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REVIEW

Advances in *Drosophila* gene targeting and related techniques

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Abstract Functional biological research has benefited tremendously by analyses of the phenotypes of mutant organisms which can be generated through targeted mutation of genes. In *Drosophila*, compared with random mutagenesis methods gene targeting has gained its popularity because it can introduce any desired mutation into a gene of interest. However, applications of gene targeting have been limited because the targeting efficiency varies with different genes, and the time and labor of targeting procedure are intensive. Nevertheless, improvement of gene targeting and development of its variant technologies have received much attention of scientists. Here we review recent progress that has been made in expanding the applications of gene targeting, which include the ΦC31 integration system and zinc-finger nucleases induced gene targeting, and new strategies that generate more efficient and reliable gene targeting.

Keywords gene targeting, ends-in, ends-out, ΦC31 integration system, zinc-finger nucleases (ZFNs), homologous recombination (HR), *Drosophila melanogaster*

1 Introduction

Drosophila melanogaster is a highly attractive model organism mostly because there are readily obtainable genetic tools that have been developed throughout the past century. Analyses of mutant phenotypes can be carried out in a relatively short time. A comprehensive range of technologies can be employed for making sophisticated genetic manipulations to study gene functions. These technologies include P-element mediated transgenesis (Rubin and Spradling, 1982), Gal4-UAS based gene overexpression system (Brand and Perrimon, 1993), Flp-*FRT* site-specific recombination system (Xu and Rubin, 1993; Golic and Golic, 1996), and the ΦC31 mediated site-specific integration system (Groth et al., 2004). Moreover, the completion of fly genome sequence (Adams et al., 2000) and the availability of numerous resources, including online databases such as Flybase and stock centers (Greenspan, 2004), have greatly facilitated functional studies of specific genes in various aspects of developmental biology, genetics, cell biology, neuroscience and behavior. The identification of novel genes and their functional characterization *in vivo* greatly depends on these available tools, and many of these researches rely on the breakthrough of reverse genetics technology: gene targeting.

Gene targeting is the modification of an endogenous gene sequence by recombination between an introduced DNA fragment and the homologous target gene. In multicellular organisms, this targeting technique was first successfully established in mouse embryonic stem cells (Capecchi, 1989) and has been extended to *Physcomitrella* (Schaefer and Zryd, 1997), sheep (McCreath et al., 2000) and human somatic cells (Hanson and Sedivy, 1995), and has enabled analyses of phenotypic consequences of specific gene mutations. In *Drosophila*, homologous recombination (HR) based gene targeting was developed by Golic and coworkers (Rong and Golic, 2000; Rong and Golic, 2001; Rong et al., 2002; Gong and Golic, 2003), and was originally carried out using two strategies: 'ends-in' or insertional gene targeting (Rong and Golic, 2000) and 'ends-out' or replacement gene targeting (Gong and Golic, 2003) (Fig. 1A and B). Ends-in and ends-out refer to the two arrangements of donor DNA constructs that are used during gene targeting. Insertional gene targeting results in an insertion of the entire targeting sequence into the region of homology (Fig. 1A). This insertion forms a duplication that can be resolved during a second round of HR. Replacement gene targeting removes the endogenous gene

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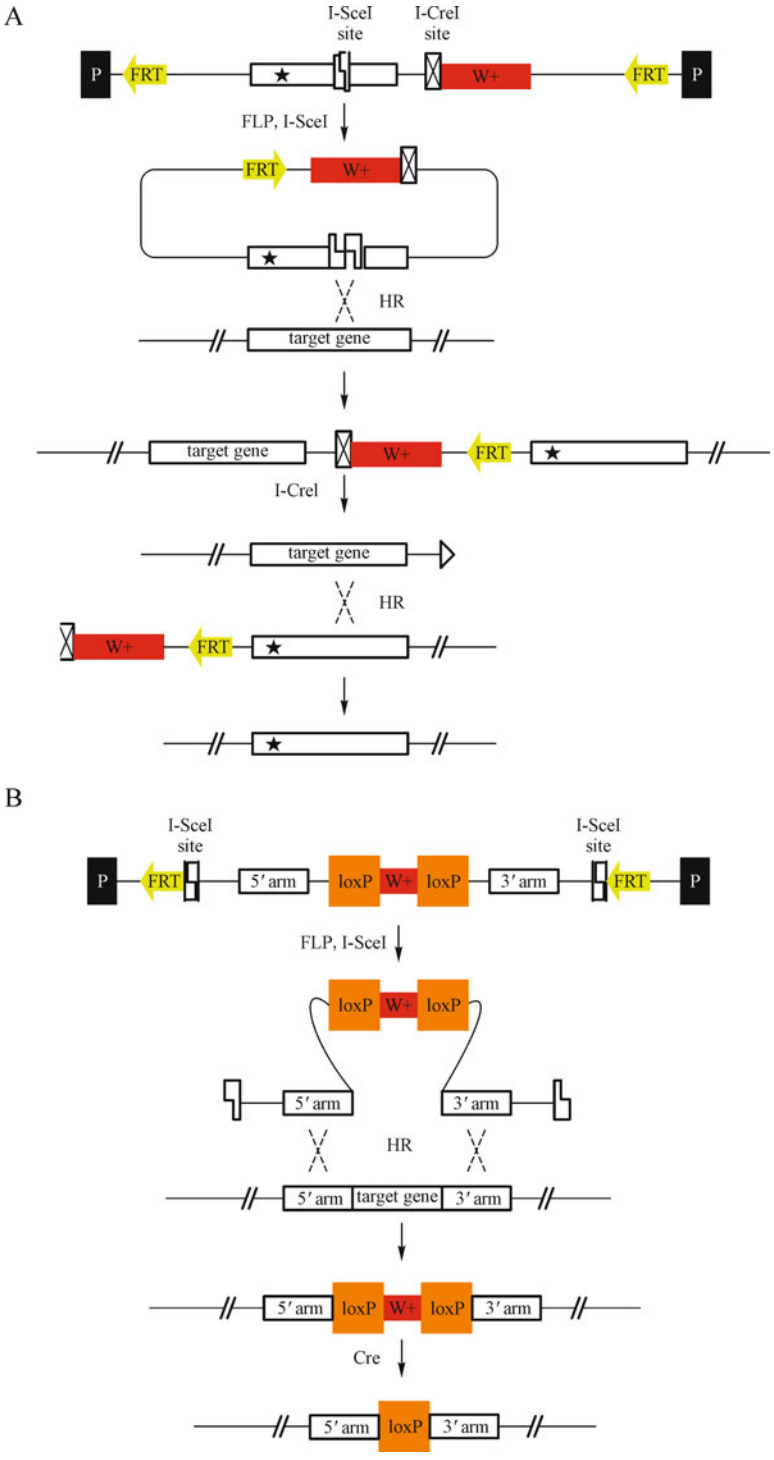

Fig. 1 Ends-in and ends-out gene targeting in *Drosophila*. A: Ends-in scheme. The relevant features of donor construct flanked by the *P* elements (black boxes): FLP recombinase target (FRT) sites (green arrows), *white* (w^+) selective marker (red boxes), I-SceI recognition sites, and I-CreI recognition sites are marked or indicated, and the asterisk represents the introduced mutation. The donor construct that contains the mutated genomic fragment is integrated into the germline by P-element-mediated transformation. A linearized targeting DNA fragment is generated *in vivo* by FLP and I-SceI. Recombination with the endogenous target sequence generates a tandem duplication of the targeted region. The duplication is reduced to a single copy of mutant gene after a double-stranded break (DSB) induced by I-CreI and repaired by homologous recombination, selected by the loss of $white^+$. B: Ends-out scheme. The donor construct flanked by the *P* elements, contains two stretches of homology arm (5′ arm and 3′ arm) interrupted by a $white^+$ marker that has two loxPs on both sides (brown box). After *P* element mediated transgenesis, linearized targeting DNA is generated *in vivo* by FLP and I-SceI. Correct targeting events are marked by $white^+$ which can be removed by Cre recombinase with only a loxP site left at the target gene locus.

through a recombination event between two stretches of homologous arms (Fig. 2B). The two strategies have different applications. Ends-in targeting allows specific mutations including deletions, insertions or point mutations. In ends-out strategy, the targeted gene is replaced or removed. With the potential of selectively disrupting a gene and being modified to be even more useful genetic tools, gene targeting has gained its popularity in *Drosophila* over the past 10 years. The improved technologies have been aiming to overcome the disadvantages of the originally developed gene targeting technique, which include (1) unable to dissect gene function repeatedly and in a versatile manner, (2) time and labor intensive, and (3) variation of targeting frequency and background mutations. Here, we summarize the major current improvements based on gene targeting that are used to efficiently generate a modified gene locus within the genome.

2 ΦC31-mediated gene targeting

Ends-in and ends-out gene targeting described above are efficient ways to make direct modifications of a target gene. However, it is time consuming and laborious; therefore, it is not practical to generate serial/multiple alleles of a given gene using these original targeting techniques. To overcome this drawback, the ΦC31-mediated site-specific integration system into the classic gene targeting strategies was reported recently (Gao et al., 2008; Huang et al., 2009). Phage integrase ΦC31 catalyzes the recombination between the phage attachment (attP) site from the phage genome and a bacterial attachment (attB) site from the bacterial host genome (Thorpe and Smith, 1998). Interestingly, attB-containing plasmids integrate more readily into attP-containing genomic docking sites than attP sites do in the reciprocal reaction, indicating that the integration is asymmetric in nature (Thyagarajan et al., 2001; Belteki et al., 2003). This integration system was soon introduced into *Drosophila* (Groth et al., 2004) and proven to be efficient to integrate transgenic constructs at defined docking sites in the fly genome. Moreover, ΦC31 integrase-mediated transgenesis allows large fragments to be integrated into the genome, greatly beyond the fragment sizes that can be introduced by P element-mediated transgenesis (Venken et al., 2006). Although the first reports used mRNA encoded ΦC31 integrase to integrate the DNA, efforts have been made to modify this technique, with very much improved efficiency of integration by direct microinjection of targeting constructs into embryos (Bischof et al., 2007).

Based on theΦC31 mediated integration system and the ends-in gene targeting, Rong and coworkers developed a method termed SIRT (site-specific integrase mediated repeated targeting) to repeatedly target a single locus in *Drosophila* (Gao et al., 2008). The first step of SIRT is the proper placement of an attP site close to the locus of interest by ends-in gene targeting scheme (Fig. 2A). The second step is the introduction of an attB containing donor plasmid into the docking sites by ΦC31-mediated integration. This attB containing donor plasmid is very similar to the one used to create an attP containing construct with designed modification(s) at the desired sites on the target gene. The ΦC31 integrase inserts the donor plasmid into the attP docking sites, creating the tandem duplication of the target gene. Finally, the expression of I-CreI endonuclease-induced reduction leads to a targeted gene with desired modification(s). The advantage of SIRT over traditional gene targeting is that it can generate a series of alleles of a target gene through two highly efficient steps: ΦC31-mediated site specific integration and I-CreI induced reduction.

Recently, Hong and coworkers combined the ΦC31 integration system and ends-out gene targeting to establish an efficient 2-step gene manipulation system termed "genomic engineering" (Huang et al., 2009). The first step is the modified ends-out gene targeting process which replaces the target gene with *white* marker flanked by two loxPs juxtaposed by an attP site (Fig. 2B). The *white* marker is removed by Cre recombinase, leaving only an attP and a loxP at the deleted locus. The second step is to introduce a modified target gene(s) into the native locus of the target gene by the ΦC31 integrase. In this step the donor plasmid contains an attB site, a modified target gene juxtaposed by a loxP site and the *white* marker. The clever attB/attP recombination will result in a *white* marker gene flanked by the newly introduced loxP and the loxP that is remained from the first step. Based on this "genomic engineering," fly researchers can generate directed and versatile modifications of genomic loci with relative less time and efforts.

3 Zinc-finger nuclease based gene targeting

In *Drosophila*, ends-in and ends-out are two ingenious gene targeting methods and has been widely used to introduce targeted mutation since its invention. However, relative low efficiency of both strategies and variations of recombination frequency between different donors and corresponding target DNA sequence have hindered the application of these targeting techniques. Recently, a new technology that is based on the use of targeted zinc-finger nucleases (ZFNs) was developed in *Drosophila* (Bibikova et al., 2002; Bibikova et al., 2003), which has been used for targeted mutagenesis in combination with HR (Beumer et al., 2006). ZFNs are hybrid proteins composed of a designed DNA binding domain of three zinc fingers that is specific for DNA target and a nonspecific FokI DNA cleavage domain (Fig. 3A). FokI requires dimerization to cut DNA. The binding of the designed heterodimeric zinc

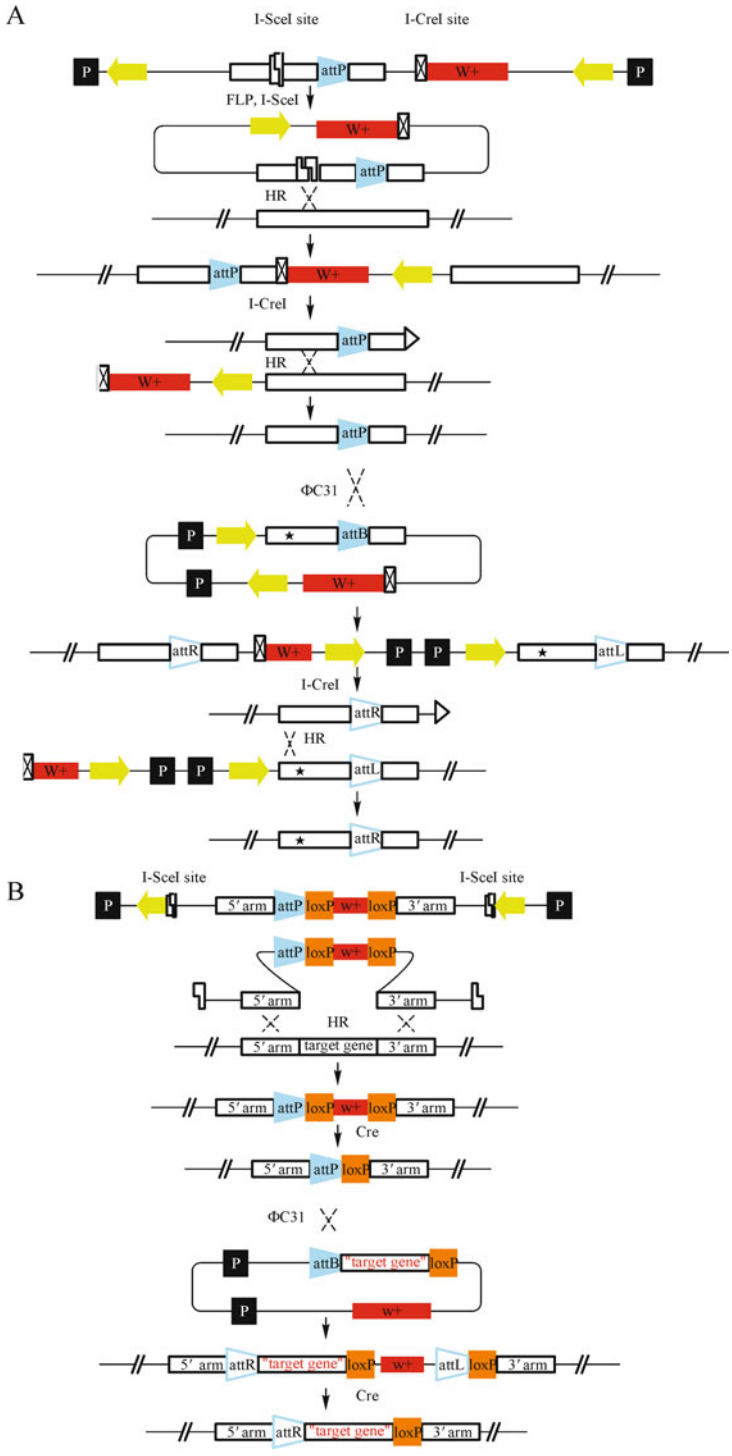

Fig. 2 Gene targeting schemes in combination with ΦC31-mediated integration. A: SIRT (site-specific integrase mediated repeated targeting) strategy. The donor construct contains some of the elements that are also present in the classic gene targeting donor construct (Fig. 1). The first step of SIRT is the ends-in targeting followed by reduction to eventually place attP (blue box) in a proper site. In the second step, the integrated donor carries FRTs and P-element, as well as the desired mutation(s) (black asterisk) and attB sites (blue box). Recombination between attP and attB creates attR and attL (blue-lined empty boxes) irreversibly and a duplication of the target gene. I-CreI induced recombination that occurs at the targeting region leads to loss of the *white*$^{+}$ gene, and remaining of the modified target gene and attR. B: "Genomic engineering" strategy. The first step of "genomic engineering" is the ends-out targeting which replaces the target gene with a transgene that is loxP-flanked, *w* + marked and juxtaposed by an attP site. The *w* + marker is removed by Cre recombinase, leaving only the attP and the loxP at the deletion locus. In the second step, the donor construct that contains the attB site and the modified target gene and a modified target gene is integrated into the deletion locus through ΦC31-mediated integration. Then the extra vector sequences will be removed by Cre recombinase to generate a final engineered target gene flanked by attR and loxP sites.

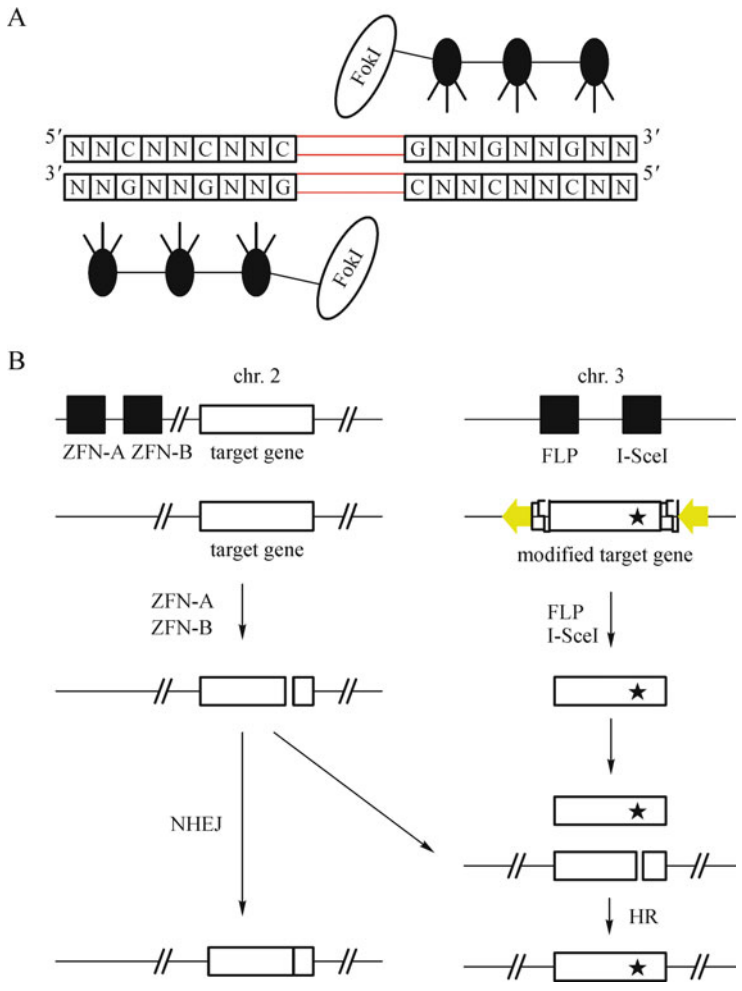

Fig. 3 zinc-finger nucleases (ZFNs) induced gene targeting in *Drosophila*. A: Diagram of the zinc-finger nucleases and its binding to the target sites. Each nuclease is composed of three zinc fingers (black balls) linked to the DNA-cleavage domain of FokI. Each finger contacts three consecutive 5′-GNN-3′ triplets. When both sets of fingers are bound, the cleavage domain will dimerize to form an active nuclease and cleave the spacer DNA (red bars) between the target sites. B: ZFNs induced gene targeting. The target gene is on chromosome 2, and the same chromosome carries ZFN transgenes: ZFN-A and ZFN-B (black boxes). FLP and I-SceI transgenes (black boxes) are on chromosome 3, and modified target gene is on the other chromosome 3. Upon heat shock, the ZFNs make a double-stranded break (DSB) in the target gene. Expression of FLP and I-SceI will result in a linear modified target DNA fragment. The break in the target gene will be restored to either wild type (homologous recombination (HR) with endogenous template) or result in a mutant target gene (non-homologous end joining (NHEJ) or HR with the linearized donor).

fingers to two contiguous target sites in each DNA strand separated by the cleavage site results in FokI dimerization and subsequent DNA cleavage. The specificity of ZFNs gene targeting is determined by the properties of the zinc-finger domains. Each finger recognizes 5′-GNN-3′ triplets, and the optimal arrangement of paired sites is in an inverted orientation with a 6 bp cleavage spacer. Based on this rule, the common sequence form is 5′-$(NCC)_3N_6(GNN)_3$-3′. In a particular case, the target gene has to be examined for a sequence of this form, and corresponding zinc finger properties will be designed accordingly by the zinc-finger platform (Liu et al., 2002; Segal, 2002).

In *Drosophila*, efficient ZFNs induced gene targeting is accomplished in combination with HR (Beumer et al., 2006). The general scheme of ZFNs gene targeting is shown in Fig. 3B. ZFN A and B are both located on the same chromosome (chr. 2) as the target gene aiming to enhance the cleavage efficiency. The marked (e.g. w^+) donor that includes the mutated target gene is on another chromosome (chr. 3). The ZFNs, FLP, and I-SceI transgenes are under the control of heat shock-inducible promoter. Expression of FLP and I-SceI excises the donor, resulting in a linear DNA fragment of the mutant target gene. When the target gene is cleaved by ZFN A + B, the following events may occur: The cleaved site can be restored by non-homologous end joining (NHEJ) without any template or by HR using the mutated donor

(linearized) as a template, both of which are mutagenic. Alternatively, the cleaved site can also be restored by simple relegation of the broken ends or by HR with the intact wild type gene on the sister chromatids, both of which will leave the target gene unmutated. Thus, cutting by the ZFN can directly create a double-strand break (DSB) at the specific target locus, leading to increased targeting efficiency when a linearized donor element is introduced. In the case of *y* locus (Beumer et al. 2006), ZFN expression in the presence of a linear donor elevated the yield of target HR product by 15-fold in the female germline and by 60-fold in the male germline. Although it appears likely that the ZFN gene targeting is an ideal alternative for the ends-in and ends-out gene targeting, especially for the low frequency recombination cases, it is not easy to construct both ZFNs in the same vector, meaning that two rounds of microinjection are needed. Also, as a relative new method, the range of effective targets may still have to be tested.

4 More efficient and more reliable gene targeting

Techniques for the targeted mutagenesis by HR have gained its popularity mostly because of its specificity. However, because the HR based gene targeting strategies need intensive labor and have relatively low recombination frequency, the original gene targeting has also met many limitations. The technical barrier seems to be unsolvable unless increasing the length of homologous arms in the donor construct. However, the modified procedure has made the original gene targeting more efficient (Huang et al., 2008). In the cases of low targeting efficiency, much of the work is spent on collecting a large number of virgins and making crosses. To break this major bottleneck in scaling up the targeting crosses, the ubiquitous expression of a cell-death gene *hid* (Grether et al., 1995) is used to eliminate all the male progeny and those female progeny carrying *hs-hid* balancer that would not generate targeting events. To eliminate the false positives which severely limit the real positive candidates before screening and mapping crosses, a negative selection marker *reaper* is introduced into the current ends-out targeting procedure. *Reaper* (*rpr*) (White et al., 1996) is another cell-death gene which causes cell death, and thus animal lethality. A *UAS-Rpr* is placed at the 3′ end of the transgenic donor DNA fragment. Once the donor DNA fragment is recombined with the endogenous target gene locus, *UAS-Rpr* will then be lost due to HR. In the non-targeting events, the donor DNA fragment will likely retain an intact *UAS-Rpr*, and the flies that carry this donor fragment will be eliminated by crossing to a Gal4 driver lines. By combining these two methods, the efficiency of ends-out gene targeting could be greatly enhanced.

Background mutations arising during HR based gene targeting have been reported (Rong et al., 2002; Greenberg et al., 2003; Lankenau et al., 2003; Radford et al., 2005); however, it is not routinely taken into account when using this technique. In a recent targeting case (O'Keefe et al., 2007), the *Drosophila* gene *Wwox* was knocked out by ends-in gene targeting strategy, and the resulted homozygous mutants ($Wwox^{1}$) were viable and fertile but showed an increased IR (ionizing radiation) sensitivity. Sensitivity to IR was also observed for another *piggyBac* transposon insertion allele of *Wwox* ($Wwox^{f04545}$) (Thibault et al., 2004). Surprisingly, sensitivity to IR was not observed in flies that were trans-heterozygous of $Wwox^{1}$ and $Wwox^{f04545}$, indicating that the IR sensitivity of the two homozygous mutant strains is independent of mutations in *Wwox*. This idea is confirmed by the fact that both alleles showed a significant decrease in sensitivity to IR after being backcrossed to their wild type parental strains. It has been reported that *piggyBac* insertion strains contain unrelated background mutation (Thibault et al., 2004), which account for the IR sensitivity in $Wwox^{f04545}$ strains. In the case of targeting procedure for *Wwox*, the authors recovered non-targeted background mutations, in addition to the desired mutations, which have significant effects on phenotypic outcomes. One might expect that in a gene targeting approach, the frequency of background mutations should be at a minimum level. Unfortunately, this is not the case in reality. The non-targeted mutations arise during HR-mediated mutagenesis procedure. First, the transgenic enzymes that are used (FLP, I-SceI, and I-CreI) in the targeting process could cleave nontargeted sites, especially because they are ectopically expressed. Second, non-targeted mutations could have been introduced directly or indirectly from the various crossing lines. Background mutation can be a considerable problem especially for some stringent tests, such as genome stability and behavior studies. However, there are simple solutions to eliminate the non-targeted background mutations. First, multiple rounds of backcrossing to the parental strain may ensure that alleles on all chromosomes will be replaced by assortment and recombination with those from the parental strains used for the targeted mutagenesis. Second, independently HR- generated alleles (i.e. after first round HR of ends-in mutagenesis; Fig. 1A) should be verified by more subsequent methods: (1) *trans*-heterozygous combination, and (2) rescue experiment following introduction of construct bearing corresponding genomic region or ectopic expression of the corresponding cDNA.

5 Perspectives

In *Drosophila*, gene targeting has been successfully applied to introduce a desired mutation(s) into a gene of interest. Gene targeting when combined with the ΦC31 site-specific integration technique becomes more powerful to carry out multiple structure-function studies at a higher

resolution with fewer transgenes. The application of ZFNs in flies makes gene targeting more efficient. Although many efforts have been made in the past 10 years to improve the targeting strategy, there are many important open questions remained. How to tackle a gene complex *in vivo*? Is there a possibility to manipulate two or three genes at one round of targeting? How to specifically delete a gene over 50 kb? How to expand the applications of ZFN induced gene targeting? How to manipulate the heterochomatic genes? How to make the HR-based machinery more efficient? It is likely that the combination of different methods and the development of new technologies will make gene targeting more powerful as one of the best *Drosophila* genetic tools to address biological questions.

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