Captured Segment Exchange: A Strategy for Custom Engineering Large Genomic Regions in *Drosophila melanogaster*

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ABSTRACT Site-specific recombinases (SSRs) are valuable tools for manipulating genomes. In *Drosophila*, thousands of transgenic insertions carrying SSR recognition sites have been distributed throughout the genome by several large-scale projects. Here we describe a method with the potential to use these insertions to make custom alterations to the *Drosophila* genome *in vivo*. Specifically, by employing recombineering techniques and a dual recombinase-mediated cassette exchange strategy based on the phiC31 integrase and FLP recombinase, we show that a large genomic segment that lies between two SSR recognition-site insertions can be "captured" as a target cassette and exchanged for a sequence that was engineered in bacterial cells. We demonstrate this approach by targeting a 50-kb segment spanning the *tsh* gene, replacing the existing segment with corresponding recombineered sequences through simple and efficient manipulations. Given the high density of SSR recognition-site insertions in *Drosophila*, our method affords a straightforward and highly efficient approach to explore gene function *in situ* for a substantial portion of the *Drosophila* genome.

MANY approaches to genetic research rely on an ability to alter DNA sequences *in vivo*. To this end, site-specific recombinases (SSRs) such as Cre, FLP, and phiC31 integrase have become invaluable tools for manipulating genes and genomes on small and large scales (reviewed by Branda and Dymecki 2004; Venken and Bellen 2005). The introduction of recognition sequences for SSRs into a genome can permit the generation of insertions, deletions, and/or inversions at precisely mapped genomic locations, facilitating analyses of genome structure and gene function.

In the model organism *Drosophila melanogaster*, SSRs play a pivotal role in several methods that make predictable changes to the genome sequence (reviewed by Venken and Bellen 2005; Venken and Bellen 2007). For example, recombination between two FRT sites located at different positions on homologous chromosomes can generate deletions, duplications, and inversions spanning several megabases depending on the relative positions and orientations of the FRTs (Golic and Golic 1996; Parks *et al.* 2004; Ryder *et al.* 2004). Several multi-step schemes have used SSRs in conjunction with the endogenous homologous recombination repair machinery to generate more precisely defined changes to the genome sequence, including small deletions, point mutations, and protein fusions (Gao *et al.* 2008; Choi *et al.* 2009; Huang *et al.* 2009; Weng *et al.* 2009). While these latter methods are of great utility to the *Drosophila* community, their efficiency and ease of use are limited by their reliance on the endogenous homologous recombination machinery during at least one step in each method. Thus, an approach to generate similarly precise changes to the genome that could circumvent the use of homologous recombination would be of great benefit.

In addition to methods that alter existing sequences in the *Drosophila* genome, several strategies that rely on SSRs have been used to improve methods of transgenesis. Much recent attention has been focused on site-specific integration of transgenes using phiC31 integrase, which catalyzes recombination between two distinct recognition sequences, attP and attB (reviewed by Venken and Bellen 2007 and Smith *et al.* 2010). Recombination between these sites creates two new sequences, attL and attR, that are not

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substrates for the integrase, resulting in a directional recombination event that is not reversible under normal conditions. Site-specific integration of a transgene can be achieved via recombination between an attP in the genome and an attB-bearing plasmid that carries the transgene of interest, resulting in a stable integrant of the plasmid at the site of the original attP (Groth *et al.* 2004; Bischof *et al.* 2007).

In an alternative strategy called recombinase-mediated cassette exchange (RMCE; reviewed by Baer and Bode 2001), a transgene of interest can be integrated into a defined site in a genome without incorporating the associated plasmid sequences. In this case, a "target cassette" carrying a selectable marker flanked by two SSR recognition sites is first inserted into the genome by other means. In the presence of the relevant SSR, introduction of a plasmid carrying a "donor" cassette flanked by compatible recognition sites can result in recombination at both ends of the cassettes, effectively exchanging the target and donor cassettes and thereby integrating the donor sequence into the genome. Methods for RMCE in *Drosophila* and other organisms have been demonstrated using Cre, FLP, or phiC31 integrase (reviewed by Baer and Bode 2001; Venken and Bellen 2007). Furthermore, "dual" RMCE strategies have been developed wherein the donor and target cassettes carry loxP recognition sites for Cre at one end and FRT sites for FLP at the other end (Lauth *et al.* 2002; Osterwalder *et al.* 2010; Anderson *et al.* 2012). In these schemes, cassette exchange relies on the combined activity of both enzymes, either simultaneously or in successive steps of plasmid insertion and subsequent deletion of the target cassette and plasmid backbone. A variant of dual RMCE has been applied to transgenesis in *Drosophila*, wherein a plasmid carrying a transgene is first integrated by recombination between phiC31 attP and attB sites, and the plasmid backbone is then deleted in a second step using Cre-mediated recombination between loxP sites (Bischof *et al.* 2007; Huang *et al.* 2009).

In parallel with the development of technologies that rely on SSRs, several large-scale projects have incorporated thousands of SSR recognition sequences throughout the *Drosophila* genome. In particular, >23,000 PiggyBac insertions carrying FRT sequences were generated as part of the Exelixis collection (Thibault *et al.* 2004), and an additional ~3000 FRT-bearing *P*-element insertions were created for the DrosDel collection (Ryder *et al.* 2004). In addition, the *Drosophila* Gene Disruption Project recently reported >1200 insertions of MiMIC, a *Minos*-based transposon carrying phiC31 attP sites, and plans to generate >6000 insertions by 2015 (Venken *et al.* 2011). These large collections are an important asset to the *Drosophila* research community and provide an invaluable resource for the development of new genetic technologies in *Drosophila*.

Here we describe a method aimed at using publicly available insertions of SSR recognition sites to generate custom alterations in the *Drosophila* genome. Our strategy is made possible by advances in recombineering, a system for manipulating large DNA fragments (*e.g.*, BACs) using homologous recombination in bacteria (reviewed by Sharan *et al.* 2009). Recombineering facilitates the introduction of insertions, deletions, and/or point mutations into DNA molecules carried by bacteria, allowing countless modifications to be applied to a cloned genomic fragment of interest in bacterial cells. In our scheme, a region of the *Drosophila* genome that is flanked on one side by an attP insertion and on the other by an FRT insertion can be exchanged for a corresponding recombineered DNA sequence from a donor BAC using a dual RMCE strategy. We demonstrate this approach by replacing a 50-kb chromosomal segment encompassing the *teashirt* (*tsh*) gene with a corresponding 50-kb BAC-derived genomic fragment that we engineered in bacteria. Furthermore, we show that this method can be used to incorporate large- and small-scale alterations into the captured segment through recombineering of the donor BAC sequence. With the vast number of attP and FRT insertions that are available to the *Drosophila* community, we believe that this method will be applicable to a substantial proportion of the genome.

Materials and Methods

Stocks and fly husbandry

A stock carrying *pBac(WH)f06252* was obtained from the Exelixis Collection at Harvard Medical School, and the stocks y^1 w^{1118} *P(70FLP)3F,* y^1 w^{1118}*; PBac(*y^+*-attP-3B)VK00003a PBac(*y^+*-attP-3B)VK00003b,* and tsh^{04319} *cn/CyO; ry* and w^{1118}*; Df(2L)BSC151/CyO* were obtained from the Bloomington Drosophila Stock Center. *PBac(*y^+*-attP-3B)VK00003a* was removed from the chromosome carrying *PBac(*y^+*-attP-3B) VK00003b* by meiotic recombination prior to the analyses described here. Stocks carrying the insertions *attP52* and *attP64* were provided by Michele Markstein. All flies were maintained at 25° on standard *Drosophila* cornmeal, yeast, sugar, and agar medium with *p*-hydroxybenzoic acid methyl ester as a mold inhibitor (Morris *et al.* 1998).

Cloning, gap repair, and recombineering

P[acman]-ApR (Venken *et al.* 2006) was obtained from the *Drosophila* Genomics Resource Center. To enhance eye pigmentation of transformants, a fragment encoding the eye enhancer *GMR* (Moses and Rubin 1991) was digested from pcr2.1-GMR (Bateman *et al.* 2012) and inserted into a unique *Eco*RI site upstream of the *mini-white* coding region of P[acman]-ApR. We then removed *Bam*HI and *Not*I sites that were carried on the GMR fragment by digesting the vector with *Bam*HI and ligating in overlapping oligonucleotides PacBK_5 and PacBK_3 (see Supporting Information, Table S1, for sequences of primers used in this study) to create GMR-P[acman].

An ~50-kb fragment of *Drosophila* genomic DNA spanning from the insertion point of *PBac(*y^+*-attP-3B)VK00003b* to that of *pBac(WH)f06252* was inserted into GMR-P[acman]

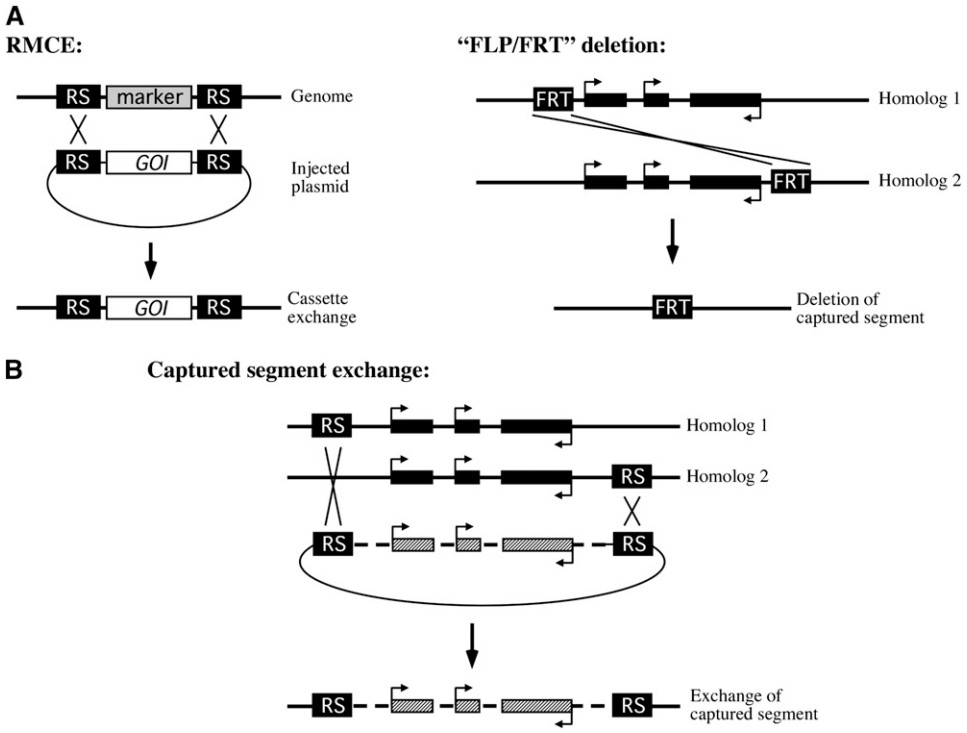

Figure 1 Conceptual view of captured segment exchange. (A) Existing technologies that form the basis of captured segment exchange. (Left) In RMCE, a marker flanked by SSR recognition sequences (RS) in the genome can be exchanged for a gene of interest (GOI) that is flanked by compatible RS on an injected plasmid. (Right) Crossovers between FRTs at nearby positions on homologous chromosomes can create a deletion of the intervening sequence on a resulting recombinant chromosome. (B) Captured segment exchange combines principles of the two technologies in A. RS at nearby positions on homologous chromosomes can effectively capture the intervening sequence such that a corresponding sequence flanked by compatible RS on an injected plasmid can replace the captured segment on a recombinant chromosome. Hatched region indicates the engineered sequence. Diagrams are not to scale.

by gap repair using existing methods (Venken *et al.* 2006; Sharan *et al.* 2009) with modifications. Briefly, 0.5-kb left and right homology arms were amplified from the BAC RP98-9H20 (BACPAC Resource Center, Children's Hospital Oakland Research Institute) using primer pairs VK3b_5_3/VK3b_3_1 and f06252_5_2/f06252_3_1; the former pair includes a 40-bp attB sequence on the forward primer, and the latter pair includes 20 bp of sequence complementary to the left homology arm on the forward primer and a 35-bp FRT sequence on the reverse primer. The two 0.5-kb arms were combined into a single 1-kb fragment via Splicing by Overlap Extension (SOEing; Horton *et al.* 1990) using primers VK3b_5_3 and f06252_3_1, and the 1-kb fragment was subcloned into pcr2.1 using a TOPO-TA cloning kit (Invitrogen). Next, the 1-kb fragment was excised from pcr2.1 and inserted into GMR-P[acman] using AscI and PacI. The resulting plasmid, GMR-P[acman]-tsh1, was linearized at a *Bam*HI site between the left and right homology arms and electroporated into heat-shocked SW102 bacteria (Warming *et al.* 2005) carrying RP98-9H20. Following selection on LB plates containing 100 μg/ml ampicillin, candidate gap-repaired plasmids were screened using the primer pairs PacmanMCS-F/tsh-5-vk3b-check-R and tsh-3-06252-check-F/PacmanMCS-R and then confirmed by fingerprinting digests with *Eco*RI. The resulting plasmid, GMR-P[acman]-tsh50, was electroporated into EPI300 bacterial cells (Epicentre Biotechnologies) for large-scale plasmid isolation.

To delete noncoding sequences downstream of the *tsh*-coding region in GMR-P[acman]-tsh50, we used a *galK*-based recombineering strategy as previously described (Warming *et al.* 2005; Sharan *et al.* 2009). Briefly, we targeted a 312-bp sequence (*tshNC1*) and an adjacent 550-bp sequence (*tshNC2*) ~10 kb downstream of the *tsh* coding region by designing galK-specific PCR primers that carried 50-bp sequences complementary to the regions flanking the intended deletions. These primers were used to amplify the *galK* gene from p*galK* (Warming *et al.* 2005), and the resulting PCR products were electroporated into heat-shocked SW102 cells carrying GMR-P[acman]-tsh50. Cells were then washed in 1× M9 salts and plated on M63 minimal media plates with galactose, leucine, biotin, and ampicillin to select for GMR-P[acman]-tsh50 clones that had incorporated the *galK* cassette. Replacement of the deleted genomic DNA by *galK* was verified using the primer pairs NC1check1-for/NC1check1rev and NC1check1for/NC2check1rev for tshNC1 and tshNC2 deletions, respectively, and the structure of each BAC was assessed by fingerprinting digests with *Eco*RI.

Drosophila transformation and captured segment exchange

GMR-P[acman] clones were injected into embryos carrying *PBac(y$^+$-attP-3B)VK00003b* and an endogenous source of the phiC31 integrase (Bischof *et al.* 2007), and transformants were identified by screening for red eye pigmentation as previously described (Venken *et al.* 2006). Injections, screening, and PCR-based confirmation of correctly targeted insertions were performed by BestGene *Drosophila* Embryo Injection Services (see Table S2 for integration rates). To exchange the captured genomic segment for the engineered sequence, we crossed *y^1 w^{1118} P(70FLP)3F*; *pBac*

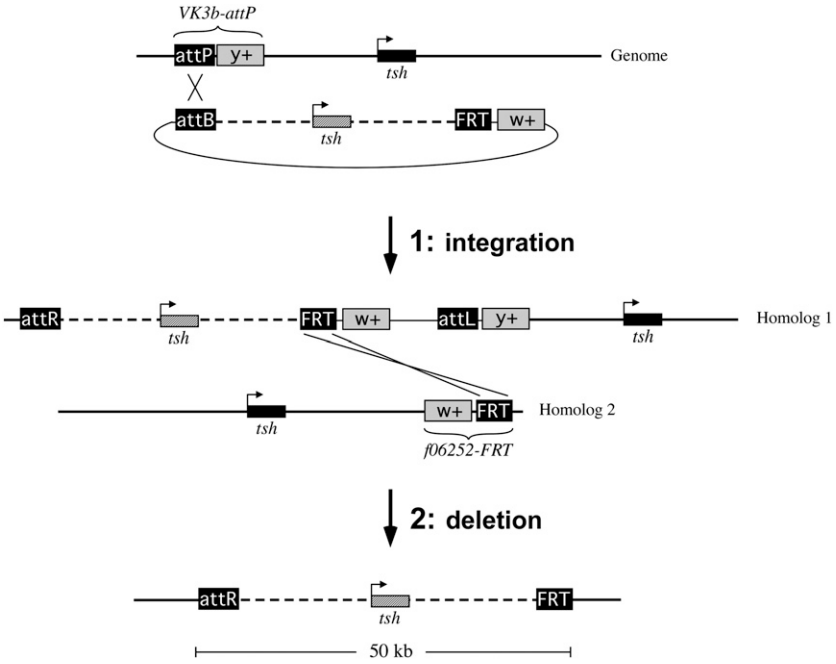

Figure 2 Two-step dual RMCE strategy for captured segment exchange at the *tsh* locus. In step 1, a P[acman] clone carrying an engineered *tsh* sequence is integrated upstream of the endogenous tsh gene via phiC31-mediated transgenesis using the attP insertion *PBac(y+-attP-3B) VK00003b* (*VK3b-attP*). The *mini-white* carried by P[acman] and *yellow* carried by *VK3b-attP* serve as markers such that successful transformants are y^+ w^+ in an otherwise y^- w^- background. In step 2, the endogenous *tsh* locus is deleted from a recombinant chromosome resulting from FLP-mediated crossing over between FRTs on the two homologs, leaving only the engineered sequence; this event also deletes all mini-*white* and *yellow* markers from the recombinant chromosome, allowing candidates to be identified by a y^- w^- phenotype. Not shown is a reciprocal chromosome resulting from the FRT exchange that will carry a tandem duplication of the captured sequence along with all *yellow* and *mini-white* markers. Diagrams are not to scale.

(WH)f06252 virgin females with males carrying a GMR-P [acman] construct inserted at *VK00003b* and heat-shocked the progeny on day 3 for 90 min at 37°. The adult males arising from this cross were mated individually to *y w*; *Sp Bl* L^{rm}*/CyO* virgin females, and the progeny were screened for y^- and w^- phenotypes.

Molecular analysis

Candidate flies in which the endogenous captured segment was exchanged using the donor GMR-P[acman]-tsh50 were verified by PCR-amplifying each end of the captured segment using primer pairs tsh_RRF3/tsh_RRR3 for the distal end and tsh_RLF/tsh_RLR for the proximal end. Genomic DNA from flies that had undergone captured segment exchange was used as a template, and amplified products were sequenced to confirm the expected structures. To identify single nucleotide polymorphisms (SNPs) in the captured segment, PCR was used to amplify several ~500-bp regions of noncoding DNA from BAC RP98-9H20, genomic DNA from flies carrying *pBac(WH)f06252*, and genomic DNA from flies carrying *PBac(y+-attP-3B)VK00003b*. Sequences were then aligned, and for the amplified regions defined by primer pairs SNP2F/SNP2R and SNP5F/SNP5R, several SNPs were detected that differentiate BAC DNA from both genomic templates. Genomic DNA from flies that had undergone captured segment exchange was then analyzed by the same method.

Candidate flies in which the endogenous captured segment was deleted via exchange using the donor GMR-P [acman]-tsh1 were verified by PCR amplification using the primer pair SOE_eF/SOE_eR and genomic DNA from flies heterozygous for the exchanged chromosome as template. These primers are complementary to genomic sequences flanking the captured segment and are predicted to generate an ~3-kb product if the captured segment is deleted, and no product if the captured segment is unaltered (due to their >50 kb spacing on an unaltered chromosome). Amplified products were sequenced to confirm the expected structure.

Candidate flies in which the endogenous captured segment was exchanged using the donors GMR-P[acman]-tsh50ΔNC1 and GMR-P[acman]-tsh50ΔNC2 were verified by PCR amplification from genomic DNA of flies homozygous for the exchanged chromosome using the primer pairs NC1check1for/NC1check1rev and NC2check1for/NC2check1rev, respectively. Genomic DNA from flies carrying *pBac(WH)f06252* or *PBac(y+-attP-3B)VK00003b* were each used as controls.

Captured segment exchange using two attP sites

The visible markers e^1 and ru^1 were recombined onto a chromosome carrying the insertion *attP52* to create flies of genotype y^1 w^{1118}; ru^1 *attP52* e^1*/TM3*. Embryos from the cross depicted in Figure 6B were injected with *piB-miniwhite*, constructed by ligating a 4-kb PCR fragment carrying *mini-white* into the vector backbone of *Xba*-digested piB-GFP (Bateman *et al.* 2006) at a concentration of ~400 ng/μl.

Results

Our goal was to establish a method for altering *Drosophila* genomic sequences that could take advantage of the large number of publicly available SSR recognition-site insertions. We developed a strategy that combined elements of two existing technologies for genome manipulation in *Drosophila*: RMCE, where a genomic DNA sequence that is flanked by SSR recognition sites can be exchanged for a donor cassette flanked by compatible recognition sequences, and the FLP/FRT method for generating deletions, where SSR

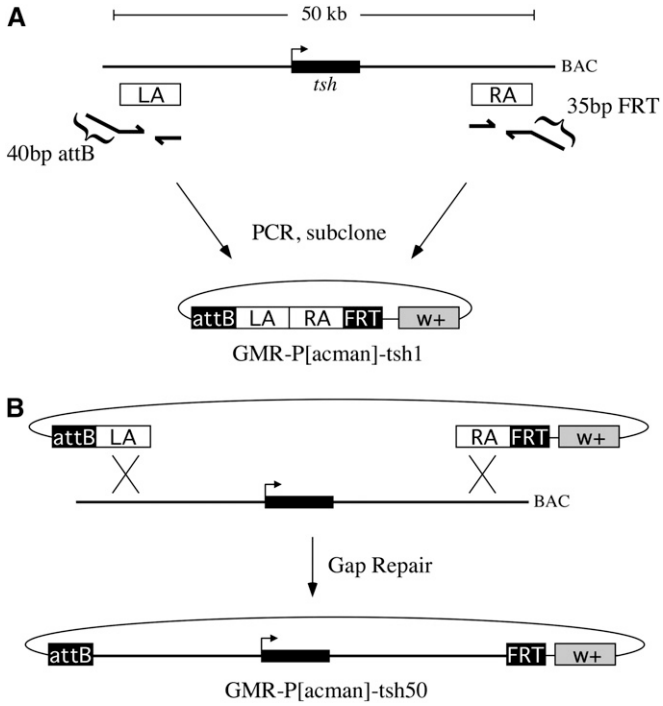

Figure 3 Recombineering strategy to generate a donor BAC for captured segment exchange of *tsh*. (A) Two "homology arms" (LA and RA) are generated by PCR and subcloned into GMR-P[acman] to create GMR-P[acman]-tsh1. The forward primer for LA incorporates a 40-bp attB sequence at its 5′ end, whereas the reverse primer for RA incorporates a 35-bp FRT sequence at its 5′ end such that the final PCR product is flanked by attP and FRT sites. Not shown is an additional 20 bp at the 5′ end of the forward RA primer that is complementary to the reverse LA primer, which was used to splice by overlap extension the two PCR fragments together prior to subcloning (Horton *et al.* 1990), and a recognition site for *Bam*HI, which was used to linearize the plasmid for gap repair. (B) GMR-P[acman]-tsh1 is linearized between the homology arms and transformed into bacteria that carry a *tsh* genomic clone (BAC) and *Red* recombination functions. The homology arms direct repair of the linearized plasmid from the BAC, creating GMR-P[acman]-tsh50, which carries a 50-kb sequence corresponding to the captured segment flanked by attP and FRT sites. Diagrams are not to scale.

recognition sites can interact between homologous chromosomes to produce a recombinant chromosome carrying a defined deficiency (Figure 1A). We imagined that the latter method could be adapted so that, rather than deleting a sequence, a genomic segment between two SSR recognition sites could instead be "captured" as a target cassette and exchanged with a donor sequence (Figure 1B). As with the FLP/FRT deletion approach, we reasoned that the SSR recognition sites could be carried on homologous chromosomes, allowing the direct use of existing *Drosophila* lines carrying SSR recognition sites without the need to recombine elements onto the same chromosome.

To demonstrate the feasibility of this method, we employed a dual RMCE strategy using a phiC31 attP site at one end of a captured segment and an FRT at the other. We reasoned that the use of an attP would maximize the efficiency of integration, while the inclusion of an FRT would provide the greatest flexibility in adapting the method to different genomic regions, given the large number of FRT insertions available. Specifically, we targeted a 50-kb genomic region defined by the attP insertion *PBac(y$^+$-attP-3B)VK00003b* (*VK3b-attP*; Venken *et al.* 2006) upstream of *tsh* and the FRT-bearing insertion *pBac(WH)f06252* (*f06252-FRT*; Thibault *et al.* 2004) downstream of *tsh*. Our strategy accomplishes exchange in two steps (Figure 2): first, integration of an engineered donor BAC into the *VK3b-attP* site via phiC31-mediated transgenesis, and second, deletion of the "endogenous" *tsh* segment via recombination between an engineered FRT in the donor BAC on one homolog and *f06252-FRT* on the other, leaving only the engineered *tsh* segment on a recombinant chromosome. Importantly, *VK3b-attP*, *f06252-FRT*, and the donor BAC are each marked with either a *mini-white* gene (BAC and *f06252-FRT*) or a *yellow* gene (*VK3b-attP*), allowing the two steps of the exchange to be monitored simply by scoring eye color and cuticle pigmentation in adult flies (Figure 2); given the positions of the *yellow* and *mini-white* markers relative to the att and FRT sites, individuals in which the endogenous segment is deleted can be identified based on their loss of y$^+$ and w$^+$ phenotypes. Accordingly, all of our crosses were carried out in a *y*$^-$ *w*$^-$ background.

To generate a compatible donor vector, we used the P[acman] BAC system that allows sequence modification by recombineering and carries a *mini-white* gene for identification of *Drosophila* transformants (Venken *et al.* 2006). In our preliminary experiments, we found that the eye pigmentation caused by *mini-white* expression was difficult to detect when P[acman] clones were integrated into the *tsh* locus, so we modified the vector to include the strong eye enhancer *GMR* (Moses and Rubin 1991) upstream of *mini-white*. The modified vector, GMR-P[acman], produces robust eye pigmentation when integrated at the *tsh* locus (data not shown) and was used for all subsequent experiments.

We used recombineering-mediated gap repair to copy a 50-kb sequence spanning the captured *tsh* segment from a publicly available genomic clone into GMR-P[acman]. In this strategy, two 0.5 kb homology arms corresponding to the ends of the captured segment were first amplified by PCR and ligated into GMR-P[acman] to create GMR-P[acman]-tsh1 (Figure 3A). We designed our primers so that the outer ends of these homology arms corresponded to the precise nucleotides of the *VK3b* and *f06252* insertions that define the boundaries of the captured segment. In addition, we incorporated a 40-bp attB sequence and a 35-bp FRT sequence as "tails" on the outermost primers (Bateman and Wu 2008) so that the homology arms would be flanked by attB and FRT sites as required for the exchange reaction. We then linearized GMR-P[acman]-tsh1 at a site between the homology arms and transformed it into bacteria carrying the corresponding *tsh* genomic region and *Red* recombination functions (Lee *et al.* 2001; Court *et al.* 2003) to create our donor BAC, GMR-P[acman]-tsh50 (Figure 3B; see *Materials and Methods*), which carries 50 kb of genomic DNA corresponding to the captured segment and is flanked by attB and FRT sites.

Table 1 Captured segment exchange at the *tsh* locus

P[acman] construct[a]	Insert size (kb)	Engineered mutation	y^- w^- (exchange)[b]	Total scored	% exchanged[c]
tsh50 #1	50	None	27	132	20.5
tsh50 #2	50	None	10	31	32.3
tsh1 #1	1	~49-kb deletion	21	151	13.9
tsh1 #2	1	~49-kb deletion	11	132	8.3
tsh50ΔNC1	~50	312-bp deletion	8	99	8.1
tsh50ΔNC2	~50	550-bp deletion	8	45	17.8

Flies were subjected to the schemes outlined in Figures 2 and 4 using the indicated GMR-P[acman] construct as a donor for captured segment exchange. Only male progeny carrying the balancer CyO were scored for y and w phenotypes.

[a] Two independent insertions of GMR-P[acman]-tsh50 and of GMR-P[acman]-tsh1 were tested.

[b] Number of individual progeny in the F_2 generation of the cross scheme in Figure 4 that had y^- cuticle and w^- eyes, indicative of a completed exchange.

[c] Percentage of all F_2 flies scored that had y^- cuticle and w^- eyes.

GMR-P[acman]-tsh50 was targeted to *VK3b-attP* via existing methods for phiC31-mediated transgenesis (Groth *et al.* 2004; Bischof *et al.* 2007; Venken and Bellen 2007) using a commercial injection service, and successful *VK3b-tsh50* integrants were identified by the eye pigmentation produced by *mini-white* expression. To carry out the second step of the exchange reaction, we crossed *VK3b-tsh50* males to virgin females carrying *f06252-FRT* and an X-chromosomal insertion of *FLP* driven by a heat-shock promoter (*hs-FLP*) (Figure 4). Heat-shocked male progeny that carried *f06252-FRT* on one homolog and *VK3b-tsh50* on the other homolog were then individually mated to virgin females carrying a second chromosome balancer, and their progeny were analyzed for evidence of a FLP-mediated crossover between homologs. As anticipated, we observed a y^- w^- phenotype, indicative of a completed exchange of the captured segment, at a high rate, with ~20–30% of the progeny scored as putative exchange candidates (Table 1). Notably, we also observed a class of y^+ w^+ progeny with darker eye pigmentation than expected (data not shown), which is consistent with a prediction for the reciprocal recombinant chromosome resulting from an FRT crossover; this chromosome would carry tandem copies of the captured segment in addition to two *mini-white* genes and the *yellow* marker. We did not analyze these candidate reciprocal chromosomes further.

To verify that the endogenous captured segment had been exchanged for the engineered sequence, we amplified and sequenced genomic DNA surrounding each end of the captured segment in one of our candidate lines and confirmed that the sequence matched that predicted for the exchange reaction. In addition, we identified SNPs within the captured segment that could differentiate the BAC-derived sequence from that of the chromosomes carrying *VK3b-attP* or *f06252-FRT*. When we analyzed these SNPs in our candidate line, we saw only the alleles corresponding to the BAC and not those from either parent chromosome (Figure S1). Thus, our data are consistent with a clean exchange of the endogenous captured segment for the engineered BAC sequence.

Flies homozygous for the engineered chromosome are viable and fertile, with no evidence of abnormal adult structures. In addition, staining of embryos with an anti-Tsh antibody shows that the overall pattern of *tsh* expression is not altered by incorporating the engineered sequence (data not shown). We conclude that captured segment exchange of *tsh* has no adverse effect on development.

Captured segment exchange can be used to custom-engineer genomic sequences

Thus far, our demonstration of captured segment exchange has used a donor sequence from a genomic clone carrying wild-type sequences. However, we imagined that our method would be most useful for making changes to genomic sequences *in situ*. Thus, we tested whether donor BACs carrying defined sequence changes could be easily exchanged for the captured segment.

We first tested whether the captured segment could be replaced with a large deletion. To do so, we completed the steps for segment exchange outlined above using the donor BAC GMR-P[acman]-tsh1, which carries only the 0.5-kb homology arms and therefore represents a deletion of ~49 kb of the *tsh* locus. Following dual RMCE, we once again observed high rates of candidate exchange events as indicated by progeny with a y^- w^- phenotype (Table 1), implying that the size of the donor DNA molecule does not significantly impact the rate of crossover between FRTs on homologous chromosomes. We verified that these candidate deletion-bearing flies carried the expected sequence by amplifying and sequencing a ~3-kb fragment spanning the engineered sequence using primers complementary to genomic DNA flanking the *VK3b-attP* and *f06252-FRT* insertions (data not shown). Notably, the resulting engineered chromosome caused lethality when homozygous, as expected for a deletion of the essential *tsh* gene. In addition, the chromosome failed to complement the independent *tsh* deficiency *Df(2L)BSC151* (Parks *et al.* 2004) and the lethal allele tsh^{04319} (Bellen *et al.* 2004) (data not shown). Thus, our method can be used to make large deletions within the captured segment.

We next used our method to make more fine-scale changes to the *tsh* locus. The *tsh* gene is situated in a small gene desert, with regions of noncoding DNA extending 33.1 kb upstream and 59.6 kb downstream from the transcription unit (Crosby *et al.* 2007). Notably, analyses from the ModEncode consortium predict that these noncoding sequences are rich in binding sites for transcription factors and other DNA-binding proteins, suggesting that much of the region plays a regulatory

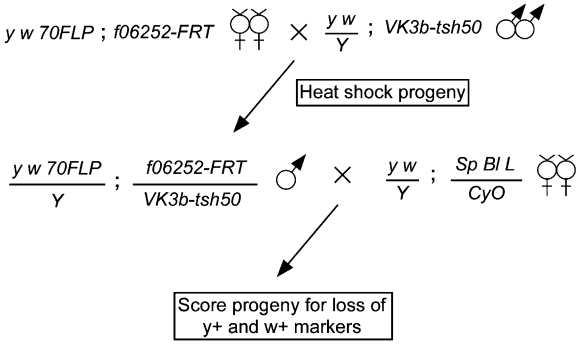

Figure 4 Cross scheme for FLP-mediated deletion of the endogenous *tsh* locus. Virgin females carrying a heat-shock-inducible *FLP* gene (*70FLP*) and *f06252-FRT* are crossed to males in which GMR-P[acman]-tsh50 was integrated into *VK3b-attP* (*VK3b-tsh50*). The resulting larvae are heat-shocked to activate FLP expression, and male progeny are mated individually to virgin females carrying a second chromosome balancer (*CyO*) and arbitrary dominant markers (*Sp Bl L*). In the F_2, male progeny carrying CyO are scored for y and w phenotypes, with y^- w^- indicative of completed exchange. All crosses were carried out in a y^- w^- background.

function (Roy *et al.* 2010; Negre *et al.* 2011). We used recombineering schemes to delete two small regions of feature-rich noncoding DNA ~10 kb downstream of *tsh* in GMR-P[acman]-tsh50, replacing each with the small bacterial gene *galK* (Warming *et al.* 2005). We then used the resulting altered BACs, GMR-P[acman]-tsh50ΔNC1 and GMR-P[acman]-tsh50ΔNC2, as donor vectors in the captured segment exchange procedure above. For both types of deletion, flies carrying the resulting engineered chromosomes were homozygous viable (the effects of these deletions on *Drosophila* development will be described in a later publication). We confirmed that the deletions were incorporated into the genome via PCR using primers flanking the altered sequences (Figure 5). In both cases, flies homozygous for chromosomes carrying the candidate-exchanged segments showed only the PCR product corresponding to the deletion, and not that of the endogenous unaltered sequence (Figure 5B; Figure S2). Thus, captured segment exchange represents a simple and efficient method to engineer custom alterations into the *Drosophila* genome.

Captured segment exchange via single-step RMCE using two attP insertions

We designed our strategy for captured segment exchange to take advantage of the large number of FRT- and attP-bearing insertions that are available to the *Drosophila* community. However, other configurations of SSR recognition sequences could also be employed to accomplish captured segment exchange. In one alternative configuration, a captured segment could be flanked at both ends by attP sites such that exchange can be accomplished by the action of phiC31 integrase alone. To explore this possibility, we used the attP insertions *attP52* and *attP64* (*Markstein et al.* 2008), which are separated by ~40 kb and are oriented such that the yellow marker of each insertion is within the captured segment defined by the attP sites (Figure 6A). We reasoned that exchange of the endogenous segment for a donor sequence would create a recombinant chromosome lacking a *yellow* marker and could therefore be detected by screening for a y^- phenotype in a *y w* mutant background. To add further evidence that a candidate exchange event is accompanied by recombination between homologs, we used the visible mutations *roughoid* (*ru*) and *ebony* (*e*), which flank the captured segment, as markers to detect recombination. According to our model, captured segment exchange will result in a recombinant chromosome with a mutant allele of *e* and a wild-type allele of *ru* (Figure 6A). Finally, for a donor plasmid, we used a construct carrying two attB sites and a *mini-white* marker gene. In this case, the orientations of the attB sites are such that captured segment exchange will integrate the plasmid backbone rather than the *mini-white* gene, whereas "single" integration events resulting from crossovers between one of the attB sites of the donor and one of the parental attP sites will incorporate the entire plasmid and produce red eye pigmentation in the resulting progeny; this serves as a useful control, as these insertions should not be accompanied by a recombination event and should therefore be either e^+ ru^+ (insertion into *attP64*) or e^- ru^- (insertion into *attP52*). In sum, captured segment exchange using this strategy will result in deletion of the endogenous segment, replacing it with bacterial plasmid sequence, and is predicted to result in flies with a y^- w^- e^- ru^+ phenotype.

We injected 390 embryos from a cross of *y M[vas-int.Dm] ZH2A w*; *attP64* virgin females to *y w*; *ru attP52 e/TM3* males, and selected male G_0 adult progeny of the genotype *y M[vas-int.Dm]ZH2A w/Y*; *ru attP52 e/attP64* to mate singly to virgin females with the third chromosome balancer *TM3* carrying *e* and *ru* markers (Figure 6B). We then scored the F_1 progeny for y, w, e, and ru phenotypes. Of the 31 vials analyzed, 10 (32%) produced y^- w^- e^- ru^+ progeny consistent with the occurrence of segment exchange. Importantly, 46/46 y^- flies that we identified among the 10 vials were also w^- e^- and ru^+, indicating that spurious loss of the *yellow* marker genes did not occur. Furthermore, of the 10 vials in which we identified candidate flies carrying a deletion (y^- w^- e^- ru^+), 8 vials also produced flies of the predicted reciprocal chromosome resulting from segment exchange with y^+ w^+ e^+ ru^- phenotypes. Finally, as predicted, 9 of the 10 vials also yielded flies consistent with a single attP/attB exchange with the chromosome carrying either *attP64* (yielding y^+ w^+ e^+ ru^+ phenotypes) or *attP52* (y^+ w^+ e^- ru^-). In sum, our genetic data are consistent with the occurrence of captured segment exchange when using insertions of attP sites on homologous chromosomes to define the captured segment.

Discussion

Captured segment exchange joins a suite of useful approaches for custom genome manipulation in *Drosophila*. Importantly, existing methods for making precise changes to *Drosophila*

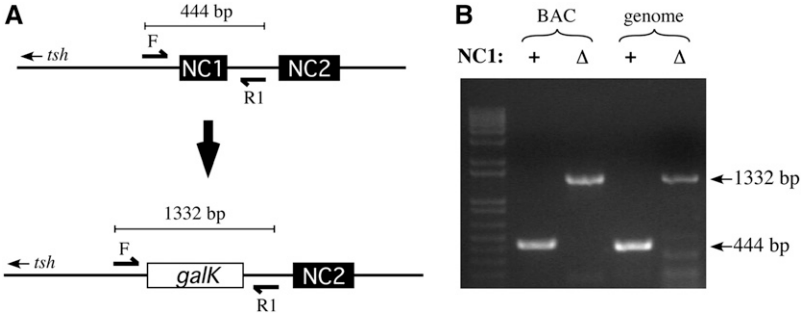

Figure 5 Confirmation of *tshNC1* deletion following captured segment exchange. (A) Schematic showing relative positions of *tshNC1*, *tshNC2*, and confirmation primers NC1check1for (F) and NC1check1rev (R1) ~10 kb downstream of the *tsh* transcription unit. Captured segment exchange using GMR-P[acman]-tsh50ΔNC1 as a donor effectively results in deletion of *tshNC1* from the chromosome, leaving the ~1-kb bacterial *galK* gene in its place. Diagrams are not to scale. (B) Ethidium-stained gel showing PCR products from templates where tshNC1 is unaltered (+) or deleted (Δ) and replaced with *galK* using purified BAC DNA or *Drosophila* genomic DNA as templates. Candidate flies homozygous for the recombinant chromosome carrying the deletion (right-most lane) show the predicted PCR product. A similar strategy was used to confirm deletion of *tshNC2* (Figure S2).

genomic sequences each employ steps that rely on the endogenous homologous recombination repair machinery and/or require pretreatment of a locus for subsequent manipulation, both of which limit efficiency and throughput (Gloor *et al.* 1991; Rong and Golic 2000; Gong and Golic 2003; Gao *et al.* 2008; Choi *et al.* 2009; Huang *et al.* 2009; Weng *et al.* 2009). In contrast, captured segment exchange relies only on highly efficient SSR activity via a dual RMCE reaction and aims to make direct use of existing stocks carrying SSR recognition-site insertions without the need for pretreatment. Furthermore, we have shown that a large segment of 50 kb is efficiently exchanged with diverse donor sequences and that necessary DNA sequence manipulations, including the incorporation of attB and FRT sites, are easily accomplished using the P[acman] recombineering system (Venken *et al.* 2006). Thus, by taking advantage of several existing resources, captured segment exchange further improves the genetic toolkit of *Drosophila* researchers.

In addition to demonstrating captured segment exchange using a wild-type donor BAC, we used two strategies to engineer changes into the genome. In the first, we used a version of our donor BAC that carried only 1 kb of the 50-kb segment to create a large deletion, while in the second, we used galK-based recombineering to delete two small regions of noncoding DNA. Although our ΔNC1 and ΔNC2 donor BACs carried the *galK* gene in place of the deleted *Drosophila* sequence, the bacterial gene can easily be removed via a simple recombineering strategy prior to the dual RMCE reaction (Warming *et al.* 2005), making it possible to incorporate "clean" deletions, point mutations, and/or gene fusions into the captured segment. The similar efficiency of exchange achieved with donor BACs carrying large *vs.* small deletions implies that captured segment exchange will be capable of incorporating diverse sequence alterations into the genome.

Our method should be easily adaptable to other genomic segments that are flanked by existing insertions of an attP and an FRT. As of this writing, the Gene Disruption Project has generated 4071 mapped insertions of the attP-bearing MiMIC transposon (Venken *et al.* 2011), which corresponds to one insertion every ~30 kb on average. In addition, a modest number of attP insertions, including the *PBac (y$^+$-attP-3B)* element used in our study, have been generated by other projects (Groth *et al.* 2004; Venken *et al.* 2006; Bischof *et al.* 2007; Markstein *et al.* 2008). Similarly, ~15,000 mapped FRT insertions are publicly available from stock collections at Harvard Medical School, Bloomington, and Kyoto (Ryder *et al.* 2004; Thibault *et al.* 2004; Crosby *et al.* 2007), making it likely that the majority of attP insertions will be within tens of kilobases of the nearest FRT insertion. The upper limit on the potential size of a captured segment will depend on whether a corresponding donor BAC can be recombineered and incorporated into the genome in the first step of the exchange protocol; notably, a 133-kb fragment of the *ten-m* gene was previously recombineered and inserted into an attP site using P[acman] (Venken *et al.* 2006), making it reasonable to assume that a captured segment of >100 kb could be easily exchanged for a recombineered sequence. Thus, our method should be applicable to much of the genome, with the number of potential captured segments increasing as new insertions of MiMIC continue to be generated. Furthermore, although we designed captured segment exchange with existing stock collections in mind, it is also possible to incorporate attP and/or FRT sequences into specific sites in the genome via homologous recombination or other methods, which may facilitate establishing a captured segment exchange strategy for those loci where appropriate insertions are unavailable.

In our demonstration at the *tsh* locus, the *yellow* and *mini-white* genes were located within the boundaries of the captured segment and were therefore lost from the recombinant chromosome, making them convenient markers to monitor the exchange reaction. In some cases, it is possible that a potentially useful insertion carrying an FRT or attP would be oriented such that the marker lies outside the captured segment. In this and other cases (*e.g.*, using an unmarked insertion), the deletion of the endogenous segment may not be identifiable by eye and body phenotypes. However, given the high efficiency of FRT-mediated recombination following step 2 of our procedure, it should be straightforward to screen for these events using a simple molecular approach such as PCR. In addition, the use of MiMIC rather than the *PBac(y$^+$-attP-3B)* element used here

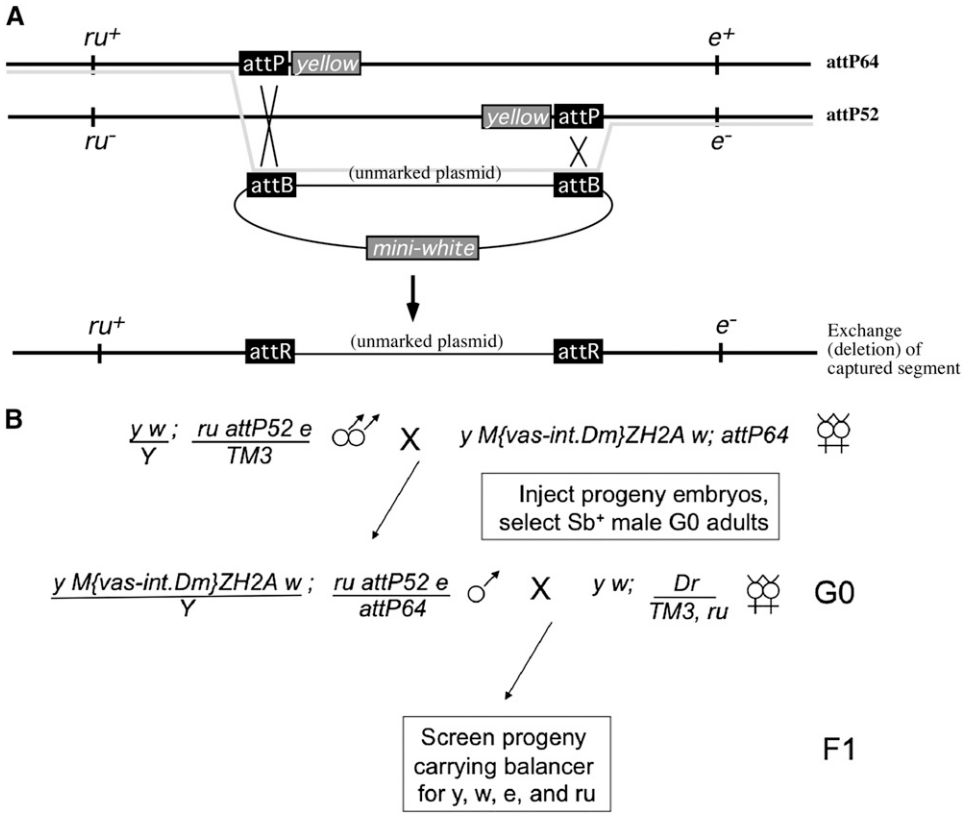

Figure 6 Captured segment exchange via single-step RMCE. (A) Schematic showing homologous chromosomes carrying *attP64* (top) and *attP52* (bottom), which are separated by ~40 kb. The chromosome carrying *attP52* is marked by mutations in *ru* to the left of the insert and in *e* to the right. Following injection of a plasmid carrying attB sites, captured segment exchange will result in a recombinant chromosome that is ru^+, e^-, lacks *yellow* markers, and carries unmarked plasmid sequence in place of the deleted captured segment (the faded line traces the regions of the parental chromosomes and the injected plasmid that are found in the final recombinant chromosome). (B) Cross scheme for accomplishing the exchange depicted in A (see text for details).

should alleviate this issue for the attP end of the exchange; since MiMIC carries two attP sites flanking a yellow gene for use in conventional RMCE transgenesis, half of all inserts following step 1 of our method will be oriented with the *yellow* marker of MiMIC and the *mini-white* marker of P[acman] within the boundaries of the captured segment, which will then be lost from the recombinant chromosome following step 2 (Figure S3).

As a final consideration in adapting our strategy to other genomic regions, some potentially useful attP and/or FRT insertions may be located within exonic sequences and therefore may cause confounding phenotypes irrespective of alterations made within the captured segment. In our demonstration, the attP and FRT insertions were located in noncoding regions and caused no visible phenotypes as homozygotes prior to our manipulations. Following exchange, a small amount of transposon sequence remains in the genome at both ends of the captured segment, which could have an adverse effect in some positions in the genome. Thus, insertions in noncoding regions will likely be most useful in defining a captured segment for study.

We chose to establish our method for captured segment exchange based on a dual RMCE strategy employing both phiC31 and FLP due to the efficiency of the former and the larger number of potential targets available for the FLP/FRT system. However, other strategies are possible, as evidenced by our demonstration of segment exchange using two nearby attP insertions on homologous chromosomes. Given the expanding number of SSRs demonstrated to function in *Drosophila* and other eukaryotes (Nern *et al.* 2011) and the adaptability of RMCE strategies to diverse enzymes in flies and other organisms, future endeavors may generate new collections of SSR recognition-site insertions that could be adapted to our protocol. Indeed, large collections of SSR recognition-site insertions in other organisms may prove useful in developing similar strategies for genome manipulation in diverse model systems.

Acknowledgments

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GENETICS

Supporting Information
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Captured Segment Exchange: A Strategy for Custom Engineering Large Genomic Regions in *Drosophila melanogaster*

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DOI: 10.1534/genetics.112.145748

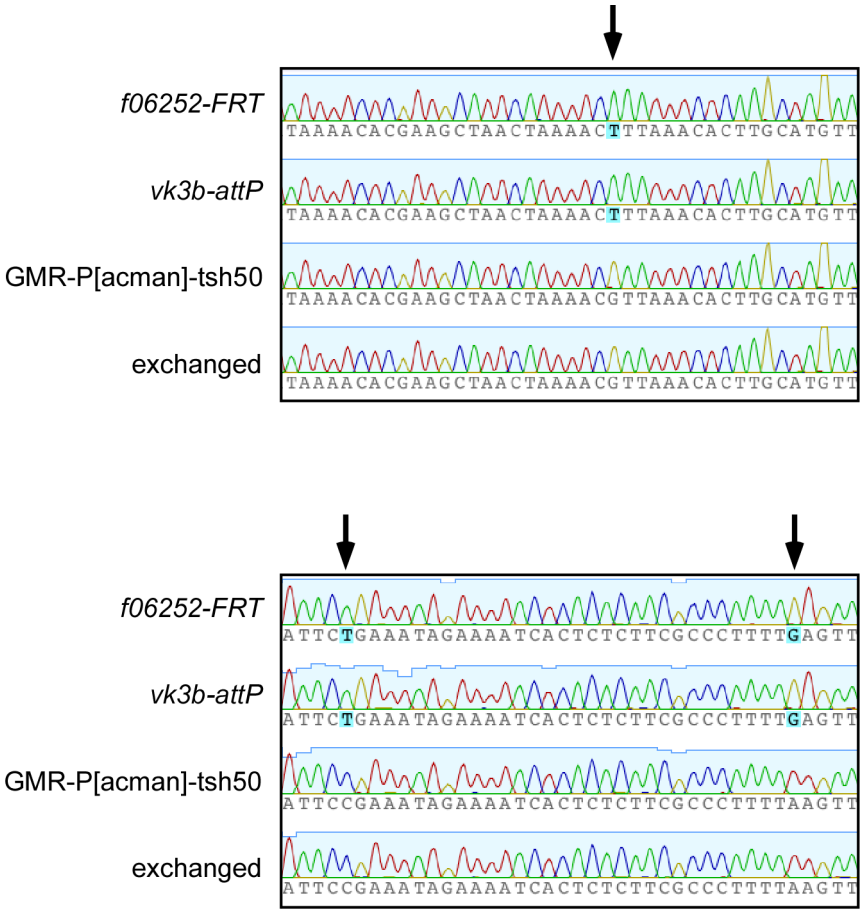

Figure S1 SNP analysis of candidate exchange flies. Shown are traces identifying three SNPs in the 706 bp genomic region amplified by the primer pair SNP2F/SNP2R within the captured segment. For each box, the templates for amplification were: Row 1, *f06252-FRT* genomic DNA (parent chromosome); Row 2, *vk3b-attP* genomic DNA (parent chromosome); Row 3, purified BAC GMR-P[acman]-tsh50 (donor plasmid); Row 4, genomic DNA from candidate $y^- w^-$ flies following exchange. For each SNP, only the BAC allele is observed in genomic DNA of candidate flies. We identified a total of 5 SNPs in this region that differentiate BAC DNA from both parent chromosomes, and in all cases, the allele observed in the BAC was the only allele observed in the amplified region from the genome of candidate flies. In addition, a total of 3 SNPs from a second independently amplified 797 bp region defined by primers SNP5F/SNP5R gave identical results.

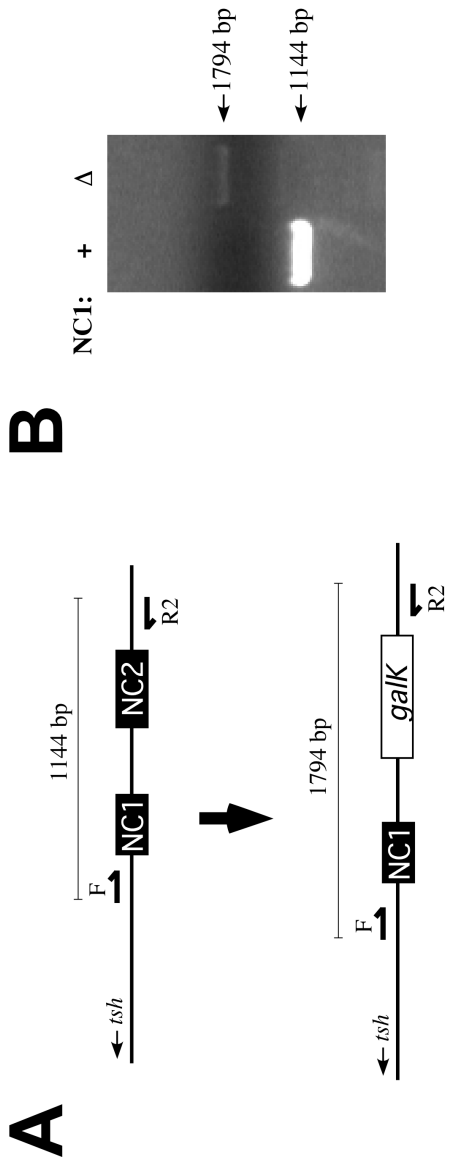

Figure S2 Confirmation of *tshNC2* deletion following captured segment exchange. A, schematic showing relative positions of primers NC1check1for (F) and NC2check1rev (R2). Diagrams are not to scale. B, Ethidium-stained gel showing PCR products from templates where tshNC2 is unaltered (+) or deleted (Δ) and replaced with *galK* using purified BAC DNA or *Drosophila* genomic DNA as templates. Candidate flies homozygous for the recombinant chromosome carrying the deletion (right-most lane) show the predicted PCR product.

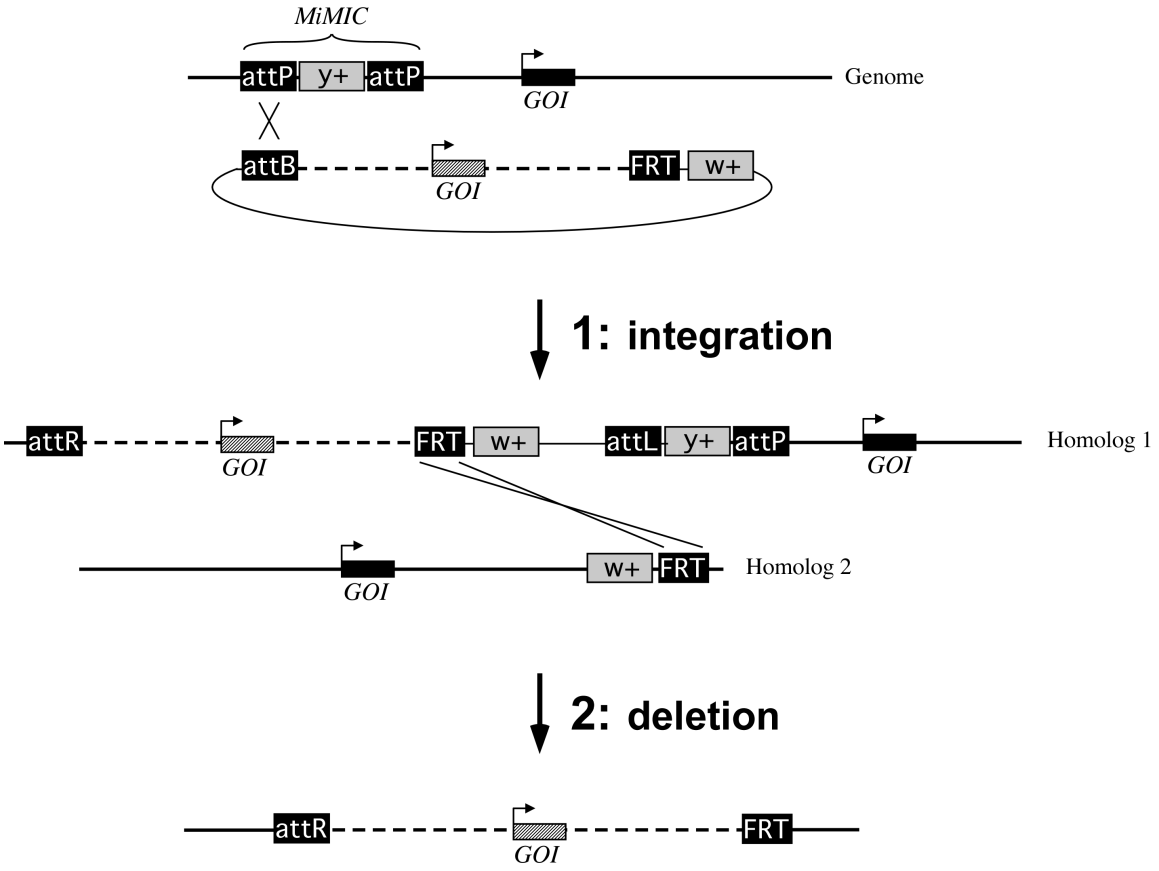

Figure S3 Schematic for two-step dual RMCE using a MiMIC element. MiMIC differs from the *VK3b-attP* element used in the present study in that it carries two attP sites in opposite orientations flanking a *yellow* marker gene. Captured segment exchange can be performed following the same steps as those demonstrated in the main text; in the initial step of inserting the donor BAC, half of all insertions will be in the correct orientation to proceed as outlined in the figure, while, by chance, half of the insertions will use the attP in the opposite orientation (not shown).

Table S1 Primers used in this study.

Primer name	Sequence (5'-3')	Additional information
VK3b_5_3	GGCGCGCCGTACGCGCCCGGGGAGCCCAAGGGCACGCCCTGGCACCCGTAAACGAGACAATTTATAAATGC	Forward primer for LA: includes AscI (orange) and 40 bp attB (blue)
VK3b_3_1	GGATCCTCTGTTTGCTATGGGCTGAA	Reverse primer for LA: includes BamHI (orange)
f06252_5_2	TTCAGCCCATAGCAAACAGAGGATCCAGAGGCTATACAAGTTAGAAAGTTGA	Forward primer for RA: includes complementary sequence to VK3B_3_1 for SOEing (orange)
f06252_3_1	TTAATTAATGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGCATTAAATAGTCGCAATTATATTTCA	Reverse primer for RA: includes PacI (orange) and 35 bp FRT (blue)
PacmanMCS-F	TTTAAACCTCGAGCGGTCCGTTATC	
tsh-5-vk3b-check-R	GGTTCAGCGGGACTAAGTGA	
tsh-3-06252-check-F	TGAACACACCCATAGGACGA	
PacmanMCS-R	CTAAAGGGAACAAAAGCTGGGTAC	
tshNC1_GalK_F	GCATCTTCTGCTTCTTCTTCATCTTCTTTTTGCTCTTCTTGATGGACAGAACCTGTTGACCAATTAATCATCGGCA	Forward primer for tshNC1 deletion: includes 50 bp homology adjacent to tshNC1 (blue)
tshNC1_GalK_R	CCACCCTCTTCCCAACCAACATCCCCTTCACTCAGTGTTGAAGAAGAATCCTCAGCACTGTCCTGCTCCTT	Reverse primer for tshNC1 deletion: includes 50 bp homology adjacent to tshNC1 (blue)
tshNC2_GalK_F	TATGTACACACTCCGGTACCAGTATTTTTGCCTGCCTTGACGTAAATGCCGCCTGTTGACAATTAATCATCGGCA	Forward primer for tshNC2 deletion: includes 50 bp homology adjacent to tshNC2 (blue)
tshNC1_GalK_R	CCACCCTCTTCCCAACCAACATCCCCTTCACTCAGTGTTGAAGAAGAATCCTCAGCACTGTCCTGCTCCTT	Reverse primer for tshNC2 deletion: includes 50 bp homology adjacent to tshNC2 (blue)
NC1check1for	TGCATCGGAGACAGAGGGAGCA	
NC1check1rev	ACCACACCCATGCCCTATGCC	
NC2check1rev	TGGCCGAAAGGAGGCAGCAAC	
tsh_RRF3	TTGGCACGCCAACTCAACGC	
tsh_RRR3	TGGCAATGGTGCGGCCATCT	

tsh_RLF	TCTTCGTTGCGTGCGGTGGG
tsh_RLR	TGCTGCAGTAGAGAGACACGGGG
SNP2F	TGCGCAGCAGTGCCTCTTGAAA
SNP2R	AAAAGCCGCAAACAGCAGGCA
SNP5F	AGCAAGCGCGGCGTGAAAAT
SNP5R	AGGATTTGGTGCGGCTTGGTGA
SOE_eF	TCGCGGTGCGAGCTGTGATT
SOE_eR	TCGCACGTTCCTGCTCGCTT
PacBK_5	GATCGGCGGCGCCGGGTACCGGGC
PacBK_3	GATCGCCCGGTACCCGGCGCCGCC

Table S2 Integration efficiency of GMR-P[acman] constructs.

Insert Name	Insert Size (kb)	Total Vials Scored	Vials With Integrants	Efficiency of Integration
tsh1	1	45	4	8.8%
tsh50	50	90	5*	5.5%*
tsh50ΔNC1	50	120	5*	4.2%*
tsh50ΔNC2	50	65	5*	7.7*

All constructs were injected by BestGene Inc. using *PBac(y^{+}-attP-3B)VK00003b* as a target site and *M[vas-int.Dm]ZH2A* as a source of the integrase. Integrants were identified based on *mini-white* eye pigmentation. Efficiency of integration is calculated as the number of vials with integrants divided by the total vials scored, expressed as a percentage.

*In cases where 5 positive vials were identified, no further screening was carried out. Thus, the efficiency in these cases represents a lower limit on the true value.

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