



RESEARCH HIGHLIGHT

CasX: a new and small CRISPR gene-editing protein

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In a recent paper published in *Nature*, Liu et al. demonstrate the DNA cleavage characteristics of CasX (a newly identified dual-RNA-guided DNA targeting nuclease) and establish its capacity for genome editing in *Escherichia coli* and human cells. Furthermore, they capture structural snapshots of CasX in different states by cryo-EM and establish a model of RNA assembly, DNA targeting and sequential cleavage.

Bacteria and archaea possess CRISPR-Cas-mediated adaptive immune systems to defend against invading foreign nucleic acids.¹ The targeting and cleavage are achieved by a single crRNA-bound Cas protein in Class 2 CRISPR-Cas systems.^{2,3} Class 2 systems can be subdivided into Type II Cas9 and Type V Cas12 that target DNA, as well as Type VI Cas13 that target RNA. Cas9 and Cas12a/Cpf1 have been well studied in the past and broadly harnessed for gene editing in various cell types and organisms in prokaryotes and eukaryotes.^{4–6} Both Cas9 and Cas12 systems discriminate self from non-self DNA targets by protospacer adjacent motifs (PAMs) and generate double strand breaks. However, the binding and cleavage of DNA by Cas9 and Cas12 are quite different. Cas9 recognizes a 3'-G-rich PAM and produces blunt ends cleaved by the RuvC and HNH domains, whereas Cas12 recognizes a 5'-T-rich PAM and produces staggered ends cleaved solely by the RuvC domain.

CasX was identified by metagenomic analysis of bacteria from groundwater and characterized as an RNA-guided DNA nuclease.⁷ It recognizes a 5'-TTCN PAM and is capable of plasmid interference in *E. coli* when presenting sgRNA (covalently linked crRNA-tracrRNA). It shares no similarity to other reported Cas endonucleases except for a RuvC domain located at the C-terminus. The above features of CasX correlate with those of type V Cas12; however, the size of CasX (~980 aa) is smaller than those of reported Cas12 (~1200 aa).

In a recent *Nature* paper, Liu et al.⁸ found that sgRNA-bound *Deltaproteobacteria* CasX (DpbCasX) contains 20-nt guide segment and recognizes a TTCN PAM element resulting in dsDNA target cleavage with 10-nt staggered ends. It has been reported that Cas12 exhibits PAM-independent non-specific ssDNA *trans*-cleavage activity when triggered by binding to a crRNA-guide-complementary ssDNA.^{7,9} Liu et al. found that the *trans*-ssDNA cleavage activity of CasX is significantly lower than those of Cas12a and Cas12b. They further investigated the potential of CasX for gene editing in *E. coli* and human cells. They found that DpbCasX has the capability to cleave the target gene in *E. coli* cells, while its deactivated counterpart is able to bind target genes and reduce gene expression. Moreover, they also show that the related *Planctomycetes* CasX (PlmCasX) has gene editing ability in human HEK293T cells. All these data shed novel insights into

the development of CasX as a new gene editing and CRISPRi tool.

The authors further determined a series of cryo-EM structures of CasX in different states to uncover targeting and cleavage mechanisms. The domain composition of CasX-sgRNA-DNA ternary complexes showed some similarity to that of Cas12; however, each structural element adopts distinct folds. They also made efforts at structure determination of apo CasX and CasX-sgRNA and obtained CasX-sgRNA maps at low resolution (7.5 Å). In combination with mass spectrometry data, they found that sgRNA assembly and DNA loading trigger domain rearrangements.

The authors used two different dsDNAs to generate CasX-sgRNA-DNA ternary complexes to investigate the cleavage mechanism. One dsDNA contains non-target DNA strand (30 bp) and the other forms a full R loop (45 bp). Interestingly, the particle sorting of both complexes revealed two states, namely target-strand DNA cleavage and non-target-strand DNA cleavage, with two thirds of particles adopting the non-target-strand DNA cleavage state. The authors then proposed a sequential model of DNA cleavage, whereby the non-target strand was cleaved first by the RuvC domain followed by cleavage of the target strand. To test this model, they designed a dsDNA containing extended target strand (45 nt) and a shorter non-target strand (20 nt) to mimic an intermediate state after non-target-strand cleavage. As expected, they found that the majority of particles were shifted to the target-strand cleavage state. Specifically, two domains are critical for DNA cleavage. The target-strand loading (TSL) domain adjacent to the target strand DNA is located at the corresponding position of the Nuc domain of Cas12 and involved in DNA strand separation. The non-target-strand binding (NTSB) domain moves towards and interacts with non-target strand DNA upon DNA binding. The NTSB domain is essential for dsDNA cleavage but not *trans*-ssDNA cleavage, indicating that this domain is involved in DNA unwinding.

Taken together, the contribution by Liu et al.⁸ has uncovered mechanisms of new Cas member CasX for DNA unwinding, cleavage of both DNA strands, and domain movements during transition between two states. Their findings raise the prospect for development of CasX-based gene editing tools. The commonly used *Streptococcus pyogenes* Cas9 (SpyCas9, 1368 aa) together with sgRNA and promoters has a molecular weight within the size limitation range of a single adeno-associated virus (AAV). To improve delivery efficiency, one solution is the usage of smaller Cas9 orthologs with similar gene editing efficiency, such as *Staphylococcus aureus* (SauCas9, 1053 aa), *Neisseria meningitidis* Cas9 (NmeCas9, 1082 aa), and *S. thermophilus* 1 (St1Cas9, 1121 aa),

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which are relatively smaller and have been successfully harnessed for gene editing in mammalian cells.¹⁰ However, unlike SpyCas9 recognition of GG PAM, these Cas9 orthologs recognize relatively longer PAMs (4–5 nt), resulting in a limited targeting scope.¹⁰ CasX (<1000 aa) is smaller than these Cas9 and typical Cas12, implying its advantage for AAV-mediated delivery and providing more room for other gene editing accessories or tools. More research remains to be done to optimize CasX-based applications ranging from the impact of PAM targeting scope, to off-target effects, DNA specificity, and editing efficiency in different cell types. The current resolution of the two CasX ternary complex states are in the 3.2 and 4.2 Å range. Further improvements in resolution are required for obtaining atomic models that could provide more detailed information and guide engineering efforts at optimization of CasX applications in the future.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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