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Study Unlocks Multiple Functions of CRISPR/Cas9 by Varying Guide RNAs

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NEW YORK (GenomeWeb) – Scientists have found a way to induce both gene activation and gene editing at the same time, in the same cell, using only one kind of Cas9 enzyme. The finding opens the door for multiplexing the various CRISPR/Cas9 applications that scientists have developed in the last few years, which could enable more complex studies of synthetic and endogenous gene circuits.

Led by Samira Kiani and Ron Weiss of MIT and Alex Chavez and George Church of Harvard University's Wyss Institute for Biologically Inspired Engineering, the researchers published their results on the flexibility of Cas9 enzymes this week in *Nature Methods*.

The key discovery was that guide RNAs (gRNA) with shorter complementary regions still allow the Cas9 protein to bind DNA, but not to cut it. "Something about the length [of the complementary section] prevents Cas9 from becoming informatically competent to cut the DNA," Chavez, a postdoc in Church's lab, told GenomeWeb. "We were able to exploit that for some really cool biotechnology applications."

The gRNA is what makes CRISPR/Cas9 a programmable and specific genome editing technology, and a full-length guide has a section 20 nucleotides long that is complementary to the target DNA. Previous studies had shown that truncated guide RNAs, with lengths (the region of the gRNA complementary to the DNA target) of 17 or 18 base pairs, resulted in less off-target cleavage activity. But with even shorter gRNAs, the Cas9 enzyme no longer makes a double-stranded break.

One hypothesis suggested that biophysics prevented the enzyme from binding to the DNA at all. But the study gives credence to a second hypothesis, that the length of the gRNA affects the function of the Cas9 protein and its ability to cut. It's possible that shorter guides affect the conformation of Cas9's nuclease domain, Kiani said, permitting it to bind to DNA but preventing it from making a double-stranded break.

Using this knowledge, the postdocs were able to get Cas9-VPR, a protein fusion engineered to cut DNA as well as promote gene expression, to do both functions in the same cell. By providing one 20 basepair-long gRNA, and one 14 basepair-long gRNA, they were able to get Cas9-VPR to cut at one target and activate expression at the other.

The finding means researchers can knock out, repress, and activate gene targets all at once using the best-characterized Cas9 available. The study's first authors make the case that it will allow scientists to

build better synthetic gene circuits and tease apart the function of endogenous ones.

The guide RNA has always been the key to CRISPR/Cas9 genome editing and related applications, telling the Cas9 enzyme where it needs to go. When Jennifer Doudna, Emmanuelle Charpentier, and Martin Jinek introduced the first single guide RNA, they gave it the 20 nucleotide region complementary to the target DNA, mimicking the CRISPR RNAs (crRNA) found in bacterial CRISPR systems. In 2014, scientists in Keith Joung's lab at Harvard discovered that shortening the complementary section of gRNA resulted in less off-target activity. But truncation had diminishing returns.

"If you keep shrinking [the gRNA length], suddenly you're no longer able to see Cas9 go where you want it to go," Chavez explained. Shrink it too much, and the enzyme doesn't cut at all. The prevailing theory was that the gRNA-Cas9 complex didn't have enough energy to bind to DNA, he said, but he and Kiani decided to see if that was really the case. It wasn't.

"The truncated gRNA actually becomes unable to activate the nuclease domain of the Cas9," Kiani explained. To demonstrate this, they used a Cas9 fused to the activation domain VPR (Cas9-VPR) and targeted a fluorescent reporter gene. With 20 nucleotide-long gRNAs, the Cas9-VPR showed nuclease activity similar to the wild-type Cas9, indicating that the enzyme could successfully cleave DNA. When they switched to much shorter gRNAs, it activated the reporter gene, indicating that the enzyme was indeed binding to DNA, but not cutting.

They discovered that the same protein can have multiple functions, depending on the length of the gRNA provided. Kiani and Chavez want to validate their findings with more detailed structural and biochemical analysis of the Cas9 enzyme, but said that their results make sense given CRISPR/Cas9's evolutionary history as a system that bacteria evolved to fight phages.

"It's like a checking mechanism," Chavez suggested. "If [the enzyme binds] to something with a little complementarity, you don't want to cut that, you need to make sure you have full complementarity. It'd be very dangerous to just cut anything. You want some sort of checks and balances."

Kiani and Chavez said that their research will vastly improve multiplexed CRISPR/Cas9 applications by allowing researchers to use the Cas9 from *Streptococcus pyogenes* (spCas9) for multiple purposes in the same cell.

The gold standard protein for CRISPR/Cas9 is spCas9, Chavez said. It's the most well-studied and most useable of the myriad Cas9s available from different bacterial species. "People have engineered spCas9 like crazy," he said, giving it the ability to activate or repress expression, make single stranded cuts, track spatiotemporal changes in the nucleus, and more.

Scientists had been able to introduce a second CRISPR/Cas9 application into a cell, but needed to use an orthologous, sub-opitmal Cas9 from a species other than *S. pyogenes*. Cutting DNA and activating gene expression at the same time had been possible, Chavez said, but difficult. The other Cas9 variants just aren't as good as spCas9, he said, and simply getting the two different Cas9 enzymes into the same cell was challenging.

Now, dual CRISPR applications won't be so hard to pull off. "With a single lentiviral vector we can do two different functions at the same time," Kiani said.

This is especially handy for cell lines and model organisms that scientists engineered to inherently express the wild-type spCas9 enzyme, which does have an active nuclease domain and lacks a way to

promote gene expression. With a shorter gRNA, the cutting enzyme can become a repressor; there's even a way to modify another section of the gRNA to turn that enzyme into an activator, they said, using RNA aptamers.

This could have major implications for research into endogenous genetic circuits. "Right now what we do is pull on one lever and say what happens, but the cell when it actually executes a function, it actually pulls 50 levers," Chavez said. "Now we can pull on two or more different levers. We can stop one pathway and activate another. Doing that, we can finally decipher these complex interactions."

He envisions a situation where a researcher constructs a gRNA library to activate a gene of interest and simultaneously knock out every other gene it may be dependent on to have its effect.

The authors demonstrated another potential application by constructing a synthetic gene circuit with a kill switch enabled by varying the gRNA length.

Synthetic biologists are always trying to increase the complexity of their circuits, Kiani explained. "What has been lacking in mammalian synthetic biology is the ability to make multilayered complex synthetic circuits that allow us to have control over the system. If you have a system where you have a gRNA expressed, and have another gRNA controlling expression of that [first] gRNA, and so on, you can have more control of your outcome."

Getting Cas9 to be both a transcriptional modulator and a DNA cleaving nuclease could open up possibilities to develop more complex circuits, she said, and could let scientists build in new kinds of important kill switches. "[Kill switches] are gaining more and more importance as we develop cell- and gene-based therapies," Kiani said.

In the study, Kiani designed a proof-of-concept series of increasingly complex kill switches. "All were designed such that that circuit can be a simple genetic part that leads to activating or repressing something," she said, and all were designed such that when the full length gRNA is added, the function of the circuit is disabled.

She didn't test the kill switches in a real biomedical problem, but suggested that it could have immediate application in chimeric antigen receptor T cells being developed as cancer therapies.

"You are always faced with the unwanted effect of when CAR T cells will kill healthy cells in normal tissue," she said. "More and more, people are looking into developing safety switches such that when adverse effects happen, they can stop it by killing the cells or killing expression of transgenes such as the chimeric antigen receptor," Kiani said.

Kiani and Chavez are extremely bullish that their findings will make CRISPR/Cas9 an even more powerful tool for basic research. "We're just on the tip of the iceberg," Chavez said. "The rules are still not well understood. [CRISPR/Cas9] does not work as you would exactly expect. It has intricacies that are waiting to be discovered."

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