

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

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17 Abstract

Bacteriophages (phages) and their preys are engaged in an evolutionary arms race 18 driving the co-adaptation of their attack and defense mechanisms. In this context, 19 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 resemblance with each other and with proteins of known function, which raises 22 intriguing questions particularly relating to their modes of action. In recent years, there 23 have been many structure-function studies shedding light on different CRISPR-Cas 24 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 on the molecular strategies deployed to inactivate distinct steps of CRISPR-Cas 27 immunity. Anti-CRISPR proteins can be orthosteric or allosteric inhibitors of CRISPR-28 29 Cas machineries, as well as enzymes that irreversibly modify CRISPR-Cas components. This repertoire of CRISPR-Cas inhibition mechanisms will likely expand 30 31 in the future, providing fundamental knowledge on phage-bacteria interactions and offering great perspectives for the development of biotechnological tools to fine-tune 32 33 CRISPR-Cas-based gene edition.

35 Introduction

In the battle for survival, bacteria and archaea have evolved mechanistically diverse 36 defense mechanisms to resist their predators [1]. The prokaryotic CRISPR-Cas 37 immune system uniquely provides a memory of past infections in the form of small 38 39 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 40 RNAs (crRNAs) that associate with Cas proteins to form effector complexes that patrol 41 the cells for potential invasion. Upon recognition and binding to nucleic acid sequences 42 complementary to crRNAs (referred to as protospacers), the Cas nuclease function is 43 44 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 45 the composition of Cas proteins, six types and twenty-five subtypes. Class 1 systems 46 47 (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 48 49 for nucleic acid binding and cleavage [3].

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

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

In recent years, the highly dynamic anti-CRISPR field of research has emerged and 70 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 [14, 64, 65, 66]. Here, we intend to provide a complementary, global perspective on 74 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 molecular tactics to inactivate CRISPR-Cas machineries, which can be differentiated 77 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-Cas inhibitors. 81

82

83 Different routes to inactivate CRISPR-Cas immunity

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

97 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 101 102 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 103 104 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 105 106 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 107 complex (glossary), associate with the Cas1-Cas2 complex for naïve, primed and 108 109 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 adaptation step [21]. 114

115 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 116 117 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 the adaptation step, although further experiments are required to confirm its anti-119 adaptation activity. The protospacer, bound to the Cascade-crRNA complex, cannot 120 121 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

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 134 their expression, remain to be uncovered. Although such anti-CRISPR proteins could 135 not rapidly inactivate CRISPR immunity as required upon virulent phage infection, they 136 137 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 138 of effector complexes. While AcrIIA1 binds to a broad-spectrum of type II-A and type 139 II-C Cas9s and triggers their degradation within infected cells [68] AcrIIC2 binds to the 140 141 apo form of Neisseria meningitidis Cas9 (unliganded NmeCas9) and blocks sgRNA 142 loading [26, 27].

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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 anti-CRISPR proteins interfere with target recognition and binding (type I-F AcrIF1, 146 AcrIF2 and AcrIF10 [28-31]; type II-A AcrIIA2, AcrIIA4, AcrIIA5 and AcrIIA6 [32-41]; 147 type II-C AcrIIC3, AcrIIC4 and AcrIIC5 [42-45]; type V-A AcrVA1, AcrV4A and AcrVA5 148 [46-52]), while only five block target cleavage (type I-E AcrIE1 [24, 53]; type III-B 149 AcrIIIB1 [10]; type I-F AcrIF3 [54, 55]; type II-C AcrIIC1 and AcrIIC3 [42-44]) (Figure 150 1). Given that DNA binding is the rate-limiting step of Cascade and Cas9-mediated 151 152 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, 153 and type II-A AcrIIA7-11 were shown to block the interference step, it is not yet known 154 155 whether they inhibit target binding or cleavage [8, 11, 12, 15].

156

Molecular mechanisms used by anti-CRISPR proteins 157

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

167 Steric occlusion of Cas effector functional sites

To date, the most common molecular tactic used by anti-CRISPR proteins is to 168 sterically block the access to the functional sites of Cas machineries, including the 169 170 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 171 172 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 173 molecular mechanisms used by anti-CRISPR proteins is required to determine 174 175 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 Cas8f and Cas5f subunits, and induces a DNA-bound conformation of the effector 180 181 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 182 183 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 convergence from structurally distinct inhibitors with overlapping binding sites. 185

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].

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].

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).

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.

AcrIIA6 and AcrVA4 both function as dimers that tightly associate with a mixed 220 221 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 each binding interface, while every subunit of the AcrV4A dimer contains one binding 223 224 interface (Figure 2B). These anti-CRISPR proteins perturb the conformational changes 225 required to bind to target DNA, thus locking St1Cas9 and LbCas12a effector complexes in a nonfunctional state. AcrIIA6 impairs St1Cas9 conformational 226 rearrangements associated with PAM binding, which leads to the inhibition of target 227 228 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 229 230 and stabilization (glossary), which prematurely stops the hybridization between the cRNA and target DNA [51]. Additionally, AcrVA4 can also associate with DNA-bound 231 232 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]. 233 234 When it binds to the post-cleavage Cas12a-crRNA-dsDNA complex, it likely blocks the recycling of the enzyme [52]. 235

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 opposite face of the catalytic site, and the NmeCas9 REC lobe (glossary) of another 238 effector complex [27, 44, 62]. The interaction between AcrIIC3 and the REC lobe, in 239 240 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 and RuvC nuclease domains (glossary) in inactive conformations [44]. These data 246 247 suggest that AcrIIC3 could inactivate NmeCas9 effector complexes in the presence or absence of target DNA. 248

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 functional effector complexes within infected cells. 255

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 overlapping regions in the PAM-interacting domain and compete with one another [49, 260 261 51]. However, their substrate and enzymatic activity are different. AcrVA1 is a multipleturnover endoribonuclease that cleaves off the Cas12a-bound crRNA spacer 262 263 sequence to irreversibly inactivate the effector assembly [49]. Noteworthy, AcrVA1 mimics the PAM to position its catalytic residues close to the crRNA substrate [51]. 264 Interestingly, the type II-A AcrIIA5 anti-CRISPR protein was recently shown to lead to 265

sgRNA cleavage at multiples sites out of the crRNA spacer sequence [39]. However, 266 267 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 268 269 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 inactivation of many interference complexes are likely beneficial to phages to decrease 272 273 the number of failed phage infections required to immunosuppress their host [60, 61], 274 and thereby rapidly evade CRISPR-Cas immunity.

275

276 **Perspectives**

• 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 finetune CRISPR-Cas-based gene edition.

• Summary of current thinking

286 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-287 288 CRISPR protein identification pipelines, our current knowledge mainly covers anti-CRISPR proteins inhibiting the CRISPR-Cas interference step. Second, the inhibition 289 290 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 that prevails over allosteric inhibition modes and enzymatic modifications. 293 294 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 295 296 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.

• Future directions

The characterization of anti-CRISPR proteins that interfere with the adaptation and 299 300 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-Cas systems. Besides, whether molecular mechanisms are favored for lytic or 304 lysogenic cycles also remains to be addressed. Our current knowledge of anti-CRISPR 305 inhibition strategies indicates that the "best" anti-CRISPR proteins from a mechanistic 306 307 and biochemical point of view, such as enzymes that rapidly modify multiple CRISPR-Cas effector complexes, may not be widely used by phages. It will be important to 308 309 examine the molecular strategy used by a given phage in light of the phage population context and of the phage-host interactions. Lastly, investigations of the mechanisms 310 311 evolved by bacteria and archaea to resist anti-CRISPR proteins will tackle the next step in the CRISPR-based molecular arms race. 312

313 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.
- 337

338 Abbreviations

- Acr, anti-CRISPR protein ; AFV1, Acidianus filamentous virus 1 ; crRNA, CRISPR
 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.

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- 352

353 Competing interests

- 354 The authors declare no competing interests.
- 355

356 Figure legends





Figure 1. Different routes to inactivate CRISPR-Cas immunity. The different steps 358 359 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 (bottom) steps. A CRISPR-array is depicted with alternated spacers (colored boxes) 361 and repeats (dark grey diamonds). The light grey arrows represent Cas-encoding 362 genes. One or several Cas proteins (shown as an ellipse) associate with crRNA or 363 364 sgRNA molecules to form the effector complex. Target binding (a dsDNA molecule is shown here) triggers the Cas nuclease activity, which leads to the destruction of foreign 365 genetic material. Anti-CRISPR proteins known to inhibit one or several of these steps 366 are listed on the right. Most of them block target binding at the interference step. The 367 368 dashed arrow indicates that anti-CRISPR proteins inhibiting the interference step could also inactivate the adaptation step. The molecular mechanisms of the underlined anti-369 CRISPR proteins have been characterized. 370



Figure 2 A. Steric occlusion of Cas effector functional sites

B. Allosteric inhibiton and clustering of effector complexes



C. Enzymatic modifications of CRISPR-Cas components

371



Figure 2. Molecular mechanisms used by anti-CRISPR proteins. A. Ribbon 372 373 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, 374 PDB 6MCB; AcrIIA4, PDB 5VZL; AcrIIC1, PDB 5VGB; AcrIIC2, PDB 6JDX; AcrIF1, 375 PDB 6B46; AcrIF2, PDB 6B47; AcrIF3, PDB 5B7I; AcrIF10, PDB 6B48. B. Ribbon 376 representation of anti-CRISPR proteins that mediate the clustering of two effector 377 complexes. AcrIIA6, AcrVA4 and AcrIIC3 are CRISPR-Cas allosteric inhibitors. 378 AcrIIA6, PDB 6RJ9; AcrVA4 PDB 6NM9; AcrIIC3, PDB 6JE9. C. Ribbon representation 379

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

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