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(Global Initiative on Sharing All Influenza Data; https://www.gisaid.org) database had only five full genome sequences from Iran where >120000 cases have been reported; by contrast, it has ~1300 genomes from ~7000 cases reported in Australia. Therefore, any interpretation of phylogenetic analyses involving undersampled regions must be made with caution. These limitations constrain certainty in interpretations obtained using phylogenetics, and a conservative approach is essential when interpreting results from complex phylogenetic models and multidimensional data.

Many countries are now investing efforts into genomic surveillance of SARS-CoV-2, and data sharing on the GISAID public database has now reached 25995 full genomes at unprecedented speed. Although the challenges of low phylogenetic resolution and biased sampling will undoubtedly remain, it is anticipated that future research into these SARS-CoV-2 genomes will take on these challenges with more robust statistical methods and cautious data interpretation, and will potentially provide important insights into SARS-CoV-2 transmission and evolution within different countries and across the world thereby aiding more effective control of the disease.

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Spotlight

SpRY: Engineered CRISPR/Cas9 Harnesses New Genome-Editing Power

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Due to protospacer adjacent motif (PAM) requirements, CRISPR/Cas9 cannot access many genetic loci. A recent study by Walton *et al.* structurally engineered *Streptococcus pyogenes* Cas9 (SpCas9) to near-PAMless SpRY that can target most DNA sequences with high editing efficiency and flexibility. This newly engineered SpRY will potentially expand genome-editing capabilities for basic and applied research.

PAM Requirements Limit the Potentials of Genome Editing

CRISPR/Cas9-based genome editing affords scientists the ability to precisely edit certain genes in a genome. Since its usability was first recognized, the CRISPR/Cas9 system has quickly become a genome-editing tool, widely used to edit specific DNA sequences across nearly all organisms [1]. However, traditional CRISPR/Cas9-based genome editing requires a PAM in the edited gene that serves as the recognition site for Cas9 enzymes. Although different Cas variants recognize different PAM sequences, the most commonly used SpCas9 recognizes NGG sequences; CRISPR/Cas9 has been widely used to edit and monitor a specific DNA sequence containing the NGG PAM sequence for a variety of purposes [2]. However, the required PAM sequences limit the sequences that CRISPR/Cas9 can target. Scientists have successfully identified Cas enzymes with differing PAMs that expand the Cas-targeted DNA sequences; however, further strategies are warranted for wider application of genome editing in gene function studies and beyond.

To relax the PAM requirements, scientists have modified Cas enzymes to recognize a wider range of PAMs using several strategies, including directed evolution and structure-guided engineering. Modified Cas9s, such as SpCas9-NG and SpCas9-VQR, have relaxed PAM preferences and recognize altered PAM sequences [3–5]. Although these modifications have expanded DNA target sites, these sites are still very limited, and most DNA sequences remain inaccessible for CRISPR/Cas-based genome editing.

Engineered CRISPR/Cas9s Relax the PAM Requirements

A recent study by Walton and colleagues [6] may completely change this field, making CRISPR/Cas9 available to edit genes at any DNA sequence. In their study, Walton and colleagues genetically modified the SpCas9 enzyme through a serial structure-guided engineering approach. They first generated a modified SpCas9 variant, named SpG, to alter the SpCas9 PAM preference and relax the Cas9 recognition sites; the newly modified SpG could target an expanded set of NGN PAMs instead of only

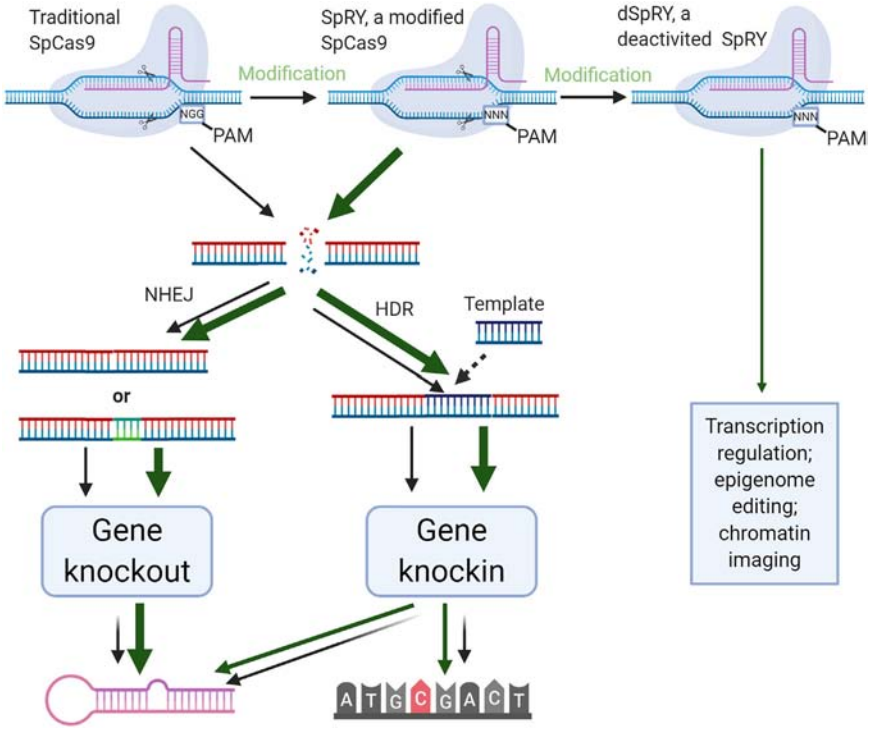

Figure 1. A Structurally Engineered CRISPR/Cas9, SpRY, Boosts the Genome-Editing Power for Gene Function Studies and Beyond. CRISPR/Cas9-based systems have revolutionized the field of gene editing. Because of the protospacer adjacent motif (PAM) proximity requirements, the traditionally used SpCas9 can edit only a limited number of genes. However, the structurally engineered *Streptococcus pyogenes* Cas9 (SpCas9), named SpRY, with almost no PAM requirement (or near-PAMless), can gain access to almost all genetic loci, including miRNA genes and SNPs. The newly engineered SpRY can potentially boost the application of the CRISPR/Cas9 genome-editing tool in both basic and applied research.

NGG PAM. This change made CRISPR/Cas9 capable of editing four times the number of genes over that of the original system (Figure 1). Compared with the traditional SpCas9 and prior modified SpCas9s (i.e., SpCas9-NG and xCas9), SpG exhibited the highest genome-editing activity, with robust targeting and editing activities across all four NGN PAMs (i.e., NGG, NGA, NGC, and NGT).

After relaxing the third nucleotide position requirements for PAM, Walton and colleagues sought to relax the PAM secondary nucleotide requirements. If so, it should be possible to target almost all DNA sequences in a genome. The authors hypothesized that changing the amino acid side chain of R1333 of SpCas9 to glutamine may enable access to sites harboring NAN PAMs. However, their initial test did not show the hypothesized results. Instead, they found that adding L1111R and A1322R to SpGR1333Q significantly enhanced the on-target activity of SpCas9. Finally, they further optimized the SpCas9 enzyme to SpRY (NRN>NYN PAMs), which can recognize almost all PAM sequences in a genome. The activities of this engineered near-PAMless SpCas9 variant SpRY were achieved by using multiple random targeting sites in human cell lines. SpRY could also successfully perform single-base editing. Because of the relaxed requirement for PAMs, the newly engineered SpG and SpRY potentially generate more off-target editing. However, these off-target effects can be eliminated without affecting the gene-editing activity by combining the newly engineered SpG and SpRY with high-fidelity variants of SpCas9 [7,8]. Overall, this work demonstrates that SpRY is capable of editing nearly all DNA sequences, including base editing, without strict PAM sequences and with minimal side effects.

Engineered CRISPR/Cas9 Boosts the Genome-Editing Power

Because CRISPR/Cas9 precisely manipulates DNA sequences, it is becoming a powerful tool for gene function studies and beyond. However, due to the PAM proximity requirements, the range of CRISPR/Cas-edited genes is very limited; the majority of genetic loci are currently inaccessible. The newly engineered SpRY will extend the ability of CRISPR/Cas for genome-wide gene editing (Figure 1). First, the new CRISPR/SpRY system makes miRNA genes editable. miRNAs are an extensive class of small regulatory RNAs that play versatile roles in plants and animals [9]. However, their short sequences, particularly the even smaller 'seed sequence', make them nearly impossible to be edited using the current CRISPR/Cas system. Additionally, SpRY makes point mutants and SNP loci accessible. SpRY holds the potential to open an era for editing miRNAs and point mutants for the treatment of disease The newly engineered CRISPR/SpRY system should also be able to access polygenes easily, to edit multiple genes at the same time, for studies of certain complicated traits, such as crop yield.

Wide adoption of CRISPR/Cas9 genome editing is based on sufficient accuracy and precision; off-target editing is a significant problem that necessitates additional research. As the requirements for PAM sequences are relaxed, the potential for undesirable off-target editing may increase. New strategies and ideas will be needed to reduce this unintended consequence. Continual discovery of new and modification of current Cas enzymes may be the best and most cost-efficient way of

eliminating off-target effects. Thus, we must carefully investigate the structures of Cas enzymes and their interaction with targeted DNA sequences. During this, many questions remain. For example, is it possible to link the Cas enzymes with a special enzyme that has a proofreading function? If so, the off-target effects can be quickly eliminated. Off-target effects associated with this new system of gene editing could also be eliminated by designing high-fidelity gRNAs. The development of new tools/strategies to deliver CRISPR/Cas may also limit the off-target impact. Delivering the CRISPR/Cas system directly to the target cells/tissues (e.g., cancer cells) will limit off-target editing and improve safety. It seems that RNP-based delivery can minimize off-target effects compared with other methods due to the limited lifespan of the CRISPR/Cas in the targeted cells [10]; further modification of RNP-based delivery and other new methods may significantly increase the delivery efficiency with unintended effects. As more knowledge and tools continue to be developed, CRISPR/Cas-based genome editing is sure to have a bright future in both basic and applied research.

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