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Supplemental Information

Anti-CRISPR AcrIIA5 Potently Inhibits All Cas9 Homologs Used for Genome Editing

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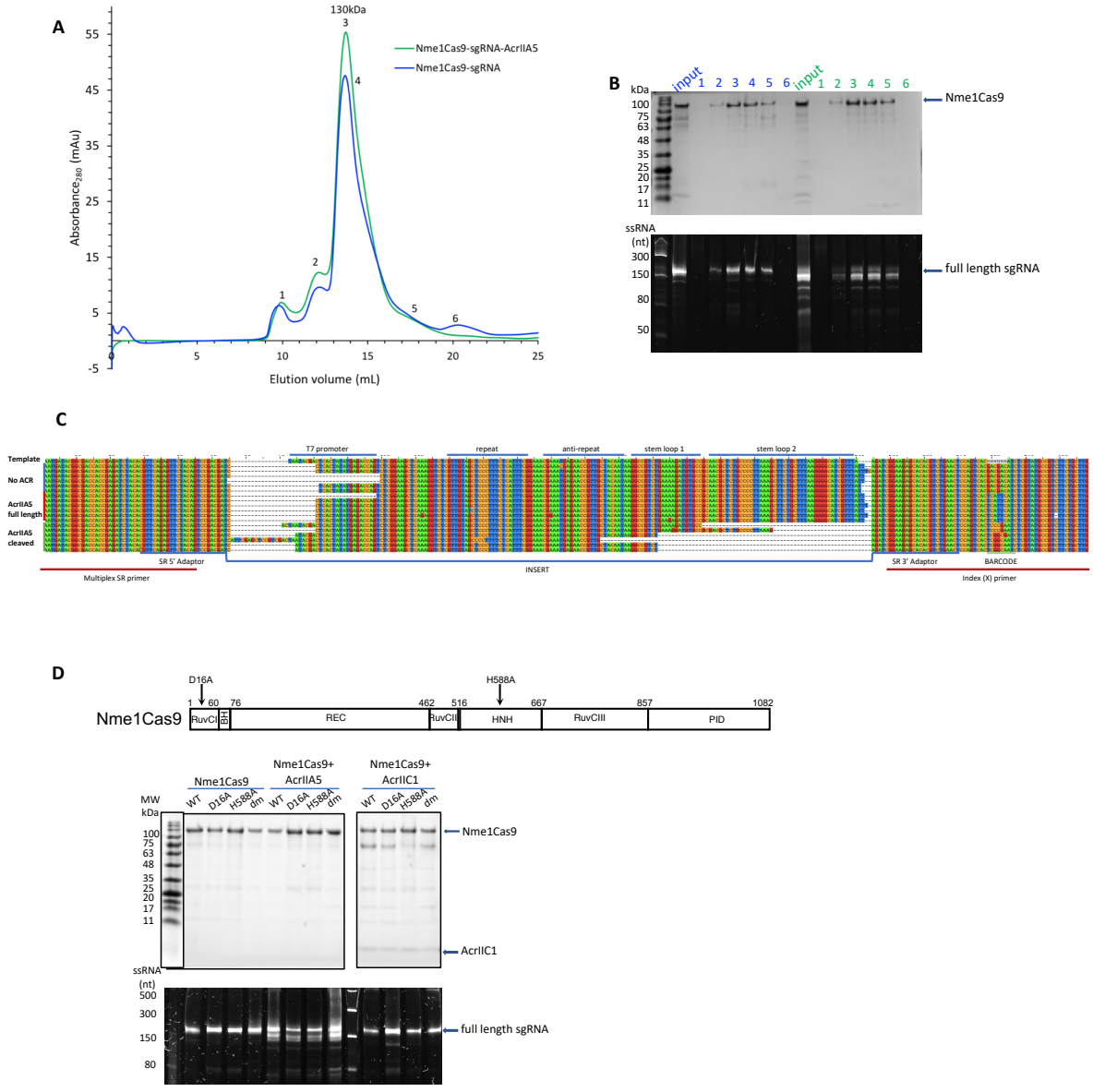

Figure S1. AcrIIA5 dependent sgRNA cleavage, Related to Figure 3.
(A) Size-exclusion chromatograms of His-tagged Nme1Cas9-sgRNA co-expressed with AcrIIA5 (green line) or no anti-CRISPR protein (blue line).
(B) Fractions indicated with numbers in (A) were analyzed on a 15% Tris-tricine polyacrylamide gel using SDS-PAGE gel followed by Coomassie staining (top panel) and a denaturing polyacrylamide/Urea gel followed by SYBR™ Gold staining (lower panel).
(C) Nucleotide sequences of reverse transcribed RNA molecules bound to Nme1Cas9 in the presence or absence of AcrIIA5, as indicated. The alignment was generated using MUSCLE (Edgar, 2004). Nucleotides are colored red (G), green (A), blue (T) or yellow (C), depending on their chemical composition, and gaps are indicated by dashes. Adaptors and primers from the NEBnext Small RNA sequencing kit are annotated below the aligned sequences. The sgRNA elements are also annotated.

(D) To ascertain whether AcrIIA5 induces Nme1Cas9 to cleave its bound sgRNA, we expressed mutant Nme1Cas9 proteins with amino acid substitutions in the domains that mediate nuclease activity; the RuvC domain (D16A), the HNH domain (H588A), and a double mutant (dm) with substitutions in both domains (D16A/H588A) (Zhang et al., 2015). Wild-type (WT) or mutant versions His_6-Nme1Cas9 were co-expressed with pCDF-1b (no Acr), AcrIIC1 or AcrIIA5, and then purified by Ni-NTA chromatography. Ribonucleoprotein complexes were analyzed by a 15% Tris-tricine polyacrylamide gel using SDS-PAGE and visualized by stain-free imaging. Complexes were also resolved on a 12.5% polyacrylamide/Urea gel and the sgRNA visualized by SYBR™ Gold staining. When the Nme1Cas9 mutants were co-expressed with AcrIIA5, the sgRNA was cleaved in the same manner as with wild-type Nme1Cas9. These results showed that Nme1Cas9 does not catalyze the AcrIIA5-induced cleavage of sgRNA by utilizing its established nuclease domains.

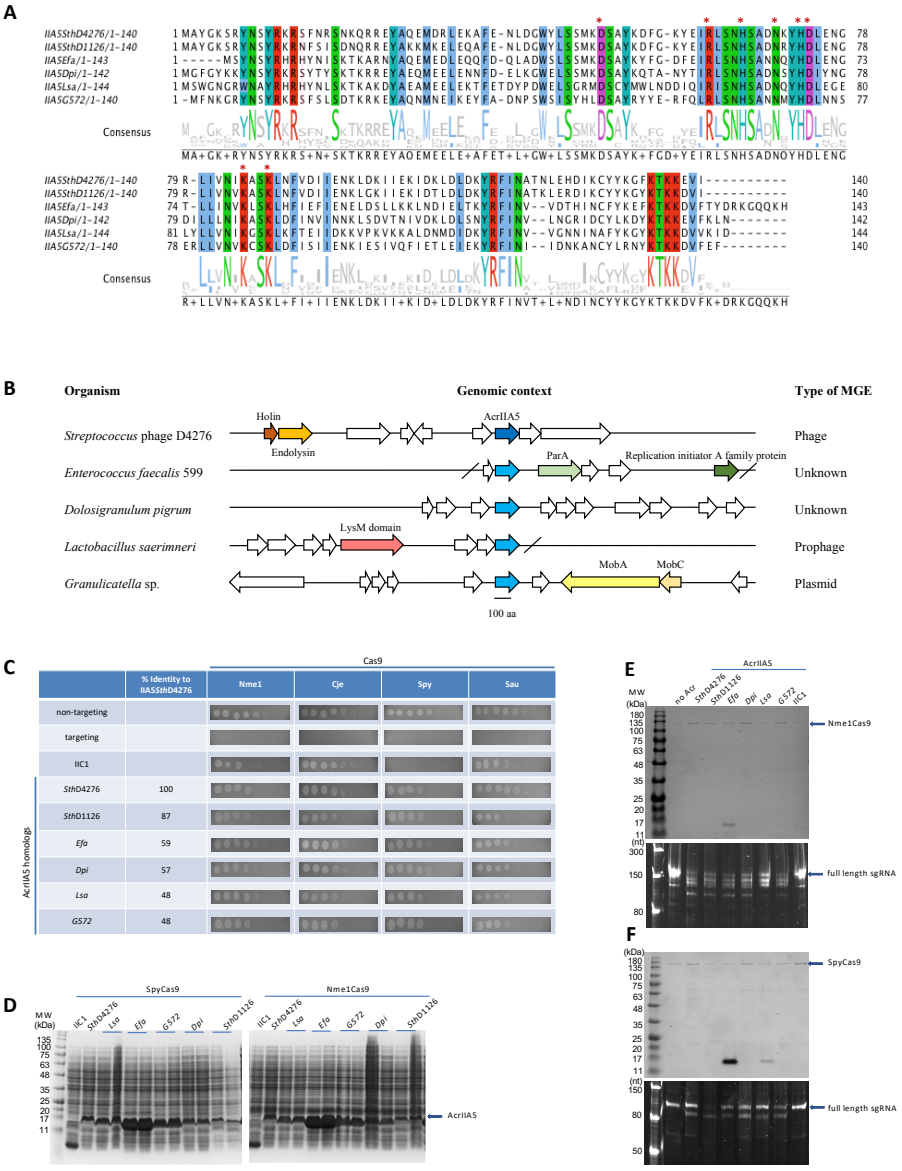

Figure S2. **AcrIIA5 homologs show broad Cas9 inhibition and sgRNA cleavage, Related to Figures 1 and 3 and STAR Methods.**

(A) A multiple sequence alignment of AcrIIA5 homologs (*Streptococcus phage* D1126 (SthD1126: AVO22762.1); *Enterococcus faecalis* (Efa: WP_002415475.1); *Dolosigranulum pigrum* (Dpi: WP_112790338.1); *Lactobacillus saerimneri* (Lsa: WP_027827173.1); *Granulicatella sp*. 572 (G572: WP_049555364.1) generated by MUSCLE. Conserved amino acid residues are highlighted and the consensus conservation logos are shown. Red asterisks indicate amino acid residues that were mutated as described in Supplementary Figure S3.

(B) Scaled diagram representing the genomic contexts of AcrIIA5 (dark blue arrow) and its homologs (light blue arrows). Open reading frames (ORFs) are depicted as arrows, and lines represent intergenic regions. Colored arrows, besides blue, reflect ORFs with NCBI annotations,

as indicated. Slanted lines indicate the ends of contigs. Mobile genetic elements (MGE) are identified where possible.
(C) Fold reduction in phage titer in response to the indicated Cas9 targeting phage Mu in the presence of AcrIIA5 homologs. Representative pictures of at least three biological replicates are shown.
(D) Representative Coomassie-stained denaturing polyacrylamide gels showing the expression levels of the AcrIIA5 homologs used for the experiments in (C).
(E) His_6-Nme1Cas9 or (F) His_6-SpyCas9 was co-expressed and co-purified with pCDF-1b (no Acr), or AcrIIA5 homologs. Ribonucleoprotein complexes were analyzed by a 15% Tris-tricine polyacrylamide gel using SDS-PAGE followed by Coomassie staining (top) and a polyacrylamide/Urea gel stained with SYBR™ Gold (bottom). It should also be noted that AcrIIA5Efa binds non-specifically to the Ni-NTA resin. Thus, the observed visible band of this protein is not a result of its binding to Cas9.

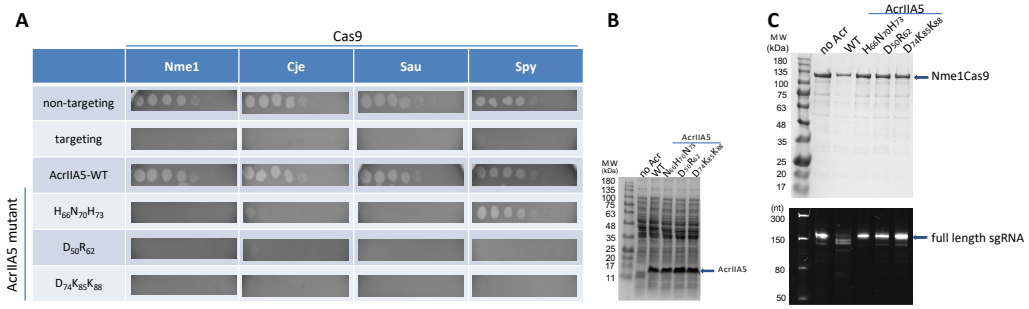

Figure S3. AcrIIA5 mutants fail to inhibit Cas9 proteins, Related to Figure 3 and STAR Methods.

(A) With the idea that AcrIIA5 might be a nuclease, we identified completely conserved residues (Supplementary Figure S2A) that could be involved in binding to metal or nucleic acids (e.g. Asp, His, Asn, Arg, or Lys). We constructed three mutants bearing amino acid substitutions at some of these positions. Ten-fold serial dilutions of phage Mu lysate were spotted on lawns of bacteria expressing the indicated AcrIIA5 mutant. The results showed that these mutations completely ($D_{50}R_{62}$ and $D_{74}K_{85}K_{88}$) or nearly completely ($H_{66}N_{70}H_{73}$) abolished the activity of AcrIIA5. Representative pictures of at least three biological replicates are shown.

(B) A representative Coomassie-stained denaturing polyacrylamide gel showing the expression levels of wild-type and mutant AcrIIA5 used for the experiments in (A). All mutants were well-expressed in the presence of the four Cas9 homologs.

(C) His_6-Nme1Cas9 was co-expressed and co-purified with pCDF-1b (no Acr), or AcrIIA5 mutants. Ribonucleoprotein complexes were analyzed by a 15% Tris-tricine polyacrylamide gel using SDS-PAGE followed by Coomassie staining (top) and a polyacrylamide/Urea gel stained with SYBR™ Gold (bottom).

Table S1. sgRNA sequences used in this study, Related to STAR Methods

sgRNA	Sequence	Size (nt)
Nme1/Boe	GUUGUAGCUCCCUUUCUCAUUUCGGAAACGAAAUGAGAACCGUUG CUACAAUAAGGCCGUCUGAAAAGAUGUGCCGCAACGCUCUGCCCC UUAAAGCUUCUGCUUUAAGGGGCAUCGUUUA	121
Hpa	GUUGUAGCUCCCUUUUUCAUUUCGCAGAAAUGCGAAAUGAAAAAC GUUGUUACAAUAAGAGAAAAGAUUUCUCGCAAAGCUCUGUCCCUU GAAAUGUAAGUUUCAAGGGACAUCUUUU	118
Cje	GUUUUAGUCCCUGAAAAGGGACUAAAAUAAAGAGUUUGCGGGACU CUGCGGGGUUACAAUCCCCUAAAACCGCUUUUUU	79
Geo	GUCAUAGUUCCCCUGAAAAGUCAGGGUUACUAUGAUAAGGGCUUU CUGCCUAAGGCAGACUGACCCGCGGCGUUGGGGAUCGCCUGUCG CCCGCUUUUGGCGGGCAUUCCCCA	113
Kla	GUUACGGCUUCCCUGCUAAUCGAUGAAAAUCGAUUAGCAGGGAUC AAGAGUCGUAAUAAGUAUUUAUUACGCAAAAUGGGGUGCUUACGG GCACCCCUUCUUCGUUUGUAAAUGGAUGUGUUAUUAGUCGCGCC UAGGGCGACAACCAGUUCCU	154
Cdi	ACUGGGGUUCAGUUCUCAAAAACCCUGAUAGACUUGAAAAGUCAC UAACUUAAUUAAAUAGAACUGAACCUCAGUAAGCAUUGGCUCGUU UCCAAUGUUGAUUGCUCCGCCGGUGCUCCUUAUUUUUAAGGGCG CCGGCUUU	142
Sau	GUUUUAGUACUCUGGAAACAGAAUCUACUAAAACAAGGCAAAAUG CCGUGUUUAUCUCGUCAACUUGUUGGCGAGAUUUUU	81
Spy	GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUA UCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU	83
Fno	GUUUCAGUUGCUGAAUUAUUUGGUAAACAGUACCAAAUAAUUAAU GCUCUGUAAUCAUUUAAAAGUAUUUUGAACGGACCUCUGUUUGAC ACGUCUGAAUAACUAAAAAUUUUUUU	116