Engineering potent, small, chimeric, synthetic, RNA-guided nucleases (sRGN) from four uncharacterized Cas9 genes

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Adeno-associated viruses (AAVs) and lipid nanoparticles (LNP) are among the methods of choice for delivery of nucleases for *in-vivo* genome editing. The widely used and highly active *Streptococcus pyogenes* (Spy) Cas9 nuclease is specific for an advantageously short and non-degenerate PAM sequence. However, the large size of SpyCas9, in combination with required sgRNA and expression elements, presents a challenge to the 4.5 kb DNA packaging limit of AAVs, as well as to the synthesis of long mRNA templates and their stable formulation into LNPs. On the other hand, the best-characterized smaller Cas9s, such as *Staphylococcus aureus* Cas9, frequently recognize degenerate and longer PAMs, which significantly reduce the number of addressable genomic target sites. To address these issues, we evaluated four related, previously uncharacterized Cas9 nucleases of ~1050 amino acids in length. Surprisingly, most were found to be specific for a common, non-degenerate, small PAM motif. Using multiple protein engineering approaches, we altered these genes to generate novel, chimeric, synthetic, RNA-guided nucleases (sRGNs) and demonstrated efficient editing in human cells. Analyses using all 60 possible, single-nucleotide mismatched off-targets of a DNA substrate indicated high overall specificity for each clone. Importantly, the different clones displayed a localized signature of higher specificity for different mismatches at different sites along the target. Thus, we have generated a set of novel, small sRGNs, from which improved nucleases can be selected for particular targets of interest. LNP packaging and *in-vivo* performance vs. SpyCas9 will be reported in a separate abstract. Because these sRGNs were not derived from Spy or S. aureus Cas9s, we are working toward assessing these alternative nucleases regarding the potential for lower occurrence of pre-existing antibodies in human populations. All in all, these engineered, chimeric, synthetic, RNA-guided nucleases are thus expected to be valuable additions to the canon of known genome-editing nucleases that can be employed for human gene therapy applications.