http://dx.doi.org/10.7124/bc.000AF5

Engineering designer-recombinases for therapeutic genome editing

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Background. Tyrosine site-specific recombinases (Y-SSRs), such as the Cre-loxP system, are widely used genome editing tools that hold potential for therapeutic application due to their precise mechanism of DNA manipulation. Y-SSRs can execute complex genome engineering operations, including excision, inversion, integration, and cassette exchange of large genomic sequences, without inducing DNA double-stranded breaks and without relying on the cellular DNA repair machinery or additional cofactors. Therefore, the DNA-editing process is predictable and works even in non-dividing cells. However, laborious step-wise directed molecular evolution and protein engineering are required to reprogram them to target defined loci. **Aims.** We aim to engineer Y-SSRs to make them suitable for therapeutic genome editing. By reprogramming their DNA-binding specificity to act on human genomic sequences, we aim to excise, integrate, invert or replace DNA sequences to develop curative therapies for genetic diseases. **Methods.** We employ substrate-linked directed evolution and structure-based protein engineering to improve the applied properties of Y-SSRs. By fusing the coding sequence of designer-recombinases to zinc-finger DNA-binding domains (ZFD) we achieve improved activity and specificity. **Results.** We perform systematic analyses to define the optimal spacing and orientation of the ZFDs with respect to the recombinase recognition sequence, as well as the optimal linker length between recombinase and ZFD. We find that N- and C-terminal ZFD-recombinase fusions increase recombination activity by up to ten-fold. We further apply pentapeptide scanning mutagenesis to identify positions that tolerate insertional fusions within the recombinase coding sequence. Notably, some insertional ZFD-recombinase fusions render recombinase activity dependent on ZFD binding to its target sequence. We show that this approach improves the properties of a recombinase with therapeutic potential and establish a molecular evolution method, which allows to improve the properties of *in silico* designed ZFDs. Moreover, we develop a prototype of a multilateral recombinase that can be programmed to recombine many target sites in a ZFD-dependent manner. **Conclusion.** The development of ZFD-dependent recombinases provides a powerful tool for genome editing applications that combines the ease of ZFD targeting with the editing precision of SSRs. Our research markedly expedites the creation of highly effective and precisely targeted designer-recombinases. Specifically, the fusion of a non-specific recombinase guided by ZFD binding to its intended target site introduces a novel concept for the development of precision genome editing tools. This approach expands the repertoire of techniques available for precise genetic modifications, holding promising implications across diverse scientific and biomedical applications. We anticipate that the developed approach could be adapted to other DNA-binding domains and other DNA-modifying enzymes. If successful, this concept would open exciting perspectives for flexible, seamless, and precise genome surgery.

Keywords: therapeutic DNA-editing, Cre-loxP system.