



# FINDING THE CRISPR OFF-SWITCH

Researchers have identified a bevy of anti-CRISPR tools that could aid in medicine and biosecurity. **By Elie Dolgin**

It started out as “sort of a stupid thing to do”, recalls Joe Bondy-Denomy, a microbiologist at the University of California, San Francisco. As a graduate student in the early 2010s, he tried to infect bacteria with viruses that, on paper, shouldn’t have stood a chance. He knew that these viruses, or phages, were susceptible to CRISPR–Cas, the bacterial defence system that scientists have harnessed as a powerful tool for gene editing. And in most cases, he was right: the CRISPR machinery chopped the incoming phages into bits. But in a few instances, against the odds, the intruders survived.

Bondy-Denomy thought he had messed up. “Then a light bulb went off,” he says. Maybe something inside the bacterial genome was disarming its defences. And maybe that self-sabotaging bit of DNA was coming from previous viral invaders.

A quick comparison of DNA sequences proved Bondy-Denomy’s intuition correct. Phage genes nestled inside the bacterial genome were completely shutting down the CRISPR–Cas system, making the bacteria vulnerable<sup>1</sup>.

“Joe got the result that changed everything,” says Alan Davidson, a phage biologist at the

University of Toronto in Canada, who was Bondy-Denomy’s PhD adviser at the time. “He found something amazing that we never expected.”

Bondy-Denomy – together with Davidson, microbiologist Karen Maxwell and fellow graduate student April Pawluk – had stumbled onto tools now known as anti-CRISPRs. These proteins serve as the rocks to CRISPR’s molecular scissors. And soon, they were popping up everywhere: more than 50 anti-CRISPR proteins have now been characterized, each with its own means of blocking the cut-and-paste action of CRISPR systems.

ILLUSTRATION BY SÉBASTIEN THIBAUT

The expansive roster opens up many questions about the archaic arms race between bacteria and the phages that prey on them. But it also provides scientists with a toolkit for keeping gene editing in check.

Some are using these proteins as switches to more finely control the activity of CRISPR systems in gene-editing applications for biotechnology or medicine. Others are testing whether they, or other CRISPR-stopping molecules, could serve as biosecurity countermeasures of last resort, capable of reining in some genome-edited bioweapon or out-of-control gene drive.

“For any reason you can think of to turn off CRISPR systems, anti-CRISPRs come into play,” says Kevin Forsberg, a microbial genomicist at the Fred Hutchinson Cancer Research Center in Seattle, Washington.

Yet, despite a growing number of proposed applications and proof-of-concept experiments in the laboratory, researchers have yet to pin down the therapeutic potential of these anti-CRISPR systems. Jennifer Doudna, a biochemist at the University of California, Berkeley, and one of the pioneers of CRISPR gene editing, voices a question that she says is on everyone’s lips: “How do you actually use these in a way that will provide meaningful control?”

“That’s certainly where that whole anti-CRISPR field needs to go,” she says. “It just hasn’t gone there yet.”

### All hell breaks loose

Despite the growing focus on anti-CRISPRs – with about one paper a week published on the topic in 2019 – the initial discovery by Davidson and his students flew under the radar.

To most scientists, it seemed like an esoteric example of evolutionary warfare – especially given that the anti-CRISPR proteins discovered were all specific to one particular form of bacterial defence, known as the type I CRISPR system. The darling of genome editing has been the type II system and its archetypal DNA-cutting protein, Cas9.

“For the wider biological audience to really take notice,” says Pawluk, now an editor at *Cell*, “it had to be Cas9”.

In December 2016, Pawluk, still working in Davidson’s lab, and Bondy-Denomy, leading his own independent research group, each identified inhibitors to the Cas9 enzyme<sup>2,3</sup>. This time, researchers around the world seized on the findings. “Like everything else in the CRISPR world, the thin edge of the wedge comes in, and the next thing you know all hell breaks loose,” says Erik Sontheimer, a molecular biologist at the University of Massachusetts Medical School in Worcester and a co-author on Pawluk’s paper<sup>2</sup>.

In less than three months, structural biologists at the Harbin Institute of Technology

in China had deciphered the molecular mechanism by which one of Bondy-Denomy’s anti-CRISPR proteins, called AcrIIA4, shut off Cas9 activity<sup>4</sup> (see ‘CRISPR correctives’). A few months later, Doudna, working with Bondy-Denomy and biochemist Jacob Corn, now at the Swiss Federal Institute of Technology in Zürich, offered the first demonstration that anti-CRISPRs had practical value: they showed not only that delivering AcrIIA4 into human cells, either alongside or right after introducing Cas9, could halt gene-editing activity in its tracks, but also that it could limit the ‘off-target’ effects that researchers and investors have fretted over since early in CRISPR’s development<sup>5</sup>.

Curbing off-target activity would be a big



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contribution to the field of CRISPR therapeutics, says David Rabuka, chief executive and cofounder with Bondy-Denomy of Acrigen Biosciences, based in Berkeley. The company’s pitch: “We’re going to make gene editing more efficient and safer,” Rabuka says.

Anti-CRISPRs could also help to confine editing activity to particular cells and tissues in the body. In 2019, research teams in Germany, Japan and the United States independently attempted to use the proteins in tandem with small regulatory molecules called microRNAs to bring about tissue-specific editing<sup>6–8</sup>. The US team, led by Sontheimer, even showed that the approach could work in mice – theirs is the only published study so far to demonstrate that anti-CRISPR proteins can work in a living animal, and not just cells<sup>8</sup>.

Sontheimer and his colleagues wanted to allow editing in the liver while suppressing it in all other tissues of the mouse. So they designed an anti-CRISPR protein that would be active everywhere except in the presence of microRNA-122, which is found only in the liver. In the mice, the anti-CRISPR successfully blocked Cas9 editing throughout the body, except in that one organ.

Although the paper focused on liver-directed editing, the platform is “plug and play,” says Sontheimer: any organs that produce a unique microRNA at high expression levels could be targeted in this way, provided

that the anti-CRISPR proteins don’t trigger unwanted immune effects.

### Not immune to challenges

Because of previous exposure to microbes harbouring CRISPR–Cas systems, many people have immune systems that are already primed to attack and disable the Cas9 protein. That could pose a challenge. In mice, just one dose of a CRISPR-based medicine can elicit a strong enough immune response to render subsequent treatments ineffective.

According to Sontheimer, anti-CRISPR proteins could be prone to the same rejection issue, potentially imperilling the technology and triggering dangerous, inflammatory reactions in patients.

Other types of CRISPR inhibitor shouldn’t have the same limitation. Last May, a team led by Amit Choudhary, a chemical biologist at the Broad Institute of MIT and Harvard in Cambridge, Massachusetts, described a new way of identifying small-molecule drugs capable of disrupting Cas9 activity<sup>9</sup>. The compounds his team identified are not as potent as natural anti-CRISPR proteins, but they are more likely to sneak past the immune system, to cross cell barriers and to allow for reversible control of Cas9 activity.

Elsewhere, researchers have designed short strings of nucleic acids that grab onto two parts of the Cas9 complex and completely shut down gene editing in human cells<sup>10</sup>. “We’re pretty sure that what we have works better than all the best anti-CRISPR proteins out there already,” says Keith Gagnon, an RNA biochemist at Southern Illinois University in Carbondale who led the research. And other groups, including virologist Brooke Harmon’s at Sandia National Laboratories in Livermore, California, have synthesized tiny protein fragments that show potential as anti-CRISPR agents. “It’s nice to have a lot of different options,” Harmon says.

That diversity could be important in medical applications: for example, in limiting the editing activity of gene-targeted medicines, or fashioning phage therapies capable of wiping out difficult-to-treat bacteria without being stymied by the pathogen’s own CRISPR defences. It might also help in other proposed applications of CRISPR-blocking technologies.

Take gene-drive systems, in which scientists deploy CRISPR gene editing to spread a DNA modification swiftly through an entire population. Some public-health officials hope that the technique might allow for the complete eradication of disease-carrying mosquitoes or ticks, for example.

But concerns over unforeseen ecological impacts abound. Many public officials and researchers also worry about gene drives being weaponized to wipe out agricultural systems or to spread a deadly disease.

Anti-CRISPRs could provide a molecular safety net against these potential bio-attacks,



says Sandia biochemist Joe Schoeniger. “You need to have an off-button,” he says.

For now, such applications are mostly hypothetical. The only published report of researchers using anti-CRISPR proteins to inhibit a gene drive comes from a proof-of-principle experiment in yeast<sup>11</sup>. However, the idea is gaining traction, including among researchers hoping to halt the spread of malaria by forcing harmful genes to spread through an entire population of mosquitoes.

Andrea Crisanti, a molecular parasitologist at Imperial College London, says that he has used anti-CRISPR genes to halt a mosquito-eradicating gene-drive system. The gene drive, which disrupts female fertility, can wipe out mosquitoes in the lab in about ten generations<sup>12</sup>. But in unpublished work, his team has added anti-drive mosquitoes to the mix, and “they can completely, 100% block the drive”, Crisanti says. “We can stop the population from crashing.”

## Insurance policy

As Crisanti looks ahead to field-testing his sterilization strategy, he imagines having cages of anti-drive mosquitoes at the ready, just in case things go awry. “It’s kind of like buying an insurance,” he says.

But the need for CRISPR containment goes beyond gene drives. “If there’s an adverse event in a clinical trial or a nefarious use of a genome editor, we’re not going to know what that looks like until it happens,” says Renee Wegrzyn, a biosecurity scientist at the US government’s Defense Advanced Research Projects Agency (DARPA) in Arlington, Virginia.

That’s why DARPA, in 2017, launched the Safe Genes programme, a four-year, US\$65-million initiative aimed at combating the dangers of CRISPR technologies. This has involved discovering new inhibitors against all types of CRISPR–Cas system and finding anti-CRISPRs that function in unique and useful ways. Bondy-Denomy, Choudhary, Crisanti, Doudna and the Sandia team, among others, are all recipients of this funding.

Beyond its biotechnology applications, the anti-CRISPR strategy is opening up fresh possibilities for basic research, too. “It’s become one of our favourite tools,” says Shawn Liu, a neuro-epigeneticist at Columbia University Medical Center in New York City. Liu studies how a modified CRISPR–Cas9 system can change the expression levels of a gene through epigenetic modifications – that is, without altering the underlying sequence. Anti-CRISPR proteins helped him to show how long the effects lasted<sup>13</sup>.

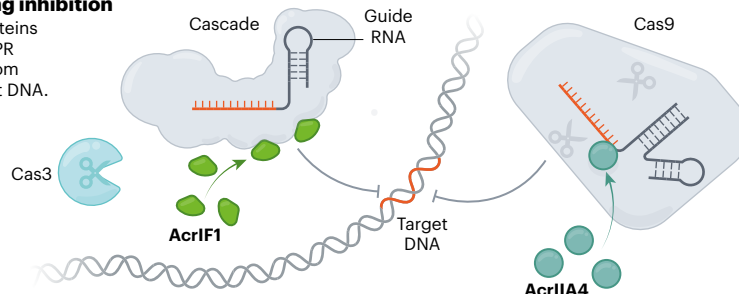
They also came in handy when researchers were looking for mutant strains of bacteria that could fend off phage attacks more effectively than standard ones. A team led by Sylvain Moineau, a phage biologist at Laval University in Quebec City, Canada, focused on *Streptococcus*

## CRISPR CORRECTIVES

There are two particularly well-studied types of CRISPR DNA editing. Type I uses a Cascade complex and guide RNA to bind a DNA target, which is then cut with the Cas3 enzyme. Type II uses a single enzyme, such as Cas9, to bind and cut the target sequence. Researchers have discovered more than 50 anti-CRISPR (Acr) proteins that turn off DNA-editing activity in a variety of ways. Here are two commonly observed mechanisms.

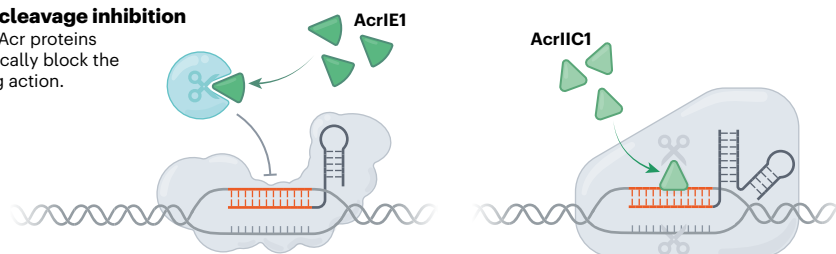
### DNA-binding inhibition

Some Acr proteins prevent CRISPR complexes from binding target DNA.



### DNA-cleavage inhibition

Some Acr proteins specifically block the cutting action.



*thermophilus*, a microbe used to make cheese and yoghurt<sup>14</sup>. “We used a phage containing an anti-CRISPR protein as a tool to find other defence mechanisms,” he explains.

Other scientists are incorporating anti-CRISPRs into tools such as biosensors that can track how much of a therapeutic gene editor is active inside cells, and optogenetic control strategies that allow researchers to switch on Cas9 genome targeting at the flick of a laser beam.

“A lot of it is still in the stage of ‘toy’ systems,” says Chase Beisel, a bioengineer at the Helmholtz Institute for RNA-based Infection Research in Würzburg, Germany. “But the concept is there, at least.”

## Open questions

As bioengineers continue to tinker with anti-CRISPRs, and as companies such as Acrigen move to introduce the inhibitors into therapeutic platforms, some biologists have also begun to grapple with more philosophical questions about the evolution of CRISPR–Cas systems in the first place. If bacteria with intact CRISPR protections commonly harbour phage-derived sequences for inhibitors that neutralize this immunity, then “CRISPR is clearly not doing its defence role in many of those cases”, says Edze Westra, who studies the ecology of CRISPR systems at the University of Exeter, UK. And yet, natural selection seems to maintain the system in working order. So, he asks, “what is its role apart from fuelling biotech start-up companies?”

Some studies point to bacteria using CRISPR–Cas systems in forming biofilms, repairing DNA and conducting other regulatory processes

involved in enhancing virulence. And it’s possible that once anti-CRISPRs have defanged Cas enzymes of their DNA-cutting abilities, bacteria will have repurposed the gene editors for other uses, says Maxwell, the University of Toronto microbiologist.

Those bedeviling mysteries won’t halt the steady march of CRISPR gene editing into human therapeutics, pest control and more. And for many, that’s why anti-CRISPRs are so important.

“There needs to be this shift to really controlling these editors so we make sure that you get the change you want and nothing else,” says Doudna. And just as the CRISPR–Cas systems that ushered in a biotechnology revolution started with a few curious observations in a laboratory, she notes, so too did the discovery of the inhibitors that could be a much-needed corrective.

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**Correction****Finding the CRISPR off-switch**

This Feature implied that the first practical demonstration of anti-CRISPR activity was in halting the gene-editing process in human cells. In fact, it was showing that anti-CRISPRs could limit off-target effects in human cells.