Methods & Technical Advances

A genomic response to the yeast transcription factor GAL4 in Drosophila

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The yeast transcription factor GAL4 is widely used in Drosophila genetics to misexpress genes that are under control of the yeast upstream activator sequence (UAS). Here we show that high levels of GAL4 change the expression of many Drosophila genes in a UAS-independent manner, including genes that encode components of important signaling pathways. We find that at least part of the genomic response to GAL4 appears to be caused by effects of GAL4 on stress and immune response pathways. Finally, using the transcription factor Senseless as an example, we demonstrate how an interaction between GAL4 and a GAL4-driven protein can impede the use of the GAL4/UAS system in experiments aimed at determining the transcriptional response to a misexpressed gene.

Introduction

The GAL4/UAS system has become an indispensable tool for genetic misexpression experiments in Drosophila.[1] A large selection of GAL4 driver lines is available that express GAL4 in specific spatiotemporal patterns, controlled by enhancers of the fly genome. Other lines carry inducible promoters for conditional, e.g., heat-shock-driven expression of GAL4. UAS-responder transgenes, encoding normal or altered proteins, can be expressed in the enhancer-specified patterns or induced at specific developmental times after crossing driver and responder lines.[2,3] Recent refinements of the system have provided additional options for the temporal control of tissue-specific GAL4 expression.[4] Although GAL4 has no ortholog in Drosophila and the Drosophila genome contains no copies of the UAS element used in the responder transgenes, it has been reported that expression of GAL4 has dosage-dependent biological effects in this species. GAL4 expression in the eye imaginal disc, driven by *GMR-GAL4*, leads to eye defects and apoptosis.[5] Whereas the eye defects are only apparent if GAL4 is expressed from two copies of the transgene, increased apoptosis is already observed in *GMR-GAL4* heterozygotes.[5] Similarly, GAL4 causes cell death and dose-dependent behavioral defects when expressed in a subset of neurons controlling rhythmic behavior.[6] Here, we show that expression of GAL4 can cause a genomic response in

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Drosophila that may explain these biological effects. Further, we present data suggesting that effects caused by high levels of GAL4 greatly complicate the use of the GAL4/UAS system in transcriptional profiling experiments.

Results and Discussion

The apparent biological effects of GAL4 observed in Drosophila[5,6] suggest that there might be a genomic response to GAL4 in this species. In an attempt to use the GAL4/UAS system in transcriptional profiling experiments (see below), we obtained data that supported this hypothesis. To substantiate these data and to determine the suitability of GAL4 for cell death studies in the larval salivary glands, we examined the genomic response to heat-shock-driven expression of GAL4 in this tissue. Affymetrix gene chips were used to analyze gene expression profiles after expression of GAL4 in prepupae of two independent lines, P{*hs-GAL4*}X1 and P{*hs-GAL4*}89. We used two lines to distinguish effects caused by GAL4 from effects caused by gene disruption at the transgene integration site. RNA samples were obtained from the salivary glands of animals carrying two copies (P{*hs-GAL4*}X1 and P{*hs-GAL4*}89) or one copy (only P{*hs-GAL4*}89) of the transgene, which allowed us to assess the dosage dependence of potential responses. The experimental samples were compared to control samples obtained from w^{1118} animals carrying no GAL4 transgene that had been subjected to the same heat treatment as the GAL4-expressing animals.

We found that 1,009 genes showed an at least 1.5-fold increase or decrease in expression in each of the two GAL4-expressing lines after heat shock induction from two copies of the transgene. These genes thus define a core set of genes responding to hs-GAL4 in Drosophila ("GAL4 core"; Table S1). As our analysis was restricted to the salivary glands, the total number of genes that can respond to GAL4 in Drosophila likely exceeds the 1,009 genes identified here. For instance, genes responding negatively to GAL4 will only appear in the list if they are expressed in prepupal salivary glands. Likewise, additional genes may positively respond to GAL4 in other tissues, for instance if a response requires cooperation of GAL4 with other transcription factors that are not present in the salivary glands. Most genes (69%) respond positively to GAL4, while the remaining 31% are downregulated. Table 1 lists those genes that show the strongest positive or negative response to the transcription factor. When GAL4 is expressed from only one copy of the transgene, most of the genes show the same positive or negative response,

Table 1   **Genes showing the strongest response to overexpression of GAL4**

FlyBase ID (FBgn)	Gene name	Fold change	FDR	Protein product
0039759	CG9733*	+ 1997/2225/1678	0.00623	serine-type endopeptidase
0004956	os*	+ 486/162/33	0.00384	receptor binding
0036875	CG9449	+ 386/356/248	0.00531	acid phosphatase
0030912	CG6023*	+ 281/203/25	0.00956	-
0010424	TpnC73F	+ 259/232/133	0.01600	calcium ion binding
0035358	CG14949*	+ 245/220/60	0.00683	-
0051781	CG31781*	+ 237/136/231	0.00564	-
0031649	hoe2*	+ 207/134/45	0.00750	tyrosine transporter
0036876	CG9451*	+ 190/74/nc	0.00421	acid phosphatase
0035084	CG15861*	+ 170/220/192	0.00650	-
0035085	CG3770	+ 159/152/144	0.00365	lipoma HMGIC fusion partner-like 2
0037163	CG11440	+ 152/151/133	0.00660	phosphatidate phosphatase
0042101	CG18744*	+ 150/206/229	0.02450	-
0050445	Tdc1	+ 140/114/34	0.00857	tyrosine decarboxylase
0036877	CG9452*	+ 131/99/9.87	0.03520	acid phosphatase
0014396	tim*	+ 131/64/11	0.00649	circadian regulator
0031261	nAcRβ-21C	+ 129/79/115	0.00750	neurotransmitter receptor
0029907	Atx-1*	+ 125/58/16	0.00857	Ataxin 1
0014469	Cyp4e2	- 132/79/3.17	0.03880	cytochrome P450
0020414	Idgf3	- 96/78/4.49	0.01660	growth factor
0053127	CG33127	- 85/85/19	0.00989	protease
0051324	CG31324	- 64/7.56/1.79	0.02800	-
0031518	CG3277	- 57/22/3.62	0.00885	protein tyrosine kinase
0036591	CG13050	- 54/39/3.32	0.03950	-
0038658	CG14292	- 52/39/3.62	0.01160	-
0028932	CG16890	- 46/57/4.67	0.01780	FRA10AC1-1 isoform
0000640	Fbp2	- 46/52/1.96	0.00932	-
0050062	CG30062	- 45/33/4.53	0.02880	lysozyme
0031162	CG34120	- 41/6.52/5.16	0.01070	ABC transporter component
0003023	otu	- 36/35/7.72	0.01710	ovarian tumor
0031747	CG9021	- 36/9.74/4.3	0.01080	-
0013765	cnn	- 31/18/1.55	0.00776	centrosomal protein
0039061	Ir	- 27/2.65/nc	0.02160	potassium channel
0038632	CG14301	- 27/64/13	0.02550	chitin binding
0034898	CG18128	- 24/4.96/4.35	0.02160	purine-nucleoside phosphorylase
0033221	CG12825	- 22/14/5.17	0.00989	-

Average fold changes in gene expression are listed in the following order: 2 copies P{hs-GAL4}X1/2 copies P{hs-GAL4}89/1 copy P{hs-GAL4}89; genes marked with an asterisk have an absent call in at least one of the w^{1118} control samples; nc, not more than 1.2-fold changed.

but the extent of the response is usually weaker, indicating dosage dependence. As the changes in expression of many of these genes are still considerable, genomic effects of GAL4 are relevant under normal experimental conditions (typically, GAL4 is expressed from only one copy of the transgene). We inspected the genes listed in Table 1, including 10 kb of flanking upstream and 5 kb of flanking downstream sequence, for matches to the GAL4 core DNA recognition element (CGGN$_5$WN$_5$CCG).[12] A comparison to a control group of GAL4 non-responsive genes did not reveal that these genes contain exceedingly high numbers or clusters of this sequence element. Both the non-responsive and responsive genes examined contain up to 4 matches to the core sequence within 10 kb of their

upstream sequence or no matches at all. This suggests that the observed effects of GAL4 do not necessarily require binding of the protein to canonical DNA-binding sites and may solely depend on protein-protein interactions.

Classification of the GAL4-responsive genes by annotation identified several groups of genes that are enriched among these genes, including genes encoding actin-binding proteins and proteins involved in protein ubiquitination (Table S2). Enrichment of the latter group suggests that cells increase their capacity to degrade protein in response to GAL4, possibly as a protection from high levels of GAL4 protein produced from the strong heat shock promoter. This observation suggests that the cells undergo a stress

response that may, at least in part, be responsible for the observed changes in gene expression. However, at least two lines of evidence suggest that GAL4 has effects on gene expression that are independent of a stress response. First, 69% of the genes that respond to GAL4 are upregulated. In contrast, the majority of the genes that respond to heat or other types of stress are downregulated, with the notable exception of heat shock genes.[13] Second, expression of GAL4 together with a second transcription factor (see below) results in the complete or partial repression of 80% of all GAL4-activated genes. If activation of these genes occurred in response to stress caused by protein overproduction, one would predict a further increase and not a reduction in their expression. These observations suggest that most of the genes activated by GAL4 do not respond to GAL4-induced stress, but are activated by GAL4 through an unknown mechanism.

One of the mechanisms by which GAL4 could influence gene expression is a selective block of protein transport into the cell nucleus. Uv et al.,[14] have shown that the nuclear import of GAL4 critically depends on the presence of the nucleoporin Dnup88. Dnup88, encoded by the *members only* (*mbo*) gene, is essential for the nuclear translocation of a subset of proteins including GAL4, but not for nuclear protein import in general. Among the proteins that depend on Dnup88 are the NFκB/Rel family members Dif and Dorsal. Together with the NFκB/Rel protein Relish, these proteins are the central transcription factors of the two signaling pathways that control innate immunity in Drosophila, the Toll and Imd pathways.[15] Consistent with a role of Dnup88 in the transport of all three proteins, *mbo* mutants exhibit a severe immune deficiency phenotype with defects in both pathways.[14] Strikingly, genes of the two pathways, including *dorsal* and *Relish*, are enriched among the GAL4-responsive genes (Table S2). *spätzle* and genes encoding peptidoglycan recognition proteins are considerably upregulated by GAL4. The products of these genes are immediate upstream components of the Toll pathway that sense bacterial or fungal infection and relay this information to the Toll receptor. Upregulation of these genes could, therefore, be caused by a feedback loop that is activated by GAL4 through competition with the nuclear transport of NFκB/Rel proteins and, consequently, a blockage of signal transduction through the Toll pathway.

Interestingly, enriched among the GAL4-responsive genes are not only genes of the Toll and Imd pathways, but also genes encoding proteins with protein tyrosine kinase activity (Table S2). This is of significance because the GAL4/UAS system is often used to experimentally dissect signaling pathways. To identify additional pathways that might be influenced by GAL4, we carried out a more thorough inspection of the microarray results for changes in the expression of genes encoding signaling and signal-transducing proteins including transcription factors. We found that GAL4 indeed changes the expression of a considerable number of regulatory genes representing various pathways, including the Wnt and JAK/STAT signaling pathways in addition to the pathways discussed above. Table 2 lists those genes that showed the strongest response. These observations raise the possibility that the observed effects of a gene of interest may result from regulatory interactions with GAL4-responsive pathways. Wild-type and GAL4-alone controls are not necessarily sufficient to uncover these false responses.

One of the goals of our study was to determine whether GAL4 changes the expression of known or potential cell death regulators,

as suggested by the apoptotic effects of GAL4 observed in some systems.[5,6] Our data indicate that apoptosis-related genes are, indeed, enriched among the GAL4-responsive genes (Table S2). Most of these genes show a significant response already to one copy of the GAL4 transgene (Table 2). The cell death gene whose expression is most strongly changed by GAL4 (~12-fold upregulated) is the Bcl-2 family member *debcl*, which has pro-apoptotic functions.[16-19] At the same time, GAL4 downregulates the second Bcl-2 family member of Drosophila, *Buffy*, which has anti-apoptotic functions and is inhibited by Debcl.[18,20] Thus, a changed balance of Bcl-2 proteins may account for cell deaths caused by GAL4 expression.[5,6] The Bcl-2 family members of Drosophila have been shown to play a role in stress-induced cell death,[18,21] suggesting that the apparent stress response to high levels of GAL4 may lead to the changed expression of these genes. Another pro-apoptotic gene strongly upregulated by GAL4 encodes the tumor necrosis factor Eiger, which is a strong inducer of cell death when expressed in the eye.[22,23] Eiger may, thus, be responsible for *GMR-GAL4*-induced apoptosis that has been observed in this tissue.[5] Other pro-apoptotic genes are strongly downregulated by GAL4, including the caspase activator Ark (Apaf-1/CED-4 homolog of the fly) and the caspase Ice (Table 2). Ark is required for death of the larval salivary glands of Drosophila during metamorphosis.[24] Thus, hs-GAL4 should be used with caution in studies of cell death in this tissue. The responses of pro- and anti-apoptotic genes to GAL4 suggest that GAL4 can have both stimulatory and inhibitory effects on cell death pathways. The phenotypic manifestation of these effects likely depends on the expression level of the transcription factor, the tissue in which GAL4 is expressed, and the developmental context.

The dosage dependence of the genomic response to GAL4 predicts that GAL4 expressed from weaker promoters may have mitigated or no effects on many of the genes identified here. To test this prediction, we expressed GAL4 from the ubiquitous tubulin promoter, alone or in the additional presence of a UAS-GAL4 element that enhances GAL4 expression. Total RNA was extracted from whole adult males and analyzed by Northern blot hybridization for the presence of the mRNA of five genes that showed a strong response to hs-GAL4 (*Chi, debcl, os, Tdc1, tra2*; see Tables 1 and 2). Although control hybridizations confirmed expression of GAL4, none of the genes tested showed significant activation (data not shown). These results suggest that, if the experimental design permits, weaker promoters should be preferred over the use of the strong heat shock promoter.

GAL4 expressed from non-heat shock promoters has been used in transcriptional profiling experiments aimed at identifying the genomic response to misexpressed transcription factors and protein kinases.[25-27] In a similar approach, we attempted to use heat shock-driven expression of GAL4 and a UAS responder to examine the genomic response to the transcription factor Senseless (Sens). However, interpretation of the results proved to be severely compromised by an apparent transcriptional interference between GAL4 and the misexpressed protein. *sens* was expressed from *UAS-sens*[8] in prepupae using P{*hs-GAL4*}89 and the same heat shock regime used for expression of GAL4 alone. RNA was extracted from dissected salivary glands and analyzed with Affymetrix gene chips. Gene expression in the presence of Sens plus GAL4 was then compared to gene expression in the presence of GAL4 alone and to gene expression

Table 2 **Regulatory genes responsive to high levels of GAL4**

FlyBase ID (FBgn)	Gene name	Fold change	FDR	Annotation
Signaling and signal transduction				
0004956	os*	+ 486/162/33	0.00384	JAK/STAT signaling pathway
0010424	TpnC73F	+ 259/232/133	0.01600	calcium signaling
0031261	nAcRβ-21C	+ 129/79/115	0.00750	neuronal synaptic transmission
0004391	shtd	+ 77/27/12	0.01200	cell cycle regulator
0005683	pie	+ 63/40/22	0.02980	eye development
0003742	tra2	+ 48/32/13	0.01290	sex determination
0053542	upd3*	+ 44/19/nc	0.01690	JAK/STAT signaling pathway
0021895	ytr	+ 34/25/21	0.01350	hemocyte development
0035083	Tina-1	+ 33/35/30	0.04970	heart development
0003495	spz	+ 28/16/6.7	0.04330	Toll pathway
0030904	upd2*	+ 27/6.71/nc	0.00623	JAK/STAT signaling pathway
0030082	HP1b	+ 20/25/3.64	0.00652	chromatin regulator
0011746	ana	+ 19/50/3.54	0.03860	neurogenesis
0031299	CG4629	+ 18/14/9.74	0.01250	serine/threonine kinase
0000275	Pka-R1	+ 16/9.57/4.27	0.01310	cAMP-dependent protein kinase
0031194	CG17598	+ 16/9.43/4.81	0.02210	serine/threonine phosphatase
0016794	dos	+ 14/11/5.67	0.00619	Sevenless signaling pathway
0000723	Fps85D	+ 14/4.86/2.62	0.01200	protein tyrosine kinase
0024329	Mekk1	+ 13/7.9/7.11	0.03650	MAP kinase signaling
0004133	blow	+ 12/10/6.4	0.01120	myoblast fusion
0038928	BG4	+ 11/11/nc	0.00501	Imd pathway
0031902	Wnt6*	+ 11/15/3.34	0.00528	Wnt signaling pathway
0000244	by	+ 11/5.42/3.09	0.01510	wing development
0034431	Tab2	+ 10/6.69/3.92	0.04810	Eiger-JNK pathway
0020414	Idgf3	- 96/78/4.49	0.01660	growth factor
0031518	CG3277	- 57/22/3.62	0.00885	protein tyrosine kinase
0003023	otu	- 36/35/7.72	0.01710	oogenesis
0036742	CG7497	- 18/18/4.79	0.00465	G-protein coupled receptor
0033988	pcs	- 13/8.88/5.74	0.02040	tyrosine kinase inhibitor
0036212	CG11597	- 11/12/6.01	0.02280	protein phosphatase
0026199	myoglianin	- 10/1.94/2.11	0.02600	TGFβ superfamily
Transcription factors and coregulators				
0014396	tim*	+ 131/64/11	0.00649	coregulator of clock protein period
0027866	CG9776	+ 93/64/77	0.04590	C_2H_2 Zn-finger
0003068	per*	+ 70/13/nc	0.02390	circadian regulator
0003330	Sce	+ 59/26/9.32	0.00861	PcG silencing
0013764	Chi	+ 49/51/47	0.00623	coregulator of homeodomain proteins
0026428	HDAC6*	+ 42/11/nc	0.00819	histone deacetylase
0002733	HLHmβ	+ 35/36/23	0.02660	bHLH/Notch signaling pathway
0005660	Ets21C*	+ 34/23/nc	0.00619	Ets domain protein
0033073	bin3	+ 28/7.62/11	0.01770	Bicoid-interacting protein
0014340	mof	+ 18/11/6.99	0.01110	histone H4 acetyl transferase
0040305	MTF-1	+ 18/29/14	0.00384	C_2H_2 Zn-finger/heavy metal homeostasis
0000462	dl*	+ 16/11/8.91	0.03370	NFkappa B transcription factor
0027567	CG8108	+ 13/2.95/1.74	0.01790	C_2H_2 Zn-finger
0039559	Mes-4	+ 12/12/4.26	0.00899	SET domain protein
0015805	Rpd3	+ 12/6.61/3.36	0.00650	histone methyltransferase
0004893	bowl	+ 10/6.87/7.56	0.00623	C_2H_2 Zn-finger
0027524	CG3909	- 20/26/6.54	0.00750	transcription factor

Table 2   Regulatory genes responsive to high levels of GAL4 (continued)

0004865	Eip78C	- 7.25/8/4.1	0.01570	nuclear receptor
Cell death regulators				
0029131	debcl	+ 12/8.42/7.2	0.00895	Bcl-2 homolog
0033483	eiger	+ 9.04/8.42/7.24	0.01530	TNF homolog
0015924	crq	+ 1.83/1.88/1.95	0.03770	apoptotic corpse engulfment
0026319	Traf1	- 7/6.44/3.03	0.01170	pro-apoptotic factor
0040491	Buffy	- 3.61/2.12/2.19	0.03520	Bcl-2 homolog
0019972	Ice	- 3.09/2.12/1.44	0.02210	effector caspase
0024252	Ark	- 2.65/2.46 /1.78	0.03120	APAF1/CED4 ortholog
0003691	thread	- 1.99/1.58/1.24	0.04980	Drosophila inhibitor of apoptosis protein 1

Average fold changes in gene expression are listed in the following order: 2 copies P{*hs-GAL4*}X1/2 copies P{*hs-GAL4*}89/1 copy P{*hs-GAL4*}89; genes marked with an asterisk have an absent call in at least one of the w^{1118} control samples; nc, not more than 1.2-fold changed.

in the absence of both GAL4 and Sens. This dual set of controls should allow the identification of genes that are both responsive to Sens and may represent biologically relevant targets of Sens in the salivary glands. Because of the genomic response to GAL4, exclusive comparison to gene expression in the presence of GAL4 alone falsely identifies many of the GAL4-activated genes as potentially relevant targets of Sens. For example, none of the genes listed in Table 1 as upregulated by GAL4 is expressed to a significant extent in the larval salivary glands under normal conditions (mean expression values <109). However, all but two of these genes (CG9733 and CG18744) are identified as downregulated by Sens in an hs-GAL4/UAS-sens versus hs-GAL4 comparison. The latter observation suggested that Sens might predominantly act as a transcriptional repressor. Indeed, when all genes of the GAL4 core set that were at least 1.2-fold activated by GAL4 in the presence of one copy of P{*hs-GAL4*}89 were examined for their responsiveness to Sens, the vast majority of these genes, 80%, turned out to be downregulated by Sens. Intrigued by this high percentage, we asked whether genes that do not respond to or are downregulated by GAL4 show a similar predominantly negative response to Sens. We found that of those genes that do not or only weakly (<1.2-fold) respond to GAL4, 52% are downregulated by Sens. A similar 56% of the genes that are at least 1.2-fold downregulated by GAL4 show an additional downregulation by Sens. Thus, Sens appears to preferentially repress GAL4-activated genes. While the mechanism of this repression is not clear, one possibility is that it is caused by competition between the two proteins for the binding of one or more transcriptional co-activators. Irrespective of the underlying mechanism, the apparent interaction between the two transcription factors greatly reduces the validity of the microarray data obtained for Sens. Interactions of this kind may not be as obvious, but still present, when hs-GAL4 is used to drive the expression of other proteins. Given the extent of the transcriptional response to high-level expression of GAL4, the likelihood of such interactions represents a serious impediment to the use of heat shock-driven expression of GAL4 in transcriptional profiling experiments.

In sum, our results show that expression of high levels of GAL4 in Drosophila can cause a dramatic genomic response. This observation leads us to infer that heat shock-driven expression of GAL4 should be avoided in experiments aimed at determining the transcriptional response to misexpressed genes. This conclusion is supported by our finding that interactions between GAL4 and the misexpressed

protein can have a considerable impact on the validity of the resulting data sets. Alternatives to heat shock-driven expression of GAL4 are the use of weaker promoters, in conjunction with control experiments that carefully monitor the effects of GAL4 alone, or the use of transgenes in which the gene of interest is under the direct control of a heat shock promoter.[28,29] The weaker genomic response to heat treatment, which mostly affects stress response genes (ref. 13; and Liu Y and Lehmann M, unpublished results), should make this a more reliable approach in transcriptional profiling experiments.

Materials and Methods

Fly stocks and crosses. Flies carrying *P{GAL4-Hsp70.PB}89-2-1*,[7] (FBti0002141) (located on the third chromosome; hereafter referred to as P{*hs-GAL4*}89) were obtained from the Thummel laboratory. A new line carrying this element on the X chromosome, P{*hs-GAL4*}X1, was generated by mobilizing the P element in P{*hs-GAL4*}89 flies using the transposase source Δ2-3 and standard procedures (using flies of the genotype *w/w; wg^{Sp-1}/CyO; ry^{506} Sb^1 P{Δ2-3}99B/TM6B, Tb^+*; FBti0000124; provided by the Bloomington stock center). Heat shock-driven expression of GAL4 in P{*hs-GAL4*}X1 strongly induces GFP expression from *UAS-2xEGFP*, indicating that the hs-GAL4 gene is functional at the new integration site (data not shown).

Flies carrying *UAS-sens* C5,[8] were provided by Hugo Bellen. For expression of *UAS-sens* in prepupae, these flies were crossed to P{*hs-GAL4*}89 flies. Prepupae of the recipient strain for P element transformation, *w^{1118}* (FBal0018186), were used as control animals and to generate progeny heterozygous for the P{*hs-GAL4*}89 element.

For the Northern analysis of gene expression after normal or UAS-GAL4-enhanced expression of GAL4 from the tubulin promoter, flies of the genotype *y^1 w*; P{tubP-GAL4}LL7/TM3, Sb^1* (FBst0005138) were crossed to *w^{1118}* flies or flies of the genotype *y^1 w^{1118}; P{UAS-Gal4.H}12B* (FBst0005939). Total RNA extracted from whole adult males of the progeny and from *w^{1118}* control males was analyzed by Northern blot hybridization as described.[9]

Developmental staging and sample preparation. Prepupae and pupae were staged by collecting freshly formed prepupae within 30 min of puparium formation and keeping them on damp filter paper at 25°C for a specified period of time. For ectopic expression of *sens* from *UAS-sens*, *UAS-sens* and P{*hs-GAL4*}89 flies were crossed, and prepupae of the progeny were collected at 9 hours after puparium

formation and heat shocked for 30 min at 37°C. w^{1118}, heterozygous P{*hs-GAL4*}89, and homozygous P{*hs-GAL4*}89 and P{*hs-GAL4*}X1 prepupae were treated the same way. After heat shock, the animals were allowed to recover at 25°C until the salivary glands were dissected for RNA preparation at 14 hours after puparium formation. Total RNA (~15–30 μg) was extracted from about 100 salivary glands using Trizol (Gibco) and purified on RNAeasy columns (Qiagen).

Microarray analyses. Hybridization to Affymetrix Drosophila Genome Arrays was carried out by the microarray facility of the University of Maryland Biotechnology Institute (UMBI). RNA samples were obtained in three biological replicates from P{*hs-GAL4*} X1 and *UAS-sens*/P{*hs-GAL4*}89 animals, in one replicate from P{*hs-GAL4*}89 animals carrying either one or two copies of the transgene, and in two replicates from w^{1118} control animals. Quantity and quality of the RNA was analyzed at UMBI using an Agilent 2100 Bioanalyzer. RNA labeling, hybridization and scanning were carried out using standard protocols recommended by Affymetrix (described in detail in GEO accession number GSE8623; http://www.ncbi. nlm.nih.gov/geo/). Raw data were normalized and compared using dChip.[10] Normalization was carried out per chip in the PM/MM mode in two separate groups (group 1: *UAS-sens*/P{*hs-GAL4*}89, P{*hs-GAL4*}89 one copy, w^{1118}, median intensity of baseline assay used for normalization was 150; group 2: P{*hs-GAL4*}X1 two copies, P{*hs-GAL4*}89 two copies, P{*hs-GAL4*}89 one copy, w^{1118}, median intensity of baseline assay used for normalization was 145). The data sets were filtered for genes that showed an at least 1.5-fold relative change in their mean expression and an absolute expression change of more than 100. In addition, genes were only selected if they had at least 2 present calls in the 2 x P{*hs-GAL4*}X1 samples and 0 to 2 present calls in the w^{1118} control samples or 0 to 3 present calls in the 2 x P{*hs-GAL4*}X1 samples and 2 present calls in the w^{1118} control samples. This restriction identified 2,899 genes in the P{*hs-GAL4*} X1 line. Genes that demonstrated statistically significant differences between the w^{1118} and 2 x P{*hs-GAL4*}X1 samples were identified in a parametric test; variances not assumed equal (Welch t-test) using a p-value cutoff of 0.05, and multiple testing correction with the Benjamini and Hochberg False Discovery Rate (FDR).[11] About 5.0% of the 1,456 identified genes would be expected to pass the restriction by chance (analysis performed with GeneSpring 7.3.1; Agilent Technologies, Santa Clara, CA). The "GAL4 core" list of GAL4-responsive genes was compiled by selecting genes (identified by FlyBase gene number) that showed an at least 1.5-fold increase or decrease in both of the GAL-4 expressing lines. Groups of genes enriched among the GAL4-responsive genes (p < 0.01) were identified using the "Classify Genes" function of dChip that uses Gene Ontology terms to search for gene function enrichment among filtered genes. Other searches for genes by annotation terms were performed using Microsoft Access. Additional searches of the data sets were carried out at a 1.2-fold relative-change threshold (data not shown). Searches for GAL4 core DNA recognition elements in selected genes were performed with NEBcutter V2.0 (New England Biolabs). The microarray data have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www. ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE8623.

Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/LiuFLY2-2-TableS1.xls
www.landesbioscience.com/supplement/LiuFLY2-2-TableS2.xls

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