Expanding the Drosophila toolkit for dual control of gene expression

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Summary

Having tools and resources for independent control of gene expression in two different tissues in the same animal is emerging as a major need, especially in the context of inter-organ communication studies. This type of study is made possible by technologies combining the GAL4/UAS and a second binary expression system such as LexA/LexAop or QF/QUAS. Here, we describe a resource of reagents that facilitate combined use of the GAL4/UAS and a second binary systems in various tissues. Focusing on genes with well-characterized GAL4 expression patterns, we generated a set of more than 40 LexA-GAD and QF2 insertions by CRISPR knock-in and verified their tissue-specificity in larvae. We also built constructs that encode QF2 and LexA-GAD transcription factors in a single vector, with each coding sequence flanked by FRT sites. Following successful integration into the fly genome, the vector generates a single polypeptide, which is separated into the individual drivers by the inclusion of T2A. If desired, one of the two coding regions can then be excised with Flp, resulting in flies that express only QF2 or LexA-GAD. We evaluated both QF2 and LexA-GAD systems for in vivo gene knockdown and are generating a compatible library of transgenic shRNA lines in our custom QUAS and LexAop vectors as a community resource. Together, these QF2/LexA-GAD and QUAS/LexAop vectors and fly lines will provide a new set of tools for researchers who need to activate or repress two different genes in an orthogonal manner in the same animal.

Introduction

Combinatorial binary systems

Most reagents available for loss of function (LOF) and gain of function (GOF) studies using RNAi or CRISPR rely on GAL4/UAS-mediated expression (Brand and Perrimon 1993; Dietzl et al. 2007; Perkins et al. 2015; Zirin et al. 2020; Port and Boutros 2022). However, some studies, such as the study of intercellular or inter-organ communication, require the simultaneous use of two independent binary transcriptional systems. For example, dual expression systems have been used to study how a Drosophila insulin-like peptide released from the wing primordium communicates with the brain to control organ growth (Colombani et al. 2015), to analyze signaling from olfactory neurons to blood cells (Shim et al. 2013), and to independently manipulate ligand-producing and ligand receiving cells (Yagi et al. 2010). Based on the need to simultaneously manipulate different sets of cells in a given tissue, the LexA/LexAop system (Lai and Lee 2006) and the QF/QUAS system (Potter et al. 2010; Potter and Luo 2011) have been developed. There have been no systematic studies comparing the two systems, with only anecdotal evidence to support one system over the other.

The numbers of available LexA and QF fly lines with tissue-specific expression domains are far lower than that represented by the thousands of GAL4 enhancer lines, which have been
developed using various approaches over the past twenty-five years. The largest existing set of LexA system driver lines were produced by the Janelia FlyLight Project and Vienna Tiles Project (Jenett et al. 2012; Tirian and Dickson 2017). However, these are brain-specific drivers and thus, not well suited for experiments targeting non-neuronal cell types. Furthermore, the FlyLight lines use a p65 transcriptional activation domain and therefore are not compatible with the Gal80 temperature sensitive Gal4 repression system. A second large collection of ~180 LexA-based enhancer trap fly stocks has been generated (the StanEx collection) (Kockel et al. 2016; Kockel et al. 2019; Kim et al. 2023). On average, each StanEx line expresses LexA activity in five distinct cell types, with only one line showing expression in just one tissue, unfortunately limiting usefulness of these reagents. These findings are consistent with prior studies indicating that enhancers very rarely produce expression patterns that are limited to a single cell type in a complex organism (Jenett et al. 2012). Regarding the Q system, there are 101 total QF lines available from the Bloomington Drosophila Stock Center (BDSC). As the original QF can be toxic when expressed at high levels (Potter et al. 2010), second generation QF2 and QF2w, which are much less toxic and can be expressed broadly in vivo were generated (Riabinina et al. 2015). Among the 51 QF2 and QF2w lines available at BDSC, most are expressed in the brain, but relatively few of these drivers are expressed specifically in other tissues. Thus, there remains an unmet need for more LexA and QF drivers with tissue-specific expression patterns. Furthermore, there are only ~260 LexA driver-compatible LexAop and ~130 QF driver-compatible QUAS stocks available at BDSC. The vast majority of these are to induce expression of fluorescent reporter genes, rather than molecular genetic reagents such as shRNAs for RNAi. This lack of fly stock reagents dramatically slows down studies that require two independent binary transcriptional systems, as custom fly stock reagents must be made by individual groups.

Rapid and efficient generation of driver lines by CRISPR-Cas9.

Several methods have been developed to generate new drivers with well-established tissue-specific patterns. However, these methods require either de novo generation of new driver lines, as for the integrase swappable in vivo targeting element (InSITE) system (Gohl et al. 2011), or PhiC31-induced insertion of a transcription factor cassette into an existing minos-mediated integration cassette (MiMIC) genomic insertion (Venken et al. 2011; Diao et al. 2015; Gnerer et al. 2015). CRISPR/Cas9 technology now makes it possible to knock-in a transcriptional activator into any locus. Some groups have also recently described tools to swap QF or LexA into the GAL4 coding region of existing GAL4 enhancer trap lines (Lin and Potter 2016; Chang et al. 2022; Karuparti et al. 2023). The conversion can be performed through genetic crosses; however, the frequency of conversion can vary greatly among different Gal4 lines, and many such swaps do not fully reproduce the original GAL4 expression patterns (Chen et al. 2019).

Despite these techniques, and the growing collection of LexA-GAD and QF2 lines, progress has been slow, and the choice of tissues depends on the specific interests of individual labs. Here, we describe the efforts of the Transgenic RNAi Project (TRiP) to facilitate combinatorial studies by building reagents that can be used alongside GAL4/UAS in a variety of tissues. Focusing on genes with well-characterized GAL4 expression patterns, we targeted 23 genes to produce 44 new highly tissue specific LexA-GAD and QF2 driver lines by CRISPR-mediated homology directed repair. Each knock-in was rigorously genotyped, and the expression pattern verified by imaging. Thus, we can systematically compare LexA-GAD vs QF2 activators inserted at precisely the same position in the genome. In addition to the new fly stocks, we provide a new set of vectors and protocols to efficiently generate LexA-GAD and/or QF2 drivers. This includes constructs that encode QF2 and LexA-GAD transcription factors in a single vector, with each coding sequence flanked by FRT sites. Following successful integration into the fly genome, the vector generates the two individual drivers by the inclusion of the T2A
ribosome-skipping peptide. If desired, one of the two coding regions can then be excised with Flp, resulting in flies that express only QF2 or LexA-GAD. Furthermore, we evaluated both QF2 and LexA-GAD systems for in vivo gene knockdown and are generating a compatible library of transgenic shRNA lines in our custom QUAS and LexAop vectors as a community resource. Together, these QF2/LexA-GAD and QUAS/LexAop vectors and fly lines will provide a new set of tools for researchers who need to activate or repress two different genes in an orthogonal manner in the same animal.

Results


We made the driver lines by inserting T2A-LexA-GAD or T2A-QF2 in the coding sequence of a target gene. We have previously reported the CRISP paint method to insert T2A-Gal4 into any gene using homology-independent repair (Bosch et al. 2020). Using this method, we showed robust gene-specific integration of donor plasmids in the fly germ line and successfully generated new driver lines. However, because of the high probability of indels at the insertion site, we opted to use traditional CRISPR homology-directed repair to insert the T2A-LexA-GAD and T2A-QF2 into the genome. We first modified the CRISP paint donor vectors to produce pHDR-T2A-LexA-GAD-Hsp70-3xP3-RFP and pHDR-T2A-QF2-Hsp70-3xP3-RFP, which contain the transcriptional activators followed by Hsp70 terminators. We also constructed pHDR-T2A-QF2-T2A-LexA-GAD-3xP3-RFP, which contains both activators flanked by FRT sites followed by SV40 terminator (Figure 1A). All the vectors contain a 3XP3-RFP transformation marker gene flanked by loxP sites. These vectors are compatible with two different cloning methods for making a CRISPR donor plasmid. In the long homology arm method (Figure 1B), ~1000bp homology arms are amplified from genomic DNA and inserted into the donor plasmid by Gibson Assembly. A separate target-gene-specific guide RNA is cloned into a U6 promoter expression vector such as pCFD3 (Port et al. 2014). In the ‘Drop-in’ cloning method (Figure 1C) (Kanca et al. 2022), a company synthesizes and clones a plasmid that contains all of the necessary features for CRISPR HDR plus a cloning site to allow ligation of T2A-LexA-GAD, T2A-QF, or T2A-LexA-GAD-T2A-QF fragments in a single step to produce the donor plasmid.

For our targets, we selected a set of genes with tissue-specific expression patterns encompassing most of the major organs of the fly (Table 1). When possible, we selected genes which had a previously characterized Gal4 insertion, and strong evidence of tissue specificity (e.g., from publicly available scRNAseq, in situ, and immunohistochemistry). Once the donors/guides were cloned, they were injected into Cas9-expressing fly embryos to induce CRISPR based HDR of the T2A-LexA-GAD or T2A-QF2 (Figure 1D-E). We selected integration sites in the most 5’ coding exon common to all or most isoforms, resulting in expression of the activators under control of the endogenous gene regulatory regions. The knock-in also produces a truncated endogenous protein and thus a strong loss-of-function allele. To verify the knock-ins, we PCR-amplified the genomic regions flanking the insertion sites and confirmed that the insertions were seamless and in-frame. Most of our knock-in stocks were made with the long homology arm method, but we transitioned to the Drop-in method as this technology became available. As Table 1 shows, we were able to successfully generate knock-ins into nearly all the target genes using these methods.

Specificity of T2A-LexA-GAD and T2A-QF2 knock-in lines.

Next, we tested the specificity of the knock-in driver lines in the 3rd instar larva (Figure 2). Each T2A-LexA-GAD and T2A-QF2 knock-in line was crossed to a LexAop-GFP and QUAS-GFP reporter, respectively. Note that most of the lines are highly tissue-specific and are comparable
between the LexA-GAD and QF2 knock-ins. Insertions in the daughterless gene (da) are an exception, as the T2A-LexA-GAD (Figure 2A), but not the T2A-QF2 (Figure 2B), gives the expected ubiquitous expression pattern. Similarly, for insertions in the nubbin gene (nub) the T2A-LexA-GAD (Figure 2LL), but not the T2A-QF2 (Figure 2MM), gives the expected expression in the wing imaginal disc. In both cases, T2A-QF is expressed in the correct tissues, but incompletely. Even with these exceptions, the patterns are remarkably consistent between the T2A-LexA-GAD and T2A-QF2 knock-in lines overall.

Our donor plasmids contain the transgenesis marker 3XP3-RFP, which expresses red fluorescence in the larval gut and anal pad, and the adult eye (Berghammer et al. 1999). Like our previous knock-ins using the CRISPaint method (Bosch et al. 2020), we sometimes observed LexA-Gad or QF2 expression in the larval anal pad and gut, coincident with expression of RFP. Figure 3 shows one such example in the repo gene, where T2A-QF2 is expressed in the expected glial cells, but also mis-expressed in RFP positive cells. Interestingly, this influence of the 3XP3-RFP is not observed in T2A-LexA-GAD repo knock-in animals (Figure 2I). Conversely, in the breathless (btl) gene, we observe misexpression of LexA-GAD, but not QF2 in RFP positive cells (Figure 2M-N). Importantly, when we removed the 3XP3-RFP cassette in the repo knock-in by cre-lox recombination, misexpression of QF2 in the gut and anal pad was completely eliminated, while the glial expression remained (Figure 3B). We therefore have removed or are in the process of removing the 3XP3-RFP marker from all the knock-in stocks. Currently, we have successfully removed it from 32 of the 41 driver lines that used this marker (Table 1).

**T2A-QF2-T2A-LexA-GAD double driver lines**

To make the T2A-LexA-GAD or T2A-QF2 knock-ins described above, each donor plasmid must be individually cloned and injected into embryos. We reasoned that by combining both drivers into a single construct we could halve the number of injections, one of the most labor-intensive and expensive parts of the process. For this combined expression of LexA-GAD and QF2 transcription factors, we built two types of vectors: 1) a CRISPR donor version, pHDR-T2A-QF2-T2A-LexA-GAD-3xP3-RFP, which is used to insert the cassette into an endogenous locus of interest (Figure 4A), and 2) a phiC31-attB version, pMCS-T2A-QF2-T2A-LexA-GAD-WALIUM20, which is used to clone an enhancer fragment of interest and is then integrated into an attP site in the fly's genome (Figure 4B). Note that the phiC31-attB compatible constructs use mini-white, not 3XP3-RFP as a marker gene. Using these vectors, we generated CRISPR knock-ins into the elav and hedgehog (hh) genes and enhancer lines for the decapentaplegic (dpp) and Insulin-like peptide 2 (Ilp2) (Figure 4F) genes. The elav knock-in (Figure 4C) expressed both T2A-QF2 and T2A-LexA-GAD in the expected pattern in the larval nervous system. However, we did see some weak non-specific expression of T2A-LexA-GAD in the somatic muscles. The hh knock-in (Figure 4D), expressed both T2A-QF2 and T2A-LexA-GAD in the expected pattern in the posterior of the imaginal discs. However, we observed that T2A-QF2, but not T2A-LexA-GAD was restricted from the wing pouch, similar to the individual nub knock-ins (Figure 4D and Figure S1LL-MM). As expected, the dpp enhancer (Figure 4E) directed expression of both T2A-QF2 and T2A-LexA-GAD along the anterior/posterior margin of the wing imaginal disc. Again, however, T2A-QF was much reduced in the wing pouch. Finally, the Ilp2 enhancer (Figure 4F) directed expression of both T2A-QF2 and T2A-LexA-GAD very specifically in the insulin-producing cells of the larval brain. Taken together, these results show that the double driver constructs can effectively drive expression of both activators simultaneously in the target tissue, with the caveat that T2A-QF2 does not express well in the wing pouch.

Next, we attempted to derive single T2A-QF2 and T2A-LexA-GAD lines from T2A-QF2-T2A-LexA-GAD double drivers. The strategy is outlined in Figure 5A. FRT3 and FRT are
mutually incompatible target sites for the Flp recombinase: FRT3 can recombine with another FRT3, and FRT with FRT, but FRT3 cannot recombine with FRT. Hence, Flp expression will result in the formation of either T2A-FRT3-LexA-GAD-FRT (caused by recombination between FRT3 sites) or T2A-FRT3-QF2-FRT (if recombination occurs between FRT sites). Both recombination products will be stable even in the presence of Flp because they now lack a pair of compatible FRT sites. This enables us to obtain the individual driver lines (QF2 and LexA-GAD) by crossing the double drivers to flies that express Flp in the germline upon heat shock. We tested this with the hh and dpp lines and observed robust generation of both T2A-QF2 and T2A-LexA-GAD from hs-Flp; T2A-QF2-T2A-LexA-GAD parents (Figure 5B). We verified the recombinants by crossing to a stock containing both LexAop-mCherry and QUAS-GFP. Figure 5C-E shows the wing disc from the hh double driver (Figure 5C), a recombinant which only expresses T2A-QF2 (Figure 5D) and a recombinant which only expresses LexA-GAD (Figure 5E). Recombinants were also independently verified by PCR (Figure 5F).

TRiP LexAop and QUAS shRNA vectors produce effective gene knockdown.

The TRiP has previously generated a QUAS version of our standard shRNA expression vector, pQUAS-WALIUM20 (Perkins et al. 2015), containing the standard five copies of the QF binding site (Potter et al. 2010). We also generated pLexAop-WALIUM20, containing thirteen LexA DNA-binding sites, previously reported to give optimal expression with minimal leakiness (Pfeiffer et al. 2010) (Figure 6A). Into these vectors, we cloned shRNAs targeting forked (f) and ebony (e) genes and assayed their phenotypes when crossed to ubiquitous LexA-GAD and QF2 drivers. The first driver tested was the T2A-LexA-GAD knock-in in the da gene (Figure 2A), generated in this study. This produced 100% penetrant forked bristle and ebony cuticle phenotypes in the adult thorax when crossed to f (Figure 6E) and e (Figure 6H) shRNA lines, respectively. For this experiment, white (w) shRNA was used as a negative control. We did not test the T2A-QF2 knock-in in the da gene, as we have already shown that the expression pattern was not ubiquitous (Figure 2G). Instead, to directly compare the two systems, we used previously described ubiquitous LexA-GAD and ubiquitous QF2 (Lai and Lee 2006) under the control of αTub84B regulatory sequences. Both Tub-LexA-GAD and Tub-QF2 drivers generated knockdown phenotypes in the thorax when crossed to f and e shRNA lines. However, the Tub-LexA-GAD phenotypes were stronger than those of Tub-QF2 (Figure 6C-D, F-G, I-J). Neither Tub-LexA-GAD or Tub-QF2 was able to achieve the strength of phenotype generated by the T2A-LexA-GAD da knock-in line.

Discussion

There have been previous efforts to make LexA and QF tools, but the availability of LexA-GAD and QF2 tissue-specific fly stocks remains a major resource gap, preventing the average fly researcher from performing the type of multi-tissue manipulations that are essential to push forward the study of organ and tissue communication. The collection of T2A-LexA-GAD and T2A-QF2 drivers described here is unique in that it is the first to focus on covering the major organ systems of the fly. Our protocol for generating these stocks is straightforward and can be easily adapted to produce driver lines for other fly tissues. These efforts will benefit from the emergence of scRNAseq datasets, which can be used to identify target genes with highly organ or tissue-specific gene expression patterns. For example, we identified the uncharacterized genes CG9458 and CG17560, as highly oenocyte-specific based on the Fly Cell Atlas Single-cell transcriptome (Li et al. 2022), and the T2A-LexA-GAD and T2A-QF2 knock-ins in these genes were indeed restricted to this tissue. Our collection of T2A-LexA-GAD and T2A-QF2 and double driver vectors can be easily adapted to target any gene for CRISPR knock-in, with a high probability that the resulting line will accurately reflect the expression of the endogenous locus.
Our vectors are compatible with both the traditional large homology arm flanked cassettes and the more streamlined drop-in approach. In our hands, the drop-in cloning strategy is particularly effective, as the cloning success rate is 100%, requires little troubleshooting, has a very high knock-in rate, and only costs ~$100 to synthesize the construct (Kanca et al. 2019; Kanca et al. 2022).

Our results also present the opportunity to directly compare the LexA-GAD and QF2 systems. While we had no difficulty obtaining knock-ins for both types of activators, we did observe that for some target genes, the T2A-QF2 was only active in a subset of the expected gene expression pattern. In particular, we found that T2A-QF2 was difficult to express in the wing pouch. It may be that toxicity is an issue, and the weaker QF2w may be a better option for generating drivers in some organs (Riabinina and Potter 2016). When we compared the knockdown efficiency of shRNAs targeting forked and ebony and, we found that the TRiP 13XLexAop vector was more effective than the 5XQUAS vector, although both were able to induce knockdown. Based on these results, the TRiP is currently generating a set of ~100 LexAop shRNA lines encompassing the genes targeted by the most commonly ordered UAS shRNA stocks at the BDSC. See Supplementary Table 1 for a list of all LexAop shRNA lines in production. In summary, we have generated a set of stocks, vectors, and protocols that when combined with the wide array of GAL4 lines will greatly expand the ability of Drosophila researchers to modulate gene expression in multiple tissues simultaneously.

Materials and methods

Generation of pHDR-T2A-LexA/QF2-Hsp70-3xP3-RFP plasmids

To generate the pHDR-T2A-LexA/QF2-Hsp70-3xP3-RFP plasmids, we replaced the SV40-3'UTR present in the pCRISPaint-T2A-LexA/QF2 vector (Bosch et al. 2020) with the hsp70-3'UTR, using Gibson assembly (Gibson et al. 2009) (NEB E2611). The hsp70-3'UTR was amplified from a pCRISPaint-Gal4-Hsp70 plasmid using the following primers: F: GTCGACTAAAGCCAAATAG, R: AAACGAGTTTTTAAGCAAAC, appended at the 5' end with appropriate homologous overhangs for Gibson assembly. To remove the two endogenous SacI sites in the QF2 coding sequence, we used Gibson assembly featuring primers that introduce synonymous SNPs which mutate the SacI binding sites without disrupting the coding sequence (GAGCTC > GAACTC).

Cloning of T2A-LexA-GAD and T2A-QF2 donor constructs

For the long-homology arm cloning method, we amplified the homology arms (HAs) by selecting ~1000kb upstream and downstream of the guide cut site, making sure that the left HA is in frame with the T2A, and that the ends of the primers contain Gibson overhangs matching the pHDR-T2A-LexA/QF2-Hsp70-3xP3-RFP plasmids. Amplification was always from genomic DNA from the nos-Cas9 injection stock. We used Phusion (NEB), Taq (Takara) or Q5 (NEB). In cases where the PCR product was faint, we set up 8 PCR samples in parallel, combined them, concentrated them using phenol-chloroform extraction followed by ethanol precipitation, and ran the concentrated sample on a gel to obtain a bright band that was then gel-purified for use in Gibson Assembly. Once the HAs were amplified, we performed Gibson assembly with the pHDR-T2A-LexA/QF2-Hsp70-3xP3-RFP plasmids digested with Ascl/Sacl. Guide RNAs were cloned separately in pCDF3 (Port et al. 2014). We chose previously designed gRNAs from https://www.flyrnai.org/crispr3/web/. Our criteria were: efficiency >5, and no U6 termination site. Primer and guide sequences are in Supplementary Table 2. We designed sense and antisense oligos for each gRNA, and then annealed them together to make a ds-oligo with overhangs for cloning: we combined 1.0 µl each 100 µM sense + antisense oligo, 1.0 µl 10x T4 ligase buffer,
0.5 µl T4 polynucleotide kinase (NEB), and 6.5 µl dH2O, and incubated at 37°C 30 min followed by 5 min at 95°C and slowly cooling down to room temperature (-5°C/min). The ds-oligos were then ligated into BbsI-digested pCFD3 vector with T4 ligase (NEB). Following cloning, plasmids were verified by sequencing with primer (GCCGAGCACAATTGTCTAGAATGC).

For the Drop-in method, we followed a modified version of the protocol described elsewhere (Kanca et al. 2022). Briefly, homology donor intermediate vectors were ordered from Genewiz in the pUC57 Kan_gw_OK2 vector backbone, containing the gene-specific guide sequence, 200 bp short homology arms flanking the genomic cut site, and a BbsI and Sacl cloning site. pHDR-T2A-LexA/QF2-Hsp70-3xP3-RFP plasmids were digested with Ascl/Sacl, producing a 2677 bp fragment for QF2 and the ~4.5kb fragment for LexA-GAD, each with overhangs compatible with the pUC57 Kan_gw_OK2 BbsI/Sacl overhangs. The digested pUC57 Kan_gw_OK2 backbone, containing the homology arms and guides was then ligated with the digested T2A-LexA/QF2-Hsp70-3xP3-RFP with 2.5 µl 10× T4 DNA ligase buffer (NEB B0202S) and 0.5 µl T4 DNA ligase (NEB M0202S). Sequences of the synthesized Drop-in fragments are in Supplementary Table 3.

Construction of T2A-QF2-T2A-LexA-GAD double driver constructs

For combined expression of LexA-GAD and QF2 transcription factors, we built two different vectors: 1) a CRISPR donor version, which we used to insert the LexA-GAD-QF2 cassette into an endogenous locus of interest, such that the expression of lexA-GAD and QF2 is driven by endogenous regulatory sequences, and 2) a φC31-attB version, which is used to clone an enhancer fragment of interest and integrated into an attP site in the fly genome.

To build the pHDR-T2A-QF2-T2A-LexA-GAD-3XP3-RFP construct, we used pCRISPaint-T2A-QF2 and pCRISPaint-T2A-LexA-GAD vectors (Bosch et al. 2020) to assemble the vector as follows (See also Supplementary Table 4):

1. A gBlock™ double-stranded DNA fragment covering the N-terminus of the QF2 ORF along with T2A, FRT3, and homology arms for Gibson assembly (BJusiak-QF2-N);
2. Part of the QF2 ORF amplified as a PCR product off the CRISPaint-T2A-QF2 vector using BJusiak-QF2-fwd+rev primer pair;
3. A gBlock™ encoding the C-terminus of QF2 and the FRTwt-T2A-FRT3 sequence between QF2 and LexA-GAD, along with homology arms for Gibson assembly (BJusiak-QF2-C);
4. Most of the LexA-GAD ORF, PCR-amplified off the CRISPaint-T2A-LexA-GAD template with the BJusiak-lexA1-fwd+rev primer pair;
5. BJusiak-FRToligo1-top+bottom, a pair of single-stranded oligos annealed to make a ds-oligo encoding the FRTwt site 3’ of LexA-GAD;
6. CRISPaint vector digested with ApaI + KpnI restriction enzymes.

gBlocks™ and oligos were ordered from Integrated DNA Technologies (IDT) and restriction enzymes were from NEB. The sequences of all gBlocks™ and oligos used to build pHDR-QF2-LexA-GAD are in Supplementary materials and methods. Homology arms and guides for CRISPR were cloned as described above for the T2A-LexA-GAD and T2A-QF2 single donor constructs.

We digested CRISPaint-T2A-QF2 with ApaI at 25°C in CutSmart buffer, followed by digestion with KpnI-HF at 37°C. We ran the digest on an agarose gel and purified the 4.9 kb vector backbone. We then set up the Gibson Assembly:

- Vector backbone (4,938 bp) 100 ng
- lexGAD PCR product (2,875 bp) 117 ng 2:1 insert:vector molar ratio
- QF2 PCR product (292 bp) 18 ng 3:1 insert:vector
We added dH₂O to 10.0 µl final volume, added 10.0 µl Gibson Assembly Mix (NEB), and incubated at 50°C for 1h. We transformed 5.0 µl of the Gibson Assembly reaction into Top10 chemically competent E. coli and plated on LB + Carbenicillin, then screened E. coli colonies by PCR with the BJusiak-Qlex-test1-fwd+rev primer pair (Supplementary Table 4), expected to give a 0.7 kb product in presence of correctly assembled CRISPaint-QF2-lexGAD. Plasmid DNA was prepared from positive colonies using the ZymoPure midiprep kit. Restriction digest fingerprinting of the plasmid midipreps produced expected band patterns, which were verified by sequencing.

To build the pMCS-T2A-QF2-T2A-LexA-GAD-WALIUM20 construct, we performed Gibson Assembly with the following fragments (See also Supplementary Table 4):

1. MCS-WAL20-START gBlock™, including the MCS, hsp70 promoter, T2A-FRT3 coding sequence, and homology arms for Gibson assembly;
2. MCS-WAL20-STOP-v2 gBlock™, including the 3’ end of LexA-GAD ORF, FRT coding sequence, and STOP codon;
3. QF2-T2A-LexA-GAD ORF PCR-amplified with FRT-QF2-fwd + FRT-lexA-GAD-rev primer pair – used Hot-Start Q5 polymerase with GC enhancer (NEB);
4. WALIUM20 vector digested with BamHI + EcoRI.

We transformed the Gibson Assembly reaction into Top10 chemically competent E. coli and plated them on LB + Carbenicillin. We screened colonies with PCR using the BJusiak-Qlex-test1-fwd+rev primer pair, same as for CRISPaint-QF2-lexGAD. Positive colonies were used for plasmid preps, which were sent for Sanger sequencing (Azenta) with the QF2-seq1-rev primer (TGTTAGTGAGATCAGCGAAC expected to read across the MCS-hsp70 region).

To make a variant pMCS-QF2-LexA-GAD-alt that lacks the Hsp70 promoter, we did Gibson assembly as above, except we replaced the MCS-WAL20-START with MCS-WAL20-START-new gBlock™, which lacks the Hsp70 sequence.

Cloning homology arms into pHDR-T2A-QF2-T2A-LexA-GAD-3XP3-RFP:

Homology arms were amplified by PCR as described above. See also Supplementary Table 5 for the PCR primers. We then digested pHDR-T2A-QF2-T2A-LexA-GAD-3XP3-RFP separately with Ascl + SaCl to release the vector backbone and with NotI + Sacl to purify the T2A-QF2-T2A-lexA-GAD-3XP3-RFP fragment. We gel-purified the backbone, T2A-QF2-T2A-lexA-GAD-3XP3-RFP fragment, and the homology arm PCR products, and we assembled all four fragments using Gibson Assembly. We used 50 ng vector backbone, 2-fold molar excess of T2A-QF2-T2A-lexA-GAD-3XP3-RFP, and 3-fold molar excess of each homology arm. We transformed the Gibson Assembly product into Top10 chemically competent E. coli, miniprepped (Qiagen) and verified by sequencing. Guides were cloned into pCFD3 as described above.

Cloning large enhancer fragments into pMCS-T2A-QF2-T2A-lexA-GAD-WALIUM20:

Ilp2-Gal4 has been described (Wu et al. 2005). The dpp-blk enhancer was described as a “4 kb BamHI fragment” (Masucci et al. 1990) that is 17 kb 3’ of the dpp transcribed region (Blackman et al. 1987)(Blackman et al 1987). The primers used to PCR these fragments, using fly genomic DNA as template, are in Supplementary Table 6. After PCR-amplifying the enhancer fragment with Q5 polymerase (+GC for Ilp2, no GC for dpp), we digested it and the destination vector with the corresponding enzymes (NotI + EcoRI for dpp-blk, PacI + EcoRI for Ilp2). We used pMCS-
T2A-QF2-T2A-lexA0GAD-WALIUM20 for dpp-blk and pMCS-T2A-QF2-T2A-lexGAD-WALIUM20-alt (which lacks the hsp70 promoter) for Ilp2, since dpp-blk does not have a basal promoter, but the Ilp2 enhancer does. We ligated the PCR fragments into the vectors using T4 ligase (NEB), transformed into E. coli, miniprepped (Qiagen) and verified by sequencing.

**Cloning shRNAs**

shRNAs (21 bp) were cloned into pQUAS-WALIUM20 and pLexAop-WALIUM20 vectors digested with EcoRI + XbaI, as described previously (Ni et al. 2011). The oligos were as follows:

<table>
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<th>Gene</th>
<th>Oligo forward</th>
<th>Oligo reverse</th>
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<tr>
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<td>CGCTGaccc</td>
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<tr>
<td></td>
<td>GGTCGAgcg</td>
<td>TCGGAactcg</td>
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</table>

All transgenic lines were sequenced to confirm the identity of the shRNA.

**Fly injections**

All CRISPR constructs were injected at 250 ng/µl along with 100 ng/µl gene-specific gRNA(s) where appropriate. 300 embryos from y w ; iso18; attP2, nos-Cas9 for genes on the X, second or fourth chromosomes and y w ; attP40, nos-Cas9; iso5 for genes on the third chromosome per genotype were injected as described previously (Lee et al. 2018). Resulting G0 males and females were crossed individually to appropriate balancer flies for 3XP3-RFP screening. Positive lines were balanced, and stocks were established. For phiC31-integration, each plasmid was injected at 50 ng/µl into y v nos-phiC31-int; attP40 (for chromosome 2 insertions) or y v nos-phiC31-int; attP2 (for chromosome 3 insertions). Injected male G0 flies were crossed with y w; Gla/CyO or y w; Dr e/TM3, Sb to identify transformants and remove the integrase from the X chromosome, and subsequently balanced.

**PCR validation of knock-ins**

PCR primers that flank the integration site were designed for each targeted gene. These primers were used in combination with primers that bind within the inserted cassette in both
orientations. 500–800 nt amplicons were amplified from genomic DNA from individual insertion lines through single fly PCR using GoTaq green master mix (Promega M7122).

Imaging

T2A-LexA-Gad, T2A-QF2, or double driver lines were crossed to y w; Sp/CyO; LexAop-GFP (BDSC 52266), y w; QUAS-GFP/CyO (BDSC 52264), or y w; QUAS-GFP (BDSC 52264)/CyO-GFP; LexAop-RFP (BDSC 52271)/TM3. Larvae were placed in PBS and sandwiched between a slide and coverslip, then live-imaged using a Zeiss (Carl Zeiss, Thornwood, NY) Stemi SVII fluorescence microscope. Wing imaginal discs from third instar larvae were dissected in PBS, fixed in 4% methanol free formaldehyde, and permeabilized in PBT, mounted on glass slides with vectashield (H-1000; Vector Laboratories) under a coverslip, and imaged on a Zeiss 780 confocal microscope.
Acknowledgements

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References


Figure Legends

Figure 1: Strategy for CRISPR knock-in of T2A-LexA-GAD and T2A-QF2. (A) Donor vectors for knock-in. pHDR-T2A-LexA-GAD-Hsp70-3xP3-RFP and pHDR-T2A-QF2-Hsp70-3xP3-RFP contain T2A-LexA-GAD or QF2 transcriptional activators followed by HSP70 terminators. pHDR-T2A-QF2-T2A-LexA-GAD-3xP3-RFP contains both activators flanked by FRT sites followed by SV40 terminator. All the vectors contain a 3XP3-RFP transformation marker flanked by loxP sites. (B) Long homology arm cloning method. ~1000bp homology arms are amplified from genomic DNA and inserted into the Ascl and SacI sites by Gibson Assembly. A separate target-gene-specific guideRNA is cloned into U6 promoter expression vector such as pCFD3. (C) Drop-in cloning method. Based on the gRNA-int200 method previously described (Kanca et al., 2022). A company synthesizes and clones a DNA fragment into the pUC57_Kan_gw_OK2 vector. The resulting plasmid contains the following elements: 1) two guide RNAs under the control of a U6 promoter, one (gRNA1) targeting the vector (pink arrows) to linearize the homology donor in vivo, and another (gRNAgeneX) targeting the gene of interest; 2) a tRNA sequence to allow liberation of the individual guides by the endogenous tRNA processing machinery; 3) 200 bp short homology arms; and 4) BbsI and SacI cloning sites. The Ascl/Sacl T2A-LexA-GAD, T2A-QF, or T2A-LexA-GAD-T2A-QF fragments can then be ligated in a single directional cloning step into the BbsI/Sacl sites to produce the donor plasmid. (D) T2A-LexA-GAD and (E) T2A-QF2 knock-in strategy. CRISPR based HDR causes integration of the T2A-LexA-GAD or T2A-QF2 in the most 5’ coding exon common to all or most isoforms, resulting in expression of the activators under control of the endogenous gene regulatory region. The knock-in also produces a truncated endogenous protein and thus a strong loss-of-function allele.

Figure 2: Tissue specificity of T2A-LexA-GAD and T2A-QF2 knock-ins. (A-KK) T2A-LexA-GAD knock-in lines crossed to a LexAop-GFP reporter and T2A-QF2 knock-in lines crossed to a QUAS-GFP reporter. Panels show 3rd instar larva. GFP shows the driver line expression pattern. RFP shows the 3XP3 transformation marker, which labels the posterior gut and anal pads of the larva. Gene names and tissues are on the left. Note that most of the lines are highly tissue-specific and are comparable between the LexA-GAD and QF2 knock-ins. (LL-MM) 3rd instar imaginal disc from the insertions in the nubbin (nub) gene. Insertions in the daughterless gene (da) and nub are the exception, as the T2A-LexA-GAD, but not the T2A-QF2, gives the expected expression pattern.

Figure 3: 3XP3-RFP can cause misexpression of T2A-LexA-GAD or T2A-QF2. (A) T2A-QF2-3XP3-RFP in the repo gene crossed to a QUAS-GFP reporter. In 3rd instar larva, the reporter is expressed in the expected glial cells, but also mis-expressed in gut and anal pad (yellow asterisk). (B) T2A-QF2 in the repo gene with the 3XP3-RFP removed by Cre-Lox recombination, crossed to a QUAS-GFP reporter. Removal of 3XP3-RFP eliminated gut and anal pad misexpression and did not affect glial cell expression.
Figure 4: T2A-QF2-T2A-LexA-GAD double driver lines. (A) CRISPR based HDR strategy for integration of the T2A-QF2-T2A-LexA-GAD-3XP3 in the most 5’ coding exon common to all or most isoforms, resulting in expression of both activators under control of the endogenous gene regulatory region. The knock-in also produces a truncated endogenous protein and thus a strong loss-of-function allele. If desired, one of the two coding regions can then be excised with Flp, resulting in flies that express only QF2 or LexA-GAD. (B) Alternative strategy allows gene enhancers to be cloned upstream of T2A-QF2-T2A-LexA-GAD. The vector backbone includes an attB site for phiC31 insertion into attP flies. (C-D) T2A-QF2-T2A-LexA-GAD knock-ins crossed to a QUAS-GFP + LexAop-mCherry double reporter line. (C) The elav\textsuperscript{T2A-QF2-T2A-LexA-GAD} knock-in drives both QUAS-GFP and LexAop-mCherry in the larval brain. There is some leakiness of mCherry in the body wall muscle (arrowheads). (D) The hh\textsuperscript{T2A-QF2-T2A-LexA-GAD} knock-in drives both QUAS-GFP and LexAop-mCherry in the posterior of the wing imaginal disc. GFP expression is much less than mCherry in the wing pouch (asterisks). (E-F) enhancer-T2A-QF2-T2A-LexA-GAD lines crossed to a QUAS-GFP + LexAop-mCherry double reporter line. (E) The dpp-blk enhancer-T2A-QF2-T2A-LexA-GAD line drives both QUAS-GFP and LexAop-mCherry along the anterior/posterior boundary of the wing imaginal disc. GFP expression is much less than mCherry in the wing pouch (stars). (F) The Ilp2 enhancer-T2A-QF2-T2A-LexA-GAD line drives both QUAS-GFP and LexAop-mCherry in the insulin-producing cells of the larval brain (arrows). The fat body mCherry expression is also present in the reporter stock and does not indicate LexA-GAD activity.

Figure 5: Generation of single drivers from T2A-QF2-T2A-LexA-GAD knock-ins by hs-FLP. (A) FLP/FRT recombination scheme. Flies containing both hs-FLP and a T2A-QF2-T2A-LexA-GAD knock-in are heat shocked during larval development to induce one of two mutually exclusive recombination events in their germline between either FRT or FRT3. (B) heat shock of hsFLP; hh\textsuperscript{T2A-QF2-T2A-LexA-GAD} and hsFLP; dpp\textsuperscript{T2A-QF2-T2A-LexA-GAD} flies produces frequent recombinants, both T2A-QF2 and T2A-LexA-GAD. The bar graph shows the proportion of heat-shocked animals that produced at least one recombinant offspring. The dot plot shows the proportion of recombinant offspring per heat-shocked parent. Mean +/- SD is indicated. (C-D) Validation of individual hh\textsuperscript{T2A-QF2} and hh\textsuperscript{T2A-LexA-GAD} derivatives by immunofluorescence. All panels show 3rd instar larval wing discs dissected from potential hh\textsuperscript{T2A-QF2-T2A-LexA-GAD} recombinants crossed to a QUAS-GFP + LexAop-mCherry reporter line. (C) Wing disc from non-recombinant hh\textsuperscript{T2A-QF2-T2A-LexA-GAD} showing expression of both GFP and mCherry in the posterior of the wing disc. (D) Wing disc from recombinant hh\textsuperscript{T2A-QF2} showing expression of GFP but not mCherry in the posterior of the wing disc. (E) Wing disc from recombinant hh\textsuperscript{T2A-LexA-GAD} showing expression of mCherry but not GFP in the posterior of the wing disc. Validation of (F) hh\textsuperscript{T2A-QF2} and hh\textsuperscript{T2A-LexA-GAD} derivatives and (G) dpp\textsuperscript{T2A-QF2} and dpp\textsuperscript{T2A-LexA-GAD} derivatives by PCR from genomic DNA from individual flies. In all panels, for brevity, T2A-QF2-T2A-LexAop, T2A-QF2 and T2A-LexAop, are notated as Q+L, Q(-L) and L(-Q), respectively.

Figure 6: TRiP LexAop and QUAS shRNA vectors produce effective gene knockdown. (A) shRNAs for knockdown or genes for overexpression were cloned into pLexAop-WALIUM20 and pQUAS-WALIUM20, derived from the TRiP WALIUM20 vector. (B-J) Dorsal view of adult fly thoraces resulting from crosses of LexAop or QUAS shRNAs to da\textsuperscript{T2A-LexA-GAD} (generated in this study), Tub-LexA-GAD (BDSC 66686), or Tub-QF2 (BDSC 51958). (B-C) white shRNA control produced no thoracic phenotypes in any of the crosses. (E-G) forked shRNA produced a forked bristles phenotype. (H-J) ebony shRNA produced a darkened cuticle phenotype. The da\textsuperscript{T2A-LexA-GAD} driver produced the strongest phenotype (compare panel H to I and J).
Table 1: T2A-LexA-GAD and T2A-QF2 knock-in lines

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<th>target gene (name)</th>
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<th>others at BDSC</th>
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*constructs were cloned using the Drop-in method. All others were cloned by PCR of long homology arms.
†constructs were cloned into double driver vectors.
Figure 1

A T2A-LexA-GAD and T2A-QF2 vectors

B Long homology arm cloning method

C Drop-in cloning method

D T2A-LexA-GAD knock-in strategy

E T2A-QF2 knock-in strategy

truncated endogenous protein
Figure 2

<table>
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<tr>
<th>Gene Combination</th>
<th>GFP</th>
<th>RFP</th>
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<td>B</td>
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- da (ubiquitous)  
- vas (germline)  
- Mef2 (muscle)  
- br (muscle)  
- repo (glia)  
- apolpp (fat)  
- btl (trachea)  
- Hand (heart)  
- CG3458 (hemocyte)  
- CG17560 (hemocyte)  
- Sgs3 (germline gland)  
- phm (germline gland)  
- mex1 (megagut)  
- Myo3DF (hemocyte)  
- Hml (hemocyte)  
- He (hemocyte)  
- PPO1 (crystal cell)  
- PPO3 (hemocyte)  
- nub (vein)  
- nub (magnified)  
- ND (not determined)
Figure 3

repo\textsuperscript{T2A-QF2-3XP3-RFP} x QUAS-GFP

repo\textsuperscript{T2A-QF2} x QUAS-GFP
Figure 4

A

CRISPR induced HDR

B

C31 induced integration

C

MERGE  mCherry  GFP

D

E

F

D''
Figure 5

**A** hs-FLP in germline → recombination
- 1. between FRT
- 2. between FRT3

**B** proportion hs-FLP parents with recombinant offspring
- hh
- Q (L)
- Q (L)
- dpp
- Q (L)

**C** MERGE mCherry GFP
- hh Q (L)
- Q (L)
- L (Q)

**D** Q (L)
- Q (L)
- Q (L)

**E** Q (L)
- Q (L)
- Q (L)

**F** hh ladder Q+L L (Q) Q (L)
- expected size
- 5741 bp
- 4561 bp
- 2762 bp

**G** dpp ladder Q+L L (Q) Q (L)
- expected size
- 4540 bp
- 3361 bp
- 1561 bp
Figure 6

A diagram showing the constructs pLexAop-WALIUM20 and pQUAS-WALIUM20, each containing elements such as gypsy, 13X LexAop, hsp70, shRNA cloning site, ftz intron, SV40, and AmpR. Below, images labeled B, C, D, E, F, G, H, I, and J show different shRNA constructs used in experiments, with annotations indicating the expression of daT2A-LexA-GAD, Tub-LexA-GAD, Tub-QF2, white shRNA, forhead shRNA, and ebony shRNA, respectively. The images depict developmental stages or phenotypes associated with each construct.