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Chimeric Cas9 allows PAM-Independent Gene Editing

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Commentary

CRISPR-Cas9 has revolutionized gene editing for the last decade, with the ability to quickly and accurately cut DNA, opening the possibility of curing many genetic diseases. Since the first paper of CRISPR to edit DNA in 2012, different enzymes have been discovered and engineered, extending its functions to be able to change DNA bases directly, insert regions of genetic material, and alter gene expression, with new developments constantly adding to its toolbox [1]. The first CRISPR therapy has currently been approved by the US Food and Drug Administration for the treatment of sickle cell *ex vivo*, with some of the first *in vivo* delivery of Cas9 trials currently underway such as for the treatment of hereditary transthyretin, a disease where toxic protein aggregates settle by nerve fibers [2]. These enzymes have been used not only in the treatment of diseases, but also to understand the genome, increase agriculture production, and develop new animal models [3, 4]. At the heart of these advances is the relatively simple mechanism of CRISPR-Cas9. For a CRISPR-Cas9 system to edit, the Cas9-gRNA complex binds the protospacer adjacent motif (PAM) which causes local unwinding of the DNA. The now exposed DNA allows for the hybridization of the chosen gRNA, and subsequent successful cleavage of the strand by the Cas9 [5]. However, a limitation with current CRISPR enzymes is that the PAM sequence is highly specific to each ortholog and prevents editing of many regions, creating the need for editors with reduced PAM requirements.

Previously, various efforts have been made to explore natural orthologs of CRISPR and to engineer existing enzymes for reduced PAM requirements. Bioinformatics pipelines such as Search for PAMs by ALignment Of Targets (SPAMALOT) and Spacer-2PAM have been used to explore the PAM sequences of Cas9 orthologs and to predict the PAM sequence given a CRISPR array [6, 7]. Additionally, extensive screening has been performed to find Cas9 and Cas12 relatives, revealing enzymes with many novel PAMs [8]. Rational engineering efforts have produced enzymes with altered or reduced PAM requirements including enFnCas9 variants which demonstrated high specificity as well as a reduced PAM requirement(5'-NRG/NGR-3') [9-11]. Other engineering efforts, such as directed evolution through random mutagenesis, phage-assisted continuous evolution (PACE), structure-guided evolution, and chimera generation have also been utilized to both relax PAM requirements and create novel variants [8]. Most notably, Chatterjee et al. have engineered Sc++, a CRISPR enzyme with a positive-charged loop, enabling a 5'-NNG-3' PAM, while Walton et al. have developed SpRY, an enzyme with mutations in the PAM interacting domain allowing for a 5'-NRN-3' PAM (R signifies either A or G can be used as a PAM) [12-14]. While these efforts have generated many enzymes that can edit a majority of the genome in combination with one another, an enzyme with the ability to edit any sequence had not previously been discovered or engineered.

Using experimental enzyme techniques and computational modeling, Zhao et al. have developed SpRYc, a chimeric enzyme consisting of SpRY and Sc++ [14]. They hypothesized that combining the Pam-interacting domain (PID) of SpRY and the N-terminus of Sc++ would allow for the reduced PAM requirements of each respective enzyme. Notably, Zhao et al. demonstrate that the enzyme has a 5'-NNN-3' PAM, allowing for genome editing of the entire genome [14]. Using PAM-SCANR, a NOT gated assay where a library of DNA sequences is introduced to the enzyme and PAM binding induces a GFP signal, SpRYc was shown to be able to edit with

only a minimal preference for the adenosine in the second position [15]. Further experimentation using HT-PAMDA, an assay where the cleavage site of a DNA library is sequenced *in vitro*, demonstrates that while this Cas9 has a wider targeting ability than previously characterized editors, the cleavage efficacy is lower, leading Zhao et al. to suggest that SpRYc may be more suited towards base editing rather than functioning as an endonuclease for direct DNA editing [14, 16]. The enzyme was then fused to the ABE8 adenine base editor and was shown to be able to edit at all PAM locations while demonstrating significantly more efficient conversion of Adenine to Guanine compared to SpRY ABE8 at 5'-NTN-3' and 5'-NNT-3' PAMs. Using both the endonuclease and the ABE8 base editor, therapeutically relevant edits were made, demonstrating the clinical applicability of SpRYc. Rett syndrome, a neurological disorder associated with impaired brain function and caused by various C to T point mutations, was corrected using the base editor (C502T) [14, 17]. Due to the 5'-NCN-3' or 5'-NTN-3' Pam required for the edit, this mutation was inaccessible to previously developed base editors [14, 17]. Additionally, Huntington's disease, a neuronal disease caused by repeats of CAG on the Huntington gene and characterized by brain impairment and neuronal death, was silenced by the SpRYc endonuclease [18]. Finally, the mechanism of action was explored using SWISS-MODEL, where the positively-charged loop of Sc++ was hypothesized to relax the PAM requirement of position 2 with sequence non-specific interactions, and the mutations of SpRY and ScCas9 were shown to result in nonspecific backbone interactions, allowing for the PAM leniency of SpRYc [14].

Going forward, it may be interesting to see further applications of this enzyme, such as expanding the base editing capabilities with a C to T base editor, or a prime editing enzyme. New applications for both disease and scientific models could be explored using this PAM-free enzyme. Additionally, the increased specificity of SpRYc despite the more lenient PAM requirement compared to SpRY raises the question of how PAM domains affect specificity. This observation emphasizes further mechanistic studies on how Cas9 interrogates DNA and how SpRYc binds *in vitro* in comparison to its predecessors. *In vivo,* assays and cryo-electron microscopy could be used to gain further insights into the activity of the enzyme to further validate the mechanism of action seen in the homology modeling. Finally, with the development of gene delivery vehicles, such as adeno-associated virus (AAV) and lipid nanoparticles (LNP), the therapeutic possibilities could be further explored *in vivo* for the diseases in this paper as well as other diseases with previously inaccessible regions. Several strategies have been used to deliver CRISPR systems in AAVs while LNPs have emerged as a viable non-viral carrier, creating the possibility of future translational work [19, 20].

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