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## Diversity of molecular mechanisms used by anti-CRISPR proteins: the tip of an iceberg?

Pierre Hardouin, Adeline Goulet

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1 **THIS IS A PRE-PUBLICATION MANUSCRIPT**

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

6  
7 Pierre Hardouin and Adeline Goulet

8  
9 Architecture et Fonction des Macromolécules Biologiques, Centre National de la  
10 Recherche Scientifique (CNRS), Campus de Luminy, Case 932, 13288 Marseille  
11 Cedex 09, France.

12 Architecture et Fonction des Macromolécules Biologiques, Aix-Marseille Université,  
13 Campus de Luminy, Case 932, 13288 Marseille Cedex 09, France.

14  
15 **Correspondence:** Adeline Goulet ([adeline.goulet@afmb.univ-mrs.fr](mailto:adeline.goulet@afmb.univ-mrs.fr))

16

17 **Abstract**

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

34

## 35 **Introduction**

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

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

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

70 In recent years, the highly dynamic anti-CRISPR field of research has emerged and  
71 accumulated important knowledge on the molecular mechanisms deployed by these  
72 natural CRISPR-Cas inhibitors. Many reviews that thoroughly present the discovery of  
73 anti-CRISPR proteins and their molecular mechanisms have recently been published  
74 [14, 64, 65, 66]. Here, we intend to provide a complementary, global perspective on  
75 their mode of action. We summarize the latest findings focusing on the different routes,  
76 and their interplay, to interfere with CRISPR-Cas immunity, and on the variety of  
77 molecular tactics to inactivate CRISPR-Cas machineries, which can be differentiated  
78 into orthosteric, allosteric, and enzymatic inhibition mechanisms. In the current context  
79 where the characterization of anti-CRISPR proteins is rapidly growing, these few  
80 functional and mechanistic parameters can be used to simply sort out diverse CRISPR-  
81 Cas inhibitors.

82

### 83 **Different routes to inactivate CRISPR-Cas immunity**

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

### 97 **Inhibition of CRISPR-Cas adaptation**

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

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

115 AcrIF3 is an example of such anti-CRISPR protein with a dual anti-interference and  
116 anti-adaptation activity [21]. This CRISPR-Cas inhibitor binds to the helicase/nuclease  
117 Cas3, and prevents its recruitment to the effector Cascade-crRNA complex [54, 55,  
118 59]. AcrIE1 inactivates the type I-E Cas3 nuclease [24] and thereby could also inhibit  
119 the adaptation step, although further experiments are required to confirm its anti-  
120 adaptation activity. The protospacer, bound to the Cascade-crRNA complex, cannot  
121 be cleaved, which aborts the interference step and, in turn, stops the generation of  
122 spacer precursors for primed and interference-driven adaptation [25].

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

### 129 **Inhibition of CRISPR-Cas expression and assembly**

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

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

### 143 **Inhibition of CRISPR-Cas interference**

144 Anti-interference activity can be achieved by the inhibition of target binding, or  
145 nuclease activity. To date, inhibition of target binding is the prevalent strategy. Thirteen  
146 anti-CRISPR proteins interfere with target recognition and binding (type I-F AcrIF1,  
147 AcrIF2 and AcrIF10 [28-31]; type II-A AcrIIA2, AcrIIA4, AcrIIA5 and AcrIIA6 [32-41];  
148 type II-C AcrIIC3, AcrIIC4 and AcrIIC5 [42-45]; type V-A AcrVA1, AcrV4A and AcrVA5  
149 [46-52]), while only five block target cleavage (type I-E AcrIE1 [24, 53]; type III-B  
150 AcrIIIB1 [10]; type I-F AcrIF3 [54, 55]; type II-C AcrIIC1 and AcrIIC3 [42-44]) (Figure  
151 1). Given that DNA binding is the rate-limiting step of Cascade and Cas9-mediated  
152 interference activities [56, 57], altering this step is therefore an efficient way to  
153 inactivate CRISPR-Cas interference. Although the type I-D AcrID1, type I-F AcrIF4,  
154 and type II-A AcrIIA7-11 were shown to block the interference step, it is not yet known  
155 whether they inhibit target binding or cleavage [8, 11, 12, 15].

156

### 157 **Molecular mechanisms used by anti-CRISPR proteins**

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

167        **Steric occlusion of Cas effector functional sites**

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

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

186        AcrIF1 and AcrIF2 anti-CRISPR proteins use different strategies to block the access  
187        to DNA binding sites of the effector Cascade-crRNA complex. Several copies of AcrIF1  
188        bind to the Cas7f backbone of Cascade and block the target DNA to access and  
189        hybridize with the crRNA [30, 31, 58]. As for AcrIF2, its binding site located between  
190        the Cas7.6f and Cas8f subunits partially overlaps with the DNA binding site (Figure  
191        2A). Moreover, AcrIF2 binding to the effector Cascade-crRNA complex induces  
192        conformational changes incompatible with target DNA binding [30].

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



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

### 207 **Allosteric inhibition and clustering of effector complexes**

208 Recently, the AcrIIA6, AcrIIC3, and AcrVA4 anti-CRISPR proteins have been shown  
209 to be allosteric inhibitors of the *Streptococcus thermophilus* CRISPR1-Cas9  
210 (St1Cas9), NmeCas9, and *Lachnospiraceae bacterium* Cas12a (LbCas12a) effector  
211 complexes, respectively. Interestingly, these anti-CRISPR proteins can also bind to  
212 and inactivate two effector complexes at a time (Figure 2B). Noteworthy, AcrID1  
213 directly interacts with Cas10d, the large subunit of the type I-D CRISPR-Cas effector  
214 complex, and induces its dimerization. However, the molecular basis of AcrID1  
215 inhibition mechanism remains to be determined [15]. Knowing that anti-CRISPR  
216 proteins gradually immunosuppress bacteria through multiple failed phage infections  
217 [60, 61], lowering the critical concentration of a given anti-CRISPR protein required for  
218 the complete inactivation of CRISPR-Cas immunity may be advantageous for a phage  
219 population to rapidly propagate.

220 AcrIIA6 and AcrVA4 both function as dimers that tightly associate with a mixed  
221 protein-RNA region distinct from the DNA-binding crevasse and catalytic domains [41,  
222 48, 51, 52]. However, regions of both subunits that compose the AcrIIA6 dimer form  
223 each binding interface, while every subunit of the AcrV4A dimer contains one binding  
224 interface (Figure 2B). These anti-CRISPR proteins perturb the conformational changes  
225 required to bind to target DNA, thus locking St1Cas9 and LbCas12a effector  
226 complexes in a nonfunctional state. AcrIIA6 impairs St1Cas9 conformational  
227 rearrangements associated with PAM binding, which leads to the inhibition of target  
228 DNA recognition and binding [41]. In contrast, AcrVA4 does not affect PAM binding but  
229 impairs LbCas12a dynamics and structural changes required for the R-loop formation  
230 and stabilization (glossary), which prematurely stops the hybridization between the  
231 cRNA and target DNA [51]. Additionally, AcrVA4 can also associate with DNA-bound  
232 effector complexes. When it binds to the LbCas12a-crRNA-dsDNA complex with a

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

236 As for AcrIIC3, one monomer is able to tether two NmeCas9 effector complexes  
237 (Figure 2B). It interacts with the NmeCas9 HNH domain of one effector complex, at the  
238 opposite face of the catalytic site, and the NmeCas9 REC lobe (glossary) of another  
239 effector complex [27, 44, 62]. The interaction between AcrIIC3 and the REC lobe, in  
240 the vicinity of the DNA-sgRNA hybridization site, likely perturbs the conformational  
241 dynamics of NmeCas9 that assists DNA binding, which would explain the reduction of  
242 NmeCas9 DNA binding affinity *in vitro* and the inhibition of DNA binding within cells  
243 [42, 43]. Besides, AcrIIC3 is able to associate with DNA-bound NmeCas9 effector  
244 complexes *in vitro* [44]. The interaction between AcrIIC3 and the HNH domain blocks  
245 the structural changes triggered by target DNA binding, and therefore locks the HNH  
246 and RuvC nuclease domains (glossary) in inactive conformations [44]. These data  
247 suggest that AcrIIC3 could inactivate NmeCas9 effector complexes in the presence or  
248 absence of target DNA.

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

## 256 **Enzymatic modification of CRISPR-Cas components**

257 The two anti-CRISPR proteins with an enzymatic activity that have been  
258 characterized so far, AcrVA1 and AcrVA5, inhibit Cas12a effector complexes through  
259 different mechanisms (Figure 2C). Interestingly, AcrVA1 and AcrVA5 bind to  
260 overlapping regions in the PAM-interacting domain and compete with one another [49,  
261 51]. However, their substrate and enzymatic activity are different. AcrVA1 is a multiple-  
262 turnover endoribonuclease that cleaves off the Cas12a-bound crRNA spacer  
263 sequence to irreversibly inactivate the effector assembly [49]. Noteworthy, AcrVA1  
264 mimics the PAM to position its catalytic residues close to the crRNA substrate [51].  
265 Interestingly, the type II-A AcrIIA5 anti-CRISPR protein was recently shown to lead to

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

275

## 276 **Perspectives**

### 277 **•Importance of the field**

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

### 285 **•Summary of current thinking**

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

### 298 **•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.

313 **Glossary**

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

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

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

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

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

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

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

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

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

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

337

338 **Abbreviations**

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

341

342 **Author contribution**

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

345

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352

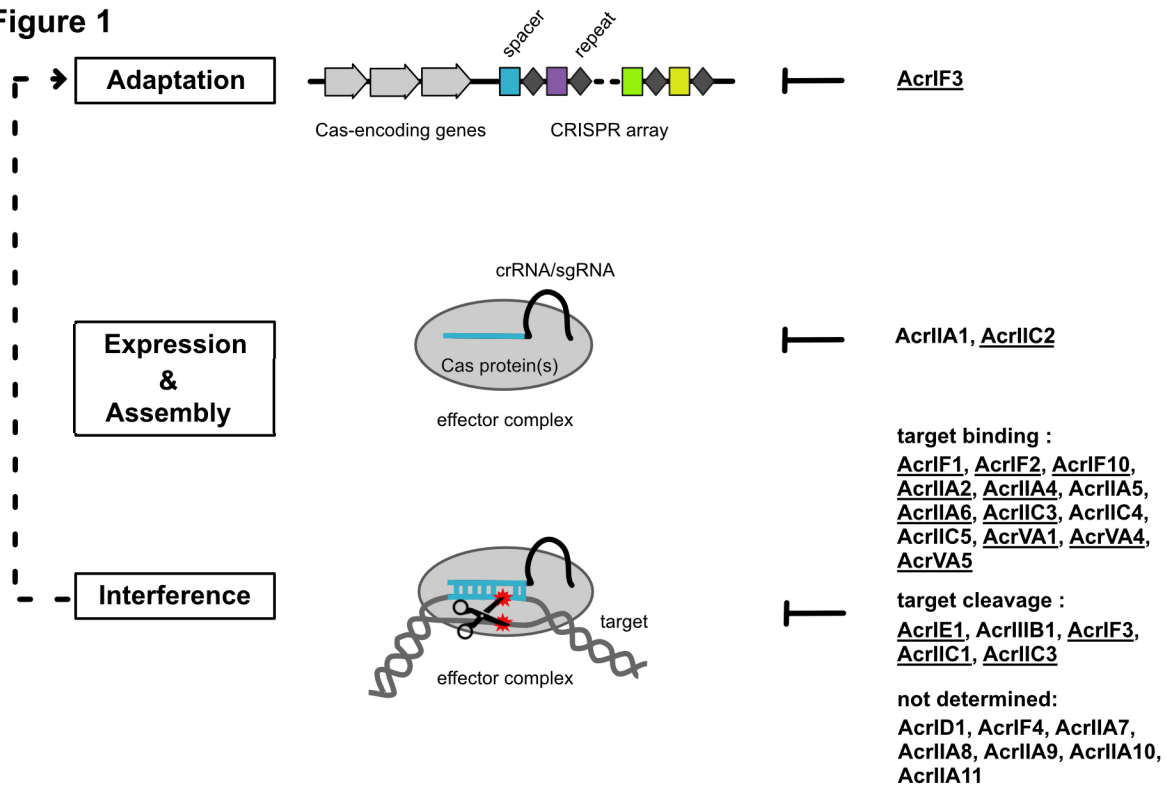
353 **Competing interests**

354 The authors declare no competing interests.

355

356 **Figure legends**

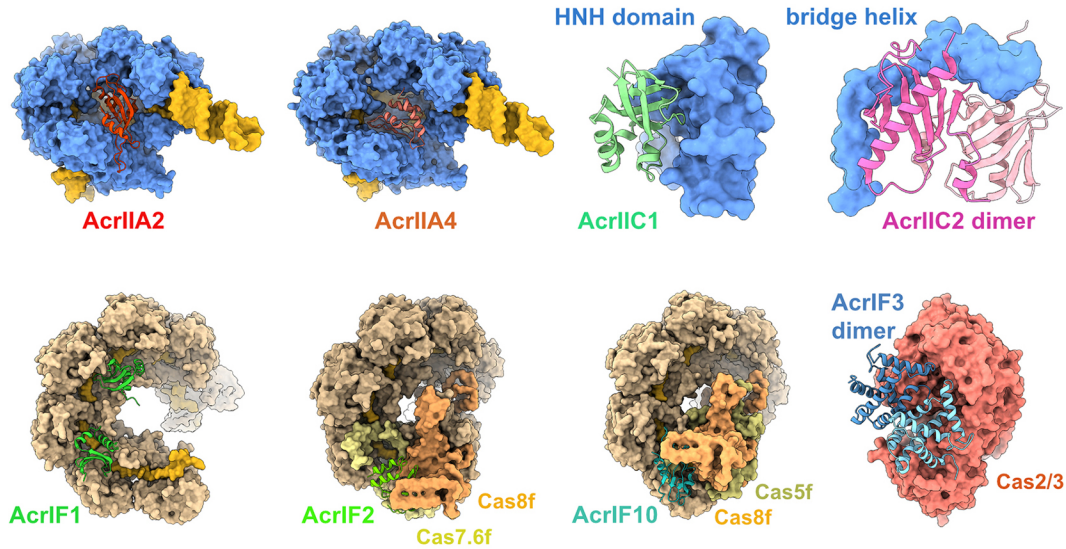
**Figure 1**



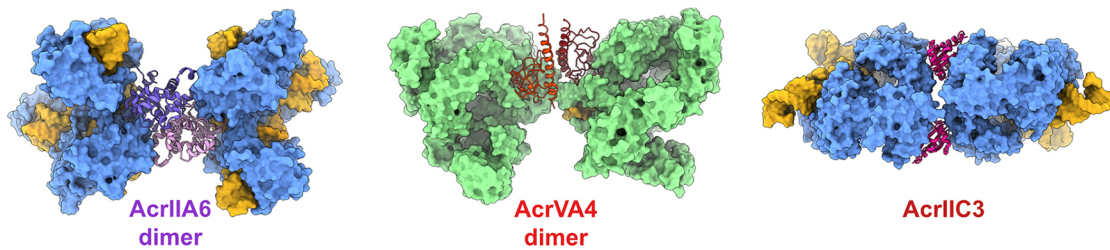
357  
358 **Figure 1. Different routes to inactivate CRISPR-Cas immunity.** The different steps  
359 of CRISPR-Cas immunity are indicated in boxes on the left. Schematics, in the center,  
360 illustrate the adaptation (top), expression\_ and assembly (middle), and interference  
361 (bottom) steps. 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

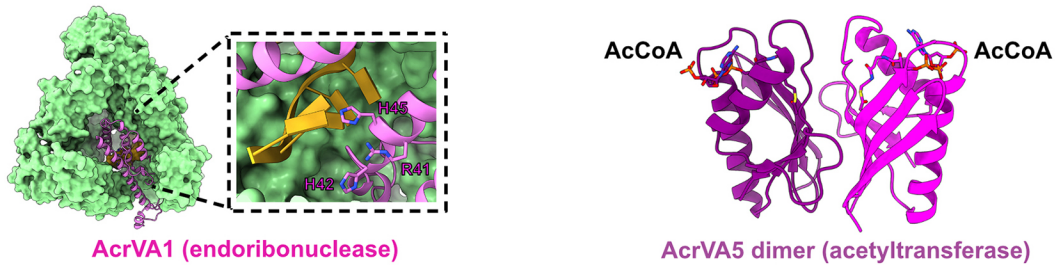
A. Steric occlusion of Cas effector functional sites



B. Allosteric inhibition and clustering of effector complexes



C. Enzymatic modifications of CRISPR-Cas components



● Cas9 ● Cascade ● Cas12a ● crRNA/sgRNA

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**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 6JDJ; 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].  
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