Enhancing Gene Targeting with Designed Zinc Finger Nucleases

Marina Bibikova,* Kelly Beumer, Jonathan K. Trautman, Dana Carroll†

Gene targeting—the process of gene replacement by homologous recombination—is a very useful but typically inefficient technique (1). A general method for improving the efficiency of gene targeting would be valuable in many circumstances, as would the extension of this genetic tool to a broader range of organisms. Model experiments have demonstrated that a double-strand break (DSB) in the chromosomal target greatly enhances the frequency of localized recombination events (2–6).

We have been developing chimeric zinc finger nucleases (ZFNs) as potential stimulators of gene targeting (7, 8). The ZFNs have a DNA recognition domain composed of three Cys-His zinc fingers linked to a nonspecific DNA cleavage domain (9). The cleavage domain must dimerize to act as a nuclease (7), and this is most readily achieved for the ZFNs by providing binding sites for two sets of zinc fingers in close proximity and in the appropriate orientations (7, 8). The ability to control the recognition specificity of zinc fingers (10, 11) opens the prospect of directing cleavage to arbitrarily chosen chromosomal sites, without prior manipulation of the target.

We designed and constructed a pair of ZFNs (yA, yB) for a site in the second exon of the yellow (y) gene on the X chromosome of Drosophila melanogaster (12) by combining zinc fingers that had been shown in other studies to bind the component triplets of the target sequence (13). Each three-finger DNA binding domain recognizes 9 base pairs (bp). The requirement for dimerization specifies an 18-bp domain that recognizes 9 base pairs (bp). The recognition site is unique in the genome.

Previously, we showed that expression of these ZFNs in larvae led to the production of both somatic and germline mutations, due to cleavage and nonhomologous end joining (NHEJ) (12). Here, we tested the ability of ZFN-induced breaks to stimulate gene targeting when a homologous donor DNA is provided.

A mutant donor (p58) was constructed, which carries 8 kb of y homology, into which the ZFN recognition site was replaced with two in-frame stop codons and an Xho I site. This mutant was inserted into a P-element vector, flanked by recognition sites for the FLP recombinase and the meganuclease I–Sce I to permit excision and linearization of the donor, and was introduced into the fly genome. Generating a linear extrachromosomal donor DNA in situ by this means has been shown to enhance its effectiveness in recombination (14, 15).

The donor was combined by appropriate crosses with transgenes for the yA and yB nuclease domains and those for FLP and/or I–Sce I, all under the control of the Drosophila HSP70 promoter. Larvae carrying these components were treated by a heat-shock protocol at 35° C 0 to 4 days after egg laying. The experiment was performed in five versions: ND, no donor; yA + yB only; ID, integrated donor; yA + yB + donor, no FLP or I–Sce I; CD, circular extrachromosomal donor; yA + yB + FLP + donor; LD, linear extrachromosomal donor: yA + yB + FLP + I–Sce I + donor; DO, linear donor only: FLP + I–Sce I + donor, but no ZFNs. Adults emerging from the heat-shock protocol were crossed to reveal germline y mutations (12). In both males and females, the frequencies of mutations rose in the presence of the donor and increased further as it became extrachromosomal.

The ZFN-induced gene targeting frequencies are about 10-fold greater than those obtained without target cleavage, and they occurred in both the male and female germ lines, whereas previously only females yielded good frequencies (14–16). The presence of a DSB in the target, apparently, activates recombination processes in males that are not efficient on intact chromosomes. The lower targeting frequencies we observed in females may reflect the possibility of repairing the break by recombination with an uncut homologous X chromosome, although the presence of this option does not preclude interaction with the extrachromosomal donor. It is possible that the efficiency of ZFN-induced gene targeting can be enhanced further with an altered heat-shock protocol or that the procedure can be simplified by direct embryo injection of the ZFNs and donor DNA.

Because stimulation of recombination by DSBs is a property of essentially all cells and organisms, the approach of enhancing gene targeting by target cleavage with designed ZFNs should be very broadly applicable. New applications will depend on the ability to produce zinc finger combinations that bind sequences in the vicinity of the desired alteration and on appropriate methods for delivery of the ZFNs.

Fig. 1. Frequency of germline y mutants and the proportion due to homologous replacement by the marked donor. For each donor configuration, the percent of all candidate offspring that were mutant is shown for both males and females. Each bar is divided to reflect the fractions resulting from homologous (HR) and nonhomologous (NHEJ) events.

References and Notes
16. Y. S. Rong et al., Genes Dev. 16, 1568 (2002).
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Table S1

1Department of Biochemistry, University of Utah School of Medicine, 20 North 1900 East, Salt Lake City, UT 84132–3201, USA.
2Present address: Illumina, Inc., San Diego, CA 92121, USA.
3To whom correspondence should be addressed. E-mail: dana@biochem.utah.edu