Broad-spectrum enzymatic inhibition of CRISPR-Cas12a

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Cas12a is a bacterial RNA-guided nuclease used widely for genome editing and, more recently, as a molecular diagnostic. In bacteria, Cas12a enzymes can be inhibited by bacteriophage-derived proteins, anti-CRISPRs (Acrs), to thwart clustered regularly interspaced short palindromic repeat (CRISPR) adaptive immune systems. How these inhibitors disable Cas12a by preventing programmed DNA cleavage is unknown. We show that three such inhibitors (AcrVA1, AcrVA4 and AcrVA5) block Cas12a activity via functionally distinct mechanisms, including a previously unobserved enzymatic strategy. AcrVA4 and AcrVA5 inhibit recognition of double-stranded DNA (dsDNA), with AcrVA4 driving dimerization of Cas12a. In contrast, AcrVA1 is a multiple-turnover inhibitor that triggers cleavage of the target-recognition sequence of the Cas12a-bound guide RNA to irreversibly inactivate the Cas12a complex. These distinct mechanisms equip bacteriophages with tools to evade CRISPR-Cas12a and support biotechnological applications for which multiple-turnover enzymatic inhibition of Cas12a is desirable.

Bacteria and archaea can protect themselves against mobile genetic elements and viruses, including bacteriophages, using CRISPR-Cas adaptive immunity. When challenged by a mobile genetic element, bacteria deploy CRISPR-associated (Cas) nucleases guided by an RNA to base-pair with the target and mediate target interference to provide immunity against reinfection. Although bacteriophages can undergo rapid mutation and selection to avoid Cas-effector targeting, genetic variation alone is insufficient to escape the potent programmability of bacterial CRISPR-Cas adaptive immunity. To effectively evade CRISPR systems, bacteriophages have evolved protein-based inhibitors—Acrs—that inactivate RNA-guided Cas nucleases and enable phage replication. In the case of CRISPR-Cas9, such inhibitory Acrs can prevent DNA cutting by blocking dsDNA binding, promoting Cas9 dimerization or preventing target DNA cleavage. Recently, Acrs were discovered that inhibit the activities of the type V-A DNA-targeting CRISPR-Cas12a system. These type V-A Acrs (AcrVA) that inhibit Cas12a might be expected to differ in mechanism, given that Cas12a has a distinct activation and DNA-cleavage pathway distinct from that of Cas9. After expression of the CRISPR array and Cas proteins, Cas12a catalyzes precursor CRISPR-RNA (pre-crRNA) processing to form a Cas12a-crRNA complex (or ribonucleoprotein, RNP). Unlike the commonly used Streptococcus pyogenes (Sp) Cas9, which utilizes two nuclease domains (HNH and RuvC) to cut dsDNA with single-turnover kinetics, Cas12a possesses a single nuclease domain (RuvC) that is activated following a crRNA targeting sequence (or spacer) binding to a complementary single-stranded DNA (ssDNA) or dsDNA target molecule. Furthermore, the Cas12a RuvC domain catalyzes both single-turnover target DNA cutting (cis-cleavage) and multiple-turnover non-target ssDNA cutting (trans-cleavage).

To determine the mechanistic basis for Cas12a inhibition, we biochemically assayed inhibition by AcrVA1, AcrVA4 and AcrVA5 on a panel of Cas12a orthologs. While biochemical experiments revealed that no AcrVA was capable of competitively inhibiting the RuvC nuclease, each AcrVA was able to robustly target dsDNA cleavage and, to some extent, target ssDNA cleavage. Further biochemical analysis revealed that each AcrVA blocked dsDNA binding, but only AcrVA4 dimerized Cas12a and, at high concentrations, outcompeted dsDNA bound to catalytically dead LbCas12a. Finally, we demonstrate that AcrVA1 triggers multiple-turnover endoribonucleolytic cleavage of a Cas12a-bound crRNA to truncate the spacer sequence and permanently inactivate the complex. Together, these data provide insights into the mechanisms of AcrVAs, shedding light on the vulnerabilities of Cas12a and the evolutionary arms race between bacteriophages and their host bacteria.

Results

AcrVAs do not inhibit all modes of DNA targeting by Cas12a. Cas12a has a distinct mechanism of activation and DNA cleavage (Fig. 1a). To determine how AcrVA proteins inhibit Cas12a, we first tested whether AcrVAs could competitively inhibit the conserved RuvC nuclease domain. To do this, we determined the velocity of an enzyme-catalyzed reaction at infinite concentration of substrate (Vmax) and the Michaelis constant (Km) for the activated Cas12a RuvC nuclease in the presence of two concentrations of each inhibitor. AcrVA1, AcrVA4 and AcrVA5 all reduced the maximum velocity of trans-ssDNA cutting by Lachnospiraceae bacterium (Lb) Cas12a without dramatically affecting the Michaelis constant for the activated RuvC (Fig. 1b and Supplementary Fig. 1a–d). These data indicated that AcrVAs were not competitive inhibitors of the activated RuvC nuclease but instead somehow depleted the pool of active trans-cleaving Cas12a enzyme. We next tested whether AcrVAs could inhibit cis-DNA cleavage by Cas12a (Fig. 1a) using radiolabeled DNA substrates and three recombinantly purified Cas12a orthologs that are phylogenetically divergent and/or have been used for genome editing (Fig. 1c and Supplementary Fig. 2a). AcrVA1 blocked dsDNA cleavage by all three Cas12a orthologs,
AcrVAs do not inhibit all modes of DNA targeting by Cas12a. a. Schematic representation of the steps in Cas12a target interference. b. Michaelis-Menten fit for 0.1 nM effective LbCas12a holoenzyme in the absence (black) or presence of 25 nM (thin line) or 50 nM (thick line) AcrVA1 (blue), AcrVA4 (red) or AcrVA5 (yellow). The mean initial velocity (V₀) is plotted against increasing DNaseAlert substrate concentrations (µM), where n = 3 replicates. The Vₘₐₓ and Kₘ are wild-type LbCas12a RuvC activity are indicated with broken lines (the calculated kinetic constants are in Supplementary Fig. 1a-d). c. Radiolabeled dsDNA-cleavage assays for (left to right) MbCas12a, LbCas12a and AsCas12a complexes with or without AcrVAs. Time courses represent 1, 2, 5, 15 and 60 min. The uncleaved and cis-cleaved fractions are indicated with black triangles. d. Quantified fraction of ssDNA cleaved by LbCas12a in the presence or absence of AcrVAs (mean ± s.d., n = 3 independent experiments). Experimental fits are shown as solid lines, and the calculated pseudo-first-order rate constants (κₘᵦₓ) (mean ± s.d.) are 2.6 ± 0.3, 0.15 ± 0.01, 0.7 ± 0.06 and 1.2 ± 0.09 min⁻¹ for LbCas12a, LbCas12a + AcrVA1, LbCas12a + AcrVA4 and LbCas12a + AcrVA5, respectively. Source data for b and d are available with the paper online. The uncropped gel images are available in Supplementary Dataset 1.

Whereas AcrV4 and AcrV5 were effective against *Moraxella bovoculi* (Mb) Cas12a and LbCas12a but not *Acidaminococcus* sp. (As) Cas12a (Fig. 1c), consistent with plasmid cleavage data. The pattern of inhibition was generally the same for Cas12a-mediated ssDNA cleavage, but activity was not completely abolished by any inhibitor (Fig. 1d and Supplementary Fig. 3a–c). Collectively, these data indicated that AcrVA1 was a broad-spectrum inhibitor of Cas12a-catalyzed cis-DNA cleavage, whereas AcrV4 and AcrV5 inhibited cis-DNA cleavage catalyzed by MbCas12a and LbCas12a.

AcrVAs block dsDNA binding and AcrV4 dimerizes Cas12a. We next tested whether AcrVAs affect DNA binding to Cas12a, the rate-limiting step of targeting by Cas12a. To test this, we assayed the binding of radiolabeled DNA to catalytically dead LbCas12a (dLbCas12a) by electrophoretic mobility-shift assay (EMSA). The dLbCas12a–crRNA complex was formed before the separation of each AcrVA and incubation with dsDNA, revealing that AcrVAs abolished dsDNA binding (Fig. 2a) while ssDNA binding was perturbed to a lesser extent (Supplementary Fig. 4a,b). Notably, we observed a slow-mobility species representing the DNA-bound dLbCas12a–crRNA complex in the presence of AcrVA4 (Supplementary Fig. 5a), hinting at a possible multimeric assembly reminiscent of inhibitor-induced *Neisseria meningitidis* (Nme) Cas9 dimerization. To test this possibility, we assayed the solution oligomeric state of each AcrVA and when mixed with LbCas12a–crRNA (Fig. 2b and Supplementary Fig. 5b,c). While AcrVA1 and AcrVA5 appeared monomeric (Supplementary Fig. 5b,c), AcrV4 appeared dimeric before complexing with Cas12a (Fig. 2b). Although neither AcrVA1 nor AcrVA5 triggered a substantial change in estimated molecular weight when complexed with LbCas12a–crRNA (Supplementary Fig. 5b,c), mixing AcrVA4 with LbCas12a–crRNA produced two higher-molecular-weight species (Fig. 2b). Using light scattering, we estimated the mass of these species to be 349 kDa and 214 kDa, consistent with a dimeric LbCas12a–crRNA–AcrVA4 complex and a monomeric LbCas12a–crRNA bound to a dimer of AcrVA4, respectively. To directly visualize the dimerization of LbCas12a–crRNA with AcrVA4, we analyzed gel filtration-purified fractions by negative-stain electron microscopy, revealing a distribution of particles including a symmetrical complex of LbCas12a–crRNA dimers (Fig. 2c and Supplementary Fig. 5d). Taken together, these results demonstrated that AcrVAs block dsDNA binding to Cas12a and that the mechanism for AcrVA4 involves dimerization of the LbCas12a–crRNA complex.

AcrVA4 can dislodge dsDNA bound to dCas12a. We next wondered whether any AcrVA was capable of disrupting dsDNA-bound complexes of Cas12a–crRNA, a mechanism that may have evolved to disable an activated and *trans*-cleaving Cas12a (Fig. 1a). To test this possibility, we attempted to displace dsDNA from a ternary complex of dLbCas12a–crRNA bound to radiolabeled dsDNA with the addition of excess AcrVA1, AcrVA4, AcrVA5 or unlabeled dsDNA and visualized the complexes by EMSA. At high concentrations, AcrVA4 triggered the release of dsDNA bound to dLbCas12a, whereas little dsDNA release occurred in the presence of AcrVA1, AcrVA5 or unlabeled dsDNA competitor (Fig. 3a and Supplementary Fig. 6a). In contrast, a stoichiometric excess of any AcrVA or ssDNA competitor had no effect on dLbCas12a–crRNA bound to radiolabeled ssDNA (Supplementary Fig. 6b). These data suggest that, at high concentrations, AcrVA4 can dislodge dsDNA after it has formed an R-loop interaction with Cas12a. Depletion or addition of ATP had no effect on dsDNA displacement from dLbCas12a–crRNA complexes by AcrVA4, suggesting an ATP-independent process (Supplementary Fig. 6c). AcrVA4 did not trigger release of ssDNA bound to dLbCas12a–crRNA, suggesting that the non-target strand (NTS) of the DNA (the strand not base-paired to the crRNA) might be required to drive re-annealing with the
target strand. In support of this possibility, addition of the NTS ssDNA molecule to the dLbCas12a–crRNA–TS DNA complex led to target strand DNA displacement in the presence of AcrV A4; a non-complementary ssDNA used in a similar experiment had no effect (Supplementary Fig. 7a). Consistent with target strand DNA release requiring base-pairing to a complementary NTS strand, AcrV A4 was unable to drive DNA release from dLbCas12a–crRNA bound to a dsDNA substrate containing mismatched nucleotides along all or some of the 20 nt NTS (Supplementary Fig. 7b,c).

The preceding experiments were conducted using catalytically inactive dLbCas12a, which prevents cutting of bound DNA and hence dLbCas12a remains associated with an intact dsDNA molecule. Given that catalytically active LbCas12a would cut and release the protospacer-adjacent motif (PAM) distal dsDNA fragment after the formation of an R-loop interaction, we reasoned that this release might prevent AcrVA4 from displacing the PAM proximal dsDNA bound to the crRNA (Fig. 1a). To test this possibility, we incubated wild-type LbCas12a–crRNA with a dsDNA substrate followed by the addition of AcrVA4 and analysis of the resulting samples by EMSA (Fig. 3b). In contrast to dLbCas12a (Fig. 3a and Supplementary Fig. 7d), AcrVA4 had no effect on DNA bound by wild-type LbCas12a (Fig. 3b). Collectively, these data demonstrated that AcrV A4 can dislodge dsDNA bound to catalytically dead but not active Cas12a, presumably due to a shift in binding equilibrium that favors dsDNA strand re-annealing.

AcrV A1 triggers endoribonucleolytic truncation of a Cas12a-bound crRNA. We next explored whether any AcrV A might prevent target DNA binding by disruption of the Cas12a–crRNA complex (Fig. 1a). To test this possibility, we incubated Cas12a orthologs with each AcrV A individually before adding radiolabeled RNA to probe crRNA integrity, the efficacy of pre-crRNA processing and the affinity of Cas12a for mature crRNA. We were surprised to observe that AcrV A1 induced rapid 3′-end truncation of both mature and pre-crRNA in the presence of any Cas12a ortholog (Supplementary Fig. 9a) and Supplementary Fig. 8a). In these experiments, neither binding to mature crRNA nor pre-crRNA processing was affected (Fig. 4b and Supplementary Fig. 8a). Notably, AcrV A1 had no effect on the integrity of mature or pre-crRNA in the absence of Cas12a, and neither AcrV A4 nor AcrV A5 had any effect on crRNA in the absence of the presence of Cas12a (Fig. 4a,b and Supplementary Fig. 8a,b). Pre-assembly of Cas12a and crRNA forms the Cas12a–crRNA complex, which was also susceptible to AcrVA1-mediated crRNA 3′-end truncation (Supplementary Fig. 8b). However, pre-assembly of a crRNA that is released by Cas12a after AcrV A1-triggered truncation (Supplementary Fig. 8c). Furthermore, AcrV A1-mediated crRNA truncation was specific for a crRNA bound by Cas12a regardless of spacer sequence (Supplementary Fig. 9a) or lengths that support Cas12a DNA targeting (Supplementary Fig. 9b) and without any detectable non-specific ribonuclease activity (Supplementary Fig. 9c). Taken together, the above data indicated that AcrV A1 triggers specific crRNA truncation on an assembled Cas12a–crRNA complex.

Interestingly, AcrV A1 is not predicted to be a nuclease nor does it have detectable RNA cleavage activity in the absence of a Cas12a–crRNA complex (Fig. 4a and Supplementary Fig. 7c). To assess the mechanism of AcrVA1-mediated RNase activity, we mapped the zzz-persistence to positions five to eight within the crRNA spacer, with some plasticity in position dependent on the Cas12a ortholog (Fig. 4c and Supplementary Fig. 9d). The activity is that of an endonuclease where catalysis generates an intact 3′ fragment of the crRNA that is released by Cas12a after AcrV A1-triggered truncation (Supplementary Fig. 9e,f). To identify the nuclease center responsible for the scissile phosphates to positions five to eight within the crRNA spacer, we conducted an endonuclease reaction that produced a mature full-length crRNA in the presence of AcrV A1 and pre-crRNA (Supplementary Fig. 9c). Taken together, the above data indicated that AcrV A1 triggers specific crRNA truncation on an assembled Cas12a–crRNA complex.
that ~95% of crRNAs were truncated, even at sub-stoichiometric concentrations of AcrVA4 (Fig. 5a). Thus, AcrVA1 activity is multiple turnover where cleavage of a crRNA will permanently inactivate Cas12a–crRNA complexes through a mode of inhibition not previously observed for any anti-CRISPR protein. However, we earlier demonstrated that AcrVA1 was not a robust inhibitor of ssDNA targeting by Cas12a (Fig. 1d and Supplementary Fig. 3), which is at odds with the observed nuclelease activity on the crRNA (Figs. 4a and 5a). We wondered whether the 5′ or 3′ fragments of the crRNA, together or separately, might still be sufficient for ssDNA targeting. To test this, we prepared RNA fragments that mimic products of AcrVA1 activity and assayed ssDNA targeting by LbCas12a. Remarkably, LbCas12a cleaved ssDNA in the presence of both the 5′ and 3′ fragments (Supplementary Fig. 10c), suggesting that the ssDNA targets can be recruited to Cas12a with a two-component crRNA. Taken together, our data demonstrate that AcrVA1 triggers crRNA truncation and release of the 3′ fragment from Cas12a, which can hybridize with a target ssDNA to activate cis- and trans-ssDNA cleavage by Cas12a.

The ability of AcrVA1 to inhibit diverse Cas12a orthologs (Fig. 1c) suggested that it might exploit an evolutionarily conserved domain of Cas12a. To determine the domains required for AcrVA1-triggered spacer truncation, we generated truncations that still allowed for crRNA binding and pre-crRNA processing. Removal of either the PAM-interacting domain (PID) or both recognition (REC) domains generated stable constructs that maintained near wild-type mature crRNA binding affinity or pre-crRNA processing (Supplementary Fig. 10b,c). However, only in the absence of the PID was AcrVA1-triggered crRNA truncation prevented (Fig. 5b). Finally, we wondered whether AcrVA4 or AcrVA5 might compete with the AcrVA1-triggered spacer truncation activity. To test this, we first incubated an LbCas12a RNP with either AcrVA4 or AcrVA5 before adding AcrVA1, and found that AcrVA5 reduced the rate of AcrVA1-triggered crRNA truncation (Fig. 5c), suggesting that AcrVA5 does compete with the spacer truncation activity of AcrVA1.

Discussion

CRISPR-Cas12a are RNA-guided DNA-targeting nucleases with robust cis-cleavage and ssDNA trans-cleavage, activities that have led to their rapid implementation as tools for genome engineering and diagnostics30. In this work, we present mechanistic insights into type V-A bacteriophage-derived anti-CRISPRs elucidating the distinct modes leveraged to inactivate Cas12a (Fig. 6). We found that AcrVA1, AcrVA4 and AcrVA5 robustly inhibited Cas12a dsDNA targeting, not unlike inhibitors that evolved to target Cas9 (ref. 4). AcrVA1 provides a uniquely potent mechanism for evading CRISPR adaptive immunity by triggering crRNA truncation with multiple-turnover kinetics to rapidly and permanently inactivate the Cas12a surveillance complex. We demonstrated that the nuclelease activity is entirely dependent on the presence of a Cas12a–crRNA complex and AcrVA1, but our data do not describe the identity of the component bearing the catalytic center for the observed nuclelease activity. Although it is probable that AcrVA1 is an RNase, we could not detect any RNase activity on free crRNA or trans-ssRNA substrates, suggesting that its activity is allosterically activated by binding to a Cas12a–crRNA complex or that Cas12a harbors the nuclease domain or a part thereof. AcrVA1 has a broad spectrum of inhibition, disabling divergent Cas12a nucleases in vitro and in mammalian cell editing31, potentially exploiting the broadly conserved PAM-interacting domain for direct access to the pre-ordered seed of the crRNA41. Interestingly, AcrVA1 displayed less robust inhibition of Cas12a ssDNA targeting, a potential artifact of working in vitro as the cleaved 3′ crRNA fragments can readily associate with ssDNA and be recruited back to Cas12a for activation. In the bacterial host, it is probable that cleavage of crRNA creates an ineffective two-component system. However, further experiments are
**Fig. 4 | AcrVA1-triggered endoribonuclease activity truncates a Cas12a-bound crRNA.** (a) Radiolabeled kinetic crRNA cleavage assays for (left to right) MbCas12a, LbCas12a and AsCas12a complexed with or without AcrVAs. Time courses represent 1, 2, 5, 15 and 60 min. Black triangles indicate full-length and truncated crRNA. (b) Quantified fraction of crRNA bound by LbCas12a in the presence or absence of AcrVAs as determined by EMSA (mean ± s.d., n = 3 independent experiments). Measured dissociation constants (K_d) are 38.9 nM ± 4.7, 17.6 nM ± 2.4, 35.8 nM ± 4.4 and 16.4 nM ± 2.1 in the absence of inhibitor or in the presence of AcrVA1, AcrVA4 or AcrVA5, respectively. Source data are available with the paper online. (c) Radiolabeled crRNA cleavage assay using LbCas12a–crRNA complexed without or with AcrVA1. Treatments in the absence of AcrVA1 are (left to right) crRNA hydrolysis ladder (OH), crRNA RNase T1 digestion (T1), untreated crRNA (-) and crRNA incubated with LbCas12a (Lb). (d) Radiolabeled crRNA cleavage assay using LbCas12a–crRNA complexed with AcrVA1 that was either untreated (Lb) or treated with PNK (Lb*). The proposed metal ion–independent mechanism of catalysis is shown in the dashed box at right. (e) Schematic representation of AcrVA1-triggered crRNA spacer cleavage activity on Cas12a. Cleavage sites for Mb (blue), Lb (green) and AsCas12a (red) are indicated by triangles. The uncropped gel images are available in Supplementary Dataset 1.
of phage-lacking Acrs given its multiple-turnover kinetics, even with the recent data suggesting that Cas12a endonuclease activity can be reset. Furthermore, Cas effectors are universally steered by programmable RNA guides, raising the possibility that all CRISPR-Cas systems are susceptible to this mode of inhibition. The unique mechanism for AcrV A1-mediated CRISPR-Cas12a inhibition may lend itself to potent control of Cas12a in gene-editing applications where it is desirable to block DNA targeting or limit unintended editing events.

We also found that AcrV A4 blocks dsDNA binding in addition to driving dimerization of Cas12a-crRNA complexes. This mechanism has also been described for AcrIIIC3, which targets NmeCas9 (ref. 17). Although mechanistically and structurally divergent, Cas9 and Cas12a are susceptible to a convergent mechanism of inhibition suggesting that higher-order assembly of Cas nucleases and the associated inhibitors offers an as yet unclear benefit to bacteriophages. AcrV A4 was also able to disrupt a dLbCas12a-crRNA complex stably associated with dsDNA, an activity that required high concentrations of the inhibitor. The disruption of dLbCas12a dsDNA-bound states may have applicability in dLbCas12a-mediated transcripational control applications, but further experiments are required to establish the off-rate for dsDNA in the presence of AcrV A4. Furthermore, while it is interesting to consider that AcrV A4 may shift the equilibrium in favor of dsDNA dissociation, this mode of action is unlikely to be biologically relevant, given that wild-type Cas12a rapidly catalyzes DNA cleavage once an R-loop is formed.

Finally, we demonstrated that AcrV A5 robustly inhibited Cas12a dsDNA targeting activity by preventing dsDNA binding. Given that AcrV A5 competed with AcrV A1 and that AcrV A1 activity is dependent on the PID, we speculate that AcrV A5 may directly exploit the PID to block PAM recognition on dsDNA substrates. If true, this raises the possibility that AcrV A5 might be leveraged as a tool to block in vivo dsDNA targeting by Cas12a to exclusively select for ssDNA targeting. Furthermore, the panel of AcrV tested in this study are more potent inhibitors of dsDNA targeting than ssDNA targeting by Cas12a, which may reflect an evolutionary pressure from dsDNA phages in the host’s microbial community. Taken together, these mechanistic insights reveal vulnerabilities in the modes of Cas12a targeting, providing scope for greater control of a rapidly expanding landscape of Cas12a and Acr applications.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41594-019-0208-z.

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Author contributions

G.J.K. conceived the study with input from K.E.W. G.J.K. designed experiments with input from B.W.T., and J.A.D. G.J.K. and B.W.T. carried out biochemical work. M.J.L. and G.I.K. carried out light-scattering experiments. J.L. carried out negative-stain electron microscopy. B.A.S. carried out bioinformatic analysis. G.J.K. drafted the manuscript and all authors edited the manuscript.

Competing interests

The Regents of the University of California have patents pending for CRISPR technologies on which the authors are inventors. J.A.D. is a co-founder of Caribou Biosciences, Editas Medicine, Intellia Therapeutics, Scribe Therapeutics and Mammoth Biosciences. J.A.D. is a scientific advisory board member of Caribou Biosciences, Intellia Therapeutics, eFFECTOR Therapeutics, Scribe Therapeutics, Synthego, Mammoth Biosciences and Inari. J.A.D. is a Director at Johnson & Johnson and has sponsored research projects by Pfizer and Biogen.

Additional information

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Methods
Phylogenetic analysis. A multiple sequence alignment of the Cas12 proteins was generated using MAFFT 1.0.3, and a maximum-likelihood phylogenetic tree was constructed using RAxML with PROTGAMMALG as the substitution model and 100 bootstrap samplings. The tree was visualized using iTOL v.3 (ref. 37) to highlight the phylogeny of Cas12a, with Cas12b-e as a collapsed outgroup.

Protein expression and purification. Plasmids encoding M. bovoculi Cas12a, L. bacterium (ND2000) Cas12a, Acidaminococcus sp. (BV36) Cas12a, AcrV A1, AcrV A4 and AcrV A5 were generated from a custom plasmid for protein expression by T7 RNA polymerase (PET-based expression vector) as described previously (7). Cas12a point mutations and truncations were introduced by either around-the-horn PCR or Gibson Assembly verified by DNA sequencing. Proteins were purified as described previously (38). Briefly, Escherichia coli Rosetta 2 (DE3) containing Cas12a or AcrV A or AcVR V plasmid expression plasmids was grown in Lysogeny broth overnight with ampicillin (100 µg·mL⁻¹). Overexpressed cultures were harvested in Terrific broth to an OD₆₀₀ of 0.6–0.8, after which they were cooled on ice for 15 min before induction with 0.5 mM isopropyl-b-D-thiogalactoside and incubated overnight at 16°C for 16 h. Cells were harvested by centrifugation and resuspended in wash buffer (20 mM Tris-Cl (pH 7.5), 500 mM NaCl, 1 mM TCEP (tris(2-carboxyethyl)phosphine), 5% (v/v) glycerol) supplemented with 0.5 mM phenylmethylsulfonyl fluoride and complete protease inhibitor (Roche), lyzed by sonication and purified over Ni-NTA Superflow resin (Qiagen) in wash buffer supplemented with either 10 mM imidazole (wash) or 300 mM imidazole (elution). Eluted proteins were digested overnight with TEV protease at 4°C in a Slide-A-Lyzer (50 mL MWCO 10,000; Pierce) buffer (20 mM HEPES-K (pH 7.5), 200 mM NaCl, 1 mM TCEP, 5% (v/v) glycerol). Digested proteins were loaded onto an MBP-Trap (GE Healthcare) upstream of a Heparin Hi-Trap (GE Healthcare, Casi12a) or a Hi-Trap Q (GE Healthcare, AcrV A) and eluted over a salt gradient (20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM TCEP, 5% (v/v) glycerol, 125 mM–1 M KCl). The eluted protein was concentrated before injection to a Superdex 200 10/300 Increase (GE Healthcare) developed in 20 mM HEPES-K (pH 7.5), 200 mM NaCl, 1 mM TCEP and 5% (v/v) glycerol. Purified proteins were concentrated and snap-frozen in liquid nitrogen for storage at −80°C. The purity and integrity of proteins used in this study were assessed by SDS–PAGE (Coomassie blue staining) (Supplementary Fig. 2b).

RNA and DNA preparation. RNA used in this study were ordered from Integrated DNA Technologies (IDT) (Supplementary Table 1). RNA substrates were purified by gel extraction from 12% (v/v) urea-denaturing PAGE (0.5X Tris-borate-EDTA (TBE)) and ethanol precipitation as described previously. All DNA substrates were synthesized by IDT and purified as described above. Radiolabeled DNA substrates were prepared by 5′-end labeling with T4 PNK (NEB) in the presence of γ²P-ATP. For 3′-end labeled substrates, the crRNA was labeled with T4 RNA Ligase 1 (NEB) in the presence of 3′-P-PcP radiolabeled DNA substrates were prepared by 5′-end labeling with T4 PNK (NEB) in the presence of γ²P-ATP. For dsDNA substrates, the non-target strand or target strand was first 5′-end labeled before annealing at a 1:2 molar excess of the complementary strand at 95°C for 3 min in 1X hybridization buffer (20 mM Tris-Cl (pH 7.5), 150 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol (DTT)) followed by slow cooling to room temperature.

DNAseAlert trans-cleavage assays. For Michaelis–Menten kinetics in the presence or absence of AcVR V, 0.1 nM of pre-activated and assembled LbCas12a–crRNA activator holozyme was prepared by complexing 5 nM Cas12a and 6.25 nM crRNA (15 min at 37°C with 25–50 nM AcVR V (30 min at 37°C) and 0.1 nM DNA activator (15 min at 37°C) in 1X trans-cleavage buffer (200 mM HEPES-K (pH 7.5), 100 mM KCl, 5 mM MgCl₂, 5% (v/v) glycerol, 1 mM dTTP, 50 µg·mL⁻¹ heparin). Reactions were initiated by adding 0.001, 0.01, 0.1, 0.25, 0.5, 1.0 or 2.0 µM of DNaseAlert substrate (IDT). Reactions were incubated in a fluorescence plate reader (BioTek) for 30 min at 37°C with fluorescence measurements taken every 20 s (λₑₓ: 535 nm; λₑ ş: 595 nm). The initial velocity (V₀) was calculated by fitting to a linear regression and plotted against the substrate concentration to determine the Michaelis–Menten constant (Kₘ, Kᵥ, Ka). According to Y = 1 [Figure 1a] (Kᵥ, Kₘ, Ka) for 3 independent experiments.

Radiolabeled DNA cleavage assays. Cas12a-mediated DNA cleavage assays were carried out in 1X cleavage buffer (20 mM Tris-Cl (pH 7.8), 150 mM KCl, 5 mM MgCl₂, 1% (v/v) glycerol, 2 mM DTT). Radiolabeled DNA cleavage assays consisted of Cas12a, crRNA and P²-P-labeled DNA substrates in the presence or absence of AcVR V at 30, 36, 1 and 300 nM, respectively. The RNP was formed at 37°C for 15 min before the addition of AcVR V (unless otherwise indicated) and incubated at 37°C for 30 min. Reactions were initiated with the addition of target DNA at 37°C and timepoints (1, 2, 5, 15, 30, 60 min) quenched in 1.5X formamide loading buffer (final concentration 45% (v/v) formamide, 15 mM EDTA, 0.1% (w/v) SDS, 200 µg·mL⁻¹ heparin and 0.25% (w/v) bromophenol blue) for 3 min at 95°C. Samples were resolved by 12% (w/v) urea-denaturing PAGE (0.5X TBE) and visualized by phosphoroimaging (Amersham Typhoon, GE Healthcare).

The percentage cleavage was calculated as the ratio of the intensity of the product band relative to the total intensity of both the product and uncleaved DNA normalized to the background within each measured substrate in ImageQuant TL (GE Healthcare) and the calculated rates were calculated by fit to a single exponential decay (Prism7, GraphPad). The rates with their associated standard deviations are included in the Figure legends (n = 3 independent experiments).

Radiolabeled crRNA cleavage assays. Assays of crRNA cleavage were carried out in 1X cleavage buffer. Radiolabeled crRNA cleavage assays consisted of Cas12a and P²-P-labeled RNA substrates in the presence or absence of AcVR V at 50, 1 and 500 nM, respectively. Complexing was carried out by incubating Cas12a and AcVR V or P²-P-labeled crRNA substrates at 37°C for 30 min before initiating the reaction with the addition of AcVR V or P²-P-labeled RNA substrates. Reaction timepoints (1, 2, 5, 15, 60 min) were quenched in 1.5X formamide loading buffer for 3 min at 95°C. Samples were resolved by 12% (w/v) urea-denaturing PAGE (0.5X TBE), visualized by phosphoroimaging (Amersham Typhoon, GE Healthcare) and quantified with ImageQuant TL Software (GE Healthcare). Where appropriate, apparent rates were calculated by fit to a single exponential decay (Prism7, GraphPad), and the calculated rates with their associated standard deviations are included in the Figure legends (n = 3 independent experiments).

Product size mapping and 3′-end chemistry identification. Cleaveage product length was determined biochemically by comparing the gel mobility with a AcVR V1-triggered cleavage products with alkaline hydrolysis and RNase T1 digestion ladders of the matched untreated crRNA. Hydrolysis ladders were generated by incubating 15 nM 5′-radiolabeled crRNA at 95°C for 10 min in 1X alkaline hydrolysis buffer (Ambion). Reactions were quenched in 1.5X formamide loading buffer and immediately loaded to a urea-denaturing PAGE (0.5X TBE) gel. For RNase T1 digestion ladders, 15 nM 5′-radiolabeled crRNA were unfolded in 1X RNA sequencing buffer (Ambion) at 65°C for 5 min and cooled to ambient temperature before the addition of 1 U of RNase T1 (Ambion). After incubation at ambient temperature for 15 min, reactions were extracted in phenol-chloroform (pH 8.0) and loaded in 1.5X formamide loading buffer to a urea-denaturing PAGE (0.5X TBE) gel and visualized by phosphoroimaging (Amersham Typhoon, GE Healthcare).

RNA electrophoretic mobility-shift assays. All experiments were equilibrated in 1X binding buffer (20 mM Tris-Cl (pH 7.5), 150 mM KCl, 5 mM MgCl₂, 1 mM DTT, 5% (v/v) glycerol, 50 µg·mL⁻¹ heparin, 0.1 µg·mL⁻¹ BS-30 (Promega) for 1 min at 90°C. Samples were resolved by 12% (v/v)  urea-denaturing PAGE (0.5X TBE) gel and visualized by phosphoroimaging (Amersham Typhoon, GE Healthcare).

DNA electrophoretic mobility-shift assays. All experiments were equilibrated in 1X binding buffer. To avoid dissociation of either the dLbCas12a–crRNA or LbCas12a–crRNA complex during DNA binding experiments, Cas12a 5′P-DNA EMSAs were prepared as an excess of Cas12a (900 nM) incubated with a titration of crRNA (0 pM, 2.4 pM, 9.7 pM, 39 pM, 0.156 nM, 0.625 nM, 2.5 nM, 10 nM, 40 nM) to pre-form the RNP for 30 min at 37°C, unless otherwise indicated. For experiments testing whether AcVR V would block ssDNA or dsDNA binding, the RNP was first incubated with an excess of AcVR V (10 µM) for 30 min at 37°C before incubation with 0.1 µM substrate (0.1 µM) for another 30 min. Affinities and their associated standard deviations are reported in the figure legends.
complex, RNP was prepared at 40 nM effective concentration as described above before pre-forming a DNA-bound state with 32P-dsDNA (0.1 nM) for 30 min at 37 °C. This was followed by the addition of AcrVA or cold DNA competitor (0 nM, 1 nM, 3 nM, 10 nM, 40 nM, 200 nM) to further incubate for 30 min at 37 °C. To test ATP dependence, an excess of Cas12a (960 nM) was incubated with a titration of crRNA (0 pM, 2.4 pM, 9.7 pM, 39 pM, 0.156 nM, 0.625 nM, 2.5 nM, 10 nM, 40 nM, 160 nM, 640 nM) to pre-form the RNP for 30 min at 37 °C, before the addition of 32P-dsDNA (0.1 nM) with or without 1 μM apyrase (NEB) or 2 μM ATP (NEB), and further incubation for 30 min at 37 °C. AcrVA4 (10 μM) was then introduced for a final incubation at 37 °C for 30 min. For all EMSAs, the resulting complexes were resolved by 6% native PAGE (0.5×TBE supplemented with 5 mM MgCl2) at 4 °C, visualized by phosphoroimaging (Amersham Typhoon, GE Healthcare) and quantified with ImageQuant (GE Healthcare). The fraction bound was determined as the ratio of intensity of the bound band relative to the total intensity of both the unbound band and the bound band, normalized to background and fit to a binding isotherm (Prism7, GraphPad) to calculate the dissociation constants (Kd) and their associated standard deviations reported in the Figure legends. All experiments were run in 20 mM HEPES.K (pH 7.5), 200 mM KCl, 1 mM TEPK and 1 mM MgCl2 on a Superdex 10/300 Increase column (GE Healthcare) at 0.5 ml min−1 using the Infinity 1260 Bio-SEC with light-scattering module (Agilent). Light scattering (LS) was collected at 15° and 90° using a 658 nm laser. The system was calibrated using a 2 mg ml−1 bovine serum albumin and refractive index increments (dn/dc) of 0.185 (ref. 41). Calibration constants were determined using a first-degree fit over the linear region of mass estimates for each peak using the Bio-SEC software V A.02.01 (Agilent).

Single-particle negative-stain electron microscopy. Purified LbCas12a RNP bound to AcrA4 was prepared at ~50 nM and negatively stained in 2% (w/v) uranyl acetate (Electron Microscopy Sciences) solution following the standard deep-stain procedure on holey carbon-coated electron microscopy copper grids covered with a thin layer of continuous carbon. Negative-stained specimens were mounted on a transmission electron microscope holder and examined with a Tecnai Spirit electron microscope operated at 120 kV acceleration voltage. Magnified digital micrographs of the specimen were taken at a nominal magnification of ×51,000, on a Gatan Ultrascan4000 CCD camera with a pixel size of 2.18 Å, at the specimen level by Leginon43. The defocus values ranged from ~0.9 to ~1.5 μm, and the total accumulated dose at the specimen was about 60 electrons per Å2. Image analysis was performed in Appion43.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Source data for Figs. 1b, d, 3a, 4b and 5a are available with the paper online. Source data for all other biochemical experiments that support the findings of this study are available from the corresponding author upon reasonable request. The uncropped images for the main text or Supplementary Figures are available in Supplementary Dataset 1.

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| Sample size       | Sample size is indicated in the figure legends where appropriate. |
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| Data exclusions   | A single data point was excluded from analysis related to main text Figure 3a, specifically dLBCas12a-cRNA-ddDNA in the presence of AcrVA1 at its highest concentration. This data point was a significant outlier. The raw data (including the excluded data point) are available in Supplementary Table 2. |
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