



RNA-guided nucleases: A new crispr subtype

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DESCRIPTION

Different enzymes have been repurposed for biotechnological applications, including programmable, RNA-guided nucleases. However, in order to broaden the therapeutic applications of these tools, targetable systems that are small enough to be delivered efficiently are required. We mined an extensive genome-resolved metagenomic database to identify families of previously unknown RNA-guided, compact nucleases. Cas9d, a new CRISPR type II subtype, has zinc-finger motifs and a high arginine content, both of which we found in nucleases related to HEARO effectors. These enzymes have a wide range of biochemical properties and can be targeted in a variety of ways. We show that natural Cas9d enzymes can edit the genome with >90% efficiency in mammalian cells, and we engineered nick variants into the smallest base editors active in *E. coli* and human cells. Cas9d and HEARO systems are expected to enable a wide range of genome editing applications due to their small size, broad targeting potential, and translatability. For guide acquisition, targeting, and DNA cleavage, RNA-guided nucleases use a variety of mechanisms. CRISPR systems, for example, are involved in microbial defence against viral infections and are widely used as genome editing tools. SpCas9, the well-studied of these enzymes, has been developed for biotechnological applications and has been used clinically to treat genetic diseases such as sickle cell disease and B-thalassemia, as well as the ocular disease Leber's congenital amaurosis.

In addition to editing via dsDNA breaks, these programmable systems have been designed to modify specific nucleotides in the genome using a technique known as base editing. These systems typically include an engineered Cas9 effector that cleaves only one strand of DNA and is fused to either cytosine or adenosine deaminase to achieve C to T or A to G conversions. However, due to their large size, current Cas9-based nuclease and base editor systems pose a challenge for therapeutic delivery. Cas9 and previously identified IscB proteins are the only proteins that contain both RuvC and HNH catalytic domains. There are numerous orthodox Cas9s with varying characteristics. In *Campylobacter jejuni*, for example, a small, 984-aa Cas9 ortholog with nuclease activity in mouse and human cells was discovered; a small SlugCas9 with a simple NGG PAM was engineered for high specificity and activity; and, most recently, PpCas9, a 1055-aa Cas9 ortholog with a NRT PAM, was shown to be active in human cells. However, little is known about smaller and more divergent homologs, such as IscB and TnpB, which have only recently been shown to be active RNA-guided nucleases. IS200/IS605 insertion sequences, were initially associated with IscB. Altae-Tran, Kannan, and colleagues recently reported that IscB is RNA-guided and programmable, that there is an evolutionary link between IscB and Cas9, and that these systems can be used for genome editing in mammalian cells, albeit with very low editing efficiencies.

Given the promise of RNA-guided systems for genome editing and other applications, we set out to identify and characterise members of nuclease families distantly related to the previously described Cas9 and IscB effectors. We discovered divergent IscB and Cas9 nuclease homologs after mining billions of proteins predicted from microbial genomic fragments assembled from metagenomic sequence data. Unlike most biochemically characterised Cas9 proteins, several of these families contain archaeal domain representatives.

We describe CRISPR Class 2 nucleases II-D and II-C2, as well as compact nucleases related to the IscB and HEARO systems. We demonstrated efficient activity in vitro and in cells for these systems, and we engineered nickases into the smallest adenine and cytosine base editing systems that have demonstrated activity in bacterial and mammalian cells to date. These systems are referred to as "SMART" because they share but have distinct biochemical properties.