effectors would specifically block CRISPR-Cas adaptation. Cas1 and Cas2 being highly conserved Cas proteins present in most known CRISPR-Cas systems [67], it is almost certain that phages have evolved anti-CRISPR proteins that directly inhibit their activity and whose discovery was limited by the approaches used so far to uncover CRISPR-Cas inhibitors. In addition, components of the interference machinery are also involved in the adaptation step. The type I helicase/nuclease Cas3 and effector Cascade-crRNA complex (glossary), and the type II effector Cas9-sgRNA complex (glossary), associate with the Cas1-Cas2 complex for naïve, primed and interference-driven spacer acquisition (glossary) [19-23]. Noteworthy, Cas2 and Cas3 are fused into a single protein (Cas2/3) in the type I-F CRISPR-Cas system [3], pointing on the functional link between the adaptation and interference steps. A consequence of this molecular crosstalk between adaptation and interference is that any anti-CRISPR protein targeting the interference machinery could also interfere with the adaptation step [21].

AcrIF3 is an example of such anti-CRISPR protein with a dual anti-interference and anti-adaptation activity [21]. This CRISPR-Cas inhibitor binds to the helicase/nuclease Cas3, and prevents its recruitment to the effector Cascade-crRNA complex [54, 55, 59]. AcrIE1 inactivates the type I-E Cas3 nuclease [24] and thereby could also inhibit the adaptation step, although further experiments are required to confirm its anti-adaptation activity. The protospacer, bound to the Cascade-crRNA complex, cannot be cleaved, which aborts the interference step and, in turn, stops the generation of spacer precursors for primed and interference-driven adaptation [25].

In type II-A CRISPR-Cas systems, the Cas9-tracrRNA complex (glossary) associates with the other components of the spacer acquisition machinery, Cas1, Cas2 and Csn2, to ensure that new spacers are flanked by the correct PAM [22]. Moreover, Cas9-mediated interference activity and protospacer cleavage have recently been shown to prime the acquisition of new spacers [23]. Therefore, type II-A anti-CRISPR proteins that inhibit Cas9 interference activity could also perturb the adaptation step.

Inhibition of CRISPR-Cas expression and assembly

Cas proteins together with crRNA molecules form effector complexes competent for the recognition and binding to specific targets. Disrupting the production or assembly of these ribonucleoprotein machineries necessarily abrogates the interference and interference-driven adaptation steps. Anti-CRISPR proteins acting as transcriptional or

translational repressors of CRISPR-Cas components, which would therefore prevent their expression, remain to be uncovered. Although such anti-CRISPR proteins could not rapidly inactivate CRISPR immunity as required upon virulent phage infection, they could be useful to protect prophages from CRISPR-Cas degradation. To date, AcrIIA1 and AcrIIC2 are the only examples of anti-CRISPR proteins that prevent the formation of effector complexes. While AcrIIA1 binds to a broad-spectrum of type II-A and type II-C Cas9s and triggers their degradation within infected cells [68] AcrIIC2 binds to the apo form of *Neisseria meningitidis* Cas9 (unliganded NmeCas9) and blocks sgRNA loading [26, 27].

Inhibition of CRISPR-Cas interference

Anti-interference activity can be achieved by the inhibition of target binding, or nuclease activity. To date, inhibition of target binding is the prevalent strategy. Thirteen anti-CRISPR proteins interfere with target recognition and binding (type I-F AcrIF1, AcrIF2 and AcrIF10 [28-31]; type II-A AcrIIA2, AcrIIA4, AcrIIA5 and AcrIIA6 [32-41]; type II-C AcrIIC3, AcrIIC4 and AcrIIC5 [42-45]; type V-A AcrVA1, AcrV4A and AcrVA5 [46-52]), while only five block target cleavage (type I-E AcrIE1 [24, 53]; type III-B AcrIIIB1 [10]; type I-F AcrIF3 [54, 55]; type II-C AcrIIC1 and AcrIIC3 [42-44]) (Figure 1). Given that DNA binding is the rate-limiting step of Cascade and Cas9-mediated interference activities [56, 57], altering this step is therefore an efficient way to inactivate CRISPR-Cas interference. Although the type I-D AcrID1, type I-F AcrIF4, and type II-A AcrIIA7-11 were shown to block the interference step, it is not yet known whether they inhibit target binding or cleavage [8, 11, 12, 15].

Molecular mechanisms used by anti-CRISPR proteins

Structure-function studies of anti-CRISPR proteins have revealed a variety of molecular mechanisms that can be grouped into three categories. First, anti-CRISPR proteins can act as inhibitors that directly bind and sterically occlude the functional sites of Cas effectors. Second, anti-CRISPR proteins can act as allosteric inhibitors that associate with regions distinct from the functional sites, and modify the conformational dynamics and structural transitions of CRISPR-Cas machineries. Third, anti-CRISPR proteins can be enzymes that degrade or permanently modify CRISPR-Cas components. Noteworthy, most of the 3D structures of anti-CRISPR proteins available to date display unique folds.

Steric occlusion of Cas effector functional sites

To date, the most common molecular tactic used by anti-CRISPR proteins is to sterically block the access to the functional sites of Cas machineries, including the target-binding site, sgRNA-binding site, and catalytic site. Such anti-CRISPR proteins that bind to highly conserved CRISPR-Cas functional sites limit the chance for the host to escape inhibition. This could be one of the reasons making these steric inhibitors the prevalent anti-CRISPR proteins so far. Nevertheless, further investigation of the molecular mechanisms used by anti-CRISPR proteins is required to determine whether this trend is biologically relevant or whether it reflects sampling bias.

The AcrIF10, AcrIIA2 and AcrIIA4 anti-CRISPR proteins act as DNA mimics to prevent target DNA from binding to effector Cascade-crRNA and Cas9-sgRNA complexes, respectively. However, they block the access to effector complexes by different means. AcrIF10 binds to the DNA binding site at the junction between the Cas8f and Cas5f subunits, and induces a DNA-bound conformation of the effector Cascade-crRNA complex [30]. As for AcrIIA2 and AcrIIA4, they are both small acidic proteins that interact with Cas9 PAM binding elements, thereby preventing the necessary primary recognition of the PAM next to the target DNA sequence [33-37] (Figure 2A). Interestingly, AcrIIA2 and AcrIIA4 illustrate a case of functional convergence from structurally distinct inhibitors with overlapping binding sites.

AcrIF1 and AcrIF2 anti-CRISPR proteins use different strategies to block the access to DNA binding sites of the effector Cascade-crRNA complex. Several copies of AcrIF1 bind to the Cas7f backbone of Cascade and block the target DNA to access and hybridize with the crRNA [30, 31, 58]. As for AcrIF2, its binding site located between the Cas7.6f and Cas8f subunits partially overlaps with the DNA binding site (Figure 2A). Moreover, AcrIF2 binding to the effector Cascade-crRNA complex induces conformational changes incompatible with target DNA binding [30].

Interestingly, AcrIF3 inactivates the type I-F CRISPR-Cas defense also through molecular mimicry. However, AcrIF3 functions as a mimic of a Cas protein, in contrast to the DNA mimics presented above. Its 3D structure is similar to the C-terminal helical bundle of Cas8, which is exposed upon target DNA binding to Cascade-crRNA complex to recruit Cas2/3 [31, 59]. AcrIF3 forms homodimers that bind to Cas2/Cas3 (Figure 2A), thereby preventing its recruitment to the target-bound Cascade-crRNA complex [54, 55].

Lastly, AcrIIC1 and AcrIIC2 are so far unique examples of inhibitors that bind to the catalytic site of the Cas9 HNH nuclease domain (glossary) and to the NmeCas9 sgRNA-binding site, respectively. AcrIIC1 directly interacts with the conserved catalytic residues of the HNH domain, thereby trapping the target-bound effector complex in a catalytically inactive state [43]. AcrIIC2 forms homodimers with an acidic groove that strongly interacts with the NmeCas9 arginine-rich bridge helix, thereby impeding sgRNA loading [26, 27] (Figure 2A).

Allosteric inhibition and clustering of effector complexes

Recently, the AcrIIA6, AcrIIC3, and AcrVA4 anti-CRISPR proteins have been shown to be allosteric inhibitors of the *Streptococcus thermophilus* CRISPR1-Cas9 (St1Cas9), NmeCas9, and *Lachnospiraceae bacterium* Cas12a (LbCas12a) effector complexes, respectively. Interestingly, these anti-CRISPR proteins can also bind to and inactivate two effector complexes at a time (Figure 2B). Noteworthy, AcrID1 directly interacts with Cas10d, the large subunit of the type I-D CRISPR-Cas effector complex, and induces its dimerization. However, the molecular basis of AcrID1 inhibition mechanism remains to be determined [15]. Knowing that anti-CRISPR proteins gradually immunosuppress bacteria through multiple failed phage infections [60, 61], lowering the critical concentration of a given anti-CRISPR protein required for the complete inactivation of CRISPR-Cas immunity may be advantageous for a phage population to rapidly propagate.

AcrIIA6 and AcrVA4 both function as dimers that tightly associate with a mixed protein-RNA region distinct from the DNA-binding crevasse and catalytic domains [41, 48, 51, 52]. However, regions of both subunits that compose the AcrIIA6 dimer form each binding interface, while every subunit of the AcrV4A dimer contains one binding interface (Figure 2B). These anti-CRISPR proteins perturb the conformational changes required to bind to target DNA, thus locking St1Cas9 and LbCas12a effector complexes in a nonfunctional state. AcrIIA6 impairs St1Cas9 conformational rearrangements associated with PAM binding, which leads to the inhibition of target DNA recognition and binding [41]. In contrast, AcrVA4 does not affect PAM binding but impairs LbCas12a dynamics and structural changes required for the R-loop formation and stabilization (glossary), which prematurely stops the hybridization between the crRNA and target DNA [51]. Additionally, AcrVA4 can also associate with DNA-bound effector complexes. When it binds to the LbCas12a-crRNA-dsDNA complex with a

complete R-loop, it induces the release of the bound DNA before cleavage [48, 52]. When it binds to the post-cleavage Cas12a-crRNA-dsDNA complex, it likely blocks the recycling of the enzyme [52].

As for AcrIIC3, one monomer is able to tether two NmeCas9 effector complexes (Figure 2B). It interacts with the NmeCas9 HNH domain of one effector complex, at the opposite face of the catalytic site, and the NmeCas9 REC lobe (glossary) of another effector complex [27, 44, 62]. The interaction between AcrIIC3 and the REC lobe, in the vicinity of the DNA-sgRNA hybridization site, likely perturbs the conformational dynamics of NmeCas9 that assists DNA binding, which would explain the reduction of NmeCas9 DNA binding affinity *in vitro* and the inhibition of DNA binding within cells [42, 43]. Besides, AcrIIC3 is able to associate with DNA-bound NmeCas9 effector complexes *in vitro* [44]. The interaction between AcrIIC3 and the HNH domain blocks the structural changes triggered by target DNA binding, and therefore locks the HNH and RuvC nuclease domains (glossary) in inactive conformations [44]. These data suggest that AcrIIC3 could inactivate NmeCas9 effector complexes in the presence or absence of target DNA.

AcrIIA6, AcrIIC3 and AcrVA4 all bind to two CRISPR-Cas effector complexes, although using different molecular strategies. What is the benefit for CRISPR-Cas allosteric inhibitors to dimerize or harbor multiple binding sites, and cluster effector complexes? One explanation could be that the “weakness” of allosteric inhibitors that leave accessible the CRISPR-Cas functional sites, would be compensated by their ability to sequester multiple effector complexes, thereby reducing the amount of functional effector complexes within infected cells.

Enzymatic modification of CRISPR-Cas components

The two anti-CRISPR proteins with an enzymatic activity that have been characterized so far, AcrVA1 and AcrVA5, inhibit Cas12a effector complexes through different mechanisms (Figure 2C). Interestingly, AcrVA1 and AcrVA5 bind to overlapping regions in the PAM-interacting domain and compete with one another [49, 51]. However, their substrate and enzymatic activity are different. AcrVA1 is a multiple-turnover endoribonuclease that cleaves off the Cas12a-bound crRNA spacer sequence to irreversibly inactivate the effector assembly [49]. Noteworthy, AcrVA1 mimics the PAM to position its catalytic residues close to the crRNA substrate [51]. Interestingly, the type II-A AcrIIA5 anti-CRISPR protein was recently shown to lead to

sgRNA cleavage at multiples sites out of the crRNA spacer sequence [39]. However, whether or not this anti-CRISPR protein also has a nuclease activity remains to be determined. In contrast, AcrVA5 is an acetyltransferase that permanently modifies one LbCas12a lysine residue required for PAM recognition [50]. This lysine acetylation not only abolishes the interaction with PAM nucleobases, but also generates steric hindrance with the whole PAM. Such enzymatic strategies allowing the permanent inactivation of many interference complexes are likely beneficial to phages to decrease the number of failed phage infections required to immunosuppress their host [60, 61], and thereby rapidly evade CRISPR-Cas immunity.

Perspectives

•Importance of the field

The highly dynamic anti-CRISPR field of research is fueled by the constant discovery of remarkably diverse CRISPR-Cas inhibitors, widespread amongst bacterial and archaeal viruses and with little similarity with proteins of known function. Deciphering their molecular modes of action is having a massive impact on our understanding of phage biology, bacterial evolution, and host-pathogen interactions. Moreover, anti-CRISPR proteins are scrutinized for their potential as biotechnological tools to fine-tune CRISPR-Cas-based gene edition.

•Summary of current thinking

Based on the structure-function studies of anti-CRISPR proteins that are currently available, four major outcomes stand out. First, because of biases associated with anti-CRISPR protein identification pipelines, our current knowledge mainly covers anti-CRISPR proteins inhibiting the CRISPR-Cas interference step. Second, the inhibition of target binding seems to be the strategy favored by anti-CRISPR proteins to inactivate both Class1 and Class 2 CRISPR-Cas systems. Third, the direct binding to CRISPR-Cas functional sites and their steric occlusion is so far the molecular tactic that prevails over allosteric inhibition modes and enzymatic modifications. Nevertheless, whether this current prevalence of steric inhibition reflects a biological reality or a sampling bias needs to be determined. To finish, nearly all 3D structures of anti-CRISPR proteins reveal new folds. All in all, anti-CRISPR proteins are remarkably diverse at the levels of their sequences, structures and inhibition mechanisms.

•Future directions

299 The characterization of anti-CRISPR proteins that interfere with the adaptation and
300 expression steps of CRISPR-Cas immunity will be key to provide a complete picture
301 of the molecular mechanisms used by these CRISPR-Cas inhibitors. Additionally,
302 phage genomes often encode for several anti-CRISPR proteins, which raises the
303 question of the interplay between different anti-CRISPR proteins to inactivate CRISPR-
304 Cas systems. Besides, whether molecular mechanisms are favored for lytic or
305 lysogenic cycles also remains to be addressed. Our current knowledge of anti-CRISPR
306 inhibition strategies indicates that the “best” anti-CRISPR proteins from a mechanistic
307 and biochemical point of view, such as enzymes that rapidly modify multiple CRISPR-
308 Cas effector complexes, may not be widely used by phages. It will be important to
309 examine the molecular strategy used by a given phage in light of the phage population
310 context and of the phage-host interactions. Lastly, investigations of the mechanisms
311 evolved by bacteria and archaea to resist anti-CRISPR proteins will tackle the next
312 step in the CRISPR-based molecular arms race.

Glossary

• **Cascade:** CRISPR-associated complex for antiviral defense. Multi-subunit Cas protein complex that associates with crRNA to form the effector complex.

• **HNH domain:** endonuclease domain named after its catalytic histidine and asparagine residues.

• **Interference-driven adaptation:** integration into CRISPR arrays of DNA fragments surrounding targeted protospacers.

• **Naïve adaptation:** integration into CRISPR arrays of DNA fragments from newly encountered mobile genetic elements.

• **Primed adaptation:** integration into CRISPR arrays of DNA fragments partially matching to spacers that are not subject to efficient interference.

• **REC lobe:** Recognition lobe. The REC lobe of Class 2 CRISPR-Cas effectors is a functional region involved in crRNA recognition, target binding, and nuclease activation.

• **R-loop:** nucleic acids structures consisting of a DNA-RNA heteroduplex and displaced ssDNA. R-loops are formed upon CRISPR-Cas effector complex binding to target dsDNA.

• **RuvC domain:** endonuclease domain named after the RuvC protein, a Holliday junction resolvase from *Escherichia coli*

• **sgRNA:** single-guide RNA. Engineered RNA construct that assembles the crRNA and tracrRNA molecules into a single sequence.

• **tracrRNA:** trans-activating CRISPR RNA. Small non-coding RNA molecule that forms base pairs with the crRNA and is required for type II and type V-B CRISPR-Cas interference.

Abbreviations

Acr, anti-CRISPR protein ; AFV1, Acidianus filamentous virus 1 ; crRNA, CRISPR RNA ; PAM, protospacer adjacent motif ; sgRNA, single-guide RNA.

Author contribution

Conceptualization, A.G.; Writing – original draft, A.G.; Writing – review and editing, P.H., A.G.; Visualization, P.H. and A.G.

Acknowledgements

A.G acknowledges the French National Research Agency for a grant related to this work (ANR-18-CE11-0016-01). UCSF ChimeraX that was used for molecular graphics is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, and receive support from NIH R01-GM129325 and P41-GM103311.

Competing interests

The authors declare no competing interests.

356 **Figure legends**

Figure 1

357

358 **Figure 1. Different routes to inactivate CRISPR-Cas immunity.** The different steps

359 of CRISPR-Cas immunity are indicated in boxes on the left. Schematics, in the center,

360 illustrate the adaptation (top), expression_ and assembly (middle), and interference

361 (bottom) steps. A CRISPR-array is depicted with alternated spacers (colored boxes)

362 and repeats (dark grey diamonds). The light grey arrows represent Cas-encoding

363 genes. One or several Cas proteins (shown as an ellipse) associate with crRNA or

364 sgRNA molecules to form the effector complex. Target binding (a dsDNA molecule is

365 shown here) triggers the Cas nuclease activity, which leads to the destruction of foreign

366 genetic material. Anti-CRISPR proteins known to inhibit one or several of these steps

367 are listed on the right. Most of them block target binding at the interference step. The

368 dashed arrow indicates that anti-CRISPR proteins inhibiting the interference step could

369 also inactivate the adaptation step. The molecular mechanisms of the underlined anti-

370 CRISPR proteins have been characterized.

Figure 2

A. Steric occlusion of Cas effector functional sites

B. Allosteric inhibition and clustering of effector complexes

C. Enzymatic modifications of CRISPR-Cas components

● Cas9 ● Cascade ● Cas12a ● crRNA/sgRNA

Figure 2. Molecular mechanisms used by anti-CRISPR proteins. A. Ribbon representation of anti-CRISPR proteins that sterically occlude the access to the Cas functional sites. The effector complexes are shown as color-coded surfaces. AcrIIA2, PDB 6MCB; AcrIIA4, PDB 5VZL; AcrIIC1, PDB 5VGB; AcrIIC2, PDB 6JDX; AcrIF1, PDB 6B46; AcrIF2, PDB 6B47; AcrIF3, PDB 5B7I; AcrIF10, PDB 6B48. B. Ribbon representation of anti-CRISPR proteins that mediate the clustering of two effector complexes. AcrIIA6, AcrVA4 and AcrIIC3 are CRISPR-Cas allosteric inhibitors. AcrIIA6, PDB 6RJ9; AcrVA4 PDB 6NM9; AcrIIC3, PDB 6JE9. C. Ribbon representation

380 of anti-CRISPR proteins that enzymatically modify CRISPR-Cas components. The
381 dashed box focuses on the AcrVA1 endoribonuclease putative catalytic residues (R41,
382 H42, H45) close to their crRNA substrate. The AcrVA5 acetyltransferase is bound to
383 acetyl-CoA cofactor (AcCoA). AcrVA1, PDB 6NMD; AcrVA5, PDB 6IUF. Pictures were
384 generated with ChimeraX [63].
385

References

- 1 Samson, J. E., Magadan, A. H., Sabri, M. and Moineau, S. (2013) Revenge of the phages: defeating bacterial defences. *Nat Rev Microbiol.* **11**, 675-687
- 2 Garneau, J. E., Dupuis, M. E., Villion, M., Romero, D. A., Barrangou, R., Boyaval, P. et al. (2010) The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature.* **468**, 67-71
- 3 Koonin, E. V., Makarova, K. S. and Zhang, F. (2017) Diversity, classification and evolution of CRISPR-Cas systems. *Curr Opin Microbiol.* **37**, 67-78
- 4 Cobian Guemes, A. G., Youle, M., Cantu, V. A., Felts, B., Nulton, J. and Rohwer, F. (2016) Viruses as Winners in the Game of Life. *Annu Rev Virol.* **3**, 197-214
- 5 Deveau, H., Barrangou, R., Garneau, J. E., Labonte, J., Fremaux, C., Boyaval, P. et al. (2008) Phage response to CRISPR-encoded resistance in *Streptococcus thermophilus*. *J Bacteriol.* **190**, 1390-1400
- 6 Semenova, E., Jore, M. M., Datsenko, K. A., Semenova, A., Westra, E. R., Wanner, B. et al. (2011) Interference by clustered regularly interspaced short palindromic repeat (CRISPR) RNA is governed by a seed sequence. *Proc Natl Acad Sci U S A.* **108**, 10098-10103
- 7 Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S. et al. (2007) CRISPR provides acquired resistance against viruses in prokaryotes. *Science.* **315**, 1709-1712
- 8 Bondy-Denomy, J., Pawluk, A., Maxwell, K. L. and Davidson, A. R. (2013) Bacteriophage genes that inactivate the CRISPR/Cas bacterial immune system. *Nature.* **493**, 429-432
- 9 Hwang, S. and Maxwell, K. L. (2019) Meet the Anti-CRISPRs: Widespread Protein Inhibitors of CRISPR-Cas Systems. *CRISPR J.* **2**, 23-30

412 10 Bhoobalan-Chitty, Y., Johansen, T. B., Di Cianni, N. and Peng, X. (2019)
 413 Inhibition of Type III CRISPR-Cas Immunity by an Archaeal Virus-Encoded Anti-
 414 CRISPR Protein. *Cell*. **179**, 448-458 e411

415 11 Uribe, R. V., van der Helm, E., Misiakou, M. A., Lee, S. W., Kol, S. and
 416 Sommer, M. O. A. (2019) Discovery and Characterization of Cas9 Inhibitors
 417 Disseminated across Seven Bacterial Phyla. *Cell Host Microbe*. **25**, 233-241 e235

418 12 Forsberg, K. J., Bhatt, I. V., Schmidtke, D. T., Javanmardi, K., Dillard, K. E.,
 419 Stoddard, B. L. et al. (2019) Functional metagenomics-guided discovery of potent
 420 Cas9 inhibitors in the human microbiome. *Elife*. **8**, e46540

421 13 Goulet, A., Spinelli, S., Blangy, S., van Tilbeurgh, H., Leulliot, N., Basta, T. et
 422 al. (2009) The thermo- and acido-stable ORF-99 from the archaeal virus AFV1.
 423 *Protein Sci*. **18**, 1316-1320

424 14 Hwang, S. and Maxwell, K.L. (2019) Meet the anti-CRISPRs: widespread
 425 protein inhibitors of CRISPR-Cas systems. *The CRISPR Journal*. **2**, 23-30

426 15 He, F., Bhoobalan-Chitty, Y., Van, L. B., Kjeldsen, A. L., Dedola, M.,
 427 Makarova, K. S. et al. (2018) Anti-CRISPR proteins encoded by archaeal lytic viruses
 428 inhibit subtype I-D immunity. *Nat Microbiol*, **3**, 461-469

429 16 Mojica, F. J., Diez-Villasenor, C., Garcia-Martinez, J. and Almendros, C.
 430 (2009) Short motif sequences determine the targets of the prokaryotic CRISPR
 431 defence system. *Microbiology*. **155**, 733-740

432 17 McGinn, J. and Marraffini, L. A. (2019) Molecular mechanisms of CRISPR-Cas
 433 spacer acquisition. *Nat Rev Microbiol*. **17**, 7-12

434 18 Nussenzweig, P. M., McGinn, J. and Marraffini, L. A. (2019) Cas9 Cleavage of
 435 Viral Genomes Primes the Acquisition of New Immunological Memories. *Cell Host*
 436 *Microbe*. **26**, 515-526 e516

437 19 Swarts, D. C., Mosterd, C., van Passel, M. W. and Brouns, S. J. (2012)
 438 CRISPR interference directs strand specific spacer acquisition. *PLoS One*. **7**, e35888

439 20 Datsenko, K. A., Pougach, K., Tikhonov, A., Wanner, B. L., Severinov, K. and
440 Semenova, E. (2012) Molecular memory of prior infections activates the
441 CRISPR/Cas adaptive bacterial immunity system. *Nat Commun.* **3**, 945

442 21 Vorontsova, D., Datsenko, K. A., Medvedeva, S., Bondy-Denomy, J.,
443 Savitskaya, E. E., Pougach, K. et al. (2015) Foreign DNA acquisition by the I-F
444 CRISPR-Cas system requires all components of the interference machinery. *Nucleic
445 Acids Res.* **43**, 10848-10860

446 22 Heler, R., Samai, P., Modell, J. W., Weiner, C., Goldberg, G. W., Bikard, D. et
447 al. (2015) Cas9 specifies functional viral targets during CRISPR-Cas adaptation.
448 *Nature.* **519**, 199-202

449 23 Nussenzweig, P. M., McGinn, J. and Marraffini, L. A. (2019) Cas9 Cleavage of
450 Viral Genomes Primes the Acquisition of New Immunological Memories. *Cell Host
451 Microbe*, **26**, 515-526

452 24 Pawluk, A., Shah, M., Mejdani, M., Calmettes, C., Moraes, T. F., Davidson, A.
453 R. et al. (2017) Disabling a Type I-E CRISPR-Cas Nuclease with a Bacteriophage-
454 Encoded Anti-CRISPR Protein. *MBio.* **8**, e01751-17

455 25 Kunne, T., Kieper, S. N., Bannenberg, J. W., Vogel, A. I., Miellet, W. R., Klein,
456 M. et al. (2016) Cas3-Derived Target DNA Degradation Fragments Fuel Primed
457 CRISPR Adaptation. *Mol Cell.* **63**, 852-864

458 26 Thavalingam, A., Cheng, Z., Garcia, B., Huang, X., Shah, M., Sun, W. et al.
459 (2019) Inhibition of CRISPR-Cas9 ribonucleoprotein complex assembly by anti-
460 CRISPR AcrIIIC2. *Nat Commun.* **10**, 2806

461 27 Zhu, Y., Gao, A., Zhan, Q., Wang, Y., Feng, H., Liu, S. et al. (2019) Diverse
462 Mechanisms of CRISPR-Cas9 Inhibition by Type IIC Anti-CRISPR Proteins. *Mol Cell*,
463 **74**, 296-309

464 28 Bondy-Denomy, J., Garcia, B., Strum, S., Du, M., Rollins, M. F., Hidalgo-
465 Reyes, Y. et al. (2015) Multiple mechanisms for CRISPR-Cas inhibition by anti-
466 CRISPR proteins. *Nature.* **526**, 136-139

467 29 Pawluk, A., Staals, R. H., Taylor, C., Watson, B. N., Saha, S., Fineran, P. C. et
468 al. (2016) Inactivation of CRISPR-Cas systems by anti-CRISPR proteins in diverse
469 bacterial species. *Nat Microbiol.* **1**, 16085

470 30 Guo, T. W., Bartesaghi, A., Yang, H., Falconieri, V., Rao, P., Merk, A. et al.
471 (2017) Cryo-EM Structures Reveal Mechanism and Inhibition of DNA Targeting by a
472 CRISPR-Cas Surveillance Complex. *Cell.* **171**, 414-426 e412

473 31 Chowdhury, S., Carter, J., Rollins, M. F., Golden, S. M., Jackson, R. N.,
474 Hoffmann, C. et al. (2017) Structure Reveals Mechanisms of Viral Suppressors that
475 Intercept a CRISPR RNA-Guided Surveillance Complex. *Cell.* **169**, 47-57 e11

476 32 Rauch, B. J., Silvis, M. R., Hultquist, J. F., Waters, C. S., McGregor, M. J.,
477 Krogan, N. J. et al. (2017) Inhibition of CRISPR-Cas9 with Bacteriophage Proteins.
478 *Cell.* **168**, 150-158 e110

479 33 Jiang, F., Liu, J. J., Osuna, B. A., Xu, M., Berry, J. D., Rauch, B. J. et al.
480 (2019) Temperature-Responsive Competitive Inhibition of CRISPR-Cas9. *Mol Cell.*
481 **73**, 601-610 e605

482 34 Liu, L., Yin, M., Wang, M. and Wang, Y. (2019) Phage AcrIIA2 DNA Mimicry:
483 Structural Basis of the CRISPR and Anti-CRISPR Arms Race. *Mol Cell.* **73**, 611-620
484 e613

485 35 Dong, Guo, M., Wang, S., Zhu, Y., Wang, S., Xiong, Z., Yang, J. et al. (2017)
486 Structural basis of CRISPR-SpyCas9 inhibition by an anti-CRISPR protein. *Nature.*
487 **546**, 436-439

488 36 Shin, J., Jiang, F., Liu, J. J., Bray, N. L., Rauch, B. J., Baik, S. H. et al. (2017)
489 Disabling Cas9 by an anti-CRISPR DNA mimic. *Sci Adv.* **3**, e1701620

490 37 Yang, H. and Patel, D. J. (2017) Inhibition Mechanism of an Anti-CRISPR
491 Suppressor AcrIIA4 Targeting SpyCas9. *Mol Cell.* **67**, 117-127 e115

492 38 Hynes, A. P., Rousseau, G. M., Lemay, M. L., Horvath, P., Romero, D. A.,
493 Fremaux, C. et al. (2017) An anti-CRISPR from a virulent streptococcal phage
494 inhibits *Streptococcus pyogenes* Cas9. *Nat Microbiol.* **10**, 1374-1380

495 39 Garcia, B., Lee, J., Edraki, A., Hidalgo-Reyes, Y., Erwood, S., Mir, A. et al.
496 (2019) Anti-CRISPR AcrIIA5 Potently Inhibits All Cas9 Homologs Used for Genome
497 Editing. *Cell Rep.* **29**, 1739-1746 e1735

498 40 Hynes, A. P., Rousseau, G. M., Agudelo, D., Goulet, A., Amigues, B., Loehr, J.
499 et al. (2018) Widespread anti-CRISPR proteins in virulent bacteriophages inhibit a
500 range of Cas9 proteins. *Nat Commun.* **9**, 2919

501 41 Fuchsbaueer, O., Swuec, P., Zimberger, C., Amigues, B., Levesque, S.,
502 Agudelo, D. et al. (2019) Cas9 Allosteric Inhibition by the Anti-CRISPR Protein
503 AcrIIA6. *Mol Cell*, **76**

504 42 Pawluk, A., Amrani, N., Zhang, Y., Garcia, B., Hidalgo-Reyes, Y., Lee, J. et al.
505 (2016) Naturally Occurring Off-Switches for CRISPR-Cas9. *Cell.* **167**, 1829-1838
506 e1829

507 43 Harrington, L. B., Doxzen, K. W., Ma, E., Liu, J. J., Knott, G. J., Edraki, A. et
508 al. (2017) A Broad-Spectrum Inhibitor of CRISPR-Cas9. *Cell.* **170**, 1224-1233 e1215

509 44 Sun, W., Yang, J., Cheng, Z., Amrani, N., Liu, C., Wang, K. et al. (2019)
510 Structures of *Neisseria meningitidis* Cas9 Complexes in Catalytically Poised and
511 Anti-CRISPR-Inhibited States. *Mol Cell*, **76**

512 45 Lee, J., Mir, A., Edraki, A., Garcia, B., Amrani, N., Lou, H. E. et al. (2018)
513 Potent Cas9 Inhibition in Bacterial and Human Cells by AcrIIC4 and AcrIIC5 Anti-
514 CRISPR Proteins. *MBio.* **9**, e02321-18

515 46 Marino, N. D., Zhang, J. Y., Borges, A. L., Sousa, A. A., Leon, L. M., Rauch, B.
516 J. et al. (2018) Discovery of widespread Type I and Type V CRISPR-Cas inhibitors.
517 *Science.* **362**, 240-242

518 47 Watters, K. E., Fellmann, C., Bai, H. B., Ren, S. M. and Doudna, J. A. (2018)
519 Systematic discovery of natural CRISPR-Cas12a inhibitors. *Science.* **362**, 236-239

520 48 Knott, G. J., Cress, B. F., Liu, J. J., Thornton, B. W., Lew, R. J., Al-Shayeb, B.
521 et al. (2019) Structural basis for AcrVA4 inhibition of specific CRISPR-Cas12a. *Elife.*
522 **8**, e49110

523 49 Knott, G. J., Thornton, B. W., Lobba, M. J., Liu, J. J., Al-Shayeb, B., Watters,
524 K. E. et al. (2019) Broad-spectrum enzymatic inhibition of CRISPR-Cas12a. *Nat*
525 *Struct Mol Biol.* **26**, 315-321

526 50 Dong, L., Guan, X., Li, N., Zhang, F., Zhu, Y., Ren, K. et al. (2019) An anti-
527 CRISPR protein disables type V Cas12a by acetylation. *Nat Struct Mol Biol.* **26**, 308-
528 314

529 51 Zhang, H., Li, Z., Daczkowski, C. M., Gabel, C., Mesecar, A. D. and Chang, L.
530 (2019) Structural Basis for the Inhibition of CRISPR-Cas12a by Anti-CRISPR
531 Proteins. *Cell Host Microbe.* **25**, 815-826 e814

532 52 Peng, R., Li, Z., Xu, Y., He, S., Peng, Q., Wu, L. A. et al. (2019) Structural
533 insight into multistage inhibition of CRISPR-Cas12a by AcrVA4. *Proc Natl Acad Sci U*
534 *S A.* **116**, 18928-18936

535 53 Pawluk, A., Bondy-Denomy, J., Cheung, V. H., Maxwell, K. L. and Davidson,
536 A. R. (2014) A new group of phage anti-CRISPR genes inhibits the type I-E CRISPR-
537 Cas system of *Pseudomonas aeruginosa*. *MBio.* **5**, e00896

538 54 Wang, J., Ma, J., Cheng, Z., Meng, X., You, L., Wang, M. et al. (2016) A
539 CRISPR evolutionary arms race: structural insights into viral anti-CRISPR/Cas
540 responses. *Cell Res.* **26**, 1165-1168

541 55 Wang, X., Yao, D., Xu, J. G., Li, A. R., Xu, J., Fu, P. et al. (2016) Structural
542 basis of Cas3 inhibition by the bacteriophage protein AcrF3. *Nat Struct Mol Biol.* **23**,
543 868-870

544 56 Gong, S., Yu, H. H., Johnson, K. A. and Taylor, D. W. (2018) DNA Unwinding
545 Is the Primary Determinant of CRISPR-Cas9 Activity. *Cell Rep.* **22**, 359-371

546 57 Szczelkun, M. D., Tikhomirova, M. S., Sinkunas, T., Gasiunas, G., Karvelis, T.,
547 Pschera, P. et al. (2014) Direct observation of R-loop formation by single RNA-
548 guided Cas9 and Cascade effector complexes. *Proc Natl Acad Sci U S A.* **111**, 9798-
549 9803

550 58 Peng, R., Xu, Y., Zhu, T., Li, N., Qi, J., Chai, Y. et al. (2017) Alternate binding
551 modes of anti-CRISPR viral suppressors AcrF1/2 to Csy surveillance complex
552 revealed by cryo-EM structures. *Cell Res.* **27**, 853-864

553 59 Rollins, M. F., Chowdhury, S., Carter, J., Golden, S. M., Miettinen, H. M.,
554 Santiago-Frangos, A. et al. (2019) Structure Reveals a Mechanism of CRISPR-RNA-
555 Guided Nuclease Recruitment and Anti-CRISPR Viral Mimicry. *Mol Cell*, **74**, 132-142

556 60 Borges, A. L., Zhang, J. Y., Rollins, M. F., Osuna, B. A., Wiedenheft, B. and
557 Bondy-Denomy, J. (2018) Bacteriophage Cooperation Suppresses CRISPR-Cas3
558 and Cas9 Immunity. *Cell*. **174**, 917-925 e910

559 61 Landsberger, M., Gandon, S., Meaden, S., Rollie, C., Chevallereau, A.,
560 Chabas, H. et al. (2018) Anti-CRISPR Phages Cooperate to Overcome CRISPR-Cas
561 Immunity. *Cell*. **174**, 908-916 e912

562 62 Kim, Y., Lee, S. J., Yoon, H. J., Kim, N. K., Lee, B. J. and Suh, J. Y. (2019)
563 Anti-CRISPR AcrIIIC3 discriminates between Cas9 orthologs via targeting the
564 variable surface of the HNH nuclease domain. *FEBS J.* **286** (23), 4661-4674

565 63 Goddard, T. D., Huang, C. C., Meng, E. C., Pettersen, E. F., Couch, G. S.,
566 Morris, J. H. et al. (2018) UCSF ChimeraX: Meeting modern challenges in
567 visualization and analysis. *Protein Sci.* **27**, 14-25

568 64 Trasanidou, D., Gerós, A.S., Mohanraju, P., Nieuwenweg, A.C., Nobrega, F.L.,
569 and Staals, R.H.J. (2019) Keeping CRISPR in check: diverse mechanisms of phage-
570 encoded anti-CRISPRs. *FEMS Microbiol Lett.* **336**, fnz098

571 65 Zang, F., Song, G., and Tian, Y. (2019) Anti-CRISPRs: The natural inhibitors
572 for CRISPR-Cas systems. *Anim Models Exp Med.* **0**, 1-7

573 66 Liu, Q., Zhang, H., and Huang, X. (2020) Anti-CRISPR proteins targeting the
574 CRISPR-Cas system enrich the toolkit for genetic engineering. *FEBS J.* **287**, 626-644

575 67 Makarova, K.S., Wolf, Y.I., Alkhnbashi, O.S., Costa, F., Shah, S.A., Saunders,
576 S.J. et al. (2015) An updated evolutionary classification of CRISPR-Cas systems, *Nat*
577 *Rev Microbiol.* **13**, 722-736

578 68 Osuna, B.A., Karambelkar, S., Mahendra, C., Christie, K.A., Garcia, B.,
579 Davidson, A.R. et al. (2019) *Listeria* phages induce Cas9 degradation to protect
580 lysogenic genomes, *BioRxiv*. 10.1101/787200