

# Temperature-sensitive control of protein activity by conditionally splicing inteins

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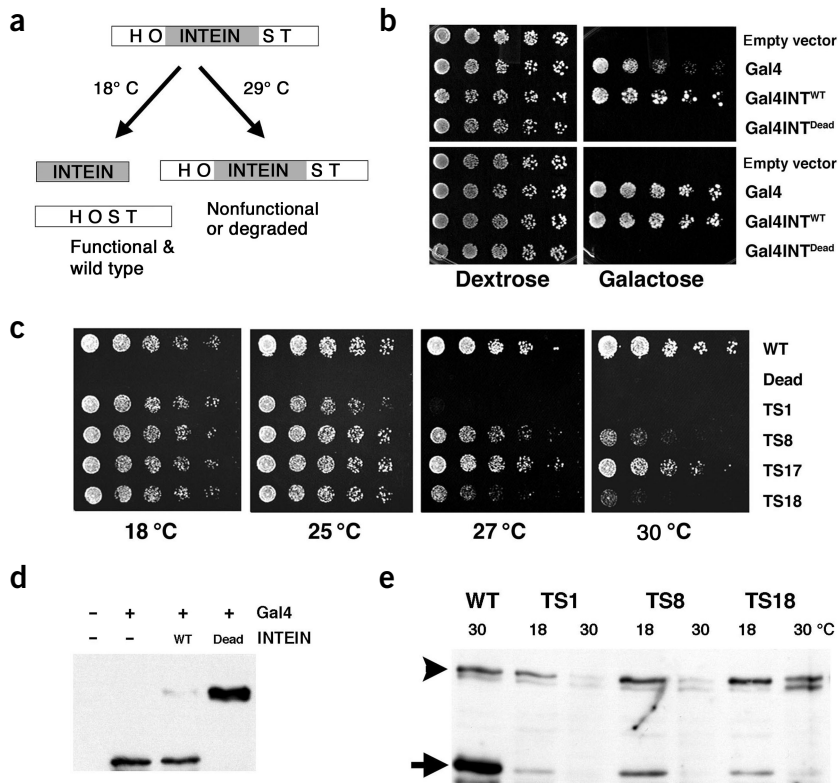
**Conditional or temperature-sensitive (TS) alleles represent useful tools with which to investigate gene function. Indeed, much of our understanding of yeast has relied on temperature-sensitive mutations which, when available, also provide important insights into other model systems. However, the rarity of temperature-sensitive alleles and difficulty in identifying them has limited their use. Here we describe a system to generate temperature-sensitive alleles based on conditionally active inteins. We have identified temperature-sensitive splicing variants of the yeast *Saccharomyces cerevisiae* vacuolar ATPase subunit (VMA) intein inserted within Gal4 and transferred these into Gal80. We show that Gal80-intein<sup>TS</sup> is able to efficiently provide temporal regulation of the Gal4/upstream activation sequence (UAS) system in a temperature-dependent manner in *Drosophila melanogaster*. Given the minimal host requirements necessary for temperature-sensitive intein splicing, this technique has the potential to allow the generation and use of conditionally active inteins in multiple host proteins and model systems, thereby widening the use of temperature-sensitive alleles for functional protein analysis.**

Our ability to understand gene functions largely relies on methods that allow the precise control of gene expression and the activity of their products. The use of temperature-sensitive alleles is a well-established method that is applicable to any organism upon which temperature changes can be imposed. These include viruses, prokaryotes, yeasts, insects, plants and even amphibia and fish<sup>1</sup>. However, identifying and recovering temperature-sensitive mutations is hindered by their rarity, a particular problem in higher organisms. It is also clear that temperature-sensitive alleles cannot be generated for all proteins and some remain refractory even following extensive mutagenesis<sup>2</sup>. In addition, the missense mutation(s) within a protein encoded by a 'classical' temperature-sensitive allele have the potential to exert an influence on the function of the protein even at the permissive temperature. Given recent advances in recombinant DNA it may be easier to generate temperature-sensitive alleles *in vitro* before reintroducing these into the experimental organisms via knock-in or rescue constructs.

Inteins have been described as 'protein introns' and function post-translationally to autonomously splice themselves from within a protein context while regenerating an intact host<sup>3-6</sup>. Temperature-sensitive splicing variants of the Gyr A and recA inteins have been identified<sup>7,8</sup> and such conditional inteins should splice themselves precisely at the permissive temperature to regenerate a wild-type host protein while remaining within the host at the restrictive temperature. Thus, inserted in a position that hinders host activity, the temperature-sensitive intein would create a defective and/or unstable product at the restrictive temperature (Fig. 1a).

The *S. cerevisiae* VMA1 gene product contains one of the best characterized inteins, Sce VMA. We established a system in which growth of a Gal4 mutant *S. cerevisiae* strain FY760 (ref. 9) on a galactose carbon source was dependent on rescue by a plasmid constitutively expressing Gal4. Although the splicing of inteins is entirely autonomous, the host protein residue immediately adjacent to the C-terminal of the intein is critical for splicing<sup>6,10,11</sup>. As this residue is a cysteine in its original protein context, we introduced the wild-type Sce VMA intein sequence into the DNA binding domain of Gal4 immediately N-terminal to the cysteine at position 21 (C21). Constitutive expression of this Gal4/wild-type intein chimera (henceforth referred to as Gal4INT<sup>WT</sup>) rescued growth of FY760 on galactose plates as well as expression of Gal4 alone (Fig. 1b). We used a mutant form of the intein (N454Q), shown to abolish all splicing activity<sup>11</sup> and henceforth referred to as Gal4INT<sup>Dead</sup>, to confirm that the intein insertion acts as a functional mutagen. As expected, Gal4INT<sup>Dead</sup> does not rescue growth of FY760 (Fig. 1b). Thus, an intein that cannot be spliced out of the host protein is sufficient to abolish all Gal4 activity. Similar results were obtained when Gal4INT<sup>WT</sup> and Gal4INT<sup>Dead</sup> were expressed from a low copy number centromeric plasmid maintained at 1–2 copies per cell (Fig. 1b top) and a high copy number vector present at 20–50 copies per cell (Fig. 1b bottom). As expected from the genetic analysis, the splicing of Gal4INT<sup>WT</sup> can be visualized at the protein level. Gal4 is present primarily in its spliced form indicating that the intein can efficiently splice itself from within this foreign host protein context whereas Gal4INT<sup>Dead</sup> is present as an exclusively unspliced protein of approximately 170 kDa (Fig. 1d).

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**Figure 1** Generation of temperature-sensitive Gal4 molecules using conditionally splicing inteins. (a) A schematic representation of the intein temperature-sensitive system for generating conditional HOST protein activity. (b) A dilution series showing growth of FY760 yeast on dextrose (left) and galactose (right) after transformation with plasmids expressing the indicated Gal4 variants. The top rows are transformed with low copy number and the bottom rows with high copy number expression plasmids. Each column represents a twofold dilution of the previous concentration. (c) A dilution series showing the growth profile of yeast rescued by Gal4-containing selected temperature-sensitive intein mutations expressed from a high copy number plasmid. Each column represents a twofold dilution of the previous concentration. (d) Western blot analysis of Gal4 variants expressed from the high copy number. Gal4INT<sup>WT</sup> is present almost exclusively in the spliced form that comigrates with Gal4 itself while Gal4INT<sup>Dead</sup> is present only as the higher molecular weight unspliced state. All lanes are loaded equally. (e) Western blot analysis of selected Gal4INT<sup>TS</sup> constructs. Protein extracts prepared at 18 °C and 30 °C show both the unspliced (arrowhead) and spliced (arrow) forms. Many Gal4INT alleles are unstable at 30 °C and no spliced Gal4 protein (arrow) is detected in these lanes. By contrast Gal4MINT<sup>TS18</sup> appears to be stable at 29 °C, but fails to splice (arrowhead). All temperature-sensitive alleles splice at lower efficiency than the WT intein control. All lanes were loaded equally, spliced Gal4 is ~100 kDa. Both 29 °C and 30 °C conditions were used and found to generate the same nonpermissive effects.

To isolate temperature-sensitive inteins, we generated a library of mutations by low fidelity PCR. Replica plates generated on galactose medium and incubated at 18 °C and 30 °C were used to identify candidate mutations, which were then isolated and recloned into high copy number plasmids to identify strong amorphic alleles at the nonpermissive temperature (Fig. 1c). Inteins showing similar temperature-dependent rescue of FY760 with both high and low copy number plasmids were analyzed further. To characterize the nature of the temperature-sensitive phenotype, we analyzed protein extracts from cells expressing Gal4INT<sup>TS</sup> alleles grown at permissive and nonpermissive temperatures. The results confirm that temperature-dependent rescue results from differential intein activity (Fig. 1e). In most cases the Gal4INT<sup>TS</sup> proved to be unstable at the nonpermissive temperature (30 °C) and very little unspliced protein and no spliced Gal4 could be detected (e.g., TS1 and TS8; Fig. 1e). By contrast Gal4INT<sup>TS18</sup>

unspliced protein is stable at 30 °C, and this intein appears to represent a true 'splicing' mutant. The nine temperature-sensitive alleles were then sequenced to identify the lesions responsible for their phenotype. No mutations were found in the DNA encoding the flanking regions of the Gal4 host gene, while 10 conservative and 27 missense mutations were identified within intein sequences (see Supplementary Fig. 1 online for details).

To be of general use as a universal temperature-sensitive technique, inteins identified must be intrinsically temperature sensitive and not dependent on host protein context or cellular milieu. We therefore transferred the temperature-sensitive intein alleles into Gal80 (ref. 12), a negative regulator of the Gal4 transcription factor<sup>13</sup> that functions in *D. melanogaster*<sup>14</sup>. Thus, at the permissive temperature, Gal80INT<sup>TS</sup> variants should produce functional Gal80, which inhibits Gal4. At the restrictive temperature, a temperature-sensitive intein would not splice, no functional Gal80 would be produced and Gal4 protein would remain fully active (see Fig. 3w). Expressing Gal80INT<sup>TS</sup> in *D. melanogaster* would serve to test the portability of temperature-sensitive inteins and potentially provide an additional level of control of the existing Gal4/UAS system<sup>15</sup>.

We therefore introduced wild-type and dead inteins immediately N-terminal of the two cysteine residues in Gal80 (C127 and C277). Gal80INT fusion proteins were then constitutively expressed from high copy number vectors in wild-type *S. cerevisiae* strain FY602 (ref. 9), and we assessed the ability of these cells to grow on a galactose carbon source. Expression of Gal80 and Gal80INT<sup>WT</sup> upstream of C127 or C277 prevented growth on galactose whereas cells containing a dead intein grew as well as empty vector controls (Fig. 2a). Because a number of inteins appear to function from positions immediately upstream of serine and threonine residues<sup>3</sup>,

we also tested the activity of the wild-type intein immediately N-terminal of S193 and T299; however, no repression of growth was observed in either case (data not shown). Inteins can therefore splice themselves from within Gal80 when upstream of either cysteine residue, and failure to splice is sufficient to abolish Gal80 activity.

We next assessed Gal80INT in *D. melanogaster* tissue culture cells using a *UAS-luciferase* reporter<sup>16</sup> and constructs expressing Gal4 and Gal80 from the constitutive *Actin5C* (*Act*) promoter<sup>17</sup>. Although results in yeast implied that inteins present upstream of both C127 and C277 splice themselves (Fig. 2a), the analysis in *D. melanogaster* cells showed that inteins inserted immediately upstream of C277 were considerably more active than those adjacent to C127 (data not shown). All further analysis therefore used Gal80INT C277 insertions. Both Gal4 and Gal80 function as expected in cells, whereas Gal80INT<sup>Dead</sup> did not affect luciferase levels and Gal80INT<sup>WT</sup> acted as a repressor

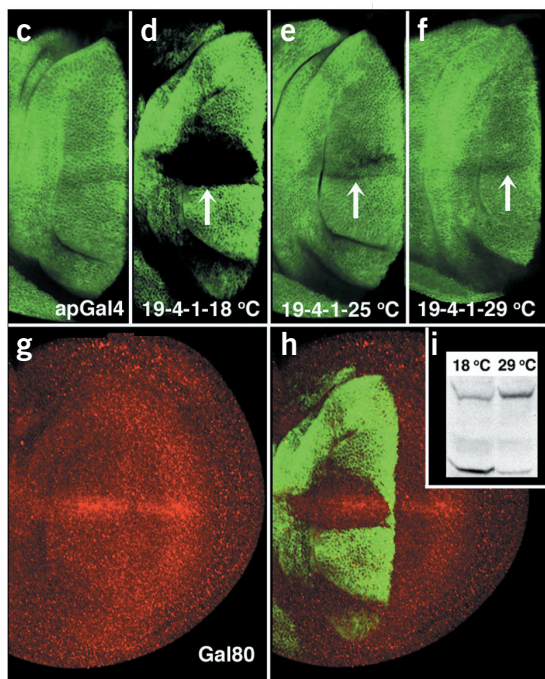
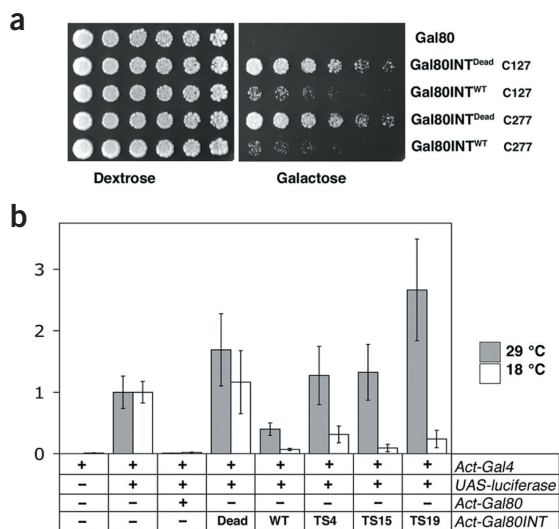
(Fig. 2b). In addition, the temperature-sensitive intein alleles TS4, TS15 and TS19 clearly function as conditional inteins in the Gal80 context, with high levels of luciferase produced at 29 °C and significantly lower levels detected at 18 °C (the permissive temperature at which functional Gal80 is generated) (Fig. 2b).

To test the temperature-sensitive intein system in animals, we then generated transgenic flies expressing Gal80INT<sup>TS19</sup> under the control of the *decapentaplegic* (*dpp*) enhancer<sup>18</sup> (*dpp-Gal80INT<sup>TS19</sup>*). Flies containing *apterous* (*ap*)-*Gal4* and *UAS-GFP* were generated and used to express green fluorescent protein (GFP) in the dorsal compartment of wing discs (Fig. 2c). In the presence of Gal80INT<sup>TS19</sup>, at 18 °C, the expression of GFP was eliminated in a broad region within the wing pouch (Fig. 2d), consistent with INT<sup>TS19</sup> splicing *in vivo*. At 25 °C, the expression of GFP was slightly reduced (Fig. 2e), whereas at the 29 °C nonpermissive temperature, Gal80INT<sup>TS19</sup> had very little effect (Fig. 2f). As expected, Gal80 protein was detected at the anterior/posterior (*a/p*) boundary (Fig. 2g,h) and western blot analysis of larval

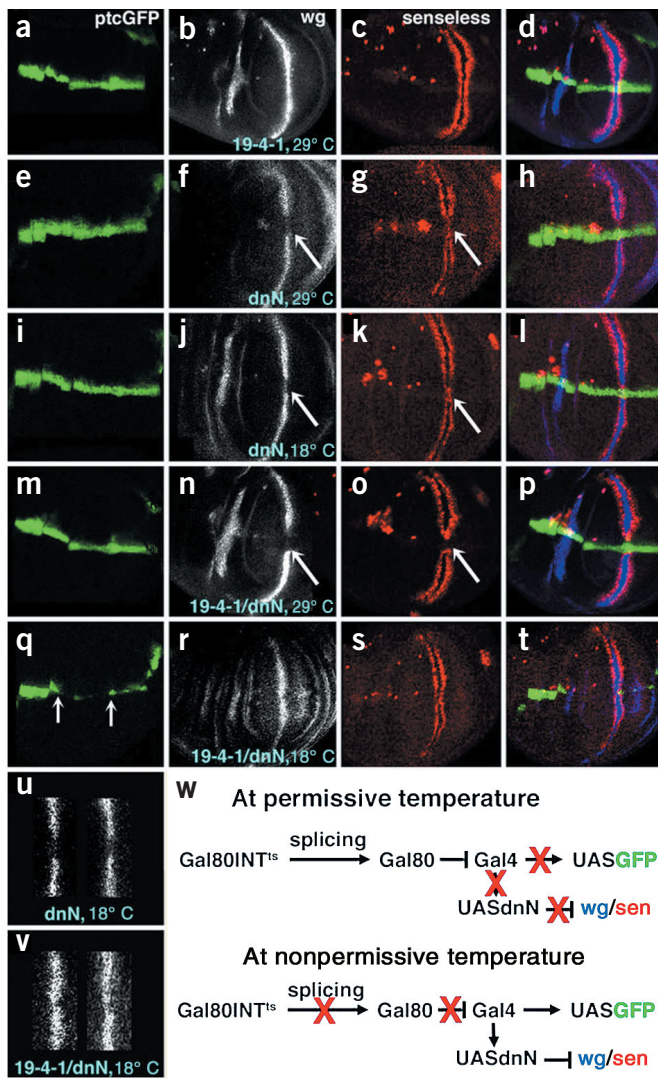
extracts showed that Gal80INT<sup>TS19</sup> spliced to generate Gal80 only at the permissive temperature (Fig. 2i). Taken together, these results demonstrate that Gal80INT<sup>TS19</sup> is fully functional in the *D. melanogaster* cellular environment and INT<sup>TS19</sup> retains its temperature-sensitive splicing activity.

We then used *dpp-Gal80INT<sup>TS19</sup>* to control the activity of the Notch (N) protein. Notch activity was monitored via the levels of *wingless* (*wg*) and its target *senseless* (*sen*)<sup>19</sup>, two genes normally expressed at the dorsal/ventral (*d/v*) compartment boundary in response to activation of N<sup>20</sup> (Fig. 3w). First, we checked that *ptc-Gal4* (Fig. 3a), *UAS-GFP*, and *dpp-Gal80INT<sup>TS19</sup>* did not affect *wg* or *sen* expression (Fig. 3b–d). However, when a dominant negative form of N (*dnN*)<sup>21</sup> is expressed by the *ptc-Gal4* driver, a striking loss of both *wg* and *sen* at the intersection of the *a/p* and *d/v* boundaries (arrows in Fig. 3f–h,j–l) is observed at both 29 °C and 18 °C. Mild temperature sensitivity of *ptc-Gal4* decreased the activity of *dnN* at the lower temperature (compare Fig. 3f with j, g with k). When *dpp-Gal80INT<sup>TS19</sup>* was introduced into *ptc-Gal4*, *UAS-GFP*, *UAS-dnN*, at 29 °C, Gal80INT<sup>TS19</sup> did not splice itself, no Gal80 was present and no effect on the inhibition by *dnN* (arrows in Fig. 3n,o) or expression of *UAS-GFP* (Fig. 3; compare a and m with e) was observed. However, at 18 °C functional Gal80 was produced, and the inhibition of Gal4 reduced the level of *dnN* such that *wg* and *sen* were restored to wild-type levels (Fig. 3r,s). The suppression of Gal4, and hence *dnN*, at permissive temperature was more obvious when focusing on the center region of the discs. At 18 °C, in the absence of Gal80INT<sup>TS19</sup>, gaps in *wg* expression at the compartment boundary were clearly present, whereas no gaps were detected in the presence of Gal80INT<sup>TS19</sup> (compare Figure 3u and v). Thus, sufficient Gal80 activity was present to suppress Gal4 despite the relatively low level of Gal80 detected in this region (Fig. 2g,h). The significant reduction of *ptc-Gal4*-driven *UAS-GFP* observed at 18 °C further supports this conclusion (Fig. 3q, arrows).

Although the temperature-sensitive inteins generated proved to be effective, a number of factors must be considered when transferring these tools into other contexts. First, it remains possible that an



**Figure 2** Activity of intein<sup>TS</sup> in Gal80 in yeast and *D. melanogaster*. (a) A dilution series of wild-type yeast cultures expressing Gal80 constructs. The Gal80, expressed from a high copy number plasmid, inhibits endogenous Gal4 activity within the cell and prevents growth on galactose-containing medium (right). All cultures grew on dextrose medium (left). Each column represents a twofold dilution of the previous concentration. (b) Levels of *UAS-luciferase* activity in *D. melanogaster* S2 cells grown at 29 °C (gray) or 18 °C (white). Transfection with *Act-Gal4*, *UAS-Luciferase*, *Act-Gal80* and *Act-Gal80INT* (including the intein variants used) are indicated. All values represent relative levels of firefly luciferase activity normalized by a cotransfected *R. reniformis* luciferase control. The values obtained in the absence of Gal80 expression (column 2) have been assigned an arbitrary value of 1. The difference in activity of Gal80INT<sup>WT</sup> at 18 °C and 29 °C are probably the result of differential expression and are not observed in other experiments. (c) *ap-Gal4* drives *UAS-GFP* (green) expression in the dorsal side of the wing discs. (d–f) Gal80INT<sup>TS19</sup> inhibits Gal4 in the wing pouch strongly at 18 °C (d), weakly at 25 °C (e), and had no effects at 29 °C (f). (g) The Gal80INT<sup>TS19</sup> (red) is expressed at the *a/p* boundary of the wing pouch. (h) is an overlay of d and g. For panels c–f, only the dorsal portion of the wing discs from third instar larvae are shown. Dorsal is left and anterior is up in all figures. (i) Western blot analysis of protein extracts from third instar larvae with antibody against Gal80 shows that Gal80INT<sup>TS19-4-1</sup> splices itself to generate Gal80 at 18 °C (the low levels of spliced Gal80 detectable in extracts derived from larvae grown at 29 °C may be a consequence of splicing that occurred during sample preparation). Genotypes are *ap-Gal4 UAS-CD8GFP/+; TM6,Tb/+* (c); *ap-Gal4 UAS-CD8GFP/+; Gal80INT<sup>TS19-4-1/+</sup>* (d–h); and *Gal80INT<sup>TS19-4-1</sup>* (i).



**Figure 3** Temperature-dependent control of Notch activity in wing imaginal discs. (a–d) *ptc-Gal4*, *UAS-eGFP*, and *dpp-Gal80INT<sup>TS19</sup>* have no effects on *sen* or *wg* expression at 29 °C and 18 °C (data not shown). (e–l) *ptc-Gal/UAS-dnN* inhibited both *wg* and *sen* expression at the junction of *a/p* and *d/v* boundary (F–H, J–L) that was stronger at 29 °C (F–H) than 18 °C (J–L). (m–v) This inhibition was not affected by *Gal80INT<sup>TS19</sup>* at 29 °C (n–p, arrows) but was diminished at 18 °C (s–v). The effects were more obvious when focusing only on the center regions of the discs as in v. Similarly, *Gal80INT<sup>TS19</sup>* abolished GFP expression where *Gal80INT<sup>TS19</sup>* expression overlaps with *Gal4* at 18 °C (q, between arrows) but has no detectable effects at 29 °C (compare a and m with e and i). Expression of *patched* (*ptc-Gal4/UAS-GFP*) (green, a, e, i, m and r), *wg* (white, b, f, j, n, s, v and w) and *sen* (red, c, g, k, o and t). d, h, l, p and u show the overlays. a–u are individual wing discs from third instar larvae, whereas u and v are only the regions around the *a/p* and *d/v* junctions. Genotypes are *ptc-Gal4 UAS-eGFP/+; Gal80INT<sup>TS19-4-1/+</sup>* (a–d); *ptc-Gal4 UAS-eGFP/+; UASdnN/+* (e–l and v); and *ptc-Gal4 UAS-eGFP/+; Gal80INT<sup>TS19-4-1/UAS-dnN</sup>* (m–u and w).

many Gal4 driver lines available in *D. melanogaster*. In other model systems it may be possible to ‘knock’ a temperature-sensitive intein directly into an appropriate position within the target gene *in vivo*. Alternatively, temperature-sensitive constructs may be generated to conditionally rescue genetically mutant backgrounds, or temperature-sensitive versions of dominant negative proteins may be used. In this context, spatial aspects of gene expression necessary for rescue can be conferred by specific promoters whereas temporal control can be achieved by temperature shifts. Finally, the temperature-sensitive intein system may also be valuable as a mutagen in screens specifically designed to generate temperature-sensitive alleles. Using a gene trap approach, lethal mutations that result could be rapidly tested for reversion at 18 °C.

## METHODS

All molecular biology, yeast techniques and *D. melanogaster* maintenance were carried out using standard procedures. Sequence positions are given such that the first residue of the first codon is 1. Yeast strains used were FY760: MATa *ura3-52 trp1Δ63 leu2Δ1 gal4Δ::LEU2 his3Δ200 lys2-128Δ*, FY970: MATa, *Gal80Δhis4-917Δ lys2-173R2: leu2Δ1 ura3-52 trp1Δ63*; and FY602: MATa *his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63* and were a gift of the Fred Winston lab (Harvard). PCR and sequencing primers used are listed in Supplementary Table 2 online.

**Cloning of Gal4 constructs.** Gal4 was amplified from pGatB(N)<sup>15</sup> using GAL401-F and Gal402-R primers to add an *EcoRI* site at the 5′ end and a single Myc tag (EQKLISEEDL) and *BamHI* site at the 3′ end. The resulting product was trimmed with *EcoRI* and *BamHI* and cloned into similarly cut pS5 and pU yeast/*Escherichia coli* shuttle vectors. pS5 is based on the low copy number centromeric YCplac33 vector<sup>23</sup> and contains a 1.6-kb fragment from the yeast ADH promoter that confers constitutive high level expression<sup>24</sup>, a *ura3* selectable marker and the ampicillin antibiotic resistance gene—the *HindIII* site within the YCplac33 polylinker was also destroyed by cutting, filling in and religating. pU is based on the high copy number 2μm YEplac195 vector<sup>23</sup> and contains the same promoter and selectable markers. The resulting pS5-Gal4 construct was then used to transform, using a standard lithium acetate technique<sup>25</sup>, the Gal4 knockout strain FY760 (ref. 9), which was tested on uracil drop-out selective medium containing 2% galactose as a carbon source.

To introduce the intein sequence, we generated silent mutations that introduce *HindIII* and *AflIII* restriction sites into positions 51–56 and 65–70 of Gal4. pS5-Gal4 was used as a template for two consecutive rounds of PCR using Gal405-R and Gal401-F to generate a 173-bp ‘mega-primer’ that contained the new restriction sites, and which was then used with Gal402-R to regenerate full-length Gal4. This was once more cloned into pS5 to generate pS5-Gal4(HA) and tested for rescue of the FY760 on galactose medium.

unspliced host containing a temperature-sensitive intein may retain some activity. Such leakiness may be minimized by inserting the temperature-sensitive intein within known functional domains of the host, whereas the instability of many unspliced Gal4INT<sup>TS</sup> molecules at nonpermissive temperatures may reduce this concern (Fig. 1e). However both wild-type and dead intein constructs should be included in any proposed experimental series to act as controls. Second, the ability of an intein to splice from within a host protein is also dependent on its context. Only inteins immediately upstream of a cysteine residue produced a detectable splicing activity (data not shown) and despite our successful generation of temperature-sensitive Gal4INT and Gal80INT proteins, variations in other flanking sequences may also influence splicing activity<sup>22</sup> (see also Supplementary Table 1 online). A number of temperature-sensitive inteins and/or host insertion sites may need to be tested before an optimal splicing combination is identified. Although such optimization may increase the complexity of generating temperature-sensitive inteins, the effort required to generate a ‘classical’ temperature-sensitive allele by genetic means is vastly greater in any multicellular organism.

To generate host temperature-sensitive alleles *in vivo* it is necessary to introduce the engineered allele into the organism of interest. Already Gal80INT<sup>TS</sup> potentially allows the temporal control of the

**Isolation and mutagenesis of intein.** The Sce VMA intein sequence was amplified from *S. cerevisiae* genomic DNA using the INT01-F and INT02-R primers (see Supplementary Table 2 online), cloned into pCRSCRIPT (Stratagene) and sequenced. The intein was then reamplified using Gal406c-F and Gal407-R primers to generate a 480-bp product in which internal *HindIII*, *KpnI* and *AflIII* sites are destroyed and a *HindIII* site inserted at the 5' end together with Gal4 sequence destined to be upstream of the intein coding sequence. This 'mega-primer' was then used in a second round of amplification together with Gal408-R to give a product that contained the Gal4 sequences destined to lie between the 3' end of the intein and the *AflIII* restriction site. After trimming with *HindIII* and *AflIII*, this product was subcloned into pS5-Gal4(HA) and cut with *HindIII* and *AflIII* to generate pS5-Gal4INT<sup>WT</sup>. This construct was then transformed into FY760 and tested for growth on galactose medium. As a negative control pS5-Gal4INT<sup>WT</sup> was mutated *in vitro* using INT05-F and G4D02 to generate a mega-primer, which was used together with Gal406c-F to generate a full-length intein<sup>Dead</sup> product, which was then cloned into pS5-Gal4(HA) using the *HindIII* and *AflIII* restriction sites. The intein within this construct (henceforth referred to as pS5-Gal4INT<sup>Dead</sup>) was sequenced, transformed into FY760 and tested for growth on galactose medium.

Using INS-1 and G4D02 primers, and pS5-Gal4INT<sup>WT</sup> as a template, four parallel low fidelity mutagenic PCR amplification reactions were done as previously described in the presence of 0.1 mM MnCl<sub>2</sub> and with the concentration of one nucleotide reduced from 200 μM to 50 μM<sup>26</sup>. The resulting PCR products were pooled, trimmed with *AflIII* and *HindIII* and subcloned into pS5-Gal4(HA) cut with the same enzymes. The resulting ligation was transformed into Ultra-max competent cells (Stratagene) and amplified in *E. coli* by allowing the transformation to grow in liquid culture to an OD<sub>600</sub> of 0.1 before DNA isolation. Plating of samples taken after transformation indicated a library with a complexity of approximately 46,000 colonies. Of 56 colonies (37.5%) tested, 21 contained intein inserts and thus the total library complexity represents ~17,250 mutated inteins.

**Gal4INT<sup>TS</sup> screening, validation and western blot analysis.** FY760 yeast was transformed by library DNA and plated on uracil drop-out selective medium containing 2% dextrose. Approximately 20% of colonies replica-plated onto galactose plates grew at both 18 °C and 29 °C. However, of approximately 8,000 colonies screened at both temperatures, a total of 19 grew at 18 °C but did not grow at 29 °C and therefore represent potential temperature-sensitive alleles. No cold-sensitive alleles were identified. After initial screening the plasmids present in temperature-sensitive colonies were recovered in *E. coli*<sup>27</sup> and used to retransform FY760 cells before rescreening. This secondary round of screening excludes background mutations within the genome of the host cell as a potential cause of temperature sensitivity and confirmed the identification of nine temperature-sensitive alleles. The intein and flanking pieces of Gal4 amplified to generate the library were then completely sequenced on both strands using primers from the INS series of primers (see Supplementary Table 2 online) and assembled using 'DNA Star' software.

Owing to low levels of protein expression from the pS5 vector all Gal4, Gal4INT<sup>WT</sup>, Gal4INT<sup>Dead</sup> and Gal4INT<sup>TS</sup> alleles were subcloned into pU high copy number vectors and rechecked for growth on galactose. Protein extracts of cells were prepared as described<sup>25</sup> using glass bead disruption and equal quantities of protein separated on 6% polyacrylamide gels. Blots were incubated in 1:8000 dilution of mouse anti-Gal4AD (BD Biosciences) and visualized using ECL (Amersham).

**Cloning of Gal80 constructs.** The Gal80 coding region was isolated from pCasper-tub-Gal80 (ref. 14) using Gal80F2 and Gal80R3, trimmed with *SacI* and *BamHI* and subcloned into pS5 vectors cut with the same enzymes. The resulting pS5-Gal80 construct was then transformed into the 'wild-type' parental strain FY602<sup>9</sup> and tested for its ability to grow on galactose medium.

Inteins were introduced into Gal80 by double recombination between two linear DNA pieces cotransfected into FY970—a yeast strain lacking endogenous Gal80 chosen to avoid possible recombination into the genomic locus. Oligos containing ~50 bp of sequence homologous to the intended Gal80 insertion site and 20 bp of sequence homologous to the intein ends were used to amplify intein<sup>WT</sup>, intein<sup>Dead</sup> and intein<sup>TS</sup> from pS5-Gal4INT templates (using the G80Px series of oligos listed in Supplementary Table 2 online). As

intein<sup>Dead</sup> contains a mutation that would otherwise be reverted to wild-type G80DPx, series oligos were used. Linearized pS5-Gal80 was generated either by cutting with *Asp718* for the P2 insertions or by using long range PCR and PxF and PXR oligos flanking the intended insertion sites and oriented to amplify around the plasmid. FY970 was transformed by the two linear pieces of DNA representing vector and intein. In theory the only colonies to grow contain circular plasmids in which a double recombination event between the two linear DNA pieces has occurred to allow plasmid propagation and expression of the *ura3* gene. Colonies recovered were tested directly for the presence of inserts using TP1F and TP1R (for position 1), TP2F and TP2R (for position 2), TP3F and TP3R (for position 3) and TP4F and TP2R (position 4) in conjunction with the intein internal primer TIE. pS5-Gal80INT plasmids identified were then recovered in *E. coli*, checked for the presence of the intein by restriction analysis, subcloned into the pU vector and used to retransform FY602. The resulting strains were then tested for growth at 18 °C and 29 °C on galactose-containing medium.

**S2 cell assays.** Fragments containing the Gal80INT<sup>WT</sup>, Gal80INT<sup>Dead</sup> and Gal80INT<sup>TS</sup> alleles were generated by digesting pS5-Gal80INT constructs with *BamHI* and *XbaI*. Gal4 was obtained by *BamHI* and *Asp718* digest of pS5-Gal4. These fragments were subcloned into the pUC-Act vector (gift of Dan Curtis), which contains the *Actin5C* promoter and 3'-UTR and drives constitutive expression in *D. melanogaster* cells. The *UAS-luciferase* reporter was a gift of D. Yamamoto. We seeded 2 × 10<sup>4</sup> S2 Schneider cells (Invitrogen) into individual wells of a 96-well plate and transfected each well with 10 ng pUC-Act-Gal4, 10 ng pUAS-luciferase, 30 ng pUC-Act-Gal80INT and 20 ng of pUp-Act-Renilla (a *Renilla reniformis* luciferase transfection control (M.P.Z. and S.H., unpublished data)) using the Effectene Transfection Reagent kit (Qiagen). Experiments undertaken without Gal80 included an equal quantity of pUC-Act empty vector. Each experiment was repeated four times. After transfection, cells were grown at 18 °C or 29 °C for 3 or 2 d respectively, lysed and assayed using a Monolight 3010 luminometer (Pharmingen) and the Dual luciferase assay kit (Promega). The overall experiment was repeated three times using the Kc and S2R+ cell lines and the effect shown to be consistent.

**In vivo experiments.** Gal80INT<sup>TS19</sup> from pUC-Act-Gal80INT<sup>TS19</sup> P4 was cloned into pCasper4 between *BamHI* and *XbaI*. The *dpp-hsp70* promoter from pBM10 (ref. 18) was then cloned by PCR into pCasper4-Gal80INT<sup>TS19</sup> between the *NotI* and *SpeI* sites to generate pCasper-dpp-Gal80INT<sup>TS19</sup>, which was injected with helper DNA according to standard protocols. Twelve independent transgenic lines containing *dpp-Gal80INT<sup>TS19</sup>* were generated. Expression of Gal80INT<sup>TS19</sup> had no discernable effect on the wing discs and the resulting wings are morphologically wild type.

Two lines inhibited *ap-Gal4/UAS-EGFP* strongly, three intermediately, two weakly and five had no effect, most likely because of positional effects. The data shown in Figs. 2 and 3 were generated using one of the strongest insertions, Gal80INT<sup>TS19-4-1</sup>.

The following crosses were set up at 25 °C:

*ap-Gal4, UAS-CD8GFP/CyO × dpp-Gal80INT<sup>TS19</sup>/TM6, Tb to give: ap-Gal4, UAS-CD8GFP/+; dpp-Gal80INT<sup>TS19</sup>/+*

*ptc-Gal, UAS-EGFP × UAS-dnN/TM3, Sb to give: ptc-Gal4, UAS-EGFP/+; UAS-dnN/+*

*ptc-Gal4, UAS-EGFP; dpp-Gal80INT<sup>TS19</sup>/CyO-TM6, Tb × UAS-dnN/TM3, Sb to give: ptc-Gal4, UAS-eGFP/+; UAS-dnN/dpp-Gal80INT<sup>TS19</sup>*

Larvae raised at 25 °C were transferred to 18 °C or 29 °C at the early third instar larval stage. Wing discs were dissected, fixed and immunostained using standard techniques at late third instar larvae. Images were taken with Leica confocal microscope with 20× objective. Protein extracts were made by homogenizing late third instar larvae in lysis buffer (150 mM NaCl, 10 mM Tris (pH 7.2), 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA) with protease inhibitor cocktails. After addition of 2× Laemmli sample buffer, boiled for 5 min, and centrifuged at full speed in a benchtop micro-centrifuge for 10 min, the resulting supernatants were used for western blot analysis. Antibodies used are against Wg (1:10 dilution, Developmental Studies Hybridoma Bank at the University of Iowa), Senseless (1:1000, a gift from Hugo J. Bellen), Gal80 (1:400 for immunostaining and 1:500 for western blot analysis, a gift from James

Hopper), Alexa 594 anti-guinea pig or rabbit IgG, Alexa 647 anti-mouse IgG (Molecular Probes) and anti-rabbit horseradish peroxidase-linked secondary antibody (1:10000, Jackson Lab).

Note: Supplementary information is available on the Nature Biotechnology website.

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#### COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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