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Transgenesis upgrades for *Drosophila melanogaster*

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Drosophila melanogaster is a highly attractive model system for the study of numerous biological questions pertaining to development, genetics, cell biology, neuroscience and disease. Until recently, our ability to manipulate flies genetically relied heavily on the transposon-mediated integration of DNA into fly embryos. However, in recent years significant improvements have been made to the transgenic techniques available in this organism, particularly with respect to integrating DNA at specific sites in the genome. These new approaches will greatly facilitate the structure-function analyses of *Drosophila* genes, will enhance the ease and speed with which flies can be manipulated, and should advance our understanding of biological processes during normal development and disease.

Introduction

During the past few years, *Drosophila melanogaster* has gained in popularity because of the availability of its genome sequence (Adams et al., 2000), its rapid life cycle, the relative ease with which it can be handled and the multitude of genetic tools that are available for its study (Greenspan, 2004). The fly's genome permits the most sophisticated manipulations of any of the known eukaryotes. Indeed, the number of existing and recently developed technological improvements, such as genome-wide transposon tagging and gene targeting (Venken and Bellen, 2005) and the availability of numerous resources, including online databases such as FlyBase and stocks from fly stock centers (see Box 1 for links to some of these resources) (Matthews et al., 2005), greatly facilitate research in the field and move it forward at a relentless pace. These technologies and resources further the study of various aspects of developmental biology, genetics, cell biology, neuroscience and behavior. Indeed, the identification of novel genes and their functional characterization in vivo greatly depends on these available tools. Moreover, as most human disease genes have a counterpart in the *Drosophila* genome, including those involved in genetic disorders and cancer (Bier, 2005; Vidal and Cagan, 2006), the fly is also becoming increasingly popular for studying the molecular mechanisms of human disease. Much of this research relies on an efficient and reliable transgenesis system.

Transgenesis in general can be defined as a group of technologies that allow DNA to be introduced into an organism of choice. The main goal of transgenesis is to integrate a foreign piece of DNA – a transgene – into an organism's genome to result in germ line transmission (see Fig. 1), in order to study gene function. Insect transgenesis, in general, has been dominated by transposon-mediated integration (Handler and James, 2000). In *Drosophila*, transgenesis mainly relies on the *P* element transposon and this has been the foundation for most of the innovative developments within the fly field (Ryder and Russell, 2003). However, various improvements in fly transgenic techniques have been recently reported that predominantly employ the site-specific integration of transgenes at specific genomic docking sites (see glossary, Box 2) via the use of different recombinases and integrases (Groth et al., 2004; Oberstein et al., 2005; Horn and Handler, 2005; Bateman et al., 2006; Venken et al., 2006; Bischof et al., 2007). Many of these advances have their origins in mouse molecular genetics (Seibler and Bode, 1997; Bethke and Sauer, 1997; Bouhassira et al., 1997; Groth et al., 2000; Thyagarajan et al., 2001) and have been very useful for developing new fly transgenic techniques, as discussed below.

Here, we summarize many of the current methods that are used to generate transgenic flies. We first review classical transposon-mediated transgenesis and site-specific integration methods, before describing a plethora of recent improvements that have their basis in site-specific integration systems.

Transposon-mediated transgenesis in *Drosophila*

Transgenesis can be performed through various techniques. In *Drosophila*, transgenesis mainly relies on the *P* element transposon, the introduction of which (Rubin and Spradling, 1982) has been one of the most important breakthroughs in germ line transgenesis in *Drosophila*. As such, *Drosophila* research has been highly dependent on *P* element-mediated transgenesis, even though it has two major drawbacks: the size of the DNA that can be integrated is limited and the insertion sites cannot be controlled.

P elements are transposable elements, or transposons, which were originally identified within the fly's own genome (Castro and Carareto, 2004). *P* elements, like other transposons, contain two terminal repeats, including inverted repeat sequences and other internally located sequence motifs absolutely required for their

Box 1. Relevant websites

***Drosophila* Genomics Resource Center:** plasmid resource center for fly transgenesis.
https://dgrc.cgb.indiana.edu
DrosDel: docking site stock center for FLP remobilization.
http://www.drosdel.org.uk
FlyBase: general online fly resource.
http://www.flybase.org
FlyC31: ΦC31 integrase system, plasmids and fly stocks.
http://www.frontiers-in-genetics.org/flyc31
P(acman): recombineering and the ΦC31 integrase system.
http://flypush.imgen.bcm.tmc.edu/lab/pacman.html
ΦC31 fly stocks at the Bloomington *Drosophila* Stock Center:
http://flystocks.bio.indiana.edu/Browse/misc-browse/phiC31.htm
ΦC31 RMCE website: ΦC31 integrase-mediated RMCE, plasmids and fly stocks.
http://genepath.med.harvard.edu/WuLab/RMCE
Recombineering website: resource for public available recombineering reagents.
http://recombineering.ncifcrf.gov
Gensat Database: resource for RecA assisted modification.
http://www.gensat.org
Vienna *Drosophila* RNAi Center: transgenic RNAi fly lines.
http://www.vdrc.at
National Institute of Genetics (Japan) RNAi Fly Stocks: transgenic RNAi fly lines.
http://www.shigen.nig.ac.jp/fly/nigfly

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mobilization or transposition (see Fig. 2 and Table 1) (Beall and Rio, 1997). Mobile or autonomous *P* element transposons encode a functional enzymatic protein called *P* transposase that catalyzes transposition through both terminal repeats of the transposon. *P* element-mediated transgenesis requires the separation of the *P* transposase and the *P* element transposon backbone (Rubin and Spradling, 1982). A plasmid that encodes *P* transposase, a so-called helper plasmid, is provided in trans with another plasmid (the transgene) that contains the transposon backbone, the sequence of interest and a marker (see Fig. 2B) (Karess and Rubin, 1984). In vitro synthesized mRNA that encodes the transposase or purified transposase protein itself (Kaufman and Rio, 1991) can also be co-injected with modified *P* elements. Co-injections limit transposase activity, which is often advantageous. Alternatively, the transposase can be expressed from a genomic source (Cooley et al., 1988), allowing the injection of a *P* element without a helper plasmid. Transgene expression can be rendered constitutive or inducible through the inclusion of a heat-shock promoter. A hyperactive form of *P* transposase has been isolated that results in increased transposition rates (Beall et al., 2002). In general, transposons are injected into fly strains that are devoid of the same transposon, avoiding unwanted mobilization events of transposons present in the genome, thereby ensuring the stable integration and maintenance of the injected transgene. The unbiased identification of integration events is crucial for transgenesis and predominantly relies on the incorporation of dominant markers, which are identified through screening or selection (see Table 2 and Box 3 for more information).

Transposition occurs by the excision or replication of the transposon from the injected plasmid and its insertion into the host genome. Different transposons have unique insertion site characteristics. Integration events of *P* elements are strongly biased towards the 5′ end of genes. Hot spots – insertion sites that attract *P* elements at a much higher frequency than others – also exist within the *Drosophila* genome (Spradling et al., 1995; Bellen et al.,

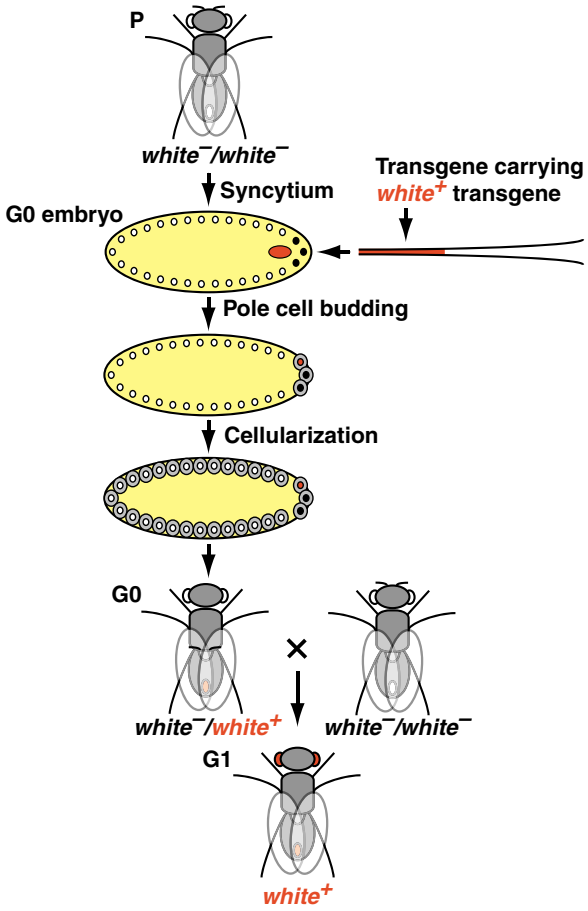


Fig. 1. *Drosophila* transgenesis. *white*⁺ transgene DNA (red) is injected into generation zero *Drosophila* embryos (G0) of less than 1 hour old, which have been obtained from a parental (P) generation. The early developmental stages of *Drosophila* embryos are characterized by rapid nuclear divisions that occur without accompanying cell divisions, creating a syncytium. Prior to cellularization, pole cells (black) bud off at the posterior end. For germ line transmission to occur, the transgenic DNA must be taken up into the pole cells that are fated to become germ cells. Transgenic DNA integrated into a pole cell (red pole cell) can be transmitted from one generation (G0) to the next (G1 progeny). The resulting integration events are identified using an appropriate marker, such as as *white*⁺. When used in a mutant *white*⁻ strain, this transgene marks transgenic flies by giving them a darker eye color (see Table 2 and Box 3 for more information on the markers used in fly transgenesis).

Box 2. Glossary of specialized terms

Acceptor site: A genomic site that receives in vivo DNA mobilized from a different location – the donor site. This occurs through FLP remobilization or *P* element replacement.

Docking site: Alternatively called a landing site. A genomic site that receives injected DNA during embryo microinjection.

Donor site: A genomic site that contains DNA sequence that will be donated for integration at another location, through FLP remobilization, *P* element replacement or gene targeting.

Episomal fragment: An independent DNA element, such as a plasmid, that can exist extrachromosomally or that can be maintained by integrating into the genome of the host.

Gal4/*UAS* system: Based on the yeast transcriptional activator GAL4 and its high-affinity binding site, the upstream activating sequence (*UAS*), this system is generally used to ectopically express a gene of interest. When a tissue-specific GAL4 line is crossed to an effector line that carries the *UAS* fused to a gene of interest, progeny with both the GAL4 and *UAS* components express the gene of interest in an activator (and often tissue)-specific manner.

Insulator: A DNA sequence that blocks the interaction between cis-acting regulatory elements. These sites are sometimes used to protect transgenes from genomic position effects.

MARCM: MARCM (mosaic analysis with a repressible cell marker) allows mutant clones generated by mitotic recombination to be identified in an otherwise wild-type unlabeled background (Lee and Luo, 1999). A recent modification of the MARCM system, dual-expression-control MARCM, has added another level of sophistication to this technique (Lai and Lee, 2006).

Mitotic recombination: A cross-over between two homologous double-stranded DNA molecules. This recombination occurs frequently during meiosis, but is relatively rare during mitosis.

Position effects: The effect of the local chromosomal environment on the level or pattern of transgene expression, owing to local chromatin configuration or nearby cis-acting regulatory elements.

Position effect variegation: A phenomenon discovered in *Drosophila* that occurs when genes placed close to large heterochromatic regions are repressed. This repression is metastable in that the silenced state can be occasionally released, giving rise to derepressed cells and a variegated phenotype.

Rescue: A condition achieved by introducing a wild-type DNA fragment that can complement a genomic mutation by producing the functional or missing protein.

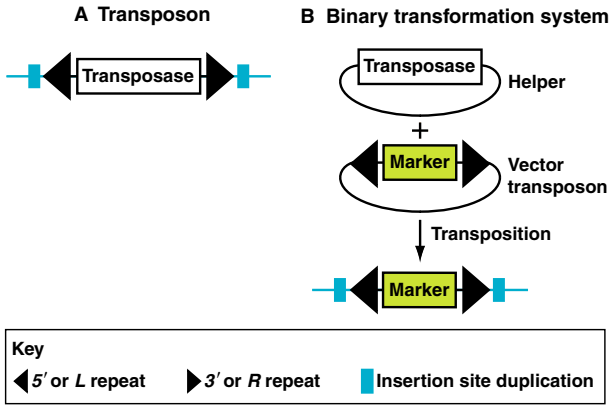


Fig. 2. Binary vector/helper transposon transformation system. (**A**) Active transposons are mobile elements that consist of two inverted terminal repeats (black) that flank an open reading frame encoding a transposase. Both features are required for transposition. The inverted repeats are commonly called *5′* or *Left* (*L*) and *3′* or *Right* (*R*). Transposition results in a duplication of the insertion site (blue). (**B**) Transposon and transposase can be separated, resulting in a binary vector/helper transposon transformation system that allows the regulated transposition of transgenes into the genome. Transposition events are identified by dominant markers (green, and see Table 2 and Box 3).

2004). Moreover, *P* elements have a narrow taxonomic activity and are non-functional outside of the Drosophilidae (Handler et al., 1993) owing to a host-specific factor that is required for transposition (Rio and Rubin, 1988). To circumvent these limitations, several other transposons with a different insertional specificity and a broader host range have been identified that are suitable for germ line transformation in *Drosophila* (see Table 1). These include *piggyBac* (Handler and Harrell, 1999), identified in the cabbage looper moth *Trichoplusia ni* (Cary et al., 1989; Handler, 2002); the Tc1/mariner-like transposons *Minos* (Loukeris et al., 1995) and *Mariner* (Lidholm et al., 1993), isolated from *Drosophila hydei* (Franz and Savakis, 1991) and *Drosophila mauritiana*, respectively (Jacobson et al., 1986); and the *hobo*, *Ac*, *Tam3* (hAT) family members *Hermes* (O'Brochta et al., 1996) and *hobo* (Blackman et al., 1989; Smith et al., 1993), isolated from the house fly *Musca domestica* (Warren et al., 1994) and *Drosophila melanogaster*, respectively (McGinnis et al., 1983). These transposons function in a variety of organisms, but their use in *Drosophila* transgenesis has been limited (O'Brochta and Atkinson, 1996; Ryder and Russell, 2003). *piggyBac* and *Minos* have been used as alternative mutagens because they have a different insertional specificity to *P* elements (Hacker et al., 2003; Horn et al., 2003; Thibault et al., 2004; Metaxakis et al., 2005). As *Mariner* elements do not remobilize efficiently (Lozovsky et al., 2002), and because *hobo* is present in most laboratory stocks, neither is commonly used. Finally, *hobo* and *Hermes* have been shown to cross-mobilize (Sundararajan et al., 1999). These features have limited the use of these transposable elements.

Applications of transposon-mediated transgenesis

The transposon-mediated integration of transgenes has been used for numerous experiments in the fly field. These experiments can be broadly subdivided into two main groups: gene disruption methods and transgenic technologies. Gene disruption occurs when a transposon insertion interferes with the function of a gene. Transgenic technologies usually involve introducing the different components of novel techniques (see below) or performing rescue experiments.

Almost all technological progress in flies depends on our ability to transform them. Indeed, *P* element-mediated enhancer detection (O'Kane and Gehring, 1987; Bellen et al., 1989; Bier et al., 1989), the use of the FLP/*FRT* system to create mutant clones by inducing mitotic recombination (see glossary, Box 2) (Xu and Rubin, 1993), the gene knockout methods in flies (Rong and Golic, 2000; Gong and Golic, 2003), the creation of molecularly defined deletions throughout the genome (Thibault et al., 2004; Ryder et al., 2004), the generation of marked mutant clones by MARCM (see glossary, Box 2) (Lee and Luo, 1999), and many other technological advances have relied on transgenesis. The recent availability of a genome-wide library of RNAi transgenic insertions that allows the knockdown of most fly genes (Dietzl et al., 2007) will also provide an invaluable tool to study gene function.

In addition, transposon-mediated phenotypic rescue of a mutation is considered to be the best and most convincing evidence that a piece of DNA contains a gene of interest. Unfortunately, traditional high-copy-number plasmids, including the *P* element-containing plasmids, have a limited cargo capacity of ~20-25 kb of DNA owing to plasmid instability in bacteria. To circumvent this, *P* elements were engineered in a medium-copy-number cosmid backbone (Haenlin et al., 1985; Steller and Pirrotta, 1985), providing a higher cargo capacity of up to 40-50 kb. Unfortunately, the difficulties associated with obtaining integration of 30-50 kb *P* element-based cosmids did not promote the use of this methodology. As a result, a transgenic cDNA rescue based on the GAL4/*UAS* system (see glossary, Box 2) (Fischer et al., 1988; Brand and Perrimon, 1993) or heat-shock induction (Basler and Hafen, 1989) became more popular.

Neutralization of position effects

One of the major drawbacks of *P* element-mediated transgenesis is that *P* elements most often integrate into the 5′ regulatory regions of genes (Bellen et al., 2004), thereby causing two unwanted consequences. First, the insertion often disrupts another gene that may or may not be relevant (e.g. within the same pathway) to the gene that is being studied (Norga et al., 2003). Second, the gene within the transposon may be subject to unwanted position effects or to position effect variegation (see glossary, Box 2) dictated by the surrounding

Table 1. Transposons for *Drosophila* transgenesis

Transposon	Inverted repeats (bp)	Insertion site preference	Target site duplication (bp)	Species compatibility
P element	31	5′ end of genes	8	Drosophilidae only
piggyBac	13	TTAA	4	Broad
Minos	255	TA	2	Broad
Mariner	28	TA	2	Broad
Hermes	17	Low sequence specificity	8	Broad
hobo	12	Low sequence specificity	8	Broad

Table 2. Dominant marker genes for *Drosophila* transgenesis

Dominant marker*	Screening/selection (compound)	Mutant line required
white	Screening	Yes
yellow	Screening	Yes
rosy	Screening	Yes
rough	Screening	Yes
vermillion	Screening	Yes
3xP3 'fluorescent protein'	Screening	No
hs-neo	Selection (G418)	No
hs-opd	Selection (paraoxon)	No
Resistant to dieldrin (*Rdl*)	Selection (dieldrin)	No
Alcohol dehydrogenase (*Adh*)	Selection (ethanol)	Yes

**white*, *rosy*, *rough* and *vermillion* are eye color markers, whereas *yellow* is a body color marker. *hs-neo* encodes a heat shock-inducible neomycin-selectable marker (Steller and Pirrotta, 1985), *hs-opd* encodes a heat shock-inducible insecticide-degrading enzyme (Benedict et al., 1995), *Resistant to dieldrin* (*Rdl*) encodes the GABA-A receptor (Stilwell et al., 1995) and *Alcohol dehydrogenase* (*Adh*) encodes an enzyme involved in ethanol catabolism (Goldberg et al., 1983).

genomic environment. Insertions in the regulatory region of a gene, on which nearby cis-acting elements typically act, bring the gene into an environment that is almost certainly subject to unwanted regulation. Indeed, position effects and position effect variegation were observed early on for markers such as *white* (Hazelrigg et al., 1984; Levis et al., 1985) and were eventually exploited in different kinds of enhancer-trap screens to identify temporally and spatially restricted expression patterns of developmentally regulated genes (O'Kane and Gehring, 1987; Bellen, 1999).

Position effects can be partially neutralized through the incorporation of insulator sequences (Roseman et al., 1995). Insulators (see glossary, Box 2) tend to shield the transgene from regulatory influences imposed by the surrounding genome. Insulators, such as *gypsy*, have been used in some *P* element vectors because they are more mutagenic than other *P* elements that do not contain insulators (Roseman et al., 1995). They were also incorporated into *P* element reporter transposons developed to analyze gene regulatory sequences (Barolo et al., 2000; Barolo et al., 2004). Insulators allow for a better comparison of different transgene insertions at different loci. Yet insulators may also influence the expression of the gene that they flank within the construct and are still somewhat subject to position effects in the genome.

There are at least four alternative genetic strategies to neutralize position effects when different transgenes are being compared at the same locus. The simplest method is transgene coplacement (Siegal and Hartl, 1996), which allows any two transgenes, such as a rescue fragment and its mutant version, to be compared in the same orientation at the same locus (Fig. 3). Both transgenes are integrated into a *P* element that contains the site-specific recognition sites *FRT* and *loxP*, the targets of FLP and Cre recombinases, respectively (see Box 4 for more information on these recombinases). After integration of the *P* element, FLP can remove one transgene and Cre can remove the other. Recognition sites are oriented such that either recombination event results in an identical configuration for either transgene. This method also introduced the use of Cre recombinase into the *Drosophila* field (Siegal and Hartl, 1996; Siegal and Hartl, 2000). One drawback of the technique is that only two transgenes can be compared at the same locus.

A second method is based on FLP recombinase-mediated transgene remobilization (Golic et al., 1997) (Fig. 4A). First, a 'donor' *P* element (see glossary, Box 2), containing a transgene together with the *white*$^+$ marker flanked by *FRT* sites, is integrated into the fly genome using *P* transposition, resulting in a donor site.

Box 3. Dominant marker genes for *Drosophila* transgenics

Identifying transgene integration events is crucial for transgenesis and relies on the incorporation of dominant markers, which are identified through screening or selection (see Table 2). The former relies on the rescue (see glossary, Box 2) of a visible mutant phenotype that minimally affects viability.

Two popular markers are the adult eye color marker *white* and body color marker *yellow* (see Table 2). The *mini-white* gene is one of the most widely used *white* markers (Pirrotta, 1988), and is present in two of the most often used *P* element plasmids, pP{UAST} for GAL4/*UAS* overexpression (see Box 2) (Brand and Perrimon, 1993) and the pP{CaSpeR} plasmid series for genomic rescue experiments (Thummel and Pirrotta, 1992; Le et al., 2007). A useful variant is *hsp70-white*, a heat shock promoter-driven *white* (Klemenz et al., 1987). Transgenic events are identified by the expression of eye color, which ranges from pale yellow to wild-type red owing to strong gene dosage and position effects (see Box 2), in a *white* mutant background. See Table 2 for more on the other eye markers, *rosy* (Rubin and Spradling, 1982), *vermillion* (Fridell and Searles, 1991) and *rough* (Lockett et al., 1992). The most frequently used body color marker is *mini-yellow*, an intron-less version of the *yellow* gene that is less subject to position effects (Patton et al., 1992) and which imparts a gray/tan color to the adult cuticle in a *yellow* mutant background (see Table 2).

Recently, fluorescent protein-based markers have been developed, which are also used in other insects and organisms (Horn et al., 2002). One popular marker is a fusion between an artificial eye-directed promoter, *3xP3*, and a fluorescent protein, such as enhanced green fluorescent protein (EGFP) (Berghammer et al., 1999; Horn et al., 2000). A combination of different fluorescent proteins permits the identification of various transgenes (Horn and Wimmer, 2000; Horn et al., 2002); transgenic events are identified visually under a stereomicroscope. The polyubiquitin promoter is also used to drive fluorescent protein expression (Handler and Harrell, 1999). Fluorescent markers are less position-dependent than *white*$^+$ (Handler and Harrell, 1999; Horn et al., 2000), especially when used with insulator sequences (see Box 2) (Sarkar et al., 2006). Because wild-type eye pigmentation quenches fluorescence, *3xP3*-driven transgenesis is often best performed on a *white* mutant background (Horn and Wimmer, 2000; Horn et al., 2000). A *3xP3-white* transgene, containing the *3xP3* promoter fused to a *white* cDNA, has recently been described (Egli et al., 2006). Four selectable markers are also available (see Table 2 for more information) (Goldberg et al., 1983; Steller and Pirrotta, 1985; Benedict et al., 1995; Stilwell et al., 1995).

Second, the transgene with the *white*$^+$ marker, flanked by *FRT* sites, is remobilized through FLP excision. This episomal fragment (see glossary, Box 2) can integrate into a second single *FRT*-containing 'acceptor' transposon (see glossary, Box 2), which also carries another dominant marker and is located elsewhere in the genome. Successful mobilization events can be identified through screening, as relocalization usually results in changes in *white*$^+$ marker expression owing to position effects. This strategy is facilitated if a split *white*$^+$ marker strategy is integrated into the system (Fig. 4B): the *white*$^+$ marker is separated into 5′ and 3′ fragments, and only becomes functional after the reconstitution of these fragments through site-specific recombination within an intron located between both fragments (Golic et al., 1997). In FLP recombinase-mediated transgene remobilization, *white* expression is only obtained after correct mobilization and site-specific integration (Fig. 4C), facilitating the screening procedure of integration events. Interestingly, thousands of *P* element insertions obtained by the

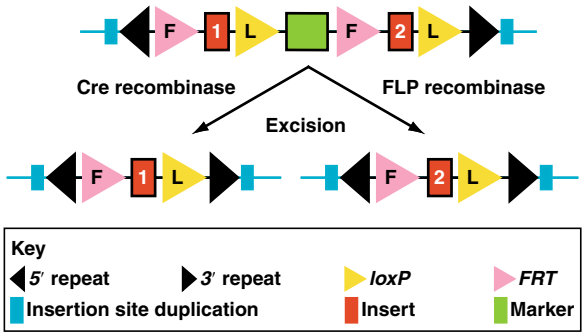


Fig. 3. Transgene coplacement. Two inserts (1 and 2, red), each containing a cloned fragment, such as a genomic rescue fragment, are integrated into a *P* element that contains appropriately positioned *loxP* (yellow) and *FRT* (pink) sites. Cre recombination results in the removal of insert 2, whereas FLP recombination results in the removal of insert 1, positioning either insert in the same orientation at the same locus (indicated in blue), thereby neutralizing position effects. In each case, recombination events are identified by the loss of a dominant marker (green).

DrosDel project (see Box 1) were generated by the mobilization of the *P{RS5}* and *P{RS3}* transposons (Golic and Golic, 1996) and were subsequently used for the generation of precise deletions (Ryder et al., 2004). Both transposons can be used as acceptor elements for in vivo FLP-mediated DNA mobilization using the split *white*$^{+}$ marker strategy, and they provide numerous docking sites that are dispersed all over the fly genome (Fig. 4D,E). A drawback of FLP recombinase-mediated transgene remobilization in general is that a second round of crossings for remobilization and screening has to be performed after an initial *P* element-mediated transformation to obtain the required integration events of donor elements.

A third genetic trick to neutralize position effects is *P* element replacement or targeted transposition (Gloor et al., 1991; Lankenau and Gloor, 1998). An ‘acceptor’ *P* element inserted at one location is replaced by a second ‘donor’ *P* element, integrated at another location, through in vivo gap repair (Fig. 5). The technique requires homologous recombination between the 10-20 bp footprints of the 31 bp inverted terminal repeat that remain after excision of the acceptor element and the homologous counterpart of the donor element. The homologous recombination event is promoted owing to a double-stranded gap that is generated after the excision of the acceptor element. Various donor *P* elements can be targeted to the same locus, allowing different transgenes to be directly compared with each other. A drawback of the technique is that replacement can occur in both directions, requiring additional molecular verification. The technique has not been used to perform structure-function analysis of differently mutagenized transgenes but has been proven useful for converting existing *lacZ* enhancer-detector

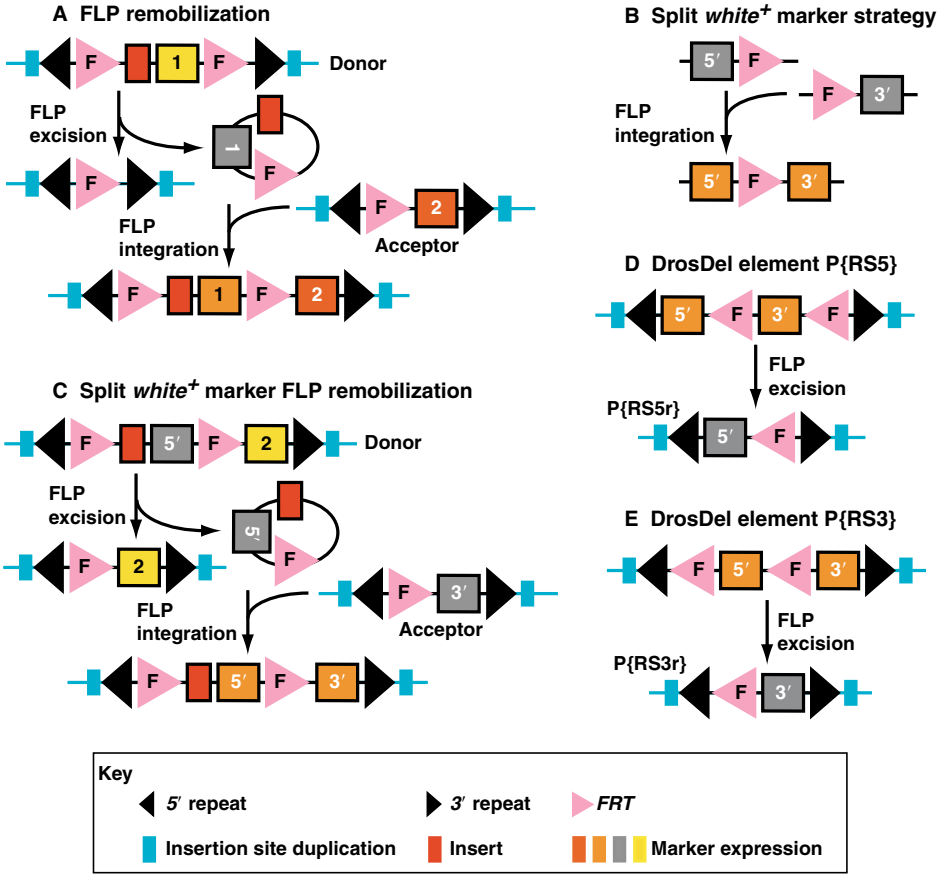
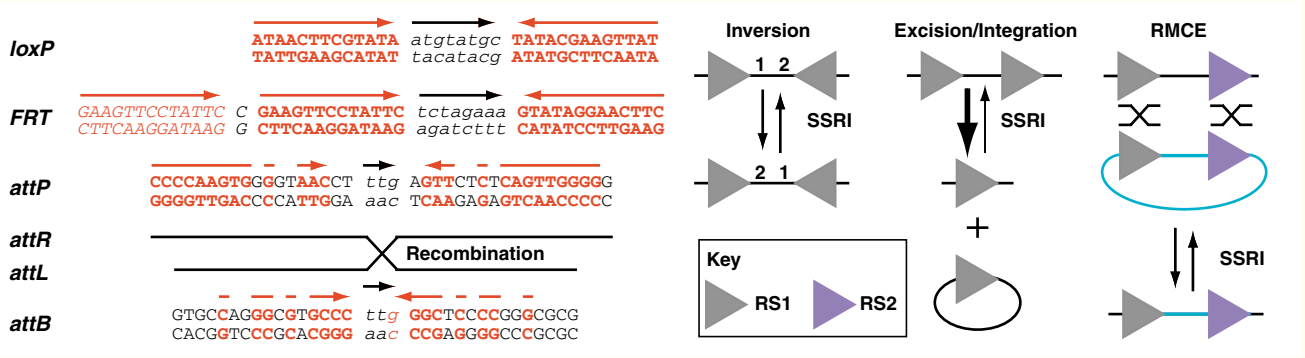


Fig. 4. FLP remobilization. (**A**) FLP remobilization technique. A donor transposon contains a transgenic insert (red) together with a marker (1) flanked by two *FRT* sites. An acceptor transposon, at a desired locus, contains a second marker (2) and one *FRT* site. Remobilization of the donor transposon by FLP results in the excision of its transgene and its potential integration into the *FRT* site of the acceptor transposon. This remobilization can be followed through changes in expression of marker 1, such as *white*, that occur because of changes in position effects (from yellow in the original site to orange in the acceptor site). Different donor transposons, each containing different transgenes, can be targeted to the same acceptor, thereby neutralizing position effects. (**B**) Split *white*$^{+}$ marker strategy. The *white*$^{+}$ marker is divided into two parts: 5′-*white*$^{+}$ (5′) and 3′-*white*$^{+}$ (3′). Neither part can produce eye pigmentation alone (indicated in gray). Recombination between appropriately localized recombination sites, *FRT* in this case, results in *white*$^{+}$ reconstitution and its expression (orange). (**C**) Integration of the split *white*$^{+}$ marker strategy into the FLP remobilization technique. The correct remobilization and integration of the transgene (red) are identified by *white*$^{+}$ reconstitution (orange). Marker 2 (yellow) identifies donor transgenes. (**D**,**E**) DrosDel elements *P{RS5}* and *P{RS3}*. FLP-mediated recombination at (D) *P{RS5}* and (E) *P{RS3}* results in chromosomal remnants, *P{RS5r}* and *P{RS3r}*, respectively. Each contains one part of the *white*$^{+}$ marker. Both remnants can be reconstituted through FLP remobilization of an appropriately designed donor transposon (see C).

Box 4. Site-specific recombinases and integrases



Site-specific recombinases and integrases (SSRIs) often require only two components: a site-specific enzyme, which, preferentially, functions without additional proteins, and a pair of DNA recombination sites (RSs) (Sorrell and Kolb, 2005). SSRIs are subdivided into the tyrosine and serine recombinases, which use a conserved tyrosine and serine residue during recombination, respectively. Commonly used tyrosine recombinases are Cre and FLP. Cre (causes recombination of the bacteriophage P1 genome) recognizes minimal *loxP* [locus of crossing-over (X) in P1] RSs of 34 bp, which consist of two 13 bp perfect inverted repeats (red) flanking an 8 bp asymmetric spacer (black) that confers directionality (Hoess et al., 1982) (see figure). Likewise, FLP (flips DNA) recognizes minimal *FRT* (FLP recombinase target) RSs of 34 bp and has a similar configuration to *loxP* but with a different sequence (McLeod et al., 1986) (see figure). A genuine *FRT* RS, absolutely required for site-specific integration, consists of 48 bp, containing an additional isolated base pair and a third 13 bp direct repeat (see figure).

A commonly used serine recombinase is the integrase from the *Streptomyces* bacteriophage ΦC31 (see figure) (Thorpe and Smith, 1998). ΦC31 integrase recognizes a minimal high-efficiency *attP* RS (attachment site in the phage genome) of 39 bp and a minimal high-efficiency *attB* RS (attachment site in the bacterial genome) of 34 bp (Groth et al., 2000). *attP* and *attB* sites contain imperfect inverted repeats (red) flanking a short recombination core (TTG, black) that provides directionality (see figure).

Recombination between two RSs can lead to an inversion, integration/excision or recombinase-mediated cassette exchange (RMCE), depending on the orientation and types of RS (see figure). A translocation can also occur if the RSs are on two different chromosomes (not shown). The presence of two compatible RSs results in a recombination event, which in the case of *FRT* or *loxP* leads to the reformation of a still functional RS, potentially resulting in additional recombination events. This problem can be overcome using RS inverted repeat variants, such as *lox71* and *lox66*, which contain mutations in the left and right inverted repeat, respectively. Recombination between *lox71* and *lox66* results in wild-type *loxP* and a double-mutant *lox72*, two sites that do not recombine with each other (Albert et al., 1995). Similar variants exist for *FRT* sites (Senecoff et al., 1988). In the case of *attP*/*attB*, new *attL* (*att* Left) and *attR* (*att* Right) sites are created (see figure), which are no longer substrates for the integrase, ensuring that recombination is irreversible.

Integration using a single RS results in the integration of the entire plasmid, including the vector backbone. This can be avoided through RMCE (Schlake and Bode, 1994; Baer and Bode, 2001). RMCE uses a double-reciprocal cross-over reaction between two cassettes, one integrated into the genome, the other episomal (circle in figure, see Box 2), to mediate transgene integration, while avoiding the integration of vector backbone (see figure). Because the use of two sets of *loxP* or *FRT* RSs favors deletion over RMCE, double reciprocal cross-over requires the use of RS spacer variants: recombination sites that react preferentially with each other but not with other variants. RS spacer variants include *lox511* (Hoess et al., 1986), *lox5171* and *lox2272* (Lee and Saito, 1998); and *m2*, *m3*, *m7* and *m11* (Langer et al., 2002). *lox2272* and *m2* seem to be the least leaky and therefore most useful for RMCE in conjunction with a wild-type *loxP* RS. An expansion of the *loxP* collection has been recently reported (Missirlis et al., 2006). Similar spacer variants also exist for *FRT*: *F3* and *F5* are useful for RMCE reactions together with a wild-type *FRT* site (Schlake and Bode, 1994). Alternatively, inverted RSs eliminate deletion but cause cassette inversion when *FRT* or *loxP* are used (Feng et al., 1999) (see figure). Because integration can occur in two orientations, additional screening is required to determine the exact nature of the integration event.

P elements into GAL4 drivers (Sepp and Auld, 1999; de Navas et al., 2006) that allow more versatile reporter analysis using the GAL4/*UAS* system (Fischer et al., 1988; Brand and Perrimon, 1993).

The best but most labor-intensive way to eliminate position effects is in vivo gene targeting through homologous recombination. Gene targeting in *Drosophila* can be performed using two strategies: 'ends-in' or insertional gene targeting (Rong and Golic, 2000) and 'ends-out' or replacement gene targeting (Gong and Golic, 2003) (Fig. 6). Insertional gene targeting results in the insertion of the entire targeting sequence into the region of homology. This results in a duplication that can be resolved during a second round of homologous recombination (Fig. 6A) (Rong et al., 2002). Replacement gene targeting results in the substitution of an endogenous DNA sequence with exogenous DNA through a double-reciprocal recombination event between two stretches of homologous sequence (Fig. 6B). Both strategies require the introduction of a 'donor' element, which contains the gene-targeting cassette, through transgenesis prior to in vivo homologous recombination, and require extensive screening. Although the techniques have not been used to compare the phenotypic outcome of different transgenes at the same locus, they are gaining in popularity for creating targeted mutations (O'Keefe et al., 2007).

Recent efforts have focused on making gene targeting more efficient in *Drosophila* through the use of site-specific zinc-finger-nuclease-stimulated gene targeting (Bibikova et al., 2003; Beumer et al., 2006). Zinc-finger nucleases are protein fusions between the Fok1 nuclease and (generally) three zinc-finger DNA-binding domains that introduce sequence specificity. Because each zinc finger recognizes 3 bp, zinc-finger nucleases can be designed to bind to a unique segment of 9 bp. As these nucleases need to dimerize at the target site before they can cut the target DNA, a recognition site of 18 bp is effectively required, a sequence that is

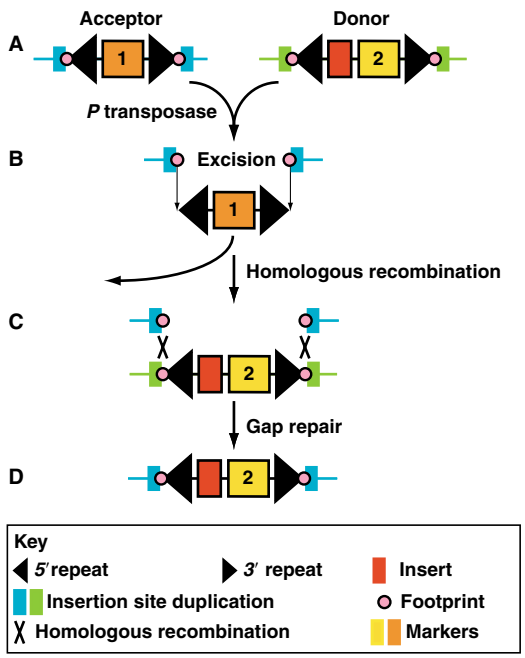


Fig. 5. *P* element replacement. (**A**) Two *P* elements, an acceptor element, containing marker 1 (orange), and a donor element, containing transgenic insert (red) and marker 2 (yellow), are brought together. (**B**) In the presence of *P* transposase, the acceptor element might excise. (**C**) This excision might promote double-stranded gap repair through homologous recombination between the 10-20 bp footprints (pink) of the 31 bp inverted terminal repeats at the acceptor site (blue) and the similar sequence at the donor site (green). (**D**) This results in the integration of the donor element into the acceptor locus.

likely to be unique in the fly genome. Thus, cutting by zinc-finger nucleases can be directed to specific target sites to create a double-stranded break, resulting in increased gene-targeting efficiency when a linearized donor targeting element is introduced.

Site-specific transgenesis for *Drosophila*

Although all the strategies of site-specific integration described above are elegant and useful, they have not been used extensively. The main drawbacks are that they allow only a limited number of transgenes to be compared and are too labor-intensive, as they require transgenesis of a donor construct prior to extensive genetic screening to obtain the required site-specific transgenic insertion event. Hence, the primary goal of true-targeted transgenesis is to achieve efficient site-specific integration upon injection of the DNA without the need for further manipulations.

This strategy was pioneered in the fly field using the bacteriophage ΦC31 integrase, which can integrate transgenic constructs at defined docking sites (Groth et al., 2004). Moreover, ΦC31 integrase-mediated transgenesis allows large DNA fragments to be integrated into the fly genome, well beyond the fragment sizes that can be introduced by *P* element-mediated integration (Venken et al., 2006). As discussed in more detail below, this approach has also introduced a user-friendly DNA modification platform, called recombineering, into *Drosophila* research.

ΦC31 integrase catalyzes the recombination between the phage attachment (*attP*) site present in its own bacteriophage genome and a bacterial attachment (*attB*) site present within the bacterial host genome (Thorpe and Smith, 1998) (see Box 4). Previous work has shown that the ΦC31 integrase can catalyze the site-specific integration of *attB*-containing plasmids into so-called *attP*-containing 'docking' or 'landing' sites that have been introduced into mammalian cell lines (Groth et al., 2000; Thyagarajan et al., 2001). Interestingly, *attB*-containing plasmids integrate more readily into *attP*-containing genomic docking sites than do *attP* sites in the reciprocal reaction, indicating that the integration reaction is asymmetric in nature (Thyagarajan et al., 2001; Belteki et al., 2003). This phenomenon was recently confirmed in *Drosophila* (Nimmo et al., 2006).

In *Drosophila*, recombination is mediated via ΦC31 integrase, provided through an mRNA source, between an *attP* docking site, previously integrated with a transposon into the fly genome, and an *attB* site present in an injected plasmid (Groth et al., 2004) (Fig. 7A). Three so-called pseudo-*attP* docking sites have been identified within the *Drosophila* genome. As one of these pseudo-sites is located in the endogenous transposable element *copia*, the true number of available pseudo-sites is likely to be high (Kaminker et al., 2002). Fortunately, these pseudo-sites were shown not to be receptive to *attB* plasmids, as all integration events were at the desired *attP* sites (Groth et al., 2004). However, rare non-specific integrations have been documented in *Drosophila* (Venken et al., 2006; Nimmo et al., 2006; Bischof et al., 2007). The ΦC31 integrase-mediated transformation technique has also recently been introduced successfully in the yellow fever mosquito *Aedes aegypti* (Nimmo et al., 2006).

After the original report describing two *attP P* element docking sites (Groth et al., 2004), numerous additional docking sites have been created. One set is embedded in a *piggyBac* backbone (Venken et al., 2006), whereas a second set is embedded in a *Mariner* backbone (Bischof et al., 2007). Venken et al. (Venken et al., 2006) observed that one out of seven docking sites tested was not receptive, suggesting that the genomic position of the docking site can affect integration efficiency. This was not observed for the 19 sites tested by Bischof et al. (Bischof et al., 2007). A detailed characterization and comparison of all the available docking sites will allow us to determine which ones are the most useful for specific purposes, such as cDNA overexpression, RNAi, genomic rescue or promoter/enhancer analysis.

Although the first reports used mRNA-encoded ΦC31 integrase to integrate the DNA (Groth et al., 2004; Bateman et al., 2006; Venken et al., 2006), Bischof et al. (Bischof et al., 2007) recently reported an efficient germ line ΦC31 integrase source that is driven by *nanos* or *vasa* regulatory elements. Interestingly, through ΦC31 integrase-mediated transgenesis, different ΦC31 integrase sources have been incorporated at the same docking sites. Additionally, the same ΦC31 integrase source was integrated into different docking sites, allowing the most efficient genomic ΦC31 integrase source to be selected. In the same study, a *Drosophila* codon-optimized ΦC31 integrase was described that performs better than the non-optimized version (Bischof et al., 2007).

Site-specific integration using a single recombination site results in the integration of the vector backbone, which may interfere with transgene expression (Chen et al., 2003). This can be minimized through marker genes strategically positioned between transgene and vector backbone (Venken et al., 2006). Alternatively, appropriately engineered recombinase sites in both the docking site and integration plasmid can be used to remove unwanted vector backbone sequence after correct integration events are isolated (Bischof et al., 2007). Finally, the integration of the backbone can be directly avoided through recombinase-mediated cassette exchange (RMCE) (Baer and Bode, 2001).

In RMCE, both docking site and transgene are flanked by a recombination site (see Box 4 and Fig. 7). Double reciprocal cross-over results in the integration of a transgene without its vector backbone. However, two sets of directly oriented *loxP* or *FRT* sites will favor deletion over RMCE. This problem can be overcome with sites, called spacer variants, that support recombination between themselves but not with others (see Box 4 for more information). The use of RMCE with spacer variants was initially utilized in the mouse in Cre- (Bethke and Sauer, 1997; Bouhassira et al., 1997) and FLP- (Seibler et al., 1998) based genetic engineering. This approach has been recently exploited in *Drosophila* for both recombinases (Oberstein et al., 2005; Horn and Handler, 2005) (Fig. 7B). For example, RMCE has been used elegantly to perform structure-function analysis of the *eve2* (*eve* – FlyBase) enhancer with a *lacZ* reporter (Oberstein et al., 2005). An alternative way to ensure that RMCE avoids the deletion or integration of plasmid backbone when employing FLP or Cre, is to use inverted recombination sites (as shown for ΦC31 integrase in Fig. 7C), which was pioneered in the mouse using Cre (Feng et al., 1999). This strategy eliminates the deletion problem but causes inversions.

Interestingly, RMCE events can be locked using the ΦC31 integrase system, as these recombination reactions are unidirectional. Pioneered in the yeast *Schizosaccharomyces pombe* (Thomason et al., 2001) and in mouse (Belteki et al., 2003), a modification of this system using inverted *att* recombination sites was recently described for *Drosophila* (Bateman et al., 2006) (Fig. 7C). This study demonstrated that unmarked constructs can be integrated through RMCE, as site-specific integration events are identified by loss of the marker (Bateman et al., 2006).

Recombineering: BAC transgenesis for *Drosophila*

Transposons are generally characterized by a low cargo capacity, limiting the amount of DNA that can be integrated and mobilized. Transgene size limitation can be overcome by the incorporation of a site-specific integration system, such as ΦC31 integrase, as shown in a chicken cell culture system (Dafhnis-Calas et al., 2005), or by gene targeting at the *Hprt1* locus in mouse embryonic stem (ES) cells (Heaney et al., 2004). Unfortunately, an intermediate cell culture system supporting both in vitro gene manipulation and subsequent germ line transmission, similar to mouse ES cells, is not available for *Drosophila*. Moreover, there

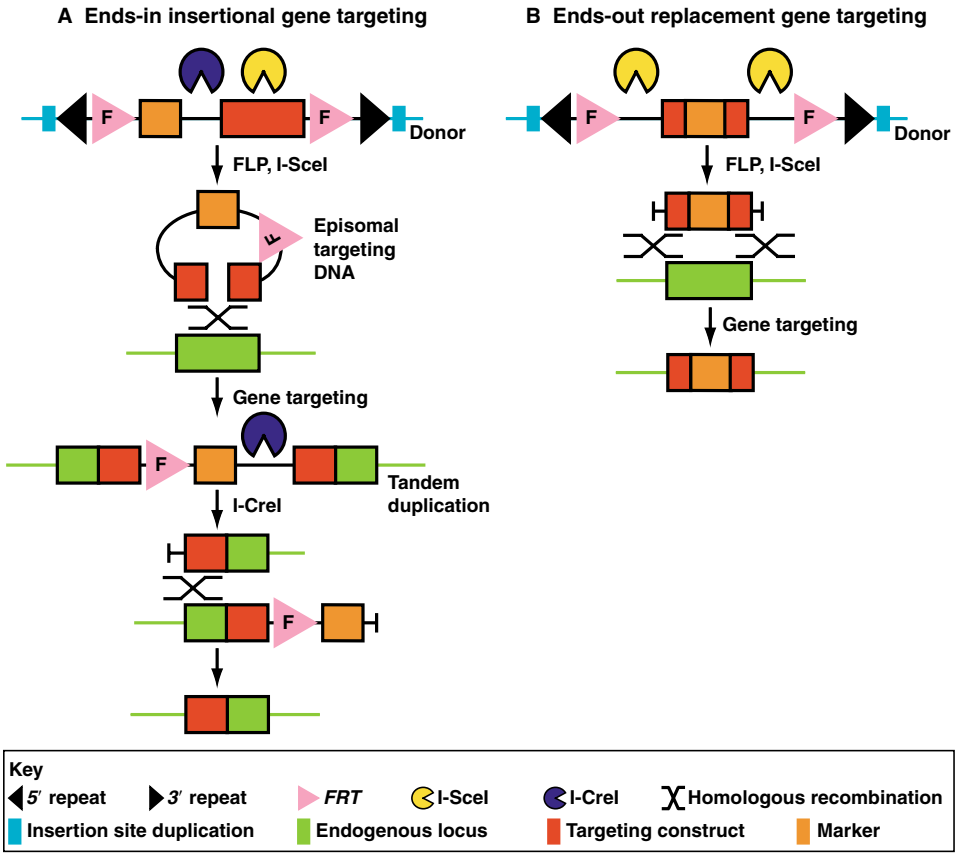


Fig. 6. Gene targeting in *Drosophila*. (**A**) Ends-in insertional gene targeting. The donor construct, within a *P* element, contains a region of homology (the targeting construct, red) interrupted by a restriction recognition site for the meganuclease I-SceI and flanked by *FRT* recognition sites for FLP recombinase. It also contains a marker (*white*$^+$) and an appropriately located restriction recognition site for the meganuclease I-CreI for a second round of homologous recombination. After *P* element transgenesis, a linearized episome is generated in vivo by FLP and I-SceI. Correct targeting results in *white*$^+$ expression and a tandem duplication of the locus. This duplication can be reduced to single copy using I-CreI, resulting in loss of *white*$^+$. (**B**) Ends-out replacement gene targeting. The donor construct, within a *P* element, contains a region of homology interrupted by a *white*$^+$ marker and is flanked by restriction recognition sites for the meganuclease I-SceI and *FRT* recognition sites for FLP recombinase. After *P* element transgenesis, identified by *white*$^+$, linearized targeting DNA is generated in vivo by FLP and I-SceI. Correct targeting results in a *white*$^+$ phenotype and replacement of part of the locus.

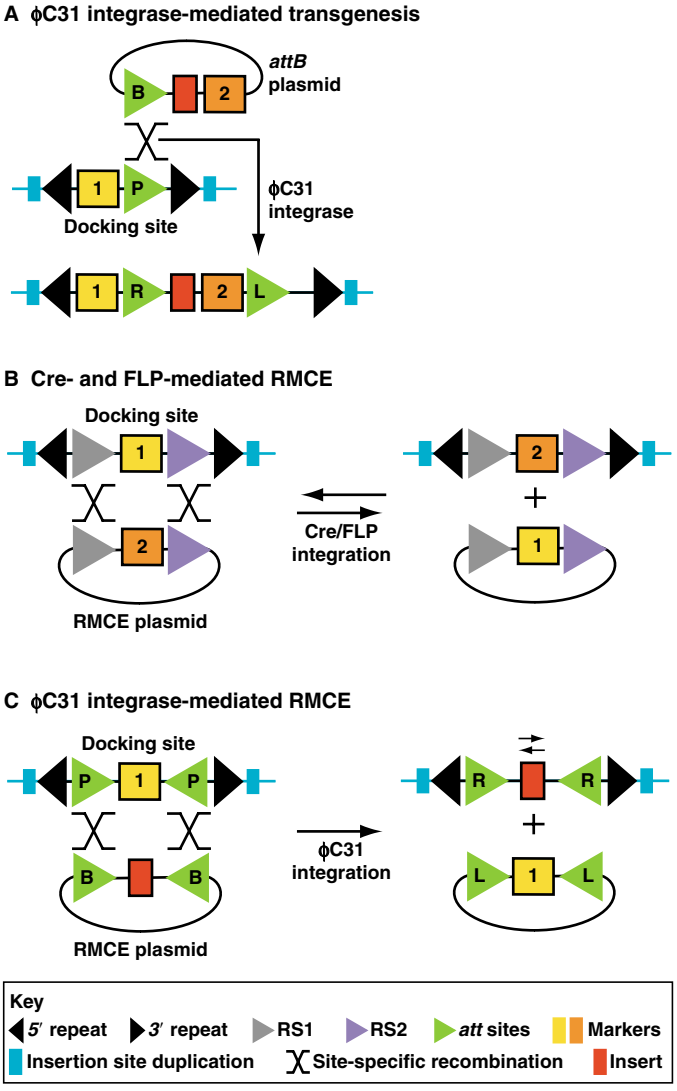


Fig. 7. Site-specific integration in *Drosophila*. (**A**) ΦC31 integrase-mediated transgenesis using single *attP* docking sites. Docking sites are transposons, such as *P* elements (Groth et al., 2004), *piggyBac* (Venken et al., 2006) or *Mariner* (Bischof et al., 2007), that contain a single *attP* recombination site and a marker 1, and that are integrated into the genome. A plasmid containing an insert, marker 2 and an *attB* recombination site, can then integrate into the docking site when ΦC31 integrase is provided. Correct recombination events between *attP* and *attB* are identified using marker 2. They result in two hybrid sites, *attL* and *attR*, that are no longer a substrate for ΦC31 integrase – the reaction is therefore irreversible. (**B**) Cre- and FLP-mediated RMCE. Docking site transposons (with *5′* and *3′* transposon termini), such as *P* (Oberstein et al., 2005) or *piggyBac* (Horn and Handler, 2005) elements, contain marker 1 flanked by heterotypic direct-oriented recombination sites (RS) 'RS1' (*loxP* or *FRT*, gray) and 'RS2' (such as *lox2272* or *F3*, purple). The RMCE plasmid, containing marker 2 flanked by a similar configuration of heterotypic recombination sites, can integrate when Cre or FLP is provided. Correct recombination events are identified by the absence of marker 1 and presence of marker 2. Recombination can be partial (single integration events are not shown) and is reversible. (**C**) ΦC31 integrase-mediated RMCE. A docking site *P* element transposon (*5′P* and *3′P* element termini) (Bateman et al., 2006) contains a marker 1 flanked by inverted *attP* recombination sites. The RMCE plasmid, containing insert flanked by inverted *attB* recombination sites, can integrate when ΦC31 integrase is provided. Correct recombination events, between both *attP* and *attB* sites, are identified through absence of marker 1 and result in hybrid sites, *attL* and *attR*, that are no longer substrates for ΦC31 integrase. The integrated DNA can be in either orientation (arrows).

is a strong negative correlation between the upper size limit of the insert and plasmid copy number: large DNA fragments are unstable when present in high-copy-number vectors in bacteria. Therefore, low-copy-number plasmids, such as P1 bacteriophage (Sternberg, 1990), bacterial artificial chromosomes (BACs) (Shizuya et al., 1992) and P1 artificial chromosomes (PACs) (Ioannou et al., 1994), were developed to maintain large inserts. Unfortunately, these plasmids interfere with both cloning and microinjection procedures, which require high DNA concentrations. This can be circumvented by the use of a specialized plasmid backbone – the conditionally amplifiable BAC – that has two origins of replication (Wild et al., 2002): an *oriS* for low-copy propagation and an *oriV* for high-copy induction. Importantly, the manipulation of large DNA fragments in these vectors has been facilitated through recent developments in in vivo recombination-mediated genetic engineering, also known as recombineering (Copeland et al., 2001; Heintz, 2001; Muyrers et al., 2001; Sawitzke et al., 2007).

Three recently developed technologies – recombineering, the ability to amplify plasmid copy number at will, and ΦC31 integrase-mediated transgenesis – have recently been integrated into a single transformation system (Venken et al., 2006). This system provides an efficient recombineering platform for *Drosophila*, permitting the integration of large DNA fragments into the fly genome. Selected fragments that encode the gene of interest are obtained in the amplifiable BAC backbone through recombineering-mediated gap repair, which can be performed at low copy number (Fig. 8A). Gap repair in high-copy and medium-copy plasmids has an upper size limit of ~30 and 80 kb, respectively (Lee et al., 2001). However, by maintaining the plasmid at low copy number, large fragments of up to 102 kb can be efficiently gap-repaired (Venken et al., 2006), as observed by others (Kotzamanis and Huxley, 2004). Interestingly, one 133 kb fragment that encodes one of the largest genes in the fly genome, *Tenascin major*, was reconstituted from two different BACs, each containing part of the gene (Venken et al., 2006). Gap-repaired DNA was induced to high copy number, isolated, and integrated into the fly genome using both *P* transposase and ΦC31 integrase: *P* transposase was used to integrate gap-repaired fragments of up to 39 kb, whereas ΦC31 integrase was used to integrate gap-repaired fragments of up to 133 kb.

A similar gap-repair approach was recently used to generate transgenes for *Drosophila* in vivo (Takeuchi et al., 2007). The gap-repaired constructs were obtained in flies through homologous recombination into the endogenous locus after the in vivo linearization of the transgene between both homology arms using the meganuclease I-SceI (Fig. 8B). The technique relies on endogenous fly enzymes, rather than on bacterial enzymes, to mediate the gap repair. The authors observed an upper size limit of 28 kb for correct gap repair.

An important reason for the development of recombineering is the ease with which DNA can be modified. Indeed, restriction enzymes and DNA ligase are not user-friendly when handling large DNA

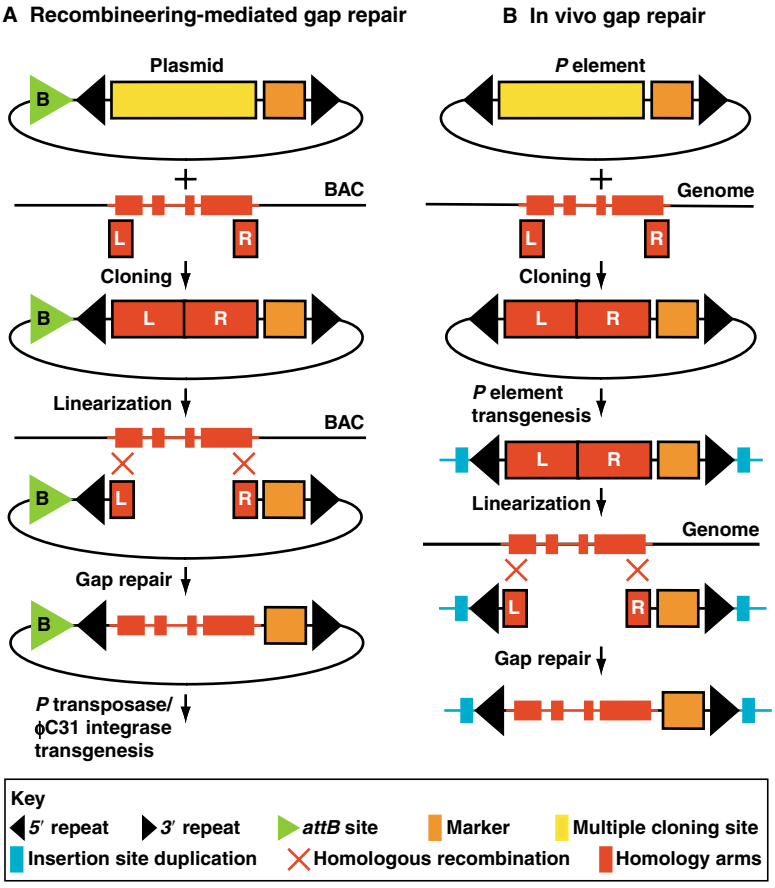


Fig. 8. Gap repair. (**A**) Recombineering-mediated gap repair. Two homology arms, located at the 5′ (Left, L) and 3′ (Right, R) end of a genomic region of interest present in a BAC, are cloned into the desired plasmid. Restriction enzyme-mediated linearization between both homology arms and subsequent transformation in bacteria competent for recombineering functions allow the selective retrieval of the desired fragment from the BAC into the plasmid through gap repair. The resulting plasmid can be used for *P* transposase- (*5′P* and *3′P* element termini) or ΦC31 integrase-mediated transgenesis (*attB* site). (**B**) In vivo gap repair. Two homology arms, located at the 5′ and 3′ ends of a genomic region of interest, are cloned within a *P* element. After *P* transposase-mediated germ line transmission, the transgene is linearized in vivo between both homology arms using the meganuclease I-SceI, potentially resulting in the selective capture of the desired fragment from a wild-type chromosome through homologous recombination-mediated gap repair.

fragments, as the occurrence of unique cutting sites decreases with increasing plasmid size. Recombineering does not suffer from those limitations and allows BACs to be modified more rapidly using PCR products or oligonucleotides that contain the desired mutation as recombination templates (Copeland et al., 2001; Court et al., 2002) (Fig. 9A). This strategy can easily be combined with positive/negative selectable markers, such as *galK* and *thyA* (Warming et al., 2005; Wong et al., 2005). Positive/negative selectable markers are targeted to the desired site for mutagenesis during a first round of recombineering using selection, and are then replaced by the desired mutation or tag during a second round of recombineering using counterselection (Fig. 9B). An alternative way to modify DNA constructs uses the recombinogenic protein RecA, also known as RecA-assisted modification (Yang et al., 1997; Gong et al., 2002), a methodology that is somewhat different from traditional recombineering. In a first recombination step, a modifying plasmid is integrated into the target plasmid, resulting in a co-integrate that becomes resolved during a second round of recombination (Fig. 9C). The technique allows the integration and deletion of fragments within a genomic fragment (Misulovin et al., 2001) and has been applied on a high-throughput level in the mouse field to create an atlas of gene expression in the mouse central nervous system (Gong et al., 2003). BAC modification was pioneered in the mouse field because most mouse genes tend to have multiple distant regulatory regions and are therefore too large to be handled using high-copy plasmid backbones (Heintz, 2001). The efficient recombineering-mediated tagging of genes in a genomic context has also been recently demonstrated in *Caenorhabditis elegans* and *C. briggsae* (Dolphin and Hope, 2006; Sarov et al., 2006).

Future applications

The advent of site-specific integration combined with recombineering and other methodologies will impact the fly field in numerous ways. These techniques make it possible to carry out structure-function studies at a higher resolution with fewer transgenes, as position effects can be mitigated using some of these approaches. Moreover, we are no longer confined to the study of single genes but can now tackle entire gene complexes that play key roles in development. Through repeated rounds of mutagenesis via recombineering, one can dissect the in vivo role of each gene and each regulatory region within a gene complex. Similar manipulations are now also possible for larger genes and for loci that have previously had no available mutations to study. These loci can now be identified and studied through the introduction of small deletions by FLP/*FRT* recombination (Parks et al., 2004; Ryder et al., 2004).

Combinations of the different methodologies described here should also greatly enhance our ability to manipulate the fly genome. For example, *P* replacement with an *attP*-containing *P* element could be used to convert many of the existing *P* elements into a useful docking site for ΦC31 integrase-mediated transgenesis or RMCE. Alternatively, gene targeting of recombination sites at desired locations might allow the site-specific integration of any DNA fragment. Finally, ΦC31 integrase-mediated transgenesis can be used to insert the donor constructs that are required for gene targeting. These are just a few examples of possible future fly manipulations.

In another vein, these technologies will also help to improve genome-wide studies of *Drosophila*. For example, one could try to identify optimal genomic sites for the integration of all RNAi

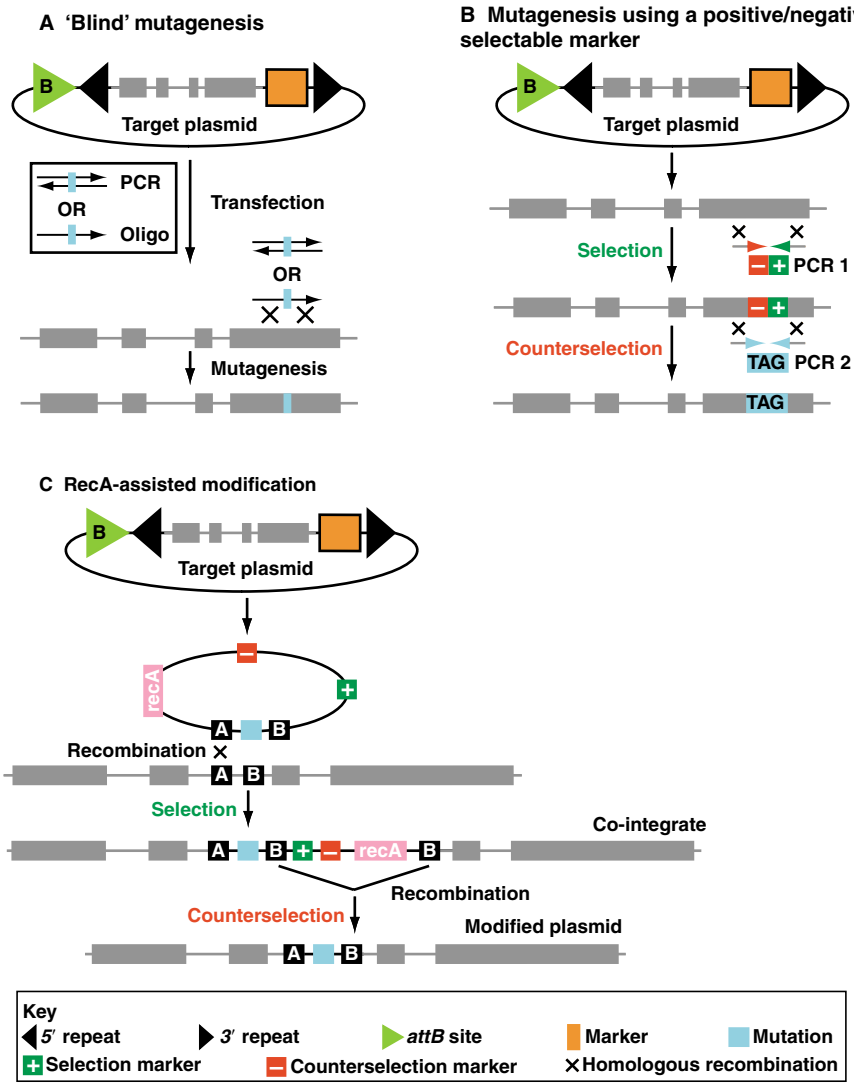


Fig. 9. Recombineering-mediated mutagenesis. (**A**) 'Blind' mutagenesis. To perform a site-specific change in a fragment within a target plasmid, a PCR fragment or oligonucleotide that contains the desired mutation is transformed into bacteria that contain recombineering functions and a target plasmid. Recombination results in the incorporation of the desired mutation: substitution, deletion or insertion. The bacteria are then screened by PCR for the proper mutagenic event. (**B**) Mutagenesis using a positive/negative selectable marker. First, in the positive-selection step, a PCR fragment containing a positive (+)/negative (–) selectable marker is transformed into bacteria that contain recombineering functions and the target plasmid. Individual colonies containing the correct recombinant plasmid are then selected. Second, during the counterselection step, a PCR fragment containing the desired change, such as a tag, might replace the positive/negative selectable marker. Counterselection or negative selection may result in the selection of a correct recombinant plasmid that can be identified through PCR. (**C**) RecA-assisted modification. A specialized plasmid that contains a selectable marker (+), a counterselectable marker (–), RecA and a mutation flanked by two homology boxes (A and B) is transformed into bacteria. During a first recombination event, identified through selection, this plasmid can integrate through homology box A (shown) or B (not shown), resulting in a co-integrate. During a second recombination event, identified through counterselection, this co-integrate can resolve to the original plasmid (not shown) or the modified plasmid (shown).

constructs. These sites should permit the optimal expression of RNA hairpin loops in all tissues at all developmental stages to allow the efficient RNAi-mediated knockdown of any gene. This approach may alleviate some of the potential drawbacks that are associated with *P* transposase-mediated transgenesis of RNAi constructs, such as poor transgene expression or misregulation (Dietzl et al., 2007). Furthermore, many different genomic DNA fragments containing cis-regulatory elements that drive GAL4 expression could be integrated at the same docking site to allow the labeling and manipulation of specific cell populations. Finally, the integration of overlapping duplications of defined areas of the X chromosome into the same docking site would be a useful way to map essential genes on the X chromosome.

Each transgenesis technique might result in unwanted side effects. Transposases might cause multiple insertion and excision events before the final transgenic insertion is stabilized. Recombinases and integrases may recognize pseudo-sites localized within the genome, as previously identified for Cre in the mammalian genome (Thyagarajan et al., 2000). Moreover, at high doses, Cre has been demonstrated to result in undesired effects in both vertebrates (Schmidt et al., 2000; Loonstra et al., 2001) and *Drosophila* (Heidmann and Lehner, 2001). Detrimental side effects have been observed for ΦC31 integrase in mammalian cell culture (Ehrhardt et al., 2006; Liu et al., 2006), although ectopic expression in vivo in mice and *Drosophila* indicates that it has minimal side effects in these organisms (Belteki et al., 2003; Raymond and Soriano, 2007; Bischof et al., 2007). Even gene targeting in *Drosophila* has not been spared from artifacts, and second-site mutations have been shown to cause interference with phenotypic characterization (O'Keefe et al., 2007). Interestingly, so far no such observations have been documented for FLP, which is widely used within the fly community (Blair, 2003).

Unfortunately, the solution to an important problem such as efficient site-specific integration, immediately results in the creation of new challenges: the handling of thousands of fly strains associated with typical high-throughput projects, as well as the maintenance of thousands of new stocks. Recent methods for the automated microinjection of fly embryos for high-throughput in vivo RNAi experiments have been developed (Zappe et al., 2006), and it should now be possible to adapt this technology for DNA microinjections. However, no solution has yet been developed to maintain numerous additional fly stocks, except through the expansion of existing or new stock centers.

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References

Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A., Galle, R. F. et al. (2000). The genome sequence of Drosophila melanogaster. *Science* **287**, 2185-2195.

Albert, H., Dale, E. C., Lee, E. and Ow, D. W. (1995). Site-specific integration of DNA into wild-type and mutant lox sites placed in the plant genome. *Plant J.* **7**, 649-659.

Baer, A. and Bode, J. (2001). Coping with kinetic and thermodynamic barriers: RMCE, an efficient strategy for the targeted integration of transgenes. *Curr. Opin. Biotechnol.* **12**, 473-480.

Barolo, S., Carver, L. A. and Posakony, J. W. (2000). GFP and beta-galactosidase transformation vectors for promoter/enhancer analysis in Drosophila. *Biotechniques* **29**, 726, 728, 730, 732.

Barolo, S., Castro, B. and Posakony, J. W. (2004). New Drosophila transgenic reporters: insulated P-element vectors expressing fast-maturing RFP. *Biotechniques* **36**, 436-440, 442.

Basler, K. and Hafen, E. (1989). Ubiquitous expression of sevenless: position-dependent specification of cell fate. *Science* **243**, 931-934.

Bateman, J. R., Lee, A. M. and Wu, C. T. (2006). Site-specific transformation of Drosophila via ϕC31 integrase-mediated cassette exchange. *Genetics* **173**, 769-777.

Beall, E. L. and Rio, D. C. (1997). Drosophila P-element transposase is a novel site-specific endonuclease. *Genes Dev.* **11**, 2137-2151.

Beall, E. L., Mahoney, M. B. and Rio, D. C. (2002). Identification and analysis of a hyperactive mutant form of Drosophila P-element transposase. *Genetics* **162**, 217-227.

Bellen, H. J. (1999). Ten years of enhancer detection: lessons from the fly. *Plant Cell* **11**, 2271-2281.

Bellen, H. J., O'Kane, C. J., Wilson, C., Grossniklaus, U., Pearson, R. K. and Gehring, W. J. (1989). P-element-mediated enhancer detection: a versatile method to study development in Drosophila. *Genes Dev.* **3**, 1288-1300.

Bellen, H. J., Levis, R. W., Liao, G., He, Y., Carlson, J. W., Tsang, G., Evans-Holm, M., Hiesinger, P. R., Schulze, K. L., Rubin, G. M. et al. (2004). The BDGP gene disruption project: single transposon insertions associated with 40% of Drosophila genes. *Genetics* **167**, 761-781.

Belteki, G., Gertsenstein, M., Ow, D. W. and Nagy, A. (2003). Site-specific cassette exchange and germline transmission with mouse ES cells expressing ϕC31 integrase. *Nat. Biotechnol.* **21**, 321-324.

Benedict, M. Q., Salazar, C. E. and Collins, F. H. (1995). A new dominant selectable marker for genetic transformation; Hsp70-opd. *Insect Biochem. Mol. Biol.* **25**, 1061-1065.

Berghammer, A. J., Klingler, M. and Wimmer, E. A. (1999). A universal marker for transgenic insects. *Nature* **402**, 370-371.

Bethke, B. and Sauer, B. (1997). Segmental genomic replacement by Cre-mediated recombination: genotoxic stress activation of the p53 promoter in single-copy transformants. *Nucleic Acids Res.* **25**, 2828-2834.

Beumer, K., Bhattacharyya, G., Bibikova, M., Trautman, J. K. and Carroll, D. (2006). Efficient gene targeting in Drosophila with zinc-finger nucleases. *Genetics* **172**, 2391-2403.

Bibikova, M., Beumer, K., Trautman, J. K. and Carroll, D. (2003). Enhancing gene targeting with designed zinc finger nucleases. *Science* **300**, 764.

Bier, E. (2005). Drosophila, the golden bug, emerges as a tool for human genetics. *Nat. Rev. Genet.* **6**, 9-23.

Bier, E., Vaessin, H., Shepherd, S., Lee, K., McCall, K., Barbel, S., Ackerman, L., Carretto, R., Uemura, T. and Grell, E. (1989). Searching for pattern and mutation in the Drosophila genome with a P-lacZ vector. *Genes Dev.* **3**, 1273-1287.

Bischof, J., Maeda, R. K., Hediger, M., Karch, F. and Basler, K. (2007). An optimized transgenesis system for Drosophila using germ-line-specific ϕC31 integrases. *Proc. Natl. Acad. Sci. USA* **104**, 3312-3317.

Blackman, R. K., Koehler, M. M., Grimaila, R. and Gelbart, W. M. (1989). Identification of a fully-functional hobo transposable element and its use for germ-line transformation of Drosophila. *EMBO J.* **8**, 211-217.

Blair, S. S. (2003). Genetic mosaic techniques for studying Drosophila development. *Development* **130**, 5065-5072.

Bouhassira, E. E., Westerman, K. and Leboulch, P. (1997). Transcriptional behavior of LCR enhancer elements integrated at the same chromosomal locus by recombinase-mediated cassette exchange. *Blood* **90**, 3332-3344.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Cary, L. C., Goebel, M., Corsaro, B. G., Wang, H. G., Rosen, E. and Fraser, M. J. (1989). Transposon mutagenesis of baculoviruses: analysis of Trichoplusia ni transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viruses. *Virology* **172**, 156-169.

Castro, J. P. and Carareto, C. M. (2004). Drosophila melanogaster P transposable elements: mechanisms of transposition and regulation. *Genetica* **121**, 107-118.

Chen, Z. Y., He, C. Y., Ehrhardt, A. and Kay, M. A. (2003). Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression in vivo. *Mol. Ther.* **8**, 495-500.

Cooley, L., Kelley, R. and Spradling, A. (1988). Insertional mutagenesis of the Drosophila genome with single P elements. *Science* **239**, 1121-1128.

Copeland, N. G., Jenkins, N. A. and Court, D. L. (2001). Recombineering: a powerful new tool for mouse functional genomics. *Nat. Rev. Genet.* **2**, 769-779.

Court, D. L., Sawitzke, J. A. and Thomason, L. C. (2002). Genetic engineering using homologous recombination. *Annu. Rev. Genet.* **36**, 361-388.

Dafhnis-Calas, F., Xu, Z., Haines, S., Malla, S. K., Smith, M. C. and Brown, W. R. (2005). Iterative in vivo assembly of large and complex transgenes by combining the activities of ϕC31 integrase and Cre recombinase. *Nucleic Acids Res.* **33**, e189.

de Navas, L., Foronda, D., Suzanne, M. and Sanchez-Herrero, E. (2006). A simple and efficient method to identify replacements of P-lacZ by P-Gal4 lines allows obtaining Gal4 insertions in the bithorax complex of Drosophila. *Mech. Dev.* **123**, 860-867.

Dietzl, G., Chen, D., Schnorrer, F., Su, K. C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S. et al. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. *Nature* **448**, 151-156.

Dolphin, C. T. and Hope, I. A. (2006). Caenorhabditis elegans reporter fusion genes generated by seamless modification of large genomic DNA clones. *Nucleic Acids Res.* **34**, e72.

Egli, D., Yepiskoposyan, H., Selvaraj, A., Balamurugan, K., Rajaram, R., Simons, A., Multhaup, G., Mettler, S., Vardanyan, A., Georgiev, O. 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.

Ehrhardt, A., Engler, J. A., Xu, H., Cherry, A. M. and Kay, M. A. (2006). Molecular analysis of chromosomal rearrangements in mammalian cells after ϕC31-mediated integration. *Hum. Gene Ther.* **17**, 1077-1094.

Feng, Y. Q., Seibler, J., Alami, R., Eisen, A., Westerman, K. A., Leboulch, P., Fiering, S. and Bouhassira, E. E. (1999). Site-specific chromosomal integration in mammalian cells: highly efficient CRE recombinase-mediated cassette exchange. *J. Mol. Biol.* **292**, 779-785.

Fischer, J. A., Giniger, E., Maniatis, T. and Ptashne, M. (1988). GAL4 activates transcription in Drosophila. *Nature* **332**, 853-856.

Franz, G. and Savakis, C. (1991). Minos, a new transposable element from Drosophila hydei, is a member of the Tc1-like family of transposons. *Nucleic Acids Res.* **19**, 6646.

Fridell, Y. W. and Searles, L. L. (1991). Vermilion as a small selectable marker gene for Drosophila transformation. *Nucleic Acids Res.* **19**, 5082.

Gloor, G. B., Nassif, N. A., Johnson-Schlitz, D. M., Preston, C. R. and Engels, W. R. (1991). Targeted gene replacement in Drosophila via P element-induced gap repair. *Science* **253**, 1110-1117.

Goldberg, D. A., Posakony, J. W. and Maniatis, T. (1983). Correct developmental expression of a cloned alcohol dehydrogenase gene transduced into the Drosophila germ line. *Cell* **34**, 59-73.

Golic, K. G. and Golic, M. M. (1996). Engineering the Drosophila genome: chromosome rearrangements by design. *Genetics* **144**, 1693-1711.

Golic, M. M., Rong, Y. S., Petersen, R. B., Lindquist, S. L. and Golic, K. G. (1997). FLP-mediated DNA mobilization to specific target sites in Drosophila chromosomes. *Nucleic Acids Res.* **25**, 3665-3671.

Gong, S., Yang, X. W., Li, C. and Heintz, N. (2002). Highly efficient modification of bacterial artificial chromosomes (BACs) using novel shuttle vectors containing the R6Kgamma origin of replication. *Genome Res.* **12**, 1992-1998.

Gong, S., Zheng, C., Doughty, M. L., Losos, K., Didkovsky, N., Schambra, U. B., Nowak, N. J., Joyner, A., Leblanc, G., Hatten, M. E. et al. (2003). A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. *Nature* **425**, 917-925.

Gong, W. J. and Golic, K. G. (2003). Ends-out, or replacement, gene targeting in Drosophila. *Proc. Natl. Acad. Sci. USA* **100**, 2556-2561.

Greenspan, R. J. (2004). *Fly Pushing: The Theory and Practice of Drosophila Genetics* (2nd edn). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Groth, A. C., Olivares, E. C., Thyagarajan, B. and Calos, M. P. (2000). A phage integrase directs efficient site-specific integration in human cells. *Proc. Natl. Acad. Sci. USA* **97**, 5995-6000.

Groth, A. C., Fish, M., Nusse, R. and Calos, M. P. (2004). Construction of transgenic Drosophila by using the site-specific integrase from phage ϕC31. *Genetics* **166**, 1775-1782.

Hacker, U., Nystedt, S., Barmchi, M. P., Horn, C. and Wimmer, E. A. (2003). piggyBac-based insertional mutagenesis in the presence of stably integrated P elements in Drosophila. *Proc. Natl. Acad. Sci. USA* **100**, 7720-7725.

Haenlin, M., Steller, H., Pirrotta, V. and Mohier, E. (1985). A 43 kilobase cosmid P transposon rescues the fs(1)K10 morphogenetic locus and three adjacent Drosophila developmental mutants. *Cell* **40**, 827-837.

Handler, A. M. (2002). Use of the piggyBac transposon for germ-line transformation of insects. *Insect Biochem. Mol. Biol.* **32**, 1211-1220.

Handler, A. M. and Harrell, R. A. (1999). Germline transformation of Drosophila melanogaster with the piggyBac transposon vector. *Insect Mol. Biol.* **8**, 449-457.

Handler, A. M. and James, A. A. (2000). *Insect Transgenesis: Methods and Applications*. Boca Raton, FL: CRC Press.
Handler, A. M., Gomez, S. P. and O'Brochta, D. A. (1993). A functional analysis of the P-element gene-transfer vector in insects. *Arch. Insect Biochem. Physiol.* **22**, 373-384.
Hazelrigg, T., Levis, R. and Rubin, G. M. (1984). Transformation of white locus DNA in drosophila: dosage compensation, zeste interaction, and position effects. *Cell* **36**, 469-481.
Heaney, J. D., Rettew, A. N. and Bronson, S. K. (2004). Tissue-specific expression of a BAC transgene targeted to the Hprt locus in mouse embryonic stem cells. *Genomics* **83**, 1072-1082.
Heidmann, D. and Lehner, C. F. (2001). Reduction of Cre recombinase toxicity in proliferating Drosophila cells by estrogen-dependent activity regulation. *Dev. Genes Evol.* **211**, 458-465.
Heintz, N. (2001). BAC to the future: the use of bac transgenic mice for neuroscience research. *Nat. Rev. Neurosci.* **2**, 861-870.
Hoess, R. H., Ziese, M. and Sternberg, N. (1982). P1 site-specific recombination: nucleotide sequence of the recombining sites. *Proc. Natl. Acad. Sci. USA* **79**, 3398-3402.
Hoess, R. H., Wierzbicki, A. and Abremski, K. (1986). The role of the loxP spacer region in P1 site-specific recombination. *Nucleic Acids Res.* **14**, 2287-2300.
Horn, C. and Wimmer, E. A. (2000). A versatile vector set for animal transgenesis. *Dev. Genes Evol.* **210**, 630-637.
Horn, C. and Handler, A. M. (2005). Site-specific genomic targeting in Drosophila. *Proc. Natl. Acad. Sci. USA* **102**, 12483-12488.
Horn, C., Jaunich, B. and Wimmer, E. A. (2000). Highly sensitive, fluorescent transformation marker for Drosophila transgenesis. *Dev. Genes Evol.* **210**, 623-629.
Horn, C., Schmid, B. G., Pogoda, F. S. and Wimmer, E. A. (2002). Fluorescent transformation markers for insect transgenesis. *Insect Biochem. Mol. Biol.* **32**, 1221-1235.
Horn, C., Offen, N., Nystedt, S., Hacker, U. and Wimmer, E. A. (2003). piggyBac-based insertional mutagenesis and enhancer detection as a tool for functional insect genomics. *Genetics* **163**, 647-661.
Ioannou, P. A., Amemiya, C. T., Garnes, J., Kroisel, P. M., Shizuya, H., Chen, C., Batzer, M. A. and de Jong, P. J. (1994). A new bacteriophage P1-derived vector for the propagation of large human DNA fragments. *Nat. Genet.* **6**, 84-89.
Jacobson, J. W., Medhora, M. M. and Hartl, D. L. (1986). Molecular structure of a somatically unstable transposable element in Drosophila. *Proc. Natl. Acad. Sci. USA* **83**, 8684-8688.
Kaminker, J. S., Bergman, C. M., Kronmiller, B., Carlson, J., Svirskas, R., Patel, S., Frise, E., Wheeler, D. A., Lewis, S. E., Rubin, G. M. et al. (2002). The transposable elements of the Drosophila melanogaster euchromatin: a genomics perspective. *Genome Biol.* **3**, RESEARCH0084.
Karess, R. E. and Rubin, G. M. (1984). Analysis of P transposable element functions in Drosophila. *Cell* **38**, 135-146.
Kaufman, P. D. and Rio, D. C. (1991). Germline transformation of Drosophila melanogaster by purified P element transposase. *Nucleic Acids Res.* **19**, 6336.
Klemenz, R., Weber, U. and Gehring, W. J. (1987). The white gene as a marker in a new P-element vector for gene transfer in Drosophila. *Nucleic Acids Res.* **15**, 3947-3959.
Kotzamanis, G. and Huxley, C. (2004). Recombining overlapping BACs into a single larger BAC. *BMC Biotechnol.* **4**, 1.
Lai, S. L. and Lee, T. (2006). Genetic mosaic with dual binary transcriptional systems in Drosophila. *Nat. Neurosci.* **9**, 703-709.
Langer, S. J., Ghafoori, A. P., Byrd, M. and Leinwand, L. (2002). A genetic screen identifies novel non-compatible loxP sites. *Nucleic Acids Res.* **30**, 3067-3077.
Lankenau, D. H. and Gloor, G. B. (1998). In vivo gap repair in Drosophila: a one-way street with many destinations. *BioEssays* **20**, 317-327.
Le, T., Yu, M., Williams, B., Goel, S., Paul, S. M. and Beitel, G. J. (2007). CaSpeR5, a family of Drosophila transgenesis and shuttle vectors with improved multiple cloning sites. *Biotechniques* **42**, 164, 166.
Lee, E. C., Yu, D., Martinez de Velasco, J., Tessarollo, L., Swing, D. A., Court, D. L., Jenkins, N. A. and Copeland, N. G. (2001). A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics* **73**, 56-65.
Lee, G. and Saito, I. (1998). Role of nucleotide sequences of loxP spacer region in Cre-mediated recombination. *Gene* **216**, 55-65.
Lee, T. and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22**, 451-461.
Levis, R., Hazelrigg, T. and Rubin, G. M. (1985). Effects of genomic position on the expression of transduced copies of the white gene of Drosophila. *Science* **229**, 558-561.
Lidholm, D. A., Lohe, A. R. and Hartl, D. L. (1993). The transposable element mariner mediates germline transformation in Drosophila melanogaster. *Genetics* **134**, 859-868.
Liu, J., Jeppesen, I., Nielsen, K. and Jensen, T. G. (2006). Phi c31 integrase induces chromosomal aberrations in primary human fibroblasts. *Gene Ther.* **13**, 1188-1190.
Lockett, T. J., Lewy, D., Holmes, P., Medveczky, K. and Saint, R. (1992). The rough (ro+) gene as a dominant P-element marker in germ line transformation of Drosophila melanogaster. *Gene* **114**, 187-193.
Loonstra, A., Vooijs, M., Beverloo, H. B., Allak, B. A., van Drunen, E., Kanaar, R., Berns, A. and Jonkers, J. (2001). Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells. *Proc. Natl. Acad. Sci. USA* **98**, 9209-9214.
Loukeris, T. G., Arca, B., Livadaras, I., Dialektaki, G. and Savakis, C. (1995). Introduction of the transposable element Minos into the germ line of Drosophila melanogaster. *Proc. Natl. Acad. Sci. USA* **92**, 9485-9489.
Lozovsky, E. R., Nurminsky, D., Wimmer, E. A. and Hartl, D. L. (2002). Unexpected stability of mariner transgenes in Drosophila. *Genetics* **160**, 527-535.
Matthews, K. A., Kaufman, T. C. and Gelbart, W. M. (2005). Research resources for Drosophila: the expanding universe. *Nat. Rev. Genet.* **6**, 179-193.
McGinnis, W., Shermoen, A. W. and Beckendorf, S. K. (1983). A transposable element inserted just 5′ to a Drosophila glue protein gene alters gene expression and chromatin structure. *Cell* **34**, 75-84.
McLeod, M., Craft, S. and Broach, J. R. (1986). Identification of the crossover site during FLP-mediated recombination in the Saccharomyces cerevisiae plasmid 2 microns circle. *Mol. Cell Biol.* **6**, 3357-3367.
Metaxakis, A., Oehler, S., Klinakis, A. and Savakis, C. (2005). Minos as a genetic and genomic tool in Drosophila melanogaster. *Genetics* **171**, 571-581.
Missirlis, P. I., Smailus, D. E. and Holt, R. A. (2006). A high-throughput screen identifying sequence and promiscuity characteristics of the loxP spacer region in Cre-mediated recombination. *BMC Genomics* **7**, 73.
Misulovin, Z., Yang, X. W., Yu, W., Heintz, N. and Meffre, E. (2001). A rapid method for targeted modification and screening of recombinant bacterial artificial chromosome. *J. Immunol. Methods* **257**, 99-105.
Muyrers, J. P., Zhang, Y. and Stewart, A. F. (2001). Techniques: recombinogenic engineering – new options for cloning and manipulating DNA. *Trends Biochem. Sci.* **26**, 325-331.
Nimmo, D. D., Alphey, L., Meredith, J. M. and Eggleston, P. (2006). High efficiency site-specific genetic engineering of the mosquito genome. *Insect Mol. Biol.* **15**, 129-136.
Norga, K. K., Gurganus, M. C., Dilda, C. L., Yamamoto, A., Lyman, R. F., Patel, P. H., Rubin, G. M., Hoskins, R. A., Mackay, T. F. and Bellen, H. J. (2003). Quantitative analysis of bristle number in Drosophila mutants identifies genes involved in neural development. *Curr. Biol.* **13**, 1388-1396.
Oberstein, A., Pare, A., Kaplan, L. and Small, S. (2005). Site-specific transgenesis by Cre-mediated recombination in Drosophila. *Nat. Methods* **2**, 583-585.
O'Brochta, D. A. and Atkinson, P. W. (1996). Transposable elements and gene transformation in non-drosophilid insects. *Insect Biochem. Mol. Biol.* **26**, 739-753.
O'Brochta, D. A., Warren, W. D., Saville, K. J. and Atkinson, P. W. (1996). Hermes, a functional non-Drosophilid insect gene vector from Musca domestica. *Genetics* **142**, 907-914.
O'Kane, C. J. and Gehring, W. J. (1987). Detection in situ of genomic regulatory elements in Drosophila. *Proc. Natl. Acad. Sci. USA* **84**, 9123-9127.
O'Keefe, L. V., Smibert, P., Colella, A., Chataway, T. K., Saint, R. and Richards, R. I. (2007). Know thy fly. *Trends Genet.* **23**, 238-242.
Parks, A. L., Cook, K. R., Belvin, M., Dompe, N. A., Fawcett, R., Huppert, K., Tan, L. R., Winter, C. G., Bogart, K. P., Deal, J. E. et al. (2004). Systematic generation of high-resolution deletion coverage of the Drosophila melanogaster genome. *Nat. Genet.* **36**, 288-292.
Patton, J. S., Gomes, X. V. and Geyer, P. K. (1992). Position-independent germline transformation in Drosophila using a cuticle pigmentation gene as a selectable marker. *Nucleic Acids Res.* **20**, 5859-5860.
Pirrotta, V. (1988). Vectors for P-mediated transformation in Drosophila. *Biotechnology* **10**, 437-456.
Raymond, C. S. and Soriano, P. (2007). High-efficiency FLP and ϕC31 site-specific recombination in mammalian cells. *PLoS ONE* **2**, e162.
Rio, D. C. and Rubin, G. M. (1988). Identification and purification of a Drosophila protein that binds to the terminal 31-base-pair inverted repeats of the P transposable element. *Proc. Natl. Acad. Sci. USA* **85**, 8929-8933.
Rong, Y. S. and Golic, K. G. (2000). Gene targeting by homologous recombination in Drosophila. *Science* **288**, 2013-2018.
Rong, Y. S., Titen, S. W., Xie, H. B., Golic, M. M., Bastiani, M., Bandyopadhyay, P., Olivera, B. M., Brodsky, M., Rubin, G. M. and Golic, K. G. (2002). Targeted mutagenesis by homologous recombination in D. melanogaster. *Genes Dev.* **16**, 1568-1581.
Roseman, R. R., Swan, J. M. and Geyer, P. K. (1995). A Drosophila insulator protein facilitates dosage compensation of the X chromosome min-white gene located at autosomal insertion sites. *Development* **121**, 3573-3582.
Rubin, G. M. and Spradling, A. C. (1982). Genetic transformation of Drosophila with transposable element vectors. *Science* **218**, 348-353.
Ryder, E. and Russell, S. (2003). Transposable elements as tools for genomics and genetics in Drosophila. *Brief. Funct. Genomic. Proteomic.* **2**, 57-71.

Ryder, E., Blows, F., Ashburner, M., Bautista-Llacer, R., Coulson, D., Drummond, J., Webster, J., Gubb, D., Gunton, N., Johnson, G. et al. (2004). The DrosDel collection: a set of P-element insertions for generating custom chromosomal aberrations in Drosophila melanogaster. *Genetics* **167**, 797-813.

Sarkar, A., Atapattu, A., Belikoff, E. J., Heinrich, J. C., Li, X., Horn, C., Wimmer, E. A. and Scott, M. J. (2006). Insulated piggyBac vectors for insect transgenesis. *BMC Biotechnol.* **6**, 27.

Sarov, M., Schneider, S., Pozniakovski, A., Roguev, A., Ernst, S., Zhang, Y., Hyman, A. A. and Stewart, A. F. (2006). A recombineering pipeline for functional genomics applied to Caenorhabditis elegans. *Nat. Methods* **3**, 839-844.

Sawitzke, J. A., Thomason, L. C., Costantino, N., Bubunenko, M., Datta, S. and Court, D. L. (2007). Recombineering: in vivo genetic engineering in E. coli, S. enterica, and beyond. *Meth. Enzymol.* **421**, 171-199.

Schlake, T. and Bode, J. (1994). Use of mutated FLP recognition target (FRT) sites for the exchange of expression cassettes at defined chromosomal loci. *Biochemistry* **33**, 12746-12751.

Schmidt, E. E., Taylor, D. S., Prigge, J. R., Barnett, S. and Capecchi, M. R. (2000). Illegitimate Cre-dependent chromosome rearrangements in transgenic mouse spermatids. *Proc. Natl. Acad. Sci. USA* **97**, 13702-13707.

Seibler, J. and Bode, J. (1997). Double-reciprocal crossover mediated by FLP-recombinase: a concept and an assay. *Biochemistry* **36**, 1740-1747.

Seibler, J., Schubeler, D., Fiering, S., Groudine, M. and Bode, J. (1998). DNA cassette exchange in ES cells mediated by Flp recombinase: an efficient strategy for repeated modification of tagged loci by marker-free constructs. *Biochemistry* **37**, 6229-6234.

Senecoff, J. F., Rossmeissl, P. J. and Cox, M. M. (1988). DNA recognition by the FLP recombinase of the yeast 2 mu plasmid. A mutational analysis of the FLP binding site. *J. Mol. Biol.* **201**, 405-421.

Sepp, K. J. and Auld, V. J. (1999). Conversion of lacZ enhancer trap lines to GAL4 lines using targeted transposition in Drosophila melanogaster. *Genetics* **151**, 1093-1101.

Shizuya, H., Birren, B., Kim, U. J., Mancino, V., Slepak, T., Tachiiri, Y. and Simon, M. (1992). Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in Escherichia coli using an F-factor-based vector. *Proc. Natl. Acad. Sci. USA* **89**, 8794-8797.

Siegal, M. L. and Hartl, D. L. (1996). Transgene Coplacement and high efficiency site-specific recombination with the Cre/loxP system in Drosophila. *Genetics* **144**, 715-726.

Siegal, M. L. and Hartl, D. L. (2000). Application of Cre/loxP in Drosophila. Site-specific recombination and transgene coplacement. *Methods Mol. Biol.* **136**, 487-495.

Smith, D., Wohlgemuth, J., Calvi, B. R., Franklin, I. and Gelbart, W. M. (1993). hobo enhancer trapping mutagenesis in Drosophila reveals an insertion specificity different from P elements. *Genetics* **135**, 1063-1076.

Sorrell, D. A. and Kolb, A. F. (2005). Targeted modification of mammalian genomes. *Biotechnol. Adv.* **23**, 431-469.

Spradling, A. C., Stern, D. M., Kiss, I., Roote, J., Laverty, T. and Rubin, G. M. (1995). Gene disruptions using P transposable elements: an integral component of the Drosophila genome project. *Proc. Natl. Acad. Sci. USA* **92**, 10824-10830.

Steller, H. and Pirrotta, V. (1985). A transposable P vector that confers selectable G418 resistance to Drosophila larvae. *EMBO J.* **4**, 167-171.

Sternberg, N. (1990). Bacteriophage P1 cloning system for the isolation, amplification, and recovery of DNA fragments as large as 100 kilobase pairs. *Proc. Natl. Acad. Sci. USA* **87**, 103-107.

Stilwell, G. E., Rocheleau, T. and Ffrench-Constant, R. H. (1995). GABA receptor minigene rescues insecticide resistance phenotypes in Drosophila. *J. Mol. Biol.* **253**, 223-227.

Sundararajan, P., Atkinson, P. W. and O'Brochta, D. A. (1999). Transposable element interactions in insects: crossmobilization of hobo and Hermes. *Insect Mol. Biol.* **8**, 359-368.

Takeuchi, H., Georgiev, O., Fetchko, M., Kappeler, M., Schaffner, W. and Egli, D. (2007). In vivo construction of transgenes in Drosophila. *Genetics* **175**, 2019-2028.

Thibault, S. T., Singer, M. A., Miyazaki, W. Y., Milash, B., Dompe, N. A., Singh, C. M., Buchholz, R., Demsky, M., Fawcett, R., Francis-Lang, H. L. et al. (2004). A complementary transposon tool kit for Drosophila melanogaster using P and piggyBac. *Nat. Genet.* **36**, 283-287.

Thomason, L. C., Calendar, R. and Ow, D. W. (2001). Gene insertion and replacement in Schizosaccharomyces pombe mediated by the Streptomyces bacteriophage phiC31 site-specific recombination system. *Mol. Genet. Genomics* **265**, 1031-1038.

Thorpe, H. M. and Smith, M. C. (1998). In vitro site-specific integration of bacteriophage DNA catalyzed by a recombinase of the resolvase/invertase family. *Proc. Natl. Acad. Sci. USA* **95**, 5505-5510.

Thummel, C. S. and Pirrotta, V. (1992). New pCaSpeR P element vectors. *Drosoph. Inf. Serv.* **71**, 150.

Thyagarajan, B., Guimaraes, M. J., Groth, A. C. and Calos, M. P. (2000). Mammalian genomes contain active recombinase recognition sites. *Gene* **244**, 47-54.

Thyagarajan, B., Olivares, E. C., Hollis, R. P., Ginsburg, D. S. and Calos, M. P. (2001). Site-specific genomic integration in mammalian cells mediated by phage ϕC31 integrase. *Mol. Cell. Biol.* **21**, 3926-3934.

Venken, K. J. and Bellen, H. J. (2005). Emerging technologies for gene manipulation in Drosophila melanogaster. *Nat. Rev. Genet.* **6**, 167-178.

Venken, K. J., He, Y., Hoskins, R. A. and Bellen, H. J. (2006). P[acman]: a BAC transgenic platform for targeted insertion of large DNA fragments in D. melanogaster. *Science* **314**, 1747-1751.

Vidal, M. and Cagan, R. L. (2006). Drosophila models for cancer research. *Curr. Opin. Genet. Dev.* **16**, 10-16.

Warming, S., Costantino, N., Court, D. L., Jenkins, N. A. and Copeland, N. G. (2005). Simple and highly efficient BAC recombineering using galK selection. *Nucleic Acids Res.* **33**, e36.

Warren, W. D., Atkinson, P. W. and O'Brochta, D. A. (1994). The Hermes transposable element from the house fly, Musca domestica, is a short inverted repeat-type element of the hobo, Ac, and Tam3 (hAT) element family. *Genet. Res.* **64**, 87-97.

Wild, J., Hradecna, Z. and Szybalski, W. (2002). Conditionally amplifiable BACs: switching from single-copy to high-copy vectors and genomic clones. *Genome Res.* **12**, 1434-1444.

Wong, Q. N., Ng, V. C., Lin, M. C., Kung, H. F., Chan, D. and Huang, J. D. (2005). Efficient and seamless DNA recombineering using a thymidylate synthase A selection system in Escherichia coli. *Nucleic Acids Res.* **33**, e59.

Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. *Development* **117**, 1223-1237.

Yang, X. W., Model, P. and Heintz, N. (1997). Homologous recombination based modification in Escherichia coli and germline transmission in transgenic mice of a bacterial artificial chromosome. *Nat. Biotechnol.* **15**, 859-865.

Zappe, S., Fish, M., Scott, M. P. and Solgaard, O. (2006). Automated MEMS-based Drosophila embryo injection system for high-throughput RNAi screens. *Lab Chip* **6**, 1012-1019.