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TECHNOLOGY REPORT

Case Studies of Ends-Out Gene Targeting in *Drosophila*

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Summary: Ends-in and ends-out gene replacement approaches have been successfully used to disrupt *Drosophila* genes involved in a variety of biological processes. These methods combine double-strand breaks and homologous recombination to replace a targeted chromosome region with a designed DNA sequence. Unfortunately, these methods require large numbers of single animal crosses, making them both time consuming and labor intensive. Here, we designed a single complete targeting vector for use in a mass crossing ends-out gene targeting study. Importantly, our gene targeting method included a balancer chromosome to block endogenous homologous chromosome pairing and to promote pairing between the foreign targeting DNA fragment and the targeted chromosome. This technique provided successful and efficient gene replacement, greatly facilitating the gene knockout procedure. genesis 1–4, 2009. © 2009 Wiley-Liss, Inc.

Key words: Drosophila; gene targeting; ends-out; balancer; reverse genetics

The great challenge of the postgenome era is to understand the functions of all sequenced genes (Adams *et al*., 2000; Venter *et al*., 2001). Despite nearly a century's effort with classical forward genetic approaches (from mutant phenotype to gene sequence), mutants are not available for more than half of the genes from multicellular model organisms, such as worms, flies, and mice (Jorgensen and Mango, 2002; Kile and Hilton, 2005; St Johnston, 2002). Reverse genetic approaches, including RNA interference (RNAi) and gene targeting by homologous recombination, greatly facilitate the functional analysis of genes with no corresponding mutations. However, reverse genetics approaches also have limitations. For instance, gene expression is never completely blocked by RNAi, and homologous recombination is time- and labor-consuming. Here, we describe a modified gene targeting procedure in *Drosophila* that is substantially less laborious compared with current techniques.

In *Drosophila*, ends-in and ends-out are two ingenious gene-targeting methods developed by Golic and coworkers (Gong and Golic, 2003; Rong and Golic, 2000, 2001; Rong *et al*., 2002). Both methods require creating a large targeting construct, making a donor transgenic fly, and carrying out two to three generations of genetic crosses that require hundreds to thousands of single-animal crosses. The key element of ends-in or ends-out is the concerted use of double-strand breaks (DSBs) and homologous recombination. First, the enzymes Flipase and I-SceI are utilized to create DSBs. Second, the replacement DNA fragment generated by DSBs is paired with the targeted chromosome to trigger homologous recombination. Therefore, the targeted chromosome region is replaced by a designed DNA sequence. The ends-in and ends-out gene targeting approaches have been successful in disrupting genes involved in many processes. However, the huge number of single-animal crosses required makes these techniques inefficient in terms of time and labor. Thus, it is not surprising that in the past 5 years, only about 50 genes have been knocked out using these two methods (Venken and Bellen, 2005; Flybase).

It is well accepted that experimentally induced homologous recombination results from pairing between a targeting DNA fragment and a targeted chromosome. Balancer chromosomes, which contain chromosomal rearrangements, can block endogenous homologous chromosome pairing to prevent crossing over. Therefore, we hypothesized that balancer chromosomes can be used to promote pairing between a foreign targeting DNA fragment and a targeted chromosome. To test this possibility, a balancer chromosome was included in the

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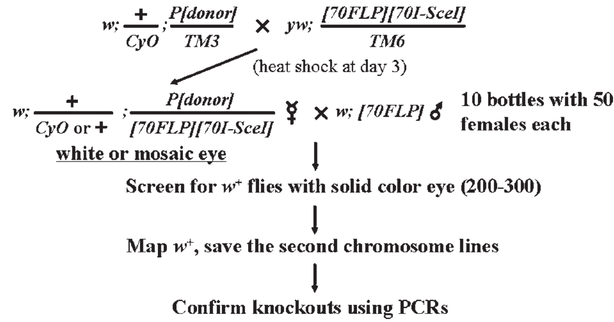

FIG. 1. Ends-out targeting procedure. We present an example of knocking out a gene on the second chromosome using a donor transgenic line from the third chromosome. The *CyO* balancer chromosome was used in the first round of crossing. In the F1 progeny, animals with *CyO* balancer were used in the next round of crosses to examine the balancer effect on targeting efficiency. As a control, animals without *CyO* balancer were used in another cross. To expand the population of flies from the crosses, we transferred flies in old bottles to new bottles every day.

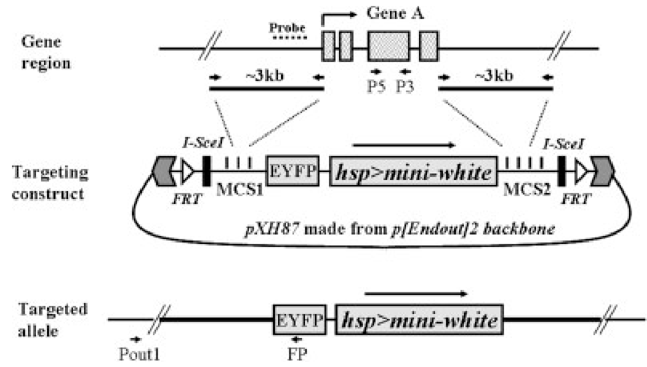

FIG. 2. Molecular design of ends-out gene targeting. *pXH87* was made from *p[Endout]2* by insertion of a *mini-white* marker and an *EYFP* reporter. Two ~3-kb regions flanking the knockout target gene (Gene A) were separately amplified by PCR and cloned into the multiple cloning sites (MCS) of *pXH87* as shown to yield the final targeting construct. During the knockout, the linear DNA excised by FRT and I-SceI from the transgene of the targeting construct pairs and recombines with the target gene region. The predicted knockout allele was distinguished from the wild-type allele and from the original donor transgene by Southern blotting using a probe specific for the ~3-kb region (as indicated in the upper diagram) or by replacement gene-specific long PCR amplification using an *EYFP* primer (FP) and a primer corresponding to a sequence outside the 3-kb homologous region (pOut1). Target gene-specific PCR amplification (internal PCR) was performed using primers corresponding to a sequence within the target gene (P5 and P3) to verify homozygous knockouts (no PCR product).

first round of cross in our *Drosophila* gene targeting procedure (see Fig. 1). We also engineered a ready-to-go targeting vector *pXH87* that contained a *mini-white* transgenic marker and an *EYFP* tag (Fig. 2 and Methods). In the F1 generation, about 500 mosaic or white-eye progenies were collected. Unlike the published ends-out procedure, in which each of the 500 white/mosaic-eye flies is singly crossed with flies constitutively expressing Flipase in individual vials, we mass-crossed the flies in large bottles. From the progeny, 200–300 flies with solid red eyes (*w*+) were collected to perform single-animal mapping crosses (see Fig. 1). The fly lines with the *w*+ marker mapping to the targeted chromosome were then isolated, and replacement gene-specific long PCR was used to identify heterozygous lines (Fig. 2, primers Pout1 and FP) and target gene-specific internal PCR was used to verify gene knockout for homozygous lines (Fig. 2, primers P5 and P3).

Using this method to analyze the isolated fly lines, we observed an amplification product of predicted size from the replacement gene-specific long PCR (Fig. 3a), indicating gene targeting had occurred where expected. The lack of amplification product from the target gene-specific internal PCR (Fig. 3b) further confirmed homozygous gene knockout. Southern blot analysis was utilized to confirm the structure of the targeted gene (Fig. 3c,d). With this procedure, many targeting events and knockout alleles for all five genes of interest were identified. For example, four knockouts from 250 single crosses were isolated for the gene *CG17119*. Twelve targeting events from 650 single crosses were also detected for *CG5582* (Table 1 and see below). *CG17119* and *CG5582* are *Drosophila* homologs of the human cystinosis gene *CTNS* and the batten disease gene *CLN3*, respectively. Sixteen knockouts from 400 single crosses were isolated for *CG3522*, a *Drosophila* homolog of mammalian *MLN64*, which encodes a lipid binding protein involved in intracellular sterol trafficking. Eighteen knockouts from 170 single crosses were isolated for *CG6708*, and nine knockouts from 180 single crosses were isolated for *CG5077* (Table 1). *CG6708* and *CG5077* encode two oxysterol-binding-protein related proteins also believed to participate in intracellular sterol trafficking. All knockout mutants obtained are viable and fertile, except the *CG6708* knockouts, which have a male sterile phenotype. Together, these results indicated that inclusion of a balancer chromosome in the ends-out targeting procedure enabled successful gene targeting.

Next, we tested whether including a balancer chromosome could increase the gene targeting efficiency. We conducted ends-out targeting using the same donor line without the balancer chromosome as a comparison. For knockout of *CG6708*, the targeting fragment was transferred to the targeted chromosome with twice the efficiency per single cross in the presence of the balancer chromosome (31% vs. 15%, $P < 0.05$), leading to a significant increase in the targeting frequency (3.6% vs. 1%, respectively, $P < 0.05$) (Table 1). We observed the same effect for knockout of *CG5582* (Table 1). We should point out that because the flies were mass-crossed, we can not rule out the possibility that the knockout alleles were derived from the same recombination event amplified through multiple rounds of germline mitosis. Therefore, the knockout alleles may not have arisen from independent recombination events. However, for *CG6708*, 18 knockout alleles isolated from crosses with the bal-

ancer chromosome originated from 10 different bottles of mass-crossed flies, indicating that there were at least 10 independent recombination events. In contrast, five knockout alleles from crosses lacking the balancer chromosome were isolated from four different bottles of flies. Together, these results suggest that the inclusion of a balancer chromosome increases targeting efficiency and supports the hypothesis that a balancer chromosome can greatly facilitate gene knockout.

Our data also support the previous notion that the efficiency of ends-out-based gene targeting varies on a gene-by-gene and case-by-case basis (Rong *et al.*, 2002). For example, two different donor lines of *CG5582* resulted in two distinct targeting efficiencies (2.4% and 0.2%, see Table 1). In addition, among the five genes tested in the present study, the targeting efficiency ranged from 0.2% to 3.6%. Unexpected results also arose from our gene targeting process. In the first round of targeting *CG5582*, we identified 27 transfer events from 650 single crosses. However, even though replacement gene-specific long PCR indicated that 12 of the 27 transfer events had occurred at the correct location, target gene-specific internal PCR revealed the presence of the *CG5582* coding region in all 12 samples, indicating that none of them were genuine knockout mutants. Gene duplication events have been reported to occur alongside gene replacement during the knockout process (Gong and Golic, 2003). Indeed, Southern blot analysis revealed the presence of DNA fragments other than those predicted from the targeting scheme, suggesting that duplication events occurred during the gene replacement process (Fig. 3d).

The balancer chromosome-based ends-out gene targeting procedure described herein required less labor and less time, and yielded targeting results comparable to the classical ends-out method. Moreover, we showed that the balancer chromosome likely increased targeting frequency. Although a rigorous comparison with previous work described by Golic and coworkers is not possible due to differences in the targeting procedures (Gong and Golic, 2003; Rong and Golic, 2000, 2001; Rong

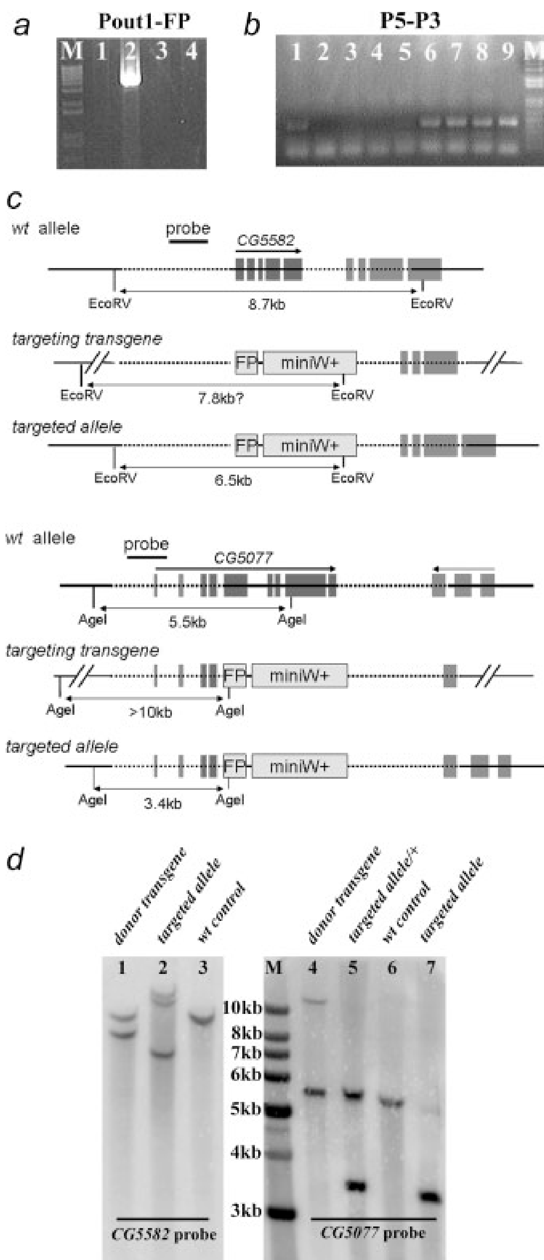

FIG. 3. Detection of targeting events via PCR amplification and Southern blotting. (**a**) Genomic DNA from four potential *CG5582* knockout lines was separately amplified by long PCR using the Pout1 and FP primers followed by agarose gel electrophoresis. A representative example of positive gene targeting is shown in Lane 2. M, 1-kb DNA ladder. (**b**) Genomic DNA from nine potential *CG5077* knockout lines was separately amplified by target gene-specific internal PCR using the P5 and P3 primers followed by agarose gel electrophoresis. Representative examples of true knockouts lacking a *CG5077* amplification product are shown in Lanes 2–5. (**c**) Schematic representation of the wild-type (*wt*) allele, the targeting transgene, and the targeted knockout allele for *CG5582* (top half) and *CG5077* (bottom half), respectively. Dashed lines represent the homologous regions used in the targeting construct (~3 kb each, see Fig. 2). Gray boxes represent exons. Positions of the probe used for Southern blotting are shown, and the expected sizes of the genomic DNA fragments that hybridize with the probe are indicated below the corresponding loci. (**d**) Representative Southern blot analysis using *CG5582* specific probe (Lanes 1–3) or *CG5077* specific probe (Lanes 4–7). Lane 1, DNA from a transgene donor for *CG5582* showing hybridization of the probe to the *wt* 8.7-kb fragment and a smaller fragment likely derived from the donor targeting transgene insertion. Lane 2, DNA from a homozygous *CG5582* knockout showing hybridization of the probe to the predicted 6.5-kb fragment plus two higher molecular weight fragments likely representing duplication events. Lane 3, DNA from *wt* (*Canton-S*) showing the *wt* 8.7-kb band. M, molecular marker with indicated sizes. Lane 4, DNA from a transgene donor for *CG5077* showing the hybridization of the probe to the *wt* 5.5-kb fragment and a larger fragment likely derived from the donor targeting transgene insertion. Lane 5, DNA from a heterozygous *CG5077* knockout showing hybridization of the probe to the predicted 3.4-kb fragment and to the *wt* 5.5-kb fragment. Lane 6, DNA from *wt* showing hybridization of the probe to the *wt* 5.5-kb fragment. Lane 7, DNA from a homozygous *CG5077* knockout showing hybridization of the probe to the predicted 3.4-kb fragment.

Table 1
Gene Targeting Results

Gene (chromosome)	Human homolog	Balancer used	Number of single crosses	Map to target chromosome (efficiency)	Targeting events	Targeting frequency (%)[a]	Phenotype
CG17119 (III)	*CTNS*	*TM3*	250	17 (6.8%)	4	0.8	Viable, fertile
CG3522 (II)	*MLN64*	*CyO*	400	20 (5%)	16	3.2	Viable, fertile
CG5077 (III)	*OSBP*	*TM3*	180	22 (12%)	9	1.8	Viable, fertile
CG6708 (III)[b]	*OSBP*	None	130	20 (15%)[c]	5	1[d]	Male sterile
CG6708 (III)[b]	*OSBP*	*TM3*	170	52 (31%)[c]	18	3.6[d]	Male sterile
CG5582 (III)[b,e]	*CLN3*	None	600	7 (1.2%)[f]	0	NA	NA
CG5582 (III)[b,e]	*CLN3*	*TM3*	650	27 (4%)[f]	12	2.4	Viable, fertile
CG5582 (III)[b,g]	*CLN3*	None	350	1 (0.3%)	0	NA	NA
CG5582 (III)[b,g]	*CLN3*	*TM3*	280	3 (1.1%)	1	0.2	Viable, fertile

[a]Targeting frequency was calculated by dividing the number of targeting events by 500 (the number of mass-crossed females). Significant changes of the efficiency of map to target chromosome and the final targeting efficiency with or without a balancer chromosome were determined.
[b]To minimize the background effect, targeting was conducted using the same donor line for each gene to compare the effect of the balancer chromosome. However, two different donor lines were used in *CG5582* targeting.
[c]Changes were statistically significant ($P < 0.05$).
[d]Changes were statistically significant ($P < 0.05$).
[e]Donor line 1 was used.
[f]Changes were statistically significant ($P < 0.05$).
[g]Donor line 2 was used. NA, not available.

et al., 2002), our data clearly demonstrated that ends-out gene targeting in *Drosophila* can be achieved in a rapid and efficient manner, greatly facilitating investigation of in vivo gene function.

METHODS

Drosophila and Genetics

Flies were raised on standard corn meal medium at 25°C. Except donor transgene, all stocks used in this study were obtained from Bloomington Stock Center. All of the donor transgenic constructs generated were injected into w^{1118} embryos using established methods to obtain transgenic lines. To induce DSBs, heat shock was performed at 38°C for 90 min at Day 3 after egg laying. Genetic markers and special balancer chromosomes are described in FlyBase (http://www.flybase.org).

Generation of the Targeting Vectors and Donor Targeting Constructs

To create the targeting vector, the *pXH86* vector was first generated by subcloning a SmaI-AflII fragment of *EYFP* from *pEYFP-N1* (Clontech Laboratories Inc., Mountain View, CA) into the SmaI site of the *pEndout2* vector, which contains FRT sites adjacent to the I-SceI sites flanking the multiple cloning sites (http://sekelsky.bio.unc.edu/Research/Vectors/pP{Ends Out2}.jpg). An EcoRI fragment of *mini-white* amplified by PCR from *pBS-70W* was then ligated into *pXH86* to yield the final constructs *pXH87* and *pXH88*, containing forward and reverse orientations of the *mini-white* cassette, respectively. The sequences of *pXH87* and *pXH88* are available upon request. Donor targeting constructs were generated by separate insertion of two ~3-kb regions (upstream and downstream of the candidate target gene) into two multiple cloning sites of *pXH87*.

Verification of Knockout Strains by Southern Blotting

Southern blot analysis was performed following a standard protocol (Rong *et al.*, 2002). Briefly, genomic DNA from 50 adult flies was digested with EcoRV (for *CG5582*) or AgeI (for *CG5077*) and was subjected to agarose gel electrophoresis before blotted to a nylon membrane. The membrane was then hybridized with a ^{32}P-labeled probe specific for *CG5582* or *CG5077*.

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