PERSPECTIVE

# Miniature genome editors derived from engineering Cas9 ancestor

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Miniature genome editors are highly desirable for gene therapy by facilitating in vivo delivery via single adeno-associated virus (AAV) vector. Recently, a group of hypercompact endonuclease named as IscB with only ~500 aa is found to be the ancestry proteins of Cas9. However, IscB exhibited only marginal genome editing activity in human cells. Our study published in *Nature Methods* on May 25, 2023 reported successfully improving the genome editing activity of IscB as efficient as Cas9. Fusion of our enhanced IscB with T5 exonuclease further boost the editing efficiency while decreased the risk of inducing chromosomal translocation. Importantly, two miniature base editors generated by fusing the enhanced IscB variant with deaminase also showed markedly high efficiency for C-to-T and A-to-G editing in human cells. Overall, these findings serve as a good basis for the community to take advantage of miniature IscB for gene editing therapy with single AAV.

CRISPR-Cas9 system has revolutionized genome editing and gene therapy technology to greatly advance biomedical research. In the past decades, numerous Cas9 orthologs with different protein sizes and protospacer adjacent motif (PAM) requirements were identified and engineered for genome editing. Moreover, fusion of Cas9 with other effector proteins generated a powerful toolbox comprising base editor, prime editor, and epigenome regulators, fulfilling needs for versatile applications. Point base mutations are the

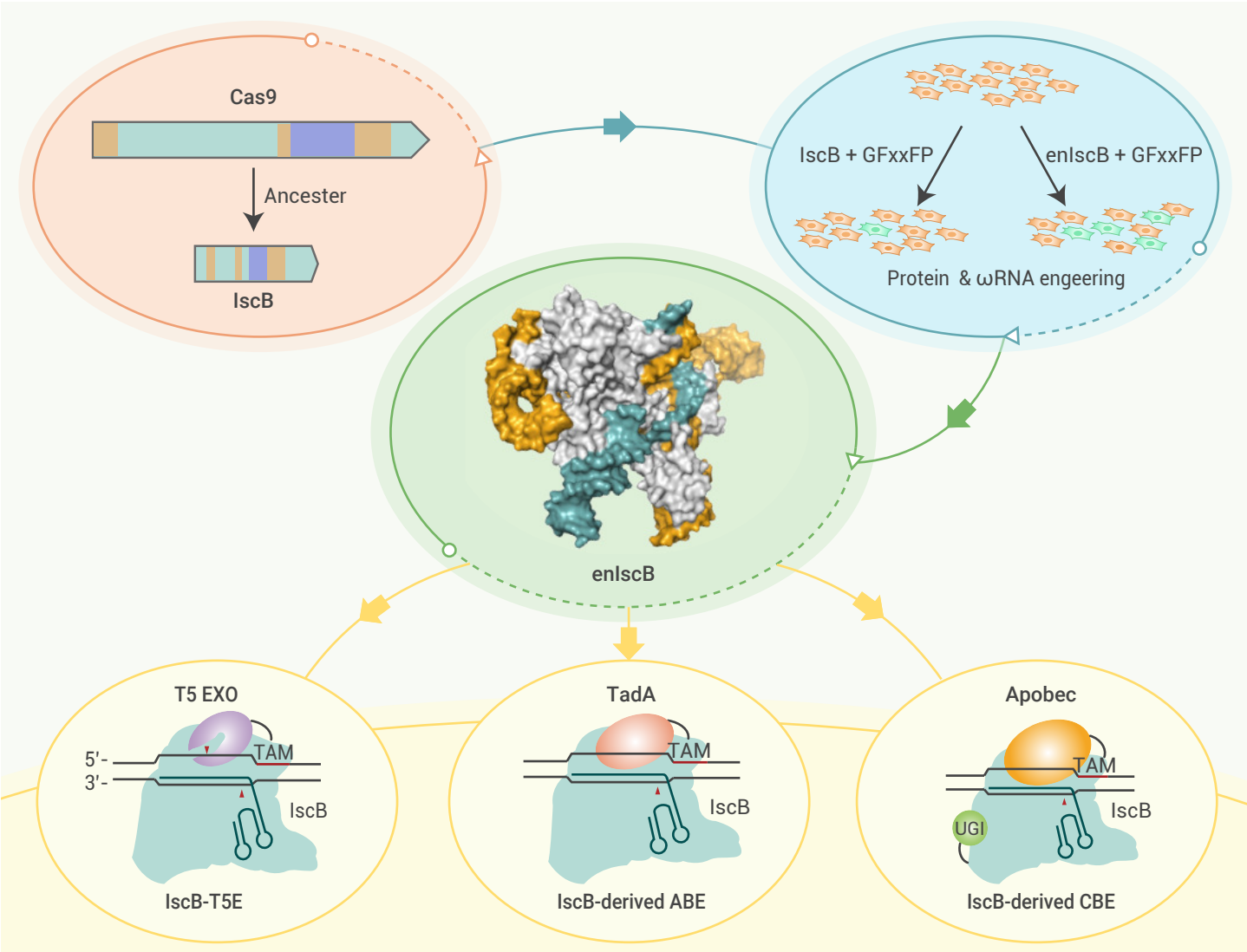


**Figure 1. Engineering miniature genome editors from IscB** Transposon-associated IscB is presumed to be ancestry protein of Cas9 but has hypercompact protein size that suitable for single AAV delivery. To improve the performance of IscB in human cells, systematic engineering of protein and ωRNA was carried out with a sensitive GFP reporter to identify a enhanced IscB (enIscB) variant. In addition, fusion of T5 exonuclease with enIscB mitigated translocation risk. Moreover, miniature base editors based on enIscB were generated by fusion with TadA and Apobec deaminase for efficient A-to-G and C-to-T editing.

most of genomic alteration types implicated in human hereditary diseases. However, the cellular repairing pathway induced by Cas9-mediated dsDNA cleavage is inefficient in the precise correction of base or other mutations. Moreover, Cas9-mediated dsDNA cleavage could result in large DNA and even chromosome rearrangements with potential detrimental effects. Therefore, base editors consisting of Cas9 nickase and deaminase or glycosylase were recently developed to induce precise base conversion without dsDNA cleavage for correcting disease-causing mutations in a safe manner.[1,2] But, therapeutic delivery of Cas9 and its different derivatives for gene therapy via single adeno-associated virus (AAV) vector widely used in clinics were often hindered by the large protein size of these genome editing agents that are beyond the maximal cargo size of AAV.[3]

In 2015, Eugene Koonin's group traced the evolutionary origin of Cas9 back to a novel group of DNA transposons and named it as ISC (insertion sequences Cas9-like) transposons.[4] Among them, IscB1 only contains a RuvC-like domain and IscB2 contains HNH nuclease domain insertion in RuvC-like domain, suggesting that IscB2 originated from IscB1, while Cas9 originated from IscB2. Recently, Zhang Feng's group proved that IscB (IscB2) protein can be guided by non-coding RNA named as ωRNA near the IscB locus, targeting double-stranded DNA for cleavage.[5] In vitro biochemical characterization demonstrated similar RNA-guided endonuclease activity for IscB as Cas9, which cleaves gRNA targeted and non-targeted strands through the HNH and RuvC catalytic domains,[6] respectively. Unlike the cleavage product of Cas9 with blunt end, the cleavage product of IscB has a 5′-sticky end. Interestingly, they also showed that IsrB (IscB1) with only RuvC domain showing dsDNA nicking rather than cleavage activity due to the lack of HNH domain,[7] implying the clear evolution path from IsrB/IscB to Cas9 in virtue of gathering different functional domains in a stepwise way. Considering that IscB has similar functions to Cas9 and is only 2/5 the size of Cas9, these works demonstrate the significant advantages of the IscB-ωRNA system as miniature genome editing tools in gene therapy applications based on AAV delivery. However, out of 89 tested IscB orthologs, only OgeuIscB that recognizes 5′-NWRNA-3′ TAM (target-adjacent motif) has been reported to exhibit marginal editing activity in human cells. Thus, it is urgently needed to develop an IscB-ωRNA system with efficient editing activity for gene therapy applications.

To make IscB work as efficiently as its Cas9 progeny proteins, our group performed systematic IscB and ωRNA engineering to markedly improve IscB activity (Figure 1), which is published in *Nature Methods* recently.[8] In the study, we also successfully developed miniature base editors with enhanced IscB variant and AAV-compatible size (Figure 1). We used a very sensitive and convenient fluorescence reporting system designed previously to detect nuclease targeted cleavage activity, and found that the OgeuIscB could be detected in 20% of EGFP positive cells on our reporting system.[9] Then we optimized the sequence and structure of ωRNA, including truncating the stem-loop region and replacing A-U base pairs with C-G pairs to enhance ωRNA stability. After multiple rounds of screening, we obtained an optimal ωRNA variant that could increase activity by approximately 120% and named it ωRNA*. Furthermore, we generated hundreds of IscB variants by mutating every amino acid into arginine that is positively charged for better interaction of IscB with negatively charged DNA. We nailed down an enhanced IscB variant with comparable activity as Cas9 on the reporting system after several rounds of screening and named it as IscB*. Finally, we obtained the enIscB system with the highest editing efficiency by combining IscB* and ωRNA*.

In previous studies, Jiazhi Hu's group found that Cas9 can increase its activity after fusion with an exonuclease, and they developed PEM-seq technology to detect that the fused exonuclease can effectively reduce chromosome translocation.[10] Considering that IscB will produce 5′-sticky end, which may rely on microhomology-mediated end joining repair (MMEJ), we fused the enIscB with exonuclease T5E (enIscB-T5E) to digest the 5′-sticky end (Figure 1). We found that the enIscB-T5E system had a higher cutting efficiency than the enIscB system. More importantly, the enIscB-T5E system has a lower risk of chromosomal translocation and is therefore safer.

To make efficient in vivo delivery of base editors via AAV, it would be highly desirable to replace large-sized Cas9 with miniature Cas proteins like IscB in base editors. In the present study, we successfully demonstrated the robust base editing activity enabled by the fusion of enhanced IscB nickase with C-to-T or A-to-G deaminase, which is termed miCBE or miABE respectively. Our results revealed that miCBE and miABE could support the high base conversion rate with up to 90%. Additional evaluation of miCBE and miABE found wider editing window than that of Cas9-derived base editors, which might be optimized by future engineering. Through systematic off-target detection experiments and analysis, including guide dependent and guide independent methods, we found that the off-target levels of miABE and miCBE were relatively low. Overall, our present work opens the door to engineering miniature base editors or prime and epigenome editors for the different applications perceived in biomedical scenarios.

At present, IscB provides us the opportunity to reduce size constraints of Cas9 on AAV for efficient and low-dose delivery of genome editors in vivo. Based on current research, more work is needed to make IscB a more popular and safer tool for gene therapy applications, such as expanding TAM sequences, narrowing base editing windows, and improving specificity. Considering the size advantage of IscB, it has the potential to be used for developing methylation editors and prime editors based on single AAV delivery. Screening for more IscB variants in nature and obtaining more IscB variants through artificial mutations are two important ways to expand the function of IscB. Due to the fact that IscB is more diverse in nature than Cas9, large-scale screening of new variants can yield more IscB proteins of different TAM types. Besides, our screening strategy and results demonstrate that manual screening of mutants is a convenient and effective way to improve the function of IscB.

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## DECLARATION OF INTERESTS

The authors declare no competing interests.