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Molecular Integration of Wingless, Decapentaplegic, and Autoregulatory Inputs into *Distalless* during *Drosophila* Leg Development

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SUMMARY

The development of the *Drosophila* leg requires both Decapentaplegic (Dpp) and Wingless (Wg), two signals that establish the proximo-distal (PD) axis by activating target genes such as *Distalless* (*Dll*). *Dll* expression in the leg depends on a Dpp- and Wg-dependent phase and a maintenance phase that is independent of these signals. Here, we show that accurate *Dll* expression in the leg results from the synergistic interaction between two *cis*-regulatory elements. The Leg Trigger (LT) element directly integrates Wg and Dpp inputs and is only active in cells receiving high levels of both signals. The Maintenance (M) element is able to maintain Wg- and Dpp-independent expression, but only when in *cis* to LT. M, which includes the native *Dll* promoter, functions as an autoregulatory element by directly binding Dll. The "trigger-maintenance" model describes a mechanism by which secreted morphogens act combinatorially to induce the stable expression of target genes.

INTRODUCTION

Drosophila leg development requires the elaboration and coordination of three body plan axes, anteroposterior (AP), dorsoventral (DV), and proximodistal (PD). The process of leg development begins during embryogenesis, when a small number of cells in each thoracic hemisegment are specified to become the leg imaginal disc. Once formed, the leg disc is comprised mainly of a single sheet of epithelial cells, which continue to proliferate during larval development (reviewed by Cohen, 1993). Both DV and PD information in the leg disc is derived from two secreted morphogens, Wg and Dpp. Wg, expressed ventrally, and Dpp, expressed dorsally, function combinatorially to create the leg's PD axis (Campbell et al., 1993; Diaz-Benjumea et al., 1994). Genetic experiments suggest that these signals are not only required to initiate PD axis formation, but that different levels of Wg and Dpp are responsible for creating different fates along the PD axis (Lecuit and Cohen, 1997). Moreover, for both the initiation and specification of PD fates, both signals are required; neither the Wg nor the Dpp pathways are sufficient, even when maximally activated (Abu-Shaar and Mann, 1998; Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997). Genetic experiments also demonstrate that the requirement for Wg and Dpp activities is transient; by ~72 hr of development, Wg and Dpp are no longer required to generate a complete PD axis (Diaz-Benjumea et al., 1994; Galindo et al., 2002). Although these results are well supported by in vivo genetic experiments, we currently have very little understanding of the underlying molecular mechanisms by which the leg's PD axis is established by Wg and Dpp.

Two targets of Wg and Dpp in the leg, *Distalless* (*Dll*) and *dachshund* (*dac*), serve as markers for different PD fates (Diaz-Benjumea et al., 1994; Mardon et al., 1994). *Dll* is activated by high levels of Wg plus Dpp signaling and, consequently, is expressed in distal regions of the leg. In contrast, *dac* is activated by lower levels of these two signals and is expressed in medial positions along the PD axis (Lecuit and Cohen, 1997). As transcriptional regulatory elements controlling *Dll* or *dac* in the leg disc have not been described, it is not known if Wg and Dpp directly regulate these genes during leg development. In fact, somewhat paradoxically, *Dll* expression in the leg disc responds to Wg and Dpp differently than it does in the embryonic leg primordia, where *Dll* is activated by Wg but repressed by Dpp (Cohen, 1990; Cohen et al., 1993; Goto and Hayashi, 1997). One scenario that would account for this difference, and that is supported by our results, is that *Dll* expression is governed by a different set of *cis*-regulatory elements in the leg disc and embryo. Consistent with this idea, the best-characterized *Dll* regulatory element, Dll304, is active only early in embryogenesis, when *Dll* is first expressed in the leg primordia (Vachon et al., 1992), but is not active in the leg disc (our unpublished data). Alternatively, it is plausible that Wg and Dpp indirectly control *Dll* expression in the imaginal disc. Further, once activated by these signals, *Dll* expression is maintained by an unknown mechanism.

To gain further insights into the control of PD target-gene expression by Wg and Dpp, we have characterized *Dll cis*-regulatory elements that are active in the leg disc. One element, which we call the Leg Trigger (LT), is active only in response to high levels of Wg plus Dpp. Consequently, an *LT-lacZ* reporter gene is expressed in a small subset of *Dll*-expressing cells in the center of the leg disc, where the Wg and Dpp expression domains

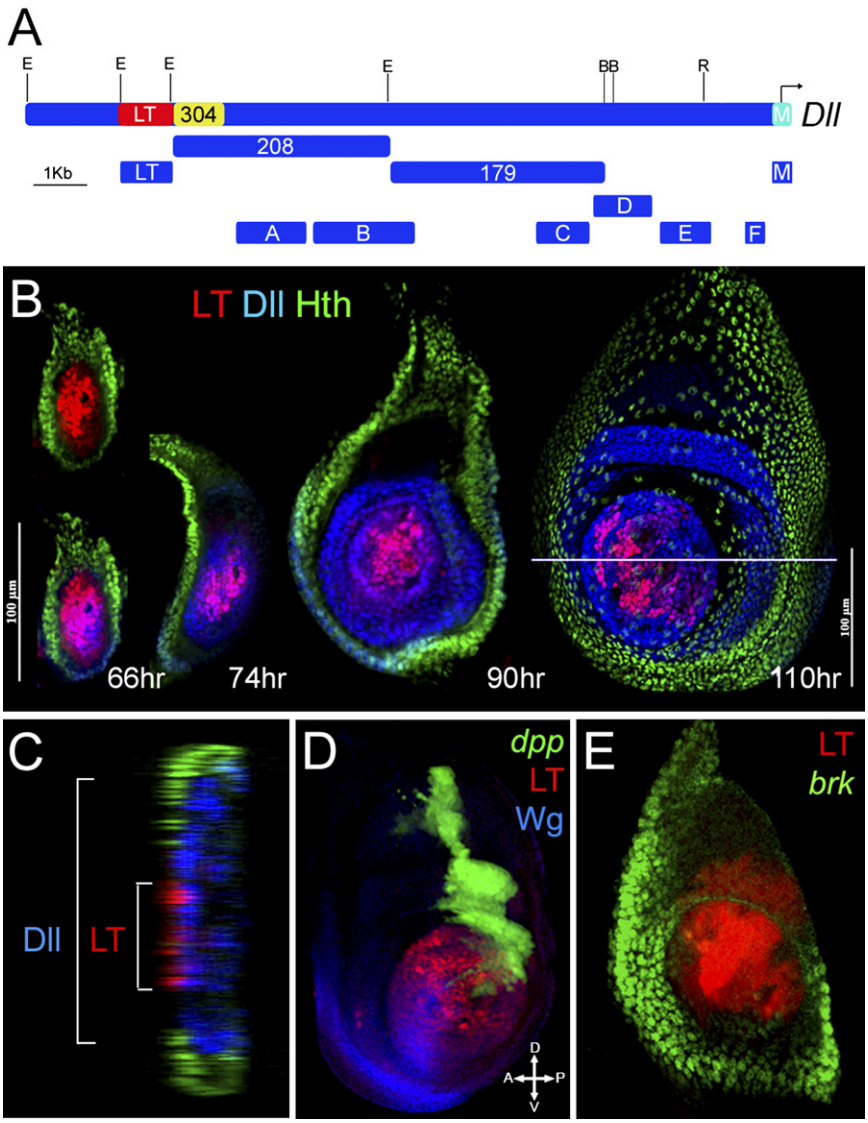

Figure 1. The LT Enhancer

(A) The *Dll* 5′ *cis*-regulatory region. DNA fragments were cloned based on sequence conservation to other *Drosophilids* and assayed in transgenic reporter genes for expression in imaginal discs. E, EcoR1; B, BamH1; and R, RsrII. LT is in red, 304 in yellow, and M in light blue. Of the fragments tested in a standard reporter gene (using the minimal promoter from the *hsp43* gene), only LT drove expression in discs. Although fragments LT (previously 215), 304, 208, and 179 were originally cloned by Vachon et al. (1992), no imaginal disc expression was reported.

(B) Wild-type leg discs at various stages of development stained for *LT-lacZ* (red), Dll (blue), and Homothorax (Hth) (green). The age of the larvae (±6 hr) is indicated below each disc. Early in development (66 hr ± 6 hr or before), LT was active in all the cells that express *Dll*. Later in development, LT was active in a subset of *Dll*-expressing cells.

(C) Cross-section image of the 110 hr leg disc from (B). LT was only active in a subset of *Dll*-expressing cells.

(D) Wild-type third instar leg disc stained for *LT-lacZ* (red), *dpp-Gal4; UAS-GFP* (green), and Wg (blue). LT was active in the center of the disc where the Wg and Dpp expression domains meet.

(E) Wild-type early third instar leg disc (~96 hr AEL) stained for *LT-Gal4; UAS-GFP* (red), and *brk-lacZ* (green). LT was active in cells that have no or very low Brk levels.

abut. We also describe a second element, called Maintenance (M), which includes the *Dll* promoter. Although M on its own is only weakly active in leg discs, it is capable of synergizing with LT to produce accurate and robust *Dll*-like expression. Consistent with genetic analyses, LT directly integrates positive inputs from Wg and Dpp by binding the signal-activated transcription factors Tcf and Mothers against Dpp (Mad), respectively. LT also directly integrates negative input from the Dpp pathway by binding Brinker (Brk), a transcription factor known to repress Dpp target genes in other contexts (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999). Further, we show that M requires direct binding by Dll for full activity, suggesting that maintenance depends in part on an autoregulatory mechanism. Thus, *Dll* expression in the leg disc is controlled in a two-step manner by separable "trigger" and "maintenance" *cis*-regulatory elements that cooperate with each other to integrate Wg and Dpp inputs during an early phase and Dll input during a maintenance phase.

RESULTS

Identification of a *Dll* Element that Integrates Wg and Dpp Signaling

We used a transgenic reporter gene assay to search for *Dll cis*-regulatory elements that were active in the leg disc. Altogether, we scanned ~14 kb 5′ to the *Dll* transcription initiation site (Figure 1A). This 14 kb is able to fully reproduce the complete Dll expression pattern in the embryo and imaginal discs except for the wing disc (data not shown). From these experiments, we identified a ~1 kb fragment located ~12 kb 5′ of the *Dll* transcription initiation site, which we named the LT element (Figure 1A). The LT element drove high levels of reporter gene (*LT-lacZ*) expression in a subset of the *Dll* domain in third instar ventral (leg, antennal, and genital) discs, but was not active in dorsal (wing and haltere) imaginal discs (Figure 1 and see Figure S1 in the Supplemental Data available with this article online). LT was the only element within this 14 kb that, when cloned into a standard reporter gene (with a heterologous, minimal promoter; see Experimental Procedures), drove strong expression in leg or antennal discs (Figure 1B and data not shown).

Early in larval development (prior to ~72 hr after egg laying [AEL]), LT drove expression in all *Dll*-expressing cells of the leg disc (Figure 1B). This time approximately coincides with the time when *Dll* is dependent on Wg and Dpp. As the leg disc continues to grow, *Dll* becomes independent of Wg and Dpp, and its

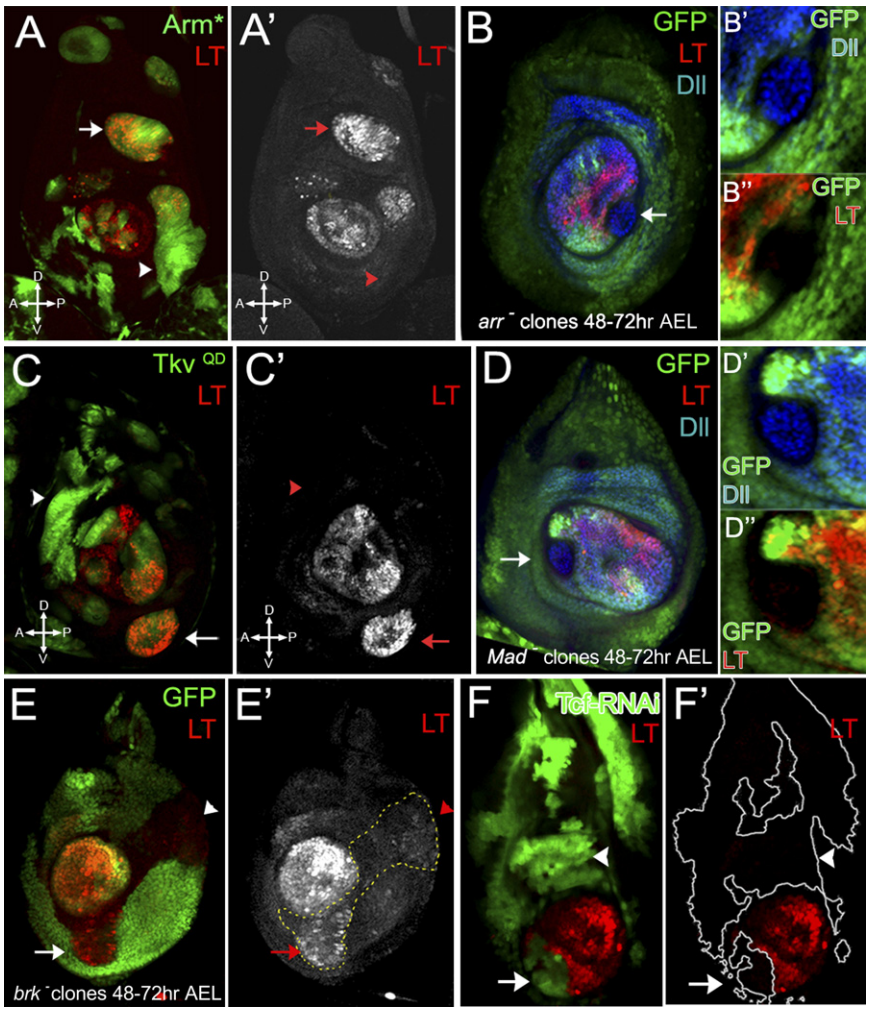

Figure 2. LT Continuously Requires the Wg and Dpp Pathways

(A and A′) Clones expressing Arm* marked by GFP (green) activated *LT-lacZ* (red) in dorsal (arrow) but not ventral (arrowhead) regions of the disc.

(B–B″) An *arr*⁻ clone induced between 48–72 hr after egg laying (hr AEL), marked by absence of GFP (green), had no *LT-lacZ* expression (red) but maintained *Dll* expression (blue).

(C and C′) Clones expressing Tkv^{QD} marked by GFP (green) activated *LT-lacZ* (red) in ventral (arrow) but not dorsal (arrowhead) regions of disc.

(D–D″) A *Mad*⁻ clone induced between 48–72 hr AEL, marked by absence of GFP (green), had no *LT-lacZ* expression (red) but maintained *Dll* expression (blue).

(E and E′) A *brk*⁻ clone induced 48–72 hr AEL, marked by absence of GFP (green), derepressed *LT-lacZ* (red) in the ventral disc, close to the source of Wg (arrow), but not in dorsal regions (arrowhead).

(F and F′) Clones expressing Tcf-RNAi, marked by GFP (green), did not derepress *LT-lacZ* expression (red) in dorsal (arrowhead) or lateral regions of the leg disc. *LT-lacZ* was not expressed in Tcf-RNAi-expressing cells (arrow).

expression expands beyond the cells in which LT was active (Figures 1B and 1C). In a mature third instar leg disc (~110 hr AEL), *Dll* expression covered the future distal leg (tibia and tarsi), whereas LT was active only at the distal tip, close to where the Wg and Dpp expression domains meet (Figures 1B–1D and Figure S2). Notably, these cells also had little or no expression of the Dpp pathway repressor, Brk, which is expressed in lateral and ventral regions of the leg disc (Figure 1E). Taken together, these results suggest that LT is only active in cells that receive both Wg and Dpp inputs.

LT Responds Continuously to Wg and Dpp Inputs

To test the idea that LT integrates Wg and Dpp inputs, we generated clones of cells expressing either an activated form of the β-catenin homolog Armadillo (Arm*) or an activated form of the Dpp receptor Thickveins (Tkv^{QD}), respectively. Activation of the Wg pathway using Arm* resulted in the cell-autonomous expression of *LT-lacZ*, but only in dorsal regions of the leg disc, where high levels of endogenous Dpp are present (Figure 2A). Likewise, activation of the Dpp pathway by Tkv^{QD} resulted in the cell-autonomous expression of *LT-lacZ*, but only in ventral regions of the leg disc, where high levels of endogenous Wg are present (Figure 2C). Thus, as for *Dll*, LT is activated only when both signaling pathways converge. Consistently, coexpression of Arm* and Tkv^{QD} resulted in *LT-lacZ* activation in both ventral and dorsal clones (Figure S3).

To test for the necessity of Wg and Dpp inputs for *LT-lacZ* expression, we generated by mitotic recombination clones of cells that were unable to transduce the Wg or Dpp signals. *LT-lacZ* expression was lost in clones mutant for the Wg coreceptor *arrow* (*arr*) or mutant for the Dpp pathway transcriptional effector *Mad* (Figures 2B and 2D). No effect on *Dll* expression was observed, because *Dll* was independent of these signals at the time these clones were generated (48 to 72 hr AEL or later) (Figures 2B and 2D). These results confirm that LT continuously requires the combined inputs of Wg and Dpp to be active, while *Dll* becomes independent of these signals by the third instar.

Due to its role in repressing Dpp target genes in *Drosophila* wing development, we next examined the role of *brk* in the control of *LT-lacZ* expression. *brk*⁻ null clones located close to the source of Wg in the ventral region of the leg disc were able to derepress *LT-lacZ* (Figure 2E), suggesting that Brk is normally a repressor of LT activity. However, the level of *LT-lacZ* derepression in *brk*⁻ clones was significantly weaker than the amount of expression seen in Tkv^{QD}-expressing clones (compare Figures 2C and 2E). As *brk* is repressed by Dpp signaling in the leg as in the wing (Figure S4), these observations suggest that to activate *LT-lacZ*, Dpp signaling does more than repress *brk*. One possibility is that, in addition to repressing *brk*, Dpp signaling is working through Mad to activate *LT-lacZ*. Consistently, *LT-lacZ* is not expressed in *Mad*⁻*; brk*⁻ double mutant clones (Figures S5A and S5B). These results suggest that Dpp signaling

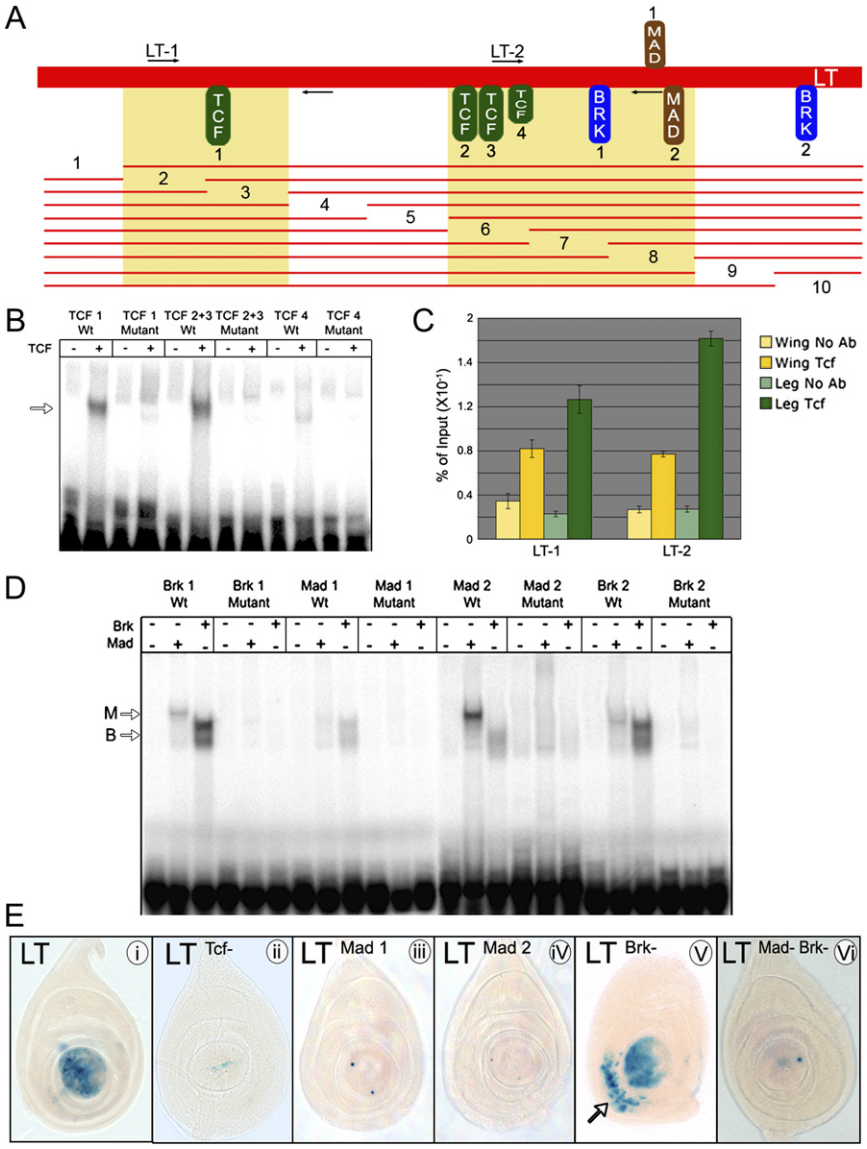

Figure 3. The Regulation of LT by the Dpp and Wg Pathways Is Direct

(A) Diagram of LT with transcription-factor-binding sites. Oval size indicates the relative affinities of these binding sites in EMSAs; ovals above and below the line indicate different binding site orientations. All of these binding sites, except for Brk2, are well conserved through *D. virilis*. The thin red lines summarize the results of a set of ~100 bp deletions tested in reporter genes. Regions shaded in yellow indicate deletions that had reduced or no reporter activity. Except for #2 and #7, all deletions that had an effect removed a Tcf- or Mad-binding site. Deletions #2 and #7 indicate that other inputs besides the mapped Tcf- and Mad-binding sites are required for LT activity.

(B) EMSAs showing binding of Tcf to probes containing wild-type or mutant binding sites (see Experimental Procedures for sequences). Arrows indicate protein-DNA complexes.

(C) ChIP experiments demonstrating specific binding of Tcf to LT in imaginal discs. Anti-Tcf antibodies pulled down LT ~twice as efficiently from leg discs as from wing plus haltere discs. Similar results were seen for two independent PCR fragments, LT-1 and LT-2 (whose positions in LT are indicated in [A]). Each column shows the averages and standard error of the mean for four independent IPs and real-time PCRs.

(D) EMSAs showing binding of Mad and Brk to probes containing wild-type or mutant binding sites (see Experimental Procedures for sequences). Arrows indicate protein-DNA complexes; M, Mad; B, Brk. Although Mad1 has a lower affinity for Mad than Mad2, its binding is sequence specific.

(E) X-Gal stains of leg discs from flies containing wild-type *LT-lacZ* (i), LT^{Tcf-}-*lacZ* (with all Tcf sites mutated; [ii]), LT^{Mad1-}-*lacZ* (with the Mad1 site mutated; [iii]), LT^{Mad2-}-*lacZ* (with the Mad2 site mutated; [iv]), LT^{Brk-}-*lacZ* (with both Brk sites mutated; [v]), and $LT^{Mad-Brk-}$-*lacZ* (with both Mad and Brk sites mutated; [vi]). Mutation of the Tcf sites or either Mad site resulted in loss of activity. Mutation of both Brk sites resulted in the ventral expansion of expression (arrow). The LT^{Brk-}-*lacZ* disc shown here has an intermediate amount of derepression; other transformant lines show stronger and more uniform ventral expression of *lacZ*. Mutation of both Mad and Brk sites resulted in no expression.

is activating LT both via repressing *brk* and activating Mad. (See Estella and Mann [2008] for a complete description of *brk*'s role in leg development.)

Analogous to the role that Brk plays in Dpp signaling, Wg pathway components, in particular the effector transcription factor Tcf, have the potential to repress Wg target genes in the absence of pathway activation (Cavallo et al., 1998). Accordingly, Tcf could potentially be a *Dll* repressor in the dorsal leg disc, away from the source of Wg. To test this idea, we generated clones of cells expressing a Tcf hairpin construct to induce RNAi and knockdown Tcf levels. In the center of the leg disc, Tcf RNAi clones eliminated *LT-lacZ* expression, demonstrating the efficacy of the Tcf RNAi and confirming the requirement for Wg input for LT activity (Figure 2F). However, we failed to observe any *LT-lacZ* derepression in Tcf RNAi clones in the dorsal or lateral regions of the leg disc (Figure 2F). This experiment suggests that Tcf is not a repressor of LT and, therefore, that Wg input into LT is not mediated by derepression. Instead, these results suggest that Wg may activate LT directly, a conclusion that is supported below.

The Dpp and Wg Pathways Directly Regulate LT

To understand how Dpp and Wg control LT expression at the molecular level, we generated a series of ~100 bp deletions of LT and searched for putative binding sites for the transcription factors Mad, Brk, and Tcf (Figure 3A). Candidate binding sites were tested for their ability to bind recombinant proteins in electrophoretic mobility shift assays (EMSAs), and sites that bound were mutated to destroy binding (Figures 3B and 3D). To assess the contribution of identified binding sites to LT's activity, each mutant or deleted LT

element was tested for its ability to drive *lacZ* expression in vivo using a standard reporter gene assay. Most of the deletions that resulted in a loss or reduction of LT activity removed either a Mad- or Tcf-binding site (Figure 3A). In all, we discovered four Tcf-binding sites (Figures 3A and 3B). Mutation of each site in isolation had weak or no impact on LT activity; however, simultaneous disruption of all four Tcf sites (LT^{Tcf-}-*lacZ*) resulted in the near elimination of LT activity (Figure 3E). In addition, consistent with the results obtained by inducing Tcf RNAi, none of the reporter genes with mutant Tcf-binding sites showed any derepression, confirming that Tcf is not repressing LT activity in the absence of Wg signaling. We also used chromatin immunoprecipitation (ChIP) to test if Tcf was bound to LT in vivo. Compared to control immunoprecipitations (IPs), an anti-Tcf antibody specifically immunoprecipitated LT DNA from *Drosophila* leg and wing imaginal discs (Figure 3C). Moreover, anti-Tcf immunoprecipitated LT from leg discs, where LT is active, significantly better than it did from wing plus haltere discs, where LT is inactive (Figure 3C). The enrichment of immunoprecipitated DNA from leg compared to wing discs was not observed for two ubiquitously expressed genes (*act5C* and *pyruvate dehydrogenase*; data not shown), suggesting that the tissue specificity of Tcf binding to LT is significant. Thus, consistent with our genetic experiments, these data indicate that the Wg pathway directly activates LT in leg discs by binding Tcf.

We discovered four candidate binding sites for the transcriptional effectors of the Dpp pathway, Mad and Brk (Figure 3A). Previous studies demonstrated that Mad and Brk bind to similar DNA sequences (Kirkpatrick et al., 2001). Consistently, all four of the sites we identified in LT bound to both Mad and Brk, although the relative affinities of these two factors differed from site to site (Figures 3A and 3D). As with the Tcf sites, the contribution of these sites to LT activity was assessed using a *lacZ* reporter gene assay in transgenic flies. Two of the sites, which we named Mad1 and Mad2, were essential for LT activity (Figures 3A and 3E). Mutation of either of these sites in isolation or in combination (LT^{Mad-}-*lacZ*) resulted in the loss of LT activity (Figure 3E and data not shown). In contrast, mutation of the other two sites, which we named Brk1 and Brk2, resulted in the ventral expansion of LT activity (LT^{Brk-}-*lacZ*) (Figure 3E). These results are consistent with the Brk expression pattern and the derepression of *LT-lacZ* seen in brk^- clones described above (Figures 1E and 2E). Mutation of either Brk1 or Brk2 on its own had no effect (data not shown). We also found that LT reporter genes with both Brk and both Mad sites mutated ($LT^{Mad-,Brk-}$-*lacZ*) were not expressed or, in some cases, had very weak expression (Figure 3E). These data suggest that LT directly integrates input from the Dpp pathway in two ways. First, Dpp directly activates LT by binding Mad at two "activator" sites, Mad1 and Mad2. Second, in ventral and lateral cells, LT activity is directly repressed due to Brk binding at two "repressor" sites, Brk1 and Brk2. The requirement for Mad input was further supported by our finding that LT reporter genes with mutant Mad-binding sites were not expressed in brk^- clones (Figure S5C and S5D). We note that the ventral expression of LT^{Brk-}-*lacZ* argues that there is activated Mad in the ventral leg disc. Consistently, although Dpp signal transduction was strongest in the dorsal disc, weaker pathway activation, visualized by anti-PMad immunostaining, was observed in the ventral region of third instar leg discs (data not shown). Moreover, ventral LT^{Brk-}-*lacZ* expression required Dpp signaling as it was lost in tkv^- clones (Figure S5E).

The Dll Promoter Region Maintains LT-Initiated Expression

We have described a *Dll* regulatory element, LT, that accurately recapitulates *Dll* expression, and its dependency on Wg and Dpp, early in leg disc development. Unlike *Dll*, LT continuously requires input from Wg and Dpp and, by the end of larval development, LT is only active in a small subset of *Dll*-expressing cells (Figure 1B). These data suggest that LT contains the information required to respond to Wg and Dpp but is lacking the information required to maintain *Dll* expression. Because promoter regions can play important roles in enhancer activities (e.g., Calhoun et al., 2002), we tested a 300 bp fragment that encompasses the transcription start site of the *Dll* gene for maintenance activity (Figure 1A). When this element, M, was used instead of the minimal promoter from the *hsp43* gene that is in our standard reporter genes, the resulting *LT+M-lacZ* reporter gene accurately reproduced the normal expression pattern of *Dll* at all stages of leg disc development. While LT was active only in the center of the mature leg disc (Figure 4A), the LT+M composite element was active in all cells that express *Dll* (Figure 4C). On its own, the *M-lacZ* reporter gene was expressed very weakly throughout the leg disc, with slightly higher activity in *Dll*-expressing cells (Figure 4B). M contains a functional promoter because, when used with another enhancer (the dpp^{discs} enhancer [Masucci et al., 1990]), a dpp^{discs}-*M-lacZ* reporter gene drove *dpp*-like expression in both wing and leg imaginal discs (data not shown).

To test if *LT+M-lacZ* was, like *Dll*, able to maintain its expression in the absence of continuous Wg and Dpp inputs, we analyzed its expression in clones that cannot transduce these signals. As with *Dll*, and in contrast to *LT-lacZ*, the expression of *LT+M-lacZ* was unaffected in arr^- clones generated between 48 to 72 hr or later (Figure 4E). Likewise, inactivation of the Dpp pathway in Mad^- clones generated at this time also had no effect on *LT+M-lacZ* expression (Figure 4D). Thus, the M element provides the information to maintain LT-initiated expression, even in the absence of continuous inputs from Wg and Dpp.

The M Element Directly Requires Dll Input

One plausible mechanism for *Dll* maintenance is through a positive autoregulatory feedback loop (Castelli-Gair and Akam, 1995). According to this idea, Dll itself may be required for maintenance. Alternatively, *Dll* expression could be maintained via the Trithorax (Trx) and/or Polycomb (Pc) groups of epigenetic regulators (reviewed by Ringrose and Paro, 2004). We found that, when generated during the maintenance phase (i.e., after 72 hr), *trx* clones had no effect on *Dll* expression (data not shown), demonstrating that *Dll* maintenance does not require this function. In contrast, Pc^- or *Sex combs on midlegs* (Scm^-) clones resulted in a loss of *Dll* expression in some regions of the Dll domain (data not shown). These data suggest that PcG functions might be playing a role in *Dll* maintenance. However, because many genes are likely to be derepressed in the absence of these PcG functions, the loss of *Dll* expression observed in these clones may be indirect (see Discussion).

To test if autoregulation contributes to *Dll* maintenance, we generated *Dll* loss-of-function clones during the maintenance

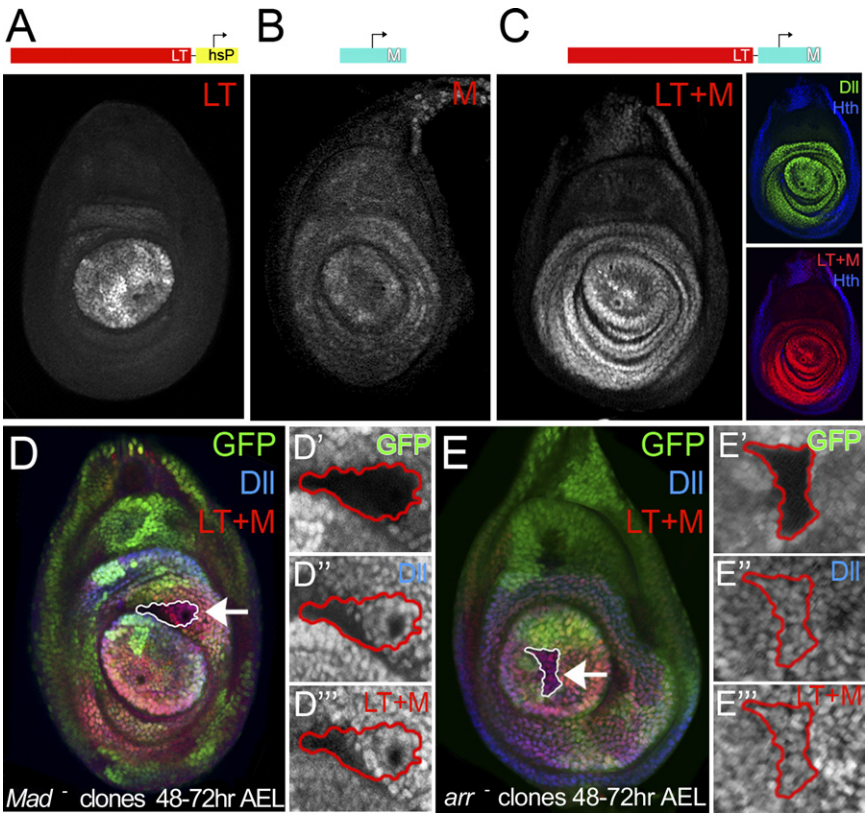

Figure 4. The *Dll* Promoter Region Has Maintenance Activity

(A) *LT-lacZ* was active in subset of *Dll*-expressing cells (with the *hsp43* promoter, hsP).

(B) *M-lacZ* is expressed nearly ubiquitously in leg discs at very low levels, with slightly higher levels in the *Dll* domain. *M-lacZ* is not expressed in wing discs (not shown).

(C) *LT+M-lacZ* was expressed in all cells that express *Dll* in the leg imaginal disc, including low-level expression in the Dll-expressing trochanter ring. *LT+M-lacZ* was not expressed in wing discs (not shown). The inset shows the expression of Dll (green) and *LT+M-lacZ* (red) in combination with Hth (blue).

(D and E) Genetic tests of *LT+M-lacZ* maintenance.

(D–D‴) A *Mad*¯ clone induced between 48–72 hr AEL, marked by absence of GFP, continues to express *LT+M-lacZ* (red) and Dll (blue).

(E) An *arr*¯ clone induced between 48–72 hr AEL, marked by absence of GFP, continues to express *LT+M-lacZ* (red) and Dll (blue). The insets (D′,D″,D‴,E, E″,E‴) show blow-ups of the clones, outlined in red.

phase and examined the effect on *LT+M-lacZ* expression. LT+M activity was eliminated in *Dll* mutant clones (Figure 5A), indicating that *Dll* is essential for its activity. To determine if the requirement for *Dll* is direct, we searched for candidate Dll-binding sites within the M element and tested the ability of wild-type and mutant sequences to bind Dll protein in vitro. Three Dll-binding sites were found in the M element (Figure 5B). Mutating all three of these binding sites together (but not individually) in the context of the *LT+M-lacZ* reporter gene strongly reduced, but did not eliminate, expression (Figure 5C). These data demonstrate that Dll is directly contributing to M's activity but suggest that there are additional inputs, and perhaps additional Dll-binding sites, that contribute to maintenance activity.

The 300 bp M element, as defined above, includes the transcription initiation site for *Dll* as well as 3′ and 5′ flanking sequences. To determine where within this element maintenance activity resides, we characterized additional reporter genes containing M variants. Combining LT with the 3′ half of the M element (including the *Dll* transcription start site; *LT-3′M-lacZ*) resulted in no reporter expression (data not shown). The 3′ fragment of M drove weak expression with the dpp^{discs} enhancer, demonstrating that it contains a functional promoter (data not shown). These data suggest that the 5′ fragment of M is essential for LT-stimulated maintenance of expression. To test for the sufficiency of 5′M, we fused it to the minimal promoter from the *hsp43* gene which, on its own, does not support maintenance (see above). Combining this chimeric fragment with LT (*LT-5′M-hsp-lacZ*) resulted in reporter gene expression that was similar to, though less uniform than, that driven by *LT+M-lacZ* (Figure 5D), suggesting that 5′M provides partial maintenance activity. Mutation of the sole Dll-binding site in 5′M reverted the expression pattern to one that is very similar to that driven by *LT-lacZ* (compare Figure 5E to Figures 5D and 4A), suggesting that this Dll-binding site is important for maintenance. Taken together, these data suggest that Dll directly regulates its own expression through binding sites located close to its own promoter, and this binding contributes to the maintenance activity displayed by the M element.

LT Is Also Required for Maintenance

We have identified two *cis*-regulatory elements that together recapitulate the Wg- and Dpp-dependent and maintenance phases of *Dll* expression during development of the *Drosophila* leg. Significantly, the LT and M elements synergize with each other to produce accurate and robust expression; neither element, on its own, is capable of generating a strong *Dll*-like expression pattern. One question that emerges from these experiments is how LT synergizes with M to elicit maintenance. One possibility is that LT transiently interacts with M and changes its properties so that it can function as a robust autoregulatory element. Alternatively, LT may have to continuously work with M to confer maintenance activity. To distinguish between these scenarios, we created a LT+M reporter gene in which LT was flanked by FRT sequences (*>LT > M-lacZ*), allowing us to delete LT at various times during development using Flp-mediated recombination (Figure 6A) (Struhl and Basler, 1993). As expected, deletion of LT during the Wg- and Dpp-dependent stage (prior to 48 hr AEL) resulted in no reporter expression (Figure 6C). Interestingly, deletion of LT during the maintenance stage (after 72 hr AEL) also caused loss of reporter gene expression

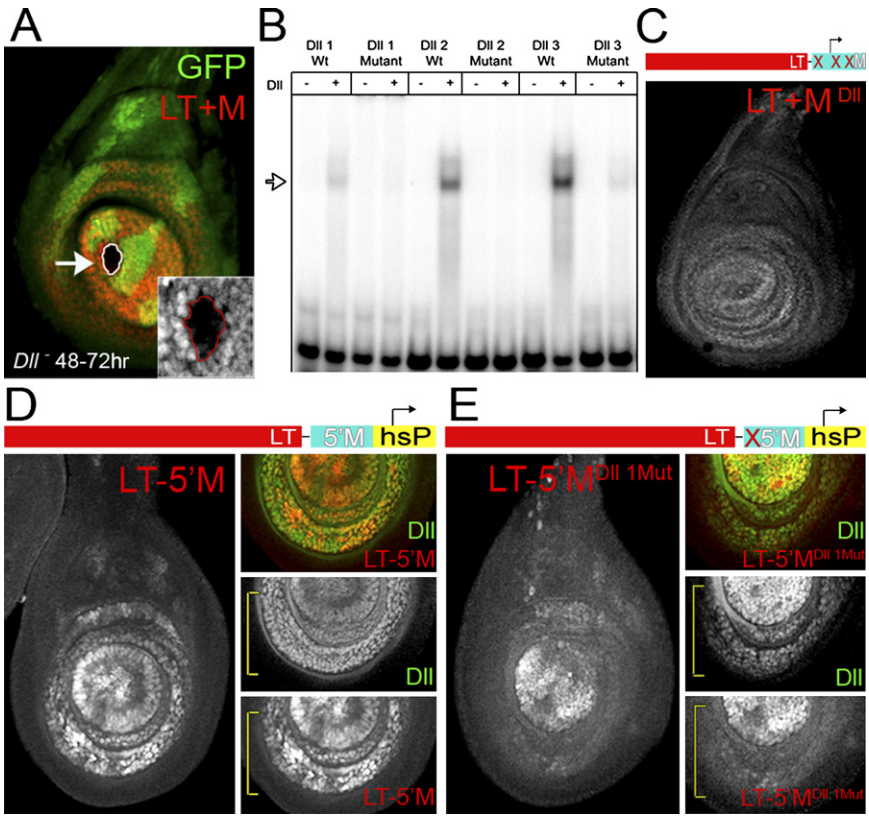

Figure 5. Dll Is Required for *Dll* Maintenance

(A) A *Dll*⁻ clone induced between 48–72 hr AEL, marked by absence of GFP, resulted in loss of *LT+M-lacZ* expression (red). The inset shows the clone outlined in red and the cell autonomous loss of *LT+M-lacZ* expression.

(B) EMSAs showing Dll binding to each of the three Dll sites in the M element (WT and mutant; see Experimental Procedures for sequences). The arrow indicates the Dll-induced complexes.

(C) The expression of a LT+M reporter gene with all three Dll-binding sites mutated (*LT+M^{Dll-}-lacZ*) was only weakly expressed in *Dll*-expressing cells of the leg disc.

(D) Expression driven by the *LT-5′M-hsP-lacZ* reporter gene. The level and pattern of expression indicates that the 5′M fragment confers partial maintenance activity. The insets and brackets compare β-gal and Dll expression.

(E) When the Dll1-binding site is mutated in this reporter (*LT-5′M^{Dll1-}-hsp-lacZ*), expression resembles that driven by *LT-lacZ* (compare with Figure 4A).

(Figure 6D). These data suggest that LT is continuously required for maintenance and, therefore, *LT+M-lacZ* expression.

DISCUSSION

Molecular Logic of *Dll* Expression during Leg Development

We have provided evidence that *Dll* expression during *Drosophila* leg development is controlled by separate, synergistically interacting *cis*-regulatory elements. The first element, LT, activates transcription only in response to high levels of Wg and Dpp signaling. The second element, M, includes the *Dll* promoter and has the ability to activate transcription in a Wg- and Dpp-independent manner, but only when in *cis* to LT. Together, these results fit well with previous genetic experiments showing that the Wg and Dpp inputs into *Dll* are only required transiently, prior to ~60 hr AEL (Galindo et al., 2002; Lecuit and Cohen, 1997). Based on our data, we hypothesize that LT, and perhaps other elements with similar properties, is responsible for activating the Wg- and Dpp-dependent phase of *Dll* expression. Further, our data suggest that the combination of LT+M executes the Wg- and Dpp-independent phase of *Dll* expression. The existence of a two-component *cis*-regulatory system for *Dll* expression has several interesting implications and provides a mechanistic understanding of how Wg, Dpp, and Dll inputs are integrated into *Dll* expression.

Signal Integration into *Dll*

The requirement for multiple inputs for gene activation is a common theme in transcriptional regulation (reviewed by Arnosti, 2003; Barolo and Posakony, 2002; Mann and Carroll, 2002). Enhancer elements can be thought of as "logic integrators" that are only active in the presence of the correct activators and in the absence of repressors (Istrail and Davidson, 2005). The LT element defined here behaves as such a logic integrator. To be active, at least three conditions must be met. First, LT must be bound to a transcriptionally active form of Tcf, a condition which indicates high levels of Wg signaling. Second, LT must be bound to a transcriptionally active form of Mad, and, third, LT must not be bound to Brk. The second and third of these three conditions both indicate high levels of Dpp signaling. This combination of inputs ensures that LT is only triggered where Wg and Dpp signaling are both active. In addition, we hypothesize that there must be another input that restricts LT's activity to the ventral discs (e.g., it is not active in other tissues where Wg and Dpp signaling intersect such as the wing disc). Such a ventral-specific input could be *Dll* itself, which is expressed before LT is active via the *Dll304* enhancer (Castelli-Gair and Akam, 1995), and/or another ventral-specific factor such as *buttonhead* (*btd*), which is also required for *Dll* expression (Estella et al., 2003). Consistent with this idea, *LT-lacZ* is lost in *Dll*⁻ clones and in *Dll* hypomorphic discs, suggesting that *Dll* input, in addition to Wg and Dpp, is required for its activity (data not shown).

As noted above, Dpp signaling uses two mechanisms (Mad binding and absence of Brk) to control LT's activity. Because Brk, a transcriptional repressor, binds directly to LT, it restricts the domain in which Wg signaling can activate this element. This conclusion is best supported by the expression pattern of the LT reporter gene in which the Brk-binding sites were mutated. Specifically, the expression of this reporter (*LT^{Brk-}-lacZ*) was expanded ventrally, indicating its potential to be activated more broadly by Wg signaling in the absence of this repressor. Thus, we suggest that the primary role of Brk is to provide spatial

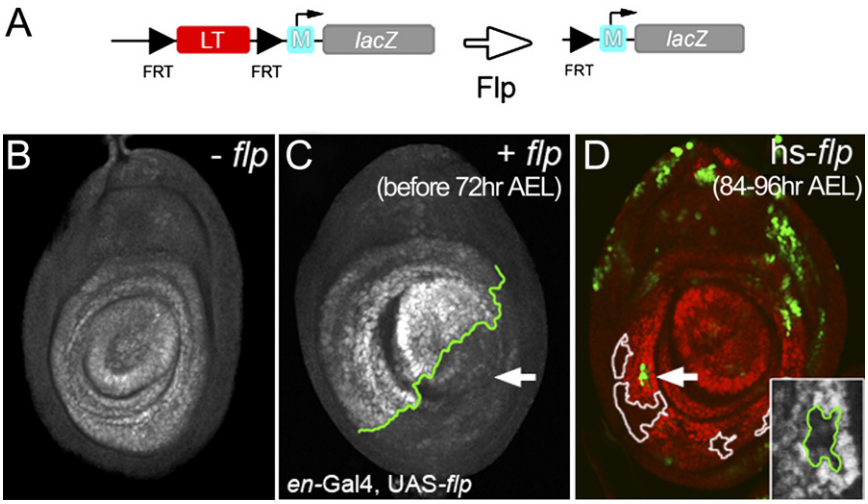

Figure 6. LT Is Required for Maintenance

(A) Diagram of a LT+M reporter gene in which LT is flanked by FRT sites (black triangles). After expression of Flp, LT is deleted, leaving a single FRT site and the M element.

(B) In the absence of Flp, > *LT* > *M-lacZ* generated a *Dll*-like expression pattern. This disc came from a larva of the same genotype as the one shown in (D) (*hs-flp122*; > *LT* > *M-lacZ*), but was not given a heat shock.

(C) Deletion of the LT enhancer in the posterior compartment (green) early in development (prior to maintenance) using *en-Gal4*, *UAS-flp* resulted in the loss of *lacZ* expression in that compartment, while leaving expression in the anterior compartment intact.

(D) Heat shock-induced expression of Flp during the maintenance stage of *Dll* expression resulted in the loss of reporter expression within the Dll domain. Due to the design of this experiment (see Experimental Procedures) only a subset of these heat-shock-induced events were marked by GFP+; other, unmarked events are outlined. The inset shows a blow-up of the GFP-marked clone. In this experiment, Flp was provided 90 ± 6 hr AEL via a 8 min heat shock, significantly after maintenance begins.

information to LT activation. The absence of Brk, however, is apparently not sufficient for LT activation; Mad input into LT appears also to be essential. Several experiments support this conclusion. Most informatively, *LT-lacZ* was not expressed in *Mad⁻; brk⁻* clones, and *LT-lacZ* reporter genes with either Mad site mutated were not expressed in *brk⁻* clones. Thus, even in the absence of Brk, LT requires Mad input. We suggest that in contrast to providing spatial information, the Mad input into LT is important for boosting the level of its activation, together with Tcf, by providing an additional potent transcriptional activator. Further, LT is unlikely to be the only *Dll cis*-regulatory element that integrates Wg plus Dpp signaling during leg development. Although LT was the only fragment within the 14 kb of 5′ DNA that drove strong expression in the leg disc in a standard reporter gene assay, thus allowing the dissection of Wg and Dpp signal integration, we identified a second fragment that was able to synergize with M to produce a *Dll*-like expression pattern (Figure S6). In summary, these data suggest that during the Wg- and Dpp-dependent stage, *Dll* expression is regulated by the direct binding of Tcf, Mad, and Brk to LT and, perhaps, additional regulatory elements (Figure 7).

Models of Maintenance

As is the case for *Dll*, there are examples of other genes that have separable initiation and maintenance phases of expression. For many of these examples, expression is maintained by the trxG

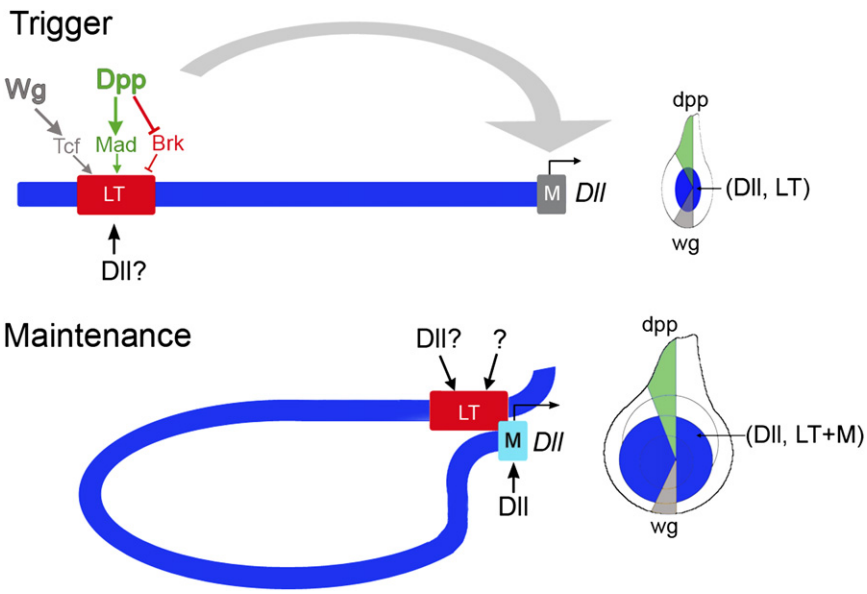

Figure 7. The Trigger-Maintenance Model

LT drives *Dll* expression early in larval development by directly integrating inputs from the Wg and Dpp signaling pathways. Tcf and Mad bind LT to activate, while Brk binds LT to repress, resulting in LT activity in the center of the young leg disc. *Dll* is also required for LT activity, although it is not known if this input is direct. We also suggest that other elements within the *Dll* locus may act redundantly with LT to integrate the Wg and Dpp signals (not indicated). As the disc grows, *Dll* becomes independent of Wg and Dpp signaling. During the maintenance phase, the composite LT+M element behaves as an autoregulatory element as it is directly activated by Dll binding to sequences close to the *Dll* promoter (M). Dll input into LT may also contribute to maintenance, as well as other currently unknown factors. Consistent with this model, a lineage-tracing experiment using *LT* demonstrates that all *Dll*-expressing cells in a third instar leg disc are derived from *LT*-expressing cells (McKay et al., unpublished data).

and PcG of epigenetic regulators (reviewed by Breiling et al., 2007; Brock and Fisher, 2005). There are also examples of genes that require enhancer-promoter communication for maintenance. For example, a regulatory element from the *Hoxb4* gene requires sequences from its own promoter for stable expression in the mouse hindbrain (Gilthorpe et al., 2002). In this case, a key input into the promoter-proximal sequences is the PcG protein, YY1. We find that *Dll* expression is unaffected in *trx* mutant clones but is lost in a subset of Pc^- and Scm^- clones, raising the possibility that PcG functions play a role in maintenance. However, PcG functions are more typically associated with maintaining genes in a repressed state, not an expressed state. Moreover, because of PcG's widespread role in gene silencing, many genes are likely to be derepressed in these clones. In fact, the Hox gene *Abd-B* is derepressed in these clones, and Abd-B has the ability to repress *Dll* (our unpublished data; Estrada and Sanchez-Herrero, 2001). Thus, on balance, it seems more likely that the loss of *Dll* expression observed in some Pc^- clones is an indirect effect. In contrast, our results strongly argue that positive autoregulation, by direct binding of Dll to the M element, plays an important role in *Dll* maintenance (Figure 7).

One conclusion we can draw from our observation that both LT and M are required for maintenance is that LT requires the *Dll* promoter to be fully active. Such promoter-specific enhancer activation has been observed previously and is generally thought to be important for remote enhancers to stimulate transcription from the correct promoter in gene-dense regions of the genome (e.g., Butler and Kadonaga, 2002; Calhoun et al., 2002; Li and Noll, 1994; Merli et al., 1996). The LT+M synergy described here is distinct from these other examples. In this case, although enhancer-promoter compatibility may be part of the reason that LT works better with M (and over large distances), our results show that the combination of the two has properties that are not exhibited by either element on its own. Specifically, while *M-lacZ* is very weakly expressed in leg discs, and *LT-lacZ* requires continuous Wg and Dpp inputs, the combination of LT+M allows *Dll* autoregulation to occur in a Wg- and Dpp-independent manner. Moreover, LT+M is not simply a Dll autoregulatory element: even though *Dll* is expressed in the wing disc, transcriptional activation by LT+M remains restricted to the ventral imaginal discs. This observation implies that the Dll input into LT+M can only occur in cells where LT was activated, which itself only happens in ventral discs. Thus, LT+M is not only a two-component Dll autoregulatory element, but is an autoregulatory element that requires the prior Wg- and Dpp activation of LT.

These observations lead us to suggest two classes of models by which maintenance may occur. In one, an activated LT element changes the chromatin structure of M, for example, by changing the position of a repressive nucleosome so that it can function as an autoregulatory element. According to this model, the continued presence of LT is required to maintain this chromatin structure. A second model that would also accommodate our data is that the combination of LT plus M is required to increase the efficacy of transcriptional activation by, for example, providing additional Dll (or other activator) binding. According to this scenario, LT activation by Wg and Dpp triggers the initial interaction between the LT and M elements, which would then be stabilized in a Wg- and Dpp-independent manner. These models are not mutually exclusive and both can be tested by analyzing the chromatin status at the M and LT elements.

Our results also raise the question of what purpose this two-step trigger-maintenance mechanism may serve. One possibility is that, by having only a transient requirement for Wg and Dpp, these morphogens are available for carrying out completely different tasks, without affecting *Dll* expression. In support of this idea, in addition to working together to create the PD axis, Wg and Dpp function independently to instruct ventral and dorsal leg fates, respectively (Morimura et al., 1996; Struhl and Basler, 1993; Theisen et al., 1996). Some of these late Wg and Dpp patterning functions may also require Dll input. The trigger-maintenance logic described here in principle allows Wg and Dpp to execute functions in collaboration with their own downstream target, *Dll*.

It is also noteworthy that the transient nature of the Wg and Dpp inputs into *Dll* is not the typical way these morphogens regulate their target genes in other tissues. In the *Drosophila* wing, for example, Dpp and Wg are required to continuously activate their targets, such as *vestigial*, *optormotor blind*, and *spalt* (de Celis et al., 1996; Grimm and Pflugfelder, 1996; Kim et al., 1996). One signficant difference between the regulation of wing and leg target genes by these morphogens is that in the wing Wg and Dpp generally act independently, whereas in the leg they act combinatorially to activate PD genes. Specifically, although they are expressed in ventral and dorsal sectors, respectively, Wg and Dpp activate *Dll* and *dac* in circular or nearly circular domains whose centers are located where the Dpp and Wg expression domains touch, in the middle of the leg disc. The trigger-maintenance mechanism defined here avoids the need for target genes such as *Dll* to continuously integrate Wg and Dpp inputs as the disc grows in size, and provides a mechanism to generate circular domains of gene expression using dorsal and ventral morphogen inputs.

EXPERIMENTAL PROCEDURES

Plasmids and Transgenes

Our standard reporter genes were built from the *hs43-nuc-lacZ* vector, which contains the minimal (TATA box) promoter from the *hsp43* gene. The *hsp43* promoter was removed for constructs containing the M element. The LT, A, B, C, D, E, F, and M fragments were selected based on sequence conservation to other *Drosophilids* (Vista Genome Browser) and cloned by PCR (details are available upon request). The 208 and 179 fragments were obtained by EcoR1 and EcoR1 and BamH1 digestion, respectively (Vachon et al., 1992). LT is essentially equivalent to the *Dll215* enhancer, although no larval expression was reported for this enhancer (Vachon et al., 1992). *LT-Gal4* was generated by cloning the LT enhancer into *hs43-Gal4*. Deletions and mutations were introduced in the LT and M elements using PCR and the QuikChange Site Directed Mutagenesis Kit (Stratagene). The *UAS-Tcf RNAi* was generated by cloning the 3′ end of *Drosophila pangolin* into the *pWIZ* vector (Lee and Carthew, 2003); this transgene was used in combination with a *UAS-Tcf RNAi* that was a gift from B. Dickson for maximal effect.

For reporter genes, multiple transformants were surveyed to select lines displaying representative expression patterns. Notably, *M-lacZ* was very sensitive to position effects; however, most lines consistently had very low-level ubiquitous expression, with slightly higher levels in the *Dll* domain. The sensitivity to position effect was eliminated in the presence of LT.

Immunostaining

Imaginal discs were prepared and stained using standard procedures. The primary antibodies used were: rabbit and mouse anti-β-Gal (Cappell and

Promega), mouse anti-Wg (DSHB), guinea pig anti-P-Mad (gift of E. Laufer and T. Jessell), guinea pig anti-Dll (generated by us against full-length protein), rabbit anti-Hth generated against full-length protein.

Protein Purification and EMSAs

GST-Mad MH1+L (Xu et al., 1998), GST-dTCF HMG (Lee and Frasch, 2000), and GST-Brk 1-100 (gift of C. Rushlow) were produced and purified by standard procedures (Amersham-Pharmacia). The full-length *Dll* cDNA was cloned in frame into *pET14b* (Novagen). His-Dll was produced and purified by standard procedures (QIAGEN). Protein concentrations were measured by Bradford assay and confirmed by SDS-PAGE and Coomassie blue analysis. EMSAs were performed as previously described (Gebelein et al., 2004). The amount of protein used in each EMSA was 25 pmol for Brk, 60 pmol for Mad, 40 pmol for dTcf, and 15 pmol for Dll. The sequences for transcription-factor-binding sites are located in the Supplemental Data.

Chromatin Immunoprecipitations

ChIP assays were based on a previously described protocol (Papp and Muller, 2006), with alterations described in the Supplemental Data.

LT Flp-Out Experiment

The > *LT* > *M-lacZ* reporter (FRT sites are indicated by >) was generated by cloning LT into plasmid J33R (Struhl and Basler, 1993); > *LT* > was subsequently cloned into *M-lacZ*. To delete LT prior to the maintenance phase (before 48 hr AEL), we drove Flp in the posterior compartment by crossing > *LT* > *M-lacZ*-containing flies to *en*-Gal4, UAS-flp, UAS-GFP. To delete LT during the maintenance phase, we crossed > *LT* > *M-lacZ* flies to *y w hs FLP122; tub* > *y+* > *Gal4 UAS-GFP; UAS-Flp* and heat shocked at 90 ± 6 hr AEL. In this experiment, some of the clones that lose *LT+M-lacZ* expression will be positively marked by GFP while others will be unmarked.

Fly Genetics

brk^{XA} is a P (lacZ) insertion and is larva lethal (Campbell and Tomlinson, 1999). Mad^{1-2} is a strong hypomorph (Wiersdorff et al., 1996), while brk^{M68} (Jazwinska et al., 1999), Pc^{XT109} (Zirin and Mann, 2004), trx^{E2} (Klymenko and Muller, 2004), Scm^{D2} (Klymenko and Muller, 2004), and tkv^{a12} (Nellen et al., 1994), which were used in the clonal analysis, are considered as nulls.

Other lines used were: *en*-Gal4, UAS-flp, UAS-GFP (gift from Laura Johnston), and *dpp*-Gal4/UAS-GFP (Staehling-Hampton et al., 1994).

For gain-of-function experiments, we used the strain *y w hs FLP122; tub* > *y+* > *Gal4 UAS-GFP* and the following UAS transgenes: UAS-tkv^{QD} (Abu-Shaar and Mann, 1998), UAS-*arm* (delta N) (Pai et al., 1997); UAS-TCF-RNAi (The Vienna *Drosophila* RNAi Center; this line is reported to have no off-target effects). Flip-out clones were originated by heat shocking the larvae for 10 min at 37°C.

For loss-of-function clones, we used the following genotypes: y f36a brkM68 FRT 19A/ ubi-GFP FRT19A; hs FLP, y w hs FLP122; Mad1-2 or tkva12 FRT 40A/ ubi-GFP FRT 40A, y w hs FLP122; FRT 42D arr2/ FRT 42D ubi-GFP, y w hs FLP122; FRT 42D Dllsa1/ FRT 42D ubi-GFP, y w hs FLP122; PcXT109 FRT2A/ ubiGFP y+ FRT2A, y w hs FLP122; FRT 82B ubiGFP/ FRT 82B trxE2, and y w hs FLP122; FRT 82B ubiGFP/ FRT 82B ScmD2.

For double mutant clones for brk and Mad we used the following genotypes: y f^{36a} brk^{M68} FRT 19A/ y w hs FLP122 ubi-GFP FRT19A; Mad^{1-2} FRT 40A/ ubi-GFP FRT 40A.

Supplemental Data

Supplemental Data include 6 figures and Supplemental Experimental Procedures and can be found with this article online at http://www.developmentalcell.com/cgi/content/full/14/1/86/DC1/.

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REFERENCES

Abu-Shaar, M., and Mann, R.S. (1998). Generation of multiple antagonistic domains along the proximodistal axis during *Drosophila* leg development. Development *125*, 3821–3830.

Arnosti, D.N. (2003). Analysis and function of transcriptional regulatory elements: insights from *Drosophila*. Annu. Rev. Entomol. *48*, 579–602.

Barolo, S., and Posakony, J.W. (2002). Three habits of highly effective signaling pathways: principles of transcriptional control by developmental cell signaling. Genes Dev. *16*, 1167–1181.

Breiling, A., Sessa, L., and Orlando, V. (2007). Biology of polycomb and trithorax group proteins. Int. Rev. Cytol. *258*, 83–136.

Brock, H.W., and Fisher, C.L. (2005). Maintenance of gene expression patterns. Dev. Dyn. *232*, 633–655.

Butler, J.E., and Kadonaga, J.T. (2002). The RNA polymerase II core promoter: a key component in the regulation of gene expression. Genes Dev. *16*, 2583–2592.

Calhoun, V.C., Stathopoulos, A., and Levine, M. (2002). Promoter-proximal tethering elements regulate enhancer-promoter specificity in the *Drosophila* Antennapedia complex. Proc. Natl. Acad. Sci. USA *99*, 9243–9247.

Campbell, G., and Tomlinson, A. (1999). Transducing the Dpp morphogen gradient in the wing of *Drosophila*: regulation of Dpp targets by *brinker*. Cell *96*, 553–562.

Campbell, G., Weaver, T., and Tomlinson, A. (1993). Axis specification in the developing *Drosophila* appendage: the role of *wingless*, *decapentaplegic*, and the homeobox gene *aristaless*. Cell *74*, 1113–1123.

Castelli-Gair, J., and Akam, M. (1995). How the Hox gene Ultrabithorax specifies two different segments: the significance of spatial and temporal regulation within metameres. Development *121*, 2973–2982.

Cavallo, R.A., Cox, R.T., Moline, M.M., Roose, J., Polevoy, G.A., Clevers, H., Peifer, M., and Bejsovec, A. (1998). Drosophila Tcf and Groucho interact to repress Wingless signalling activity. Nature *395*, 604–608.

Cohen, B., Simcox, A.A., and Cohen, S.M. (1993). Allocation of the thoracic imaginal primordia in the *Drosophila* embryo. Development *117*, 597–608.

Cohen, S.M. (1990). Specification of limb development in the *Drosophila* embryo by positional cues from segmentation genes. Nature *343*, 173–177.

Cohen, S.M. (1993). Imaginal disc development. In The Development of *Drosophila melanogaster*, M. Bate and A. Martinez Arias, eds. (Cold Spring Harbor, NY: CSH Lab Press), pp. 747–842.

de Celis, J.F., Barrio, R., and Kafatos, F.C. (1996). A gene complex acting downstream of dpp in *Drosophila* wing morphogenesis. Nature *381*, 421–424.

Diaz-Benjumea, F.J., Cohen, B., and Cohen, S.M. (1994). Cell interaction between compartments establishes the proximal-distal axis of *Drosophila* legs. Nature *372*, 175–179.

Estella, C., Rieckhof, G., Calleja, M., and Morata, G. (2003). The role of buttonhead and Sp1 in the development of the ventral imaginal discs of *Drosophila*. Development *130*, 5929–5941.

Estella, C., and Mann, R.S. (2008). Logic of Wg and Dpp induction of distal and medial fates in the *Drosophila* leg. Development *135*, in press.

Estrada, B., and Sanchez-Herrero, E. (2001). The Hox gene Abdominal-B antagonizes appendage development in the genital disc of *Drosophila*. Development *128*, 331–339.

Galindo, M.I., Bishop, S.A., Greig, S., and Couso, J.P. (2002). Leg patterning driven by proximal-distal interactions and EGFR signaling. Science *297*, 256–259.

Gebelein, B., McKay, D.J., and Mann, R.S. (2004). Direct integration of Hox and segmentation gene inputs during *Drosophila* development. Nature *431*, 653–659.

Gilthorpe, J., Vandromme, M., Brend, T., Gutman, A., Summerbell, D., Totty, N., and Rigby, P.W. (2002). Spatially specific expression of Hoxb4 is dependent on the ubiquitous transcription factor NFY. Development *129*, 3887–3899.

Goto, S., and Hayashi, S. (1997). Specification of the embryonic limb primordium by graded activity of Decapentaplegic. Development *124*, 125–132.

Grimm, S., and Pflugfelder, G.O. (1996). Control of the gene optomotor-blind in *Drosophila* wing development by decapentaplegic and wingless. Science *271*, 1601–1604.

Istrail, S., and Davidson, E.H. (2005). Logic functions of the genomic cis-regulatory code. Proc. Natl. Acad. Sci. USA *102*, 4954–4959.

Jazwinska, A., Kirov, N., Wieschaus, E., Roth, S., and Rushlow, C. (1999). The *Drosophila* gene *brinker* reveals a novel mechanism of Dpp target gene regulation. Cell *96*, 563–573.

Kim, J., Sebring, A., Esch, J.J., Kraus, M.E., Vorwerk, K., Magee, J., and Carroll, S.B. (1996). Integration of positional signals and regulation of wing formation and identity by *Drosophila* vestigial gene. Nature *382*, 133–138.

Kirkpatrick, H., Johnson, K., and Laughon, A. (2001). Repression of dpp targets by binding of brinker to mad sites. J. Biol. Chem. *276*, 18216–18222.

Klymenko, T., and Muller, J. (2004). The histone methyltransferases Trithorax and Ash1 prevent transcriptional silencing by Polycomb group proteins. EMBO Rep. *5*, 373–377.

Lecuit, T., and Cohen, S.M. (1997). Proximal-distal axis formation in the *Drosophila* leg. Nature *388*, 139–145.

Lee, H.H., and Frasch, M. (2000). Wingless effects mesoderm patterning and ectoderm segmentation events via induction of its downstream target sloppy paired. Development *127*, 5497–5508.

Lee, Y.S., and Carthew, R.W. (2003). Making a better RNAi vector for *Drosophila*: use of intron spacers. Methods *30*, 322–329.

Li, X., and Noll, M. (1994). Compatibility between enhancers and promoters determines the transcriptional specificity of gooseberry and gooseberry neuro in the *Drosophila* embryo. EMBO J. *13*, 400–406.

Mann, R.S., and Carroll, S.B. (2002). Molecular mechanisms of selector gene function and evolution. Curr. Opin. Genet. Dev. *12*, 592–600.

Mardon, G., Solomon, N.M., and Rubin, G.M. (1994). dachshund encodes a nuclear protein required for normal eye and leg development in Drosophila. Development *120*, 3473–3486.

Masucci, J.D., Miltenberger, R.J., and Hoffmann, F.M. (1990). Pattern-specific expression of the *Drosophila* decapentaplegic gene in imaginal disks is regulated by 3′ cis-regulatory elements. Genes Dev. *4*, 2011–2023.

Merli, C., Bergstrom, D.E., Cygan, J.A., and Blackman, R.K. (1996). Promoter specificity mediates the independent regulation of neighboring genes. Genes Dev. *10*, 1260–1270.

Minami, M., Kinoshita, N., Kamoshida, Y., Tanimoto, H., and Tabata, T. (1999). brinker is a target of Dpp in *Drosophila* that negatively regulates Dpp-dependent genes. Nature *398*, 242–246.

Morimura, S., Maves, L., Chen, Y., and Hoffmann, F.M. (1996). decapentaplegic overexpression affects *Drosophila* wing and leg imaginal disc development and wingless expression. Dev. Biol. *177*, 136–151.

Nellen, D., Affolter, M., and Basler, K. (1994). Receptor serine/threonine Kinases implicated in the control of *Drsophila* body pattern by *decapentaplegic*. Cell *78*, 225–237.

Pai, L.M., Orsulic, S., Bejsovec, A., and Peifer, M. (1997). Negative regulation of Armadillo, a Wingless effector in Drosophila. Development *124*, 2255–2266.

Papp, B., and Muller, J. (2006). Histone trimethylation and the maintenance of transcriptional ON and OFF states by trxG and PcG proteins. Genes Dev. *20*, 2041–2054.

Ringrose, L., and Paro, R. (2004). Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins. Annu. Rev. Genet. *38*, 413–443.

Staehling-Hampton, K., Jackson, P.D., Clark, M.J., Brand, A.H., and Hoffmann, F.M. (1994). Specificity of bone morphogenetic protein-related factors: cell fate and gene expression changes in *Drosophila* embryos induced by decapentaplegic but not 60A. Cell Growth Differ. *5*, 585–593.

Struhl, G., and Basler, K. (1993). Organizing activity of wingless protein in *Drosophila*. Cell *72*, 527–540.

Theisen, H., Haerry, T.E., O'Connor, M.B., and Marsh, J.L. (1996). Developmental territories created by mutual antagonism between Wingless and Decapentaplegic. Development *122*, 3939–3948.

Vachon, G., Cohen, B., Pfeifle, C., McGuffin, M.E., Botas, J., and Cohen, S. (1992). Homeotic genes of the bithorax complex repress limb development in the abdomen of the *Drosophila* embryo through the target gene *Distal-less*. Cell *71*, 437–450.

Wiersdorff, V., Lecuit, T., Cohen, S.M., and Mlodzik, M. (1996). Mad acts downstream of Dpp receptors, revealing a differential requirement for dpp signaling in initiation and propagation of morphogenesis in the *Drosophila* eye. Development *122*, 2153–2162.

Xu, X., Yin, Z., Hudson, J.B., Ferguson, E.L., and Frasch, M. (1998). Smad proteins act in combination with synergistic and antagonistic regulators to target Dpp responses to the *Drosophila* mesoderm. Genes Dev. *12*, 2354–2370.

Zirin, J.D., and Mann, R.S. (2004). Differing strategies for the establishment and maintenance of teashirt and homothorax repression in the *Drosophila* wing. Development *131*, 5683–5693.