Refined LexA transactivators and their use in combination with the *Drosophila* Gal4 system

Ryohei Yagi, Franz Mayer, and Konrad Basler¹

Institute of Molecular Life Sciences, University of Zurich, CH-8057 Zurich, Switzerland

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The use of binary transcriptional systems offers many advantages for experimentally manipulating gene activity, as exemplified by the success of the Gal4/UAS system in *Drosophila*. To expand the number of applications, a second independent transactivator (TA) is desirable. Here, we present the optimization of an additional system based on LexA and show how it can be applied. We developed a series of LexA TAs, selectively suppressible via Gal80, that exhibit high transcriptional activity and low detrimental effects when expressed in vivo. In combination with Gal4, an appropriately selected LexA TA permits to program cells with a distinct balance and independent outputs of the two TAs. We demonstrate how the two systems can be combined for manipulating communicating cell populations, converting transient tissue-specific expression patterns into heritable, constitutive activities, and defining cell territories by intersecting TA expression domains. Finally, we describe a versatile enhancer trap system that allows swapping TA and generating mosaics composed of Gal4 and LexA TA-expressing cells. The optimized LexA system facilitates precise analyses of complex biological phenomena and signaling pathways in *Drosophila*.

genetic tools | binary transcriptional system

A powerful strategy to gain mechanistic insights into biological phenomena is to manipulate gene activity in model organisms and to monitor the resulting phenotypic consequences. The modularity of binary transcriptional systems based on a transactivator (TA) driving effector gene (*EG*) expression (Fig. S1A) is often advantageous to directly driving the expression of a gene of interest by a specific promoter. In binary systems, such as the Gal4, the LexA, and the tetracycline-regulatable systems, the TA is under specific promoter-control, whereas the *EGs* are regulated by TA-binding sites. This setup enables combinatorial use of TA drivers and *EGs*, facilitates the repeated analysis of lethal phenotypes, and results in higher gene expression levels due to transcriptional amplification (Fig. S1A).

The Gal4/Upstream Activating Sequence (*UAS*) system is the most extensively used binary transcriptional system in *Drosophila* (1). Thousands of Gal4 driver lines have already been established, which exhibit distinct Gal4 expression profiles during development. Moreover, several tools have been introduced that control the expression or activity of Gal4 (2), such as the yeast repressor Gal80, which efficiently represses Gal4 (3); a temperature-sensitive Gal80 mutant (Gal80^{ts}) that allows temporal control (4); and the yeast *Flp/FRT* recombinase system to render the Gal4 system inducible in an irreversible manner (5, 6).

Despite its power, the Gal4/*UAS* system alone is insufficient to perform a number of sophisticated experiments in developmental biology. Three examples for situations that would require a second, independent binary transcriptional system in conjunction with Gal4 are depicted in Fig. 1A and described in its legend. For all these cases, the activity of the second TA should be high, whereas potential side effects should be low.

The LexA system is a binary transcriptional system that has been used extensively in yeast for two-hybrid assays. It is based on a bacterial transcription factor that binds to specific sequences called *lexA operator* (*lexO*). When fused with a transcriptional activation domain from eukaryotic species, LexA TA activates *EGs* that are preceded by multimerized *lexO* sites and a basal

promoter. Several attempts (7–9) to use LexA in *Drosophila* have been reported, the most extensive of which (8) described two chimeric proteins, LexA::VP16 (LV16) and LexA::GAD (LG), containing the activation domains of herpes simplex virus VP16 and yeast Gal4, respectively. However, despite their introduction several years ago, LexA-based binary transcriptional systems never enjoyed significant success, possibly for one or several of the following reasons: (i) deleterious effects with artificial TAs containing the VP16 complete activation domain (VPcAD) in cells or model organisms (10–12); (ii) lower transcriptional activity of LG compared with full-length Gal4; (iii) paucity of drivers and tools; and (iv) lack of an enhancer trap system.

Here, we report our attempts to rectify this situation. We first improved the transcriptional activity of the Gal80-suppressible LG by introducing the hinge region (H) of Gal4. Using multimeric forms of a minimal activation domain of VP16 (VPmAD) (13), which is less deleterious than VPcAD, we then engineered two series of Gal80-insuppressible LexA TAs, LV_n and LHV_n (with *n* ranging from 1 to 3, denoting the copy number of intact VPmADs). By altering the Gal4 transactivation domain of LG and LHV by a point mutation, we obtained two additional Gal80-insuppressible LexA TAs. These TAs exhibit different strengths, depending on the number of the VPmADs and the presence of H. All LexA TAs were compared in terms of their transcriptional and possible detrimental activities to Gal4 and in terms of their thermosensitivity. We demonstrate the usefulness of the optimized LexA system in conjunction with Gal4 by conducting experiments representative for the three situations in Fig. 1A: (i) programming two cell populations by different TAs to manipulate Dpp morphogen gradient formation; (ii) converting a Dpp-dependent Gal4 driver into a constitutively active LexA driver by means of CONVERT (conversion of an enhancer activity, regulated in a temperature-sensitive manner); (iii) manipulating Dpp levels in an intersectional domain formed by overlapping Gal4 and LexA activities. Finally, we developed a versatile Gal4-enhancer trap system termed G-MARET (Gal4-based Mosaic-inducible And Reporter-exchangeable Enhancer Trap), which has two distinct functions: The initial Gal4 reporter can be changed to a LexA TA, and with the resulting transgene, genetic mosaics can be produced that are composed of two types of cells expressing either Gal4 or LexA TA in a tissue-specific manner. We show how G-MARET can be used to increase the number of LexA TA drivers and to perform analyses of cell-cell communication between two distinct cell populations. Together, the optimized LexA system and G-MARET enable sophisticated functional analyses of signaling pathways and other complex biological phenomena.

Results

System for Evaluating LexA TAs in the Wing Disc. To compare individual LexA TAs in terms of activity and tissue tolerance, we

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¹To whom correspondence should be addressed. E-mail: konrad.basler@imls.uzh.ch.

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Fig. 1. (A) Three examples of experiments that require two independent binary transcriptional systems. (i) The areas A (green) and B (blue) represent two interacting cell populations defined and manipulated by *a-Gal4* and *b-LexA TA*, respectively. Variations in the balance between the activities of Gal4 and a LexA TA allow manipulation of the interaction. (ii) If the *a* promoter activity is influenced by the manipulation of A then *a-Gal4* (green) could be affected. This problem can be circumvented if the *a-Gal4* activity is irreversibly converted to a constitutive *c-LexA TA* activity by using the *Flp/FRT* technique: e.g., Flp driven by *a-Gal4* removes the *FRT*-flanked transcriptional termination cassette (>stop>) from *c>stop>LexA TA*, giving rise to expression of *c-LexA TA* (orange) in the area A independent of the *a-Gal4* activity. (iii) Flp driven by *d-LexA TA* removes the >stop> from *UAS>stop>X* in D (magenta). The *UAS>X* is activated in E (yellow) at the intersection of A ∩ D because of Gal4 expression in A. (B) Schematics of the Gal4 (G4) and LexA TAs used in this study. GAD (G), G4 AD; GDBD, G4 DBD; H, G4 hinge region; L, LexA; TP, Thr860 to Pro modification in Gal4; VPcAD, VP16 complete AD; VPmAD, VP16 minimal AD; V_n, n tandem copies of the VPmAD; V₁, one copy of the VPmAD flanked by two mutated VPmADs (VPmAD^{FG} and VPmAD^{FY} indicate substitutions of phenylalanine to a glycine or a tyrosine) (13).

generated a transgenic platform based on *dpp* imaginal disc enhancer sequences and site-specific $\phi C31$ -integration (14) at chromosomal position 86Fb (Fig. S1B). As monitored by a *UAS-GFP EG*, the *dpp-Gal4-86Fb* transgene showed Gal4 activity in the *dpp* domain of the wing disc, a narrow stripe of cells in the anterior compartment along the anteroposterior (A/P) compartment boundary (Fig. S1C). The *dpp-86Fb* system has several properties making it suitable for the comparative analysis of exogenous TAs. First, because all TA transgenes are integrated at the 86Fb site, they are all under identical genomic influence. Second, the coding sequences (cds) of the TAs are all flanked by the same 5' and 3' UTR from an *hsp70* gene. Third, the intensity and spatial extent of EG expression (e.g., intensity and width of the GFP stripe) reflect activity and protein levels of the TA. Fourth, the system can report a range of detrimental effects caused by the TA; e.g., the heterozygous *dpp-Gal4-86Fb* insertion is viable and fertile at 18 °C but lethal at pharate adult stage at 29 °C. Finally, embryonic lethality does not complicate the analysis of potential deleterious effects because the *dpp* disc enhancer is restricted to larval stages (15). Some LexA TAs showed lethality in late pupal or adult stages. To circumvent this problem and to establish transgenic lines of such LexA TAs, we inserted an *FRT*-flanked transcriptional termination cassette between the promoter and the coding region (Fig. S1B).

For quantitative comparisons of TA activities, we established a dual luciferase assay in wing discs. The system comprises *dpp-Renilla-luciferase-86Fa* (*dpp-Rluc*), which constitutively expresses Rluc in the *dpp* domain, and either *lexO-* or *UAS-Firefly-luciferase-22A* (*-Fluc*), which can be induced by a *dpp-TA-86Fb* (Fig. S2A). This setup allowed us not only to compare activities of the LexA TAs, but also to compare the LexA TAs with Gal4.

Engineering Refined LexA TAs. First, we examined the two previously reported LexA TAs, LG and LV16, by generating *dpp-LG-86Fb* and *dpp-LV16-86Fb* transgenics. *dpp-LG-86Fb* animals exhibit less reporter gene activity than *dpp-Gal4-86Fb* (Fig. 2A; see below). Combined with the fact that the *lexO* transgenes contain more TA binding sites than the *UAS* transgenes (eight versus five, respectively, throughout this work), the data show that LG is a weaker TA than Gal4. *dpp-LV16-86Fb* showed signifi-

cantly higher activity (Fig. 2E; see below), but even at 18 °C, such animals died soon after eclosion or were unhealthy and sterile. They also exhibit truncated legs (Fig. 2L) and a slightly reduced eye size.

We then set out to generate improved LexA TAs. To obtain Gal80-suppressible LexA TAs with increased activity, we modified LG by inserting either the SV40 NLS (Fig. S1D) as done in other systems (7), or the Gal4 hinge region (H, corresponding to the middle part of Gal4), between the LexA and the GAD domains (Fig. 1B). The H region contains a short stretch enriched in acidic amino acids (aa 148–196) that serves as an auxiliary activation domain in yeast (16). Unexpectedly, the NLS had the negative effect of reducing the activity of the TA (Fig. S1F). The insertion of H, however, increased the activity of LG by ≈ 3.5 -fold (LHG, Fig. 2B; see below) while still preserving Gal80-suppressibility (Fig. S1J).

To engineer Gal80-insuppressible LexA TAs, we used two strategies: first, the addition of different transcriptional activation domains and second, to convert LG and LHG into Gal80-insuppressible forms by mutation. In our attempts to use less harmful activation domains, we first used a portion of the human NFkB subunit p65 (aa 283–551) (10) (Fig. S1D). This domain has been used for the progesterone-inducible Gal4 system in *Drosophila* (17). We generated *Lp65* in the *dpp-86Fb* platform, but the resulting transgenic animals died soon after eclosion and stable lines could not be established. *dpp-Lp65-86Fb* flies displayed truncated legs and an irregular eye-shape (Fig. S1H), suggesting that *Lp65* is deleterious to flies, like *LV16*. A shortened derivative of the VP16 activation domain, VPmAD, is tolerated at higher intracellular concentrations (13). This motif (referred to as V) is only 12 amino acids in length (aa 436–447) and was grafted in different copy numbers onto LexA, with and without the H domain, to generate LV₁, LV₂, and LV₃, as well as LHV₁, LHV₂, and LHV₃ (Fig. 1B). Although most of these distinct LexA TAs (LV_n and LHV_n) show high transcriptional activities (Fig. 2 F–K; see below), none of them caused detectable morphological phenotypes in the *dpp-86Fb* system (Fig. 2M).

Gal80-insuppressible forms of LG and LHG (Fig. 1B) were obtained by the use of an amino acid substitution, which was shown in yeast to abolish suppression by Gal80 (Gal4^{T860P}) (18). The modified TAs, LG^{TP} and LHG^{TP}, were resistant to the

Fig. 2. (A–K) The activity of the different *dpp*-LexA TAs. Representative fluorescence images showing expression of *rCD2::GFP* (mGFP; green) in a wing disc heterozygous for *lexO-rCD2::GFP*; *dpp*-LexA TA at 18 °C. Nuclei were stained with DAPI (DNA; blue). (Scale bars: 50 μ m.) (L and M) *dpp*-LV16-86Fb heterozygous animals display a morphological defect, whereas *dpp*-LHV3-86Fb heterozygotes do not. Arrows show leg truncation. (N) Relative activities of less detrimental LexA TAs and G4 compared with LG, analyzed by dual luciferase assay in wing discs. Activities of LexA TAs at 25 °C and that of G4 at 18 °C are shown. Numbers above the bars indicate activity relative to LG. Gal80-suppressible LexA TAs are orange.

presence of Gal80 (Fig. S1 K and L), while retaining significant transcriptional activities (Fig. 2 C and D; see below).

Finally, we quantitatively assessed the activities of the various refined LexA TAs in the *dpp*-86Fb luciferase system in a systematic manner against each other and in comparison with the benchmark activator Gal4. These results are shown in Fig. S2 B–E and allow at least two conclusions: (i) Detrimental effects do not appear to correlate with strength of transcriptional activation; for example, although the entire LHV_n series shows higher activity than LV16, none of them causes any observable phenotypic side effects. (ii) The various LexA TAs provide a wide spectrum of transcriptional potential (Fig. 2N), i.e., activities below and above that of Gal4, with the selectable option of Gal80 sensitivity.

Characterization of LexA TAs for Dual Binary Transcriptional Systems.

As a further step toward designing sophisticated setups with two dual binary transcriptional systems, we characterized the LexA TAs in terms of protein levels, detrimental effects when combined with Gal4, and temperature sensitivity. We analyzed expression of each LexA TA in the wing discs by immunostaining with an anti-LexA antibody (Fig. S3). Protein levels vary among distinct LexA TAs: e.g., LHG and LG are present at higher levels than the TAs of the LHV_n series and LV3. Because all *dpp*-LexA-TA-86Fb constructs bear the same *hsp70* UTR and were integrated at the same chromosomal position, the differences in protein levels probably reflect distinct protein stabilities.

High levels of exogenous TAs such as GDBD::VPcAD are known to give rise to nonspecific inhibition of host-cell transcription (19), which may result from titrating away components of the basal transcriptional machinery. Concomitantly expressing

two distinct exogenous TAs (e.g., at the intersection of two domains) and the resulting high levels could thus be more harmful than expressing either one alone. To address this possibility, we examined whether the combined expression of Gal4 and a LexA TA has detrimental effects on imaginal disc cells. Using the strong *apterous* (*ap*)-Gal4 driver in conjunction with various *dpp*-LexA-TA-86Fb transgenes, high Gal4 levels overlapped with LexA TAs in the *dpp* domain of the dorsal wing compartment. Of the five different intersections examined (*ap*-Gal4 with LV3, LHV2, LG, LHG), only *dpp*-LHG-86Fb showed elevated levels of activated Caspase 3. This elevation of apoptosis, however, turned out to be due to the high levels of LHG alone, because it was also observed in the dorsal *dpp* domain without coexpression of *ap*-Gal4.

Gal4 exhibits a striking temperature dependency in *Drosophila* (2), having a higher activity at 29 °C than at 18 °C. To examine whether LexA TAs also show this property, we compared the activity of a representative selection of LexA TAs, LV3, LHV2, LG, and LHG at two temperatures, 18 and 29 °C, by using the *dpp*-86Fb system. Except for LG, which had a slightly higher activity at 29 °C than at 18 °C, the activity of LexA TAs tested did not significantly differ at the two temperatures (Fig. S2F).

Independent Manipulation of Dpp-Producing and -Receiving Cells.

Using the information and tools derived so far, we set out to test how the LexA reagents could be used in conjunction with the Gal4 system. In the first scenario, we wanted to establish a system in which fluorescently tagged Dpp could be followed through tissue that has been genetically manipulated during wing development. Dpp is produced in a stripe of cells adjacent to A/P-compartment boundary and spreads to regulate patterning and growth of the wing through activating its intracellular signaling pathway (Fig. S4A). How the Dpp gradient is formed is an issue of great interest in developmental biology (20). One of the obstacles to investigate the mechanisms of Dpp spread has been the absence of a suitable system to screen for genes involved in this process. The combined use of two dual binary transcriptional systems could be applied to overcome this hurdle. In our setup (Fig. 3A), *dpp*-LHG-86Fb and *tub*-Gal80^{ts} (4) are used to induce the expression of a *lexO*-Egfp::*dpp* transgene, and *engrailed* (*en*)-Gal4 are used to manipulate the Dpp-receiving cells of the P compartment with a *UAS*-EG. The system is fully regulatable: inactive at 18 °C due to the Gal80^{ts} repressor and active at 29 °C, allowing to control the period of activation by shifting the temperature. Once the dual binary transcriptional system is activated, Egfp::Dpp is expressed and secreted under the control of *dpp*-LHG-86Fb and can be visualized by fluorescence microscopy. The P compartment cells express Gal4, and a *UAS*-EG can be used to manipulate them. Using the A compartment as a control, even slight changes in Egfp::Dpp transport can be detected in the manipulated P compartment.

As a proof of principle, to manipulate the Dpp-receiving cells in the P compartment, we used a *UAS*-RNAi construct for the *thickveins* (*tkv*) gene that encodes the Dpp type I receptor (*UAS*-*tkv*^{IR}). After a temperature shift, detectable levels of Egfp::Dpp were expressed in the *dpp* domain of the wing discs. The Egfp::Dpp is biologically active (21) and expressed at elevated levels causes an overgrowth phenotype toward the lateral sides (Fig. 3B). When the *UAS*-*tkv*^{IR} was driven by *en*-Gal4, the size of the posterior wing pouch was significantly reduced (Fig. 3C and Fig. S4C). Correspondingly there was also a severe reduction of the phosphorylation levels of the signal transducer Mad (pMad). The results suggest that the system works as we designed and can be applied to dissect the transport and signaling of Dpp and for conducting large-scale screens; for example, with *UAS*-EG or -RNAi libraries.

Converting Transient *brk* Enhancer Activity into Constitutive Expression by the CONVERT Technique.

brinker (*brk*) is a target of Dpp signaling and is negatively regulated by the pathway (Fig. S4A): *brk* is expressed at high levels in the lateral regions where Dpp signaling activity is low, but repressed in the center where

Dpp is high. The laterally active *brk-Gal4* driver, a useful tool to manipulate cells receiving low levels of Dpp, is, however, subjected to artificial feedback regulation when used in conjunction with EGs encoding Dpp pathway components, limiting its use. For example, ectopic activation of Dpp signaling by the dominantly active form of *tkv* (*tkv^{QD}*) in lateral regions, where *brk* is expressed, decreases *brk* expression (22), leading to suppression of *brk-Gal4* activity and variable *tkv^{QD}* levels. To avoid such unwanted feedback loops, we devised the CONVERT technique for switching tissue-specific enhancer activity into constitutive promoter activity. The method uses the two dual binary transcriptional systems, *Gal80^{ts}* and the *Flp/FRT* method (Fig. 3D and Fig. S4D). Inactivating *Gal80^{ts}* by a temperature shift leads to the tissue-specific activity of a Gal4 driver of interest, inducing expression of a *UAS-flp* transgene. The Flp recombinase excises a *>stop, yellow⁺>* flp-out cassette (*>y⁺>*) present between a constitutive promoter and the cds of a *Gal80*-insuppressible LexA TA, such as *LHV_n* or *LV_n*. High levels of Flp are required for this reaction, necessitating amplification of Flp expression via a binary transcriptional system. Once the cassette is removed, the constitutive promoter activates the LexA TA, independent of temperature and Gal4 activity, and, most importantly, in a heritable manner that is no longer signaling-dependent.

To test whether the CONVERT system can be used to “freeze” an expression pattern and liberate it from unwanted feedback loops, we attempted to express *tkv^{QD}* in the *brk* domain (Fig. 3D). Upon a temperature shift to 29 °C, *brk-Gal4* became active as monitored by *UAS-GFP* expression and caused Flp-mediated removal of the stop-cassette from the *actin5C(act)>y⁺>LHV₂*

transgene. After a shift back to 18 °C, although GFP expression was reduced, cells in the *brk* domain constitutively expressed *LHV₂*, as evidenced by the *lexO-RFP* and *lexO-tkv^{QD}* transgenes, which caused RFP expression and overgrowth in the lateral disc (Fig. 3E).

Manipulating Dpp Levels in an Intersectional Domain. The availability of two independent binary transcriptional systems will allow researchers to genetically target a set of cells defined by the intersection of two larger overlapping sets of cells. As a proof of principle, we selected the *apterous* and *dpp* expressing populations and attempted to drive a Dpp transgene just in the intersection (Fig. S4E). Larvae were generated that carry *ap-Gal4*, *tub-Gal80^{ts}*, *dpp-LG-86Fb*, *lexO-flp*, and *UAS > CD2,y⁺>dpp*. To drive Dpp transgene expression in just the dorsal *dpp* domain of the wing disc, we increased the temperature to 29 °C, which inactivates *Gal80^{ts}* and, thereby, activates Gal4 and LG. In the *dpp* domain, LG drives Flp that, in turn, removes the *>CD2,y⁺>* flp-out cassette from the *UAS-dpp* transgene. Because Gal4 is expressed exclusively in the dorsal compartment (*ap-Gal4*), the *dpp* transgene and, consequently, Dpp levels, are only raised in the dorsal *dpp* domain (Fig. S4F). Because Dpp is secreted, the effect of this manipulation can also be observed in the ventral compartment as manifested by ectopic phosphorylation of the signal transducer Mad (Fig. S4G).

G-MARET: A Versatile Gal4-Enhancer Trap System That Enables Reporter Exchange and Induction of Mosaics. To generate a variety of LexA TA drivers, we set up a versatile *Gal4*-enhancer trap

Fig. 3. Using the LexA system in conjunction with Gal4. (A–C) A screening system for genes involved in Dpp signaling/transport. At the restrictive temperature for *tub-Gal80^{ts}*, *dpp-LHG* drives *lexO-Egfp::dpp* and *en-G4* drives *UAS-X^{IR}* (IR, inverted repeat to induce RNAi). (B and C) Third instar wing discs from larvae with the indicated genotypes. The system was activated by a temperature shift to 29 °C at the early larval stages. *Egfp::Dpp* (green) driven by *dpp-LHG* causes overgrowth of the wing discs. *pMad* (red) reports the status of Dpp signaling. *En* (yellow) defines P compartment. (C) *tkv^{QD}* driven by *en-G4* results in reduction of *pMad* levels in the P compartment and of size in the pouch. (D and E) Using the CONVERT technique to fix an expression pattern. In the conventional G4-based system, expression of *tkv^{QD}* by *brk-G4* decreases *brk* promoter activity, leading to suppression of the *brk-G4* activity and variable *tkv^{QD}* levels due to an artificial feedback loop. The CONVERT system converts *brk-G4* activity to constitutive *LHV₂* activity. At the restrictive temperature for *tub-Gal80^{ts}*, Flp expressed by *brk-G4* irreversibly removes the *>y⁺>* from *act>y⁺>LHV₂*. The resulting *act>LHV₂* clones in the *brk* domain will now constitutively express *tkv^{QD}*, irrespective of the later status of *brk-G4*. (E) Third instar wing discs in which Dpp signaling in the *brk* domain is manipulated. *mGFP* (GFP; green) and *mRFP* (mCherry::CAAX; red) are expressed under control of *brk-G4* and *act>LHV₂*, respectively. Genotypes of the larvae, and the conditions for the temperature shift and cycles are shown. In the temperature shift conditions, most cells of the *brk* domain with *tkv^{QD}* lost mGFP expression because of suppression of *brk-G4* activity. Nuclei were stained with DAPI (DNA; blue). Schematics in A and D: red and blue lines represent positive and negative signals, respectively. (Scale bars: 50 μm.)

cation, cell competition, or regeneration, processes in which the behavior of one population depends on that of another.

In addition to the technical tools described above, we have established proof of principle for three applications in which the LexA and Gal4 systems are used simultaneously to enhance experimental precision. The first of these applications is a system enabling the analysis of Dpp gradient formation. Independent manipulation of signal sending and receiving cells allows the visualization of the Dpp signal, by means of the fluorescent Egfp::Dpp, in a context where a large portion of the tissue, one compartment of the wing disc, is subjected to genetic manipulation. The availability of *UAS-RNAi* libraries will enable high-throughput in vivo screens for factors involved in movement, transport, and signaling of Dpp.

Despite the large number of tissue-specific Gal4 drivers mentioned above, many of these have been used to manipulate gene function without paying attention to potential artificial feedback loops. With the CONVERT technique, one can now circumvent such potential problems. Additionally, CONVERT can also be used for fate mapping. The conversion of a transient expression pattern into strong heritable marker gene activity allows the lineage tracing of cell populations. Particularly useful for this purpose is the temporal control feature of this system, which enables transient expression patterns to be fixed.

The third application uses the two TA systems to restrict expression to a subset of cells within an enhancer domain. One TA drives Flp recombinase in a particular domain, whereas the other TA drives the biological effector transgene, which only becomes functional upon Flp exposure. As more LexA enhancer trap lines are established, a vast number of intersectional cell populations can be targeted by the combinatorial use of Gal4 and LexA drivers.

Finally, the Gal4 system has already been used in zebrafish (25) and mice (26). Because our optimized LexA TAs are composed of protein domains well characterized in vertebrate systems, it is likely that the described LexA system will also be useful in higher model organisms to conduct sophisticated experiments in conjunction with Gal4.

Very recently, a new binary expression system, termed Q system, was reported (27). This system uses regulatory genes from the *Neurospora crassa qa* gene cluster and permits sophisticated experi-

ments owing to its own repressor, which functions independently of the Gal4 system (Fig. S5). Nonetheless, as described above, the refined LexA system has some advantages compared with the Q system (Fig. S5), such as the choice of TA activity from a wide range. Together, both the Q and the LexA systems not only complement the use of Gal4, but they are also ideally suited to be used concomitantly, opening up an even wider spectrum of experimental manipulations.

Materials and Methods

Plasmid Construction. Unless otherwise noted, the plasmids were constructed by using standard molecular cloning methods. When plasmids contain newly synthesized nucleotide (nt) sequences via PCR, oligonucleotide synthesis, or mutagenesis, the sequences were verified by DNA sequencing. A detailed description of each construct is provided in *SI Materials and Methods*.

Histology. Antibodies used are Rabbit anti-LexA DBD (1:500; Upstate Biotechnology), Rabbit anti-cleaved Caspase 3 (1:100; Cell Signaling), Rabbit anti-phosphorylated Mad (1:1,000; gift from Ed Laufer, Columbia University, New York), Mouse anti-En (4D9, 1:20), Mouse anti-Wg (4D4, 1:500; DSHB), Mouse anti-rCD2 (1:200; Serotec) and Alexa Fluor 555 F(ab')₂ fragments of goat anti-mouse and -rabbit IgGs (all 1:1,000; Invitrogen).

Dual Luciferase Assays. *UAS-* or *lexO-Fluc-22A*; *dpp-Rluc-86Fa/SM5^ΔTM6b* virgin females were crossed to *dpp-TA-86Fb/TM6b* males at temperatures described in Fig. 2 and Fig. S2. Eight wing discs from staged third instar larvae were collected and processed according to the protocol described in *SI Materials and Methods* with reagents from the Promega Dual-Luciferase Reporter Assay System.

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