Targeted Gene Replacement in Drosophila Goes the Distance

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Increasingly we rely on reverse genetics to knock out genes initially identified in genome sequences. The discovery of new classes of non-coding RNA genes reveals a large number of genes, some very small, that have been largely refractory to traditional forward genetic approaches. Knocking out these genes requires an ability to target specific regions of the genome. Similarly, structure-function studies of genes and proteins are most clearly interpreted when the endogenous copy of a locus is altered because it maintains the chromosomal context of that locus. For this type of analysis it would therefore be beneficial to have methods for repeatedly making alterations to the same locus. Two articles in this issue of *Genetics* describe new methods for gene targeting in *Drosophila* that are simpler and faster than existing approaches, can be applied repeatedly to the same region, and allow large regions (up to at least 80kb) to be replaced.

Among metazoan model organisms, *Drosophila melanogaster* has stood out for the availability of innovative tools to manipulate the genome (reviewed in VENKEN and BELLEN 2005). The advent of *P*-element transgenesis thirty years ago (RUBIN and SPRADLING 1982) opened up numerous approaches to studying gene function, such as structure-function studies and *in vivo* expression of proteins tagged with epitopes or fused to fluorescent proteins (reviewed in ROMAN 2004). A critical limitation to this technology is that some of its most valuable uses require that a mutation in the target gene be available. In addition, there is no control over where transposons insert. This means that each insertion may be idiosyncratic due to its unique location in the genome (HAZELRIGG *et al.* 1984; WAKIMOTO *et al.* 1986), a limitation typically dealt with by analyzing several independent insertions. Another limitation is that transformation efficiency decreases drastically with construct length (HAENLIN *et al.* 1985), thereby limiting the sizes of genes that can be analyzed in this way.

More than ten years ago Rong and Golic (2000) introduced a method for gene targeting by homologous recombination. DNA ends are recombinogenic, but recombination is not efficient enough to make direct injection of linear DNA feasible as a targeting strategy. The key to the Rong and Golic method is that linear targeting DNA is generated *in vivo* by using the sequence-specific recombinase FLP to excise a circle from an integrated transgene and the meganuclease *I-Sce*I to cut this circle into a linear fragment. Although this method brought a powerful way to manipulate genes *in situ*, the process can be arduous (Fig. 1A). After building the targeting construct, one has to get it inserted into the genome. Different insertions of the same construct can result in vastly different rates of targeting, so several different insertions are necessary. Each of these is crossed at a reasonably large scale to a stock that expresses FLP and *I-Sce*I, with the hope that targeting will occur in the germlines of the progeny. To detect germline targeting events, these progeny are crossed to an appropriate stock and their offspring are screened for those that appear to have a new insertion of the targeting DNA. Stocks generated from different independent integration events are tested to identify any in which the targeting DNA has inserted into the target locus by homologous recombination, which in some cases is a small minority of the events (e.g., RADFORD *et al.* 2005). In the original "ends-in" method, homologous recombination results in a tandem duplication. This must be collapsed to a single copy, followed by another round of screening for retention of the desired copy. In the end, success is not guaranteed; some loci appear to be refractory to targeting (or some targeting constructs are ineffective). For these reasons, many Drosophila researchers still rely on transgenes inserted at ectopic locations to study gene and protein function.

There have been substantial improvements to Drosophila gene targeting technologies. The "ends-out" method results in a direct replacement rather than a tandem duplication, eliminating the need for a duplication reduction step (Fig. 1B) (GONG and GOLIC 2003). Employment of a negative selection increased the efficiency of screening (HUANG *et al.* 2008). These improvements have led to numerous successful targeting experiments (reviewed in WESOLOWSKA and RONG 2010), but many researchers still avoid this method because it remains labor intensive and must repeated for each new mutation in a gene.

Development of new technologies led to additional advances in gene targeting in Drosophila. Foremost among these is the phiC31 integration system (GROTH *et al.* 2004; BISCHOF *et al.* 2007). phiC31 integrase catalyzes recombination between *attP* and *attB* sequences with high efficiency. Multiple large-scale efforts have been made to insert transgenes carrying *attP* sequences throughout the Drosophila genome, greatly expanding the ability to target DNA insertions to specific genomic locations (reviewed in VENKEN and BELLEN 2012).

Combining gene targeting and phiC31 technologies allowed methods for generating multiple different alleles of the same gene, in its endogenous location. In "genomic engineering" (HUANG *et al.* 2009) and "site-specific integrase-mediated repeated targeting" (SIRT) (GAO *et al.* 2008), gene targeting is used to introduce an *attP* site into or near a gene of interest (Fig. 1C). This site can then be used to insert any desired number of constructs individually. Each insertion produces a tandem duplication that can be reduced to a single copy as in the initial ends-in gene targeting scheme. This approach allows multiple independent changes to be made to the same gene, but it still relies on an initial gene targeting step. The targeting step is unnecessary if one is fortunate enough to have an *attP* site in or near the gene of interest. However, at least as initially conceived, changes could be made only within about 10 kb of the *attP* site, severely restricting the ability to use *attP* sites introduced by genome projects.

The high efficiency of transformation with the phiC31 system has made it more feasible to introduce larger DNA fragments into the genome. To facilitate working with larger fragments, Venken *et al.* (2009) made two genomic bacterial artificial chromosome (BAC) libraries, with average insert sizes of 21 kb and 83 kb. The libraries were constructed using the pP[acman] vector, which has an *attB* site for transformation. *In vitro* manipulation of large fragments can be challenging, but recombineering techniques, in which mutagenesis is carried out in bacteria using lambda phage recombination enzymes to catalyze recombination between the BAC and a PCR product or oligonucleotide, make this possible (COURT *et al.* 2002). Recombineering can be used to make diverse alterations, including gene fusions, point mutations, or insertions and deletions.

The two papers in this issue of GENETICS take conceptually similar approaches to making gene targeting in Drosophila more efficient. First, by making use of previously integrated sequence-specific recombinase recognition sites, they bypass the need for an initial gene targeting step based on endogenous homologous recombination processes. Second, they demonstrate that replacement of a large genomic region is possible, thereby increasing the probability of finding a site or sites close enough to the gene of interest to be of use. Both methods rely on recombineering of large BACs to engineer changes and the efficient phiC31 integrase to target the modified construct to the desired location. There are differences, however, in the strategies presented to replace the endogenous genomic region with the recombineered version. Integration in the "long-range SIRT" method of Wesolowska and Rong generates a tandem duplication that is then reduced to a single copy by an *I-Sce*I induced double-strand break. The "captured segment exchange" method of Bateman *et al.* is similar to recombination mediated cassette exchange (RMCE) (BATEMAN *et al.* 2006), in which the region between two sequence-specific recombinase sites is replaced. Importantly, these methods allow the efficient replacement of and/or clean deletion of any endogenous region of interest, as long as it is within range of a phiC31 recognition site. These strategies effectively increase the size of the area that is targetable by any given recognition site, and because of the large number of sites at which phiC31 recognition sequences have been integrated into the Drosophila genome, a large fraction of the genome is currently (or soon will be) within targeting distance of a recognition site.

Wesolowska and Rong modified the pP[acman] vector to carry two FLP recombination target (FRT) sequences and an *I-Sce*I recognition sequence; the resulting vector is called pP[Walkman]. The target genomic region is cloned into this vector and modified by recombineering. After integration into an existing *attP* site (or one generated by gene targeting, as in standard SIRT) there is a tandem duplication (Fig. 2). A double-strand break made by *I-Sce*I promotes reduction to a single copy, most likely through the single-strand annealing repair pathway. The location of the break greatly influences whether the endogenous copy or the modified copy is retained. The authors tested several configurations and found one that favors retention of the modified copy (the *I-Sce*I sequence between the genomic DNA and the *white* gene from the vector). An advantage of this long-range SIRT strategy

is that only one *attP* site is required, and it can be 70 kb, possibly more, from the region of interest; the largest reported BAC insertion is 146 kb (VENKEN *et al.* 2006), raising the possibility of even longer distance targeting. The authors suggest that if an *attP* site is not within targeting distance, this strategy can be used to insert *attP* sites by walking down the chromosome until a site is placed near enough to the region of interest.

The captured segment exchange method of Bateman and colleagues can be used to replace a large target locus with minimal crossing and screening. In the two-step version of their procedure, the target locus (50 kb in their test case) is flanked by an *attP* site on one chromosome and an FRT site on the homologous chromosome (Fig. 3A). A donor BAC with the corresponding genome fragment flanked by *attB* and FRT sequences and carrying a designed modification is generated by recombineering. The first step is to integrate this BAC into the genomic *attP* site by standard phiC31-mediated transformation, generating a tandem duplication. To reduce this to a single copy, a FLP recombination reaction is induced between the FRT inserted from the BAC and the flanking FRT on the homologous chromosome, producing a recombinant chromosome with only the recombineered version of the target locus. Recombinants are easily identified by loss of *white* from the BAC and FRT-carrying transgene or by exchange of flanking markers.

The method was further simplified to a one-step approach (Figure 3B) for cases with two *attP* sites in inverted orientation flanking the region of interest. In this version, the BAC is engineered to contain the desired modifications along with flanking inverted *attB* sites. This DNA is injected directly into embryos that have the two *attP* landing sites on homologous chromosomes. Upon integration, the endogenous locus is replaced with the modified version in one step. These events are recognized by loss of markers on the *attP* transgenes or exchange of flanking markers, and are then screened for insertions in the correct orientation.

Both long-range SIRT and captured segment exchange are viable options when attempting gene targeting, but which method is the most suitable will depend on the specific genomic location and the integration sites that are available. One-step captured segment exchange involves the fewest integration and screening steps (Figure 1F), but it requires a very specific arrangement of attP sites

(two *attP* sites in an inverted orientation) that may not be available. Furthermore, the recombineered BAC is injected into embryos containing different *attP* sites on homologous chromosomes, so these embryos have to be generated from a cross instead of a stock. Two-step captured segment exchange gets around this extra round of crossing because the BAC can be injected into a stock that is homozygous or heterozygous for one *attP* site (Figure 1E). Two-step captured segment exchange again requires two available sequence specific recombinase recognition sites, decreasing the chances that a particular genome region is readily targetable without additional work. Long-range SIRT has the advantage that only one *attP* site is used to integrate the recombineered BAC; however, the modified version of the region of interest is kept only about half of the time (see Table 1 in Wesolowska and Rong) and screening procedures are less straightforward than for captured segment exchange. The ability to do repeated rounds of insertion and reduction, each putting a new *attP* landing site further from the original, further increases the versatility of this method.

The strategies presented in this issue of *Genetics* are a large step forward in the ability to carry out targeted replacement in the Drosophila genome. These strategies use efficient transformation methods and a minimal amount of crossing and screening, making them more versatile, efficient, and rapid than existing methods for gene replacement. The time-limiting step for either method is likely to be the recombineering, but there are sure to be advances in this technology as it becomes more widely adopted. Ongoing genome projects are providing an increasing number of *attP* and FRT integrations, opening up more and more of the Drosophila genome to manipulation through these new methods. FLP and phiC31 have become widely used in Drosophila research, but they work in other organisms as well, so it should be possible to develop similar methods for other organisms.

ACKNOWLEDGEMENTS

KNC is supported by T32 CA009156. Research in the Sekelsky laboratory is supported by R01 GM-61252.

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FIGURE LEGENDS

Figure 1. Steps to achieve targeted gene replacement. Each panel lists the steps typically taken to achieve gene replacement. Many factors can impact the total time to complete each procedure, but readers with a basic knowledge of Drosophila genetics may be able to estimate the minimum time for each.

Figure 2. Long-range SIRT. In step 1, a recombineered vector (pP[Walkman]) containing the modified region of interest, an *attB* site (53 bp), an *I-Sce*I recognition site (18 bp), two FRT sites (34 bp) and two P element ends (100-500 bp) is injected into stocks that contain an attP site within approximately 70 kb of the region of interest. Recombination between *attP* and *attB* results in *attL* and *attR* sites flanking the insertion. In step 2, a double-strand break is induced at the *I-Sce*I site, and repair of that break results in collapse of the duplication. Either the modified or the endogenous region of interest can be retained. The pP[Walkman] backbone can be removed through a FLP/FRT recombination reaction.

Figure 3. Captured segment exchange. A. 2-step captured segment exchange. In step 1, a BAC containing the region of interest with the desired modifications, an *attB* site, an FRT site, and *white* to identify positive transformants, is injected into a stock that has an *attP* site within targeting distance of the genomic region of interest. In step 2, a FLP/FRT recombination reaction is induced between the newly inserted FRT and an FRT on the homologous chromosome that flanks the opposite site of the region of interest. This results in a new recombinant chromosome containing only the modified region of interest at the endogenous location. B. 1-step captured segment exchange. A BAC containing the modified region of interest and two flanking inverted attB sites is injected into embryos with two attP sites on homologous chromosomes. Upon integration into the genome, the endogenous region of interest is swapped out for the modified region of interest.

A Ends-In Gene Targeting

1) Build targeting transgene *in vitro*
2) Generate transgenic donor lines
3) Cross in FLP and *I-Sce*I transgenes
4) Outcross to remove donor transgene
5) Screen progeny for integration of targeting DNA
6) Make stocks for each independent integration
7) Screen for cases of homologous recombination
8) Cross in *I-Cre*I to induce reduction of duplication
9) Screen progeny for reduction to single copy
10) Screen for retention of desired copy

B Ends-Out Gene Targeting

1) Build targeting transgene *in vitro*
2) Generate transgenic donor lines
3) Cross in FLP and *I-Sce*I transgenes
4) Outcross to remove donor transgene
5) Screen progeny for integration of targeting DNA
6) Make stocks for each independent integration
7) Screen for cases of homologous recombination

C SIRT / Genomic Engineering

1) Do steps 1-10 from A. to integrate *attP* site
2) Build targeting construct with *attB* site
3) Do phiC31-mediated transformation
4) Cross in *I-Sce*I or *I-Cre*I to induce reduction.
5) Screen progeny for retention of desired copy
6) (optional) Cross in FLP to remove reporter gene

D Long-Range SIRT

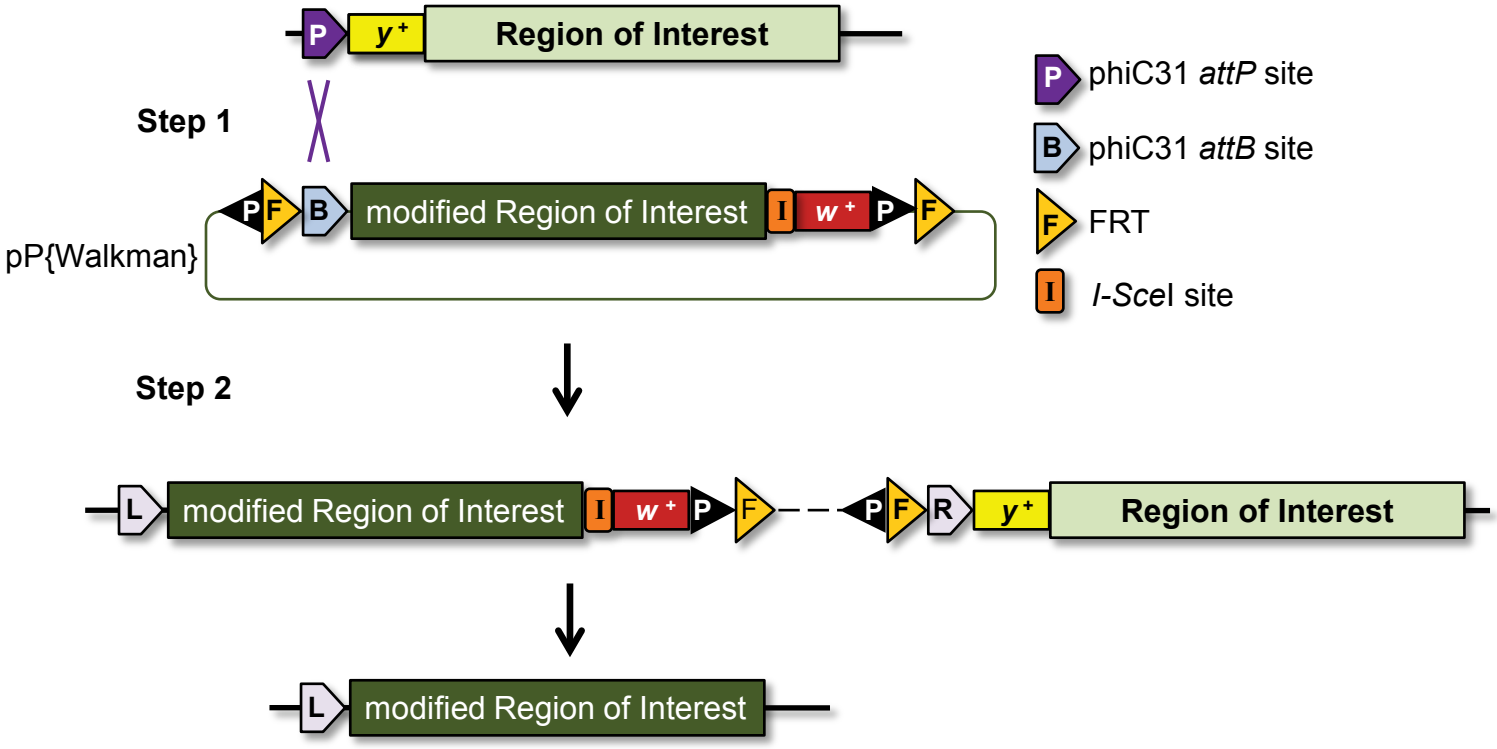
1) Identify *attP* within 80-100 kb of region of interest
2) Modify *P*{acman} clone by recombineering
3) Do phiC31-mediated transformation
4) Cross in *I-Sce*I to induce reduction.
5) Screen progeny for retention of desired copy
6) (optional) Cross in FLP to remove reporter gene

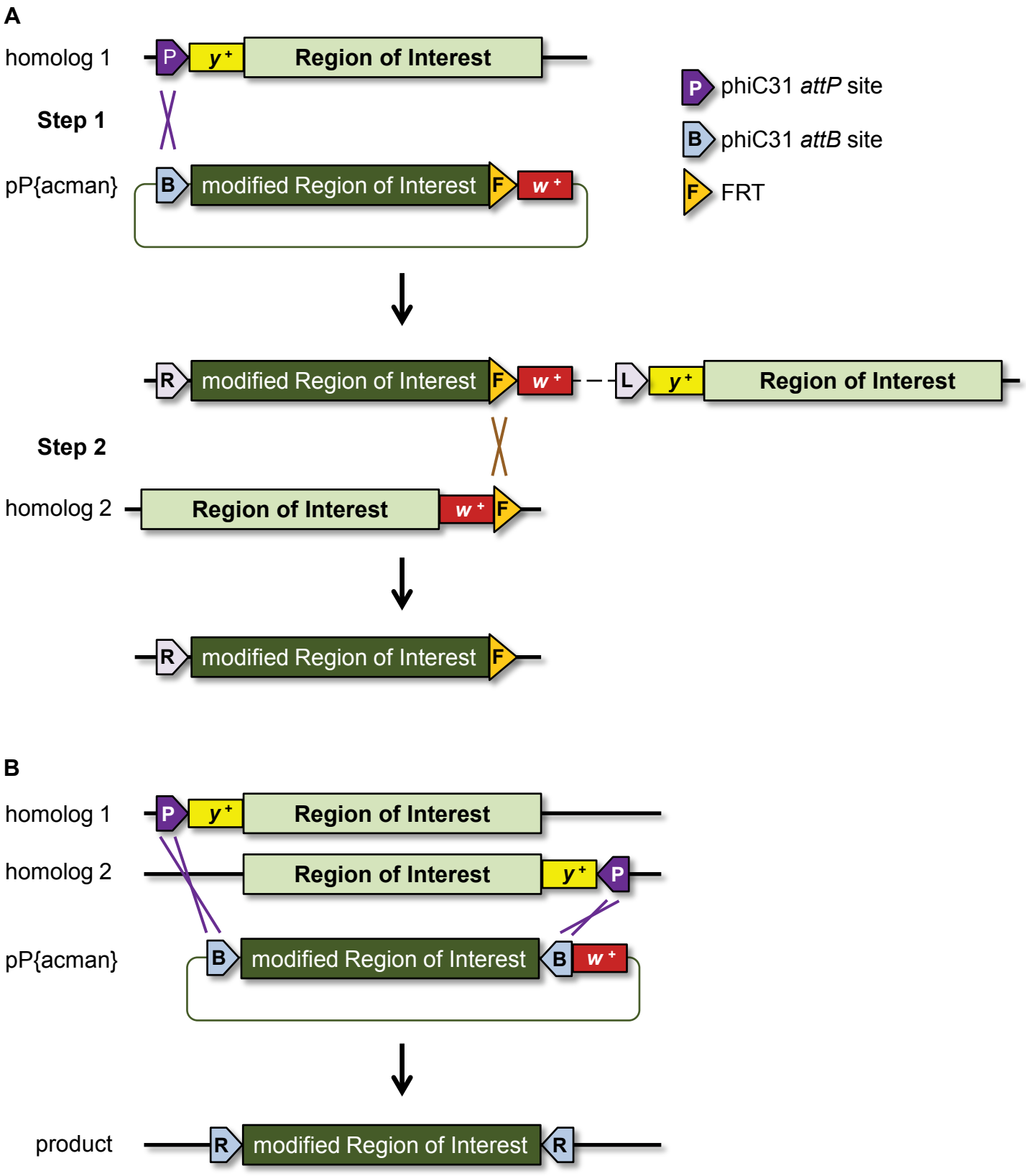
E Captured Segment Exchange (2-step)

1) Identify *attP* and FRT flanking region of interest
2) Build BAC clone of region by recombineering
3) Do phiC31-mediated transformation into *attP*
4) Cross in FRT *in trans* and FLP
5) Screen for excision and/or crossover

F Captured Segment Exchange (1-step)

1) Identify inverted *attP* flanking region of interest
2) Build BAC clone of region by recombineering
3) Do phiC31-mediated transformation into embryos heterozygous for *attP* sites
4) Screen progeny of injected individuals for replacement and/or crossover


P
y+
Region of Interest
Step 1
P phiC31 attP site
B phiC31 attB site
F FRT
I I-SceI site
pP{Walkman}
P F B
modified Region of Interest
I w+ P F
Step 2
L
modified Region of Interest
I w+ P F
P F R y+
Region of Interest
L
modified Region of Interest


A
homolog 1
P
y+
Region of Interest
Step 1
pP{acman}
B
modified Region of Interest
F
w+
R
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Step 2
homolog 2
phiC31 attP site
phiC31 attB site
FRT
B
product