CRISPR

Enzymatic anti-CRISPRs improve the bacteriophage arsenal

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Bacteriophage-encoded anti-CRISPR (Acr) proteins have previously been thought to inhibit CRISPR-mediated immunity by acting as physical barriers against DNA binding or cleavage. Two new studies report that recently discovered type V Acr proteins use enzymatic activities to shut down the Cas12a endonuclease, providing a multi-turnover off-switch for CRISPR-based immunity and technology.

Bacteria and phages are in a constant war for survival. In this fight, bacteria have developed an arsenal to defend against infections, including sophisticated CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPR associated) systems that enable adaptive immunity[1]. The basis for immunity is the activity of CRISPR RNA (crRNA)-guided Cas effectors, which find and destroy nucleic acids from invader phages (Figure 1). To thwart this attack, phages deploy anti-CRISPR (Acr) proteins that directly inhibit Cas effectors to shut down CRISPR-mediated defense[2–4]. Acr proteins can also be used as off-switches for CRISPR-based technology, in which Cas effectors, such as Cas9 and Cas12a, are used for genomic manipulation[5,6]. Although Acr proteins against Cas9 have been extensively characterized[4], Acrs against Cas12a were discovered only recently[7,8]. In this issue, Knott *et al.*[9] and Dong *et al.*[10] provide the first mechanistic characterization of these type V-A Acr (AcrVA) proteins. Surprisingly, each study uncovers a unique AcrVA activity, in which enzymatic cleavage of the crRNA or covalent modification of Cas12a renders the Cas effector complex

inactive. These enzymatic inhibitors of CRISPR-Cas systems are the first of their kind and may provide significant advantages over previously characterized Acr mechanisms.

Acr proteins generally inhibit CRISPR-Cas immunity by preventing either target binding or cleavage by the Cas effector[3,4]. Attacking phages must cooperate to match each Cas effector already present in the host cell with a copy of an Acr protein to completely shut down immunity[11,12]. In contrast, it has been proposed that enzymatic Acr proteins would more effectively deactivate CRISPR-Cas immunity by turning over and neutralizing many Cas effectors per Acr copy (Figure 1)[12].

In studies of recently discovered type V-A Acr proteins, Knott *et al*. and Dong *et al.* now demonstrate that such enzymatic Acr proteins do indeed exist. By collectively characterizing three of Cas12a inhibitors, AcrVA1, AcrVA4 and AcrVA5, the authors discover that all three AcrVA proteins block DNA binding by Cas12a, similar to other Acr proteins. However, AcrVA1 and AcrVA5 act through novel mechanisms (Figure 2).

Knott *et al.* discovered that AcrVA1 truncates the 3'-end of the guide crRNA, which is required for binding to the DNA target through complementary base pairing (Figure 2)[9]. While AcrVA1 alone lacks RNA cleavage activity, it robustly cleaves the crRNA within the effector complex. Intriguingly, when the authors tested AcrVA1 activity at sub-stoichiometric concentrations, crRNA cleavage was still observed at maximal levels. These data demonstrate that AcrVA1 is a bona fide enzyme that releases the Cas12a-crRNA complex following RNA cleavage, allowing for multi-turnover activity.

The authors demonstrate that neither Cas12a active site residues nor metal ions are required for AcrVA1-dependent crRNA truncation, although the exact mechanism of AcrVA1 activity remains unclear. Like most Acr proteins, AcrVA1 lacks conservation with any proteins

of known function, obscuring the identification of a putative active site. The data presented in Knott *et al.* strongly suggest that AcrVA1 activity is linked to binding to a complete Cas12a-crRNA complex. Importantly, AcrVA1 has previously been shown to potently inhibit a broad range of Cas12a orthologs, suggesting that this activation can occur across several Cas effector sequences.

Dong *et al.* discovered that AcrVA5 is an acetyltransferase that covalently modifies lysine residues within Cas12a (Figure 2)[10]. One particular target of this acetylation is the lysine residue involved in reading out the PAM (protospacer adjacent motif), a short sequence located next to the target that is required for dsDNA binding[13,14]. Modification of this residue to acetyllysine nullifies its ability to interact with the PAM, and thus renders Cas12a unable to unwind and bind dsDNA targets (Figure 2). The authors show that AcrVA5 activity relies on acetyl Co-A (AcCoA) and that acetylation activity can be enhanced by providing excess AcCoA *in vitro*. These results suggest an inherent multi-turnover capacity of AcrVA5 within bacterial cells where AcCoA is abundant (Figure 2).

Intriguingly, many Cas12a orthologs contain a PAM-interacting arginine, rather than a lysine residue. Dong *et al.* show that this substitution renders AcrVA5 ineffective, enabling continued DNA targeting by Cas12a. Surprisingly, AcrVA5 acetylation activity does not appear to be highly sequence specific, as many lysine residues within Cas12a were also acetylated. Although only a single acetylation of the PAM-sensing lysine is required for Cas12a inhibition, it will be interesting in the future to determine whether other AcrVA5 acetylation events have consequences on the bacterial cell.

The discovery of Acr enzymes continues to illuminate the evolutionary battle for survival between prokaryotes and viruses. However, although enzymatic Acr proteins are theoretically

advantageous, they appear to be relatively uncommon. Is the rarity of Acr enzymes a consequence of the complex set of requirements for evolution of enzymatic activities? Do Acr proteins that act as physical blocks actually provide better long-term inhibition by remaining bound to the Cas effector? It is possible that the two enzymatic activities of AcrVA1 and AcrVA5 may provide only temporary Cas12a inactivation. Recent studies have suggested that Cas12a can efficiently replace its guide to reset the complex, which could reduce the efficacy of crRNA truncation by AcrVA1[15]. Similarly, bacterial cells could employ a deacetylase to counter AcrVA5. Nevertheless, if Acr enzymes have evolved robust turnover kinetics, they may overcome such counteractive measures. With Acr enzymes now in hand, scientists can begin to determine whether these mechanisms truly provide phages a stronger weapon, and whether humans can take advantage of these enzymes for tighter control of CRISPR technology.

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Competing interests

The authors declare not competing interests.

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Figure legends

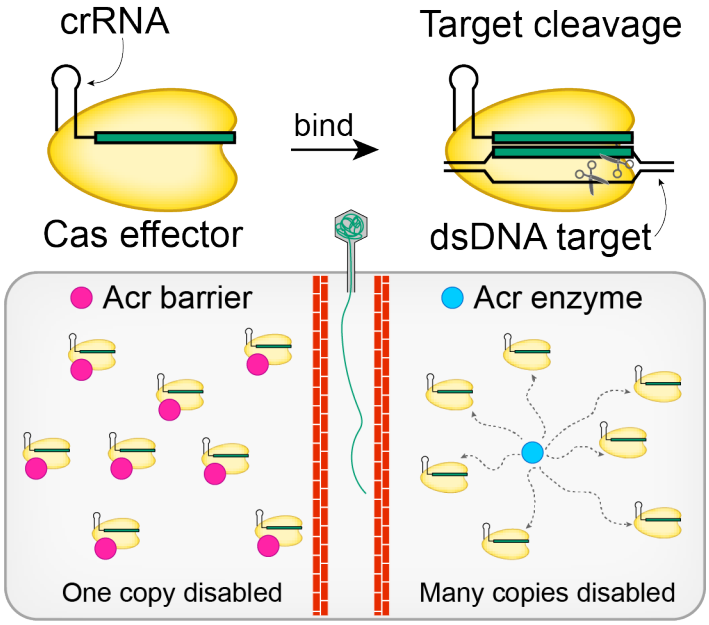

Figure 1: Alternative mechanisms for disabling Cas effectors. (top) Cas effector proteins (yellow) use crRNA guides to bind to complementary double-stranded DNA (dsDNA) targets, leading to target cleavage. (bottom) Previously characterized Acr proteins act as physical barriers (magenta) to block DNA binding or cleavage, requiring a 1:1 stoichiometry between Acr protein and Cas effector. Acr enzymes (blue) can theoretically inactivate multiple Cas effectors for each copy of Acr protein, increasing the rate of CRISPR-Cas nullification.

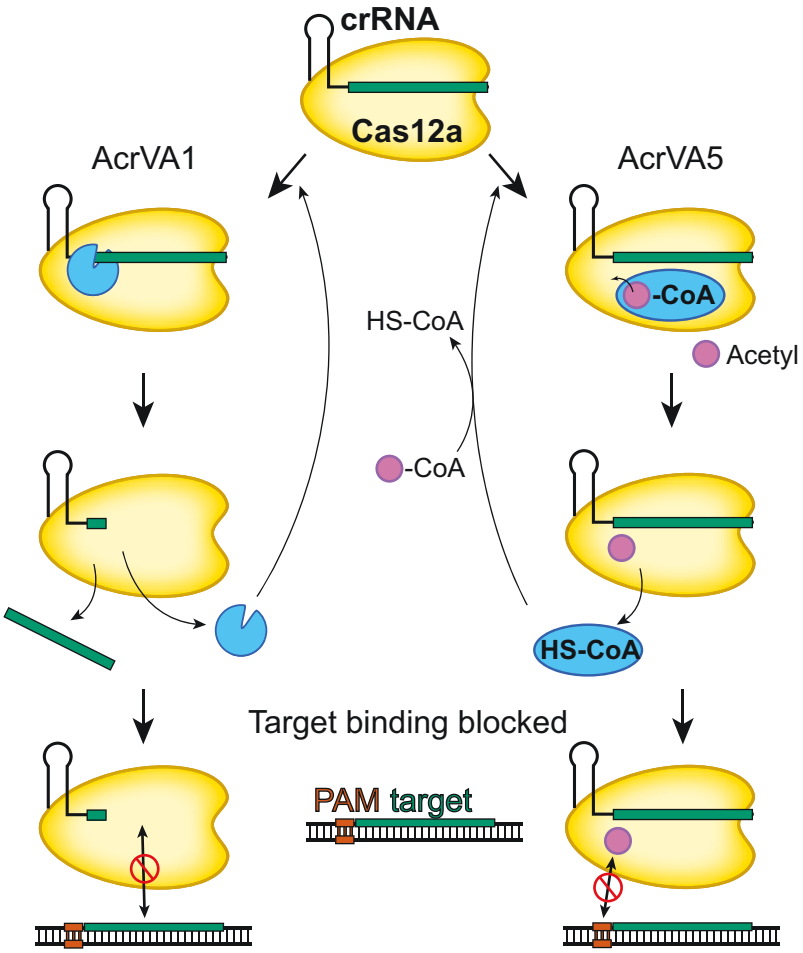

Figure 2: AcrVA1 and AcrVA5 are enzymatic Cas12a inhibitors. (left) AcrVA1 cleaves the crRNA within the guide region (green) when the crRNA is bound to Cas12a. Following cleavage, the cleaved guide fragment and AcrVA1 are released. The shortened crRNA is not competent for dsDNA binding as it lacks complementarity to the target region of the dsDNA (green). AcrVA1 can turn over and cleave crRNA in other copies of the Cas12a effector complex. (right) AcrVA5 uses an acetyl-CoA cofactor to add an acetyl group (purple) to the PAM-sensing lysine in the Cas12a PID. This chemical modification renders Cas12a unable to recognize the PAM (brown) and unwind dsDNA. Following replacement of the HS-CoA with a new acetyl-CoA cofactor, AcrVA5 can turn over and inactivate more copies of Cas12a.