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Methods for Homologous Recombination in *Drosophila*

Keith A. Maggert, Wei J. Gong, and Kent G. Golic

Summary

We present detailed protocols for two methods of gene targeting in *Drosophila*. The first, ends-out targeting, is identical in concept to gene replacement techniques used routinely in mammalian and yeast cells. In *Drosophila*, the targeted gene is replaced by the marker gene *white*$^+$ (although options exist to generate unmarked targeted alleles). This approach is simple in both the molecular cloning and the genetic manipulations. Ends-out will likely serve most investigators' purposes to generate simple gene deletions or reporter gene "knock-ins."

The second method, ends-in targeting, targets a wild-type gene with an engineered mutated copy and generates a duplication structure at the target locus. This duplication can subsequently be reduced to one copy, removing the wild-type gene and leaving only the introduced mutation. Although more complicated in the cloning and genetic manipulations (*see* **Note 1**), this approach has the benefit that the mutations may be introduced with no other remnant of the targeting procedure. This "surgical" approach will appeal to investigators who desire minimal perturbation to the genome, such as single nucleotide mutation.

Although both approaches appear to be approximately equally efficient (*see* **Note 2**), each method has separate strengths and drawbacks. The choice of which approach is best depends on the researcher's goal.

Key Words: Ends-in; ends-out; gene targeting; homologous recombination; mutation; replacement.

1. Introduction

Genetic manipulations have long been a recognized strength for research utilizing *Drosophila*, despite the late arrival of techniques for targeted gene disruption or replacement. We present two protocols for generating linear DNA molecules in vivo, capable of stimulating homologous recombination and incorporating desired sequence into specific locations in the genome. Targeting allows an investigator great flexibility by allowing the design of specific alleles, including amorphic or

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antimorphic alleles. A growing number of cases of successful targeting by many groups demonstrates the utility of this approach ***(1–32)***.

The chief requirement for homologous recombination as we describe is the generation of in vivo linear DNA molecules. The linear molecule is generated from an integrated transgenic donor construct using the enzymatic activity of *FLP* recombinase (to excise a circular molecule of DNA from the construct) and *I-Sce*I homing meganuclease (to convert the liberated circle to a linear molecule). Two arrangements of the linear molecule relative to the target genomic sequence are possible—ends-out and ends-in (**Figs. 1** and **2**)—generating distinctly different products after recombination and repair (*see* **Note 3**) ***(33,34)***.

The efficiency of pairing and recombination in *Drosophila* may be similar to the high rate of targeting in fungal cells, but because *Drosophila* does not allow in vitro introduction to cultured germ cells, and positive/negative selection is not used, more complicated genetic manipulation and screening are required for *Drosophila*. In the following protocols, we attempt to make the considerations in construct design clear, and the genetic crosses straightforward. For those who wish to understand the gritty details, we provide ample references, and for those who merely wish to follow the instructions and get their mutant, we provide a step-by-step protocol.

There are three steps of targeting that require some attention—construct design (*see* **Subheadings 3.1.1.** and **3.2.1.**), targeting (*see* **Subheadings 3.1.2.** and **3.2.2.**), and either marker removal (*see* **Subheading 3.1.3.**, for ends-out) or reduction (*see* **Subheading 3.2.3.**, for ends-in). Marker removal is optional and likely unnecessary for most purposes. We assume that a laboratory that attempts these procedures is sufficiently equipped for molecular biology (DNA cloning, the polymerase chain reaction (PCR), Southern blotting, electrophoresis, sequencing, and so on) and *Drosophila* husbandry (incubators, fly food, microscopes, experience with simple genetic crosses, P-element-mediated germline transformation, and so on) to complete the procedures.

2. Materials

2.1. Ends-Out Targeting

1. *FLP* and *I-Sce*I on chromosome *2* (y^1 w^*; $P\{ry^{+t7.2}$ *70FLP*$\}11$ $P\{v^{+t1.8}$ *70I-Sce*I$\}$2B noc^{Sco}/*CyO*, S^2) or on chromosome *3* (y^1 w^*; $P\{ry^{+t7.2}$ *70FLP*$\}23$ $P\{v^{+t1.8}$ *70I-Sce*I$\}$4A/*TM6*).
2. *FLP* on chromosome *2* (w^{1118}; $P\{ry^{+t7.2}$ *70FLP*$\}10$; Sb^1/*TM6*, *Ubx*).
3. One or more of: pP[w25.2], pP[w30], pP[w35] ends-out targeting vectors.
4. Optional: *Cre* recombinase on the *X* chromosome (y^1 w^{67c23} $P\{y^{+mDint2}$ *Crey*$\}$1b; noc^{Sco}/*CyO* or y^1 w^{67c23} $P\{y^{+mDint2}$ *Crey*$\}$1b; D^*/*TM3*, Sb^1) or on a chromosome *2* balancer (y^1 w^{67c23}; noc^{Sco}/*CyO*, $P\{w^{+mC}$ *Crew*$\}$DH1).
5. Optional: an in vivo source of Δ2,3 transposase (e.g., the *TMS* balancer, *TMS*, $P\{ry^{+t7.2}$ Δ*2-3*$\}$99B, *see* **Note 4**).

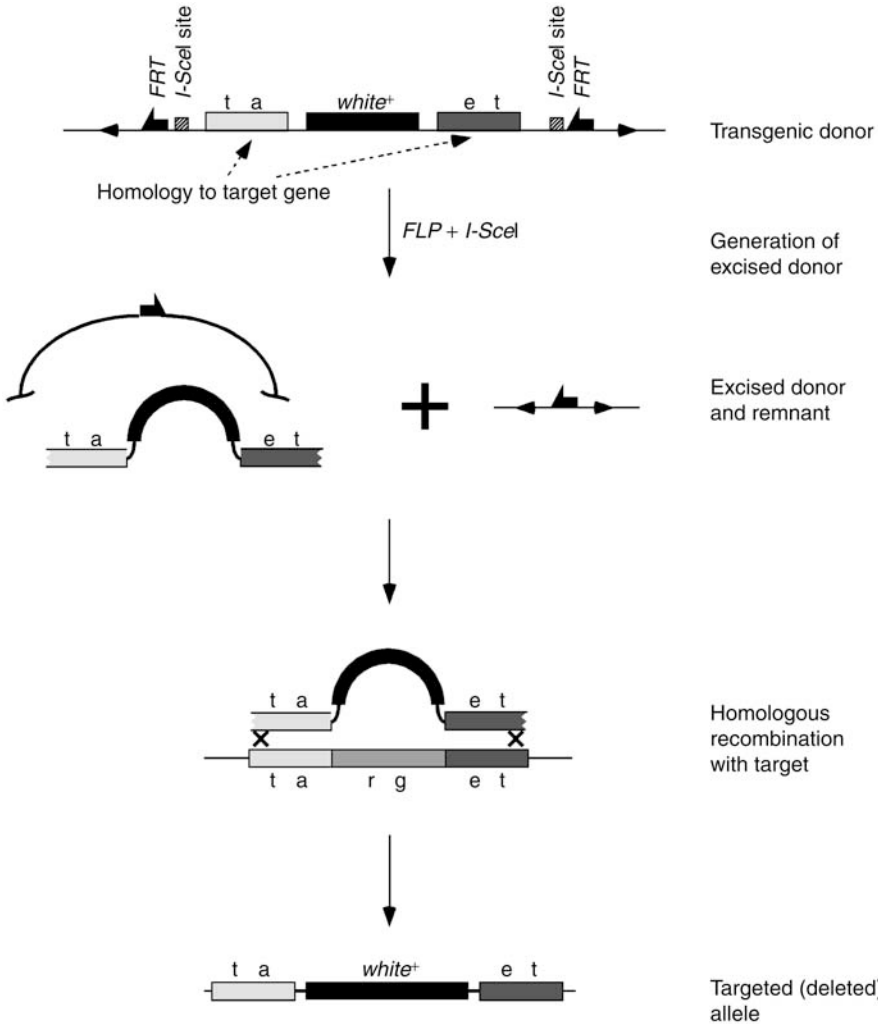

Fig. 1. Diagrammatic representation of ends-out targeting. *Transgenic donor* shows a clone using P[w35] as a basis for targeting. Hatched boxes are *I-Sce*I recognition sites, half-arrows are *FRT*s, arrowheads are *P*-element ends. Black box is the *white*$^+$ marker, used for both transformation and targeting. Gray bars, labeled with "ta" and "et," show cloned homology to target sequence. *FLP* and *I-Sce*I induction is responsible for the *generation of the excised donor*, leaving the *remnant* at the site of original transformant integration. Homology matches between the *excised donor* and *target* sequence cause *homologous recombination with the target*, replacing endogenous "rg" sequence with the *white*$^+$ gene and generating the *targeted (deletion) allele*.

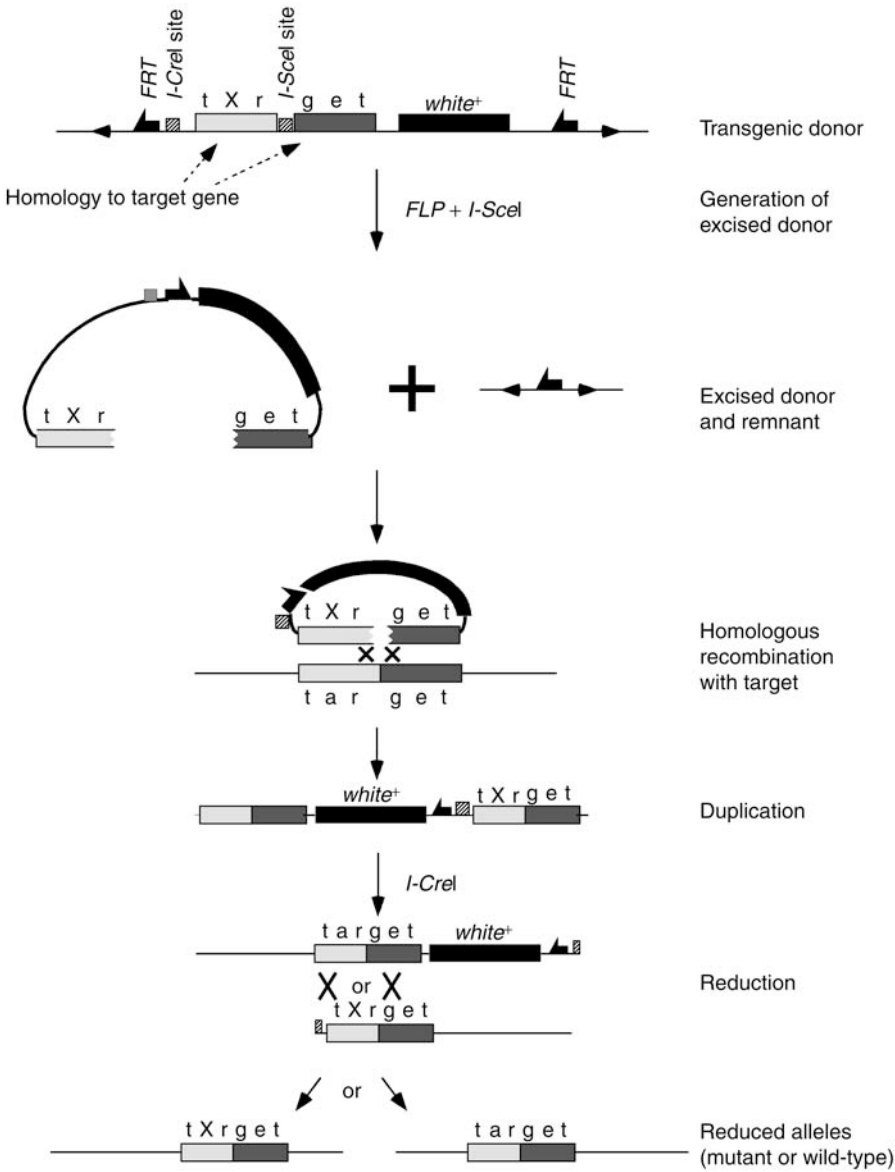

Fig. 2. Diagrammatic representation of ends-in targeting. In the *transgenic donor*, hatched boxes are *I-Sce*I and *I-Cre*I recognition sites, half-arrows are *FRT*s, arrowheads are *P*-element ends. Black box is the *white*$^+$ marker, used for both transformation and targeting. Gray bars, labeled with "tXr" and "get," show cloned homology to *target* sequence, where the "X" represents an introduced mutation. *FLP* and *I-Sce*I induction is responsible for the *generation of the excised donor*, leaving the *remnant* at the site of original transformant integration. Homology matches between the *excised donor* and

6. Optional: a balancer or double-balancer stock to establish a stock of your targeted allele (e.g., w^1; noc^{Sco}/*CyO*; D^*/*TM3*, Sb^1).

2.2. Ends-In Targeting

1. *FLP* and *I-Sce*I on chromosome *2* (y^1 w^*; $P\{ry^{+t7.2}$ *70FLP*$\}11$ $P\{v^{+t1.8}$ *70I-Sce*I$\}2B$ noc^{Sco}/*CyO*, S^2) or on chromosome *3* (y^1 w^*; $P\{ry^{+t7.2}$ *70FLP*$\}23$ $P\{v^{+t1.8}$ *70I-Sce*I$\}4A$/*TM6*).
2. *FLP* on chromosome *2* (w^{1118}; $P\{ry^{+t7.2}$ *70FLP*$\}10$; Sb^1/*TM6*, *Ubx*).
3. *I-Cre*I on the *X* chromosome ($P\{v^{+t1.8}$ *hs-I-Cre*I.R$\}2A$, v^1; ry^{506}) or on chromosome *3* (w^{1118}; $P\{v^{+t1.8}$ *hs-I-Cre*I.R$\}1A$ Sb^1/*TM6*, *Ubx*).
4. The pP[TV2] (*see* ref. ***33***) ends-in targeting vector.
5. Optional: an in vivo source of Δ2,3 transposase (e.g., the *TMS* balancer, *TMS*, $P\{ry^{+t7.2}$ Δ*2-3*$\}99B$, *see* **Note 4**).
6. Optional: a balancer or double-balancer stock to establish a stock of your targeted allele (e.g., w^1; noc^{Sco}/*CyO*; D^*/*TM3*, Sb^1).

All fly stocks are available at the Bloomington Drosophila Stock Center, Indiana (http://flystocks.bio.indiana.edu/), and DNA vectors are available from the Drosophila Genomics Resource Center (Indiana University, Bloomington, Indiana.) (http://dgrc.cgb.indiana.edu/).

3. Methods

3.1. Ends-Out Targeting

Ends-out targeting is so-named because the paired arrangement of donor and target DNA places the cut ends of the donor at the left and right sides of the recombining structure **(Fig. 1)** ***(34)***. Through heat-shock-induced expression of *FLP* recombinase and *I-Sce*I homing endonuclease, you will generate a linear excised donor DNA molecule whose ends are homologous to your target, but whose middle is not. Homologous recombination at both ends will replace the genomic target with the desired donor sequence. For screening, either a sequence alteration of your design (using P[w30], *see* **Subheading 3.1.1.**) or the $white^+$ marker (using P[w25.2] or P[w35]) may be used. The chief advantages of ends-out targeting are that cloning and genetic manipulations are simple. Ends-out is the best approach if your application requires the simple removal of a sequence (for instance, to create a deletion allele of a gene under investigation) or insertion of a sequence to create a disruption allele.

Fig. 2. *(Continued) target* sequence cause *homologous recombination with the target*, generating a *duplication* of the wild-type and mutated sequence separated by the $white^+$ marker gene. Subsequent *I-Cre*I induction generates a double-stranded break and induces recombination between the two copies of the gene. *Reduction* by recombination generates *reduced alleles (mutant or wild-type)*, depending on the location of the recombination relative to the mutation.

The final product of ends-out usually leaves some exogenous DNA at the targeted locus, often a *white*$^+$ marker gene. However, using the *Cre-loxP* site-specific recombination system ***(35)***, the *white*$^+$ gene may subsequently be removed, leaving only a single 34 bp *loxP* site behind. If the targeted gene is expected to have an easily scored phenotype (e.g., reversion of a mutant phenotype to wild-type, or generation of an allele with expected morphological phenotype), you may choose to target without a marker gene.

3.1.1. Construct Design for Deletion

1. Select the appropriate ends-out targeting vector **(Fig. 3)**.
   a. P[w35]: this vector has two cloning sites for cloning of the sequences that flank the region that you have targeted for insertion or removal **(Fig. 1)**. DNA between these flanking sequences in the target will be replaced with the *white*$^+$ gene during the targeting procedure. *white*$^+$ serves as both a transformation marker and a marker for monitoring the targeting procedure. P[w35] is the simplest vector for targeting.
   b. P[w25.2]: this vector, like P[w35], is designed to generate disruption or deletion alleles, but with a few additions. Two six-frame stop codons are included to assure minimal "read-through" from the *white*$^+$ gene, as well as two *loxP* sites flanking the *white*$^+$ marker. These *loxP* sites may be subsequently used to remove the *white*$^+$ gene (and its regulatory elements) and generate "unmarked" alleles. P[w25.2] may be useful if the presence of *white*$^+$ would interfere with subsequent analyses. Examples may include cases where other alleles of *white* are used as reporters, or when the presence of a marker gene is undesirable.
   c. P[w30]: this vector is designed with a *white*$^+$ transformation marker, but no targeting marker. *white*$^+$ is not flanked by FLP recombination target (FRTs) and will not become part of the excised donor during targeting—targeting with P[w30] does not allow secondary screening with *FLP* (*see* **Subheading 3.1.2., steps 9** and **10**). Note that only one cloning site is present in P[w30]; the sequence cloned into this site will replace the endogenous sequence, so P[w30] may be used to introduce site-directed mutations or specific allele structures created by the investigator. The targeting reaction will not be marked with a convenient marker, other than a phenotype introduced by the targeting itself. Examples of application of P[w30] include the generation of an allele with an obvious phenotype (e.g., a GFP fusion gene or a known morphological phenotype).
2. Clone sequences of homology into the appropriate vector, using high fidelity PCR or cloning from a DNA library, to minimize polymorphism between donor and target sequence (*see* **Note 5**). We recommend using as much homology as is practical to increase targeting efficiency. We prefer to use a minimum of 3 kb of homology on each side when using P[w35] or P[w25.2], or 6 kb of total homology when using P[w30] *(34)*.
3. Generate transformed lines carrying this donor construct.
4. Map the insertion site using meiotic segregation.

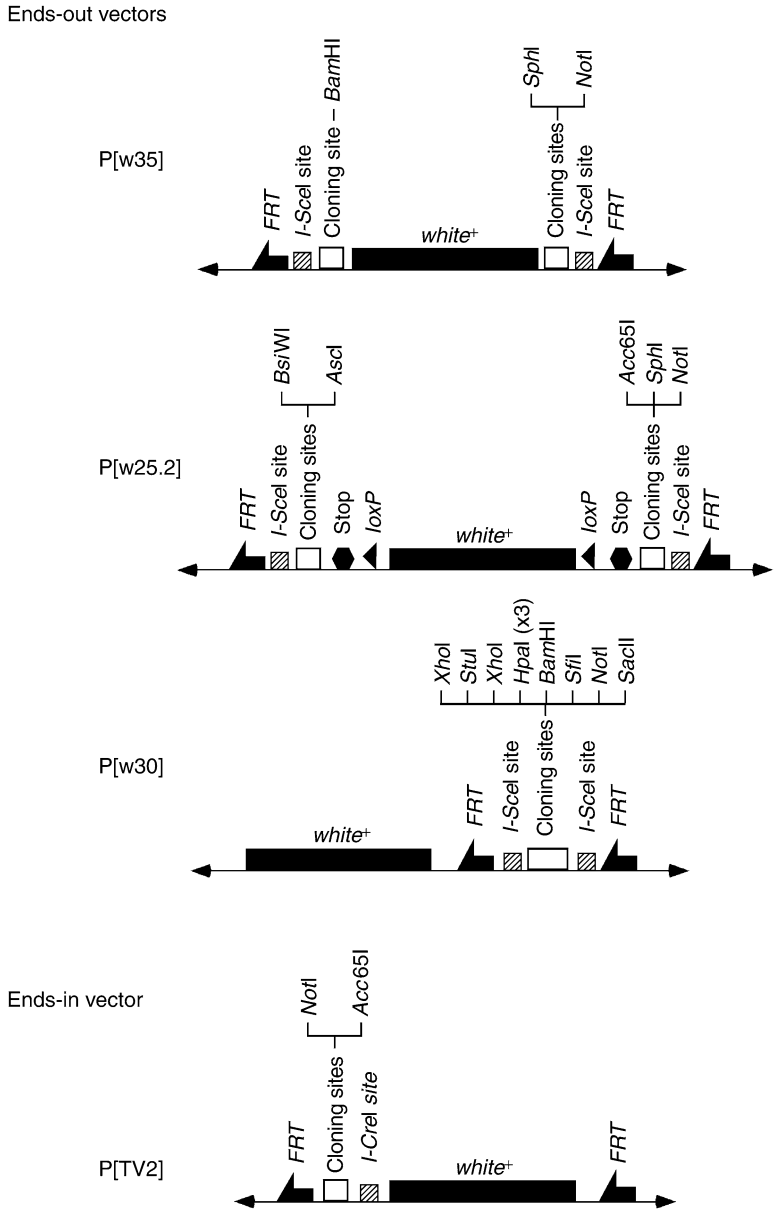

Fig. 3. Vectors used for ends-out and ends-in targeting. Arrowheads are *P*-element ends, half-arrows are *FRT* sequences, hatched boxes are *I-Sce*I and *I-Cre*I recognition sites, open boxes are sites for cloning DNA homologous to targeted sequence, hexagons are six-frame translation stop codons, triangles are *loxP* sites, and black boxes are *white*$^+$ marker gene.

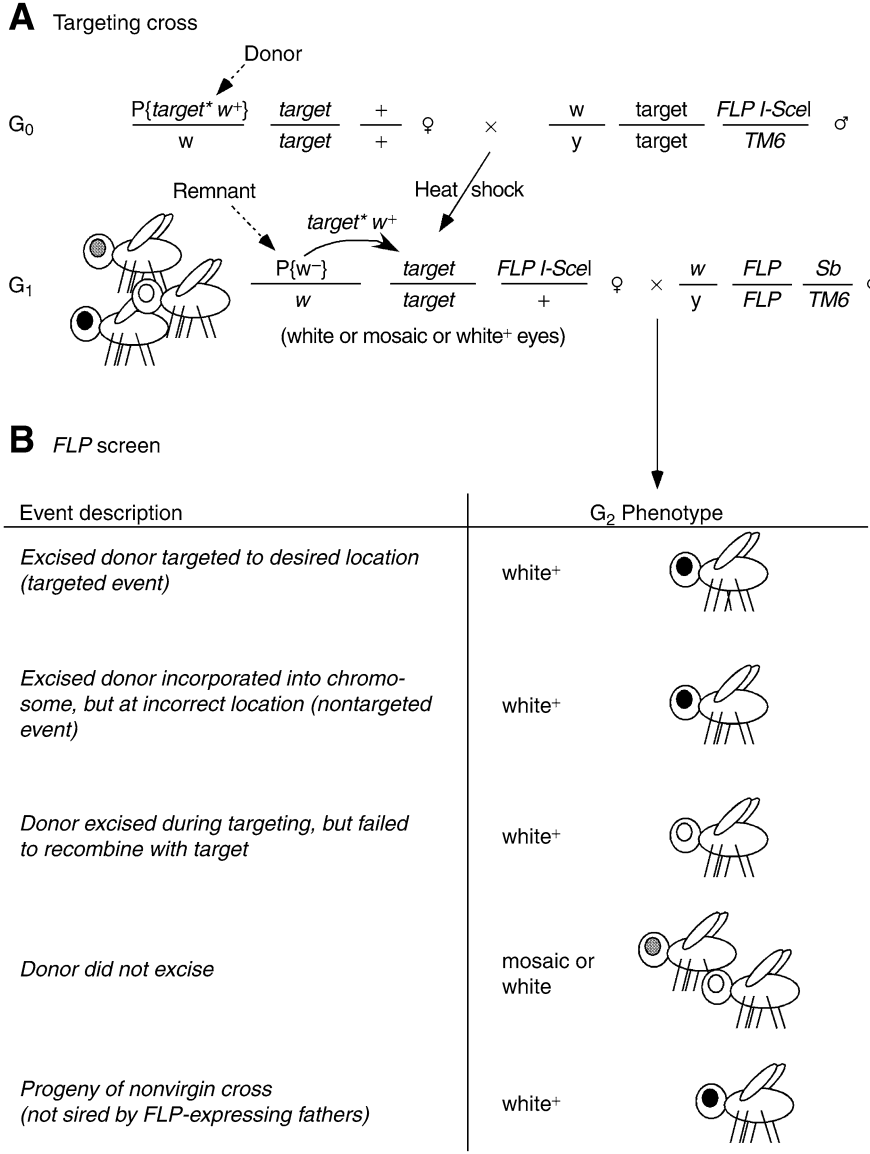

Fig. 4. Genetic crosses for targeting. **(A)** Ends-out and ends-in targeting (*see* **Subheading 3.1.2.**). G_0: transgenic flies, carrying the donor construct (P{*target* w*$^+$}) are crossed to flies expressing *FLP* and *I-Sce*I and heat-shocked (*see* **Subheading 3.1.2.**, **steps 1–7**). G_1: female progeny will have red, mosaic, or white eyes, and are collected and crossed to males expressing *FLP* (*see* **Subheading 3.1.2.**, **steps 8** and **9**). Red-eyed progeny (G_2) are collected and analyzed for proper targeting (*see* **Subheading 3.1.2.**, **steps 10–12**). **(B)** Offspring of the *FLP*-screened potential targeted flies (*see*

5. Confirm that the structure of the donor is unaltered using PCR and Southern-based techniques.

3.1.2. Targeting

The first cross (**Figs. 4** and **5**) is the same, for both ends-out or ends-in targeting. During this cross, portions of the donor are liberated from the chromosome to form the excised donor (*see* **Note 3**), which will recombine with the endogenous target gene. In the second generation, a constitutively expressed *FLP* gene is used as a secondary screen to exclude and discard flies that are white$^+$ because the excised donor was not generated, greatly reducing the amount of genetic and molecular analysis ***(36)***.

1. G_0 generation: cross flies containing your donor transgene to flies carrying *FLP* and *I-Sce*I transgenes **(Fig. 4)**—establish about 20 vials, each with five females and three males. Label this set of vials "A."
   a. The choice of which *FLP*- and *I-Sce*I-expressing lines to use will depend on your genetic scheme. It is best to use one different from your target chromosome, as you don't want to target the gene on the *FLP*- and *I-Sce*I-containing chromosome.
   b. y^1 w^*; $P\{ry^{+t7.2}$ *70FLP*$\}11$ $P\{v^{+t1.8}$ *70I-Sce*I$\}$2B noc^{Sco}/*CyO*, S^2.
   c. y^1 w^*; $P\{ry^{+t7.2}$ *70FLP*$\}23$ $P\{v^{+t1.8}$ *70I-Sce*I$\}$4A/*TM6.*
2. After 2 or 3 d, when you see first and second instar larvae crawling in the food, transfer the adults to new vials, labeling the new set of vials "B."
3. The following day, push the cotton plugs in the "A" vials to just above the food, preventing the larvae from crawling up the walls of the vial.
4. Heat-shock "A" vials at 38°C for 1 h in a circulating water bath, immersed with the water level above the level of the cotton plug.
5. Raise the cotton plugs and return the flies in the incubator.
6. Continue transferring and heat-shocking, labeling successive transfers "C," "D," and so on. Transfer flies 6–10 times **(Fig. 5)**.
7. After heat-shock, allow the flies to grow under standard culture conditions.

Fig. 4. *(Continued)* **Subheading 3.1.2., step 10**) will be of five types. The first type are targeted events, generating the expected replacement (for ends-out) or duplication (for ends-in) structures. These will be *white*$^+$ and may be Stubble (*see* **Note 11**). The second type are nontargeted events, where the excised donor is incorporated at random in the genome, not at the desired location. These will also be white$^+$ and may be Stubble, and must be discriminated from targeted events using molecular or genetic methods. The third type, typically representing the vast majority of offspring, are cases where the donor was excised, but did not target, and was lost. These flies will have white eyes and may be Stubble. The fourth type are cases where the donor was not excised from the chromosome. These flies will possess white or mosaic eyes and may be Stubble. The fifth type are products of nonvirgin matings. Your crossing scheme may vary, depending on the chromosomes that contain your donor and target genes.

Example timeline for targeting

Day	Activity
1	Set “A” vials with G0 parents
2	
3	Transfer flies from “A” vials to “B” vials
4	Heat shock “A” vials
5	Transfer flies from “B” vials to “C” vials
6	Heat shock “B” vials
7	Transfer flies from “C” vials to “D” vials
8	Heat shock “C” vials
9	Transfer flies from “D” vials to “E” vials
10	Heat shock “D” vials
11	Transfer flies from “E” vials to “F” vials; begin collecting G1 females from “A” vials, cross to *FLP* males
12	Heat shock “E” vials
13	Discard flies from “F” vials; begin collecting females from “B” vials, cross to *FLP* males
14	Heat shock “F” vials
15	Begin collecting females from “C” vials, cross to *FLP* males
16	
17	Begin collecting females from “D” vials, cross to *FLP* males
18	
19	Begin collecting females from “E” vials, cross to *FLP* males
20	
21	Begin collecting females from “F” vials, cross to *FLP* males
22	
23	
24	
25	Begin screening G2 offspring from “A” females for white^{+} eyes
26	
27	Begin screening offspring from “B” females
28	
29	Begin screening offspring from “C” females
30	
31	Begin screening offspring from “D” females
32	
33	Begin screening offspring from “E” females
34	
35	Begin screening offspring from “F” females

Fig. 5. Typical timeline for targeting (ends-out or ends-in) crosses. Transfer of parents (*see* **Subheading 3.1.2.**, **steps 2** and **6**) should occur when you see first and second instar larvae in the food. Heat-shock **(steps 3–5)** should occur when third instar larvae are visible. Actual time may differ based on your culture conditions.

8. Collect female flies (*see* **Notes 6** and **7**) as they eclose—remember that “B” vials will begin to eclose 2 or 3 d after the “A” set, and so on.
9. G_1 generation: cross *all* females, two at a time (*see* **Note 8**), to *FLP*-expressing males (*see* **Fig. 4B** and **Note 9**). Set up about 1000 vials.
   a. w^{1118}; $P\{ry^{+t7.2}\ 70FLP\}10$; $Sb^{1}/TM6$, *Ubx*.

10. G_2 generation: screen for nonwhite-eyed progeny (*see* **Note 10**). These flies will be targeted events, nontargeted events, or the progeny of nonvirgin mothers (*see* **Note 11**).
11. Cross to appropriate flies (e.g., w^1; noc^{Sco}/*CyO*; D^*/*TM3*, Sb^1) to establish a stock.
12. Confirm successful targeting using molecular analyses (*see* **Note 12**). PCR-based techniques are sufficient for quick screening, but Southern blot-based analyses are ultimately necessary, as many alternate structures may be produced during targeting ***(36)***.

3.1.3. Marker Removal

If you have used P[w25.2] for targeting and do not wish to have your engineered allele marked with *white*$^+$, it can be removed using the *loxP* sites flanking the *white*$^+$ marker gene ***(37)***. Targeted events can be crossed to *Cre* recombinase-expressing flies according to the following cross **(Fig. 6A)**.

1. G_0 generation: cross *Cre*-expressing females (listed below) to targeted males (*see* **Note 13**)—establish 5–10 vials, each with five females and three males.
   a. The choice of which *Cre* recombinase-expressing line to use will depend on your genetic scheme. Choose a stock to balance your targeted allele because after this cross, it will be unmarked.
   b. y^1 w^{67c23} $P\{y^{+mDint2}$ *Crey*$\}$1b; noc^{Sco}/*CyO*.
   c. y^1 w^{67c23} $P\{y^{+mDint2}$ *Crey*$\}$1b; D^*/*TM3*, Sb^1.
   d. y^1 w^{67c23}; noc^{Sco}/*CyO*, $P\{w^{+mC}$ *Crew*$\}$DH1.
2. Grow the flies in standard culture conditions. Heat-shock is not necessary because the *Cre* recombinase exhibits considerable maternal expression ***(35)***.
3. G_1 generation: select male (or, if your targeted gene is *X*-linked, female) progeny that possess *Cre* recombinase and the targeted allele.
4. Cross to appropriate flies (e.g., w^1; noc^{Sco}/*CyO*; D^*/*TM3*, Sb^1) to balance your targeted allele.
5. G_2 generation: select white-eyed progeny. These should possess the targeted allele now devoid of the *white*$^+$ marker. Collect individuals and cross them to appropriate flies (e.g., w^1; noc^{Sco}/*CyO*; D^*/*TM3*, Sb^1) to establish stocks (G_3 generation).
6. Confirm marker removal using molecular analyses (e.g., PCR or Southern blot-based analyses).

3.2. Ends-In Targeting

This approach uses a two-step method to introduce a mutant gene copy in tandem to the endogenous (wild-type) allele, and subsequently, to remove a portion from each of the two copies to leave a single-copy mutated allele in the chromosome **(Fig. 2)** ***(33,36,38)***. The benefit of this approach is that alleles carrying only the desired mutation, and no other alterations, can be recovered. Some have used this approach to make single nucleotide mutations within a gene ***(39)***. Ends-in targeting typically adds one additional genetic step compared with ends-out targeting, and construct design is somewhat more involved (though not always ***[36]***). Nevertheless, for some needs, it is the best solution.

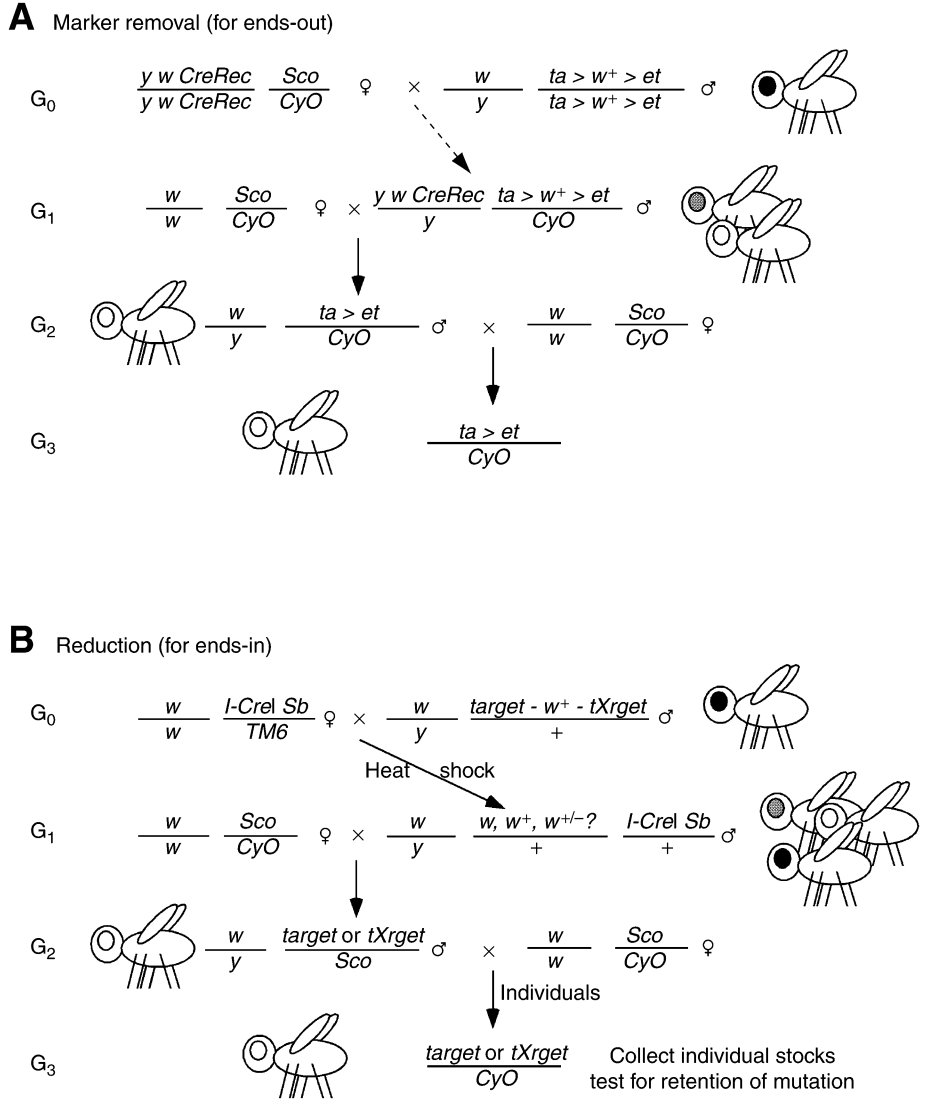

Fig. 6. Genetic crosses for marker removal and reduction. **(A)** Marker (*white*$^+$) removal when using P[w25.2] as an ends-out targeting vector (*see* **Subheading 3.1.3.**). G_0: *white*$^+$ targeted males (containing the target gene disrupted by *loxP*-flanked *white*$^+$, "ta > w^+ > et") are crossed to *Cre* recombinase-expressing females (*see* **Subheading 3.1.3.**, **step 1**). G_1: progeny will have mosaic or white eyes, and are collected and crossed to balancer-containing flies (*see* **Subheading 3.1.3.**, **steps 3** and **4**). G_2: white-eyed flies have lost the *loxP*-flanked *white*$^+$ gene, retaining only a *loxP* site (>) and are crossed to establish a stock (G_3, *see* **Subheading 3.1.3.**, **step 5**). **(B)** Reduction of anends-in targeted allele to a mutant allele (*see* **Subheading 3.2.3.**). G_0: *white*$^+$ targeted

3.2.1. Construct Design

1. Clone sequences of homology into a convenient plasmid (e.g., pBluescript [Strategene, La Jolla, CA], pGEM [Promega, Madison,WI]) for easy manipulation, using high fidelity PCR or cloning from a DNA library, to minimize polymorphism between donor and target sequence (*see* **Note 5**). We typically use DNA sequence with 5–6 kb of total homology or more.
2. Introduce an *I-Sce*I recognition site in the middle of the sequence (*see* **Note 14**).
3. Introduce your mutation—a small deletion or insertion, a point mutation, and so on. This mutation should preferably be at least approx 1 kb from the *I-Sce*I recognition site, although shorter distances may also produce successful alterations (*see* **Notes 1, 15**, and **16**).
4. Clone the constructed allele into P[TV2] **(Fig. 3)**. It possesses the other features necessary for ends-in targeting.
   a. A *white*$^+$ transformation and targeting marker gene.
   b. Two *FRT* sequences.
   c. An *I-Cre*I recognition site for Reduction (*see* **Subheading 3.2.3.**).
5. Generate transformed lines carrying this donor construct.
6. Map the insertion site using meiotic segregation.
7. Confirm that the structure of the donor is unaltered using PCR and Southern-based techniques.

3.2.2. Targeting

The procedure to induce targeting, and subsequently, screen with *FLP*-expressing males, is identical to that of ends-out targeting (*see* **Subheading 3.1.2.**). However, the final product differs in structure **(Fig. 2)**.

3.2.3. Reduction

With ends-in targeting, the theoretically expected product is a duplication at the targeted locus **(Fig. 2)**: the wild-type gene followed by the donor (mutated) gene with the *white*$^+$ marker in between. However, because recombinants with unexpected structures do arise ***(38)***, the structure should be confirmed by molecular methods, including Southern blotting. The duplication is reduced by

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Fig. 6. *(Continued)* males (containing the duplication of wild-type gene and mutant gene separated by *white*$^+$, "target - w^+ - tXrget") are crossed to *I-Cre*I-expressing females and heat-shocked during development (*see* **Subheading 3.2.3.**, **steps 1–7**). G_1: progeny will have red, mosaic, or white eyes, and are collected and crossed to balancer-containing flies (*see* **Subheading 3.2.3.**, **steps 8** and **9**). G_2: individual white-eyed flies are again crossed to balancer-containing flies to establish many independent lines (*see* **Subheading 3.2.3.**, **step 10**). G_3: each line, once established, is tested for reduction to wild-type or to mutant allele **(step 11)**. Genetic markers are listed in **Subheading 2.**

a recombination event that removes one copy of the gene and the marker ***(33)***. The result is the replacement of the wild-type copy with a specifically designed mutation, and no other alterations to the genome.

1. G_0 generation: cross *I-Cre*I females to targeted (duplication) males **(Fig. 6B)**—establish five vials, each with five females and three males.
   a. The choice of which *I-Cre*I-expressing line to use will depend on your genetic scheme.
   b. $P\{v^{+t1.8}$ *hs-I-Cre*I.R$\}$2A, v^1; ry^{506}.
   c. w^{1118}; $P\{v^{+t1.8}$ *hs-I-Cre*I.R$\}$1A Sb^1/*TM6*, *Ubx*.
2. After 2 or 3 d, when you see first and second instar larvae crawling in the food, transfer the adults to new vials, labeling the new set of vials "B."
3. The following day, push the cotton plugs in the "A" vials to just above the food, preventing the larvae from crawling up the walls of the vial.
4. Heat-shock "A" vials at 36°C (*see* **Note 17**) for 1 h in a circulating water bath, immersed with the water level above the level of the cotton plug.
5. Raise the cotton plugs and return the flies in the incubator.
6. Continue transferring and heat-shocking, labeling successive transfers "C," "D," and so on. Transfer flies approximately four times.
7. After heat-shock, allow the flies to grow under standard culture conditions.
8. Collect males as they eclose—remember that "B" vials will begin to eclose 2 or 3 d after the "A" set, and so on.
9. G_1 generation: cross males, two or three at a time, to appropriate flies (e.g., w^1; noc^{Sco}/*CyO*; D^*/*TM3*, Sb^1) to introduce marker mutations so that you may monitor potential reduced targeted alleles.
10. G_2 generation: select an *individual* white-eyed male (or, if your targeted gene is *X*-linked, individual females) from each vial, which will assure that each collected event is independent, and cross to appropriate flies (e.g., w^1; noc^{Sco}/*CyO*; D^*/*TM3*, Sb^1) to establish stocks (G_3 generation). Establish at least 20 (or more) individual lines to test for reductions to the mutant allele.
11. Confirm successful reduction using molecular analyses (e.g., PCR and Southern blot-based analyses).

4. Notes

1. The orientation of elements in the donor, the resulting duplication, and final reduction allele of ends-in can be very confusing, and a poorly designed donor construct may decrease efficiency or prevent targeting altogether. We recommend diagramming the structure of each step. The orientation of the $white^+$ gene relative to the donor allele sequence, the location of the *I-Sce*I recognition site, and the location of the introduced mutation are all critical factors **(Fig. 2)**. A few minutes of drawing can save months of work leading to a dead end.
2. It is difficult to compare frequencies because variance in targeting efficiency is high, between different laboratories, target genes, and donor transformant lines (*see* **Note 4**). We and others have seen frequencies of targeting as high as one event

per three females, and as low as one event per 1000 females, depending on the gene being targeted and the donor transformant line ***(32)***.

3. For ease and consistency, we define the following nomenclature: *donor*—a *P*-element-based transgene, carrying sequence homologous to the gene to be targeted, as well as necessary homing endonuclease sites (i.e., *I-Cre*I and *I-Sce*I), *FRT* sites, and marker genes used during targeting. *Excised donor*—The linear DNA, from the *donor*, that will recombine with the endogenous gene; *FLP* excises the DNA from the *donor* site and *I-Sce*I creates the double-stranded break(s) to expose the ends that stimulate recombination. *Remnant*—The exogenous DNA left behind by the *donor* during the targeting reaction. In most cases (except ends-out targeting with P[w30]), it consists of a single *FRT* flanked by *P*-element ends. *Targeted event*—desired movement of a portion of the *donor* to the target endogenous location, based on homology. *Nontargeted event*—although initially appearing as a *targeted event*, this is an undesirable movement of the *Donor*, perhaps to a random location in the genome. *Marker removal*—For ends-out targeting using P[w25.2], the removal of the *white*$^+$ marker gene, using Cre recombinase, to generate an unmarked but targeted allele. *Duplication*—the product of a *targeted event* during ends-in targeting; the juxtaposition of the *donor* (mutated) copy of the gene of interest and the endogenous copy (*see* **Fig. 2**). *Reduction*—For ends-in targeting, the *I-Cre*I-induced recombination between the two copies of the targeted gene, resulting in a single copy (wild-type or mutant, *see* **Fig. 2**). The crosses that we show (**Figs. 4** and **6**) illustrate a target gene on chromosome *2*. The details of your genetic scheme may differ and will be based on the chromosome on which your target gene is found.
4. Chromosomal position effects—proximity of a transformed transgene to regulatory elements, heterochromatic chromosome features, and so on—may affect the ability of a transgene to act as a donor for targeting. Although we have not methodically tested different insertion sites for their efficacy in targeting, we routinely notice that some insertions fail to lead to targeting events, whereas others do so at high frequency. Hence, use of Δ2,3 Transposase to mobilize a targeting transgene to other locations within the genome (or use of multiple independent transformation events as targeting donors) is recommended.
5. In genomic regions where there are nearby genes, some parts of the donor may include coding regions of neighboring genes. To prevent inadvertently introducing mutations to these genes, we recommend the use of high-fidelity polymerases during PCR to clone the gene from flies. Some problematic genes may require PCR amplification and cloning, or simply direct subcloning, from a bacterial artificial chromosome or bacteriophage P1 genomic clone. Although no controlled comparison has been made to our knowledge, DNA polymorphism appears to have little effect on targeting efficiency in *Drosophila* ***(10,39)***.
6. Females have a higher rate of targeting than do males, and so we recommend using only females. Males may be used and crossed to *FLP*-expressing females, but we find that it is not worth the extra effort owing to an appreciably lower frequency of targeting. Females do not need to be virgin, although use of virgins will make subsequent crosses to identify targeting events easier. Nonvirgins may have mated with

white$^+$ male siblings, but progeny from nonvirgin matings will be obvious in the next generation (*see* **Note 7**). We feel that it is better to cross all females, whether virgin or not, and screen through the progeny later.

7. Expect to see all sorts of patterns of your marker gene: white eyes, red eyes, and eyes with patches of both. We have not seen a strong correlation between expression of *white*$^+$ in the eyes (variegation caused by *FLP*-mediated *white*$^+$ removal in the soma) and successful targeting (where *FLP*-mediated excision is in the germline). *Be safe*: cross every female, regardless of eye phenotype, to *FLP*-expressing males.
8. Most researchers have reported frequencies of targeting to be relatively low, approx 1/100 vials of progeny from the cross of heat-shocked females to *FLP*-expressing males. For this reason, we routinely culture the progeny of two (or three) females together: the vials are healthier and we rarely encounter multiple independent targeting events from two females in the same vial.
9. The *FLP*-expressing stock appears to be an enhancer-trap line and does not require heat-shock to express efficiently. Other labs use eye-specific expression of *FLP* with similar effect ***(40)***.
10. The *white*$^+$ marker used during targeting may come under the influence of chromosomal position effects, and thus properly targeted alleles may have red, orange, or yellow eyes. Any nonwhite eyed fly should be treated as a potential targeting event.
11. As the *FLP*-expressing stock is marked with *Sb*, it is possible to discriminate some of the white$^+$ flies that represent targeting events from those that are products of nonvirgin mothers by the presence of the Stubble phenotype. However, only 50% of the progeny of *FLP*-expressing fathers will be Stubble (their siblings will be Ultrabithorax, which could also come from nonvirgin mothers). Although white$^+$ *Sb* flies can only be from transgene movement, and indicate a targeted (or nontargeted) allele, excluding *white*$^+$ Stubble$^+$ flies may result in discarding 50% of your targeting events. Although some germ cells may show targeting, many more will not. Typically, we see fewer than five *white*$^+$ flies from a vial, the remaining white$^-$ siblings are derived from germ cells where targeting did not occur.
12. After recovery of white$^+$ individuals, and before more tedious molecular analyses, it is often worthwhile to repeat the *FLP* screen (*see* **Subheading 3.1.2.**, **steps 9** and **10**, **Fig. 4A** generation G_1, **Fig. 4B**) to confirm that the recovered *white*$^+$ gene is not flanked by *FRT*s.
13. Cre recombinase expression is controlled by an *Hsp70-Mos1* promoter fusion, and is expressed efficiently without heat-shock ***(35)***. In our experience, nearly 100% of the flies with a *loxP*-flanked *white*$^+$ gene and expressing *Cre* recombinase give offspring with white eyes.
14. The *I-Sce*I recognition sequence is 5′-TAGGGATAACAGGGTAAT-3′. Note that the consensus is nonpalindromic, and has a T_m of approx 50°C. Although orientation of the *I-Sce*I site does not affect targeting (in most cases the *I-Sce*I sequence will be removed before or during targeting by cellular exonucleases), PCR can be used to determine orientation. We use versions of this sequence that can be cloned into blunt-ended or cohesive restriction sites. Remember that oligonucleotides must be 5′ phosphorylated (chemically during synthesis, or afterwards using polynucleotide kinase) in order to be efficient substrates for ligation.

15. For mutagenesis, we often use annealed oligonucleotides that introduce translational stop codons in all three reading frames. This sequence may be designed with cohesive ends to suit your cloning scheme. In addition to stop codons in all three frames, we recommend making the oligonucleotides of correct length to introduce a frame shift. Easy molecular analysis can be accomplished by simultaneous introduction of a diagnostic restriction site.
16. As the "homologous recombination" during Reduction (*see* **Subheading 3.2.3.**) may involve single strand annealing and mismatch repair, multiple mutations on a donor may not all appear in the final mutated allele. Although this may be a problem for some, it has been used to great advantage by one laboratory to generate a suite of alleles with different combinations of lesions that otherwise would have taken significant time and effort ***(39)***. Additionally, another laboratory has used the "unexpected" structures generated during ends-in targeting as additional alleles ***(40)***.
17. *I-Cre*I endonuclease recognizes a sequence in the *Drosophila rDNA* arrays on the *X* and *Y* chromosomes. High levels of expression can cause *rDNA* deletion or translocation between the *X* and *Y* chromosomes ***(41)***. Even higher levels of expression can cause death. You will note that offspring with the *I-Cre*I transgene are underrepresented in the second generation. A reduced heat-shock temperature will facilitate survival if this is a problem.

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References

1. Beall, E. L., Bell, M., Georlette, D., and Botchan, M. R. (2004) Dm-myb mutant lethality in *Drosophila* is dependent upon mip130: positive and negative regulation of DNA replication. *Genes Dev.* **18,** 1667–1680.
2. Bi, X., Wei, S. C., and Rong, Y. S. (2004) Telomere protection without a telomerase; the role of ATM and Mre11 in *Drosophila* telomere maintenance. *Curr. Biol.* **14,** 1348–1353.
3. Brankatschk, M. and Dickson, B. J. (2006) Netrins guide *Drosophila* commissural axons at short range. *Nat. Neurosci.* **9,** 188–194.
4. Donaldson, T. D., Noureddine, M. A., Reynolds, P. J., Bradford, W., and Duronio, R. J. (2004) Targeted disruption of *Drosophila* Roc1b reveals functional differences in the Roc subunit of Cullin-dependent E3 ubiquitin ligases. *Mol. Cell Biol.* **15,** 4892–4903.
5. Demir, E. and Dickson, B. J. (2005) fruitless splicing specifies male courtship behavior in *Drosophila*. *Cell* **121,** 785–794.
6. Egli, D., Yepiskoposyan, H., Selvaraj, A., et al. (2006) A family knockout of all four *Drosophila* metallothioneins reveals a central role in copper homeostasis and detoxification. *Mol. Cell Biol.* **26,** 2286–2296.

7. Elmore, T., Ignell, R., Carlson, J. R., and Smith, D. P. (2003) Targeted mutation of a *Drosophila* odor receptor defines receptor requirement in a novel class of sensillum. *J. Neurosci.* **23,** 9906–9912.
8. Funk, N., Becker, S., Huber, S., Brunner, M., and Buchner, E. (2004) Targeted mutagenesis of the Sap47 gene of *Drosophila*: flies lacking the synapse associated protein of 47 kDa are viable and fertile. *BMC Neurosci.* **5,** 16.
9. Gao, Z., Joseph, E., Ruden, D. M., and Lu, X. (2004) *Drosophila* Pkd2 is haploid-insufficient for mediating optimal smooth muscle contractility. *J. Biol. Chem.* **279,** 14,225–14,231.
10. Greenberg, A. J., Moran, J. R., Coyne, J. A., and Wu, C. I. (2003) Ecological adaptation during incipient speciation revealed by precise gene replacement. *Science* **302,** 1754–1757.
11. Haines, N. and Irvine, K. D. (2005) Functional analysis of *Drosophila* beta1,4-N-acetlygalactosaminyltransferases. *Glycobiology* **15,** 335–346.
12. Han, Z., Li, X., Wu, J., and Olson, E. N. (2004) A myocardin-related transcription factor regulates activity of serum response factor in *Drosophila*. *Proc. Acad. Sci. USA* **101,** 12,567–12,572.
13. Han, Z., Peng, Y., Li, X., and Olson, E. N. (2006) Hand, an evolutionarily conserved bHLH transcription factor required for *Drosophila* cardiogenesis and hematopoiesis. *Development* **133,** 1175–1182.
14. Hirosawa-takamori, M., Chung, H. R., and Jackle, H. (2004) Conserved selenoprotein synthesis is not critical for oxidative stress defence and the lifespan of *Drosophila*. *EMBO Rep.* **5,** 317–322.
15. Hittinger, C. T., Stern, D. L., and Carroll, S. B. (2005) Pleiotropic functions of a conserved insect-specific Hox peptide motif. *Development* **132,** 5261–5270.
16. Huang, J., Wu, S., Barrera, J., Matthews, K., and Pan, D. (2005) The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating yorkie, the *Drosophila* homolog of YAP. *Cell* **122,** 421–434.
17. Klymenko, T., Papp, B., Fischle, W., et al. (2006) A Polycomb group protein complex with sequence-specific DNA-binding and selective methyl-lysine-binding activities. *Genes Dev.* **20,** 1110–1122.
18. LaLonde, M. M., Janssens, H., Rosenbaum, E., et al. (2005) Regulation of phototransduction responsiveness and retinal degeneration by a phospholipase D-generated signaling lipid. *J. Cell Biol.* **169,** 471–479.
19. Larsson, M. C., Domingos, A. I., Jones, W. D., Chiappe, M. E., Amrein, H., and Vosshall, L. B. (2004) Or83b encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. *Neuron* **43,** 703–714.
20. Liu, H. and Kubli, E. (2003) Sex-peptide is the molecular basis of the sperm effect in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **100,** 9929–9933.
21. Manoli, D. S., Foss, M., Villella, A., Taylor, B. J., Hall, J. C., and Baker, B. S. (2005) Male-specific fruitless specifies the neural substrates of *Drosophila* courtship behaviour. *Nature* **436,** 395–400.
22. O'Keefe, L. V., Liu, Y., Perkins, A., Dayan, S., Saint, R., and Richards, R. I. (2005) FRA16D common chromosomal fragile site oxido-reductase (FOR/WWOX)

protects against the effects of ionizing radiation in *Drosophila*. *Oncogene* **24,** 6590–6596.
23. Radford, S. J., Goley, E., Baxter, K., McMahan, S., and Sekelsky, J. (2005) *Drosophila* ERCC1 is required for a subset of MEI-9-dependent meiotic crossovers. *Genetics* **170,** 1737–1745.
24. Sears, H. C., Kennedy, C. J., and Garrity, P. A. (2003) Macrophage-mediated corpse engulfment is required for normal *Drosophila* CNS morphogenesis. *Development* **130,** 3557–3565.
25. Sogame, N., Kim, M., and Abrams, J. M. (2003) *Drosophila* p53 preserves genomic stability by regulating cell death. *Proc. Natl. Acad. Sci. USA* **100,** 4696–4701.
26. Sokol, N. S. and Ambros, V. (2005) Mesodermally expressed *Drosophila* microRNA-1 is regulated by Twist and is required in muscles during larval growth. *Genes Dev.* **19,** 2343–1354.
27. Stockinger, P., Kvitsiani, D., Rotkopf, S., Tirian, L., and Dickson, B. J. (2005) Neural circuitry that governs *Drosophila* male courtship behavior. *Cell* **121,** 795–807.
28. Sun, S., Ting, C. -T., and Wu, C. –I. (2004) The normal function of a speciation gene, *odysseus*, and its hybrid sterility effect. *Science* **305,** 81–83.
29. Swan, A., G Barcelo, G., and Schupbach, T. (2005) *Drosophila* Cks30A interacts with Cdk1 to target Cyclin A for destruction in the female germline. *Development* **132,** 3669–3678.
30. Teleman, A. A., Maitra, S., and Cohen, S. M. (2006) *Drosophila* lacking microRNA miR-278 are defective in energy homeostasis. *Genes Dev.* **20,** 417–420.
31. Watnick, T. J., Jin, Y., Matunis, E., Kernan, M. J., and Montell, C. (2003) A flagellar polycystin-2 homolog required for male fertility in *Drosophila*. *Curr. Biol.* **13,** 2179–2184.
32. Xie, H. B. and Golic, K. G. (2004) Gene deletions by ends-in targeting in *Drosophila melanogaster*. *Genetics* **168,** 1477–1489.
33. Rong, Y. S., Titen, S. W., Xie, H. B., et al. (2002) Targeted mutagenesis by homologous recombination in *D. melanogaster*. *Genes Dev.* **16,** 1568–1581.
34. Gong, W. J. and Golic, K. G. (2003) Ends-out, or replacement, gene targeting in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **100,** 2556–2561.
35. Siegal, M. L. and Hartl, D. L. (1996) Transgene coplacement and high efficiency site-specific recombination with the Cre/lox system in *Drosophila*. *Genetics* **144,** 715–726.
36. Rong, Y. S. and Golic, K. G. (2001) A Targeted Gene Knockout in *Drosophila*. *Genetics* **157,** 1307–1312.
37. Gong, W. J. and Golic, K. G. (2004) Genomic deletions of the *Drosophila melanogaster* Hsp70 genes. *Genetics* **168,** 1467–1476.
38. Rong, Y. S. and Golic, K. G. (2000) Gene Targeting by Homologous Recombination in *Drosophila*. *Science* **288,** 2013–2018.
39. Dolezal, T., Gazi, M., Zurovec, M., and Bryant, P. J. (2003) Genetic analysis of the ADGF multigene family by homologous recombination and gene conversion in *Drosophila*. *Genetics* **165,** 653–666.

40. Egli, D., Selvaraj, A., Yepiskoposyan, H., et al. (2003) Knockout of 'metal-responsive transcription factor' MTF-1 in *Drosophila* by homologous recombination reveals its central role in heavy metal homeostasis. *EMBO J.* **22,** 100–108.
41. Maggert, K. A. and Golic, K. G. (2005) Highly efficient sex chromosome interchanges produced by I-CreI expression in *Drosophila*. *Genetics* **171,** 1103–1114.