The Q System: A Repressible Binary System for Transgene Expression, Lineage Tracing, and Mosaic Analysis

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SUMMARY

We describe a new repressible binary expression system based on the regulatory genes from the Neurospora qa gene cluster. This “Q system” offers attractive features for transgene expression in Drosophila and mammalian cells: low basal expression in the absence of the transcriptional activator QF, high QF-induced expression, and QF repression by its repressor QS. Additionally, feeding flies quinic acid can relieve QS repression. The Q system offers many applications, including (1) intersectional “logic gates” with the GAL4 system for manipulating transgene expression patterns, (2) GAL4-independent MARCM analysis, and (3) coupled MARCM analysis to independently visualize and genetically manipulate siblings from any cell division. We demonstrate the utility of the Q system in determining cell division patterns of a neuronal lineage and gene function in cell growth and proliferation, and in dissecting neurons responsible for olfactory attraction. The Q system can be expanded to other uses in Drosophila and to any organism conducive to transgenesis.

INTRODUCTION

The ability to introduce engineered transgenes with regulated expression into organisms has revolutionized biology. A popular strategy for regulating expression of an effector transgene is to use a binary expression system. In this strategy, one transgene contains a specific promoter driving an exogenous transcription factor, while the other transgene uses the promoter activated only by that transcription factor to drive the effector gene. As a result, the effector gene is controlled exclusively by the chosen transcription factor, and the expression pattern of the effector transgene corresponds to the expression pattern of the exogenous transcription factor (Figure 1A). A number of binary expression systems have been established in genetic model organisms, including tetracycline-regulable tTA/TRE in mice (Gossen and Bujard, 1992) and GAL4/UAS in flies (Fischer et al., 1988; Brand and Perrimon, 1993). Compared to effector transgenes driven directly by a promoter, binary systems offer several advantages. First, binary systems usually result in higher levels of effector transgene expression as a result of transcription factor-mediated amplification. Second, expression of some effectors directly by a promoter may cause lethality and thus prevent the generation of viable transgenic animals; in binary systems, the effector transgene is not expressed until the exogenous transcription factor is introduced into the same animal, usually through a genetic cross. Third, some transcription factors used in binary systems can be additionally regulated by small molecule ligands and thus offer temporal control of transgene expression. Lastly, libraries of transgenes expressing a transcription factor and/or corresponding effectors can be established, such that the transcription factor and effector transgenes can be systematically combined by genetic crosses to enable expression of the same effector transgene in different patterns, or different effector transgenes in the same pattern, thereby enabling a variety of genetic screens in vivo.

The impact of the budding yeast-based GAL4/UAS binary expression system on studies of Drosophila biology cannot be overstated. Thousands of GAL4 lines have been characterized for expression in specific tissues and developmental stages (Brand and Perrimon, 1993; Hayashi et al., 2002; Pfeiffer et al., 2008). Tens of thousands of UAS-effector lines have also been established (Rørth et al., 1998; Fischer et al., 1988; Brand and Perrimon, 1993). Compared to effector transgenes driven directly by a promoter, binary systems offer several advantages. First, binary systems usually result in higher levels of effector transgene expression as a result of transcription factor-mediated amplification. Second, expression of some effectors directly by a promoter may cause lethality and thus prevent the generation of viable transgenic animals; in binary systems, the effector transgene is not expressed until the exogenous transcription factor is introduced into the same animal, usually through a genetic cross. Third, some transcription factors used in binary systems can be additionally regulated by small molecule ligands and thus offer temporal control of transgene expression. Lastly, libraries of transgenes expressing a transcription factor and/or corresponding effectors can be established, such that the transcription factor and effector transgenes can be systematically combined by genetic crosses to enable expression of the same effector transgene in different patterns, or different effector transgenes in the same pattern, thereby enabling a variety of genetic screens in vivo.

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a repressible cell marker) (Lee and Luo, 1999). With MARCM, mosaic animals can be created that contain a small population of genetically defined cells labeled by a transgenic marker (such as GFP). At the same time, these labeled cells can be homozygous mutant for a gene of interest and/or modified with additional effector transgenes. MARCM has been widely used for lineage analysis, for tracing neural circuits, and for high-resolution mosaic analysis of gene function (Luo, 2007).

The versatile GAL4/UAS system still has limitations. The GAL4 expression patterns from enhancer trap lines or promoter-driven transgenes often include cells other than the cells of interest. It is thus difficult to assign the effect of transgene expression to a specific cell population, especially when phenotypes, such as behavior, are assayed at the whole organism level. Additionally, analysis of gene function and dissection of complex biological systems in multicellular organisms often requires independent genetic manipulations of separate populations of cells. For the improvement of the precision of transgene expression, intersectional expression methods such as the split GAL4 system (Luan et al., 2006) or the combined use of GAL4/UAS and FLP/FRT (Stockinger et al., 2005) have been introduced. To enable independent manipulation of separate populations of cells, additional binary systems such as the lexA/lexAO system have been developed (Lai and Lee, 2006). Here, we describe a new repressible and small molecule-regulable binary expression system, the Q system, which offers significant advantages and versatility compared to the existing systems.

The Q system utilizes regulatory genes from the Neurospora crassa qa gene cluster. This cluster consists of five structural genes and two regulatory genes (qa-1F and qa-1S) used for the catabolism of quinic acid as a carbon source (Giles et al., 1991). QA-1F (shortened as QF hereafter) is a transcriptional activator that binds to a 16 bp sequence present in one or more copies upstream of each qa gene (Patel et al., 1981; Baum et al., 1987). QA-1S (shortened as QS hereafter) is a repressor of QF that blocks its transactivation activity (Huiet and Giles, 1986) (Figure 1A). Here, we explore the properties of the Q system in cultured fly and mammalian cells, and demonstrate its utility for transgene expression, lineage tracing and genetic mosaic analysis in Drosophila in vivo.

RESULTS AND DISCUSSION

Characterization of the Q System in Drosophila and Mammalian Cells

To test whether qa cluster genes function in biological systems besides Neurospora, we created expression constructs for transient transfection of Drosophila and mammalian cells. We used the same ubiquitous promotors to drive QF and QS: actin 5c for Drosophila and CMV for mammalian cells. We generated...
a reporter plasmid containing the synthetic firefly luciferase (luc2) gene under the control of five copies of the QF binding site, which we termed QUAS, and the Drosophila hsp70 minimal promoter. We also created the GAL4 system equivalents as controls and for quantitative comparisons with the Q system. Transfection of Drosophila S2 cells with QF and QUAS-luc2 resulted in ~3300-fold enhancement of luc2 expression compared with QUAS-luc2 alone (Figure 1B). For comparison, GAL4 induced luc2 expression from UAS-luc2 by ~5300-fold (Figure 1B) and therefore had ~1.6-fold higher inducibility than QF/QUAS. GAL4/UAS also reached ~1.8-fold higher level of reporter expression than QF/QUAS. Cotransfection of QS with QF and QUAS-luc2 resulted in dosage-dependent suppression of luc2 expression (Figure 1B). Full suppression was not observed with equimolar ratios of QF and QS (similar lack of full suppression was observed with GAL4/GAL80). Quinic acid, which relieves suppression of QS in Neurospora (Giles et al., 1991), significantly suppressed QS to restore QF-based transcription (Figure S1A available online). Finally, QF and GAL4 showed minimal cross-activation of UAS and QUAS, respectively (Figure 1B, middle)—QF activation of UAS was ~1500-fold less than that of QUAS; GAL4 activation of QUAS was ~200-fold less than that of UAS.

In human HeLa cells (Figure 1C), the Q system behaved similarly as in Drosophila S2 cells, but with the following distinctions. First, QF induced expression from QUAS by ~24,000-fold, compared to ~1000-fold induction of UAS by GAL4. Therefore, in human cells, QF/QUAS achieves ~24-fold higher inducibility and ~30-fold higher level of reporter expression than GAL4/UAS. Second, higher QS:QF or GAL80:GAL4 molar ratios are required for effective suppression in HeLa cells compared with Drosophila S2 cells. Third, quinic acid does not suppress QS in mammalian cells, but seems to activate it further to make it an even better repressor (Figure S1B); the reasons for this unexpected behavior in mammalian cells are unknown. All these distinctions were also observed in COS cells (data not shown). Taken together, these experiments demonstrate that the Q repressible binary expression system is effective in Drosophila and mammalian cells.

Repressible Binary Transgene Expression with the Q System in Drosophila In Vivo

To test whether the Q system functions in Drosophila in vivo, we generated transgenic flies that express (1) different markers under the control of QUAS, (2) QF under the control of a specific promoter or in enhancer trap vectors, and (3) QS under the control of a ubiquitous tubulin promoter (tubP-QS) (Table S1).

Figures 2A and 2B (left panels) show low basal fluorescence in whole mount Drosophila adult brains harboring only reporter transgenes, QUAS-mCD8-GFP (full-length mouse CD8 followed by GFP) or QUAS-mdtT-HA (myristoylated and palmitoylated tandem dimer Tomato followed by three copies of the HA epitope). The low basal expression of QUAS and UAS reporters provides significant advantage over the lexA binary expression system (Lai and Lee, 2006). All QUAS-mCD8-GFP transgenic flies have basal reporter expression comparable to or lower than the lexA-mCD2-GFP line with the lowest reporter expression (Figure S2A). Low basal expression was also observed in other QUAS reporters such as QUAS-mdtT-HA (Figure S2A, data not shown). These observations suggest that the QUAS promoter is not easily influenced by genomic enhancers near the transgene insertion site and that flies do not contain endogenous proteins capable of inducing significant expression from QUAS-transgenes at least within the tissues we examined.

Introduction of transgenes expressing QF into flies containing QUAS-markers results in strong marker expression. For example, QF driven by the GH146 enhancer (Stocker et al., 1997; Berdnik et al., 2008) drives strong reporter gene expression in olfactory projection neurons (PNs; Figures 2A2, 2A3, 2B2, and 2B3). We also isolated enhancer trap lines that drive strong reporter expression in imaginal discs and adult tissues including large subsets of neurons and glia (Figure 2C, middle; Figures S2B and S3). Expression of these transgenes was effectively suppressed by ubiquitous expression of QS (Figures 2A4, 2B4, and 2C, right; Figure S2B). These experiments show that the Q repressible binary system is as effective in vivo as the widely used GAL80/GAL4/UAS system (Brand and Perrimon, 1993; Lee and Luo, 1999).

The Q system provides an additional level of control compared to the GAL4 system: inhibition of QS by quinic acid. Addition of increasing doses of quinic acid to fly food on which flies developed increasingly reverted the QS inhibition of enhancer trap ET40-QF driven QUAS-mdtT-HA expression (Figure 2D). When adult flies were transferred to quinic acid-containing food, reversion of suppression could be seen after 6 hr, with marked reversion after 24 hr and saturation by day 5 (Figure 2E, data not shown). Flies kept for nine generations on food containing high doses of quinic acid, a natural product present at ~1% in cranberry juice (Nollet, 2000), exhibited no noticeable abnormalities. Quinic acid can thus be used to temporally regulate QF-driven transgene expression. For instance, one can suppress developmental expression of a transgene and allow reactivation in adult for behavioral analysis, analogously to the GAL80(PA0 strategy (McGuire et al., 2003). This manipulation can be achieved without changing the temperature, thereby avoiding complications with temperature-sensitive behaviors.

Q-MARCM

An incentive to develop the Q repressible binary system is the potential to build a new GAL4-independent MARCM system. The Q system-based MARCM (Q-MARCM) can then be used to mark and genetically manipulate a single cell or a small population of cells, while GAL4/UAS can be used to genetically manipulate a separate population of cells in the same animal. To test Q-MARCM, we placed tubP-QS distally to an FRT site and used FLP/FRT to induce mitotic recombination, so that one of the two daughter cells would lose tubP-QS, thus permitting QF to drive QUAS-marker expression (Figure 3A).

Using GH146-QF to label olfactory PNs in Q-MARCM experiments, we found single-cell and neuroblast clones labeled by QUAS-mCD8-GFP (Figure 3B) or QUAS-mdtT-HA (see below). In single-cell clones, the dendritic innervation of individual glomeruli in the antennal lobe and stereotyped projections of single axons in the lateral horn appeared indistinguishable from previously characterized single-cell clones labeled by GH146-GAL4-based MARCM (Jeffers et al., 2001; Marin et al., 2002;
We have validated tubP-QS transgenons on all five major chromosome arms (Table S1), thereby allowing GAL4-independent MARCM analysis for a vast majority of Drosophila genes using the Q system.

GAL4 and QF showed minimal cross-activation of their respective upstream activating sequences in cultured cells (Figures 1B and 1C). Moreover, we could not detect any cross-activation (Figure S4A) or cross-repression (Figure S4B) of the GAL4 and QF systems in vivo. Therefore, QF- and GAL4-based MARCM (G-MARCM) can be combined in the same fly. If tubP-GAL80 and tubP-QS transgenes are placed distally to the same FRT site in trans (Figure 3C), sister MARCM (G-MARCM) can be combined in the same fly. If tubP-GAL80 and tubP-QS are placed distally to FRT sites on different chromosome arms (Figure S4C), independently generated clones can be labeled by Q- and G-MARCM. This arrangement, which we term “independent double MARCM,” can be used to study interactions between two separate populations of cells that have undergone independent mitotic recombination and genetic alteration. If tubP-GAL80 and tubP-QS transgenons are placed distally to the same FRT site in trans (Figure 3C), sister MARCM (G-MARCM) can be combined in the same fly. If tubP-GAL80 and tubP-QS are placed distally to FRT sites on different chromosome arms (Figure S4C), independently generated clones can be labeled by Q- and G-MARCM. This arrangement, which we term “independent double MARCM,” can be used to study interactions between two separate populations of cells that have undergone independent mitotic recombination and genetic alteration. If tubP-GAL80 and tubP-QS transgenons are placed distally to the same FRT site in trans (Figure 3C), sister MARCM (G-MARCM) can be combined in the same fly. If tubP-GAL80 and tubP-QS are placed distally to FRT sites on different chromosome arms (Figure S4C), independently generated clones can be labeled by Q- and G-MARCM. This arrangement, which we term “independent double MARCM,” can be used to study interactions between two separate populations of cells that have undergone independent mitotic recombination and genetic alteration. If tubP-GAL80 and tubP-QS transgenons are placed distally to the same FRT site in trans (Figure 3C), sister MARCM (G-MARCM) can be combined in the same fly. If tubP-GAL80 and tubP-QS are placed distally to FRT sites on different chromosome arms (Figure S4C), independently generated clones can be labeled by Q- and G-MARCM. This arrangement, which we term “independent double MARCM,” can be used to study interactions between two separate populations of cells that have undergone independent mitotic recombination and genetic alteration. If tubP-GAL80 and tubP-QS transgenons are placed distally to the same FRT site in trans (Figure 3C), sister
cells resulting from the same mitotic recombination can be labeled by Q- and G-MARCM respectively. We call the latter case “coupled MARCM.”

Figure 3D illustrates an example of coupled MARCM in the third-instar larval eye disc. Sister cells and their descendants, derived from a single mitotic recombination event based on clone frequency and the proximity of labeled cells, are marked by tubP-GAL4 driven UAS-mCD8-GFP and ET40-QF driven QUAS-mtdT-HA. The photoreceptor cell bodies and their axonal projections into the brain were clearly visualized by both G-MARCM and Q-MARCM.

Analysis of Lineage and Cell Division Patterns with Coupled MARCM

The ability to label both progeny of a dividing cell with different colors via coupled MARCM (Figure 3C) can be used to characterize two important aspects of a developmental process: cell lineage and division patterns. As an example to illustrate such utility, we investigated the cell division pattern of a central nervous system neuroblast that gives rise to a subset of adult olfactory PNs.

The cell division patterns of neuroblasts that generate adult insect central nervous system (CNS) neurons are thought to follow the scheme shown in Figure 4A: a neuroblast undergoes asymmetric divisions to produce a new neuroblast and a ganglion mother cell (GMC), which divides once more to produce two postmitotic neurons (Nordlander and Edwards, 1969). A previous GAL4-based MARCM analysis of the mushroom body lineage supports this model: neuroblast, two-cell, and single-cell clones can be produced (Figure 4B), and the frequency of the neuroblast and two-cell clones is roughly equal, reflecting the random segregation of the GAL80-containing chromosomes into the neuroblast or the GMC (Lee et al., 1999; Lee and Luo, 1999). However, when we analyzed PN lineages using MARCM and GH146-GAL4 (Jeffersis et al., 2001) or GH146-QF (data not shown), we obtained either neuroblast or single-cell clones, but no two-cell PN clones. Three different models can account for these data (Figure 4C). In model I, the stereotypical division pattern (Figure 4A) does not apply to this lineage: GH146-positive PNs are direct descendants of the neuroblasts. In models II and III, the general division pattern still applies, but the sibling for the GH146-positive PN either is a GH146-negative cell (model II) or dies (model III).

We used coupled MARCM to distinguish among these models, focusing on the best-characterized anterodorsal lineage in which all progeny are PNs (Lai et al., 2008) and where birth order has been determined for most GH146-positive PNs (Jeffersis et al., 2001; Marin et al., 2005). We used GH146-QF to label PNs derived from one progeny of a cell division, and the ubiquitous tubP-GAL4 to label the sibling progeny (Figure S5). We
induced clones by heat shock at different time windows within 0–100 hr after egg laying and recovered a total of 91 coupled MARCM clones. We sorted the clones according to their labeling by GH146-QF and tubP-GAL4 (Figure 4D).

If model I were true, a single PN should always have a neuroblast sibling (Figure 4C1). However, we found 19 out of 44 single PNs labeled by GH146-QF without a tubP-GAL4-labeled neuroblast clone (Figure 4D; Figure 4E1) and five out of 38 single PNs labeled by tubP-GAL4 without a GH146-QF-labeled neuroblast clone (Figure 4D; Figure 4E2). Thus, model I does not apply.

If model II were true, GH146-QF-labeled neuroblast clones should be coupled with a two-cell clone labeled by the ubiquitous tubP-GAL4 (regardless of them being GH146 positive or GH146 negative; Figure 4C2, left). However, of the 40 GH146-QF-labeled neuroblast clones, none of the tubP-GAL4-labeled siblings were two-cell clones (Figure 4D). Instead, in 33 cases, the siblings were single-cell clones (Figure 4E3), and in the other seven cases, there were no labeled siblings (Figure 4E4). In addition, model II would predict pairs of sister cells each labeled by tubP-GAL4 or GH146-QF as a result of mitotic recombination in the GMC (Figure 4C2, right), but such an event was never observed (Figure 4D).

These experiments therefore support model III: the sibling of each PN dies during development and is no longer present in the adult brain (Figure 4C3). The frequent occurrence of single singly labeled PNs without labeled siblings could result from mitotic recombination that occurs either in the neuroblast or in the GMC.

Comparisons with Other Methods

While this manuscript was in preparation, two other twin-spot labeling methods were reported. “Twin-spot MARCM” uses...
**UAS-Inverse Repeat** transgenes as repressors against two fluorescent proteins and places these transgenes on the same chromosome arm in *trans* such that the FLP/FRT-mediated mitotic recombination creates two sibling cells, each losing one of the RNAi repressor genes (Yu et al., 2009). “Twin-spot generator” (TSG), which is analogous to the MADM method in mice (Zong et al., 2005), places two chimeric fluorescent proteins on the same chromosome arm in *trans*. Upon FLP/FRT-mediated recombination, two fluorescent proteins are reconstituted and can be segregated to daughter cells. (Upon GAL4- and QF-labeled siblings are wild-type. Genotype: *hsFLP, QUAS-mtdT-HA, UAS-mCD8-GFP (X); ET40-QF, QUAS-mtdT-HA/+ (II); tubP-GAL4, 82B*; tubP-QS (III)).

**Combining GAL4 and QF** allows for the ability to examine clones shortly after induction since there is no perdurance of a repressor; however, marker expression is low because of the lack of binary system-based amplification. In addition, both markers are driven by a ubiquitous promoter, thereby limiting the utility for tracking lineages in complex tissues such as the nervous system as a result of frequent interference by a large number of background mitotic clones. Twin-spot MARCM uses fewer transgenes than coupled MARCM. However, both progeny are labeled by the same GAL4 driver, thereby limiting the power for resolving cell division patterns (for example, siblings of a particular neuron may not be labeled by the same GAL4 line) and lacking the flexibility for selective manipulation of different siblings. Coupled MARCM offers robust marker expression and versatility as it can combine all available GAL4 and QF lines, whether cell-type-specific or ubiquitous. The combined use of ubiquitous *tubP-GAL4* and PN-specific *GH146-QF* was key to resolving cell division patterns in the PN lineage, and it could not have been achieved with TSG or twin-spot MARCM. Furthermore, coupled MARCM can be used for independent gain- and loss-of-function genetic manipulations of both progeny. An example is illustrated in the next section.

**Analyzing Cell Proliferation and Growth with Coupled MARCM**

Coupled MARCM allows direct comparision of two cell populations that arise from a single cell division within the same animal. Here, we illustrate its use to study cell proliferation and growth in the wing imaginal disc (Figure 5A).

The ~50,000 epithelial cells of the wing disc are produced by exponential cell division from less than 40 progenitor cells during the larval stages of *Drosophila* development (Bryant and Simpson, 1984). Clonal analysis in the wing imaginal disc is a sensitive strategy for studying the effects of genetic perturbations on cell growth or proliferation. To verify that QF expression does not affect normal cell growth or proliferation, we used coupled MARCM to label wild-type clones in the larval wing imaginal disc (Figure 5B). Clones were induced by heat shock at 48 hr after egg laying and examined 72 hr later. The area of the GAL4- and QF-labeled clones, their cell number, and cell size (Figures 5D, 5E, and 5F, respectively) were indistinguishable from one another. These results indicate that G-MARCM and Q-MARCM do not differentially affect cell proliferation or growth of wing disc cells. Additional control experiments indicated that high levels of QF expression did not interfere with growth and patterning of imaginal discs and the corresponding adult structures (Figure S6).

To show the utility of coupled MARCM in mutant analysis, we generated wing imaginal disc clones in which control cells were labeled by GAL4 and *Tuberous Sclerosis 1 (Tsc1)* homozygous mutant cells were labeled by QF. Tsc1, along with its partner Tuberous Sclerosis 2 (Tsc2), forms a complex that negatively regulates the Tor pathway to affect both cell size and cell proliferation (Ito and Rubin, 1999; Potter et al., 2001; Tapon et al., 2001). We found that Tsc1 mutant clones (labeled red via QF) were significantly larger than wild-type clones (labeled green...
via GAL4 (Figure 5C), covering on average 2.9-fold larger area than their control sister clones (Figure 5D). To determine whether the increase in clone area is due to an increase in cell proliferation or cell size, we counted the number of cells within these labeled clones. We found a 2-fold increase in cell numbers in Tsc1 mutant clones compared to the sister clones, yet only a 26% increase in cell size (Figures 5E and 5F), suggesting that mutation of Tsc1 in rapidly dividing cells primarily leads to an increase in proliferative capacity. This example, although largely confirmatory of previous findings, illustrates the utility of coupled MARCM for investigating gene function in developmental processes.

**Refining Transgene Expression by Intersecting GAL4 and QF Expression Patterns**

A major power of the GAL4/UAS system is its ability to manipulate many cell types through thousands of GAL4 lines generated by enhancer trapping or GAL4 driven from specific promoters. Despite the abundance of GAL4 lines, their expression patterns are often too broad to establish the causality between the expression of a transgene in a particular cell type and a phenotype, especially if the phenotype is assayed at the organismal level. Combining GAL4- and QF-based binary systems into logic gates can create new expression patterns (Figure S7). Below we provide proof-of-principle examples for some of these strategies (Figure 6).

**QF NOT GAL4**

Like the previously characterized GH146-GAL4 (Jeffers et al., 2001), GH146-QF is expressed in PNs that are derived from the anterodorsal, lateral and ventral neuroblast lineages (Figure 2B). The POU transcription factor Acj6 is expressed only in anterodorsal but not in lateral or ventral GH146-positive PNs (Komiyama et al., 2003). Acj6, and acj6-GAL4, an enhancer trap line inserted into the acj6 locus, are also expressed in some GH146-negative anterodorsal PNs, in many olfactory receptor neurons (ORNs), in atypical PNs, and in lateral horn output neurons (Clyne et al., 1999; Komiyama et al., 2003; Suster et al., 2003; Komiyama et al., 2004; Jeffers et al., 2007; Lai et al., 2008).

As shown in Figure 6A, when GH146-QF and acj6-GAL4 are present in the same fly, and are detected via QUAS-mtdT-HA and UAS-mCD8-GFP, respectively, a large subset of anterodorsal PNs is labeled by both mCD8-GFP and mtdT-HA, whereas lateral and ventral PNs express mtdT-HA but not mCD8-GFP.

By introducing a UAS-QS transgene, we subtracted the GAL4-expressing cells from the QF-expressing cells such that the QUAS-mtdT-HA reporter was only expressed in the lateral and ventral, but not the anterodorsal, PNs (Figure 6B; compare Figure 6B3 with Figure 6A3). In this manner, we created “QF NOT GAL4,” a new QF-dependent expression pattern. Using this logic gate, we observed nonoverlapping glomeruli labeled by Acj6-expressing anterodorsal PNs in green and QF-expressing lateral PNs in red (Figure 6B3). This observation confirms directly in the same animal a previous finding that PNs from the anterodorsal and lateral lineages project dendrites to complementary and nonoverlapping glomeruli in the antennal lobe (Jeffers et al., 2001).

Expression pattern subtraction can also be visualized at the level of axon terminals. Both anterodorsal and lateral PNs project their axonal collaterals into the mushroom body calyx, where they terminate in large presynaptic boutons. In the absence of the UAS-QS transgene, these individual terminal boutons are labeled green, yellow, and red, representing axon terminals of PNs that are Acj6+/GH146- anterodorsal PNs, Acj6+/GH146+ anterodorsal PNs, and GH146+/Acj6- lateral PNs, respectively (Figure 6A3). In the presence of UAS-QS, yellow terminal boutons are no longer present (Figure 6B3), indicating that the cells labeled by acj6-GAL4 have been subtracted from the GH146-QF expression pattern. This experiment allows a direct comparison of axon terminal distributions of anterodorsal and lateral PNs innervating the same mushroom body.

**QF AND GAL4**

By introducing two additional transgenes, QUAS-FLP, UAS > stop > effector (Figures 6C1 and 6D1; “>” represents FRT), or UAS-FLP, QUAS > stop > effector (Figure 6E), into an animal containing a GAL4 and a QF line, only cells that express both QF and GAL4 (“QF AND GAL4”) can be selectively visualized and genetically manipulated. Below we show three examples.

First, we studied the intersection of GH146-QF and acj6-GAL4. With the introduction of UAS-FLP and QUAS > stop > mCD8-GFP, anterodorsal PNs that are both Acj6+ and GH146+ were labeled (Figure 6C), as confirmed by the glomerular identity of dendritic projections of these neurons (data not shown). A previously described Acj6/GH146 double-positive cell from a separate lineage (Komiyama et al., 2003) was also labeled (Figure 6C4, arrowhead). All other lateral and all ventral GH146+ PNs, which do not express Acj6, no longer expressed the marker. The marker was also not expressed in ORNs or lateral horn neurons, which express Acj6 but not GH146. Thus, we can express transgenes only in cells that express both GH146 and Acj6: a subset of anterodorsal PNs.

In the second and third examples, we studied the intersection between GH146-QF and NP21-GAL4 using two AND gate strategies. NP21-GAL4 is an enhancer trap line inserted near the promoter of fruitless (fru) (Hayashi et al., 2002) that drives the expression of the male-specific isoform of Fru (FruM), which is essential for regulating mating behavior (Demir and Dickson, 2005; Manoli et al., 2005). NP21-GAL4 labels many neurons in the brain (Kimura et al., 2005) (Figure 6D), including PNs that project dendrites to the DA1 glomerulus (Figure 6E). In our first strategy (Figure 6D3), we used UAS-FLP and QUAS > stop > mCD8-GFP and found that approximately ten PNs that innervated several glomeruli were selectively labeled (Figures 6D3 and 6D3). In our second strategy (Figure 6E1), we used QUAS-FLP and UAS > stop > mCD8-GFP and found that the labeled PNs were restricted to only approximately five cells that project their dendrites to the DA1 glomerulus (Figures 6E3 and 6E3). The difference between these two strategies reflects the fact that in these intersectional strategies, the binary system used to drive FLP reports the cumulative developmental history, rather than only the adult expression, of the driver. Our data suggest that NP21-GAL4 (and by inference fruM) is expressed in more PN classes during development than in the adult. In both cases, the complex NP21-GAL4 expression pattern outside of PNs has been reduced to very few cells. The comparison of expression patterns from the two strategies can pinpoint the cells that are at the intersection of GH146-QF and NP21-GAL4 adult expression patterns. Future use of a perturbing effector could...
Figure 6. Intersectional Methods to Refine Transgene Expression

(A1) Schematic showing two partially overlapping cell populations: one expressing an acj6-GAL4-driven green marker (within the left rectangle) and the other expressing a GH146-QF-driven red marker (within the right rectangle). Cells in the center express both GAL4 and QF and appear yellow.

(A2–A4) Single confocal sections (A2 and A4) or a Z projection (A3) of the adult antennal lobe (A2 and A3) or mushroom body calyx (A4) from flies with the genotype shown in (A1). Green, red, and yellow cells in (A2) represent PNs that express acj6-GAL4 only, GH146-QF only, or both, respectively. Their dendrites form green, yellow, and red glomeruli (A2). Their axons form green, red, and yellow terminal boutons in the mushroom body (A4). (A3) is the Z projection of the red channel for (A2); the oval highlights cell bodies of anterodorsal PNs. Green, anti-CD8 staining for UAS-mCD8-GFP; red, anti-HA staining for QUAS-mtdT-HA; blue, neuropil marker.

(B1) Schematic for “QF NOT GAL4” for acj6-GAL4 and GH146-QF. UAS-QS is added to (A1), resulting in the repression of QF activity in cells that express both QF and GAL4 (center). QF reporter expression is thus subtracted from the overlapping population of cells.

(B2–B4) Equivalent samples as (A2)–(A4), except with UAS-QS added. Compared to (A3), anterodorsal PNs no longer express QUAS-mtdT-HA (dotted oval in B3). There are no yellow cells and glomeruli in the antennal lobe (B2), or yellow terminal boutons in the mushroom body (B4).

(A and B) Note that in the experiments shown, for clear visualization of only non-ORN processes in the antennal lobe, antennae and maxillary palps were removed 10 days prior to staining, causing all Acj6-expressing ORN axons to degenerate.
lead to functional characterization of this small genetically
defined group of cells.

**Comparisons with Other Methods**

An AND gate can be achieved by utilizing the split-GAL4 system
(Luan et al., 2006). The benefit of our method is that it can take
advantage of the thousands of available and well-characterized
GAL4 lines, whereas the split-GAL4 system needs to generate
new split N-GAL4 and C-GAL4 lines. In addition, reconstituted
GAL4 from the split GAL4 system is not as strong as wild-type
GAL4 in driving transgene expression (Luan et al., 2006).

The intersection between FLP/FRT and GAL4/UAS can also be
used directly as an AND gate without going through a second
binary system to express FLP (Stockinger et al., 2005; Hong
et al., 2009). Both this method and our method have the caveats
of transient FLP expression during development, as well as the
possibility that FLP/FRT-mediated recombination may not occur
in all cells that express FLP. Although our method requires one
additional transgene, it offers several advantages over
promoter-driven FLP. First, our method does not require the
generation of separate tissue or cell type-specific FLP lines.
Second, by inducing higher FLP levels due to transcriptional
amplification of binary expression, our method should more
readily overcome problems of incomplete recombination.
Indeed, counts of the number of DA1-projecting PNs that are
part of the NP21-GAL4 expression pattern with or without the
AND gate with GH146-QF are similar (NP21-GAL4: 5.2 ± 0.1,
n = 48; GH146-QF/QUAS-FLP AND NP21-GAL4: 5.1 ± 0.1,
n = 10), suggesting nearly complete FLP/FRT mediated recom-
bination. Third, our method offers two complementary AND
gate strategies, which together can be used to overcome the
ambiguities arising from transient developmental expression.
Fourth, transient developmental expression mediated by
QUAS-FLP could in principle be suppressed by introduction of
tubP-QS, and the suppression could be reversed by supplying
the flies with quinic acid at appropriate developmental stages.

The “QF NOT GAL4” or “GAL4 NOT QF” (Figure S7) strategies
are conceptually similar to GAL80 subtraction of GAL4 expres-
sion (Lee and Luo, 1999). If one were to generate a large number
of GAL80 enhancer trap or promoter driven lines, one could use
this set to subtract their expression patterns from GAL4 expres-
sion patterns. One limitation of this approach is that the GAL80
expression pattern is difficult to determine at high resolution
because it is based on suppression of GAL4-induced gene
expression. In addition, GAL80 levels must be sufficiently high
to ensure proper suppression of GAL4, which may not be true
for many enhancer trap or promoter-driven GAL80 transgenes.
By contrast, the NOT gate we describe here utilizes the expres-
sion patterns of two transcription factors, which express the
appropriate repressor through binary amplification, and should
therefore circumvent both limitations above.

A major limitation of our intersectional strategies for refine-
ment of gene expression is the availability of QF drivers with
different expression patterns. So far, we were unsuccessful in
generating tubP-QF transgenic animals, suggesting that QF is
toxic to flies when highly expressed in a ubiquitous manner or
in a particular developmental stage or tissue (see the
Extended Experimental Procedures). Nonetheless, we isolated many QF
enhancer traps that express strongly in imaginal discs, epithelial
tissues, glia, and neurons (Figures S2B and S3). We hope
that our proof-of-principle examples here will stimulate the
Drosophila community to generate large numbers of enhancer
trap and promoter-driven QF lines in the future. The number of
new expression patterns created by intersections between GAL4
and QF should be multiplicative. For instance, 100 QF lines in
combination with 10,000 GAL4 lines, given sufficient expression
overlap and utilizing different logic gates (Figure S7), should in
principle generate millions of new effector expression patterns.

**Defining PNs Responsible for Olfactory Attraction**

By expressing an effector that alters neuronal activity, intersec-
tional approaches can be used to dissect the function of
neuronal circuits. We used this approach to assay the function
of PNs in an olfactory attraction behavior. Instead of expressing
a marker in specific populations of neurons, we expressed
*shibire*<sup>sh2</sup> (sh2), a temperature-sensitive variant of the protein dy-
namin that dominantly interferes with synaptic vesicle recycling

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(C1) Schematic for “QF AND GAL4” for acj6-GAL4 and GH146-QF. GAL4 driven FLP results in the removal of a transcrip
tional stop (!) from a QUAS reporter (within the left rectangle), but the reporter can only be expressed in cells where QF is expressed (within the right rectangle). Thus, only the cells in the overlap (center) express the reporter.

(C2) Confocal stack of a whole mount central brain showing reporter (mCD8-GFP) expression driven by acj6-GAL4, which labels many types of neurons including most ORNs, olfactory PNs and optic lobe neurons.

(C3 and C4) The AND gate between GH146-QF and acj6-GAL4 (genotype as in C1) limits mCD8-GFP expression to a cluster of anterodorsal PNs and a single lateral neuron (arrowhead in C3). Arrow in (C4), axons of anterodorsal PNs.

(D1) Schematic for “QF AND GAL4” similar to (C1), but for NP21-GAL4 and GH146-QF.

(D2) Confocal stack of whole-mount central brain showing reporter (mCD8-GFP) expression driven by NP21-GAL4.

(D3 and D4) The AND gate between GH146-QF and NP21-GAL4 limits reporter expression to a few classes of PNs that project to several glomeruli including DA1 (arrow in D3) and to neurons that project to the ellipsoid body (arrow in D4).

(E1) Schematic for an alternative approach to “GAL4 AND QF” for NP21-GAL4 and GH146-QF. Here, FLP is driven by QF, and the reporter is driven by GAL4.

(E2) High magnification of the NP21-GAL4 expression pattern centered at the antennal lobe. In the adult, only one class of lateral PNs projecting to the DA1 glomerulus (arrow) is evident.

(E3 and E4) This AND gate between GH146-QF and NP21-GAL4 limits expression to a single class of lateral PNs that project to the DA1 glomerulus (arrow in E3). Occasional expression is also found in a few cells in the anterior lateral region of the brain.

Genotypes: acj6-GAL4, GH146-QF, UAS-mCD8-GFP, QUAS-mtdT-HA (A), acj6-GAL4, GH146-QF, UAS-mCD8-GFP, QUAS-mtdT-HA. UAS-QS (B), acj6-GAL4, UAS-mCD8-GFP (C1), acj6-GAL4, GH146-QF, UAS-FLP, QUAS > stop > mCD8-GFP (C2 and C3), NP21-GAL4, UAS-mCD8-GFP (D1 and D2), NP21-GAL4, GH146-QF, UAS-FLP, QUAS > stop > mCD8-GFP (D3 and D4), NP21-GAL4, GH146-QF, UAS > stop > mCD8-GFP, QUAS-FLP (E2 and E4). Yellow triangle or “>,” FRT site; “!” or “stop,” transcriptional stop. Scale bars represent 20 μm. Figure S7 shows strategies to generate 12 QF and GAL4 intercalation logic gates.
(Kitamoto, 2001). At the nonpermissive temperature, synaptic transmission of neurons that express shi is reversibly inhibited. This approach allowed us to selectively inhibit different populations of PNs—lateral and ventral (GH146-QF NOT acj6-GAL4; Figure 6B) or anterodorsal (GH146-QF AND acj6-GAL4; Figure 6C)—and then assay behavioral attraction to the fruity odorant ethyl acetate using a modified trap assay (Larsson et al., 2004) (Figure 7). Similar to controls, flies containing only GH146-QF or QUAS-shi exhibited strong attraction to ethyl acetate. When all GH146+ PNs were inhibited (GH146-QF+QUAS-shi or GH146-QF+QUAS-FLP+QUAS > stop > shi), there was a significant deficit in olfactory attraction. However, when only anterodorsal GH146+ PNs were inhibited, attraction remained normal. In contrast, when lateral/ventral GH146+ PNs were inhibited, there was a deficit in olfactory attraction akin to the inhibition of all GH146+ PNs. These results suggest that, in this behavioral context, attraction to ethyl acetate is mediated by the lateral/ventral, and not anterodorsal, subpopulations of PNs.

Conclusions and Perspectives

In conclusion, we demonstrate that the Q repressible binary expression system functions well outside its native Neurospora, from cultured Drosophila and mammalian cells to Drosophila in vivo. We have generated and validated a substantial number of tools (Table S1, Figures S2 and S3) that can be used for many applications, as illustrated by the examples given above. Below, we discuss a few future developments and applications.

Genetic Dissection of Neural Circuits

Drosophila has emerged as an attractive model system to establish causal links between the functions of individual classes of neurons, information processing within neural circuits, and animal behavior. A bottleneck in this endeavor is the genetic access to specific populations of neurons with reproducible precision, such that one can label them with markers for anatomical analysis, express genetically encoded indicators to record their activity, and silence or activate these neurons to examine the consequences to circuit output or to animal behavior (Luo et al., 2008). The intersectional methods we describe should greatly increase the precision of genetic access to specific neuronal populations, especially as more QF drivers are characterized.

High-Resolution Mosaic Analysis

Although MARCM is a powerful tool for identification and functional studies of genes that act cell autonomously, it is less adaptable to studies of genes that act non-cell autonomously. The ability to perform MARCM analysis independently from GAL4/UAS should expand the power of mosaic analysis for genes that function in intercellular communication. For example, using GAL4/UAS, one can perturb the function of a group of cells, while using Q-MARCM to examine the consequences of the perturbation on a small subset of interacting cells. Furthermore, both systems can be used in the same animal for independent perturbations of two populations of interacting cells, via both loss- and gain-of-function approaches. Finally, these approaches can be expanded into genetic screens where, for example, the GAL4 binary system is used to drive an RNAi library in a large group of cells while the Q system is used to label a small population of neurons with high resolution.

Beyond the Nervous System and Drosophila

The Q system should be widely applicable beyond the Drosophila nervous system. We have provided an example of clonal phenotypic analysis in the wing disc for cell growth and proliferation. Similar studies could be used for the identification and characterization of tumor suppressors or oncopgenes that function cell autonomously or non-cell autonomously. The Q system should in principle permit transgene expression, lineage and mosaic analysis in many other Drosophila tissues. Finally, QF/QUAS-induced transgene expression is ~30-fold more effective in mammalian cells compared with GAL4/UAS. This fact may make the Q binary expression system more effective than GAL4/UAS for transgene expression in mice (Ornitz et al., 1991; Rowitch et al., 1999). Indeed, the Q system could be extended to all organisms conducive to transgenesis.

EXPERIMENTAL PROCEDURES

QF and QS cDNAs were obtained by PCR with a cosmids, pLorist-HO35F3 from the Fungal Genetics Stock Center, as the template. QUAS was constructed with five copies of naturally occurring QF binding sites (each 16 bp long, shown in capital letters, with spacer sequences in small letters): GGGTAATCGCTTATCCtcGGATAAACAATTATCCtcacGGGTAATCGCTTATCCgctcGGGTAATCGCTTATCCtcGGGTAATCGCTTATCCgctcGGGTAATCGCTTATCCtcGGGTAATCGCTTATCCgctcGGGTAATCGCTTATCCtcGGGTAATCGCTTATCCgctcGGGTAATCGCTTATCCtcGGGTAATCGCTTATCCgctcGGGTAATCGCTTATCCtcGGGTAATCGCTTATCCgctcGGGTAATCGCTTATCCtcGGGTAATCGCTTATCCgctcGGGTAATCGCTTATCCc.

See the Extended Experimental Procedures for details on the construction of plasmids and transgenic flies, cell transfection, Drosophila genetics, mosaic analysis, imaging, and behavior.

All plasmids and sequence files have been deposited to Addgene. Most fly stocks in Table S1 have been deposited to the Bloomington Stock Center. Other fly stocks are available upon request.

ACCESSION NUMBERS

Sequences for representative QF, QS, and QUAS plasmid constructs were deposited to GenBank and have the following accession numbers:
SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and one table and can be found with this article online at doi:10.1016/j.cell.2010.02.025.

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Recombinant DNA Construction

Plasmids were constructed by standard subcloning, synthesis and/or PCR. For PCR amplifications, we usually used Phusion Taq polymerase (Finnzymes, Catalog # F530L), and in a few cases Platinum Pfx polymerase (Invitrogen, Catalog # 11708-021). When synthetic oligos or PCR were involved in plasmid construction, the sequence of the construct was verified by DNA sequencing. Please note that four constructs described below (pattB-QF-SV40, pattB-QF-hsp70, pattB-DSCP-QF-SV40, pBS-SK-QS) were not used in the experiments described in this report but are included here as they simplify the generation of additional Q system reagents.

Three parent plasmids were used for a number of constructs below:

1) pPAC5C-PL (gift of Rui Zhou, Harvard Medical School), which contains Drosophila actin 5c promoter and its polyadenylation sequence.

2) pCDNA3.1-myc-His-A (Invitrogen), which contains human cytomegalovirus (CMV) immediate-early promoter, myc and six-Histidine tags for C-terminal fusion, and bovine growth hormone polyadenylation signal sequence. All cDNAs that were cloned into this plasmid contained a stop codon preceding the Myc and His tags, thereby preventing the fusion of the cDNAs with the tags.

3) pBluescript SK (+) (Stratagene).

pQUAS-GG

This plasmid contains 5 copies of naturally occurring QF binding sites (each 16 bp long, shown in capital letters, with spacer sequences in small letters): GGGTAATCGCTTATCCtcGGATAAACAATTATCTcaccGGGTAATCGCTTATCCgtcctGGGTAATCGCTTATCCtt

This sequence was assembled from overlapping primers and cloned using XhoI and HindIII into pBluescript containing hsp70 minimal promoter (Brand and Perrimon, 1993) and an optimized GFP split with an intron (GG) (Zong et al., 2005).

pQUAS-luc2

The XhoI/NcoI fragment containing QUAS-Pmin was subcloned from pQUAS-GG into pGL4.23 (Promega) to replace its minimal promoter. pGL4.23 contains synthetic firefly luciferase gene (luc2), which has been codon optimized for high expression in mammalian cells.

pAC-QF

QF cDNA was obtained by PCR using primers PR50 (aatggatcccaatccgctcaagac) and PR51 (aatgcggccgcctattgtcatactgtattatatcg), and the cosmid, pLorist-HO35F3 from the Fungal Genetics Stock Center, as the template. The PCR fragment was cloned into pPAC5C-PL using BamHI and NotI. The QF gene is intronless.

pCMV-QF

Obtained by subcloning QF cDNA using BamHI and NotI from pAC-QF into pCDNA3.1-myc-His-A (Invitrogen).

pCMV-QS

QS cDNA was obtained by PCR using primers PR53 (aatggtacccaacatgaacaccatcccggcac) and PR54 (aatgcggccgctcaagatttgcgttgcaattc) using a cosmid, pLorist-HO35F3 from the Fungal Genetics Stock Center, as the template. The PCR fragment was initially cloned into pPAC5C-PL using Acc65 I and NotI to obtain the QS gene containing a single intron. The intron was subsequently removed by creating two PCR products that were cloned using 3-way ligation into pCDNA3.1-Mys-His-A (Invitrogen) using Acc65I and NotI and blunt ends at the exon-exon junction. The primers used to create the first exon are: PR53 (see above) + PR190 (agagcctagttactctggtg); and the second exon are: PR58 (tggctgccgcccaattcc) + PR54 (see above).

pAC-QS

Obtained by subcloning intronless QS from pCMV-QS using Acc65I and NotI into pPAC5C-PL.

pUAS-GG

The BamHI/BglI fragment from pUAST (Brand and Perrimon, 1993), containing 5 copies of a GAL4 binding site and the Drosophila hsp70 minimal promoter was subcloned into BamHI site of pBluescript containing an optimized GFP split with an intron (GG) (Zong et al., 2005). This strategy generates a hybrid BglII/BamHI site in front of the GFP.

pUAS-luc2

Obtained by subcloning 5 copies of GAL4-binding UAS sequence and the hsp70 minimal promoter from pUAS-GG into pGL4.23 using HindIII and NotI. The 5xUAS+Pmin fragment from pUAS-GG originates from pUAST (Brand and Perrimon, 1993).

pAC-GAL4 and pCMV-GAL4

GAL4 cDNA was PCR amplified using primers PR509 (CCCCGGATCCCAAatgagactgtctctctatgacca) and PR510 (CGGTAAACCGGCCGCgtactctttttttgggtttgtg), and a lab vector, pCA-GAL4 as the template. The PCR product was cloned using BamHI and NotI into pPAC5C-PL and pCDNA3.1-myc-His-A, respectively.
pAC-GAL80 and pCMV-GAL80

GAL80 cDNA was PCR amplified using primers PR511 (CCCCCCGATCCCAACaggtcactacaacaagatctcgg) and PR512 (CGGTAACGGGCGCGGCGGtataactatagtcagagatgccag), and a lab vector, pCA-GAL80 as the template. The PCR product was cloned using BamHI and NotI into pACSC-PL and pCDNA3.1-myc-His-A, respectively.

pAC-hRluc

Synthetic Renilla luciferase that was codon optimized for expression in mammalian cells (hRluc) was amplified using primers PR444 (aaatGGTACCaaagATGGGCTTCCAAGGTAAGCA) and PR445 (aaatGGTACCCTTACTGCTGTCTTGAGCA), and pGL4.75 (hRluc/CMV, Promega, catalog # E6931) as the template. The PCR product was cloned into pACSC-PL using Acc65I and NotI.

pQUAST

This vector was designed to mimic the multi-cloning site of the pUAST vector (Brand and Perrimon, 1993) thereby allowing easy exchange of inserts between pUAST and pQUAST. pQUAST was digested with SphI and EcoRI and blunted with Klenow to remove the 5xUAS and hsp70 minimal promoter (minP). pQUAS-GG was digested with BamHI and EcoRI to excise the 5xQUAS and Pmin promoter and then blunted. The 5xQUAS-Pmin promoter was then ligated into the modified pUAST vector to generate pQUAST. Any gene X can be subcloned from pUAST-geneX into pQUAST using the same restriction sites that were originally utilized for pUAST-geneX construction. If the pUAST-geneX plasmid is not available, genomic DNA from flies containing the geneX can be subcloned from pUAST-geneX into pQUAST using the same restriction sites that were originally utilized for pUAST-geneX and then blunted. The 5xQUAS-Pmin promoter was then ligated into the modified pUAST vector to generate pQUAST. Any gene X can be subcloned from pUAST-geneX into pQUAST using the same restriction sites that were originally utilized for pUAST-geneX construction. If the pUAST-geneX plasmid is not available, genomic DNA from flies containing the UAS-geneX transgene can be used. In this case, pQUAST-geneX can be constructed as follows:

1) PCR amplify the UAS insert from UAS-geneX genomic DNA by using the primer pairs genUASFOR (GCTTCGTCTACGGGACG GACAATTCATTTCAAC) and genUASREV (GCGAGATCCATACATCAGTATGCGGATTTTCTTCT) that were used for cloning the insert into the UAS vector.
2) If the restriction sites used for cloning of the UAS insert are unknown, sequence the PCR fragment using UASFOR-SEQ (TCAATCAACAGGCAAAGTGAACACG) and SV40REV-SEQ (CCATTTACATTTCCAAGGTTGCG) primers.
3) Digest the PCR product with appropriate enzymes for cloning into pQUAST.

pQUAS-DSCP

pQUAS-DSCP was digested with EcoRI, which removed the hsp70 minimal promoter. A PCR fragment containing the DSCP promoter was PCR amplified from pBPGRw (Pfeiffer et al., 2008) with 5' EcoRI and 3' MfeI site, and was ligated into pQUAST to generate pQUAS-DSCP. Expression levels between pQUAS-DSCP and pQUAST vectors have not been directly compared (for example, by having the reporter constructs integrated at the same attP location). Nonetheless, pQUAS-DSCP-FLPo and pQUAST-FLPo transgenic flies both showed strong FLP activity in the intersectional studies shown in Figure 6E.

pmCD8-GFP,y+

This enhancer trap vector is based on the pGalW vector (Gerlitz et al., 2002). The GAL4 insert from pGalW was removed by complete NotI and XbaI digestion and replaced with the GAL80 ORF isolated as a NotI/XbaI fragment from pCasper-tubP-GAL80 (Lee and Luo, 1999), to generate the plasmid pG80,y+. To generate pmCD8-GFP,y+, the GAL80 insert was removed by digestion with NotI/XbaI.

pQUAST-mCD8-GFP

The Notl fragment containing CD8-GFP-SV40 from the enhancer trap construct pCD8-GFP,y+ was cloned into pQUAST.

ptubP-QS

The QS cDNA was excised from pAC-QS with Acc65I and NotI, blunted, and ligated into a blunt pCasper-tubulin-GAL80, from which the GAL80 insert was removed by digestion with NotI/XbaI.

pBac-GH146-QF

pBac-GH146-QF with FseI/AscI/AvrII multi-cloning site has been previously described (Hong et al., 2009). QF cDNA with 5' FseI and 3' AvrII restriction sites was amplified from pAC-QF by PCR, and cloned into the FseI/AvrII restriction sites of pBAC-GH146 to yield pBac-GH146-QF.

pQUAST-mtD7-3xHA

The Notl fragment containing mCD8-GFP-SV40 from the enhancer trap construct pCD8-GFP,y+ was cloned into pQUAST.

pBAC-3xPDsRed-GH146-MCS with FseI-AscI-AvrII restriction sites was amplified from pAC-QS with Acc65I and NotI, blunted, and ligated into a blunt pCasper-tubulin-GAL80, from which the GAL80 insert was removed by digestion with NotI/XbaI.

pQUAST-mtD7-3xHA, pUASTattB-mtD7-3xHA

The N-terminal membrane tag on tdTomato contains 8 amino acids that direct myristoylation and palmitoylation (Muzumdar et al., 2007). 3 copies of the HA tag were PCR amplified from pTHW (Drosophila Genomics Resource Center) and included a 5' BsrGI restriction site and a 3' EcoRI restriction site preceded by the TAA stop codon (5' oligo:TTATGTAACATCCGATGCTGTTCTTGTCATCGTAC; 3' oligo: TAAAGAATTCTTAAGCTGTTGAAAGCTATGCAAGTGG). pSN20-mtD7 (Muzumdar et al., 2007) was digested with BsrGI and EcoRI and the HA PCR fragment was ligated into this vector to generate pSN20-mtD7-3xHA. This vector was then digested with XhoI and partially digested with BamHI and cloned into pQUAST and pUASTattB (Bischof et al., 2007) to generate pQUAST-mtD7-3xHA and pUASTattB-mtD7-3xHA respectively. The mtD7-3xHA reporter in vivo is as good as mCD8-GFP in labeling dendritic and axonal processes, or in labeling imaginal disc tissues. However, it does not label neuronal cell bodies as well as mCD8-GFP as most of the mtD7-3xHA signal is localized to the plasma membrane surface whereas mCD8-GFP, which also localizes to intracellular membranes, allows for the cell soma to be better visualized.
pQUAST-nucLacZ
The nuclear LacZ insert was PCR amplified from UAS-nucLacZ genomic flies (Bloomington Stock Center) using oligos genUASFOR and genUASrev40REV. To determine which cloning sites were used in the cloning of UAS-nucLacZ, the PCR fragment was sequenced using UASFOR-SEQ and SV40REV-SEQ. The KpnI/Xbal digested nucLacZ PCR fragment was then ligated into pQUAST.

pQUAST-FLPo
Mammalian codon-optimized FLP recombinase (FLPo; Addgene plasmid 13792) (Raymond and Soriano, 2007) was PCR amplified to include BglII/NotI restriction sites, and cloned into pQUAST to generate pQUAST-FLPo.

pQUAS-DSCP-FLPo
Mammalian codon-optimized FLP recombinase (FLPo; Addgene plasmid 13792) (Raymond and Soriano, 2007) was PCR amplified to include EcoRI/XbaI restriction sites, and cloned into pQUAS-DSCP to generate pQUAS-DSCP-FLPo.

pCa4B2G-QUAS-DSCP-FLPo
The QUAS-DSCP-FLPo-SV40 region was excised from pQUAS-DSCP-FLPo by BamHI digestion, and subcloned into pCa4B2G at the BamHI site that is flanked by gypsy insulators (Markstein et al., 2008). This construct was used for PhiC31-mediated integration into the attP2 locus (Groth et al., 2004).

pUAST > stop > mCD8-GFP
The widely used FLP-Out reporter, UAS > CD2.y+ > mCD8-GFP (Wong et al., 2002) contains non-optimal FRT sites (‘ > ’ represents FRT), which were chosen in order to increase the percentage of small FLP-out clones during FLP-out labeling experiments (G. Struhl, personal communication). As such, in control FLP experiments, there was some variability in the extent of the excision of the ‘CD2,y+’ cassette in all expected target neurons, presumably due to incomplete excision of the FLP-out cassette. To reduce this variability, optimal FRT sites were used to flank two transcription stops. Direct comparison between UAS > stop > mCD8-GFP and UAS > CD2,y+ > mCD8-GFP transgenic flies—in test crosses with GH146-FLP (Hong et al., 2009) and elav-GAL4—verified that the new FLP-out reporter is more effective in removing the cassette between the FRT sites, resulting in decreased variability in mCD8-GFP reporter expression (data not shown). Construction of pUAST > stop > mCD8-GFP has been previously described (Hong et al., 2009).

pQUAST > stop > mCD8-GFP
The FRT-Stop-FRT cassette from pUAST > Stop > mCD8-GFP was excised by BglII and KpnI digestion. The two inserts were ligated into the BglII/NotI sites of pQUAST vector.

pQUAST-shibire	\textsuperscript{#11}
Shibire\textsuperscript{#11} was PCR amplified from genomic DNA of UAS-shibire\textsuperscript{#11} transgenic flies (Kitamoto, 2001) using genUASFOR and genUAS-REVs40 oligos, and ligated into the NotI/KpnI sites of pQUAST. To test for functional transgenic QUAS-shibire\textsuperscript{#11} insertions, we crossed flies containing a particular insertion with flies carrying ET49-QF, which broadly expresses QF in many neurons, and then placed the progeny in a 37°C water bath. Flies with a functional QUAS-shibire\textsuperscript{#11} became paralyzed within 20 s, followed by full recovery within 5 min.

pQUAST > stop > shibire	\textsuperscript{#11}
The NotI fragment containing the mCD8-GFP cassette was excised from pQUAS > stop > mCD8-GFP, the vector was blunted, and ligated to a blunted NotI/KpnI shibire\textsuperscript{#11} isolated from pQUAST-shibire\textsuperscript{#11}. Functional transgenic QUAS > stop > shibire\textsuperscript{#11} insertions were tested by crossing them to hsFLP122 and ET49-QF. The progeny containing all three transgenes were heat shocked at least once during development to excise the > stop > cassette, and then tested as described for QUAS-shibire\textsuperscript{#11}.

pQF-ET42 (Swappable QF Enhancer Trap)
An enhancer trap was constructed that allows recombination mediated cassette exchange (Oberstein et al., 2005). A loxM2 site was inserted immediately following the 5P promoter, and a LoxP site was placed after the selectable white marker to generate the vector pDonor-M2, which contains elements in the following order: 5P-Ppromoter-LoxM2-MCS-SV40 terminator-white-LoxP-3P. QF was PCR amplified from pAC-QF adding 5’ EcoRI and 3’ AatII restriction sites, and inserted into the EcoRI/AatII cloning sites of pDonor-M2 to yield pQF-ET42. Of the 14 original insertions of pQF-ET42 from the initial injection, 5 showed only tracheal expression, 8 showed tracheal expression and expression in additional tissues, and only 1 (ET40) showed very minor tracheal expression and high expression in additional tissues (e.g., all imaginal discs, and some adult brain structures). The reason for the tracheal expression is currently unknown. We are generating additional enhancer trap vectors to circumvent the tracheal expression. The ET40 enhancer trap is inserted into the 5’ upstream region of the posterior sex combs (psc) gene located at 49E6. The name, cytological location, and associated gene of the pQF-ET42 insertions in Figure S2B are: #6, 78A2, skulid; #8, 47A7, lola; #31, 52E11, Ext2.

pQF-ET (QF Enhancer Trap)
The QF-SV40polyA fragment was excised from pQF-ET, w+ by digestion with EcoRI and BamHI, blunted by Klenow and inserted into blunted pGalW (Brand and Perrimon, 1993) from which the GAL4 hp70polyA fragment had been excised by NotI digestion. The name, cytological location, and associated gene of the pQF-ET insertions in Figure S2B are: #17, 85C3, CG11033; #9, 49E7, Su(z)2; #12, 34A10, snoRNA:U2; #13, 44B5, kermit; #14, 39A5, intergenic; #10, 34A10, CG9426; #49, 70C15, Hsc70b.

pUAStatBb-QS
The QS cDNA was excised from pAC-QS with Acc65I and NotI, blunted, and inserted into the EcoRI/AatII cloning sites of pDonor-M2 to yield pQF-ET42. Of the 14 original insertions of pQF-ET42 from the initial injection, 5 showed only tracheal expression, 8 showed tracheal expression and expression in additional tissues, and only 1 (ET40) showed very minor tracheal expression and high expression in additional tissues (e.g., all imaginal discs, and some adult brain structures). The reason for the tracheal expression is currently unknown. We are generating additional enhancer trap vectors to circumvent the tracheal expression. The ET40 enhancer trap is inserted into the 5’ upstream region of the posterior sex combs (psc) gene located at 49E6. The name, cytological location, and associated gene of the pQF-ET42 insertions in Figure S2B are: #6, 78A2, skulid; #8, 47A7, lola; #31, 52E11, Ext2.

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The QF-SV40polyA fragment was excised from pQF-ET, w+ by digestion with EcoRI and BamHI, blunted by Klenow and inserted into blunted pGalW (Brand and Perrimon, 1993) from which the GAL4 hp70polyA fragment had been excised by NotI digestion. The name, cytological location, and associated gene of the pQF-ET insertions in Figure S2B are: #17, 85C3, CG11033; #9, 49E7, Su(z)2; #12, 34A10, snoRNA:U2; #13, 44B5, kermit; #14, 39A5, intergenic; #10, 34A10, CG9426; #49, 70C15, Hsc70b.

pUAStatBb-QS
The QS cDNA was excised from pAC-QS with Acc65I and NotI, blunted, and ligated into a blunted pUAStatBb (Bischof et al., 2007) containing elements in the following order: 5P-Ppromoter-LoxM2-MCS-SV40 terminator-white-LoxP-3P. QF was inserted immediately following the 5P promoter, and a LoxP site was placed after the selectable white marker to generate the vector pQUAST-FLPo.
pattB-QF-SV40

The QF-SV40 cassette from pQF-M2ET was PCR amplified to include EcoRI/NotI restriction sites and inserted into the pattB vector (Bischof et al., 2007).

pattB-QF-hsp70

The QF-hsp70 cassette from pBac-GH146-QF was PCR amplified to include EcoRI/NotI restriction sites and inserted into the pattB vector (Bischof et al., 2007). The hsp70 terminator results in reduced QF expression (in comparison to QF-SV40) and has enabled fly transgenesis using constructs that proved toxic with QF-SV40 (CJP and LL, unpublished results).

pattB-DSCP-QF-SV40

The DSCP from pBPGUw (Pfeiffer et al., 2008) was PCR amplified to include EcoRI/MfeI restriction sites and ligated into the EcoRI site of pattB-QF-sv40.

The previous three vectors contain convenient multi-cloning sites preceding the QF ORF for cloning of promoter/enhancer regions to drive QF expression. These QF constructs can be integrated into the fly genome using qC31-mediated integration (Bischof et al., 2007). The DSCP variant can be used if the cloned enhancer region does not contain a promoter (Pfeiffer et al., 2008). These constructs can also be used for convenient isolation of the QF ORF by restriction digestion.

pBS-SK-QS

The QS ORF was PCR amplified from pAC-QS to include EcoRI/XbaI restriction sites, and cloned into pBluescript-SK (Strategene). This construct contains convenient restriction sites before and after QS and can be used for cloning of QS promoter/enhancer constructs or for isolation of the QS ORF.

Expression Studies in Cultured Cells

S2 Cell Transfection

S2 cells (gift of M. Simon, Stanford University) were maintained in Shields and Sang M3 insect medium (Sigma, Catalog # S8398), prepared according to the manufacturer’s instructions and supplemented with 10% heat inactivated fetal bovine serum (FBS) and antibiotics (from 100x penicillin/streptomycin stock, Invitrogen, Catalog # 15140). FBS was heat inactivated at 56°C for 30 min.

The cells were grown in an air incubator at 25°C. For transfection, 0.4 ml of complete M3 medium containing 5*10^5 Drosophila S2 cells were plated into individual wells of 24-well plates several hours before the transfection. The DNA for transfection was mini-prepped (QiAprep Miniprep kit, QIAGEN, Catalog # 27106), DNA concentrations were determined using the NanoDrop 1000 spectrophotometer (Thermo Scientific), diluted in 10 mM TRIS-HCl pH 7.5, 0.1 mM EDTA to 25 ng/µl and filtered through a 13-mm 0.2 µm filter (Nalgene, Catalog # 180-1320). For individual transfections, we used 0.2 µg of total DNA (total of 8 µl from 25 ng/µl stocks) including one or more of the following: 25 ng of reporter, 12.5 ng of a transcription factor plasmid and the amount or repressor plasmid that was adjusted according to the molarity of the corresponding transcription factor plasmid to get either equimolar, 3-fold or 5-fold higher molar concentration, as indicated. Each sample also contained 2.5 ng of pPAC5C-hRluc, which was used for normalization of the firefly luciferase signal for each sample (see below) and pBluescript to supplement the total DNA amount to 0.2 µg. The cells were transfected using Effectene (QIAGEN, Catalog # 301425) according to the manufacturer’s protocol (for each sample we used: 59 µl EC buffer; 1.6 µl enhancer solution; 4 µl Effectene and 350 µl M3 medium). 12 hr after the addition of transfection mixes to cells, quinic acid was added where indicated from 50x stocks. The starting quinic acid stock solution (250 mg/ml) was prepared from D-(−)-quinic acid (Sigma-Aldrich, 98%, Catalog # 138622) in sterile Milli-Q water and neutralized with NaOH to pH ~7. All other 50x stocks were made from this stock by dilution in water. Cells were lysed 24 hr after the addition of quinic acid (36 hr since the start of transfection), by spinning the 24-well plates for 5’ in a table top centrifuge at ~1000 g, removing the supernatant by aspiration and incubation with of 200 µl passive lysis buffer (from the Dual Luciferase Reporter Assay System by Promega, Catalog # E1910) with shaking at room temperature for ~30 min. The lysates were transferred into 1.5 ml tubes and frozen at ~80°C. The lysates were analyzed using the Dual Luciferase Reporter Assay System, according to the manufacturer’s instructions, and the single tube Turner Biosystems luminometer, model 20/20n. The luminescence light signal for both firefly and Renilla luciferases was collected for 10 s. All samples that were transfected with reporters had at least 100-fold higher signal than the background (lysates from pBluescript-only transfected cells). For each sample “X,” the relative luciferase activity (RLA) was calculated according to the following formula:

\[
RLA_X = \frac{(F_X/R_X)}{(F/R)_{QUAS}}.
\]

where

\[
(F/R)_{QUAS} = \left( \sum_{i=1}^{n} \frac{F_{QUAS}^i}{R_{QUAS}^i} \right) / n.
\]

where \( n \) = number of QUAS-only samples; \( F \) = Firefly luciferase luminescence signal; \( R \) = Renilla luciferase luminescence signal. Each condition was executed at least in triplicate, the average and SEM were determined for each condition, and statistical significance was evaluated using Student’s t test. Plasmids used for transfection in S2 cells were: pUAS-luc2, pQUAS-luc2, pAC-QF, pAC-QS, pAC-GAL4, pAC-GAL80, pAC-hRluc, pBluescript.
Full suppression was not observed with equimolar ratios of QF and QS (similar lack of full suppression was observed with GAL4/ GAL80). This result could be a consequence of transient transfections, where individual cells do not necessarily uptake the same amounts of activator and repressor plasmids. Additionally, somewhat higher QS/QF ratios compared to GAL80/GAL4 ratios are required to reach similar degree of repression. The difference between the two systems probably lies in the specific nature of the individual genes or the corresponding proteins (activity, codon choice, mRNA or protein stability, etc.).

**HeLa Cell Transfection**

HeLa cells (gift of K. Wehner, Stanford University) were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen, Catalog # 10566), supplemented with 10% FBS and antibiotics (from 100x penicillin/streptomycin stock, Invitrogen, Catalog # 15140) in a 37°C air incubator with 5% CO₂. At least 5 hr before transfection, cells were detached and dissociated from the plate using TrypLE Express (Invitrogen, Catalog # 12605) and plated into 24-well plates at 7*10⁴ cells in 0.5 ml medium with FBS, but without antibiotics. Cells were transiently transfected using Lipofectamine 2000 (Invitrogen, Catalog # 11668-019), according to the manufacturer’s instructions. Plasmid DNA for transfection was prepared as described in the S2 cell transfection protocol above. For each sample we used 0.25 μg DNA (total of 10 μl from 25 ng/μl stocks) including one or more of the following: 50 ng of reporter, 25 ng of a transcription factor plasmid and the amount or repressor plasmid that was adjusted according to the molarity of the corresponding transcription factor plasmid.

**Drosophila Genetics and Manipulations**

**Transgenes**

Transgenes were generated by standard P-element (Spradling and Rubin, 1982) or PhiC31 integrase-mediated transformation (Groth et al., 2004), as noted in Table S1. All fly transgenes were mapped using splinkerette PCR (Potter and Luo, 2010). When necessary, transgenes were recombinated onto the same chromosome using standard meiotic recombination techniques. To check for Tsc1⁰⁶⁰⁰X (Potter et al., 2001) recombinants, a genomic region containing the Tsc1 point mutation was PCR amplified from genomic DNA using oligos gTsc1-1-REV (GCTGCAGTTTGTGGCGAGTG) and gTsc1-1-REV (AAACGCCATCCCGGTCCCATTCT) and cut with the restriction enzyme SnaBI. The SnaBI site was created by the C⁻ > T mutation in the Tsc1⁰⁶⁰⁰X allele. Wild-type Tsc1 gives an uncut band of 719 bp and the Tsc1⁰⁶⁰⁰X mutant gives bands of sizes 322 bp and 397 bp.

**Toxicity of QF**

We were unsuccessful in generating tubP-QF transgenic animals by either site-directed integration using PhiC31 integrase or P-element mediated transformation. These observations suggest that QF is toxic to flies when highly expressed in a ubiquitous manner or in a particular developmental stage or tissue. We note that in our previous effort to generate tubP-GAL4, we encountered similar difficulty in obtaining transgenes from microinjection or transposition. In parallel experiments, we obtained a single tubP-GAL4 transgene but more than a dozen tubP-GAL80 transgene insertions (Lee and Luo, 1999). Furthermore, repeated experiments of remobilizing the tubP-GAL4 transgene resulted in only one additional line on the same chromosome. Both tubP-GAL4 transgenes are homozygous lethal, but become homozygous viable in the presence of a tubP-GAL80 transgene, suggesting that high-level ubiquitous expression of GAL4 is toxic. Preliminary results indicate that QF-induced toxicity can also be suppressed by a tubP-QS transgene (CJP and LL, unpublished results). We suspect that high-level ubiquitous expression of strong transcription factors such as GAL4 and QF is harmful to flies — probably due to squelching of the transcriptional machinery. It is also possible that high-level expression of QF in some tissues and developmental stages is more harmful than equivalent expression of GAL4.

We are currently experimentally addressing this toxicity issue. As noted in the Recombinant DNA Construction section above, terminator choice can make a significant impact on QF expression and toxicity: by exchanging the SV40 terminator with an hsp70 terminator in QF constructs, we were able to generate transgenic animals containing promoter-QF-hsp70 that previously proved difficult to produce using an SV40 terminator (CJP and LL, unpublished results). In addition, we have generated a mutant QF protein (QFm) that exhibits ~5 fold less activity in S2 transfection assays compared to wild-type QF (BT and LL, unpublished results). We were able to obtain tubP-QFm transgenic animals, as well as > 100 QFm enhancer trap lines, but reporter expression levels were not sufficiently strong in many tissues (CJP and LL, unpublished results). We are in the process of isolating a QF variant that drives reporter expression sufficiently well in all tissues. We are also conducting experiments to address the possibility that...
an particular developmental stage or a particular tissue is especially sensitive to QF expression. We will report these findings to the fly community in a timely fashion. Nonetheless, we have isolated many QF enhancer trap lines that express strongly in many tissues without adverse effects, including imaginal discs (ET40-QF), glia (ET31-QF), trachea (ET14-QF) and neurons (GH146-QF and ET49-QF), suggesting that high-level expression of QF is not toxic to many cell types. We further note that the toxicity associated with high-level ubiquitous expression of GAL4 does not prevent the widespread use of the GAL4/UAS system.

**Coupled MARCM Analysis of Projection Neuron Lineage**

Vials containing approximately 15 males and 25 females were allowed to lay eggs for 6-12 hr. Animals were heat shocked in a 37°C water bath for 1 hr from 0 to 100 hr after egg laying (AEL). Brains from adult animals were dissected 3-5 days after eclosion. The genotype of GH146 coupled MARCM flies is: hsFLP, QUAS-mtdT-3xHA, UAS-mCD8-GFP (X); GH146-QF#53, 82B^FRT, tub-QS/tubP-GAL4, 82B^FRT, tubP-GAL80 (III)

**Coupled MARCM Analysis of Wing Disc Clones**

Vials containing approximately 15 males and 25 females were allowed to lay eggs for 6-8 hr. The progeny were heat shocked in a 37°C water bath for 30 min at 48 ± 3 hr AEL, and dissected 72 ± 3 hr after the heat shock. This 30 min heat shock leads to about 1-2 wild-type coupled MARCM wing clones per disc. (A 1 hr heat shock leads to approximately 5-10 clones per disc.) In control coupled MARCM experiments, coupled MARCM clones in the wing imaginal disc could be visualized in live samples (unfixed/ unstained) as early as 28 hr after the clone-inducing heat shock (clones were induced by a 2 hr heat shock at 60 hr AEL). This observation suggests that perdurance of GAL80 and QS is minimal 28 hr after clone induction.

**Quinic Acid Feeding of Flies**

Quinic acid containing medium was made as follows: a few holes were made in standard fly medium with wooden sticks, and 0.3 ml of freshly made D-(-)-quinic acid (Sigma-Aldrich, 98%, Catalog # 138622) solution dissolved in water was added per 10 ml of medium. Vials were allowed to air-dry overnight. Quinic acid appears to be stable in food stored for at least a week at 18°C, as judged by its derepression activity (e.g., Figure 2D). For assaying the effect of quinic acid on developing animals, approximately 10 males and 15 females per vial of the genotype ET40-QF, QUAS-mtdT-3xHA (II); 82B^FRT tubP-QS (III) were allowed to lay eggs for 6 hr on quinic acid containing medium. Animals fed upon and developed in the quinic acid containing food. Adult flies were imaged within 3 days of eclosion. For assaying the effect of quinic acid feeding on adult flies, approximately 30 adult flies per vial of genotype ET40-QF, QUAS-mtdT-3xHA (II); 82B^FRT tubP-QS (III) were allowed to feed on quinic acid medium for the listed times in Figure 2D. For feedings over multiple days, flies were transferred to fresh quinic acid containing food every two days. Starving flies for 24 hr did not lead to increased suppression, suggesting that both well-fed and hungry flies ingested the quinic acid food at similar levels. Flies fed with quinic acid food for up to 10 days exhibited no more derepression than those fed for 5 days.

**Imaging and Image Processing**

**Immunohistochemistry**

Confocal images were taken on a LSM 510 Confocal Microscope (Zeiss). The procedures for fixation, immunohistochemistry and imaging were as described previously (Wu and Luo, 2006). Primary antibodies used were Rat anti-CD8 (Caltag Laboratories, 1:200), Mouse anti-Repo (8D12, DSHB, 1:50), Mouse 24B10 (1:25), Rabbit anti-β-galactosidase (1:100), Chicken anti-GFP (Aves Labs, 1:100), Mouse anti-acj6 (DSHB, 1:50), Mouse anti-fibrillarin (72B9, 1:20), Rat anti-ELAV (7E8A10, DSHB, 1:100), Mouse anti-Repo (8D12, 1:50), Mouse 24B10 (1:25).

**Imaging of Adult Flies**

Adult flies were imaged on a QImaging Retiga 2000R Cooled Monochrome Digital camera using a Discovery V8 Pentavluor System (Zeiss) with a DS RED filter cube.

**GH146 Expression Pattern**

Both GH146-QF transgenic lines described in this study have approximately equal PNs expression patterns. The major difference is that GH146-QF#11 expresses QF in fewer ventral PNs than GH146-QF#53. GH146-QF#53 was used for all GH146 coupled MARCM experiments.

**Wing Imaginal Disc Quantification**

A customized Matlab script was used for the automated calculation of clone size and cell number in confocal stacks. Cell size (area) was calculated by dividing clone area by cell number. Confocal images were initially processed so that only one set of coupled MARCM clones was present in a confocal stack. The clone area was defined by first thresholding the red channel (mtdT-HA staining) or green channel (mCD8-GFP staining) to delineate the clone, and then calculated by summing the number of pixels above the set threshold. Clone areas in the red and green channels were independently calculated throughout the image stack. The z plane containing the largest clone area and two adjacent planes were recorded. The final clone size, cell number and cell size for each image were the average values of these three z planes. For automated counting of cell numbers in the stack, the thresholded red or green clone areas were used as a mask to define the region of interest in the blue channel (anti-fibrillarin staining which marks the single nucleolus in each cell). The algorithm for counting cell numbers was adapted from the ITCN Matlab script by Thomas Kuo and Jiyun Buyn (Center for Bioimage Informatics). The parameters for automated measurements were set according to test measurements of clone area and cell number, which were obtained by manually counting a small randomly selected image set. The obtained numbers were compared for statistical significance using Student’s paired t test.
Behavioral Analysis

Olfactory attraction was measured using a modified two choice trap assay (Larsson et al., 2004). 2-3 day old adult flies were starved for 40–42 hr in collection cages containing water moistened Kimwipes. Approximately 100 flies per assay were anaesthetized by cold and placed into a small culture dish at the bottom of a 85 mm x 170 mm, 1000 ml glass jar (Fisher, 02-912-305) covered by a 150 mm Petri dish (Falcon, 351058) that had three nylon mesh screened holes inserted for ventilation. Odor traps were constructed from 40 ml glass vials (National Scientific B7999-6) to which a custom-built polyethylene top containing a cut pipette tip was securely placed. Traps contained a cotton foam plug to which either 0.5 ml of 1% ethyl acetate (Sigma, > 99.5% purity) dissolved in mineral oil, or mineral oil alone, were added. The behavioral tests were conducted for two hours in a dark humidified room at 34 °C.

SUPPLEMENTAL REFERENCES

Figure S1. The Effect of Quinic Acid on Transiently Transfected Drosophila and Mammalian Cells, Related to Figure 1

(A) The effect of quinic acid on transiently transfected Drosophila S2 cells. Relative luciferase activity (normalized as described in Extended Experimental Procedures) is plotted on a logarithmic scale on the y axis, with QUAS-luc2 alone set to 1. Quinic acid (QA) was added where indicated to the final concentration of: QA1 = 12.5 µg/ml; QA2 = 250 µg/ml; QA3 = 5 mg/ml. All data are presented as average ± SEM. Statistical significance was evaluated using Student’s t test; ns, not significant; *p < 0.05; ***p < 0.001. QA, at the highest concentration tested, results in ~24-fold re-activation of QUAS, compared to the control sample with QUAS, QF and 5xQS. At this high concentration, QA has no effect on QF activation of QUAS or Gal4 activation of UAS, and it has a small but significant effect on GAL80 repression of GAL4: it derepresses GAL4 by ~1.5-fold. Plasmids used for transfections are noted below the x axis. QUAS, pQUAS-luc2 reporter; QF, pAC-QF; QS, pAC-QS; x5, five-fold molar excess of QS over QF.

(B) The effect of quinic acid on transiently transfected human HeLa cells. The data are presented, and statistical significance was evaluated, as in (A). QA has no effect on QF-dependent transcription of QUAS, but in the presence of QF and 5xQS, it further significantly suppresses QUAS activation by ~13-fold at the two higher QA concentrations tested. Explanations and abbreviations as in (A) except: QF, pCMV-QF; QS, pCMV-QS.
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Cell 141, 536–548, April 30, 2010 ©2010 Elsevier Inc.  S9
Figure S2. Further Characterization of the Q System In Vivo, Related to Figure 2

(A) Comparison of basal expression levels for UAS-, lexAO- and QUAS-reporter transgenes in the adult Drosophila brain. All images show maximum z-projections of confocal stacks. A1-A10 show anti-GFP antibody staining of adult brains of: (A6) a white^{1118} control fly, (A6) a commonly used UAS-mCD8-GFP fly (Lee and Luo, 1999), (A1-A4) four independent insertions of lexAO-CD2-GFP (Lai and Lee, 2006), (A7-A10) four independent insertions of QUAS-mCD8-GFP (this study). A11-A14 show anti-HA staining of adult brains for four independent insertions of QUAS-mtdT-HA (this study).

All brains that were stained against GFP (A1-A10) were processed using identical staining and imaging conditions, which were set such that the anti-GFP signal in control animals (GH146-GAL4 + UAS-mCD8-GFP) was fully saturated (not shown). All brains that were stained against HA (A11-A14) were processed using identical conditions. Imaging conditions were set such that the anti-HA signal in control animals (GH146-QF + QUAS-mtdT-HA) was fully saturated (not shown). All brains were heterozygous for the driver and reporter transgenes. Line numbers or genotypes are indicated on the bottom of each image. Scale bar = 50 μm.

(B) Expression of QF enhancer trap lines and their suppression by ubiquitous expression of QS. Fluorescence images of adult flies of QF enhancer traps (ET) lines expressing the same QUAS-mtdT-HA reporter are shown. ET line numbers are indicated in the first panel for each set of four panels. All images were taken at the same exposure (400 ms), except for ET6-QF, which was taken with a longer exposure. Inset panels for ET6-QF and ET12-QF are higher magnifications of the abdomen showing that each bristle socket is labeled. The animals are ordered from lowest expressing line (ET6-QF) to highest expressing line (ET49-QF) from top to bottom and continuing to the right column. Expression of each ET line can be suppressed by tubP-QS, although occasionally a few cells remained labeled with ET49-QF (not shown). White outlines mark the positions of the adult flies and magnified heads in the presence of tubP-QS. These QF enhancer trap lines exhibited no gross defects caused by QF expression. Line ET10-QF exhibited a crumpled wing, but this is likely due to a mutational insertion - other QF enhancer trap lines expressing more highly in similar tissues appeared normal (e.g., ET40-QF, ET9-QF, ET49-QF).
Figure S3. Expression of QF Enhancer Trap Lines in the Adult Brain, Related to Figure 2

(A) Representative limited z-projections of confocal stacks of whole mount Drosophila brains containing a QF enhancer trap (line number indicated on top right) and QUAS-mCD8GFP. The brains were immunostained for a general neuropil marker (monoclonal antibody nc82) in magenta, and for mCD8 in green. The QF enhancer trap line number is indicated within each panel. The lines are organized as in Figure S2B. All lines except ET40-QF express in trachea to varying degrees.

Notable expression patterns are:
- ET6-QF, ET10-QF, ET14-QF, and ET12-QF—mostly trachea with few neurons;
- ET31-QF—ensheathing glia (see Figure S3C);
- ET11-QF—AL, CC, and MB;
- ET8-QF—general expression in brain tissues, especially in the AL, CC, MB, OL and SOG;
- ET13-QF—expression throughout the brain;
- ET40-QF—AL, MB, OL and SOG;
- ET9-QF and ET49-QF—strong expression throughout the brain including AL, CC, MB and SOG. Full confocal stack files available upon request. Abbreviations: AL, antennal lobe; CC, central complex; MB, mushroom bodies; OL, optic lobe; SOG, subesophageal ganglion. Scale bars: 50 μm.

(B) GH146-QF labels olfactory PNs. The PN nuclei are labeled by GH146-QF driven QUAS-nuclear-LacZ (nucLacZ, anti-LacZ in green) and PN morphology is outlined by QUAS-mtdT-HA (anti-HA in red) transgenes. Note that all red neurons contain green nuclei. There are some scattered green-only cells (LacZ-positive, mtdT-HA-negative). As nuclear-LacZ is likely a more stable marker than mtdT-HA (our unpublished observations), the green-only cells might represent those that expressed GH146-QF transiently during development. Dotted lines represent the antennal lobes, which contain PN dendrites. Scale bars: 20 μm.

(C) ET31-QF labels ensheathing glia. (TOP) Limited z-projection confocal images of ET31-QF driving mCD8-GFP expression (left panel). The labeled cells ensheath neuronal tissues, such as the glomeruli of the antennal lobe (anterior section, middle panel) and mushroom body lobes (posterior section, right panel). (MIDDLE) Full z-projection confocal image of ET31-QF driving nuclear LacZ expression. The brain was stained against lacZ (green) and against the glial marker Repo (red); cells expressing both markers appear yellow (left panel). Limited z-projection confocal images of anterior (middle panel) and posterior (right panel).
sections show that many Repo-positive cells are labeled by ET31-QF. (BOTTOM) Full z-projection confocal image of ET31-QF driving nuclear LacZ expression (green), co-stained with an antibody against the neuronal marker ELAV (red); cells expressing both markers appear yellow (left panel). Limited z-projection confocal images of anterior (middle panel) and posterior (right panel) sections show that ET31-QF rarely labels ELAV-positive cells. Cells labeled by ET31-QF that are both Repo-negative and ELAV-negative most likely represent tracheal cells. The white boxes in the left panels show the brain regions that are magnified in the middle and right panels. Scale bars: 50 μm for left panels; 20 μm for middle and right panels.

(D and E) Enhancer trap lines ET9-QF and ET49-QF label many neurons. Full z-projections of confocal images of Drosophila brains containing ET9-QF or ET49-QF driving nuclear LacZ expression. The brains were stained against lacZ (green) and against the neuronal marker ELAV (red) (left panels). Limited z-projection confocal images of anterior (middle panels) and posterior (right panels) sections show that many neurons are labeled by these ET lines. Mushroom body neurons are outlined in images on the right. Scale bars: 50 μm for left panels; 20 μm for middle and right panels.
Figure S4. The QF and GAL4 Systems Do Not Cross-Activate or Cross-Repress In Vivo, Related to Figure 3

(A) QF and GAL4 systems do not cross-activate in vivo.
(First to Third row) Representative confocal projections of whole mount Drosophila brains immunostained for a general neuropil marker (monoclonal antibody nc82) in magenta, and for mCD8 in green. The genotypes are represented by the schematics in the left panels. Higher magnification images centered at the antennal lobe are shown in the right panels. Staining and imaging conditions were similar to those of Figure 2A. Scale bars: 50 μm for middle panels; 20 μm for right panels.
(First row) GH146-QF expression does not activate a UAS-mCD8-GFP reporter.
(Second row) GH146-GAL4 expression does not activate a QUAS-mCD8-GFP reporter.
(Third row) GH146-QF expression does not activate UAS-FLP since mCD8-GFP was not expressed from the QUAS > stop > mCD8-GFP reporter. Yellow triangle or ‘>’, FRT site; ‘!’ or ‘stop’, transcriptional stop.
(Fourth and Fifth row) Representative confocal projections of wing imaginal discs immunostained for mCD8 in green and nuclei using DAPI in blue. The genotypes...
are represented by the schematics in the left panel. Staining and imaging conditions were similar to those of Figure 5 and S6C. Scale bars: 50 μm. Yellow triangle, FRT site; ‘!’, transcriptional stop.

(Fourth row) QF expression throughout the wing imaginal disc (driven by ET40-QF) does not activate UAS-FLP since mCD8-GFP was not expressed from the QUAS > stop > mCD8-GFP reporter. 50 wing imaginal discs were examined. Eye imaginal discs (n = 30) also showed no reporter expression (not shown). Yellow triangle or ‘>’, FRT site; ‘!’ or ‘stop’, transcriptional stop.

(Fifth row) GAL4 expression throughout the wing imaginal disc (driven by tubP-GAL4) does not activate QUAS-FLP since mCD8-GFP was not expressed from the UAS > stop > mCD8-GFP (right panel) reporter. 20 wing imaginal discs were examined. Eye imaginal discs (n = 6) also showed no reporter expression (not shown). Yellow triangle or ‘>’, FRT site; ‘!’ or ‘stop’, transcriptional stop.

(B) QF and GAL4 systems do not cross-repress in vivo.

Representative confocal projections of whole mount Drosophila brains immunostained for a general neuropil marker (monoclonal antibody nc82) in magenta, and for mCD8 in green. The genotypes are represented by the schematics on the left. Higher magnification images centered at the antennal lobe are shown on the right. Comparison of first and second rows indicates that GH146-QF expression, as reported by QUAS-mCD8-GFP, is not affected by ubiquitous expression of GAL80. Comparison of third and fourth rows indicate that GH146-GAL4 expression, as reported by UAS-mCD8-GFP, is not affected by ubiquitous expression of QS. All experimental conditions (staining protocol, antibody concentrations, number of brains per staining, imaging conditions) were identical. Scale bars: 50 μm for middle panels; 20 μm for right panels.

(C) Schematic of independent double MARCM. tubP-GAL80 and tubP-QS are placed on two different chromosome arms. Two types of mitotic recombination events (1 and 2) are independent of each other and result in differently labeled progeny. Cells homozygous for a single mutation (x or *) are singly colored green or red, respectively, while cells homozygous for both mutations appear yellow. Additional UAS or QUAS transgenes can be included into the scheme, so that progeny containing active GAL4 or QF can express additional markers or effector genes. Centromeres are represented as circles on the chromosomes. This type of manipulation will be useful for studying gene function in cell-cell interactions and in comparing phenotypes of single and double mutants in the same animal.
Mitotic recombination followed by specific chromosome segregation produces two distinct progeny. One progeny is devoid of the QS transgene and is therefore capable of expressing a red fluorescent protein (R, QUAS-mtdT-HA) via GH146-QF in PNs. The other progeny is devoid of the GAL80 transgene and is therefore capable of expressing a green fluorescent protein (G, UAS-mCD8-GFP) via tubP-GAL4 in any cell that is the sibling of the red cell above. FLP expression during development is mediated by a 1 hr heat-shock induction of a hs-FLP transgene (not diagrammed).
Figure S6. Expression of QF, or Coexpression of QF and GAL4, Does Not Affect Imaginal Disc Development, Related to Figure 5

(A) Eye disc differentiation is not affected by QF expression. Third instar eye imaginal discs were stained for nuclei (DAPI), HA (from QUAS-mtdT-HA), 24B10 (photoreceptor specific marker), and ELAV (neuronal differentiation marker) to monitor normal differentiation. Marker expression appears indistinguishable in eye imaginal discs that express QF throughout eye disc development (ET40-QF, rows three and four) and in control wild-type imaginal discs (Canton S, rows one and two). Scale bars: 50 μm for rows 1 and 3; 20 μm for rows 2 and 4. Rows 2 and 4 show higher magnification images of eye discs from Rows 1 and 3.

(B) Adult eye section of ET40-QF animals, which expressed QF throughout eye disc development, exhibits no defects. The dashed line labels the dorsal/ventral equatorial border.

(C) Expression of QF and GAL4 together in wing imaginal discs does not affect expression levels of QF and GAL4 reporters. Third instar eye imaginal discs of the genotypes listed on the left were stained for nuclei (DAPI), mCD8 (from UAS-mCD8-GFP), and HA (from QUAS-mtdT-HA). Expression of QF does not affect the levels of GAL4-mediated reporter expression (compare anti-mCD8 signal in top row and third row), and expression of GAL4 does not affect QF-mediated reporter expression (compare anti-HA signal in second and third rows). To limit experimental variability during immunostaining, 15 wings discs for each of the three genotypes were stained together in the same tube. Scale bars: 50 μm.

(D) Wing disc expression of QF, or coexpression of QF and GAL4, does not affect adult wing morphogenesis. Light microscope images of whole adult wings and magnified images of the anterior wing regions are shown for the genotypes listed on top. The development and morphogenesis of the adult wing (as monitored by vein pattern, bristle orientation and wing size) is unaffected by expression of QF, GAL4, or QF+GAL4. For each genotype, we examined at least 20 wings and all wings appeared normal with the exception that one out of 46 wings in the QF+GAL4 condition exhibited an incomplete L3–4 cross vein. Wing sizes were calculated in ImageJ by measuring the wing area (excluding the wing hinge). No significant differences in wing size were found. Error bars are ± SEM.
<table>
<thead>
<tr>
<th>Logic Gate</th>
<th>A-GAL4</th>
<th>B-QF</th>
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<td></td>
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Figure S7. Reporter Expression Patterns Derived from Logic Gates of QF and GAL4 Expression Patterns, Related to Figure 6

Grey squares represent the original expression pattern of GAL4 or QF (top line), or the reporter (R) expression resulting from the listed logic gate. The transgenes required for the intersectional logic gates are listed in the right column. Not listed here are four remaining logic gates (A, B, FALSE, TRUE), which do not create new expression patterns. Transgenes not yet constructed are italicized. Although there are multiple ways to construct each logic gate, we show only one for each except for the AND gate, for which we have experimentally demonstrated two alternative methods. ‘>’ represents an FRT site.