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## Disabling Cas9 by an anti-CRISPR DNA mimic

Jiyung Shin<sup>1,2\*</sup>, Fuguo Jiang<sup>2\*</sup>, Jun-Jie Liu<sup>2,5\*</sup>, Nicolas L. Bray<sup>1,2</sup>, Benjamin J. Rauch<sup>3</sup>,  
Seung Hyun Baik<sup>1,2</sup>, Eva Nogales<sup>2,5</sup>, Joseph Bondy-Denomy<sup>3</sup>, Jacob E. Corn<sup>1,2†</sup> and  
Jennifer A. Doudna<sup>1,2,4,5†</sup>

<sup>1</sup>Innovative Genomics Institute, <sup>2</sup>Department of Molecular and Cell Biology, University of California, Berkeley, California 94720; <sup>3</sup>Department of Microbiology and Immunology, and Quantitative Biosciences Institute, University of California, San Francisco, CA 94158; <sup>4</sup>Howard Hughes Medical Institute; <sup>5</sup>Department of Chemistry, University of California, Berkeley, California 94720; <sup>5</sup>Molecular Biophysics and Integrated Bioimaging Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720

\*These authors contributed equally to the project.

†Correspondence should be addressed to J.E.C. ([jcorn@berkeley.edu](mailto:jcorn@berkeley.edu)) and J.A.D. ([doudna@berkeley.edu](mailto:doudna@berkeley.edu))

22 **CRISPR-Cas9 gene editing technology is derived from a microbial adaptive**  
23 **immune system, where bacteriophages are often the intended target. Natural**  
24 **inhibitors of CRISPR-Cas9 enable phages to evade immunity and show promise**  
25 **in controlling Cas9-mediated gene editing in human cells. However, the**  
26 **mechanism of CRISPR-Cas9 inhibition is not known and the potential**  
27 **applications for Cas9 inhibitor proteins in mammalian cells has not fully been**  
28 **established. We show here that the anti-CRISPR protein AcrIIA4 binds only to**  
29 **assembled Cas9-single guide RNA (sgRNA) complexes and not to Cas9 protein**  
30 **alone. A 3.9 Å resolution cryo-EM structure of the Cas9-sgRNA-AcrIIA4 complex**  
31 **revealed that the surface of AcrIIA4 is highly acidic and binds with 1:1**  
32 **stoichiometry to a region of Cas9 that normally engages the DNA protospacer**  
33 **adjacent motif (PAM). Consistent with this binding mode, order-of-addition**  
34 **experiments showed that AcrIIA4 interferes with DNA recognition but has no**  
35 **effect on pre-formed Cas9-sgRNA-DNA complexes. Timed delivery of AcrIIA4 into**  
36 **human cells as either protein or expression plasmid allows on-target Cas9-**  
37 **mediated gene editing while reducing off-target edits. These results provide a**  
38 **mechanistic understanding of AcrIIA4 function and demonstrate that inhibitors**  
39 **can modulate the extent and outcomes of Cas9-mediated gene editing.**

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42 Phage-encoded inhibitors of CRISPR-Cas bacterial immune systems evolved to enable  
43 phage escape from destruction in bacterial cells (1) and have the potential to control  
44 CRISPR-Cas enzymes that are deployed for gene editing applications in various cell  
45 types (2, 3). Determination of the molecular basis for Cas9 inhibition could shed light on  
46 the evolutionary “arms race” between phage and bacteria, as well as suggest new  
47 approaches to regulate genome editing in eukaryotic cells. The 87 amino acid anti-  
48 CRISPR-Cas9 protein AcrIIA4 is notable in both respects: it inhibits multiple Cas9  
49 proteins including the widely used Cas9 ortholog from *Streptococcus pyogenes* (Spy),  
50 and it blocks Cas9-mediated gene editing in human cells (3). To investigate the  
51 molecular basis for AcrIIA4-mediated Cas9 inhibition, we first tested whether  
52 recombinant AcrIIA4 protein interacts directly with SpyCas9 (Fig. 1A). Purified AcrIIA4  
53 was incubated with SpyCas9 in the presence or absence of a single-guide RNA  
54 (sgRNA) that assembles with Cas9 to provide sequence-specific DNA recognition (4).  
55 Size exclusion chromatography showed that AcrIIA4 binds to SpyCas9 only in the  
56 presence of sgRNA (Fig. 1B and C, fig. S1), implying that AcrIIA4 recognizes a protein  
57 surface created upon sgRNA-triggered conformational rearrangement (5, 6).  
58 Furthermore, the AcrIIA4-bound Cas9-sgRNA complex is more resistant to proteolytic  
59 digestion than the Cas9-sgRNA complex alone, implying that AcrIIA4 stabilizes a  
60 particular Cas9 conformation (fig. S2).

61 To elucidate the molecular basis of AcrIIA4-mediated inhibition of Cas9 activity,  
62 we performed cryo-electron microscopy (cryo-EM) single-particle analysis on a  
63 SpyCas9-sgRNA complex bound to AcrIIA4. Cryo-EM images were collected on a Krios

64 microscope using zero-loss energy-filtered imaging and a K2 direct electron detector.  
65 After unsupervised 3D classification of 840,000 particle images, refinement of a class  
66 containing 285,600 particles resulted in an EM reconstruction of the SpyCas9-sgRNA-  
67 AcrIIA4 complex with an overall resolution of 3.9 Å (fig. S3-S4). Subsequent local 3D  
68 classification that was focused on Cas9's HNH nuclease domain ultimately yielded two  
69 EM reconstructions, one at 3.9 Å resolution where the HNH was poorly resolved due to  
70 flexibility (reconstruction 1, obtained as a combination of two 3D classes) (Fig. 2a and  
71 fig. S5a) and one with better defined density for the HNH domain at an average  
72 resolution of 4.5 Å (reconstruction 2) (fig. S4d and S5a; see Methods section and fig.  
73 S3). In both cryo-EM reconstructions, the density for the HNH domain of Cas9 was  
74 weaker than for the rest of the structure, consistent with the previously observed  
75 conformational plasticity of the HNH nuclease domain in the pre-targeting state. An  
76 atomic model for AcrIIA4 was built from reconstruction 1. The EM density map displays  
77 excellent main-chain connectivity and side-chain densities for almost all residues of  
78 AcrIIA4 (fig. S5). We then built an atomic model for the Cas9-sgRNA complex based on  
79 the crystal structure of SpyCas9-sgRNA (PDB ID 4ZT0) and refined the entire SpyCas9-  
80 sgRNA-AcrIIA4 model in real space to good stereochemistry (Fig. 2B and table S1).  
81 Overall, Cas9 bound to AcrIIA4 resembles the pre-target state rather than the DNA-  
82 bound state (fig. S6).

83 *De novo* model building demonstrated that AcrIIA4 binds to Cas9 with 1:1  
84 stoichiometry and comprises a three-stranded antiparallel  $\beta$  sheet flanked by one alpha-  
85 helix at the amino-terminal end and two  $\alpha$  helices on the carboxy-terminal end

86 ( $\alpha_1\beta_1\beta_2\beta_3\alpha_2\alpha_3$ ). Remarkably, superposition of the AcrIIA4-bound Cas9 structure with  
87 DNA-bound Cas9 revealed that AcrIIA4 sits exactly in the PAM-interacting cleft formed  
88 between the alpha-helical recognition (REC) lobe and the nuclease lobe (Fig. 2B and  
89 fig. S6). AcrIIA4 completely occupies the PAM binding pocket and thus blocks DNA  
90 recognition through contacts between the  $\beta_3$  strand of AcrIIA4 and Cas9 PAM-binding  
91 residues (R1333 and R1335) (Fig. 2D-E). In addition, AcrIIA4 wedges into the DNA  
92 melting region immediately upstream of the PAM sequence and sits on top of +1  
93 phosphate on the target strand and the flipped nucleotides on the nontarget strand,  
94 indicating AcrIIA4 could also prevent DNA binding/unwinding. Consistent with this DNA-  
95 mimicking binding mode, AcrIIA4 is extremely acidic (Fig. 2E). Interestingly, AcrIIA4 also  
96 occupies the same space as the DNA-bound HNH domain and the linker connecting the  
97 HNH and RuvC domains (Fig. 2D), suggesting AcrIIA4 could block the HNH movement  
98 required for catalysis (Fig. 2B, C).

99         Collectively, our structural studies show that AcrIIA4 is a highly acidic DNA mimic  
100 that blocks target DNA recognition through multiple mechanisms: 1) competitive  
101 inhibition of PAM binding; 2) inhibition of DNA unwinding upstream of the PAM  
102 sequence; and 3) inactivation of HNH domain movement from the inactive to active  
103 conformation. These structural findings help explain the effectiveness of AcrIIA4 as an  
104 inhibitor of Cas9-mediated DNA cleavage and cell-based genome editing. Previous  
105 studies revealed the importance of Cas9's interactions with targeted DNA, mediated via  
106 the PAM in an interaction preceding base pairing between the sgRNA and its target (7).  
107 Transient Cas9-sgRNA association with PAM sequences in DNA is thought to enable a

108 rapid target sequence search. By mimicking the structure and electrostatic properties of  
109 the DNA PAM sequence, AcrIIA4 might compete for initial DNA binding and thereby  
110 prevent target recognition and cleavage (Fig. 2E-G).

111 To determine the functional effects of AcrIIA4 binding to Cas9-sgRNA  
112 complexes, we subjected DNA substrates possessing a target sequence to Cas9-  
113 sgRNA-catalyzed DNA cleavage assays. A linearized plasmid possessing a target  
114 sequence and PAM was incubated with combinations of Cas9, sgRNA and AcrIIA4, and  
115 the products were resolved by gel electrophoresis. Cas9-sgRNA alone completely cut  
116 the target DNA within five minutes, while AcrIIA4 limited cleavage at even later time  
117 points (Fig. 3A). To confirm that AcrIIA4 would also inactivate Cas9 at low enzyme and  
118 inhibitor concentrations, we performed similar experiments with a radiolabeled DNA  
119 target for increased sensitivity (Fig. 3B). Near-stoichiometric concentrations of AcrIIA4  
120 inhibited DNA cleavage when titrated against 10 nM Cas9-gRNA complex, indicating an  
121 apparent dissociation constant of less than 10 nM. These data show that AcrIIA4  
122 functions as a robust Cas9 “off-switch” that can inhibit most Cas9 activity at low  
123 concentrations.

124 The structure of Cas9 bound to AcrIIA4 suggested that the inhibitor competes for  
125 the initial DNA recognition event of PAM binding. Yet we previously found that Cas9  
126 binds so tightly to target DNA that its off-rate is negligible (7, 8). This implies an unusual  
127 non-equilibrium mode of inhibition, in which AcrIIA4 requires access to Cas9-sgRNA  
128 prior to formation of the Cas9-sgRNA-DNA complex. To test this prediction, we used  
129 bilayer interferometry to measure the binding of catalytically inactivated Cas9 (dCas9) to

130 a DNA target in the presence of inhibitor. These experiments were performed under  
131 stoichiometric binding conditions in which Cas9 was present at concentrations greater  
132 than the dissociation constant of the Cas9-AcrIIA4 interaction. Pre-incubation of Cas9  
133 with AcrIIA4 markedly inhibited Cas9 binding to DNA (on-rate) in a dose-dependent  
134 fashion, including complete prevention of target engagement (Fig. 3C, fig. S7). An  
135 electrophoretic mobility shift assay (EMSA) further confirmed that AcrIIA4 does not  
136 impact sgRNA loading into Cas9 protein alone (fig. S8), but does inhibit Cas9-sgRNA  
137 complex binding to a target DNA (fig. S9). Strikingly, allowing the Cas9-sgRNA-DNA  
138 complex to form and then adding AcrIIA4 had no effect on Cas9's release of target DNA  
139 (off-rate). These order-of-addition results are consistent with both the structure of  
140 AcrIIA4 bound to Cas9 and Cas9's extremely slow dissociation from DNA. Together,  
141 these data support a mechanism for AcrIIA4 inhibition in which the inhibitor blocks  
142 Cas9's ability to bind and cut target DNA by obscuring the PAM-interacting domain.

143 To determine the ability of AcrIIA4 to regulate gene editing in human cells, we  
144 first utilized human K562 erythroleukemia cells stably expressing a chromosomally-  
145 integrated blue fluorescent protein (BFP) reporter (8). Nucleofection of Cas9-sgRNA  
146 (Cas9 RNP) complexes targeting BFP resulted in loss of BFP fluorescence in almost all  
147 cells as measured by flow cytometry (fig. S10). Simultaneous delivery of Cas9 RNP and  
148 AcrIIA4 protein inhibited Cas9-mediated gene targeting by up to 80% (Fig. 4A blue  
149 symbols; fig. S10). Simultaneous delivery of AcrIIA4 encoded in a plasmid inhibited  
150 gene editing to a lesser extent, possibly due to a delay in expression of the inhibitor  
151 from a plasmid relative to immediate nucleofection of Cas9 RNP (Fig. 4A green

152 symbols; fig. S10, S11). This implied that the order-of-addition effects we observed in  
153 biophysical experiments may play a role during gene editing. To test this idea, we  
154 nucleofected AcrIIA4 plasmid 24 hours prior to introducing Cas9 RNP into cells. The  
155 presence of the AcrIIA4-encoding plasmid 24 hours prior to Cas9 RNP introduction  
156 inhibited Cas9-mediated gene targeting to a far greater extent than was observed  
157 during co-introduction, and to a similar extent as observed during co-introduction of  
158 AcrIIA4 protein and Cas9 RNP (Fig. 4B, fig. S12). Remarkably, we found that addition of  
159 AcrIIA4 six hours after Cas9-RNP reduced editing by ~50%, demonstrating the utility of  
160 inhibitors for revealing *in vivo* gene editing kinetics (Fig. 4C, fig. S13). In sum,  
161 controlling the timing of AcrIIA4 inhibition in human cells strongly affects the frequency  
162 of gene editing at a given locus.

163         Using a given guide RNA, Cas9 may target both an on-target site and off-target  
164 loci (9-12). But several lines of evidence suggest that off-target sites may be bound  
165 without being immediately cleaved (13-15). Cas9 displaced from uncleaved sites (e.g.  
166 by cellular factors) would be available for inhibition by AcrIIA4. Since inhibitor timing  
167 experiments suggested that at least 50% of on-target Cas9 gene editing takes place  
168 within the first 6 hours (Fig. 4C), we asked whether off-target editing could be reduced  
169 by properly timed addition of inhibitor.

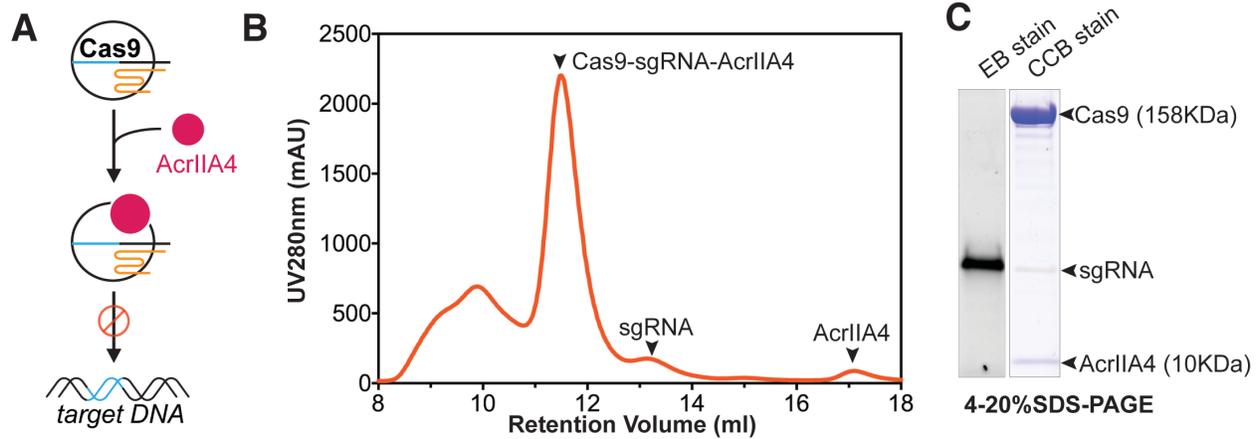
170         We examined on- and off-target editing using guide RNAs targeting the EMX1  
171 and HBB loci. The off-target sites for both the EMX1 and HBB guides have been  
172 previously described (10, 16, 17). Notably, the HBB guide is of therapeutic interest to  
173 edit the causative mutation of sickle cell disease, but has an off-target that is nearly

174 identical to the on-target site. We edited the EMX1 and HBB loci using Cas9 RNPs with  
175 and without pre-introduction of AcrIIA4 protein or plasmid. Consistent with experiments  
176 at the BFP locus, we found that timed addition of AcrIIA4 retained substantial levels of  
177 on-target editing. Strikingly, both T7E1 and TIDE analysis showed that timed addition of  
178 AcrIIA4 almost completely abolished off-target editing at both HBB and EMX1 (Fig. 4D).  
179 This suggests that Cas9 inhibitors have the potential to be useful in reducing off-target  
180 events during research and therapeutic applications.

181         The recent and rapid expansion of the Cas9 toolkit for gene editing applications  
182 has lacked an inducible “off-switch” to prevent undesired gene editing. Newly  
183 discovered protein inhibitors, encoded by bacteriophages, provide an attractive solution  
184 to this problem as these proteins are small and function well in human cells (3). Here,  
185 we demonstrate that AcrIIA4, the most potent SpyCas9 inhibitor in human cells, acts as  
186 a DNA mimic to block PAM recognition. The deployment of DNA mimics to inhibit a  
187 DNA-binding protein is an elegant solution that is often deployed in the phage-host arms  
188 race. A recent structural study revealed that a Class 1 anti-CRISPR (AcrF2) uses a  
189 similar strategy (18). Further, phage-encoded restriction enzyme inhibitor proteins have  
190 long been known to mimic the DNA target (19). Our results further show that Cas9  
191 inhibitors delivered as either protein or expressed from a plasmid can modulate the  
192 efficacy of gene editing at multiple loci in human cells. Although pre-addition of inhibitor  
193 almost completely abolishes overall gene editing, timed addition of inhibitor after  
194 initiating Cas9-sgRNA-based gene editing can adjust the amount of time that Cas9 is  
195 active in the nucleus, thereby selectively limiting off-target editing. We anticipate that

196 Cas9 inhibitors could be broadly useful in situations where precise control of either on-  
197 or off-target gene editing is desirable, such as during allele-specific therapeutic editing.  
198  
199

200 **Figures**



201

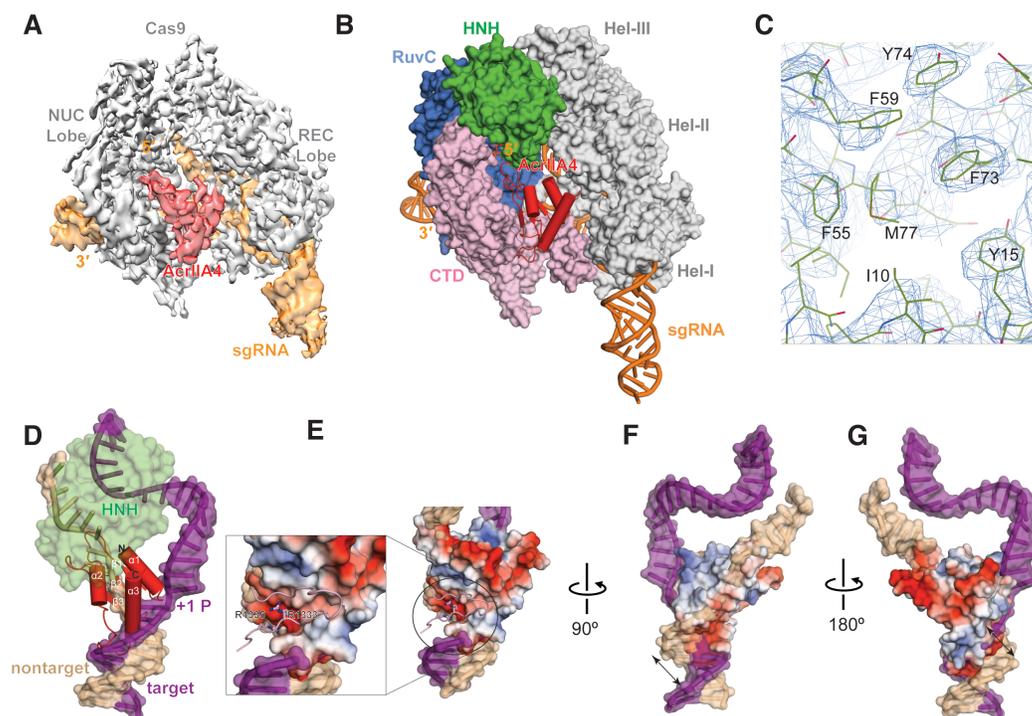
202 **Fig 1. AcrIIA4 binds to the *S. pyogenes* Cas9-sgRNA complex.**

203 A) A cartoon depiction of Cas9 protein loaded with the sgRNA binding to AcrIIA4  
204 (pink). Cas9-sgRNA complexed with AcrIIA4 is unable to bind to the target DNA.

205 B) Size exclusion chromatogram of SpyCas9-sgRNA after preincubation with  
206 AcrIIA4. Relevant peaks are indicated with arrowheads.

207 C) Coomassie and Ethidium Bromide (EB) stained polyacrylamide gel showing the  
208 commigration of AcrIIA4 with Cas9 in the presence of guide RNA.

209



210

211 **Fig. 2. Architecture of the *S. pyogenes* Cas9-sgRNA in complex with AcrIIA4**

212 A) Cryo-EM reconstruction of the AcrIIA4-bound SpyCas9. The electron density map  
213 was contoured at high threshold levels showing distinct features for each subunit.

214 B) The atomic model of SpyCas9-sgRNA-AcrIIA4. AcrIIA4 (red) and sgRNA  
215 (orange) are shown in ribbon diagram and SpyCas9 is displayed as surface  
216 representation.

217 C) Representative region (hydrophobic core) of the cryo-EM density for AcrIIA4 with  
218 the refined model superimposed.

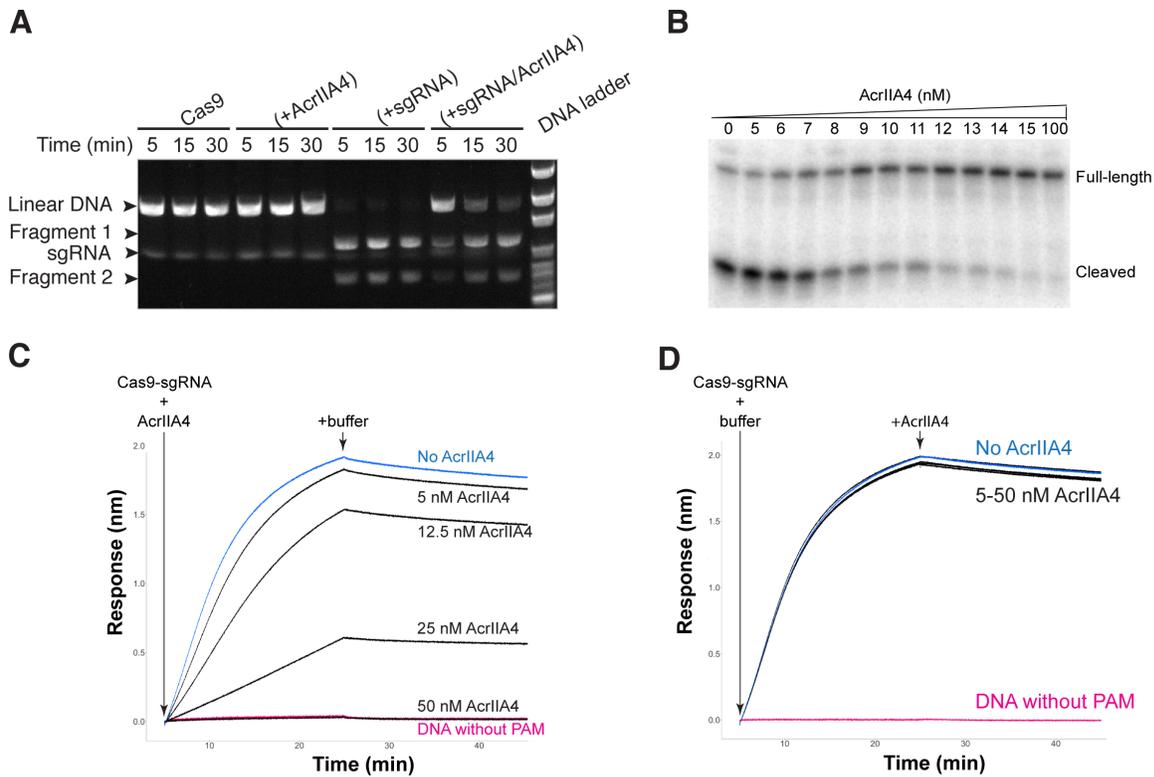
219 D) Superposition with Cas9-guideRNA-dsDNA structure (PDB ID 5F9R). For clarity,  
220 Cas9 is omitted except the HNH domain. Target and nontarget DNA strand is is

221 colored purple and beige, respectively.

222 E) Electrostatic surface potential of AcrIIA4. The inset shows the PAM recognition  
223 residues (R1333 and R1334) are largely buried in an acidic pocket within  
224 AcrIIA4.

225 F) and G) Close-up of AcrIIA4 binding pocket at different views showing AcrIIA4 is a  
226 dsDNA mimic.

227



228

229 **Fig. 3. AcrIIA4 inhibits DNA cleavage *in vitro***

230 A) A linearized plasmid was incubated with Cas9 alone, AcrIIA4 alone, Cas9 +  
 231 sgRNA, or Cas9 + sgRNA + AcrIIA4 for 5, 15, or 30 minutes. Reactions were  
 232 resolved via agarose gel electrophoresis and visualized by ethidium bromide  
 233 staining.

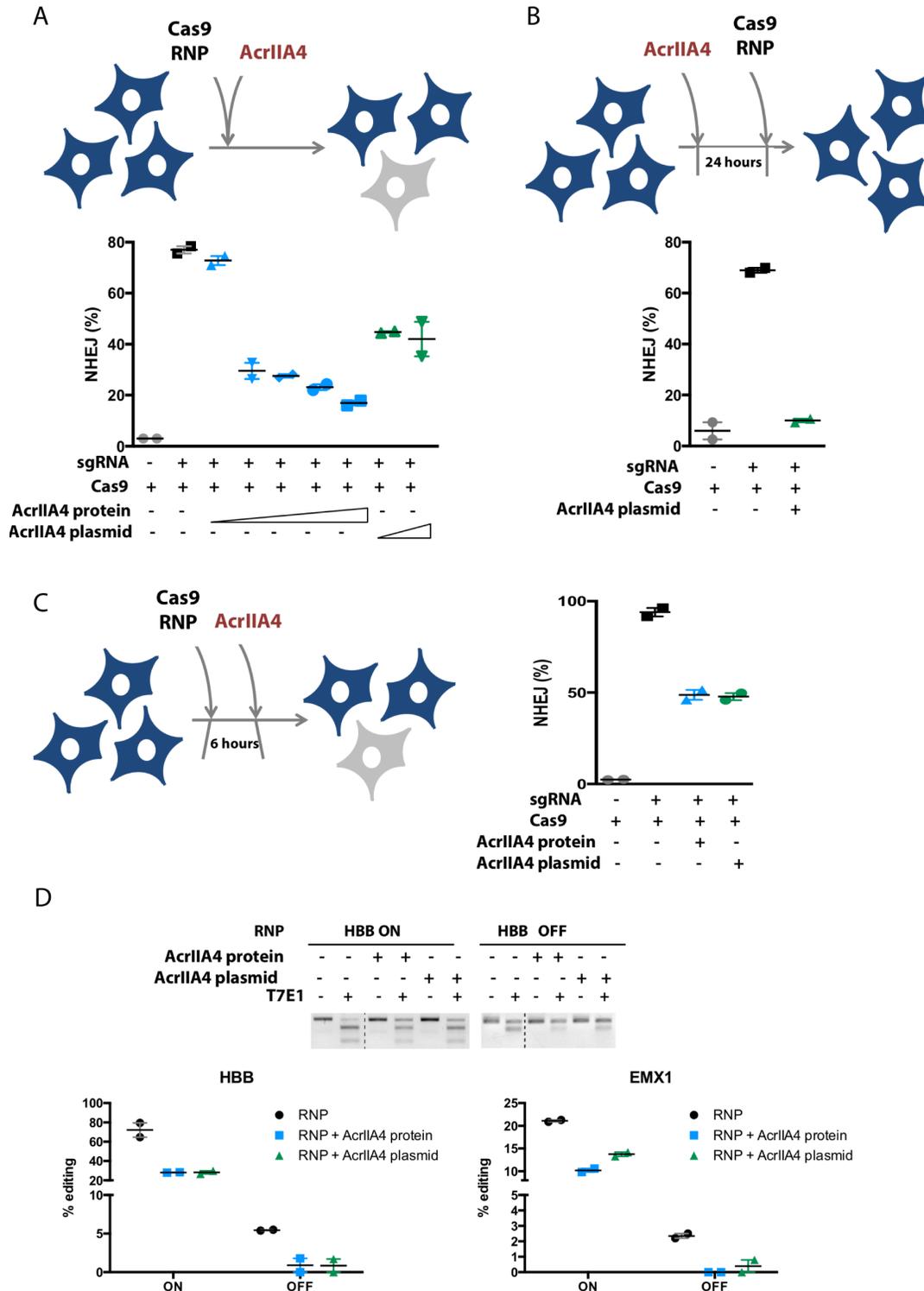
234 B) AcrIIA4 inhibits SpyCas9 cleavage of radiolabeled target DNA *in vitro*. 10 nM  
 235 SpyCas9\_crRNA\_tracrRNA complex were pre-incubated with increasing  
 236 concentrations (0-100 nM) of AcrIIA4. Sub-stoichiometric synthetic oligonucleotide  
 237 duplexes (2.5 nM) bearing a radiolabel at the 5' end of the complementary strand,  
 238 were introduced for 6-minute cleavage reactions. Reactions were resolved by  
 239 denaturing polyacrylamide gel electrophoresis and visualized by

240 phosphorimaging.

241 C) AcrIIA4 inhibits dCas9-sgRNA binding to a DNA target but does not affect target  
242 release, as measured by biolayer interferometry. Pre-incubating increasing  
243 concentrations of AcrIIA4 with dCas9-sgRNA reduces the on-rate of association  
244 with a DNA target relative to no inhibitor (blue). Maximal inhibition is identical to  
245 dCas9-sgRNA added to target DNA with a PAM mutation (red).

246 D) Addition of increasing concentrations of AcrIIA4 with pre-formed dCas9-sgRNA-  
247 DNA complex has no effect upon the off-rate of dissociation.

248



249

250 **Fig. 4. Timed delivery of AcrIIA4 differentially inhibits on- and off-target genome**

251 **editing in human cells.**

- 252 A) Simultaneous delivery of Cas9 RNP and AcrIIA4 inhibits Cas9-mediated gene  
253 targeting in human cells. K562 cells with a chromosomally integrated BFP (BFP-  
254 K562) were nucleofected with Cas9 RNP and AcrIIA4 protein (Cas9:AcrIIA4  
255 (molar ratio), 1:0.5, 1:1, 1:2, 1:3, 1:5) or plasmid (0.7 $\mu$ g and 2.8 $\mu$ g). NHEJ  
256 frequencies are quantified by loss of BFP expression in BFP-K562 cells 96 hours  
257 post nucleofection via flow cytometry. Data presented as mean  $\pm$  SEM from at  
258 least two biological replicates.
- 259 B) Administration of AcrIIA4 prior to Cas9 RNP completely inhibits Cas9-mediated  
260 gene targeting. BFP-K562 cells were nucleofected with AcrIIA4 plasmid (0.7 $\mu$ g)  
261 24 hours prior to Cas9 RNP delivery. Data presented as mean  $\pm$  SEM from at  
262 least two biological replicates.
- 263 C) Delivery of AcrIIA4 after introduction of Cas9 RNP yields intermediate inhibition  
264 of Cas9 activity. BFP-K562 cells were nucleofected with AcrIIA4 protein  
265 (Cas9:AcrIIA4 (molar ratio), 1:5) or plasmid (0.7 $\mu$ g) 6 hours post Cas9 RNP  
266 delivery. Data presented as mean  $\pm$  SEM from at least two biological replicates.
- 267 D) Proper timing of AcrIIA4 delivery diminishes off-target editing events while largely  
268 retaining on-target editing. K562 cells were nucleofected with either HBB or  
269 EMX1 targeting Cas9 RNP 6 hours prior to AcrIIA4 protein (Cas9:AcrIIA4 (molar  
270 ratio), 1:5) or plasmid (0.7 $\mu$ g) delivery. (Top) Representative T7 endonuclease I  
271 assay for visualization of HBB on- and off- target editing. (Bottom) Quantification  
272 of on- and off-target editing at HBB and EMX1, as measured by TIDE analysis.

273 **References**

- 274 1. J. Bondy-Denomy, A. Pawluk, K. L. Maxwell, A. R. Davidson, Bacteriophage genes that  
275 inactivate the CRISPR/Cas bacterial immune system. *Nature* **493**, 429-432 (2013).
- 276 2. A. Pawluk *et al.*, Naturally Occurring Off-Switches for CRISPR-Cas9. *Cell* **167**, 1829-  
277 1838 e1829 (2016).
- 278 3. B. J. Rauch *et al.*, Inhibition of CRISPR-Cas9 with Bacteriophage Proteins. *Cell* **168**,  
279 150-158 e110 (2017).
- 280 4. M. Jinek *et al.*, A programmable dual-RNA-guided DNA endonuclease in adaptive  
281 bacterial immunity. *Science* **337**, 816-821 (2012).
- 282 5. M. Jinek *et al.*, Structures of Cas9 endonucleases reveal RNA-mediated  
283 conformational activation. *Science* **343**, 1247997 (2014).
- 284 6. H. Nishimasu *et al.*, Crystal structure of Cas9 in complex with guide RNA and target  
285 DNA. *Cell* **156**, 935-949 (2014).
- 286 7. S. H. Sternberg, S. Redding, M. Jinek, E. C. Greene, J. A. Doudna, DNA interrogation by  
287 the CRISPR RNA-guided endonuclease Cas9. *Nature* **507**, 62-67 (2014).
- 288 8. C. D. Richardson, G. J. Ray, M. A. DeWitt, G. L. Curie, J. E. Corn, Enhancing homology-  
289 directed genome editing by catalytically active and inactive CRISPR-Cas9 using  
290 asymmetric donor DNA. *Nat Biotechnol* **34**, 339-344 (2016).
- 291 9. Y. Fu *et al.*, High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases  
292 in human cells. *Nat Biotechnol* **31**, 822-826 (2013).
- 293 10. P. D. Hsu *et al.*, DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat*  
294 *Biotechnol* **31**, 827-832 (2013).

- 295 11. P. Mali *et al.*, CAS9 transcriptional activators for target specificity screening and  
296 paired nickases for cooperative genome engineering. *Nat Biotechnol* **31**, 833-838  
297 (2013).
- 298 12. V. Pattanayak *et al.*, High-throughput profiling of off-target DNA cleavage reveals  
299 RNA-programmed Cas9 nuclease specificity. *Nat Biotechnol* **31**, 839-843 (2013).
- 300 13. S. Kiani *et al.*, Cas9 gRNA engineering for genome editing, activation and repression.  
301 *Nat Methods* **12**, 1051-1054 (2015).
- 302 14. S. H. Sternberg, B. LaFrance, M. Kaplan, J. A. Doudna, Conformational control of DNA  
303 target cleavage by CRISPR-Cas9. *Nature* **527**, 110-113 (2015).
- 304 15. X. Wu *et al.*, Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian  
305 cells. *Nat Biotechnol* **32**, 670-676 (2014).
- 306 16. T. J. Cradick, E. J. Fine, C. J. Antico, G. Bao, CRISPR/Cas9 systems targeting beta-  
307 globin and CCR5 genes have substantial off-target activity. *Nucleic Acids Res* **41**,  
308 9584-9592 (2013).
- 309 17. M. A. DeWitt *et al.*, Selection-free genome editing of the sickle mutation in human  
310 adult hematopoietic stem/progenitor cells. *Sci Transl Med* **8**, 360ra134 (2016).
- 311 18. S. Chowdhury *et al.*, Structure Reveals Mechanisms of Viral Suppressors that  
312 Intercept a CRISPR RNA-Guided Surveillance Complex. *Cell* **169**, 47-57 e11 (2017).
- 313 19. M. D. Walkinshaw *et al.*, Structure of Ocr from bacteriophage T7, a protein that  
314 mimics B-form DNA. *Mol Cell* **9**, 187-194 (2002).

315

## 316 **Acknowledgments**

317 EM-derived maps and atomic coordinates of the Cas9-sgRNA-AcrIIA4 structure have  
318 been deposited in the EM Databank (EMDB) and the Protein Data Bank (PDB) with  
319 accession codes **EMD-XXXX and YYYY, respectively**. The EM data was collected in  
320 the EM facility of HHMI Janelia Research Campus. We thank Rick Huang and Zhiheng  
321 Yu for expert electron microscopy assistance; and members of the Bondy-Denomy,  
322 Corn, Doudna and Nogales labs for helpful discussions and critical reading of the  
323 manuscript. F.J. is a Merck Fellow of the Damon Runyon Cancer Research. J.E.C. is  
324 supported by the Li Ka Shing Foundation and the Heritage Medical Research Institute.  
325 J.S. is supported by the National Institute on Aging of the National Institutes of Health  
326 under Award Number T32 AG000266. J.B.-D. and B.R. are supported by the University  
327 of California San Francisco Program for Breakthrough in Biomedical Research, funded  
328 in part by the Sandler Foundation, and an NIH Office of the Director Early  
329 Independence Award (DP5-OD021344). J.A.D. and E.N. are Investigators of the  
330 Howard Hughes Medical Institute. This work was supported in part by HHMI. The  
331 content is solely the responsibility of the authors and does not necessarily represent the  
332 official views of the National Institutes of Health.  
333