midline represses Dpp signaling and target gene expression in Drosophila ventral leg development

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ABSTRACT
Ventral patterning in Drosophila is controlled by the expression of the redundant T-box Transcription factors midline (mid) and H15. Here, we show that mid represses the Dpp-activated gene Daughters against decapentaplegic (Dad) through a consensus T-box binding element (TBE) site in the minimal enhancer, Dad13. Mutating the Dad13 DNA sequence results in an increased and broadening of Dad expression. We also demonstrate that the engrailed-homology-1 domain of Mid is critical for regulating the levels of phospho-Mad, a transducer of Dpp-signaling. However, we find that mid does not affect all Dpp-target genes as we demonstrate that brinker (brk) expression is unresponsive to mid. This study further illuminates the interplay between mechanisms involved in determination of cellular fate and the varied roles of mid.

KEY WORDS: Tissue patterning, Limb development, Dpp-signaling, T-box transcription factors

INTRODUCTION
Proper organization of tissue is crucial for maintaining the body plan of animals. This organization occurs during development when multiple factors cooperate to determine cell fate. In particular, the Drosophila melanogaster leg is organized in part by the interplay between signaling molecules and transcription factors. Dorsal and ventral fates in Drosophila legs are dependent on the action of morphogens and selector genes. The morphogens decapentaplegic [dpp, a bone morphogenetic protein (BMP) homolog] and wingless (wg, a fly Wnt) are induced by Hedgehog signaling. Wg is induced in the ventral domain and controls ventral fate through induction of the redundant Tbx20 class T-box transcription factor homologs midline (mid) and H15, that specify ventral fate (Svendsen et al., 2009). Dorsal fate is dictated by dpp, which is expressed at high levels in the dorsal domain and low levels in the ventral domain, though it has no role in ventral fate aside from joint formation (Held and Heup, 1996; Manjón et al., 2007). Dpp-signaling is mediated by transcription factors Mothers against dpp (Mad), a fly Smad1/5 activator and Medea (Med), a fly Co-Smad (Hudson et al., 1998; Kim et al., 1997).

mid and H15 control ventral patterning being both necessary and sufficient to specify the fate in the ventral region of fly legs (Svendsen et al., 2009). They act as selector genes, transcription factors that specify cell fate for a particular developmental region, with expression restricted to the region in which they specify cell fate (Mann and Morata, 2000). The ventral specific expression of mid and H15 is controlled through a combination of Wg activation and Dpp repression (Svendsen et al., 2009, 2015). When mid and H15 function is lost in the ventral leg, tissues are transformed into dorsal while ectopic expression of mid or H15 induces ventral fate in dorsal regions (Svendsen et al., 2009).

How does mid control ventral development? We have shown that one role of mid is to block Dpp signaling in the ventral domain (Svendsen et al., 2019). The distribution of phosphorylated Mad (pMad) follows the same pattern as dpp, with significant levels of staining in the dorsal domain and weaker staining in ventral region, indicating that the Dpp pathway is activated at lower levels in ventral cells. While Dpp does not contribute to ventral patterning, double-mutant analysis shows that the ventral to dorsal transformation of mid H15 mutant clones in some regions of the leg is rescued to ventral phenotype if they are simultaneously blocked for Dpp signaling. This indicates an inhibitory effect of mid and H15 on Dpp signaling that is necessary for ventral patterning. This is further demonstrated by the inhibition of pMad accumulation by mid (Svendsen et al., 2019). Additionally, mid-expressing clones repress the Dpp-target genes Dad, Upd3, and mid itself in an eh1-dependent manner, and using chromatin-immunoprecipitation (ChIP) assays, Mid has been shown to localize to enhancers for these genes (Svendsen et al., 2019).

Here, we study further the way mid antagonizes Dpp signaling, showing that it represses the regulation of Dad, a target gene activated by Dpp/pMad, but has no effect on brk, a gene repressed by Dpp/pMad. We show that Mid repression of Dad depends on a predicted T-box binding element (TBE) in the Dad13 enhancer, and that Mid-inhibition of Dpp-dependent pMad accumulation and tissue re-patterning depends on the eh1 repressor domain.

RESULTS
mid blocks Dpp activation of Dad13 reporter expression
Our previous work showed that the eh1 repressor binding domain was required for Mid selector gene function. We also showed that Mid acted by antagonizing Dpp signaling, reducing the levels of pMad accumulation in the ventral domain of the fly leg (Svendsen et al., 2019). Here, we further investigate how mid affects Dpp-target gene expression. The Dpp-target genes Dad and brk are canonical examples of genes activated or repressed by Dpp signaling, respectively (Gao et al., 2005; Tsuneizumi et al., 1997). Since mid antagonizes Dpp signaling, we sought to understand how mid regulates both genes. We showed previously that an enhancer-trap reporter of Dad (Dad-lacZ) was weakly repressed by Mid in an eh1-dependent manner (Svendsen et al., 2019). Mid likely antagonizes Dpp-target genes through direct repression because Mid has been shown to bind to several enhancer fragments of Dad in...
ChIP assays (Svendsen et al., 2019). One of these fragments was a well-characterized 520 bp enhancer, Dad13. The Dad13-driven expression pattern is similar to that of Dad-lacZ, with strong expression in the dorsal domain and weaker expression in the ventral domain. However, the ventral Dad13-driven expression is weaker compared to wild-type Dad. We first confirmed that a Dad13 reporter was regulated by Mid. We investigated whether loss of H15 and mid function would affect Dad13-driven expression by generating H15 mid loss-of-function clones via mitotic recombination in imaginal discs of second instar larvae. Dad13-driven expression was detected by RFP expression while clones null for H15 mid were marked by the absence of GFP. Ventrally located H15 mid loss-of-function clones showed either an increase of Dad13-driven expression or an expansion into the lateral region of the leg imaginal disc (Fig. 1A,B). Because the mid expression domain completely encompasses the ventral Dad13 domain (Fig. S1C), we induced ectopic Dpp-signaling in lateral regions of the imaginal disc in order to induce Dad13-reporter expression outside the mid-expression domain. We then introduced mid expression to assess the ability of Mid to repress Dad13. By using AyGal4, a construct that generates random clones expressing Gal4 under the control of the actin5C promoter, we induced clones marked by GFP expression that also expressed a constitutively active form of the Dpp receptor, thickveins (UAS-thickveins) and/or expressed Mid. As expected, UAS-thickveins expressing clones, which are constitutively activated for Dpp-signaling, had increased Dad13-nRFP reporter expression. However, co-expressing UAS-mid+ and UAS-thickveins blocked the ectopic activation of Dad13-nRFP in clones located throughout the disc (Fig. 1C,D). This indicates that the presence of Mid blocks the effects of Dpp-signaling on Dad13-driven expression. Together these results confirm that Mid does indeed regulate the Dpp-target gene Dad via its enhancer fragment, Dad13.

The TBE in Dad13 is necessary for mid repression of Dad

To further investigate the role of mid in Dad regulation, we examined how Mid influences Dad13-reporter expression. The Dad13 enhancer contains multiple binding sequences for Mad that are responsible for driving Dad13 expression (Weiss et al., 2010). Dad13 also contains tandem Smad binding element (SBE) sequences (GTCTGTCT) that have a minor role in activating Dad13-reporters in embryos but which have not been tested in leg imaginal discs (Weiss et al., 2010). Adjacent to the SBE sites and separated by a single nucleotide is a consensus T-box binding element (TBE) (AGGTGA) similar to the consensus binding site for Mid (Najand et al., 2012). To test whether the TBE is necessary for Mid regulation of Dad13, we mutated the Dad13-TBE site (Dad13TBE) and tested expression with a lacZ reporter (Fig. 2A). Dad13TBE-lacZ reporter expression was stronger and broader in the ventral domain compared to the Dad13-lacZ control (Fig. 2B). This clearly indicates that the TBE is required for repression of Dad. Furthermore, the Dad13TBE-driven expression in dorsal regions outside the mid expression domain was more intense than Dad13-driven expression, suggesting that factors expressed outside the ventral domain may also regulate Dad through the TBE site. The proximity of the activating SBE next to the potential Mid-binding element suggested a model in which the activation of Dad13 by Dpp may be blocked by Mid or other factors interfering with the SBE site. However, mutating the SBE (Dad13SBE) had no observable effect on Dad13-driven expression and mutating both the TBE and SBE (Dad13SBE-TBE) produced effects on reporter expression that were similar to mutating the TBE alone (Fig. S3). Thus, the SBE site does not appreciably affect Dad13-driven expression in the leg imaginal disc and the TBE site must affect Dad13 enhancer through another mechanism.

To test whether Mid could still regulate Dad13TBE-driven expression, we generated UAS-mid+ gain-of-function clones and measured Dad13TBE-lacZ expression. Consistent with our previous

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**Fig. 1. Mid blocks Dpp activation of Dad13-driven expression.** (A–B) Third-instar leg discs expressing Dad13-nRFP (red, single channel) and loss-of-function H15 and mid (lack of green) clones. Clones lacking H15 and mid have (A) increased Dad13-nRFP reporter expression (white arrowhead, lower inset), or (B) expanded Dad13-nRFP reporter expression (white arrowhead, lower inset; n=7). (C) Discs with UAS-thickveins gain-of-function clones (green) driven by AyGal4 driver showed ectopic Dad13-driven expression (red, single channel, lower inset; n=12), while (D) clones co-expressing UAS-thickveins and UAS-mid+ (green) did not induce ectopic Dad13-driven expression (red, single channel, lower inset clone outline; n=13). All imaginal discs in this report are orientated dorsal up, anterior left.
work (Svendsen et al., 2019), Dad13-reporter expression was repressed in most mid-expressing clones in the ventral domain (7/8) (Fig. 2C,C′). In contrast, few mid-expressing clones in the ventral domain of discs a decreased Dad13TBE-driven expression (1/11 clones; Fig. 2E,E′). To further validate that mid-expressing clones had weaker effects on Dad13TBE-reporter expression, we measured the expression in pairs of adjacent cells located inside and outside of the mid-expressing clone. The ratio of reporter expression of the outside cell divided by the reporter expression of the inside cell was 1.95 for Dad13 and 1.19 for Dad13TBE (Fig. 2F). These results suggest that the wild-type TBE in the Dad13 enhancer fragment is an essential element for the mid-mediated repression of Dad.

**mid does not substantially affect Dpp repression of brk**

brk is negatively regulated by Dpp signaling. Unlike Dad, which is activated by a complex of pMad and Med, brk is repressed by a
complex of pMAD and Med binding along with a third protein, Schnurri, through a repressive binding element (Hamaratoglu et al., 2014; Marty et al., 2000; Saller and Bienz, 2001; Weiss et al., 2010). In leg imaginal discs, high levels of brk and pMAD expression are reciprocal, demonstrating their antagonistic activities (Müller et al., 2003; Fig. S1). We found that neither H15 mid loss-of-function clones nor mid\(^{−}\)—expressing gain-of-function clones had any effect on brk expression. In loss-of-function experiments, endogenous brk expression was detected by a brk antibody and remained unchanged in ventrally located clones lacking H15 mid (Fig. 3A, insets). Moreover, mid\(^{−}\) gain-of-function clones marked by GFP (UAS-mid\(^{+}\)) and located throughout the imaginal disc did not affect brk-lacZ expression (Fig. 3B, insets). Because Dpp-signaling represses brk and mid represses Dpp-signaling, we tested if ectopic expression of Dpp signaling with mid would increase or otherwise affect brk expression. As expected, gain-of-function clones expressing UAS-tkv\(^{O/D}\) gain-of-function strongly repressed brk-lacZ reporter expression leaving only residual expression (Fig. 3C, insets). However, clones co-expressing UAS-mid\(^{−}\) with UAS-tkv\(^{O/D}\) had little influence on the tkv\(^{O/D}\) gain-of-function effect on brk-reporter expression, which remained almost as strongly repressed (Fig. 3D, insets). This was surprising given that clones co-expressing UAS-mid\(^{−}\) and UAS-tkv\(^{O/D}\) have markedly reduced pMAD activation compared to clones expressing UAS-tkv\(^{O/D}\) alone (Fig. S2; Svendsen et al., 2019). This suggests that clones co-expressing UAS-mid\(^{−}\) and UAS-tkv\(^{O/D}\) have sufficient residual pMAD activation to repress brk despite UAS-mid\(^{−}\) expression. Taken together, these results suggest that mid does not regulate brk and thus mid does not affect all Dpp-target genes.

**Mid antagonizes Dpp signaling in an eh1-dependent manner**

Mid acts as a repressor through its engrailed-homology-1 (eh1) domain, which recruits the co-repressor groucho (gro) (Formaz-Preston et al., 2012). The eh1 domain is essential for Mid-mediated repression of genes in the ventral leg including the Dpp-target gene Dad (Svendsen et al., 2019). Here we asked if the eh1 is also necessary to inhibit the effects of ectopic Dpp signaling, including phenotypic defects and pMAD accumulation. We generated gain-of-function UAS-tkv\(^{O/D}\) clones in developing imaginal discs in second instar larvae. In adult legs, these clones gave rise to rounded outgrowths characteristic of ectopic Dpp-signaling (Fig. 4A; Svendsen et al., 2019). Next, we co-expressed UAS-tkv\(^{O/D}\) and a Flag-tagged mid (UAS-mid\(^{−}\)–Flag) in adult legs. These clones induced fewer defects, which were less severe with generally smaller outgrowths (Fig. 4C). However, when we co-expressed UAS-tkv\(^{O/D}\) with UAS-mid\(^{eh1}\)–Flag, a transgene in which the eh1 domain is mutated and has reduced Gro-binding (Formaz-Preston et al., 2012), the adult legs (Fig. 4E) displayed outgrowths similar in size and severity to clones expressing UAS-tkv\(^{O/D}\) alone (Fig. 4A). Overall, more defects were detected in legs containing either UAS-tkv\(^{O/D}\) or UAS-tkv\(^{O/D}\) and UAS-mid\(^{eh1}\)–Flag clones as compared to legs expressing UAS-tkv\(^{O/D}\) and UAS-mid\(^{−}\)–Flag clones.

In addition to the different patterning defects induced in adult legs, we also saw changes in the pMAD accumulation in leg imaginal discs. Clones in third-instar discs expressing gain-of-function UAS-tkv\(^{O/D}\) have increased pMAD staining (Fig. 4B,B′,B″). Similar levels of pMAD were detected in clones co-expressing UAS-tkv\(^{O/D}\) and UAS-mid\(^{−}\)–Flag (Fig. 4F,F′,F″). In comparison, clones expressing the UAS-mid\(^{−}\)–Flag and UAS-tkv\(^{O/D}\) had lower levels of pMAD staining relative to the other two genotypes (Fig. 4D,D′,D″,G). We note that the suppression of pMAD by Flag-tagged UAS-mid\(^{−}\)–Flag is less pronounced than the suppression by untagged UAS-mid\(^{−}\) (Fig. S2; Svendsen et al., 2019). This is consistent with our observation that all Flag-tagged Mid gain-of-function phenotypes are weaker compared to untagged Mid (Formaz-Preston et al., 2012; Svendsen et al., 2019). However, the UAS-mid\(^{−}\)–Flag is able to rescue mid mutants, and the UAS-mid\(^{−}\)–Flag and UAS-mid\(^{eh1}\)–Flag transgenes are well matched for expression levels, with UAS-mid\(^{eh1}\)–Flag expressed approximately twofold higher than UAS-mid\(^{−}\)–Flag (Formaz-Preston et al., 2012; Svendsen et al., 2019). Thus, despite being expressed at a higher level than the UAS-mid\(^{−}\)–Flag strain, the UAS-mid\(^{eh1}\)–Flag expressing clones have much weaker effects on pMAD levels. Together, these results suggest that the eh1 domain is implicated in both of Mid’s repressive roles: direct repression of Dpp-target genes and interference with the Dpp-signaling cascade.
When UAS-mid-Flag was co-expressed with UAS-mid+−Flag, the outgrowths were less severe (arrowhead) indicating suppression of the tkv gain-of-function phenotype. The effect was also less frequent with only 1.9% of legs having ectopic outgrowths (n=309). (E) Clones co-expressing UAS-tkvQD and UAS-mid−eh1-Flag had deformities (arrowhead) similar to clones expressing UAS-tkvQD alone, where 9.4% of legs had ectopic outgrowths (n=106). The clones scored in adult cuticles in panels A, C and E are not marked in these experiments but resemble the effect of marked tkvQD clones in other experiments (data not shown). pMad staining was less elevated (D,D″; n=22). (G) The difference in pMad levels staining between UAS-tkvQD clones and UAS-tkvQD, UAS-mid−eh1-Flag was not significant. However, clones expressing UAS-tkvQD, UAS-mid−Flag had significantly lower pMad compared to the other two conditions. The mean values were UAS-tkvQD 1.22×10^7, UAS-tkvQD, UAS-mid−Flag 6.73×10^6 and UAS-tkvQD, UAS-mid−eh1-Flag 1.16×10^7. Statistical analysis used the Tukey’s multiple comparisons test, with bars representing the mean, s.d., and significance indicated, *P-value<0.05 and **P-value<0.01.

FIG. 4. The eh1 domain is involved in Dpp repression. (A) AγGal4 gain-of-function clones expressing UAS-tkvQD result in outgrowths and dorsal transformation (arrowhead), 12.5% of legs had ectopic outgrowths under these conditions (n=181). (C) When UAS-tkvQD was co-expressed with UAS-mid+−Flag, the outgrowths were less severe (arrowhead) indicating suppression of the tkv gain-of-function phenotype. The effect was also less frequent with only 1.9% of legs having ectopic outgrowths (n=309). (E) Clones co-expressing UAS-tkvQD and UAS-mid−eh1-Flag had deformities (arrowhead) similar to clones expressing UAS-tkvQD alone, where 9.4% of legs had ectopic outgrowths (n=106). The clones scored in adult cuticles in panels A, C and E are not marked in these experiments but resemble the effect of marked tkvQD clones in other experiments (data not shown). pMad staining was less elevated (D,D″; n=22). (G) The difference in pMad levels staining between UAS-tkvQD clones and UAS-tkvQD, UAS-mid−eh1-Flag was not significant. However, clones expressing UAS-tkvQD, UAS-mid−Flag had significantly lower pMad compared to the other two conditions. The mean values were UAS-tkvQD 1.22×10^7, UAS-tkvQD, UAS-mid−Flag 6.73×10^6 and UAS-tkvQD, UAS-mid−eh1-Flag 1.16×10^7. Statistical analysis used the Tukey’s multiple comparisons test, with bars representing the mean, s.d., and significance indicated, *P-value<0.05 and **P-value<0.01.

DISCUSSION

In this study, we investigated how the ventral selector gene mid antagonizes Dpp signaling. Specifically, we showed that mid, which antagonizes Dpp signaling and pMad accumulation, represses the Dpp-activated gene Dad but has minimal effects on the regulation of the Dpp-repressed gene brk. Furthermore, mid antagonizes dorsal fate by repressing Dad and by inhibiting Dpp signaling induced pMad accumulation via the eh1 domain.

We showed previously that Mid localizes to several Dad enhancers, including the Dad13 enhancer, and represses a Dad enhancer trap in an eh1-dependent manner (Svendsen et al., 2019; unpublished data). Here we show that mid regulates Dad13-driven expression through a TBE site. Ventral Dad13-driven expression is increased and expanded in mid loss-of-function, while ectopic expression of mid blocks Dad13-reporter expression. Mutating the TBE in the Dad13 enhancer fragment increased Dad13 expression levels and expanded the ventral domain of expression compared with controls. Furthermore, the TBE mutation rendered the construct less sensitive to mid gain-of-function. The localization of Mid to the Dad13 enhancer by ChIP (Svendsen et al., 2019) and the genetic results suggesting that the TBE is required for Mid repression of Dad13-expression supports direct repression of Dad by Mid through the TBE.

However, a surprising result in light of this proposal was that the dorsal Dad13TBE-driven expression outside the mid expression domain was also increased compared to Dad13. This suggests that the TBE sequence binds factors in dorsal cells to control the activity of the Dad13 enhancer. Recent work on Tc-omb, the optomotor-blind (omb) homolog in the red flour beetle, Tribolium castaneum, suggests one possible explanation. Like the Drosophila T-box factor omb, Tc-omb is expressed in the dorsal region of developing beetle legs and is required for dorsal patterning. Loss of function of dorsal Tc-omb results in increased pSmad levels in dorsal cells (Pechmann and Prpic, 2022). This is analogous to the increase in ventral pMad levels we find in mid H15 loss of function (Svendsen et al., 2019). Although it is not known if Tc-omb loss of function also results in the increase of Dpp target genes in T. castaneum, it is interesting to speculate that perhaps dorsal T-boxes like omb play parallel roles to mid and H15, dorsal Dpp signaling and gene expression.

A second finding is that Mid suppresses pMad accumulation in a manner that is dependent on the eh1 domain. Mid mutants in which eh1 is compromised are unable to suppress Dpp gain-of-function effects. When co-expressed with Dpp-signaling overexpression in imaginal discs, clones of UAS-mid−eh1-Flag maintain strong pMad staining and adult cuticles display outgrowths and deformities that resemble the effects of Dpp-signaling overexpression alone. Conversely, clones activated for Dpp signaling that express wild-type mid (UAS-mid−Flag) have decreased pMad levels in imaginal discs, while adult legs have fewer and milder defects. This demonstrates that in addition to blocking Dpp-target genes such as Dad, mid represses genes that act to increase pMad levels and Dpp signaling. What these repressed target genes may be remains a subject for further study.
Our results showing that brk expression was not affected by mid were surprising to us because brk is a sensitive readout of Dpp signaling and because mid alters PMad levels. However, mid loss-of-function clones, which increase PMad levels, do not affect brk expression, even though brk is regulated by Dpp-signaling in ventral mid-expressing cells. It seems likely that brk is simply not sensitive to the modulations in PMad seen in mid/H15 mutant clones. Additionally, mid expression was unable to substantially reverse the repression of brk by Dpp gain-of-function, despite mid dramatically decreasing PMad accumulation in this genetic background, suggesting that brk repression is sensitive to repression at low thresholds of Dpp signaling. This suggests that, overall, mid and H15 do not contribute to the regulation of brk.

The lack of interaction between mid and brk in this study is consistent with previous work, where it was reported that the expression of the ventral genes H15 and wg, and the dorsal genes omb and dpp, were normal in brk mutant leg discs, indicating that brk does not participate in the formation of the DV axis (Estella and Mann, 2008). Instead, brk functions to form the proximo-distal (PD) axis by antagonizing the Wg target genes Distalless (Dll) and dachshund (dac), helping to establish their expression in the PD axis. This leads to a model in which Dpp induces the PD and DV leg axes through two separate modes: Dpp inhibits brk repression of Wg targets to establish the PD axis and antagonizes Wg targets in ventral development through pMad/Med/Shn mediated repression (Estella and Mann, 2008). Thus, mid and brk may play parallel roles repressing Dpp-target genes in the D/V and P/D axes of the leg imaginal disc, respectively.

MATERIALS AND METHODS

Fly stocks and constructs
All flies were maintained on standard media containing cornmeal, agar, yeast, glucose, and water (Deliu et al., 2017) and housed at between 18°C-25°C. Stocks UAS-devOP, brk-lacZ, and Dad-lacZ were obtained from Bloomington Indiana Stock Center. brk-lacZ (BM315) was a gift from Dr. Konrad Basler, University of Zurich (Müller et al., 2003). H15X4 mid1, Bloomington Indiana Stock Center. UAS midV5, (Svendsen et al., 2009) and UAS-mid2.12 (Buescher et al., 2004) were generated previously.

UAS-mid strains
In this study, mid* may refer to either UAS-mid2 or UAS-mid2.12 and both lines have very similar levels of mid* activity. For quantitative comparisons of mid* and mutant mid*j, previously generated strains with Flag-tagged constructs, UAS-mid*-Flag and UAS-mid*-Flag, were used (Formaz-Preston et al., 2012). The relative expression levels of these lines are such that mid*j is expressed at roughly two-fold higher levels than mid* (Formaz-Preston et al., 2012; Svendsen et al., 2019). UAS-mid* strains have generally stronger effects than UAS-mid*-Flag in gain-of-function experiments, although both UAS-mid* and UAS-mid*-Flag are able to rescue mid H15 loss-of-function (Formaz-Preston et al., 2012; Svendsen et al., 2019).

Dad13 reporter strains
Dad13 constructs (Dad13, Dad13TBE, Dad13SBE, and Dad13SBE-TBE) were generated for this study using the pGL3basic-lsp70-dad13 (331) plasmid gifted by Dr. Giorgos Pyrowolakis, University of Freiburg, and mutations were made with an adapted splice protocol (Warrens et al., 1997) in a lcaZ-2, attB vector. All transgenes were inserted into P[CaryP]attP2 located at 68A4. The Dad13nRFP strain was a gift from Dr. Doug Allan, University of British Columbia.

Loss-of-function and gain-of-function genetic mosaics
Heat shocking larvae 48-72 h after egg laying activates heat-shock-inducible hs-FLP, which is implemented in both gain-of-function and
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