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Design and Isolation of Temperature-sensitive Mutants of Gal4 in Yeast and *Drosophila*

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Little is known about mechanisms responsible for the temperature-sensitive (ts) phenotype, or of the transferability of ts mutants of a specific gene between organisms. Using a structure-based approach, nine ts mutants of Gal4 were generated in yeast by mutating four DNA binding residues. Two of these nine yeast ts mutants were cloned into *P* element vectors under control of the *Elav* and *GMR* promoters and transgenic Drosophila lines were generated. These were crossed to UAS reporter lines and progeny were characterized for reporter gene expression as a function of temperature. Both of these yeast ts mutants show a ts phenotype in Drosophila and result in rapid induction of reporter gene expression upon shifting to the permissive temperature. Exposed, functional residues involved in protein–ligand or protein–protein interactions appear to be attractive candidate sites for generating ts mutants that are transferable between organisms.

Keywords: temperature-sensitive; design; transferability; yeast; *Drosophila*

Introduction

Rapid advances in DNA sequencing technology have resulted in the determination of complete genome sequences for many organisms, including human.[1] An appreciable fraction of such sequenced genes cannot be assigned a function because they lack homologs of known function. Gene knockouts, a powerful tool to study gene function, have limitations in the study of development when the early phenotypes are lethal for the cell or the organism. Conditional mutants, of which temperature-sensitive (ts) mutants are an important class, are valuable in these situations. Organisms homozygous for a ts allele of an essential gene can survive and display a wild-type (wt) phenotype at lower (permissive) temperatures where the mutant phenotype is not seen. Above a certain temperature (restrictive temperature) the ts phenotype is seen. In contrast, the product of the wt allele is functional and active at both the permissive and the restrictive temperatures. Since wt and ts alleles have similar phenotypes at permissive temperatures, it might be expected that the wt and ts gene products also have similar activities at such temperatures, though this remained to be tested.

Although ts mutants are widely used to study gene function *in vivo*, little is known about the molecular mechanisms responsible for generating a ts phenotype. The ts mutants are typically generated by random mutagenesis followed by, often laborious, screening procedures.[2,3] It is believed that ts alleles cannot be generated for all proteins and some remain refractory even following extensive mutagenesis.[4] In recent years, a few alternative approaches have been proposed for the generation of ts mutants. It was shown that ts mutants can be generated by appropriate destabilizing substitutions of buried, hydrophobic residues.[5] In yeast, it has been shown that ts mutants can be generated by fusion to a heat-sensitive degron.[6,7] However, this

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Abbreviations used: fGal4, full-length Gal4; *hid*, head involution defective; mgal4, miniGal4; *nlacZ*, *lacZ* gene fused to a nuclear localization signal; tubP, tubulin 1 promoter; SC-U, synthetic complete media; SC-UAH, SC-U without histidine and adenine; TNTG, tetanus toxin (G); ts, temperature-sensitive; UAS, upstream activating sequence; wt, wild-type.

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methodology has not been applied extensively to other organisms.[8] Zeidler *et al.* have generated temperature-dependent splicing mutants of the *Saccharomyces cerevisiae* vacuolar ATPase subunit (VMA) intein.[9] These temperature-dependent inteins can be transferred to other host proteins to make them ts. However, the possibility remains that an unspliced host containing a ts intein may retain some activity. The ability of an intein to splice from within a host protein is also dependent on its context, as variations in the flanking sequences near intein insertion may influence splicing activity. In addition, splicing is an irreversible process and hence, this system cannot be used for reversible control of gene expression. Yet another potential method to generate ts mutants might be to mutate functionally important residues involved in ligand binding. To our knowledge, this possibility has not been explored, and here we have employed this approach to generate several ts mutants of yeast Gal4.

In higher eukaryotes, such as Drosophila, where simple, plate-based screens do not exist, screening becomes a rate-limiting step in the generation of ts mutants. It would therefore be useful if a library of putative ts mutants could be screened in a lower eukaryote such as *S. cerevisiae* and selected ts mutants then transferred to other organisms of interest. It was observed in a small number of cases that ts mutants isolated in one organism are also ts in another organism.[9–11] On the basis of the sequences of ts alleles of $pp60^{v\text{-}src}$ of Rous sarcoma virus isolated from chick cells,[12] Simon *et al.* constructed five mutations in a homologous region of Drosophila *sev*.[10] Two of the five mutants were ts; however, the molecular basis for the ts phenotype was not known and not the focus of the study. It has been shown that ts mutants of inteins[9] and Gal80[11] genes generated in yeast were ts in Drosophila, but the molecular basis of the ts phenotypes in either organism was not known. Hence, it is unclear if ts mutants of a gene generated in one organism will also show a ts phenotype in a completely different organism, and what molecular features might be responsible for transferable ts phenotypes. We have examined the transferability from yeast to Drosophila of the ts phenotype caused by two of the Gal4 ts mutants isolated in this study. We show that both of these yeast ts mutants show a ts phenotype in Drosophila. These ts mutants of Gal4 can be used for conditional and reversible expression of a number of other genes of Drosophila and other organisms using the UAS/Gal4 system described previously.[13]

Results

Design and isolation of ts *gal4* mutants in yeast

Gal4 is an 881 amino acid residue protein that functions as a transcriptional activator in yeast (*S. cerevisiae*). Gal4 binds a sequence called UAS (upstream activating sequence consisting of tandem 17 bp imperfect repeats) and activates transcription of genes downstream of this sequence.[14–16] Gal4 is absent from higher eukaryotes. However, when expressed, in many instances Gal4 is functionally active and able to activate transgene expression of genes downstream of UAS. DNA binding (residues 1–65) and dimerization (residues 50–94) functions are localized to the N terminal region of Gal4. Residues 94–106, 148–196 and 768–881 are involved in transcriptional activation. It has been shown that a construct (*minigal4* or *mgal4*) consisting of residues 1–147 and 840–881 connected by a seven residue linker can activate transcription of reporter genes in yeast.[17,18] This construct is expressed under control of the alcohol dehydrogenase 1 promoter in the plasmid *pGBA*. Several ts mutants of mGal4 have been isolated from yeast. Mutants were constructed by site-directed mutagenesis at predicted buried residues in the putative dimerization region of the protein. Several of these ts mutants also showed a ts phenotype in yeast when transferred into full-length *gal4* (*fgal4*).[17] In the present work, an alternative approach was utilized to generate additional ts mutants of Gal4 in yeast, with the goal of transferring these mutants into Drosophila. Residues from Gal4 that contact the UAS DNA site were identified from the crystal structure of the DNA-bound form of the DNA binding domain of Gal4 (Table 1).[19] Four of these residues (see Materials and Methods) were selected for mutagenesis (Figure 1). Each of these four residues was individually randomized using overlap PCR. The mutated PCR products were transformed along with digested *pGBA* vector into the yeast reporter strain PJ69-4A,[20] and plated onto SC plates lacking uracil. Transformants were replicated onto SC plates lacking both uracil and adenine (the strain contains a *UAS ade* reporter) at 21 °C, 30 °C and 37 °C. Putative strong ts mutants were identified by growth on these plates only at 21 °C. Weak ts mutants grew at 21 °C and 30 °C only. The wt and active mutants grew at all three temperatures, whereas inactive mutants did not grow at any temperature (Figure 2). Plasmid from putative strong and weak ts mutants was recovered and retransformed into the PJ69-4A reporter strain to confirm the ts phenotype. The entire *mgal4*-coding region was sequenced to identify the mutation. The

Table 1. Hydrogen bonding and residue burial for DNA binding residues of Gal4

Residue	Number of hydrogen bonds with DNA	ΔASA (Å^2)
Gln9	1	6
Ala10	1	19
Arg15	1	60
Lys17	2	13
Lys18	5	98
Leu19	–	26
Cys21	3	6
Lys23	1	17
Arg46	2	58
Leu49	1	22
Thr50	2	22

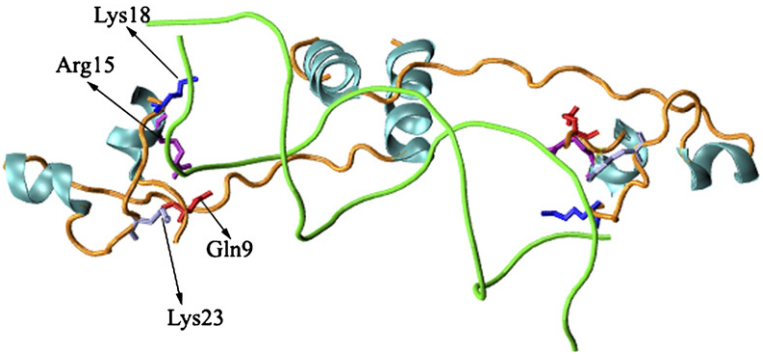

Figure 1. Structure of DNA binding domain (amino acid residues 1–65) of Gal4 bound to its target upstream activating sequence (UAS).[19] Residues, which have been mutated, are labeled. This Figure was generated using VMD.[64]

ts mutants could be identified at only two of the four positions selected for mutagenesis (R15 and K23) (Table 2). Both K23 and Q9 are located close to backbone DNA phosphate groups. However, the interaction between the positively charged K23 and the negatively charged phosphate group is presumably stronger than the corresponding interaction of Q9. R15 is also hydrogen bonded to the DNA backbone and buries a larger amount of surface than K23 (60 Å^2 *versus* 17 Å^2). Consistent with this, the R15W mutant shows lower activity than mutants at K23 in both yeast and Drosophila (see below). K18 forms very extensive interactions with DNA (Table 1) and all mutants at this position were found to show an inactive rather than a ts phenotype. The activity in liquid culture was assayed for wt full-length Gal4 (fGal4), wt mGal4 as well as the ts mutants (Table 2). The data demonstrate clearly that the activity of the ts mutants at the permissive temperature is measurably lower than that of the wt protein under similar conditions. At restrictive temperatures, the activity of the wt protein is slightly decreased, while the mutant activity is lowered to background levels. Both mGal4 and fGal4 appear to have similar activity in yeast,

Figure 2. Verification of the ts phenotype of mGal4 mutants at 21 °C, 30 °C, and 37 °C on SC-UAH plates. From section 1 to section 8 (clockwise) are WT, K23N, K23P, R15W, K23V, K23I, K23L and K23T grown at 21 °C, 30 °C, and 37 °C. K23N, K23P, R15W, K23V, K23L and K23I are strong ts mutants and K23T is a mild ts mutant.

consistent with reports from earlier studies.[18] In a separate study, we have shown that the K23P ts mutant can be used to study the function of essential genes involved in RNA splicing in yeast and offers much tighter control than the conventional galactose/glucose switch used to regulate Gal4 in this organism (R.V., unpublished results).

Temperature-independence of wt fGal4 activity in *Drosophila*

fGal4 has been widely used to activate expression of a variety of *UAS-transgene* constructs in a number of different organisms including yeast, fruit flies, zebrafish, mice and frogs.[21–26] It would therefore be useful to generate ts mutants of Gal4 that would enable ts expression of such constructs in diverse organisms. Before examining putative ts mutants of Gal4 in Drosophila, it was necessary to confirm that wtGal4 activity itself does not show an intrinsic temperature-dependence. To this end, wt*fgal4* was cloned into the Drosophila *P* element vectors *pPTGMR* (see Materials and Methods) and *pElav*.[27] GMR stands for glass multiple reporter and drives expression in the developing eye of Drosophila. *pPTGMR* contains a multimer of binding sites for Glass protein as well as a minimal constitutive promoter derived from *pHLw2*.[28] The *glass* gene encodes a DNA binding transcriptional activator and is required for normal development of photoreceptor cells in the larval Bolwig organ, the adult compound eye and the adult simple eyes.[29,30] The *pElav* vector contains the *Elav* promoter. The *Drosophila melanogaster* vital gene, *embryonic lethal, abnormal vision* (*elav*), is required for post determinative development of the nervous system.[31,32] Its gene product encodes an RNA binding protein that was found to be expressed in all neurons immediately after birth. Like GMR, the domain of expression includes the developing eye.

The Drosophila eye is a convenient organ to monitor effects of changes in gene expression. It is non-essential for viability or fertility of the fly, so changes in eye morphology as a result of expressing different genes under *GMR* or *Elav* can be easily scored by visual inspection of the adult eye. Differentiation of the cells that will form the adult Drosophila eye begins in larval monolayer epithelial tissue called the eye imaginal disc, as an indentation called the morphogenetic furrow sweeps across the

Table 2. Activity of mutant Gal4s in yeast and Drosophila

Mutant[a]	Reporter *lacZ* activity in yeast (Miller units)		Phenotype of *mGal4* mutant in yeast	Phenotype of *fGal4* in Drosophila[b] under	
	21 °C	30 °C		*Elav*	GMR
Wt	607	676	wt	wt	wt
R15W	22	6	Strong ts	Inactive	ts
K23P	38	6	Strong ts	ts	ts
K23L	20	8	Strong ts	–	–
K23I	22	7	Strong ts	–	–
K23V	28	10	Strong ts	–	–
K23N	37	9	Strong ts	–	–
K23T	68	17	Mild ts	–	–
K23M	64	14	Mild ts	–	–
K23W	49	23	Mild ts	–	–

[a] All liquid culture assay data shown are for mGal4 derivatives. wt mGal4 and fGal4 had very similar activity. Miller units for fGal4 were 684 at 21 °C and 703 at 30 °C. The average errors in LacZ activity estimates from multiple determinations were about 20%. The background value for inactive mutants/host strain is about five units.
[b] Assayed using various reporter genes listed in the text.

disc. Cells posterior to the furrow are sequentially recruited into ommatidial clusters. *glass* as well as *elav* RNA and protein are strongly expressed in the morphogenetic furrow and in cells posterior to the furrow.[30,33] Hence, as an alternative to visual examination of the adult eye, expression of reporters controlled by the GMR and Elav drivers can be readily monitored by staining/antibody labeling of third instar larval imaginal discs. Following microinjection, a number of transgenic lines expressing wt fGal4 under control of either *GMR* or *Elav* were obtained and crossed with the reporter lines *UAS-hid* and *UAS-nlacZ*. *hid* (head involution defective) is a toxic reporter that induces apoptosis by activating a caspase pathway.[34] The *UAS-nlacZ* line has the *lacZ* gene fused to a nuclear localization signal. In this work, *hid* expression was monitored by examining adult eye morphology, while *lacZ* expression was monitored by histochemical staining/antibody labeling of larval imaginal discs as described below.

It is widely believed by Drosophila developmental biologists that wt Gal4 in flies has substantially higher activity at higher temperature.[13,35] However, we show here that wt Gal4 activity controlled by both GMR and Elav drivers is independent of temperature. This is apparent from the Drosophila data in Figures 3(b), (f), and (j) and 4(b), (h), and (n) (and Supplementary Data Figure 1) and is consistent with the yeast data in Table 2. Expression of the lacZ reporter in late third instar imaginal discs was detected either by probing the discs with anti-LacZ antibodies (Figures 3 and 4) or staining with the chromogenic LacZ substrate X-gal, as described in Materials and Methods. Discs are also probed with anti-Elav antibodies to show the regions of the discs thst have undergone differentiation (Figures 3(a), (e), and (i) and 4(a), (g), and (m)). As expected, the domains of expression of lacZ and Elav are identical. The strong GMR driver results in very high levels of lacZreporter gene expression (Figure 4(b), (h), and (n))

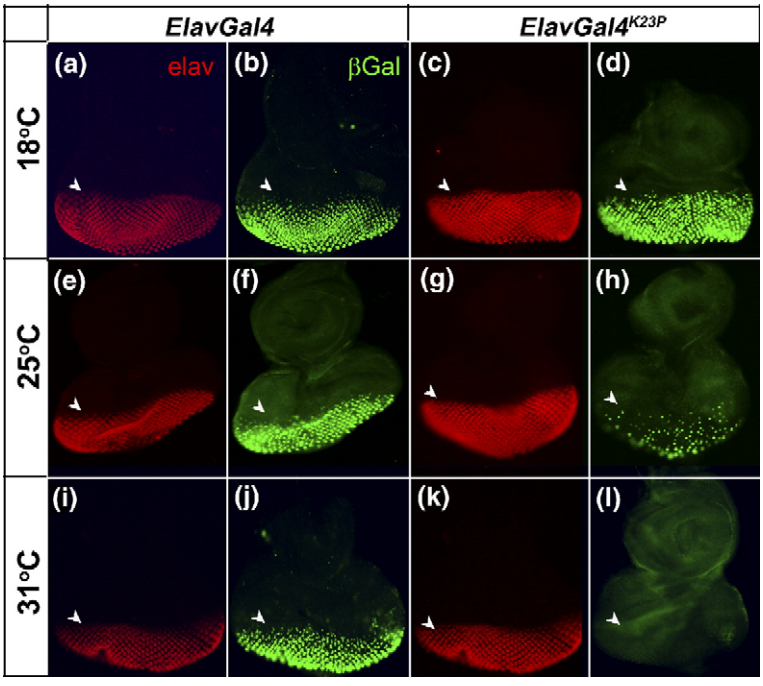

Figure 3. Expression of *UAS-nlacZ* reporter under control of *Elav-* wt/mutant fGal4's at 18 °C, 25 °C and 31 °C. Eye imaginal disc (anterior is up, arrow points posterior to the furrow) labeled with mouse anti-Elav antibody ((a), (c), (e), (g), (i), and (k)); rabbit anti β-galactosidase antibody ((b), (d), (f), (h), (j) and (l)). *Elav- fGal4* ((a), (b), (e), (f), (i) and (j)), *Elav-K23PfGal4* ((c), (d, (g), (h), (k) and (l)).The K23P mutant of fGal4 at 25 °C shows ts reporter gene expression (appreciable nlacz expression at 18 °C and little or no expression at 25 °C or 31 °C).

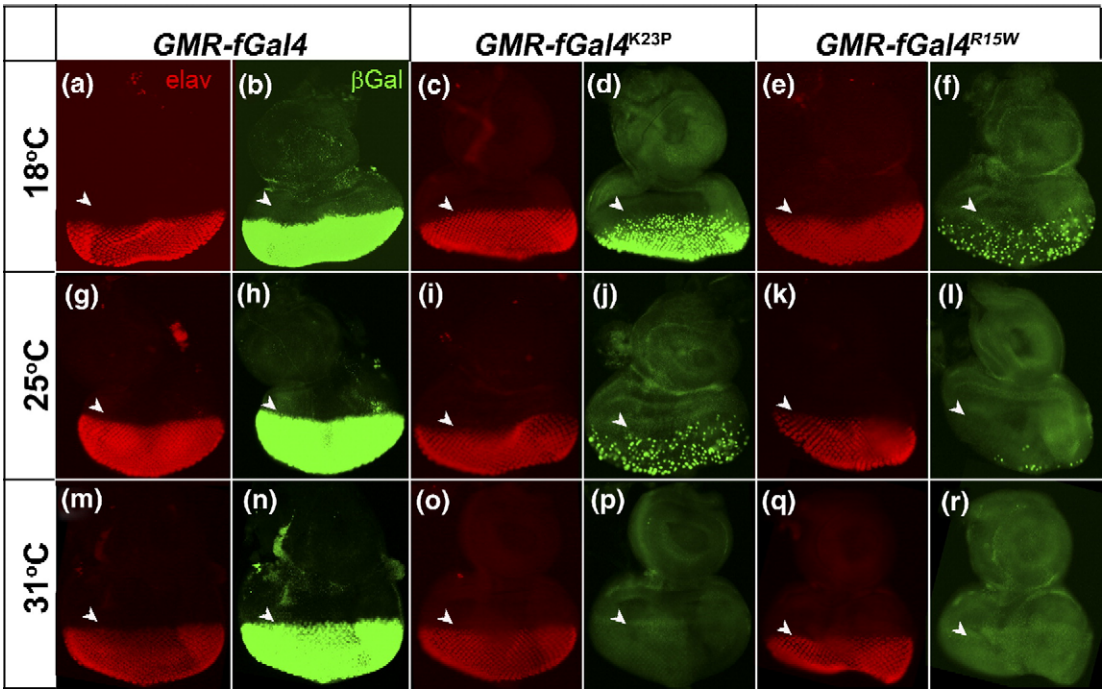

Figure 4. Expression of *UAS-nlacZ* reporter under control of *GMR-* wt/mutant fGal4's at 18 °C, 25 °C and 31°C. Eye imaginal disc (anterior is up, arrow points posterior to the furrow) labeled with mouse anti-Elav antibody ((a), (c), (e), (g), (i), (k), (m), (o) and (q)); rabbit anti β-galactosidase antibody ((b), (d), (f), (h), (j), (l), (n), (p) and (r)). *GMR- fGal4* ((a), (b), (g), (h), (m), and (n)), *GMR-K23PfGal4* ((c), (d), (i), (j), (o) and (p)), *GMR-R15W fGal4* ((e), (f), (k), (l), (q) and (r)). Both the K23P and R15W mutants show a ts phenotype with the GMR driver. However the R15W mutant is much less active than the K23P mutant at all temperatures.

which are outside the linear detection range. Hence, it was difficult to rule out a weak temperature-dependent expression with antibody staining alone. Hence, to confirm that expression is temperature-independent, discs were stained with X-gal and the reaction stopped at various intervals of time (See Supplementary Data Figure 1). These data confirm that reporter gene expression levels are relatively independent of temperature with the GMR driver also.

The incorrect notion that wild-type Gal4 in flies has appreciably higher activity at higher temperature is presumably because many of the enhancer trap Gal4 vectors contain heat shock elements (FlyBase IDs: FBmc0000380, FBms0003205 and FBtp0000352).[13] Expression of several *UAS* reporters from such enhancer-trap Gal4 constructs has been shown to result in temperature-activated reporter gene expression in Drosophila.[23,35] This has given rise to the possibly erroneous belief that the activity of Gal4 in Drosophila increases substantially over the temperature range 18–31 °C. Since this does not occur to a significant extent in yeast, we suggest that the apparent enhancement is due to the presence of hsp binding sites in the enhancer-trap vectors.

Temperature-dependence of K23P and R15W fGal activity in Drosophila

Two of the yeast ts mutants, K23P and R15W, were cloned into the vectors *pPTGMR* and *pElav* described above. The mutants K23P and R15W were chosen for these studies because both of these showed appreciable activity at the permissive temperature in yeast (21 °C), and close to background activity at the restrictive temperature (30 °C). However, at the permissive temperature, the K23P mutant was 15-fold and R15W mutant was 30-fold less active than the wt (Table 2). Following microinjection into Drosophila embryos, a number of transgenic lines expressing either the K23P or R15W mutant under control of either *GMR* or *Elav* were obtained and crossed with the reporter lines *UAS-hid* and *UAS-nlacZ* (Table 3).

The K23P *fGal4* mutant showed the desired ts phenotype when driven by *Elav* or *GMR* (Figures 3 and 4). In both cases, appreciable wt-like expression of the *lacZ* reporter was observed in late third instar eye imaginal disks of larvae grown at 18 °C, while little or no expression was seen in larvae grown at 31 °C. This is in contrast to the situation in yeast, where the K23P mutant showed lower reporter gene

Table 3. Phenotypes of transgenic Drosophila lines generated

Gal4 drivers/mutants in Drosophila	No. independent lines screened	Active	Inactive	ts
GMR- fGal4	15	14	1	–
GMR-K23P fGal4	15	–	1	14
GMR-R15W fGal4	15	–	2	13
GMR-R15W/K23P fGal4	15	–	15	–
Elav- R15W fGal4	11	2	9	–
Elav- K23P fGal4	15	–	5	10

expression than wt at the permissive temperature. This is probably because the slower growth rate and longer timescale in Drosophila allow sufficient accumulation of the reporter gene product to occur. Flies carrying *Elav-K23P fGal4* along with the toxic *hid* reporter do not survive at 18 °C but show a normal eye phenotype at 25 °C and 31 °C (Figure 5). Similar ts phenotypes could be observed when this Gal4 line was combined with the following *UAS* reporters (*UAS-TNTG*, *UAS-rpr*, *UAS-Antp* and *UAS-Notch^{intra}*; data not shown). The *Elav-R15W fGal4* flies showed an inactive phenotype with most of the reporters studied. The only exception was the *hid* reporter, which showed an erect wing phenotype at all the three temperatures.[36] The lack of activity of the *R15WfGal4* mutant in *Drosophila* with the *Elav* driver is possibly because the activity of R15W even at the permissive temperature in yeast is approximately twofold lower than for K23P (Table 2). When expressed under control of the stronger *GMR* driver, both the K23P and the R15W mutants showed a ts phenotype with both *lacZ* and *hid* reporters. R15W shows weaker reporter gene expression as compared to K23P (Figures 4 and 5). The R15W/K23P double mutant in combination with the *GMR* driver shows an inactive phenotype with both *lacZ* and *hid* reporters. As controls, all the parental Gal4 lines, as well as the *UAS-nlacZ* line, have been tested for the presence of β-galactosidase by immunohistochemistry, but no activity was detected (Supplementary Data Figure 2). Neither the parental wt/ts Gal4 lines nor the (wt/ts) Gal4/UAS-nlacZ flies showed any obvious phenotypic abnormality, indicating the absence of off-target effects in the Gal4 ts mutants.

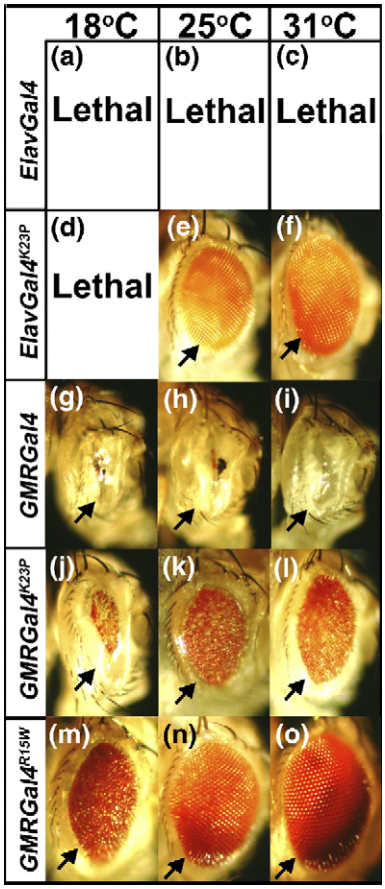

Figure 5. Adult eye phenotypes observed by expressing the apoptotic *UAS-hid* reporter under control of *GMR* and *Elav wt/mutant Gal4* drivers at (a) 18 °C, (b) 25 °C and (c) 31 °C. (d)–(f) E*lav-fGal4*. (g)–(i) *Elav-K23PfGal4*. (j)–(l) *GMR-fGal4*. (m)–(o) *GMR-K23PfGal4*. *GMR-R15W fGal4*. *Elav-K23P fGal4* shows a ts phenotype (lethal at 18 °C, normal eyes at 25 °C and 31 °C). *GMR-wt fGal4* shows severe eye defects at all three temperatures. *GMR-K23P fGal4* shows a ts phenotype (abnormal eyes at 18 °C, greatly reduced abnormality at 25 °C and 31 °C). *GMR-R15W fGal4* also shows a ts phenotype (abnormal eyes at 18 °C, nearly normal at 25 °C and normal eyes at 31 °C). The arrow points to a similar position in all the eye photographs.

Comparison with the Gal80ts system

Methods for conditional expression of target genes in *Drosophila* have been reported.[9,11,37–39] Of these, one of the most convenient systems uses a ts allele of Gal80,[11] referred to as Gal80ts. Gal80 is an inhibitor of Gal4. In this approach, flies containing the desired Gal4 driver are crossed with tubulin 1 promoter (tubP)-driven, constitutively expressing Gal80ts lines. Flies homozygous for both *gal4* and *gal80ts* were isolated. These flies can subsequently be crossed with flies containing a *UAS* transgene. The resulting progeny will thus express the transgene under Gal4 control. The presence of the gal80ts allele should permit expression to be induced only at or above the restrictive temperature of the gal80ts allele. It was therefore of interest to compare control of reporter gene expression in this system with the Gal4 ts mutants described above. To this end, flies expressing *UAS-hid* or *UAS-nlacZ* were crossed with flies expressing Gal80ts. Progeny homozygous for both the reporter and gal80ts were obtained. These were crossed with flies expressing wt fGal4 under control of either the *Elav* or *GMR* drivers. Crosses were carried out at 18 °C and 31 °C. With the *Elav* driver, flies expressing the toxic *hid* reporter did not survive at either 18 °C or 31 °C (Figure 6(a) and (c)) in the Gal80ts background. In contrast, with the same reporter and driver, *K23P fGal4* ts mutants showed normal eyes at 31 °C but did not survive at 18 °C (Figure 5(d) and (f)), suggesting tighter control of expression with ts *fGal4* than with *Gal80ts*. With the *GMR* driver, Gal80ts flies containing *UAS-hid* had abnormal eyes at 18 °C and extensive eye defects at 31 °C (Figure 6(b) and (d)). In comparison, the corresponding *K23P* and *R15W fGal4* flies exhibited abnormal eyes at 18 °C and close to normal eyes at 31 °C (Figure 5(j), (l), (m), and (o)). With the *lacZ* reporter, the *Gal80ts* flies showed no reporter expression at 18 °C and appreciable expression at 31 °C with the *Elav* driver (Figure 6(f) and (h)). However, with the *GMR* driver, some reporter expression is seen at 18 °C, while there was appreciable expression at 31 °C (Figure 6(j) and (l)), indicating incomplete repression by Gal80ts at 18 °C.

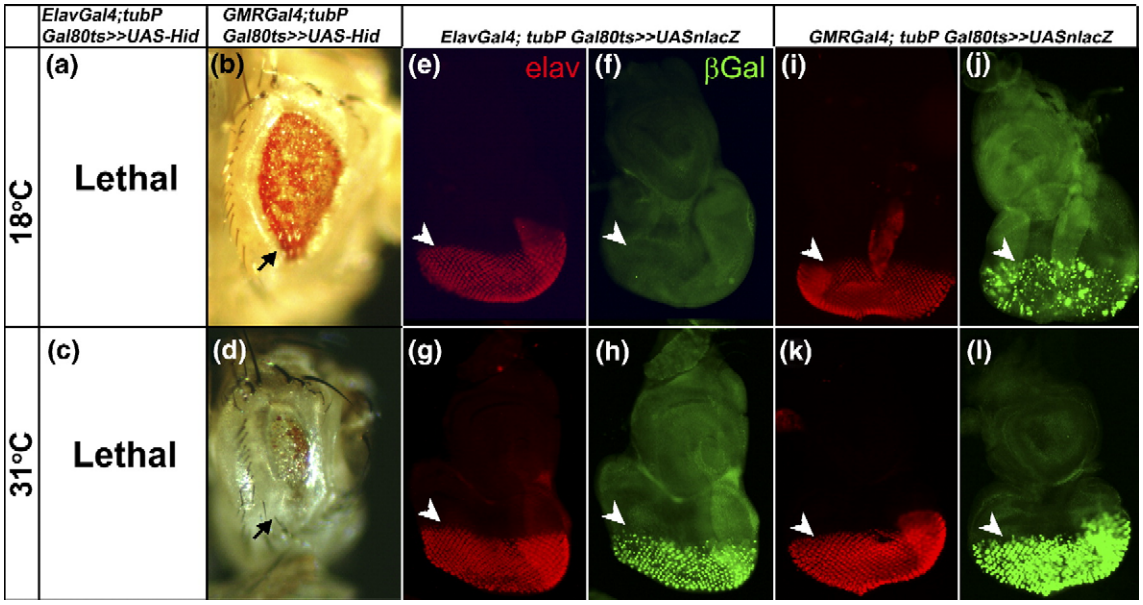

Figure 6. Effects of Gal80ts on reporter gene expression as a function of temperature. (a)–(c) *Elav-fGal4* flies were crossed to *UAS-hid; tubP-Gal80ts.* All progeny died at late third instar larval stage at both (a) 18 and (c) 31 °C. (b) and (d) *GMR- fGal4* flies were crossed to the same reporter, at (b) 18 °C progeny had abnormal eyes (the arrow points to a similar position relative to other adult eye photographs in other Figures). (d) At 31 °C progeny had severe eye defects. (e)–(l) *Elav* or *GMR- fGal4* flies were crossed to *UAS-nlacZ; tubP-Gal80ts*. Eye imaginal discs (anterior is up, the arrow points posterior to the furrow) (e), (g), (i), and (k) are labeled with anti-Elav antibody; (f), (h), (j), and (l) are labeled with anti-β-galactosidase antibody. Drivers and temperatures are as follows. (e) and (f) *Elav- fGal4* at 18 °C and (g) and (h) *Elav fGal4* at 31 °C. (i) and (j) *GMR- fGal4* at 18 °C and (k) and (l) *GMR- fGal4* at 31 °C. In combination with Gal80ts, fGal4 shows ts *UAS-nlacZ* expression when driven by either *Elav* or *GMR* drivers. However, expression with the stronger *GMR* driver is leaky in this system.

In order to compare the kinetics of reporter gene expression in the Gal80ts and ts Gal4 systems, flies were grown to the late second instar larval stage at the restrictive temperature. At various time intervals between late second and early third instar, larvae were transferred to the permissive temperature and dissected at the late third instar larval stage. *LacZ* expression was monitored using anti-β-galactosidase antibodies. Data for transfers at the early third instar are shown in Figure 7. With the *Elav* driver, the amount of induction with the Gal80 system is appreciably lower than with the ts *gal4* mutant. With the *GMR* driver, the pattern of induction with the Gal80 system is non-uniform, with a high level of expression just ahead of the morphogenetic furrow. In contrast, with the K23P ts Gal4 expression is more uniform and colocalized with the Elav expression. In addition, control of expression *via* ts Gal4 requires only two components, *gal4* and *UAS-transgene*, while that *via* the Gal80ts system requires one additional component, Gal80. Moreover, the ts Gal4 system is used to drive reporter gene expression at lower temperatures than the Gal80ts (18–21 °C *versus* 29–31 °C). This may be preferable when the flies already contain other mutations that often reduce viability, especially when combined with transgene expression at higher temperatures. The disadvantage of using ts Gal4 mutants is that a fresh set of transgenic lines needs to be generated for each new driver that is examined. In contrast, for the Gal80 system, the Gal80ts needs to be crossed with the Gal4 strain of interest, before crossing with *UAS*-containing strains.

Discussion

We had shown that ts mutants can be generated by destabilizing mutations at buried hydrophobic positions,[5] and we had generated several ts mutants of Gal4 in yeast using this strategy.[17] In the present work, we have shown that it is also possible to generate ts mutants at functionally important, substrate binding residues. In addition to the Gal4 ts mutants described above, we have applied this strategy of altering exposed functional residues, to mutate residues involved in a protein–protein interaction, to generate several ts mutants of the *Escherichia coli* toxin CcdB. In that study, by doing a separate large-scale characterization of ~1400 site-directed mutants of the *E. coli* toxin CcdB, we have shown that ts mutants were found primarily at either buried residues or at exposed, functional residues involved in interaction with its binding partner DNA gyrase. ts mutants could be isolated at six out of seven functional residues involved in this protein–protein interface (data not shown) These data along with the Gal4 data suggest that targeting functional residues involved in a protein–ligand interaction is a useful strategy for generating ts mutants. We anticipate that this strategy is likely to generate ts mutants that are transferable from one organism to another since

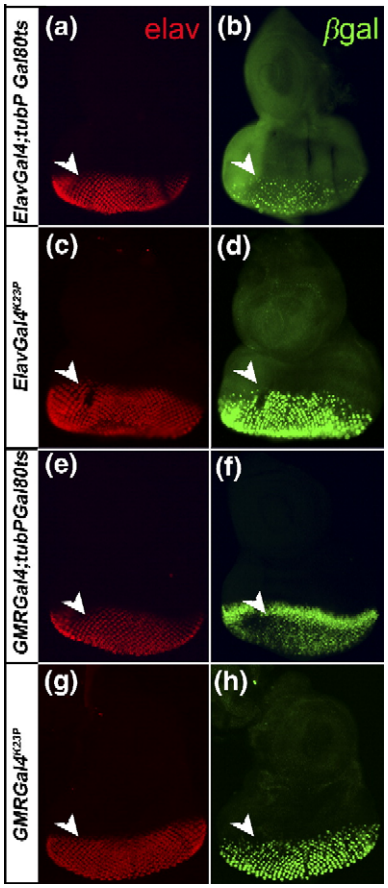

Figure 7. *UAS-nlacZ* reporter gene expression (a), (b), (e), and (f) in the presence and (c), (d), (g), and (h) in the absence of Gal80ts upon temperature shifts of early third instar larvae from non-permissive to permissive temperatures. Eye imaginal disc labeled with (a), (c), (e), and (g) mouse anti-Elav antibody or (b), (d), (f), and (h) rabbit anti β-galactosidase antibody. Shifts at early third instar larval stage from 18–31 °C for (a) and (b) *Elav-fGal4,tubP-* and (e) and (f) *GMR- fGal4, Gal80ts*, and from 31–18 °C for (c) and (d) *Elav-K23P fGal4,* and (g) and (h) *GMR-K23PfGal4*. Both *GMR-K23P fGal4 and Elav-K23PfGal4* flies show significant induction of *lacZ* expression after transfer from 31 °C to 18 °C. In both cases, no expression was visible before transfer (see Figures 3(l) and 4(p)). For *Elav-fGal4* flies in the presence of *Gal80ts*, there is much weaker induction of reporter gene expression (compare (b) and (d)) upon transfer from 18 °C to 31 °C compared to *Elav-K23P fGal4* flies transferred from 31 °C to 18 °C. For *GMR- fGal4* flies in the presence of *Gal80ts*, there is induction of reporter gene upon transfer from 18 °C to 31 °C; however, (f) the expression pattern is non-uniform with high expression close to the furrow and lower expression below it. (g) and (h) In contrast, for *GMR-K23P fGal4* flies, induction is high and uniform and co-localizes with Elav expression.

substrate binding affinities are likely to be dependent primarily on the molecular structures of the binding partners rather than the organism or cell type employed. Also, unlike buried site mutations, mutations at exposed functional residues are unlikely to affect protein stability/levels and hence induction kinetics upon temperature shift is likely to be extremely rapid. However, this approach requires prior knowledge of functionally important residues. In the present study, both of the ts mutants at exposed, functional sites also showed a ts phenotype in Drosophila. In contrast, ts mutations at buried positions affect protein stability. Such mutants normally have lowered levels of expression, presumably because of increased degradation. The exact relationship between lowered thermodynamic stability and intracellular protein levels is complicated. An ubiquitin-dependent proteosomal pathway mediates most protein degradation. In yeast, it has been shown recently that two distinct F-box-containing proteins are involved in ubiquitinylation of Gal4.[40] Since these specific proteins are not present in Drosophila, it is likely that the degradation mechanism and pathways will differ in the two organisms and, therefore, that ts mutants at buried positions will not be as readily transferable, as they affect stability rather than activity. This issue will be addressed in future studies.

While ts mutants are extensively used to study gene function, little is known about the molecular mechanisms responsible for the ts phenotype. We outline below possible mechanisms that may contribute to this phenomenon. The thermodynamic stability of a protein is typically quantified in terms of its free energy of unfolding (ΔG^0_u). A plot of ΔG^0_u as a function of temperature is called the stability curve. ΔG^0_u's for most proteins display a bell-shaped dependence on temperature and have a temperature of maximal stability (T_{ms}) close to room temperature.[41,42] This is possibly because the strength of the hydrophobic driving force may be maximal close to room temperature.[43] Destabilizing mutations decrease the magnitude of $\Delta G^0_u(T_{ms})$ and T_{ms} as well as the width of the stability curve.[44] A similar logic applies to the temperature-dependence of the free energy of intermolecular interactions such as protein–protein or protein–DNA interactions. We suggest that ts phenotypes occur as a result of threshold effects. Such mutations lower the activity and/or level of the protein relative to the wt at all temperatures. Since maximal stability temperatures are rarely in excess of room temperature, with an increase in temperature, the activity of an already marginally active mutant can fall below the threshold required for function. An idealized notion of a ts mutant is one that has near wt activity at the permissive temperature and no activity at the restrictive temperature. However, in practice, with few exceptions, ts mutants show a wt-like phenotype at the permissive temperature and that of a loss of function mutant at the restrictive temperature. There are few studies that involve molecular level characterization of activities of ts mutants *in vitro* and functional protein levels *in vivo*. However, most of these have shown that ts mutants typically have appreciably lower activity than the wt even at the permissive temperature and/or the difference in activity of the ts mutant between permissive and restrictive temperatures is not large. Recent examples include those of *E. coli* peptidyl tRNA hydrolase[45] ts inteins of Gal4[9] and a ts paralytic mutant of triosephosphate isomerase in Drosophila.[46] Sev-

eral ts mutants have substantially lower thermodynamic stability than wt[47,48] at both permissive and restrictive temperatures, and this presumably results in lowered *in vivo* levels at both permissive and restrictive temperatures. It is hard to imagine any plausible molecular mechanism by which a protein can show a large change in activity over a small change in temperature (~2 deg.C for *sev* ts mutants) at temperatures close to room temperature.[10] A much more plausible scenario is that ts mutants have an activity slightly above the threshold required to show a phenotype at the permissive temperature. At the restrictive temperature, this level falls below the threshold (Figure 8). The difference in activity between wt and ts at the permissive temperature will therefore be a function of how much above the threshold the wt activity is.

In the case of yeast Gal4, when expressed under the strong ADH1 promoter, the total activity is much higher than the activity required to observe a reporter phenotype. Consequently, all selected ts mutants (whether at exposed (Table 2) or buried positions (data not shown)) show reduced activity relative to wt at the permissive temperature. For a different gene product, where the wt activity itself is only marginally above the level required to show a ts phenotype, then the corresponding selected ts mutant would have a similar activity to wt at the permissive temperature but somewhat lower activity at the restrictive temperature. Mutations that lower the activity can do so by two distinct mechanisms. Mutations at buried sites in a protein structure will affect the value of $\Delta G^0_{u.}$ We suggest this will typically result in a reduced, steady-state level of soluble, functional protein relative to wt.[48–50] The total activity will be lowered because the total amount of functional protein present has decreased. Mutations at exposed residues involved in function will generally have little effect on $\Delta G^0_{u.}$ Instead, such mutations are likely to lower the specific activity and hence the total activity of the protein relative to wt.[51] Future biophysical studies with purified proteins will attempt to validate these ideas. In summary, we have outlined a simple and novel strategy for generating ts mutants by targeting functional, ligand binding residues, described a plausible mechanism for why such mutants show a ts phenotype and we have shown that these ts mutants are transferable from yeast to Drosophila. In addition, we outline a view of how ts mutants function that is chemically reasonable and quite different from the general idea that ts mutants have activity virtually identical with that of the wt at the permissive temperature and by some mysterious process lose activity within a temperature span of a few degrees.

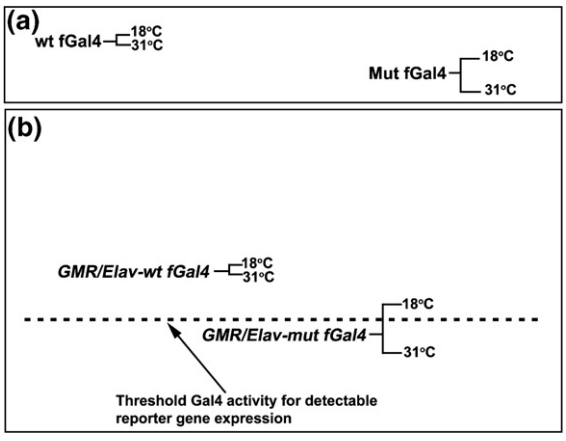

Figure 8. (a) Model for ts Gal4 expression. The specific activity at 31 °C and 18 °C is similar for wt fGal4. For the mutant fGal4s, this activity is lower at 31 °C than at 18 °C and is lower than the wt at the corresponding temperature. (b) In the case of Elav or GMR promoter, Gal4 activity is independent of temperature, and at both temperatures the total activity of Gal4 is above the threshold (dotted line) required for detectable reporter gene expression. For the mutant, the total activity is above the threshold at 18 °C but falls below the threshold at 31 °C.

Materials and Methods

Plasmids and yeast strains, transformation and media

Plasmids used in this study were *pPTGal*,[52] *pElav-Gal4*,[27] *pGBA*.[17] The yeast strain PJ69-4A was used.[20] This contains three reporter genes (*his3, ade2*, and *lacZ*) whose expression is Gal4-dependent. Media was prepared as described.[53] All yeast transformations were done by the high-efficiency lithium acetate method as described.[54] Gap repair cloning was done by following the protocol as described.[55] All mutations were confirmed by DNA sequencing.

Predicting the probable sites in the Gal4 DNA binding domain used to generate ts mutants

The accessible surface areas (*ASA*)[56] of residues in the Gal4 DNA binding domain,[19] PDB ID 1D66 were calculated in the presence and in the absence of DNA. All residues that had an increase in *ASA* (Δ*ASA*) of greater than 5 Å^2 in the free form relative to the bound form were considered to be the DNA interacting residues. These were Gln9, Ala10, Arg15, Lys17, Lys18, Leu19, Cys21, Lys23, Arg46, Leu49, and Thr50. Of these residues, four were selected for mutagenesis. They were Gln9, Arg15, Lys18 and Lys23. These residues were chosen because they span a wide range in ΔASA as well as in the number of hydrogen bonds made with DNA (Table 1).

Site-directed mutagenesis of mGal4

MiniGal4 (mGal4) consists of amino acid residues 1–147 from the DNA binding domain of Gal4 connected to residues 840–881 from the C-terminal activation domain by a seven residue linker. The plasmid *pGBA* expresses wt mGal4 under control of the ADH1 promoter.[17] Primers were designed to replace each of the predicted residues individually with all 20 residues, by replacement of the wt codon by NNK (where N = A/C/G/T and K = G/T). Mutagenesis was carried out by using overlap PCR followed by gap repair as described.[17] Transformants (Ura+) were obtained in synthetic complete media without uracil (SC-U) plates. These were subsequently screened for ts induction of *His-3*, *Ade2* and *LacZ* reporters, as described.[17] Colony plasmid rescue of putative ts mutants was performed and the entire gal4 coding region was sequenced to identify the mutation and to confirm that no additional mutation had

occurred. Quantitative measurement of Gal4 activity in liquid culture was carried out as described.[57] For this, cells were grown until the absorbance at 600 nm was 0.8–1. An equal number of cells was pelleted for each sample and resuspended in Z-buffer. The cells were then lysed by using glass beads (vortex mixed for10 min at 4 °C). A 25 μl sample of the soluble portion of the cell lysate was assayed at 37 °C for β-galactosidase activity in 975 μl of Z-buffer with 200 μl of 4 mg/ml orthonitrophenyl-β,d-galactoside as substrate. The intensity of the color developed was measured at 420 nm. Total protein concentration of the soluble portion of the lysate was determined by Lowry's method.

Construction of mutant full-length *gal4* (*fgal4*) in *pElav*

A DNA fragment containing the entire coding region of *fgal4* was excised from pElav-Gal4[27] by NotI digestion and cloned into *pZErO*™-2 (Invitrogen) at the NotI site to give *pZErO2-fgal4*. Site-directed mutagenesis was carried out to generate the single mutants K23P and R15W using the Stratagene QuikChange site-directed mutagenesis protocol. Each single mutant was recloned into the NotI site of *pElav*.

Construction of wt and mutant *fgal4* in *pPTGMR*

The *GMR* element and the K23P, R15W and R15W/K23P double mutations of *gal4* were cloned in to the *pPTGal* vector[52] using a three-fragment overlap PCR strategy. Fragment 1 consisted of the *GMR* element (a pentamer of truncated Glass-binding sites, residues 4816–4984 of *pGMR*,[58] fragment 2 was the *pPTGal* minimal promoter, and fragment 3 was the wt/mutated DNA binding domain of *fgal4*. These three PCR fragments were subjected to overlap PCR amplification using N and C-terminal primers with EcoRI and BamHI sites, and then cloned into EcoRI/BamHI-digested *pPTGal* to obtain *pPTGMRGal4* and its mutant derivatives.

Generation of transgenic flies

Transgenic flies were generated by injecting the desired *gal4* derivatives of the *P* element containing vector *pPTGMR* and *pElav* (0.5 μg/μl) into *yw*; +; *Ki P*^{p} *[Δ2-3]* flies according to standard procedures.[59,60] Individual flies carrying a single copy of each *P* element were used to generate stocks. For each construct, insertions were obtained in second, third and X chromosomes and homozygous stocks were made using standard procedures.

Drosophila stocks and genetics

Elav-Gal4 flies and *UAS-nuclear lacZ* (*UAS-nlacZ*) flies were available in the laboratory. The lines *UAS-nlacZ; tubP-Gal80ts* and *UAS-hid; tubP-Gal80ts* were generated by using the line w [*]; P{w [+mC]=tubP-Gal80 [ts]}2/TM2.[11] This latter strain was obtained from the Bloomington Stock Center.

Screening of mutant *Elav-fGal4*/*GMR-fGal4* lines for temperature sensitivity

The transgenic lines carrying the *Elav-K23P fGal4* or *Elav-R15W fGal4* constructs were screened with the reporters *UAS-hid/CyO*,[61] *UAS-TNTG* and *UAS-nuclear lacZ* (*UAS-nlacZ*).[62] Here, *CyO* represents a balancer chromosome containing a marker giving rise to a curly winged phenotype. Transgenic lines carrying the *GMR-K23P fGal4* or *GMR-R15W fGal4* constructs were screened with the reporters *UAS-hid/CyO*[61] and *UAS-nuclear lacZ* (*UAS-nlacZ*).[62] The flies were kept at three experimental temperatures, 31 °C, 25 °C and 18 °C. Wild-type *Elav-fGal4* or *GMR-fGal4* flies were crossed with each reporter line as controls. Survival of the progeny was checked in the case of the cross with the *UAS-TNTG* reporter. Adult eye phenotypes were examined in progeny expressing the toxic *UAS-hid* reporter. Adult Drosophila eyes were photographed under a Nikon E-1000 microscope. In the case of crosses with *UAS-nlacZ*, wandering third instar larval eye antennal discs were dissected and prepared for histochemistry/immmunohistochemistry as described.[63] The discs were assayed histochemically with X-gal and immunohistochemically with anti-β-galactosidase and anti-Elav antibodies. Rabbit anti-β-galactosidase antibody (Molecular Probes) was used at a dilution of 1:500, for *Elav-Gal4* and at a dilution of 1:1000 for *GMR-Gal4* crosses. Mouse anti-Elav (Molecular Probes) was used at a dilution of 1:200 in all cases. Secondary antibodies conjugated to Alexa Fluor dyes (Molecular Probes) were used at dilutions of 1:200. Alexa rabbit 488 and Alexa mouse 568 were used for green and red labeling, respectively. Stained preparations were mounted in 80% (v/v) glycerol. The dissected and mounted tissues were visualized under a Nikon E1000 microscope and images were obtained at a magnification of 20×.

Temperature-shift experiments

Elav-fGal4 and *Elav-K23P/R15W fGal4* were crossed with reporter lines *UAS-hid/CyO*, *UAS-TNTG*, and *UAS-nlacZ*. *GMR-fGal4* and *GMR-K23P/R15W fGal4* were crossed with reporter lines *UAS-nlacZ* and *UAS-hid/Cyo*. Progeny were subjected to temperature-shift experiments. Early third instar larvae were collected in vials from 31 °C crosses and transferred to an 18 °C incubator. The *UAS-hid/CyO* and *UAS-TNTG* reporter cross larvae were kept at the transferred temperature until eclosion, while the *UAS-nlacZ* reporter cross larvae were kept at the transferred temperature till the late third instar larval stage and dissected thereafter for histochemical and immunohistochemical assays of β-galactosidase expression in the eye-antennal discs as described above.

Cross of Gal80ts lines with *Elav-fGal4* and *GMR-fGal4*:

The lines *Elav-fGal4* and *GMR-fGal4* were crossed with the reporter lines *UAS-nlacZ; tubP-Gal80ts* and *UAS-hid; tubP-Gal80* described above. The flies were kept at two experimental temperatures, 18 °C and 31 °C, and subjected to temperature-shift experiments as described above. The progeny of the *UAS-hid*; *tubP-Gal80ts* crosses were checked for adult eye phenotypes. In the case of the cross with *UAS-nlacZ*; *tubP-Gal80ts*, late third instar larval eye-antennal discs were dissected and assayed immunohistochemically with fluorescent rabbit anti-β-galactosidase and mouse anti-Elav antibodies as described above.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2007.05.035

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