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.


**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 co-workers (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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isolated for the gene on 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.

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 Flpase 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 CG5077 (Table 1 and see below). CG17119 and CG5077 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 CG5322, 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 CG53582 (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...
CG5582 (II)  MLN64  CyO  400  20 (5%)  16  3.2  Viable, fertile
CG5582 (II)  OBSP  TM3  180  22 (12%)  9  1.8  Viable, fertile
CG6708 (III)  OBSP  None  130  20 (15%)  5  1  Male sterile
CG6708 (III)  OBSP  TM3  170  52 (31%)  18  3.6  Male sterile
CG5582 (III) CLN3  None  600  7 (1.2%)  0  NA  NA
CG5582 (III) CLN3  TM3  650  12 (7.3%)  2  1.2  Viable, fertile
CG5582 (III) CLN3  None  350  1 (0.3%)  0  NA  NA
CG5582 (III) CLN3  TM3  280  3 (1.1%)  1  0.2  Viable, fertile

Identification 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 32P-labeled probe specific for CG5582 or CG5077.

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