CRISPR-Cas9 gene editing: Check three times, cut once

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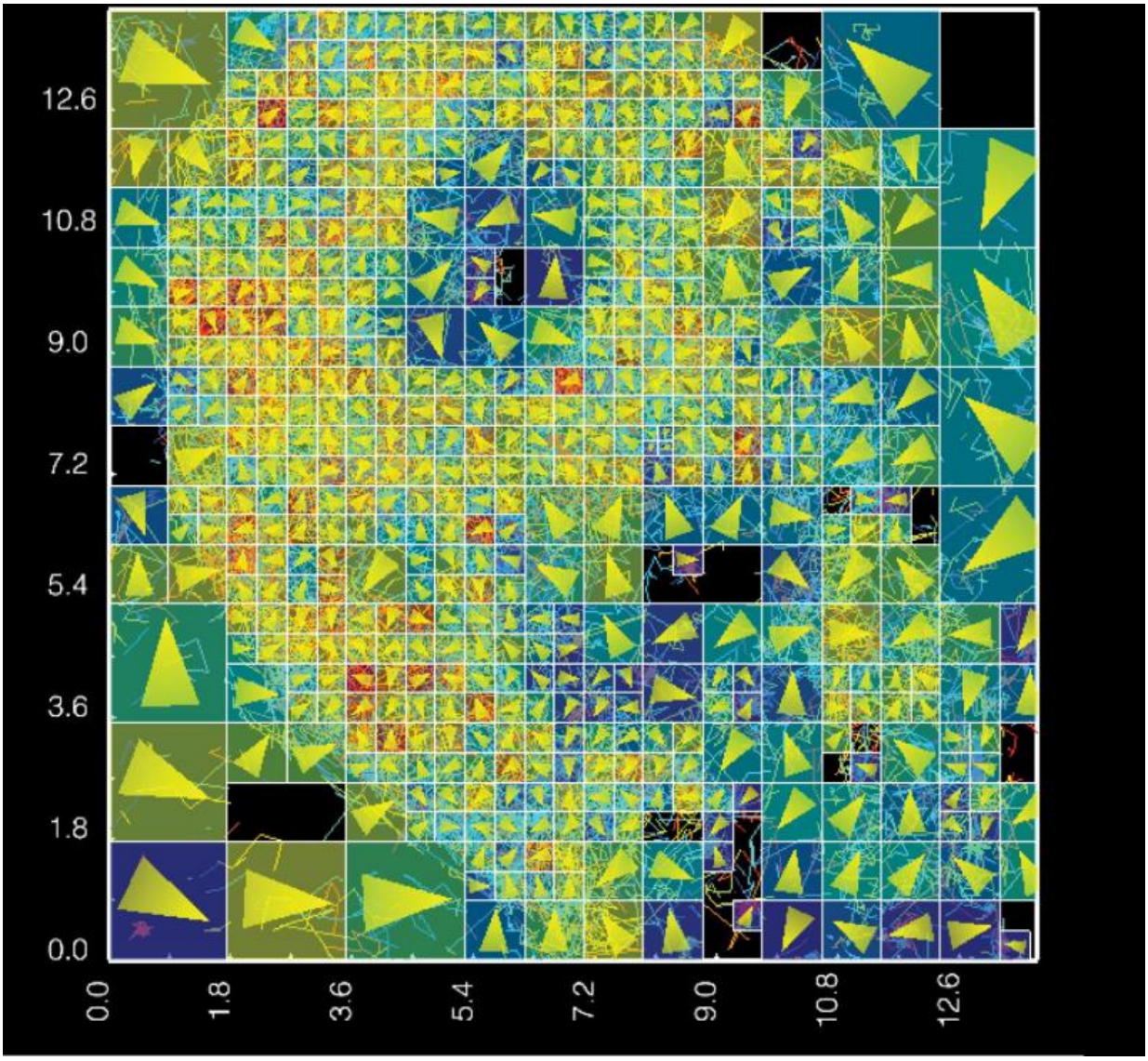

Bayesian color map depicting Cas9 particle movement in different regions of a mammalian cell nucleus. Red/orange denotes faster movement, and blue denotes

slower movement. Cas9 movement is generally more restricted in silenced/lowly expressed regions of the genome known as heterochromatin. Credit: UC Berkeley/HHMI

Two new studies from the University of California, Berkeley, should give scientists who use CRISPR-Cas9 for genome engineering greater confidence that they won't inadvertently edit the wrong DNA.

The gene editing technique, created by UC Berkeley biochemist Jennifer Doudna and her European colleague Emmanuelle Charpentier, has taken the research and clinical communities by storm as an easy and cheap way to make precise changes in DNA in order to disable genes, correct genetic disorders or insert mutated genes into animals to create models of human disease.

The two new reports from Doudna's lab and that of UC Berkeley colleague Robert Tjian show in much greater detail how the Cas9 protein searches through billions of base pairs in a cell to find the right DNA sequence, and how Cas9 determines whether to bind, or bind and cut, thereby initiating gene editing. Based on these experiments, Cas9 appears to have at least three ways of checking to make sure it finds the right target DNA before it takes the irrevocable step of making a cut.

"CRISPR-Cas9 has evolved for accurate DNA targeting, and we now understand the molecular basis for its seek-and-cleave activity, which helps limit off-target DNA editing," said Doudna, a Howard Hughes Medical Institute investigator at UC Berkeley and professor of molecular and cell biology and of chemistry. Tjian is president of the Howard Hughes Medical Institute and a UC Berkeley professor of molecular and cell biology.

The studies also illustrate how well CRISPR/Cas9 works in human and animal cells - eukaryotes - even though "the technique was invented by bacteria to protect themselves from getting the flu," Doudna said.

CRISPR-Cas9 is a hybrid of protein and RNA - the cousin to DNA - that functions as an efficient search-and-snip system in bacteria. It arose as a way to recognize and kill viruses, but Doudna and Charpentier realized that it could also work well in other cells, including humans, to facilitate genome editing. The Cas9 protein, obtained from the bacteria Streptococcus pyogenes, functions together with a "guide" RNA that targets a complementary 20-nucleotide stretch of DNA. Once the RNA identifies a sequence matching these nucleotides, Cas9 cuts the double-stranded DNA helix.

One study, published in the Nov. 13 issue of *Science*, tracked Cas9-RNA molecules though the nucleus of mammalian cells as they rapidly searched through the entire genome to find and bind just the region targeted and no other.

"It's crazy that the Cas9 complex manages to scan the vast space of eukaryotic genomes," said graduate student Spencer Knight, first author of the *Science* paper.

Previous studies had suggested that there are many similar-looking DNA regions that Cas9 could bind and cut, which could limit its usefulness if precision were important. These off-target regions might share as few as four or five nucleotides with the 20-nucleotide primer, just enough for Cas9 to recognize.

"There is a lot of off-target binding by Cas9, but we found that these interactions are very brief - from milliseconds to seconds - before Cas9 moves on," he said.

Because these exploratory bindings - perhaps as many as 300,000 of them - are often very short-lived, a few thousand CRISPR-Cas9 complexes can scour the entire genome to find one targeted stretch of DNA. Cas9 must also recognize a short three-base-pair DNA sequence immediately following the primer sequence, dubbed PAM, which occurs about 300 million times within the human genome.

"If Cas9 bound for tens of seconds or minutes at each off-target site, it would never, ever be able to find a target and cut in a timely manner," Knight said.

Cas9's final checkpoint

The other study, published online Oct. 28 in *Nature*, showed that once Cas9 binds to a region of DNA, it performs another check before two distant sections of the Cas9 protein complex come together, like the blades of a scissors, to precisely align the active sites that cut double-stranded DNA.

"We found that RNA-guided Cas9 can bind some off-target DNA sequences, which differ from the correct target by just a few mutations, very tightly. Surprisingly, though, the region of Cas9 that does the cutting is inhibited because of the imperfect match. But when the correctly matching DNA is located, Cas9 undergoes a large structural change that releases this inhibition and triggers DNA cutting," said first author Samuel Sternberg, who recently received his Ph.D. at UC Berkeley. He was able to observe these changes using a fluorescently labeled version of the Cas9 complex.

"We think that this structural change is the last checkpoint, or proofreading stage, of the DNA targeting reaction," he said. "First, Cas9 recognizes a short DNA segment next to the target - the PAM - then the target DNA is matched up with the guide RNA via Watson-Crick base-

pairing. Finally, when a perfect match is identified, the last part of the protein swings into place to enable cutting and initiate genome editing."

A smaller Cas9 protein from a different species of bacteria, Staphylococcus aureus, likely exploits the same strategy to improve the precision of DNA targeting, suggesting that "this important feature has been preserved throughout evolutionary time," he added.

"This is good news, in that it suggests that you have more than one checkpoint to ensure correct Cas9 binding," Knight said. "There's not just sequence regulation, there is also temporal regulation: it has to engage with the DNA and park long enough that it can actually rearrange and cut."

The discoveries from Doudna, Tjian and their teams shed light on the molecular basis of off-target effects during genome editing applications, and may guide the future design of more accurate Cas9 variants.

More information: "Dynamics of CRISPR-Cas9 genome interrogation in living cells," *Science*, www.sciencemag.org/lookup/doi/… 1126/science.aac6572

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