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Discovery of widespread Type I and Type V CRISPR-Cas inhibitors

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Abstract

Bacterial CRISPR-Cas systems protect their host from bacteriophages and other mobile genetic elements. Mobile elements, in turn, encode various anti-CRISPR (Acr) proteins to inhibit the immune function of CRISPR-Cas. To date, Acr proteins have been discovered for type I (subtypes I-D, I-E, and I-F) and type II (II-A and II-C) but not other CRISPR systems. Here we report the discovery of 12 *acr* genes, including inhibitors of type V-A and I-C CRISPR systems. Notably, AcrVA1 inhibits a broad spectrum of Cas12a (Cpf1) orthologues including MbCas12a, Mb3Cas12a, AsCas12a, and LbCas12a when assayed in human cells. The *acr* genes reported here

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Author contributions:

N.D.M. conducted *Moraxella* AcrVA and AcrI discovery, characterization, and bioinformatics, J.Y.Z. and A.L.B. conducted *Pseudomonas* AcrI and *aca* discovery, characterization, and bioinformatics. L.M.L. built the *Pseudomonas* type I-C strain and J.D.B. and N.D.M. built the *Pseudomonas* type V-A strain. B.J.R. conducted self-targeting analysis. J.B.D conceptualized the project and supervised all bioinformatics and bacterial experiments. B.P.K., A.A.S., and R.T.W. constructed AcrVA expression plasmids and performed human cell experiments. Funding for this work was procured by J.B.D., B.P.K., and J.K.J. The manuscript was written by N.D.M., A.L.B., and J.B.D. with editing and contributions from all authors.

Competing interests: A patent has been filed pertaining to AcrVA genes and their applications. J.K.J. has financial interests in Beam Therapeutics, Blink Therapeutics, Editas Medicine, Endcadia, Inc., Monitor Biotechnologies (formerly known as Beacon Genomics), Pairwise Plants, Poseida Therapeutics, and Transposagen Biopharmaceuticals. J.K.J.'s interests were reviewed and are managed by Massachusetts General Hospital and Partners HealthCare in accordance with their conflict of interest policies.

Data and materials availability: All data are available in the main text or the supplementary materials. Plasmids described in this work are available through Addgene. Phages and bacterial strains will be made available upon request to Joseph.Bondy-Denomy@ucsf.edu.

provide useful biotechnological tools and mark the discovery of *acr* loci in many bacteria and phages.

One Sentence Summary:

A widespread anti-CRISPR gene enables the discovery of novel type V-A CRISPR-Cas12a inhibitors that block gene editing in human cells.

The discovery of bacterial CRISPR-Cas systems that prevent infection by bacterial viruses (phages) has opened a paradigm for bacterial immunity while yielding exciting tools for targeted genome editing. CRISPR systems destroy phage genomes, and in turn, phages express anti-CRISPR (Acr) proteins that directly inhibit Cas effectors (1, 2). Six distinct types (I-VI) of CRISPR systems are spread widely across the bacterial world (3), but Acr proteins have only been discovered for type I and II CRISPR systems (1, 3–6). Given the prevalence and diversity of CRISPR systems, we predict that Acr proteins against other types await discovery.

Anti-CRISPR proteins do not have conserved sequences or structures and only share their relatively small size, making *de novo* prediction of *acr* function challenging (6). However, *acr* genes often cluster together with other *acr* genes or are adjacent to highly conserved anti-CRISPR associated genes (*aca* genes) in "*acr* loci" (7, 8). In this work, we sought to identify *acr* genes in bacteria and phages that are not homologous to previously identified *acr* or *aca* genes.

Acr proteins were first discovered in *Pseudomonas aeruginosa*, inhibiting type I-F and I-E CRISPR systems (1, 9). *P. aeruginosa* strains also encode a third CRISPR subtype (type I-C), which lacks known inhibitors (10). We engineered *P. aeruginosa* to target phage JBD30 with type I-C CRISPR-Cas (fig. S1A) and used it in parallel with existing type I-E (strain SMC4386) and I-F (strain PA14) CRISPR strains to screen for additional *acr* candidates.

Homologs of *aca1* were searched for in *Pseudomonas* genomes, and 7 gene families not previously tested for anti-CRISPR function were identified upstream of *aca1* (Fig. 1A). Three genes inhibited the type I-E CRISPR-Cas system (*acrIE5–7*), one inhibited type I-F (*acrIF11*), restoring the plaquing of a targeted phage, and two genes had no inhibitory activity (*orf1, orf2)* (Fig. 1B, fig. S1B, table S1, S2). Another gene exhibited dual I-E and I-F inhibition, and domain analysis revealed a chimera of previously identified *acrIE4* and *acrIF7* (*acrIE4-F7*). No type I-C inhibitors were identified. The type I-F inhibitor *acrIF11* was commonly represented in both the *P. aeruginosa* mobilome and in over 50 species of diverse Proteobacteria (fig. S2, Table S2). *acrIF11* is often associated with genes encoding DNA-binding motifs, which we have designated *aca4–7* (fig. S2, table S1, S3, S4). To confirm that these *aca* genes can be used to facilitate *acr* discovery, we used *aca4* to discover an additional *Pseudomonas* anti-CRISPR, *acrIF12* (Fig. 1A, 1B).

Given the widespread nature of *acrIF11*, we next used it to discover Acr proteins against CRISPR systems where they have not yet been found: type I-C, a minimal Class 1 system and type V-A CRISPR-Cas12a (Cpf1), a Class 2 single effector system that has high efficiency in genome editing (11–13). To find AcrIC and AcrVA proteins, we first searched

for genomes encoding CRISPR spacers that match a target protospacer elsewhere in the same genome (Fig. 2A). The tolerance of this "self-targeting" in viable bacteria indicates potential inhibition of the CRISPR system (4), since genome cleavage would result in bacterial death.

The Gram negative bovine pathogen *Moraxella bovoculi* (14, 15) is a Cas12a–containing organism (11) where four of the seven genomes feature Type V-A self-targeting (table S5), and one strain (58069) also features self-targeting by type I-C (table S6). Although no previously described *acr* or *aca* genes were present in this strain, an *acrIF11* homolog was found in phages infecting the human pathogen *M. catarrhalis* (16), a close relative of *M. bovoculi*. Genes adjacent to *acrIF11* in *M. catarrhalis* had homologs in the self-targeting *M. bovoculi* strains (Fig. 2B), and together these genes were selected as candidate *acr* genes. Each gene was first tested against the type I-C and I-F systems introduced above, as both subtypes are found in *Moraxella*. Gene AAX09_07415 (now *acrIC1*) inhibited the type I-C system, explaining the tolerance of self-targeting in strain 58069 (Fig. 2C). Additionally, gene E9U_08473 (*acrIF13*) from the *Moraxella catarrhalis* BC8 prophage completely inhibited I-F function, as did AKI27193.1 (*acrIF14*), found in phage Mcat5 at the same genomic position as *acrIF11* in BC8 (Fig. 2B, 2D). Notably, these Acr proteins possess broad spectrum activity as the type I-C and I-F systems in *Moraxella* and *Pseudomonas* only share an average pairwise identity of 30% and 36%, respectively (fig. S3)

Due to the limited tools available for the genetic manipulation of *Moraxella* sp., the remaining genes were tested for type V-A anti-CRISPR function in *P. aeruginosa* PAO1 engineered to express MbCas12a and a crRNA targeting *P. aeruginosa* phage JBD30. Two distinct crRNAs were used, showing strong reduction of titer by >4 orders of magnitude (Fig. 2E). The first gene in the *M. bovoculi* 58069 *acr* locus, AAX09_07405 (*acrVA1*), restored phage titers nearly to levels seen with the crRNA-minus control, indicating it robustly inhibits Cas12a. This is in good agreement with the independent discovery of AcrVA1 reported in a companion paper (Watters et al.). The adjacent gene, *acrVA2*, also inhibited targeting, as did its orthologue (*acrVA2.1*) (Fig. 2E). An additional gene from this locus, *acrVA3*, possessed subtle anti-Cas12a activity but was toxic to cells and adversely affected JBD30 phage growth independently of Cas12a (fig. S5). We therefore tested an orthologue with 43% sequence identity, B0181_04965 (*acrVA3.1*), which showed stronger Cas12a inhibition with no toxicity or adverse effects on phage growth (Fig. 2E, fig. S5). Surprisingly, *acrVA3.1* also showed partial restoration of phage titer during type I-C targeting, suggesting that it may inhibit the type I-C as well as type V-A system (Fig. 2C, 2E). Although these two CRISPR subtypes do not share any protein components, a dual-specificity inhibitor may use distinct protein interaction interfaces or modulate an undiscovered host process required for CRISPR immunity. In sum, we used the anti-CRISPR "key" *acrIF11* to unlock *acr* loci encoding seven distinct *acr* genes inhibiting type I-C, I-F, and V-A CRISPR. Below, we focus on the evolutionary analysis of type V-A inhibitors, and their function in mammalian cells.

acrVA1 encodes a 170-amino acid protein found only in *Moraxella* sp. and *Eubacterium eligens* (fig. S6), both type V-A CRISPR-containing organisms. By contrast, *acrVA2* (322 aa) and *acrVA3* (168 aa) orthologues are found broadly distributed throughout multiple

classes of bacteria. For example, *acrVA2* orthologues are present in *Lachnospiraceae* and *Leptospira* (fig. S7), which contain type V-A CRISPR, as well as in *Moraxella, Leptospira*, and *Lactobacillus* phages. Distant orthologues of *acrVA2* were also identified on plasmids and conjugative elements in *E. coli* (fig. S7), although the significance of a bacterium lacking type V-A CRISPR encoding a putative *acrVA* gene is unknown. Orthologues of *acrVA3* were identified in many Proteobacteria, and *Eubacterium* and *Clostridium* species, which encode type V-A CRISPR (fig. S8).

Given the inhibitory effect of *acrVA1–3.1* on MbCas12a in bacteria, we sought to determine whether AcrVA proteins could block MbCas12a activity in human cells. Human U2-OS-EGFP cells (17) transiently expressing MbCas12a, *EGFP*-targeting crRNA, and human codon-optimized *acrVA1–3.1* were assessed for EGFP disruption using flow cytometry. Co-expression of MbCas12a and crRNA resulted in ~60–70% disruption of *EGFP* expression relative to background (Fig. 3A). AcrVA1 expression reduced *EGFP* disruption to background levels, indicating inhibition of MbCas12a, while the other *acrVA* genes showed little evidence of activity here (Fig. 3A). We additionally found that *acrVA1* inhibited another Cas12a orthologue, (Mb3Cas12a), while having no impact on SpyCas9 editing in the same assay (Fig. 3B). Titration of the Acr plasmid relative to the Cas12a expression plasmid revealed comparable dose-dependent responses to inhibition between MbCas12a or Mb3Cas12a with AcrVA1 and SpyCas9 with AcrIIA4 (fig. S9). Furthermore, AcrVA1 was found to be a broad-spectrum inhibitor of other commonly used Cas12a orthologues (11), providing strong inhibition of AsCas12a and LbCas12a, and modest inhibition of FnCas12a (Fig. 3C).

Finally, to determine whether AcrVA1 could inhibit Cas12a-mediated modification of endogenous loci in human cells, U2-OS cells were co-transfected with plasmids expressing *acrVA1*, Cas12a and crRNAs targeting endogenous genes (*RUNX1, DNMT1, or FANCF*) and assessed for gene disruption by T7 endonuclease I (T7E1) assay. We found that AcrVA1 completely inhibited gene disruption by MbCas12a and Mb3Cas12a, with modest to strong inhibition of As-, Lb-, and FnCas12a orthologues (Fig. 3D, fig. S10).

Here we report the discovery of a broadly distributed type I-F Acr protein (AcrIF11) that served as a marker for *acr* loci and led to the identification of type I-C and V-A CRISPR inhibitors. One of these *acrVA* genes (*acrVA1*) potently inhibits Cas12a in bacteria and in human cells, providing a new tool for Cas12a regulation. Our findings show that mobile genetic elements can tolerate bacteria with more than one CRISPR-Cas type by possessing multiple Acr proteins in the same locus. The strategy described herein enabled the identification of many widespread anti-CRISPR proteins, which may prove useful in future anti-CRISPR discovery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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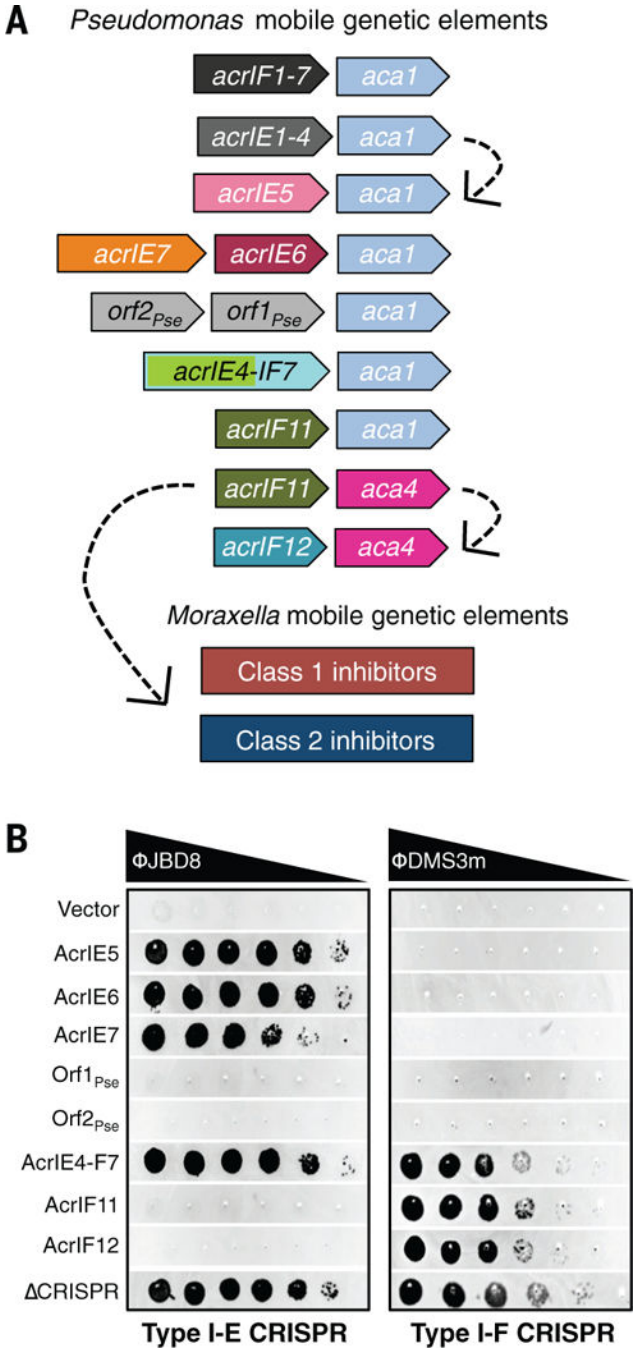

Figure 1: The discovery of a widespread type I inhibitor

(A) Schematic of type I-E and type I-F anti-CRISPRs with anti-CRISPR associated (*aca1, aca4*) genes in *Pseudomonas sp.* mobile genetic elements, with dotted lines indicating the "guilt-by-association" relationships used to discover new *acr* genes in *Pseudomonas sp.* and *Moraxella sp.* from known *acr* genes (top two rows). (B) Phage plaque assays to assess CRISPR-Cas inhibition. Ten-fold serial dilutions of a type I-E or type I-F CRISPR-targeted phage (JBD8 or DMS3m, respectively) titered on lawns of *Pseudomonas aeruginosa* with

naturally active type I-E or type I-F CRISPR-Cas systems expressing candidate inhibitors. Δ*CRISPR* strains measure phage replication in the absence of CRISPR immunity (top row).

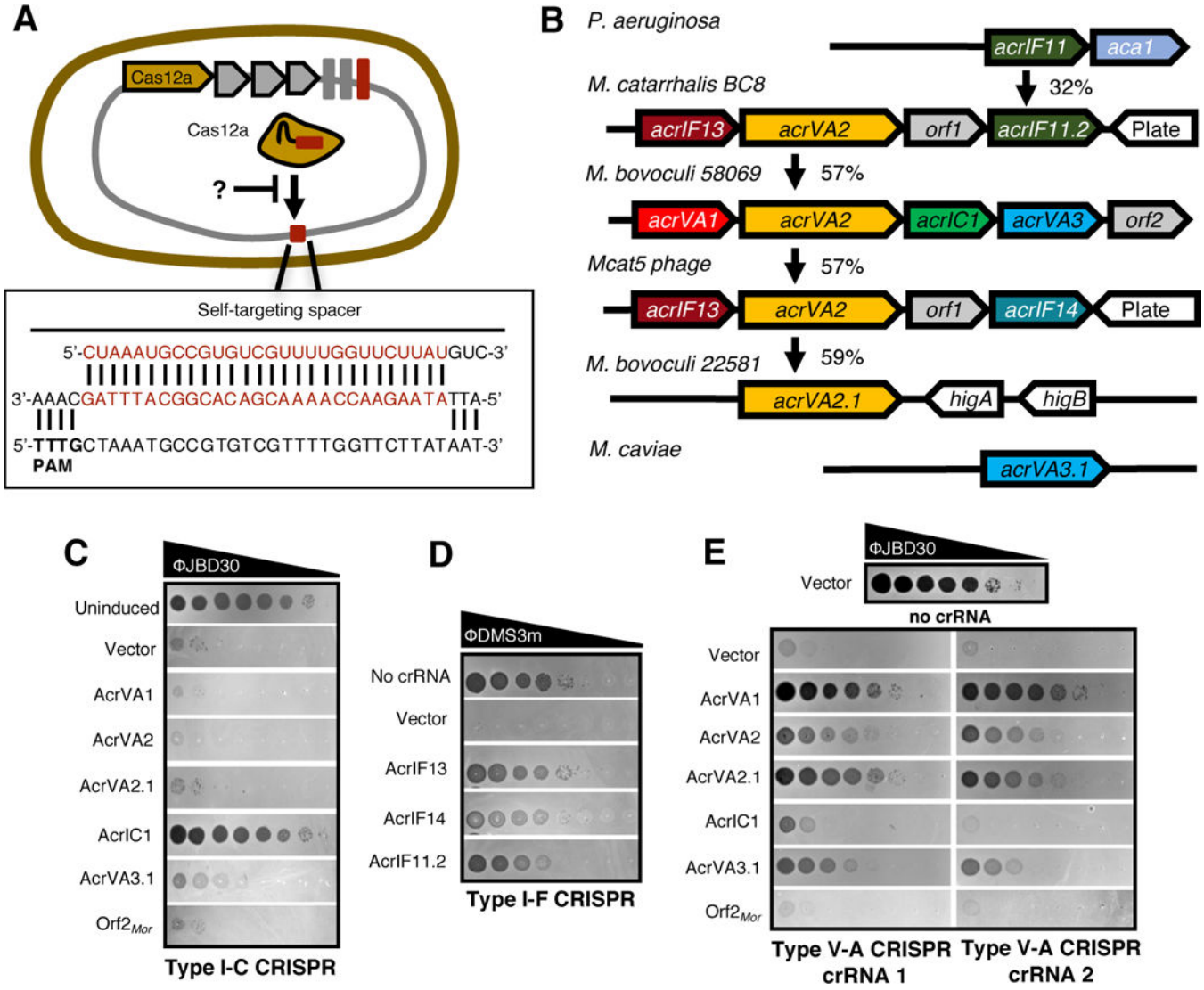

Figure 2: Type I-C and type V-A anti-CRISPR proteins identified in *Moraxella*

(A) Schematic of *Moraxella bovoculi* intragenomic self-targeting, wherein spacers encoded by CRISPR-Cas12a system and their target protospacers exist within the same genome. (B) Schematic showing type V-A (*acrVA1-VA3*), type I-C (*acrIC1*), and type I-F (*acrIF11-IF14*) inhibitors in *Moraxella*. *orf1* and *orf2* are genes of unknown function. Vertical arrows indicate the % protein sequence identity. Phage plaque assays with ten-fold serial dilutions of the indicated phage to assess inhibition of CRISPR-Cas type I-C (C), type I-F (D), and type V-A (E). Bacterial clearance (black) indicates phage replication. “Uninduced” panel (C) and “no crRNA” (D, E) indicate full phage titer.

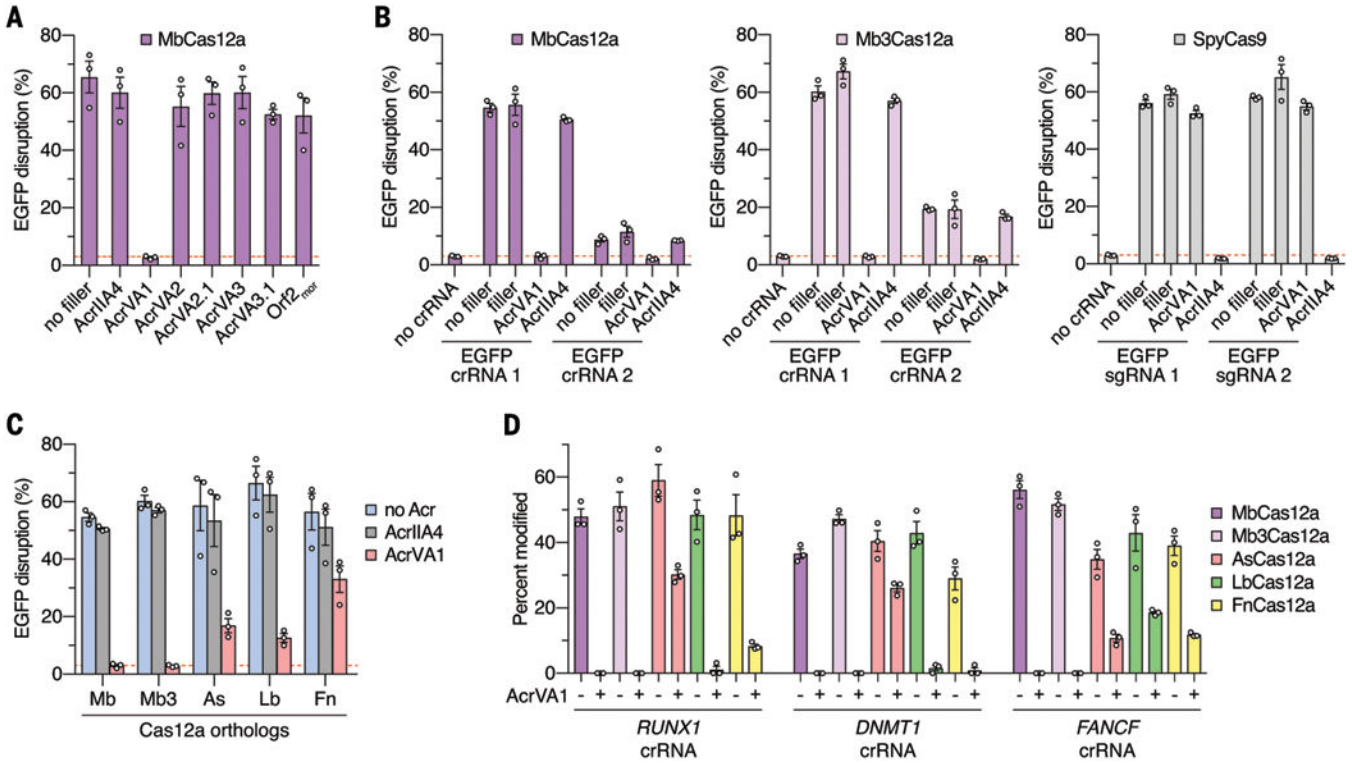

Figure 3: AcrVA1 blocks Cas12a-mediated gene editing in human cells.

(A-C) Human cell U2-OS-EGFP disruption experiments to assess AcrVA-mediated inhibition of Cas12a activities. (A) Inhibition of MbCas12a activity with various AcrVA constructs; the “no filler” condition contained only plasmids for Cas12a and crRNA expression. (B) Comparisons between the inhibitory activities of AcrVA1 and AcrIIA4 against MbCas12a, Mb3Cas12a, and SpyCas9. Controls using “filler” plasmid in lieu of Acr plasmids were included to equalize amounts of DNA. (C) Assessment of AcrVA1 activity against Cas12a orthologues, with AcrIIA4 used as control. Background EGFP disruption is indicated by the red dashed line; error bars indicate s.e.m. for n = 3. (D) Inhibition of activity of Cas12a orthologues against endogenous sites in human cells (*RUNX1, DNMT1, or FANCF* genes). Gene modification assessed by T7E1 assay 72 hours post-transfection; error bars indicate s.e.m. for n = 3.