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## A versatile *in vivo* system for directed dissection of gene expression patterns

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### Abstract

Tissue-specific gene expression using the *UAS/GAL4* binary system has facilitated genetic dissection of many biological processes in *Drosophila melanogaster*. Refining *GAL4* expression patterns or independently manipulating multiple cell populations using additional binary systems are common experimental goals. To simplify these processes, we have developed a convertible genetic platform, called the Integrase Swappable *In vivo* Targeting Element (InSITE) system. This approach allows *GAL4* to be replaced with any other sequence, placing different genetic effectors under the control of the same regulatory elements. Using InSITE, *GAL4* can be replaced with *LexA* or *QF*, allowing an expression pattern to be repurposed. *GAL4* can also be replaced with *GAL80* or split-*GAL4* hemi-drivers, allowing intersectional approaches to refine expression patterns. The exchanges occur through efficient, *in vivo* manipulations, making it possible to generate many swaps in parallel. Furthermore, this system is entirely modular, allowing future genetic tools to be easily incorporated into the existing framework.

### Introduction

Many *in vivo* manipulations rely on directing gene expression to a specific tissue, or to a particular developmental time. Two basic methods exist to do this. In one approach, transposable elements carrying genetic effectors with minimal promoters are inserted into the genome and expression is driven by local gene regulatory elements<sup>1–2</sup>. Alternatively, regulatory elements can be fused to genetic effectors *in vitro* and reinserted in the genome<sup>3–</sup>

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#### Author Contributions

D.M.G. and T.R.C. designed the experiments; D.M.G., M.A.S., X.J.G., F.J.L., C-C. L., C.J.P., and T.R.C. performed the experiments; S.B. provided critical reagents; D.M.G. and T.R.C. wrote the manuscript.

#### Competing Financial Interests

The authors declare no competing financial interests.

5. Such enhancer traps and enhancer fusions have been powerful tools in cell biology, development, physiology, and neuroscience<sup>6–9</sup>.

In *Drosophila melanogaster*, the *UAS/GAL4* system allows for expression of *UAS*-linked genes in cells expressing the transcription factor *GAL4*. Two additional binary systems, using the *LexA* and *QF* transcriptional activators, allow independent manipulation of multiple populations of cells<sup>10–11</sup>. However, while many enhancer trap and enhancer fusion lines exist, particularly for the *UAS/GAL4* system, the expression of such lines is seldom confined to a single tissue or cell type, limiting the resolution of these approaches<sup>4,12–13</sup>.

Several strategies exist for using the intersections of partially overlapping expression patterns to generate increased specificity. For example, *FLP*, *GAL4*, and *QF* have been used with *FRT*-flanked interruption or flip-out cassettes to target subsets of expression patterns<sup>11,13–15</sup>. The split-*GAL4* system allows *GAL4* activity to be reconstituted in cells that express both halves of the hemi-driver<sup>16</sup>. Finally, the *GAL80* repressor can be used to subtract the overlap between two expression patterns<sup>12,17</sup>. While these intersectional methods are useful for generating lines with more specific gene expression patterns, a drawback of these approaches is that with each elaboration or extension of the *GAL4* system, new combinations of these regulatory elements, or their reporters frequently need to be designed, and in the worst case, whole libraries need to be re-generated.

Recombinase-Mediated Cassette Exchange (RMCE) methods allow a sequence cassette to be replaced *in vivo*. Several strategies for RMCE, typically using a microinjected plasmid as the substrate for cassette exchange, have been developed<sup>18–24</sup>. Several of these approaches rely on *FLP*-mediated recombination using wild-type or mutant *FRT* sites to target an *FRT*-flanked cassette to a specific locus<sup>20–21,23</sup>. Another uses Cre recombinase and a pair of incompatible *loxP* sites<sup>22</sup>. The *Streptomyces* phage  $\Phi$ C31 integrase, which catalyzes irreversible site-specific recombination between two sites (*attP* and *attB*)<sup>25–26</sup>, has had a profound impact on animal transgenesis and has made possible several novel RMCE strategies<sup>18–19</sup>.

We have combined these recombination systems into a versatile platform to facilitate the segmentation of complex expression patterns, and to allow *GAL4* expression patterns to be repurposed. The Integrase Swappable *In vivo* Targeting Element (InSITE) system allows an enhancer trap or enhancer fusion to be rapidly converted from *GAL4* to any other sequence (Fig. 1a). The InSITE system uses an RMCE strategy in which the substrate for RMCE can be genetically-derived, allowing replacement of *GAL4* simply by crossing flies. We demonstrate that such swaps can be done either entirely genetically, using genomic donor lines, or with a microinjected donor plasmid. As this strategy is highly efficient, it is possible to perform high-throughput swapping of many enhancer trap lines to multiple different effector molecules in parallel. In addition, we describe an enhancer fusion vector that is compatible with this replacement strategy, allowing a single transgene to be diversified *in vivo*. Finally, because the InSITE system allows *GAL4* to be converted into any other sequence, this platform is forward compatible with currently unanticipated future technologies.

## Results

### A convertible enhancer trap platform

The InSITE enhancer trap contains a minimal (*P* transposase) promoter, the *GAL4* transcriptional activator, and an *attP* recognition sequence for  $\Phi$ C31 integrase (Fig. 1b)<sup>18–19,25–26</sup>. Swappable enhancer traps were constructed using two different transposons, the *Drosophila melanogaster*-specific *P element* and the *piggyBac* element, which has been used to transform a wide range of species, from insects to mammals<sup>27–30</sup> (Supplementary Fig. 1). *PiggyBac* elements also have a different insertion spectrum from *P elements*, facilitating the generation of lines with distinct expression patterns<sup>31</sup>. Transformants were established, and mobile, X-linked insertions, which allow the isolation of additional lines for enhancer trap screens, were identified for both transposons. Both the *P element* and *piggyBac* transposons (referred to as *P{IT.GAL4}* or *PBac{IT.GAL4}*, respectively, where “*IT*” denotes InSITE Target) functioned as enhancer traps, and were expressed in diverse patterns in the adult brain (Fig. 1c–e, Supplementary Fig. 2).

The InSITE system uses a trio of site-specific recombinases to exchange *GAL4* with any other sequence (Fig. 1b)<sup>19</sup>. First, the original enhancer trap is treated with Cre, which removes the *loxP*-flanked *mini-white* marker<sup>32</sup>. Next, an *FRT*-flanked genomic donor line is introduced, together with FLP and  $\Phi$ C31 integrase. In a strategy similar to that used to generate knock-out alleles<sup>33</sup>, upon treatment with FLP, a circular, *attB*-containing molecule is excised from the donor chromosome (Fig. 1b). Targeting of a genomic *FRT*-flanked cassette to a second *FRT*-containing locus using FLP has been previously described<sup>20</sup>. We reasoned that the irreversible nature of  $\Phi$ C31 integrase-mediated insertion would allow for high efficiency re-integration of the FLP-liberated donor molecule into the *attP* site of the InSITE enhancer trap (Fig. 1b). Integration events are detected by the re-appearance of the *mini-white* marker. Both constructs contain *loxP* sites oriented such that when treated with Cre, the original *GAL4* and *mini-white* marker in the integrated donor are deleted, leaving just the donor sequence. Thus, *GAL4* can be replaced with any other sequence, which should then be expressed under the control of the same regulatory elements.

In addition to the purely genetic swap, a microinjected plasmid analogous to the circular molecule that is liberated by FLP, can also be integrated into the *attP* site of the enhancer trap (Supplementary Fig. 3). A similar strategy using a microinjected *LexA* donor plasmid has recently been described<sup>24</sup>.

### An InSITE-compatible enhancer fusion vector

Another approach to segmenting complex expression patterns is to use enhancer fusion constructs, where a DNA fragment corresponding to an endogenous regulatory region is cloned upstream of *GAL4* or another effector. This approach has the advantage that lines containing enhancer subfragments are often expressed in fewer cells than enhancer traps, and enhancers can be further subdivided *in vitro* or the effector molecule can be switched by generating new constructs<sup>4</sup>. In order to take advantage of the enhancer fusion approach, but bypass the need to clone and microinject each new construct, we made an InSITE-compatible enhancer fusion vector (Fig. 1f). We cloned an enhancer fragment with a known

expression pattern, *ortc234*, into this vector, and inserted this construct into the *attP2* landing site<sup>25</sup>, and one of the Cre-reduced InSITE enhancer trap lines (*PBac{IT.GAL4.w-}0096*). While the presence of tandem *attB* and *attP* sites in this vector reduces the rate of transformation, likely through vector suicide via intramolecular recombination, integrants were obtained in both cases using standard injection procedures and verified by PCR. The insertion into *attP2* drove the expected *ortc2* expression pattern (data not shown)<sup>34</sup>. Such enhancer fusions are fully compatible with the InSITE system.

### InSITE genetic donor lines and plasmids

A collection of *attB*-containing genetic donor lines and injectable donor plasmids have been made (Supplementary Fig. 1). All of the reagents described here are publicly available (see Methods section for details). Transgenic donor lines, referred to as *P{ID.X}* (“X” is the inserted effector gene and “ID” denotes InSITE Donor), were established for the *GAL80* repressor<sup>17</sup>, the *GAL4DBD*, *GAL4AD*, and *VP16AD* split-*GAL4* hemi-drivers<sup>16</sup>, as well as the *LexA10* and *QF* transcriptional activators<sup>11</sup> (Supplementary Fig. 4). Taken together, this collection of donor constructs represents a versatile toolkit for manipulating gene expression.

### Enhancer trap lines are genetically swappable

To test the efficiency of the conversion procedure, four enhancer trap lines (*PBac{IT.GAL4}1.1*, *PBac{IT.GAL4}3.1*, *PBac{IT.GAL4}5.1*, and *PBac{IT.GAL4}6.1*) were crossed to Cre-expressing flies to remove the *loxP*-flanked *mini-white* marker<sup>32</sup>. Deletions were identified by loss of *mini-white* expression, and confirmed by PCR (Fig. 2a, b). This step of the conversion procedure was highly efficient, as no *white+* flies were recovered after Cre treatment. Using *eyFLP*, we also confirmed that the donor molecules were readily excised by FLP, as followed by the loss of *mini-white* expression (Fig. 2c)<sup>35</sup>.

Next, eight donor lines, representing all six effectors, were crossed to one or more of three different recipient lines, *PBac{IT.GAL4.w-}3.1*, *PBac{IT.GAL4.w-}5.1*, and *PBac{IT.GAL4.w-}6.1*, in flies that also carried a heat-shock inducible FLP and expressed  $\Phi$ C31 integrase in the germ-line<sup>19</sup> (Supplementary Fig. 5). Larvae were heat shocked to liberate the circular donor molecule, and the resulting flies were crossed to flies carrying *eyFLP* (Fig. 2d, e)<sup>35</sup>. The presence of *eyFLP* in the following generation ensured that flies in which the *FRT*-flanked donor cassette had not been excised were not scored as false positives. For all thirteen recipient-donor pairs tested, many *white+* putative genetic swap flies were recovered (ranging from 16.7% to 56.6% of crosses giving at least one *white+* progeny, Table 1, Fig. 2f).

Several putative genetic swaps were selected for molecular and functional validation. For twelve out of thirteen recipient-donor pairs, successful genetic swaps were identified by PCR analysis (Fig. 2a), referred to as *PBac{IS.X.GAL4}* (“X” is the swapped effector gene, and “IS” denotes InSITE Swap). For one of the recipient-donor pairs, only aberrant events were recovered (see below). In addition to having the PCR products diagnostic for the novel junctions created by integration of the genetic donor constructs, the swaps lost PCR products specific to the intact donor transposon (Fig. 2a). The junction between the *mini-white* and

*GAL4* genes was sequenced and, as expected, a single *FRT* site from the genomic donor element was retained between the *loxP* and *attL* sites in the integrated constructs (Fig. 2g). To complete the swap, these lines were treated with Cre, and removal of *GAL4* and *mini-white* was confirmed by PCR (Fig. 2a). The final, reduced constructs are referred to as *PBac{IS.X.w-}*.

In addition to verifying the genetic swap lines, a set of *attB*-containing donor plasmids was microinjected, along with a  $\Phi$ C31 integrase helper plasmid, into one of the three InSITE enhancer traps (Supplementary Fig. 3). *White+* integrants were obtained for all injections, with integration frequencies between 9.5% and 22.0%. Integration of all five donor plasmids was confirmed by PCR. Finally, the integrants were treated with Cre to remove *GAL4* and *mini-white*, and the final conversion products were confirmed by PCR.

### Avoiding genetic swap aberrant events

Typically, all integrants into a single *attP* landing site have similar eye colors<sup>36</sup> (Supplementary Fig. 6). However, aberrant events with different eye colors were observed for many of the above genetic swap crosses (Table 1). Most of these events retained the original donor transposon, but one *FRT* site was inactivated (Supplementary Fig. 7). In addition, one reciprocal translocation was recovered, in which the recipient *attP* site and the donor *attB* site were directly fused (Supplementary Fig. 7). Such events have been described previously with  $\Phi$ C31 integrase-mediated recombination in human cell lines<sup>37</sup>. However, by selecting appropriate recipient and donor pairs, and following specific markers in the crosses (see Methods), aberrant events can be essentially eliminated.

### Functional validation of the enhancer trap swaps

To determine whether the swapped enhancer trap lines were functional, a number of Cre-reduced swaps were crossed to appropriate reporters. First, swaps to *QF* and *LexA* were tested. In *PBac{IT.GAL4.w-}6.1* flies, expression was observed in the antennae and maxillary palps (Fig. 3a), and widely in the adult brain (Fig. 3b, c). The *PBac{IT.GAL4}1.1* enhancer trap drove expression in a group of neurons in the subesophageal ganglion (Fig. 3d, e), as well as a small number of neurons in the central brain. In *PBac{IS.QF.w-}6.1*, expression of *QUAS-mCD8:GFP* recapitulated the pattern seen with the *UAS*-driven expression of *PBac{IT.GAL4.w-}6.1* (Fig. 3f–h). In *PBac{IS.LexA.w-}1.1* flies, expression from *LexAOp-rCD2:GFP* was detected in the subesophageal ganglion, but not in the central brain neurons (Fig. 3i, j). Thus the *PBac{IS.LexA.w-}1.1* swap recapitulated some, but not all, features of the original *GAL4* expression pattern.

Next, the conversion to split-*GAL4* hemi-drivers and *GAL80* was tested. Swaps of *PBac{IT.GAL4.w-}3.1* to the *GAL4DBD*, *VP16AD*, and *GAL4AD* hemi-drivers and *GAL80* were generated, and expression of these lines was assayed in the antennae (Fig. 4a–e) and antennal lobes (Fig. 4f–y). In *PBac{IT.GAL4.w-}3.1*, expression was observed in the antennae of adult flies (Fig. 4a), as well as in several olfactory glomeruli (Fig. 4f, k), and in a small number of central brain neurons. As expected, both *PBac{IS.GAL4DBD.w-}3.1* and *elav-VP16AD16* were incapable of driving *UAS-mCD8:GFP* expression on their own (Fig. 4b, c, g, h, l, m). Together, *PBac{IS.GAL4DBD.w-}3.1/elav-VP16AD* drove robust

expression in a pattern similar to the original *GAL4* line (Fig. 4d, i, n, Supplementary Table 1). As with *PBac{IS.LexA.w-}1.1*, a minor difference was detected between the two patterns, with no expression seen in the small number of central brain neurons in the *PBac{IS.GAL4DBD.w-}3.1/elav-VP16AD* brains (Fig. 4f, i, k, n).

Neither of the activation domain hemi-driver lines drove *UAS-mCD8:GFP* expression on their own (Fig. 4p, q, u, v), nor did *elav-GAL4DBD16* (Fig. 4r, w). *PBac{IS.VP16AD.w-}3.1/elav-GAL4DBD* drove expression in a pattern that was similar to, but somewhat broader than the original *PBac{IT.GAL4}3.1* (Fig. 4s, x, Supplementary Table 1), while *PBac{IS.GAL4AD.w-}3.1/elav-GAL4DBD* drove weak expression in a subset of the glomeruli detected in the parent *GAL4* line (Fig. 4t, y, Supplementary Table 1). Such differences are consistent with previous reports that the *VP16AD* hemi-driver is a stronger transcriptional activator than *GAL4AD* 16,38. Similarly, the *PBac{IS.VP16AD.w-}3.1/PBac{IS.GAL4DBD.w-}3.1* pair drove expression in an essentially identical set of glomeruli as the parent *GAL4* line, while *PBac{IS.GAL4AD.w-}3.1/PBac{IS.GAL4DBD.w-}3.1* was expressed weakly in an overlapping but restricted subset of glomeruli (Supplementary Table 1). Likewise, *PBac{IS.VP16AD.w-}6.1*, in combination with *elav-GAL4DBD*, largely recapitulated the original *PBac{IT.GAL4}6.1* pattern (Supplementary Fig. 8).

As expected, *PBac{IS.GAL80.w-}3.1* eliminated most expression driven by the original *PBac{IT.GAL4}3.1* line (Fig. 4e, j, o). Similar to the split-*GAL4* lines, *GAL80* failed to target the small group of central brain neurons (Fig. 4f, j, k, o), suggesting that this expression was influenced by sequences inside *GAL4* itself.

For most of the swaps, the resultant expression was strongly predicted by the original *GAL4* pattern. In three out of ten cases, the expression patterns overlapped with the original pattern, but also displayed substantial differences. Expression of *PBac{IS.QF.w-}3.1* was similar to the original *PBac{IT.GAL4.w-}3.1* line in the antennae and olfactory glomeruli, but new expression was observed in a single glial subtype (cortex glia)<sup>39</sup> and in trachea (Supplementary Fig. 8, Supplementary Table 1). In a second case, *PBac{IS.GAL4DBD.w-}6.1*, the pattern of expression overlapped with *PBac{IT.GAL4}6.1* in both the antennae and the adult brain, but was much sparser and was not detected in the maxillary palps. Finally, *PBac{IS.GAL80.w-}6.1* repression of *PBac{IT.GAL4}6.1* was incomplete (Supplementary Fig. 8). In this case, since *GAL4* and *GAL80* are presumably being expressed at similar levels, it is not clear that complete repression would be expected.

## Discussion

Here we introduce a versatile platform designed to facilitate the functional diversification of expression patterns. The InSITE system uses  $\Phi$ C31 integrase, FLP, and Cre-mediated recombination to convert a *GAL4* effector in an enhancer trap or enhancer fusion line to another sequence. We have demonstrated that InSITE enhancer traps can be efficiently swapped and made to express a split-*GAL4* hemi-driver, *GAL80*, *LexA*, or *QF*. This will allow lines with overlapping expression patterns to be used intersectionally to target restricted subsets of the original patterns. Using this system, it should be possible to generate highly specific driver lines targeting small populations of cells or tissues. This will be useful



in a wide range of developmental and cell biological experiments, allowing the tissue-specific requirements of genes to be mapped with high precision, and the dissection of neural circuits at the level of single neuron types.

One advantage of the InSITE system is that the effector swap can be done genetically, through a series of crosses, bypassing the labor intensive step of DNA microinjection. While other approaches exist for repurposing enhancer expression patterns, such as constructed enhancer fusions, these strategies have previously required that new constructs must be cloned and transformed for each new desired fusion<sup>4</sup>. The InSITE-compatible enhancer fusion vector we describe allows one to make a single *GAL4* construct, and then swap it for any of the available donor lines *in vivo*. In addition, the InSITE genetic swap approach is easily scalable, making it possible to simultaneously exchange large numbers of lines with multiple donor constructs.

The majority of the swaps captured most features of the original *GAL4* expression patterns. However, in some cases, either prominent features of the *GAL4* pattern were lost, or new expression patterns were observed. These changes may have resulted from differences in the strength or responsiveness of reporter lines. Alternately, the swap may have modified some combination of enhancer spacing and sequence composition flanking the promoter. Since the InSITE donors are essentially identical outside the effector sequence, these effects are likely intrinsic properties of the effector sequence itself. Therefore, any approach using these effectors would also likely encounter such context-specific cryptic regulatory activities. These occasional discrepancies between the original and swapped patterns underscore the importance of having a versatile, high-throughput system that allows many orthogonal intersectional strategies to be quickly tested in parallel.

The modular nature of the InSITE platform makes this system forward compatible. Recent work has described refinements to the existing binary systems<sup>13,24,38</sup>. Such new reagents and many unanticipated future technologies can be readily incorporated into the InSITE system, and will be useful in designing additional intersectional strategies.

While we have focused primarily on binary system expression and the refinement of expression patterns, many additional elaborations of this system are possible due to the fact that *GAL4* can be replaced with any other sequence. For instance, it is possible to design strategies to mutate or modify loci near the transposon insertion site. In particular, because the genetic swap leaves behind a single *FRT* site, it is possible to use genetic swap lines together with other *FRT*-containing transposons (like the Exelixis and Drosdel collections) to make deletions<sup>31</sup>. These deletions, which could disrupt nearby genes and regulatory elements, can be designed to leave behind either *GAL4* or the swapped effector. This provides another approach to segmenting complex expression patterns, by altering the surrounding regulatory elements. Thus, the InSITE system is a versatile toolkit both for capturing and manipulating gene activity. As the methods described here use recombinases that work in other model organisms, this system should be easily adaptable.

## Methods

### Cloning enhancer trap transposons

pXL-BacII-attPGal4LWL: First, the *attP* site was excised from pUASTP2 (obtained from J. Bateman18) with *EcoRI*, and ligated into the *EcoRI* site of pBluescript, to make pBS-attP. Next a linker fragment, SPpolyF/R, was made by annealing and phosphorylating the SPpolyF and PSpolyR oligos (all oligos were obtained from Integrated DNA Technologies, see Supplemental Table 2 for oligo sequences) and this linker was ligated into *PstI* and *SacI* cut pBS-attP, destroying the *SacI* site, to make pBS-attPAS3. A second linker fragment, HKpolyF/R, was made with the HKpolyF and KHpolyR oligos. pBS-attPAS3 was cut with *HindIII* and *KpnI*, and the HKpolyF/R linker was inserted, to generate pBS-attPAS+S1.

Next, a fragment containing the P promoter was amplified from pLAPVPRA (D. Gohl and M. Müller, unpub. data) using the pPromF and pPromR primers (see Supplemental Table 3 for sequences of PCR and sequencing primers). The P promoter fragment was cloned into pCRII-TOPO (Invitrogen), to generate pCRII-Pprom1–2. This vector and pBS-attPAS+S1 were both digested sequentially with *BamHI* and *HindIII*, and the P promoter fragment was cloned into pBS-attPAS+S1 to make pBS-attPAS+SPprom1. This vector was cut with *BamHI* and *SacI*. A *BamHI* and *SacI* fragment containing *GAL4* and the *hsp70* 3' UTR was isolated from pGaTB (obtained from M. Wernet1) and ligated into pBS-attPAS+SPprom1, to generate pBS-attPPpromGal4.

In parallel, the *mini-white* gene was obtained as an *EcoRI* fragment from pC4YM (from M. Müller40), and ligated between two *loxP* sites into the *EcoRI* site of pBLLA (from M. Müller, 40) to make pBLLA-miniwhite1. A *NotI*, *BamHI* fragment containing *mini-white* flanked by *loxP* sites was isolated and cloned into the *NotI*, *BamHI* sites of pXL-BacII-ECFP30 (obtained from L. Luo41) to make pXL-BacII-lox-miniwhite-lox. Finally, in order to make pXL-BacII-attPGal4LWL, a *NotI* fragment containing *attP-Pprom-GAL4-hsp70* 3' UTR was isolated from pBS-attPPpromGal4 and ligated into the *NotI* site of pXL-BacII-lox-miniwhite-lox. The correct insert orientation was determined by diagnostic digests and by sequencing with primers: pPromF, pPromR, MW300Up, and 9-1.

pXN-attPGal4LWL: A *KpnI*, *BamHI* fragment from pXL-BacII-attPGal4LWL was ligated into the *KpnI*, *BamHI* sites of pXN (from Paul Schedl42), between the P element ends. This vector was re-cut with *NotI* and *BamHI*, and a *NotI*, *BamHI* fragment containing *mini-white* flanked by *loxP* sites (see above) was inserted, to make pXN-LWL. pXN-LWL was then cut with *NotI*, and was ligated to the *NotI* fragment containing *attP-Pprom-GAL4-hsp70* 3'UTR isolated from pBS-attPPpromGal4 (see above), to make pXN-attPGal4LWL. The correct orientation of the insert was confirmed by diagnostic digests and sequencing with the P5'inF and MW5'R primers.

### Cloning injectable donor plasmids

**pBPHLWL**—Two pairs of oligos which encoded a multiple cloning site with the following restriction sites: *KpnI-HindIII-BamHI-NotI-MluI-AscI-XbaI-ClaI-NheI-PacI-SphI-SpeI-BglII-SacI*, were annealed and phosphorylated to make Oligo1F/R and Oligo2F/R. Next, pBluescriptKSII+ was cut with *KpnI* and *XbaI* and Oligo1F/R was inserted to generate pBS-



Oligo1. pBS-Oligo1 was cut with *Xba*I and *Sac*I, and Oligo2F/R was ligated into this backbone to make pBSO1O2. Next, a *Cla*I, *Spe*I fragment containing the *hsp70* 3' UTR was isolated from pGaTB and cloned into the *Cla*I, *Spe*I sites of pBSO1O2. The resulting vector was cut with *Spe*I and *Bgl*III, and was ligated to an *Xba*I, *Bam*HI fragment containing *loxP-mini-white-loxP* from pBLLA-miniwhite1, generating the plasmid pBSO1O2hsp70LWL.

In parallel, the *attB* site from piB-GFP (obtained from Jack Bateman18) was isolated as a *Kpn*I, *Hind*III fragment and cloned into pBluescriptKSII+, to make pBS-attB. A *Hind*III, *Bam*HI fragment containing the P minimal promoter (from pCRII-Pprom1–2, see above) was ligated into the *Hind*III, *Bam*HI sites of pBS-attB, to make pBS-attBPprom.

Finally, a *Kpn*I, *Bam*HI fragment containing *attB* and the minimal P promoter was isolated from pBS-attBPprom, and ligated into the *Kpn*I, *Bam*HI sites of pBSO1O2hsp70LWL, to generate the pBPHLWL donor plasmid. pBPHLWL was sequenced with the following primers: M13For, M13Rev, hsp3'F, MW5'R, MW3'F, hsp3'R(seq), PpromF(seq), MW5'F(seq), MW2F(seq), MW2R(seq), MW3F(seq), MW3R(seq), MW4F(seq), MW4R(seq), MW6F(seq), and MW6R(seq).

pBPHLWL-Gal80: A *Not*I, *Xba*I fragment containing *GAL80* from pCASPTubGAL80 (obtained from T. Schwabe17), was cloned into the *Not*I, *Xba*I sites of pBPHLWL. The insert was verified by sequencing using the following primers: PpromF(seq), MW5'R, Gal80F1(seq), and Gal80R1(seq).

pBPHLWL-Gal4AD: A *Not*I, *Asc*I fragment containing the *GAL4AD*-leucine zipper fusion from pActPL-Gal4AD (obtained from Addgene16) was inserted in the MCS of pBPHLWL and verified by sequencing with the PpromF(seq) and MW5'R primers.

pBPHLWL-VP16AD: A *Not*I, *Asc*I fragment containing the *VP16AD*-leucine zipper fusion from pActPL-VP16AD (obtained from Addgene16) was inserted in the MCS of pBPHLWL and verified by sequencing with the PpromF(seq) and MW5'R primers.

pBPHLWL-Gal4DBD: A *Not*I, *Asc*I fragment containing the *GAL4DBD*-leucine zipper fusion from pActPL-Gal4DBD (obtained from Addgene16) was inserted in the MCS of pBPHLWL and verified by sequencing with the PpromF(seq) and MW5'R primers.

pBPHLWL-LexA: A *Not*I, *Xba*I fragment containing *LexA* from pGD319 (obtained from G. Dietzl) was cloned into the MCS of pBPHLWL and verified by sequencing with the following primers: PpromF(seq), MW5'R, LexA1F, and LexA1R.

pBPHLWL-QF: A *Bam*HI and *Spe*I fragment containing *QF11* from pXN-FBLWLF-QF (see below) was ligated into the *Bam*HI, *Xba*I sites of pBPHLWL and verified by sequencing with the PpromF(seq) and MW5'R primers.

### Cloning genetic donor transposons

**pXN-FBLWLF**—Two sets of FRT site-containing oligos were used to make 5'FRT(*Kpn*I)F/R and 3'FRT(*Not*-*Sac*)F/R. Next, pBS-attBPprom was cut with *Kpn*I and 5'FRT(*Kpn*I)F/R was inserted to generate the plasmid pBSattBPprom+5'FRT5, which had a

tandem insertion of the oligo. The orientation of the insert was verified by sequencing with M13For. To reduce this tandem insert, pBSattBPprom+5'FRT5 was cut with *AscI*, gel purified, and re-ligated to generate pBSFattBPprom. This plasmid was then cut with *NotI* and *SacI*, and 3'FRT(Not-Sac)F/R was inserted to make pBSFattBPpromF. Next, a *KpnI*, *XhoI* FattBPpromF fragment from pBSFattBPpromF was ligated into the *KpnI*, *SalI* sites of the pXN vector, to make pXN-FattBPpromF2-1. A linker containing specific restriction sites (*NotI-EcoRV-PacI-SphI-AvrII-NheI-MluI-BglII*) was made from a pair of oligos (GCNotI F (dXho) and GCNotI R (dXho)) and ligated into the *NotI* site of pXN-FattBPpromF2-1. This plasmid was cut with *BglII* and *NheI*, and an *XbaI*, *BamHI loxP-mini-white-loxP* from pBLLΔ-miniwhite1 was inserted to make pXN-FBLWLF. pXN-FBLWLF was confirmed by sequencing with the P5'inF and MW5'R primers.

pXN-FBLWLF-Gal80: A *BamHI*, *SphI* fragment containing *GAL80* and the *hsp70* 3' UTR from pBPHLWL-Gal80 was cloned into the MCS of pXN-FBLWLF and confirmed by diagnostic digests and sequencing with the P5'inF primer.

pXN-FBLWLF-GAL4AD: A *BamHI*, *SphI* fragment containing *GAL4AD* and the *hsp70* 3' UTR from pBPHLWL-GAL4AD was cloned into the MCS of pXN-FBLWLF and confirmed by diagnostic digests and sequencing with the P5'inF primer.

pXN-FBLWLF-VP16AD: A *BamHI*, *PacI* fragment containing *VP16AD* and the *hsp70* 3' UTR from pBPHLWL-VP16AD was cloned into the MCS of pXN-FBLWLF and confirmed by diagnostic digests and sequencing with the P5'inF primer.

pXN-FBLWLF-Gal4DBD: A *BamHI*, *PacI* fragment containing *GAL4DBD* and the *hsp70* 3' UTR from pBPHLWL-Gal4DBD was cloned into the MCS of pXN-FBLWLF and confirmed by diagnostic digests and sequencing with the P5'inF and MW5'R primers.

pXN-FBLWLF-LexA: A *NotI*, *PacI* fragment containing *LexA* and the *hsp70* 3' UTR from pBPHLWL-LexA was cloned into the MCS of pXN-FBLWLF and confirmed by diagnostic digests and sequencing with the P5'inF primer.

pXN-FBLWLF-QF: Using pBac-GH146-QF-hsp7011 as substrate, *QF* and *hsp70* were separately PCR amplified with a shared sequence (TAAGCACTAGTGCAGATCTTATCGATAC) between the *QF* and *hsp70* regions. For *QF*, the following primers were used: BHI-QF-FOR/QFREY-hsp70-LNKR. For *hsp70*, the following primers were used: pGaTn-hsp70REV-NotI-BH1/hsp70-FOR-QF-LNKR. The *QF* and *hsp70* PCR products were annealed and the BHI-QF-FOR and pGaTn-hsp70REV-NotI-BH1 primers were used to PCR amplify a *BamHI-QF-SpeI-BglII-hsp70-NotI-BamHI* cassette, which was cloned into the *BamHI* site of pXN-FBLWLF. An insert in the correct orientation was verified by sequencing.

### Cloning the enhancer fusion vector

**pBMPGal4LWL**—pBSO102 was cut with *BamHI* and *BglII*, and a *BamHI* fragment containing *GAL4*, the *hsp70* 3' UTR and *loxP*-flanked *mini-white* from pXL-BacII-attPGal4LWL was ligated into these sites to make pBSO102-Gal4LWL. A correctly

oriented insert was cut with *NotI*, treated with mung bean nuclease and re-ligated to destroy the *NotI* site. Next, to make a multi-cloning site, a pair of oligos (PstI-SacI EF linker For and PstI-SacI EF linker Rev) were phosphorylated, annealed, and ligated into the *SacI* and *PstI* sites of pBS-attP. Next, the multi-cloning site and *attP* site were liberated using *HindIII*, and ligated into the *HindIII* site of pBPHLWL. An insert with the correct orientation was identified and a fragment containing *attB*, the multi-cloning site, and *attP* was cut out of this plasmid with *KpnI* and *BamHI*. Finally, this fragment was ligated into the *KpnI*, *BamHI* sites of pBSO1O2-Gal4LWL to make pBMPGal4LWL.

**pBMPGal4LWL-ortc2b**—A fragment containing the *ortc2b* regulatory region was amplified from pChs-ATTB-OrtC2-Gal4 (obtained from C.-H. Lee)<sup>34</sup> using the *ortc2b* For and *ortc2b* Rev primers. A *NotI* site was added to the 5' end of the PCR product, which was then cloned into pCRII-TOPO (Invitrogen). The *ortc2b* fragment was cut out of pCRII-TOPO with *NotI* and *BamHI* and cloned into *NotI*, *BglIII* cut pBMPGal4LWL.

### Fly Methods

Flies were grown on molasses food at 22–25°C. Injections were performed as previously described by Rainbow Transgenic Flies<sup>43</sup>. P element constructs were transformed using a standard  $\Delta 2-3$  helper plasmid. *PiggyBac* constructs were transformed using the pBSII-hs-orf30 helper plasmid (obtained from L. Luo). Injected *attB* donor constructs (pBPHLWL derivatives) were transformed using the  $\Phi C31$  integrase helper plasmid pBS130. Flies were obtained from the Bloomington Drosophila Stock Center (BDSC) (*y, w; P{CaryP}attP2*) to test the integration of pBMPGal4LWL.

### Cre reductions

To prepare swappable enhancer trap lines for injection of the donor plasmid (or genetic conversion), females containing a convertible enhancer trap line were crossed to males carrying the Cre recombinase (*y, w; MKRS, P{hsFLP}86E/TM6B, P{w[+mC]=Crew}DH2, Tb[1]*, or *y, w; noc[ScO]/CyO, P{w[+mC]=Crew}DH1*, from BDSC). Males containing both the enhancer trap and the *Cre* chromosome were crossed to the appropriate balancer line, and single male progeny were used to establish a balanced *white* minus enhancer trap stock (Supplementary Fig. 6). The clean excision of *mini-white* was confirmed by PCR (see below). The same procedure was used to remove the *mini-white* marker and original *GAL4* gene from integrant flies. Excision using Cre was highly efficient, approaching 100%.

### Genetic conversion crosses

To carry out the genetic swap procedure (see Fig. 1b, Supplementary Fig. 5), first a *y, w, hs-FLP, vas- $\Phi C31$*  integrase recombinant X chromosome was established. Recombinants were scored for the presence of GFP (marking the *J15, vas- $\Phi C31$*  integrase transgene<sup>19</sup>), and then tested by PCR for the presence of FLP (using the FLP Set1 Forward/FLP Set1 Reverse and FLP Set2 Forward/FLP Set2 Reverse primers). *y, w, hs-FLP, vas- $\Phi C31$ ; Recipient(enhancer trap)w-/Balancer* stocks were made, and virgins were crossed to genetic donor males (Supplementary Fig. 5). These flies were allowed to lay eggs for 2–4 days, then flipped onto fresh food. The vials containing the progeny of this cross were then heat-shocked in a 37°C

water bath for 1 hour, every day, until most of the progeny had pupated. The resulting progeny had eyes which were either completely white, or had small patches of orange or red variegation (Fig. 2d–e). Heat-shocked *y, w, hs-FLP, vas-ΦC31; Donor/+; Recipient(w-)/+* males were crossed to *y, w, eyFLP2; Balancer* virgins, and *white* plus putative genetic swaps were balanced. Integration of the genetic donor construct in the enhancer trap was confirmed by PCR (see below). To complete the swap, the enhancer trap containing the integrated genetic donor construct was reduced using Cre (see above). Genetic donor constructs lose *mini-white* expression when crossed to *eyFLP2*, which excises the *FRT*-flanked donor cassette in the eye. At least eight independent lines were tested for the *VP16AD*, *GAL80*, *GAL4AD*, *GAL4DBD*, *LexA*, and *QF* constructs, and every line had white eyes in the presence of *eyFLP2* (Fig. 2c), with the exception of one *VP16AD* line (*P{ID.VP16AD}39.1D1*), which was pupal lethal with *eyFLP2*.

Note: Maintaining the genetic donor lines together with the *y, w, hs-FLP, vas-ΦC31* chromosome for many generations may lead to degradation of the donor construct.

### Avoiding genetic swap aberrant events

Since all aberrant events were associated with the original donor site, crosses should be set up with recipient and donor lines on different chromosomes. This way, putative genetic swap lines that do not map to the recipient chromosome can be immediately discarded. In addition, by using recipient and donor lines with distinct eye colors, the level of *mini-white* expression can facilitate the identification of *bona fide* swaps. To avoid reciprocal translocations, recipient and donor lines can be molecularly mapped (Supplementary Fig. 4)44–45 and selected such that any translocations will be lethal (for instance, by generating a dicentric or acentric chromosome). Finally, by selecting *white+* flies in the final step that also contain the balancer or dominant marker that was opposite the donor chromosome in the previous generation, it is possible to select against aberrant events. In our experience, selecting matched donors and recipients in this way eliminates all aberrant events. Even if no effort is made to select against aberrant events, the frequency of true events among *w+* putative swaps is quite high (averaging 51.7%, Table 1).

### Functional tests of enhancer trap swaps

The following lines were used to functionally test the split-*GAL4* enhancer trap swaps: *w; P{UAS:2xEGFP}; P{elav-GAL4.DBD}H4A1, w; P{UAS:2xEGFP}; P{elav-GAL4.AD}IIA1, w; P{UAS:2xEGFP}; P{elav-VP16.AD}G3A1* (from BDSC)16. For testing the enhancer trap swaps, the *UAS:2xEGFP* chromosome present in the *elav* split-*GAL4* lines was replaced with *UAS:mCD8-GFP17. w; LexAop:rCD2-GFP10* (obtained from Chi-Hon Lee) reporter lines was used to monitor LexA driven gene expression. *w; P{QUAS-mCD8-GFP.P}5J* was used to monitor QF expression11. The following injected enhancer trap swap lines were functionally tested: *PBac{IS.VP16AD.w-}6.1(11.1)*, *PBac{IS.GAL80.w-}6.1(39.1)*, *PBac{IS.GAL4AD.w-}3.1(33.1)*, *PBac{IS.GAL4DBD.w-}3.1(51.1)*, *PBac{IS.LexA.w-}1.1(44.1)*. The following genetic swap lines were functionally tested: *PBac{IS.VP16AD.w-}6.1(D37-6)*, *PBac{IS.GAL80.w-}6.1(E17-30)*, *PBac{IS.GAL4DBD.w-}6.1(F32-2)*, *PBac{IS.GAL80.w-}3.1(E17-12)*, *PBac{IS.VP16AD.w-}3.1(D33-16)*, *PBac{IS.QF.w-}6.1(Q10B-7)*, and *PBac{IS.QF.w-}3.1(Q12A-10)*. GFP expression in adult animals was

visualized on a Zeiss M2Bio stereomicroscope and fluorescence images were acquired using a SPOT-RT digital camera (Diagnostic Instruments, Inc.).

### Confirming Cre-mediated reductions and injected integration events by PCR

To confirm the deletion of *mini-white* by Cre, the subsequent integration of the microinjected *attB*-containing donor construct, and the Cre-mediated reduction of the integrant, a set of diagnostic PCR primers was used (see Fig. 2b and Supplementary Fig. 3 for primer locations and Fig. 2a and Supplementary Fig. 3 for examples). The following primer pairs were used on the *piggyBac* constructs: 1) MW3'F2/pBAC3'R4, 2) Gal4 3'F2/MW5'R2, 3) pBAC5'F1/attP R1, 4) Gal4 3'F2/pBAC3'R4, 5) pBSF1/attP R1, 6) pBAC5'F1/attB R1, 7) construct-specific primer/pBAC3'R4. Construct-specific primers: Gal80 3'F1, Gal4AD 3'F1 (note: also works on *VP16AD*), Gal4DBD 3'F1, LexA 3'F1, or QF 3'F1.

To confirm integration into the *P element* constructs (data not shown), the above primer pairs can be used, with two modifications. P5'inF can be used in place of pBAC5'F1, and P3'Rnew2R can be used in place of pBAC3'R4.

### Confirming genetic swaps by PCR

The primers that were used to confirm the injection-based enhancer trap swaps were also used to validate the genetic swaps, with one exception. In the genetic swaps, the sequence corresponding to the vector backbone in the injected swaps does not exist, so primer MW3'F2 was substituted for pBSF1 in primer pair number 5. In addition, another set of PCRs using the primer pairs P5'inF/attBR1 (primer pair 8 in Fig. 2b) and MW3'F2/P3'Rnew2R (primer pair 9 in Fig. 2b) were done on the genetic swap lines, to confirm the loss of the donor element 5' and 3' junctions.

### Confirming insertion of the pBMPGal4LWL-ortc2 enhancer fusion vector

To confirm the insertion of pBMPGal4LWL into *P{CaryP}attP2*, the following primers were used: 1) pBSF1/attP R1, 2) yellow 3'F2/attB R1, 3) yellow 3'F2/ortc2 5'R1.

### Splinkerette mapping of transposon insertion sites

Fifteen of the *piggyBac* enhancer trap lines and twenty four genetic donor *P element* lines were mapped using Splinkerettes<sup>44</sup>, and localized using Flybase to BLAST search the *Drosophila melanogaster* genome<sup>46</sup>. Line *PBac{IT.GAL4}1.1* was inserted in an intron of the *sarah* (*sra*) gene. Line *PBac{IT.GAL4}3.1* was inserted in an intron of the *desaturase1* (*desat1*) gene. Line *PBac{IT.GAL4}5.1* was inserted within a microRNA cluster in *mir-2498*. Line *PBac{IT.GAL4}6.1* was inserted in an intergenic region between the *Gr93a-d* cluster and the *gliolectin* (*glec*) gene. Insertion sites and orientations of the genetic donor lines are shown in Supplementary Fig. 4.

### Immunohistochemistry and Imaging

Brains were dissected and fixed for one hour in 2% paraformaldehyde in phosphate buffered lysine (50 mM lysine, 100 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4), then washed 3x5 minutes in PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, 0.1% Triton X-100, pH 7.4).

Following the washes, the brains were blocked for 30 minutes in 10% Normal Goat Serum in PBST, and incubated with primary antibody overnight at 4°C. Brains were then washed 3×5 minutes in PBST and incubated with secondary antibody for two hours at room temperature. After secondary incubation, the brains were washed 3×5 minutes in PBST, and transferred to 70% glycerol. When staining for mCD8, the following primary and secondary antibodies were used: mouse anti-Bruchpilot (nc82)47 (1:30, Developmental Studies Hybridoma Bank), rat anti-mCD817 (1:100, Invitrogen), goat anti-rat Alexa 488 (1:200, Molecular Probes), and goat anti-mouse Alexa 594 (1:200, Molecular Probes). When staining lines containing the *LexAop:rCD2-GFP* construct (and corresponding controls) chicken anti-GFP (1:2000, Abcam) primary, and goat anti-chicken Alexa 488 (1:200, Molecular Probes) secondary antibodies were used. Brains were mounted in Vectashield (Vector Laboratories) for imaging. Images were acquired on a Leica TCS SP2 AOBS confocal microscope with either a 20x (N.A. = 0.7) or 40x (N.A. = 1.25) lens. Confocal images were rendered in three dimensions using Imaris (Bitplane) and adjusted as necessary in Photoshop (Adobe) using cropping and thresholding tools, and assembled into figures using Illustrator (Adobe).

### InSITE reagents

*Drosophila* stocks, including the mobile, X-linked *P element* and *piggyBac* enhancer trap lines (*P{IT.GAL4}A134.3* and *PBac{IT.GAL4}0315*), the *y, w, hs-FLP, vas-ΦC31* line, and all of the genetic donor lines shown in Supplementary Fig. 4 have been deposited with the Bloomington *Drosophila* Stock Center. A collection of 100 InSITE enhancer trap lines is available upon request. All plasmids have been deposited with Addgene.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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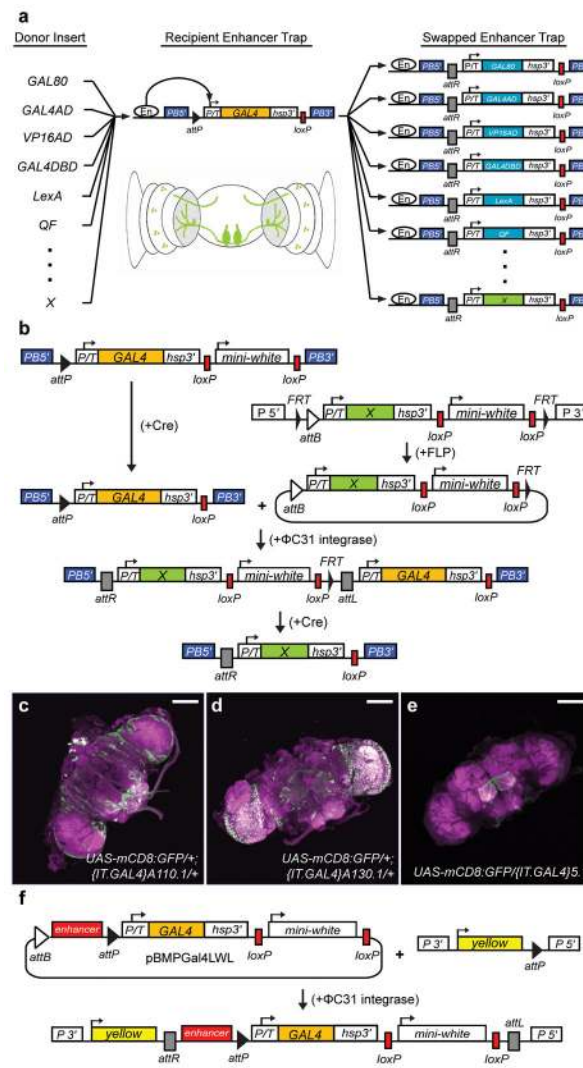
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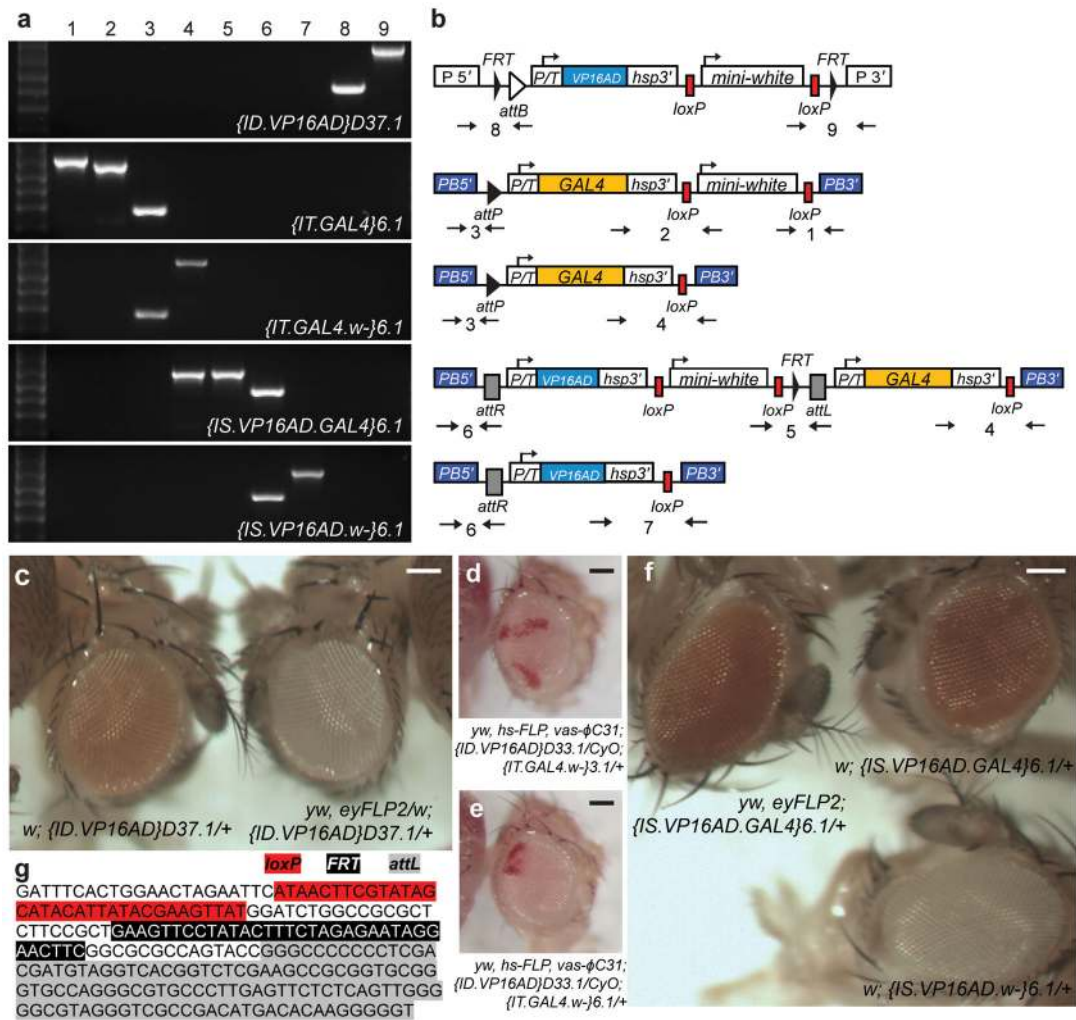


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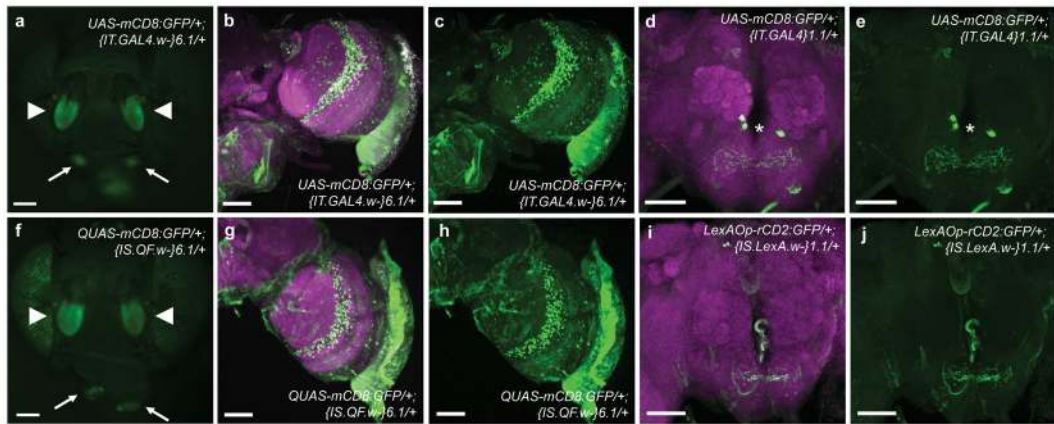


**Figure 1.** The InSITE system. **(a)** Schematic illustration of the InSITE system, which can convert a *GAL4* enhancer trap to another sequence (“X”), which will then be expressed under the control of local enhancers (En). **(b)** Schematic illustration of the procedure for genetically swapping *GAL4* with another sequence (“X”). **(c-e)** InSITE enhancer trap expression in the adult brain. Green: anti-mCD8. Magenta: anti-Bruchpilot (nc82). Scale bar: 100  $\mu$ m. **(c)** *P element* line *P{IT.GAL4}A110.1*. **(d)** *P element* line *P{IT.GAL4}A130.1*. **(e)** *PiggyBac* line *PBac{IT.GAL4}5.1*. **(f)** The InSITE-compatible enhancer fusion vector, pBMPGal4LWL.



**Figure 2.**

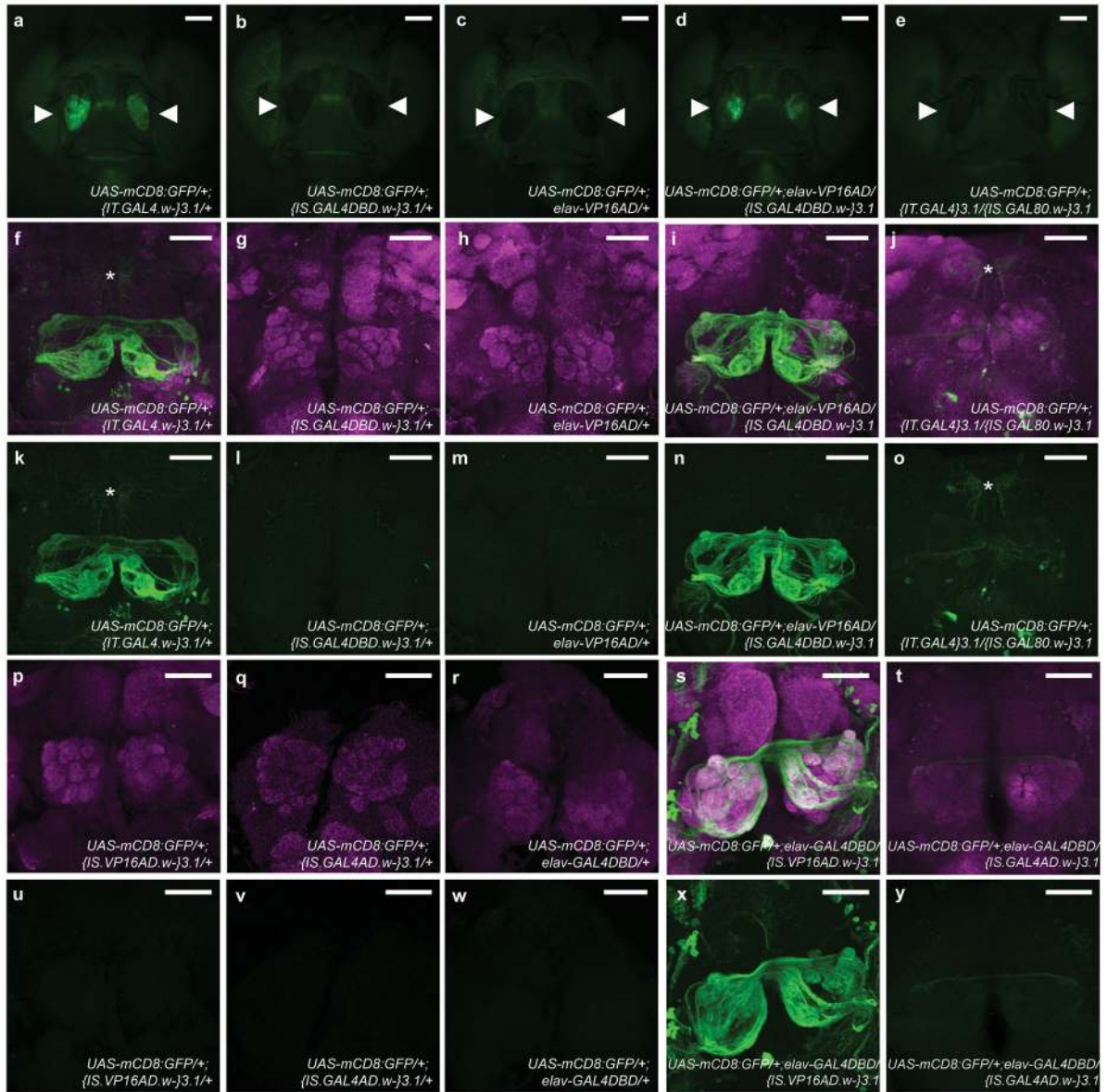
Molecular and genetic validation of the enhancer trap exchange. **(a)** PCR confirmed each step of the genetic conversion of line *PBac{IT.GAL4}6.1* to the *VP16AD* hemi-driver. Numbers refer to primer pairs shown in panel **(b)**. **(b)** Schematics depicting the structure of the insertion being amplified in each set of PCR reactions. Locations of PCR primers are shown beneath each construct. **(c)** Genetic donor constructs lose *mini-white* expression when crossed to *eyFLP2*. Left: *P{ID.VP16AD}D37.1/+*, Right: *y*, *w*, *eyFLP2*; *P{ID.VP16AD}D37.1/+*. Scale bar: 100  $\mu$ m. **(de)** When crossed to *y*, *w*, *hs-FLP*, *vas-ΦC31* and heat shocked periodically throughout larval development, flies containing both recipient and genetic donor elements displayed either completely white eyes (not shown), or a small amount of variegated *mini-white* expression. Scale bar: 100  $\mu$ m. **(d)** *y*, *w*, *hs-FLP*, *vas-ΦC31*; *P{ID.VP16AD}D33.1/CyO*; *PBac{IT.GAL4.w-}3.1/+*. **(e)** *y*, *w*, *hs-FLP*, *vas-ΦC31*; *P{ID.VP16AD}D33.1/CyO*; *PBac{IT.GAL4.w-}6.1/+*. **(f)** Correct integration events (genetic swaps) became resistant to *eyFLP2* because one FRT site was lost during excision and re-integration. Top Right: *PBac{IS.VP16AD.GAL4}6.1/+*, Bottom: *PBac{IS.VP16AD.w-}6.1/+*, Top Left: *y*, *w*, *eyFLP2*; *PBac{IS.VP16AD.GAL4}6.1/+*. Scale bar: 100  $\mu$ m. **(g)** Sequence of the PCR product of primer pair 5.



**Figure 3.**

Functional validation of the *QF* and *LexA* enhancer trap swaps. (**a-e**) Expression of the *PBac{IT.GAL4.w-}6.1* and *PBac{IT.GAL4}1.1* enhancer traps. (**a, f**) Both *PBac{IT.GAL4.w-}6.1* and *PBac{IS.QF.w-}6.1* were expressed in the antennae (arrowheads) and maxillary palps (arrows) of adult flies. Scale bar: 100  $\mu$ m. (**b, g**) Adult brains stained with anti-mCD8 (green) and anti-Bruchpilot (nc82, magenta). Scale bar: 50  $\mu$ m. *PBac{IT.GAL4.w-}6.1* and *PBac{IS.QF.w-}6.1* were expressed in similar patterns in the adult brain. (**c, h**) mCD8 (green) channel only of images in panels (**b**) and (**g**). Scale bar: 50  $\mu$ m. (**d-e, i-j**) Comparison of *PBac{IT.GAL4}1.1* and *PBac{IS.LexA.w-}1.1* lines. Scale bar: 50  $\mu$ m. Asterisk denotes a group of cells where GFP expression was observed in *PBac{IT.GAL4}1.1*, but not in the *PBac{IS.LexA.w-}1.1* swap. (**d, i**) Adult brains stained with anti-GFP (green) and anti-Bruchpilot (nc82, magenta). (**e, j**) GFP (green) channel only of images in (**d**) and (**i**).



**Figure 4.**

Functional validation of the split-*GAL4* and *GAL80* enhancer trap swaps. (a-e) GFP expression in the antennae (arrowheads) of adult flies. Scale bar: 100  $\mu$ m. (f-j, p-t) Adult brains stained with anti-mCD8 (green) and anti-Bruchpilot (nc82, magenta). Scale bar: 50  $\mu$ m. (k-o, u-y) mCD8 (green) channel only of images in panels (f-j) and (p-t). Scale bar: 50  $\mu$ m. *PBac{IT.GAL4.w-}3.1* was expressed in the antennae of adult flies (a), as well as in several olfactory glomeruli (f, k). Weak expression was also seen in *PBac{IT.GAL4.w-}3.1* in a small number of central brain neurons (marked with an asterisk in panels (f) and (k)). No GFP or CD8 expression was seen in any of the samples carrying only one half of split-*GAL4* (b, c, g, h, l, m, p-r, u-w). However, patterns similar to the original *PBac{IT.GAL4.w-}3.1* were seen when both halves of split-*GAL4* were present (d, i, n, s, t, x, y). *PBac{IT.GAL4.w-}3.1* expression was repressed by *PBac{IS.GAL80.w-}3.1* in both the



antennae (**e**) and in the adult brain (**j**, **o**). In the adult brain, repression was strongest in the antennal lobe neurons and *PBac{IS.GAL80.w-}3.1* failed to repress *GAL4* in the small cluster of central brain neurons (marked with asterisk in panels (**j**) and (**o**)).

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Efficiency of the InSITE genetic swap procedure. At least one line, chosen at random, for each of the six genetic donor elements was tested for the ability to excise and re-integrate into an *attP*-containing recipient site. Three different enhancer trap target lines were tested. Rates of *eyFLP*-resistant *white+* flies and true integration events, as assayed by PCR, are shown.

Table 1

Recipient	Donor (Line #)	# of crosses	# of crosses with w+ flies	% of crosses with w+ flies	# tested by PCR	# of true integrations	% of true integrations	Recovered swap
<i>PBac</i> [ <i>IT.GAL4.w-</i> ]/6.1	<i>P</i> { <i>ID.VP</i> [6AD]/D33.1	26	9	34.6	9	7	77.8	yes
<i>PBac</i> [ <i>IT.GAL4.w-</i> ]/3.1	<i>P</i> { <i>ID.VP</i> [6AD]/D33.1	39	9	23.1	8	1	12.5	yes
<i>PBac</i> [ <i>IT.GAL4.w-</i> ]/6.1	<i>P</i> { <i>ID.VP</i> [6AD]/D37.1	34	8	23.5	no data	no data	no data	yes
<i>PBac</i> [ <i>IT.GAL4.w-</i> ]/3.1	<i>P</i> { <i>ID.VP</i> [6AD]/D37.1	30	7	23.3	7	0	0.0	no
<i>PBac</i> [ <i>IT.GAL4.w-</i> ]/6.1	<i>P</i> { <i>ID.GAL4</i> [DBD]/F32.1	30	5	16.7	5	3	60.0	yes
<i>PBac</i> [ <i>IT.GAL4.w-</i> ]/3.1	<i>P</i> { <i>ID.GAL4</i> [DBD]/F32.1	30	10	33.3	9	4	44.4	yes
<i>PBac</i> [ <i>IT.GAL4.w-</i> ]/6.1	<i>P</i> { <i>ID.GAL80</i> }/E17.1	29	10	34.5	no data	no data	no data	yes
<i>PBac</i> [ <i>IT.GAL4.w-</i> ]/3.1	<i>P</i> { <i>ID.GAL80</i> }/E17.1	30	17	56.7	no data	no data	no data	yes
<i>PBac</i> [ <i>IT.GAL4.w-</i> ]/6.1	<i>P</i> { <i>ID.QF</i> }/Q10B	20	4	20.0	4	3	75.0	yes
<i>PBac</i> [ <i>IT.GAL4.w-</i> ]/3.1	<i>P</i> { <i>ID.QF</i> }/Q12A	18	5	27.8	5	3	60.0	yes
<i>PBac</i> [ <i>IT.GAL4.w-</i> ]/5.1	<i>P</i> { <i>ID.LexA</i> }/L34.2L	19	6	31.6	6	6	100.0	yes
<i>PBac</i> [ <i>IT.GAL4.w-</i> ]/5.1	<i>P</i> { <i>ID.GAL4</i> AD}/G12.1	19	5	26.3	5	3	60.0	yes
<b>Total</b>	-	<b>324</b>	<b>95</b>	<b>29.3</b>	<b>58</b>	<b>30</b>	<b>51.7</b>	<b>12 of 13</b>