BIOCHEMISTRY

CRISPR snapshots of a gene-editing tool

From single-molecule functional studies to atomic-resolution structures, a windfall of data sheds light on the Cas9 mechanism of targeted DNA scission.

An inexhaustible source of research tools for genetic trickery, bacteria have recently delivered another revolutionary technology. The Cas9 endonuclease, which in nature uses short guide RNAs to protect bacteria against genome invaders, has been successfully harnessed for on-demand DNA targeting in multiple cell types and organisms. Now three separate studies take a closer look at Cas9 structure and function.

For Jennifer Doudna, a biochemist and RNA-interference aficionado at the University of California (UC), Berkeley, it all started in the early 2000s with a basic scientific question: could bacterial clustered, regularly interspaced, short palindromic repeats (CRISPR) and their associated (*cas*) genes represent an RNA interference–like immune pathway?

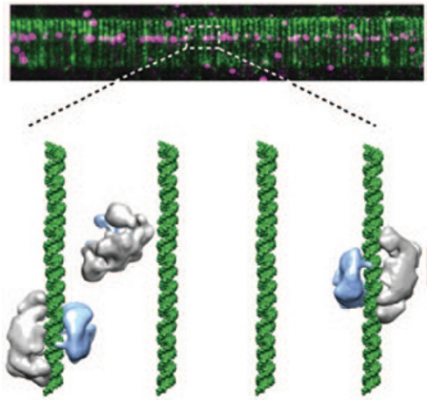
"We certainly did not set out to discover a genome engineering technology," says Doudna. But when she and Emmanuelle Charpentier at the Helmholtz Center for Infection Research later realized that Cas9 not only provides adaptive immunity in many bacteria but can also be reprogrammed by a single RNA to target virtually any DNA sequence, the potential for genome engineering was too blatant to ignore. Unlike zinc-finger nucleases and transcription activator–like effector nucleases, "this system utilizes a single protein whose specificity is changed by simply altering the guide RNA, and it is very amenable to targeting multiple sequences in the same cell," explains Doudna.

Despite the simplicity and broad adoption of the technology, however, the molecular mechanisms of Cas9 function have remained enigmatic. To study the enzyme in action, Doudna's group joined forces with that of Eric Greene at Columbia University. The team generated a quantum dot–labeled Cas9 variant and used a DNA-curtain assay to monitor the action of single Cas9-RNA complexes on tethered DNA substrates by total-internal-reflection fluorescence microscopy (Sternberg *et al.*, 2014). Using unlabeled substrate competitors, the researchers were able to probe binding kinetics and homology requirements for target recognition.

Both DNA binding and cleavage were shown to require the recognition of a trinucleotide sequence (protospacer-adjacent motif, or PAM) that is not part of but is adjacent to the RNA-targeted sequence. Whereas Cas9-RNA bound to PAM-enriched regions even in the absence of target sequences, it did not detect even perfectly homologous targets without an intact PAM. DNA melting and RNA-DNA hybridization were shown to originate at the PAM and then spread towards the distal end of the target. And although this master role for PAMs was unexpected, Doudna hopes that scientists can use the findings to minimize off-target effects by monitoring PAM distribution and PAM-adjacent sequences in engineered genomes.

In a separate study, Doudna's group along with that of her UC Berkeley colleague Eva Nogales set out to reveal the structural basis for Cas9 function (Jinek *et al.*, 2014). They solved crystal structures of the apo forms of two different Cas9 enzymes, each representing a major Cas9 subtype. The structures revealed bi-lobed enzyme architecture, with a common catalytic core hosted in one of the lobes and a divergent α-helical domain in the other. The latter is substantially smaller in one of the Cas9 enzymes, and this evolutionary variability could be further exploited to engineer more compact Cas9 variants or to insert additional functionalities, explains Doudna. The authors identified the putative nucleic acid binding clefts and PAM recognition motifs, as well as the structural requirements for PAM sequence specificity.

Interestingly, in both structures the enzymes appeared autoinhibited, with catalytic residues facing away from the target-binding clefts. To capture the conformation of the guide- and target-bound forms, the authors turned to single-particle electron microscopy. The three-dimensional structure reconstructions showed that nucleic acid–bound Cas9 exhibited a massive conformational change that repositions the two lobes to clasp around the target sequence. Such a conformational rearrangement was confirmed by an independent report from the groups of Feng Zheng at the Broad Institute and Osamu Nureki at the University of Tokyo that presented the crystal structure of a Cas9-RNA-DNA complex (Nishimasu *et al.*, 2014). "What we really now want to understand is whether this large conformational change is actually part of the mechanism to open the double-stranded DNA duplex," says Doudna, explaining that the enzyme has no intrinsic or coupled ATPase activity. Hopefully further mechanistic snapshots will follow.



Structural and single-molecule fluorescence studies provide insight into the Cas9 mechanism. Figure reproduced with permission from J. Doudna, D. Taylor and S. Sternberg.

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RESEARCH PAPERS

Jinek, M. *et al.* Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. *Science* doi:10.1126/science.1247997 (6 February 2014).

Nishimasu, H. *et al.* Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell* **156**, 935–949 (2014).

Sternberg, S.H. *et al.* DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature* **507**, 62–67 (2014).