Developmental Cell
Article

Establishment of Medial Fates along the Proximodistal Axis of the *Drosophila* Leg through Direct Activation of *dachshund* by Distalless

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DOI 10.1016/j.devcel.2011.03.017

SUMMARY

The proximodistal (PD) axis of the *Drosophila* leg is thought to be established by the combined gradients of two secreted morphogens, Wingless (Wg) and Decapentaplegic (Dpp). According to this model, high [Wg+Dpp] activates *Distalless* (*Dll*) and represses *dachshund* (*dac*) in the distal cells of the leg disc, while intermediate [Wg+Dpp] activates *dac* in medial tissue. To test this model we identified and characterized a *dac cis*-regulatory element (*dac* RE) that recapitulates *dac*'s medial expression domain during leg development. Counter to the gradient model, we find that Wg and Dpp do not act in a graded manner to activate RE. Instead, *dac* RE is activated directly by Dll and repressed distally by a combination of factors, including the homeodomain protein Bar. Thus, medial leg fates are established via a regulatory cascade in which Wg+Dpp activate *Dll* and then Dll directly activates *dac*, with Wg+Dpp as less critical, permissive inputs.

INTRODUCTION

Animals use multiple mechanisms to establish unique cell types within developing tissues. One well-characterized mechanism depends on morphogens, molecules that trigger distinct responses in responding cells in a concentration-dependent manner. For example, Sonic hedgehog (Shh), secreted from the ventral-most cells of the vertebrate neural tube, exists as a ventral-to-dorsal concentration gradient that in turn establishes five discreet cellular domains along the dorsoventral (DV) axis (Jessell, 2000). Positional information can also arise from cascades of cross-regulating transcription factors. In the early *Drosophila* embryo, for example, a network of interacting segmentation genes provides positional information to the preblastoderm nuclei along the anteroposterior (AP) axis (Schroeder et al., 2004). In many cases, both mechanisms—morphogen gradients and transcription factor networks—work in concert with each other. Once Shh regulates the expression of an initial set of transcription factors in the neural tube, cross-regulation is required to fully define cellular fates (Briscoe et al., 2000). Analogously, the segmentation gene network in the fly requires an initial asymmetric input that is provided, at least in part, by an anterior-to-posterior gradient of the morphogen Bicoid (Ephrussi and St Johnston, 2004). These and other examples suggest that biological systems often use both mechanisms to generate positional information in developing tissues.

Compared to the vertebrate neural tube and the early *Drosophila* embryo, the formation of animal appendages requires an additional layer of complexity. In addition to having AP and DV axes, appendages also have a proximodistal (PD) axis, which forms orthogonally to the two main body axes. Unlike the AP and DV axes, the PD axis is established de novo for each appendage, during embryogenesis. Classical grafting experiments carried out in the cockroach provided important insights into how the PD axis is initiated (French, 1978, 1980). Juxtaposition of nonadjacent leg fragments (e.g., dorsal next to ventral) lead to the formation of supernumerary legs with new PD axes. At the time, the formation of these supernumerary legs was interpreted as resulting from the juxtaposition of different positional values followed by extensive tissue growth to fill in the missing positional values. More recent experiments carried out in *Drosophila* established that a new PD axis in the leg could be generated by the juxtaposition of two populations of cells, one that expresses the morphogen Wingless (Wg) and one that expresses the morphogen Decapentaplegic (Dpp) (Campbell et al., 1993; Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997). Moreover, by activating these pathways in a cell-autonomous manner, Lecuit and Cohen (1997) demonstrated that Wg and Dpp have the ability to induce a new PD axis directly, without the induction of another nonautonomous signal. Because Wg and Dpp are expressed in ventral and dorsal sectors, respectively, of developing insect legs (Figure 1A), these observations provided a molecular explanation for the cockroach-grafting experiments: the juxtaposition of nonadjacent leg fragments likely resulted in new juxtapositions of Wg and Dpp-expressing cells, which in turn led to the production of a new PD axis.

In addition to initiating PD axis formation, Wg and Dpp have also been proposed to establish positional information along the PD axis of the fly leg in a concentration-dependent manner (Lecuit and Cohen, 1997). In support of this idea, which we refer to here as the gradient model, two readouts of the PD axis were examined. *Distalless* (*Dll*), which encodes a homeodomain transcription factor, is expressed in cells that will give rise to the distal-most portion of the leg, from the tip of the tarsus to the middle of the tibia (Figure 1A). In contrast, *dachshund*

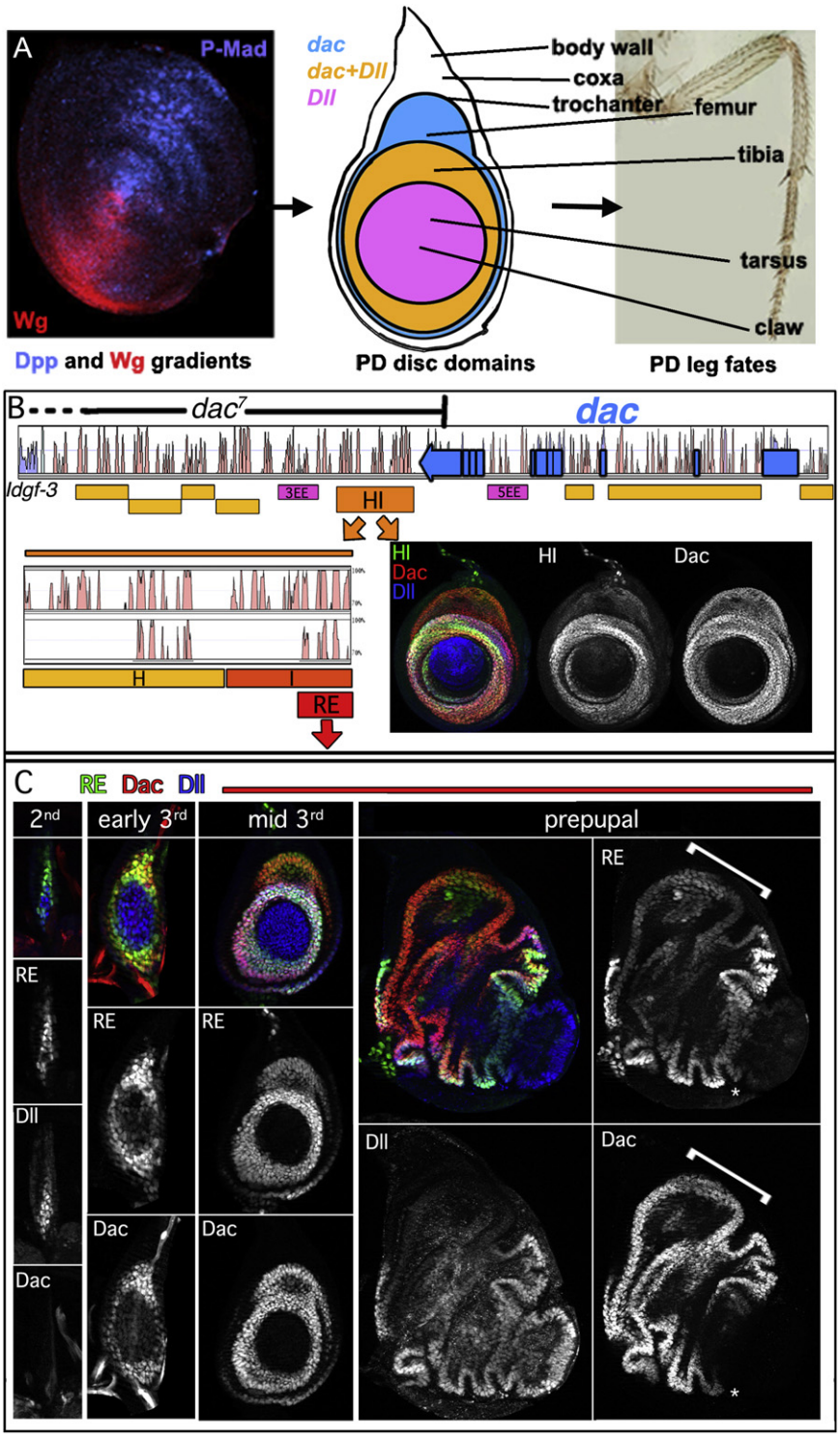

Figure 1. Identification of the Dac RE

(A) In the left panel Wg and Dpp gradients in the leg disc are shown by staining for Wg (red) and the activated form of the downstream effector of Dpp signaling, phospho-Mad (blue). The middle and right panels show a schematic of a third-instar disc and the corresponding PD fates in the adult leg.

(B) VISTA plot alignment of *D. melanogaster dac* locus compared to *D. pseudoobscura* (*dac* coding region shown in blue). Yellow and orange boxes represent cloned regions tested for the ability to drive reporter gene expression. Pink boxes represent enhancers active in the eye identified previously (Pappu et al., 2005). dac^7 is a deletion allele that begins in *dac*'s last exon, and extends 3′ to the gene, but its 3′ endpoint has not been mapped (Pappu et al., 2005). HI was further subdivided based upon sequence homology (VISTA alignments show *D. melanogaster* HI compared to *D. pseudoobscura* [top] and *D. virilis* [bottom]).

(C) Leg discs stained for *dac RE-lacZ* (green), Dac (red), and Dll (blue). *lacZ* expression was first apparent in the second instar, slightly before Dac protein was detectable. *dac* RE maintains a ringed pattern throughout development. RE expression is weaker in the Dac-only domain (bracket), compared to the Dac+Dll domain. *dac* RE is active at high levels at its distal edge where Dac protein is only weakly detected (asterisk).

(*dac*), which also encodes a transcription factor, is expressed in a medial domain of the leg's PD axis, from the tibia to the trochanter (Figure 1A). According to the gradient model, high concentrations of Wg+Dpp activate *Dll* and repress *dac*, intermediate concentrations of Wg+Dpp activate *dac* but not *Dll*, and lower levels activate neither gene (Lecuit and Cohen, 1997). Three experiments were carried out to support the idea that *dac* is responsive to lower levels of these signals compared to *Dll*. First, although both *Dll* and *dac* could be induced in cells in which the Dpp pathway was activated cell autonomously, the outcome depended on the distance the cells were from the endogenous source of Wg: *Dll* was induced in clones closer to the source of Wg compared to *dac* induction, and neither readout was induced in clones located far from the source of Wg. Analogously, when the Wg pathway was activated cell autonomously, *dac* was induced in cells farther from the endogenous source of Dpp compared to *Dll* induction. Third, when a secreted form of Wg was expressed in dorsal clones (close to the endogenous source of Dpp), it resulted in a nested pattern of PD gene expression, with *Dll* expressed closest to the source of Wg and *dac* expressed further from the source (Lecuit and Cohen, 1997). Together, these results suggested that *dac* and *Dll* are induced by different levels of Wg and Dpp signaling. However, the expression patterns of Wg and Dpp in the growing leg imaginal disc—the progenitor of the adult leg—raise several questions about how this would

happen in molecular terms: because Wg and Dpp are expressed in ventral and dorsal sectors of the leg disc, respectively (Figure 1A), it is unclear how cells might read and integrate a combined Wg+Dpp gradient.

One way to test the gradient model is to dissect the mechanisms that activate *Dll* and *dac* during leg development. *Dll* expression in the leg discs is mediated at least in part by two separable *cis*-regulatory elements (Estella et al., 2008; McKay et al., 2009; Galindo et al., 2011). In stage 14 embryos and leg imaginal discs, the "leg-trigger" (LT) enhancer is specifically activated in cells that receive high levels of Wg and Dpp signaling. Consequently, on its own, LT is only active in a small number of cells in the center of the leg disc, close to where the Wg and Dpp expression sectors come into contact. In contrast, when LT is in *cis* with the *Dll* maintenance (M) element, which includes the native *Dll* promoter, an accurate *Dll* expression pattern is generated. These observations led to the "trigger-maintenance" model, which posits that *Dll* is first activated by LT or LT-like elements in a small number of cells, and then maintained by the M element in these cells and their progeny (Estella et al., 2008). Notably, due to subsequent growth and maintenance of the *Dll*-expression domain, this mechanism does not require *Dll* to interpret graded Wg or Dpp inputs to be accurately expressed. Only high Wg and high Dpp inputs are required to initially activate *Dll*, and once activated, Wg and Dpp inputs are no longer required. Direct binding of the downstream transcription factors in the Wg and Dpp pathways (Pangolin [Pan], Mothers against Dpp [Mad], and Brinker [Brk]) is required for LT activity (Estella et al., 2008). LT activity also requires *Sp1*, a ventral selector gene that ensures that Wg and Dpp only activate *Dll* in ventral leg tissue, but not dorsal tissue such as the wing (Estella and Mann, 2010). Together, these results suggest that a gradient mechanism is not required for generating the *Dll* expression pattern during leg development.

Although the gradient model may not apply to *Dll* activation, it could still account for the establishment of medial fates along the PD axis, where *dac* is activated. Here, we test this possibility by characterizing a *dac* *cis*-regulatory element that is active in a medial domain along the PD axis. We show that Wg and Dpp inputs play a surprisingly minor role in the direct activation of this element. Consistent with lineage-tracing studies showing that the entire *dac* expression domain is derived from *Dll*-expressing cells (McKay et al., 2009), we find that direct *Dll* input is essential for the activation of this element and *dac* expression. Together, these and other data suggest that *dac* is activated by a regulatory cascade whereby Wg and Dpp activate *Dll*, which in turn activates *dac*. According to this revised model, gradients of Wg and Dpp activities are not required for *Dll* or *dac* activation or, therefore, the establishment of positional information along the PD axis. Instead, the combination of Wg and Dpp initiates a regulatory cascade that, coupled to the growth of the leg disc, establishes the PD axis.

RESULTS

Identification of a *dac* Enhancer Element

dac^7 is a deletion allele of *dac* that removes DNA 3′ to *dac* beginning in its last exon (Pappu et al., 2005) (Figure 1B). dac^7 completely removes *dac* expression and function in the leg (see below) but only partially removes *dac* function in the eye (Pappu et al., 2005), suggesting that *cis*-regulatory elements essential for leg expression are deleted by this allele. Therefore, we searched the DNA deleted by dac^7 for a *cis*-regulatory element that is able to drive expression in medial leg fates along the PD axis during leg development (Figure 1B). We focused our attention on sequences between *dac* and the neighboring gene, *Idgf-3*, in part because of the binding of insulator proteins close to *Idgf-3* (Negre et al., 2010). We discovered a 3.6 kb fragment, *dac* HI, that was able to drive reporter gene expression in a medial leg ring that is very similar to the pattern of Dac protein (Figure 1B). *dac* HI was further dissected to a 567 bp fragment that we call the *dac* Ring Enhancer (RE), which retained strong activity in the medial leg and is well conserved in multiple *Drosophila* species (Figures 1B and 1C). We also looked for, but failed to find, additional leg elements in the *dac* introns (Figure 1B). In sum, although we cannot exclude that there is an additional element distal to *Idgf-3*, these results suggest that *dac* RE is essential for *dac* expression and function in the leg.

Reporter gene expression driven by *dac* RE was robust when *dac* is first activated in the second instar and continued through the remainder of leg development. In the mid to late third instar, reporter gene expression became weaker in the dorsal, Dll-non-expressing, region of the *dac* expression domain, which gives rise to the femur (referred to here as the "Dac-only domain"). Additionally, although Dac protein levels were relatively weak in the first tarsal segment, which is the distal-most extent of its domain, expression driven by RE remained strong. *dac* RE also drove expression in a medial ring in the antenna but was not active in any of the other imaginal discs (data not shown).

dac RE Regulation Is Similar to *dac*

Based on previous genetic experiments, *dac* RE should be regulated by Wg and Dpp in two ways: (1) high levels of Wg and Dpp signaling, which are normally present in the region of the leg disc that will give rise to the distal tarsal segments, should repress RE; and (2) intermediate levels of both signals should activate RE. Consistently, in dorsal clones expressing an activated form of Armadillo ($Arm^{\Delta N}$), which mimics high Wg activity, both *dac* and *dac* RE were repressed, while more lateral $Arm^{\Delta N}$ clones activated *dac* and *dac* RE (Figure 2A). As previously suggested (Lecuit and Cohen, 1997), the different behavior of these clones is likely due to how close they are to the source of Dpp. Analogously, clones overexpressing a constitutively active Dpp coreceptor, Thickveins (Tkv^{QD}), repressed both *dac* and *dac* RE in ventral regions of the disc, where endogenous Wg signaling is strong, while more lateral Tkv^{QD} clones activated *dac* and *dac* RE (Figure 2B).

Loss-of-function analysis of the Wg and Dpp pathways supports the idea that *dac* RE interprets these signals similarly to *dac*. Distal clones mutant for the Wg coreceptor, *arrow* (arr^-), derepressed both *dac* and *dac* RE, demonstrating that Wg signaling is required to repress *dac* (Figure 2C). arr^- clones within the *dac* domain generated after the initiation of *dac* expression had no effect on *dac* or *dac* RE (Figure 2D). Similarly, distal clones mutant for the transcriptional effector of Dpp signaling, *mothers against Dpp* (*mad*), derepressed both *dac* and *dac* RE (Figure 2E). mad^- clones within the *dac* domain also did not affect *dac* expression (Figure 2F).

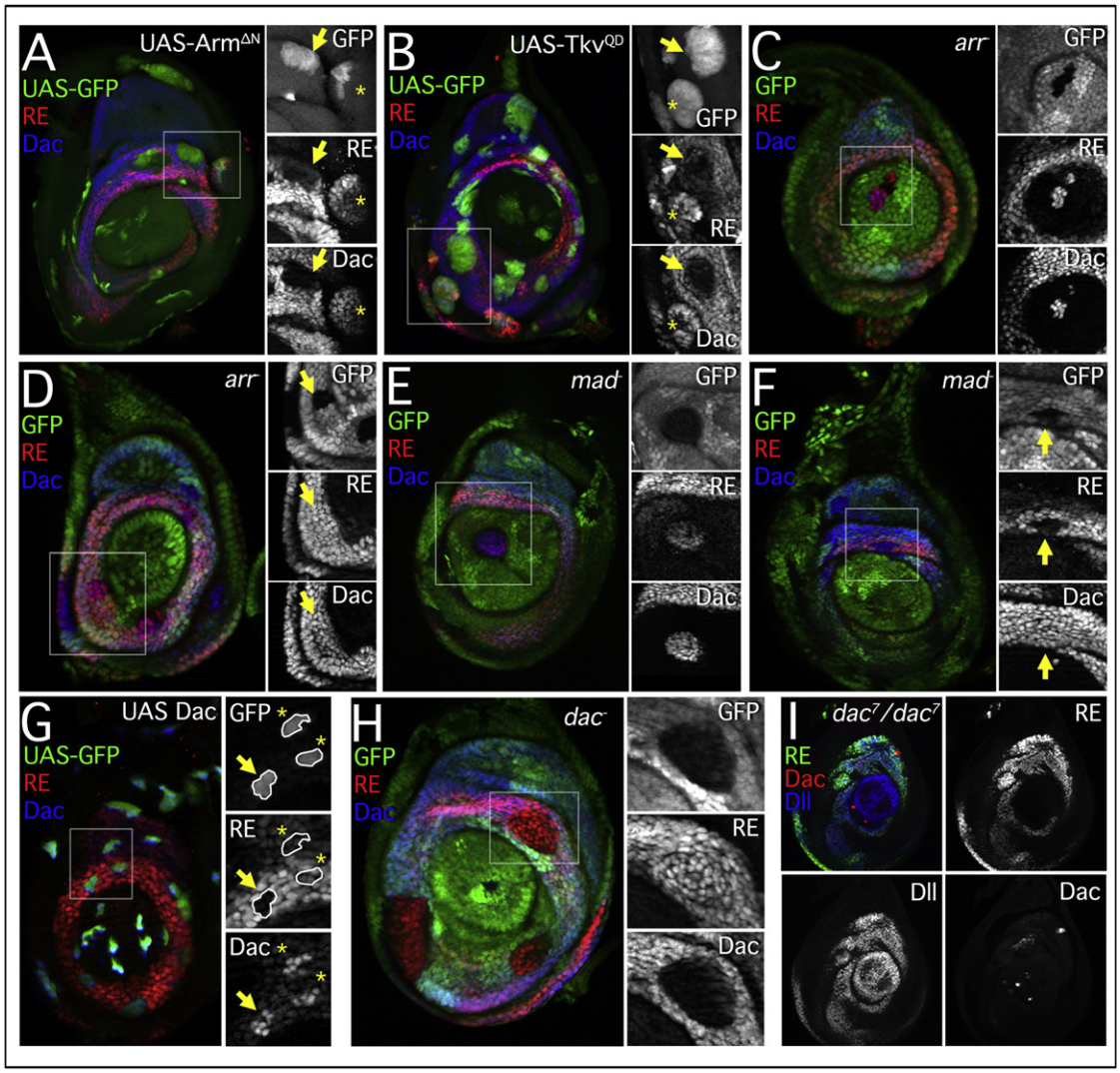

Figure 2. *dac* RE Is Regulated in a Manner Similar to *dac*

(A–H) Leg discs stained for *RE-lacZ* (red), Dac (blue), and Gal4-expressing clones (A, D, and G; marked by GFP) or mutant clones (B, C, E, F, and H; marked by the absence of GFP). For this and subsequent panels, smaller images show the individual staining patterns of the boxed regions. All clones were examined after growth for 48 hr at 25°C unless otherwise indicated. *arr*$^-$ and *mad*$^-$ clones were generated in the early second instar.

(A) Clones expressing activated Arm, Arm$^{\Delta N}$, create new domains of *dac* and *dac* RE activation in lateral tissue (asterisks) and repress both *dac* and *dac* RE in medial tissue where high levels of Dpp signaling are present (arrow).

(B) Clones expressing an activated form of the Dpp receptor, Tkv^{QD}, repress both *dac* and *dac* RE medially (arrow) while activating both *dac* and *dac* RE in proximal clones (asterisk).

(C) Distal clones mutant for *arr* derepress both *dac* and *dac* RE.

(D) Medial clones mutant for *arr* have no effect on either *dac* or *dac* RE (arrow).

(E) Distal clones mutant for *mad* derepress both *dac* and *dac* RE.

(F) Medial clones mutant for *mad* have no effect on Dac protein, but *dac* RE activity is absent.

(G) UAS-Dac expression clones. The extent of *dac* RE repression is variable (compare arrow and asterisks). Discs stained 24 hr after heat shock.

(H) *dac* null clones.

(I) *dac*$^{7/7}$ mutant disc stained for *dac RE*-lacZ (green), Dac (red), and Dll (blue). In *dac*^{7} mutant discs, *dac* RE activity is still present in a ring-like pattern, while Dac protein is virtually undetectable. See also Figure S1.

However, *dac* RE activity was lost in some *mad*$^-$ clones (Figure 2F), indicating a role for continuous Dpp input in maintaining *dac* RE activity. For both readouts, distal derepression in *mad*$^-$ or *arr*$^-$ clones was only observed when the clones were induced prior to the start of the third instar (72 hr after egg laying [AEL]); *mad*$^-$ or *arr*$^-$ clones generated in the third instar did not show distal derepression of *dac* or *dac RE-lacZ* (Figure S1 available online).

The near-identical behaviors of *dac* and *dac* RE-*lacZ* raised the possibility that *dac* RE is simply a Dac-responsive autoregulatory element. However, clones overexpressing Dac failed to activate *dac RE-lacZ* outside the *dac* domain and showed

a reduction in *dac* RE activity within the *dac* domain (Figure 2G). We also tested *dac* RE activity in clones and leg discs mutant for *dac*. In both cases *dac RE-lacZ* expression was unaffected (Figures 2H and 2I), demonstrating that *dac* function is not required for *dac* RE activity. We also observed that *dac* mutant clones within the Dac-only domain, where *dac* RE expression is normally low, resulted in the upregulation of *dac RE-lacZ* (Figure S1H). Together, these results show that *dac* RE is not activated by Dac and suggest that *dac* exhibits negative autoregulation, perhaps to fine-tune its expression levels.

Mutation of Multiple Binding Sites for Wg and Dpp Pathway Effectors Has Only a Minor Effect on *dac* RE Activation

To initially define the direct inputs into *dac* RE, we characterized the in vivo activities of a series of RE deletions (Figure S2). No individual deletion was sufficient to eliminate the ring pattern or to fully derepress distal expression. One deletion, REΔ5, drove ectopic expression in a distal ring at the tarsal/pretarsal boundary and is discussed below. We also tested smaller, 100–200 bp, subfragments of RE and found that none of these were sufficient to reproduce a ringlike expression pattern (Figure S2). Some fragments produced faint arcs of expression in the ventral Wg-expressing domain during late larval stages, suggesting some Wg responsiveness within RE. These results indicate that the control of *dac* RE activity is distributed along the full element, likely through multiple inputs.

If *dac* RE is directly activated or repressed in response to the amount of Wg and Dpp signaling, we would expect multiple binding sites for the Wg and/or Dpp transcriptional effectors Pan and Mad, respectively. Using Target Explorer (Sosinsky et al., 2003), we found that *dac* RE has multiple putative binding sites for both transcription factors (Figure 3A). We performed electrophoretic mobility shift assays (EMSAs) to determine whether or not these candidate sites were able to bind Mad or Pan proteins in vitro (data not shown). Of the seven candidate Pan sites, four showed specific binding to Pan protein that was lost upon mutation of three core nucleotides in the Pan-binding site (Figure 3A). Of the seven predicted Mad-binding sites, binding to two was lost when the sites were mutated (Figure 3A). As Brk recognizes similar sequences as Mad, we also checked the predicted Mad-binding sites for their ability to bind Brk. Four of the seven sequences showed specific binding to Brk that was sequence specific (Figure 3A).

We next tested the role of these binding sites in vivo. If intermediate levels of Wg and Dpp inputs activate *dac* RE in the medial leg, reducing or eliminating these direct inputs should reduce or eliminate *dac* RE activity during leg development. Single mutants of Mad or Pan sites had no effect on *dac* RE activity (data not shown). Even when the seven predicted Pan-binding sites (RE mP^{all}) or the seven predicted Mad/Brk-binding sites (RE M^{all}) were mutated, enhancer activity was not significantly compromised at the third larval stage (Figures 3C″–3G″). RE M^{all} drove expression in a largely normal pattern (Figure 3C″), while RE P^{all} drove expression in an imperfect ring, with some gaps in the pattern evident in third-instar leg discs (Figure 3G″). Although we cannot rule out that some Pan or Mad binding remains intact in these multiply mutated fragments, these observations do not support models in which *dac* regulation—both medial activation and distal repression—is sensitive to graded levels of Wg and Dpp inputs.

In contrast to the relatively normal expression patterns these mutant fragments drove in the third instar (Figures 3B″–3G″), some of their activities were not wild-type in younger leg discs, e.g., in the second instar (Figures 3B–3G). At this stage, RE M^{all} is expressed like wild-type RE, suggesting that Dpp is indirectly required for both distal repression and medial activation (Figures 3B and 3C). In contrast, when Pan sites were mutated, we observed derepression in distal cells in second-instar discs (Figures 3B and 3D–3G). Although the distal derepression driven by these fragments is transient (i.e., is not observed by the third instar), these observations suggest that Pan is directly required for the repression of *dac* in distal cells when it is first activated in the second instar. Although additional Pan sites not identified by Target Explorer may also contribute to distal repression, below we provide evidence that other factors are required for repression of *dac* in the third instar.

Although the above findings are inconsistent with the gradient model, they do not rule out that Mad and/or Pan inputs contribute to *dac* activation. Support for this idea comes from the analysis of *dac* RE subfragments, in which Pan or Mad-binding sites have been mutated. For example, REΔ45, which is missing subregions 4 and 5, drove a patchy pattern of expression in the medial leg disc (Figure 3H). When all predicted Mad or Pan-binding sites were mutated in this compromised context, expression was strongly reduced, particularly when the Pan-binding sites were mutated (Figures 3I and 3J). Together, these observations suggest that Wg and Dpp promote *dac* RE activity but that these signaling pathways are not being used in a graded manner to establish positional information along the PD axis.

Dll Is Essential for *dac* Expression

The above results support a model in which the Wg and Dpp inputs into *dac* activation are primarily indirect, raising the question of what factor(s) directly activates *dac* during leg development. Importantly, lineage-tracing experiments demonstrated that the entire *dac* domain is derived from cells that expressed *Dll* earlier in development (McKay et al., 2009). Consistent with these observations, Dac protein was first observed in cells that also have low levels of Dll protein (Figure 4A). Dll levels declined in *dac*-expressing cells until the early third instar when they became undetectable (Figures 4A and 4B). These observations are consistent with the idea that Dll plays a positive role in *dac* activation. As a first test of this idea, we examined clones overexpressing Dll, which resulted in an upregulation of both *dac* and *dac RE-lacZ*. Upregulation was observed in all parts of the leg disc except in distal regions that normally do not express *dac* (Figure 4C). Conversely, clones mutant for *Dll* in the *dac* domain showed a loss of both *dac* and *dac* RE-*lacZ* expression (Figure 4D). Together, these results demonstrate that *Dll* is required for the activation of *dac*.

A central observation in support of the gradient model is that de novo juxtapositions of Wg and Dpp-expressing cells activate either *Dll* or *dac* in proximal tissues, depending on where in the disc these clones arise (Abu-Shaar and Mann, 1998; Lecuit and Cohen, 1997) (Figure 2). If *Dll* is an essential activator of *dac*, we would expect that Wg+Dpp would be unable to activate *dac* in Dll^{-} cells. To test this prediction we used the MARCM

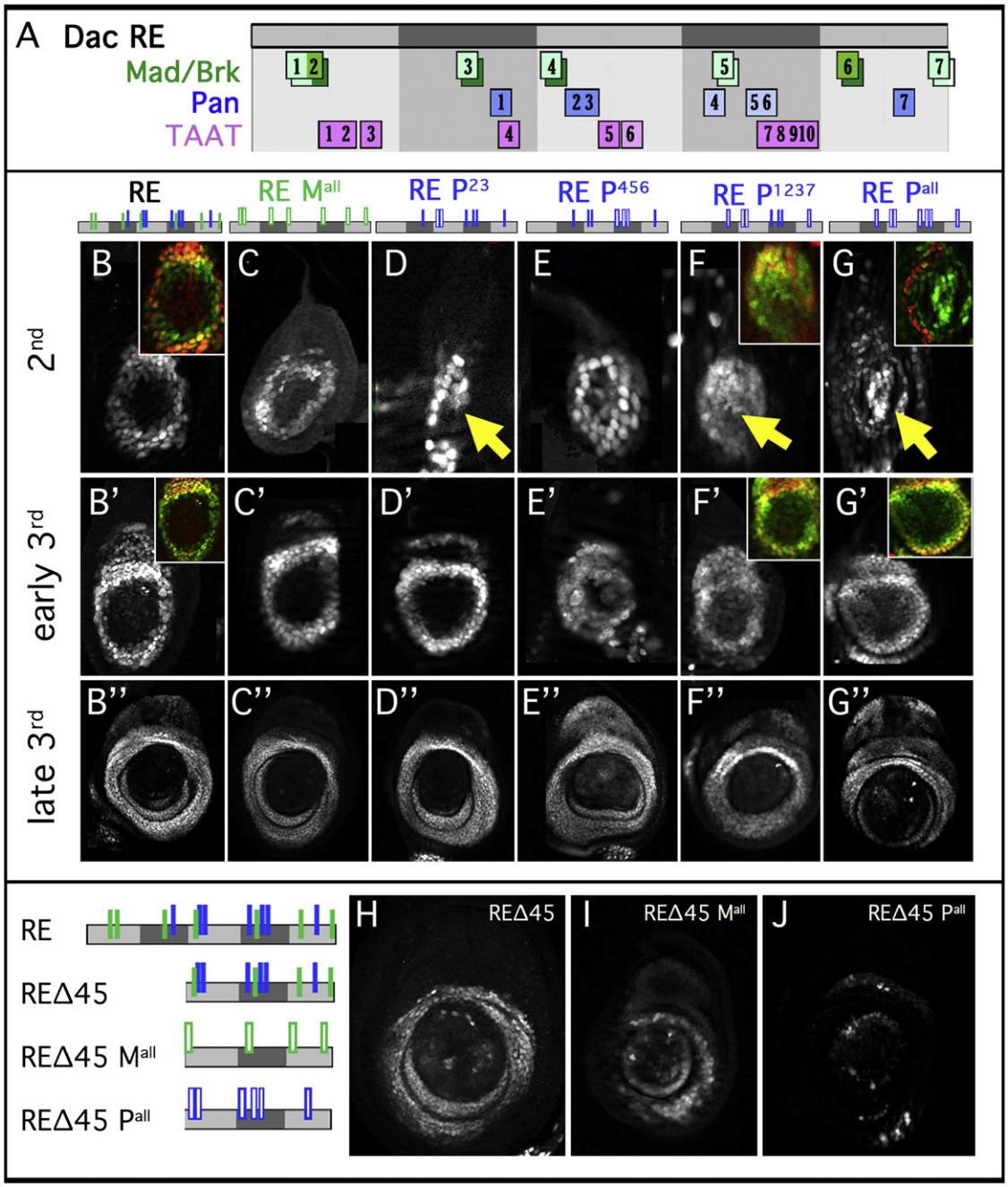

Figure 3. Normal *dac* RE Activation When Direct Inputs by Mad or Pan Are Compromised

(A) Schematic of *dac* RE showing binding sites for Mad (green), Pan (blue), and TAAT sequences (pink). Binding sites that show specificity in EMSAs have darker shading. Brk binding is shown in boxes dropped down from the Mad boxes. (B–G) Leg discs at second (B–G), early third (B′–G′), and late third (B″–G″) instar stages stained for the activities of *dac* RE and mutant versions of *dac* RE. Mutant elements are schematized above with the mutant sites represented by open bars. (B) *dac RE*. The inset shows RE (green) and Dac (red). (C) *dac RE* M^{all}. RE with seven Mad sites mutated.

(D) *dac RE* $P^{2,3}$. Mutating two Pan-binding sites gives initial expression in some cells distal to the normal Dac domain (arrow).

(E) *dac RE* $P^{4,5,6}$. Mutation of the three weak Pan-binding sites results in little effect on the RE ring pattern.

(F) *dac RE* $P^{1,2,3,7}$. Mutating these four binding sites results in distal expression in the second instar (arrow), in addition to RE's normal expression domain. A ring-like pattern is eventually formed, but distal expression remains into the third instar. The inset shows RE (green) and Dac (red).

(G) *dac RE* P^{all}. Mutation of seven candidate Pan sites results in distal expression in early discs (arrow) but does not significantly affect RE activity in older discs. The inset shows RE (green) and Dac (red).

(H–J) Direct Wg and Dpp inputs are required for expression of a truncated *dac* RE fragment, REΔ45 (schematics on left). (H) REΔ45. (I) REΔ45 fragment with seven Mad-binding sites mutated. (J) REΔ45 fragment with seven Pan-binding sites mutated.

See also Figure S2 and Table S1.

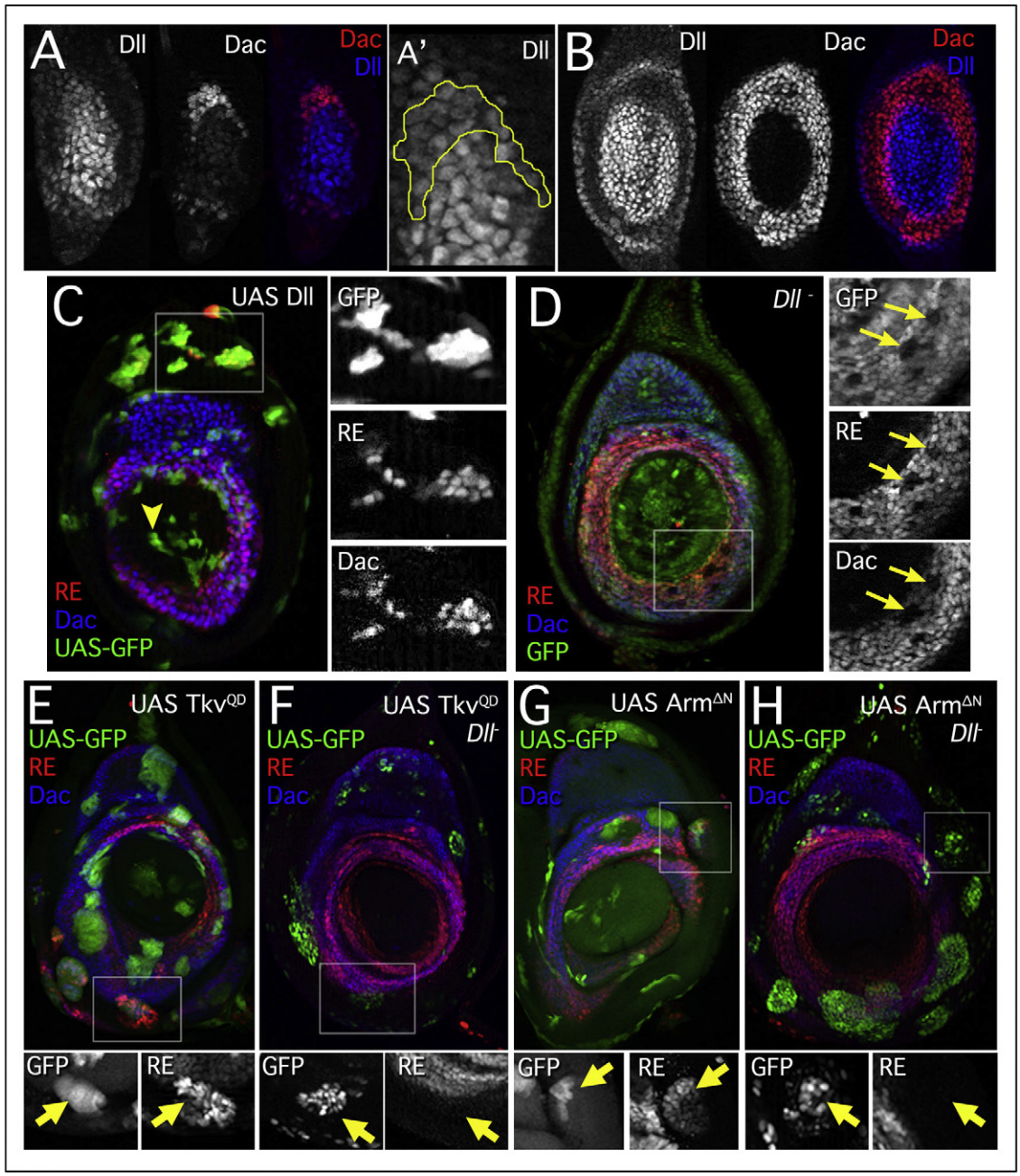

Figure 4. *Dll* Is Required for *dac* and *dac* RE Activation

(A) Late second-instar leg disc stained for Dac (red) and Dll (blue). Dac protein is first observed in cells that have lower levels of Dll, consistent with lineage-tracing experiments. (A′) shows a close-up of the Dac-expressing cells (outlined) with lower levels of Dll.

(B) Early third-instar leg disc showing that Dll (blue) is no longer observed in most of the Dac (red) expressing cells. Dac and Dll have a one to two cell overlap at this stage.

(C) Leg disc with clones expressing Dll (green), which activates both *dac* (blue) and *dac RE lacZ* (red) in proximal tissue. There is no effect on *dac* or *dac* RE activity distally (arrowhead). Larvae heat shocked in early third instar (72–96 hr AEL) and stained 24 hr after heat shock.

(D) *Dll* mutant clones (lack of GFP, green). Larvae were heat shocked in early third instar (72–96 hr AEL) and stained 24 hr after heat shock.

(E–H) Clones are marked by GFP+ expression (green), and discs are stained for *dac RE lacZ* (red) and Dac (blue). Larvae heat shocked between 24 and 72 hr AEL, then fixed and stained 48 hr after heat shock.

(E) UAS-Tkv^{QD} clones activate both *dac* and *dac RE lacZ* in ventral-proximal tissue.

(F) MARCM clones expressing Tkv^{QD} and mutant for *Dll* do not activate the distal leg program or result in ectopic expression of *dac* or *dac RE lacZ*.

(G) UAS-Arm$^{\Delta N}$ clones express both *dac* and *dac RE lacZ* in dorsal-proximal tissue.

(H) MARCM clones expressing Arm$^{\Delta N}$ and mutant for *Dll* do not activate *dac* or *dac RE-lacZ*.

method (Lee and Luo, 2001) to express either Tkv^{QD} or $Arm^{\Delta N}$ in Dll^{-} clones. We found in both cases that Wg or Dpp pathway activation was unable to activate *dac* or *dac RE-lacZ* in Dll^{-} clones (Figures 4E–4H). Thus, *Dll* is essential for *dac* activation, even at new sites of Wg and Dpp pathway activation.

Dll Acts through Multiple Binding Sites to Directly Activate *dac* RE

Dll is a homeodomain protein whose predicted target sequence, like many homeodomains, is based around a core TAAT sequence (Berger et al., 2008; Noyes et al., 2008). There are ten TAAT sequences in *dac* RE (Figure 3A). We tested all ten of these for their ability to bind Dll protein in a series of EMSA experiments. Dll bound specifically to seven of the ten sites (Figures 3A and 5A). To test if Dll is bound to these sites in vivo, in leg discs, we performed chromatin immunoprecipitation (ChIP) with third-instar leg discs using an anti-Dll antibody. We found that anti-Dll was able to specifically immunoprecipitate *dac* RE, but not control chromatin, consistent with a direct role for Dll in *dac* regulation (Figure 5B).

To test the importance of the Dll-binding sites for *dac* RE activity, we mutated the TAAT core sequences singly and in combination in the context of the full *dac* RE reporter gene. In contrast to mutation of the predicted Mad or Pan sites, mutation of all ten TAAT sites eliminated activity at all stages of disc development (Figure 5C). Mutation of the first four TAAT sequences (RE $TAAT^{1-4}$) did not significantly affect the ring pattern (Figure 5D), while mutation of the latter six TAAT sites (RE $TAAT^{5-10}$) resulted in delayed expression and a weaker ring pattern (Figure 5E). Mutation of the two central TAAT sites (RE $TAAT^{5,6}$) also significantly delayed the onset of reporter gene expression (Figures 5F and 5G), and deletion of these two sites in an already compromised fragment resulted in the near-elimination of RE activity (Figures S2F–S2H). Interestingly, multimerization of a small portion of RE that contains TAAT sites 5 and 6 was sufficient to drive a ringlike expression pattern in third-instar leg discs (4×(3b); Figure S2O). These findings suggest that Dll activates *dac* RE by binding to multiple TAAT-containing binding sites and that multiple sites are required for the correct timing and levels of *dac* RE activity.

Bar Directly Represses *dac* RE

The above results argue that Wg and Dpp do not act in a graded fashion to activate *dac* RE or *dac* because eliminating the predicted Pan and Mad-binding sites had only a minimal effect on enhancer activity. Notably, by the third instar, mutation of seven Pan or seven Mad-binding sites also did not result in significant expression in the distal leg disc (Figures 3B″–3G″). These observations raise the question of what factor(s) directly mediates repression in older discs. In addition to activating *Dll* and *dac*, Wg and Dpp activate components of the epidermal growth factor receptor (EGFR) pathway, which are important for patterning the tarsal segments. One of the early downstream targets of this pathway is the homeodomain protein, Bar, which is initially expressed in a ring just distal to the *dac* domain starting in the third instar (Kojima et al., 2000). When this ring of Bar expression initiates, it is adjacent to the distal edge of the *dac* domain. Thus, Bar is a good candidate for maintaining *dac*'s distal expression boundary in the early third instar.

To test Bar's ability to repress *dac* RE, we examined clones overexpressing Bar. Consistent with previous results (Kojima et al., 2000), ectopic Bar was able to repress both *dac* and *dac RE-lacZ* (Figure 6A). However, Bar-mediated repression of *dac* was not observed in all clones, suggesting that it is not sufficient for repression in all contexts (Figure S3). When Bar was ectopically expressed prior to *dac*, for example using *Dll-Gal4*, repression was robust, suggesting that the timing of *Bar* expression is important. Similar results were observed in clones expressing an activated form of the EGFR (λTop) (Figure 6B and data not shown). These results suggest that the repression of *dac* in the third instar is mediated in part by the EGFR pathway through its activation of *Bar*.

As noted above, one of the *dac* RE subfragments (REΔ5) drove expression distal to the normal *dac* domain during the second and early third instar, eventually giving rise to a strong additional distal ring of expression (Figures 6C and 6D). This ectopic ring of expression coincided with Bar expression (Figure 6E), also supporting a role for Bar in the distal repression of *dac*. Furthermore, Bar may repress RE through the 5′ end of the element. Consistently, a smaller RE subfragment comprised of just RE's 3′ half (REΔ45) also drove ectopic expression in a distal ring in the third instar (Figures 6F and 3H). Interestingly, REΔ45 was unaffected in clones that overexpress Bar (Figure 6G).

Like Dll, Bar is a homeodomain protein that is likely to bind DNA-binding sites that contain a TAAT core. There are four TAAT sequences missing from REΔ45. Strikingly, when those TAATs are mutated (RE $TAAT^{1-4}$), there is a similar derepression in a distal ring that coincides with the Bar expression domain (Figure 6H and data not shown). Mutation of just the first two TAAT sites ($TAAT^{1,2}$) results in a slim ring of distal derepression (Figure 6I). Together, these data provide strong evidence that *Bar* directly binds to and represses *dac* RE through multiple TAAT-binding sites located in the 5′ portion of the enhancer.

DISCUSSION

Activation of *dac* in the Medial Leg by a Nongradient Mechanism

The PD axis of the *Drosophila* leg is initiated by two secreted morphogens, Wg and Dpp. The process begins when these two pathways collaborate to induce the expression of *Dll* in a small group of ventral cells in each thoracic hemisegment of stage 14 embryos, in part by directly activating the *Dll* LT enhancer (Estella et al., 2008). Soon after *Dll* LT is activated, these cells invaginate from the main epithelium, thus establishing the leg imaginal disc. Wg and Dpp continue to be expressed next to the AP compartment boundary in ventral and dorsal cells, respectively, until the end of larval development. As the disc grows during larval stages, Wg and Dpp are secreted and diffuse from the cells that express them, resulting in gradients of both morphogens. These gradients help to pattern the DV axis of the adult leg (Struhl and Basler, 1993).

The question we address here is whether the ventral and dorsal gradients of Wg and Dpp are also used to establish distinct cell fates along the PD axis of the growing leg. To test this idea, we characterized an enhancer element from the *dac* locus, *dac* RE, which directs expression in a medial domain along the PD axis. If *dac* was interpreting intermediate levels of

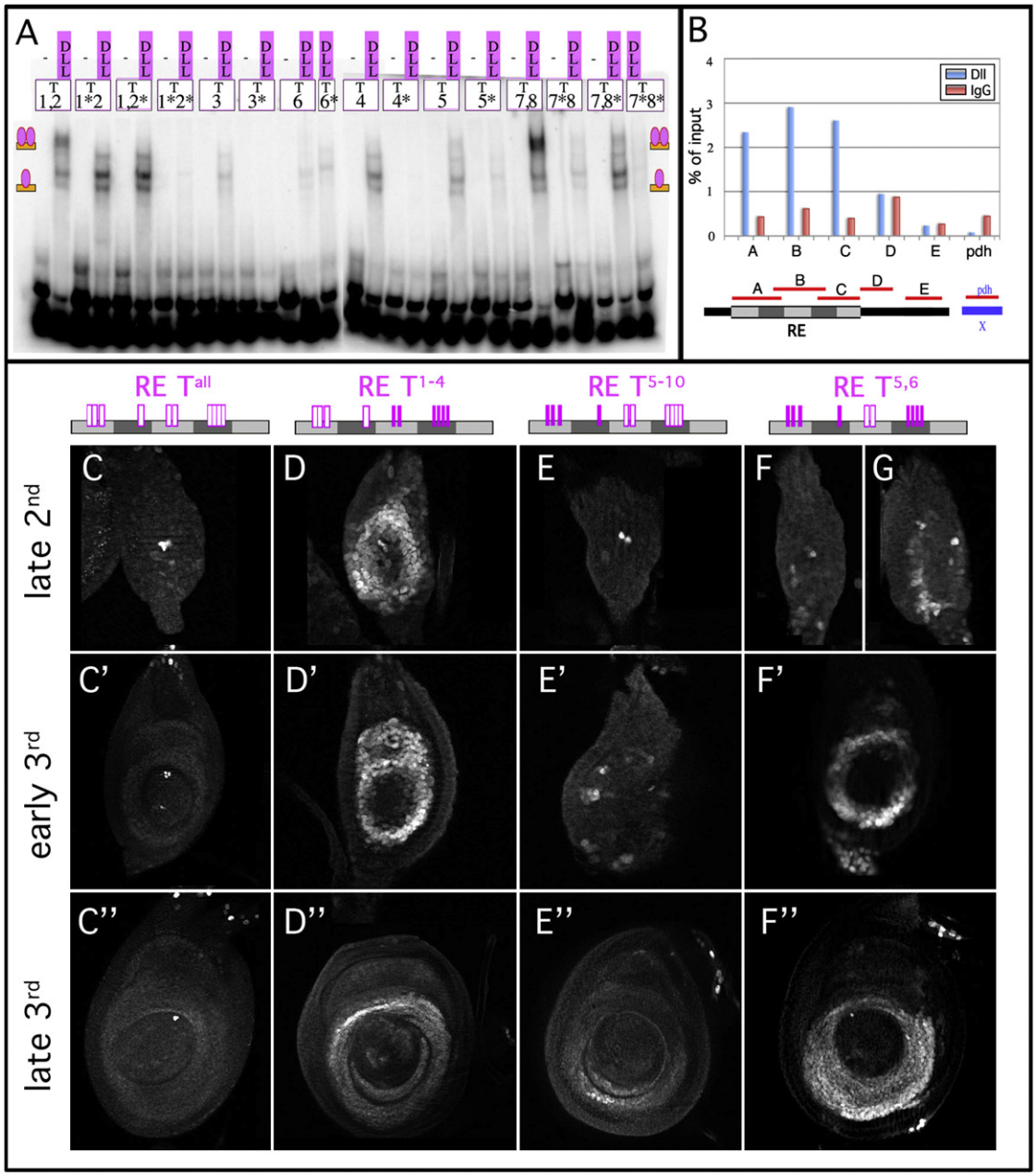

Figure 5. Dll Acts through TAAT Sequences in *dac* RE to Directly Activate Expression

(A) EMSAs with Dll protein on TAAT-containing oligos from *dac* RE. All TAAT sites except t6 show specific binding to Dll that is lost upon mutation. Oligos that contain two TAAT sequences (taat1,2 and taat7,8) show a slower mobility band that is due to the occupancy of both sites.

(B) Representative ChIP of third-instar leg discs using anti-Dll. Real-time PCR of primer sets at *dac* RE are pulled down specifically relative to IgG controls. Primer sets #1–3 are contained within *dac* RE, #4 is located just downstream, and #5 is located 400 bp further downstream. *pdh* is a negative control amplicon of the *pdh* gene on the X chromosome. (C–G) Leg discs at late second (D–G), early third (D′–F′), and late third (D″–F″) instar stained for *dac* RE reporter genes with mutated TAAT sequences. Mutant *dac* REs are schematized above with the mutant sites represented by open bars.

(C) *dac RE taat*^{all}. RE with all ten TAAT sequences mutated is nearly inactive. Expression is limited to a few distal cells and in a few proximal cells of the trochanter.

(D) *dac RE taat*$^{1-4}$. RE with the four 5′ TAAT sequences mutated is expressed in a normal RE ring. In the late third instar, there is an ectopic distal ring of expression at the boundary of the pretarsus (see Figure 6).

(E) *dac RE taat*$^{5-10}$. Mutation of the six 3′ TAAT sequences results in severely limited expression. By the third instar, expression is limited to a few cells in a weak ring-like pattern. In the late third instar, there is staining in some ventral medial cells, but expression is weak elsewhere. Apparent differences in expression in dorsal and ventral regions of the RE domain are largely due to folds in the discs and slight differences in the focal plane. (F and G) *dac RE taat*^{5,6}. Mutation of TAAT sites 5 and 6 results in a delayed expression of the Dac ring. By the third instar, expression is largely normal.

See also Table S1.

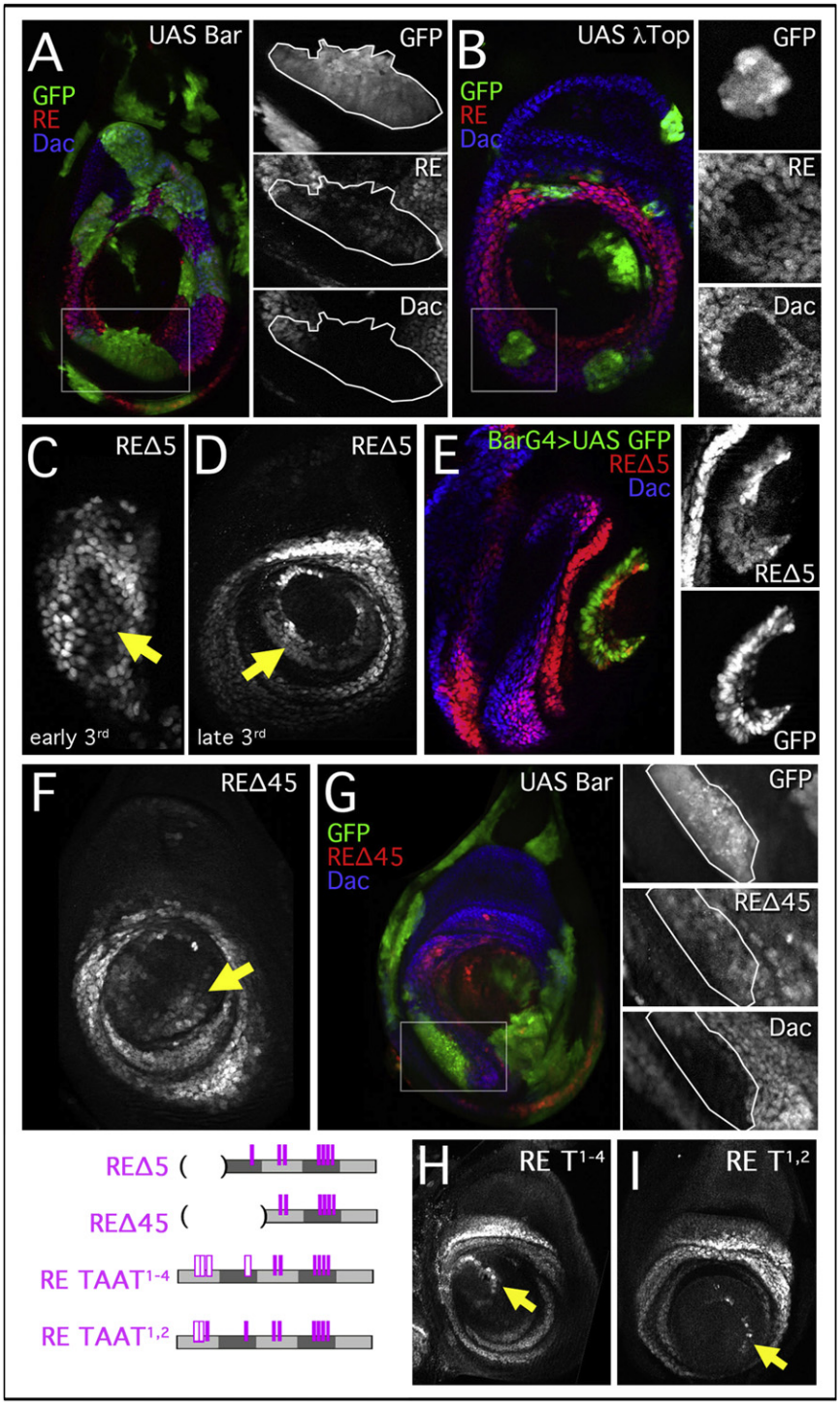

Figure 6. Late-Stage Distal Repression Elements in *dac* RE

(A and B) Leg discs stained for *dac RE lacZ* (red) and Dac (blue), with Gal4-expressing clones marked by GFP+. Boxed regions are blown up in the right-hand panels.

(A) Ectopic bar clones. Larvae heat shocked 24–72 hr AEL and stained 64 hr after heat shock.

(B) Clones expressing a constitutive EGFR (λTop, green). Larvae heat shocked 24–72 hr AEL and stained 48 hr after heat shock.

(C–E) Activity of *dac REΔ5 lacZ*, a truncated fragment of RE (schematized below).

(C) *dac REΔ5 lacZ* is expressed in cells distal (arrow) to the normal Dac domain in the early third instar.

(D) By the late third instar, there is a prominent ring (arrow) of expression at the fifth tarsal segment at the boundary with the pretarsus.

(E) Late third instar everting leg disc with *Bar-Gal4* driving GFP (green) stained for *dac REΔ5 lacZ* (red) and Dac (blue). The distal ring of REΔ5 is coincident with *Bar-Gal4* expression. The distal region of the disc is shown blown up in boxes on right.

(F and G) Activity of *dac REΔ45 lacZ*, a truncated fragment of RE (schematized below).

(F) *dac REΔ45 lacZ* is ectopically expressed in a distal ring (arrow).

(G) Clones expressing *Bar* repress *dac* (blue) but do not significantly affect *dac REΔ45 lacZ* expression. Larvae heat shocked 24–72 hr AEL and stained 64 hr after heat shock. The boxed region is blown up in the right-hand panels.

(H and I) Expression of *lacZ* from mutant RE constructs in late third-instar discs. Mutant REs are schematized at left with the mutant sites represented by open bars.

(H) *dac RE taat*$^{1-4}$. Mutation of the four 5′ TAAT sequences results in a thin ring of expression at the tarsal/pretarsus boundary (arrow).

(I) *dac RE taat*^{1,2}. Mutating two of the 5′ TAAT sequences results in a partial ring of expression at the tarsal/pretarsus boundary (arrow).

See also Figure S3 and Table S1.

the Wg and Dpp gradients for its activation, two expectations should be fulfilled: (1) binding sites for Pan and Mad should be present in the *dac* enhancer; and (2) reducing the amount of Pan or Mad binding to *dac* RE should reduce the proximal extent of its activity; in other words, the domain of *dac* RE activity should shrink as the amount of Pan and Mad input is reduced. However, our data only support the first of these two expectations. Counter to the prediction of the gradient model, eliminating most, if not all, of the Pan or Mad-binding sites in *dac* RE had virtually no effect on its ability to activate this element. Even if some residual Pan or Mad binding remains in these heavily mutated RE reporter genes, the gradient model predicts that they should have had significantly reduced activities. Based on these observations, we conclude that this element is not directly integrating intermediate levels of Wg and Dpp activities to be activated.

If Wg and Dpp are not the primary activators of *dac*, what is? Because lineage-tracing experiments demonstrated that all *dac*+ cells derive from cells that previously expressed *Dll*, we tested the idea that Dll is an activator of *dac*. Both genetic and molecular studies support this idea: *Dll*$^{-}$ clones in the *dac*

domain lose *dac* expression; gain-of-function *Dll*+ clones proximal to the *dac* domain activate *dac*; and Dll protein binds to *dac* RE both in vivo (in leg discs) and in vitro. Furthermore, mutating Dll-binding sites in *dac* RE eliminates its activity. Because ectopic activation of the Wg and Dpp pathway can induce a new PD axis and new sites of *dac* expression, we asked if this de novo activation of *dac* also required *Dll*. We found that Wg or Dpp pathway activation was incapable of inducing *dac* or *dac RE-lacZ* expression when the cells were also mutant for *Dll*. Together, these data provide strong evidence that *Dll* is an essential and direct activator of *dac* and *dac* RE.

In light of these observations, do Wg and Dpp play any direct role in the activation of *dac*? Based on the finding that compromised subfragments of *dac* RE (e.g., *dac* REΔ45) require Pan and Mad-binding sites for their activities, we suggest that they play a permissive role in *dac* activation. However, the nearly wild-type activities of *dac* RE P^{all} and *dac* RE M^{all} in the third instar imply that this contribution to *dac* activation is relatively minor compared to Dll.

Distal Repression of *dac* by Wg, EGFR, and Bar

Another question our results raise is, if *Dll* is an activator of *dac*, why is *dac* not activated in the center of the leg disc? Even when *Dll* is ectopically expressed, it cannot activate *dac* or *dac RE-lacZ* in this distal-most domain, although it can activate both of these readouts in proximal cells. We suggest that there are at least two mechanisms that repress *dac* in the distal leg disc. The first mechanism is due to direct binding of Pan to *dac* RE. The evidence in support of this idea is that mutating the Pan-binding sites (e.g., *dac* RE P^{all}) results in distal derepression in second-instar discs. The direct repression of *dac* RE by Pan is noteworthy because it apparently occurs in the presence of active Wg signaling, which is usually associated with transcriptional activation in a mechanism that depends on Arm binding to Pan (Stadeli et al., 2006). However, an alternative DNA-binding mode by Pan has been defined that mediates Wg- and Arm-dependent transcriptional repression (Blauwkamp et al., 2008). Although such a mechanism may be operating at *dac*, we note that Wg signaling cannot be sufficient for *dac* repression because Wg fails to repress *dac* in the ventral leg disc, despite high levels of signaling. In the future it will be interesting to explore this repression mechanism further and to specifically test the idea that Wg signaling collaborates with distally expressed transcription factors such as Bar to repress *dac*.

Soon after *dac* is first activated, the EGFR pathway is triggered in distal cells, due to the expression of EGFR ligands, such as *vein*, and EGFR pathway proteases, such as *rhomboid*, that are required for ligand activation (Campbell, 2002; Galindo et al., 2002). Our data suggest that this pathway also contributes to *dac* repression. First, expressing a constitutively activated form of the EGFR (λTop) resulted in the repression of *dac* and *dac* RE activity. Second, at least one downstream target of the EGFR pathway, *Bar*, is required for a subset of the distal repression of *dac* and *dac* RE. Our mutagenesis studies of *dac* RE suggest that Bar mediates repression directly, by binding to multiple homeodomain binding sites in the 5′ end of the enhancer. EGFR signaling results in the patterned expression of several transcription factors in addition to *Bar*, including *aristaless* and *rotund* (Kojima, 2004). Following our evidence for *Bar*, it is plausible that these, and perhaps other, distally expressed transcription factors also play a role in *dac* repression. It is also possible that the EGFR pathway directly represses *dac*, or that there are noncanonical Pan-binding sites that also contribute to repression in the third instar.

Interestingly, *dac* and *dac RE-lacZ* are derepressed in distal mad^{-} or arr^{-} clones, but only when these clones are induced prior to the start of the third instar. These observations suggest that timing plays a critical role in *dac* repression. Similarly, the RE P^{all} mutant reporter gene only shows distal derepression in second-instar discs, but not in third-instar discs. We suggest that these temporal differences are due to different phases of *dac* regulation (Figure 7). Specifically, distal *dac* repression early in development, prior to the activation of the EGFR pathway, depends primarily on Wg. At later stages, other mechanisms come into play. In addition to *Bar* and other distal transcription factors, the Polycomb group (PcG) of transcriptional repressors may also contribute to the maintenance of *dac* repression. Consistent with this idea, in flies mutant for the PcG gene *pleiohomeotic* (*pho*), *dac* is derepressed in the distal tip (Kim et al., 2008).

A Temporal Cascade of Regulatory Inputs Establishes Medial Fates during Leg Development

These findings support a revised view of PD axis formation in the *Drosophila* leg (Figure 7). In the second-instar leg disc, we propose that the combination of high levels of Wg signaling and high levels of Dpp signaling, which only occur in cells close to the center of the disc, results in two outputs: (1) activation of *Dll*, in part via the LT enhancer (Estella et al., 2008); and (2) repression of *dac* RE. As the disc grows in size, Dll activates *dac* in all cells that are not receiving high levels of Wg+Dpp signaling. Soon thereafter, in the early third instar, *Dll* expression is maintained, and high Wg+Dpp activities begin to activate the EGFR pathway (Campbell, 2002; Galindo et al., 2002). Genes downstream of the EGFR pathway, such as *Bar*, continue to repress *dac* in distal cells through the rest of leg disc development. The fundamental difference between this model and the gradient model is that graded activities of Wg and Dpp are not relevant for the regulation of either *dac* or *Dll*. Instead, by proposing that *dac* is activated by Dll and repressed by high levels of Wg+Dpp signaling, only peak levels of Wg and Dpp pathway activation are relevant to forming the PD axis. In one case (*Dll*), activation is direct (mediated by Mad and Pan binding to *Dll*), while in the other case (*dac*), activation is indirect, mediated by Dll binding to *dac* RE.

As described so far, this model would predict that the proximal extent of *dac* expression should coincide with the proximal extent of *Dll* expression. (For this discussion, we do not include the proximal trochanter ring, which expresses both *Dll* and *dac* but is under separate control.) However, this is not the case: in third-instar leg discs, there is a *Dll*-nonexpressing Dac-only domain that surrounds the Dll expression domain. This Dac-only domain is larger in the dorsal portion of the disc due in part to the absence of Brk expression (Estella and Mann, 2008). Lineage-tracing experiments show that the progenitors of all *dac*-expressing cells, including the Dac-only domain, expressed *Dll* (McKay et al., 2009). Thus, Dll is a transient activator of *dac* in these cells: it must be expressed to initially

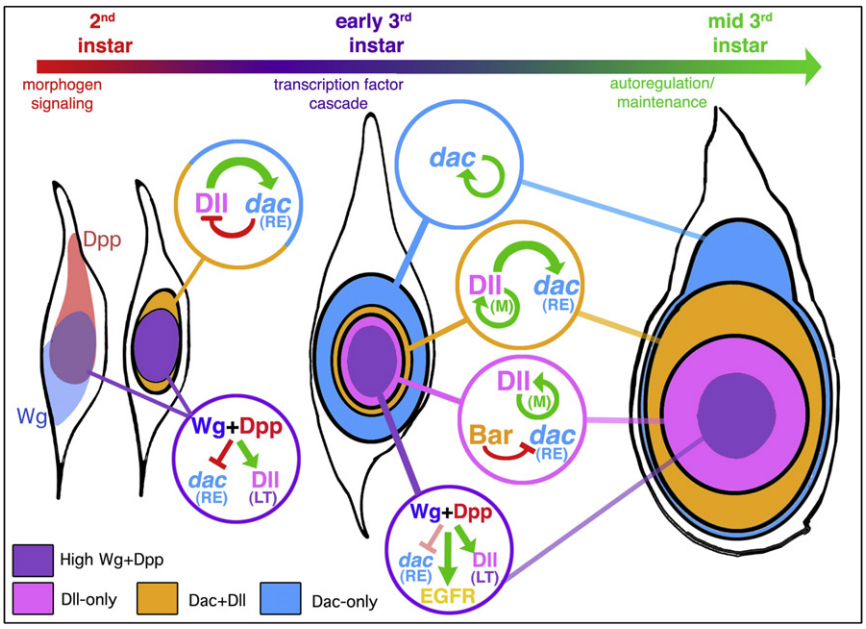

Figure 7. Establishment and Elaboration of the PD Axis of the Leg

Shown are schematic diagrams of second-instar (left), early third-instar (middle), and mid-third-instar leg imaginal discs. In second-instar discs, there is a ventral sector of high Wg signaling (light blue) and a dorsal sector of high Dpp signaling (red). Although not shown, these patterns of Wg and Dpp signaling remain the same throughout the remainder of leg development. Only cells in the center of the leg disc (purple) are receiving high inputs for both Wg and Dpp. The combination of high Wg signaling and high Dpp signaling results in the activation of the *Dll LT*-enhancer element and the repression of the *dac RE* element. As the disc grows, some *Dll*-expressing cells move out of this *dac*-repression domain, allowing *Dll* to activate *dac* (Dac+Dll domain; orange). Once *dac* is activated, it may repress *Dll*, thus contributing to the establishment of the initial Dac-only domain (blue). By the early third instar, the three primary gene expression domains (Dll-only, Dac+Dll, and Dac-only) become fixed by a maintenance mechanism that is independent of Wg and Dpp signaling. For *Dll*, this maintenance mechanism involves autoregulation mediated by the M element; for *dac*, autoregulation may also be involved, but this is not yet known. Also during the early third instar, the EGFR pathway is activated in distal cells, leading to the expression of *Bar* as well as other downstream transcription factors. *Bar* continues to repress *dac* in distal cells, thus helping to maintain the Dll-only domain. High levels of Wg and Dpp signaling, still limited to the center of the disc (purple), may continue to contribute to the repression of *dac* in distal cells.

activate *dac* but then is only maintained in a subset of *dac*-expressing cells. We suggest that the growth of the leg disc, coupled with a transition to autoregulation or a transcriptional maintenance mechanism, accounts for the formation of the Dac-only domain (Figure 7). In the second instar, *dac* is first activated in *Dll*-expressing cells that are not also receiving high levels of Wg and Dpp. As cells in the young disc divide, some of the *Dll* and *dac*-expressing cells will move out of the range of Wg and Dpp signaling that is minimally required for *Dll* activation. Consequently, some of these cells still express *dac* but lose *Dll* expression. In addition, *dac* may downregulate *Dll* in a negative feedback loop (Figure 7). This suggestion is consistent with previous observations showing that small dac^{-} clones in the Dac-only domain derepress *Dll* (Abu-Shaar and Mann, 1998).

The above regulatory steps will result in an early third-instar disc that contains all three primary expression domains: Dll-only, Dac+Dll, and Dac-only. At about the same time, we suggest that the *dac* and *Dll* expression status begins to be maintained independently of these earlier regulatory steps, either by autoregulation or by a transcriptional memory system. For *Dll*, autoregulation plays a critical role beginning in the early third instar (Estella et al., 2008). Notably, *dac* RE is not activated by Dac, so it does not contain an autoregulatory component, and interestingly, *dac RE-lacZ* is poorly expressed in the Dac-only domain. These observations are consistent with our model because it predicts that *dac* expression in the Dac-only domain should be more dependent on autoregulation than in the "Dac+Dll" domain, where Dll is constantly available to activate *dac*. In summary we suggest that the distinct proximal extents of the *dac* and *Dll* domains in the third-instar leg disc are a consequence of the *dac* and *Dll*-expression status at the time when autoregulation/maintenance initiates. In effect the Wg- and Dpp-independent maintenance phases of *dac* and *Dll* expression lock into place the three domains that are initially established in the late second/early third imaginal disc, and maintained throughout the remainder of development.

The Role of Temporal Transcriptional Cascades in Development

In more general terms, the logic of gene regulation revealed here highlights the importance of developmental timing and tissue growth in eventually generating stable domains of gene expression. Developmental processes such as PD axis formation are dynamic, with cells changing their states in a directional manner over time, typically from a less-differentiated state toward a more differentiated state. Such developmental programs are fundamentally distinct from other transcriptional regulatory scenarios that are used to toggle between bistable states, which may be more typical in terminal differentiation programs (Mikeladze-Dvali et al., 2005; Poole and Hobert, 2006). The regulatory steps in *Drosophila* leg development—from morphogen signaling (Wg+Dpp → *Dll*), through a transcription factor cascade and negative feedback (Dll → *dac*, Dac –| *Dll*), to autoregulation/maintenance (e.g., Dll → *Dll*)—represent a series of subcircuits or subroutines that gradually transition from one to another as the tissue grows (Figure 7). This transition through a series of subcircuits is also seen in other dynamic developmental processes such as specification of cell types in the vertebrate neural tube and in the early *Drosophila* embryo (Ben-Tabou de-Leon and Davidson, 2006; Kutejova et al., 2009; von Dassow et al., 2000). The progression through a series of transient, inherently unstable states may be typical for developmental programs in which cells are continually refining their fates over time and as tissues grow in size.

EXPERIMENTAL PROCEDURES

Transgenes

The initial *dac* reporter fragments, as well as HI, RE, REΔ5, and REΔ45, were inserted into a standard *P* element *nuc-lacZ* vector (Estella et al., 2008) for which multiple transformants were surveyed for position effects. In addition all RE reporter genes were inserted into an *attB-nuc-lacZ* vector modified from Bischof et al. (2007) with an *hsp43* promoter. All *attB* constructs were inserted into the second chromosome at *51D attP* site (gift of K. Basler) via phi-C31 mediated transgenesis (Bischof et al., 2007). Expression at 51D was comparable to the representative first generation standard *P* element transformants. MARCM and mutant clone analyses used a *P* element RE reporter inserted in the third chromosome. Putative *dac* regulatory fragments were selected based on sequence conservation to other Drosophilids (VISTA Genome Browser, UCSC) and cloned by PCR (*dac* RE was cloned with primers: CCAACTGAAAAAGGAGCAGCTTTC and ACAAAATTTATACGCCA GATG; all other primer sequences and details are available upon request). 4×(3b) was synthesized as a single oligo with the sequence, TCCAATAA TAAAGTTAAATCGATAATTGAGGTCA, repeated four times. *dac* RE deletion constructs were formed by fusing two PCR fragments made with primers tagged with Spe I digest sites. We used Target Explorer (Sosinsky et al., 2003) to locate potential binding sites for Mad, Brk, and Pan, by generating matrices for each protein based upon published binding sites. Mad/Brk matrices were built around a MGCCGCCGM consensus sequence. Pan matrices used the canonical Pan site SCTTTGW and HMG-domain site WTTGWW. Mutant *dac* RE constructs were generated by PCR. TAATs were mutated to TGGG. For Mad/Brk-binding sites the CGC or GCC core was mutated to ATA; for Pan-binding sites the AAA or TAA core was mutated to CCC or GGG. The binding sequences are listed in Table S1.

Immunostaining

Imaginal discs were prepared and stained using standard procedures. The primary antibodies used were: rabbit anti-β-Gal (Cappell); mouse anti-Wg & mouse anti-Dac (Developmental Studies Hybridoma Bank; http://dshb.biology.uiowa.edu); guinea pig anti-P-Mad (gift of E. Laufer and T. Jessell); and guinea pig anti-Dll.

Protein Purification and EMSAs

GST-Mad MH1+L (Xu et al., 1998), GST-dTCF HMG (Lee and Frasch, 2000), GST-Brk 1-100 (gift of C. Rushlow), and His-Dll (Estella et al., 2008) were produced and purified by standard procedures (Amersham-Pharmacia or 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. Oligo and primer sequences are available upon request.

ChIPs

ChIP assays were carried out using a previously described protocol (Estella et al., 2008).

Fly Genetics

For gain-of-function experiments, we used the strain *yw hsFLP122*; *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-Dac* (Shen and Mardon, 1997); UAS-Bar (Sato et al., 1999); UAS-Dll (Dong et al., 2000); and UAS-λTop$^{4.2}$ (Queenan et al., 1997). Flip-out clones were originated by heat shocking larvae aged 24–48 hr for 7–10 min at 37°C. Larvae were dissected following 48 hr growth at 25°C unless otherwise indicated. For loss-of-function clones, we used the following genotypes: *mad$^{1-2}$ FRT40A/ ubi-GFP FRT40A*, *yw hsFLP122; FRT42D arr^{2}/ FRT42D ubi-GFP, yw hsFLP122; FRT42D Dll^{sa1}/ FRT42D ubi-GFP, yw hsFLP122; dac^{3} FRT40A/ ubi-GFP y+ FRT40A*. Clones were generated by a 1 hr heat shock at 37°C. Larvae were dissected 48 hr after heatshock unless otherwise indicated. The *dac^{7}* allele is a 3′ deletion that encompasses the RE element and has virtually no Dac expression in the leg (Pappu et al., 2005). To monitor Bar expression we used *yw Bar-Gal4; FM7c* (Kyoto Stock Center) with *dac REΔ5*; *UAS-GFP*. We used the MARCM technique (Lee and Luo, 1999) to express *UAS-Tkv^{QD}* or *UAS-Arm$^{\Delta N}$* in *Dll^{-}* cells using: *FRT42D Dll^{Sa1}*; *UAS-ArmΔN* (over compound SM6^TM6B), *FRT42D Dll^{Sa1}*; *UAS-Tkv^{QD}* (SM6^TM6B), *yw hsFLP122 tub-Gal4 UAS-GFP*; *FRT42D tub-Gal80; dac RE-nucLacZ*, *yw hsFLP122 tub-Gal4 UAS-GFP*; *tub-Gal80 FRT40A*; *dac RE-nucLacZ*.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at doi:10.1016/j.devcel.2011.03.017.

ACKNOWLEDGMENTS

We thank K. Basler, T. Jessell, E. Laufer, G. Mardon, G. Rubin, and G. Struhl for reagents, and James Briscoe, Claude Desplan, Carlos Estella, and Roumen Voutev for comments on the manuscript. We thank Wen Zhang for generating the transgenic lines. This work was supported by NIH grant GM058575 awarded to R.S.M. and an NRSA fellowship awarded to M.W.G.

Received: February 7, 2011
Revised: March 21, 2011
Accepted: March 24, 2011
Published: April 18, 2011

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.

Ben-Tabou de-Leon, S., and Davidson, E.H. (2006). Deciphering the underlying mechanism of specification and differentiation: the sea urchin gene regulatory network. Sci. STKE *2006*, pe47.

Berger, M.F., Badis, G., Gehrke, A.R., Talukder, S., Philippakis, A.A., Pena-Castillo, L., Alleyne, T.M., Mnaimneh, S., Botvinnik, O.B., Chan, E.T., et al. (2008). Variation in homeodomain DNA binding revealed by high-resolution analysis of sequence preferences. Cell *133*, 1266–1276.

Bischof, J., Maeda, R.K., Hediger, M., Karch, F., and Basler, K. (2007). An optimized transgenesis system for *Drosophila* using germ-line-specific phiC31 integrases. Proc. Natl. Acad. Sci. USA *104*, 3312–3317.

Blauwkamp, T.A., Chang, M.V., and Cadigan, K.M. (2008). Novel TCF-binding sites specify transcriptional repression by Wnt signalling. EMBO J. *27*, 1436–1446.

Briscoe, J., Pierani, A., Jessell, T.M., and Ericson, J. (2000). A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. Cell *101*, 435–445.

Campbell, G. (2002). Distalization of the *Drosophila* leg by graded EGF-receptor activity. Nature *418*, 781–785.

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.

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.

Dong, P.D., Chu, J., and Panganiban, G. (2000). Coexpression of the homeobox genes Distal-less and homothorax determines *Drosophila* antennal identity. Development *127*, 209–216.

Ephrussi, A., and St Johnston, D. (2004). Seeing is believing: the bicoid morphogen gradient matures. Cell *116*, 143–152.

Estella, C., and Mann, R.S. (2008). Logic of Wg and Dpp induction of distal and medial fates in the *Drosophila* leg. Development *135*, 627–636.

Estella, C., and Mann, R.S. (2010). Non-redundant selector and growth-promoting functions of two sister genes, buttonhead and Sp1, in *Drosophila* leg development. PLoS Genet. *6*, e1001001.

Estella, C., McKay, D.J., and Mann, R.S. (2008). Molecular integration of wingless, decapentaplegic, and autoregulatory inputs into Distalless during *Drosophila* leg development. Dev. Cell *14*, 86–96.

French, V. (1978). Intercalary regeneration around the circumference of the cockroach leg. J. Embryol. Exp. Morphol. *47*, 53–84.

French, V. (1980). Positional information around the segments of the cockroach leg. J. Embryol. Exp. Morphol. *59*, 281–313.

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.

Galindo, M.I., Fernandez-Garza, D., Phillips, R., and Couso, J.P. (2011). Control of Distal-less expression in the Drosophila appendages by functional 3′ enhancers. Dev. Biol., in press. Published online February 12, 2011. 10.1016/j.ydbio.2011.02.005.

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.

Jessell, T.M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat. Rev. Genet. *1*, 20–29.

Kim, S.N., Jung, K.I., Chung, H.M., Kim, S.H., and Jeon, S.H. (2008). The pleiohomeotic gene is required for maintaining expression of genes functioning in ventral appendage formation in *Drosophila melanogaster*. Dev. Biol. *319*, 121–129.

Kojima, T. (2004). The mechanism of *Drosophila* leg development along the proximodistal axis. Dev. Growth Differ. *46*, 115–129.

Kojima, T., Sato, M., and Saigo, K. (2000). Formation and specification of distal leg segments in *Drosophila* by dual Bar homeobox genes, BarH1 and BarH2. Development *127*, 769–778.

Kutejova, E., Briscoe, J., and Kicheva, A. (2009). Temporal dynamics of patterning by morphogen gradients. Curr. Opin. Genet. Dev. *19*, 315–322.

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, T., and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron *22*, 451–461.

Lee, T., and Luo, L. (2001). Mosaic analysis with a repressible cell marker (MARCM) for *Drosophila* neural development. Trends Neurosci. *24*, 251–254.

McKay, D.J., Estella, C., and Mann, R.S. (2009). The origins of the *Drosophila* leg revealed by the cis-regulatory architecture of the Distalless gene. Development *136*, 61–71.

Mikeladze-Dvali, T., Wernet, M.F., Pistillo, D., Mazzoni, E.O., Teleman, A.A., Chen, Y.W., Cohen, S., and Desplan, C. (2005). The growth regulators warts/lats and melted interact in a bistable loop to specify opposite fates in *Drosophila* R8 photoreceptors. Cell *122*, 775–787.

Negre, N., Brown, C.D., Shah, P.K., Kheradpour, P., Morrison, C.A., Henikoff, J.G., Feng, X., Ahmad, K., Russell, S., White, R.A., et al. (2010). A comprehensive map of insulator elements for the *Drosophila* genome. PLoS Genet. *6*, e1000814.

Noyes, M.B., Christensen, R.G., Wakabayashi, A., Stormo, G.D., Brodsky, M.H., and Wolfe, S.A. (2008). Analysis of homeodomain specificities allows the family-wide prediction of preferred recognition sites. Cell *133*, 1277–1289.

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

Pappu, K.S., Ostrin, E.J., Middlebrooks, B.W., Sili, B.T., Chen, R., Atkins, M.R., Gibbs, R., and Mardon, G. (2005). Dual regulation and redundant function of two eye-specific enhancers of the *Drosophila* retinal determination gene dachshund. Development *132*, 2895–2905.

Poole, R.J., and Hobert, O. (2006). Early embryonic programming of neuronal left/right asymmetry in *C. elegans*. Curr. Biol. *16*, 2279–2292.

Queenan, A.M., Ghabrial, A., and Schupbach, T. (1997). Ectopic activation of torpedo/Egfr, a *Drosophila* receptor tyrosine kinase, dorsalizes both the eggshell and the embryo. Development *124*, 3871–3880.

Sato, M., Kojima, T., Michiue, T., and Saigo, K. (1999). Bar homeobox genes are latitudinal prepattern genes in the developing *Drosophila* notum whose expression is regulated by the concerted functions of decapentaplegic and wingless. Development *126*, 1457–1466.

Schroeder, M.D., Pearce, M., Fak, J., Fan, H., Unnerstall, U., Emberly, E., Rajewsky, N., Siggia, E.D., and Gaul, U. (2004). Transcriptional control in the segmentation gene network of *Drosophila*. PLoS Biol. *2*, E271.

Shen, W., and Mardon, G. (1997). Ectopic eye development in *Drosophila* induced by directed dachshund expression. Development *124*, 45–52.

Sosinsky, A., Bonin, C.P., Mann, R.S., and Honig, B. (2003). Target Explorer: an automated tool for the identification of new target genes for a specified set of transcription factors. Nucleic Acids Res. *31*, 3589–3592.

Stadeli, R., Hoffmans, R., and Basler, K. (2006). Transcription under the control of nuclear Arm/beta-catenin. Curr. Biol. *16*, R378–R385.

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

von Dassow, G., Meir, E., Munro, E.M., and Odell, G.M. (2000). The segment polarity network is a robust developmental module. Nature *406*, 188–192.

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.